BACTERIOCIN RESISTANCE AMONG DAIRY PATHOGENS AND SPOILAGE BACTERIA

THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY
IN
DAIRY MICROBIOLOGY

BY
Gurpreet Kaur
M.Sc (Microbiology)

DIVISION OF DAIRY MICROBIOLOGY
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132 001 (HARYANA), INDIA

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Regn. No. 1010702

Dedicated

to

My Family

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Approved by

EXTERNAL EXAMINER

(Dr. R. K. MALIK) 21st Nov. 2011
MAJOR ADVISOR & CHAIRMAN
(GUIDE)

Members of Advisory Committee

1. **Dr. S.K Tomar**
   Senior Scientist,
   Dairy Microbiology Division
2. **Dr. Naresh Kumar**
   Senior Scientist
   Dairy Microbiology Division
3. **Dr. S. De**
   Senior Scientist,
   Animal Biotech Division
4. **Dr. (Mrs.) Kapila**
   Senior Scientist, Animal Biochemistry Division
5. **Dr. Sirohi**
   Senior Scientist, Dairy Cattle Nutrition Division

DAIRY MICROBIOLOGY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL-132001 (HARYANA), INDIA

Dr. R.K. Malik
Principal Scientist

CERTIFICATE

This is to certify that the thesis entitled, **"BACTERIOCIN RESISTANCE AMONG DAIRY PATHOGENS AND SPOILAGE BACTERIA"** submitted by **Ms. Gurpreet Kaur** towards the partial fulfilment of the award of the degree of **Doctor of Philosophy (Dairy Microbiology)** of the **National Dairy Research Institute (Deemed University)**, Karnal (Haryana), India, is a bonafide research work carried out by her under my supervision, and no part of the thesis has been submitted for any other degree or diploma.

Dated: 29th November 2011

(R.K. Malik)
Major Advisor
(Guide)

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Dated: August, 2011
Place: Karnal *(Gurpreet Kaur)*

ABSTRACT

The Gram-positive bacterial species studied in this work differed considerably in their sensitivity to nisin, pediocin 34 and enterocin FH99. A greater antibacterial effect was observed against *Listeria monocytogenes* ATCC 53135*, L. monocytogenes* MTCC 657*, Enterococcus faecium* DSMZ 20477 *and E. faecium* VRE when the bacteriocins were combined in pairs. Bacteriocin resistant variants of *L. monocytogenes* ATCC 53135*, L. monocytogenes* MTCC 657*, E. faecium* DSMZ 20477*, E. faecium* VRE and *E. faecalis* ATCC 29212 were developed. Bacteriocin cross-resistance and the antibiotic susceptibility of wild type and their corresponding resistant variants were assessed and the results showed that the acquired resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes and can also alter the antibiotic susceptibility/resistance profile of bacteria. In order to determine whether resistance to bacteriocins confers cross-resistance to these common food preservatives, sensitivity of bacteriocin resistant variants to common food preservatives viz. sodium chloride, low pH, sodium nitrite and potassium sorbate was compared with the wild (sensitive-type) strains. It was observed that nisin, pediocin 34 and enterocin FH99 resistant strains did not have intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. The heat resistance of wild type and the nisin, pediocin 34 and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* VRE and *E. faecalis* ATCC 29212 was assayed at 45, 50 and 55°C. The results suggested that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of target organisms. In an attempt to clarify the possible mechanisms underlying bacteriocin resistance in *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212, surface properties such as cell surface hydrophobicity were analyzed and compared between the wild types and the bacteriocin resistant variants. The addition of divalent cations significantly reduced the inhibitory activity of nisin, pediocin 34 and enterocin FH99 against resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* VRE and *E. faecalis* ATCC 29212. The addition of EDTA however restored this activity. Ultra structural profiles of bacteriocin sensitive *L. monocytogenes* ATCC 53135*, E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their bacteriocin resistant counterparts revealed that the cells of wild type strain of *L. monocytogenes* ATCC 531315 were maximally in pairs or short chains, whereas, nisin, pediocin 34, and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135 tend to form aggregates. Similarly, the cells of wild type *E. faecium* VRE were observed to be in short chains. However nisin, pediocin 34, and enterocin FH99 resistant variants of *E. faecium* VRE displayed clumping of cells into compact mass. Nisin resistant variants of *E. faecalis* ATCC 29212 also displayed clumping and fusion of cells. When the cell wall was removed, the wild type strains of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477*, E. faecium* VRE and *E. faecalis* ATCC 29212 and their resistant variants to nisin, pediocin 34 and enterocin FH99 showed identical sensitivity to the three bacteriocins. However, the protoplasts of the resistant variants appeared to be more resistant to bacteriocins than the protoplasts of their wild type counterpart. An increase in amino group containing phospholipids in the nisin resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, pediocin 34 and enterocin FH99 resistant variant of *E. faecium* DSMZ 20477, pediocin 34 and enterocin FH99 resistant variant of *E. faecium* VRE was observed. Results also showed that mannose, glucose, fructose and cellobiose have an effect on sensitivity of *L. monocytogenes, E. faecium* and *E. faecalis strains* to nisin, pediocin 34 and enterocin FH99. It underlines that the level of sensitivity is tightly linked to sugar availability, suggesting that sugars (mannose, glucose, fructose and cellobiose) directly causes expression of a molecule responsible for sensitivity to nisin, pediocin 34 and enterocin FH99. From Real Time PCR studies, it is evident that σ^{54} is involved in conferring resistance to nisin, pediocin 34 and enterocin FH99. In case of strains of *L. monocytogenes* and *E. faecium and E. feacalis*, *IIC* and *IID* genes were down-regulated. Gene *glpQ* which encodes a putative glycerophosphoryl diester phosphoesterase was also down regulated.

सारांश

[illegible] अध्ययन में ग्राम पॉजिटिव बैक्टीरिया की प्रजातियों ने नाइसिन, पीडीयोसिन 34 एवं एनटैरोसिन एफ. [illegible] 99 के प्रति संवेदनशीलता में काफी अंतर दिखाया। अधिक जीवाणुरोधी प्रभाव लिस्टेरिया मोनोसासटोजीन्स [illegible] सी. 53135, लिस्टेरिया मोनोसासटोजीन्स एम.टी.सी.सी. 657, एनटैरोकोकस फीसीयम डी. [illegible] जेड. 20477 एवं एनटैरोकोकस फीसीयम वी.आर.ई. के प्रति पाया गया जब बैक्टीस्योसिन्स को [illegible] रूप में उपयोग किया गया। लिस्टेरिया मोनोसासटोजीन्स ए.टी.सी.सी. 53135, लिस्टेरिया [illegible] टोजीन्स एम.टी.सी.सी. 657 एनटैरोकोकस फीसीयम डी.एस.एम.जेड. 20477 के बैक्टीरियोसिन [illegible] वेरिएंट विकसित किए गए। जंगली प्रकार और उनके प्रतिरोध वेरिएंट के बैक्टीरियोसिन पार [illegible] एवं एंटीबायोटिक संवेदनशीलता का मूल्यांकन किया और नतीजे बताते हैं कि एक बैक्टीरियोसिन [illegible] अधिग्रहित प्रतिरोध का विस्तार एक ही वर्ग के दूसरे बैक्टीरियोसिन या यहां तक कि अन्य वर्गों [illegible] सिन्स तक हो सकता है और बैक्टीरियोसिन्स प्रतिरोध जीवाणुओं की एंटीबायोटिक [illegible]/प्रतिरोध प्रोफाइल को भी बदल सकता है। यह निर्धारित करने के लिए कि बैक्टीरियोसिन्स [illegible] खाना परिरक्षकों के लिए प्रतिरोध प्रदान कर रहा है या नहीं, बैक्टीरियोसिन्स [illegible] की आम खाना परिरक्षकों जैसे कि सोडियम क्लोराइड, कम पी.एच., सोडियम नाइट्राइट [illegible] सारबेट के प्रति संवेदनशीलता की तुलना उनके संवेदनशील उपभेदों के साथ की गई। यह [illegible] कि नाइसिन, पीडीयोसिन 34 और एनटैरोसिन एफ.एच. 99 प्रतिरोधी उपभेदों ने कम पी.एच. [illegible] क्लोराइड, पोटेशियम सारबेट या सोडियम नाइट्राइट के प्रति आंतरिक प्रतिरोध नही किया। [illegible] साइटोजीन्स ए.टी.सी.सी. 53135, लिस्टेरिया मोनोसाइटोजीन्स एम.टी.सी.सी. 657, [illegible] फीसीयम डी.एस.एम.जैड 20477, एनटैरोकोकस फीसीयम वी.आर.इ. एवं एन्टैरोकोकस [illegible] ए.टी.सी.सी 29212 के संवेदनशील और नाइसिन, पीडीयोसिन 34 एवं एन्टैरोसिन एफ.एच. [illegible] वेरिएंट की गर्मी प्रतिरोध क्षमता का आंकलन 45, 50 और 55 डिग्री सेल्सियस पर किया [illegible] ने दर्शाया कि नाइसिन, पीडीयोसिन 34 और एन्टैरोसिन एफ.एच.99 प्रतिरोध ने जीवों [illegible] संवेदनशीलता को नहीं बदला। लिस्टेरिया मोनोसाइटोजीन्स ए.टी.सी.सी. 53135, लिस्टेरिया [illegible] एम.टी.सी.सी. 657, एनटैरोकोकस फीसीयम डी.एस.एम.जैड. 20477, एनटैरोकोकस [illegible] आर.इ. एवं एनटैरोकोकस फीकेलिस ए.टी.सी.सी 29212 में संभव अंतर्निहित तंत्र को स्पष्ट [illegible] कोशिका की सतह गुण विष्लेषण किया गया और संवेदनशील प्रकार और बैक्टीरीयोसिन [illegible] के बीच तुलना की गई। द्विसंयोजक कैटायनों ने लिस्टेरिया मोनोसाइटोजीन्स ए.टी.सी.सी. [illegible] लिस्टेरिया मोनोसाइटोजीन्स एम.टी.सी.सी. 657, एनटैरोकोकस फीसीयम डी.एस.एम.जैड. [illegible] एन्टैरोकोकस फीसीयम वी.आर.इ. एवं एन्टैरोकोकस फीकेलिस ए.टी.सी.सी 29212 के [illegible] पीडीयोसिन 34 और एनटेरोसिन एफ.एच.99 की निरोधात्मक गतिविधि को कम किया [illegible] ने इस गतिविधि को बहाल किया। बैक्टीरीयोसिन संवेदनशील लिस्टेरिया मोनोसाइटोजीन्स [illegible] 53135, लिस्टेरिया मोनोसाइटोजीन्स एम.टी.सी.सी. 657, एनटैरोकोकस फीसीयम डी. [illegible] 20477, एनटैरोकोकस फीसीयम वी.आर.इ. एवं एन्टैरोकोकस फीकेलिस ए.टी.सी.सी [illegible] और उनके प्रतिरोधी समकक्षों की संरचनात्मक प्रोफाइल से पता चला कि एल. मोनोसाइटोजीन्स [illegible] 53135 के जंगली प्रकार ज्यादातार जोड़ों या छोटी जंजरो में थे। जबकि नाइसिन,

[illegible] 34 और एन्टैरोसिन एफ.एच.99 प्रतिरोधी वेरिएंट समुच्चय फार्म में थे। इसी प्रकार जंगली [illegible] वी.आर.इ. छोटी जंजीरों में थे। हालांकि नाइसिन, पीडीयोसिन 34 एवं एन्टैरोसिन एफ.एच. [illegible] वेरिएंटस ने कॉम्पैक्ट मास या कलम्पिंग का प्रर्दशन किया। इ. फीकेलिस ए.टी.सी.सी. [illegible]2 के नाइसिन प्रतिरोधी वेरिएंट ने भी कलम्पिंग और संल्यन का प्रदर्शन किया। जब सैल वाल को [illegible] गया तब लिस्टेरिया मोनोसाइटोजीन्स ए.टी.सी.सी. 53135, लिस्टेरिया मोनोसाइटोजीन्स एम.टी. [illegible] 657, एनटैरोकोकस फीसीयम डी.एस.एम.जैड. 20477, एनटैरोकोकस फीसीयम वी.आर.इ. [illegible]कोकस फीकेलिस ए.टी.सी.सी 29212 के जंगली प्रकार उपभेदों और उनके प्रतिरोधी वेरिएंट [illegible] बैक्टीरियोसिन्स के लिए समान संवेदनशीलता दिखाई। हालांकि प्रतिरोधी वेरिएंट के प्रोटोप्लास्ट [illegible] जंगली प्रकार समकक्ष के प्रोटोप्लास्ट की तुलना में बैक्टीरियोसिन्स के लिए प्रतिरोधी दिखाई दिए। [illegible] मोनोसायटोजीन्स ए.टी.सी.सी. 53135, एल. मोनोसायटोजीन्स एम.टी.सी.सी. 657 के नाइसिन [illegible] वेरिएंट, इ. फीसीयम डी.एस.एम.जैड. 20477 और इ. फीसीयम वी.आर.इ. के पीडीयोसिन 34 [illegible] एन्टैरोसिन एफ.एच.99 प्रतिरोधी वेरिएंट में एमीनो समूह युक्त फासफोलिपिड में वृद्धि पाई गई। [illegible] ने यह भी दिखाया कि मैनोज, ग्लूकोज, फ्रक्टोज और सैलोबायोज का लिस्टेरिया मोनोसाइटोजीन्स [illegible]सी. 53135, लिस्टेरिया मोनोसाइटोजीन्स एम.टी.सी.सी. 657, एनटैरोकोकस फीसीयम डी. [illegible] जैड. 20477, एनटैरोकोकस फीसीयम वी.आर.इ. एवं एनटैरोकोकस फीकेलिस ए.टी.सी.सी [illegible]212 उपभेदों की नाइसिन, पीडीयोसिन एवं ऐन्टैरोसिन एफ.एच.99 के प्रति संवेदनशीलता पर एक [illegible] है। यह रेखांकन है कि संवेदनशीलता का स्तर शर्करा उपलब्धता से जुड़ा हुआ है। सुझाव है कि [illegible] (मैनोज, ग्लूकोज, प्रक्टोरज और सैलोबायोज) सीधे नाइसिन, पीडीयोसिन 34, एन्टोरोसिन एफ. [illegible] 99 संवेदनशीलता के लिए जिम्मेदार अणु के अभिव्यक्ति का कारण बनता है। रीयल टाइम पी.सी. [illegible] अध्ययन से यह स्पष्ट है कि σ^{54} नाइसिन, पीडीयोमिन 34 और ऐनटैरोसिन एफ.एच. 99 प्रतिरोध [illegible] करने में शामिल है। एल. मोनोसायटोजीन्स एवं ऐनटैरोकोकस के विभिन्न वर्णो के मामलों में II [illegible] और II डी. जीन विनियमित पाए गए। जीन *glpQ* जो एक ख्यात glycerophosphory [illegible]ester phosphoesterase को एनकोड करता है भी विनियमित किया गया था।

ABBREVIATIONS

DNA	:	Deoxyribonucleic Acid
RNA	:	Ribonucleic Acid
PCR	:	Polymerase Chain Reaction
RT-PCR	:	Real Time PCR
bp	:	Base Pair
TLC	:	Thin Layer Chromatography
NCDC	:	National Collection of Dairy Cultures
spp.	:	Species
ssp.	:	Subspecies
cfu	:	Colony forming units
l	:	Litre
ml	:	Millilitre
µl	:	Microlitre
g	:	Gram
mg	:	Milligram
µg	:	Microgram
ng	:	Nanogram
g^{-1}/l^{-1}	:	Per gram/ Per litre
m	:	Meter
mm	:	millimeter
nm	:	nanometer
N	:	Normal
OD	:	Optical Density
w	:	Weight
vol	:	Volume
rpm	:	Rotations per minute
s	:	Second
min	:	Minute
h	:	Hour
d	:	Days

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1. INTRODUCTION

For the past few decades, food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. Biopreservation of foods offers an alternative control measure for improving the stability and safety of mildly processed food products and has, therefore, been the focus of increased attention in the last few years. Biopreservation reduces the amount of chemical preservatives to be added to the food as well as the intensity of heat treatments, both of which can otherwise negatively affect the food quality (Galvez *et al.,* 2007; Kalchayanand *et al.,* 1992). Lactic acid bacteria (LAB) are critical for the production of a great multitude of fermented foods. The most important contribution of these bacteria to fermented products is to preserve the nutritive qualities of the raw material and inhibit the growth of spoilage and pathogenic bacteria. Protection of food from spoilage and pathogenic microorganisms by LAB is through the production of organic acids, hydrogen peroxide, diacetyl (Menssens & De Vugst, 2002), antifungal compounds such as fatty acids (Corsetti *et al.,* 1996) or phenullactic acid (Lavermicocca *et al.,* 2000) and/or bacteriocins (De Vugst & Vandamme, 1994)

Bacteriocins produced by lactic acid bacteria have been the subject of considerable research and industrial interest due to their potential as food biopreservatives. Although bacteriocins may be found in many Gram positive and Gram negative bacteria (Riley & Wertz, 2002), those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives (Ennahar *et al.,* 1999). Compared to classical peptide antibiotics, which are synthesized through enzymatic condensation of free amino acids, the bacteriocins are proteinaceous antibacterial compounds, which constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides with antimicrobial activity against closely related bacteria (De Vugst & Vandamme, 1994). In general these substances are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity (Moll *et al.,* 1999).

Although the use of bacteriocins for preservation (biopreservation) is a novel approach to eliminate or control pathogens in food, the development of highly tolerant or resistant strains remains the main concern and decreases the efficiency of bacteriocins as biopreservatives. Resistant food-borne pathogens are posing a global problem which is further facilitated by international trade of raw and processed foods (Bower & Daeschel, 1999). In foods with a long shelf life, even a small number of these resistant cells can multiply to very high number and thus may lead to food-borne outbreaks and food spoilage. Among bacteriocins produced by LAB, nisin, a class I bacteriocin, has demonstrated antilisterial activity. It was the first bacteriocin to be characterized and is the only one approved for use in food applications (Chung *et al.,* 1989). The emergence of nisin-resistant *L. monocytogenes* mutants has already been reported (Davies & Adams, 1994; Gravesen *et al.,* 2000). The emergence of nisin resistant mutants has been described for several *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bacillus* spp., and *Clostridium* spp. and for *Staphylococcus aureus*, and *L. monocytogenes* (Daeschel *et al*., 1991; Ming & Daeschel, 1993; Ming & Daeschel, 1995; Breuer & Radler, 1996; Davies *et al.,* 1996).

The resistance mechanism is complex and may be due to three major factors: (i) variation of peptidoglycan composition (Maisnier & Richard, 1996), which should make it possible to increase the binding of divalent cations that should interact with the cationic peptide; (ii) modification of the electric charge of the membrane by changes in the phospholipids content, thereby preventing pore formation (Crandall & Montville, 1998); and (iii) increase in membrane rigidity, preventing peptide insertion and association (Ming & Daeschel, 1993). However, resistance to bacteriocins has also been correlated with an altered fatty acid composition (Mazzotta & Montville, 1997) and an altered phospholipids composition (Ming & Daeschel, 1995). It has been suggested that resistance to class IIa bacteriocins occurs at either a low (2-4 fold increase in Minimum Inhibitory Concentration i.e. MIC) or a high level (1000-fold increase in MIC) (Nes *et al.,* 2007). High-level resistance of *E. faecalis*, *L. monocytogenes* and some other Gram-positive bacteria to class IIa bacteriocins has been reported to result from the loss of expression of

Mannose-Specific Phosphotransferase System (*mpt*), either in defined mutants or in spontaneous resistant strains (Gravesen *et al.,* 2002). The *mpt* operon encodes a mannose permease (EII_t^{Man}) which belongs to the Mannose Specific Phosphotrnsferase System and the mpt –PTS transporter family is responsible for the import and phosphorylation of carbohydrates such as mannose and glucose in bacteria. Sigma 54 (σ54) has been described to be involved in expression of Phosphotransferase systems. It is unique since it targets conserved -24/-12 promoter sequences and requires an activator protein for transcription initiation, referred to as σ54-associated activator.

Combinations of different bacteriocins have been reported to produce a more effective antibacterial effect against food-borne pathogens in comparison to the bacteriocins used alone. It has been assumed that a mixture containing more than one bacteriocins would have greater bactericidal effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. Moreover, synergistic effects have been reported when the interactions between pairs of bacteriocins from lactic acid bacteria were tested. One possible explanation for the different effectiveness of bacteriocin pairs would be that the bacteriocins used in this study belonged to different classes, which vary considerably in the nature and sequence of amino acid residues. The simultaneous use of two or more bacteriocins could be useful not only to lower the added bacteriocin doses, but also to avoid regrowth of bacteriocin-resistant/adapted cells. The synergistic action of combinations of two different bacteriocins with different structures produced by the same strain has also been reported (Limonet *et al.,* 2004). It has been shown that the bacteriocins from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems (Jamuna *et al.*, 2005).Vignolo *et al.* (2000) also reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT in meat slurry showed no viable counts after incubation for 3 h. A recent study has also reported the synergistic effect of Nisin and bacteriocin from *Pediococcus acidilactici* to be more effective in inhibiting the growth

of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav (Jamuna & Jeevaratnam, 2009).

Though there are several reports showing that combination of different bacteriocins have a greater antibacterial effect against food-borne pathogens and spoilage bacteria some reports suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. A nisin resistant strain of *L. monocytogenes* was shown to be cross resistant to the Class IIa bacteriocin, pediocin PA-1 and the Class IV leuconocin S. Cross-resistance between bacteriocins has been observed when the sensitivity of *Listeria* variants to lactocin 705, enterocin CRL35, and nisin was tested. Similar results were obtained by Rekhiff *et al.* (1994), who reported that mutants of *L. monocytogenes* ATCC 15313 resistant to one of three bacteriocins tested (mesenterocin 52, curvaticin 13, and plantaricin C19) displayed more resistance to the two other, but not to nisin.

These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the other bacteriocins. Many reports have suggested that the ultimate failure of bacteriocin based preservation systems is due to the eventual growth of resistant strains.

However, the application of bacteriocins as part of hurdle technology has received great attention in recent years (Chen & Hoover, 2003; Ross *et al.,* 2003; Deegan *et al.,* 2006), since bacteriocins can be used purposely in combination with selected hurdles in order to increase microbial inactivation. Bacteriocins can be used to reduce the intensity of heat treatments in foods without compromising microbial inactivation (Ananou *et al.,* 2004; Grande *et al.,* 2006). Though, several studies have focused on the application of nisin's activity with potential synergists such as nitrites, low pH, pasteurization, controlled atmosphere, and food ingredients these, however, did not consider the possible emergence of nisin resistant strains.

Keeping in view the foregoing, the present investigation was undertaken with the following objectives:

1. Minimum inhibitory concentrations (MIC) of different bacteriocins against some common dairy pathogens and spoilage bacteria
2. Isolation and molecular & biochemical characterization of bacteriocin resistant variants
3. Effect of mild heat treatment, pH and combination of bacteriocins on the susceptibility of resistant variants

2. REVIEW OF LITERATURE

2.1 PROLOGUE

Biopreservation refers to the extended storage life and enhanced safety of foods using their natural or controlled microflora and/or their antibacterial products (Stiles, 1996). Among the wide spectrum of antibacterial products released by microorganisms, the bacteriocins, especially those produced by lactic acid bacteria (LAB), have attracted the greatest attention as tools for food biopreservation. Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack *et al.*, 1995). Bacteriocins are nontoxic to eukaryotic cells and are generally recognized as safe substances. Lactic acid bacteria are well known for their capacity to produce bacteriocins. Given the high prevalence and pivotal roles that this group of bacteria plays in food and health, structure, biosynthesis, genetics, and food application of LAB bacteriocins have all been studied extensively (Cleveland *et al.*, 2001; O'Sullivan *et al.*, 2002; Chen & Hoover, 2003; Cotter *et al.*, 2005; Fimland *et al.*, 2005; Deegan *et al.*, 2006; Drider *et al.*, 2006; Franz *et al.*, 2007; G´alvez *et al.*, 2007).

The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (i) are generally recognized as safe substances, (ii) are not active and nontoxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation (G´alvez *et al.*, 2007).

The accumulation of studies carried out in recent years clearly indicate that the application of bacteriocins in food preservation can offer several benefits: (i), an extended shelf life of foods, (ii) provide extra protection during temperature abuse

conditions, (iii) decrease the risk for transmission of food borne pathogens through the food chain, (iv) ameliorate the economic losses due to food spoilage, (v) reduce the application of chemical preservatives, (vi) permit the application of less severe heat treatments without compromising food safety: better preservation of food nutrients and vitamins, as well as organoleptic properties of foods, (vii), permit the marketing of "novel" foods (less acidic, with a lower salt content, and with a higher water content), and (viii) they may serve to satisfy industrial and consumers demands (Thomas *et al.*, 2000).

However, the development of highly tolerant and/or bacteriocin resistant strains may decrease the efficiency of bacteriocins as biopreservatives. Several mechanisms of bacteriocin resistance development have been proposed among various food borne pathogens. The acquiring of resistance to bacteriocins can significantly affect physiological activity profile of bacteria, alter cell-envelope lipid composition and also modify the antibiotic susceptibility/resistance profile of bacteria. This review presents some background on the scientific research about the various possible mechanisms involved in the development of resistance to nisin and Class IIa bacteriocins among the food borne pathogens.

2.2 BACTERIOCINS

Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins (Jack *et al.*, 1995). The bacteriocins produced by LAB offer several desirable properties that make them suitable for food preservation: (i) are generally recognised as safe substances, (ii) are not active and nontoxic on eukaryotic cells, (iii) become inactivated by digestive proteases, having little influence on the gut microbiota, (iv) are usually pH and heat-tolerant, (v) they have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (vi) they show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (vii) their genetic determinants are usually plasmid-encoded, facilitating genetic manipulation. Bacteriocins are a heterogeneous group of peptides and proteins and were classified into five main classes of LAB bacteriocins (Fig 2.1) (Klaenhammer, 1993; Nes *et al.*, 1996). The bacteriocins,

however, have so far been grouped into two major classes, which display great diversity with regard to their mode of action, structure, genetics, mode of secretion and choice of target organisms (Nes *et al*., 2007):

Class I: Lantibiotics; which are small, posttranslationally modified peptides that contain unusual amino acids such as lanthionine, and

Class II: The heat-stable nonlantibiotics. A third class of bacteriocins has been suggested that includes secreted heat-labile, cell-wall degrading enzymes, but the classification of such enzymes as being bacteriocins has recently been thrown into doubt (Cotter *et al*., 2005).

In contrast to this, Heng *et al*. (2007) proposed to retain this class for large bacteriocins, which is subdivided into IIIa (bacteriolysins) and IIIb (nonlytic proteins). The lantibiotics have been divided into two subgroups: type A, elongated molecules with a flexible structure in solution, and type B, which tend to have a more rigid, globular structure (although there are some exceptions). Lack of consensus also exists in the distinction between subgroups of the nonlantibiotics belonging to class II, which include a large number of small peptides that only have in common their marked thermostability and a lack of modified residues (Cleveland *et al*., 2001; Eijsink *et al*., 2002; Ross *et al*., 2002; Cotter *et al*., 2005; Drider *et al*., 2006). In the new classification scheme, Nes *et al*. (2007) subdivided Class II into four subclasses:

1) Class IIa (antilisteria pediocin-like bacteriocins)
2) Class IIb (two-peptide bacteriocins)
3) Class IIc (leaderless peptide bacteriocins) and
4) Class IId (circular bacteriocins).

Nevertheless, according to the recommendations of several research groups (Kemperman *et al*., 2003; Kawai *et al*., 2004; Maqueda *et al*., 2004; Heng & Tagg, 2006; Heng *et al*., 2007), the circular, posttranslationally modified bacteriocins deserve to be upgraded to a new class IV. Nonetheless, this classification is

continuously being reviewed and it is evolving with the accumulation of knowledge and the appearance of new bacteriocins (Ennahar *et al.,* 2000; Cleveland *et al.,* 2001; Riley & Wertz, 2002; Rodriguez *et al.,* 2003; Cotter *et al.,* 2005).

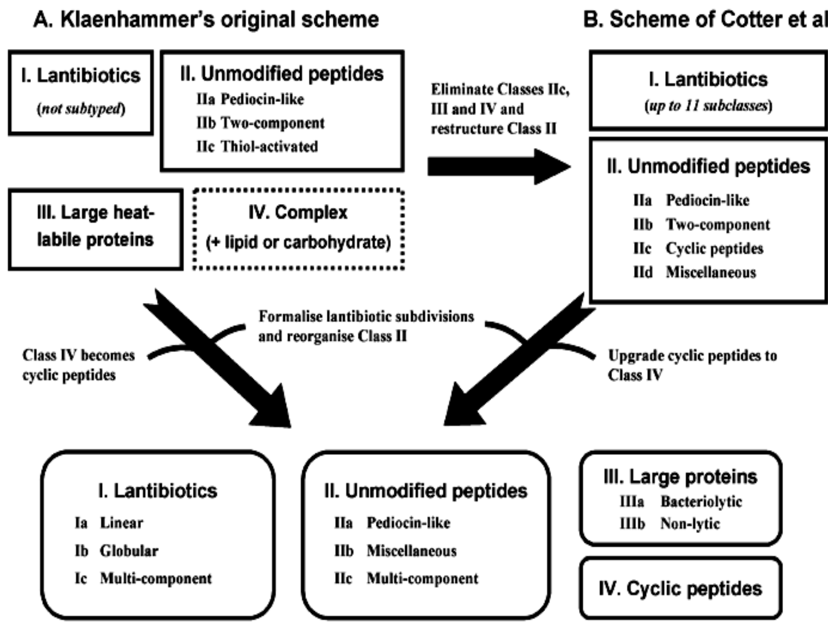


Fig. 2.1. Proposed "Universal" bacteriocin classification scheme.

Bacteriocins are usually formed as prepeptides in the cell and mature bacteriocins are generated by processing during export. This is generally accomplished by removal of an N-terminal leader peptide either by signal peptidase in sec-pathway secreted bacteriocins, or more often at a specific double glycine position (-2,-1) in the prepeptide, concomitant with externalization by a dedicated ABC-transporter protein and an accessory protein. With the exception of disulfide bridge formations, post translational modifications are rare among the non-lantibiotic bacteriocins (Nes *et al.,* 1996; Ennahar *et al.,* 1999; Garneau *et al.,* 2002). At least four different genes are required to achieve the production of bacteriocins by Gram-positive bacteria: 1) a structural gene (or two structural genes in two-peptide bacteriocins) encoding the prepeptide; 2) dedicated immunity gene; 3) a gene encoding a dedicated ABC transporter; and 4) a gene encoding an accessory protein which is necessary for bacteriocin transport (Nes *et al.,* 1996; Ennahar *et al.,* 1999). Usually, the

bacteriocin structural and immunity genes occur in an operon, as do the transport genes. For most non-lantibiotics, the bactericidal effect of bacteriocins occurs as a result of permeabilisation of the cell membrane (Nes & Holo, 2000). This pore forming ability leads to cell leakage and efflux of K^+ ions, which in turn leads to dissipation of membrane potential and inhibition of amino acid uptake. Cell death is finally affected by futile cycle of ATP-driven potassium uptake and bacteriocin-mediated potassium release in combination with increased ATP hydrolysis by an ATPase (Fig. 2.2) (Jack *et al.,* 1995; Garneau *et al.,* 2002)

Mature bacteriocins are generally cationic, amphiphilic species and the environment in which they are found can strongly dictate their secondary structure. In cases where the structure has been examined, they usually exist as random coils under aqueous conditions, but in charged, weakly ionic solution they exist in ordered conformation (β-sheet and α-helices) (Garneau *et al.,* 2002).

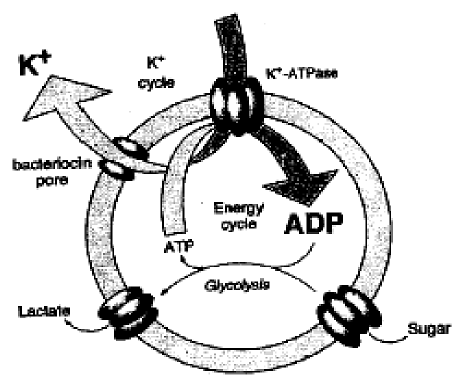


Fig. 2.2. Bactericidal action of bacteriocin by potassium ion efflux and increased ATP demand from K+ ATPase (adapted from Garneau *et al.,* 2002)

The bacteriocins produced by LAB belong to the large group of ribosomally synthesised, small, cationic, amphiphilic (rather hydrophobic), antimicrobial peptides, naturally produced by microorganisms, which vary in spectrum and mode of activity, molecular structure and molecular mass, thermostability, pH range of activity, and genetic determinants (Klaenhammer, 1993; De Vuyst & Vandamme,

1994; Nes *et al.,* 1996; Ennahar *et al.,* 2000; Cleveland *et al.,* 2001; McAuliffe *et al.,* 2001; Riley & Wertz, 2002).

2.3 APPLICATION OF BACTERIOCINS IN THE CONTROL OF FOOD-BORNE PATHOGENIC AND SPOILAGE BACTERIA

2.3.1 Application of Nisin, Pediocin and Enterocin in Dairy Foods

The main bacterial pathogens of concern in the dairy industry such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella* spp are able to survive and multiply in the raw materials as well as in certain types of cheese (De Buyser *et al.*, 2001). Spoilage of semihard and hard cheeses (e.g., Kasseri, Emmental, Gruy`ere, Grana, Edam, Gouda) due to gas formation by *Clostridium tyrobutyricum* is also of great economical concern (Berg´ere & Lenoir, 2000). Therefore, most investigations concerning application of bacteriocins in dairy foods have focused in this direction. Nisin (in the commercial form Nisaplin) has been tested extensively in dairy foods. One of the earliest applications was to prevent gas blowing in cheese caused by *C. tyrobutyricum* (Hirsch *et al.*, 1951; De Vuyst & Vandamme, 1994). Nisin has found many other applications in the dairy industry, especially in processed cheeses and cheese products (e.g., block cheese, soft white cheeses, slices, spreads, sauces, dips) to prevent proliferation of surviving endospore formers, mainly the gas-producing clostridia and *Clostridium botulinum*, as well as other post-process contaminating bacteria such as *L. monocytogenes* (Davies & Delves-Broughton, 1999; Thomas & Delves-Broughton, 2001). It is also used in other pasteurized dairy products, such as chilled desserts, flavored milk, clotted cream, or canned evaporated milks (Thomas *et al.*, 2000). In heat treated cream, growth of *Bacillus cereus* during storage was completely inhibited by low concentrations of nisin (Nissen *et al.*, 2001). In sliced cheese, immobilized nisin in a polyethylene/polyamide packaging was shown to reduce the population of LAB, *Listeria innocua* and *S. aureus* (Scannell *et al.*, 2000). However, the use of nisin in cheese fermentation may interfere with growth of starter cultures, and have detrimental effects on acidification and/or aroma formation. To protect

starters from the detrimental action of nisin during cheese production (and also to enhance nisin stability), nisin Z encapsulated into liposomes was successfully tested and found to inhibit listeria in Cheddar cheese (Benech *et al.*, 2002a, 2002b).

In milk, addition of nisin is permitted in certain countries to avoid shelf-life problems associated with hot weather temperature and/or long-distance transport and inadequate refrigeration systems (Davies & Delves-Broughton, 1999; Thomas *et al.*, 2000). In skim milk, whey, or simulated milk ultrafiltrate media, increased nisin activity in combination with pulsed electric fields (PEFs) has been reported against several bacteria such as *L. monocytogenes, S. aureus, B. cereus,* and *E. coli* (Calder´on-Miranda *et al.*, 1999; Pol *et al.*, 2001; Sobrino-Lopez & Mart´ın Belloso, 2006; Terebiznik *et al.*, 2002). In combination with high hydrostatic pressure (HHP) treatments, nisin increased the inactivation of several bacteria associated with milk (i.e., *E. coli, Pseudomonas fluorescens, L. innocua*, *Lactobacillus viridescens*) (Garc´ıa-Graells *et al.*, 1999; Black *et al.*, 2005) and improved the inactivation of endospores and mesophilic bacteria in cheese (Capellas *et al.*, 2000; L´opez-Pedemonte *et al.*, 2003; Arqu´es *et al.*., 2005a & 2005b). This is of special relevance in the case of cheeses made from raw milk, which includes many traditional cheeses. Lacticin 3147 is another lactococcal bacteriocin with a high potential for application in the preservation of dairy foods (Ross *et al.*, 1999).

Application of enterococcal bacteriocins in dairy foods has been the focus of many investigations (Giraffa, 1995; Foulqui´e Moreno *et al.*, 2006). An early report indicated that bacteriocin from *E. faecium* DPC1146 had a rapid bactericidal effect on *L. monocytogenes* in milk (Parente & Hill, 1992). Enterocin CCM 4231 reduced the viable counts of *S. aureus* SA1 in skim milk, Sunar (milk nourishment for suckling babies), and yogurt (Laukov´a *et al.*, 1999a, 1999b). A decrease of viable *L. monocytogenes* Ohio bacterial cells was also reported in enterocin-added yogurt and in Saint-Paulin cheese (Laukov´a *et al.*, 1999b, 2001). However, after 6 weeks and at the end of the experiment, the

difference of surviving listeria was only 1 or 0.7 log cycle compared to the control cheese (Laukov´a *et al.*, 2001). In “bryndza” (a traditional Slovak dairy product from sheep milk), added enterocin CCM 4231 reduced the levels of *L. monocytogenes* Li1 during a 7-day ripening period, although the listeria were not completely eliminated (Laukov´a & Czikkov´a, 2001). In contrast, concentrated enterocin CRL35 added to goat cheese reduced the population of *L. monocytogenes* by 9 log units by the end of the ripening period without affecting the cheese quality (Far´ıas *et al.*, 1999). Similarly, cultured broths obtained from raw ewe’s milk containing enterocin 4 (enterocin AS-48) significantly reduced viable counts of *L. monocytogenes* (Rodr´ıguez *et al.*, 1997). Enterocin AS-48 added to a rice-based infant formula dissolved in whole milk completely inactivated *B. cereus* and prevented its growth for at least 15 days at 37◦C (Grande *et al.*, 2006b). These results are illustrative of the varying efficacy of enterocins in dairy foods and the need to completely inactivate the target bacteria at early stages on bacteriocin addition in order to avoid regrowth of survivors during storage.

Though pediocins have been tested mainly in meat products, pediocin PA 1/AcH was shown to inhibit *L. monocytogenes* in several dairy systems, such as dressed cottage cheese, half-andhalf cream, and cheese sauce (Pucci *et al.*, 1988), as well as in other dairy products (Motlagh *et al.*, 1992; Degnan *et al.*, 1993). Pediocin 5 from *P. acidilactici* UL5 was also tested in milk, with bactericidal effects against *L. monocytogenes* (Huang *et al.*, 1994).

The use of bacteriocin-producing starter or adjunct cultures in fermented dairy foods may solve many of the problems associated with the addition of bacteriocin, including the processing costs. Application of nisin-producing starter cultures to prevent gas-blowing defects in cheese was proposed as early as 1951 (Hirsch *et al.*, 1951). Nisin-producing strains have been reported to inhibit *L. monocytogenes* in several types of cheeses, such as cottage cheese (Bankerroum & Sandine, 1988) or Camembert (Sulzer & Busse, 1991; Maisnier-Patin *et al.*, 1992). In Manchego cheese made from raw ewe’s milk, *L. lactis*

subsp. *lactis* ESI 515 reduced viable counts of *L. innocua* by 4.08 log units after 60 days of ripening, and the produced nisin was detected in cheese through the ripening period (Rodr´ıguez *et al*., 1998). Similarly, a reduction of *S. aureus* viable counts was detected in a semihard cheese (Rodr´ıguez *et al*., 2000). Enterococci are prevalent in dairy foods (especially in traditional cheeses). Most of the bacteriocinogenic enterococci can grow and produce bacteriocins in milk and in cheeses, which makes them good candidates as adjunct cultures for protection against food borne pathogens. Enterococcal strains producing enterocin AS-48 have been tested in milk and dairy systems with satisfactory results. Strain *E. faecium* 7C5 (Torri Tarelli *et al*., 1994; Folli *et al*., 2003) produced bacteriocin during Taleggio cheese making, and bacteriocin activity was stable in the cheese until the end of ripening for at least 40 days (Giraffa *et al*., 1995). Similarly, bacteriocin activity produced by strain *E. faecalis* INIA 4 in Manchego and Hispano cheeses was inhibitory to *L. monocytogenes* in cheese (N´u˜nez *et al*., 1997). In a nonfat hard cheese, the strain *E. faecalis* A-48-32 produced enough enterocin AS-48 to inhibit *B. cereus* and reduce the cell count of bacilli by 5.6 log units after 30 days of ripening (Mu˜noz *et al*., 2004). *S. aureus* was inhibited to a lesser extent in the cheese (Mu˜noz *et al*., 2007). Growth of starter cultures used in cheese making was not affected by the bacteriocin-producing strain. The same A-48-32 strain successfully inhibited *B. cereus* and *S. aureus* in skim milk (Mu˜noz *et al*., 2004, 2007).

Several other bacteriocinogenic enterococci have also been reported to produce antagonistic activities in dairy foods. *E. faecalis* 226 (producer of enterocin 226NWC) inhibited *L. monocytogenes* during cocultivation at 30◦C in skim milk (Villani *et al*., 1993). *E. faecium* CCM 4231 was able to produce bacteriocin in Saint-Paulin cheese (Laukov´a *et al*., 2001), and *E. faecium* DPC 1146 was reported to produce enterocin 1146 (enterocin A) during cheddar cheese manufacture (Foulqui´e Moreno *et al*., 2002). Similarly, strain *E. faecium* RZS C5 (a natural cheese isolate carrying the structural genes for enterocins A, B, and P) was also reported to be effective as an antilisteria bacteriocin producing coculture in Cheddar cheese manufacture (Foulqui´e Moreno *et al*., 2002; Leroy

et al., 2003). In contrast, the bacteriocinogenic strain *E. faecium* FAIR-E 198 isolated from Greek feta cheese failed to produce bacteriocin in skim milk or in feta cheese when used as an adjunct starter (Sarantinopoulos *et al.*, 2002). This was an indication of how the complex food environment may interfere with bacteriocin production levels.

Bacteriocin-producing enterococci have also been tested in traditional fermented milks. The enterocin L50-producing strain *E. faecium* F58 isolated from goat Jben cheese produced in Morocco reduced the number of viable *L. monocytogenes* in the cheese below detectable levels after 1 week of storage at 22°C. Addition of this strain as an adjunct culture in cheese was proposed as an additional hurdle to control contamination and growth of *L. monocytogenes* by *in situ* enterocin production (Achemchem *et al.*, 2006).

2.3.2. Application of Nisin, Pediocin, Enterocin in Vegetable Foods and Drinks

Bacteriocins and bacteriocinogenic strains could be applied in the preservation of vegetable foods and drinks with several purposes. Competitive exclusion techniques, where nonpathogenic microorganisms are used to repress the growth of pathogenic bacteria during sprouting, have been suggested on several occasions. Isolation of naturally occurring microbes that produce antimicrobial substances against pathogens in fresh produce has been reported (Carlin *et al.*, 1996; Buchanan & Bagi, 1999; Liao & Fett, 2001; Wilderdyke *et al.*, 2004), and LAB strains have been shown to be effective in suppressing the growth of pathogens on ready-to-use vegetables (Vescovo *et al.*, 1996). Nisin-producing lactococci isolated from bean sprouts reduced the levels of *L. monocytogenes* after refrigeration storage (Cai *et al.*, 1997). A mundticin-producing *Enterococcus mundtii* strain isolated from minimally processed vegetables inhibited the growth of *L. monocytogenes* on sterile vegetable medium but not on fresh mungbean sprouts (Bennik *et al.*, 1999).

Pediocin treatment alone or in combination with any of the organic acid tested was more effective in reducing *L. monocytogenes* populations than the nisin

treatment alone (Bari *et al.*, 2005). Enterocin AS-48 may also be an interesting bacteriocin for decontamination of raw vegetables. In lettuce juice, enterocin AS-48 caused a strong inhibition of *S. aureus* and completely inactivated *L. monocytogenes* and *B. cereus* (Grande *et al.*, 2005b). Application of immersion treatments with AS- 48 solutions markedly reduced the concentrations of listeria on sprouts and green asparagus. To solve the problem of regrowth of survivors during storage, the bactericidal effect of AS-48 was potentiated by several antimicrobial agents, providing an effective method for decontamination of sprouts (Cobo Molinos *et al.*, 2005). Other bacteriocins produced by LAB strains on a lettuce extract (nisin Z, coagulin, and a nisin:coagulin cocktail) reduced viable cell counts of *L. monocytogenes* on fresh-cut iceberg lettuce stored in microperforated plastic bags when applied as washing solutions, but did not prevent further growth of survivors during refrigeration storage of the treated samples (Allende *et al.*, 2007). In both canned and cooked vegetables, endospore-forming bacteria represent the main risk for spoilage due to the high thermal resistance of endospores and the frequent endospore contamination of raw materials. Several studies indicate that bacteriocins could be applied in this category of foodstuffs to inhibit endospore outgrowth and also to increase the efficacy of thermal treatments against endospores. Incorporation of nisin in canned vegetables can prevent spoilage caused by nonaciduric (*Bacillus stearothermophilus* and *Clostridium thermosaccharolyticum*) and aciduric (*Clostridium pasteurianum, Bacillus macerans, Bacillus coagulans*) spore formers (Thomas *et al.*, 2000). Nisin is also an effective preservative in fresh pasteurized "home-made"–type soups (Thomas *et al.*, 2000) and in the control of *Bacillus* and *Clostridium* in cooked potato products (Thomas *et al.*, 2002).

Enterocin AS-48 was also able to suppress *B. coagulans* vegetative cells in tomato paste, syrup from canned peaches, and juice from canned pineapple for at least 15 days of storage at 37°C (Lucas *et al.*, 2006). In boiled rice and in a rice gruel inoculated with vegetative cells and endospores of *B. cereus,* enterocin AS-48 completely eliminated the bacilli and prevented enterotoxin production during storage under refrigeration and under ambient temperatures

(Grande *et al.*, 2006b). Although the bacterial endospores were resistant to this bacteriocin, application of AS-48 in combination with heat treatments decreased the thermal death *D*- values for endospores (Grande *et al.*, 2006a; Lucas *et al.*, 2006). Enterocin AS-48 completely inactivated *Alicyclobacillus acidoterrestris* inoculated in several types of fruit juices for up to 3 months (Grande *et al.*, 2005a). Nisin and enterocin AS-48 were also able to inactivate endospores of *A. acidoterrestris* (Komitopoulou *et al.*, 1999; Yamazaki *et al.*, 2000; Grande *et al.*, 2005a). Similarly, *E. coli* O157:H7 cells sublethally injured by other treatments (EDTA, sodium tripolyphosphate, pH 5.0, pH 8.6, or sublethal heat) were inhibited significantly by enterocin AS-48 in apple juice, providing a means to lower the intensity of juice processing treatments (Ananou *et al.*, 2005a).

In soy milk, enterocin CCM 4231 completely eliminated *L. monocytogenes* and markedly reduced the counts of *S. aureus*. The enterococcal strainCCM4231was able to grow and produce enterocin in soy milk (Laukova & Czikkova, 1999). Nisin was also tested with satisfactory results to inhibit proliferation of *B. stearothermophilus* in soy milk, as well as thermophilic clostridia and bacilli in coconut milk/water (Thomas *et al.*, 2000).

In fruit juices and fruit drinks, nisin has been tested to prevent spoilage caused by *Alicyclobacillus* (Komitopoulou *et al.*, 1999; Yamazaki *et al.*, 2000). Because unpasteurized fruit juices may act as vehicles for transmission of enteric pathogens, bacteriocins have been tested against *E. coli* and *Salmonella* in combination with outer membrane-permeabilizing treatments. In apple juice, a combination of nisin and cinnamon accelerated death of *Salmonella* Typhimurium and *E. coli* O157:H7, enhancing the safety of the product (Yuste & Fung, 2004). When nisin and lysozyme were tested for inactivation of *Salmonella* typhimurium in orange juice in combination with PEFs, the combination of the two antimicrobials had a more pronounced bactericidal effect than either nisin or lysozyme alone (Liang *et al.*, 2002). Similarly, *E. coli* O157:H7 cells sub lethally injured by other treatments (EDTA, sodium tripolyphosphate, pH 5.0, pH 8.6, or sub lethal heat) were inhibited

significantly by enterocin AS-48 in apple juice, providing a means to lower the intensity of juice processing treatments (Ananou *et al.*, 2005a). In soy milk, enterocin CCM 4231 completely eliminated *L. monocytogenes* and markedly reduced the counts of *S. aureus*. The enterococcal strain CCM4231 was able to grow and produce enterocin in soy milk (Laukova & Czikkova, 1999). Nisin was also tested with satisfactory results to inhibit proliferation of *B. stearothermophilus* in soy milk, as well as thermophilic clostridia and bacilli in coconut milk/water (Thomas *et al.*, 2000).

2.3.3 Application of Nisin, Pediocin and Enterocin in Meat and Poultry Products

Meat and meat products provide the necessary nutrients for growth of a wide variety of microorganisms, depending on the storage conditions. Under refrigeration, oxygen availability will allow the growth of aerobic Gram-negative bacteria (especially *Pseudomonas*), whereas LAB (mainly, *Carnobacterium, Lactobacillus,* and *Leuconostoc*) predominate under anaerobic conditions (Borch *et al.*, 1996; Gram *et al.*, 2002).

Several reports have described the effects of added bacteriocin preparations on inhibition of spoilage and pathogenic bacteria in fresh meats. In beef meat, addition of pediocin PA-1 retarded growth of meat spoilage Gram-positive bacteria (Kalchayanand, 1990) and reduced the *L. monocytogenes* population (Nielsen *et al.*, 1990). Similar studies confirmed the antilisterial protection of pediocins in raw meats (Motlagh *et al.*, 1992; Taalat *et al.*, 1993; Goff *et al.*, 1996; Nieto-Lozano *et al.*, 2006). Although the application of nisin in meats is limited due to several factors such as its poor solubility, interaction with phospholipids, and inactivation by glutathione (Rayman *et al.*, 1983; Montville *et al.*, 1995; Rose *et al.*, 1999; Stergiou *et al.*, 2006), addition of nisin has been shown to extend the lag phase of *L. monocytogenes* inoculated into minced buffalo meat (Pawar *et al.*, 2000). Activity of nisin against *L. monocytogenes* in minced beef was potentiated in combination with thyme essential oil, decreasing the impact of the oil on the meat organoleptic properties (Solomakos

et al., 2008). Addition of bacteriocins to meat mixtures may also reduce the intensity of HHP treatments (Hugas *et al.*, 2002).

In a cooked ham meat model mixture, although the final effect as measured by microbial inactivation and inhibition of proliferation during further storage largely depended on the bacteriocin and the target bacteria, several bacteriocins (enterocinsA and B, sakacin K, pediocin AcH, or nisin) were shown to act synergistically in combination with HHP, (Garriga *et al.*, 2002). Application of an HHP treatment in combination with a nisin: pediocin cocktail extended the shelf-life of refrigerated roast beef artificially contaminated with clostridial spores (Kalchayanand *et al.*, 2003). Enterococci are frequently found in meat fermentations. Bacteriocinogenic enterococci could be used as co-cultures for preservation of meat products (e.g., fermented sausages and sliced vacuum-packed cooked meat products) and for the control of emergent pathogenic and spoilage bacteria (Hugas *et al.*, 2003; Foulqui´e Moreno *et al.*, 2006). When used as starter cultures in sausage fermentation, the bacteriocinogenic strains *E. faecium* CCM 4231 and *E. faecium* RZS C13 were partially competitive and strongly inhibited the growth of *Listeria* spp. (Callewaert *et al.*, 2000). *E. faecium* CTC492 (producer of enterocins A and B) partially prevented ropiness due to *L. sakei* CTC746 in sliced vacuum-packaged cooked ham (Aymerich *et al.*, 2002). The strain *E. casseliflavus* IM 416K1 (producer of enterocin 416 K1) was able to eliminate *L. monocytogenes* in artificially inoculated “cacciatore” Italian sausages (Sabia *et al.*, 2003). The cyclic bacteriocin enterocin AS-48 produced in situ by an *E. faecalis* strain or a food-grade *E. faecium* transconjugant controlled growth of *L. monocytogenes* and *S. aureus* in a meat model system (Ananou *et al.*, 2005b, 2005c). Addition of nisin to pasteurized liquid whole egg reduced the viable counts of *L. monocytogenes*, increased the product refrigerated shelf-life, and protected the liquid egg from growth of *L. monocytogenes* and *B. cereus* during storage (Delves-Broughton *et al.*, 1992; Knight *et al.*, 1999; Schuman & Sheldon, 2003). Both nisin and pediocin Pa1/Ach acted synergistically with heat treatments against *L. monocytogenes* (Muriana, 1996; Knight *et al.*, 1999), and nisin addition increased the heat

sensitivity of *Salmonella enteritidis* PT4 in liquid whole egg and in egg white during pasteurization (Boziaris *et al.*, 1998).

In fermented sausages, bacteriocinogenic LAB (especially lactobacilli and pediococci) may be selected as functional starter cultures to improve the product safety and quality (Leroy *et al.*, 2006). Addition of *L. sakei* starter cultures may reduce *Listeria* levels in fermented sausages (Schillinger *et al.*, 1991; Hugas *et al.*, 1995, 1996, 1998; De Martinis & Franco, 1998; Liserre *et al.*, 2002; Leroy *et al.*, 2005a, 2005b). A sakacin-P producing *L. sakei* strain performed satisfactorily as a starter during sausage fermentation, and significantly decreased the total bacterial counts and fecal enterococci and listeria (Urso *et al.*, 2006). Similarly, Drosinos *et al.* (2006) reported that addition of bacteriocinogenic *L. sakei* strains reduced the time required for a 4 log reduction of *L. monocytogenes* in fermented sausages. Antilisterial effects have also been demonstrated for curvacin-producing *L. curvatus* in sausage fermentations (Vogel *et al.*, 1993; Hugas *et al.*, 1996; Leroy *et al.*, 2005a) and in ostrich meat salami (Dicks *et al.*, 2004). *L. plantarum* was useful as a bacteriocinogenic sausage starter culture (Campanini *et al.*, 1993; Dicks *et al.*, 2004). Among the cocci, pediocin producing *P. acidilactici* strains have been introduced in some commercial starter cultures for fermentation of meat products, thanks to their capacity to reduce the numbers of *L. monocytogenes* in the final product (Berry *et al.*, 1990; Degnan *et al.*, 1992; Foegeding *et al.*, 1992; Baccus-Taylor *et al.*, 1993; Farber *et al.*, 1993; Lahti *et al.*, 2001; Amezquita & Brashears, 2002). Bacteriocinogenic *P. acidilactici* were proposed as indigenous starter cultures in the fermentation of Urutan, a Balinese traditional dry fermented sausage (Antara *et al.*, 2004).

2.4 NISIN AND CLASS IIA BACTERIOCIN RESISTANCE AMONG *LISTERIA* AND OTHER FOOD BORNE PATHOGENS AND SPOILAGE BACTERIA

Although the use of bacteriocins (biopreservation) is a novel approach for eliminating or controlling pathogens in food, the development of highly tolerant or resistant strains remains the main concern and decrease the efficiency of

bacteriocins as biopreservatives. Resistant food-borne pathogens are posing a global problem which is further facilitated by international trade of raw and processed foods. In foods with a long shelf life, even a small number of these resistant cells can multiply to very high number and thus may lead to food-borne outbreaks and food spoilage. Among bacteriocins produced by LAB, nisin, a class I bacteriocin, has demonstrated antilisterial activity, was the first bacteriocin to be characterized and is the only one approved for use in food applications (Chung *et al.*, 1989). Therefore, most of the research in this area has focused on specific bacteriocins, such as nisin and some of the class IIa peptides. The emergence of nisin-resistant *L. monocytogenes* mutants has already been reported (Davies & Adams, 1994; Gravesen *et al.*, 2001). In the current report, extensive study is presented to review the proposed mechanisms of nisin resistance and also class II bacteriocin resistance developed among the food borne pathogens.

2.4.1. Emergence of Nisin Resistance

The resistance responses of microorganisms to antimicrobials may be innate, apparent, or acquired. Innate resistance is a chromosomally controlled property that is naturally associated with a microorganism. Differences in resistance to antimicrobials occurring among different types, genera, species, and strains of microorganisms under identical environmental conditions and antimicrobial concentrations are most likely controlled innately. Mechanisms of innate resistance may include cellular barriers preventing entry of the antimicrobial (e.g., the outer membrane of Gram-negative bacteria and teichoic acids contained within Gram-positive bacteria), cellular efflux (i.e., mechanisms that pump compounds out of the cell), lack of a biochemical target for antimicrobial attachment or microbial inactivation, and inactivation of antimicrobials by microbial enzymes (Bower & Daeschel, 1999). Apparent resistance is related to assay or application conditions. As with any preservation technique, susceptibility to antimicrobials is dependent upon the conditions of the application. The presence of interacting stress conditions (e.g., low pH, high temperatures, high pressure) may increase or decrease the measured resistance of a microorganism. Food composition may also have a major influence on

the apparent activity of food antimicrobials, especially organic acids. Acquired resistance results from genetic changes in the microbial cell through mutation or acquisition of genetic material from plasmids (Russell, 1991).

The emergence of nisin resistant mutants, which are generated when nisin-sensitive cells are exposed to relatively high nisin concentrations, has been described for several *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bacillus* spp., and *Clostridium* spp. and for *Staphylococcus aureus*, and *L. monocytogenes* (Daeschel *et al*., 1991; Ming & Daeschel, 1993, Ming & Daeschel, 1995; Breuer & Radler, 1996, Davies *et al*., 1996). Harris *et al*. (1991) detected mutant strains of *L. monocytogenes*, at frequencies of 10^{-6} and 10^{-8}, which were able grow at nisin concentrations 5 to 10 times higher than was the original population, indicating the potential for nisin resistant variants to arise from widespread application of the lantibiotic.

2.4.2. Proposed Mechanisms for Development of Nisin Resistance

The acquisition of nisin resistance is reported to be a complex and may differ among strains, as suggested by reports on alterations in fatty acid composition and cell wall changes in nisin-resistant *Listeria*. Nisin resistance may result from the presence of a nisin-degrading enzyme, nisinase, which has been reported in several Gram-positive bacteria (Galesloot, 1956; Alifax & Chevalier, 1962; Jarvis, 1967; Lipinska 1977; Hoover & Hurst, 1993). The nisinase from several *Bacillus* species specifically reduced the C-terminal dehydroalanyl lysine of nisin to alanyllysine (Jarvis, 1970; Jarvis & Farr, 1971). Alternatively, nisin resistance genes have been reported in both nisin-producing and non producing strains of *Lactococcus lactis* ssp. *lactis;* as yet the characteristics of the products of these nisin resistance genes have not been determined. The resistance mechanism seems to be complex. In 1998, Crandall & Montville put forward a model for the nisin resistance of *Listeria monocytogenes* ATCC 700302 which included three factors: (i) variation of peptidoglycan composition (Maisnier & Richard, 1996), which should make it possible to increase the binding of divalent cations that should interact with the cationic peptide; (ii) modification of the electric charge of the membrane by change in the phospholipids

content, thereby preventing pore formation (Ming &Daeschel, 1993; Revol *et al*., 1996; Crandall & Montville, 1998); and (iii) increase in membrane rigidity, preventing peptide insertion and association (Ming & Daeschel, 1993). Resistance has been correlated with both an altered fatty acid composition (Ming & Daeschel, 1993; Mazzotta & Montville, 1997) and an altered phospholipids composition (Ming & Daeschel, 1995).

2.4.2.1 Nisin resistance through cell wall modification

Crandall & Montville (1998) studied the changes in the cell wall of the nisin resistant strain by evaluating the strain's sensitivity to cell wall-acting compounds. The nisin resistant strain was reported to be more resistant than the wild type to lysozyme, which catalyzes the hydrolysis of the β-1, 4 glycosidic bond between *N*-acetylmuramicglucosamine and *N*-acetylglucosamine of cell wall peptidoglycan, and more sensitive to the cell wall-acting antibiotics benzyl penicillin and ampicillin, which block the cross-linking reaction of peptidoglycan synthesis. These altered sensitivities suggested compositional changes in the cell wall of the nisin resistant strain.

The cell wall has also been implicated in nisin-resistant *Listeria innocua*, in which altered sensitivities to cell wall-acting antibiotics and enzymes and a thickened cell wall were observed (Maisnier & Richard, 1996). In addition, removal of the cell wall from nisin-resistant *L. monocytogenes* F6861 resulted in the loss of nisin resistance, suggesting that differences in the cell wall were responsible for resistance in this strain (Davies & Adams, 1994). Similarly a *Staphylococcus aureus* mutant reported to be defective in D-alanine incorporation to lipoteichoic acids was more sensitive to nisin than wild-type cells, and it was suggested that the positively charged D-alanine residue was excluding the positively charged nisin molecule (Peschel *et al*., 1999). Experiments with *S. bovis* indicated that the nisin-resistant cells had an increased positive (i.e., less negative) charge than nisin-sensitive cells. Nisin-resistant cells (i) bound less cytochrome *c* than nisin-sensitive cells, (ii) were more lysozyme resistant and (iii) were less hydrophobic (Mantovani & Russell, 2001).

Moreover, it has been reported that several of the class I lantibiotics as well as non lantibiotic lactococcin 972 employ lipid II- a precursor in cell wall synthesis as a docking molecule and interaction between a bacteriocin and lipid II leads to inhibition of cell wall synthesis and/or pore formation, depending on the structure of the bacteriocin and its concentration (Brotz *et al*., 1998; Wiedemann *et al*., 2001; Martinez *et al*., 2008).

2.4.2.2 Role of divalent cations in bacterial resistance against the inhibitory activity of bacteriocins

Divalent cations are required to sufficiently stabilize the altered nisin resistant cell's cytoplasmic membrane against disruption by nisin. This stabilization might involve interferance with nisin's binding or a specific interaction of the cations with membrane components or some combination of the two. Divalent cations interact extensively with cell wall components, especially the negatively charged teichoic acids. Perhaps the change in the cell wall of the nisin resistant strain requires additional divalent cations to either stabilize extra negative charge or prevent nisin from binding to anionic sites. In the presence of divalent cations, a tighter packing of the acyl chains is introduced, which can hinder nisin insertion (Harwood & Russell, 1984, Killian *et al*., 1994).

Abee *et al*. (1994) found that di- and trivalent cations (Mg^{2+}, Ca^{2+}, and Gd^{3+}) decreased the nisin Z-induced rate of K+ efflux from whole cells of *L. monocytogenes* Scott A. Alternatively or additionally, the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane. the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane. The Nisin resistant strain of *L. monocytogenes* was reported to require divalent cations to resist the inhibitory effect of nisin Crandall & Montville (1998). The effect of divalent cations was shown to be dependent on the concentration of divalent cations. Crandall & Montville (1998) suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipid head groups.

2.4.2.3 Nisin resistance through modifications of membrane phospholipid composition

The development of nisin resistance has also been correlated with both an altered fatty acid composition (Ming & Daeschel, 1993; Mazzotta & Montville, 1997,) and an altered phospholipids composition (Ming & Daeschel, 1995). Ming & Daeschel (1995) reported that the stable nisin-resistant mutant of *L. monocytogenes* contained a greater proportion of straight chain fatty acids whereas the parent contained more branched chain fatty acids; no changes in unsaturation of lipid acyl chains were reported.

In a study conducted by Verheul *et al.* (1997), it was shown that higher nisin concentrations were required to dissipate the proton motive force in a nisin resistant variant of *L. monocytogenes* Scott A compared to its parent wild type strain, which correlated with the nisin sensitive status of the strains in Brain Heart Infusion (BHI) broth. This suggested a possible role for the energy transducing cytoplasmic membrane in the acquisition of resistance in *L. monocytogenes* Scott A. The phenomenon of nisin resistance in *L. monocytogenes* Scott A was studied in detail with a focus on membrane composition. The study conducted by Verheul *et al.* (1997), also provided additional understanding of the mechanism of nisin resistance in *L. monocytogenes*. The results demonstrated that phospholipid head group alterations, particularly in the content of Diphosphatidylglycerol (DPG), were the basis of a nisin resistant variant of *L. monocytogenes* Scott A. They also reported that the nisin resistant strain produce relatively more phosphatidylglycerol (PG) and less Diphosphatidylglycerol (DPG) than does the wild type strain. It was suggested that this could be due to decreased activity of the enzyme DPG synthetase, which forms DPG by condensation of two PG molecules (Raetz, 1986). In a study conducted by Demel *et al.* (1996), it has been demonstrated that nisin penetrates more deeply into monolayers of DPG than into those of other lipids including PG, phosphatidylcholine, phosphatidylethanolamine, monogalactosyl-diacylglycerol and digalactosyldiacylglycerol.

Similar changes in membrane fatty acid composition, including increased long-chain fatty acids, decreased short chain acids, and a lower C15/C17 ratio, were found for nisin resistant *L. monocytogenes* ATCC 700302 (Mazzotta & Montville, 1997). Both the fatty acid composition change and the alteration in membrane phospholipids were observed only when nisin resistant *L. monocytogenes* ATCC 700302 was grown in the presence of nisin, indicating that nisin induced these changes (Mazzotta & Montville, 1997). Crandall & Montville (1998) confirmed that the major phospholipids in nisin resistant strain of *L. monocytogenes* ATCC 700302 contained significantly more zwitter ionic phosphatidyl-ethanolamine and less anionic phosphatidylglycerol and cardiolipin. Given the role that anionic phospholipids play in nisin's interaction with membranes (Garcera *et al.*, 1993; Driessen *et al.*, 1995; Martin *et al.*, 1996), a decrease in the net negative charge of the lipid bilayer was reported to hinder nisin's ability to bind and interact with the membrane. Also resistance to sakacin P has been reported to introduce distinct changes mainly in the polysaccharide, fatty acid, and protein regions, which represented the major cell membrane components in *L. monocytogenes* strains (Tessema *et al.*, 2009).

2.4.3 Resistance to Class IIa Bacteriocins from the Loss of *mpt* Expression

It has been suggested that resistance to class IIa bacteriocins (Nes *et al.*, 2007) occurs at either a low (2-4 fold increase in Minimum Inhibitory Concentration i.e.MIC) or a high level (1000-fold increase in MIC). High-level resistance of *E. faecalis*, *L. monocytogenes* and some other Gram-positive bacteria to class IIa bacteriocins has been reported to result from the loss of expression of Mannose-Specific Phosphotransferase System (*mpt*), either in defined mutants or in spontaneous resistant strains (Gravesen *et al.*, 2002).

Some reports suggest that spontaneous resistance of *L. monocytogenes* is accompanied by a respective increase and decrease of two phosphoenolpyruvate-dependent Phospho-transferase systems, which are responsible for sugar uptake and phosphorylation in Gram-negative and Gram-positive bacteria (Postma *et al.*, 1993; Saier & Reizer, 1994). The *mpt* operon encodes a mannose permease (EII_t^{Man}) which belongs to the Mannose Specific Phosphotrnsferase System and the mpt –PTS

transporter family is responsible for the import and phosphorylation of carbohydrates such as mannose and glucose in bacteria (Postma *et al.*, 1993). A phosphate moiety is transferred from phosphoenolpyruvate (PEP) to the transported sugar via the PTS proteins EI, HPr and EII. The latter is a complex permease, made of three or four subunits which can be fused: IIA and IIB are cytoplasmic subunits responsible for phosphorylation, whereas IIC is an integral membrane subunit involved in sugar transport. An IID membrane subunit, associated with IIC, is found specifically in permeases of the mannose family. Sigma 54 (σ^{54}) has been described to be involved in expression of Phosphotransferase systems. Sigma (σ) factors are subunits of the RNA polymerase holoenzyme involved in the initial step of transcription. Among them, σ^{54} is unique since it targets conserved -24/-12 promoter sequences and requires an activator protein for transcription initiation, referred to as σ^{54}-associated activator. Also, as the genes encoding these four subunits are clusterd in one operon, their expression has been reported to be coordinated (Morett & Segovia 1993; Shingler 1996).

Based on the observations that resistant mutants have an altered expression pattern of man-PTS and that heterologous expression of cloned man- PTS genes renders resistant cells sensitive, some studies have shown that the mannose-phosphotransferase system (man-PTS) might serve as a receptor for some pediocin-like bacteriocins, (Ramnath *et al.*, 2000; Dalet *et al.*, 2001; He´chard *et al.*, 2001; Gravesen *et al.*, 2002;, Ramnath *et al.*, 2004). Also, it has been demonstrated that the membrane components (ManM/PtnC and ManN/PtnD) of the man-PTS are directly involved as receptors for several bacteriocins from subclass IIa and some from subclass IIc, and that in bacteriocin producing cells, a cognate immunity protein tightly binds the receptor in a bacteriocin-dependent manner, to prevent killing by the bacteriocin (Diep *et al.*, 2007). The level of mpt expression has been shown to correlate with the level of bacteriocin sensitivity (Dalet *et al.*, 2001; He´chard *et al.*, 2001), which implies that EII_t^{Man} permease might be a target molecule for class IIa bacteriocins. Two bacteriocin resistant spontaneous mutants of *L. monocytogenes* were linked to phosphotransferase systems (PTSs). In the first mutant, resistant to leucocin A, a two dimensional SDS-PAGE protein analysis

revealed absence of a IIAB subunit of a PTS permease (Ramnath *et al.*, 2000). In the second mutant, resistant to pediocin PA-1, a *β*-glucoside PTS permease was described to be over-expressed (Gravesen *et al.*, 2000). Finally, Dalet *et al.* (2001) described that interruption of genes encoding a σ^{54}- associated activator (MptR) and a 1 σ^{54} dependent PTS permease of the mannose family (termed EII_t^{Man}) led to resistance of *E. faecalis* to Mesentericin Y105. To find a link between σ^{54} and sensitivity of *L. monocytogenes* to Mesentericin Y105, Dalet *et al.* (2001) searched for σ^{54}-associated activators and σ^{54} dependent genes in the *L. monocytogenes* EGDe genome. They found an activator and a PTS permease of the mannose family that are required for sensitivity of *L. monocytogenes* to Mesentericin Y105. Dalet et al. 2001 also hypothesized that the transcriptional factor σ^{54} (encoded by *rpoN*) could direct the expression of a receptor since *rpoN* mutants of *L. monocytogenes* (Robichon et al. 1997) and *E. faecalis* (Dalet et al. 2000) were shown to be resistant to mesentericin Y105 and related subclass IIa bacteriocins.

Attempts were made to unravel the resistance of *L. monocytogenes* to DivercinV41 (DvnV41) which involved a proteomic approach, comparing protein profiles of *L. monocytogenes* wild-type and a mutant resistant to DvnV41. *L. monocytogenes* with a DvnV41-resistant phenotype displayed differential protein synthesis (Duffes *et al.*, 2000). This mutant strain was reported to lack at least nine protein spots, two of which were shown to have a molecular mass and pI matching those of the *mptA* cluster, which is controlled by the σ^{54} transcription factor in coordination with the ManR regulator (Dalet *et al.*, 2001). It was subsequently proposed that high level of resistance to class IIa bacteriocins was acquired through one general mechanism: the lack of EII_t^{Man} expression (Gravesen *et al.*, 2002). Vadyvaloo *et al.* (2004) reported that the presence of glucose or mannose induced the sensitivity of *L. monocytogenes* and *E. faecalis* to Mesentericin Y105. These sugars also induced expression of the *mpt* operon of *L. monocytogenes*, indicating that EII_t^{Man} transports glucose and mannose in accordance with previous observations showing a specific inducible effect of the transported sugar on PTS permease expression (Postma *et al.*, 1993). These correlated results suggested that the level of EII_t^{Man} expression is directly linked to sensitivity of *L. monocytogenes* to Mesentericin Y105 (MesY105).

Vadyvaloo *et al.* (2004) investigated the effect of the missing *MptA* subunit of the EII_t^{Man} on glucose metabolism in Class IIa bacteriocin-resistant *L. monocytogenes* strains. They focused on glucose consumption rates and analysis of the end products of glucose metabolism. The observation that the specific growth rate in Class IIa bacteriocin-resistant strains, B73-MR1 and EGDe-mptA, on media containing glucose was lower than that of the corresponding wild-type strains has also been described for another class IIa bacteriocin resistant *L. monocytogenes* strain, 412P, also showing loss of MptA expression (Gravesen *et al.*, 2002). The decreased growth rate in 412P, and in other Class IIa bacteriocin-resistant B73 strains (Dykes & Hastings, 1998) has been interpreted as a fitness cost associated with class IIa bacteriocin resistance. This fitness cost was thought to be due to energy expensive metabolic pathways in resistant strains (Dykes & Hastings, 1998). By taking a closer look at the physiology of these resistant strains, one can suggest explanation for the reduction in specific growth rate, namely the reduced consumption rate of glucose. It was reported that EII_t^{Man} may be the major transporter of glucose for *L. monocytogenes* considering the greater than 50% decrease in glucose consumption rate observed for the resistan strains lacking MptA and evidence for the existence of only the glucose-specific enzyme IIA component and no other functional components of the glucose specific PTS in *L. monocytogenes* EGDe (Glaser *et al.*, 2001). Furthermore, Vadyvaloo *et al.* (2004) suggested that the lower activity of glucose transporting enzymes causing extensive decrease in the glucose consumption rate was responsible for the decrease in specific growth rate. In contrast to the results obtained in media containing glucose, they observed an increased specific growth rate for the resistant strains compared to the wild type strains in the absence of glucose. They suggested that it might due to the missing glucose transporter, an up-regulation of metabolic routes for other substrates has occurred which gives these cells an advantage in the absence of glucose. During the physiological characterization Vadyvaloo *et al.* (2004) noted that, in addition to the apparent disadvantage of a lower growth rate in the presence of glucose, the resistant strains had a higher biomass yield on glucose. A product analysis revealed that the resistant strains had more of a mixed acid type of fermentation as compared to the homolactic fermentation in the wild-type strains. In a homolactic fermentation, 2 mol ATP is

formed per mole of glucose fermented and in a pure mixed acid fermentation (i.e. no lactate formed and acetate and ethanol formed in a 1: 1 ratio), 3 mol ATP is formed per mole of glucose fermented. Their results indicated that physiological responses related to the absence of MptA in class IIa bacteriocin-resistant strains could further compromise the potential use of class IIa bacteriocins as biopreservatives. Although resistant strains, in the presence of glucose, showed a lower specific growth rate than the wild-type strains, it was reported that the biomass yield on glucose (and potentially other energy sources) was significantly increased. Vadyvaloo *et al.* (2004) also reported that together with the inactivation of the MptA, a shift in metabolism occurs that could significantly alter the final concentrations of the fermentation products suggesting a strong possibility that the end product of metabolism in lactic acid bacteria starter cultures could change as a result of acquiring this type of resistance to class IIa bacteriocins .The shift in metabolism and subsequent change in the end product would profoundly influence both the organoleptic qualities and spoilage potential of the food product.

Besides the high resistance strains, two spontaneous mutants of *L. monocytogenes*, 1 B73-V1 and B73- V2, were also described to have an intermediate resistance level (2–4 times the IC50 for wild type sensitive strain) to leucocin A, a class IIa bacteriocin (Vadyvaloo *et al.*, 2002). Some modifications of membrane phospholipids were associated to this intermediate resistance phenotype, although the authors suggested that other mechanisms are likely to be involved. To characterize the intermediate resistance, Arous *et al.* (2004) studied a new intermediate resistant strain that was interrupted in a mannose PTS operon similar to mpt, namely *mpo.* The mpo operon is composed of four open reading frames, *mpoA, mpoB, mpoC and mpoD.* They putatively encode four proteins, MpoA (124 aa), MpoB (161 aa), MpoC (295 aa) and MpoD (292 aa), which show greatest similarities with PTS permease subunits of the mannose family. Mpo is located just downstream of the gene encoding ManR, the σ54-dependent activator that is responsible for *mptACD* expression. While characterizing the intermediate resistance mechanism at the molecular level, the expressions of both *mptACD* and *mpoABCD* were followed by real time PCR in each mutant strain compared to the wild type as a reference. The

highly resistant strains, namely EGK54 and B73-MR1were reported to exhibit an induced *mpo* expression (11.3- and 3.6-fold, respectively), while no significant changes were observed in strains B73-V1 and B73-V2. The highly resistant strains were dramatically affected in EII_t^{Man} expression, suggesting that *mpo* was induced by the loss of EII_t^{Man} expression. A similar mechanism of regulation has been described for a β-glucoside permease gene, which is highly expressed in these mutants (Gravesen *et al.*, 2000) as well as in a *rpoN* mutant (Arous *et al.*, 2004). Arous *et al.* (2004) also hypothesized that *mpo* is regulated by carbon catabolite regulation (CCR), as a putative catabolite responsive element (CRE) sequence was located upstream from *mpoABCD*, Given that the IIC and IID subunits of *mpt* operon are probably present in the membrane, these two subunits were reported to be the potential targets of Class IIa bacteriocins. The *mptACD* operon of *L. monocytogenes* heterologously expressed in an insensitive species, such as *Lactococcus lactis* (Ramnath *et al.*, 2004), rendered this strain sensitive to various Class IIa bacteriocins. Each gene of the *mptACD* operon was expressed independently in *Lactococcus lactis* and the expression of *mptC* alone was found to be sufficient to confer sensitivity. The IIC subunit was therefore proposed as the target molecule of the Class IIa bacteriocins (Ramnath *et al.*, 2004). Enhanced nisin resistance in some mutants of *Listeria monocytogenes* has also been associated with increased expression of three genes, *pbp2229*, *hpk1021*, and *lmo2487*, encoding a penicillin binding protein, a histidine kinase, and a protein of unknown function, respectively. (Gravesen *et al.*, 2004).

Calvez *et al.* (2007) also identified three genes associated with the resistance of *E. faecalis* JH2-2 to Divercin V41 (DvnV41). The first one was the *rpoN* gene, which encodes the σ^{54} factor, an alternative subunit of RNA polymerase responsible for the transcription of a specific set of genes. It has been postulated that *rpoN* is involved in the expression of a target molecule for Class IIa bacteriocins, loss of whose expression leads to resistance (Drider *et al.*, 2006). The second gene identified encoded a putative glycerophosphoryl diester phosphoesterase (GlpQ), an exoprotein found to participate in fatty acid and phospholipids degradation in many bacteria (Antelmann *et al.*, 2000). A hypothesis that could be drawn from data given by

Calvez *et al.* (2007) was that the absence of GlpQ activity in mutant 36H4 led to an intact fatty acid and phospholipids composition of the cell membrane, which contributed to the resistant phenotype of the mutant. Moreover, the GlpQ protein was originally suggested to belong to the Pho regulon in *Bacillus subtilis* (Antelmann *et al.*, 2000), and to be governed by a pleiotropic two-component regulatory system PhoP-PhoR (Groisman, 2001). Interestingly, PhoP and Mg2+ 11 were also reported to control the resistance of many Gram negative bacteria to antimicrobial peptides, as supported by a number of lines of evidence (Groisman *et al.*, 1992; Moss *et al.*, 2001). Similarly, in Gram-positive bacteria, different studies highlighted the role of a two-component regulatory system in the resistance of *L. monocytogenes* (Cotter *et al.*, 2002), *E. faecalis* (Comenge *et al.*, 2003) and *Staphylococcus aureus* (Kuroda *et al.*, 2003) to antimicrobial peptides and antibiotics. In order to gain a more comprehensive view of the role of a two-component signal transduction system pathway in resistance to pediocin-like bacteriocins, Calvez *et al.* (2007), examined the resistance of each insertional mutant characterized so far in *E. faecalis* JH2-2 (Le Breton *et al.*, 2003) and *E. faecalis* V583 (Hancock & Perego, 2004). The results obtained rejected any relationship between a two-component regulatory system and resistance to DvnV41. Finally, the third gene identified encoded a putative phosphoesterase (PDE). Overall, it was reported that the *rpoN* gene was associated with the high level resistance and the newly identified genes with the intermediate resistance of *E. faecalis* JH2-2 to DvnV41, MesY105 and Ped PA 1/Ach.

Listeria orthologs of enterococcal *pde* and *glpQ* genes, i.e. two ORFs named *lmo0052* and *lmo1292*, exhibiting the highest similarity (65.4 and 60.7 %) to enterococcal *pde* and *glpQ* gene respectively were identified in the genome of *L. monocytogenes* EGDe (Calvez *et al.*, 2008a). Also, the relative expression of *mptABCD* operon, *glpQ, pde, rpoN, mptR* and *gap*-1 was studied by reverse transcription combined with the real time polymerase chain reaction to understand the role of each gene in resistance/sensitivity of *E. faecalis* JH-2 to class IIa bacteriocins such as recombinant Divercin V41 (DvnRV41) (Calvez *et al.*, 2008b). DvnRV41 was reported to up-regulate the expression of *gap*-1 gene, which codes for glyceraldehyde 3

phosphate dehydrogenase, an enzyme involved in glycolysis and gluconeogenesis. The up-regulation of *mptC* and *gap*-1 genes indicated that *E. faecalis* JH2-2 could activate sugar metabolism. The down-regulation of *glpQ* gene by DvnRV41 was not expected and resulted in contradictory to the former results (Calvez *et al.*, 2007), which ruled out that resistant phenotype of *E. faecalis* JH2-2 to DvnV41 was acquired after inactivation of *glpQ* gene. Finally, the down regulation of *glpQ* gene was attributed to interplay and/or energy compensation between down- and up-regulated genes. Also it was suggested that the expression of glpQ could depend on the growth conditions, amount of DvnV41 and growth phase (Calvez *et al.*, 2008b).

2.5 COMBINED EFFECT OF BACTERIOCINS ON THE EMERGENCE OF BACTERIOCIN RESISTANCE

Several studies have shown that the combinations of different bacteriocins produce a more effective antibacterial effect against food-borne pathogens in comparison to the bacteriocins used alone. Hanlin *et al.* (1993) while studying the antibacterial efficiency of pediocin AcH and nisin against several Gram-positive bacterial strains assumed that a mixture containing more than one bacteriocins would have greater bactericidal effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. Moreover synergistic effects were reported when the interactions between pairs of bacteriocins from lactic acid bacteria were tested (Mullet-Powell *et al.*, 1998). One possible explanation for the different effectiveness of bacteriocin pairs would be that the bacteriocins used in this study belonged to different classes, which vary considerably in the nature and sequence of amino acid residues as earlier suggested by Moll *et al.* (1999). Simultaneous or sequential additions of nisin (50 IU/ml) and curvaticin 13 (160 AU/ml) also induced a greater inhibitory effect against *L. monocytogenes* than each bacteriocin individually (Bouttefroy & Millière, 2000). The simultaneous use of two or more bacteriocins could be useful not only to lower the added bacteriocin doses, but also to avoid regrowth of bacteriocin-resistant/adapted cells. The synergistic action of combinations of two different bacteriocins with different structures produced by the same strain has also been reported in agar medium by Limonet *et al.* (2004). Similar results have been reported by Jamuna *et al.* (2005) who showed that the bacteriocins

from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems. Vignolo *et al.* (2000) also reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT in meat slurry showed no viable counts after incubation for 3 h. Jamuna & Jeevaratnam (2009) have also reported the synergistic effect of Nisin and bacteriocin from *Pediococcus acidilactici* to be more effective in inhibiting the growth of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav.

2.6 CROSS RESISTANCE TO BACTERIOCINS

Resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *L. monocytogenes* was shown to be cross resistant to the Class IIa bacteriocin, pediocin PA-1 and the Class IV leuconocin S. Pediocin PA-1 is a 44-amino acid peptide whose sequence has been determined (Henderson *et al.*, 1992) and modeled into a three dimensional structure (Chen *et al.*, 1997) which predicts that initial binding to membranes is through electrostatic interactions. Leuconocin S is a small (molecular weight, 10,000) glycoprotein. Both leuconocin S and pediocin PA-1, like nisin, act against *L. monocytogenes* by depleting the Proton Motive Force (Bruno & Montville, 1993). *L. monocytogenes* mutants resistant to mesenterocin 52, curvaticin 13, and plantaricin were also reported to be cross-resistant to the other bacteriocins (Rekhiff *et al.*, 1994). In addition, piscicolin 126-resistant mutants of *L. monocytogenes* which emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02 (Wan *et al.*, 1997). These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy (Hanlin *et al.*, 1993) might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the others.

Cross-resistance between bacteriocins has been observed when the sensitivity of *Listeria* variants to lactocin 705, enterocin CRL35, and nisin was tested. Similar results were obtained by Rekhif *et al.* (1994), who reported that mutants of *L. monocytogenes* ATCC 15313 resistant to one of three bacteriocins tested

(mesenterocin 52, curvaticin 13, and plantaricin C19) displayed more resistance to the two other, but not to nisin. Insensitivity of a variant to lactocin 705 and enterocin CRL35 while retaining sensitivity to nisin, and vice versa, may be associated with the mechanism by which a bacteriocin enters the cell following binding to the cell surface, as well as with the ability to form pores in bacterial membranes.

2.7 EPILOGUE

The use of bacteriocins in biopreservation is a novel approach in eliminating or controlling *L. monocytogenes* and other pathogens in food while the development of resistant variants of the organism remains the main concern and limits this use. There have been systematic studies to investigate bacteriocin resistance in bacterial pathogens. Most of the data exist on *Listeria* spp. while the number of reports on development of bacteriocin resistance among other pathogens or food spoilage bacteria is limited. The acquiring of resistance to bacteriocins can significantly affect physiological activity, alter cell-envelope lipid composition and modify the antibiotic susceptibility/resistance profile of bacteria. Improved control of the target microorganisms and inhibition of bacteriocin-resistant strains and species can be obtained by using a combination of one or more bacteriocins. However, resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes.

Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage. Among the food borne pathogens, knowledge of the characteristics of bacteriocin resistant variants and the conditions that prevent their emergence will help in determining the optimal conditions for application of bacteriocins in foods and minimize the incidence of resistance.

3. MATERIALS AND METHODS

The composition of all the bacteriological media, buffers and reagents used in this study are given in Appendix I, II and III.

The media and AR or reagent grade chemicals and reagents, used in this study (except molecular biology work), were procured from different manufacturers viz HiMedia (Mumbai), SRL (Mumbai), Qualigens (Mumbai), and Sigma-Aldrich (CA, USA) while Molecular Biology Grade chemicals were purchased from Bangalore Genei (Bangalore) and Fermentas (Hanoveer, USA).

3.1 SELECTION OF CULTURES

3.1.1 Bacterial Strains and Culture Conditions:

E. faecium FH99, bacteriocinogenic strain was an isolate from human faeces (Gupta *et al*., 2010). *Pediococcus acidilactici* LB 42 (an indicator strain used for the detection of bacteriocin producers), was obtained from Prof Bibek ray, Department of Animal Science, University of Wyoming, Laramie Wyoming, USA. *E. faecalis* DSMZ 20477 was obtained from Dr. Ulrich Schillinger, Institute of Biotechnology, Federal Research Centre for Nutrition and Food (BFEL), Karlsruhe, Germany. *E. faecium* VRE (a vancomycin resistant strain isolated from human faeces). *E. faecalis* 29212 and *L. monocytogenes* ATCC 53135 were procured from American Type Culture Collection (ATCC) while *L. monocytogenes* MTCC 657 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Culture conditions and the culture medium used for the bacterial cultures are given in Table 3.1.

3.2 BACTERIOCINS

One liter aliquots of MRS broth (pH 6.5) (HiMedia, Mumbai) were inoculated with active cultures of *E. faecium* FH 99 (Gupta et al., 2010) and *Pediococcus pentosaceus* 34 (1%) and incubated at 37°C for 24 h. Cell free culture supernatants (CFCS) were prepared by centrifugation of the cultures in refrigerated centrifuge at 10,000 rpm

for 10 minutes, filter sterilized by passing through a 0.2μ, 45 mm diameter membrane filter and used for partial purification after neutralization. To 1000 ml of cell free culture supernatant, ammonium sulfate was added slowly with constant stirring to achieve 60% saturation and stirring was continued for another one hour in a cold room at 5-7ºC. The mixture was then kept overnight in the cold room. It was then centrifuged at 10,000 rpm for 20 min and the precipitates were recovered. The supernatant was subsequently adjusted to 80% saturation levels by further addition of solid ammonium sulfate. The pellet in each case was dissolved in Milli Q water. Nisin A (Nisaplin ®) was procured from Danisco (Gurgaon, India). Nisin stock solutions were prepared from pure nisin in 0.02 N HCl and autoclaved.

Table 3.1 Bacterial strains used in the study and culture conditions

Bacteria	Strains	Culture Conditions
Pediococcus pentosaceous 34	Bacteriocinogenic strain ; Lab. Isolate	37°C, MRS
Enterococcus faecium FH99	Bacteriocinogenic strain ; Lab. Isolate	37°C, MRS
Pediociccus acidilactici	LB 42 (Indicator strain)	37°C, MRS
Enterococcus feacalis	ATCC 29212	37°C, BHI
Listeria monocytogenes	ATCC 53132	37°C, BHI
Listeria monocytogenes	MTCC 657	37°C, BHI
Enterococcus faecium	DSMZ 20477	37°C, BHI
Enterococcus faecium VRE	Vancomycin Resistant Strain (VRE) Lab. isolate	37°C, BHI

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures); MTCC- Microbial Type Culture Collection (Chandigarh, India); ATCC- American Type Culture Collection; BHI-Brain Heart Infusion Broth; MRS- de Mann Rogosa and Sharpe

3.3 MEASUREMENT OF ACTIVITY UNITS (AU)

The inhibitory spectrum of activity was determined using the spot on lawn assay as described by Ulhman *et al.*1992, against the indicator strain *Ped. acidilactici* LB 42. Five microlitres of the partially purified bacteriocin of *Enterococcus faecium* FH 99

and *Pediococcus pentosaceus* 34 grown in MRS broth (De Man *et al.*, 1960) was spotted on the plates TGE agar plates (Biswas *et al.*, 1991) (1.5% agar). Before spotting, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism. Plates were kept undisturbed for 3-4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of *Ped. acidilactici* LB 42. was evaluated by checking for clear zones around the spots. Three independent replicates of experiment were done. The activity units of the culture broth were calculated using the following formula and expressed as activity units per ml:

Activity Units per ml (AU/ml) = 200 × Reciprocal of highest dilution that gave a clear zone

3.4 BACTERIOCIN SUSCEPTIBILITY TEST AND DETERMINATION OF MINIMUM INHIBITORY CONCENTRATIONS (MICs)

The inhibitory spectrum of activity was obtained using the spot on lawn assay as described by Ulhman *et al.*, (1992) against *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657 *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212. Five microlitres of the partially purified bacteriocin of *Enterococcus faecium* FH 99 and *Pediococcus pentosaceus* 34 grown in MRS broth (De Man *et al.*, 1960) was spotted on TGE agar plates (1.5% agar) overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism (Biswas *et al.*, 1991). Plates were kept undisturbed for 3-4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. For MIC determinations, 5μl of a 1:2 dilution series of a bacteriocin solution was spotted. The MIC was defined as the lowest concentration of bacteriocin that induced an inhibition zone. Three independent replicates of experiment were done.

3.5 KINETICS OF CELL GROWTH INHIBITION BY BACTERIOCINS

Overnight cultures of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 were inoculated into fresh BHI broth tubes (1%) containing nisin, pediocin 34 or enterocin

FH99 individually or in combination (the mixture contained the calculated MICs of each bacteriocin). Additionally, the efficacy of nisin, pediocin 34 and enterocin FH99 in combination (half the concentration of MICs for respective bacteriocin) was also evaluated. The survivors were enumerated after different time intervals (1h, 2h, 4h, 6h and 24h) by plating the appropriate dilutions on Brain Heart Infusion (BHI) agar. The colonies were counted after 24-48 h of incubation at 37°C. Three independent replicates of the experiment were done.

3.6 ISOLATION OF SPONTANEOUS BACTERIOCIN RESISTANT VARIANTS

Spontaneous resistant mutants of strains *L. monocytogenes* ATCC 53135*, L. monocytogenes* MTCC 657*, E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to nisin, pediocin 34 and enterocin FH99 were isolated after sequential exposure to a bacteriocin concentration 10-fold higher the MIC. Only nisin resistant variant for *E. faecalis* ATCC 29212 was developed since it was already resistant to pediocin 34 and enterocin FH99. The stability of these resistances in cultures without bacteriocins was checked and determined by MICs.

3.7 BACTERIOCIN CROSS-RESISTANCE BY AGAR WELL DIFFUSION METHOD

The sensitivity of *L. monocytogenes* ATCC 53135*, L. monocytogenes* MTCC 657*, E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their resistant variants to nisin, pediocin 34 and enterocin FH99 were qualitatively determined by the agar well diffusion method. Five milliliter of molten TGE agar containing 0.75% (w/v) agar medium was cooled to 47°C and seeded with 1% (v/v) overnight BHI culture of *E. faecium* DSMZ 20477, *E. faecium* (VRE), *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 or their nisin, pediocin 34 or enterocin FH99 resistant variants. Seeded agar was then poured onto TGE agar plate and allowed to solidify at room temperature. Wells (8 mm) were cut in the solidified agar using a sterile metal cork borer and filled with 80µl of sample. The plates were left at 5°C for 2 h to allow diffusion of the tested aliquot and then incubated for 18 h at 37°C. Absence or presence of inhibition zones

as well as their diameters were recorded. Three independent replicates of experiment were done.

3.8 ANTIBIOTIC DISC DIFFUSION SUSCEPTIBILITY TEST

Pattern of resistance/susceptibility to antibiotics of wild type and bacteriocin resistant variants isolates was studied by disc diffusion method as recommended by National Committee for Clinical Laboratory Standards (CLSI; Wayne, PA, USA). Antibiotic discs containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin G (10 units), novobiocin (30 μg), bacitracin (10 μg), streptomycin (10 μg), tetracycline (30 μg), vancomycin (30 μg), rifampicin (5 μg), nalidixic acid and kanamycin (30 μg) were obtained from HiMedia. All the measurements were done in triplicate.

Approximately twenty millilitre of TGE agar was poured in Petri plate and allowed to solidify. Then it was overlaid with a 5 ml of TGE soft agar (0.75% w/v) seeded with 0.5 ml of the overnight culture of the sensitive *E. faecium* DSMZ 20477, *E. faecium* (VRE), *and E. faecalis* ATCC 29212*, L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 or their nisin / pediocin 34 / enterocin FH99 resistant variants. Antibiotic discs were placed on the surface of each of the plates. Growth inhibition zone diameter was measured in millimeters after incubation for 24-72 h at 37°C. Diameter (mm) of zone of inhibition was measured using antibiotic zone scale and results are expressed in terms of resistance, moderate susceptibility or susceptibility by comparing with the interpretative zone diameters given by Performance Standards for Antimicrobial Disk Susceptibility tests (CLSI, 2007) for disc diffusion antibiotic susceptibility test. Three independent replicates of experiment were done.

3.9 CROSS RESISTANCE TO LOW pH, POTASSIUM SORBATE, SODIUM CHLORIDE AND SODIUM NITRITE.

Experiments were conducted to examine the sensitivity of bacteriocin resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to

common food preservatives i.e. sodium chloride, low pH, sodium nitrite and potassium sorbate in comparison to the parent bacteriocin *sensitive* strains and to determine if resistance to bacteriocins confers cross-resistance to these common food preservatives. BHI broth was supplemented with NaCl to final concentrations of 3%, 4%, 4.5%, 5%, 5.5%, 6%, 12%, and 13% (wt/vol) (control, BHI without additional NaCl); (ii) BHI broth was acidified with concentrated HCl to final pH values of 5.2, 5.0, 4.8, 4.6 and 4.4 (control, BHI pH 7.4); BHI broth was supplemented with sodium nitrite to final concentrations of 20, 25, 30, 35, 40, 45, 50 and 55µg/ml (control, BHI without additional sodium nitrite) and potassium sorbate to final concentrations of 2, 2.5, 3.0, 3.5, 4, 4.5, 5, 6, 5.5 mg/ml (control: BHI without additional potassium sorbate). Three independent replicates of experiment were done.

3.10 MEASUREMENT OF HEAT RESISTANCE

3.10.1 Effect of Heat on Wild Type and Nisin Resistant, Pediocin 34 and Enterocin FH99 Resistant Variants

The heat resistance of sensitive (wild type) and the nisin resistant, pediocin 34 and enterocin FH99 variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 was assayed at 45, 50 and 55°C. Sensitive strains and their corresponding nisin, pediocin 34 and enterocin FH99 resistant variants were inoculated into 100ml of BHI broth in 250ml flasks. Cells were incubated without aeration at 37°C and were grown until late exponential phase. Cells were then harvested by centrifugation for 10min at 13,000 g at 4°C. The cells were washed twice with 0.1mol/l 2- (N-Morpholino) ethanesulphonic acid buffer (MES), pH 7.0. The heat treatment was performed by transferring 1ml of the cell suspension into 9ml of 0.1mol/l MES buffer pre- heated to the appropriate temperature. Samples were taken at preset time intervals (5, 10, 15 and 20 min) and immediately cooled on ice. Heat-treated samples were diluted 10-fold. Appropriate dilutions were plated on BHI agar plates. All plates were incubated at 37°C for 24-48 h and colonies were counted. Three independent replicates of experiment were done.

3.10.2 Synergistic effect of Heat + Nisin and Heat + Pediocin 34 and Heat +Enterocin FH99 on Wild Type Strains and Their Bacteriocin Resistant Variants

The sensitivity of wild type, nisin resistant, pediocin 34 and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to the combination of heat (45, 50 and 55°C), nisin, pediocin 34 and enterocin FH99 was determined. The menstrum for all experiments was 0.1mol/l 2- (N-Morpholino) ethanesulphonic acid buffer (MES), pH 7.0. Bacteriocins were added to the heating menstrum before the heat treatment. Cells were incubated without aeration at 37°C and were grown until late exponential phase. Cells were then harvested by centrifugation for 10min at 13,000 g at 4°C. The cells were washed twice with 0.1mol/l 2- (N-Morpholino) ethanesulphonic acid buffer (MES), pH 7.0. The heat treatment was performed by transferring 1ml of the cell suspension into 9ml of 0.1mol/l MES buffer pre- heated to the appropriate temperature. Samples were taken at preset time intervals (5, 10, 15 and 20 min) and immediately cooled on ice. Heat-treated samples were diluted 10-fold. Appropriate dilutions were plated on BHI agar plates. All plates were incubated at 37°C for 24-48 h and colonies were counted. Three independent replicates of experiment were done.

3.11 COMPARITIVE EVALUATION OF CHANGES THAT OCCUR BETWEEN RESISTANT AND WILD TYPE STRAINS AT CELLULAR AND MOLECULAR LEVEL

3.11.1 Scanning Electron Microscopy of Wild Type Strains and Their Nisin, Pediocin 34 and Enterocin FH99 resistant variants

To visualize differences occurring in the morphology between wild type *Listeria monocytogenes* ATCC 53135 grown and its nisin, pediocin 34 and enterocin FH99 resistant variants were grown overnight BHI broth. Bacterial cells were washed in 0.1 M phosphate buffered saline (pH 7.0) and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0). After 2 h on a rotator (2 rpm) at room temperature, the samples were washed three times with the same buffer and post fixed with 2% osmium tetroxide in 0.1M sodium cacodylate buffer (pH 7.0). After 1 h at room temperature, the samples were washed three times in the same buffer and

for dehydration of cells, graded series of ethanol water mixtures of 30%, 50%, 70% and absolute alcohol were used. After dehydration in absolute alcohol, these were subsequently dehydrated in propylene oxide for 15 min and allowed to dry. Dried samples were mounted on aluminium stubs with silver paint and sputter coated with gold at approximately 200 A° thickness. The samples were visualized with a Scanning Electron Microscopy (SEM), Model 501, Philips - Holland with EDAX and EBIC attachments.

3.11.2 Divalent-Cation Requirement of Nisin resistant, Pediocin 34 resistant and Enterocin FH99 Resistant Variants

Overnight cultures of wild-type or nisin, pediocin 34 and enterocin FH99 resistant variants were harvested by centrifugation (10,000 rpm/min), washed once with phosphate-buffered saline, and resuspended in 50 mM MES (morpholineethanesulfonic acid) buffer (pH 6.5) containing either divalent cations alone, divalent cations plus EDTA, EDTA alone, or none of these. The metal ions tested, (final concentration of 10 mM), were $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$. The final concentration of EDTA was 20 mM. The cells were then treated with a final concentration of 50 IU of Nisin, 500AU/ml of Pediocin 34 and 120 AU/ml of Enterocin FH99 for 20 min. Control samples were not treated with Nisin or Pediocin 34. The cells were then diluted in saline and plated on BHI agar plates. The plates were incubated at 37°C, and colonies were counted after 48 h. The results are reported as log reductions in cell viability relative to an untreated control. Three independent replicates of the experiment were done.

3.11.3 Lysozyme Sensitivity of Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

Overnight cultures of wild-type cells and nisin, pediocin 34 and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212, were inoculated at 1% (vol/vol) into fresh BHI containing 2 mg of lysozyme (Sigma) per ml. The cultures were incubated at 37°C, and samples were drawn at different time intervals (1h, 2h, 4h, 6h and 24h). The survivors, at each time interval were enumerated on BHI agar medium after appropriate dilutions in saline, and colonies

were counted after 24-48 h of incubation at 37°C. Three independent replicates of experiment were done.

3.11.4 Role of Various Sugars on the Sensitivity of Wild and Resistant Variants to the Bacteriocins

The effect of different sugars on the sensitivity of parent (wild type) and resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to Nisin, Pediocin34 and Enterocin FH99 was also evaluated. In order to see whether sugars influence the sensitivity in a dose-dependent manner, the wild type strains and their respective bacteriocin resistant variants were grown in medium supplemented with sugars viz. glucose, mannose, fructose and cellobiose at various concentrations (5mM, 10mM, 15mM, 20mM and 25mM) followed by the measurement of optical density at 600nm. The composition of the medium is shown in Appendix I. The experimental control included the medium without additional sugars. Three independent replicates of experiment were done.

3.11.5 Role of the Cell Envelope of *L. monocytogenes* MTCC 657*, L. monocytogenes* ATCC 53135*, E. faecium* DSMZ 20477*, E. faecium* (VRE*)* and *E. faecalis* ATCC 29212 in the Acquisition of Bacteriocin Resistance

3.11.5.1 Protoplast formation

Protoplasts of wild type *L. monocytogenes* MTCC 657*, L. monocytogenes* ATCC 53135*, E. faecium* DSMZ 20477*, E. faecium* (VRE*) and E. faecalis* ATCC 29212 and their respective bacteriocin resistant variants were prepared using the method of Ghosh and Murray (1967). *L. monocytogenes* MTCC 657*, L. monocytogenes* ATCC 53135*, E. faecium* DSMZ 20477*, E. faecium* (VRE*) and E. faecalis* ATCC 29212 and their nisin, pediocin 34 and enterocin FH99 resistant variants were grown in 100ml BHI broth (pH 7.3) at 37°C for 20h to get 10^9 cfu/ml. Cell concentrations were determined using spread plates. Cells were harvested by centrifugation at 7000g for 20 min (at 4"C), washed with water and weighed. The cell pellets were then resuspended in lysozyme incubation buffer (0.015mol.l^{-1} NaCl; 0.03 mol^{-1} Tris-HC1 (pH 6.7) (Sigma) and 0.4mol^{-1} sucrose at 20 mg ml^{-1} wet weight before addition of

lysozyme (Sigma) solution (6 mg ml^{-1}) to give a final concentration of 600μg lysozyme ml^{-1}. The samples were shaken for 15 min at 37°C and 1 mol. l^{-1} $MgCl_2$ added to give a final concentration of 0.02mol.l^{-1} $MgC1_2$. After a 45-min incubation at 37°C without shaking, the samples were centrifuged at 1500g for 15 min and the pellets washed in protoplast buffer (0.03 mol.$l^{-1'}$ Tris-HC1 (pH 6.7); 0.01 mol l^{-1} $MgC1_2$ and 0.5 mol.1^{-1} sucrose). The pellets were then resuspended to their original volume (100 ml) with protoplast buffer and stored briefly on ice before use.

3.11.5.2 Determination of the efficiency of protoplast formation

The total number of cfu ml^{-1} present in each protoplast preparation was determined using spread plates. All enumerations were conducted using protoplast buffer as a diluents and BHI that had been hydrated with protoplast buffer. To determine the percentage of protoplasts present, a small volume of each protoplast suspension was centrifuged (1500g for 15 min) and resuspended in an equal volume of water. The cells were incubated at 30°C for 30 min with constant shaking to ensure lysis. Control whole cells were treated in the same manner.

3.11.5.3 Inactivation of whole cells and protoplasts by nisin, pediocin 34 and enterocin FH99

Overnight cultures of *L. monocytogenes* MTCC 657, *L. monocytogenes* ATCC 53135, *E. faecium* DSMZ 20477, *E. faecium* (VRE) *and E. faecalis* ATCC 29212 and their nisin resistant, pediocin 34 resistant and enterocin FH99 resistant variants were grown to 10^9 cfu ml^{-1} in BHI broth (10ml) at 30°C. After centrifugation (1500g for 15 min), they were washed and resuspended to their original volume in protoplast buffer (0.03 mol 1^{-1} Tris-HC1 (pH 6.7); 0.01 mol 1^{-1} $MgCl_2$ and 0.5 mol 1^{-1} sucrose). After 15 min equilibration at 30°C, samples (100 p1) were removed, diluted in protoplast buffer and cells enumerated on BHI. Bacteriocin, as per their MICs were added to the remaining cultures at the required concentration and samples taken for cell enumeration periodically over 3 h. Protoplasts were treated in exactly the same way as whole cells, except for the use of buffered diluent and agar. Three independent replicates of experiment were done.

3.11.6 Cell Surface Hydrophobicity

Cell surface hydrophobicity of wild type *E. faecium* DSMZ 20477*, E. faecium* (VRE), *E. faecalis* ATCC 29212, *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 and their bacteriocin resistant variants was determined according to the method described by Rosenberg e*t al.* (1980) with slight modification using n-Hexadecane. Cultures of the strains were grown in BHI broth overnight at 37ºC. The cells (8 log_{10} cfu/ml) were harvested in their early log phase by centrifugation at 12,000 x g for 5 min at 5ºC, washed twice and resuspended in 5 ml phosphate urea magnesium (PUM) buffer (K_2HPO_4: 22.2 g; KH_2PO_4: 7.26 g; Urea: 1.8 g; $MgSO_4.7H_2O$: 0.2 g; in 1000 ml of distilled water; pH 7.1) and the cell suspension was adjusted to an absorbance value (A_{610}) of approx. 0.8 - 1.0. Three ml aliquots of the bacterial suspension were put in contact with 1 ml of each of n-Hexadecane. The cells were pre-incubated at 37ºC for 10 min and then vortexed for 120 s. The suspension was then kept undisturbed at 37°C for 1h to allow phase separation and the hydrocarbon layer was allowed to rise completely. After 1h, aqueous phase was removed carefully with a Pasteur pipette and the absorbance (A_{610}) was measured using Spectrophotometer (Jenway Genova, Jenway Ltd. Gransmore Green, Felsted, Dunmow, UK). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity. Percent hydrophobicity was calculated using the following equation:

$$\%\text{Hydrophobicity} = \frac{(\text{OD initial} - \text{OD final})}{(\text{OD initial})} \times 100$$

Where $OD_{initial}$ and OD_{final} are the absorbance (at 610 nm) before and after extraction with the n-Hexadecane. All the measurements were done in triplicate.

3.12 MEASUREMENT OF CELLULAR PHOSPHOLIPIDS

The analysis of phospholipids was done as follows. The wild-type or the bacteriocin resistant variants were grown in BHI medium with or without bacteriocins to early stationary phase (OD600=1.7). The cells were harvested, washed and then resuspended in 0.9% NaCl. Total phospholipids were extracted from the cells by the method of Bligh & Dyer (1959). Individual lipids were separated from the total lipid extracts TLC. Individual lipids on TLC were visualized using the following stains:

iodine vapour (general lipid detection), ninhydrin (amino group) and ammonium molybdate.

3.12.1 Extraction of Lipids:

Total cellular lipids were extracted from sensitive strains as well as resistant variants using the following protocol. Proportions of chloroform, methanol and water to be added for different volumes of sample is shown in Table 3.2

i. For each 1 ml of sample, add 3.75 ml 1:2 (v/v) CHCl3: MeOH and vortex well.

ii. Then added 1.25 ml chloroform and vortexed well.

iii. Finally added 1.25 ml distilled water and vortexed well.

iv. Centrifuged at 1000 RPM in IEC table-top centrifuge for 5 min at room temperature to give a two-phase system (aqueous top, organic bottom).

v. Recovered the bottom phase as follows: inserted Pasteur pipette through the upper phase with gentle positive-pressure (*i.e.*, gentle bubbling) so that the upper phase did not get into the pipette tip. When pipette tip was at the bottom of the tube, carefully withdrew bottom phase through the pipette, making sure to avoid interface or upper face (should only try to recover ~90% of bottom phase, not all of it).

Table 3.2 Proportions of chloroform ($CHCl_3$), methanol (MeOH) and water for different volumes of sample

Sample	0.2 ml	0.5 ml	1 ml	1.5 ml	2 ml	3.5 ml
1:2 $CHCl_3$:MeOH	0.750	1.9	3.75	5.7	7.5	13.125
$CHCl_3$	0.250	0.625	1.25	1.875	2.5	4.375
Distilled Water	0.250	0.625	1.25	1.875	2.5	4.375
Total volume	1.45	3.65	7.25	10.95	14.50	25.375

3.12.2 Thin Layer Chromatography (TLC)

Different classes of lipids from total lipid extracts obtained from sensitive strains and mutant were resolved using TLC for identification.

3.12.2.1 Preparation of TLC plates

Ninety millilitre of distilled water was poured into 40g of silica gel G in a mortar and mixed well using pestle to get uniform slurry without air bubbles. Four dry grease free, glass plates (20x 20 cm) were placed on an ebonite board in a row to provide a smooth surface. The Desaga applicator was filled with silica gel slurry and rolled over plates with uniform slow motion to completely cover the plates with 0.5mm thickness. The plates were allowed to dry for 15 min and activated at 110°C for 1 hr in an oven. Activated plates were not kept in exposed air for more than an half before spotting.

3.12.2.2 Solvent mixture and equilibration

One hundred ml of mobile phase solvent mixture was prepared by mixing chloroform: methanol: distilled water: glacial acetic acid (65: 25: 4:1 V/V). It was poured into TLC developing chamber (20 cm x 20 cm x 10 cm) lined inside with filter paper on three sides. The tank was covered with glass lid and kept undisturbed for 2 hr for equilibration to take place.

3.12.2.3 Spotting of TLC plates

Known quantities of phospholipids standards and lipid extracts from sensitive strain and resistant variants were dissolved in chloroform: methanol (2:1 v/v) to give final concentration of 5-10µg/µl. With a micropipette, 4µl of each standard was spotted on the plate in each lane. Ten µl of mixture containing standard phospholipids was also spotted in a separate lane. Similarly, extracted lipids from sensitive strains and resistant variants were spotted in separate lanes. All the spots were allowed to dry before developing the TLC plates.

3.12.2.4 Developing TLC plates

Spotted plates were gently lowered into the TLC tank and lid was replaced immediately. The level of the mobile phase in the tank was not allowed to touch the spots directly. The solvent front started moving upward and reached less than 1 cm from the top in approximately 55 min. Subsequently the plate was taken out and the solvent front was marked with the marker. The mobile phase was allowed to evaporate to dry the plate before staining.

3.12.2.5 Identification of lipids

Different classes of resolved lipids were visualized using specific stains viz. Iodine vapours for general lipids, ammonium molybdate for phospholipids and ninhydrin for ACPs. The various classes of lipids were identified in comparison to standard lipids.

3.12.2.5.1 General lipids

Developed plates were kept in iodine chamber (A TLC tank with a few iodine crystals) for visualization of general lipids. Plate was removed and spots marked with a marker on back side of the plate. The distance of spots from the spotting line was measured. Retardation factor (R_f) value of the spots was calculated using the formula:

$$\text{Retardation factor } (R_f) = \frac{\text{Distance moved by analyte from origin}}{\text{Distance moved by solvent front from origin}}$$

3.12.2.5.2 Phospholipids

Phospholipids were visualized using ammonium molybdate reagent, which was prepared by dissolving 0.25g ammonium molybdate in 1 ml of warm distilled water. Eighty mg of metallic copper was placed in solution. This was chilled over ice and 1 ml concentrated sulphuric acid was added which resulted in a deep blue colored solution. It was kept at room temperature for 2 hr with occasional shaking. Forty ml distilled water was then added and shaken to give a light brown solution. Copper

metal was then removed and 3.2 ml of concentrated sulphuric acid added. Resulting solution remained light brown and was stored at 2-8°C. The solution was freshly prepared every week.

Spots on TLC plates were initially identified with iodine vapors and position of spots marked. Iodine was allowed to evaporate and then using a nebulizer, ammonium molybdate spray reagent was uniformly sprayed. Plates were then kept in an oven, set at 90°C, for 5 min or until the blue colored spots appeared.

3.12.2.5.3 Amino group containing phospholipids

Two hundred mg ninhydrin was dissolved in 100ml of 95% ethanol to give ninhydrin spray reagent. This solution was stored in dark. Spots on TLC plates were initially identified with iodine vapors and position of spots marked. Iodine was allowed to evaporate and then using a nebulizer, ninhydrin spray reagent was uniformly sprayed. The plate was kept in an oven, set at 110°C, for 10 min or until dark pink spots appeared.

3.13 MOLECULAR CHARACTERIZATION

3.13.1 Isolation of Genomic DNA

All the cultures used in this study along with standard strains were grown for 18 to 20 h in BHI broth at 37ºC and the extraction of their genomic DNA was done as described by Pospiech & Neumann (1995). The procedure in brief is as follows:

1. The cells were harvested from 1.5 ml of overnight grown culture in a microcentrifuge tube by centrifugation in a refrigerated centrifuge (Sigma-Aldrich, St. Louis, Missouri, USA) at 8000 rpm for 10 min.
2. The supernatant was discarded carefully without disturbing the pellet and the pellet was resuspended in 0.5 ml of SET buffer (pH 7.5).
3. Lysozyme was added to the above cell suspension at a concentration of 1 mg/ml and incubated at 37ºC /1 h.

4. This was followed by the addition of 1/10th (one- tenth) volume of 10% SDS and further incubation at 37ºC with occasional inversion for 30 min.

5. After this one third volume of 5 M NaCl and one volume of chloroform: iso-amyl alcohol (24:1) were then added to the above mixture and incubated at room temperature for 30 min with frequent inversions for 30 min.

6. The samples were centrifuged at 4,000 rpm for 15 min and the aqueous phase was transferred to a new tube.

7. The DNA was precipitated by adding one and half volume of isopropanol and the tubes were inverted gently to allow proper mixing and kept on ice for 30 min overnight at -20ºC for.

8. The DNA pellet thus obtained was recovered after centrifugation at 12,000 rpm for 10 min at 4ºC in a refrigerated centrifuge.

9. The pellet was washed with 70% ethanol and dried for 10 min and finally dissolved in 50 µl of TE buffer (pH 8.0).

10. The extracted genomic DNA along with tracking dye was run on 1% agarose in gel electrophoresis unit (Mini submarine, Hoeffer, USA).

3.13.2 Agarose Gel Electrophoresis

DNA was electrophoresed on the agarose gels by following the standard procedure as given by Sambrook *et al.* (1989). Agarose gels (1% concentration) were prepared by dissolving the appropriate quantity of agarose in 1 X TAE buffer (pH 8.0) (Appendix II) followed by addition of ethidium bromide stock solution (Appendix II) at the rate of 0.5 µg/ ml before casting the gel.

Molten agarose was cooled to 50°C and poured into respective moulds of minigel using appropriate comb. The surface was leveled before pouring the gel. After complete setting of the gel, the comb was removed carefully and the gel plate was mounted on respective horizontal electrophoresis tank (Mini submarine, Hoeffer, USA). The electrophoresis tank was filled with 1 X TAE electrophoresis buffer to cover the gel to a depth of about 1 mm. The DNA samples were mixed with 5 µl of

tracking dye and were loaded slowly into the slots of submarine gel using micropipette. Electrophoresis was carried out at 80 V (60 mA current) for one hour in mini gel electrophoresis apparatus. After completion of electrophoresis, the gels were taken out of the chamber and examined using UV transilluminator (Foto/UV-21, Fotodyne Inc., USA) and the gels were subsequently photographed by Bio RadGel Doc system (Bio-Rad, Hercules, CA, USA).

3.13.3 Determination of DNA Concentration by Spectrophotometric Analysis

The concentration of DNA samples was determined by the measurement of optical density (OD) in a UV spectrophotometer (DU 640, Beckman, USA) at 260 and 280 nm. The purity of DNA was ascertained by measuring as ratios at 260 to 280 nm. The concentration and quality of DNA was calculated by the following equivalents as suggested by Sambrook *et al.* (1989).

DNA mg/ ml = A_{260} x dilution x 50.0

A_{260} = Absorbance as OD at 260 nm.

OD (=1) = 50 μg/ml (double stranded DNA) or 30 μg/ml (Single stranded DNA)

3.13.4 Primer Designing

The primers for GAPDH genes were designed for Real time PCR analysis using Primer3 plus software based on the genomic sequence of *L. monocytogenes* (Genebank accession number: AARY02000001) and *Enterococcus* sp(Genebank accession number: ABQJ01000041). The primers for σ^{54} genes were designed for Real time PCR analysis using Primer3 plus software based on the genomic sequence of *L. monocytogenes* (Genebank accession number: NZ_AARP03000017) and *Enterococcus* sp(Genebank accession number: AF210458) and their specificity was ensured insilico by Blast analysis. The primers for IIC component of *m* operon were designed for Real time PCR analysis using Primer3 plus software based on the genomic sequence of *L. monocytogenes* (Genebank accession number: AF397145) and

Enterococcus sp(Genebank accession number: CP002621) and their specificity was ensured insilico by Blast analysis. The primers for IID component of *m* operon were designed for Real time PCR analysis using Primer3 plus software based on the genomic sequence of *L. monocytogenes* (Genebank accession number: AF397145) and *Enterococcus* sp(Genebank accession number: CP002491) and their specificity was ensured insilico by Blast analysis.The primers for *glpQ* genes were designed for Real time PCR analysis using Primer3 plus software based on the genomic sequence of *L. monocytogenes* (Genebank accession number: NZ_AARZ02000002) and *Enterococcus* sp(Genebank accession number: ABQJ01000041) and their specificity was ensured insilico by Blast analysis. The primers were got custom synthesized form Sigma Aldrich Chemicals, USA. The appropriate selection criteria followed during designing of primers included small amplicon size (100bp-250bp), a GC content ranging between 40 and 60% and Tm from 55 to 60°C. Table 3.3 shows the genes for which the primers were designed. The sequences of the primers are enlisted in Table 3.4.

Table 3.3 Genes studied

Sr No.	Abbreviations	Name of Gene
1	Gapdh	Glyceraldehyde 3-phosphate Dehydrogenase
2	σ^{54}	Sigma 54 encoded by *rpoN*
3	II C	Membrane-located proteins of the mannose-phosphotransferase system (man-PTS)
4	II D	Membrane-located proteins of the mannose-phosphotransferase system (man-PTS)
5	*glpQ*	Glycerophosphoryl diester phosphodiesterase

Table 3.4 Primers designed for the Real Time PCR.

Product Name	Sequence	Product Size	Reference
L. mono σ^{54} (F)	TGCCTATGACGTGATCCAAA	**161 bp**	This Study
L. mono σ^{54} (R)	CGCGTTCAGACTCAAACTCA		
L. mono IIC (F)	ATTCCAGCTGCAGCACTTTT	**102 bp**	This Study
L. mono IIC (R)	TCCTCCACCGATAGCCATAC		
L. mono IID (F)	TAGCTGGTGTTGGTGATCCA	**123bp**	This Study
L. mono IID (R)	CGTTCCAAGCAACGAAGAAT		
L. mono *glpQ* (F)	CAGGGATTACAGACGCCAAT	**150bp**	This Study
L. mono *glpQ* (R)	CTTTCTCCCCGCAATTGATA		
E. fm σ^{54} (F)	TCCGGGTATTAGTGGACGAC	**188bp**	This Study
E. fm σ^{54} (R)	CCCACGTTGTAGGATCGTTT		
E. fm IIC (F)	CGTTCATTTGATGGATGCAG	**100bp**	This Study
E. fm IIC(R)	GCGATACGAACCCCTTGTAA		
E. fm IID (F)	GTGGCGCTCT ACCTTCATTC	**220bp**	This Study
E. fm IID (R)	ACCATTCGCACGTTCTTCTT		
E. fm *glpQ* (F)	AATGCAGGAGGCAAATTCAG	**191 bp**	This Study
E. fm *glpQ* (R)	TGAAAAGGCCAATCTCTGCT		
Gapdh (F)	GGACGTATCGGACGTCTAGC	**234 bp**	This Study
Gapdh (R)	TAGGTCACCCCATGGAAGTT		

F= Forward primer; R= Reverse Primer; L. mono= *Listeria monocytogenes*; E. Fm = *Enterococcus faecium*

3.13.5 PCR with Selected Primers

Before setting up of PCR, all the reagents were thawed (except Taq DNA polymerase), mixed and spinned. Reagents were always kept on ice bath during the period of setting up of the reaction. The reaction mixture comprising 10 X PCR buffer (containing $MgCl_2$), dNTPs and primers was prepared and distributed to reaction tubes according to the requirements shown below in Table 3.5.

Table 3.5. Details of the PCR mix used in the study

Reagents	Concentration	Volume (μl)
Sterile Nuclease Free Water	....	Variable
10X Reaction buffer with $MgCl_2$ (15 mM)	1X	10
dNTP mix	10 mM	0.8
Primer forward	0.1-0.5 μM	1.0
Primer reverse	0.1-0.5 μM	1.0
Taq DNA polymerase	0.03 – 0.05 U μl^{-1}	1.2
Template DNA	200 pg μl^{-1}	2

The final volume of PCR mix was adjusted to 25μl. All the reagents except template DNA were added to the master mix, and then it was given a short spin, and distributed equally into the PCR tubes. The template DNA was added just prior to transferring the tubes into the thermal cycler. PCR tubes were given a final spin again and at once transferred to the heating block of the thermo cycler (Eppendorf Master Cycler, Germany).

3.13.6 Extraction of RNA

3.13.6.1 Preparation of reagents, glasswares and plasticwares for RNA isolation

All the reagents and materials to be used during RNA extraction were prepared in 0.1% DEPC (diethyl pyrocarbonate) treated sterilized MilliQ water (incubated overnight) and autoclaved at 121°C for 15 minutes at 15lbs pressure. The glasswares and plasticwares used fro RNA work were also DEPC treated and autoclaved at 121°C for 15 min. at 15lbs pressure. Diethylpyrocarbonate (DEPC), also called diethyl dicarbonate, diethyl oxydiformate, ethoxyformic anhydride, or pyrocarbonic acid diethyl ester, is used in the laboratory to inactivate the RNase enzymes from water and other laboratory utensils. It inactivates the RNases by the covalent modifications of the histidine residues. Water is usually treated with 0.1% v/v diethylpyrocarbonate for at least 1 hour at 37°C and then autoclaved (at least 15 min) to inactivate traces of DEPC. Inactivation of DEPC in this manner yields CO_2,

H_2O and ethanol. The chemicals reserved fro RNA work were handled by wearing gloves to avoid contamination of RNases. The work was conducted in an isolated room confined only for RNA work.

3.13.6.2 Procedure for extraction RNA and treatment of RNA samples with DNase

Total RNA was isolated from a mid-exponential phase culture (10 ml) using the RNeasy kit (Qiagen), according to the manufacturer's instructions. The extracted RNA was treated with DNase-RNase free (Qiagen).

3.13.6.3 Electrophoresis of RNA samples

The quality of RNA was assessed by running samples on a 1% formaldehyde agarose gel (Appendix III).

3.13.7 Preparation of cDNA

3.13.7.1 Quantitation and quality check of RNA

RNA was quantified spectrophotometrically. Purity of RNA was judged on the basis of optical density ratio at 260: 280 nm.

3.13.7.2 Confirmation of DNase treatment of RNA by PCR

Positive control (without DNase treated)

MilliQ	17.0 µl
PCR buffer (10X)	2.5 µl
dNTP's (10mM)	2.0 µl
Forward Primer (10pmol/µl)	1.0 µl
Reverse Primer (10pmol/µl)	1.0 µl
Taq Polymerase(3U/µl)	0.5 µl
Template (without DNase treated)	1.0 µl
Total	25.0 µl

Negative Control (DNase Treated)

MilliQ	17.0 µl

PCR buffer (10X)	2.5 µl
dNTP's (10mM)	2.0 µl
Forward Primer (10pmol/µl)	1.0 µl
Reverse Primer (10pmol/µl)	1.0 µl
Taq Polymerase(3U/µl)	0.5 µl
Template (without DNase treated)	1.0 µl
Total	25.0 µl

Negative control (without template)

MilliQ	18.0 µl
PCR buffer (10X)	2.5 µl
dNTP's (10mM)	2.0 µl
Forward Primer (10pmol/µl)	1.0 µl
Reverse Primer (10pmol/µl)	1.0 µl
Taq Polymerase(3U/µl)	0.5 µl
Total	25.0 µl

The PCR mixes were prepared as shown above and following PCR conditions were used for the confirmation of the desired amplicon.

Initial Denaturation	94ºC/ 4 min
Denaturation	94ºC/ 30 s
Annealing	55°C/ 30s
Extension	72ºC/ 30s
Final Extension	72ºC/ 7 min
No. of cycles	30

PCR was carried out using Bio-Rad thermal cycler and the amplified products were run on 2% agarose gel as described in section 3.8.2.

3.13.8 Reverse transcription –PCR (RT-qPCR)

cDNA was synthesized from 2 mg total RNA using the Revertaid Kit Premium First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer's instructions. The reverse transcription reaction mix was prepared by combining the

components shown in the table given below in a sterile 1.5 ml microcentrifuge tube on ice. Sufficient mix was made to allow 10µl for each cDNA synthesis reaction to be performed. The volumes needed for each component were determined, and combined them in the order as listed. It was then mixed gently and kept on ice prior to dispensing into the reaction tubes.

The following protocol was used to generate first strand cDNA for use in two step RT-qPCR. A reaction containing all the components but omitting reverse transcriptase was included in order to assess DNA contamination.

Template RNA	1pg-5µg
Primers (Random hexamer primer)	25 pmol
10mM dNTP Mix	1µl
Water Nuclease free	To 15µl

- Incubated the reaction mix at 65°C for 5 min. Chilled on ice, briefly centrifuged again and placed on ice.
- The following components were pipette into the reaction tube in the indicated order:

5X RT Buffer	4 µl
Ribonuclease inhibitor	1 µl
Reverse transcriptase enzyme (20U/ µl)	2 µl

- Mixed gently, centrifuged and Incubated for 5 min at 25°C.
- Incubated for 60 min at 37°C and then terminated the reaction at 70°C for 5 min.

3.13.8.1 Control Reactions for RT-PCR

The following negative control reactions were used to verify the results of the first strand cDNA synthesis.

Reverse transcriptase minus (RT-) control: The control RT- reaction contained all the reagents for reverse transcription reaction except for the reverse transcriptase enzyme.

Template RNA	1pg-5µg
Primers (Random hexamer primer)	25 pmol
10mM dNTP Mix	1µl
Water Nuclease free	To 15µl
5X RT Buffer	4 µl
Ribonuclease inhibitor	1 µl

No template control (NTC): This control is necessary to assess for reagent contamination. The NTC reaction contained all reagents necessary for the reverse transcription reaction except for the RNA template.

Primers (Random hexamer primer)	25 pmol
10mM dNTP Mix	1 µl
Water Nuclease free	To 15 µl
5X RT Buffer	4 µl
Ribonuclease inhibitor	1 µl
Reverse transcriptase enzyme (20U/ µl)	2 µl

3.13.8.2 Optimization of Real Time –quantitative PCR (RT-qPCR) conditions-annealing temperature

Optimizing the annealing temperature of real- time PCR assay is one of the most critical parameters for reaction specificity. Setting the annealing temperature too low may lead to amplification of non-specific PCR products. On the other hand, setting the annealing temperature too high may reduce the yield of the desired PCR product. Even after calculating the T_m (melting temperature of a primer, we need to determine the annealing temperature empirically. This was done by repeating a reaction at different temperatures. In order to find the optimal annealing temperature for reaction, a range of temperatures above and below the calculated T_m

of the primers were tested by gradient PCR. The optimal annealing temperature is the one that results in the lowest C_T with no nonspecific amplification. Because SYBR Green I binds to all ds DNA, it is necessary to check the specificity of qPCR assay by analyzing the reaction product(s). To do this, we used the melt curve function on real time instrument and also electrophoresed products on an agarose gel. An optimized SYBR Green I qPCR reaction should have a single peak in the melt curve, corresponding to the single band on the agarose gel.

3.13.8.3 Standard Curve for target and house keeping genes

The relative quantification approach first calculates the absolute value of the target investigation and the value of the housekeeping gene in the sample. To obtain the concentration of these two parameters, an external standard curve is prepared. Standard curve is used to calculate the amplification efficiency of both the target and housekeeping (reference) genes at a particular experimental condition. For this serial dilution is done with typical nucleic acid i.e. cDNA or RNA from the calibrator (control). Dilutions were prepared covering the respective dynamic ranges of detection for both target and reference genes. The cDNA samples were diluted in a range of 100, 50, 25, 5, 1 and 0.25 ng/μl and were used as RT-qPCR templates. The standard curves were generated by plotting the log cDNA values against Cq values obtained over the range of the dilutions. The amplification efficiency was determined by running serial dilutions of standards on the Light cycler. The slope of the standard curve was converted to the amplification efficiency E by the following algorithm: $E= 10^{[-1/slope]}$

The amplification efficiency of target and housekeeping gene should differ by no more than +/- 0.05.

3.13.8.4 Real time PCR

3.13.8.4.1 Materials

- Sample material- cDNA
- Multiwell Plate or PCR tube strips
- PCR tube caps

- Primers
- PCR mix

3.13.8.4.2 Preparation of Real Time PCR (qPCR) mix

i. One vial each of Maxima® SYBR Green I qPCR Master Mix (2X) and Nuclease free water was thawed. Briefly centrifuged all the solutions after thawing.
ii. Prepared a reaction master mix by adding the following components (except template DNA) for each 25 µl reaction to a tube at room temperature:

Maxima® SYBR Green I qPCR Master Mix (2X)	12.5 µl
Forward Primer	10 pmol/µl
Reverse Primer	10 pmol/µl
Template	≤ 500 ng
Water nuclease free	To 25 µl

iii. Mixed the master mix thoroughly and dispense appropriate volumes into PCR tubes or plates.
iv. Added template DNA to the individual PCR tubes or wells containing the master mix
v. Gently mixed the reactions without creating bubbles. Centrifuged briefly. bubbles can interfere with fluorescence detection.

3.13.8.4.3 Statistical analysis of relative gene expression

Data were analyzed by the comparative critical threshold (ΔΔ CT) method in which the target RNA was adjusted to a reference (RNA of an internal control gene); CT is the cycle number at which the fluorescence emission due to PCR products exceeded the threshold. The equations used to determine ΔCT and ΔΔCT were described by Desroche (2005). Overall, ΔCT = CT of reference control - CT of target gene, ΔΔCT = ΔCT of stress condition - ΔCT of environmental condition, relative expression level = $2^{\Delta\Delta CT}$

3.14 STATISTICAL ANALYSIS

The data were subjected to various statistical analyses as and when needed, using SYSTAT 6.0.1., Statistical Software Package, 1996, SPSS, Inc., USA, Microsoft R excel 2000 Software Package, Microsoft Corporation, USA and GraphPad 3.02, 1999, GraphPad Software Inc., San Diego CA.

3.14.1 Determination of Mean and Standard Error of the Mean (SEM)

The experimental data, as and when necessary, is presented as the mean and standard error of the mean (SEM) of different parameters studied in the present investigation. The mean and SEM were determined running Microsoft Excel 2000 Software Package, Microsoft Corporation, USA.

3.14.2 Graphical Presentation

The mean ± SEM of different parameters studied were graphically presented using Graph Pad 3.02, 1999, GraphPad Software Inc., San Diego CA.

4. RESULTS AND DISCUSSION

4.1 PROLOGUE

For the past few decades, food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. Illness caused due to the consumption of contaminated foods has a wide economic and public health impact worldwide. Therefore, the need to avoid economic losses due to microbial spoilage of raw materials and food products, the preservation of foods by natural, biological methods may be a satisfactory approach to solve many of the current food-related issues.

To inactivate food borne pathogens, various novel technologies, such as biopreservation systems, have been studied. Bacteriocins of Lactic Acid Bacteria (LAB) have been widely studied due to their potential use in food preservation as natural preservatives. Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced mainly by lactic acid bacteria (LAB). Several LAB bacteriocins with broad spectra of inhibitory activity offer potential applications in food biopreservation (Galvez *et al.*, 2008). Bacteriocins, such as nisin, are already used by food industries as adjuncts to minimal food preservation techniques (Delves-Broughton *et al.*, 1996; Smid & Gorris, 1999). However, similar to the use of antibiotics, the concern with the use of bacteriocins is the development of resistance in foodborne pathogens. A promising method of food protection by using class I and class IIa bacteriocins could be compromised by the development of resistant strains. Gravesen *et al.* (2002) investigated the frequency of resistance development in *L. monocytogenes* to two bacteriocins, pediocin PA-1 and nisin A, along with the effects of strain differences and environmental conditions. Resistant food-borne pathogens are posing a global problem which is further facilitated by international trade of raw and processed foods. In foods with a long shelf life, even a small number of these resistant cells can multiply to very high number and thus may lead to food-borne outbreaks and food spoilage. Studies aimed at characterizing the resistance mechanisms of bacterial targets have revealed the stability of this

phenomenon (Rekhif *et al*., 1994; Dykes & Hastings, 1998), which occurs at either a low or a high level. In *L. monocytogenes* and *Enterococcus faecalis*, low-level resistance has been attributed to alterations in membrane lipid composition (Vadyvaloo *et al*., 2002, 2004; Naghmouchi *et al*., 2006) and high-level resistance has been attributed to the inactivation of the *mptACD* operon, which encodes the EII_t^{Man} mannose permease of the phosphotransferase system (PTS) (Dalet *et al*., 2001; He´chard *et al*., 2001).

4.2 SUSCEPTIBILITY OF PATHOGENS (WILD TYPE STRAINS) TO NISIN, PEDIOCIN 34 AND ENTEROCIN FH99

Antibacterial efficiency of three bacteriocins from lactic acid bacteria viz., nisin, pediocin 34 (produced by *Pediococcus pentosaceous* 34) and enterocin FH99 (produced by *Enterococcus faecium* FH99), was evaluated against several Gram positive food spoilage and pathogenic bacteria (Table 4.1). The Gram positive bacterial species studied in this work differed considerably in their sensitivity to nisin, enterocin FH99, and pediocin 34. *E. faecalis* ATCC 29212, *Bacillus cereus* ATCC 13061, *B. cereus* NCDC 66*, S. aureus* NCDC 110 were sensitive to nisin only. *E. faecium* DSMZ 20477, *E. faecium* (VRE), *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 were sensitive to all the bacteriocins used in the study. Earlier Bankerroum & Sandine (1988) and Ukuku & Shelef (1997) observed that the *Listeria* strains differed considerably in their sensitivity to nisin. They indicated that genera, species and strain differed in the degree of inhibition and thus showing that some *Listeria* strains are more sensitive to nisin than others. Our findings are very much in accordance with the bacteriocins of other lactic acid bacteria (LAB) that are mainly active against Gram-positive organisms but cannot penetrate through the lipopolysaccharide layer of Gram-negative bacteria (Aymerich *et al.,* 1996; Nes *et al.,* 1996; Franz *et al.,* 2003). A few reports, however, indicated enterocins to be active against Gram-negative bacteria such as *E. coli* (Galvez *et al.,* 1989; Tomita *et al.,* 1997), *Shigella sonnei, Shigella flexneri* (Sparo *et al.,* 2006) and *Vibrio cholera* (Simonetta *et al.,* 1997). But in the present investigation, neither nisin nor pediocin 34 or enterocin FH99 were observed to be effective against Gram-negative organisms.

Table 4.1 Susceptibility of wild type strains to nisin, pediocin 34 and enterocin FH99.

Culture	Strain	Nisin	Pediocin 34	Enterocin FH99
Enterococcus. fecalis	ATCC 29212	+	-	-
Bacillus cereus	ATCC 13061	+	-	-
Bacillus cereus	NCDC 66	+	-	-
Staphylococcus aureus	NCDC 110	+	-	-
Listeria monocytogenes	ATCC 53135	+	+	+
Listeria monocytogenes	MTCC 657	+	+	+
Enterococcus faecium	DSMZ 20477	+	+	+
Enterococcus faecium	VRE	+	+	+
Escherichia coli	O157:H7	-	-	-
Escherichia coli	ATCC 25922	-	-	-
Salmonella typhi	NCDC 113	-	-	-
Salmonella arbutus	NCTC 6017	-	-	-
Pseudomonas aeruginosa	NCDC 104	-	-	-

Inhibition = +, No inhibition = -

4.3 MINIMUM INHIBITORY CONCENTRATION (MIC) OF WILD TYPE STRAINS:

The MICs of the wild type strains *E. faecalis* ATCC 29212, *Bacillus cereus* ATCC 13061, *B. cereus* NCDC 66*, S. aureus* NCDC 110, *B. cereus* 110, *E. faecalis* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657, as determined by the spot-on –lawn assay, are given in Table 4.2. The MIC was determined as the minimal concentration giving a visible zone of inhibition after 24 h at 37 °C (mean of three independent experiments).

4.4 KINETICS OF CELL GROWTH INHIBITION BY BACTERIOCINS (ALONE AND IN DIFFERENT COMBINATIONS)

The antibacterial efficacy of the bacteriocins viz., nisin, pediocin 34 and enterocin FH99 was evaluated singly as well as in different combinations against several Gram-positive bacteria i.e. *E. faecalis* ATCC 29212, *Bacillus cereus* ATCC 13061, *B.*

cereus NCDC 66, *S. aureus* NCDC 110, *B. cereus* 110, *E. faecalis* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 in BHI broth. Table 4.2 and Table 4.3 show the concentrations of the bacteriocins used when tested against the target organism alone and in different combinations, respectively

Fig. 4.1a, 4.1b and 4.2a, 4.2b show the bactericidal effectiveness of nisin against *E. faecalis* ATCC 29212, *B. cereus* ATCC 13061, *B. cereus* NCDC 66 and *S. aureus* NCDC 110, respectively. In case of *E. faecalis* ATCC 29212, maximum viability loss of 4.3 log cycles was observed after 2h incubation though no significant ($p > 0.05$) decrease was observed in case of *B. cereus* ATCC 13601 after 1h incubation with nisin. However, after 1h a significant decrease ($P<0.001$) in viable count was observed.

Table 4.2: Minimum Inhibitory Concentrations (MIC) of wild type strains

Culture	Strain	Nisin (IU/ml)	Pediocin 34 (AU/ml)	Enterocin FH99 (AU/ml)
Enterococcus faecalis	ATCC 29212	26.5	-	-
Bacillus cereus	ATCC 13061	1250	-	-
Bacillus cereus	NCDC 66	106.25	-	-
Staphylococcus aureus	NCDC 110	53	-	-
Listeria monocytogenes	ATCC 53135	13.2	540	120
Listeria monocytogenes	MTCC 657	50	600	700
Enterococcus faecium	DSMZ 20477	53.5	70	937.5
Enterococcus faecium	VRE	14	2187.5	3750

Maximum loss of 5 log cycles was observed in case of *B. cereus* ATCC 13061. Similarly in case of *B. cereus* NCDC 66, a significant decrease of 5 log cycles ($P<0.001$) was observed on incubation with nisin. The two strains of *B. cereus* thus

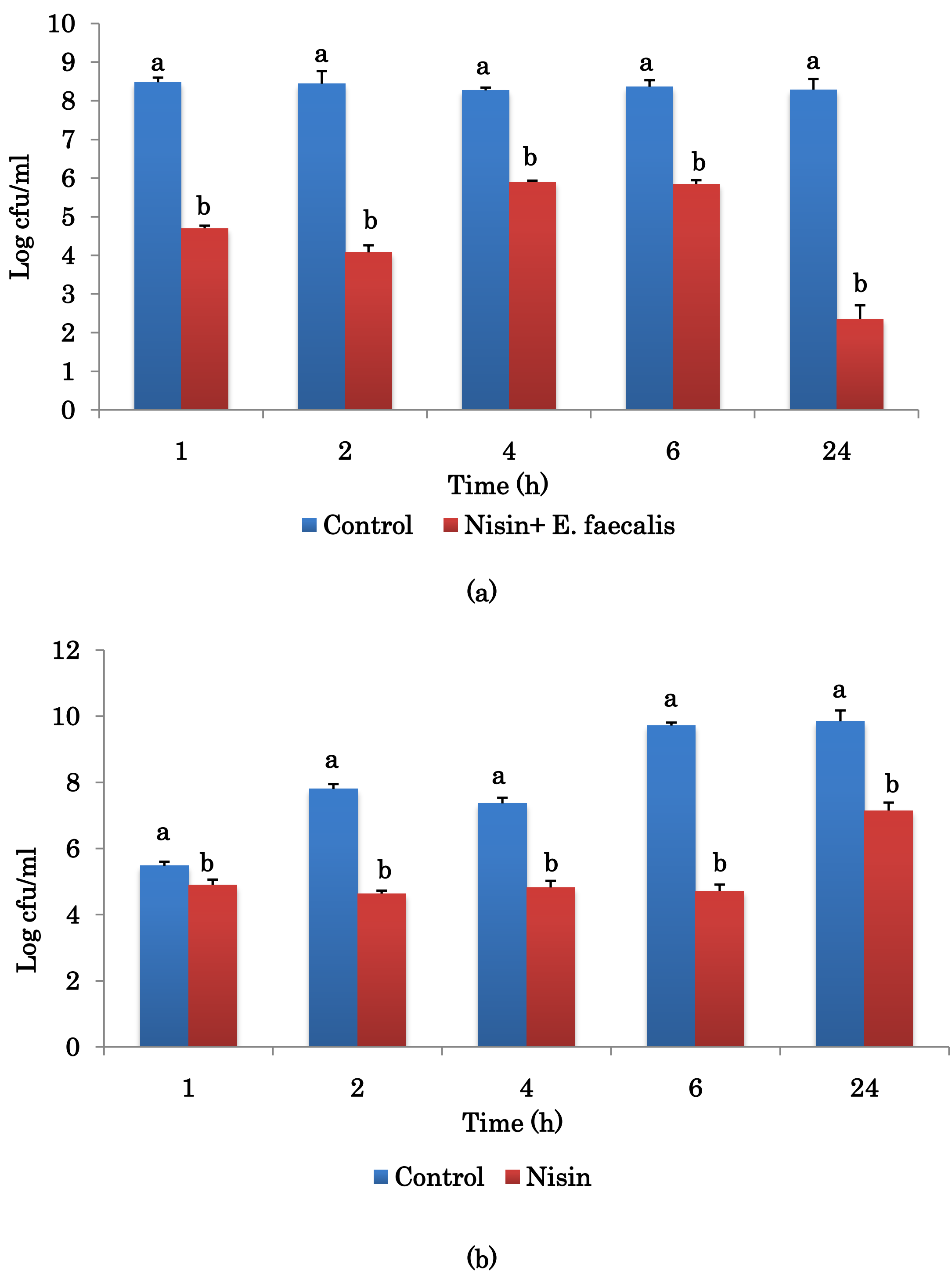


Fig 4.1. Effect of Nisin on a) *E. faecalis* ATCC 29212 and b) *B. cereus* ATCC 13061. Values are presented as mean ±SEM; n=3. [a,b] Values with different superscripts differ significantly at the level of P<0.01 at the corresponding period between control (in the absence of Nisin) and in presence of Nisin.

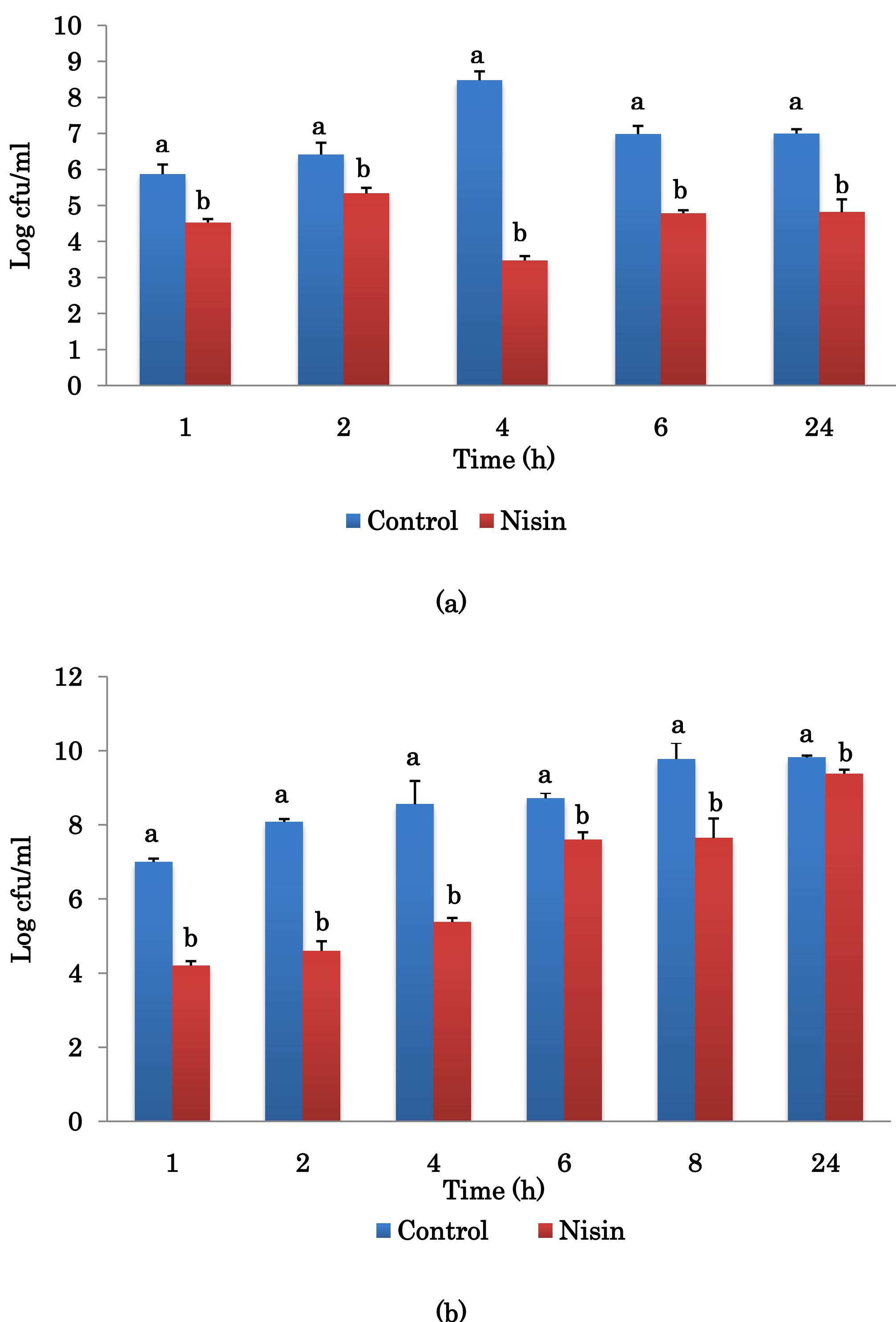


Figure 4.2. Effect of Nisin on a) *B. cereus* NCDC 66 and b) *S. aureus* NCDC 110. Values are presented as mean ±SEM; n=3. [a,b] Values with different superscripts are differ significantly at the level of P<0.01 at the corresponding period between control (in the absence of Nisin) and in presence of Nisin.

differed considerably with regard to their sensitivity towards Nisin. Both *B. cereus* ATCC 13061 and *B. cereus* NCDC 66 were, however, resistant to enterocin FH99 and pediocin 34.

Table 4.3 Concentrations of Nisin (N), Pediocin 34(P) and Enterocin FH99 (E) used alone and in different combinations for evaluating the inhibition of test organisms by the bacteriocins

	ADDITIVE				SYNERGISTIC			
Culture	**N + P**	**N + E**	**E + P**	**N + P + E**	**N + P**	**N + E**	**E + P**	**N + P + E**
Listeria monocytogenes ATCC 53135	13.2 + 540	13.2 + 120	540 + 120	13.2 + 540 + 120	6.6 + 270	6.6 + 60	270 + 60	6.6 + 270 + 60
Listeria monocytogenes MTCC 657	50 + 600	50 + 700	600 + 700	50 + 600 + 700	25 + 300	25 + 350	300 + 350	25 + 300 + 350
E. faecium DSMZ 20477	53.5 + 70	53.5 + 937.5	937.5 + 70	53.5 + 70 + 937.5	26.25 + 35	26.25 + 468.75	468.75 + 35	26.25 + 35 + 468.75
E. faecium VRE	14 + 2187.5	14 + 3750	2187.5 + 3750	14 + 2187.5 + 3750	7 + 1093	7 + 1875	1093 + 1875	7 + 1093 + 1875

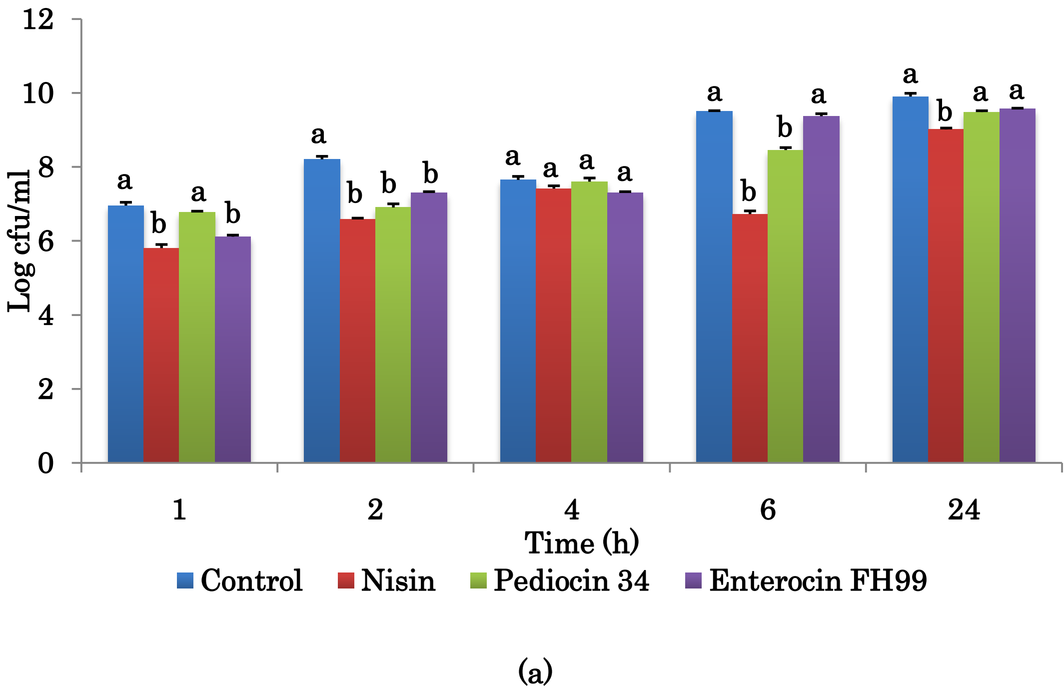
N= Nisin, P= Pediocin 34, E= Enterocin FH99
Units for concentrations: Nisin=IU/ml, Pediocin 34= AU/ml, Enterocin FH99= AU/ml
The data were given as mean values from the duplicate experiments

Similar results were observed in case of *S. aureus* NCDC 110 with maximum viability loss of 3.47 log cycles after 2h incubation. Though there was a significant decrease ($P<0.001$) in viable count of *S. aureus* NCDC 110 on incubation with nisin after 1h, the number of survivors increased after 24h incubation.

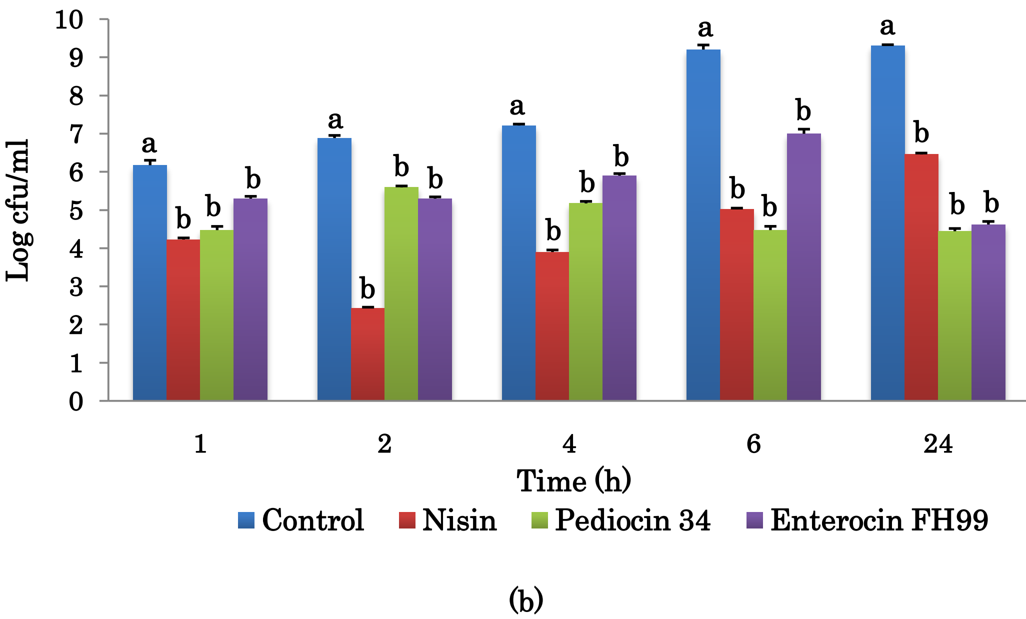
Nisin was most effective in inhibiting the *E. faecium* DSMZ 20477 followed by pediocin 34. Enterocin FH99, however, was least effective in inhibiting *E. faecium* DSMZ 20477. No significant decrease ($P>0.05$) in viable count of *E. faecium* DSMZ 20477 was observed with enterocin FH99 after 4h incubation. In case of *E. faecium* (VRE), nisin, followed by pediocin 34, were observed to be more effective as compared to Enterocin FH99.

Significant decrease ($P<0.001$) in viable count of *E. faecium* (VRE) was observed on incubation with nisin, pediocin 34 and enterocin FH99. With Nisin, maximum viability loss of 2.7 log cycles was observed after 6h. A maximum viability loss of 0.9 log cycles after 2h was observed with Enterocin FH99 in case of *E. faecium* DSMZ 20477. The number of survivors, however, increased after 24h incubation after treatment with Nisin (Fig. 4.3a).

In case of *E. faecium* VRE, maximum viability loss of 1.94 log cycles was observed after 1 h incubation with Nisin followed by 1.69 log cycles in case of Pediocin 34 and 0.87 log cycles in case of Enterocin FH99 (Fig. 4.3b). Combination of all the three bacteriocins i.e. Nisin+ Pediocin 34 + Enterocin FH 99 was observed to be most effective in preventing the growth of both *E. faecium* DSMZ 20477 (Fig. 4.4a) and *E. faecium* VRE (Fig. 4.4b) in contrast to the Nisin + Pediocin 34, Nisin + Enterocin FH99 and Enterocin FH99+ Pediocin 34. In case of *E. faecium* DSMZ 20477 loss of 3.3 log cycles was observed after 6h. Significant differences ($p<0.001$) were observed between the control and the combinations of bacteriocins used to evaluate their additive effect at different time intervals as shown in (Fig. 4.4a). A significant decrease was observed in viable count of *E. faecium* DSMZ 20477 during 1h incubation with Nisin + Pediocin 34, Nisin + Enterocin FH99 and Nisin+ pediocin 34 + enterocin FH 99. There was no significant decrease ($P>0.05$) in viable count of *E. faecium* DSMZ 20477 during 1h incubation with enterocin FH99 + pediocin 34.



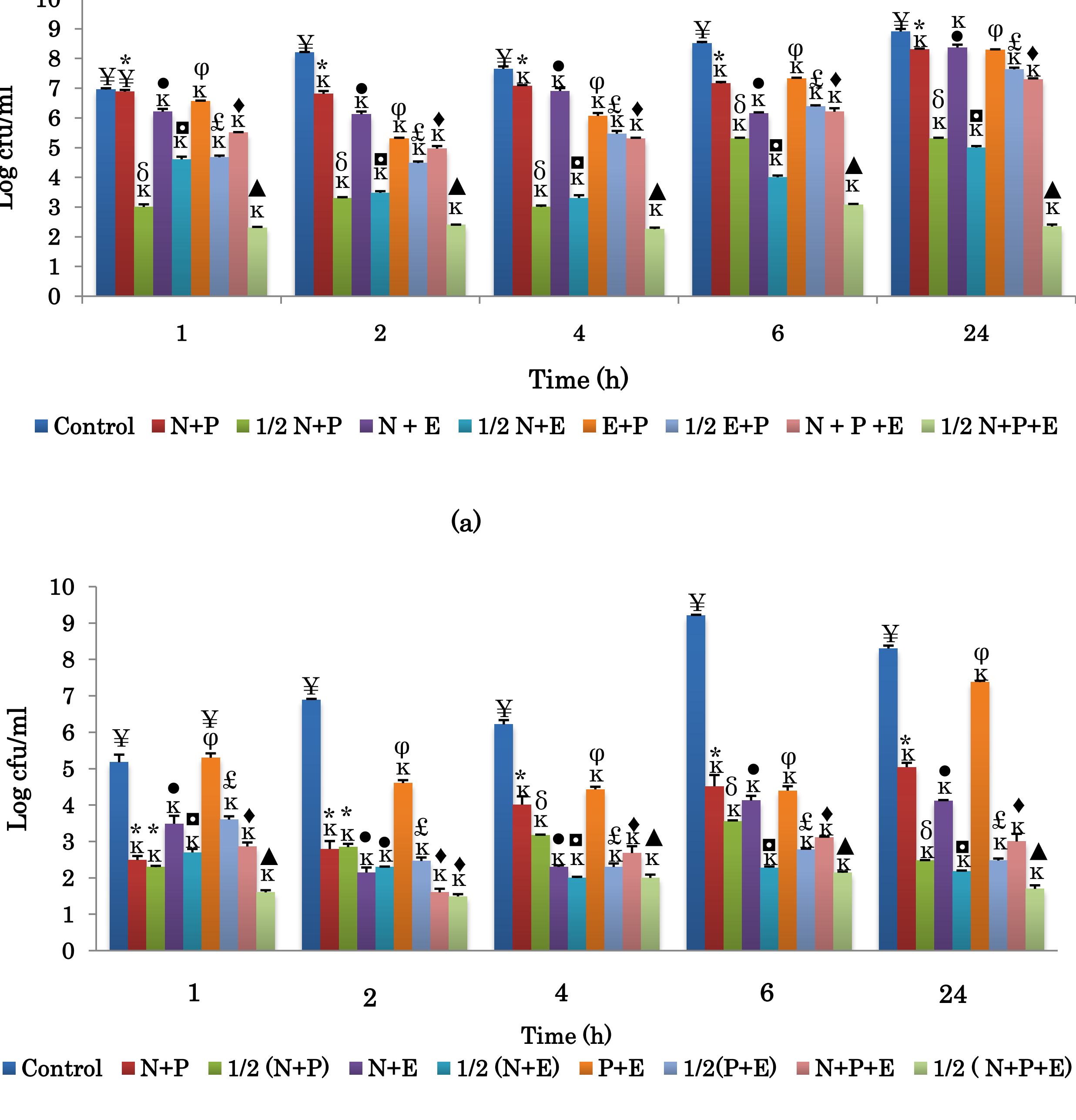
(a)



(b)

Fig 4.3. Effect of Nisin, Pediocin 34 and Enterocin FH99 alone on a) *E. faecium* DSMZ 20477 and b) *E. faecium* VRE.

Values are presented as mean ±SEM; n=3. [a,b] Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between control (without bacteriocin treatment) and in presence of bacteriocins (Nisin, Pediocin 34, Enterocin FH99).



(b)

Fig. 4.4. Effect of different combinations of Nisin, Pediocin 34 and Enterocin FH99 on a) *E. faecium* DSMZ 20477 and b) *E. faecium* VRE. Values are presented as mean ±SEM; n=3. ¥, κ Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between control and in presence of different combinations of bacteriocins. *, δ Values show significant difference between (N+P) and ½(N+P). φ, £ Values show significant difference between (N+E) and ½(N+E). •, ◘ Values show significant difference between (P+E) and ½(P+E). ♦, ▲ Values show significant difference between (N+P+E) and ½(N+P+E). N= Nisin, P= Pediocin, E= Enterocin.

But thereafter, a significant decrease ($P<0.001$) in viable count of *E. faecium* DSMZ 20477 was. The number of survivors of *E. faecium* DSMZ 20477 increased considerably after 24h incubation with all the three bacteriocins in case of. When the two non nisin bacteriocins were used together against the two strains of *E. faecium,* a higher number of survivors were detected as compared to the pairs containing nisin.

When the bacteriocins were used at half the concentration of their MICs in combinations against *E. faecium* DSMZ 20477 a significant difference ($p<0.001$) were observed between the control and the combinations of bacteriocins used to evaluate additive effect of bacteriocins at different time intervals (Fig. 4.4a). A combination of nisin + pediocin 34 + enterocin FH99 resulted in a viability loss of 4.65 and 5.43 log cycles after 1h and 6h incubation, respectively. A combination of nisin + enterocin FH99 resulted in a 2.35 and 4.51 log cycle reductions after 1h and 6h incubation, respectively, whereas nisin + pediocin 34 resulted in 3.95 log reduction after 1h and 3.20 log reduction after 6h incubation. A combination of enterocin FH99 and pediocin 34 resulted in viability loss of 2.28 and 2.13 log units after 1h and 6h, respectively. Also significant differences ($p<0.001$) were observed when the combinations of bacteriocins using half the concentration of MICs for each bacteriocin were compared with the combinations of bacteriocins using concentrations of MICs for each bacteriocins used to evaluate additive effect of bacteriocins on *E. faecium* DSMZ 20477 (Fig. 4.4a).

When the bacteriocins were used at half the concentration of their MICs in combinations against *E. faecium* VRE, a significant difference ($p<0.001$) was observed as compared to the control and the combinations of bacteriocins used to evaluate additive effect of bacteriocins at different time intervals (Fig. 4.4b). A combination of nisin + pediocin 34 + enterocin FH99 resulted in a viability loss of 3.57 and 7.05 log cycles after 1 and 6h incubation, respectively. On the other hand a combination of nisin + enterocin FH99 resulted in a reduction of 2.47 and 6.92 log cycle after 1 and 6h incubation, respectively. However, a combination of pediocin 34 + enterocin FH99 resulted in the viability loss of 3.60 and 2.77 log cycles after the

same intervals. Also significant differences ($p<0.001$) were observed when the combinations of bacteriocins using half the concentration of MICs for each bacteriocin were compared with the combinations of bacteriocins used to evaluate additive effect of bacteriocins on *E. faecium* VRE at different time intervals (Fig. 4.4b). Nisin + pediocin 34 resulted in 2.87 log reduction after 1h and 5.64 log reduction after 6h incubation whereas no significant difference was observed when the combinations of nisin + pediocin 34 using half the concentration of MICs for each bacteriocin were compared with the combinations of nisin + pediocin 34 using concentrations of MICs for each of the bacteriocin used to evaluate the additive effect of bacteriocins on *E. faecium* VRE during first two hours of incubation (Fig. 4.4b).

Nisin was also observed to be most effective in inhibiting the *Listeria monocytogenes* ATCC 53135, followed by pediocin 34 and enterocin FH99 (Fig. 4.5a). Significant decrease ($P<0.001$) in viable count of *Listeria monocytogenes* ATCC 53135 was observed with nisin, pediocin 34 and enterocin FH99. However, in case of *L. monocytogenes* ATCC 53135, the number of survivors increased during 6h incubation after treatment with nisin. Maximum viability loss of 3.7 log cycles was observed in 1 h incubation and about 5.9 log reduction after 4h with nisin followed by 2.3 log reduction after 1 h and 3.9 log reduction after 4h incubation with enterocin FH99. With pediocin 34 the respective viability losses were 2.2 and 3.4 log cycles (Fig. 4.5a). As shown in Fig. 4.5b, when bacteriocins were tested in combination, the mix of nisin, pediocin 34 and enterocin FH99, was observed to be most effective in preventing the growth with viability loss of 5.6 and 7.04 log cycles after 1 and 4h of incubation, respectively. The combination of enterocin FH99 + pediocin 34 resulted in a viability loss of 2.3 and 3.5 log units after 1 and 4h incubation, respectively. A combination of enterocin FH99+ nisin resulted in a 4.8 log and 6.2 log reductions after 1 and 4h incubation, respectively while pediocin 34 + nisin resulted in 4.32 log reductions after 1h and 5.78 log reduction after 4h incubation. Though a significant decrease ($P<0.001$) in viable cell count was observed in the culture treated with different combination of bacteriocins, there was no significant decrease ($P>0.05$) in the cell count after 24h incubation on treatment

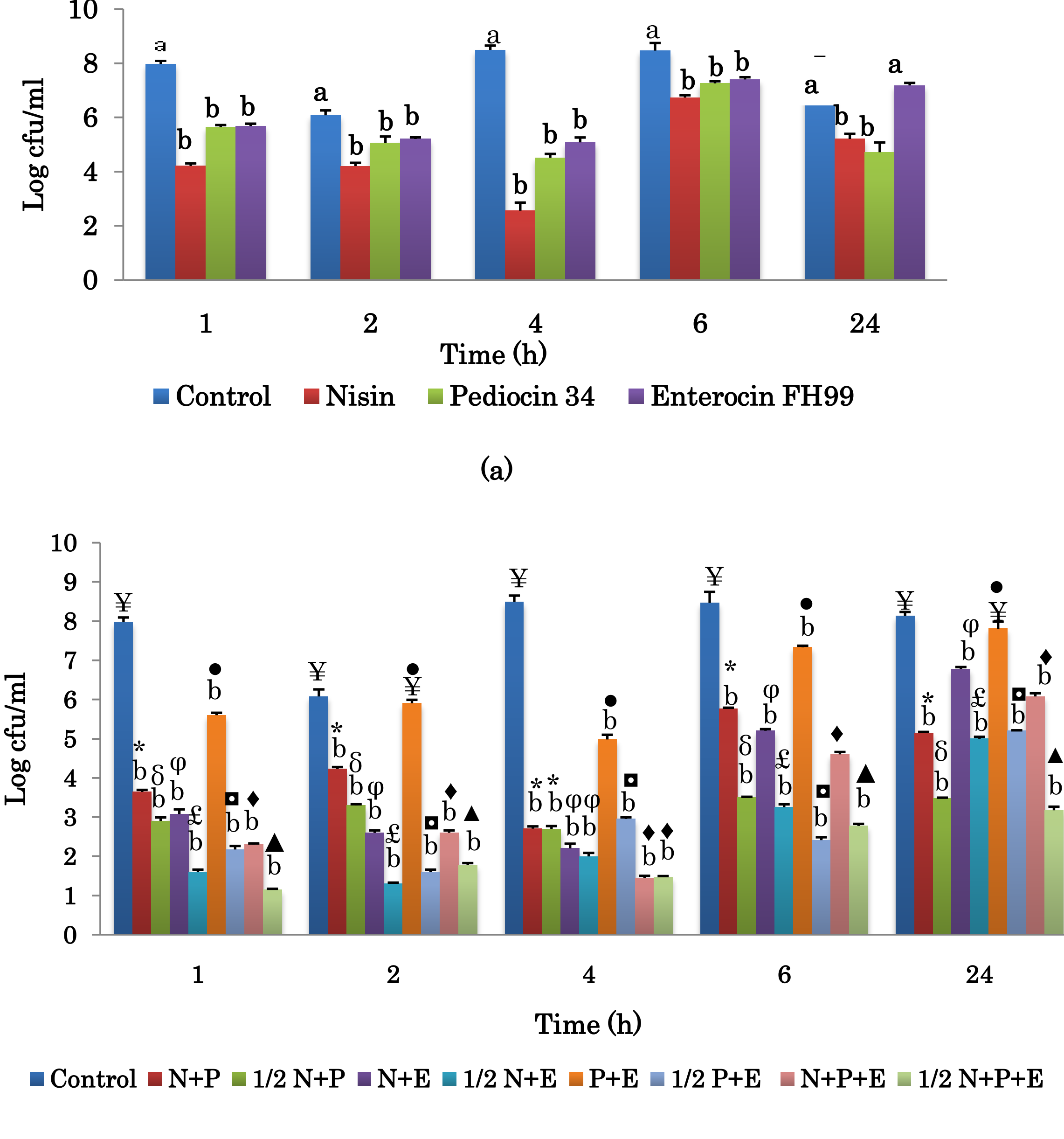


Fig. 4.5. a) **Effect of Nisin, Pediocin 34 and Enterocin FH99 alone on *L. monocytogenes* ATCC 53135. b) Effect of different combinations of Nisin, Pediocin 34 and Enterocin FH99 on *Listeria monocytogenes* ATCC 53135.** Values are presented as mean ±SEM; n=3. ^{a,b} Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between control and in presence of bacteriocins (in fig a). $^{¥, b}$Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between control and in presence of different combinations of bacteriocins.$^{*, δ}$ Values show significant difference between (N+P) and ½(N+P).$^{φ, £}$ Values show significant difference between (N+E) and ½(N+E). $^{•, ▫}$Values show significant difference between (P+E) and ½(P+E). $^{♦, ▲}$Values show significant difference between (N+P+E) and ½(N+P+E). N= Nisin, P= Pediocin 34, E= Enterocin FH99

with a mix of pediocin 34 and enterocin FH99. When bacteriocins were used at ½ the concentrations of their calculated MICs, relatively a greater antibacterial effect was observed. When bacteriocins were tested in combination, the mix of nisin, pediocin 34 and enterocin FH99, was observed to be most effective in preventing the growth with viability loss of 6.8, 4.3, 7.04, 5.6 and 4.9 log cycles after 1 h, 2h, 4h, 6h and 24h incubation respectively (Fig. 4.5b). The combination of enterocin FH99+ pediocin 34 resulted in a viability loss of 5.7 and 5.5 log units after 1 and 4h of incubation, respectively.

When the bacteriocins were used at half the concentration of their MICs in combinations against *L. monocytogenes* ATCC 53135 significant differences ($p<0.001$) were observed compared between the control and the combinations of bacteriocins used to evaluate additive effect of bacteriocins at different time intervals as shown in Fig. 4.5b. Also significant differences ($p<0.001$) were observed when the combinations of bacteriocins using half the concentration of MICs for each bacteriocin were compared with the combinations of bacteriocins used to evaluate additive effect of bacteriocins on *L. monocytogenes* ATCC 53135 at different time intervals as shown in Fig. 4.5b. A combination of nisin + pediocin 34 + enterocin FH99 resulted in a viability loss of less than 6.82 and 5.68 log cycles after 1 and 6h incubation, respectively. A combination of nisin + enterocin FH99 bacteriocin resulted in a 6.37 and 5.21 log cycle reductions after 1 and 6h incubation, whereas nisin + pediocin 34 resulted in 5.07 log reduction after 1h and 4.96 log reduction after 6h incubation. A combination of pediocin 34 and enterocin FH99 resulted in viability loss of 5.79 and 6.05 log units after 1 and 6h, respectively.

Pediocin 34 proved to be more effective than nisin and enterocin FH99 in inhibiting *L. monocytogenes* MTCC 657. A significant difference ($P<0.001$) was observed in the corresponding period for the viability loss of *L. monocytogenes* MTCC 657 in presence of nisin, pediocin 34 and enterocin FH99 as compared to the control (without bacteriocin treatment). Maximum viability loss of 1.85 log units was observed in 1 h incubation and about 2.26 log cycle reduction after 4h with pediocin 34 followed by 0.79 log reduction after 1 h and 0.34 log cycle reduction after 4h

incubation with enterocin FH99. With nisin, however, a viability loss of 0.63 and 0.56 log cycles, respectively, was observed (Fig. 4.6a) whereas with nisin the reduction in viable cell count was not significant (P>0.05) after 24h.

When bacteriocins were tested in combination, the mix of pediocin 34 and enterocin FH99 was observed to be most effective in preventing the growth with viability loss of 4.5 log cycles after 1 h incubation and 4.7 log cycles after 6h incubation. Significant differences (p<0.001) were observed as compared to the control and the combinations of bacteriocins used to evaluate additive effect of bacteriocins as shown in Fig. 4.6b. The combination of nisin + pediocin 34+ enterocin FH99 resulted in a viability loss of 3.5 and 2.7 log cycles after 1 and 6h incubation, respectively. A combination of nisin + enterocin FH99 resulted in a 2.7 and 4.5 log cycle reductions after 1 and 6h incubation, whereas nisin + pediocin 34 resulted in a reduction of 2.2 log cycles after 1h and 2.6 log cycles after 6h incubation (Fig. 4.6b).

When the bacteriocins were used at half the concentration of their MICs in combinations against *L. monocytogenes* MTCC 657, significant differences (p<0.001) were observed as compared to the control and the combinations of bacteriocins used to evaluate additive effect of bacteriocins at different time intervals (Fig. 4.6b). Also significant differences (p<0.001) were observed when the combinations of bacteriocins using half the concentration of MICs for each bacteriocin were compared with the combinations of bacteriocins used to evaluate additive effect of bacteriocins on *L. monocytogenes* MTCC 657 at different time intervals as shown in Fig. 4.6b. A combination of nisin + pediocin 34 + enterocin FH99 resulted in a viability loss of 4.12 and 4.04 log cycles after 1 and 6h incubation, respectively. A combination of nisin + enterocin FH99 bacteriocin resulted in 4.12 and 5.69 log cycle reductions after 1 and 6h incubation respectively, whereas nisin + pediocin 34 resulted in 3.7 log reduction after 1h and 4.24 log reduction after 6h incubation. A combination of pediocin 34 and enterocin FH99 resulted in viability loss of 5.2 and 6.42 log units after 1 and 6h, respectively. No significant differences was observed when the combinations of nisin + enterocin FH99 using half the concentration of MICs for each bacteriocin were compared with the combinations of nisin + enterocin

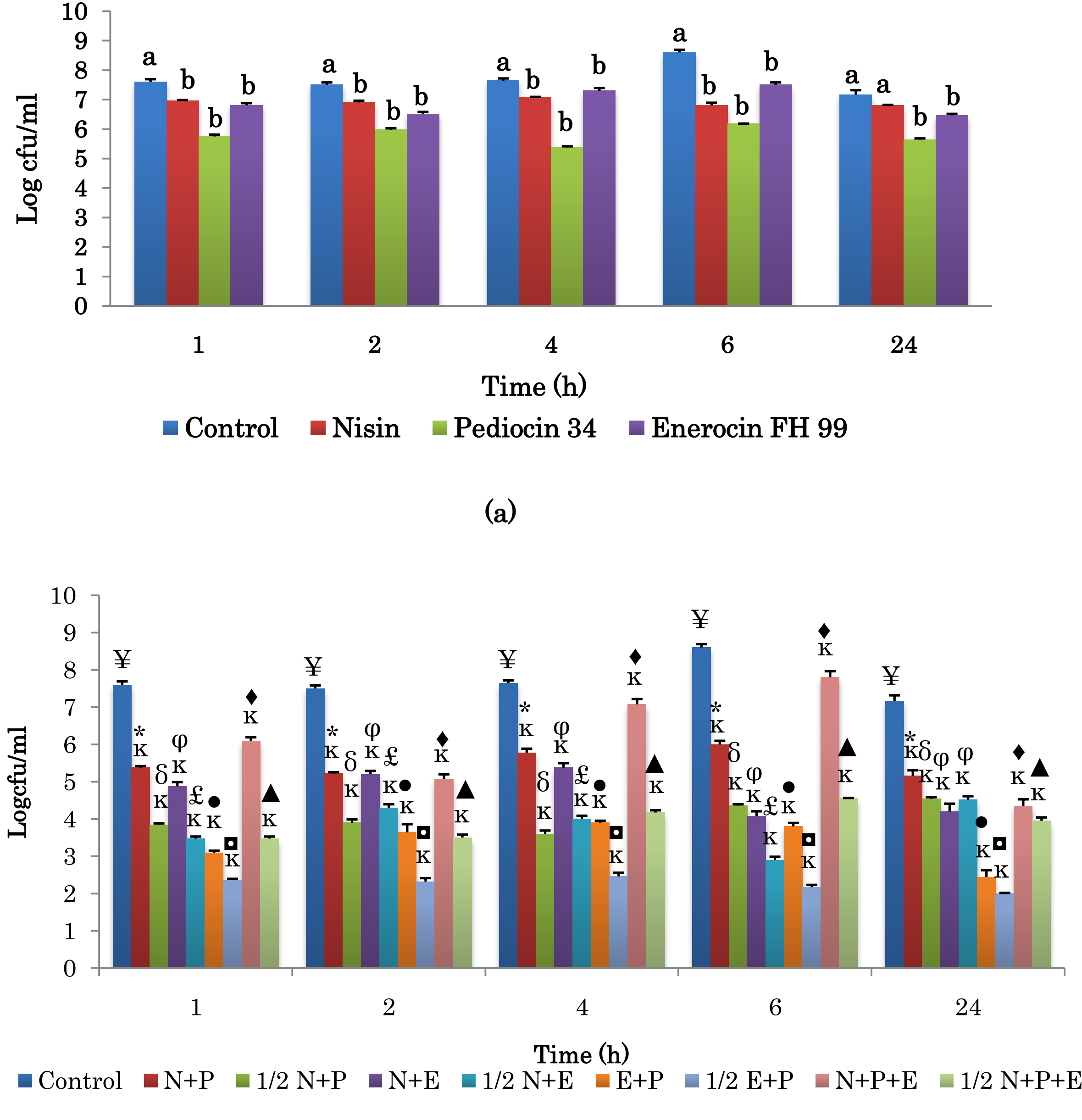


Fig 4.6. a) Effect of Nisin, Pediocin 34 and Enterocin FH99 alone on *L. monocytogenes* MTCC 657. b) Effect of different combinations of Nisin, Pediocin 34 and Enterocin FH99 on *Listeria monocytogenes* MTCC 657. Values are presented as mean ±SEM; n=3. ^{a,b} Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between control and in presence of bacteriocins. $^{¥,\kappa}$Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between control and in presence of different combinations of bacteriocins.$^{*,\delta}$ Values show significant difference between (N+P) and ½(N+P).$^{\varphi,£}$ Values show significant difference between (N+E) and ½(N+E). $^{•,◘}$Values show significant difference between (P+E) and ½(P+E). $^{♦,▲}$Values show significant difference between (N+P+E) and ½(N+P+E). N= Nisin, P= Pediocin 34, E= Enterocin FH99

FH99 using concentrations of MICs for each of the bacteriocins used to evaluate additive effect of bacteriocins on *L. monocytogenes* MTCC 657 after 24h incubation.

Synergistic action of bacteriocins was observed among different combinations of nisin + pediocin 34, nisin + enterocin FH99, pediocin 34 + enterocin FH99 and nisin + pediocin 34 + enterocin FH99 when tested against *E. faecium* DSMZ 20477*, E. faecium* (VRE) *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657. Though, a combination of nisin + pediocin 34 + enterocin FH99 was most effective against *E. faecium* DSMZ 20477 and *E. faecium* (VRE), a combination of pediocin 34 + enterocin FH99 was most effective in inhibiting *L. monocytogenes* MTCC 657.

Though nisin was most effective in inhibiting *L. monocytogenes* ATCC 53135, but it was not effective in inhibiting *L. monocytogenes* MTCC 657. Effectiveness of nisin in inhibiting the *Listeria monocytogenes ATCC* 53135 was followed by pediocin 34 and enterocin FH99. These results have been found to be similar to those reported earlier for the kinetics of cell destruction of *L. innocua*, where nisin proved to be the most effective inhibitor in comparison to the pediocin AcH and enterococcin EFS2, but nisin was also the bacteriocin that allowed the highest cell counts at 24 h (Schillinger *et al*., 1998). Pediocin 34 proved to be more effective as compared to nisin and enterocin FH99 in inhibiting *L. monocytogenes* MTCC 657. Cintas *et al*. (1998) has also reported pediocin to be more effective than nisin against some food borne pathogens such as *L. monocytogenes*.

In case of *Listeria* strains, it was observed that even though nisin displayed the most rapid inhibitory activity after 1 h, the survivors resumed growth, reaching the highest cell counts after 24 h. Similar observations have also been made by Schillinger *et al*. (1998), who reported a regrowth of survivors of *L. monocytogenes* Scott A after exposure to nisin concentrations between 10 and 500 IU/ml. Earlier Song & Richard (1997) also reported that survivors of *L. innocua* resumed growth after the addition of nisin, pediocin AcH, and enterococcin EFS2 into TSBYE broth.

The results of the present study indicate that the combinations of different bacteriocins produce a more effective antibacterial effect against *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* ATCC 53135 and *L. monocytogenes*

MTCC 657 in comparison to the bacteriocins used alone. Similar results were reported by Hanlin *et al.* (1993) while studying the antibacterial efficiency of pediocin AcH and nisin against several Gram-positive bacterial strains. It was concluded that a mixture containing more than one bacteriocins would have greater bactericidal effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. In our study, synergistic effects are reported when the interactions between pairs of bacteriocins from lactic acid bacteria are tested and these findings are in accordance with the results obtained by Mullet-Powell *et al.* (1998). However, different effectiveness of bacteriocin pairs could be due to the bacteriocins used in this study belonged to different classes, which vary considerably in the nature and sequence of amino acid residues (Moll *et al.*, 1999).The synergistic action of combinations of two different bacteriocins with different structures produced by the same strain has also been reported in agar medium by Limonet *et al.* (2004). Similar results have been reported by Jamuna *et al.* (2005) who showed that the bacteriocins from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems. Vignolo *et al.* (2000) also reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT in meat slurry resulted in complete killing of cells after incubation for 3 h. Jamuna & Jeevaratnam (2009) also reported the synergistic effect of nisin and bacteriocin from *Pediococcus acidilactici*, as they were more effective in inhibiting the growth of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav. When pediocin 34 and enterocin FH99 were used alone or in combination, a higher number of survivors were detected than with the pairs containing nisin for the strains of *E. faecium* and *L.monocytogenes*. Similar results were reported by Vignolo *et al.* (2000) while studying the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT. Similar results have been reported by Jamuna *et al.* (2005) who showed that the bacteriocins from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems.

4.5 STABILITY OF DEVELOPED RESISTANCE IN TERMS OF MINIMUM INHIBITORY CONCENTRATION (MIC) OF BACTERIOCIN RESISTANT VARIANTS

Spontaneous resistant mutants of strains *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecalis* ATCC 29212, *E. faecium* DSMZ 20477 and *E. faecium* VRE to nisin, pediocin 34 and enterocin FH99 were isolated after sequential exposure to a bacteriocin concentration 10-fold higher the MIC. Only nisin resistant variant for *E. faecalis* ATCC 29212 was developed since it was already resistant to pediocin 34 and enterocin FH99. The stability of these resistances in cultures without bacteriocins was checked and determined by MICs.

The stability of nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecalis* ATCC 29212, *E. faecium* DSMZ 20477 and *E. faecium* VRE was determined by studying the susceptibility towards the given bacteriocin (Table 4.4). The nisin resistance phenotype in *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* VRE and *E.faecalis* ATCC 29212 was stable during at 60, 50, 40, 20 and 30 successive cultures, respectively, without nisin. The pediocin resistance phenotype in *L. monocytogenes* ATCC 53135, *L.monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE was stable during at 40, 20, 30 and 10 successive cultures, respectively, without pediocin 34. The enterocin resistance phenotype in *L. monocytogenes* ATCC 53135, *L.monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE was stable during at 10, 30, 30 and 20 successive cultures, respectively, without pediocin 34.

Table 4.5 shows the MICs of the wild type and the nisin, pediocin 34 and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135*, Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 as determined by the spot- on –lawn assay. The MIC was determined as the minimal concentration giving a visible zone of inhibition after 24 h at 37 °C, as the mean of three independent experiments. Increase in the MIC was observed for the bacteriocin resistant variants. The MICs of the developed

bacteriocin resistant variants was assessed by the spot on lawn assay as described by Ulhman *et al.* (1992).

Table 4.4 Comparison of susceptibility of wild type strains and resistant variants to nisin, pediocin 34 and enterocin FH99

Culture	Strain	Nisin	Pediocin 34	Enterocin FH99
L. monocytogenes ATCC 53135	WT	+	+	+
	Nr	-	+	+
	Pr	+	-	-
	Er	+	+	-
L. monocytogenes MTCC 657	WT	+	+	+
	Nr	-	+	+
	Pr	+	-	-
	Er	+	+	-
E. faecium DSMZ 20477	WT	+	+	+
	Nr	-	-	-
	Pr	+	-	-
	Er	+	-	-
E. faecium VRE	WT	+	+	+
	Nr	-	-	-
	Pr	+	-	-
	Er	+	-	-
E. feacalis ATCC 29212	WT	+	-	-
	Nr	-	-	-

WT= wild type, Nr= nisin resistant, Pr= pedioicn 34 resistant, Er= enterocin FH99 resistant. No inhibition = - , Inhibition= +

Nisin resistant variants of *E. faecium* DSMZ 20477, *E. faecium* (VRE), *E. faecalis* ATCC 29212, *L. monocytogenes* MTCC 657 and *L. monocytogenes* ATCC 53135 were 60, 300, 100, 200 and 92 fold more resistant to nisin than their corresponding wild type strains. Pediocin 34 resistant variants of *E. faecium* DSMZ 20477, *E. faecium* (VRE), *L. monocytogenes* MTCC 657 and *L. monocytogenes* ATCC 53135 were 80, 2, 6 and 64-fold more resistant to pediocin 34, respectively, than their corresponding wild type strains whereas enterocin FH99 resistant variants of *E. faecium* DSMZ 20477, *E. faecium* (VRE), *L. monocytogenes* MTCC 657 and L. monocytogenes ATCC

53135 were 70, 2, 5 and 1250 -fold more resistant to enterocin FH99 than their corresponding wild type strains.

Table 4.5 MICs of wild type and the nisin, pediocin 34 and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC , *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212

Culture	Strains	Nisin	Pedioicn 34	Enterocin FH99
L. monocytogenes ATCC 53135	WT	13.2	540	120
	Nr	1250	540	120
	Pr	400	35000	7500
	Er	400	35000	150000
L. monocytogenes MTCC 657	WT	50	600	700
	Nr	10,000	600	700
	Pr	5000	35000	3750
	Er	10,000	3500	3750
E. faecium DSMZ 20477	WT	53.5	68.5	937.5
	Nr	3200	5600	4800
	Pr	5000	5600	4800
	Er	3200	5600	4800
E. faecium VRE	WT	14	2187.5	3750
	Nr	3200	5600	4800
	Pr	100	5600	4800
	Er	100	5600	4800
E. faecalis ATCC 29212	WT	26.5	-	-
	Nr	3200	-	-

WT= wild type, Nr= nisin resistant, Pr= Pedioicn 34 resistant, Er= Enterocin FH99 resistant.

4.6. ANTIBIOTIC RESISTANCE

An undesirable consequence of an extended use of natural antimicrobials such as nisin in food might be cross-resistance to clinically used antibiotics in foodborne

pathogens. Only a few studies have comprehensively addressed this issue (Crandall & Montville, 1998; Gravesen *et al.*, 2001). In this work, we analyzed the antibiotic susceptibility of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium DSMZ 20477*, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 along with their nisin, pediocin 34 and enterocin FH99 resistant variants (Table 4.6).

The sensitivity of *L. monocytogenes* ATCC 53135, and its bacteriocin-resistant variants to antibiotics, as measured by agar inhibition zone diameter, is given in Table 4.6. Strain *L. monocytogenes* ATCC 53135 was highly sensitive to chloramphenicol, rifampicin, and tetracycline, moderately sensitive to streptomycin, ampicillin, kanamycin, erythromycin, gentamycin, vancomycin, bacitracin, polymyxin, novobiocin, and penicillin G and showed resistance towards nalidixic Acid. Nisin resistant variant of *L. monocytogenes* appeared to be more susceptible to all the antibiotics except bacitracin, streptomycin and kanamycin and was resistant to nalidixic. However, the Pediocin resistant variant of *L. monocytogenes* ATCC 53135 showed increased sensitivity to only tetracycline than the wild type *L. monocytogenes* ATCC 53135. The highest increase in sensitivity for Nisin resistant variant was to penicillin, which showed inhibition zone diameters higher (12 mm) than those obtained for wild type strain. Enterocin resistant variant showed sensitivity to nalidixic acid than their wild type counterparts as well as nisin and pediocin 34 resistant variants. Also, the enterocin resistant variant displayed resistance to polymyin B. The sensitivity of *L. monocytogenes* MTCC 657, and its bacteriocin-resistant variants to antibiotics, as measured by agar inhibition zone diameter, is given in Table 4.6.

Strain *L. monocytogenes* MTCC 657 was highly sensitive to chloramphenicol, rifampicin, tetracycline, streptomycin, ampicillin, kanamycin, erythromycin, gentamycin, vancomycin, bacitracin, polymyxin, and penicillin G and showed resistance towards novobiocin and nalidixic acid. Nisin resistant variant of *L. monocytogenes* appeared to be more susceptible to all the antibiotics. However, the pediocin 34 resistant variant of *L. monocytogenes* MTCC 657 showed resistance to erythromycin and gentamycin than the wild type *L. monocytogenes* MTCC 657.

Enterocin as well as nisin and pediocin resistant variant, however showed sensitivity to novobiocin in contrast to their wild type counterparts.

Strain *E. faecium* DSMZ 20477 was highly sensitive to chloramphenicol, rifampicin, tetracycline, vancomycin, bacitracin and polymyxin; moderately sensitive to erythromycin and chloramphenicol but showed resistance towards streptomycin , kanamycin, gentamycin, nalidixic acid, ampicillin, rifampicin, penicillin g and novobiocin. Nisin resistant variant of *E. faecium* DSMZ 20477 appeared to be more susceptible to the antibiotics ampicillin, rifampicin, penicillin G, chloramphenicol and novobiocin. However, the pediocin 34 resistant variant of *E. faecium* DSMZ 20477 showed no change in the antibiotic susceptibility profile as compared to the wild type counterpart. Enterocin resistant variant showed sensitivity to rifampicin, penicillin G, and chloramphenicol and moderate sensitivity to novobiocin than their wild type counterparts.

Strain *E. faecium* VRE was, however, highly sensitive to bacitracin, rifampicin, chloramphenicol, novobiocin and polymyxin; moderately sensitive to tetracycline and showed resistance towards vancomycin, streptomycin, kanamycin, gentamycin, nalidixic acid, ampicillin and penicillin G. Nisin resistant variant of *E. faecium* VRE appeared to be more susceptible to the antibiotics vancomycin and penicillin G, and resistant to polymyxin B. However, the pediocin 34 resistant variant of *E. faecium* VRE displayed sensitivity to novobiocin and resistance to polymyxin B as compared to its wild type counterpart. Enterocin FH99 resistant variant showed sensitivity to vancomycin and penicillin G, and resistance to polymyxin B in contrast to its wild type counterpart.

Strain *E. faecalis* ATCC 29212 was highly sensitive to tetracyclin, vancomycin bacitracin, gentamycin, nalidixic acid, ampicillin, rifampicin, penicillin G, chloramphenicol and novobiocin, moderately sensitive to kanamycin and showed resistance towards streptomycin, erythromycin, nystatin and polymyxin B. Nisin resistant variant of *E. faecalis* ATCC 29212 appeared to be resistant to streptomycin, kanamycin, gentamycin and nalidixic acid. However, the Nisin

resistant variant displayed moderate sensitivity to polymyxin B as compared to its wild type counterpart.

Table 4.6 Susceptibility[a] of wild type strains and their bacteriocin resistant variants to antibiotics

Bacterial culture & Strains		Antibiotics													
		T	**V**	**B**	**S**	**K**	**E**	**G**	**Na**	**A**	**R**	**P**	**C**	**Nv**	**Pb**
L. monocytogenes ATCC 53135	**WT**	S	M	M	M	M	M	M	R	M	S	M	S	M	M
	Nr	S	S	M	M	M	S	S	R	S	S	S	S	S	S
	Pr	S	M	M	M	M	M	M	R	M	S	M	S	M	M
	Er	S	M	M	M	M	M	M	S	M	S	M	S	M	R
L. monocytogenes MTCC 657	**WT**	S	S	S	S	S	S	S	R	S	S	S	S	R	S
	Nr	S	S	S	S	S	S	S	S	S	S	S	S	M	S
	Pr	S	S	S	M	M	R	R	R	S	S	M	S	R	S
	Er	S	S	S	S	S	S	S	R	S	S	S	S	S	S
E. faecium DSMZ 20477	**WT**	S	S	S	R	R	M	R	R	R	R	R	M	R	S
	Nr	S	S	S	R	R	M	R	R	S	S	S	S	S	S
	Pr	S	S	S	R	R	M	R	R	R	R	R	M	R	S
	Er	S	S	S	R	R	M	M	R	R	S	S	S	M	S
E. faecium VRE	**WT**	M	R	S	R	R	R	R	R	R	S	R	S	S	S
	Nr	S	S	S	R	R	R	R	R	R	S	S	S	S	R
	Pr	S	S	S	R	R	R	R	R	R	S	R	S	S	R
	Er	S	S	S	R	R	R	R	R	R	S	S	S	S	R
E. faecalis ATCC 29212	**WT**	S	S	S	R	M	R	S	S	S	S	S	S	S	R
	Nr	S	S	S	R	R	R	R	R	S	S	S	S	S	M

WT= wild type, Nr= nisin resistant, Pr= Pedioicn 34 resistant, Er= Enterocin FH99 resistant
T=Tetracycline, V=Vancomycin, B=Bacitracin, K=Kanamycin, E=Erythromycin, G=Gentamycin, N=Nystatin, Na= Nalidixic acid, A= Ampicillin, R=Rifampicin, P=PenicillinG, C= Chloramphenicol, Nv= Novobiocin, Pb=Polymyxin B
S= sensitive; M= moderately sensitive; R= resistant
[a]Zone of Inhibition calculated according to the table given by NCCLS (2001)

The nisin resistant strains of *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) were more susceptible to most of the antibiotics tested than their wild type counterparts. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998). Only cross-resistance to the membrane disturbing polymixin B was exhibited by the three resistant variants of *E. faecium* (VRE). This seems to be quite reasonable as nisin shares the same primary target: the cytoplasmic membrane, with polymixin B. Also, the resistance has been partially correlated with changes in the membrane composition which potentially interfere with the pore forming ability of nisin in the cytoplasmic membrane (Mazzotta & Montville, 1997; Crandall & Montville, 1998). Moreover, changes in the cell envelope such as a thickened cell wall, polysaccharide production or a higher degree of D-alanine substitution in the teichoic acids have been described as resistance strategies to avoid killing by cationic antimicrobial peptides (Davies *et al.*, 1996; Peschel *et al.,* 1999). Basically these mechanisms lower the net negative surface charge and restrict the accessibility of nisin and, hence, of other cationic drugs such as polymixin B and amino glycosides, to their targets. Pediocin 34 resistant strain of *L. monocytogenes* MTCC 657 and Nisin resistant strain of *E. faecalis* ATCC 29212 were observed to be resistant to gentamicin. Nisin resistant variant of *E. faecalis* ATCC 29212 was also resistant to nalidixic acid. The site of action of gentamicin is protein synthesis and nalidixic acid inhibits a subunit of DNA gyrase and induces formation of relaxation complex analogue. It also inhibits the nicking dosing activity on the subunit of DNA gyrase that releases the positive binding stress on the supercoiled DNA. The increase in resistance to gentamicin and nalidixic acid might result from an alteration in the cell wall which prevents these compounds from reaching their targets (Crandall & Montville, 1998).

4.7 BACTERIOCIN CROSS RESISTANCE

Resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The sensitivity of *Listeria monocytogenes ATCC* 53135*,*

Listeria monocytogenes MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their resistant variants to nisin, pediocin 34 and enterocin FH99 was determined qualitatively by the agar diffusion test (Plate 4.1 to 4.5).

L. monocytogenes ATCC 53135 showed sensitivity to nisin, pediocin 34 and enterocin FH99. However, its pediocin 34 resistant variant appeared to be sensitive to nisin but resistant to enterocin FH99, indicating that the development of resistance to one of these bacteriocins may show resistance to the other bacteriocins. However, its nisin resistant variant showed the sensitivity to pediocin 34 as well as enterocin FH99. On the other hand, resistance to enterocin FH99 conferred cross resistance to pediocin 34 but not to nisin (Plate 4.1).

Pediocin 34 resistant variant of *L. monocytogenes* MTCC 657 displayed cross resistance to Enterocin FH99 but not to nisin. However, its Enterocin FH99 resistant variant was sensitive to both nisin and as well as pediocin 34 (Plate 4.2).

Nisin resistance in *E. faecium* DSMZ 20477 conferred cross resistance to both pediocin 34 and enterocin FH99. Enterocin FH99 resistant variant displayed cross resistance to pediocin 34 whereas pediocin 34 resistant variant of *E. faecium* DSMZ 20477 showed cross resistance to enterocin FH99 (Plate 4.3). Similar results were observed in case of *E. faecium* (VRE). *L. monocytogenes* ATCC 53135 showed sensitivity to nisin, pediocin 34 and enterocin FH99. However, pediocin 34 resistant variant appeared to be sensitive to nisin but resistant to enterocin FH99, indicating that the development of resistance to one of these bacteriocins may show resistance to the other bacteriocins. Nisin resistant variant retained the sensitivity to pediocin 34 as well as enterocin FH99. But resistance to Enterocin FH99 conferred cross resistance to pediocin 34 but not to nisin (Plate 4.4). Nisin resistant *E. faecalis* ATCC 29212 was resistant to pediocin 34 and enterocin FH99 (Plate 4.5).

Several reports suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *L. monocytogenes* was observed to be showing cross resistance to the class IIa

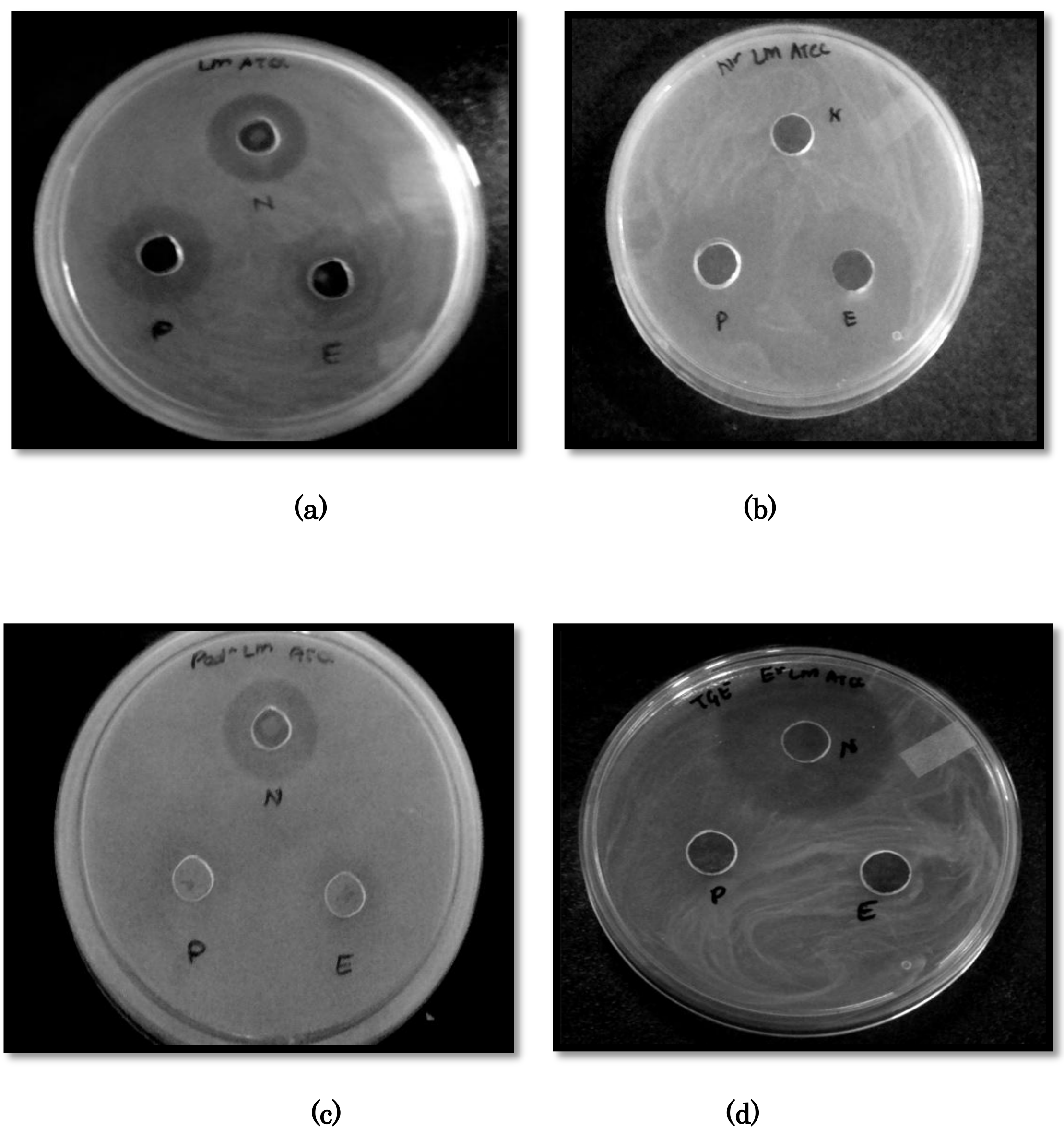


Plate 4.1: Agar diffusion test showing cross resistance among bacteriocin resistant variants of *L. monocytogenes* ATCC 53135. Agar was seeded with a) Wild type *L. monocytogenes* ATCC 53135 and its variants resistant to b) Nisin (N), c) Pediocin 34 (P) and d) Enterocin FH99 (E).

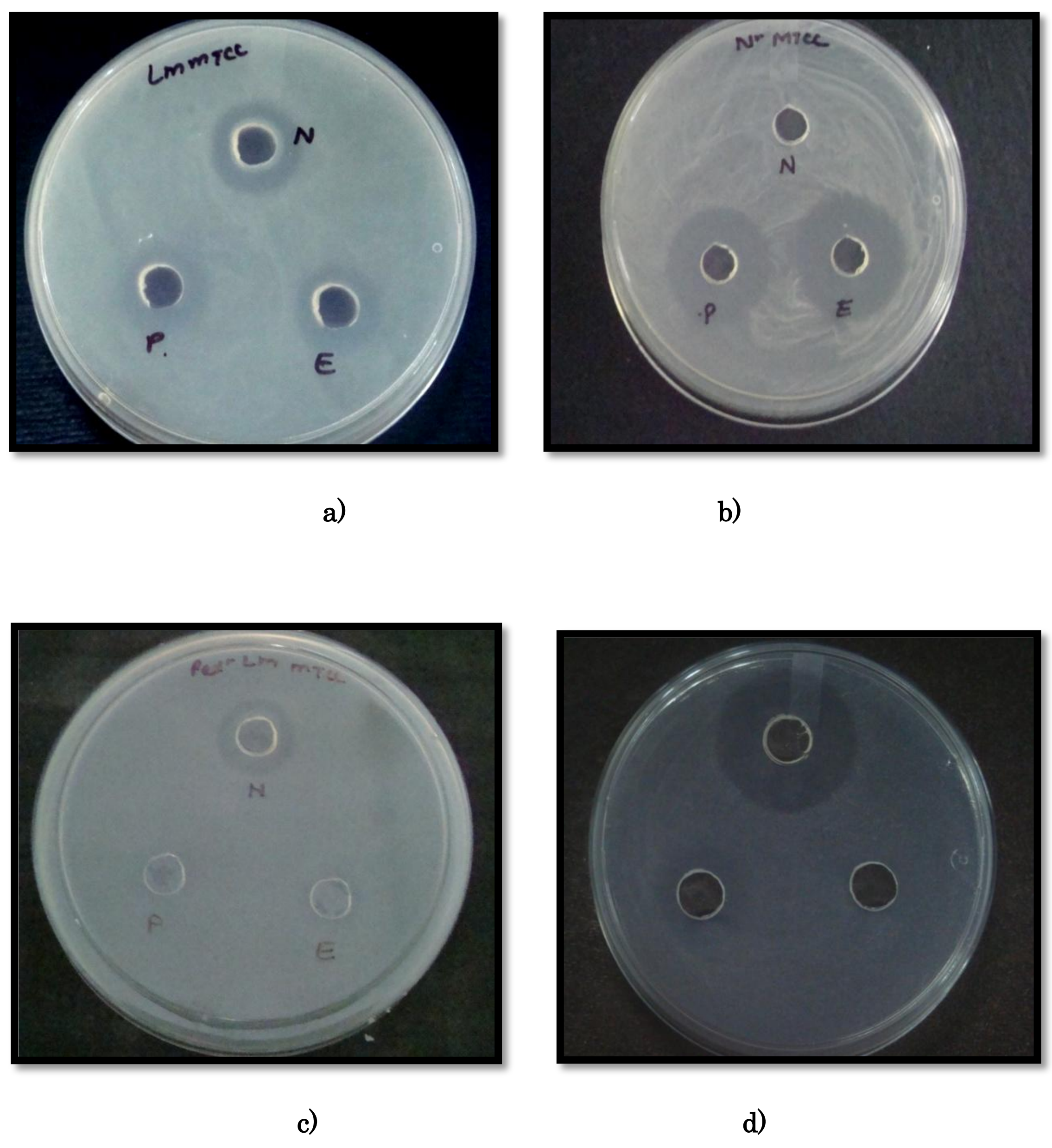


Plate 4.2: Agar diffusion test showing cross resistance among bacteriocin resistant variants of *L. monocytogenes* MTCC 657. Agar was seeded with a) Wild type *L. monocytogenes* MTCC 657 and its variants resistant to b) Nisin (N), c) Pediocin 34 (P) and d) Enterocin FH99 (E).

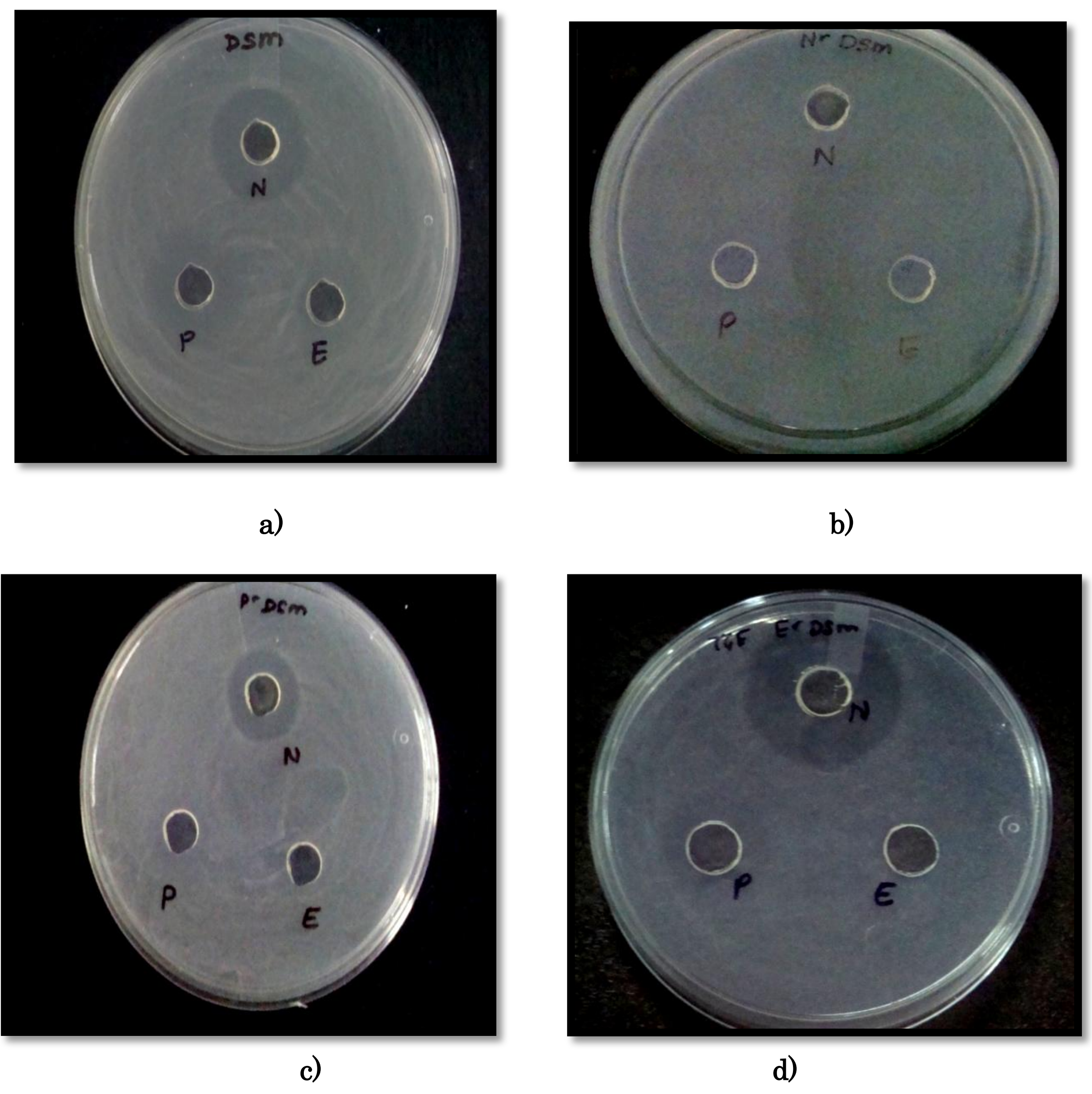


Plate 4.3: Agar diffusion test showing cross resistance among bacteriocin resistant variants of *E. faecalis* DSMZ 20477. Agar was seeded with a) Wild type *E. faecalis* DSMZ 20477 and its variants resistant to b) Nisin (N), c) Pediocin 34 (P) and d) Enterocin FH99 (E)

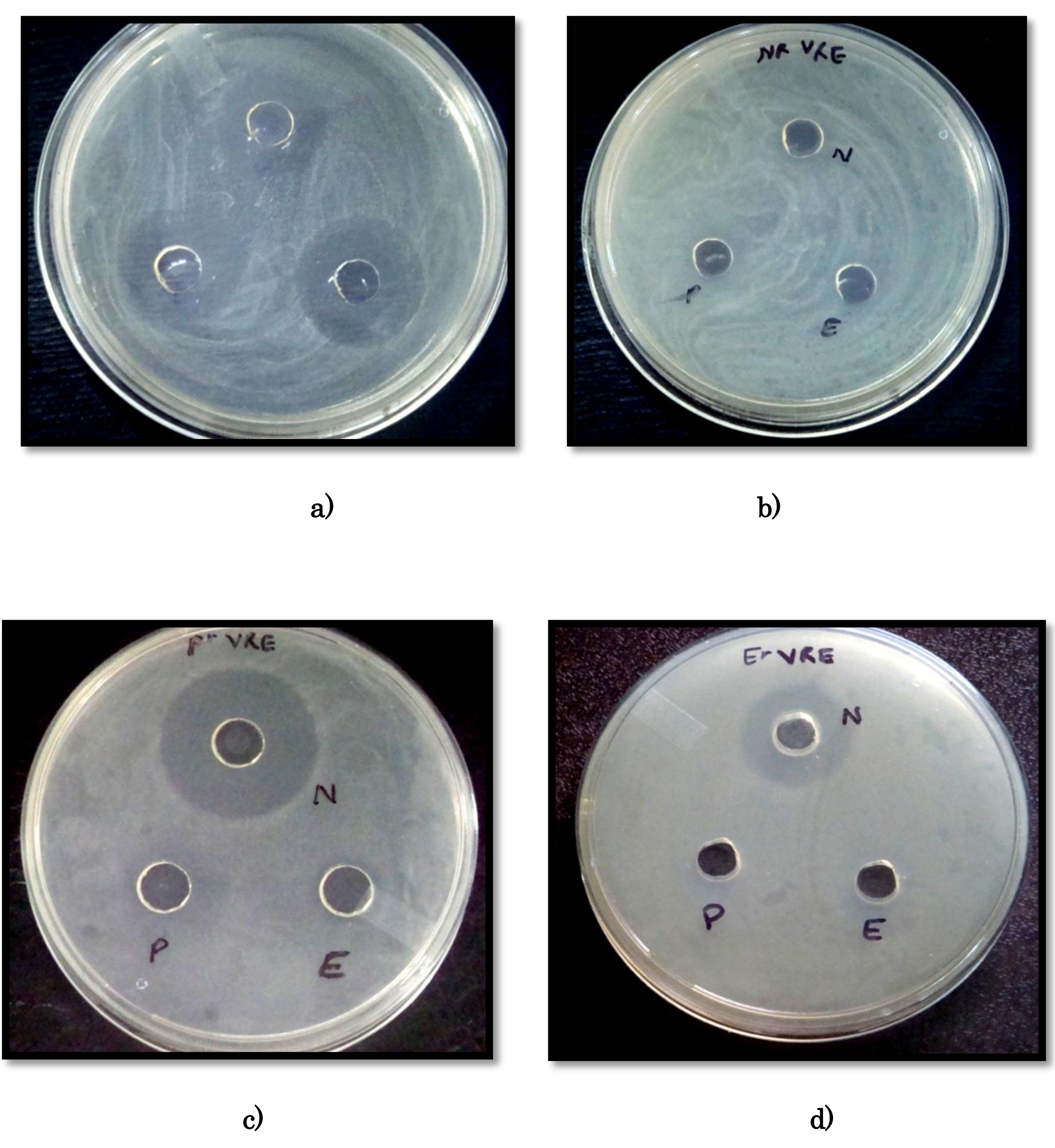
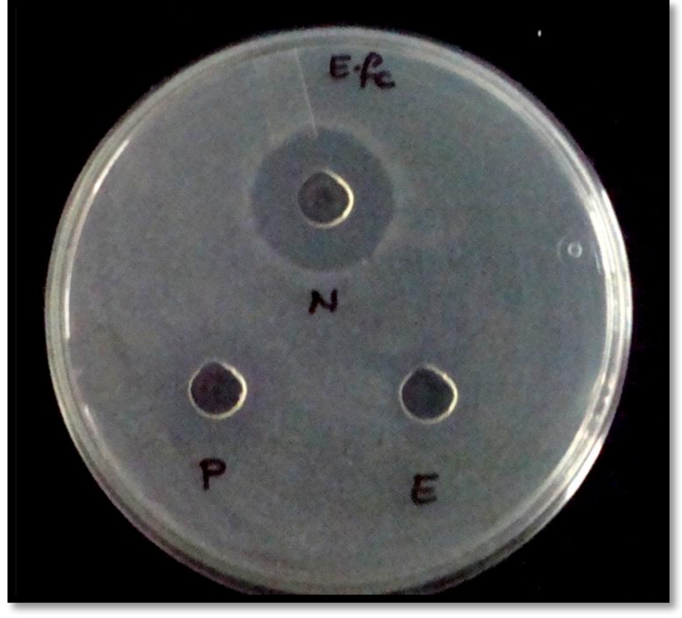


Plate 4.4: Agar Diffusion test showing cross resistance among bacteriocin resistant variants of *E. faecium* VRE. Agar was seeded with a) Wild type *E. faecium* VRE and its variants resistant to b) Nisin (N), c) Pediocin 34 (P) and d) Enterocin FH99 (E)



a)

b)

Plate 4.5: Agar Diffusion test showing cross resistance among bacteriocin resistant variants of *E. faecalis* ATCC 29212. Agar was seeded with a) Wild type *E. faecalis* ATCC 29212 and its variants resistant to b) Nisin (N)

bacteriocin pediocin PA-1 and the class IV leuconocin S (Crandall & Montville, 1998). *L. monocytogenes* mutants resistant to mesenterocin 52, curvaticin 13, and plantaricin were also reported to show cross-resistance to the other bacteriocins (Rekhif *et al.*, 1994). In addition, piscicolin 126-resistant mutants of *L. monocytogenes* which emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02 (Wan *et al.*, 1997). These reports of cross-resistance thus indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy (Hanlin *et al.*, 1993) might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the others too.

Cross-resistance among bacteriocins has also been observed when the sensitivity of *Listeria* variants to lactocin 705, enterocin CRL35, and nisin was tested. Similar results were obtained by Rekhif *et al.* (1994) who reported that mutants of *L. monocytogenes* ATCC 15313 resistant to one of three bacteriocins tested (mesentericin 52, curvaticin 13, and plantaricin C19), displayed more resistance to the other two, but not to nisin. Insensitivity of a variant to lactocin 705 and enterocin CRL35 while retaining sensitivity to nisin, and vice versa, was associated with the mechanism by which a bacteriocin enters the cell following binding to the cell surface, as well as with the ability to form pores in bacterial membranes.

4.8 CROSS RESISTANCE OF WILD TYPE AND RESISTANT STRAINS TO COMMON FOOD PRESERVATIVES

The concept of hurdle technology began to apply in the food industry in a rational way after the observation that survival of microorganisms greatly decreased when they were confronted with multiple antimicrobial factors (Leistner, 1978; Leistner & Gorris, 1995; Leistner, 2000). The application of bacteriocins as part of hurdle technology has received great attention in recent years, since bacteriocins can be used purposely in combination with selected hurdles in order to increase microbial inactivation (Chen & Hoover, 2003; Ross *et al.*, 2003; Deegan *et al.*, 2006). Examples of those factors are traditional food-preservation hurdles like acidification and addition of salt (Harris *et al.*, 1991;

Mazzotta & Montville, 1997), heat treatment, refrigeration, (Roberts & Hoover, 1996; De Martinis *et al.*, 1997) or incorporation of additives (Taylor *et al.*, 1985). Bacteriocin-resistant strains of pathogens and food spoilage bacteria may arise as bacteriocins become more widely used as an additional safety barrier in minimally-processed foods. Reports suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *L. monocytogenes* was observed to be showing cross resistance to the class IIa bacteriocin pediocin PA-1 and the class IV leuconocin S (Crandall & Montville, 1998).

Stable nisin-resistant strains of *L. monocytogenes* and *C. botulinum* have been reported to be cross-resistant to bacteriocins that are chemically different than nisin (Mazzotta & Montville, 1997; Crandall & Montville, 1998), but there are no reports on the development of intrinsic resistance to other preservation factors. These additional factors that act in combination with bacteriocins to decrease the emergence of bacteriocin-resistant strains may not be effective once nisin-resistant isolates emerge as stable strains.

Therefore, the sensitivity of wild type *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 along with their nisin, pediocin 34 and enterocin FH99 resistant variants to low pH, salt, sodium nitrite, and potassium sorbate was assayed to determine if resistance to nisin, pediocin 34 and enterocin FH99 confers cross resistance to these common food preservatives (Tables 4.7 to 4.26).

4.8.1. Effect of Low pH on Wild Type and Bacteriocin Resistant Variants

The sensitivity of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 along with their nisin, pediocin 34 and enterocin FH99 resistant variants to low pH (5.2, 5.0, 4.8, 4.6, 4.4) was assessed (Table 4.7 to 4.11).

4.8.1.1 Effect of low pH on wild type *L. monocytogenes* ATCC 53135 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 is given in Table 4.7. At pH 4.4 wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 4.45, 6.3 and 4.59 after 1, 8 and 24h, respectively (Table 4.7). Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.75, 6.78 and 5.43 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 5.88. 7.83 and 6.00 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.60, 6.03 and 4.86 log cycles after 1, 8 and 24h, respectively.

At pH 4.6 wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.65, 4.32 and 4.03 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.14, 4.67 and 4.96 after 1h, 8h and 24h respectively. Pediocin 34 resistant variant showed a reduction of 4.8, 5.04 and 4.00 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.25, 4.23 and 5.00 log cycles after 1, 8 and 24h, respectively.

At pH 4.8 wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 1.79, 3.07 and 2.21 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.50, 5.12 and 4.00 after 1, 8 and 24h, respectively.

Pediocin 34 resistant variant showed a reduction of 3.21, 5.45 and 3.55 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.34, 5.54 and 4.96 log cycles after 1, 8 and 24h, respectively.

At pH 5.0 wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 2.59, 2.32 and 0.83 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.13, 4.15 and 4.04 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.21,

4.52 and 3.85 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.47, 5.09 and 4.49 log cycles after 1, 8 and 24h, respectively.

Table 4.7 Viable cell count (log cfu/ml) of wild type *L. monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant and enterocin FH99 resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3)

pH	Strain	TIME (h)					
		1	2	4	6	8	24
Control	WT	7.97±0.03	7.74±0.03	8.49±0.13	7.86±0.03	8.95±0.02	8.11±0.09
	Nr	7.95±0.04	7.71±0.03	8.47±0.01	7.83±0.09	8.93±0.08	8.04±0.03
	Pr	7.99±0.05	7.72±0.06	8.86±0.09	7.83±0.09	8.94±0.03	8.00±0.13
	Er	7.94±0.09	7.71±0.03	8.47±0.01	7.85±0.02	8.93±0.09	8.04±0.03
pH 4.4	WT	3.51±0.85	4.81±0.29	3.60±0.20	3.39±0.79	2.25±0.35	3.51±0.13
	Nr	4.20±0.04	2.47±0.12	2.62±0.32	2.11±0.81	2.14±0.61	2.60±0.20
	Pr	2.11±0.39	1.60±0.20	2.27±0.60	1.77±0.15	1.11±0.39	2.00±0.83
	Er	3.34±0.24	2.34±0.81	2.46±0.23	2.32±0.22	2.90±0.30	3.17±0.60
pH 4.6	WT	4.32±0.22	4.71±0.60	4.54±0.40	4.79±0.05	4.63±0.12	4.07±0.91
	Nr	4.80±0.61	3.69±0.04	3.66±0.27	4.54±0.40	4.25±0.05	3.07±0.18
	Pr	3.14±0.61	3.07±0.24	3.30±0.10	3.84±0.04	3.90±0.08	4.00±0.38
	Er	3.14±0.61	3.07±0.18	3.30±0.10	3.84±0.50	3.90±0.30	4.00±0.56
pH 4.8	WT	6.17±0.12	5.73±0.23	6.04±0.13	6.68±0.12	5.88±0.08	5.90±0.30
	Nr	4.44±0.31	4.49±0.13	3.83±0.25	4.25±0.05	3.81±0.13	4.04±0.26
	Pr	4.77±0.51	3.90±0.30	3.77±0.15	3.62±0.23	3.49±0.13	3.44±0.31
	Er	3.60±0.20	3.51±0.13	3.50±0.51	3.69±0.04	3.39±0.79	3.07±0.91
pH 5.0	WT	5.38±0.61	5.70±0.01	5.50±0.14	6.55±0.30	6.63±0.34	7.27±0.60
	Nr	4.81±0.16	5.00±0.85	5.41±0.49	5.25±0.27	4.77±0.25	4.00±0.49
	Pr	4.77±0.12	4.69±0.04	4.60±0.20	4.54±0.40	4.41±0.49	4.14±0.61
	Er	4.47±0.71	4.17±0.60	3.84±0.50	4.00±0.82	3.84±0.50	3.54±0.44
pH 5.2	WT	6.39±0.09	6.44±0.31	6.30±0.10	5.77±0.12	5.57±0.83	5.43±0.13
	Nr	4.68±0.12	4.83±0.25	4.47±0.12	4.67±0.20	4.63±0.34	4.56±0.36
	Pr	5.17±0.60	4.68±0.12	5.36±0.17	5.49±0.13	5.54±0.40	4.44±0.71
	Er	4.47±0.12	4.53±0.14	4.43±0.13	4.61±0.27	4.77±0.25	5.23±0.04

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At pH 5.2, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 1.57, 3.37 and 2.68 after 1, 8 and 24h, respectively. Nisin resistant variant of *L.*

monocytogenes ATCC 53135 showed a reduction of 3.27, 2.88 and after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.81, 3.40 and 3.55 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.47, 4.16 and 2.81 log cycles after 1, 8 and 24h, respectively.

Results clearly showed that resistance to nisin, pediocin 34 and enterocin FH99 in *L. monocytogenes* ATCC 53135 did not confer intrinsic resistance to low pH. In contrast, resistant variants were more or equally sensitive to low pH as compared to the wild-type strain. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.1.2 Effect of low pH on wild type *L. monocytogenes* MTCC 657 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *L. monocytogenes* MTCC 657 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium with pH 4.4, 4.6, 4.8, 5.0 and 5.2 is given in Table 4.8. At pH 4.4 wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.05, 2.59 and 3.24 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 3.80, 6.36 and 7.19 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.84. 6.24 and 7.00 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.54, 5.53 and 6.78 log cycles after 1, 8 and 24h, respectively.

At pH 4.6, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.25, 2.72 and 3.96 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 1.82, 5.19 and 5.79 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.87, 5.17 and 6.02 log cycles whereas enterocin FH99 resistant variant showed a reduction of 1.97, 5.48 and 6.16 log cycles after 1, 8 and 24h, respectively.

Table 4.8 Viable cell count (log cfu/ml) of wild type *L. monocytogenes* MTCC 657 (WT) and its nisin (Nr) resistant, pediocin 34 resistant and enterocin FH99 resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3)

pH	Strain	TIME (h)					
		1	2	4	6	8	24
Control	WT	6.00±0.34	6.57±0.19	6.54±0.24	7.39±0.01	7.79±0.55	8.50±0.51
	Nr	6.07±0.81	6.56±0.14	6.53±0.17	7.36±0.23	7.79±0.20	8.49±0.13
	Pr	5.95±0.42	6.54±0.41	6.51±0.14	7.38±0.21	7.77±0.15	8.47±0.21
	Er	5.97±0.21	6.55±0.33	6.50±0.15	7.34±0.24	7.78±0.11	8.46±0.23
pH 4.4	WT	4.95±0.24	5.14±0.28	5.41±0.49	5.17±60	5.20±0.41	5.25±0.52
	Nr	2.27±0.54	2.07±0.91	2.17±0.60	2.07±0.81	1.43±0.13	1.30±0.10
	Pr	2.11±0.39	2.04±0.13	2.04±0.33	2.25±0.52	1.53±0.14	1.47±0.21
	Er	2.43±0.13	2.30±0.10	2.47±0.12	2.30±0.03	2.25±0.52	1.68±0.12
pH 4.6	WT	4.74±0.18	4.95±0.13	5.27±0.53	5.00±0.49	5.07±0.18	4.54±0.40
	Nr	4.25±0.55	3.30±0.10	3.17±0.69	2.39±0.49	2.60±0.20	2.69±0.37
	Pr	4.07±0.91	3.11±0.39	2.65±0.32	2.69±0.52	2.60±0.26	2.41±0.49
	Er	4.00±0.91	2.77±0.15	2.69±0.13	2.47±0.10	2.30±0.52	2.30±0.12
pH 4.8	WT	4.77±0.13	5.00±0.89	5.32±0.22	5.11±0.39	5.17±0.60	4.60±0.20
	Nr	4.2±0.04	3.39±0.79	3.25±0.52	2.47±0.71	2.69±0.89	2.69±0.97
	Pr	4.07±0.91	3.11±0.39	3.65±0.25	3.69±0.89	3.60±0.20	3.41±0.73
	Er	4.07±0.92	3.84±0.50	3.77±0.13	3.55±0.32	3.39±0.94	2.46±0.23
pH 5.0	WT	5.14±0.61	5.01±0.18	5.17±0.60	4.69±0.97	5.55±0.63	5.57±0.20
	Nr	4.32±0.22	4.47±0.71	4.14±0.62	3.69±0.89	3.60±0.20	5.20±0.41
	Pr	4.04±0.41	4.20±0.41	4.25±0.52	4.07±0.91	2.55±0.63	3.07±0.79
	Er	4.34±0.24	4.49±0.13	4.50±0.51	4.25±0.52	4.17±0.60	2.69±0.97
pH 5.2	WT	4.74±0.03	4.76±0.34	5.23±0.44	5.41±0.49	5.43±0.31	5.47±0.23
	Nr	4.17±0.87	4.20±0.41	4.60±0.20	432±0.93	4.25±0.13	5.39±0.39
	Pr	4.77±0.13	5.14±0.61	5.20±0.41	5.75±0.87	5.46±0.34	5.36±0.25
	Er	4.90±0.30	4.80±0.61	5.32±0.22	5.63±0.34	5.34±0.25	5.14±0.89

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At pH 4.8, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.22, 2.62 and 3.90 after 1h, 8h and 24h respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 1.84, 5.09 and 5.79 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of

1.87, 4.17 and 5.06 log cycles whereas enterocin FH99 resistant variant showed a reduction of 1.89, 4.38 and 6.00 log cycles after 1, 8 and 24h, respectively.

At pH 5.0, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 0.85, 2.24 and 2.92 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 1.75, 4.19 and 3.28 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.26, .522 and 5.39 log cycles, whereas enterocin FH99 resistant variant showed a reduction of 1.63, 3.60 and 5.76 log cycles after 1, 8 and 24h, respectively.

At pH 5.2, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.25, 2.36 and 3.02 after 1h, 8h and 24h respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 1.90, 3.53 and 3.09 after 1h, 8h and 24h respectively. Pediocin 34 resistant variant showed a reduction of 1.17, 2.31 and 3.11 log cycles whereas enterocin FH99 resistant variant showed a reduction of 1.07, 2.44 and 3.31 log cycles after 1h, 8h and 24h respectively.

Results clearly showed that resistance to nisin, pediocin 34 and enterocin FH99 in *L. monocytogenes* MTCC 657 did not confer intrinsic resistance to low pH. In contrast, resistant variants were more or equally sensitive to low pH as compared to its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.1.3 Effect of low pH on wild type *E. faecium* DSMZ 20477 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in media at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3) is given in Table 4.9.

Table 4.9 Viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3)

pH	Strain	TIME (h)					
		1	2	4	6	8	24
Control	WT	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
	Nr	6.43±0.04	6.83±0.01	6.46±0.08	7.61±0.07	8.51±0.04	8.69±0.04
	Pr	6.73±0.06	6.77±0.05	6.62±0.09	6.77±0.05	6.65±0.04	5.72±0.07
	Er	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
pH 4.4	WT	3.60±0.09	3.47±0.05	2.80±0.04	2.81±0.06	2.74±0.09	3.11±0.02
	Nr	2.20±0.03	2.30±0.06	2.32±0.05	2.67±0.08	2.77±0.05	2.47±0.05
	Pr	2.47±0.05	2.47±0.07	2.30±0.06	2.00±0.09	2.30±0.06	2.69±0.04
	Er	2.90±0.07	2.95±0.09	2.69±0.04	2.04±0.50	2.20±0.08	2.00±0.09
pH 4.6	WT	3.90±0.07	3.8±0.03	3.84±0.04	4.38±0.02	3.60±0.05	4.25±0.05
	Nr	3.23±0.04	3.84±0.09	3.36±0.17	3.30±0.10	3.39±0.09	3.14±0.06
	Pr	2.77±0.05	2.69±0.04	2.60±0.01	2.00±0.09	2.59±0.07	2.30±0.06
	Er	3.90±0.07	4.04±0.05	4.07±0.06	3.68±0.07	3.53±0.07	3.00±0.02
pH 4.8	WT	4.81±0.07	4.81±0.06	4.77±0.05	4.60±0.01	4.59±0.07	4.81±0.07
	Nr	2.43±0.04	3.59±0.01	3.27±0.08	3.04±0.07	3.11±0.04	3.00±0.07
	Pr	2.74±0.03	2.69±0.07	2.65±0.01	2.17±0.05	2.60±0.05	2.63±0.06
	Er	4.95±0.01	4.07±0.09	4.17±0.06	4.69±0.07	4.60±0.02	4.30±0.10
pH 5.0	WT	5.92±0.06	5.99±0.08	6.30±0.01	6.81±0.07	6.77±0.05	6.53±0.07
	Nr	4.44±0.01	5.69±0.04	5.77±0.02	5.86±0.06	5.30±0.01	3.00±0.03
	Pr	4.60±0.01	4.30±0.06	4.34±0.01	4.25±0.05	4.51±0.09	5.32±0.05
	Er	4.55±0.01	4.47±0.05	4.95±0.09	4.56±0.04	4.60±0.01	6.23±0.02
pH 5.2	WT	5.00±0.03	5.17±0.09	5.36±0.03	5.41±0.08	4.83±0.03	4.90±0.07
	Nr	4.62±0.09	4.63±0.06	4.69±0.04	5.14±0.06	5.71±0.04	4.69±0.04
	Pr	4.41±0.08	4.54±0.04	4.84±0.04	4.90±0.07	4.53±0.07	4.50±0.08
	Er	5.07±0.01	4.46±0.08	4.30±0.06	4.60±0.01	5.61±0.04	5.60±0.04

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At pH 4.4, wild type *E. faecium* DSMZ 20477 showed a log reduction of 3.34, 4.73 and 5.42 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 4.22, 5.74 and 6.22 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 4.25, 4.35 and

5.02 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.04, 6.27 and 7.78 log cycles after the same intervals, respectively.

At pH 4.6, wild type *E. faecium* DSMZ 20477 showed a log reduction of 3.04, 4.87 and 5.53 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 3.20, 5.12 and 5.55 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.95, 4.06 and 5.42 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.04, 4.94 and 6.78 log cycles after 1, 8 and 24h, respectively.

At pH 4.8, wild type *E. faecium* DSMZ 20477 showed a log reduction of 2.13, 2.59 and 3.88 after 1h, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 4.00, 5.40 and 5.69 log cycles after the same intervals. Pediocin 34 resistant variant showed a reduction whereas of 3.99, 4.05 and 5.09 log cycles whereas, enterocin FH99 resistant variant showed a reduction 1.99, 3.87 and 5.48 log cycles after 1, 8 and 24h, respectively.

At pH 5.0, wild type *E. faecium* DSMZ 20477 strain showed a log reduction of 1.02, 1.69 and 3.25 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 1.98, 3.21 and 5.69 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.13, 2.13 and 3.12 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.39, 3.15 and 3.55 log cycles after 1, 8 and 24h, respectively.

At pH 5.2, wild type *E. faecium* DSMZ 20477 showed a log reduction of 1.80, 2.80 and 4.00. Similar respective log reduction after 1, 8 and 24h for nisin resistant variant of this culture showed a reduction of 1.94, 3.58 and 4.88 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.31, 2.12 and 3.21 log cycles, whereas enterocin FH99 resistant variant showed a reduction of 1.87, 2.86 and 4.18 log cycles after 1, 8 and 24h, respectively.

Results clearly showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecium* DSMZ 20477 did not confer intrinsic resistance to low pH. In contrast, resistant variants were more or equally sensitive to low pH than wild-

type strain. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.1.4 Effect of low pH on wild type *E. faecium* VRE and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* VRE (WT) and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3) is shown in Table 4.10.

At pH 4.4, wild type *E. faecium* VRE showed a log reduction of 1.92, 5.47 and 4.93 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 3.31, 6.19 and 6.62 after 1, 8 and 24h, respectively. No growth was observed for pediocin 34 resistant variant at pH 4.4 whereas enterocin FH99 resistant variant showed a reduction of 3.84, 6.17 and 5.89 log cycles after 1, 8 and 24h, respectively.

At pH 4.6, wild type *E. faecium* VRE showed a log reduction of 2.42, 5.16 and 5.15 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 3.45, 6.17 and 6.36 log cycles, respectively, after identical intervals. No growth was observed for pediocin 34 resistant variant at pH 4.6 whereas enterocin FH99 resistant variant showed a reduction of 3.66, 6.12 and 5.85 log cycles during the same periods of incubation.

At pH 4.8, wild type *E. faecium* VRE showed a reduction of 1.79, 4.49 and 4.22 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.45, 5.38 and 6.00 after the same periods of incubation whereas no growth was observed for pediocin 34 resistant variant at pH 4.8, the enterocin FH99 resistant variant showed a reduction of 2.86, 6.08 and 5.84 log cycles after 1, 8 and 24h, respectively at the same pH.

Table 4.10 Viable cell count (log cfu/ml) of wild type *E. faecium* VRE (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard error, n = 3)

pH	Culture	TIME (h)					
		1	2	4	6	8	24
Control	WT	6.17±0.6	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Nr	6.14±0.02	6.89±0.09	7.21±0.08	9.17±0.10	8.89±0.07	8.32±0.19
	Pr	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Er	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.32±0.19
pH 4.4	WT	4.25±0.52	3.04±0.13	3.95±0.42	3.38±0.21	3.41±0.49	3.36±0.17
	Nr	2.84±0.50	2.64±0.34	2.17±0.60	2.68±0.18	2.69±0.89	1.69±0.97
	Pr	ND	N D	ND	ND	ND	ND
	Er	2.30±0.10	2.75±0.58	2.73±0.23	2.78±0.53	2.71±0.60	2.43±13
pH 4.6	WT	3.74±0.18	3.14±0.12	3.67±0.20	4.14±0.12	3.72±0.27	3.14±0.61
	Nr	2.69±0.01	2.74±0.03	2.63±0.34	2.70±0.02	2.71±0.60	1.95±0.90
	Pr	ND	N D	ND	ND	ND	ND
	Er	2.47±0.21	2.82±0.60	2.77±0.08	2.83±0.25	2.77±0.85	2.46±0.23
pH 4.8	WT	4.38±0.21	4.39±0.79	4.41±0.49	4.34±0.24	4.39±0.79	4.07±0.81
	Nr	3.69±0.09	3.74±0.03	3.63±0.34	3.61±0.27	3.50±0.51	2.32±0.22
	Pr	ND	N D	ND	ND	ND	ND
	Er	3.27±0.36	3.23±0.44	3.34±0.22	2.60±0.20	2.81±0.29	2.47±0.71
pH 5.0	WT	5.00±0.39	4.95±0.42	4.50±0.05	4.36±0.17	4.60±0.20	5.62±0.12
	Nr	4.14±0.61	3.84±0.50	3.95±0.42	3.04±0.13	3.84±0.50	3.82±0.07
	Pr	3.30±0.10	3.36±0.17	3.84±0.50	3.61±0.27	3.23±0.44	3.04±0.13
	Er	3.43±0.13	3.56±0.02	3.30±0.10	3.50±0.51	3.54±0.40	3.49±0.13
pH 5.2	WT	5.61±0.27	5.62±0.32	5.63±0.34	5.25±0.52	4.57±0.97	4.46±0.23
	Nr	3.84±0.50	4.11±0.39	4.14±0.61	3.84±0.50	3.67±0.20	3.83±0.25
	Pr	3.69±0.89	3.22±0.22	3.47±0.71	3.30±0.10	3.60±0.26	3.44±0.58
	Er	3.56±0.82	3.53±0.14	3.43±0.13	3.61±0.27	3.77±0.81	4.23±0.04

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

At pH 5.0, wild type *E. faecium* VRE showed a log reduction of 1.17, 4.29 and 2.68 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.00, 5.04 and 4.49; pediocin 34 resistant variant showed a reduction of 2.26, .522 and 5.39 log cycles and enterocin FH99 resistant

variant showed a reduction of 2.87, 5.66 and 5.25 log cycles after 1, 8 and 24h, respectively.

At pH 5.2, wild type *E. faecium* VRE showed a log reduction of 0.56, 4.31 and 3.83 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 1.91, 5.22 and 4.48. Pediocin 34 resistant variant 2.47, 5.29 and 4.85 whereas enterocin FH99 resistant variant showed a reduction of 2.57, 5.11 and 4.09 log cycles after 1, 8 and 24h, respectively.

Results clearly showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecium* VRE did not confer intrinsic resistance to low pH. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to low pH when compared with its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.1.5 Effect of low pH on wild type *E. faecalis ATCC* 29212 and its bacteriocin resistant variants

The viable cell counts (log cfu/ml) of wild type *E. faecalis* ATCC 29212 and its nisin resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard deviation, n = 3) are shown in Table 4.11.

At pH 4.4, wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.05, 3.34 and 4.50 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 4.77, 5.00 and 5.44 after 1, 8 and 24h, respectively.

At pH 4.6, wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.03, 3.35 and 4.49 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 4.69, 4.86 and 5.33 after the same periods of incubation.

At pH 4.8, wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.02, 3.39 and 4.45 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 2921.2 showed a reduction of 4.00, 4.18 and 4.68 after 1, 8 and 24h, respectively.

Table 4.11 Viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 (WT) and its nisin (Nr) resistant variant at different time intervals after growth in medium at pH 4.4, 4.6, 4.8, 5.0 and 5.2 (mean ± standard deviation, n = 3).

pH	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	8.47±0.12	8.43±0.33	8.26±0.17	8.36±0.27	8.30±0.53	8.28±0.31
	Nr	8.47±0.02	8.43±0.01	8.26±0.09	8.36±0.03	8.30±0.05	8.28±0.01
pH 4.4	WT	4.43±0.13	4.38±0.21	4.34±0.24	4.90±0.03	4.95±0.25	3.77±0.81
	Nr	3.69±0.97	4.04±0.13	3.32±0.22	4.00±0.30	3.30±10	2.84±0.50
pH 4.6	WT	4.43±0.33	4.39±0.40	4.36±0.17	4.92±0.18	4.94±0.26	3.79±0.23
	Nr	3.77±0.51	4.07±0.18	3.44±0.07	4.27±0.54	3.43±0.13	2.95±0.42
pH 4.8	WT	4.44±0.15	4.43±0.13	4.38±0.02	4.95±0.42	4.90±0.30	3.82±0.75
	Nr	4.47±0.71	4.25±0.52	4.50±0.14	4.14±0.12	4.11±0.39	3.60±0.20
pH 5.0	WT	5.11±0.39	6.34±0.24	5.30±0.10	6.84±0.50	5.96±0.37	5.23±0.44
	Nr	4.77±0.81	4.77±0.08	4.50±0.15	4.55±0.63	4.57±0.97	4.47±21
pH 5.2	WT	4.47±0.12	4.27±0.87	5.27±0.87	7.84±0.50	5.55±0.63	4.74±0.63
	Nr	4.95±0.02	4.78±0.15	4.69±0.08	4.60±0.20	4.53±0.14	4.60±0.20

WT= Wild Type, Nr= Nisin resistant variant

At pH 5.0, whereas, wild type *E. faecalis* ATCC 29212 showed a log reduction of 3.36, 2.33 and 3.05 after 1, 8 and 24h, respectively its nisin resistant variant showed a reduction of 3.69, 3.72 and 3.80 after the same interval. At pH 5.2 strain of this culture showed a log reduction of 4.00, 2.74 and 3.54 while its

nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 3.52, 3.76 and 3.68 after 1, 8 and 24h, respectively

Results clearly showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecalis* ATCC 29212 did not confer intrinsic resistance to low pH. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to low pH when compared with its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.2 Effect of Sodium Chloride (Nacl) On Wild Type and Bacteriocin Resistant Variants

4.8.2.1 Effect of different concentrations of NaCl on wild type *L. monocytogenes* ATCC 53135 and its bacteriocin resistant variants

The results of viable cell count (log cfu/ml) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 9, 10, 11, 12, 14 and 16% (w/v) are presented in Table 4.12.

At 1% (w/v) NaCl concentration, wild type *L. monocytogenes* ATCC 53135 showed a reduction of 2.63, 1.72 and 0.93 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of this culture showed a 3.00, 1.96 and 1.64 log cycles, pediocin 34 resistant variant 3.29, 1.83 and 1.37 log cycles whereas enterocin FH99 resistant variant 3.31, 3.25 and 2.67 log cycles of reduction after 1h, 8h and 24h, respectively.

At 2% (w/v) NaCl concentration, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 2.57, 1.84 and 1.33 after 1h, 8h and 24h respectively, its nisin resistant variant showed a reduction of 3.10, 2.42 and 2.08 log cycles, pediocin 34 resistant variant 3.31, 2.96 and 1.69 log cycles and enterocin FH99

resistant variant showed a reduction of 3.48, 3.49 and 3.04 log cycles after 1h, 8h and 24h respectively.

Table 4.12 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) (mean ± standard error, n = 3)

Concentration (% w/v)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	7.97±0.03	7.74±0.03	8.49±0.13	7.86±0.03	8.95±0.02	8.11±0.09
	Nr	7.95±0.04	7.71±0.03	8.47±0.01	7.83±0.09	8.93±0.08	8.04±0.03
	Pr	7.99±0.05	7.72±0.06	8.86±0.09	7.83±0.09	8.94±0.03	8.00±0.13
	Er	7.94±0.09	7.71±0.03	8.47±0.01	7.85±0.02	8.93±0.09	8.04±0.03
1	WT	5.34±0.13	6.07±0.08	6.30±0.10	6.78±0.05	7.22±0.05	7.17±0.09
	Nr	4.95±0.03	4.90±0.09	5.11±0.03	7.32±0.14	6.97±0.08	6.39±0.04
	Pr	4.69±0.07	4.77±0.06	4.90±0.09	6.74±0.08	7.11±0.06	6.62±0.09
	Er	4.63±0.08	4.59±0.15	5.66±0.08	6.43±0.04	5.68±0.07	5.36±0.17
2	WT	5.39±0.03	6.08±0.06	6.14±0.10	6.30±0.03	7.11±0.12	6.77±0.08
	Nr	4.84±0.08	4.84±0.09	4.77±0.08	5.50±0.05	6.50±0.10	5.95±0.09
	Pr	4.68±0.04	4.39±0.07	4.98±0.01	5.96±0.10	5.97±0.04	6.30±0.03
	Er	4.46±0.02	4.65±0.03	5.82±0.05	5.50±0.15	5.44±0.08	5.00±0.09
8	WT	4.20±0.12	4.23±0.09	4.20±0.13	4.00±0.06	4.00±0.19	3.60±0.06
	Nr	3.69±0.07	3.60±0.06	3.30±0.03	3.60±0.14	3.07±0.06	3.04±0.03
	Pr	3.68±0.04	3.39±0.07	3.98±0.07	3.96±0.08	3.97±0.04	3.30±0.04
	Er	3.94±0.09	3.71±0.11	3.47±0.08	3.85±0.06	3.93±0.09	3.04±0.07
10	WT	3.39±0.04	3.46±0.08	3.71±0.03	3.69±0.07	3.07±0.08	3.07±0.10
	Nr	3.30±0.03	3.36±0.11	3.30±0.06	2.78±0.05	2.90±0.09	2.95±0.03
	Pr	3.27±0.04	3.25±0.03	3.20±0.02	2.95±0.03	2.99±0.05	2.07±0.08
	Er	3.14±0.08	3.17±0.09	3.49±0.09	3.36±0.08	2.95±0.03	2.81±0.04
12	WT	ND	ND	ND	ND	ND	ND
	Nr	ND	ND	ND	ND	ND	ND
	Pr	ND	ND	ND	ND	ND	ND
	Er	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

Wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.76, 4.95 and 4.51 after 1, 8 and 24h, respectively, at 8% NaCl concentration. At this salt concentration nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 4.25, 5.85 and 5.00 after 1h, 8h and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 4.31, 4.96 and 4.69 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.00, 5.00 and 5.00 log cycles after 1, 8 and 24h, respectively.

Wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 4.57, 5.87 and 5.03 after 1, 8 and 24h, respectively in the medium having NaCl concentration 10% (w/v). Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 4.65, 6.03 and 5.08 ; pediocin 34 resistant variant 4.71, 5.94 and 5.92 log cycles and enterocin FH99 resistant 4.80, 5.98 and 5.22 log cycles after 1h, 8h and 24h respectively. No growth was detected at NaCl concentration of 12% (w/v).

Results of this experiment showed that resistance to nisin, pediocin 34 and enterocin FH99 in *L. monocytogenes* ATCC 53135 did not confer intrinsic resistance to sodium chloride. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to the different concentrations of sodium chloride tested when compared with its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.2.2 Effect of different concentrations of NaCl on wild type *L. monocytogenes* MTCC 657 and its bacteriocin resistant variants

The results of the viable cell count (log cfu/ml) of wild type *L. monocytogenes* MTCC 657 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) are shown in Table 4.13.

Table 4.13 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* MTCC 657 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) (mean ± standard error, n = 3).

Concentration (% w/v)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.00±0.34	6.57±0.19	6.54±0.24	7.39±0.01	7.79±0.55	8.50±0.51
	Nr	6.07±0.81	6.56±0.14	6.53±0.17	7.36±0.23	7.79±0.20	8.49±0.13
	Pr	5.95±0.42	6.54±0.41	6.51±0.14	7.38±0.21	7.77±0.15	8.47±0.21
	Er	5.97±0.21	6.55±0.33	6.50±0.15	7.34±0.24	7.78±0.11	8.46±0.23
1	WT	4.49±0.09	6.07±0.13	6.00±0.11	6.47±0.10	6.83±0.09	6.69±0.07
	Nr	3.65±0.13	3.69±0.07	4.11±0.13	5.32±0.19	5.28±0.12	5.09±0.19
	Pr	3.81±0.03	3.64±0.06	4.25±0.07	5.19±0.05	6.11±0.09	5.15±0.08
	Er	3.63±0.08	3.79±0.10	4.19±0.05	5.10±0.09	6.17±0.12	5.08±0.11
2	WT	4.39±0.07	5.34±0.14	5.60±0.16	6.30±0.10	6.17±0.06	6.50±0.15
	Nr	3.43±0.14	3.46±0.08	4.10±0.09	5.12±0.04	4.50±0.15	4.28±0.13
	Pr	3.54±0.09	3.39±0.11	3.99±0.08	4.27±0.24	4.29±0.05	5.07±0.08
	Er	3.35±0.05	3.38±0.06	4.22±0.16	4.12±0.07	5.25±0.09	5.05±0.10
8	WT	4.27±0.08	3.51±0.04	3.60±0.06	3.77±0.11	3.92±0.19	3.30±0.03
	Nr	3.17±0.10	3.16±0.08	3.07±0.11	3.11±0.03	3.23±0.09	3.25±0.07
	Pr	3.44±0.08	3.39±0.06	3.29±0.06	3.28±0.09	3.46±0.12	3.12±0.04
	Er	3.27±0.12	3.18±0.14	3.11±0.09	3.23±0.08	3.27±0.02	3.20±0.09
10	WT	3.07±0.10	2.95±0.03	2.77±0.12	2.69±0.07	2.95±0.04	3.00±0.08
	Nr	2.65±0.13	2.47±0.04	2.84±0.08	2.78±0.03	2.50±0.05	ND
	Pr	2.84±0.08	2.77±0.01	2.90±0.09	2.77±0.04	2.47±0.01	ND
	Er	2.97±0.07	2.95±0.03	2.94±0.03	2.77±0.06	2.60±0.06	2.88±0.09
12	WT	ND	ND	ND	ND	ND	ND
	Nr	ND	ND	ND	ND	ND	ND
	Pr	ND	ND	ND	ND	ND	ND
	Er	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

At 1% (w/v) NaCl concentration wild type, *L. monocytogenes* MTCC 657 showed a reduction of 1.50, 0.96 and 1.80 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of

2.42, 2.50 and 3.39 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.14, 1.66 and 3.32 log cycles while enterocin FH99 resistant variant showed a reduction of 2.34, 1.61 and 3.37 log cycles after 1, 8 and 24h, respectively.

At 2% (w/v) NaCl concentration wild type, *L. monocytogenes* MTCC 657 showed a log reduction of 1.60, 1.62 and 2.00 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 2.64, 3.28 and 4.21; pediocin 34 resistant variant 2.41, 3.48 and 3.39 whereas enterocin FH99 resistant variant showed a reduction of 2.61, 2.52 and 3.40 log cycles after 1, 8 and 24h, respectively.

Similarly, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.72, 3.86 and 5.20 after 1, 8 and 24h, respectively, in the medium supplemented with 8% (w/v) NaCl. Nisin resistant variant of *this culture* showed a reduction of 2.90, 4.56 and 5.23; pediocin 34 resistant variant showed a reduction of 2.50, 4.30 and 5.35 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.70, 4.51 and 5.26 log cycles after 1, 8 and 24h, respectively in this concentration of salt supplemented medium.

At 10% (w/v) NaCl, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 2.92, 4.84 and 5.50 after 1, 8 and 24h, respectively. Its nisin resistant variant showed a reduction of 3.42 and 5.28 cycles, whereas, pediocin 34 resistant variant showed a reduction of 3.10 and 5.30 log cycles after 1 and 8h and no growth after 24h, respectively. Enterocin FH99 resistant variant showed a reduction of 3.00, 5.18 and 5.57 log cycles after 1, 8 and 24h, respectively. No growth was detected for wild as well as nisin, pediocin 34 and enterocin resistant variant of *L. monocytogenes* MTCC 657 at salt concentration of 12% and beyond.

Results of this experiment showed that resistance to nisin, pediocin 34 and enterocin FH99 in *L. monocytogenes* MTCC 657 did not confer intrinsic resistance to sodium chloride. In contrast, nisin, pediocin 34 and enterocin

FH99 resistant variants were more or equally sensitive to the different concentrations of sodium chloride tested when compared with its wild-type counterpart.

4.8.2.3 Effect of different concentrations of NaCl on wild type *E. faecium* DSMZ 20477 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) is given in Table 4.14.

At 1% (w/v) NaCl concentration wild type *E. faecium* DSMZ 20477 showed a reduction of 1.25, 0.64 and 0.09 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 0.78, 2.23 and 0.06 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 0.92, 0.54 and 0.54 log cycles, whereas, enterocin FH99 resistant variant showed a reduction of 1.31, 2.39 and 2.60 log cycles after same intervals.

At 2% (w/v) NaCl concentration wild type *E. faecium* DSMZ 20477 showed a log reduction of 1.41, 0.74 and 1.44 log cycles; nisin resistant variant 1.52, 2.43 and 1.57 log cycles; pediocin 34 resistant variant showed a reduction of 1.82, 0.57 and 1.56 log cycles and enterocin FH99 resistant variant 1.99, 2.00 and 3.00 log cycles each after 1, 8 and 24h, respectively.

At 8% (w/v) NaCl, wild type *E. faecium* DSMZ 20477 showed a log reduction of 1.53, 2.51 and 2.78 after 1, 8 and 24h, respectively. Its nisin resistant variant showed a reduction of 1.52, 4.43 and 4.22 log; pediocin 34 resistant variant 2.13, 2.53 and 2.36 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.22, 3.75 and 4.29 log cycles after 1, 8 and 24h, respectively.

At 10% (w/v) NaCl wild type *E. faecium* DSMZ 20477 and its nisin resistant variant showed a reduction of 2.67, 4.17 and 5.30 and 2.43, 5.67 and 5.92 log cycles after 1h, 8h and 24h, respectively. Similarly pediocin 34 resistant variant

showed a reduction of 2.77, 3.65 and 4.61 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.17, 4.87 and 6.78 log cycles after 1, 8 and 24h, respectively.

Table 4.14 Viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) (mean ± standard error, n = 3)

Concentration (% w/v)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
	Nr	6.43±0.04	6.83±0.01	6.46±0.08	7.61±0.07	8.51±0.04	8.69±0.04
	Pr	6.73±0.06	6.77±0.05	6.62±0.09	6.77±0.05	6.65±0.04	6.72±0.07
	Er	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
1	WT	5.69±0.09	6.07±0.13	7.10±0.11	7.47±0.10	7.83±0.09	9.69±0.07
	Nr	5.65±0.13	5.69±0.07	6.11±0.13	6.32±0.19	6.28±0.12	8.09±0.19
	Pr	5.81±0.03	5.64±0.06	6.25±0.07	6.19±0.05	6.11±0.09	6.15±0.08
	Er	5.63±0.08	5.79±0.10	6.19±0.05	6.10±0.09	6.17±0.12	7.18±0.11
2	WT	5.53±0.19	5.77±0.18	7.05±0.15	6.93±0.12	7.73±0.14	8.34±0.13
	Nr	4.90±0.09	5.03±0.12	5.84±0.11	6.77±0.10	6.07±0.08	7.12±0.12
	Pr	4.90±0.15	4.83±0.09	5.86±0.12	5.92±0.09	6.08±0.15	6.15±0.11
	Er	4.95±0.13	4.55±0.11	5.64±0.09	6.51±0.15	6.47±0.10	6.78±0.03
8	WT	5.41±0.06	5.60±0.06	5.30±0.10	5.60±0.06	5.96±0.08	7.00±0.21
	Nr	4.90±0.09	4.95±0.13	5.00±0.05	4.84±0.08	4.07±0.11	4.47±0.12
	Pr	4.60±0.20	4.90±0.09	5.17±0.16	4.69±0.18	4.11±0.17	4.36±0.16
	Er	4.72±0.14	4.47±0.12	5.34±0.11	4.64±0.13	4.72±0.06	5.49±0.13
10	WT	4.27±0.09	4.47±0.10	4.30±0.08	4.25±0.05	4.30±0.03	4.47±0.11
	Nr	4.00±0.06	3.89±0.07	3.84±0.09	3.34±0.08	2.83±0.12	2.77±0.09
	Pr	3.95±0.13	3.90±0.09	3.69±0.08	3.30±0.13	3.00±0.09	3.11±0.08
	Er	3.77±0.08	3.55±0.06	3.30±0.03	3.47±0.09	3.60±0.06	3.00 ±0.14
12	WT	ND	ND	ND	ND	ND	ND
	Nr	ND	ND	ND	ND	ND	ND
	Pr	ND	ND	ND	ND	ND	ND
	Er	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

No viable cells were detected for wild type, as well as nisin, pediocin 34 and enterocin resistant variants of *E. faecium* DSMZ 20477 at salt concentration of 12%.

Results of this experiment showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecium* DSMZ 20477 did not confer intrinsic resistance to sodium chloride. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to the different concentrations of sodium chloride tested when compared with its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.2.4 Effect of different concentrations of NaCl on wild type *E. faecium* VRE and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* VRE and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) is given in Table 4.15.

At 1% and 2% (w/v) NaCl concentration wild type *E. faecium* VRE showed a reduction of 1.33, 0.87 and 0.83 and 1.54, 2.66 and 1.56 log cycles after 1h, 8h and 24h respectively. Similarly, nisin resistant variant of *E. faecium* VRE showed a reduction of 1.52, 3.76 & 3.22 and 1.81, 4.50 & 3.26 log; pediocin 34 resistant variant showed a reduction of 1.47, 3.53 and 3.20 and .84, 4.51 and 3.22 log At 1 as well as 2% (w/v) NaCl concentrations wild type *E. faecium* VRE showed a reduction of 1.33, 0.87 & 0.83 and 1.54, 2.66 & 1.56 log cycles after 1, 8 and 24h, cycles whereas enterocin FH99 resistant variant showed a reduction of 1.84, 3.80 & 3.26 and 2.05, 3.84 and 3.28 log cycles after 1, 8 and 24h, respectively.

Table 4.15 Viable cell count (log cfu/ml) of wild type *E. faecium* VRE (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) (mean ± standard error, n = 3)

Concentration (% w/v)	Culture	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Nr	6.14±0.02	6.89±0.09	7.21±0.08	9.17±0.10	8.89±0.07	8.32±0.19
	Pr	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Er	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.32±0.19
1	WT	4.84±0.08	5.25±0.13	6.23±0.19	7.61±0.14	8.01±0.13	7.46±0.18
	Nr	4.62±0.19	4.30±0.10	5.99±0.12	5.09±0.12	5.12±0.15	5.09±0.20
	Pr	4.69±0.17	4.41±0.09	5.24±0.08	5.10±0.21	5.35±0.12	5.09±0.11
	Er	4.30±0.10	4.36±0.17	5.13±0.34	5.11±0.13	5.08±0.25	5.05±0.24
2	WT	4.63±0.18	4.88±0.14	6.17±0.10	7.02±0.11	6.23±0.19	6.73±0.12
	Nr	4.33±0.09	4.30±0.12	4.34±0.11	4.53±0.09	4.39±0.07	5.05±0.05
	Pr	4.33±0.14	4.32±0.08	4.34±0.13	4.64±0.07	4.38±0.11	5.07±0.09
	Er	4.08±0.12	4.33±0.04	4.41±0.13	4.46±0.08	5.04±0.08	5.04±0.13
8	WT	4.57±0.04	4.72±0.06	4.50±0.05	4.77±0.10	4.68±0.11	4.47±0.21
	Nr	4.04±0.18	4.04±0.13	4.04±0.09	4.35±0.05	4.53±0.09	4.39±0.04
	Pr	4.32±0.02	4.04±0.18	4.13±0.09	4.11±0.03	4.16±0.08	4.17±0.10
	Er	4.04±0.13	4.31±0.04	4.16±0.13	4.07±0.12	4.27±0.07	4.25±0.03
10	WT	3.92±0.19	4.01±0.17	3.47±0.11	4.00±0.09	4.04±0.13	3.69±0.17
	Nr	3.17±0.11	3.12±0.07	3.14±0.08	3.18±0.07	3.12±0.11	3.14±0.15
	Pr	3.25±0.13	3.29±0.15	3.00±0.09	3.07±0.08	3.27±0.08	3.01±0.13
	Er	3.21±0.08	3.25±0.12	3.14±0.09	3.27±0.09	3.03±0.09	3.26±0.05
12	WT	3.25±0.13	3.11±0.13	3.17±0.11	3.14±0.08	2.90±0.09	3.14±0.11
	Nr	2.69±0.06	2.77±0.08	2.60±0.12	2.81±0.14	2.63±0.08	2.65±0.13
	Pr	2.74±0.09	2.60±0.16	2.77±0.15	2.86±0.05	2.81±0.12	2.72±0.06
	Er	2.60±0.06	2.69±0.07	2.84±0.09	2.90±0.09	2.77±0.08	2.95±0.17

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant; Er= Enterocin FH99 resistant variant, ND= Not Detected

At 8% (w/v) NaCl wild type *E. faecium* VRE showed a reduction of 1.59, 4.21 and 3.82 log cycles after 1h, 8h and 24h respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.09, 4.36 and 3.92; pediocin 34 resistant

variant 1.85, 4.73 and 4.12 whereas enterocin FH99 resistant variant showed a reduction of 2.10, 4.62 and 4.06 log cycles after 1, 8 and 24h, respectively.

At 10% (w/v) NaCl wild type *E. faecium* VRE showed a log reduction of 2.25, 4.85 and 4.60 after 1, 8 and 24h, respectively. Its nisin resistant variant showed a reduction of 2.97, 5.77 and 5.17 log cycles, respectively; pediocin 34 resistant 2.92, 5.61 and 5.28 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.93, 5.85 and 5.05 log cycles after 1, 8 and 24h, respectively.

At 12% (w/v) NaCl wild type *E. faecium* VRE showed a log reduction of 2.92, 5.99 and 5.16 after 1, 8 and 24h, respectively. Its nisin resistant variant showed a reduction of 3.45, 6.26 and 5.65 log cycles, respectively; pediocin 34 resistant 3.43, 6.08 and 5.58 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.57, 6.12 and 5.37 log cycles after 1, 8 and 24h, respectively.

Results of showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecium* VRE did not confer intrinsic resistance to sodium chloride. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to the different concentrations of sodium chloride tested when compared with its wild-type counterpart

4.8.2.5 Effect of different concentrations of NaCl on wild type *E. faecalis* ATCC 29212 and its nisin resistant variant

Table 4.16 shows the viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 and its nisin resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v).

At 1% (w/v) NaCl concentration wild type *E. faecalis* ATCC 29212 showed a reduction of 4.47, 1.88 and 1.24 log cycles after 1h, 8h and 24h respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 5.36, 4.22 and 3.81 log cycles after 1h, 8h and 24h respectively.

At 2% (w/v) NaCl concentration wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.69, 3.86 and 2.13 after 1h, 8h and 24h respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 5.43, 5.00 and 4.31 log cycles after 1h, 8h and 24h respectively. At 8% (w/v) NaCl these reduction values were slightly higher after respective time intervals. For wild type and 4.76, 4.44 and 3.39, 5.98, 5.39 and 5.17 log cycles for nisin resistant variants.

At 10% (w/v) NaCl wild type *E. faecalis* ATCC 29212 showed a log reduction of 5.80, 5.68 and 5.81 after 1, 8 and 24h, respectively; No viable growth was detected for nisin resistant variant after same time intervals. No viable growth was detected for wild type *E. faecalis* ATCC 29212 and its nisin resistant variant at salt concentration of 12%.

Table 4.16 Viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 (WT) and its nisin (Nr) resistant variant at different time intervals after growth in medium supplemented with Sodium Chloride at concentration of 1, 2, 8, 10 and 12% (w/v) (mean ± standard error, n = 3)

Concentration (% w/v)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	8.47±0.12	8.43±0.33	8.26±0.17	8.36±0.27	8.30±0.53	8.28±0.31
	Nr	8.47±0.02	8.43±0.01	8.26±0.09	8.36±0.03	8.30±0.05	8.28±0.01
1	WT	4.00±0.12	4.77±0.08	4.73±0.09	5.85±0.08	6.41±0.07	7.04±0.09
	Nr	3.11±0.07	3.10±0.04	3.97±0.07	4.08±0.09	4.07±0.09	4.46±0.08
2	WT	3.78±0.03	4.47±0.07	4.44±0.08	5.19±0.09	4.43±0.04	6.14±0.06
	Nr	3.04±0.09	2.93±0.05	3.17±0.09	3.36±0.07	3.30±0.03	3.96±0.03
8	WT	3.71±0.03	3.07±0.18	3.30±0.03	4.07±0.08	3.85±0.12	4.89±0.09
	Nr	2.49±0.06	2.67±0.09	2.69±0.07	2.47±0.06	2.90±0.09	3.11±0.08
10	WT	2.67±0.08	2.60±0.26	2.56±0.19	2.69±0.12	2.62±0.23	2.47±0.15
	Nr	ND	ND	ND	ND	ND	ND
12	WT	ND	ND	ND	ND	ND	ND
	Nr	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, ND= Not Detected

Results of this experiment showed that resistance to nisin, pediocin 34 and enterocin FH99 in *E. faecalis* ATCC 29212 did not confer intrinsic resistance to sodium chloride. In contrast, nisin, pediocin 34 and enterocin FH99 resistant variants were more or equally sensitive to the different concentrations of sodium chloride tested when compared with its wild-type counterpart. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

4.8.3 Effect of Potassium Sorbate on Wild Type and Bacteriocin Resistant Variants

4.8.3.1 Effect of different concentrations of potassium sorbate on wild type *L. monocytogenes* ATCC 53135 and its bacteriocin resistant variants

The results of the viable cell count (log cfu/ml) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml are given in Table 4.17.

When grown in the medium supplemented with potassium sorbate at a concentration of 2.0 mg/ml wild type *L. monocytogenes* ATCC 53135 showed a reduction of 2.74, 2.26 and 0.15 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.36, 2.61 and 0.26 log cycles; pediocin 34 resistant variant 2.74, 2.22 and 0.36 log cycles and enterocin FH99 resistant variant of 3.04, 3.35 and 1.25 log cycles after 1, 8 and 24h, respectively.

At a concentration of 3.0 mg/ml, while wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.76, 2.65 and 2.11 after 1, 8 and 24h, respectively. Similarly, reduction for nisin resistant variant of *L. monocytogenes* ATCC 53135 was 4.43, 3.63 and 2.86 log cycles after identical time intervals. Pediocin 34 resistant variant showed a reduction of 4.69, 5.64 and 4.09 log cycles

whereas enterocin FH99 resistant variant showed a reduction of 4.38, 5.16 and 4.22 log cycles after 1, 8 and 24h, respectively.

Table 4.17 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml (mean ± standard error, n = 3)

Concentration (mg/ml)	Culture	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	7.97±0.03	7.74±0.03	8.49±0.13	7.86±0.03	8.95±0.02	8.11±0.09
	Nr	7.95±0.04	7.71±0.03	8.47±0.01	7.83±0.09	8.93±0.08	8.04±0.03
	Pr	7.99±0.05	7.72±0.06	8.86±0.09	7.83±0.09	8.94±0.03	8.00±0.13
	Er	7.94±0.09	7.71±0.03	8.47±0.01	7.85±0.02	8.93±0.09	8.04±0.03
2.0	WT	5.23±0.04	5.23±0.09	5.55±0.03	6.57±0.04	6.69±0.06	7.95±0.04
	Nr	4.59±0.05	4.83±0.09	5.44±0.08	6.53±0.09	6.32±0.09	7.77±0.01
	Pr	5.25±0.03	5.47±0.02	5.50±0.05	6.57±0.04	6.71±0.03	7.63±0.08
	Er	4.90±0.09	5.07±0.08	4.95±0.03	5.53±0.09	5.57±0.04	6.78±0.05
3.0	WT	4.20±0.02	4.95±0.03	5.11±0.03	5.90±0.09	6.30±0.03	6.00±0.13
	Nr	3.51±0.04	3.30±0.03	4.30±0.03	4.00±0.12	5.30±0.11	5.17±0.01
	Pr	3.30±0.10	3.38±0.11	3.47±0.01	3.50±0.05	3.30±0.03	3.90±0.90
	Er	3.56±0.02	3.41±0.03	3.67±0.08	3.75±0.01	3.77±0.01	3.81±0.13
4.0	WT	3.44±0.08	3.60±0.06	4.00±0.12	4.20±0.02	4.25±0.03	4.47±0.01
	Nr	2.90±0.09	2.69±0.02	3.30±0.03	3.59±0.05	3.77±0.01	3.25±0.03
	Pr	3.20±0.02	3.27±0.04	3.11±0.02	2.71±0.03	2.43±0.04	2.87±0.06
	Er	2.36±0.08	2.00±0.09	ND	ND	ND	ND
5.0	WT	2.39±0.04	2.53±0.09	2.60±0.06	2.65±0.03	2.69±0.07	2.77±0.01
	Nr	1.90±0.09	1.95±0.03	2.30±0.03	2.39±0.04	2.30±0.03	1.95±0.03
	Pr	2.00±0.16	2.07±0.01	2.30±0.03	2.54±0.08	2.18±0.04	2.65±0.06
	Er	2.25±0.03	1.74±0.05	ND	ND	ND	ND
6.0	WT	1.87±0.05	1.60±0.02	1.47±0.01	1.54±0.08	1.60±0.06	1.47±0.01
	Nr	1.60±0.06	1.47±0.01	1.60±0.06	1.47±0.01	ND	ND
	Pr	1.90±0.09	1.47±0.01	ND	ND	ND	ND
	Er	1.95±0.01	1.60±0.06	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

Wild type *L. monocytogenes* ATCC 53135 showed a progressive increase in reduction of cell count with increase in the concentration of the potassium sorbate in the medium i.e from 4.0 to 6.0 mg/ml. Nisin resistant variant of *L. monocytogenes* ATCC 53135 also showed a similar progress in reduction of cell count with increase in the concentration of the potassium sorbate in the medium from 4.0 to 6.0 mg/ml after same1, 8 and 24h intervals.

In the medium with potassium sorbate at a concentration of 6.0 mg/ml, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 6.09, 7.35 and 6.63 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 6.35, 8.93 and 8.04 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 6.09, 8.94 and 8.00 log cycles whereas enterocin FH99 resistant variant showed a reduction of 5.99 and 6.11 log cycles after 1h and 2h, respectively, however, no growth was observed after 4h incubation.

4.8.3.2 Effect of different concentrations of potassium sorbate on wild type *L. monocytogenes* MTCC 657 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *L. monocytogenes* MTCC 657 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0, and 6.0 mg/ml are shown in **Table 4.18**.

When grown in the medium supplemented with potassium sorbate at a concentration of 2.0 mg/ml, wild type, *L. monocytogenes* MTCC 657 showed a reduction of 1.38, 1.05 and 1.69 log cycles after 1, 8 and 24h, respectively. Whereas, nisin resistant variant of *L. monocytogenes* MTCC 657 showed a showed a relatively higher reduction (1.75, 2.61 and 3.19 log cycles) after identical periods while pediocin 34 resistant variant showed a reduction of 1.33, 1.93 and 2.73 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.19, 1.97 and 2.61 log cycles after 1, 8 and 24h, respectively.

Table 4.18 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* MTCC 657 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml (mean ± standard error, n = 3)

Concentration (mg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.00±0.34	6.57±0.19	6.54±0.24	7.39±0.01	7.79±0.55	8.50±0.51
	Nr	6.07±0.81	6.56±0.14	6.53±0.17	7.36±0.23	7.79±0.20	8.49±0.13
	Pr	5.95±0.42	6.54±0.41	6.51±0.14	7.38±0.21	7.77±0.15	8.47±0.21
	Er	5.97±0.21	6.55±0.33	6.50±0.15	7.34±0.24	7.78±0.11	8.46±0.23
2.0	WT	4.61±0.04	4.84±0.08	5.53±0.09	6.69±0.07	6.74±0.13	6.81±0.11
	Nr	4.32±0.08	4.57±0.04	4.95±0.03	5.00±0.09	5.17±0.10	5.30±0.03
	Pr	4.62±0.09	4.80±0.08	4.81±0.06	5.81±0.04	5.84±0.08	5.74±0.06
	Er	3.77±0.05	3.79±0.09	5.70±0.07	5.77±0.11	5.81±0.03	5.84±0.08
3.0	WT	4.47±0.10	4.65±0.03	5.14±0.08	5.38±0.11	6.60±0.06	6.66±0.08
	Nr	4.17±0.09	4.62±0.09	4.44±0.05	4.41±0.03	4.69±0.07	5.00±0.11
	Pr	4.30±0.03	4.43±0.04	4.54±0.08	5.41±0.04	5.47±0.04	5.39±0.08
	Er	3.69±0.07	3.77±0.05	4.32±0.09	4.25±0.03	4.17±0.09	4.30±0.07
4.0	WT	4.14±0.18	3.75±0.05	4.65±0.13	4.70±0.17	4.60±0.16	4.66±0.18
	Nr	3.47±0.02	3.62±0.09	3.55±0.12	3.61±0.04	3.69±0.07	3.04±0.13
	Pr	3.53±0.09	3.77±0.08	3.84±0.05	3.69±0.07	3.30±0.03	4.00±0.12
	Er	3.11±0.03	3.47±0.02	3.25±0.07	3.07±0.08	3.54±0.06	3.90±0.02
5.0	WT	3.30±0.03	3.14±0.08	3.20±0.02	3.25±0.05	3.27±0.04	3.30±0.10
	Nr	2.51±0.04	2.64±0.04	2.68±0.09	2.69±0.08	2.69±0.09	2.69±0.07
	Pr	3.00±0.08	2.97±0.04	3.00±0.12	3.07±0.04	3.17±0.06	2.84±0.02
	Er	2.69±0.07	3.04±0.04	3.11±0.03	2.90±0.09	2.95±0.04	2.97±0.07
6.0	WT	2.00±0.11	2.47±0.14	2.55±0.09	2.57±0.04	2.59±0.06	2.47±0.08
	Nr	ND	ND	ND	ND	ND	ND
	Pr	ND	ND	ND	ND	ND	ND
	Er	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

At a concentration of 3.0 mg/ml, wild type, *L. monocytogenes* MTCC 657 showed a log reduction of 1.52, 1.19 and 1.84 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 similarly showed a relatively higher reduction of 1.90, 3.09 and 3.49 after 1, 8 and 24h, respectively while

pediocin 34 resistant variant showed a reduction of 1.65, 2.30 and 3.07 log cycles, enterocin FH99 resistant variant showed a reduction of 2.27, 3.60 and 4.16 log cycles after 1, 8 and 24h, respectively.

At a concentration of 4.0 mg/ml wild type, *L. monocytogenes* MTCC 657 showed a log reduction of 1.85, 3.19 and 3.84 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 2.60, 4.09 and 5.44 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.42, 4.47 and 4.47 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.86, 4.24 and 4.55 log cycles after 1, 8 and 24h, respectively.

At a concentration of 5.0 mg/ml wild type, *L. monocytogenes* MTCC 657 showed a higher reduction as compared to previous concentrations. Its nisin resistant variant showed relatively higher reduction values after 1, 8 and 24h intervals (Table 4.18). Pediocin 34 resistant variant showed a reduction of 2.95, 4.60 and 5.63 log cycles, whereas enterocin FH99 resistant variant showed a reduction of 3.27, 4.83 and 5.48 log cycles after 1, 8 and 24h, respectively.

No viable growth was detected for nisin, pediocin 34 and enterocin FH99 resistant variants in broth medium supplemented with potassium sorbate at a concentration of 6.0 mg/ml.

4.8.3. 3 Effect of different concentrations of potassium sorbate on wild type *E. faecium* DSMZ 20477 and its bacteriocin resistant variants

The results of the the viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml are given in Table 4.19.

When grown in the medium supplemented with potassium sorbate at a concentration of 2.0 mg/ml wild type *E. faecium* DSMZ 20477 showed a reduction of 3.04, 2.76 and 4.18 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 2.65, 3.90 and 4.09 log

cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.13, 3.35 and 4.72 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.58, 3.73 and 5.24 log cycles after 1, 8 and 24h, respectively.

At a concentration of 3.0 mg/ml, wild type *E. faecium* DSMZ 20477 showed a relatively higher log reduction (3.45, 2.94 and 4.30 after 1, 8 and 24h, respectively) as compared to potassium sorbate at a concentration of 2.0 mg/ml. Nisin resistant variant of *E. faecium* DSMZ 20477 also showed a reduction of 3.58, 5.04 and 5.01 after 1h, 8h and 24h respectively. Similarly, pediocin 34 resistant variant showed a reduction of 4.03, 3.90 and 4.97 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.36, 4.60 and 5.94 log cycles after 1, 8 and 24h, respectively which are relatively higher in contrast to potassium sorbate at a concentration of 2.0 mg/ml.

At a concentration of 4.0 mg/ml, wild type *E. faecium* DSMZ 20477 showed a log reduction of 3.64, 3.81 and 4.58 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 3.79, 5.31 and 6.69 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 4.10, 4.35 and 5.72 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.18, 5.43 and 6.78 log cycles after 1h, 8h and 24h, respectively.

However in the presence of potassium sorbate, at a concentration of 5.0 mg/ml, wild type *E. faecium* DSMZ 20477 showed quite high reduction (3.80, 5.47 and 6.22 after 1, 8 and 24h, respectively). Nisin resistant variant of *E. faecium* DSMZ 20477 similarly also showed higher reduction in the cell count after the given incubation periods. Pediocin 34 resistant variant showed a reduction of 4.43, 4.47 and 6.24 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.47, 5.63 and 7.83 log cycles after 1, 8 and 24h, respectively.

At a concentration of 6.0 mg/ml, wild type *E. faecium* DSMZ 20477 showed a log reduction of 5.34, 5.69 and 7.48 after 1, 8 and 24h, respectively. Nisin resistant

variant of *E. faecium* DSMZ 20477 showed a reduction of 4.95, 6.91 and 7.22 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 5.25, 5.05 and 6.24 log cycles whereas enterocin FH99 resistant variant showed a reduction of 5.45, 6.63 and 8.18 log cycles after 1, 8 and 24h, respectively.

Table 4.19 Viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml (mean ± standard error, n = 3)

Concentration (mg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
	Nr	6.43±0.04	6.83±0.01	6.46±0.08	7.61±0.07	8.51±0.04	8.69±0.04
	Pr	6.73±0.06	6.77±0.05	6.62±0.09	6.77±0.05	6.65±0.04	5.72±0.07
	Er	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
2.0	WT	3.90±0.09	4.07±0.08	4.55±0.03	5.17±0.09	5.71±0.03	5.60±0.06
	Nr	3.77±0.05	3.00±0.10	4.04±0.13	4.43±0.06	4.61±0.08	4.60±0.06
	Pr	3.60±0.06	3.76±0.12	4.54±0.08	3.77±0.10	3.30±0.13	3.00±0.12
	Er	3.36±0.08	3.04±0.09	4.04±0.13	3.30±0.01	4.74±0.06	4.54±0.08
3.0	WT	3.49±0.06	3.55±0.03	4.34±0.15	5.44±0.08	5.53±0.14	5.47±0.02
	Nr	2.84±0.09	2.82±0.07	3.47±0.02	3.36±0.18	3.47±0.02	3.68±0.12
	Pr	2.69±0.08	2.84±0.09	4.32±0.13	3.71±0.06	2.74±0.08	2.74±0.12
	Er	2.57±0.04	3.67±0.10	3.70±0.07	3.25±0.13	3.87±0.06	3.84±0.08
4.0	WT	3.30±0.03	3.47±0.10	4.04±0.13	4.00±0.05	4.66±0.08	5.20±0.12
	Nr	2.63±0.08	2.68±0.01	3.17±0.02	3.14±0.09	3.20±0.02	2.00±0.10
	Pr	2.62±0.09	2.75±0.05	3.87±0.01	3.17±0.01	2.30±0.03	2.00±0.16
	Er	2.76±0.08	3.04±0.01	3.32±0.19	3.07±0.08	3.04±0.03	3.00±0.19
5.0	WT	3.14±0.08	2.61±0.04	3.60±0.06	3.77±0.01	3.00±0.08	3.55±0.06
	Nr	2.30±0.03	2.39±0.09	2.47±0.01	2.95±0.03	3.00±0.02	1.90±0.09
	Pr	2.30±0.17	2.43±0.04	3.47±0.02	2.87±0.06	2.17±0.01	1.47±0.10
	Er	2.47±0.01	2.84±0.08	2.27±0.04	2.77±0.01	2.84±0.08	1.95±0.03
6.0	WT	1.60±0.06	2.04±0.03	2.14±0.08	2.47±0.02	2.77±0.01	2.30±0.03
	Nr	1.69±0.07	1.77±0.01	1.47±0.02	1.84±0.08	1.95±0.03	1.65±0.03
	Pr	1.47±0.01	2.07±0.08	1.47±0.01	1.74±0.03	1.60±0.06	1.47±0.01
	Er	1.49±0.02	1.84±0.08	2.27±0.04	1.77±0.01	1.84±0.08	1.60±0.06

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

4.8.3.4 Effect of different concentrations of potassium sorbate on wild type *E. faecium* VRE and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* VRE and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml are shown in **Table 4.20**.

Table 4.20 Viable cell count (log cfu/ml) of wild type *E. faecium* VRE (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml (mean ± standard error, n = 3)

Concentration (mg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Nr	6.14±0.02	6.89±0.09	7.21±0.08	9.17±0.10	8.89±0.07	8.32±0.19
	Pr	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Er	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.32±0.19
2.0	WT	5.44±0.08	5.20±0.12	6.25±0.11	6.72±0.06	6.73±0.14	6.74±013
	Nr	4.69±0.07	4.69±0.14	5.07±0.09	4.66±0.08	5.20±0.02	5.30±0.03
	Pr	4.30±0.10	4.25±0.13	4.23±0.07	5.00±0.11	6.07±0.10	6.14±0.08
	Er	5.41±0.03	5.46±0.08	6.23±0.09	6.38±0.07	6.41±0.03	6.44±0.10
3.0	WT	5.11±0.03	4.64±0.05	4.69±0.07	5.61±0.04	5.73±0.09	5.74±0.06
	Nr	3.95±0.02	4.25±0.07	4.38±0.11	4.38±0.08	4.55±0.03	4.90±0.09
	Pr	3.93±0.09	3.90±0.01	4.04±0.15	3.95±0.05	4.07±0.08	5.00±0.06
	Er	4.41±0.07	4.49±0.06	4.74±0.08	4.38±0.02	4.41±0.04	4.44±0.07
4.0	WT	4.47±0.10	4.07±0.08	4.11±0.11	5.00±0.16	4.84±0.03	5.07±0.08
	Nr	3.47±0.02	3.77±0.05	3.84±0.03	4.04±0.10	4.00±0.09	3.95±0.06
	Pr	3.56±0.05	3.69±0.07	3.77±0.06	3.74±0.06	3.69±0.07	4.60±0.05
	Er	3.90±0.09	3.95±0.03	4.00±0.08	3.95±0.03	3.77±0.01	3.84±0.08
5.0	WT	3.17±0.09	2.77±0.10	2.84±0.08	3.77±0.05	3.60±0.06	3.90±0.09
	Nr	2.17±0.14	2.47±0.02	2.60±0.05	2.95±0.04	2.90±0.03	2.60±0.06
	Pr	2.23±0.04	2.60±0.06	2.47±0.07	2.54±0.08	2.30±0.03	3.00±0.10
	Er	2.60±0.11	2.77±0.03	2.90±0.09	2.69±0.07	2.47±0.02	2.30±0.03
6.0	WT	ND	ND	ND	ND	ND	ND
	Nr	ND	ND	ND	ND	ND	ND
	Pr	ND	ND	ND	ND	ND	ND
	Er	ND	ND	ND	ND	ND	ND

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant, ND= Not Detected

When grown in the medium supplemented with potassium sorbate at a concentration of 2.0 mg/ml, wild type *E. faecium* VRE showed a reduction of 0.72, 2.16 and 1.56 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 1.44, 3.69 and 3.02 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.87, 2.81 and 2.15 log cycles whereas enterocin FH99 resistant variant showed a reduction of 0.73, 2.47 and 1.87 log cycles after 1, 8 and 24h, respectively.

At a concentration of 3.0 mg/ml, wild type *E. faecium* VRE showed a log reduction of 1.06, 3.16 and 2.56 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.19, 4.33 and 3.41 after 1, 8 and 24h, respectively.

Pediocin 34 resistant variant showed a reduction of 2.23, 4.81 and 3.30 log cycles, whereas enterocin FH99 resistant variant showed a reduction of 1.73, 4.47 and 3.87 log cycles after 1, 8 and 24h, respectively.

At a concentration of 4.0 mg/ml, wild type *E. faecium* VRE showed a log reduction of 1.69, 4.08 and 3.22 after 1h, 8h and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.66, 4.89 and 4.36 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.60, 5.19 and 3.69 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.24, 5.11 and 4.47 log cycles after 1, 8 and 24h, respectively

At a concentration of 5.0 mg/ml wild type *E. faecium* VRE showed a log reduction of 4.00, 6.29 and 5.39 after 1h, 8h and 24h, respectively. No viable growth was detected for nisin, pediocin 34 and enterocin FH99 resistant variants.

When grown in the medium supplemented with potassium sorbate at a concentration of 6.0 mg/ml no viable growth was detected for wild type, nisin, pediocin 34 and enterocin FH99 resistant variants.

4.8.3.5 Effect of different concentrations of potassium sorbate on wild type *E. faecalis* ATCC 29212 and its bacteriocin resistant variants

Table 4.21 shows the viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 and its nisin resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0, and 6.0 mg/ml.

Table 4.21 Viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 (WT) and its nisin (Nr) resistant variant at different time intervals after growth in medium supplemented with potassium sorbate at concentration of 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml (mean ± standard error, n = 3)

Concentration (mg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	8.47±0.12	8.43±0.33	8.26±0.17	8.36±0.27	8.30±0.53	8.28±0.31
	Nr	8.47±0.02	8.43±0.01	8.26±0.09	8.36±0.03	8.30±0.05	8.28±0.01
2.0	WT	4.07±0.01	4.73±0.04	5.20±0.02	5.74±0.08	5.77±0.01	5.74±0.03
	Nr	3.47±0.02	3.34±0.03	4.00±0.11	4.25±0.10	5.69±0.07	5.65±0.01
3.0	WT	4.04±0.03	4.70±0.07	3.95±0.03	4.98±0.01	4.00±0.03	5.17±0.09
	Nr	3.00±0.15	3.07±0.12	3.38±0.01	4.00±0.02	4.30±0.05	4.39±0.04
4.0	WT	3.66±0.08	3.63±0.02	3.20±0.05	3.21±0.04	3.47±0.01	3.36±0.08
	Nr	2.47±0.01	2.69±0.07	2.38±0.01	2.54±0.09	3.39±0.04	3.54±0.08
5.0	WT	3.20±0.12	3.30±0.03	2.95±0.03	2.90±0.09	2.81±0.03	2.89±0.05
	Nr	2.00±0.01	2.07±0.06	1.90±0.09	2.19±0.02	2.79±0.09	2.77±0.02
6.0	WT	2.90±0.09	3.00±0.02	2.65±0.07	2.54±0.08	2.47±0.02	2.54±0.08
	Nr	1.90±0.09	1.89±0.05	1.69±0.06	1.875061	1.477121	1.544068

WT= Wild Type, Nr= Nisin resistant variant

When grown in the medium supplemented with potassium sorbate at a concentration of 2.0 mg/ml wild type *E. faecalis* ATCC 29212 showed a reduction of 4.39, 2.52 and 2.54 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 5.00, 2.60 and 2.63 log cycles after 1, 8 and 24h, respectively.

At a concentration of 3.0 mg/ml wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.43, 4.30 and 3.10 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 5.47, 4.00 and 3.88 after 1, 8 and 24h, respectively.

However, at a concentration of 4.0 mg/ml wild type *E. faecalis* ATCC 29212 showed a log reduction of 4.81, 4.82 and 4.92 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 6.00, 4.90 and 4.74 after 1, 8 and 24h, respectively.

At a concentration of 6.0 mg/ml wild type *E. faecalis* ATCC 29212 showed a log reduction of 5.57, 5.82 and 5.74 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 6.57, 6.82 and 6.74 after 1, 8 and 24h, respectively.

4.8.4. Effect of Sodium Nitrite on Wild Type and Bacteriocin Resistant Variants

4.8.4.1 Effect of different concentrations of sodium nitrite on wild type *L. monocytogenes* ATCC 53135 and its bacteriocin resistant variants

Table 4.22 shows the viable cell count (log cfu/ml) of wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with sodium nitrite at concentrations of 20, 30, 40 and 50μg/ml.

When grown in the medium supplemented with sodium nitrite at a concentration of 20μg/ml, wild type *L. monocytogenes* ATCC 53135 showed a reduction of 1.59, 1.95 and 1.48 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 2.96, 3.28 and 1.78 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.59, 2.93 and 1.90 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.83, 2.64 and 2.03 log cycles after 1, 8 and 24h, respectively.

Supplementing the medium with sodium nitrite at a concentration of 30μg/ml, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.37, 4.35 and 3.15 after 1, 8 and 24h, respectively which are quite higher as compared to the nitrite concentration at 20μg/ml. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 3.87, 4.40 and 3.66 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.99, 4.49 and 3.60 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.64, 4.57 and 3.92 log cycles after 1h, 8h and 24h respectively.

Table 4.22 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* ATCC 53135 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Nitrite at concentration of 20, 30, 40 and 50μg/ml (mean ± standard error, n = 3).

Concentration (μg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	7.97±0.03	7.74±0.03	8.49±0.13	7.86±0.03	8.95±0.02	8.11±0.09
	Nr	7.95±0.04	7.71±0.03	8.47±0.01	7.83±0.09	8.93±0.08	8.04±0.03
	Pr	7.99±0.05	7.72±0.06	8.86±0.09	7.83±0.09	8.94±0.03	8.00±0.13
	Er	7.94±0.09	7.71±0.03	8.47±0.01	7.85±0.02	8.93±0.09	8.04±0.03
20	WT	6.38±0.11	6.19±0.13	6.28±0.07	6.00±0.16	7.00±0.15	6.63±0.08
	Nr	4.99±0.06	5.17±0.09	5.04±0.09	5.34±0.03	5.65±0.13	6.25±0.07
	Pr	5.39±0.04	6.25±0.03	6.30±0.03	6.15±0.06	6.01±0.07	6.09±0.02
	Er	5.11±0.03	5.90±0.09	6.17±0.01	5.32±0.09	6.29±0.03	6.00±0.06
30	WT	4.60±0.16	4.84±0.05	4.14±0.12	4.14±0.18	4.60±0.20	4.95±0.04
	Nr	4.07±0.09	4.30±0.10	4.92±0.09	4.39±0.04	4.53±0.10	4.38±0.11
	Pr	4.00±0.12	4.41±0.03	4.87±0.06	3.90±0.09	4.44±0.05	4.39±0.09
	Er	4.30±0.03	4.36±0.08	4.54±0.18	4.47±0.03	4.36±0.06	4.11±0.08
40	WT	3.39±0.04	3.47±0.07	3.90±0.09	4.27±0.04	4.17±0.04	3.90±0.09
	Nr	3.07±0.08	3.00±0.06	3.04±0.04	3.44±0.07	3.60±0.06	3.69±0.07
	Pr	3.04±0.03	3.08±0.08	3.55±0.03	3.47±0.01	3.65±0.03	3.69±0.06
	Er	3.00±0.10	3.19±0.05	3.65±0.07	3.25±0.03	3.94±0.08	3.90±0.09
50	WT	3.27±0.02	3.11±0.03	3.23±0.09	3.89±0.05	3.81±0.03	3.44±0.08
	Nr	2.69±0.07	2.50±0.05	2.60±0.06	2.73±0.04	2.50±0.05	2.59±0.06
	Pr	2.90±0.09	2.97±0.04	3.00±0.07	3.07±0.01	3.27±0.08	3.01±0.03
	Er	2.80±0.08	2.89±0.05	3.14±0.05	2.95±0.03	3.03±0.06	2.91±0.08

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At a concentration of 40µg/ml, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 4.57, 4.77 and 4.21 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 4.87, 5.33 and 4.34 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 4.95, 5.29 and 4.30 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.94, 4.99 and 4.13 log cycles after 1, 8 and 24h, respectively.

At a concentration of 50 µg/ml wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 4.69, 5.14 and 4.66 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed a reduction of 5.25, 6.42 and 5.45 after 1, 8hand 24h, respectively. Pediocin 34 resistant variant showed a reduction of 5.09, 5.67 and 4.98 log cycles whereas enterocin FH99 resistant variant showed a reduction of 5.14, 5.90 and 5.12 log cycles after 1, 8 and 24h, respectively.

4.8.4.2 Effect of different concentrations of sodium nitrite on wild type *L. monocytogenes* MTCC 657 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *L. monocytogenes* MTCC 657 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with sodium nitrite at concentration of 20, 30, 40 and 50µg/ml is given in Table 4.23.

When grown in the medium supplemented with sodium nitrite at a concentration of 20 µg/ml, wild type *L. monocytogenes* MTCC 657 showed a reduction of 0.52, 0.70 and 1.68 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 1.04, 1.83 and 2.12 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 0.55, 1.76 and 2.38 log cycles whereas enterocin FH99 resistant variant showed a reduction of 0.80, 1.78 and 2.41 log cycles, respectively, after identical time periods.

At a concentration of 30 μg/ml, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.33, 2.65 and 3.22 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 2.17, 3.64 and 4.11 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.37, 3.22 and 4.17 log cycles whereas enterocin FH99 resistant variant showed a reduction of 1.50, 3.12 and 4.04 log cycles after 1, 8 and 24h, respectively.

Table 4.23 Viable cell count (log cfu/ml) of wild type *Listeria monocytogenes* MTCC 657 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in medium supplemented with Sodium Nitrite at concentration of 20, 30, 40 and 50μg/ml (mean ± standard error, n = 3)

Concentration (μg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.00±0.34	6.57±0.19	6.54±0.24	7.39±0.01	7.79±0.55	8.50±0.51
	Nr	6.07±0.81	6.56±0.14	6.53±0.17	7.36±0.23	7.79±0.20	8.49±0.13
	Pr	5.95±0.42	6.54±0.41	6.51±0.14	7.38±0.21	7.77±0.15	8.47±0.21
	Er	5.97±0.21	6.55±0.33	6.50±0.15	7.34±0.24	7.78±0.11	8.46±0.23
20	WT	5.47±0.10	5.74±0.03	5.96±0.13	7.20±0.07	7.08±0.05	6.81±0.04
	Nr	5.03±0.04	5.39±0.09	5.07±0.01	5.70±0.08	5.95±0.03	6.36±0.08
	Pr	5.39±0.09	6.25±0.07	6.30±0.03	6.15±0.06	6.01±0.07	6.09±0.12
	Er	5.17±0.01	6.00±0.08	6.00±0.04	6.07±0.08	6.00±0.09	6.04±0.08
30	WT	4.66±0.08	4.69±0.07	5.01±0.13	5.09±0.02	5.14±0.08	5.27±0.04
	Nr	3.90±0.09	4.00±0.01	4.53±0.09	4.60±0.06	4.14±0.01	4.38±0.11
	Pr	4.57±0.08	4.74±0.04	4.90±0.06	4.20±0.12	4.55±0.03	4.30±0.06
	Er	4.47±0.01	4.66±0.08	4.77±0.01	4.44±0.08	4.66±0.06	4.41±0.03
40	WT	3.77±0.05	3.84±0.08	4.20±0.02	4.59±0.05	4.47±0.06	4.00±0.04
	Nr	3.34±0.03	3.61±0.11	3.17±0.09	3.25±0.02	3.90±0.10	3.67±0.09
	Pr	3.62±0.09	3.50±0.05	3.87±0.04	3.44±0.08	3.97±0.04	3.69±0.07
	Er	3.50±0.04	3.47±0.09	3.92±0.19	3.25±0.11	3.99±0.05	3.90±0.02
50	WT	3.27±0.05	3.11±0.09	3.23±0.03	3.89±0.05	3.81±0.01	3.44±0.08
	Nr	2.69±0.07	2.50±0.05	2.60±0.06	2.73±0.04	3.65±0.03	3.14±0.05
	Pr	3.25±0.03	3.04±0.02	3.46±0.08	2.90±0.09	2.94±0.04	3.15±0.03
	Er	3.11±0.09	3.17±0.01	3.59±0.05	2.95±0.03	3.45±0.02	3.21±0.06

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At a concentration of 40 µg/ml, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 2.22, 3.32 and 4.50 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 2.73, 3.88 and 4.81 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.33, 3.80 and 4.77 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.47, 3.78 and 4.55 log cycles after 1, 8 and 24h, respectively.

Further, at a concentration of 50 µg/ml, wild type *L. monocytogenes* MTCC 657 showed a log reduction of 2.72, 3.98 and 5.05 after 1, 8 and 24h, respectively. Nisin resistant variant of *L. monocytogenes* MTCC 657 showed a reduction of 3.38, 4.13 and 5.34 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 2.69, 4.83 and 5.32 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.86, 4.32 and 5.25 log cycles after 1, 8 and 24h, respectively.

4.8.4.3 Effect of different concentrations of sodium nitrite on wild type *E. faecium* DSMZ 20477 and its bacteriocin resistant variants

The viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals growth in medium supplemented with sodium nitrite at concentration of 20, 25, 30, 35, 40, 45, 50 and 55µg/ml is given in Table 4.24.

When grown in the medium supplemented with sodium nitrite at a concentration of 20 µg/ml wild type *E. faecium* DSMZ 20477 showed a reduction of 1.62, 0.76 and 0.02 log cycles after 1, 8 and 24h, respectively.

Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 2.28, 2.12 and 0.35 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.58, 0.75 and 0.42 log cycles, whereas, enterocin FH99 resistant variant showed a reduction of 1.94, 1.69 and 2.18 log cycles after 1, 8 and 24h, respectively.

Table 4.24 Viable cell count (log cfu/ml) of wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variant at different time intervals after growth in supplemented with Sodium Nitrite at concentration of 20, 30, 40 and 50µg/ml (mean ± standard error, n = 3)

Concentration (µg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
	Nr	6.43±0.04	6.83±0.01	6.46±0.08	7.61±0.07	8.51±0.04	8.69±0.04
	Pr	6.73±0.06	6.77±0.05	6.62±0.09	6.77±0.05	6.65±0.04	5.72±0.07
	Er	6.94±0.07	7.41±0.04	7.64±0.07	9.51±0.01	8.47±0.05	9.78±0.05
20	WT	5.32±0.19	5.34±0.23	7.00±0.17	8.03±0.26	7.70±0.14	9.76±0.02
	Nr	4.14±0.08	4.47±0.06	5.89±0.06	6.14±0.08	6.39±0.09	8.34±0.13
	Pr	5.14±0.11	5.30±0.10	6.19±0.08	5.84±0.12	5.90±0.06	7.30±0.08
	Er	5.00±0.09	5.07±0.13	7.00±0.03	6.54±0.08	6.77±0.01	7.60±0.06
30	WT	5.14±0.08	5.30±0.03	6.62±0.09	7.47±0.07	7.20±0.02	7.23±0.08
	Nr	3.14±0.11	4.60±0.06	5.47±0.06	6.34±0.03	6.74±0.08	6.41±0.07
	Pr	5.07±0.08	4.38±0.08	5.30±0.03	5.16±0.05	5.20±0.12	5.00±0.17
	Er	4.65±0.03	4.77±0.05	5.77±0.08	5.07±0.09	5.30±0.10	6.17±0.09
40	WT	4.84±0.05	4.90±0.03	5.00±0.08	5.07±0.08	6.00±0.05	6.14±0.08
	Nr	3.00±0.06	4.23±0.04	4.60±0.06	4.53±0.09	4.60±0.06	5.25±0.03
	Pr	3.20±0.09	3.82±0.05	3.57±0.04	3.93±0.05	3.85±0.02	3.59±0.07
	Er	3.30±0.03	3.74±0.04	4.47±0.01	3.95±0.23	4.00±0.07	4.90±0.09
50	WT	4.67±0.09	4.84±0.02	4.57±0.04	4.14±0.08	5.43±0.04	5.60±0.06
	Nr	2.60±0.06	3.59±0.05	3.20±0.02	3.11±0.03	3.47±0.01	3.84±0.08
	Pr	2.74±0.03	3.03±0.04	3.11±0.15	3.72±0.16	3.69±0.07	3.63±0.02
	Er	2.90±0.02	3.30±0.03	3.77±0.01	3.90±0.09	3.30±0.03	3.76±0.03

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At a concentration of 30µg/ml, wild type *E. faecium* DSMZ 20477 showed a log reduction of 1.80, 1.27 and 2.55 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 3.28, 1.77 and 2.28 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.65, 1.44 and 2.72 log cycles whereas enterocin FH99 resistant variant showed a reduction of 2.29, 3.17 and 3.60 log cycles after 1, 8 and 24h,

respectively. However, at a concentration of 40 μg/ml, wild type *E. faecium* DSMZ 20477 showed a log reduction of 2.10, 2.47 and 3.63 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 3.43, 3.91 and 3.44 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.52, 2.79 and 4.13 log cycles whereas, enterocin FH99 resistant variant showed a reduction of 3.64, 4.47 and 4.48 log cycles after 1, 8 and 24h, respectively.

At a concentration of 50 μg/ml, wild type *E. faecium* DSMZ 20477 showed a log reduction of 2.27, 3.05 and 4.18 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* DSMZ 20477 showed a reduction of 3.82, 5.04 and 4.85 after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 3.99, 2.95 and 4.09 log cycles whereas enterocin FH99 resistant variant showed a reduction of 4.04, 5.17 and 6.02 log cycles after 1, 8 and 24h, respectively.

4.8.4.4 Effect of different concentrations of sodium nitrite on wild type *E. faecium* VRE and its bacteriocin resistant variants

The viable cell counts (log cfu/ml) of wild type *E. faecium* VRE and its nisin, pediocin 34 and enterocin FH99 resistant variants at different time intervals after growth in medium supplemented with sodium nitrite at concentration of 20, 30, 40 and 50μg/ml are given in Table 4.25.

When grown in the medium supplemented with sodium nitrite at a concentration of 20 μg/ml wild type *E. faecium* VRE showed a reduction of 1.22, 0.95 and 0.15 log cycles after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 2.54, 2.71 and 0.28 log cycles after 1, 8 and 24h, respectively. Pediocin 34 resistant variant showed a reduction of 1.57, 3.29 and 1.12 log cycles whereas, enterocin FH99 resistant variant showed a reduction of 1.24, 2.41 and 1.02 log cycles after 1, 8 and 24h, respectively.

At a concentration of 30μg/ml, wild type *E. faecium* VRE showed relatively more reduction (1.39, 1.06 and 0.52 log cycles) after 1, 8 and 24h, respectively as

compared to growth in 20µg/ml supplementation. Similarly, relatively higher reduction of growth of nisin resistant variant *E. faecium* VRE; pediocin 34 resistant variant and enterocin FH99 resistant variant was observed after 1, 8 and 24h of incubation.

Table 4.25 Viable cell count (log cfu/ml) of wild type *E. faecium* VRE (WT) and its nisin (Nr) resistant, pediocin 34 resistant (Pr) and enterocin FH99 (Er) resistant variants at different time intervals after growth in medium supplemented with Sodium Nitrite at concentration of 20, 30, 40 and 50µg/ml (mean ± standard error, n = 3)

Concentration (µg/ml)	Strain	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Nr	6.14±0.02	6.89±0.09	7.21±0.08	9.17±0.10	8.89±0.07	8.32±0.19
	Pr	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.30±0.10
	Er	6.17±0.60	6.88±0.64	7.21±0.48	9.20±0.41	8.89±0.32	8.32±0.19
20	WT	4.95±0.13	5.25±0.25	7.19±0.08	8.85±0.12	7.93±0.18	8.14±0.18
	Nr	3.60±0.20	3.25±0.13	4.27±0.14	5.60±0.16	6.17±0.11	8.04±0.13
	Pr	4.60±0.16	5.17±0.10	5.69±0.07	5.54±0.08	5.60±0.16	7.17±0.09
	Er	4.90±0.09	4.77±0.05	6.95±0.03	6.23±0.09	6.47±0.21	7.30±0.12
30	WT	4.77±0.05	5.07±0.09	6.00±0.02	7.04±0.03	7.83±0.09	7.77±0.05
	Nr	3.00±0.09	3.17±0.06	4.17±0.09	5.25±0.13	5.46±0.08	6.20±0.12
	Pr	4.30±0.10	5.00±0.08	5.07±0.09	4.86±0.03	5.25±0.03	5.00±0.09
	Er	4.54±0.08	4.47±0.03	5.47±0.11	5.00±0.09	5.04±0.09	5.84±0.08
40	WT	4.30±0.10	4.60±0.06	5.00±0.03	5.17±0.05	6.00±0.06	6.74±0.12
	Nr	2.90±0.09	2.84±0.08	3.17±0.06	4.38±0.11	4.54±0.08	4.90±0.09
	Pr	2.97±0.04	3.23±0.09	3.90±0.09	3.77±0.01	4.49±0.02	4.27±0.04
	Er	3.47±0.01	3.39±0.02	4.00±0.13	4.32±0.19	4.00±0.21	4.60±0.10
50	WT	4.23±0.09	4.00±0.06	4.25±0.03	4.00±0.09	5.23±0.04	5.00±0.04
	Nr	2.60±0.06	3.39±0.04	2.77±0.05	3.07±0.07	3.17±0.09	4.23±0.06
	Pr	2.74±0.03	2.99±0.06	3.00±0.11	3.11±0.03	3.00±0.16	3.30±0.10
	Er	2.90±0.03	3.30±0.08	3.77±0.09	3.90±0.09	3.30±0.03	3.76±0.08

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin FH99 resistant variant

At a concentration of 40µg/ml, wild type *E. faecium* VRE showed a log reduction of 1.87, 2.89 and 1.55 after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 3.24, 4.35 and 3.41 after 1, 8 and 24h,

respectively. Pediocin 34 resistant variant showed a reduction of 3.19, 4.40 and 4.02 log cycles whereas, enterocin FH99 resistant variant showed a reduction of 2.66, 4.89 and 3.72 log cycles after 1, 8 and 24h, respectively.

At a concentration of 50μg/ml, wild type *E. faecium* VRE showed a log reduction of 1.94, 3.66 and 3.30 after 1h, 8h and 24h respectively. Nisin resistant variant of *E. faecium* VRE showed a reduction of 3.54, 5.71 and 4.09 after 1h, 8h and 24h respectively. Pediocin 34 resistant variant showed a reduction of 3.43, 5.89 and 5.00 log cycles whereas enterocin FH99 resistant variant showed a reduction of 3.24, 5.59 and 4.55 log cycles after 1h, 8h and 24h respectively.

4.8.4.5 Effect of different concentrations of sodium nitrite on wild type *E. faecalis* ATCC 29212 and its bacteriocin resistant variants

Table 4.26 shows the viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 and its nisin resistant variant at different time intervals after growth in medium supplemented medium supplemented with sodium nitrite at concentration of 20, 30, 40 and 50 μg/ml.

When grown in the medium supplemented with sodium nitrite at a concentration of 20μg/ml wild type *E. faecalis* ATCC 29212 showed a reduction of 3.69, 1.69 and 0.42 log cycles after 1h, 8h and 24h respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 3.77, 2.30 and 1.17 log cycles after 1, 8 and 24h, respectively.

At a concentration of 25μg/ml, wild type wild type *E. faecalis* ATCC 29212 showed a log reduction of 3.92, 1.85 and 0.96 after 1, 8 and 24h respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 showed a reduction of 4.00, 2.04 and 1.24 log cycles after 1, 8 and 24h, respectively.

At a concentration of 30, 40 and 50μg/ml, wild type wild type *E. faecalis* ATCC 29212 showed progressively higher log reductions of cells after 1, 8 and 24h, respectively. Nisin resistant variant of *E. faecalis* ATCC 29212 similarly showed more cell death after 1, 8 and 24h, respectively.

Table 4.26 Viable cell count (log cfu/ml) of wild type *E. faecalis* ATCC 29212 (WT) and its nisin (Nr) resistant variant at different time intervals after growth in supplemented with Sodium Nitrite at concentration of 20, 30, 40 and 50µg/ml (mean ± standard error, n = 3)

Concentration (µg/ml)	Culture	TIME (h)					
		1	2	4	6	8	24
CONTROL	WT	8.47±0.12	8.43±0.33	8.26±0.17	8.36±0.27	8.30±0.53	8.28±0.31
	Nr	8.47±0.02	8.43±0.01	8.26±0.09	8.36±0.03	8.30±0.05	8.28±0.01
20	WT	4.77±0.08	5.14±0.12	7.34±0.13	7.96±0.18	6.60±0.20	7.86±0.23
	Nr	4.69±0.07	5.00±0.10	7.00±0.15	7.07±0.09	6.00±0.17	7.11±0.06
30	WT	4.47±0.03	4.23±0.09	5.76±0.08	6.83±0.09	6.27±0.04	6.27±0.04
	Nr	4.30±0.10	3.84±0.08	5.25±0.03	6.25±0.03	5.84±0.08	5.95±0.03
40	WT	4.30±0.10	4.00±0.12	5.39±0.04	5.53±0.09	5.60±0.06	5.95±0.09
	Nr	4.00±0.23	3.95±0.03	4.77±0.15	4.65±0.16	4.97±0.12	4.95±0.04
50	WT	3.75±0.05	3.80±0.18	4.67±0.08	4.84±0.08	4.92±0.09	5.64±0.07
	Nr	3.23±0.09	3.14±0.08	4.23±0.06	4.00±0.12	4.14±0.12	4.14±0.09

WT= Wild Type, Nr= Nisin resistant variant

After exposure of a bacterial population to a single antimicrobial factor there is often a heterogeneous response, depending on the intensity of the treatment besides many other factors. For a fraction of the population the antimicrobial could be a lethal dose leading to the cell death. The remaining fraction however, may survive as the given concentration of the antimicrobial could be a sub-lethal dose; increased resistance of the organism because of its physiological state (e.g. stationary phase cells, cells are already stressed in response to other unfavourable environmental conditions), and/or cells are naturally resistant to the antimicrobial agent. Sub-lethally injured cells as well as cells with

increased resistance may repair the damage caused by the antimicrobial agent and thus survive. Also, s fraction having good mechanisms of resistance or adaptation would make them immune to further challenges with the particular antimicrobial factor. By contrast, when cells are exposed to a combination of antimicrobial factors, the intensity of damage may be higher since some of the antimicrobial factors may act on the same cellular target. The repair of multiple damages may also require much higher energy costs, leading to energy exhaustion and cell death. Therefore, the probabilities for survival and proliferation for cells confronted with multiple hurdles are very low. In addition, the synergy between different antimicrobial factors may allow the use of lower doses compared to their individual application. The application of bacteriocins as part of hurdle technology has received great attention in recent years (Chen & Hoover, 2003; Ross *et al.*, 2003; Deegan *et al.*, 2006), since bacteriocins can be used purposely in combination with selected hurdles in order to increase microbial inactivation. The combination of hurdles to be applied will depend greatly on the type of food and its microbial composition.

However, the emergence of bacteriocin-resistant microorganisms is a major concern in bacteriocin-preserved, minimally-processed foods. Many reports have suggested that the ultimate failure of bacteriocin based preservation systems is due to the eventual growth of resistant strains (Harris *et al.*, 1992; Ming & Daeschel, 1993; Motlagh *et al.*, 1992). Nisin-resistant isolates of *Listeria monocytogenes*, *C. botulinum*, *Bacillus subtilis*, *B. licheniformis*, *B. cereus* and *Staphylococcus aureus* have been generated (Harris *et al.*, 1992; Jarvis, 1967; Jones, 1974; Ming & Daeschel, 1993; Mazzotta & Montville, 1997; Mazzotta *et al.*, 1999). Several studies have focused in the application of potential synergists of nisin's activity. The use of nitrites, low pH, pasteurization, controlled atmosphere, and food ingredients has been tried (Scott & Taylor, 1981a,b; Somers & Taylor, 1981; Taylor *et al.*, 1985 & 1990; Rayman *et al.*, 1981; Rogers & Montville, 1994). These reports, however, did not consider the possible emergence of nisin resistant strains.

Crandall & Montville (1998) and Mazzotta *et al.*, (1998) observed that nisin resistance conferred cross-resistance to other chemically and structurally different bacteriocins (). Moreover, Mesentericin 52-, curvaticin-, and plantaricin-resistant *L. monocytogenes* were also cross resistant to other bacteriocins (Rehkhif & others, 1994) However; nisin resistant *L. monocytogenes* was not more resistant than the parent strain to a variety of antibiotics (Crandall & Montville, 1998).

The current study examined whether nisin, pediocin 34 and enterocin FH99 resistance confers intrinsic resistance to preservatives like low pH, sodium chloride, sodium nitrite and potassium sorbate. Our results clearly show that resistance to nisin, pediocin 34 and enterocin FH99 did not confer intrinsic resistance to any of the preservatives tested. In contrast, resistant variants were more or equally sensitive when compared with the wild-type strain. This was true for *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212. This could be related to the fitness cost commonly associated with the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes & Hastings, 1998).

The resistance mechanism(s) of the nisin, pediocin 34 and enterocin FH99 resistant variants was observed to be specific to bacteriocin resistance and do not confer general resistance. Bacteriocins act on sensitive cells by a common mechanism that dissipates the chemical and energy gradient across the cytoplasmic membrane (Montville & Bruno, 1994; Okereke & Montville, 1991). Changes in the membrane composition of nisin-resistant strains that interfere with the action of one bacteriocin affect the antimicrobial activity of other bacteriocins that act at the cytoplasmic membrane. Studies have shown that resistance to nisin-conferred cross-resistance to other antimicrobial peptides but did not confer intrinsic resistance to heat (Mazzotta & Montville, 1999;

Modi, 1999) or the other preservative tested in this study, which act on the cell by different mechanisms.

The nisin, pediocin 34 and enterocin FH99 resistant strains did not have intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In no case were the bacteriocin resistant *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 variants examined, resistant to inhibitors than the parental strains. Together, these findings suggest that rational design of multiple-hurdle preservation systems containing bacteriocins can improve food safety without being undermined by resistance-related phenomena.

4.9 COMBINED EFFECTS OF TEMPERATURE AND BACTERIOCIN ON WILD TYPE AND RESISTANT VARIANTS

Bacteriocins can be used to reduce the intensity of heat treatments in foods without compromising microbial inactivation. Nisin and heat have been reported to act synergistically against *L. plantarum* and *L. monocytogenes* (Mahadeo & Tatini, 1994; Ueckert *et al.*, 1998), reducing the heat resistance of *L. monocytogenes* in milk (Maisnier-Patin *et al.*, 1995) and in cold-pack lobster meat (Budu-Amoako *et al.*, 1999). Nisin-resistant *L. monocytogenes* cells grown in the presence of nisin were more sensitive to heat at 55 °C than wild-type cells (Modi *et al.*, 2000). The efficacy of enterocin AS-48 has been reported to be higher on *S. aureus* cells sub-lethally injured by heat due to the lower concentration of remaining viable cells and to the cell damage induced by the heat treatment (Ananou *et al.*, 2004). Bacteriocins can also provide an additional protection during food storage against proliferation of endospores surviving heat treatments. Moreover, it has been demonstrated that the intensity of heat treatments against bacterial endospores can be lowered in combination with nisin as well as with enterocin AS-48 (Beard *et al.*, 1999; Wandling *et al.*, 1999; Grande *et al.*, 2006a), saving costs in the heat treatment and decreasing the impact of heat on the food. Sub-lethal heat has been shown to sensitize Gram-negative bacteria to several bacteriocins such as nisin or

pediocin AcH (Kalchayanand *et al.*, 1992; Boziaris *et al.*, 1998), enterocin AS-48 (Abriouel *et al.*, 1998; Ananou *et al.*, 2005a), or jenseniin G (Bakes *et al.*, 2004), extending their spectrum of action. Highest sensitization was reported for combined treatments of bacteriocins, heat and a chelating agent (Abriouel *et al.*, 1998; Ananou *et al.*, 2005a). The synergistic effect of nisin with heat could lead to decreased thermal process requirements and improved food quality. However, the emergence of bacteriocin resistant variants may hinder the application of bacteriocins in food preservation in combination with mild heat treatments if in any case the resistant strains develop cross resistance to heat.

Therefore, the objective of the research was to evaluate whether nisin resistance in *L. monocytogenes* leads to increased resistance to heat relative to the wild type cells. The heat resistance of wild type and the nisin resistant, pediocin 34 and enterocin FH99 variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 was determined at 45, 50 and 55°C. The concentrations of bacteriocins used to evaluate synergistic effect of heat and bacteriocins are presented in Table 4.27.

4.9.1 Synergistic Effect of Heat + Nisin, Heat + Pediocin 34 and Heat + Enterocin FH99 on Wild Type *L. Monocytogenes* ATCC 53135 and Its Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

Effect of heat alone and also combined effect of heat + nisin and heat + pediocin 34 and heat + enterocin FH99 on wild type *L. monocytogenes* ATCC 53135 and its nisin, pediocin 34 and enterocin FH99 resistant variants was evaluated at 45, 50 and 55°C. At 45°C log reduction of 2.17, 2.66, 3.07 and 3.60 was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99. In the presence of nisin, a log reduction of 3.00, 4.30, 4.60 and 4.07 was observed after 5, 10, 15 and 20 min, respectively.

In the presence of pediocin 34 loss of 3.30, 3.73, 4.17 and 4.03 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin

FH99 loss of 2.26, 2.72, 3.12 and 3.01 log cycles was observed after 5, 10, 15 and 20 min, respectively.

Table 4.27 The concentrations of bacteriocins used to evaluate synergistic effect of heat and bacteriocins

Culture	Strains	Nisin (IU/ml)	Pedioicn 34 (AU/ml)	Enterocin FH99 (AU/ml)
L. monocytogenes ATCC 53135	WT	13.2	540	120
	Nr	13.2	-	-
	Pr	-	540	-
	Er	-	-	120
L. monocytogenes MTCC 657	WT	50	600	700
	Nr	50	-	-
	Pr	-	600	-
	Er	-	-	700
E. faecium DSMZ 20477	WT	53.5	68.5	937.5
	Nr	53.5	-	-
	Pr	-	68.5	-
	Er	-	-	937.5
E. faecium VRE	WT	14	2187.5	3750
	Nr	14	-	-
	Pr	-	2187.5	-
	Er	-	-	3750
E. faecalis ATCC 29212	WT	26.5	-	-
	Nr	26.5	-	-

WT= Wild Type, Nr= Nisin resistant variant, Pr= Pediocin 34 resistant variant, Er= Enterocin resistant variant

For nisin resistant variant of *L. monocytogenes* ATCC 53135, a log reduction of 3.60, 4.00, 4.08 and 3.90 was observed after 5, 10, 15 and 20 min, respectively at 45°C, whereas in presence of nisin, log reduction of 3.66, 4.33, 4.50 and 4.57 was observed after 5, 10, 15 and 20 min, respectively. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 3.05, 3.67, 3.94 and 3.98 log cycles was observed after 5, 10, 15 and 20 min, respectively. In presence of pediocin 34, loss 3.60, 4.60, 4.94 and 5.16 log cycles was observed after 5, 10, 15 and 20 min, respectively. In absence of enterocin FH99, loss of 3.18, 3.78, 4.07 and 4.09

log cycles was observed after 5, 10, 15 and 20 min, respectively In presence of enterocin FH99, reduction of 3.75, 3.67, 4.08 and 4.30 log cycles in cell count was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.7).

At 50°C, log reduction of 2.75, 3.18, 3.98 and 4.63 in cell count was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99, whereas in the presence of nisin log reduction of 3.66, 4.03, 4.75 and 4.90 was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, loss of 3.06, 3.62, 4.58 and 4.62 log cycles in cell count was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99 loss of 4.5, 4.33, 2.5 and 2.4 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the absence of nisin, at 50°C, log reduction of 5.26, 5.45, 5.34 and 5.91 was observed after 5, 10, 15 and 20 min whereas, in the presence of nisin reduction of 5.38, 6.29, 6.51 and 7.34 log cycles was observed after 5, 10, 15 and 20 min, respectively for nisin resistant variant of *L. monocytogenes* ATCC 53135. There was no significant difference in the heat sensitivity of the pediocin 34 resistant variant when grown in the absence or presence of pediocin 34 at 50°C. In the absence of pediocin 34, a reduction of 3.79, 4.24, 4.39 and 4.41 log cycles was observed after 5, 10 and 15 min, respectively. In the presence of pediocin 34 a reduction of 3.33, 3.33 and 3.47 log cycles was observed after 5, 10, 15 and 20 min, respectively. In case of enterocin FH99 resistant variant, at 50°C, log reduction of 3.75, 3.90, 4.08 and 4.11 was observed after 5, 10, 15 and 20 min, respectively, whereas in the presence of enterocin FH99 log reduction of 4.8, 5.02, 5.27 and 5.20 was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.8).

At 55°C reduction of 3.75, 4.88, 5.60 and 6.06 log cycles was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99, whereas in the presence of nisin, log reduction of 3.83 and 4.96 after 5 and 10 min, respectively was observed while no growth was observed after 15 and 20 min. In presence of pediocin 34, loss of 4.06, 4.96, 6.43 and 5.88 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence

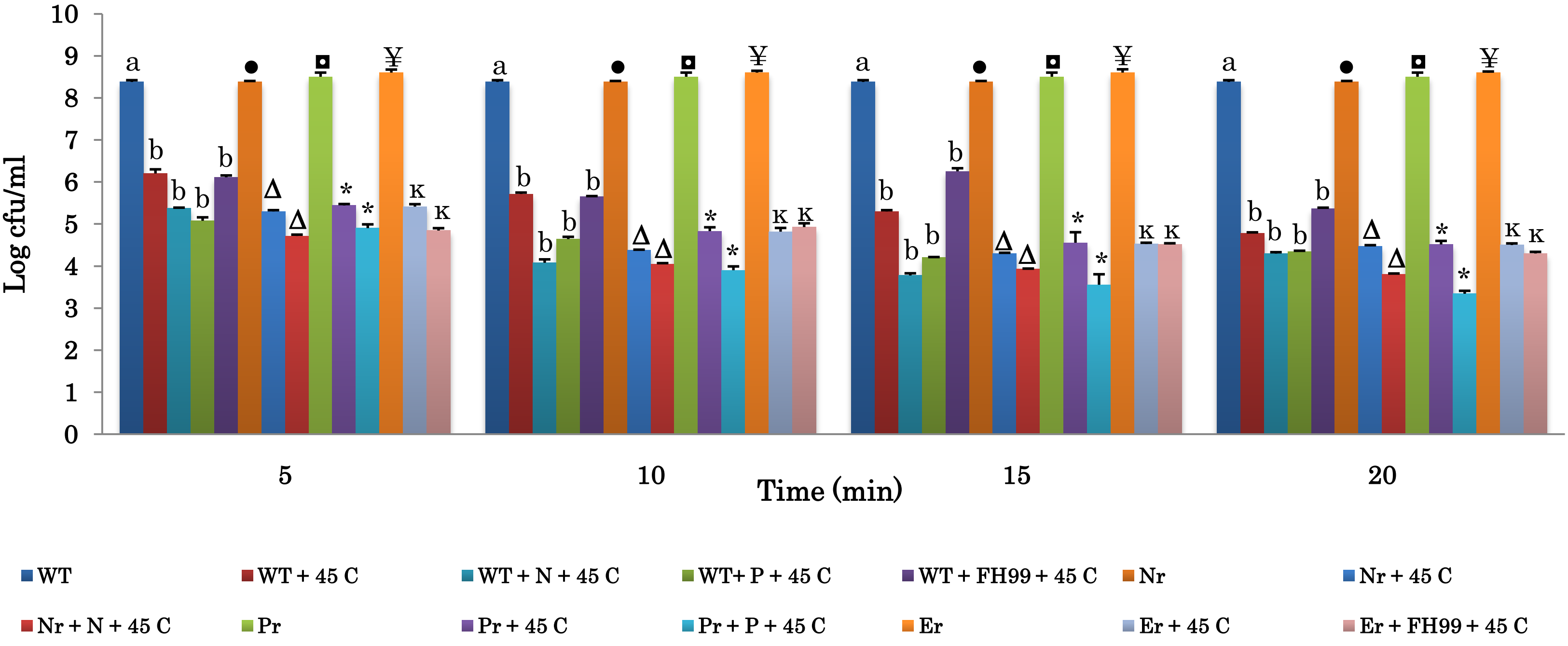


Fig. 4.7. Effect of heat (45°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* ATCC 53135 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of $P<0.001$ at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, ĸ] Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+45 C: *L. monocytogenes* treated with heat (45 °C), WT+N+45C:*L.monocytogenes* treated with nisin at 45°C, WT+P+45 C: *L.monocytogenes* treated with pediocin 34 at 45°C, WT+FH99+ 45C:*L.monocytogenes* treated with enterocin FH99 at 45°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +45 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 45C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+45C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+45 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 45 C: ENTEROCIN FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Entercocin FH99

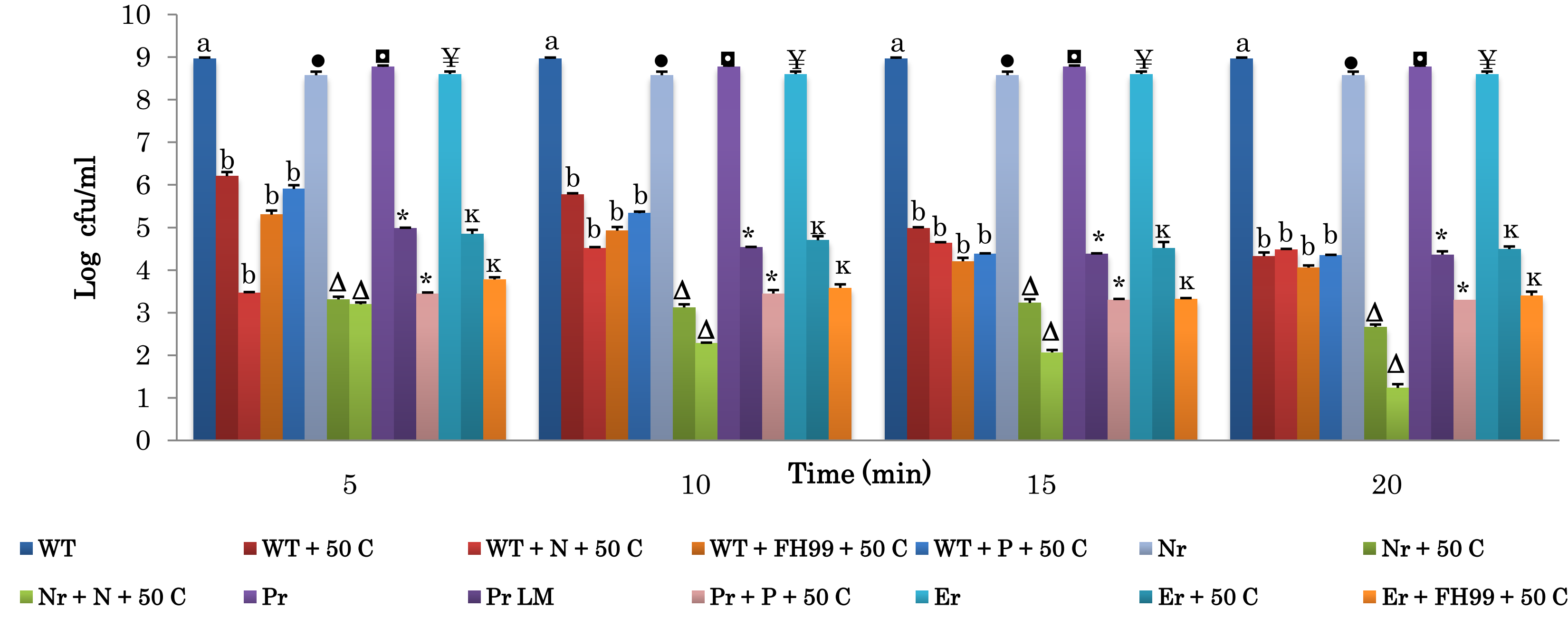


Fig. 4.8. Effect of heat (50°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* ATCC 53135 (WT) and its Nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+50 C: *L. monocytogenes* treated with heat (50 °C), WT+N+50C:*L. monocytogenes* treated with nisin at 50°C, WT+P+ 50C: *L. monocytogenes* treated with pediocin 34 at 50°C, WT+FH99+ 50C:*L. monocytogenes* treated with enterocin FH99 at 50°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +50 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 50C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+50C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+50 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 50 C: Enterocin FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Entercocin FH99

of enterocin FH99, loss of 4.96, 5.47 and 6.11 log cycles was observed after 5, 10 and 15, respectively, and no growth was observed after 20 min. At 55°C log reduction of 4.85, 5.45 and 5.75 was observed after 5, 10 and 15 and no growth was observed after 20 min in the absence of nisin, whereas, in the presence of nisin, log reduction of 5.66, 6.06 was observed after 5 and 10 min, respectively. No growth was observed after 15 and 20min for nisin resistant variant of *L. monocytogenes* ATCC 53135 in presence of nisin. In case of Pediocin 34 resistant variant, log reduction of 4.47, 5.63 and 6.77 was observed after 5, 10 and 15 min, respectively whereas in presence of pediocin 34 log reduction of 4.57 and 5.77 after 5 and 10 min respectively and no growth was observed after 15 and 20 min. In the absence of enterocin FH99 resistant variant, log reduction of 3.90, 5.45 and 5.78 was observed. Whereas, in the presence of enterocin FH99, log reduction of 5.39, 5.69, and 5.86 was observed after 5, 10 and 15 min, respectively no growth was observed was observed after 20 min both, in absence and presence of enterocin FH99 (Fig. 4.9).

This experiment shows that nisin, pediocin 34 or enterocin FH99 resistance does not alter the heat sensitivity of *L. monocytogenes* ATCC 53135. Additionally, the nisin, pediocin 34 and enterocin FH99 resistant cells were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also clearly show that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of thermal processes against *L. monocytogenes*.

4.9.2 Synergistic Effect of Heat + Nisin, Heat + Pediocin 34 and Heat + Enterocin FH99 on Wild Type *L. monocytogenes* MTCC 657 and Its Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

Effect of heat alone and also the combined effect of heat & nisin, heat & pediocin 34 and heat & enterocin FH99 on wild type *L. monocytogenes* MTCC 657 and its nisin, pediocin 34 and enterocin FH99 resistant variants was also evaluated at 45, 50 and 55°C.

At 45°C, log reduction of 2.97, 3.04, 3.09 and 3.20 was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99. In presence of nisin, log reduction of 4.41, 4.58, 4.79 and 5.00 was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34 a reduction of 2.97, 3.05, 3.14 and 5.62 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99, loss of 2.92, 3.19, 3.41 and 3.54 log cycles was observed after 5, 10, 15 and 20 min, respectively. For nisin resistant variant of *L. monocytogenes* MTCC 657, reduction of 2.69, 3.04, 3.22 and 3.30 log cycles was observed after 5, 10, 15 and 20 min, respectively, at 45°C in the absence of nisin, whereas in the presence of nisin, log reduction of 5.22, 5.30, 5.30 and 5.52 was observed after 5, 10, 15 and 20 min, respectively. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 2.84, 3.05, 3.10 and 3.17 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, loss of 4.25, 4.47, 4.65 and 4.95 log cycles was observed after 5, 10, 15 and 20 min, respectively. For enterocin FH99 resistant variant, in the absence of enterocin FH99, a reduction of 3.30, 3.47, 3.63 and 3.77 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99, loss of 4.47, 4.63, 4.87 and 5.47 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.10).

At 50°C, reduction of 3.10, 3.11, 3.19 and 3.30 log cycles was observed after 5, 10, 15 and 20 min respectively in the absence of nisin, pediocin 34 and enterocin FH99; whereas in the presence of nisin, log reduction of 4.39, 6.02 and 6.58 was observed after identical periods and no growth was observed after 20 min. In the presence of pediocin 34 loss of 3.20, 3.34, 4.02 and 4.54 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.11). In the presence of enterocin FH99, loss of 3.22, 3.30, 3.54 and 4.17 log cycles was observed after 5, 10, 15 and 20 min, respectively. At 50°C log reduction of 3.22, 3.30, 3.52 and 3.61 was observed after 5, 10, 15 and 20 min, respectively, whereas in the presence of nisin log reduction of 3.20 and 3.88 was observed after 5 and 10min, respectively, for nisin resistant variant of *L. monocytogenes* MTCC 657. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 3.05, 3.25,

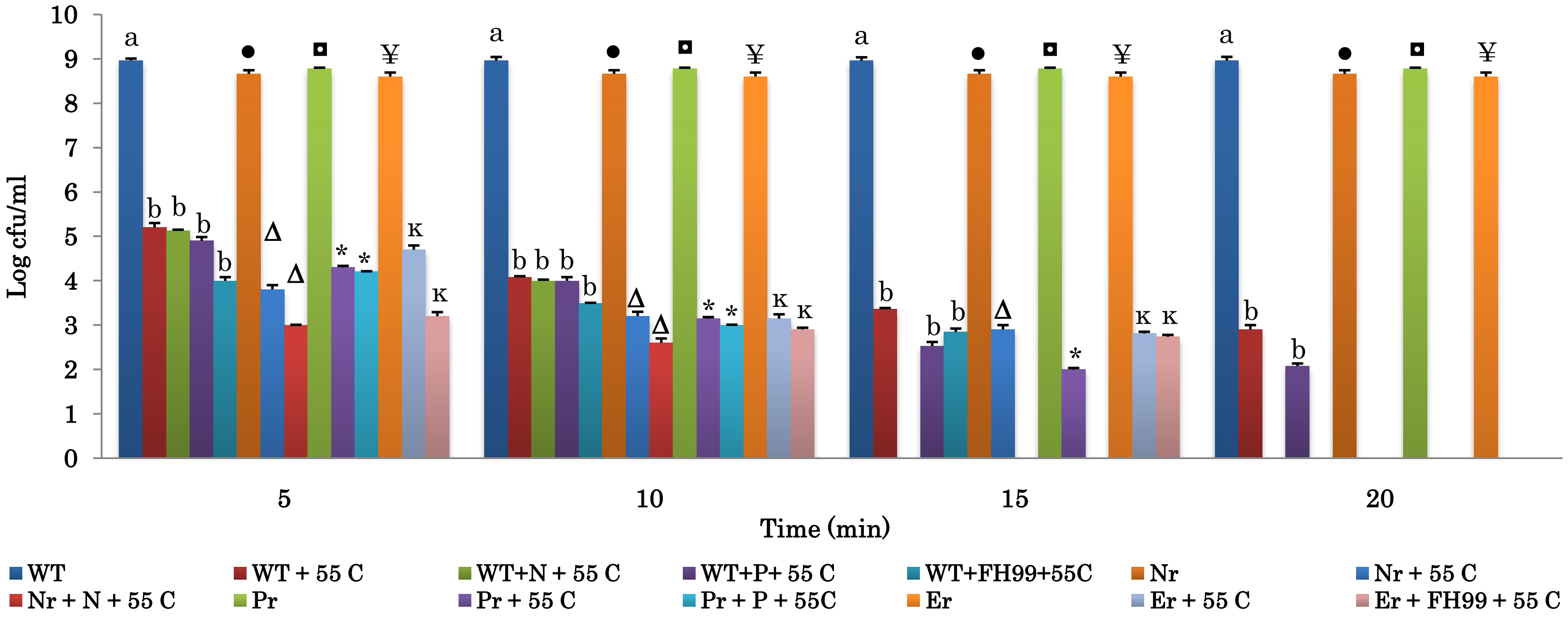


Fig. 4.9. Effect of heat (55°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* ATCC 53135 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+55 C: *L. monocytogenes* treated with heat (55 °C), WT+N+55C:*L. monocytogenes* treated with nisin at 55°C, WT+P+ 55C: *L. monocytogenes* treated with pediocin 34 at 55°C, WT+FH99+ 55C:*L. monocytogenes* treated with enterocin FH99 at 55°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +55 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 55C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+55 C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+55 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 55 C: Enterocin FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Entercocin FH99

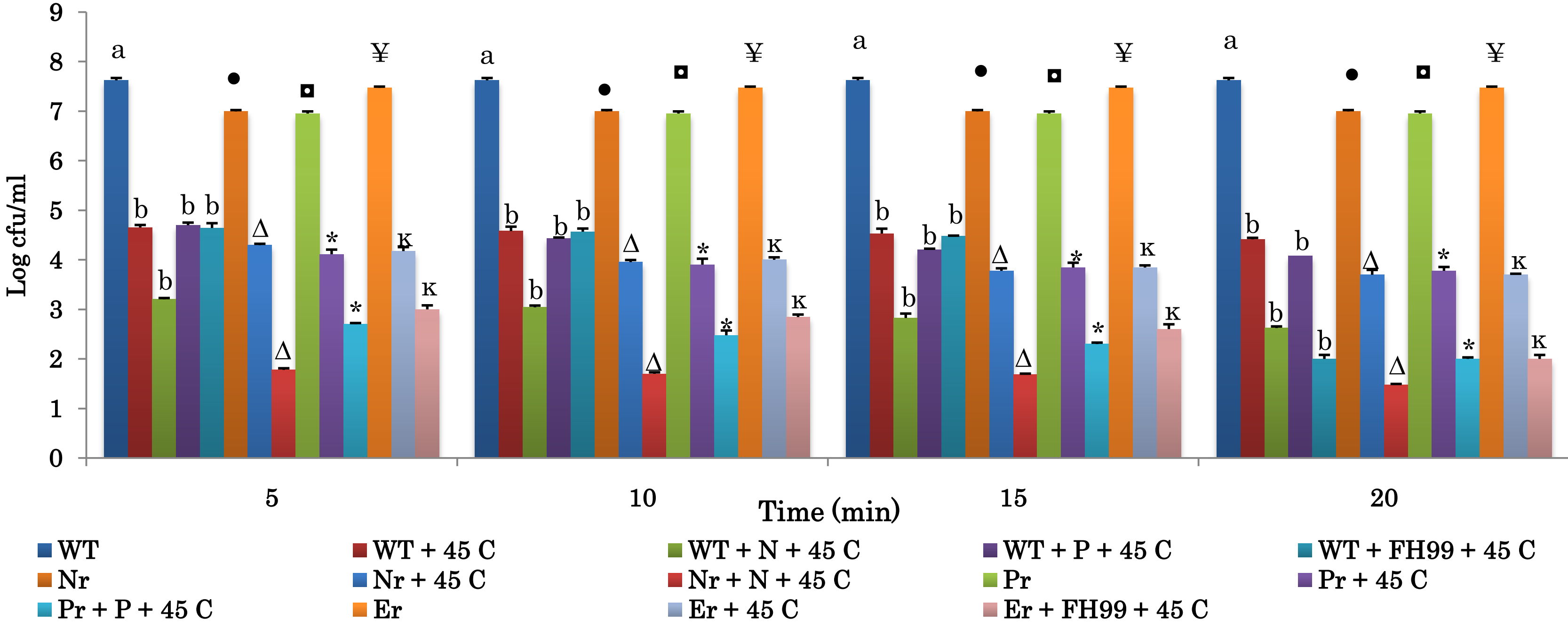


Fig. 4.10. Effect of heat (45°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* MTCC 657 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+45 C: *L. monocytogenes* treated with heat (45 °C), WT+N+45C: *L. monocytogenes* treated with nisin at 45°C, WT+P+45 C: *L. monocytogenes* treated with pediocin 34 at 45°C, WT+FH99+ 45C: *L. monocytogenes* treated with enterocin FH99 at 45°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +45 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 45C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+45C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+45 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 45 C: Enterocin FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Entercocin FH99

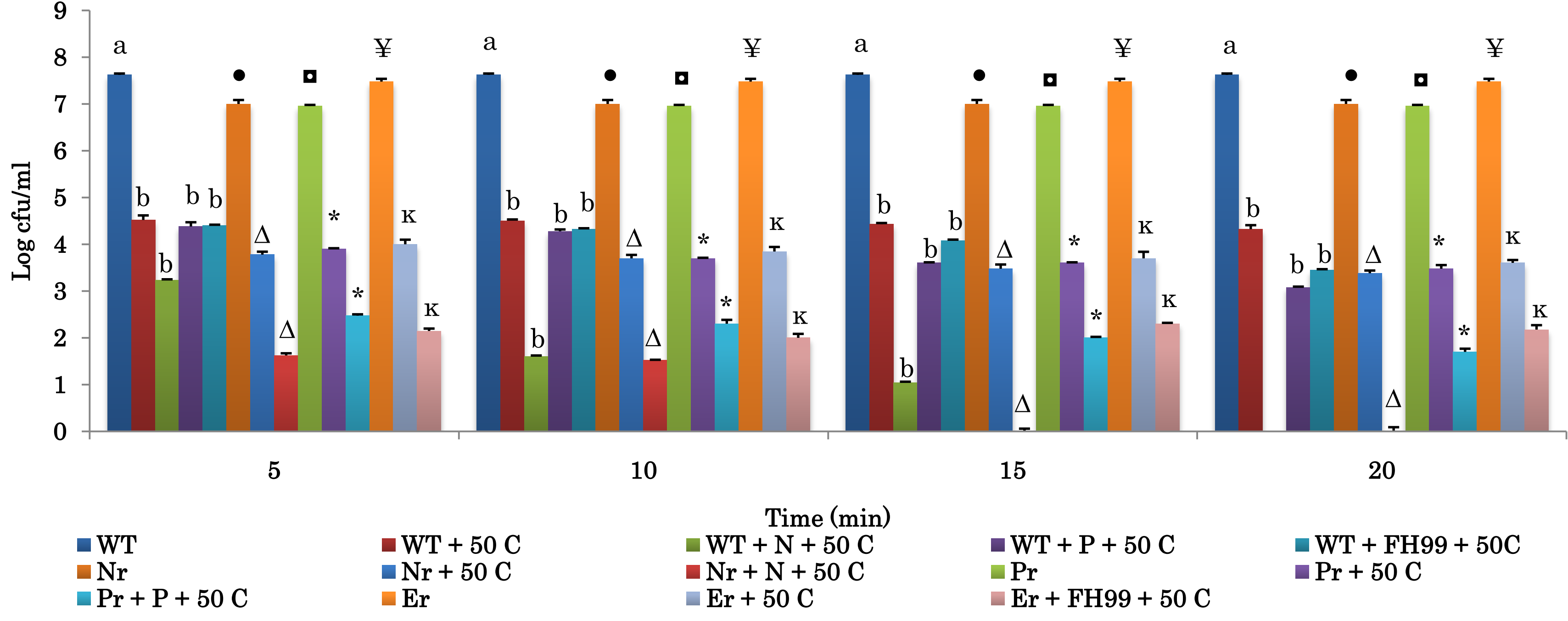


Fig. 4.11. Effect of heat (50°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* MTCC 657 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+50 C: *L. monocytogenes* treated with heat (50 °C), WT+N+50C:*L. monocytogenes* treated with nisin at 50°C, WT+P+ 50C: *L. monocytogenes* treated with pediocin 34 at 50°C, WT+FH99+ 50C:*L. monocytogenes* treated with enterocin FH99 at 50°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +50 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 50C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+50C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+50 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 50 C: Enterocin FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

3.35 and 3.40 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, loss of 4.47, 4.65, 4.95 and 5.25 log cycles was observed after 5, 10, 15 and 20 min, respectively. For enterocin FH99 resistant variant, while in the absence of enterocin FH99, loss of 3.47, 3.63, 3.77 and 3.87 log cycles was observed after 5, 10, 15 and 20 min, respectively, relatively a higher loss i.e. 5.33, 5.47, 5.17 and 5.30 log cycles after 5, 10, 15 and 20 min, respectively, was observed in the presence of enterocin FH99 (Fig. 4.11).

In the absence of nisin, pediocin 34 and enterocin FH99, log reduction of 4.14, 5.47, 6.14 after 5, 10 and 15 min, respectively was observed at 55°C whereas, no growth was observed after 20 min. However, in the presence of nisin, log reduction of 6.14, 6.26 and 6.84 after 5, 10 and 15 min respectively and no growth after 20 min was observed at this temperature. In the presence of pediocin 34, loss of 4.14 and 6.66 log cycles was observed after 5 and 10min, respectively (Fig. 4.12). No growth was observed after 15 and 20 min. In the presence of enterocin FH99, loss of 3.05, 5.72 and 7.14 log cycles was observed after 5, 10 and 15min, respectively and no growth was observed after 20 min. At 55°C reduction of 4.58, 4.92 and 5.00 log cycles was observed after 5, 10 and 15min, respectively. No growth was observed after 20 min incubation at 55°C, whereas, in the presence of nisin log reduction of 5.09 after 5min was observed and no growth was observed after 10, 15 and 20 min for nisin resistant variant of *L. monocytogenes* MTCC 657. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 4.63, 4.87 and 5.00 log cycles was observed after 5, 10 and 15, respectively (Fig. 4.12). In the presence of pediocin 34 loss of 4.69 was observed after 5min, whereas no growth was observed after 10, 15 and 20min incubation at 55°C. In the absence of enterocin FH99, enterocin FH99 resistant variant showed a loss of 4.39 and 5.69 log cycles after 5 and 10min, respectively. In the presence of enterocin FH99, loss of 5.39 log cycles was observed after 5min. No growth was observed for enterocin FH99 resistant variant after 10, 15 & 20 min incubation at 55°C both in the presence as well as absence of enterocin FH99 (Fig. 4.12).

This experiment shows that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of *L. monocytogenes* MTCC 657. In addition, the nisin, pediocin 34 and enterocin FH99 resistant cells were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also depict that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of thermal processes against *L. monocytogenes*.

4.9.3 Synergistic Effect of Heat + Nisin and Heat + Pediocin 34 and Heat + Enterocin FH99 on Wild Type *Enterococcus faecium* DSMZ 20477 and Its Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

Effect of heat alone and also combined effect of heat & Nisin, heat & Pediocin 34 and heat & Enterocin FH99 on wild type *E. faecium* DSMZ 20477 and its nisin, pediocin 34 and enterocin FH99 resistant variants was evaluated at 45, 50 and 55°C and the results are given in Figures 4.13 to 4.15.

At 45°C, a reduction of 2.75, 2.73. 2.77 and 2.76 log cycles was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99, whereas in the presence of nisin, log reduction of 3.70, 3.52, 3.84 and 3.65 was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, loss of 2.80, 2.97, 2.98 and 2.80 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99, loss of 2.78, 2.97, 2.98 and 2.98 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.13). For nisin resistant variant of *E. faecium* DSMZ 20477, log reduction of 4.33, 4.39, 4.20 and 4.21 was observed after 5, 10, 15 and 20 min, respectively at 45°C, whereas, in the presence of nisin, a log reduction of 4.41, 4.41, 4.37 and 4.41 was observed after 5, 10, 15 and 20 min, respectively. In the absence of pediocin34, pediocin 34 resistant variant showed a reduction of 3.20, 3.31, 3.39 and 3.32 log cycles after 5, 10, 15 and 20 min, respectively. In presence of pediocin 34, a loss of 3.25, 3.33, 3.64 and 4.05 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.13). In the absence of enterocin FH99, enterocin resistant variant showed a reduction of 3.21, 3.26, 3.33 and 3.41 log cycles after 5, 10, 15 and 20 min, respectively, however, in the

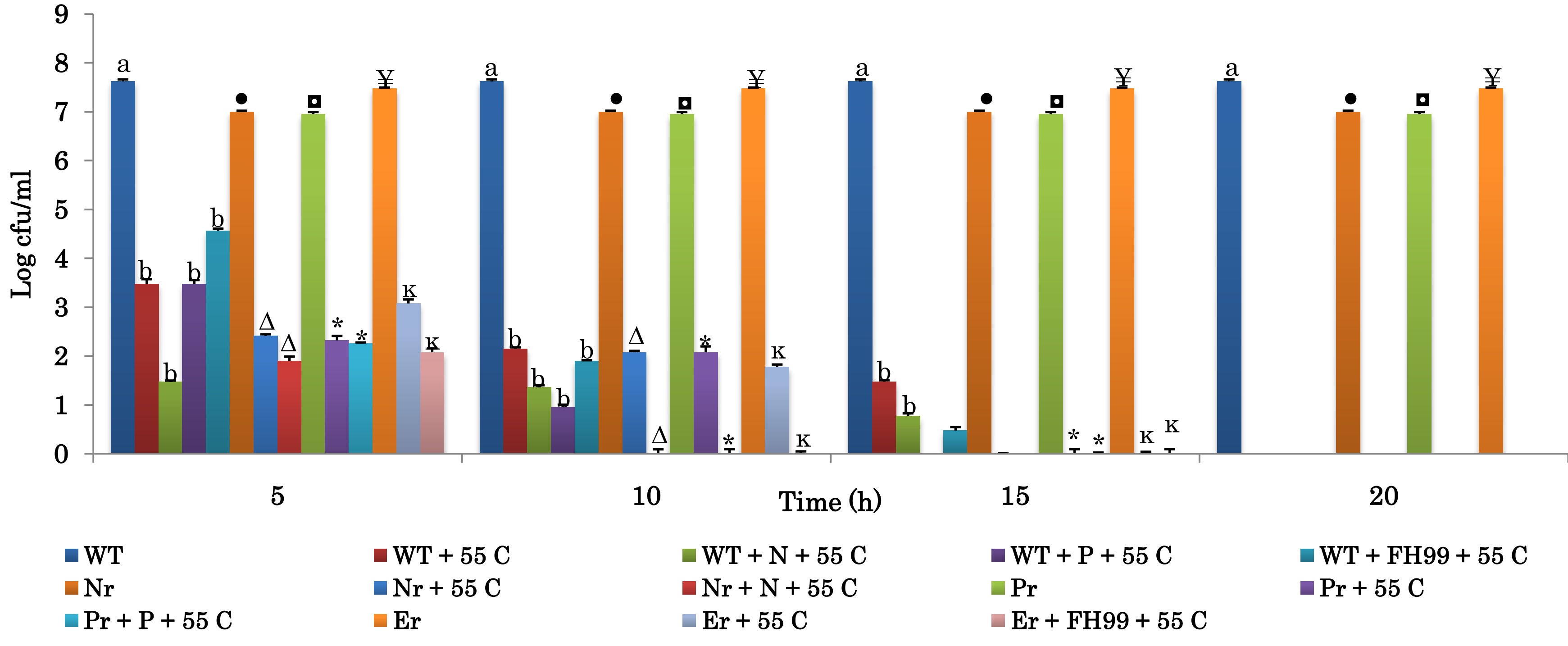


Fig. 4.12. Effect of heat (55°C) alone and in combination with bacteriocins on wild type *L. monocytogenes* MTCC 657 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *L. monocytogenes* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *L. monocytogenes* untreated by heat and bacteriocins, WT+55 C: *L. monocytogenes* treated with heat (55 °C), WT+N+55C:*L. monocytogenes* treated with nisin at 55°C, WT+P+ 55C: *L. monocytogenes* treated with pediocin 34 at 55°C, WT+FH99+ 55C:*L. monocytogenes* treated with enterocin FH99 at 55°C, Nr : Nisin resistant *L. monocytogenes* untreated by heat & nisin, Nr +55 C: Nisin resistant *L. monocytogenes* treated by heat, Nr +N+ 55C: Nisin resistant *L. monocytogenes treated* by heat & nisin, Pr: Pediocin 34 resistant *L. monocytogenes* untreated by heat & nisin, Pr +P+55 C: Pediocin 34 resistant *L. monocytogenes* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *L. monocytogenes* untreated by heat & enterocin FH99, Er+55 C: Enterocin FH99 resistant *L. monocytogenes treated* by heat, Er + FH99+ 55 C: Enterocin FH99 resistant *L. monocytogenes* treated by heat & enterocin FH99

N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

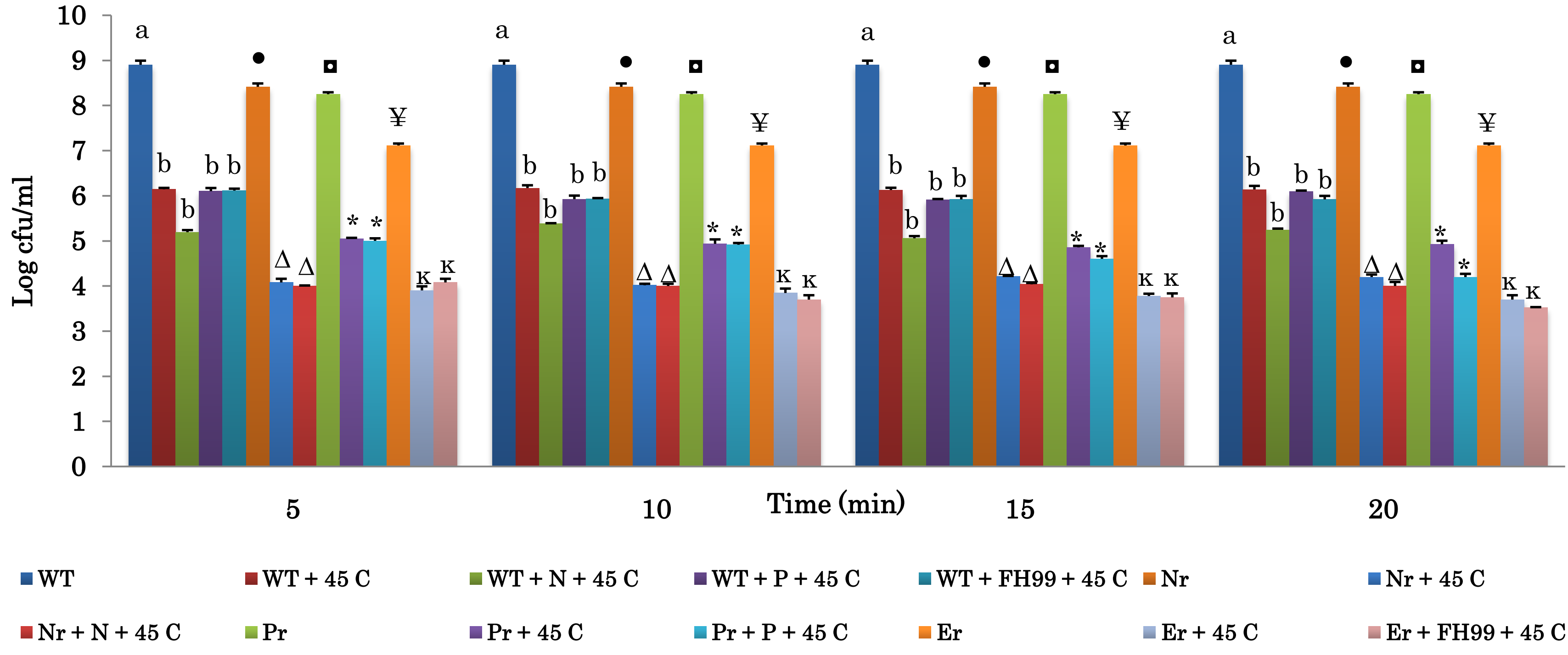


Fig. 4.13. Effect of heat (45°C) alone and in combination with bacteriocins on wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecium* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, ĸ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+45 C: *E. faecium* treated with heat (45 °C), WT+N+45C: *E. faecium* treated with nisin at 45°C, WT+P+45 C: *E. faecium* treated with pediocin 34 at 45°C, WT+FH99+ 45C:*L. monocytogenes* treated with enterocin FH99 at 45°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +45 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 45C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+45C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+45 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 45 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

presence of enterocin FH99 loss of 3.03, 3.41, 3.36 and 3.59 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.13).

At 50°C log reduction of 3.05, 3.54, 4.54 and 4.84 was observed after 5, 10, 15 and 20 min, respectively in the absence of nisin, pediocin 34 and enterocin FH99. In the presence of nisin a log reduction of 4.54, 4.73, 5.05 and 5.36 was observed after 5, 10, 15 and 20 min respectively. In the presence of pediocin 34 a loss of 4.02, 4.02, 4.69 and 4.89 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99 loss of 3.54, 3.73, 4.05 and 4.36 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.14). At 50°C, log reduction of 4.41, 4.26, 4.48 and 4.51 was observed after 5, 10, 15 and 20 min, respectively, whereas in the presence of nisin log reduction of 5.03, 5.47, 5.51 and 5.81 was observed after 5, 10, 15 and 20 min, respectively, for nisin resistant variant of *E. faecium* DSMZ 20477. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 3.26, 3.90, 4.86 and 5.07 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin loss of 4.40, 5.38, 5.41 and 5.86 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.14). In the absence of enterocin FH99, enterocin resistant variant showed a reduction of 3.38, 3.45, 3.47 and 3.56 log cycles after 5, 10, 15 and 20 min, respectively, however, in the presence of enterocin FH99 loss of 3.51, 3.63, 4.15 and 4.33 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.14).

At 55°C log reduction of 4.93, 4.98, 4.97 and 5.11 was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99; whereas in the presence of nisin, log reduction of 5.24, 5.93, 6.01, 7.02 was observed after 5, 10, 15 and 20 min,, respectively (Fig. 4.15). In the presence of pediocin 34 loss of 4.65, 4.82, 4.97 and 6.01 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99, loss of 4.64, 4.78, 4.97 and 6.00 log cycles was observed after 5, 10, 15 and 20 min, respectively. At 55°C reduction of 5.11, 5.33, 5.46 and 5.59 log cycles was observed after 5, 10, 15 and 20 min, respectively, whereas, in the presence of

nisin, log reduction of 5.60, 5.68, 6.75 and 6.83 after 5, 10, 15 and 20 min, respectively, was observed for nisin resistant variant of *E. faecium* DSMZ 20477 (Fig. 4.15). For pediocin 34 resistant variant, in the absence of pediocin 34 loss of 4.47, 4.64, 4.94 and 5.04 log cycles was observed after 5, 10, 15 and 20 min, respectively. In presence of pediocin 34, loss of 4.64, 5.13, 5.34 and 5.77 log cycles was observed after 5, 10, 15 and 20 min respectively (Fig. 4.15). In the absence of enterocin FH99, enterocin resistant variant showed a reduction of 4.15, 4.21, 4.63 and 4.90 log cycles after 5, 10, 15 and 20 min, respectively, however, in the presence of enterocin FH99 loss of 5.56, 6.00, 5.75 and 6.03 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.15).

This experiment shows that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of *E. faecium* DSMZ 20477. In addition, the nisin resistant, pediocin 34 and enterocin FH99 resistant cells were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also depict that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of the thermal processes against *E. faecium* DSMZ 20477.

4.9.4 Synergistic Effect of Heat + Nisin and Heat + Pediocin 34 and Heat + Enterocin FH99 on Wild Type *Enterococcus faecium* VRE and Its Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

Effect of heat alone and also combined effect of heat + Nisin, heat + Pediocin 34 and heat+ Enterocin FH99 on wild type *E. faecium* VRE and its nisin, pediocin 34 and enterocin FH99 resistant variants was evaluated at 45, 50 and 55°C.

At 45°C, log reduction of 1.75, 1.73, 1.77 and 1.76 was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99. Whereas, in the presence of nisin, reduction of 2.70, 2.52, 2.84 and 2.65 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.16). In the presence of pediocin 34, a loss of 1.80, 1.97, 1.98 and 1.80 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99, loss of 1.78, 1.97, 1.98 and 1.98 log cycles was observed after 5, 10, 15

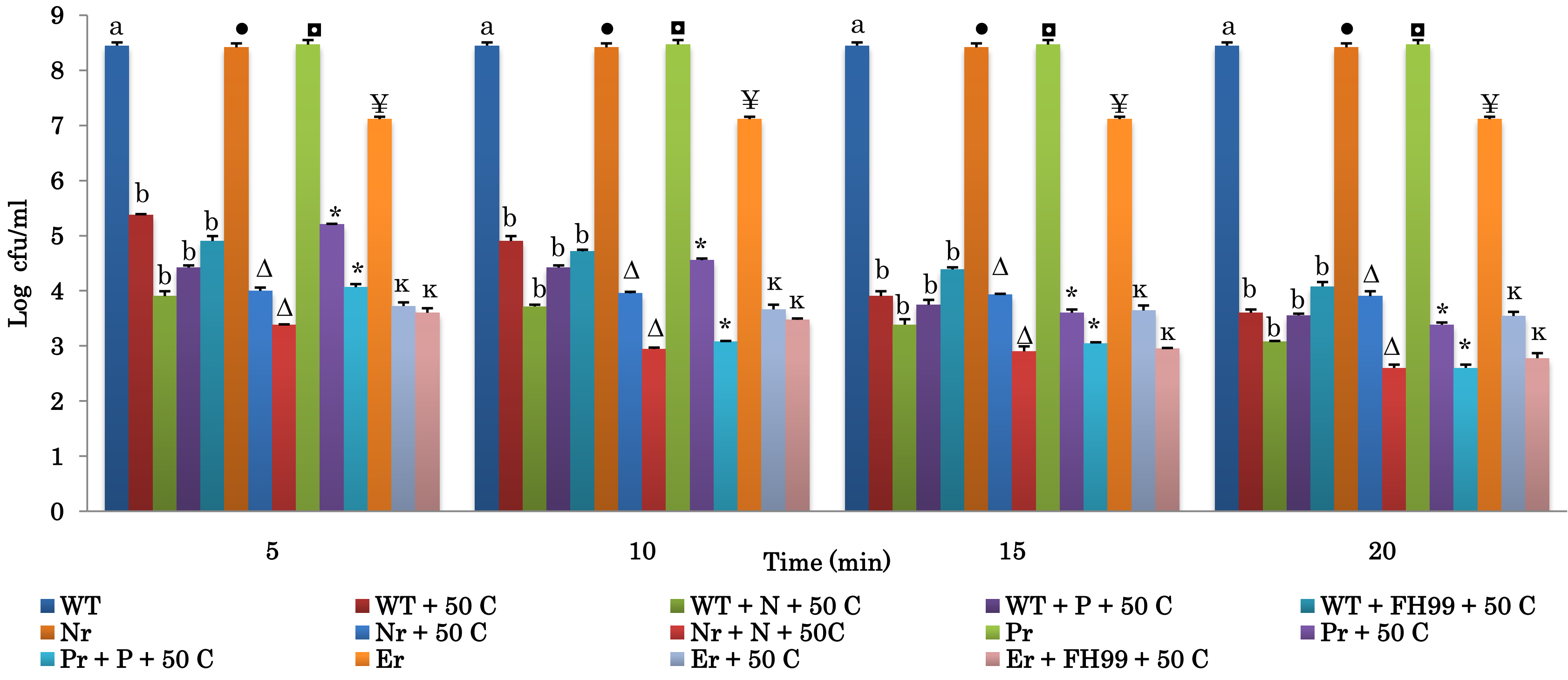


Fig. 4.14. Effect of heat (50°C) alone and in combination with bacteriocins on wild type *E. faecium* DSMZ 20477 (DSM) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecium* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+50 C: *E. faecium* treated with heat (50 °C), WT+N+50C: *E. faecium* treated with nisin at 50°C, WT+P+50 C: *E. faecium* treated with pediocin 34 at 50°C, WT+FH99+ 50C:*L. monocytogenes* treated with enterocin FH99 at 50°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +50 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 50C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+50 C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+ 50 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 50 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

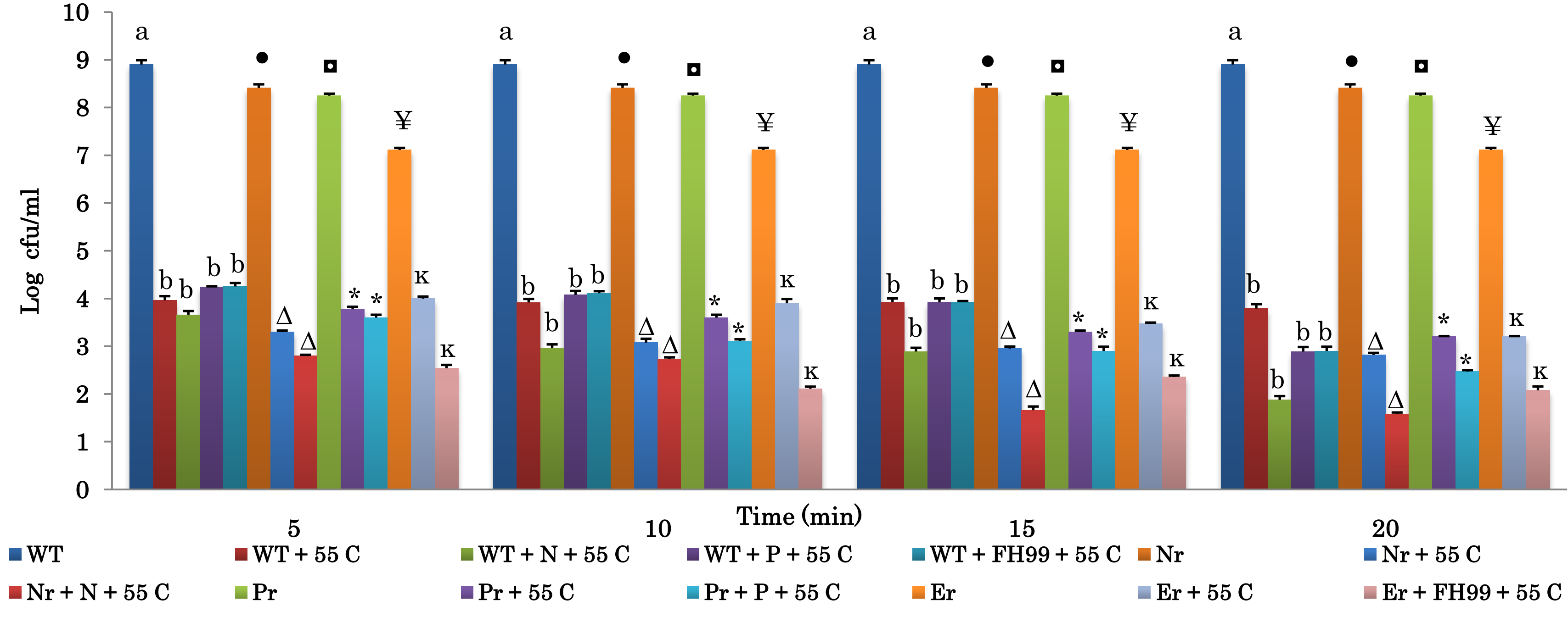


Fig. 4.15. Effect of heat (55°C) alone and in combination with bacteriocins on wild type *E. faecium* DSMZ 20477 (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecium* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+55 C: *E. faecium* treated with heat (55 °C), WT+N+55 C: *E. faecium* treated with nisin at 55°C, WT+P+55 C: *E. faecium* treated with pediocin 34 at 55°C, WT+FH99+ 55 C:*L. monocytogenes* treated with enterocin FH99 at 55°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +55 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 55 C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+55 C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+ 55 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 55 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

and 20 min, respectively. For nisin resistant variant of *E. faecium* VRE, a log reduction of 2.36, 2.17, 2.21 and 2.30 cycles after 5, 10, 15 and 20 min, respectively was observed at 45°C, where as in the presence of nisin log reduction of 3.17, 3.03, 2.93 and 3.12 was observed after 5, 10, 15 and 20 min, respectively. For pediocin 34 resistant variant, in the absence of pediocin 34, loss of 2.00, 2.05, 2.20 and 2.42 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin, loss of 3.00, 3.05, 3.20 and 3.42 log cycles was observed after 5, 10, 15 and 20 min, respectively. In thevabsence of enterocin FH99, enterocin FH99 resistant variant showed a loss of 2.21, 2.26, 2.3 and 2.4 log cycles after 5, 10, 15 and 20 min, respectively. However, in the presence of enterocin FH99a log reduction of 3.03, 3.4, 4.3 and 4.5 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.16).

At 50°C, 2.54, 2.73, 3.05 and 3.36 log cycle reduction was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99. In the presence of nisin, log reduction of 3.54, 3.73, 4.05 and 4.36 was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, a loss of 3.02, 3.02, 3.69 and 3.89 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.17). In the presence of enterocin FH99, loss of 3.06, 3.24, 3.54 and 3.84 log cycles was observed after 5, 10, 15 and 20 min respectively. At 50°C, log reduction of 3.22, 3.23, 3.30 and 4.35 was observed after 5, 10, 15 and 20 min, respectively, whereas in presence of Nisin log reduction of 4.00, 4.06, 4.14 and 5.36 was observed after 5, 10, 15 and 20 min, respectively, for nisin resistant variant of *E. faecium* VRE. For pediocin 34 resistant variant, in the absence of pediocin 34, a loss of 3.12, 3.20, 3.72 and 3.82 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin, loss of 4.12, 4.20, 4.72 and 4.82 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the absence of enterocin FH99, enterocin FH99 resistant variant showed a loss of 3.38, 3.45, 3.47 and 3.56 log cycles after 5, 10, 15 and 20 min, respectively. However, in the presence of enterocin FH99, a log reduction of 3.63, 3.81, 4.15 and 4.33 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.17).

At 55°C, log reduction of 2.60, 3.53, 3.97 and 3.98 after 5, 10, 15 and 20 min, respectively, in the absence of nisin, pediocin 34 and enterocin FH99 was observed, whereas in the presence of nisin respective loss of growth after 5, 10, 15 and 20 min was 4.24, 4.04, 4.13 and 4.24 log cycles (Fig. 4.18). In the presence of pediocin 34, loss of 3.65, 3.82, 4.00 and 4.01 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of enterocin FH99 loss of 3.64, 3.78, 3.97 and 4.00 log cycles was observed after 5, 10, 15 and 20 min, respectively. At 55°C log reduction of 4.14, 4.25, 4.26 and 4.36 was observed after 5, 10, 15 and 20 min, respectively, whereas in the presence of nisin, log reduction of 5.02, 5.24 and 5.39 after 5, 10 min and 15 min and 20 min, respectively, for nisin resistant variant of *E. faecium* VRE. No cells were observed after 20 min incubation with nisin at 55°C for nisin resistant variant. For pediocin 34 resistant variant, in the absence of pediocin 34, a loss of 4.20, 4.67, 4.72 and 4.86 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the presence of pediocin 34, loss of 4.90, 5.05, 5.35 and 5.60 log cycles was observed after 5, 10, 15 and 20 min, respectively. In the absence of enterocin FH99, enterocin FH99 resistant variant showed a loss of 4.11, 4.21, 4.63 and 4.90 log cycles after 5, 10, 15 and 20 min, respectively at 55°C. However, in the presence of enterocin FH99, a log reduction of 4.56, 5.00, 5.75 and 6.03 log cycles was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.18).

This experiment shows that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of *E. faecium* VRE. In addition, the nisin resistant, pediocin 34 and enterocin FH99 resistant cells were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also depict that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of thermal processes against *E. faecium* VRE.

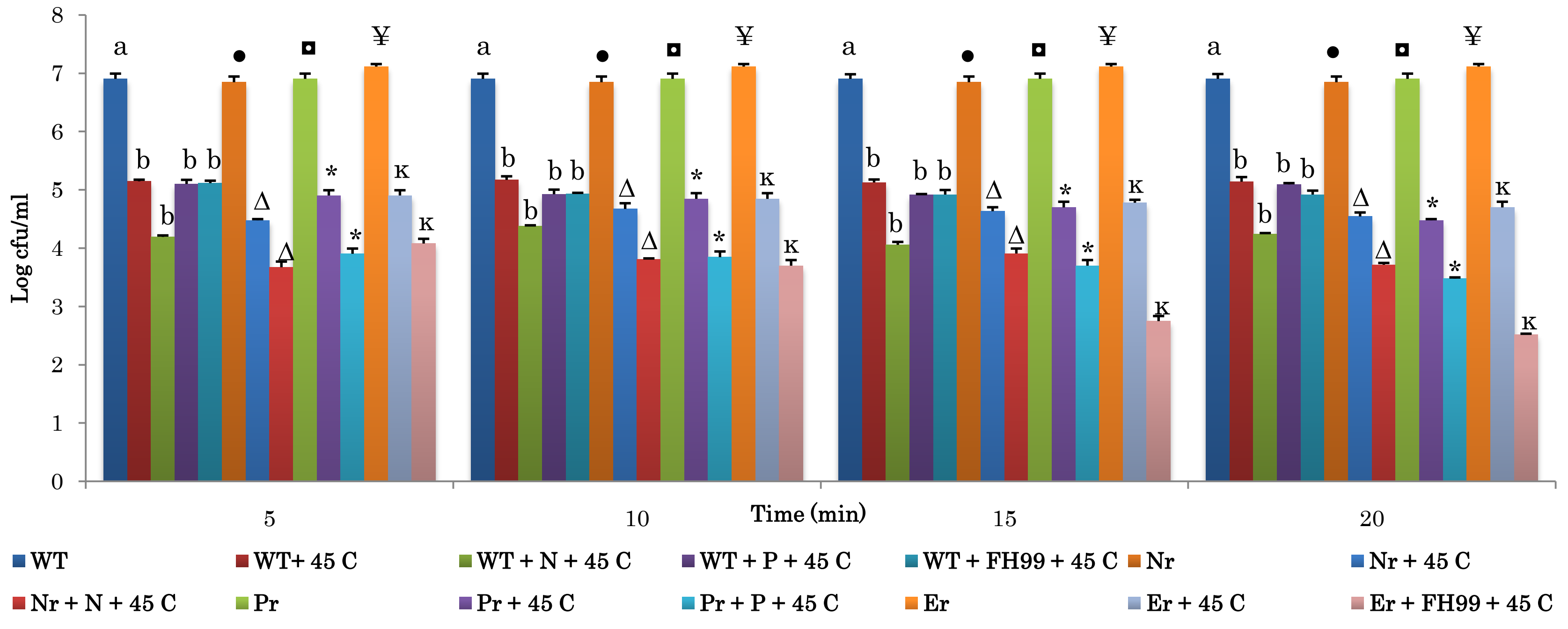


Fig. 4.16. Effect of heat (45°C) alone and in combination with bacteriocins on wild type *E. faecium* VRE (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. ^{a,b} Values with different superscripts are differ significantly at the level of $P<0.001$ at the corresponding period between WT *E. faecium* (untreated with heat & bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. $^{\bullet,\Delta}$Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. $^{¤,*}$ Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. $^{¥,\kappa}$ Values with different superscripts differ significantly at the level of $P<0.001$ at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+45 C: *E. faecium* treated with heat (45 °C), WT+N+45C: *E. faecium* treated with nisin at 45°C, WT+P+45 C: *E. faecium* treated with pediocin 34 at 45°C, WT+FH99+ 45C:*L. monocytogenes* treated with enterocin FH99 at 45°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +45 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 45C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+45C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+45 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 45 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

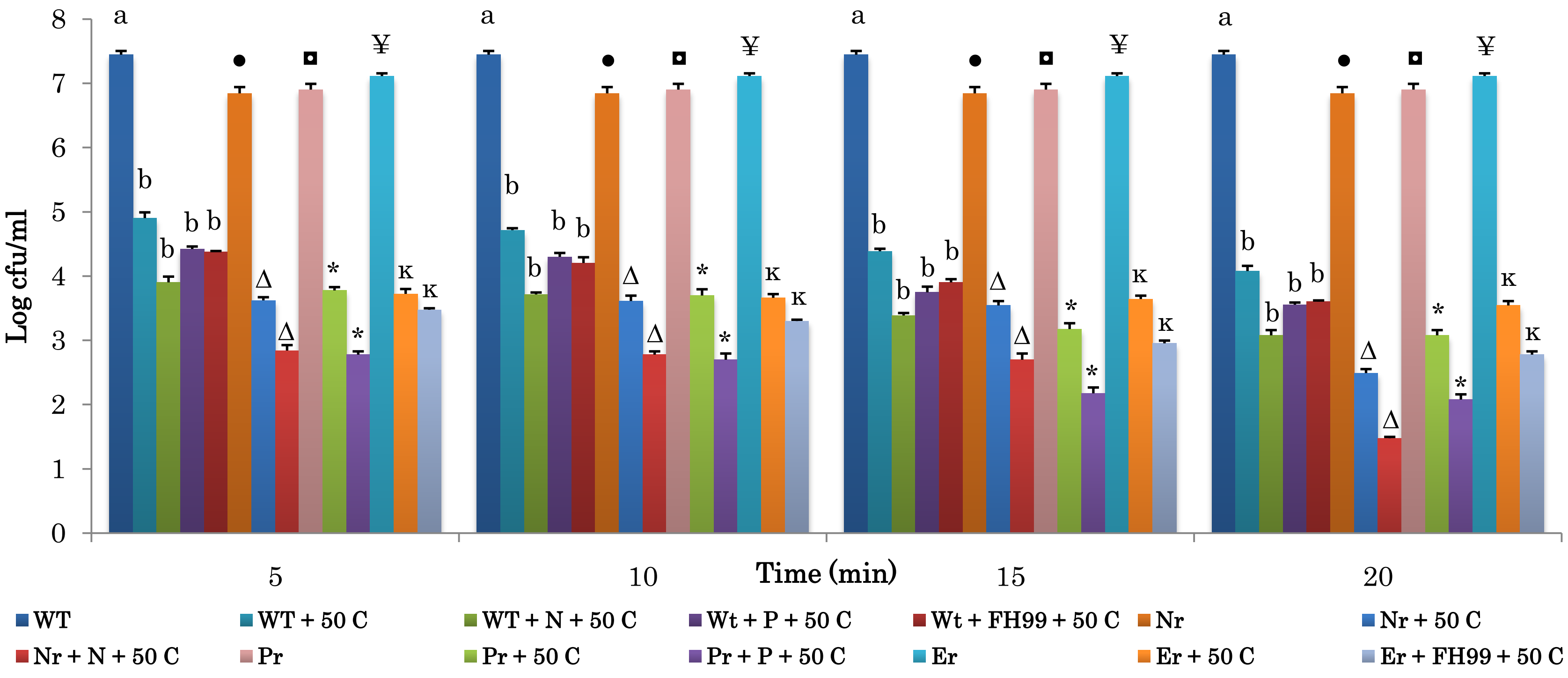


Fig. 4.17. Effect of heat (50°C) alone and in combination with bacteriocins on wild type *E. faecium* VRE (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecium* (untreated with heat & Bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+50 C: *E. faecium* treated with heat (50 °C), WT+N+50C: *E. faecium* treated with nisin at 50°C, WT+P+50 C: *E. faecium* treated with pediocin 34 at 50°C, WT+FH99+ 50C:*L. monocytogenes* treated with enterocin FH99 at 50°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +50 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 50C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+50 C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+ 50 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 50 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

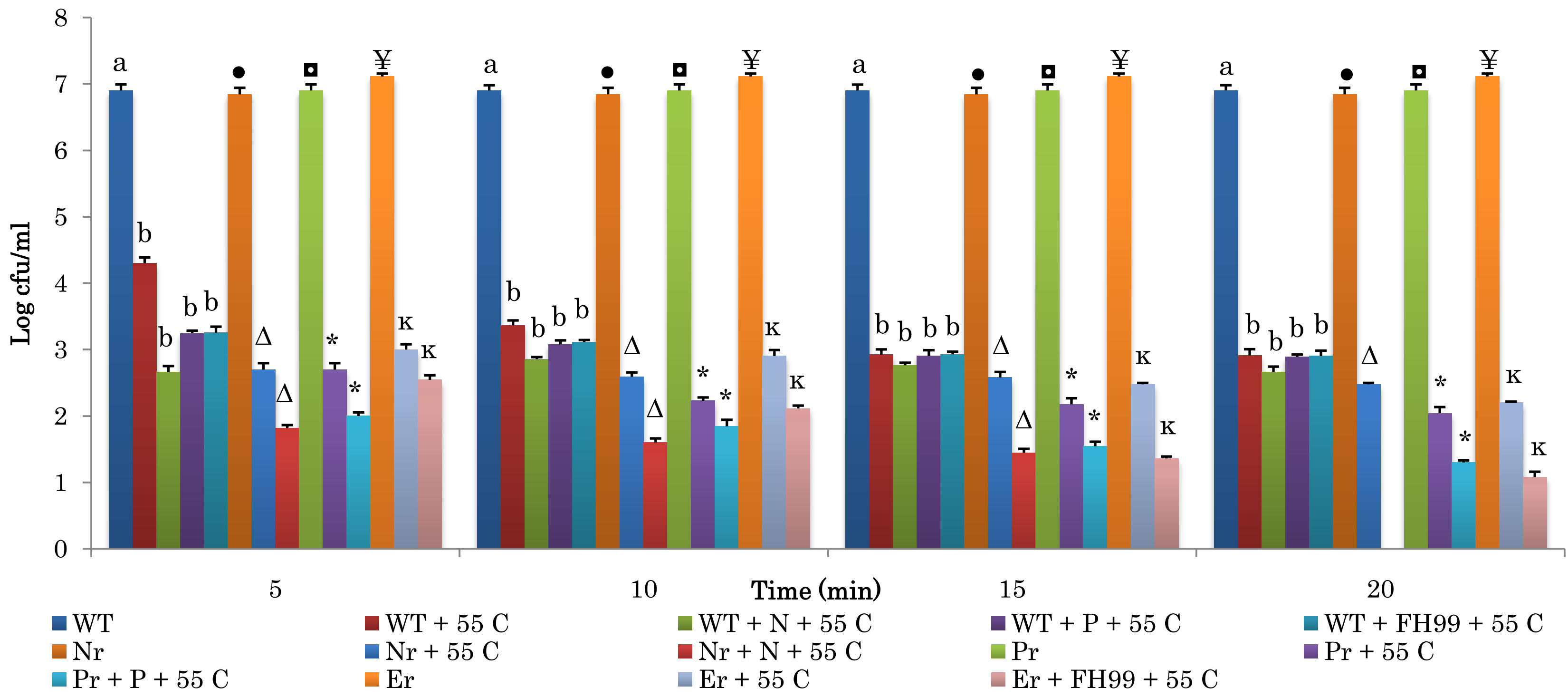


Fig. 4.18. Effect of heat (55°C) alone and in combination with bacteriocins on wild *E. faecium* VRE (WT) and its nisin (Nr), pediocin 34 (Pr) and enterocin FH99 (Er) resistant variants.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecium* (untreated with heat & Bacteriocins) and WT treated with heat in the absence of bacteriocins, respectively. [•, Δ]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr variant (untreated with heat & nisin) and Nr variant treated with heat in the absence & presence of nisin, respectively. [¤, *] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Pr variant (untreated with heat & pediocin 34) and Pr variant treated with heat in the absence & presence of pediocin, respectively. [¥, κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Er variant (untreated with heat & nisin) and Er variant treated with heat in the absence & presence of enterocin FH99, respectively.

WT: *E. faecium* untreated by heat and bacteriocins, WT+55 C: *E. faecium* treated with heat (55 °C), WT+N+55 C: *E. faecium* treated with nisin at 55°C, WT+P+55 C: *E. faecium* treated with pediocin 34 at 55°C, WT+FH99+ 55 C:*L. monocytogenes* treated with enterocin FH99 at 55°C, Nr : Nisin resistant *E. faecium* untreated by heat & nisin, Nr +55 C: Nisin resistant *E. faecium* treated by heat, Nr +N+ 55 C: Nisin resistant *E. faecium treated* by heat & nisin, Pr: Pediocin 34 resistant *E. faecium* untreated by heat & nisin, Pr +P+55 C: Pediocin 34 resistant *E. faecium* treated by heat & pediocin 34, Er: Enterocin FH99 resistant *E. faecium* untreated by heat & enterocin FH99, Er+ 55 C: Enterocin FH99 resistant *E. faecium* treated by heat, Er + FH99+ 55 C: Enterocin FH99 resistant *E. faecium* treated by heat & enterocin FH99. N: Nisin; P: Pediocin 34; FH99:Enterocin FH99

4.9.5 Synergistic Effect of Heat + Nisin on Wild Type *Enterococcus faecalis* ATCC 29212 and Its Nisin Resistant Variants

Effect of heat alone and also combined effect of heat + Nisin, on wild type *E. faecalis* ATCC 29212 and its nisin resistant variant was evaluated at 45, 50 and 55°C.

At 45°C, of 1.73, 1.79, 1.80 and 1.89 log reduction was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin, whereas in the presence of nisin a log reduction of 2.76, 3.05, 3.07 3nd 3.74 was observed after identical periods. For nisin resistant variant of *E. faecalis* ATCC 29212, a log reduction of 2.41, 2.47, 3.47 and 3.91 was observed after 5, 10, 15 and 20 min, respectively, at 45°C, whereas in the presence of nisin log reduction of 3.72, 3.77, 3.89 and 4.25 was observed after 5, 10, 15 and 20 min respectively (Fig. 4.19). In the absence of nisin, a reduction of 1.79, 1.67, 1.82 and 2.55 was observed after 5, 10, 15 and 20 min, respectively, at 50°C,. In the presence of nisin, a log reduction of 2.87, 2.89, 2.92 and 3.09 was observed after 5, 10, 15 and 20 min, respectively. For nisin resistant variant of *E. faecalis* ATCC 29212, at 50°C a log reduction of 2.81, 3.28, 3.79 and 3.93 was observed after 5, 10, 15 and 20 min, respectively, whereas in presence of nisin log reduction of 3.65, 4.71, 4.75 and 6.07 was observed after 5, 10, 15 and 20 min, respectively (Fig. 4.19).

At 55°C log reduction of 2.03, 2.07, 2.74 and 2.86 was observed after 5, 10, 15 and 20 min, respectively, in the absence of nisin; whereas in the presence of nisin the respective log reductions were 4.59, 4.69, 4.79 and 4.92 cycles after identical timings (Fig. 4.19). At 55°C log reduction of 4.30, 4.63, 4.68 and 5.14 was observed after 5, 10, 15 and 20 min, respectively, whereas in the presence of nisin log reduction of 5.67, 5.87 and 5.95 after 5, 10 min and 15 min was observed. However, no growth was observed after 20 min for nisin resistant variant of *E. faecalis* ATCC 29212 (Fig. 4.19).

The results of this experiment show that the nisin resistance does not alter the heat sensitivity of *E. faecalis* ATCC 29212. In addition, the nisin, pediocin 34

and enterocin FH99 resistant cells are further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also depict that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of thermal processes against *E. faecalis* ATCC 29212. This study also suggested that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212. In addition, the corresponding nisin, pediocin 34 and enterocin FH99 resistant cells of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat. The results of this study also show that the development of resistance to nisin, pediocin 34 and enterocin FH99 should not adversely affect the efficacy of thermal processes against *L. monocytogenes*. Also, it was observed that the synergistic effect of heat and bacteriocins (nisin, pediocin 34 and enterocin FH99) was more pronounced for the nisin, pediocin 34 and enterocin FH99 resistant cells of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212.

Mazzotta & Montville (1999) had earlier reported that nisin resistant *Clostridium botulinum* 169B were not more heat resistant than the wild type cells. The synergistic effect of heat-treatment (55°C) and nisin (500IU/ml) on the Nisin resistant cells and the wild type *L. monocytogenes* ScottA was also studied by Modi *et al.* (2000). They reported that when the nisin resistant cells were grown in the presence of nisin, they were more sensitive to heat at 55 °C than the wild type cells. In conclusion, our study showed that emergence of nisin, pediocin 34 and enterocin FH99 resistant mutants of *L. monocytogenes* strains and *Enterococcus* strains should not be a threat to the existing thermal processes.

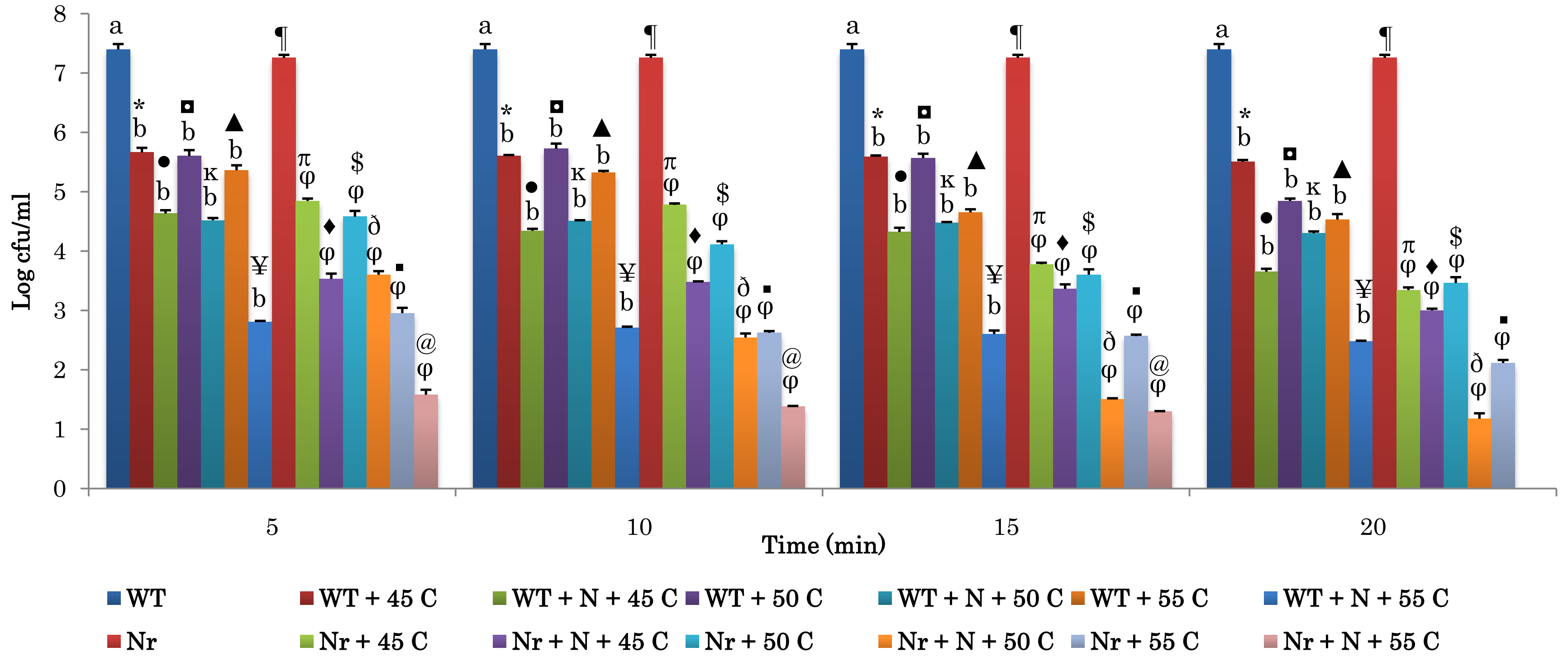


Fig. 4.19. Effect of heat (45, 50 & 55°C) alone and in combination with nisin (N) on wild type *E. faecalis* ATCC 29212 (WT) and its nisin (Nr) resistant variant.

Values are presented as mean ±SEM; n:3. [a,b] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between WT *E. faecalis* (untreated with heat & nisin) and WT treated with heat in the absence and presence of nisin, respectively. [*,•]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between WT treated at 45°C and WT treated with 45°C & nisin. [¤,κ] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between WT treated at 50°C and WT treated with 50°C & nisin. [▲,¥] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between WT treated at 55°C and WT treated with 55°C & nisin, respectively. [¶,φ] Values with different superscripts are differ significantly at the level of P<0.001 at the corresponding period between Nr *E. faecalis* (untreated with heat & nisin) WT treated with heat (45, 50 & 55°C) in the absence and presence of nisin. [π, ♦]Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr treated at 45°C and Nr treated with 45°C & nisin. [$,ð] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr treated at 50°C and Nr treated with 50°C & nisin. [▪,@] Values with different superscripts differ significantly at the level of P<0.001 at the corresponding period between Nr treated at 55°C and Nr treated with 55°C & nisin, respectively

WT: *E. faecalis* untreated by heat and nisin, WT+45 C: *E. faecalis* treated with heat (45 °C), WT+N+45C: *E. faecalis* treated with nisin at 45°C, WT: *E. faecalis* untreated by heat and nisin, WT+50 C: *E. faecalis* treated with heat (50 °C), WT+N+50C: *E. faecalis* treated with nisin at 50°C, WT: *E. faecalis* untreated by heat and nisin, WT+55 C: *E. faecalis* treated with heat (55 °C), WT+N+55C: *E. faecalis* treated with nisin at 55°C, Nr: Nisin resistant *E. faecalis* untreated by heat and nisin, Nr+45 C: Nisin resistant *E. faecalis* treated with heat (45 °C), Nr+ N+45C: Nisin resistant *E. faecalis* treated with nisin at 45°C, Nr+50 C: Nisin resistant *E. faecalis* treated with heat (50 °C), Nr+N+50C: Nisin resistant *E. faecalis* treated with nisin at 50°C, Nr+55 C: Nisin resistant *E. faecalis* treated with heat (55 °C), Nr+N+55C: Nisin resistant *E. faecalis* treated with nisin at 55°C. N: Nisin.

4.10 ASSESSMENT OF CHANGES THAT OCCUR IN BACTERIOCIN RESISTANT STRAINS AT CELLULAR AND MOLECULAR LEVEL:

4.10.1 Bacteriocin Induced Changes in Bacterial Cell Morphology by Electron Microscopy

In the present investigation attempts were made to study ultra structural profiles of bacteriocin sensitive *Listeria monocytogenes* ATCC 53135*, E. faecium* (VRE), *E. faecalis* 29212 and their bacteriocin resistant counterparts. The results of the bacteriocin induced changes in the bacterial cell morphology by electron microscopy are given in Plates 4.6 to 4.8.

The cells of wild type strain were maximally in pairs or short chains. On the other hand nisin, pediocin 34, and enterocin FH99 resistant variants of *Listeria monocytogenes* ATCC 53135 tend to form aggregates (Plate 4.6). Similarly, the cells of wild type *E. faecium* (VRE) were observed to be in short chains. However nisin, pediocin 34, and enterocin FH99 resistant variants of *E. faecium* (VRE) displayed clumping of cells into compact mass (Plate 4.7). Similarly, cells of wild type *E. faecalis* ATCC 29212 were observed to be in short chains. However, nisin resistant variant of this organism displayed clumping of cells into compact mass (Plate 4.8). The formation of bacterial cell aggregates observed in resistant cells may be the prime mechanism of resistance because, overall, a smaller cell surface in aggregated cells is exposed to bacteriocins. Similar results have been reported by Mehla & Sood (2011).

4.10.2 Divalent-Cation Requirement of Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants

A major component of growth media which could be required by the bacteriocin resistant strain to resist bacteriocin are divalent cations. Therefore, the Nisin, Pediocin 34 and Enterocin FH99 sensitivity of the resistant strains of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 in the absence and presence of different divalent cations was assessed.

The effect of $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$ on sensitivity of nisin, pediocin 34 and enterocin FH99 resistant *L. monocytogenes* ATCC 53135 variants to nisin, pediocin 34 and enterocin FH 99 in the presence or absence of EDTA is given in Fig. 4.20. In case of *L. monocytogenes* ATCC 53135, the viability of the nisin resistant cells suspended in MES buffer supplemented with 10mM $MgSO_4$,and treated with 50 IU of Nisin/ml was reduced by 0.50 log cycles only. The effect was confirmed to be due to the divalent cation through an experiment involving EDTA, a chelator of divalent cations. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to a 2.51 log reduction. The inhibition of pediocin 34 and enterocin FH99 resistant variants was also affected by the addition of Mg^{2+} ions. In the presence of 10mM $MgSO_4$ a reduction of 0.35 and 0.08 log cycles log cycles was caused by pediocin 34 and enterocin FH99, but the inclusion of 20 mM EDTA increased the lethality caused by pediocin 34 by 2.52 log cycles and enterocin FH99 by 3.87 log cycles (Fig. 4.20a). In the presence of $MgCl_2$, inclusion of EDTA resulted in the increase of lethality for nisin and pediocin34 resistant variant, caused by nisin and pediocin 34 to about 1.40 and 1.63 log cycles, respectively. However, for eneterocin resistant variant, reduction of about 1.94 log cycles was observed after inclusion of EDTA in the system (Fig. 4.20b).

For nisin and pediocin resistant variant, in the presence of $CaCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 3.17 and 3.50 log cycles, respectively. However, for enterocin resistant variant, reduction of about 2.21 log cycles was observed after inclusion of EDTA in the system (Fig. 4.20c). For nisin and pediocin resistant variant, in the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality by nisin and pediocin 34 to about 3.65 and 3.02 log cycles, respectively. However, for enterocin resistant variant reduction of about 3.19 log cycles was observed after inclusion of EDTA in the experimental system (Fig. 4.20d).

Supplementation of 10 mM $MgSO_4$, $MgCl_2$, $CaCl_2$ or $MnSO_4$ in the medium reduced the lethality caused by Nisin, pediocin 34 and enterocin FH99. In the

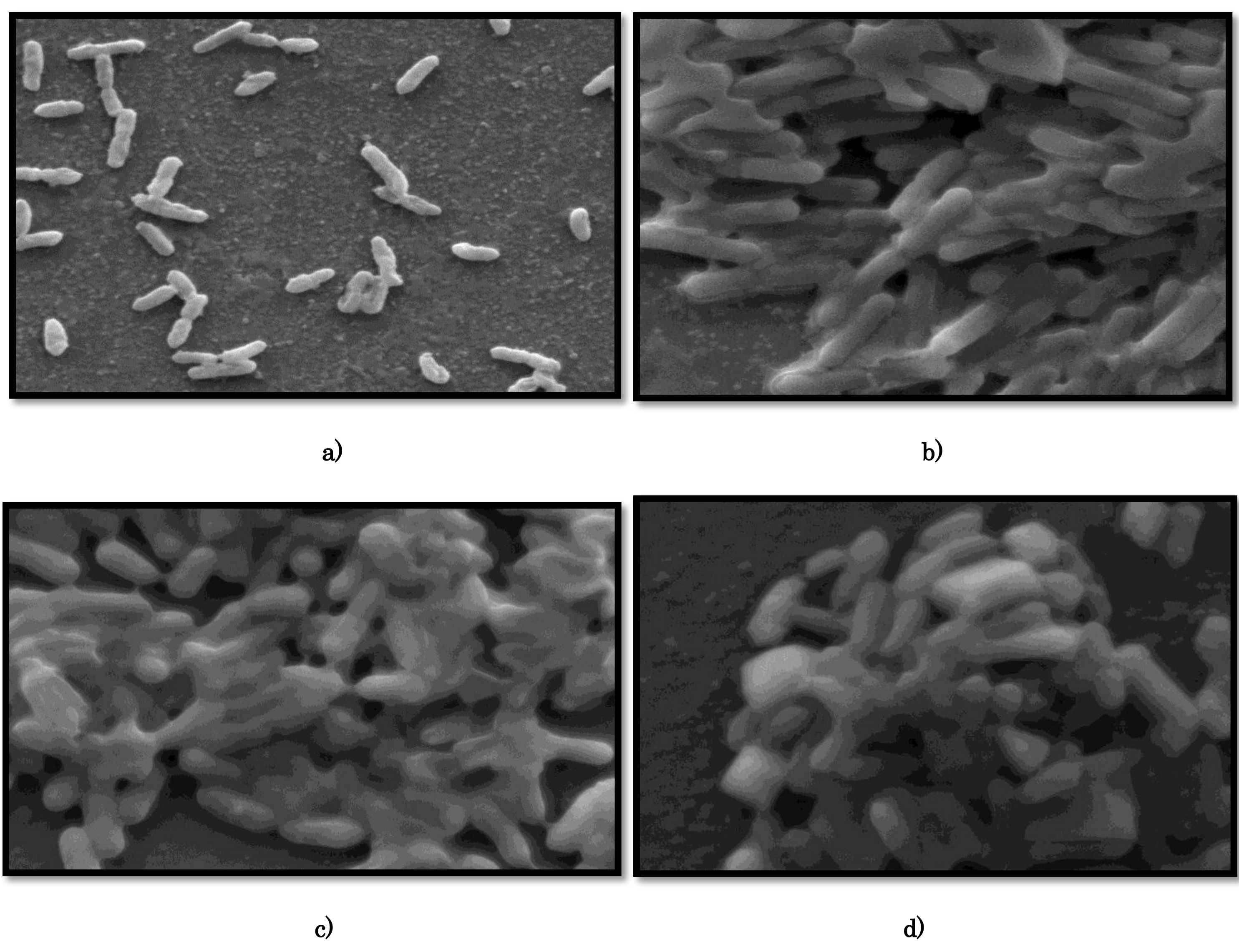


Plate 4.6 Scanning Electron Micrographs of a) Wild Type *L. monocytogenes* ATCC 53135 and its b) Nisin; c) Pediocin 34 and d) Enterocin FH99 resistant variants

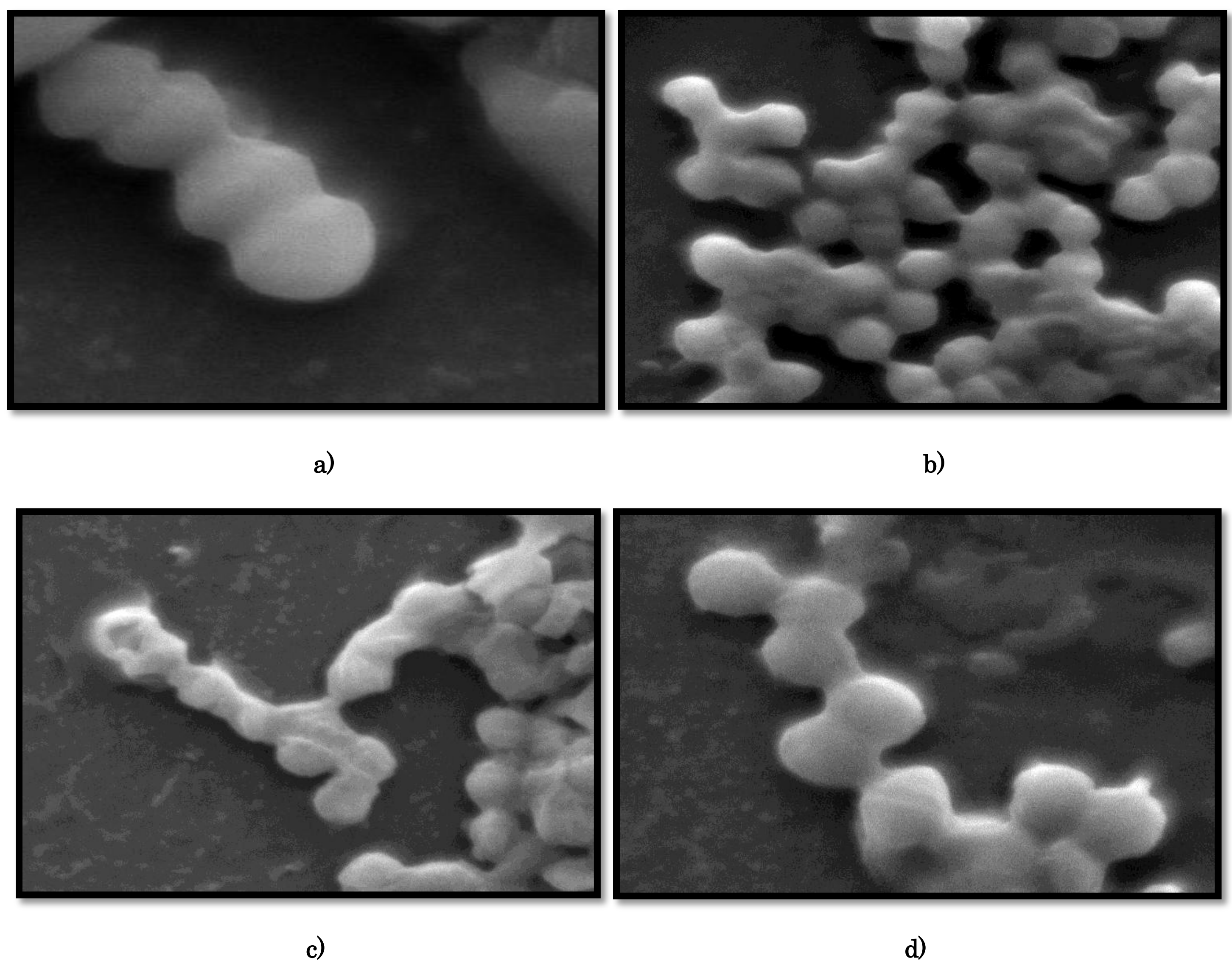


Plate 4.7 Scanning Electron Micrographs of a) Wild Type *E. faecium* VRE and its b) Nisin; c) Pediocin 34 and d) Enterocin FH99 resistant variants

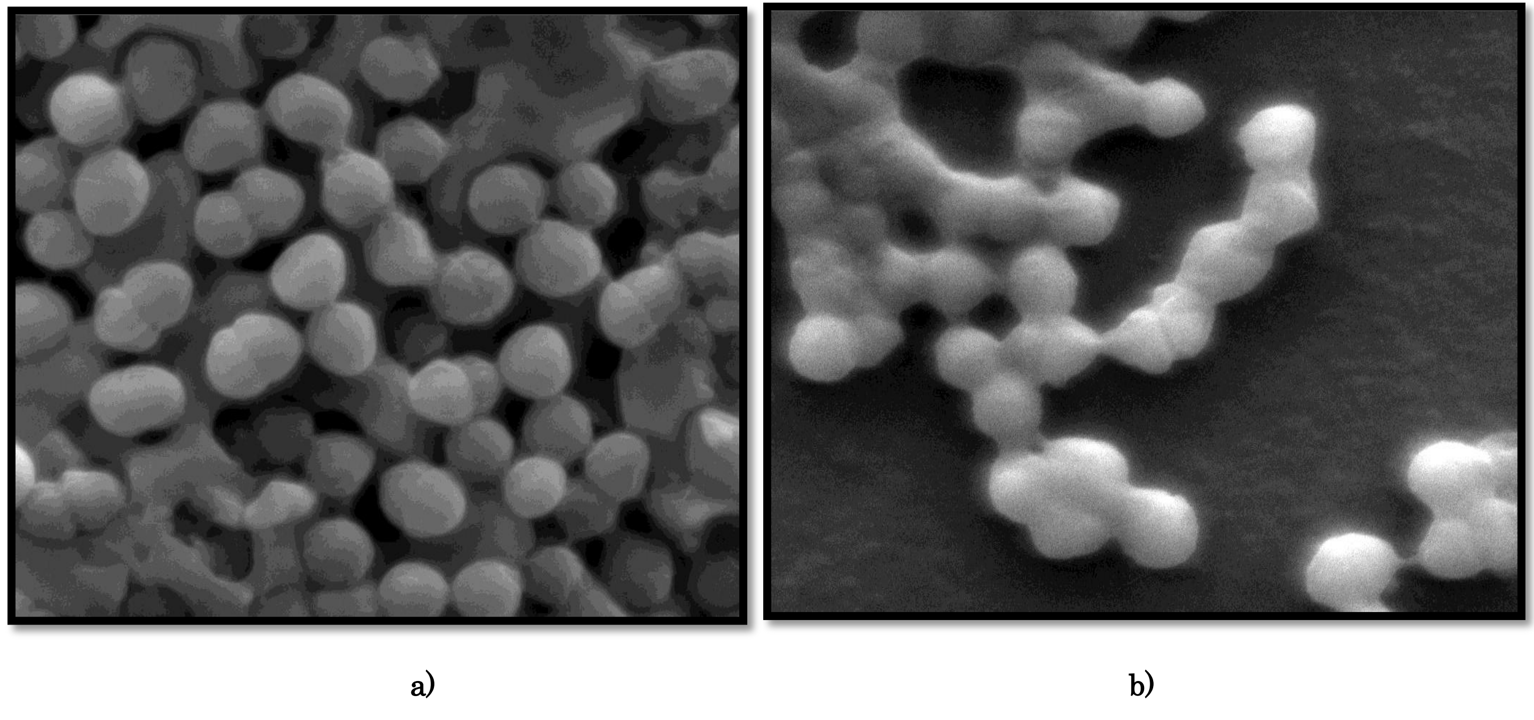
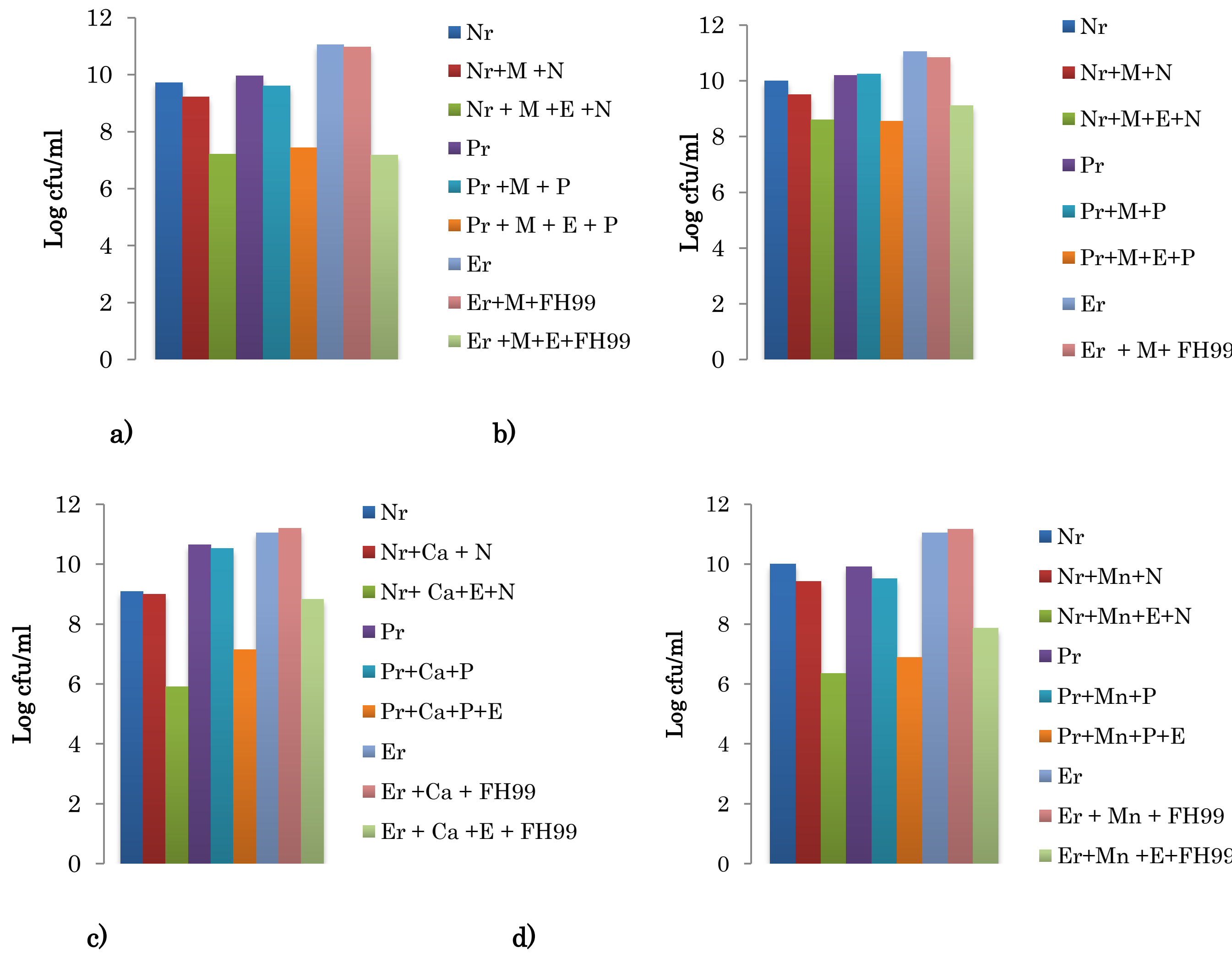


Plate 4.8 Scanning Electron Micrographs of a) Wild Type *E. faecalis* ATCC 29212 and its b) Nisin; c) Pediocin 34 and d) Enterocin FH99 resistant variants

Fig. 4.20 Effect of a) $MgSO_4$ (M) b) $MgCl_2$ (M) c) $CaCl_2$ (Ca) and d) $MnSO_4$ (Mn) on sensitivity of Nisin Resistant (Nr), Pediocin 34 (Pr) resistant and Enterocin FH99 (Er) resistant *L. monocytogenes* ATCC 53135 to Nisin (N), Pediocin 34 (P) and Enterocin FH 99 (FH 99) in presence or absence of EDTA (E)



absence of bacteriocins, the divalent cations had no effect on cell viability. The effect due to divalent cations was further confirmed by experiments involving EDTA, a chelator of divalent cations. Inclusion of 20 mM EDTA in any of the systems containing divalent cations increased the lethality caused by nisin, pediocin 34 and enterocin FH99.

Effect of $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$ on sensitivity of nisin resistant, pediocin 34 resistant and enterocin FH99 resistant *L. monocytogenes* MTCC 657 to nisin, pediocin 34 and enterocin FH 99 in presence or absence of EDTA is shown in Fig. 4.21. In case of *L. monocytogenes* MTCC 657, of nisin resistant variants were able to resist the inhibitory effect of nisin in presence of 10mM $MgSO_4$. The effect was confirmed to be due to the divalent cations by experiment involving EDTA. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to a 1.93 log cycles. Pediocin 34 and enterocin resistant variants of *L. monocytogenes* MTCC 657 were also able to resist the inhibitory activities of the pediocin 34 and enterocin FH99. It was, however, confirmed when the inclusion of 20 mM EDTA increased the lethality caused by pediocin 34 by 1.62 log cycles and enterocin FH99 by 1.63 log cycles (Fig. 4.21a). For nisin and pediocin resistant variant, in the presence of $MgCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 3.60 and 1.37 log cycles respectively. However for enterocin resistant variant reduction of about 2.00 log cycles was observed after inclusion of EDTA in the system (Fig. 4.21b).

Inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 1.75 and 1.80 log cycles, respectively, for nisin and pediocin 34 resistant variant, in the presence of $CaCl_2$. However, for enterocin resistant variant reduction of about 2.14 log cycles was observed after inclusion of EDTA in the system (Fig. 4.21c). For nisin and pediocin resistant variant, in the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 2.72 and 1.37 log cycles,

respectively. However, for enterocin resistant variant reduction of about 2.77 log cycles was observed after inclusion of EDTA in the experimental system (Fig. 4.21d).

The effect of $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$ on sensitivity of nisin resistant, pediocin 34 resistant and enterocin FH99 resistant *E. faecium* DSMZ 20477 to nisin, pediocin 34 and enterocin FH99 in presence or absence of EDTA is presented in Fig. 4.22. In case of *E. faecium* DSMZ 20477, the nisin resistant variant was able to resist the inhibitory effect of nisin in the presence of 10mM $MgSO_4$. The effect was confirmed to be due to the divalent cations by experiment involving EDTA. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to a 2.31 log cycles. Pediocin 34 and enterocin resistant variants of *E. faecium* VRE were also able to resist the inhibitory activities of the Pediocin 34 and Enterocin FH99. This was confirmed when the inclusion of 20 mM EDTA increased the lethality caused by pediocin 34 by 2.86 log cycles and enterocin FH99 by 2.17 log cycles (Fig. 4.22a). Inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 1.63 and 3.74 log cycles, respectively, for nisin and pediocin 34 resistant variant, in the presence of $MgCl_2$. However, for enterocin resistant variant reduction of about 2.69 log cycles was observed after inclusion of EDTA in the system (Fig. 4.22b).

For nisin and pediocin 34 resistant variant, in the presence of $CaCl_2$, inclusion of EDTA resulted in the increase of lethality by nisin and pediocin 34 to about 1.76 and 3.39 log cycles, respectively. However, for enterocin resistant variant reduction of about 2.93 log cycles was observed after inclusion of EDTA in the system (Fig. 4.22c). For nisin and pediocin resistant variant, in the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 2.76 and 3.36 log cycles, respectively. However, for enterocin resistant variant reduction of about 1.63

Fig. 4.21 Effect of a) $MgSO_4$ (M) b) $MgCl_2$ (M) c) $CaCl_2$ (Ca) and d) $MnSO_4$ (Mn) on sensitivity of Nisin Resistant (Nr), Pediocin 34 (Pr) resistant and Enterocin FH99 (Er) resistant *L. monocytogenes* MTCC 657 to Nisin (N), Pediocin 34(P) and Enterocin FH 99 (FH 99) in presence or absence of EDTA (E)

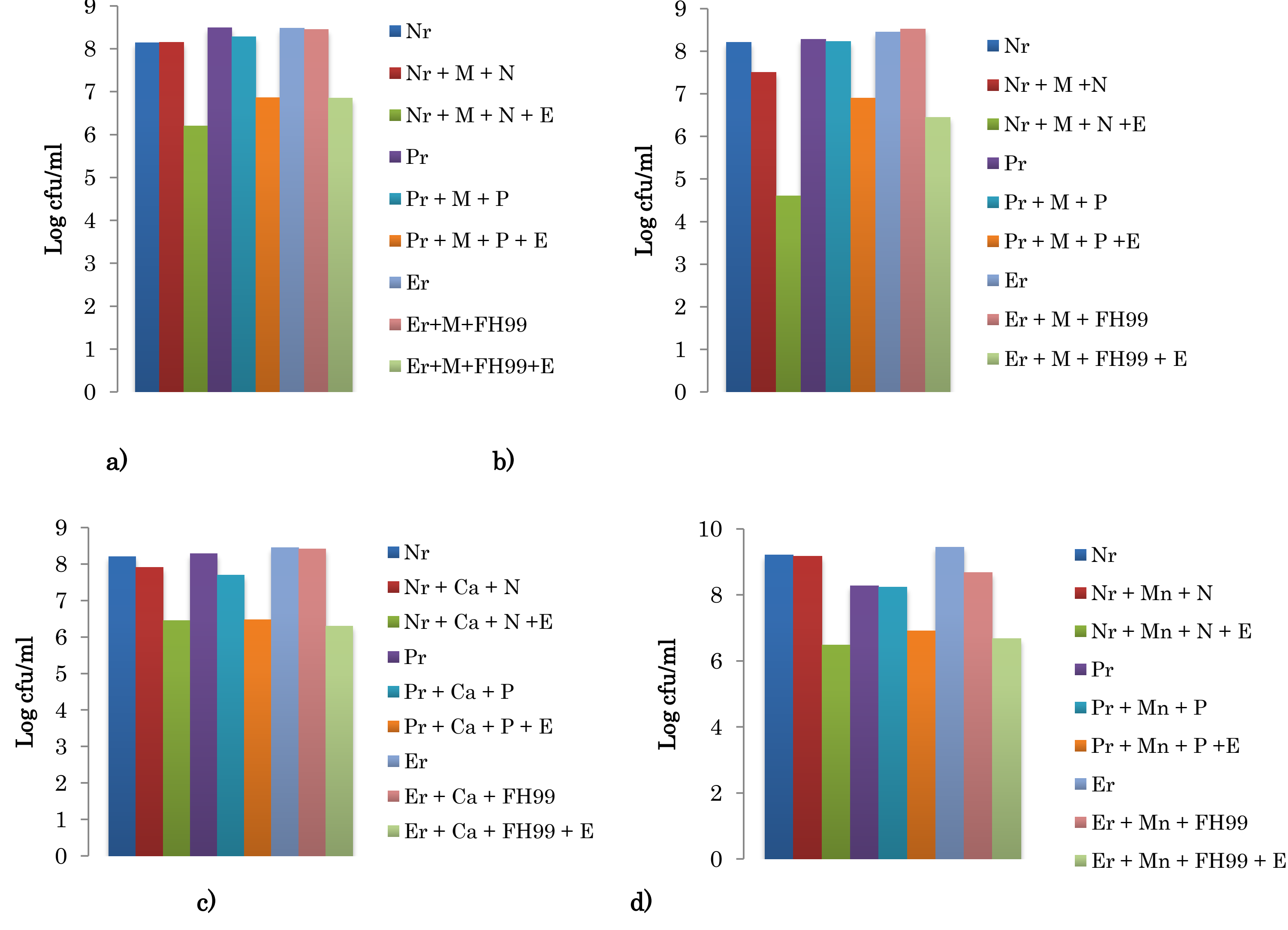
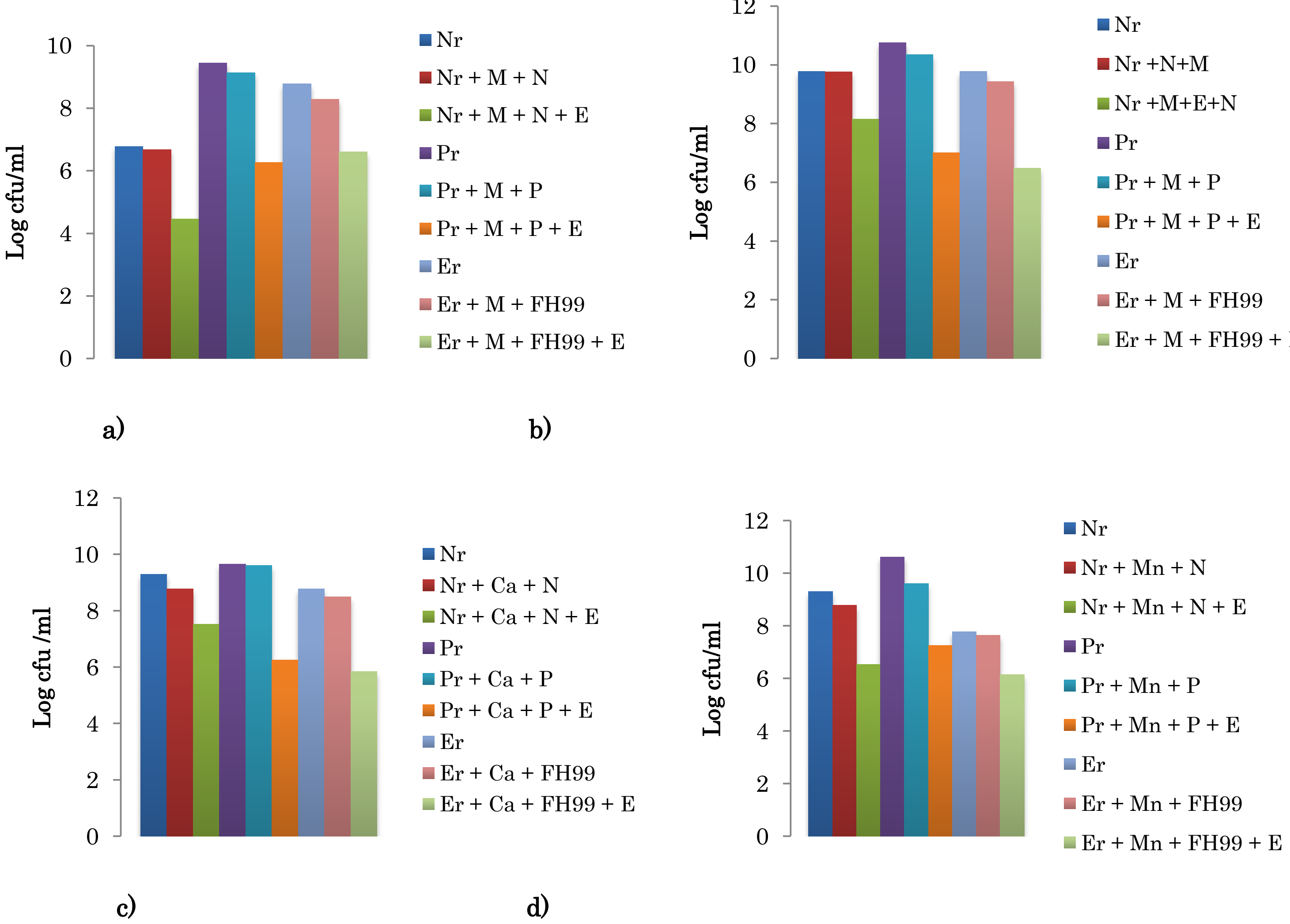


Fig. 4.22 Effect of a) $MgSO_4$ (M) b) $MgCl_2$ (M) c) $CaCl_2$ (Ca) and d) $MnSO_4$ (Mn) on sensitivity of Nisin Resistant (Nr), Pediocin 34 (Pr) resistant and Enterocin FH99 (Er) resistant *E. faecium* DSMZ 20477 to Nisin (N), Pediocin 34(P) and Enterocin FH 99 (FH 99) in presence or absence of EDTA (E)



log cycles was observed after inclusion of EDTA in the experimental system (Fig. 4.22d).

The effect of $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$ on sensitivity of nisin resistant, pediocin 34 resistant and enterocin FH99 resistant *E. faecium* VRE to Nisin, Pediocin 34 and Enterocin FH99 in the presence or absence of EDTA is shown in Fig. 4.23. In case of *E. faecium* VRE, the nisin resistant variants were able to resist the inhibitory effect of nisin in presence of 10mM $MgSO_4$. The effect was confirmed to be due to the divalent cation by experiment involving EDTA. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to a 1.63 log cycles. Pediocin 34 and enterocin resistant variants of *E. faecium* VRE were also able to resist the inhibitory activities of the pediocin 34 and enterocin FH99. This was confirmed when the inclusion of 20 mM EDTA increased the lethality caused by pediocin 34 by 1.39 log cycles and enterocin FH99 by 2.39 log cycles (Fig. 4.23a). For nisin and pediocin resistant variant (in the presence of $MgCl_2$), inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 2.17 and 2.22 log cycles respectively. However, for enterocin resistant variant reduction of about 2.69 log cycles was observed after inclusion of EDTA in the system (Fig. 4.23b).

For nisin and pediocin resistant variant, in the presence of $CaCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 1.87 and 2.78 log cycles, respectively. However, for enterocin resistant variant, reduction of about 2.54 log cycles was observed after inclusion of EDTA in the system (Fig. 4.23c). For nisin and pediocin resistant variant, in the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 2.87 and 1.63 log cycles, respectively. However, for enterocin resistant variant, reduction of about 3.30 log cycles was observed after inclusion of EDTA in the experimental system (Fig. 4.23d).

The effect of divalent cations $MgSO_4$, $MgCl_2$, $CaCl_2$ and $MnSO_4$ on sensitivity of nisin resistant *E. faecalis* ATCC 29212 to nisin in the presence or absence of EDTA is shown in Fig. 4.24. In case of *E. faecalis* ATCC 29212, supplementation of 10 mM $MgSO_4$, $MgCl_2$, $CaCl_2$, and MnSO4 reduced the lethality caused by nisin. The nisin resistant variant was able to resist the inhibitory effect of nisin in presence of 10mM $MgSO_4$. The effect was confirmed to be due to the divalent cation by experiment involving EDTA. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to 2.83 log cycles. In the presence of $MgCl_2$ inclusion of EDTA resulted in the increase of lethality caused by nisin to about 2.08 log cycles. In the presence of $CaCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin to about 2.47 log cycles. In the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality caused by nisin to about 4.29 log cycles.

The addition of divalent cations significantly reduced the inhibitory activity of nisin, pediocin 34 and enterocin FH99 against cells of resistant variants of test culture *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212. The addition of EDTA, however, restored this activity. Similar results have earlier been reported by Abee *et al.* (1994) who found that di- and trivalent cations (Mg^{2+}, Ca^{2+}, and Gd^{3+}) decreased the nisin Z-induced rate of K1 efflux from whole cells of *L. monocytogenes* Scott A. They suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipids head groups. Alternatively or additionally, the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane (Abee *et al.,* 1994). In conclusion the impact of divalent cations on bactericidal activity of nisin, pediocin 34 and enterocin FH99 revealed that Mg^{2+} Mn^{2+} and Ca^{3+} cations were able to reduce the binding of antimicrobial peptide to the cell membrane. Divalent cations seem to affect the initial

Fig. 4.23 Effect of a) $MgSO_4$ (M) b) $MgCl_2$ (M) c) $CaCl_2$ (Ca) and d) $MnSO_4$ (Mn) on sensitivity of Nisin Resistant (Nr), Pediocin 34 (Pr) resistant and Enterocin FH99 (Er) resistant *E. faecium* VRE to Nisin (N), Pediocin 34(P) and Enterocin FH 99 (FH 99) in presence or absence of EDTA (E)

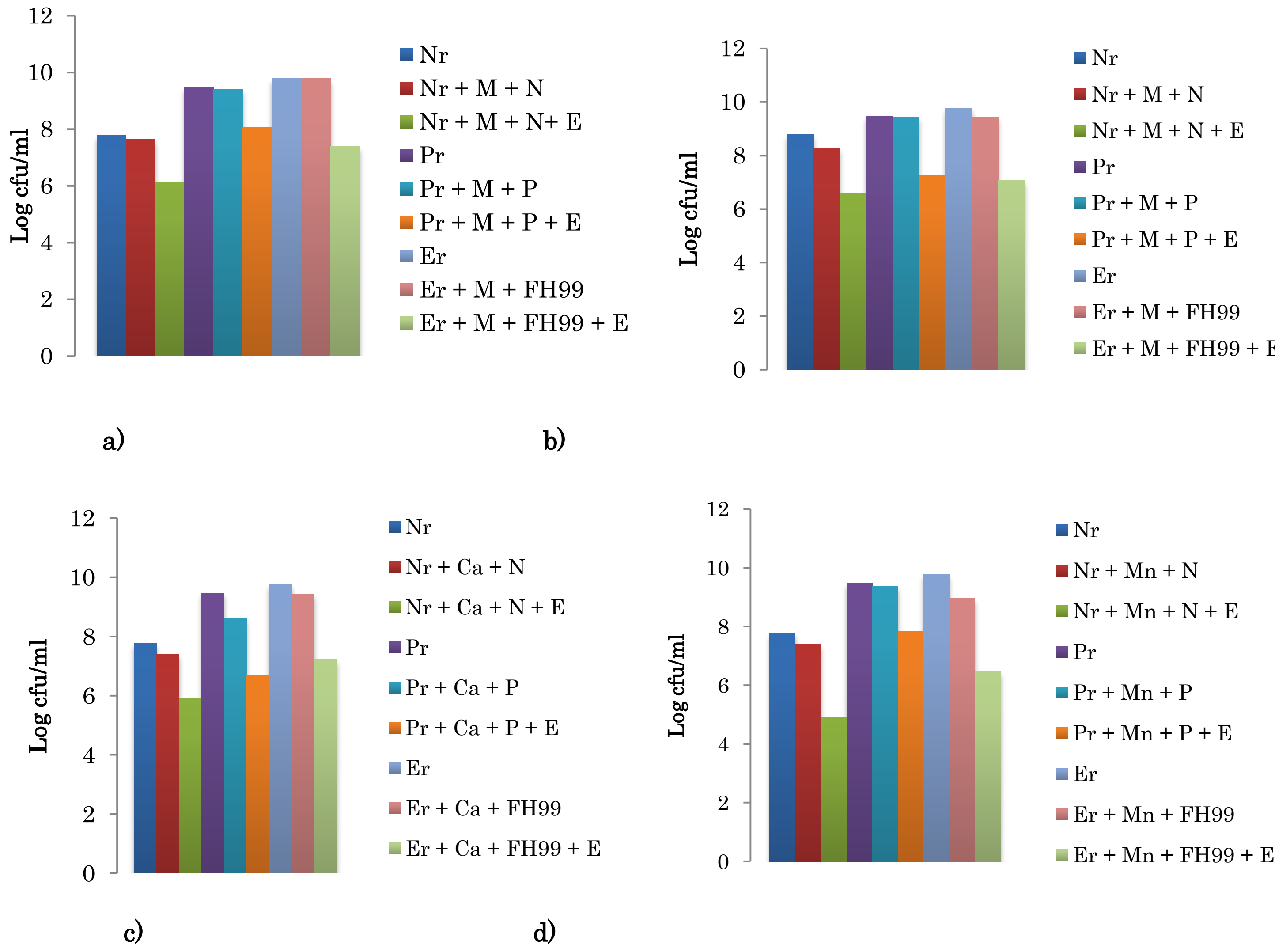
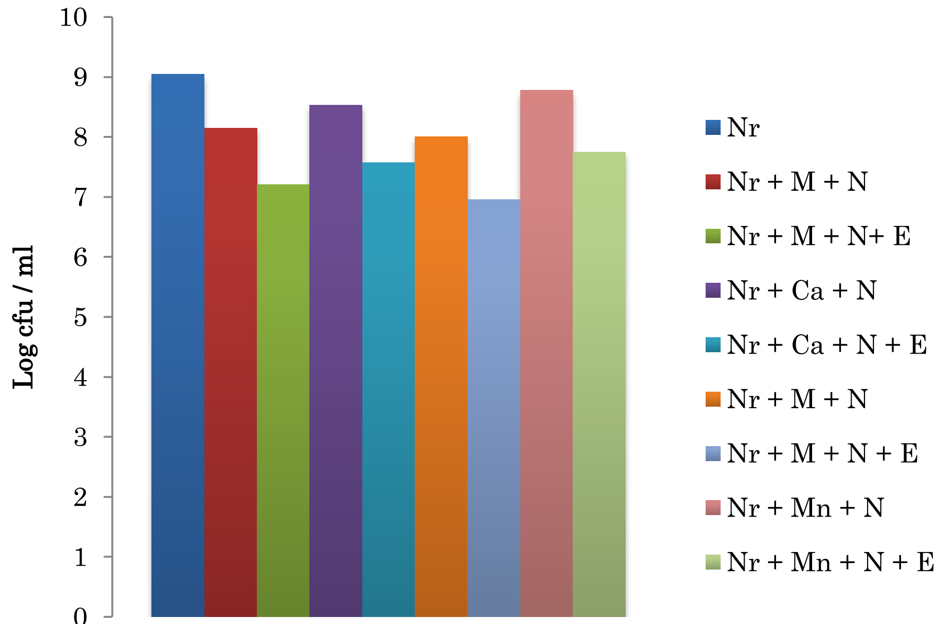


Fig. 4.24 Effect of a) $MgSO_4$ (M) b) $MgCl_2$ (M) c) $CaCl_2$ (Ca) and d) $MnSO_4$ (Mn) on sensitivity of Nisin Resistant (Nr), Pediocin 34 (Pr) resistant and Enterocin FH99 (Er) resistant *E. faecalis* ATCC 29212 to Nisin (N) in presence or absence of EDTA (E)



electrostatic interaction between the positively charged bacteriocin and the negatively charged phospholipids of the membrane.

4.10.3 Lysozyme Sensitivity of Nisin, Pediocin 34 and Enterocin Fh99 Resistant Variants

In the presence of lysozyme the nisin resistant variant showed an increase of 0.62 log cycles after 4h, whereas, the wild type strain showed a decrease of about 2.5, 2.9 and 2.08 log cycles after 4h, 6h and 8h, respectively. Pediocin 34 resistant variant of *L. monocytogenes* ATCC 53135 depicted a log reduction of 0.58, 2.88 and 2.04 log cycles after 4, 6 and 8h, respectively. Enterocin FH99 resistant variant showed an increase of 0.44 log cycles after 4h. However, after 6h and 8h, a decrease of approximately 0.57 and 0.82 log cycles, respectively, was observed (Fig. 4.25a).

The effect of lysozyme on both the wild type and nisin resistant, pediocin 34 resistant and enterocin FH99 resistant strain of *L. monocytogenes* ATCC 53135 variant in the presence as well as in the absence of nisin, pediocin 34 resistant and enterocin FH99 was also determined. Wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.07 and 3.86 log cycles after 1h and 2h , whereas no growth was observed after 2h incubation with lysozyme and nisin whereas, nisin resistant variant showed a log reduction of about 0.57, 1.09 and 1.64 log cycles after 2h, 6h and 24h, respectively (Fig. 4.25b). In the presence of pediocin 34 and lysozyme, wild type *L. monocytogenes* ATCC 53135 showed a log reduction of 1.9 and 3.6 log cycles after 4h and 8h, respectively, and no growth was observed after 24h, whereas, pediocin 34 resistant variant showed a log reduction of 3.11 after 6h and no growth after 8h and 24h (Fig. 4.25c). Wild type *L. mononocytogenes* ATCC 53135, in the presence of both lysozyme and enterocin FH99, showed a log reduction of 0.35 and 4.0 log cycles after 4 and 8h, respectively and no growth after 24h incubation. Enterocin resistant variant showed an increase of approximately 0.89 log cycles after 6h whereas a log reduction of 1.27, 1.23 and 0.09 log cycles was observed after 6h, 8h and 24h incubation (Fig. 4.25d). In conclusion, it was observed that *L. monocytogenes*

ATCC 53315 nisin, pediocin 34 and enterocin resistant variants were more resistant to lysozyme when compared with the wild type strain both in the presence as well as absence of either nisin, pediocin 34 and enterocin FH99.

The effect of lysozyme on both the wild type and nisin resistant, pediocin 34 resistant and enterocin FH99 resistant variants of *L. monocytogenes* MTCC 657 in the presence as well as in the absence of nisin, pediocin 34 resistant and enterocin FH99 was also determined (Fig. 4.26). Wild type *L. monocytogenes* MTCC 657 showed a log reduction of 1.45 and 2.07 log cycles after 1 and 24h incubation with lysozyme. In the presence of lysozyme, the nisin resistant variant of *L. monocytogenes* MTCC 657 showed an increase of 0.30 0.044, 0.27, 0.076 and 0.430 log cycles after 1, 2, 4, 6 and 8h, respectively; whereas the wild type strain showed a decrease of about 1.4, 1.6, 1.3, 1.49, 2.4 and 2.07 log cycles after 1, 2, 4, 6, 8 and 24h, respectively. Pediocin 34 resistant variant of *L. monocytogenes* MTCC 657 depicted a log reduction of 0.19, 0.35 and 1.01, 0.43 and 1.27 log cycles after 1, 2, 4 , 6, 8 and 24h, respectively. Enterocin FH99 resistant variant showed a decrease of 0.57, 0.84, 0.522, 0.43 and 1.22 log cycles after 1, 4, 6, 8 and 24h, respectively (Fig. 4.26a).

In the presence of both nisin and lysozyme no growth was observed after 2h for wild type *L .monocytogenes* MTCC 657. Nisin resistant variant showed a log reduction of about 0.62, 0.87 and 0.98 log cycles after 6, 8 and 24h, respectively, in the presence of both lysozyme and nisin (Fig. 4.26b). On incubation with both lysozyme and pediocin 34, pediocin resistant variant showed a reduction of about 2.75 and 2.07 after 6 and 24h, respectively; whereas, wild type strain showed a reduction of 2.79 and 3.84 log cycles after 6h and 8h and no growth was observed after 24h (Fig. 4.26c). In the presence of both lysozyme and enterocin FH99, wild type *L. monocytogenes* MTCC 657 showed a reduction of 2.92 and 4.10 log cycles after 6 and 8h and no growth after 24h, whereas, enterocin resistant variant showed a reduction of approximately 2.11, 1.13 and 2.95 log cycles after 6, 8 and 24h incubation with lysozyme and enterocin FH99 (Fig. 4.26d). It was, thus observed that nisin, pediocin 34 and enterocin

Fig. 4.25. a) Growth of wild-type (WT), Nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant(Er) variants of *L. monocytogenes* ATCC 53135 in presence of lysozyme (Lyso), b) Growth of WT and Nr variant in presence of both lysozyme and nisin, c) Growth of WT and Pr variant in presence of both lysozyme and pediocin 34 and d) Growth of WT and Er variant in presence of both lysozyme and enterocin FH99

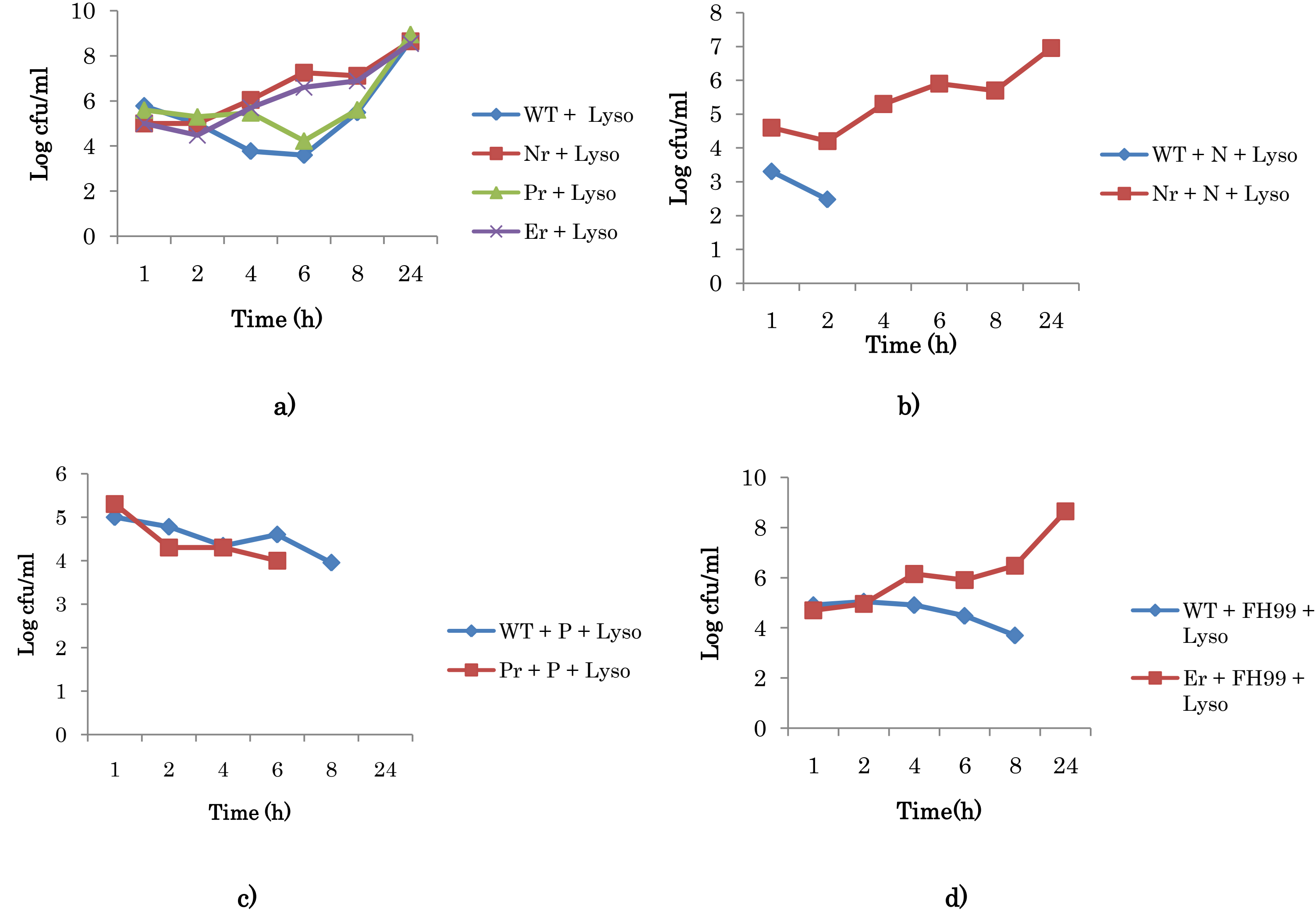
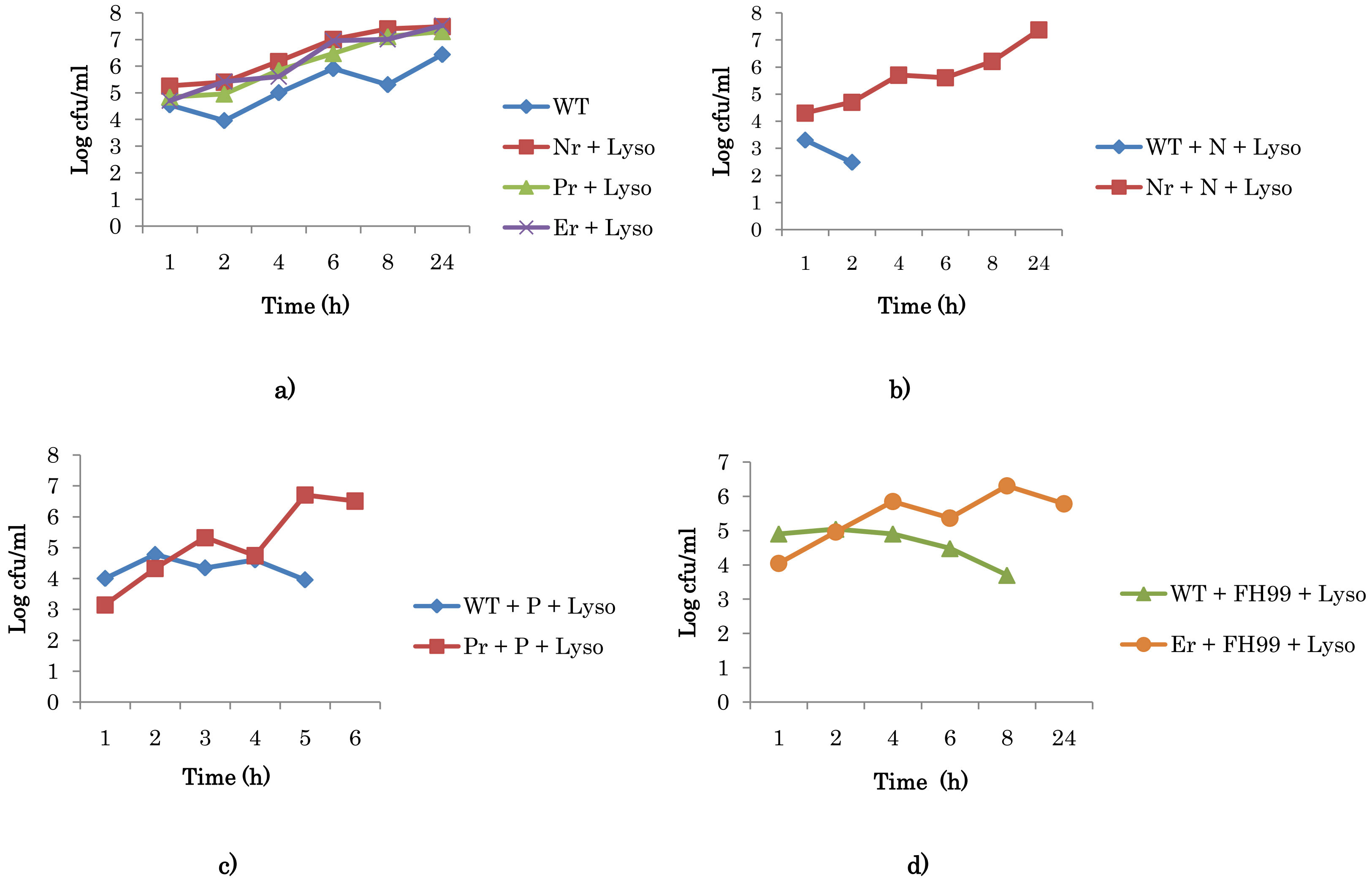


Fig. 4.26. a) Growth of wild-type (WT), Nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant(Er) variants of *L. monocytogenes* MTCC 657 of lysozyme (Lyso), b) Growth of WT and Nr variant in presence of both lysozyme and nisin, c) Growth of WT and Pr variant in presence of both lysozyme and pediocin 34 and d) Growth of WT and Er variant in presence of both lysozyme and enterocin FH99



resistant variants of *L. monocytogenes* MTCC 657 were more resistant to lysozyme when compared with the wild type strain both in the presence as well as absence of either nisin, pediocin 34 or enterocin FH99, as shown in the Fig. 4.26d.

Wild type *E. faecium* DSMZ 20477 showed a decrease of about 1.04, 0.15, 0.60, 1.25, 0.51 log cycles after 1h, 2h, 4h, 6h, and 8h, respectively and increase of about 0.02 log cycles after 24h in the presence of lysozyme. In the presence of lysozyme, the nisin resistant variant of *E. faecium* DSMZ 20477 showed a decrease of 1.13, 0.81, 3.41 and 2.71 log cycles after 1h, 2h, 4h, and 6h, respectively, whereas increase of 0.17 log cycles was observed after 24h. Pediocin 34 resistant variant of *E. faecium* DSMZ 20477 showed a log reduction of 0.41 and 1.00 log cycles after 1 and 24h, respectively. Enterocin FH99 resistant variant showed a decrease of 0.05, 0.30 and 0.06 log cycles after 2, 4 and 24h incubation with lysozyme in the absence of enterocin FH99 (Fig. 4.27a). The nisin resistant variant was able to resist the inhibitory effect of lysozyme in the presence of nisin. It showed an increase of about 0.35, 0.12, 1.05, 1.11 and 0.25 log cycles after incubation period of 1, 2, 4, 6, 8 and 24h, respectively, (Fig. 4.27b). Pediocin 34 resistant variant of *E. faecium* DSMZ 20477 in the presence of both pediocin and lysozyme the pediocin resistant variant was able to resist the activity of lysozyme showing an increase of about 0.28, 0.43 and 1 log cycle after 1h, 6h and 8h, respectively(Fig. 4.27c). In the presence of both lysozyme and enterocin FH99, enterocin resistant variant showed a decrease of about 1.17 1.07 and 1.22 log cycles after 1, 6 and 24h, respectively, (Fig. 4.27d). Thus, it was observed that nisin and enterocin resistant variants were sensitive to lysozyme both in presence as well as absence of bacteriocins as compared to the wild type strains; whereas, pediocin 34 resistant variant resisted the effect of lysozyme in the presence of pediocin 34. However, in the absence of pediocin 34, pediocin resistant variant of *E. faecium* DSMZ 20477 was sensitive to the action of lysozyme.

The effect of lysozyme on both the wild type and nisin resistant, pediocin 34 resistant and enterocin FH99 resistant strain of *E. faecium* VRE in the presence as well as in the absence of nisin, pediocin 34 resistant and enterocin FH99 was also determined (Fig. 4.28). In the presence of lysozyme the nisin resistant variant showed a decrease of 0.54, 1.59, 0.51, 2.27, 0.41 and 0.02 log cycles after 1, 2, 4, 6, 8 and 24h, respectively; whereas, the wild type strain showed a decrease of about 1.5, 2.28, 0.73, 2.35, 2.23 and 1.30 log cycles after 1, 2, 4, 6, 8h and 24h, respectively. Pediocin 34 resistant variant of *E. faecium* VRE depicted a log reduction of 0.64, 1.02, 0.43, 1.30, 0.89 and 0.07 log cycles after 1, 2, 4, 6, 8 and 24h, respectively, in the presence of lysozyme. Enterocin FH99 resistant variant showed a decrease of 0.89, 1.74, 0.13, 1.27, 0.44 and 0.32 log cycles after 1, 2, 4, 6, 8 and 24h, incubation with lysozyme (Fig. 4.28a). It was observed that the nisin, pediocin 34 and enterocin resistant variants of *E. faecium* VRE were resistant to lysozyme in the absence of bacteriocins as compared to their wild type counterpart. Also the effect of lysozyme on both the wild type and Nisin, Pediocin 34 and enterocin FH99 resistant strain of *E. faecium* VRE variant in the presence of nisin, pediocin 34 resistant and enterocin FH99 was determined. Wild type *E. faecium* VRE showed a log reduction of 2.17, 1.93, 1.91, 2.90, 2.99 and 1.97 log cycles after 1h, 2h, 4h, 6h, 8h and 24h, respectively on incubation with both lysozyme and nisin. In the presence of nisin, nisin resistant variant showed a log reduction of about 2.54, 2.63, 2.91, 4.27, 4.19 and 2.3 log cycles after 1, 2, 4, 6, 8 and 24h, respectively (Fig. 4.28b). In the presence of pediocin 34 and lysozyme, wild type *E. faecium* VRE showed a log reduction of 2.17, 1.93, 1.91, 2.90, 2.99 and 1.97 log cycles after 1, 2, 4, 6, 8 and 24h. Pediocin 34 resistant variant showed a log reduction of 1.87, 2.58, 2.91 and 5.20 after 1, 2, 4 and 6h and no growth after 8 and 24h in the presence of pediocin 34 (Fig. 4.28c). With Enterocin FH99, a log reduction of 3.17, 1.98, 2.21, 2.90, 2.77 and 2.04 log cycles after 1, 2, 4, 6, 8 and 24h, respectively for wild type *E. faecium* VRE. Enterocin resistant variant showed an reduction of 2.84, 2.85, 3.31, 4.69 and 5.19 log cycles after 1, 2, 4, 6 and 8h; whereas, no viable growth was observed after 24h incubation with both lysozyme and enterocin FH99 (Fig. 4.28d). However, nisin, pediocin 34 and enterocin resistant

Fig. 4.27. a) Growth of wild-type (WT), Nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant(Er) variants of *E. faecium* DSMZ 20477 of lysozyme (Lyso), b) Growth of WT and Nr variant in presence of both lysozyme and nisin, c) Growth of WT and Pr variant in presence of both lysozyme and pediocin 34 and d) Growth of WT and Er variant in presence of both lysozyme and enterocin FH99

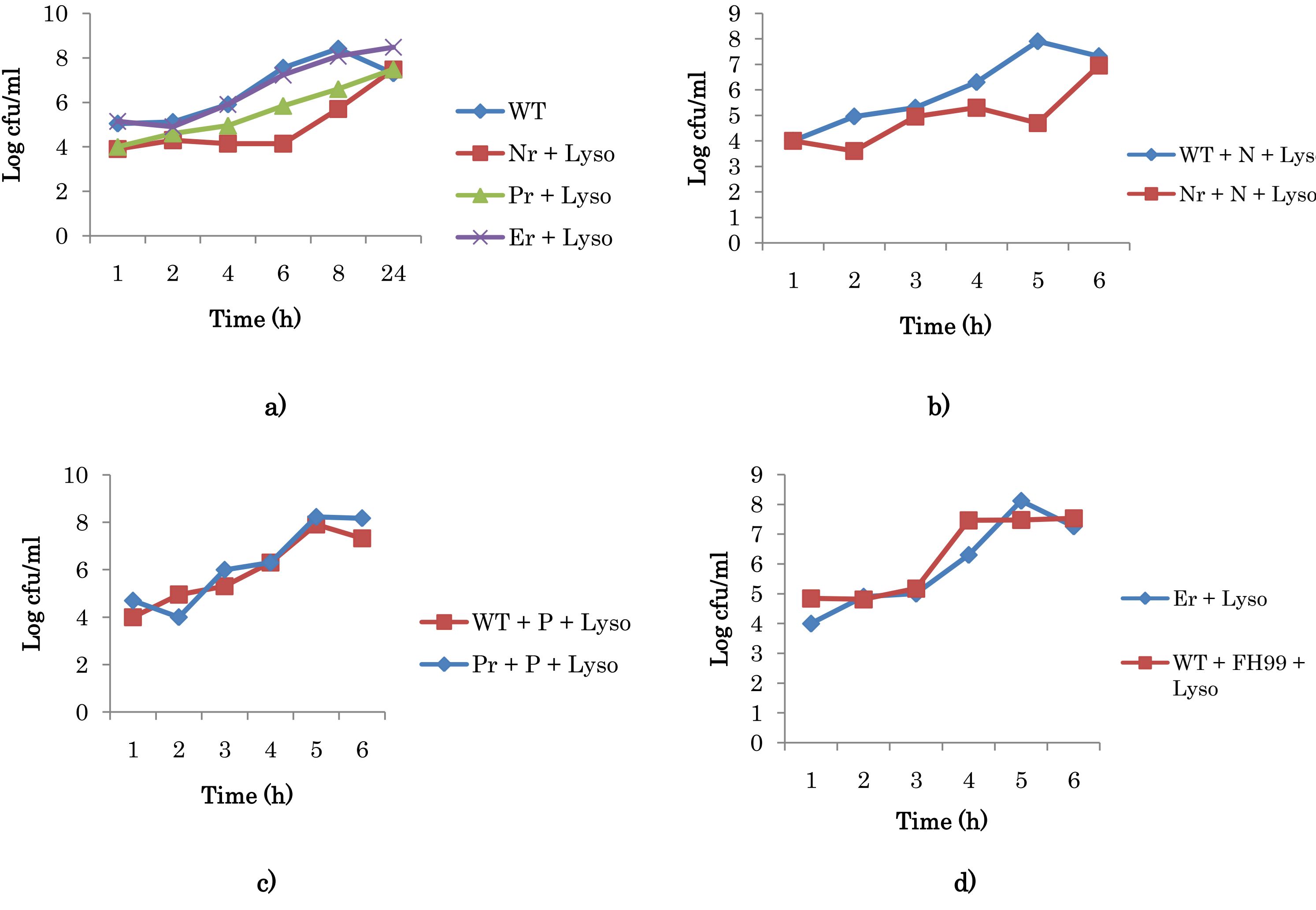
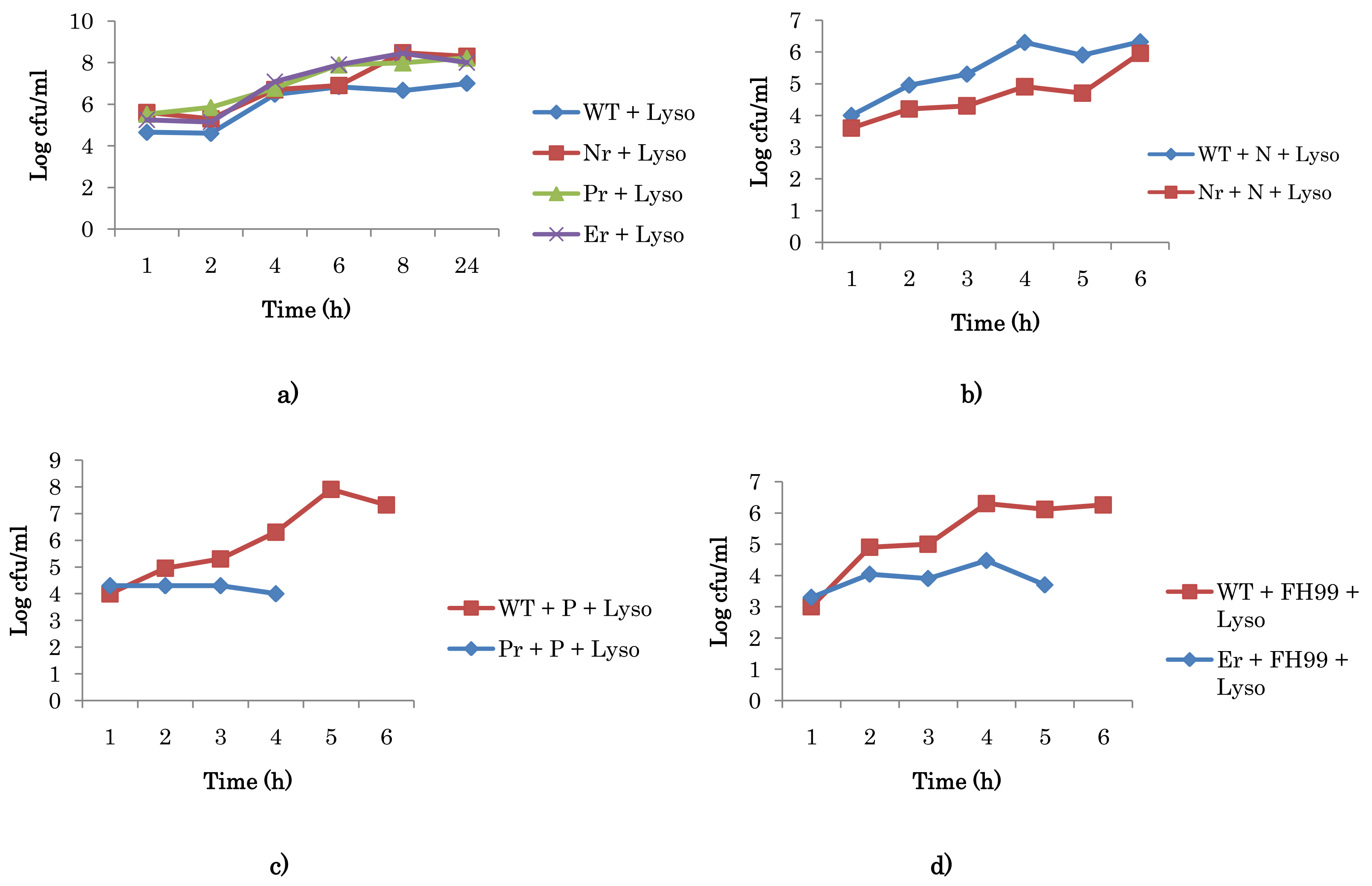


Fig. 4.28. a) Growth of wild-type (WT), Nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant(Er) variants of *E. faecium* VRE of lysozyme (Lyso), b) Growth of WT and Nr variant in presence of both lysozyme and nisin, c) Growth of WT and Pr variant in presence of both lysozyme and pediocin 34 and d) Growth of WT and Er variant in presence of both lysozyme and enterocin FH99



variants of *E. faecium* VRE were more resistant to lysozyme when compared with the wild type strain both in the absence of either nisin, pediocin 34 and enterocin FH99 as shown in the Fig. 33a. In the presence of bacteriocins the resistant variants were more sensitive to the effect of lysozyme as compared to their wild type counterparts.

The effect of lysozyme on both the wild type and nisin resistant strain of *E. faecalis* ATCC 29212 variant in the presence as well as in the absence of Nisin was determined (Fig. 4.29). There was a reduction of approximately 0.80 log cycles and 0.16 log cycles after 1 and 24 h, respectively in wild type *E. faecalis* ATCC 29212 when grown with lysozyme in the absence of nisin as compared to 0.98 and 1.10 log cycle reduction after 6 and 24h, respectively of Nisin resistant variant of *E. faecalis* ATCC 29212 (Fig. 4.29a). In the presence of both lysozyme and nisin, the wild *E. faecalis* ATCC 29212 showed a reduction of about 1.07 and 1.04 log cycles after 1 and 24h, respectively. However, in the presence of both lysozyme and nisin, the nisin resistant variant showed a decrease of 0.79 and 0.21 log cycles after 6 and 24h, respectively (Fig. 4.29b). It was observed that the nisin resistant variant was able to resist the action of lysozyme both in the presence as well as absence of nisin as compared to its wild type counterpart.

Lysozyme has two modes of action: (1) enzymatic lysis of bacterial cell wall, (2) membrane perturbation inducing cell death (Ibrahim *et al.,* 2002) The efficacy of lytic activity of lysozyme is preferentially directed to Gram-positive bacteria because of the membrane composition (peptidoglycan, the target of lysozyme). Nisin, pediocin 34 and enterocin resistant variants of *L. monocytogenes* ATCC 53315 were more resistant to lysozyme as compared to the wild type strain both in the presence as well as absence of nisin, pediocin 34 and enterocin FH99. Similarly, nisin, pediocin 34 and enterocin resistant variants of *L. monocytogenes* MTCC 657 were more resistant to lysozyme as compared to the wild type strain both in the presence and absence of either nisin, pediocin 34 or enterocin FH99. In case of *E. faecium* DSMZ 20477, it was observed that

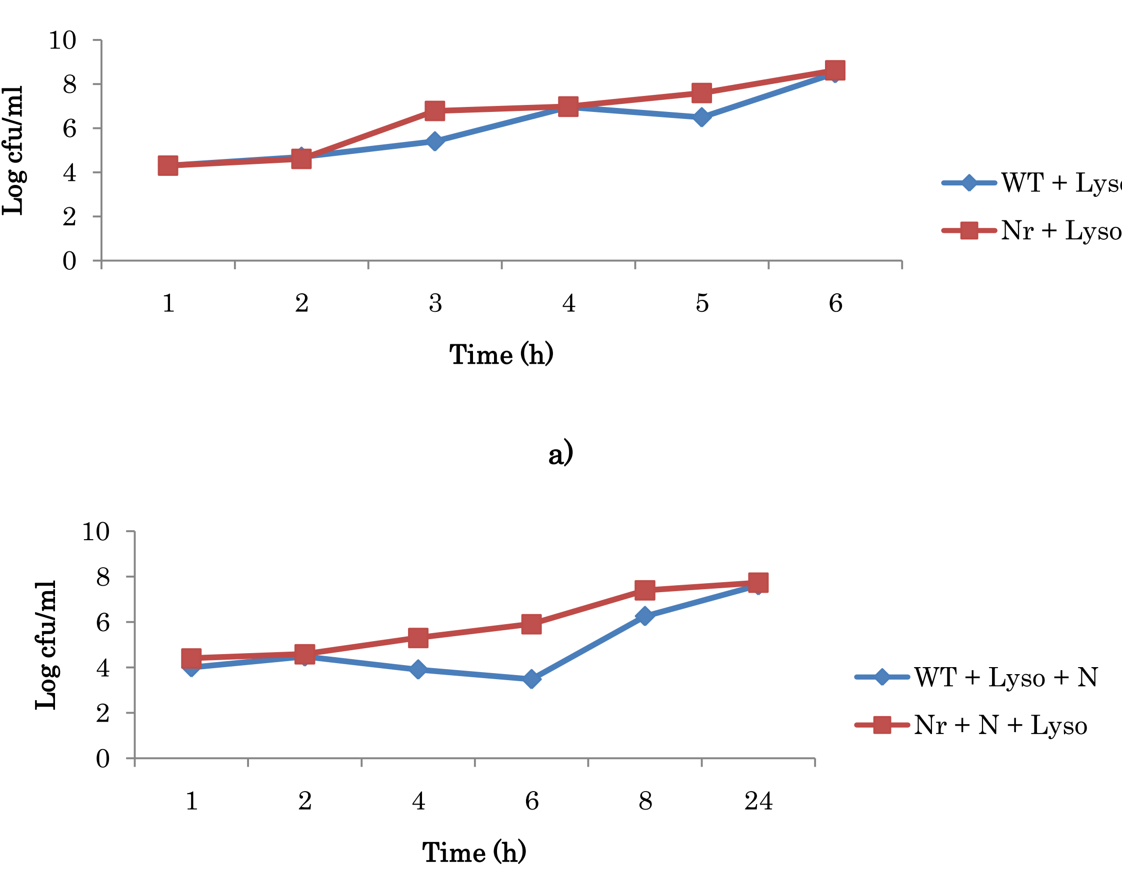
pediocin resistant variant resisted the effect of lysozyme in the presence of pediocin 34. It was observed that the nisin resistant variant was able to resist the action of lysozyme both in the presence as well as absence of nisin as compared to its wild type counterpart. These results suggest that certain cell wall associated modifications might have occurred in these resistant variants that made them resistant towards the lytic effect of lysozyme and bacteriocins. The results of our study are in contrast to the studies conducted by **Calvez *et al.*** (2010) where they showed that the resistant variant to divercin RV41 did not confer any cross-resistance but exhibited an additive effect ascribed to the combined action of lysozyme and (P)-DvnRV41.

In the presence of bacteriocins the resistant variants of *E. faecium* VRE were more sensitive to the effect of lysozyme as compared to their wild type counterparts. Also, in case of *E. faecium* VRE, it was observed that nisin and enterocin resistant variants were sensitive to lysozyme both in the presence as well as absence of nisin and enterocin, respectively, as compared to the wild type strains. This phenomenon could be explained by the mode of action of each substance. The lysozyme causes cell wall disruption and stress in cell. Both events could facilitate access to IIC subunit encoded by *mptC* gene of the mannose transport system, which thereby could constitute a putative target of pediocin34. Furthermore, this target is localized in the plasmic membrane as described by Ramnath *et al.*, 2004. Similar observations have been made recently by Calvez *et al.* (2010).

4.10.4. Role of the Cell Envelope in the Acquisition of Bacteriocin Resistance

The role of the cell wall in the acquisition of bacteriocin resistance in strains of wild type and resistant variants was investigated. For this, protoplasts were prepared using the method of Ghosh & Murray (1967). Efficiency of protoplast formation and inactivation of whole cells and protoplasts by bacteriocins was also determined.

Fig. 4.29. Growth of wild-type (WT), Nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant(Er) variants of *E. faecalis* ATCC 29212 of a) lysozyme (Lyso), b) Growth of WT and Nr variant in presence of both lysozyme and nisin



4.10.4.1. Protoplast formation

The inactivation of whole cells and protoplasts of *L. monocytogenes* ATCC 53135*, L. monocytogenes* MTCC 657*, E. faecium* DSMZ 20477*, E. faecium* (VRE*) and E. faecalis* ATCC 29212 and their nisin, pediocin 34 and enterocin FH99 resistant variants was studied using a nisin, pediocin 34 and enterocin FH99 (MICs of each bacteriocin were used).

In the presence of nisin, the number of wild type *L. monocytogenes* ATCC 53135 whole cells decreased by 1.6 log cycles over a period of 3h as compared with a decrease of 0.5 log cycles in the nisin resistant variant whole cell population. After a 3-h incubation with nisin, the nisin resistant *L. monocytogenes* ATCC 53135 protoplasts had decreased in number by 3.09 log cycles compared to a 0.50 log cycle reduction of the nisin resistant variant of *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in cell number of the *L. monocytogenes* ATCC 53135 wild type protoplasts was about a 5.23 log cycles after 3h (Fig. 4.30a). After a 3-h incubation with pediocin 34, the pediocin resistant *L. monocytogenes* ATCC 53135 protoplasts had decreased in concentration by 2.00 log cycles compared to a 0.44 log cycle reduction of the pediocin 34 resistant *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* ATCC 53135 wild type protoplasts was about a 4.55 log cycles compared to a reduction of 1.20 log cycles of wild type whole cells after 3 h (Fig. 4.30b). After a 3-h incubation with enterocin FH99, the enterocin resistant *L. monocytogenes* ATCC 53135 protoplasts decreased in concentration by 2.06 log cycles compared to only 0.24 log cycle reduction of the enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* ATCC 53135 wild type protoplasts was about a 4.85 log cycles compared to a reduction of 2.24 log cycles of wild type whole cells after 3h (Fig. 4.30c).

In case of *L. monocytogenes* MTCC 657, wild type whole cell numbers decreased by 2.82 log cycles over a period of 3h as compared to a decrease of 4.32 log

cycles of protoplasts of wild type L. monocytogenes ATCC 53135. After a 3-h incubation with nisin, the nisin resistant *L. monocytogenes* MTCC 657 protoplasts had decreased in concentration by 5.71 log cycles compared to a 0.87 log cycle reduction of the nisin resistant *L. monocytogenes* MTCC 657 whole cells (Fig. 4.31a). After 3h incubation with pediocin 34, the pediocin resistant *L. monocytogenes* MTCC 657 protoplasts had decreased in concentration by 3.73 log cycles compared with a 0.53 log cycle reduction of the pediocin 34 resistant *L. monocytogenes* MTCC 657 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* MTCC 657 WT protoplasts and whole cells after 3h was about a 3.62 and 1.92 log cycles, respectively (Fig. 4.31b). After 3h incubation with enterocin FH99, the enterocin resistant *L. monocytogenes* MTCC 657 protoplasts had decreased in concentration by 2.77 log cycles in contrast to a 1.38 log cycle reduction of the enterocin FH99 resistant *L. monocytogenes* MTCC 657 whole cells. However, the reduction in concentration of the protoplasts of wild type *L. monocytogenes* MTCC 657 was about a 3.77 as compared to a 1.97 log cycle after 3h in case of *L. monocytogenes* MTCC 657 wild type whole cells. (Fig. 4.31c).

In case of *E. faecium* DSMZ 20477, wild type whole cell numbers decreased by 0.69 log cycles over a period of 3h compared to a decrease of 0.01 log in the nisin resistant variant whole cell population. After a 3-h incubation with nisin, the nisin resistant *E. faecium* DSMZ 20477 protoplasts had decreased in concentration by 2.19 log cycles compared to a 0.01 log cycle reduction of the nisin resistant *E. faecium* DSMZ 20477 whole cells. In contrast, the reduction in concentration of the *E. faecium* DSMZ 20477 WT protoplasts was about a 3.69 log cycles after 3h (Fig. 4.32a). After 3h incubation with pediocin 34, the pediocin resistant *E. faecium* DSMZ 20477 protoplasts had decreased in concentration by 4.39 log cycles compared to a 0.10 log cycle reduction of whole cells of the pediocin 34 resistant variant *E. faecium* DSMZ 20477. In contrast, the reduction in concentration of the *E. faecium* DSMZ 20477 WT protoplasts was about a 4.00 log cycles compared to a log reduction of 0.82 log cycles of whole cells of wild type *E. faecium* DSMZ 20477 after 3 h incubation with

Fig. 4.30. The effect of (a) Nisin (N) on whole cells and protoplast cells of wild type (WT) and nisin resistant variant cells(Nr), (b) Pediocin 34 (P) on WT protoplast cells and protoplast of pediocin 34 resistant variant, (c) Enterocin FH99 (FH99) on whole cells and protoplast cells of WT and enterocin FH99 resistant variant cells(Er) of *L. monocytogenes* ATCC 53135. Mean and range of triplicate experiments indicated

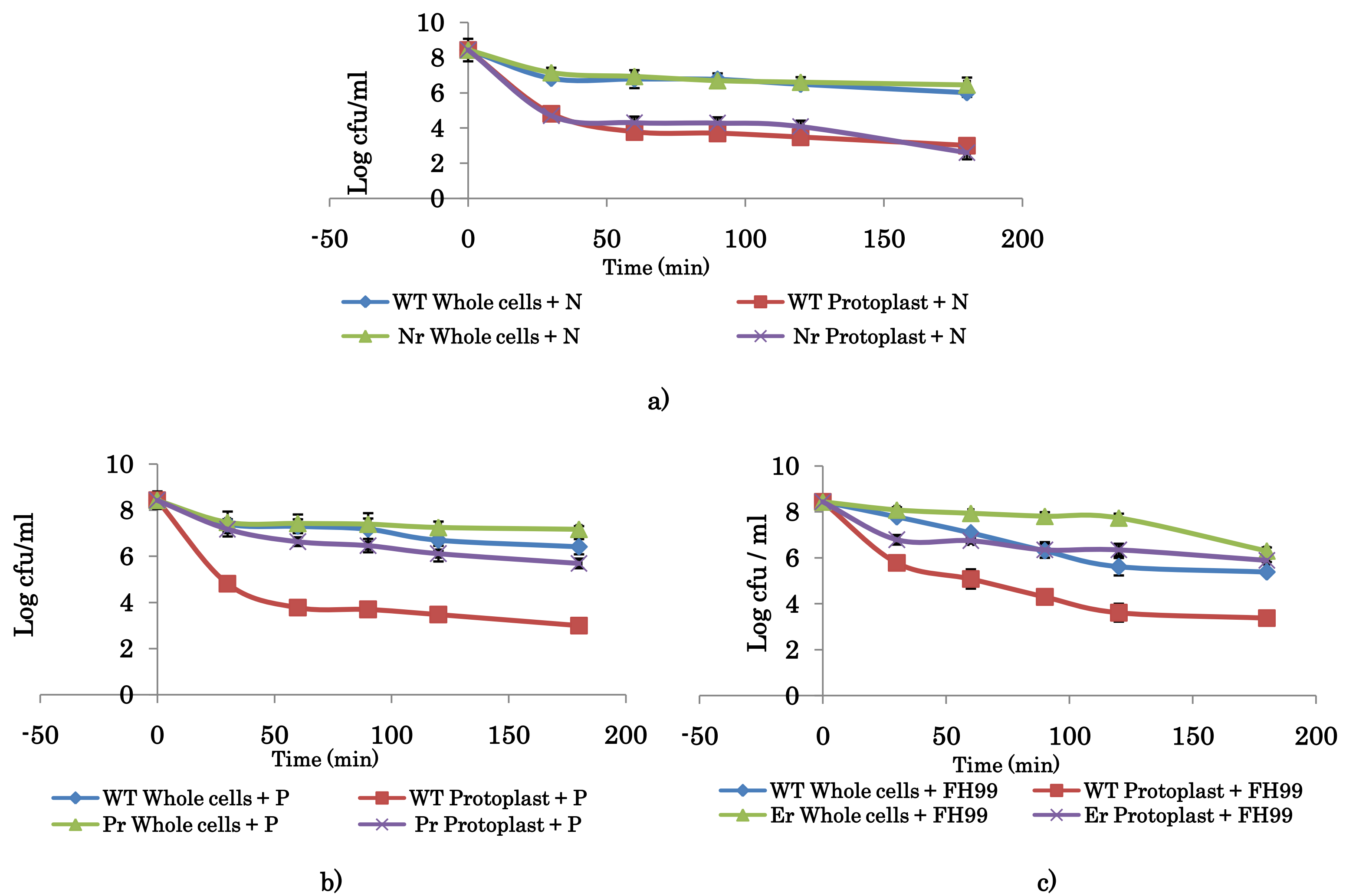


Fig. 4.31. The effect of (b) Nisin (N) on whole cells and protoplast cells of wild type (WT) and nisin resistant variant cells(Nr), (b) Pediocin 34 (P) on WT protoplast cells and protoplast of pediocin 34 resistant variant, (c) Enterocin FH99 (FH99) on whole cells and protoplast cells of WT and enterocin FH99 resistant variant cells(Er) of *L. monocytogenes* MTCC 657. Mean and range of triplicate experiments indicated

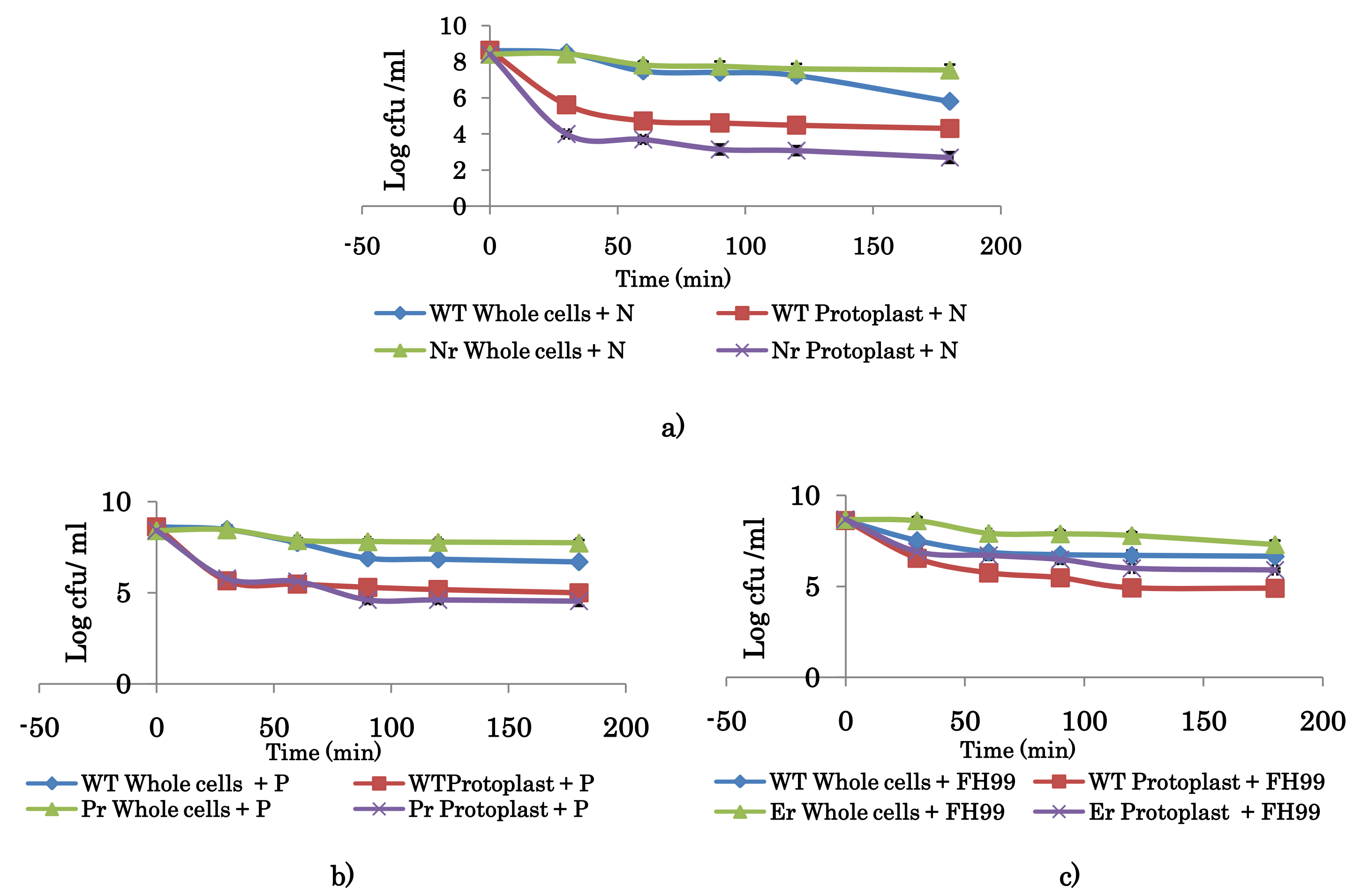
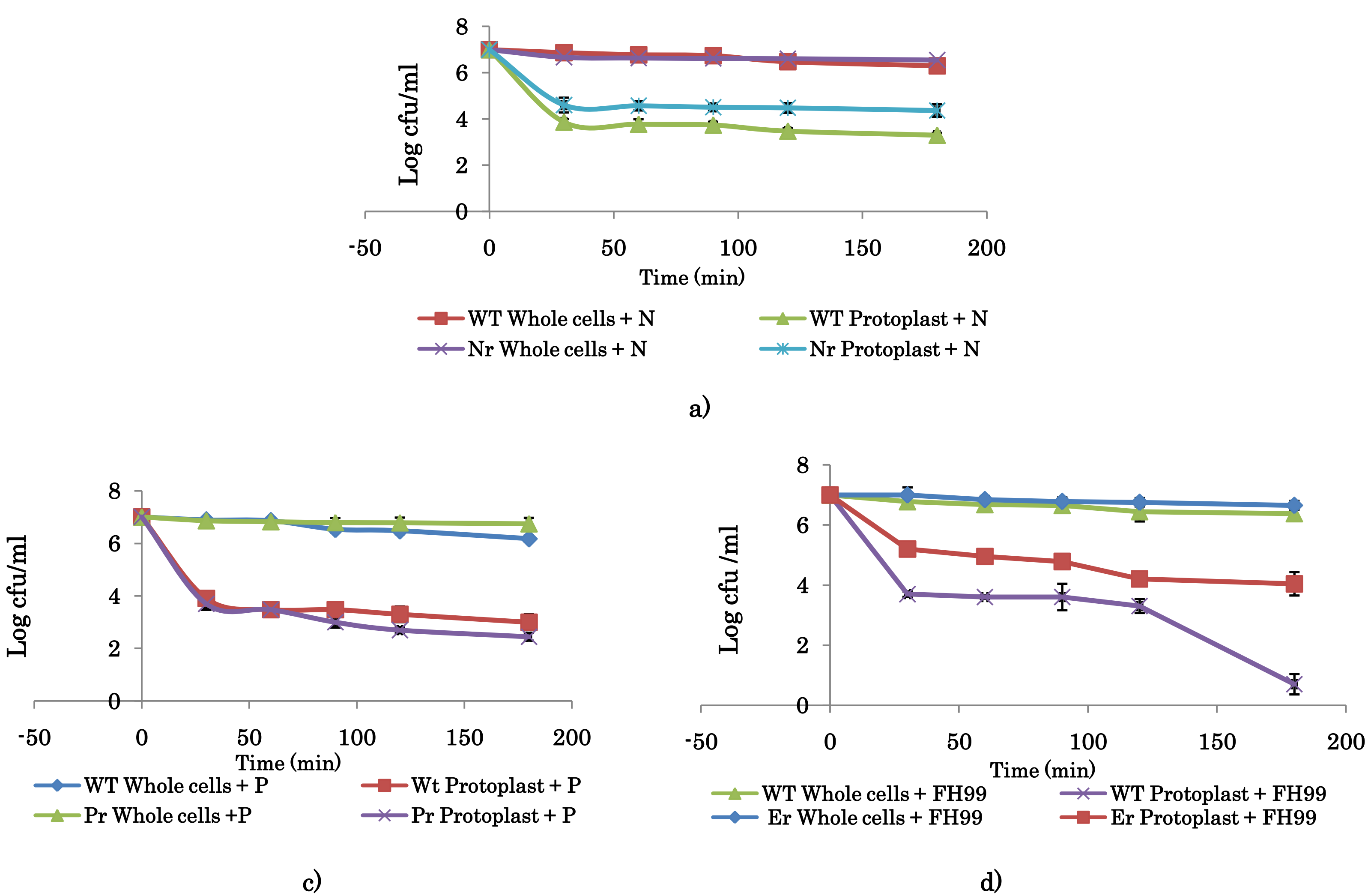


Fig. 4.32. The effect of (a) Nisin (N) on whole cells and protoplast cells of wild type (WT) and nisin resistant variant cells (Nr), (b) Pediocin 34 (P) on WT protoplast cells and protoplast of pediocin 34 resistant variant, (c) Enterocin FH99 (FH99) on whole cells and protoplast cells of WT and enterocin FH99 resistant variant cells(Er) of *E. faecium* DSMZ 20477. Mean and range of triplicate experiments indicated.



pediocin 34 (Fig.4.32b). After a 3h incubation with enterocin FH99, the enterocin resistant *E. faecium* DSMZ 20477 protoplasts had decreased in concentration by 3.91 log cycles as compared to a 1.3 log cycle reduction of the enterocin FH99 resistant *E. faecium* DSMZ 20477 whole cells. In contrast, the reduction in concentration of the *E. faecium* DSMZ 20477 WT protoplasts was about a 6.3 log cycles after 3 h. Also, a reduction of 0.61 log cycles was observed in whole cells of wild type *E. faecium* DSMZ 20477 after 3 h incubation with enterocin FH99 (Fig. 4.32c).

In case of *E. faecium* VRE, wild type whole cell numbers decreased by 2.47 log cycles in 3h compared to a decrease of 0.95 log in the nisin resistant variant whole cell population. After 3h incubation with nisin, the nisin resistant *E. faecium* VRE protoplasts decreased in concentration by 4.11 log cycles compared to a 0.95 log cycle reduction of the nisin resistant *E. faecium* VRE whole cells. In contrast, the reduction in concentration of the *E. faecium* VRE WT protoplasts was about a 5.14 log cycles after 3 h incubation with nisin (Fig. 4.33a). After 3h incubation with pediocin 34, the pediocin resistant *E. faecium* VRE protoplasts had decreased in concentration by 5.96 log cycles compared to a 1.67 log cycle reduction of the whole cells of pediocin 34 resistant variant of *E. faecium* VRE. In contrast, the reduction in concentration of the wild type *E. faecium* VRE protoplasts was about 5.44 log cycles after 3h (Fig. 4.33b). After a 3h incubation with enterocin FH99, the enterocin resistant *E. faecium* VRE protoplasts decreased in concentration by 4.03 log cycles compared to a 1.42 log cycle reduction of the enterocin FH99resistant *E. faecium* VRE whole cells. In contrast, the reduction of the wild type *E. faecium* VRE whole cells was about 1.76 log cycle after 3h incubation with enterocin FH99 (Fig. 4.33c).

In the presence of nisin, wild type whole cells of *E. faecalis* ATCC 29212, decreased by 0.66 log cycles over a period of 3h in contrast to a decrease of 0.87 log cycles in the nisin resistant variant whole cell population (Fig. 4.34). After a 3h incubation with nisin, the nisin resistant *E. faecalis* ATCC 29212 protoplasts decreased in concentration by 4.38 log cycles compared with a 0.87 log cycle

reduction of the nisin resistant *E. faecalis* ATCC 29212 whole cells. In contrast, the reduction in concentration of the *E. faecalis* ATCC 29212 WT protoplasts was about a 3.72 log reduction after 3h (Fig. 4.34).

Resistance to bacteriocins can be due to three factors: (i) variation of peptidoglycan composition (Maisnier-Patin & Richard, 1996), (ii) modification of the electric charge of the membrane because of changes in the phospholipids content, therefore preventing pore formation (Crandall & Montville, 1998; Ming & Daeschel, 1993; Verheul *et al.*, 1997) and (iii) increase in membrane rigidity. Studies have also revealed that nisin-resistant listerial strains contained more lipoteichoic acids than nisin-sensitive strains (Montovani & Russell, 2001). Therefore, the involvement of the cell wall in the acquisition of nisin resistance by *L. monocytogenes* MTCC 657*, L. monocytogenes* ATCC 53135*, E. faecium* DSMZ 20477*, E. faecium* (VRE) *and E. faecalis* ATCC 29212 and their bacteriocin resistant variants was investigated.

Results indicated that without a cell wall, the acquired nisin, pediocin 34 and enterocin FH99 resistance of the variants was lost. When the cell wall was removed, from the wild type strains, the nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477*, E. faecium* (VRE) and *E. faecalis* ATCC 29212 showed sensitivity to the three bacteriocins. Although the bacteriocin resistant variants appeared to lose their acquired resistance towards nisin, pediocin 34 and enterocin FH99, the protoplasts of the resistant variants appeared to be more resistant to bacteriocins than the protoplasts of their wild type counterparts. This may be due to non-specific adsorption of the bacteriocins on to freshly exposed hydrophobic sites on the protoplast. These results concur with Schved *et al.* (1994) who attributed the resistance of *Lactobacillus plantarum* strains to pediocin SJ-1, to the barrier properties of the cell wall. However our results are in contrast to the observations of Zajdel *et al.* (1985) who in their study stated that the bacteriocin lactostrepsin (Las) **5** did not kill protoplasts prepared from either sensitive or resistant bacterial cells. The authors suggested that

Fig. 4.33. The effect of (a) Nisin (N) on whole cells and protoplast cells of wild type (WT) and nisin resistant variant cells(Nr), (b) Pediocin 34 (P) on WT protoplast cells and protoplast of pediocin 34 resistant variant, (c) Enterocin FH99 (FH99) on whole cells and protoplast cells of WT and enterocin FH99 resistant variant cells(Er) of *E. faecium* VRE. Mean and range of triplicate experiments indicated.

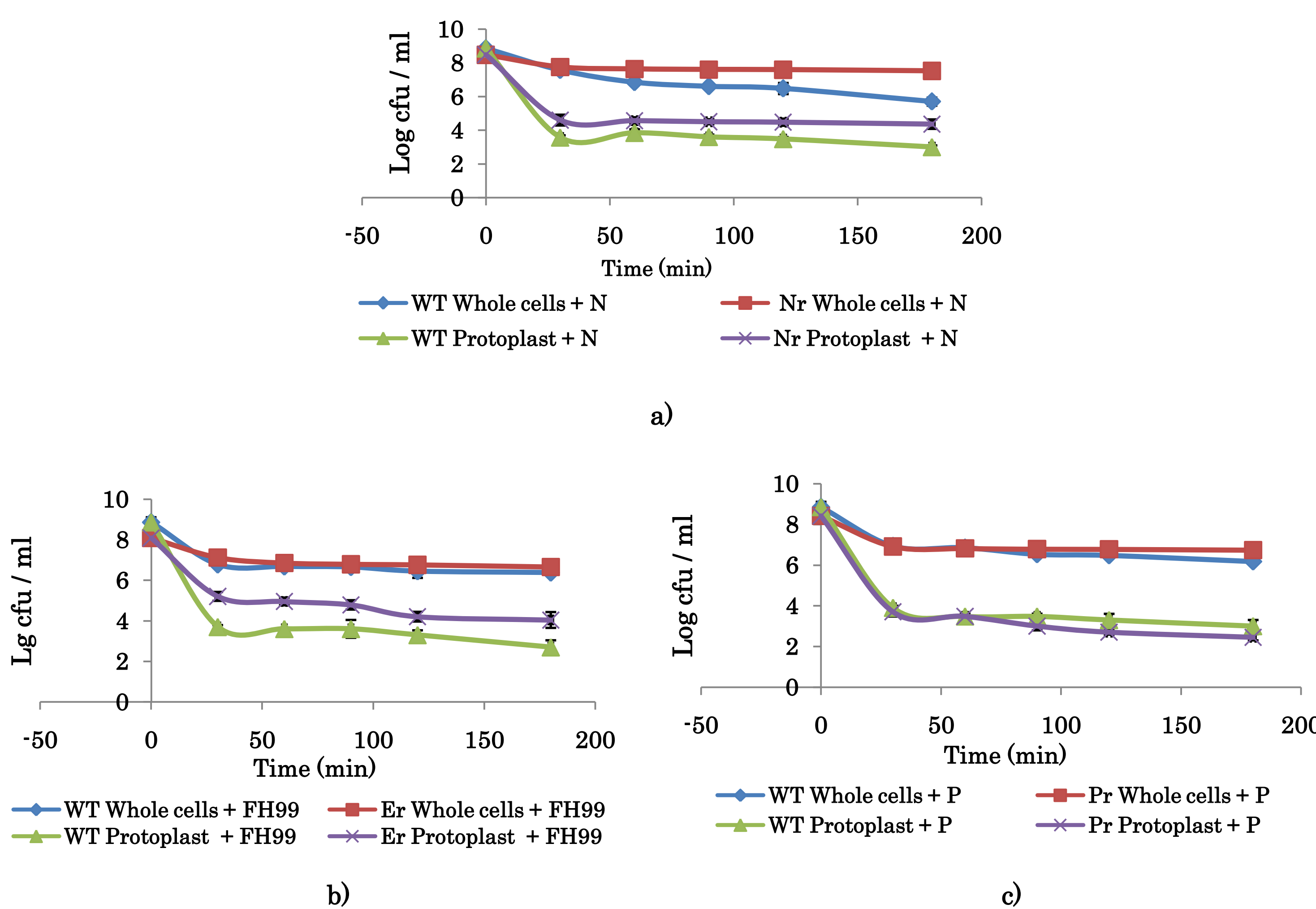
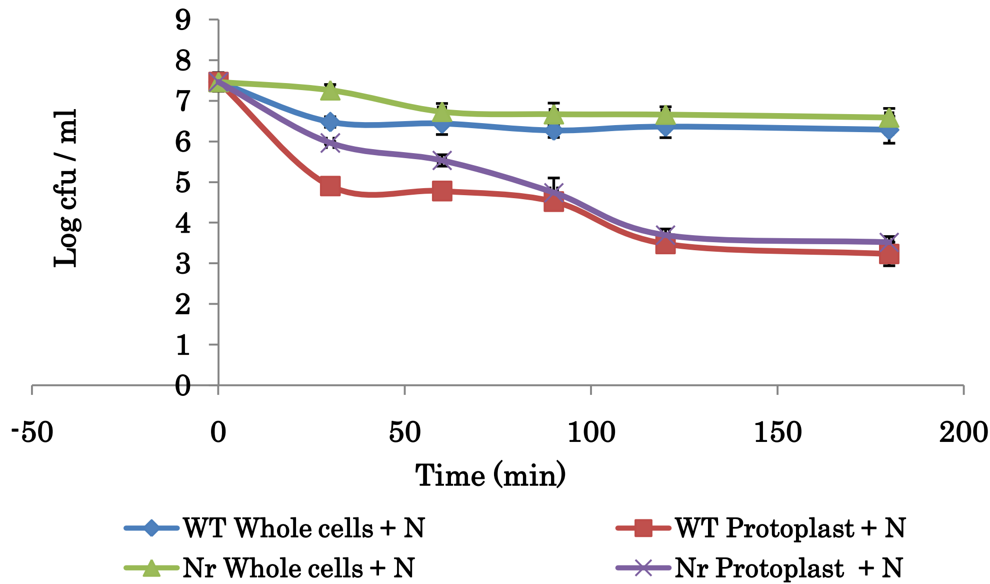


Fig. 4.34. The effect of Nisin (N) on whole cells and protoplast cells of wild type (WT) and nisin resistant variant cells (Nr) of *E. faecalis* ATCC 29212. Mean and range of triplicate experiments indicated.



interaction with the cell wall is a condition necessary for Las **5** activity. The observations with protoplasts, however, indicate that the cell wall architecture plays a major role in the development of bacteriocin resistance.

4.10.4.2. Cell Surface Hydrophobicity

The strains of *L. monocytogenes* and *E. faecium* in this study were evaluated for their cell surface hydrophobicity (CSH) towards hydrocarbon i.e. n-hexadecane. The results pertaining to the surface hydrophobicity of the selected cultures and their bacteriocin resistant variants are given in Table 4.28 and Fig. 4.35.

Table 4.28 Cell surface hydrophobicities of the selected *Listeria monocytogenes* and *Enterococcus faecium* strains and their bacteriocin resistant variants

Culture	Isolates	% Hydrophobicity
L. monocytogenes ATCC 53135	WT	11.59±0.29
	Nr	2.8±.088
	Pr	13.74±0.079
	Er	18.45±0.073
L. monocytogenes MTCC 657	WT	5.33±0.033
	Nr	6.99±0.033
	Pr	2.3±0.028
	Er	0.64±0.0005
E. faecium DSMZ 20477	WT	6.16±0.04
	Nr	4.37±0.031
	Pr	2.38±0.012
	Er	0.75±0.02
E. faecium VRE	WT	2.46±0.017
	Nr	0.23±0.001
	Pr	0.99±0.005
	Er	2.27±0.014
E. faecalis ATCC 29212	WT	30.646±0.029
	Nr	28.95±0.02

WT= wild type, Nr= nisin resistant, Pr= Pedioicn 34 resistant, Er= Enterocin FH99 resistant

Significant differences ($p < 0.05$) in hydrophobicity were observed between wild type *L. monocytogenes* ATCC 53135 and its nisin resistant, pediocin 34 resistant and

enterocin FH99 resistant counterparts, respectively (Fig. 4.35a). On the contrary, pediocin 34 and enterocin FH99 resistant variants were more hydrophobic ($p < 0.05$) than the corresponding wild type, whereas the nisin resistant variant was less hydrophobic than the wild type strain. Also, significant differences ($p < 0.001$) in hydrophobicity were observed between wild type *E. faecium* DSMZ 20477*, E. faecium* (VRE*), E. faecalis* ATCC 29212 *and L. monocytogenes* MTCC 657 and their nisin resistant, pediocin 34 resistant and enterocin FH99 resistant counterparts, respectively. On the contrary, nisin resistant variant of *L. monocytogenes* MTCC 657 was more hydrophobic ($p < 0.001$) than the corresponding wild type, whereas the pediocin 34 and enterocin FH99 resistant variants were less hydrophobic than the wild type strain (Fig. 4.35b). Nisin, Pediocin 34 and Enterocin FH99resistant variants of *E. faecium* DSMZ 20477 were less hydrophobic than their wild type counterparts (Fig. 4.35c). Similar results were obtained for *E. faecium* VRE and its bacteriocin resistant variants (Fig. 4.35d). Also, nisin resistant *E. faecalis* 29212 was less hydrophobic than its wild type counterpart (Fig. 4.35e).

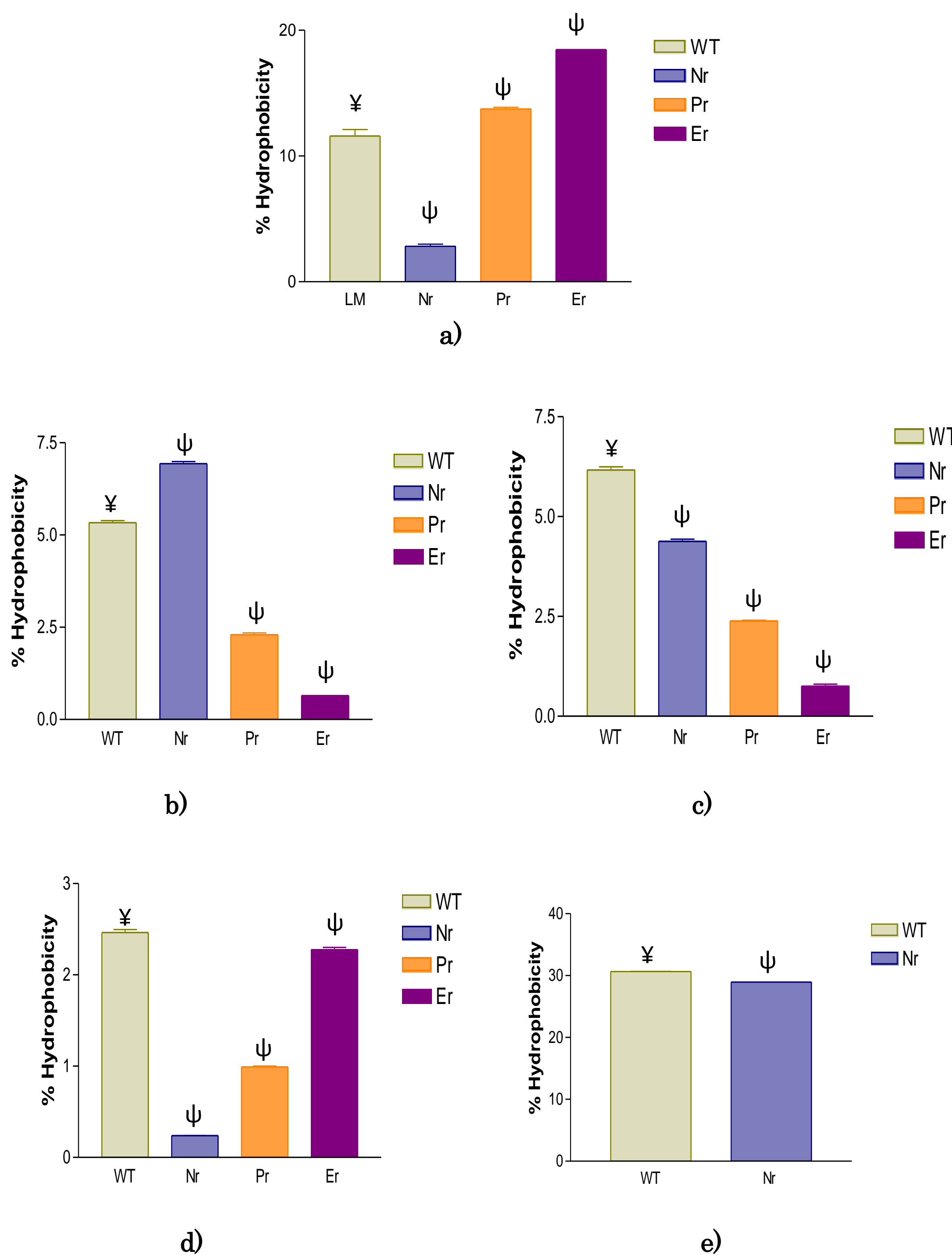
These results indicate cell surface hydrophobicity differences between the wild type strains and their bacteriocin resistant variants which may be involved in nisin, pediocin 34 and enterocin FH99 resistance. Nisin itself is predominantly hydrophobic and so the decreased hydrophobicity of the more resistant cells may be related to their reduced avidity for nisin observed earlier (Davies & Adams, 1994). Resistance to nisin, pediocin 34 and enterocin FH99 may be due to a substantial change in the surface architecture of the resistant variants which might involve a different protein display at the surface. These results are in accordance with those reported by Martinez & Rodriguez (2005).

4.10.5 Modifications of Membrane Phospholipids Composition in Bacteriocin-Resistant Strains

4.10.5.1 Measurement of cellular phospholipids

The studies on phospholipids analysis of bacteriocin sensitive and resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 were carried out to observe the

Fig. 4.35. Surface hydrophobicity of the wild type (WT) a) *Listeria monocytogenes* ATCC 53135, b) *L. monocytogenes* MTCC 657 c) *E. faecium* DSMZ 20477, d) *E. faecium* VRE, e) *E. faecalis* ATCC 29212 and their nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant (Er) variants.



*p < 0.001. Values are presented as mean ±SEM; n=3. ¥, ψ Values with different superscripts differ significantly at the level of P<0.001 between wild type and bacteriocin resistant strains.

changes in composition of phospholipids in acquiring resistance to bacteriocins nisin, pediocin 34 and enterocin FH99.

4.10.5.2 Comparitive Analysis of Phospholipids between Nisin, Pediocin 34 and Enterocin FH99 Resistant Variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212

4.10.5.2.1 Changes in phospholipids head group

Differences in the phospholipid content of the resistant variants compared to their wild type counterparts were observed (Plates 4.9 to 4.11).

Total cellular lipid extract of sensitive strain of *L. monocytogenes* ATCC 53135 could be resolved into three spots which were detectable with ninhydrin but not with iodine vapours and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP). Similarly, total cellular lipid extract of nisin resistant variant of *L. monocytogenes* ATCC 53135 could be resolved into five spots which were detectable with ninhydrin but not with iodine vapours and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP). Total cellular lipid extract of pediocin 34 resistant variant could be resolved into one spot only which was detectable only with ammonium molybdate reagent spray and could be tentatively identified as amino group lacking phospholipid. Total cellular lipid extract of enterocin FH99 resistant variant could be resolved into three spots which were detectable with ninhydrin but not with iodine vapors and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP) (Plate 4.9a).

Total cellular lipid extract of sensitive strain of *L. monocytogenes* MTCC 657 could be resolved into two spots which were detectable with molybdate spray reagent but not with iodine vapors. R_f value of spot 2 was close to dimyristol phosphaidyl glycerol (DMPG) and since it was not detectable with ninhydrin, it may be phosphatidylglycerol. Spot 1 was detectable only with ninhydrin. Tentatively these spots may be identified as amino containing phospholipids. Similarly, total cellular

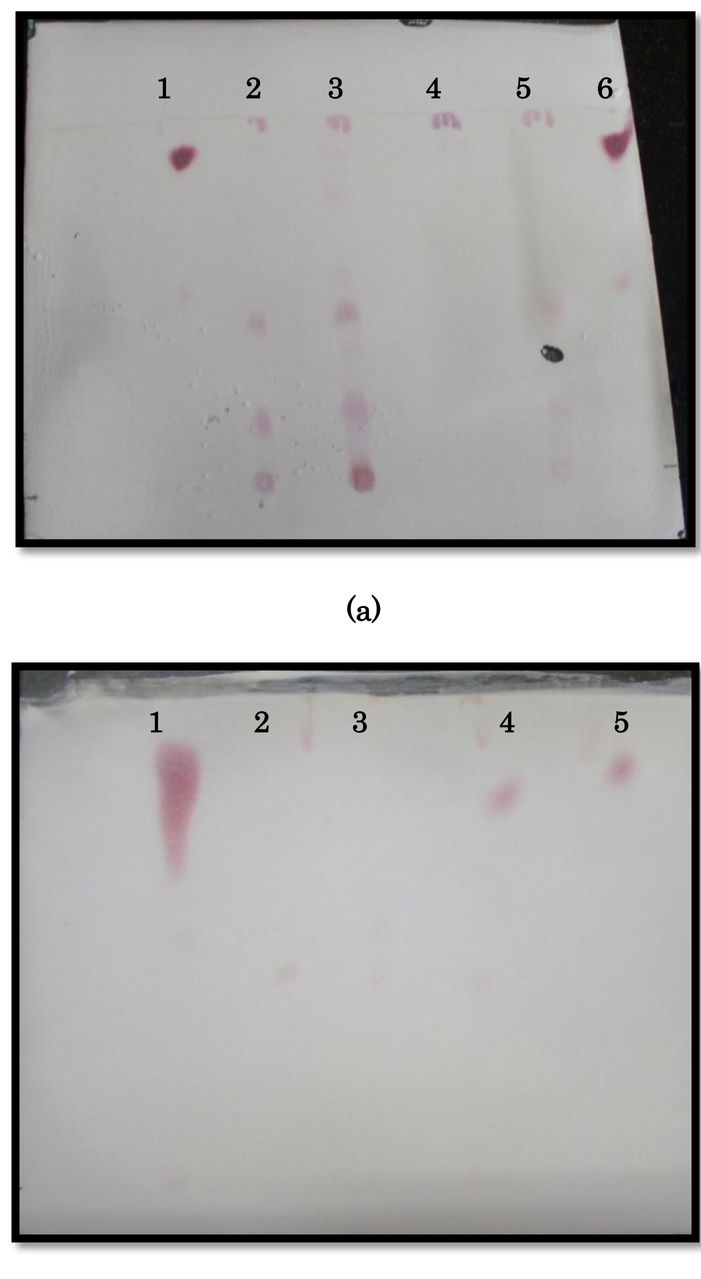
lipid extract of nisin resistant variant of *L. monocytogenes* MTCC 657 could be resolved into one spot only which was detectable with ninhydrin but not with iodine vapors and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP). Total cellular lipid extract of pediocin 34 resistant variant could be resolved into two spots which were detectable with ninhydrin. Spot 2 was detectable with molybdate reagent also. Total cellular lipid extract of enterocin FH99 resistant variant could be resolved into one spot which was detectable with ninhydrin and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP) (Plate 4.9b).

Total cellular lipid extract of sensitive strain of *E. faecium* DSMZ 20477 and nisin resistant variant of *E. faecium* DSMZ 20477 could be resolved into one spot only which was detectable with ninhydrin but not with iodine vapors and molybdate reagent. Tentatively this spot may be identified as amino containing phospholipids. Total cellular lipid extract of pediocin 34 resistant variant could be resolved into four spots which were detectable with ninhydrin only. Similarly, total cellular lipid extract of enterocin FH99 resistant variant could be resolved into three spots which were detectable with ninhydrin and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP) (Plate 4.10a).

Total cellular lipid extract of sensitive strain of *E. faecium* VRE and nisin resistant variant of E*. faecium* VRE could be resolved into one spot only which was detectable with ninhydrin but not with iodine vapours and molybdate reagent. Tentatively this spots may be identified as amino containing phospholipids. Total cellular lipid extract of pediocin 34 resistant variant could be resolved into three spots which were detectable with ninhydrin only. Similarly, total cellular lipid extract of enterocin FH99 resistant variant could be resolved into three spots which were detectable with ninhydrin and molybdate spray reagent. Tentatively these spots may be identified as amino group containing phospholipids (ACP) (Plate 4.10b).

Total cellular lipid extract of sensitive strain of *E. faecalis* ATCC 29212 and its nisin resistant variant could be resolved into one spot only which were detectable

Plate 4.9 TLC analysis of lipids extracted from bacteriocin sensitive and resistant variants of (a) *L. monocytogenes* ATCC 53135 (b) *L. monocytogenes* MTCC 657

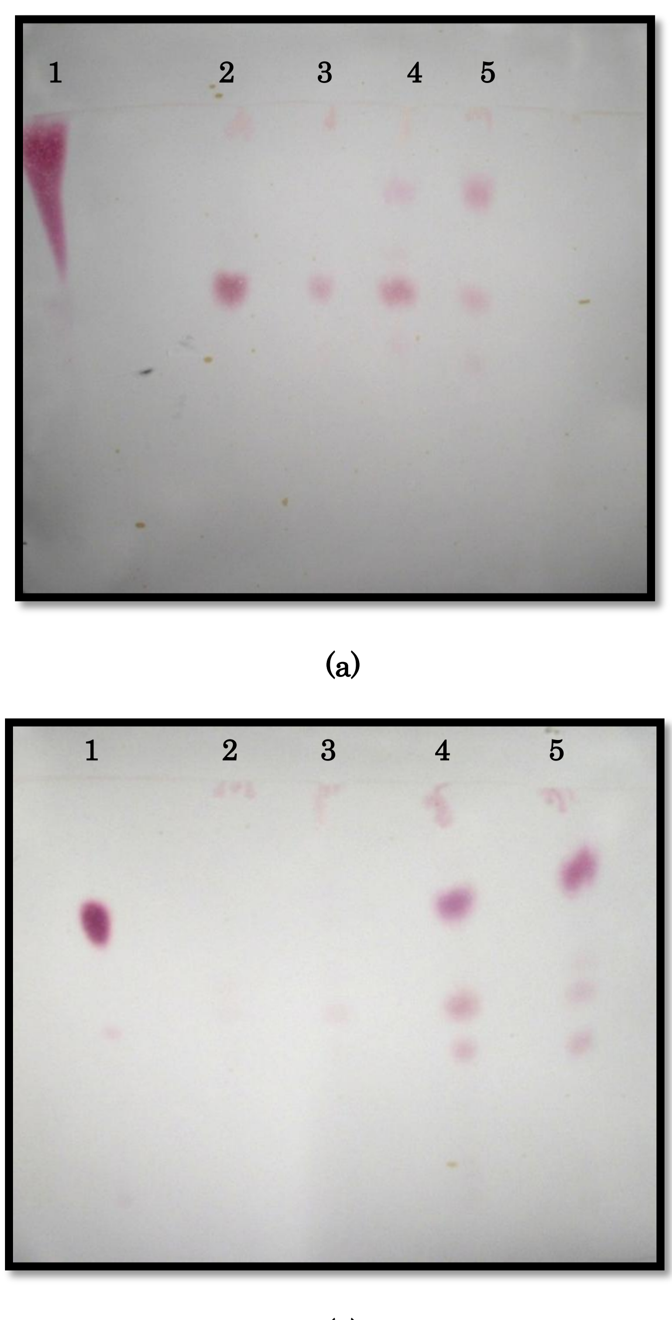


(b)

(a) 1= dimyristol phosphatidyl glycerol (DMPG), 2= Wild Type *L. monocytogenes* ATCC 53135, 2 =Nisin resistant variant of *L. monocytogenes* ATCC 53135, 3= Pediocin 34 resistant variant and 4= Enterocin FH99 resistant variant of *L. monocytogenes* ATCC 53135

(b) 1= dimyristol phosphatidyl glycerol (DMPG), 2= Wild Type *L. monocytogenes* MTCC 657, 2 =Nisin resistant variant of *L. monocytogenes* MTCC 657, 3= Pediocin 34 resistant variant of *L. monocytogenes* MTCC 657, 4= Enterocin FH99 resistant variant of *L. monocytogenes* MTCC 657.

Plate 4.10 TLC analysis of lipids extracted from bacteriocin sensitive and resistant variants of (a) *E. faecium* DSMZ 20477 and (b) *E. faecium* (VRE)

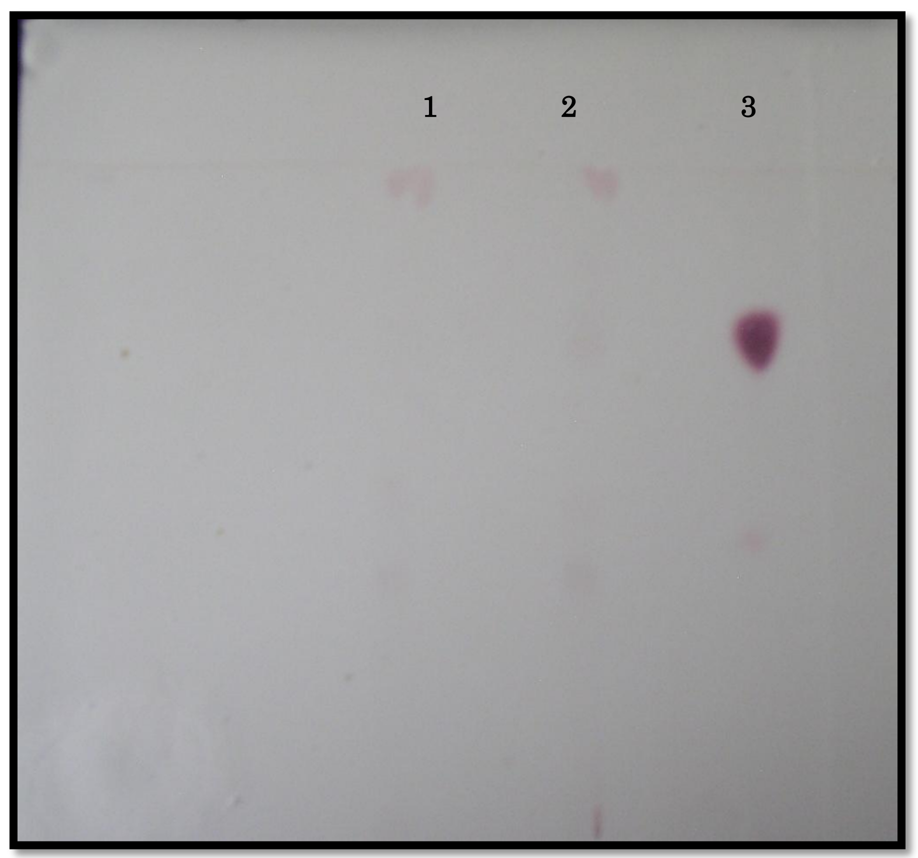


(b)

(a) 1= dimyristol phosphatidyl glycerol (DMPG), 2= Wild Type *E. faecium* DSMZ 20477, 2 =Nisin resistant variant of *E. faecium* DSMZ 20477, 3= Pediocin 34 resistant variant of *E. faecium* DSMZ 20477, 4= Enterocin FH99 resistant variant of *E. faecium* DSMZ 20477

(b) 1= dimyristol phosphatidyl glycerol (DMPG), 2= Wild Type *E. faecium* VRE, 2 =Nisin resistant variant of *E. faecium* VRE, 3= Pediocin 34 resistant variant of *E. faecium* VRE, 4= Enterocin FH99 resistant variant of *E. faecium* VRE.

Plate 4.11 TLC analysis of lipids extracted from bacteriocin sensitive and resistant variants of *E. faecalis* ATCC 29212



1= Wild Type *E. faecalis* ATCC 29212, 2 =Nisin resistant variant of *E. faecalis* ATCC 29212, 3= dimyristol phosphatidyl glycerol (DMPG)

with ninhydrin but not with but not with iodine vapours and molybdate reagent. Tentatively this spot may be identified as amino containing phospholipids (Plate 4.11).

An increase in amino group containing phospholipids in the nisin resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, pediocin 34 resistant variant and enterocin FH99 resistant variant of *E. faecium* DSMZ 20477, pediocin 34 resistant variant and enterocin FH99 resistant variant of *E. faecium* VRE indicates an increase in the net positive charge. In general bacteriocins are cationic peptides that display hydrophobic or amphiphilic properties and the bacterial membrane is in most cases the target for their activity. Models of modes of class IIa bacteriocin activity have been proposed (Ennahar *et al.*, 2000). In brief, the modes of bacteriocins consist of at least three steps: (1) electrostatic interaction between the N terminus of bacteriocin and membrane phospholipids, (2) reorientation and insertion into the membrane, and (3) pore formation.

At present, no evidence for a protein receptor for binding of class IIa bacteriocin has been reported (Chen *et al.*, 1997b). Conditions favorable to electrostatic interactions in the membrane are important for binding class IIa bacteriocins to phospholipid vesicles (Chen *et al.*, 1997a). Because ACP could have zwitterionic properties, an increased level of ACP might inhibit binding of bacteriocin by positively charging the membrane.

It has been reported that changes in the phospholipids composition occur in nisin-resistant strains of *L. monocytogenes Scott A* (Verheul *et al.*, 1997). Specific changes include more zwitterionic phosphatidylethanolamine and less anionic PG and CL (Verheul *et al.*, 1997; Crandall & Montville, 1998). Changes in the composition of phospholipids in resistant variants that have been observed in the study are similar to these changes in phospholipids in *L. monocytogenes*. Thus the level of zwitterionic phospholipids is a critical determinant in the resistance to both class I and class IIa bacteriocins. Vadyvaloo *et al.* (2002) reported that class-IIa-resistant *L. monocytogenes* strains showed increased levels of desaturated PG.

4.10.6 Role of Various Sugars on the Sensitivity of Wild and Resistant Strains to the Bacteriocin Alone or Combination of Bacteriocins

The effect of sugars on the sensitivity of wild type *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their resistant variants to Nisin, Pediocin 34 and Enterocin FH99 to bacteriocins was also evaluated. To see whether sugars influence the sensitivity in a dose-dependent manner, the wild type strains and the resistant variants were grown in medium supplemented with sugars at various concentrations followed by the measurement of optical density at 600nm.

The presence of glucose, mannose, fructose or cellobiose induced sensitivity of wild type *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to nisin, pediocin 34 and enterocin FH99 (Fig. 4.36 to 4.61).

It was observed that in the absence of bacteriocins (i.e nisin, pediocin 34 and enterocin FH99, growth of *L. monocytogenes* ATCC 53135 increases along with mannose concentration whereas, in the presence of Nisin, pediocin 34 and enterocin FH99 growth inhibition increases on addition of mannose concentration. In the presence of nisin, maximum growth inhibition was observed with 20mM mannose, 15mM glucose, and 10mM fructose whereas, maximum growth inhibition was observed with 10mM cellobiose in the presence of nisin (Fig. 4.36). In the presence of pediocin 34, wild type *L. monocytogenes* ATCC 53135 showed maximum growth inhibition with 20mM mannose and glucose, 15mM cellobiose and 10mM fructose (Fig. 4.37). In presence of enterocin FH99, wild type *L. monocytogenes* ATCC 53135 showed maximum growth inhibition with 20mM mannose, 25mM glucose, 10mM fructose and 15mM cellobiose (Fig. 4.38). In the absence of bacteriocins, the nisin, pediocin 34 and enterocin resistant variants of *L. monocytogenes* ATCC 53135 were insensitive to the bacteriocins but in the presence of sugars, they regained their sensitivity towards the bacteriocins. Nisin resistant variant regained its

Fig. 4.36 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on wild type *L. monocytogenes* ATCC 53135 (WT)

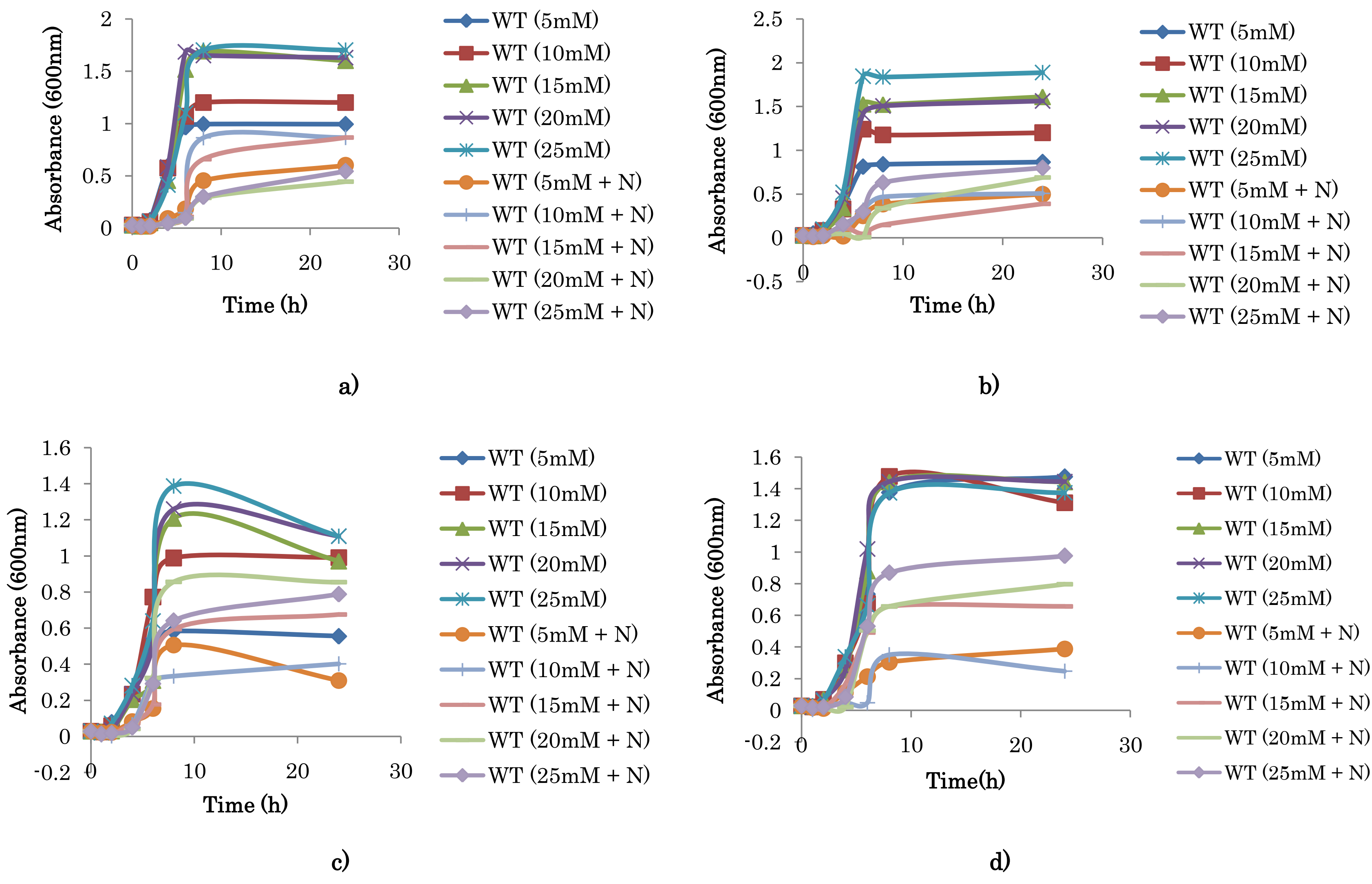


Fig. 4.37 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on wild type *L. monocytogenes* ATCC 53135 (WT)

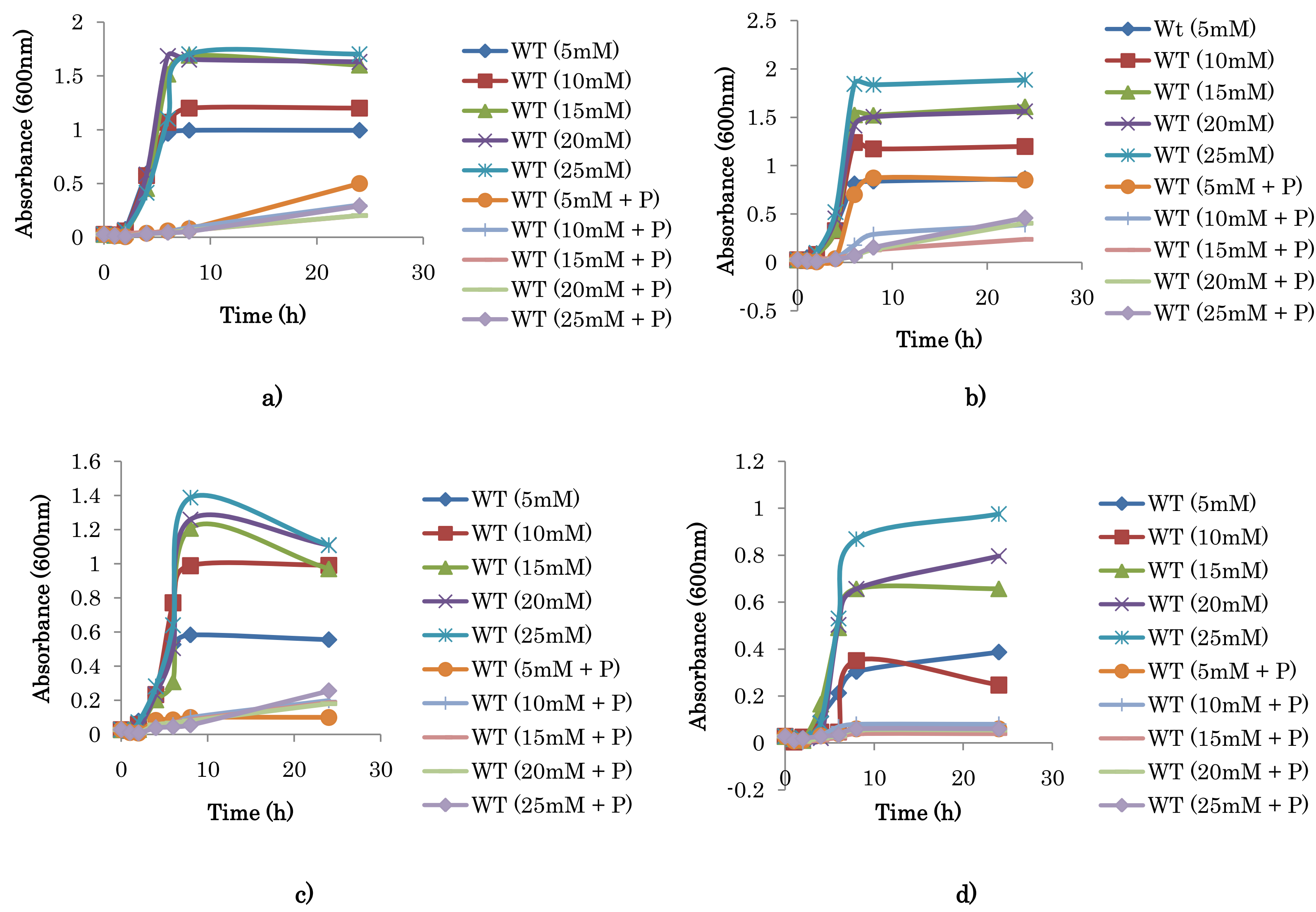
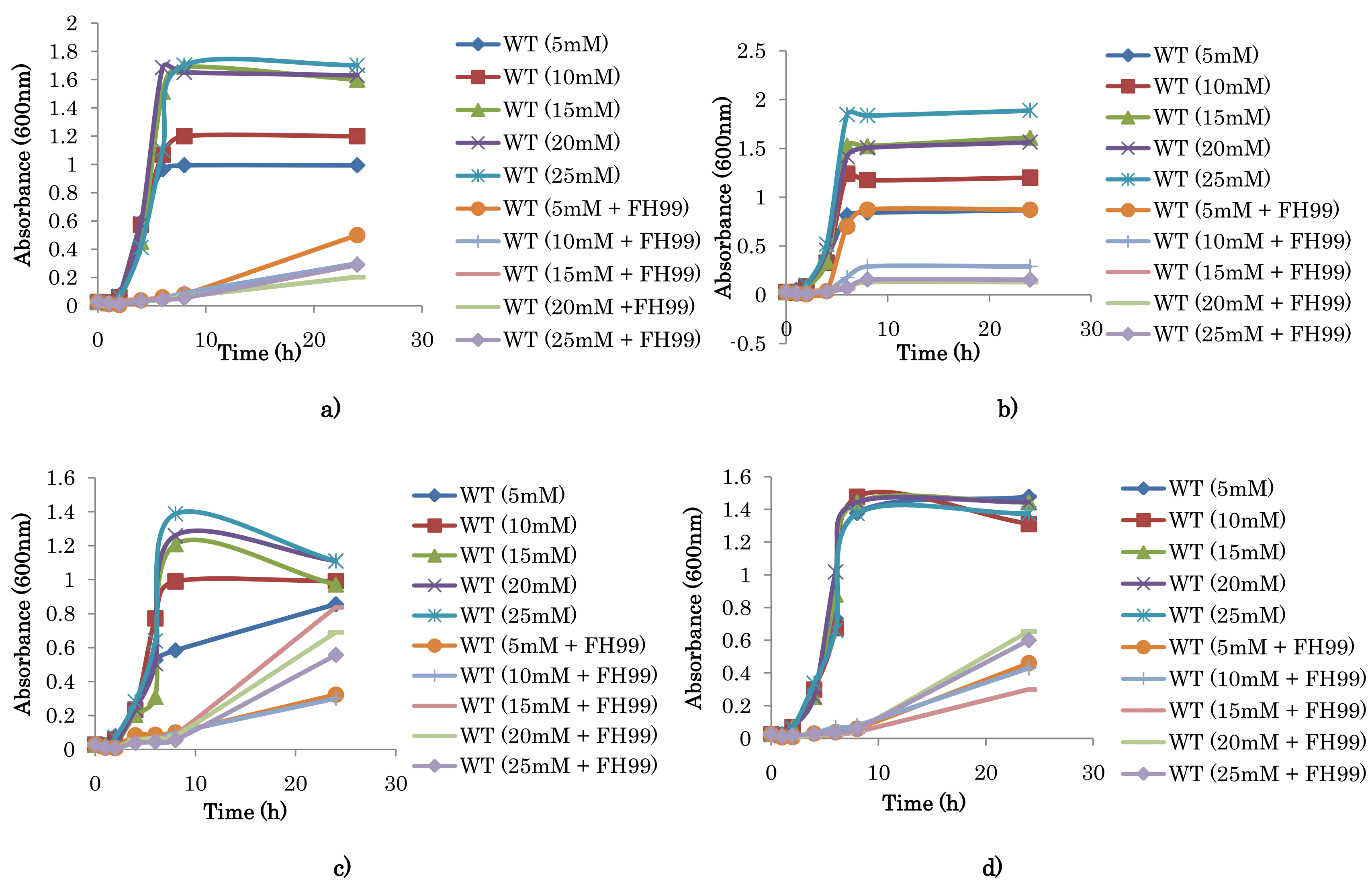


Fig. 4.38 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on wild type *L. monocytogenes* ATCC 53135 (WT)



sensitivity to nisin in presence of mannose, glucose, fructose and cellobiose as shown in Fig. 4.39. Similar results were obtained for pediocin 34 resistant, variant. Maximum inhibiton being observed in the presence of fructose and cellobiose in presence of pediocin 34 as shown in Fig. 4.40. Also, enterocin FH99 resistant variant showed in creased sensitivity towards enterocin FH99 in presence of mannose, glucose, fructose and cellobiose (Fig. 4.41).

In the absence of bacteriocins (i.e. nisin, pediocin 34 and enterocin FH99), growth of *L. monocytogenes* MTCC 657 increased along with mannose concentration, whereas, in the presence of nisin, pediocin 34 and enterocin FH99 growth inhibition increased along with mannose concentration. Similar results were observed for glucose, fructose and cellobiose. In presence of nisin, maximum growth inhibition was observed at glucose, mannose and fructose concentration 5mM whereas, maximum growth inhibition was observed at 10mM cellobiose in the presence of nisin. In presence of pediocin 34, maximum growth inhibition was observed with 20mM mannose, 15mM cellobiose and 10mM fructose (Fig. 4.42). In presence of pediocin 34, wild type *L. monocytogenes* ATCC 53135 showed maximum growth inhibition with 20mM mannose, 15mM glucose, 15mM cellobiose and 10mM fructose (Fig. 4.43). In presence of enterocin FH99, wild type *L. monocytogenes* ATCC 53135 showed maximum growth inhibition with 20mM mannose, 15mM glucose, 10mM fructose and 15mM cellobiose (Fig. 4.44). In the absence of sugars, the nisin, pediocin 34 and enterocin resistant variants of *L. monocytogenes* ATCC 53135 were insensitive to the bacteriocins but in the presence of sugars, they regained their sensitivity towards the bacteriocins. Nisin resistant variant regained it sensitivity to nisin in presence of mannose, glucose, fructose and cellobiose as shown in Fig. 4.45. Similar results were obtained for pediocin 34 resistant variant maximium inhibiton being observed in presence of fructose and cellobiose in presence of pediocin 34 as shown in Fig. 4.46. Also, enterocin FH99 resistant variant showed an increased sensitivity towards enterocin FH99 in the presence of mannose, glucose, fructose and cellobiose (Fig. 4.47)

It was observed that in the absence of test bacteriocins (nisin, pediocin 34 and enterocin FH99), growth of *E. faecium* DSMZ 20477 increased along with mannose concentration whereas, in the presence of Nisin, pediocin 34 and enterocin FH99 growth inhibition increased on addition of mannose concentration. In presence of nisin, maximum growth inhibition was observed at 20mM mannose, 25mM glucose, and 20mM fructose whereas maximum growth inhibition was observed with 25mM cellobiose (Fig. 4.48). In presence of pediocin 34, wild type *E. faecium* DSMZ 20477 showed maximum growth inhibition with 25mM mannose, glucose, fructose and 15mM cellobiose (Fig. 4.49). In presence of enterocin FH99, wild type *E. faecium* DSMZ 20477 showed maximum growth inhibition with 15mM mannose, 25mM glucose, 25mM fructose and 15mM cellobiose (Fig. 4.50). In the absence of bacteriocins the nisin, pediocin 34 and enterocin resistant variants of *E. faecium* DSMZ 20477 were insensitive to the bacteriocins but in the presence of sugars, they regained their sensitivity towards the bacteriocins. Nisin resistant variant regained its sensitivity to nisin in presence of mannose, glucose, fructose and cellobiose as shown in Fig. 4.51. Similar results were obtained for pediocin 34 resistant variant maximium inhibiton being observed in presence of 25mM mannose, glucose, fructose and cellobiose in presence of pediocin 34 as shown in Fig. 4.52. Also, enterocin FH99 resistant variant showed increased sensitivity towards enterocin FH99 in presence of mannose, glucose, fructose and cellobiose (Fig. 4.53)

It was observed that in the absence of test bacteriocins (nisin, pediocin 34 and enterocin FH99), growth of *E. faecium* VRE increased along with mannose concentration whereas, in the presence of Nisin, pediocin 34 and enterocin FH99 growth inhibition increased on addition of mannose concentration. In presence of nisin, maximum growth inhibition was observed at with 20mM mannose, 25mM glucose, and 20mM fructose whereas maximum growth inhibition was observed with 25mM cellobiose (Fig. 4.54). In presence of pediocin 34, wild type *E. faecium* VRE showed maximum growth inhibition with 25mM mannose, glucose, fructose and 15mM cellobiose (Fig. 4.55). In presence

Fig. 4.39 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N)on wild type *L. monocytogenes* ATCC 53135 (WT) and its Nisin (Nr) resistant variants.

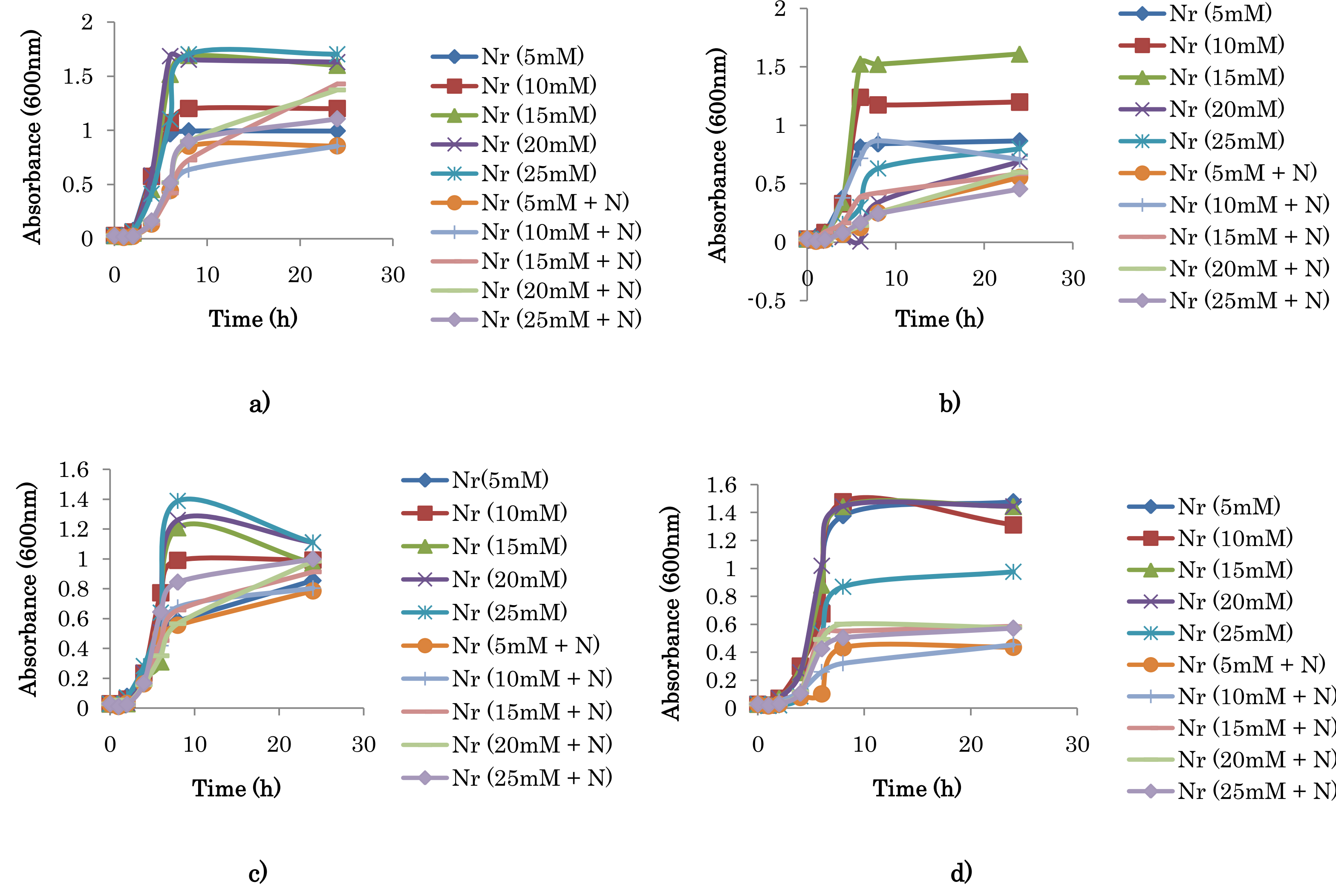


Fig. 4.40 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on Pediocin 34 (Pr) resistant variant of *L. monocytogenes* ATCC 53135.

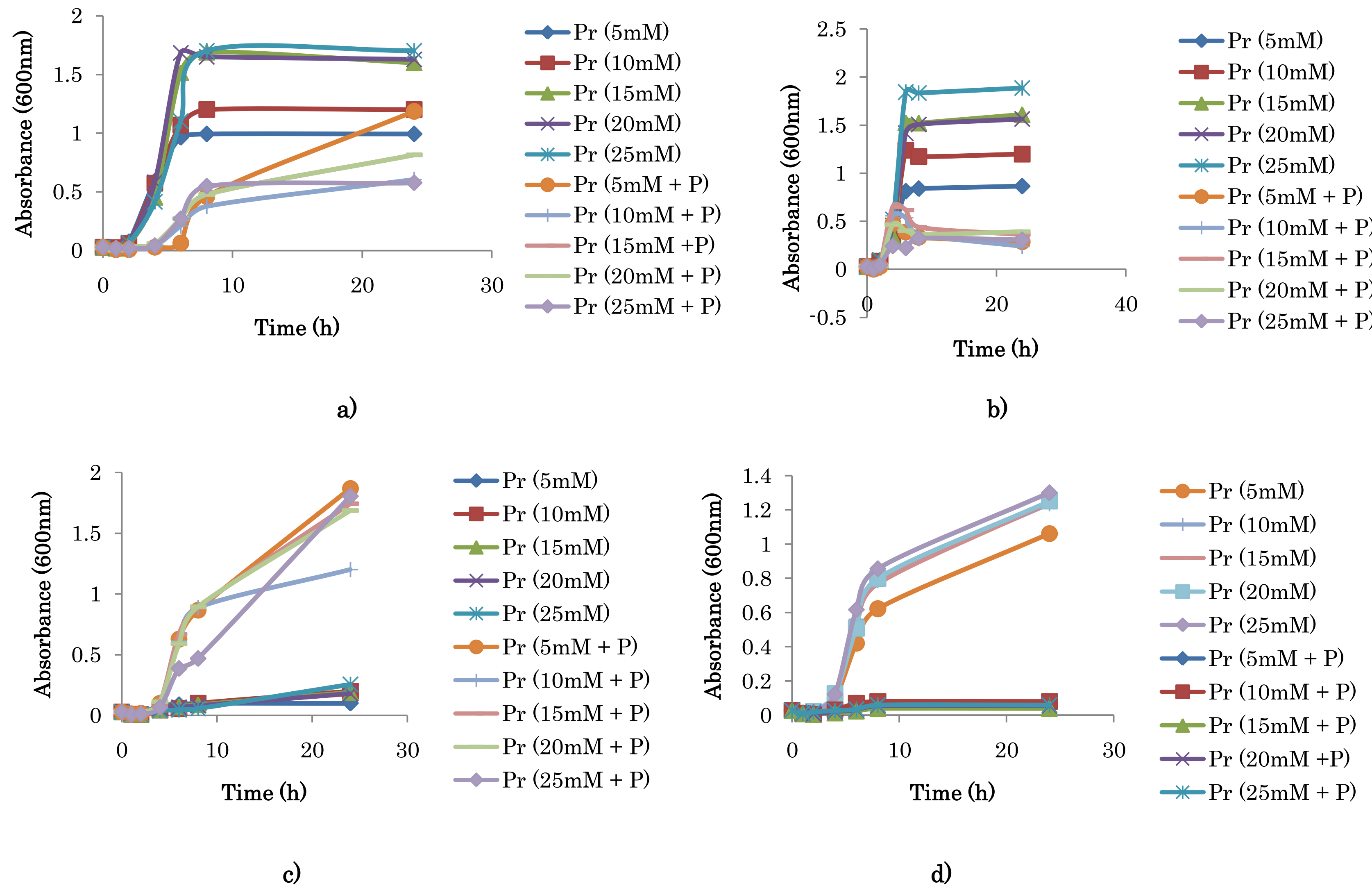


Fig. 4.41 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) its Enterocin FH99 (Er) resistant variant of *L. monocytogenes* ATCC 53135.

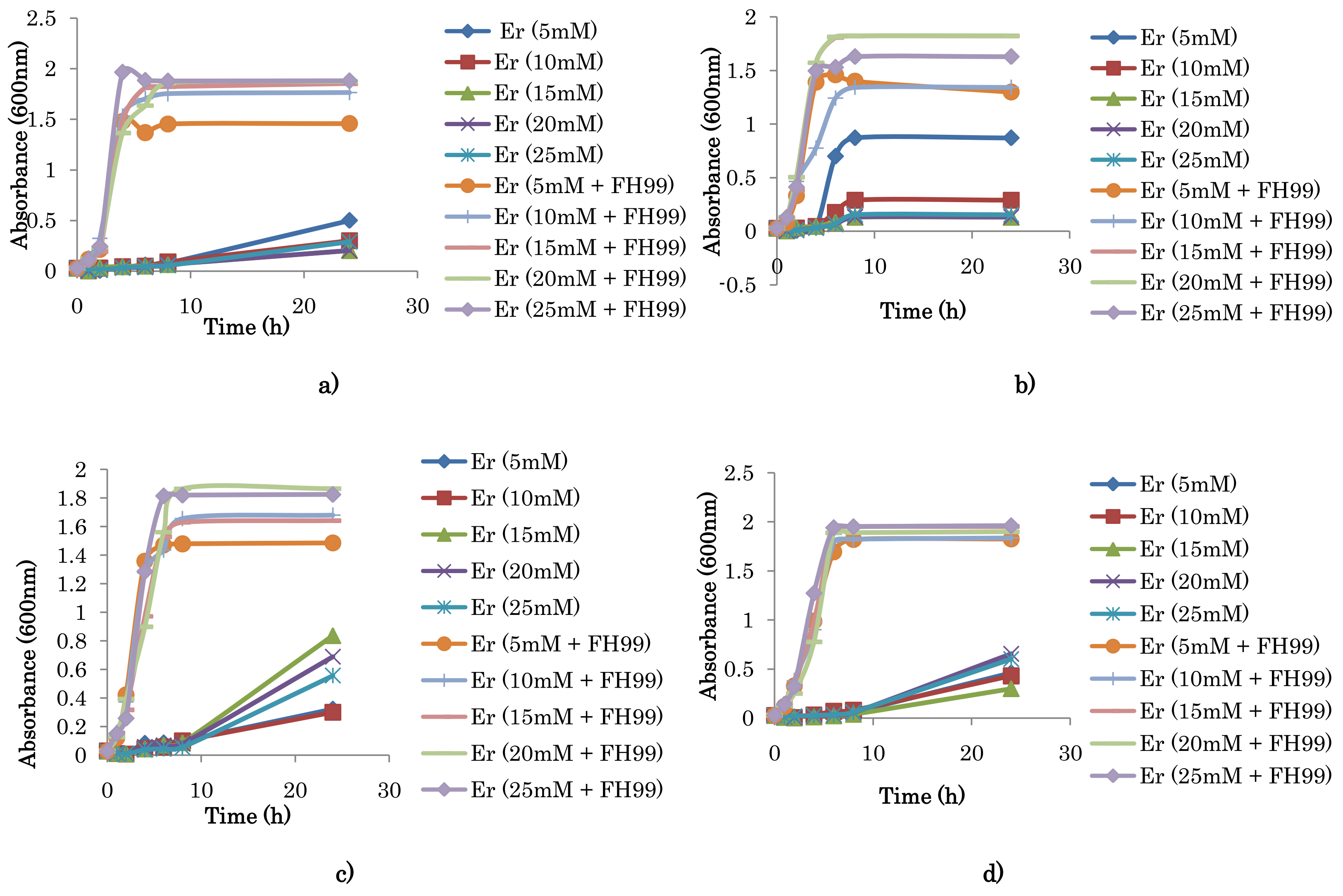


Fig. 4.42 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on wild type *L. monocytogenes* MTCC 657 (WT)

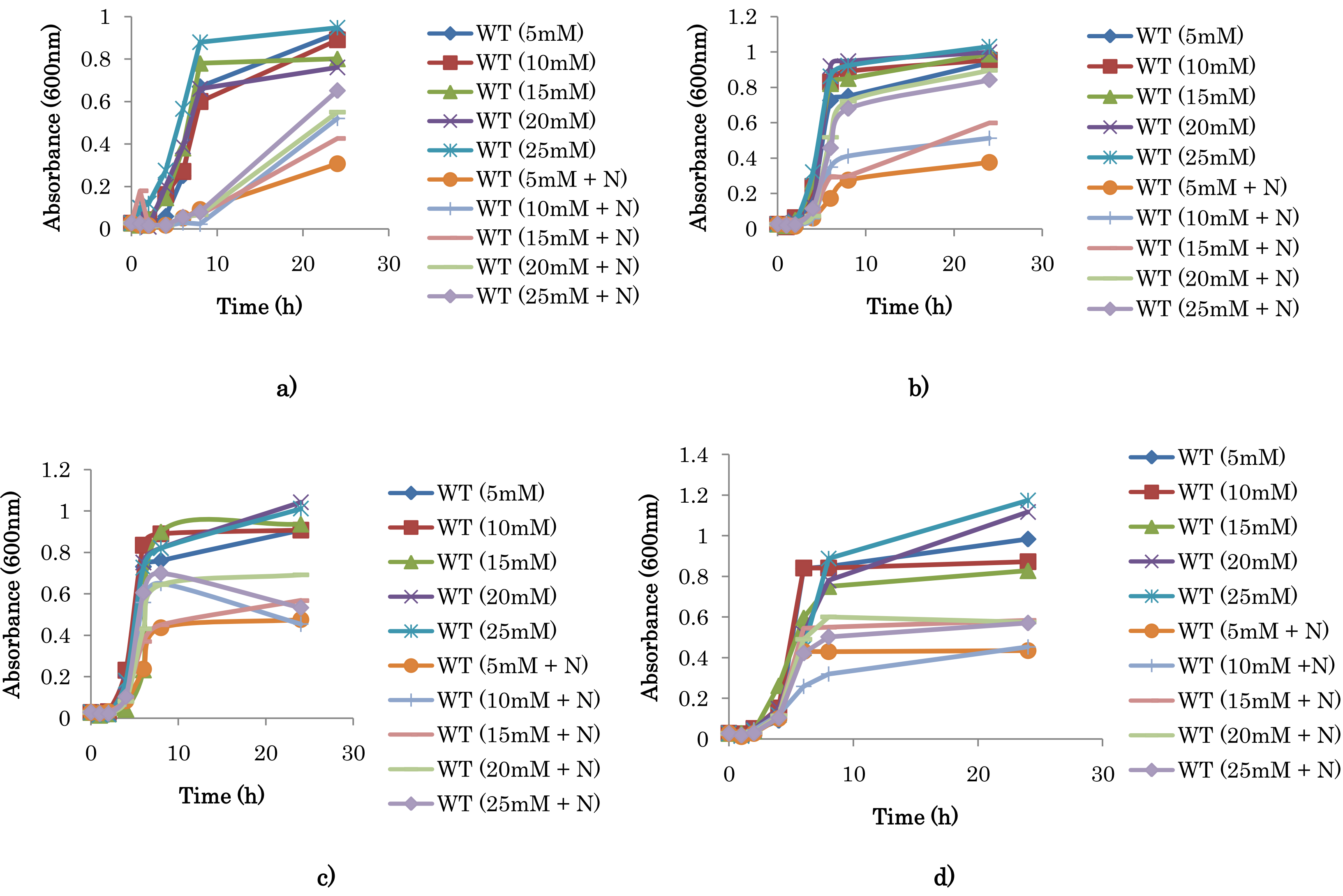


Fig 4.43 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on wild type *L. monocytogenes* MTCC 657 (WT)

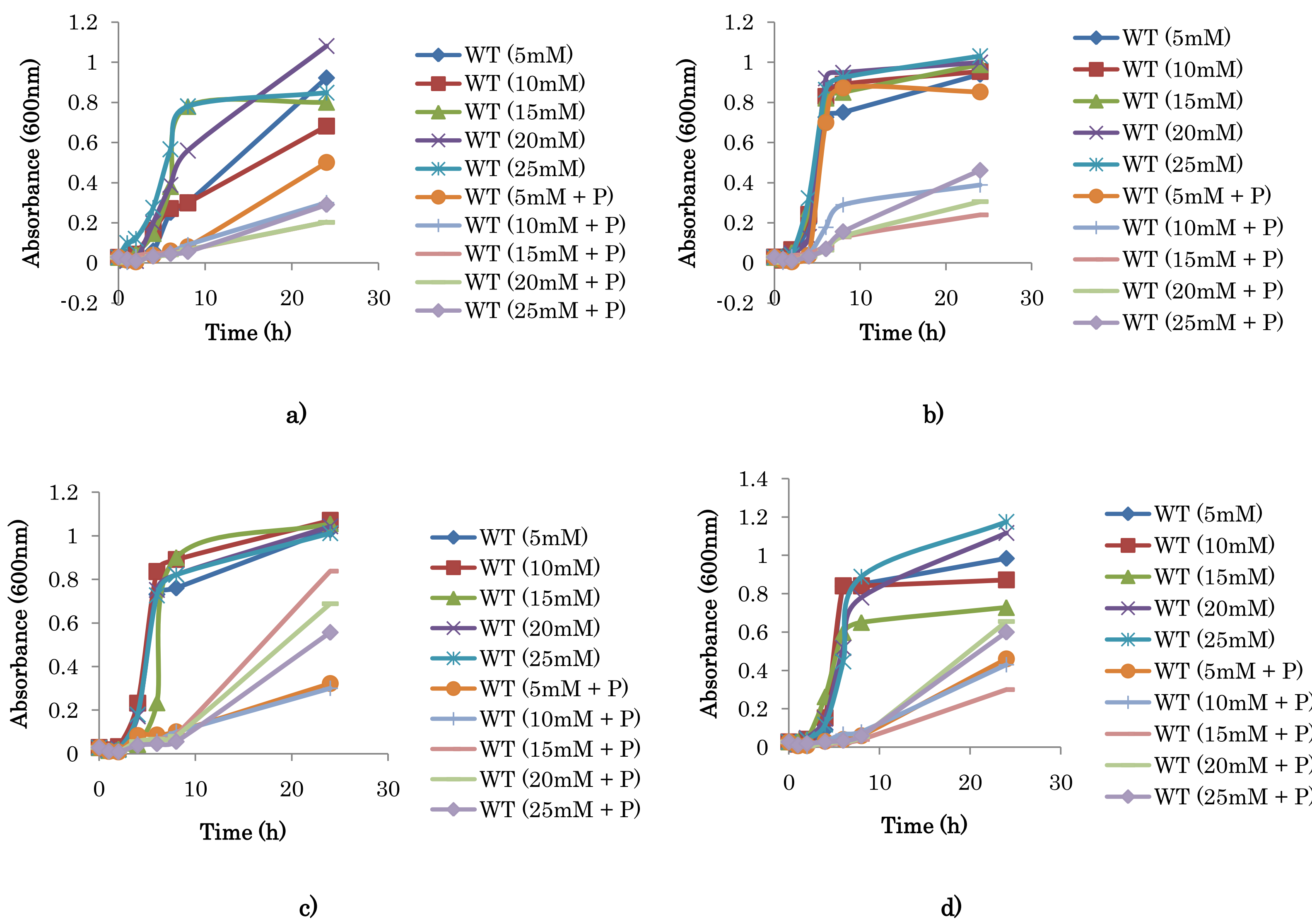


Fig. 4.44 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on wild type *L. monocytogenes* MTCC 657 (WT)

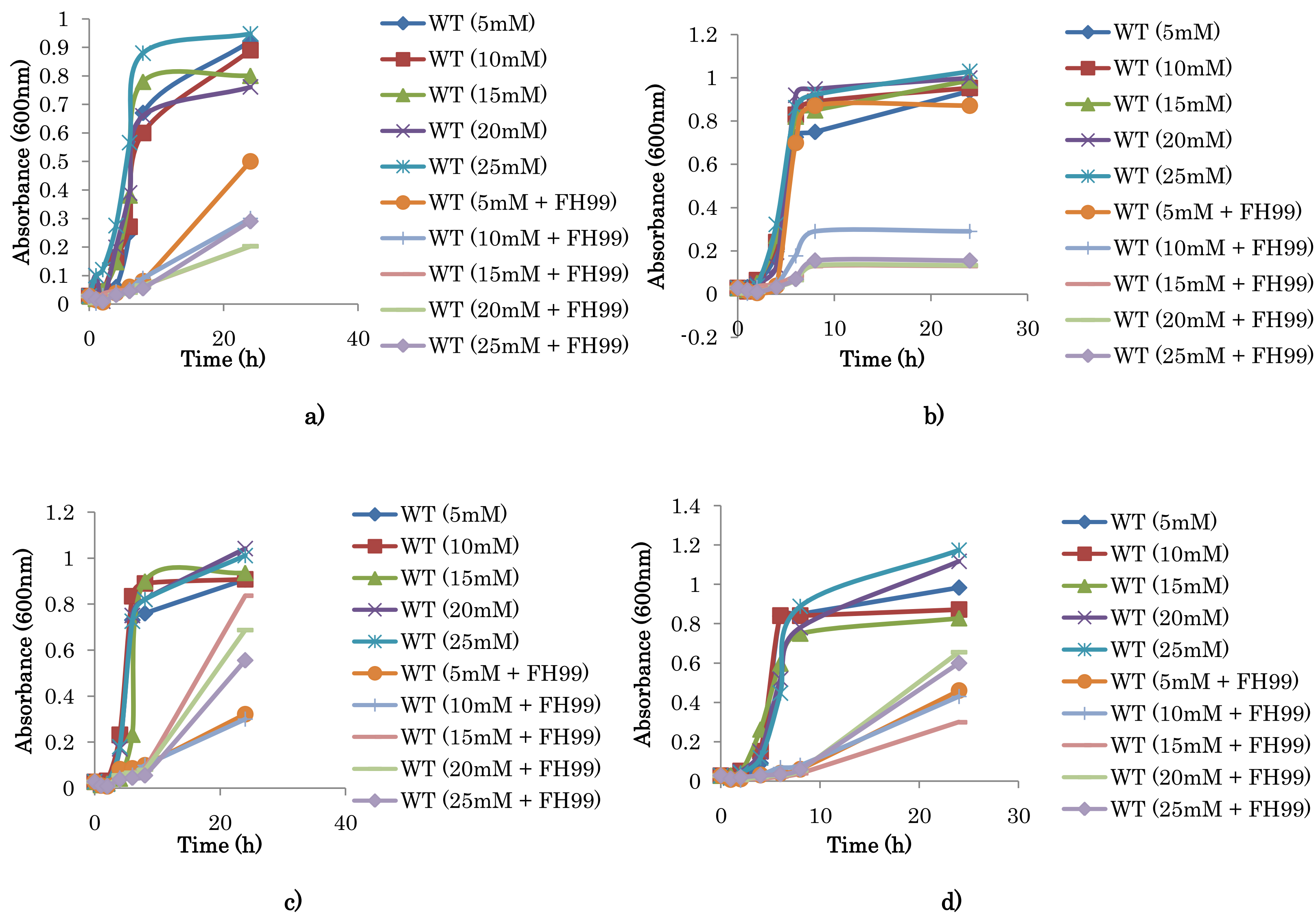


Fig. 4.45 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on Nisin (Nr) resistant variants *L. monocytogenes* MTCC 657

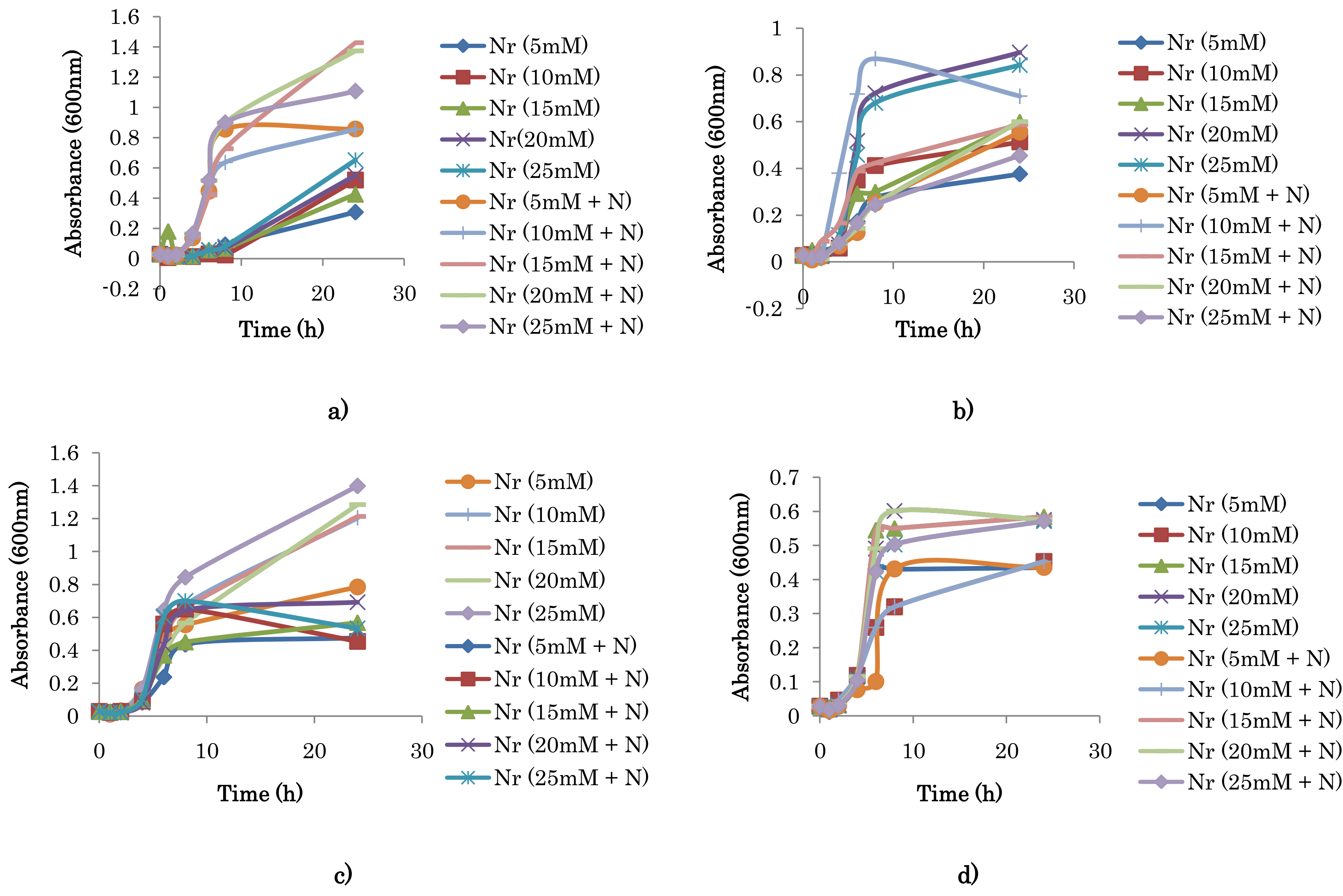


Fig. 4.46 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on Pediocin 34 (Pr) resistant variant of *L. monocytogenes* MTCC 657.

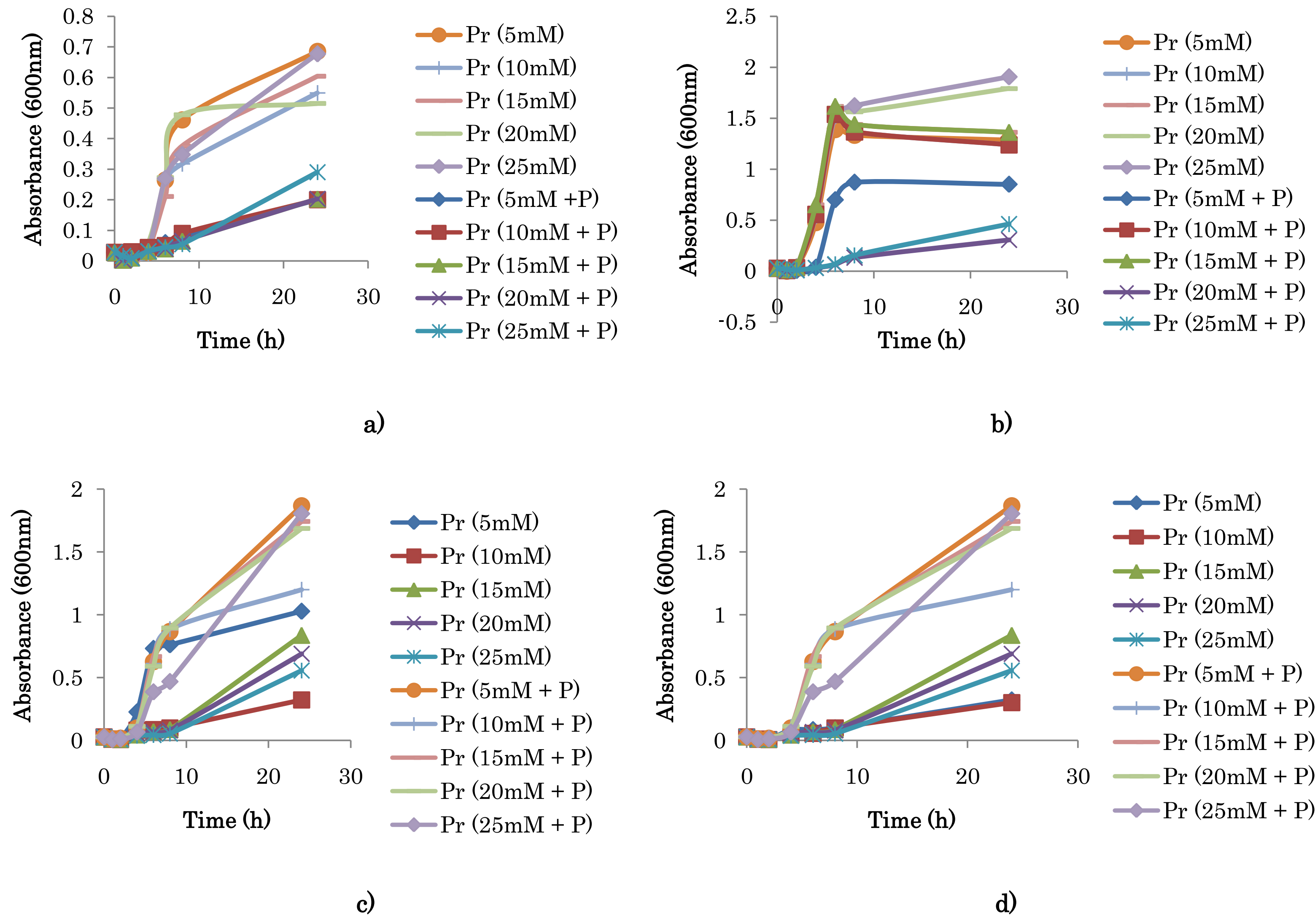


Fig. 4.47 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with enterocin FH99 (FH99) on Enterocin FH99 (Er) resistant variant of *L. monocytogenes* MTCC 657.

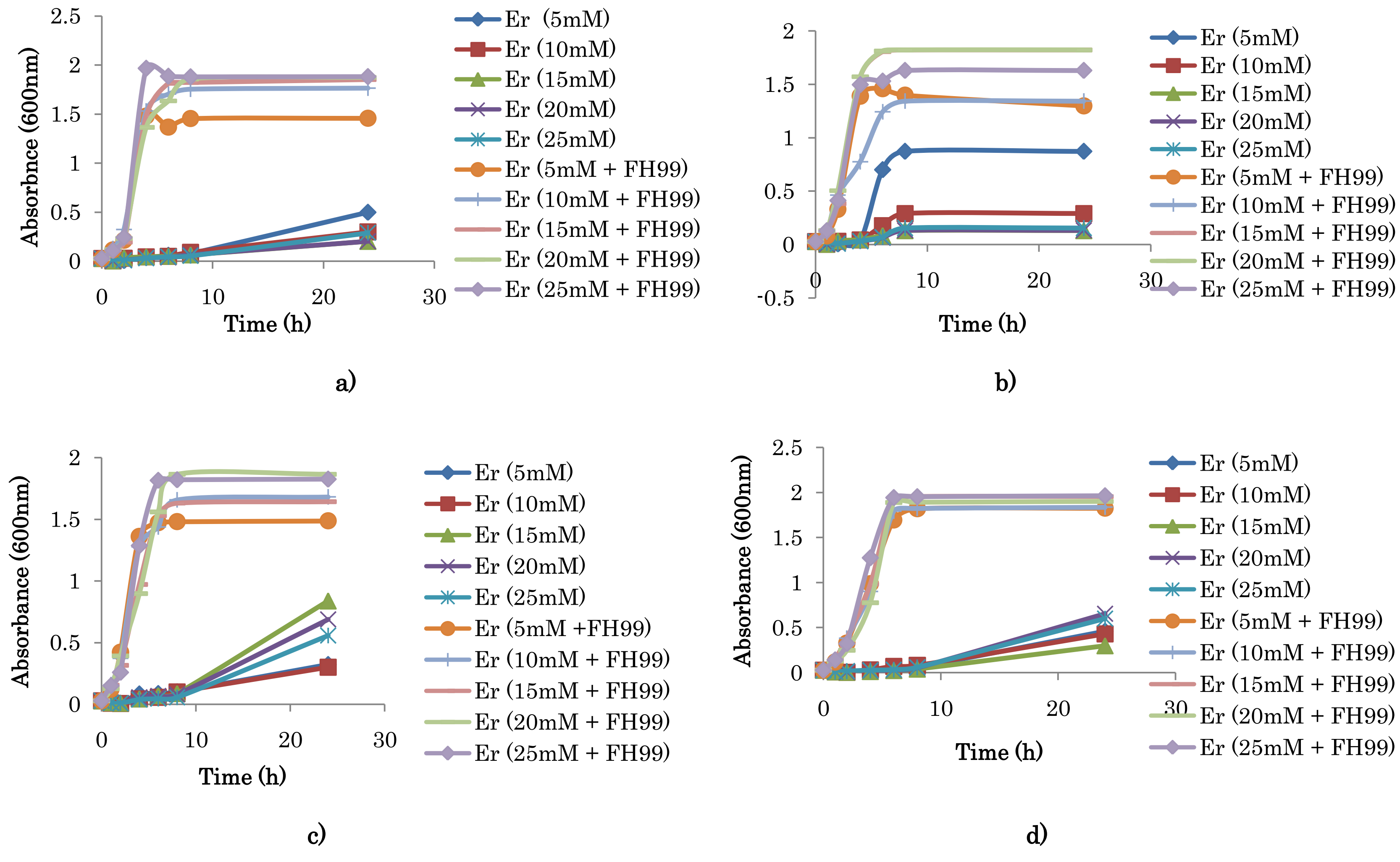


Fig. 4.48 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin(N) on wild type *E. faecium* DSMZ 20477 (WT)

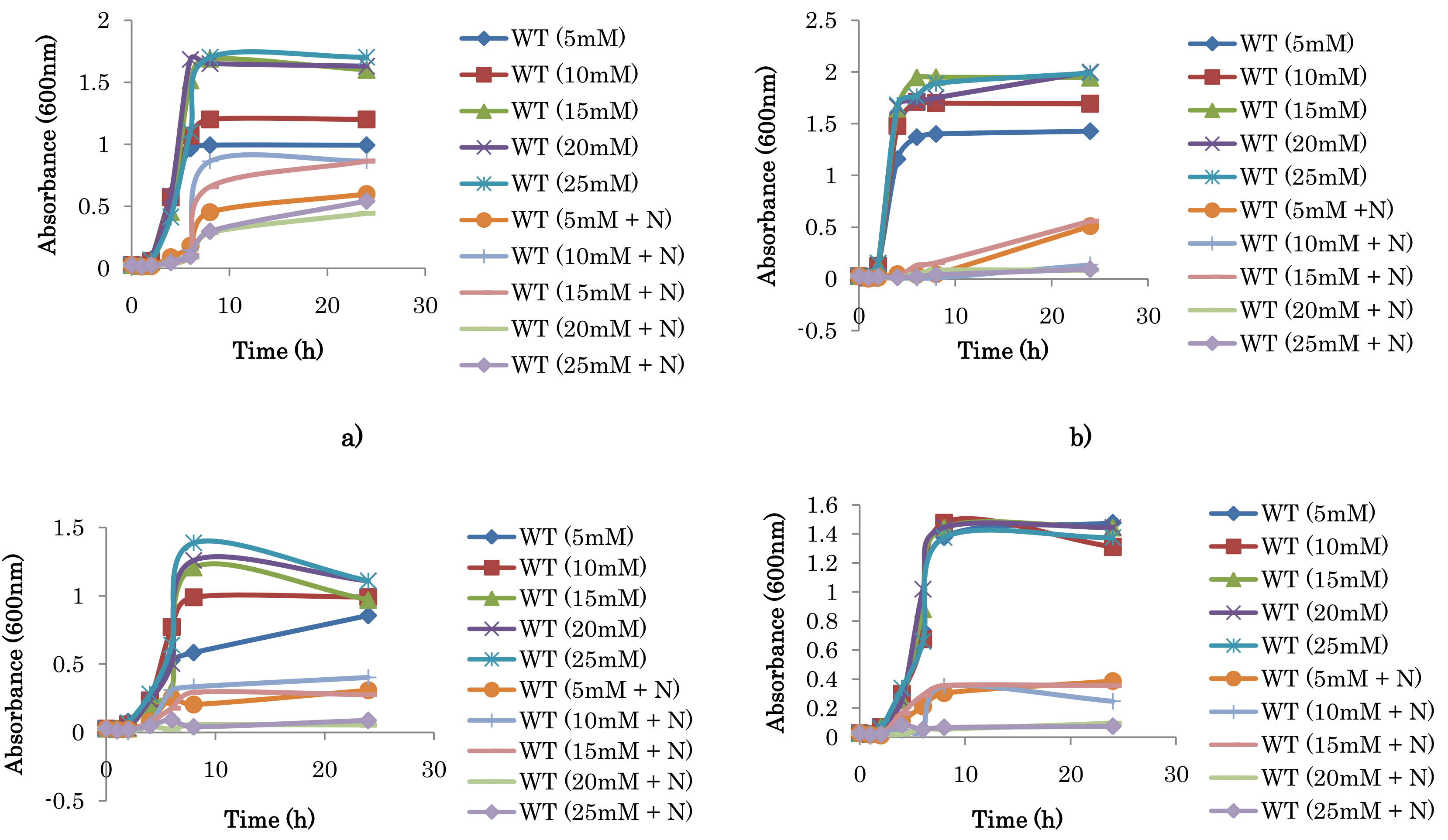


Fig. 4.49 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34(P) on wild type *E. faecium* DSMZ 20477 (WT)

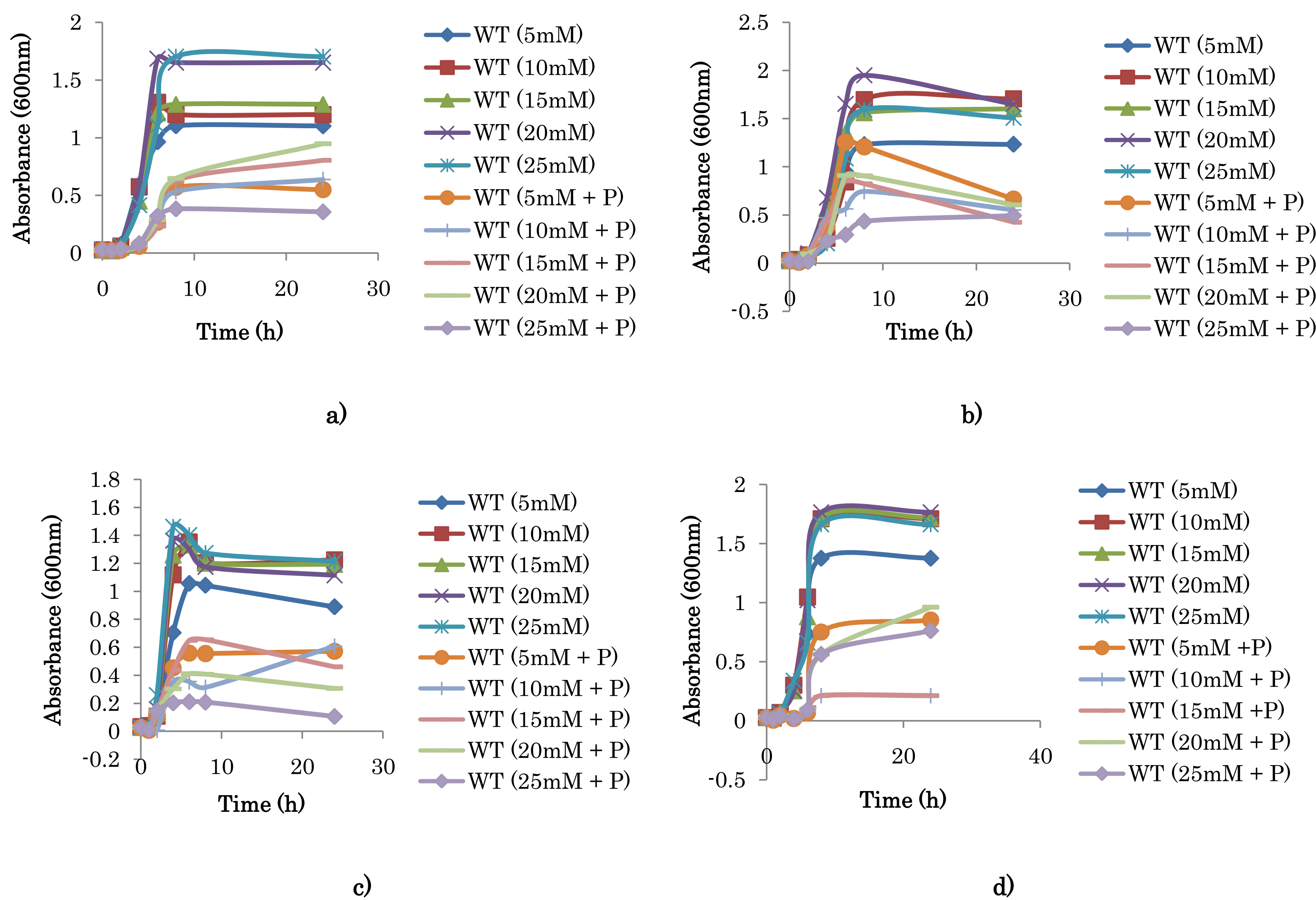


Fig. 4.50 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on wild type *E. faecium* DSMZ 20477 (WT)

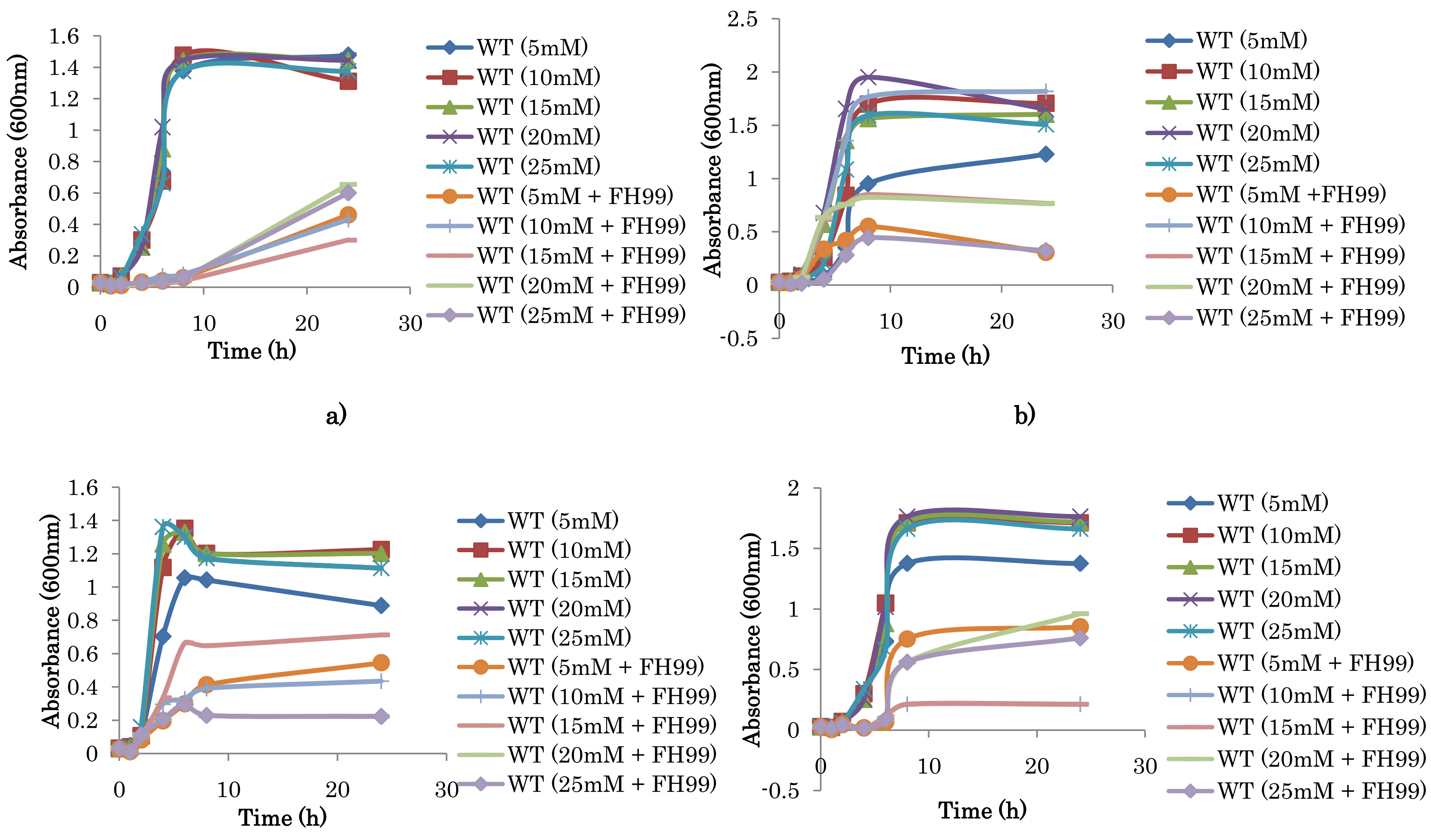


Fig. 4.51 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on Nisin (Nr) resistant variant of *E. faecium* DSMZ 20477

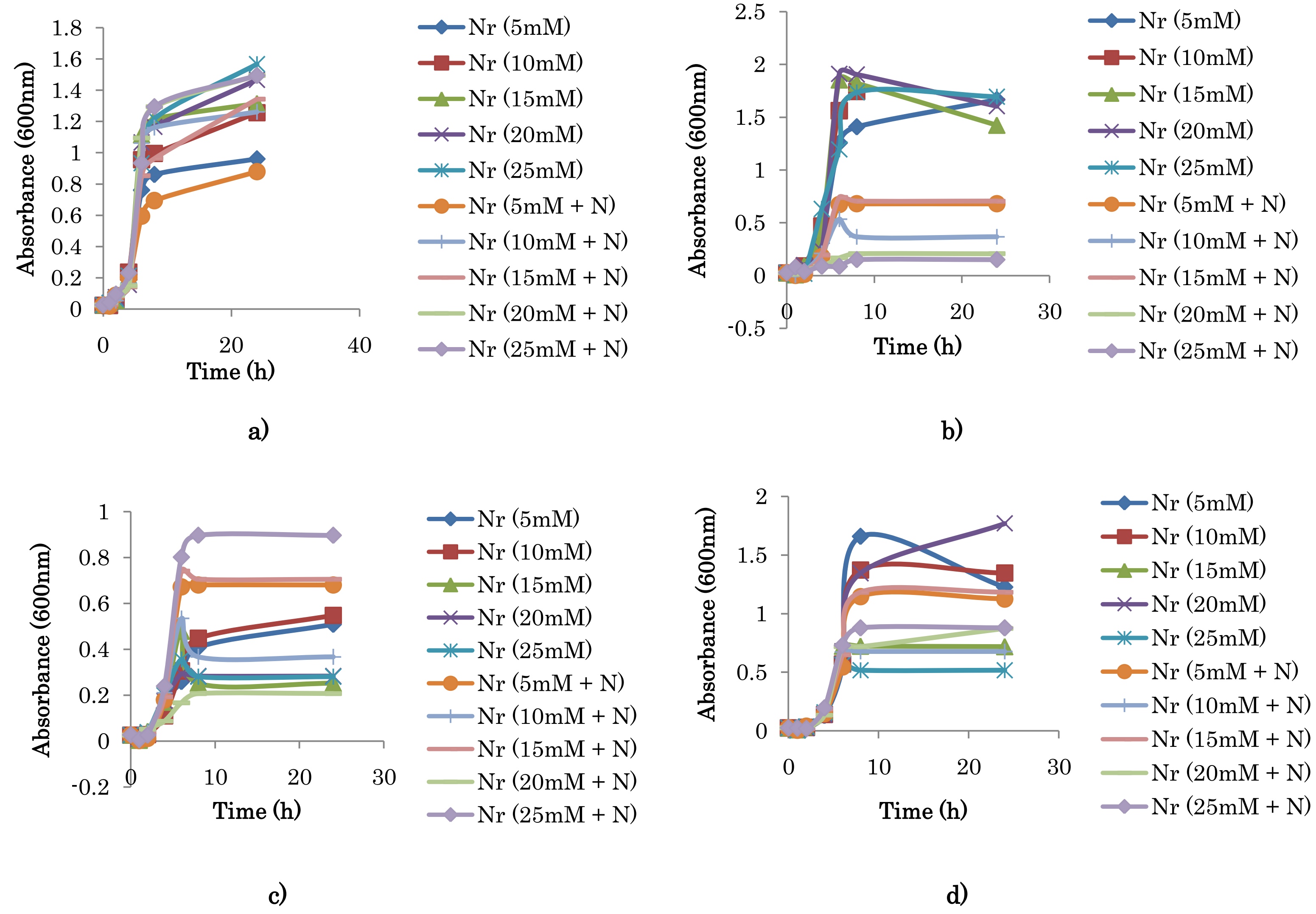


Fig. 4.52 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on Pediocin 34 (Pr) resistant variant of *E. faecium* DSMZ 20477

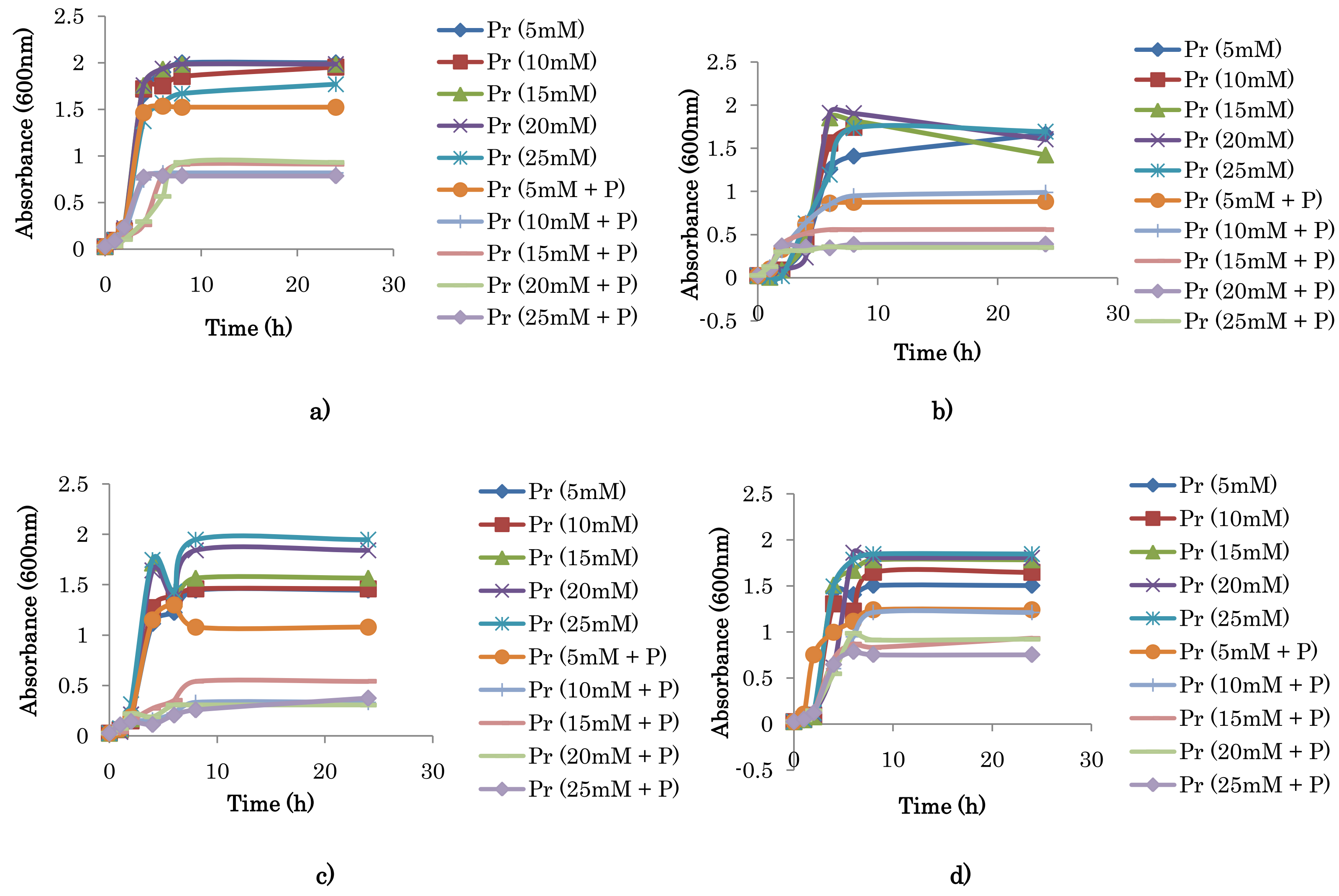


Fig. 4.53 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on Enterocin FH99 (Er) resistant variant of *E. faecium* DSMZ 20477

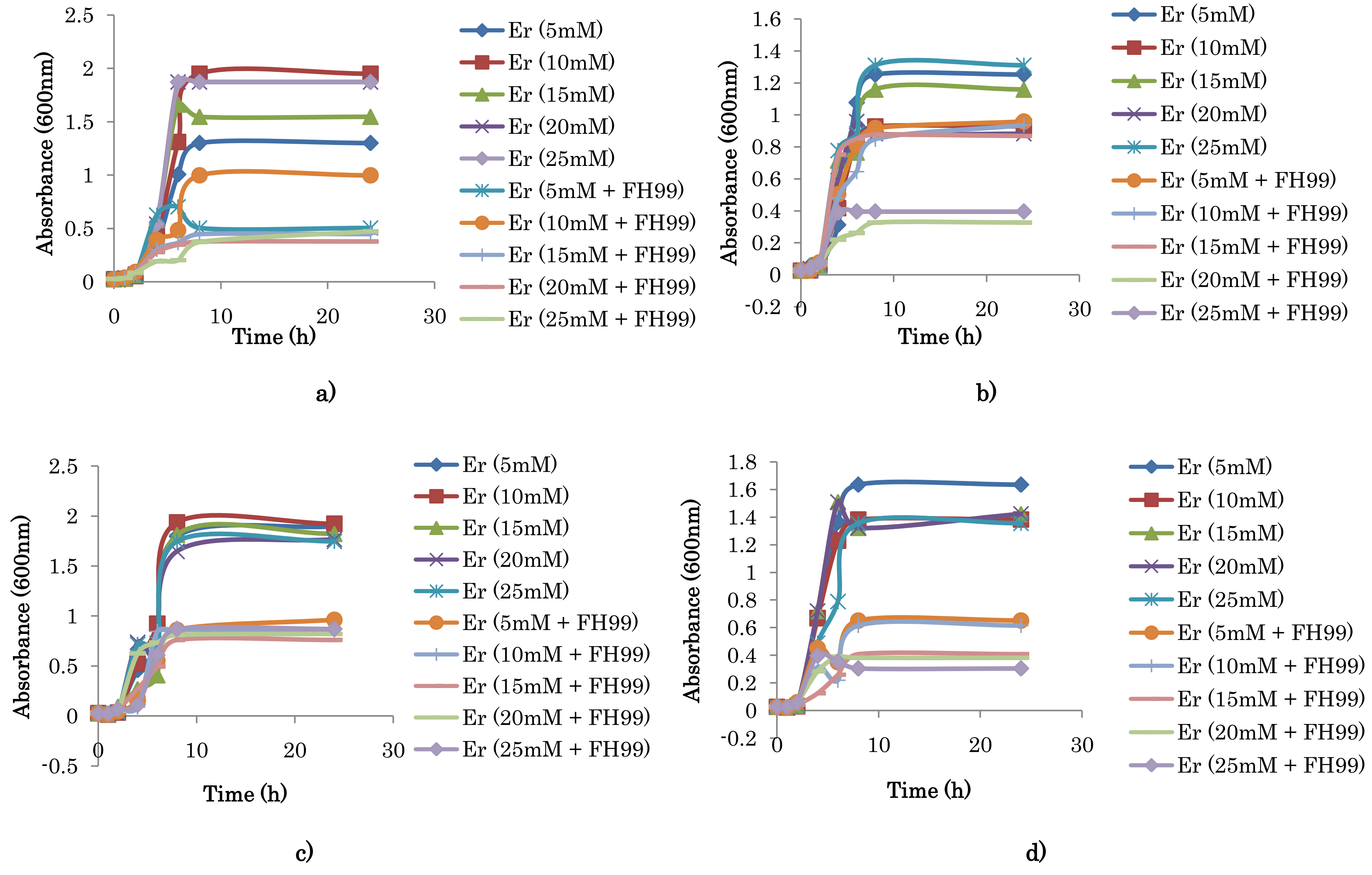
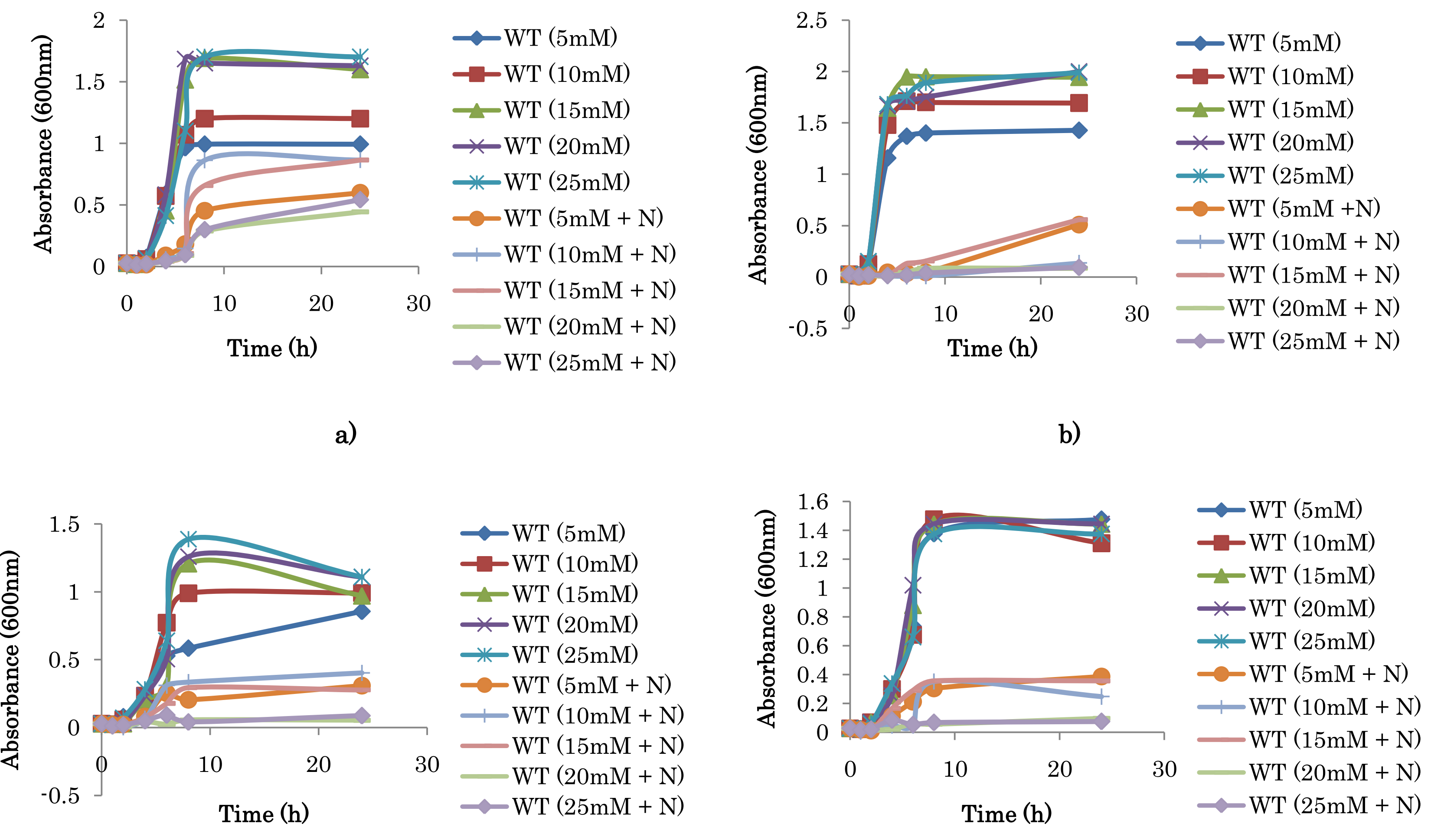


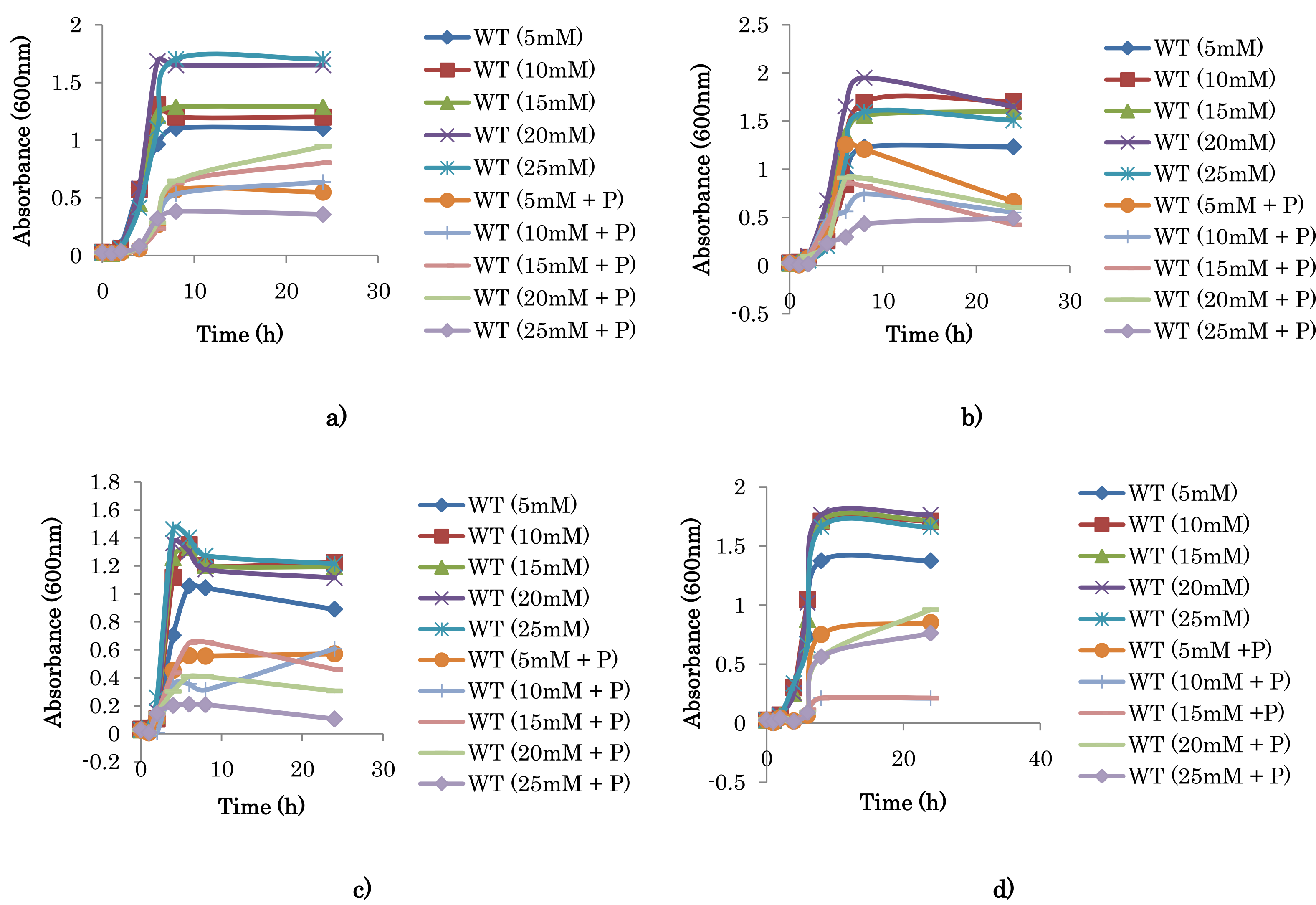
Fig. 4.54 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on wild type *E. faecium* VRE (WT)



c)

d)

Fig. 4.55 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on wild type *E. faecium* VRE (WT)



of enterocin FH99, wild type *E. faecium* VRE showed maximum growth inhibition with 15mM mannose, 25mM glucose, 25mM fructose and 15mM cellobiose (Fig. 4.56). In the absence of bacteriocins the nisin, pediocin 34 and enterocin resistant variants of *E. faecium* VRE were insensitive to the bacteriocins but in the presence of sugars, they regained their sensitivity towards the bacteriocins. Nisin resistant variant regained its sensitivity to nisin in the presence of mannose, glucose, fructose and cellobiose as shown in Fig. 4.57. Similar results were obtained for pediocin 34 resistant variant, maximum inhibition being observed in presence of 25mM mannose, glucose, fructose and cellobiose in presence of pediocin 34 as shown in Fig. 4.58. Also, enterocin FH99 resistant variant showed increased sensitivity towards enterocin FH99 in presence of mannose, glucose, fructose and cellobiose (Fig. 4.59).

Fig. 4.60 shows that, in the absence of nisin, growth of *E. faecalis* ATCC 29212 increases along with mannose, glucose, fructose and cellobiose concentration whereas, in the presence of nisin growth inhibition increases. In the presence of nisin, maximum growth inhibition was observed at 5mM glucose, mannose and fructose whereas maximum growth inhibition was observed with 25mM cellobiose in the presence of nisin. Fig. 4.61 shows that in the absence of nisin, growth of nisin resistant variant increased with the addition of sugars, however, in the presence of nisin, the nisin resistant variant regained its sensitivity towards nisin.

These results show that mannose, glucose, fructose and cellobiose have an effect on *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 sensitivity to nisin, pediocin 34 and enterocin FH99. It underlines that the level of sensitivity is tightly linked to sugar availability, suggesting that sugars such as mannose, glucose, fructose and cellobiose directly caused expression of a molecule responsible for sensitivity to nisin, pediocin 34 and enterocin FH99.

These sugars also induced expression of the *mpt* operon of *L. monocytogenes* (not shown in *E. faecalis*), indicating that EII_t^{Man} transports glucose and mannose in accordance with previous observations showing a specific inducible effect of the transported sugar on PTS permease expression (Postma *et al.*, 1993). These correlated results suggest that the level of EII_t^{Man} expression is directly linked to sensitivity of *L. monocytogenes* to mesentericin Y105. Similar results have also been reported by Dalet *et al.* (2001) wherein glucose and mannose specifically induce sensitivity of *L. monocytogenes* EGDe to mesentericin Y105. In our study we observed that the level of sensitivity of nisin resistant variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 is also linked to sugar availability.

4.10.7 Identification of Genes Associated With Resistance Strains to Bacteriocins by RT-PCR Analysis

Class IIa bacteriocins are antibacterial peptides produced by lactic acid bacteria that inhibit the food-borne pathogen *Listeria monocytogenes* active bacteriocins from the subclass IIa; share a consensus YGNGV peptidic motif and an anti-*Listeria* activity. Moreover, most of them are also active against *Enterococcus faecalis*. (Ennahar *et al.*, 2000). These peptides have been shown to exert their activity by dissipation of the proton motive force of susceptible cells, possibly via membrane pore formation (Chikindas *et al.*, 1993; Montville & Chen, 1998; He´chard & Sahl, 2002). Several mechanisms of interaction with the target cell have been proposed, including electrostatic binding to the membrane and/or specific binding to a membrane-associated component (Abee, 1995; He´chard & Sahl, 2002; Montville & Chen, 1998). Protease treatment of membrane vesicles, derived from sensitive cells, resulted in an increased resistance to pediocin PA-1 (Chikindas *et al.*, 1993). This was the first indication of a proteinaceous membrane component that may interact with the bacteriocin. The requirement of a chiral interaction for leucocin A activity further strengthens the hypothesis that a specific interaction between a bacteriocin and a membrane compound in the target strain is required for inducing sensitivity (Yan *et al.*, 2000). The subunit IIAB of a mannose permease of the phosphotransferase

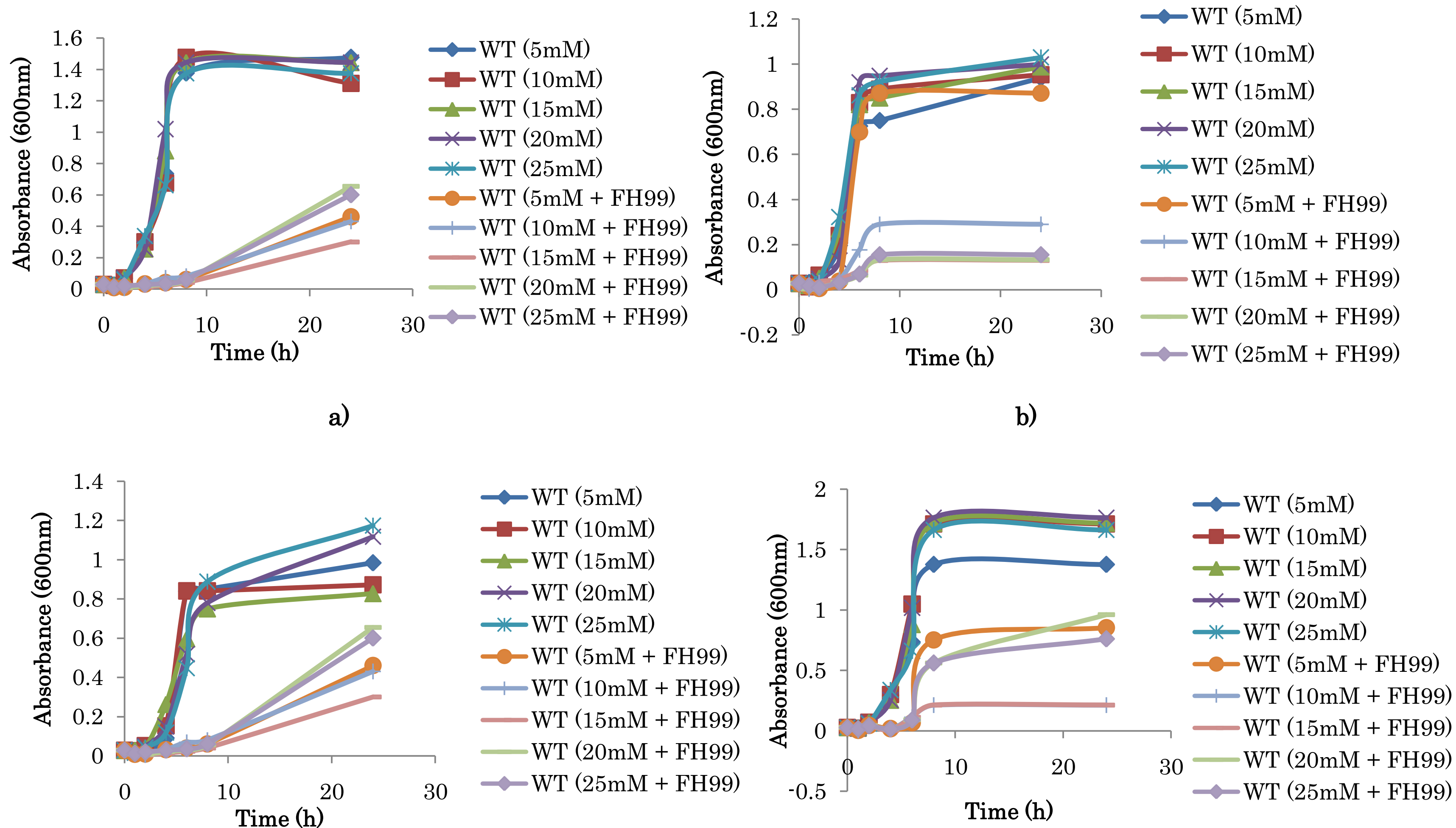


Fig. 4.56 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on wild type *E. faecium* VRE (WT)

Fig. 4.57 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on Nisin (Nr) resistant variant of *E. faecium* VRE

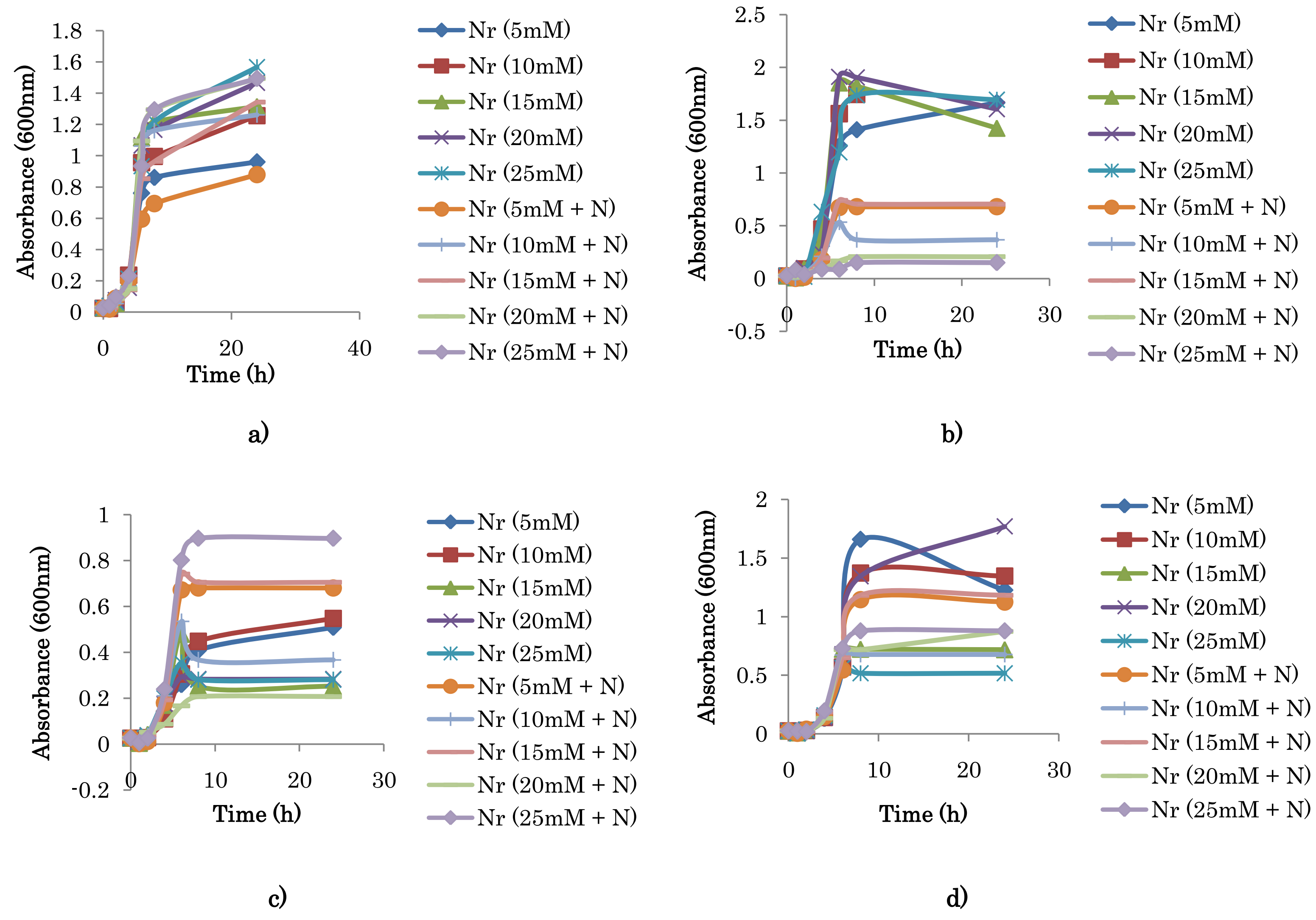


Fig. 4.58 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on Pediocin 34 (Pr) resistant variant of *E. faecium* VRE

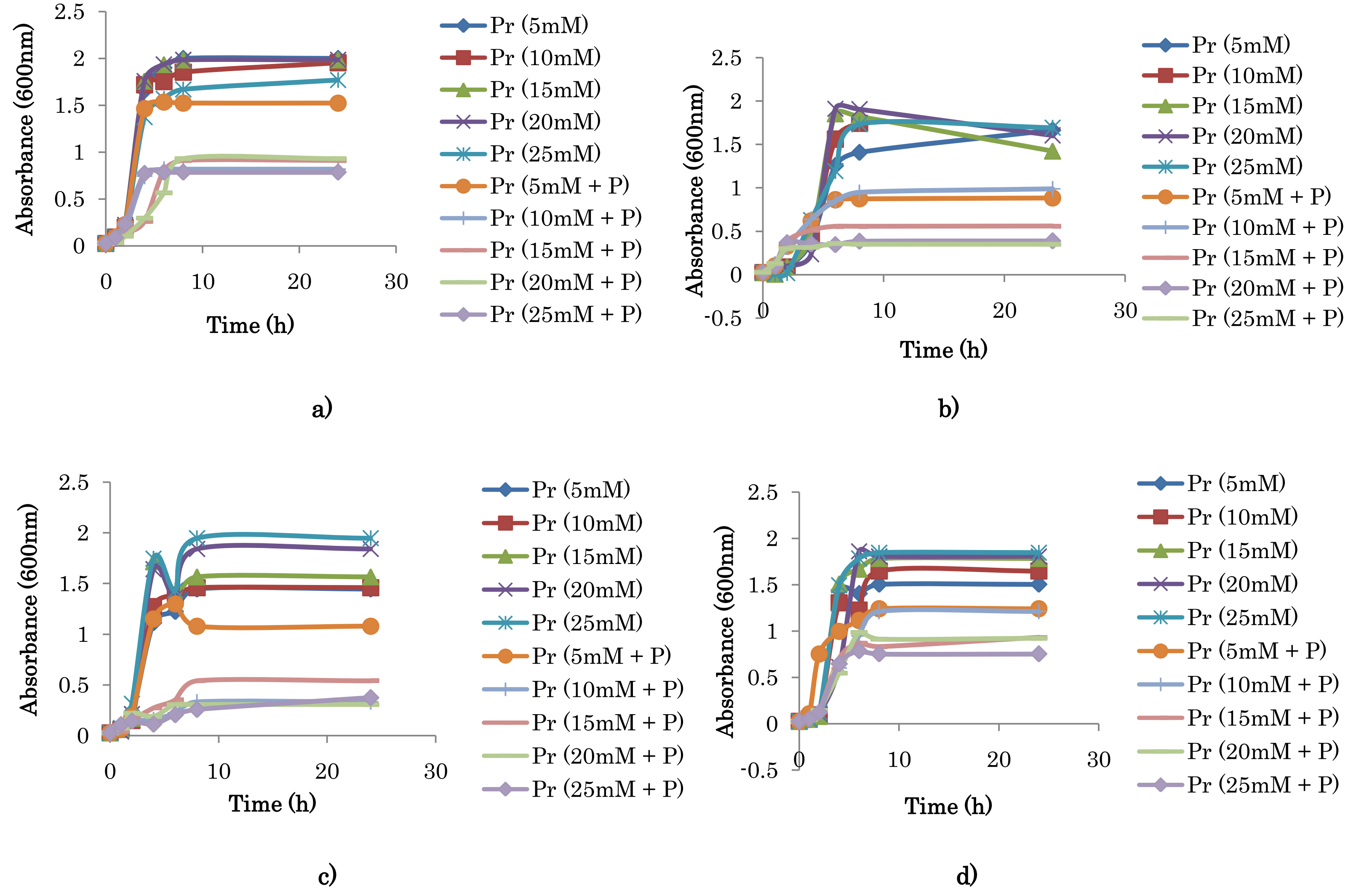


Fig. 4.59 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on Enterocin FH99 (Er) resistant variant of *E. faecium* VRE.

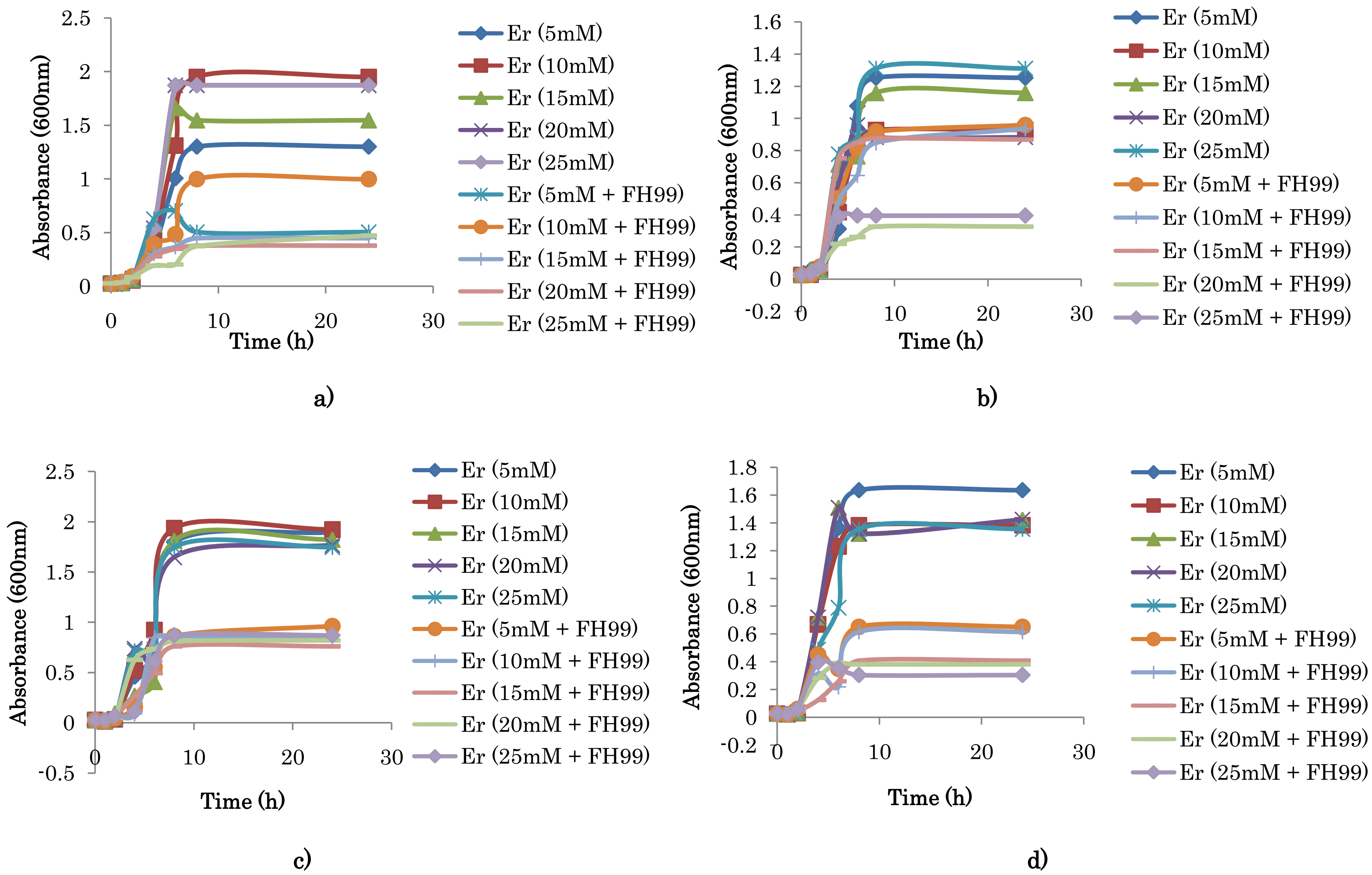


Fig. 4.60 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on wild type *E. feacalis* ATCC 29212 (WT)

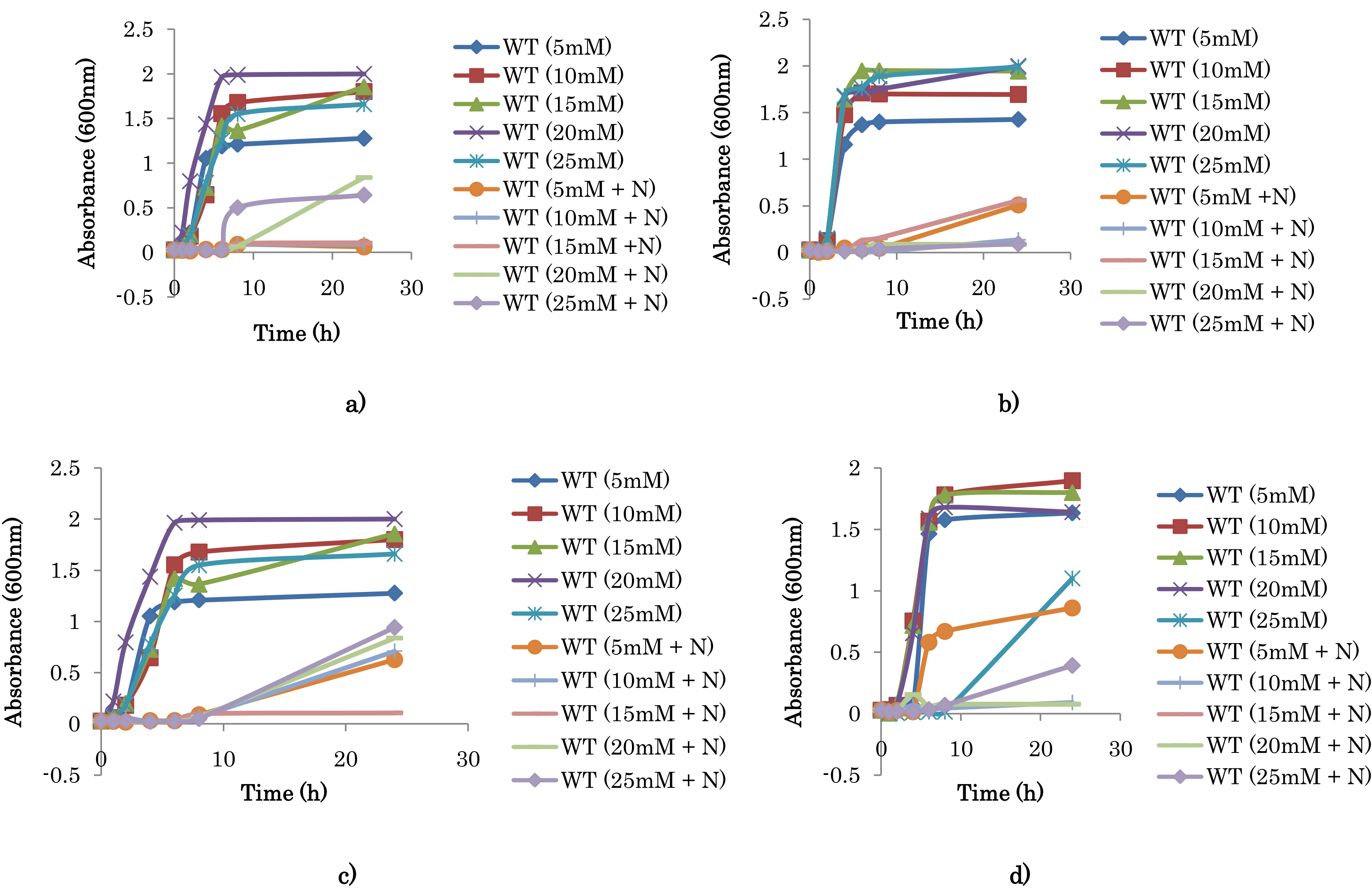
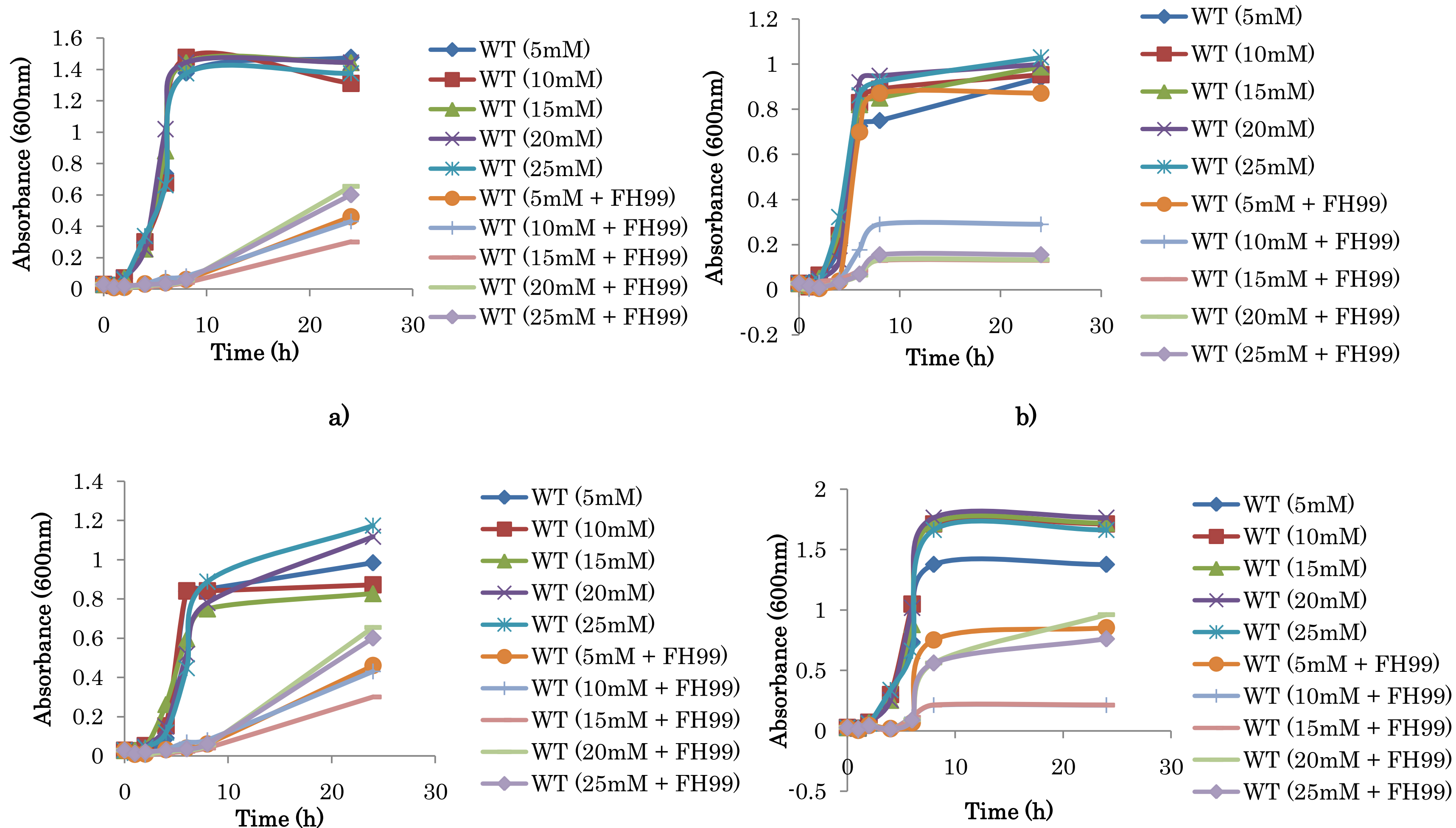


Fig. 4.56 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on wild type *E. faecium* VRE (WT)



c) d)

Fig. 4.57 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on Nisin (Nr) resistant variant of *E. faecium* VRE

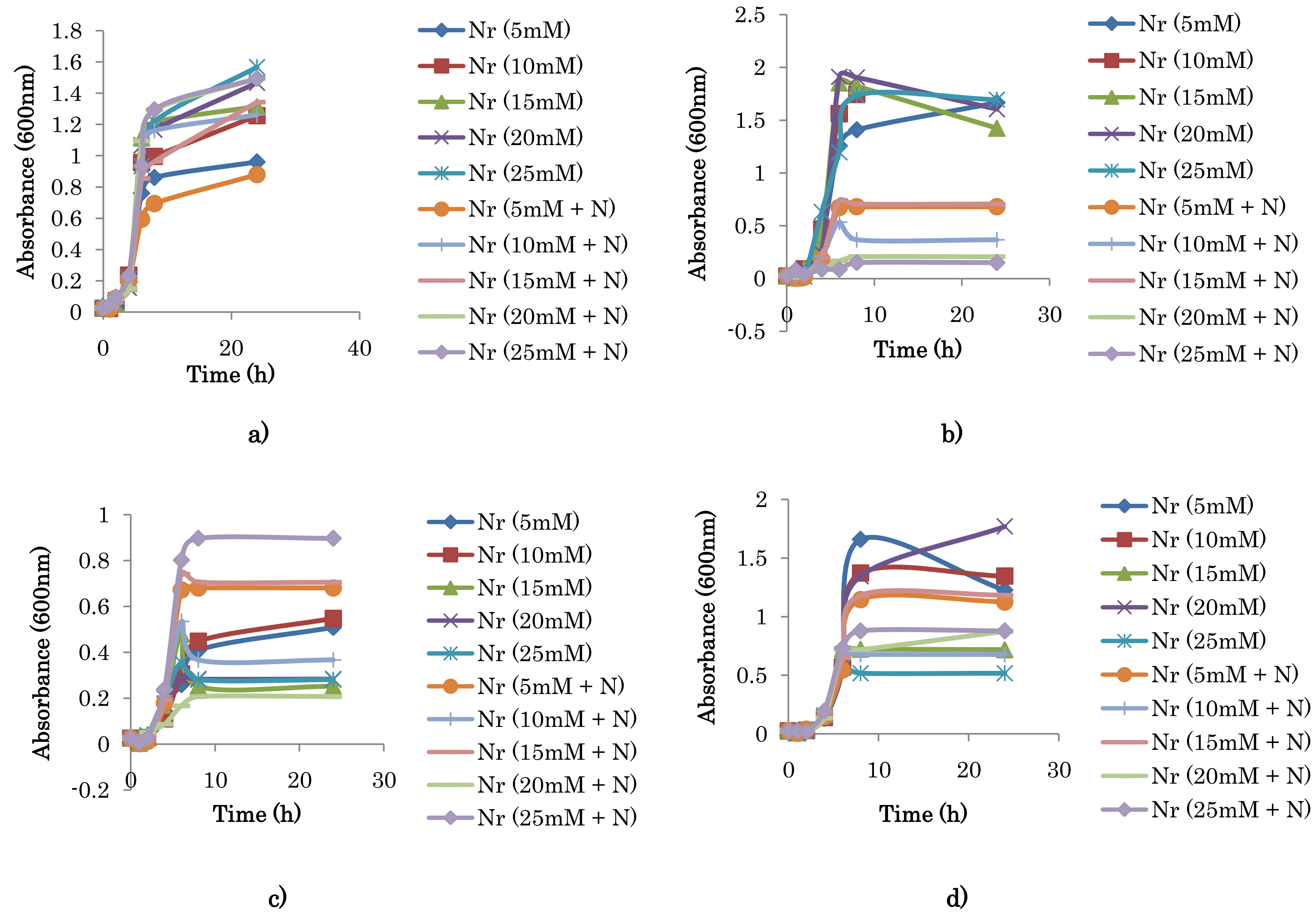


Fig. 4.58 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Pediocin 34 (P) on Pediocin 34 (Pr) resistant variant of *E. faecium* VRE

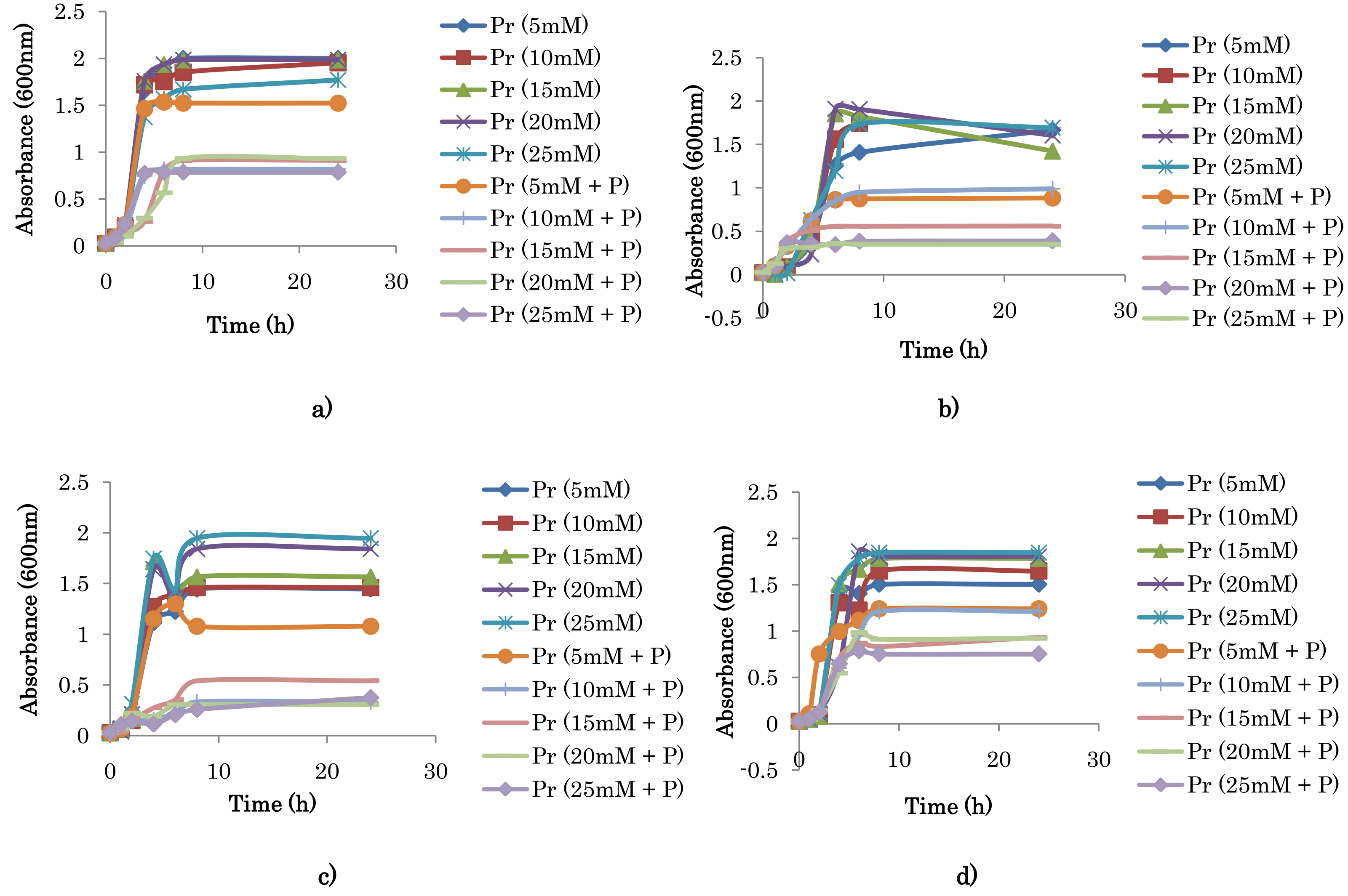


Fig. 4.59 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Enterocin FH99 (FH99) on Enterocin FH99 (Er) resistant variant of *E. faecium* VRE.

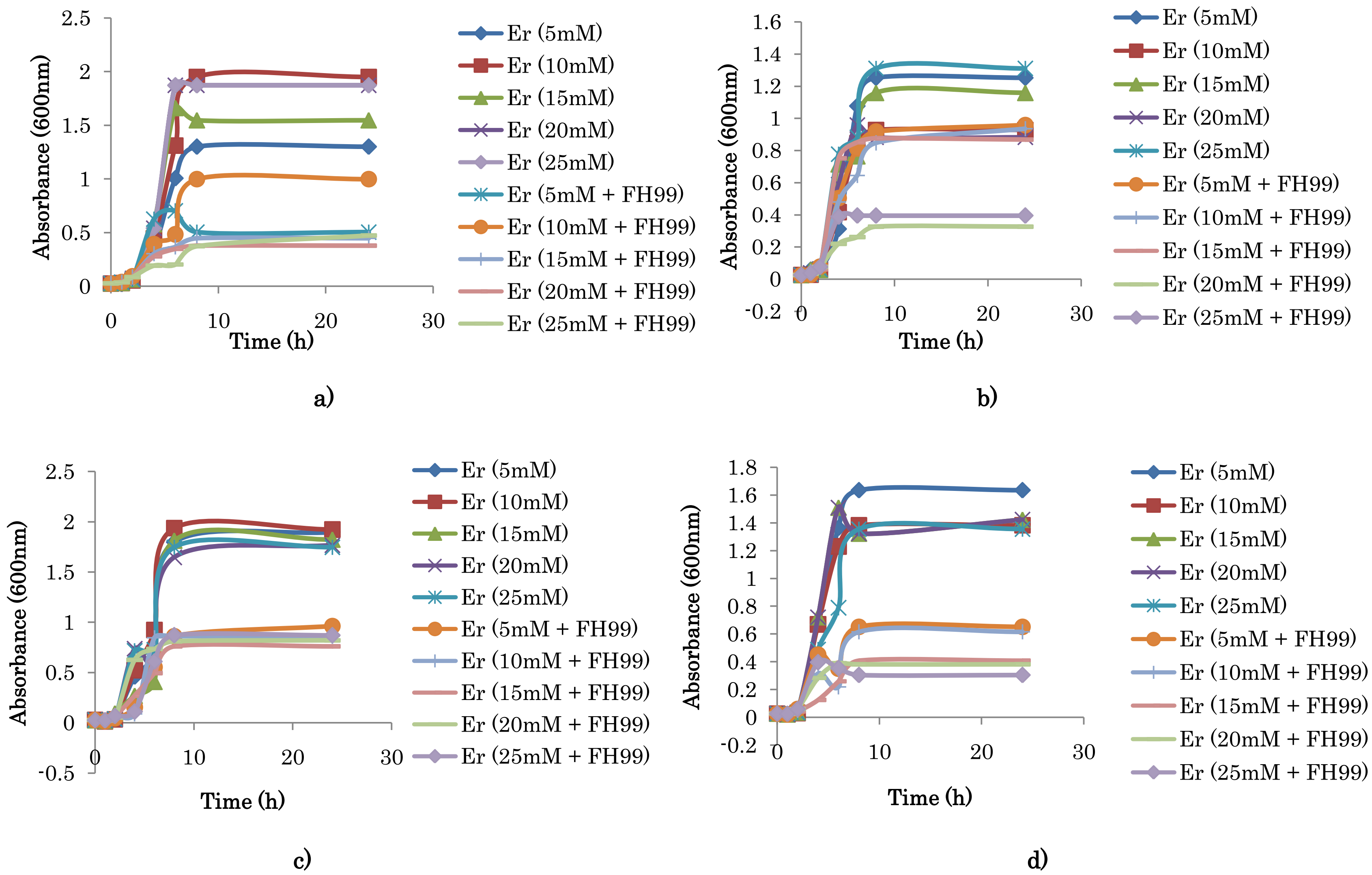


Fig. 4.60 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on wild type *E. feacalis* ATCC 29212 (WT)

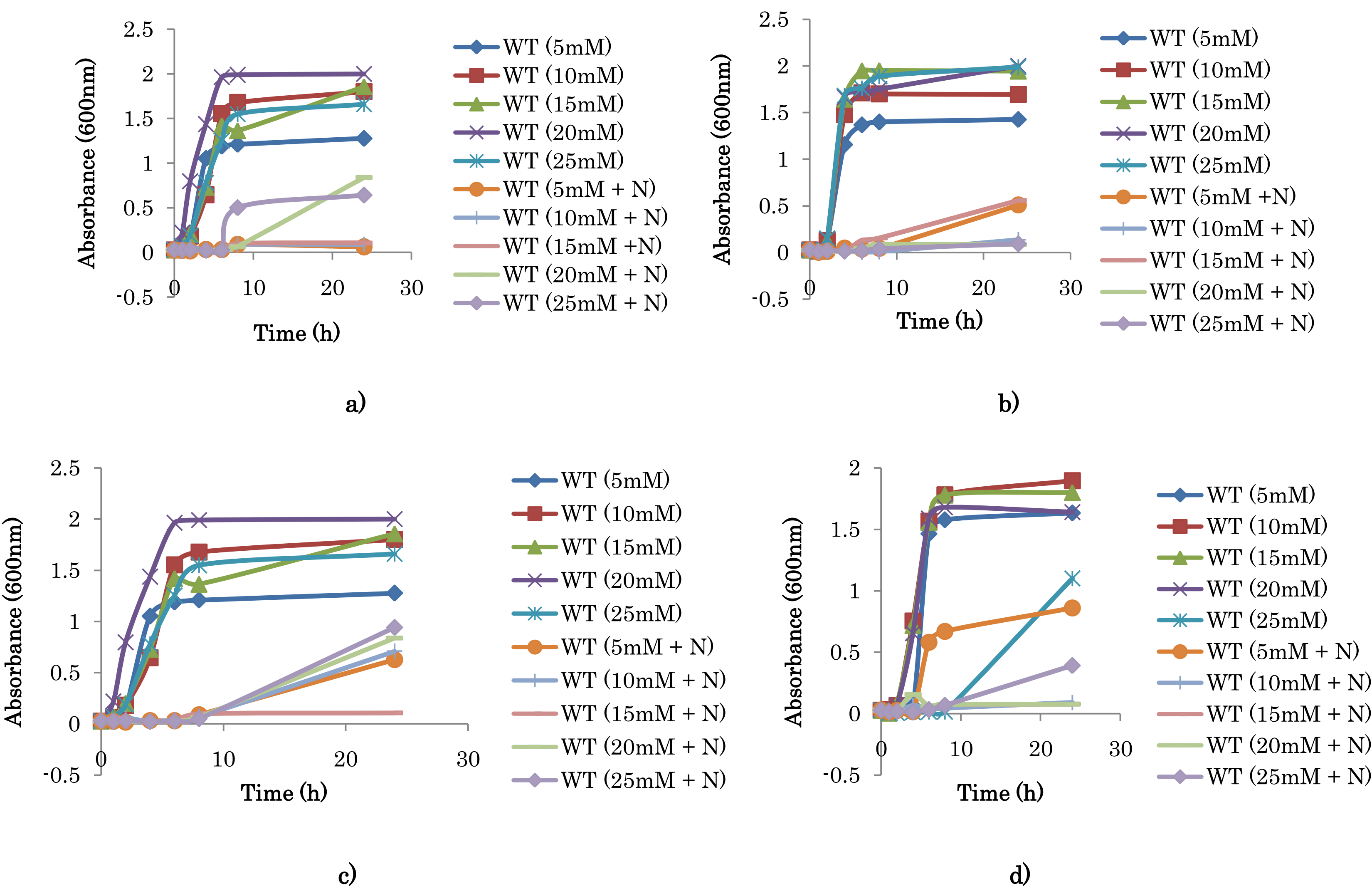
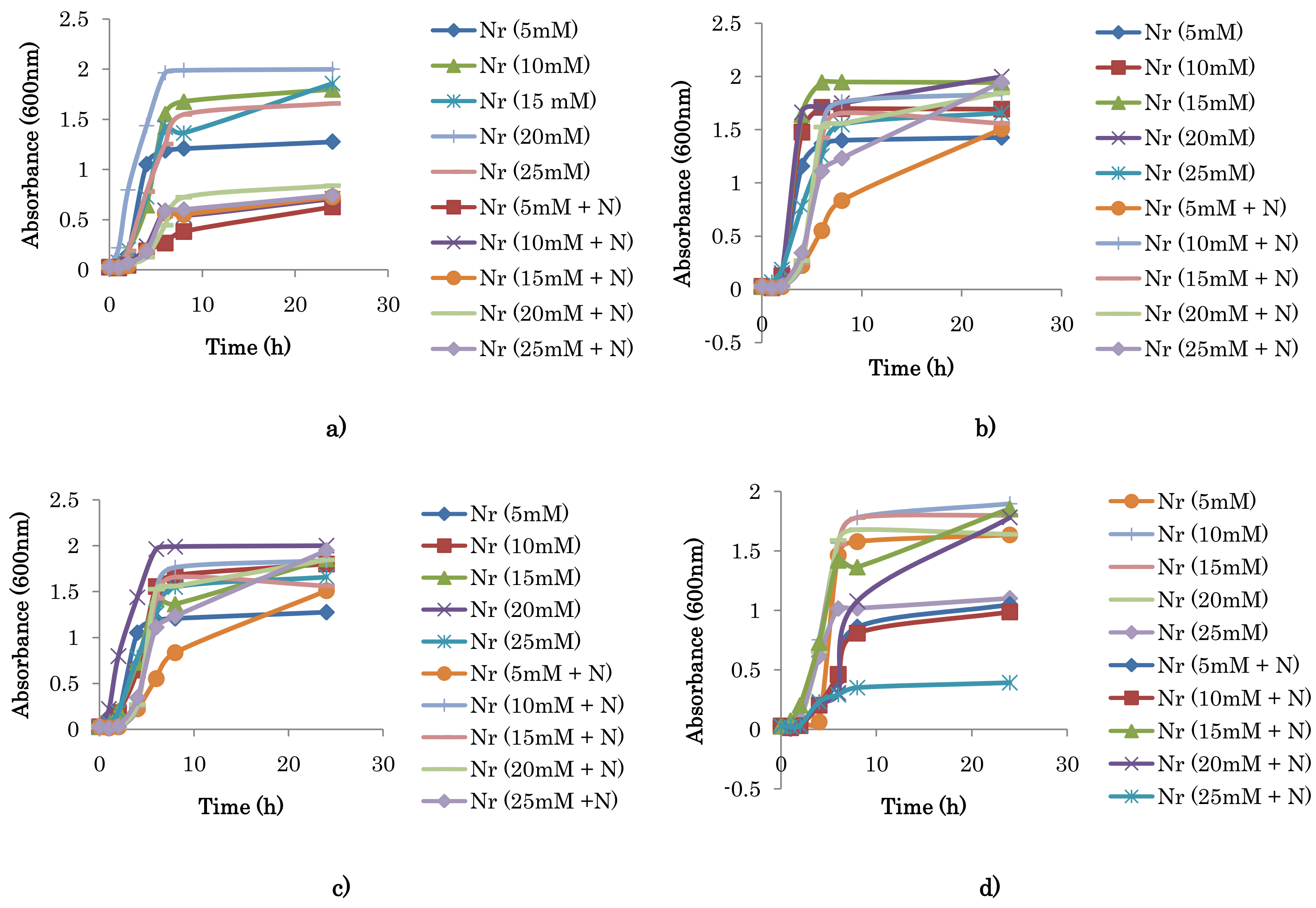


Fig. 4.61 Effect of different concentrations (5mM, 10mM, 15mM, 20mM and 25mM) of a) Mannose b) Glucose c) Fructose and d) Cellobiose alone and in combination with Nisin (N) on nisin resistant variant (Nr) of *E. feacalis* ATCC 29212



system (PTS) was found to be missing in a spontaneous mutant of *L. monocytogenes* with resistance to leucocin A, a class IIa bacteriocin (Ramnath *et al*., 2000). The same observation was made for a number of spontaneous mutants of *L. monocytogenes* showing high-level resistance to class IIa bacteriocins (Gravesen *et al*., 2002). This IIAB is part of the EII_t^{Man} permease encoded by the mptACD operon (Dalet *et al*., 2001). Genetic inactivation of the mptACD operon resulted in resistance to mesentericin Y105 in *Listeria monocytogenes* and *Enterococcus faecalis* (Dalet *et al*., 2001; He´chard *et al*., 2001). Thus EII_t^{Man} has been proposed as a requirement for inhibition of the target cell by class IIa bacteriocins.

The aim of this part of the study was to further understand the underlying mechanisms of class IIa resistance in, *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE).

4.10.7.1. RNA Isolation and cDNA Synthesis

The total RNA from each culture was extracted with Rneasy mini kit as per manufacturer's instruction. Purity of the total RNA was determined as the 260/280 nm ratio and the integrity of RNA was checked by electrophoresing on 1.2% agarose gel. Some of the representative samples of RNA extracted from the wild type *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their nisin, pediocin 34 and enterocin FH99 resistant variants is shown in Plate 4.12. Residual DNA was removed by treating RNA with RNase free DNAse by on column digestion in Rneasy kit as per manufacturer's instruction. An aliquot of one µg of the DNase treated RNA was transcribed into cDNA, using Revertaid Kit Premium First Strand cDNA Synthesis Kit (Fermentas), and hexamer random primers. The specificity of the primers was checked

4.10.7.2. Specificity of primers

DNA was extracted from the wild type i.e bacteriocin sensitive strains of type *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE and their nisin, pediocin 34 and enterocins FH99 resistant

variants. The specificity of the primers was checked by PCR. It is evident from Plate 4.13 that the primers designed for GAPDH (housekeeping gene) for *L. monocytogenes* strain and *Enterococcus* strain resulted into amplicons of expected size i.e. 234 bp. It is evident from Plate 4.14 that the primers designed for IIC, IID, σ^{54} and *glpQ* genes of *mpt* operon for *L. monocytogenes* strains resulted into amplicons of expected sizes i.e. 102, 123, 161 and 150 bp, respectively. Similarly, the primers designed for IIC, IID, σ^{54} and *glpQ* genes of *mpt* operon for *Enterococcus* strains resulted into amplicons of expected sizes i.e. 100, 220, 188, 191 bp, respectively as shown in Plate 4.15

4.10.7.3 Expression of σ^{54}, IIC, IID and *glpQ* genes in *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* (VRE)

In order to gain insight to the mechanism of action of nisin, pediocin 34 and enterocin FH99, comparative analysis of the expression of *mpt* operon was studied by real-time PCR in bacteriocin sensitive strains of type *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 and their nisin, pediocin 34 and enterocins FH99 resistant variants. The experiments were performed in triplicate from each of two independent total RNA extractions. The real-time PCR experiments were performed using the Sybr Master Mix to quantify the transcription level of the mpt operons. Primers and probes were designed from the IIC, IIB, IID and gene encoding sigma 54 sequences of the *L. monocytogenes* and *Enterococcus faecium* genome using Primer 3 software. All the selected primers and probes are listed in the Table 2. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The $2^{-\Delta\Delta CT}$ method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments. Real time analysis generates the threshold cycle value (CT), which is defined as the cycle at which a significant increase in amplification product occurs. The CT value is inversely proportional to the amount of starting template RNA. For each target gene, the average CT value was calculated from triplicate reactions for RNA samples from each of two independent

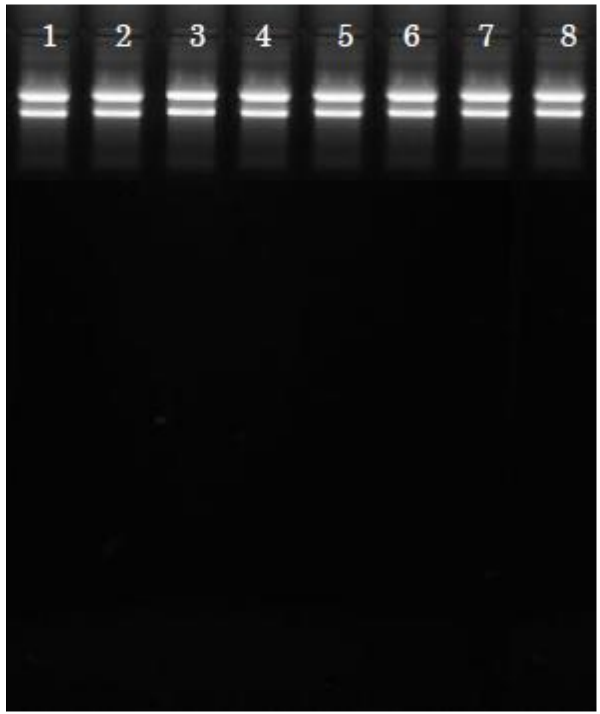


Plate 4.12. Electrophoretic profile of RNA isolated using Qiagen RNeasy Kit

Lane 1: Wild Type *L. monocytogenes* ATCC 53135; **Lane 2**: Nisin resistant variant of *L. monocytogenes* ATCC 53135; **Lane 3:** Pediocin 34 resistant variant of *L. monocytogenes* ATCC 53135; **Lane 4:** Enterocin FH99 resistant variant of *L. monocytogenes* ATCC 53135; **Lane 5:** Wild Type *E. faecium* VRE; **Lane 6:** Nisin resistant variant of *E. faecium* VRE; **Lane 7:** Pediocin 34 resistant variant of *E. faecium* VRE, **Lane 8:** Enterocin FH99 resistant variant of *E. faecium* VRE

a)

b)

Plate 4.13. Electrophoretic profile of housekeeping gene of a) *Enterococcus* spp. and b) *Listeria* spp. by PCR with primers targeted to GAPDH

1: 100bp ladder; 2: *Listeria* (positive control); 3: *Listeria monocytogenes* ATCC 53135 4: Nisin resistant variant of *Listeria*; 5: Pediocin 34 resistant variant of *Listeria*; 6: Enterocin FH99 resistant variant of *Listeria*

1: 100bp ladder; 2: *E. faecium* (positive control); 3: Nisin resistant variant of *E. faecium*; 4: Pediocin 34 resistant variant of *E. faecium*; 5: Enterocin FH99 resistant variant of *E. faecium*

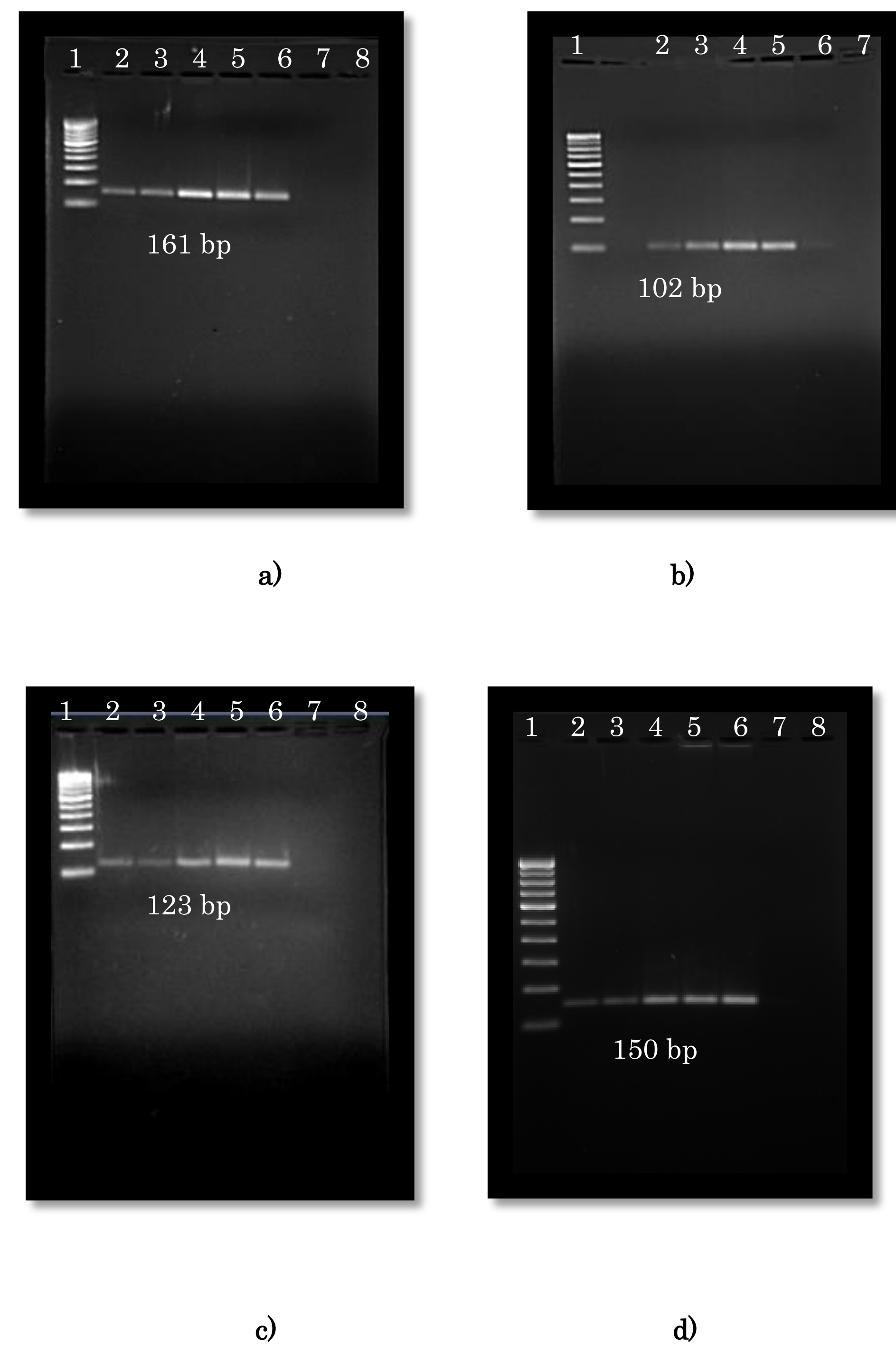


Plate 4.14. Electrophoretic profile of genes of *mpt* operon of *Listeria monocytogenes* spp. by PCR with primers targeted to a) σ⁵⁴, b) IIC, c) IID and d) *glpQ*

1: 100 bp ladder; 2: *Listeria monocytogenes* (positive control); 3: Nisin resistant variant of *Listeria monocytogenes*; 4: Pediocin 34 resistant variant of *Listeria monocytogenes*; 5: Enterocin FH99 resistant variant of *Listeria monocytogenes*; 6: Negative control

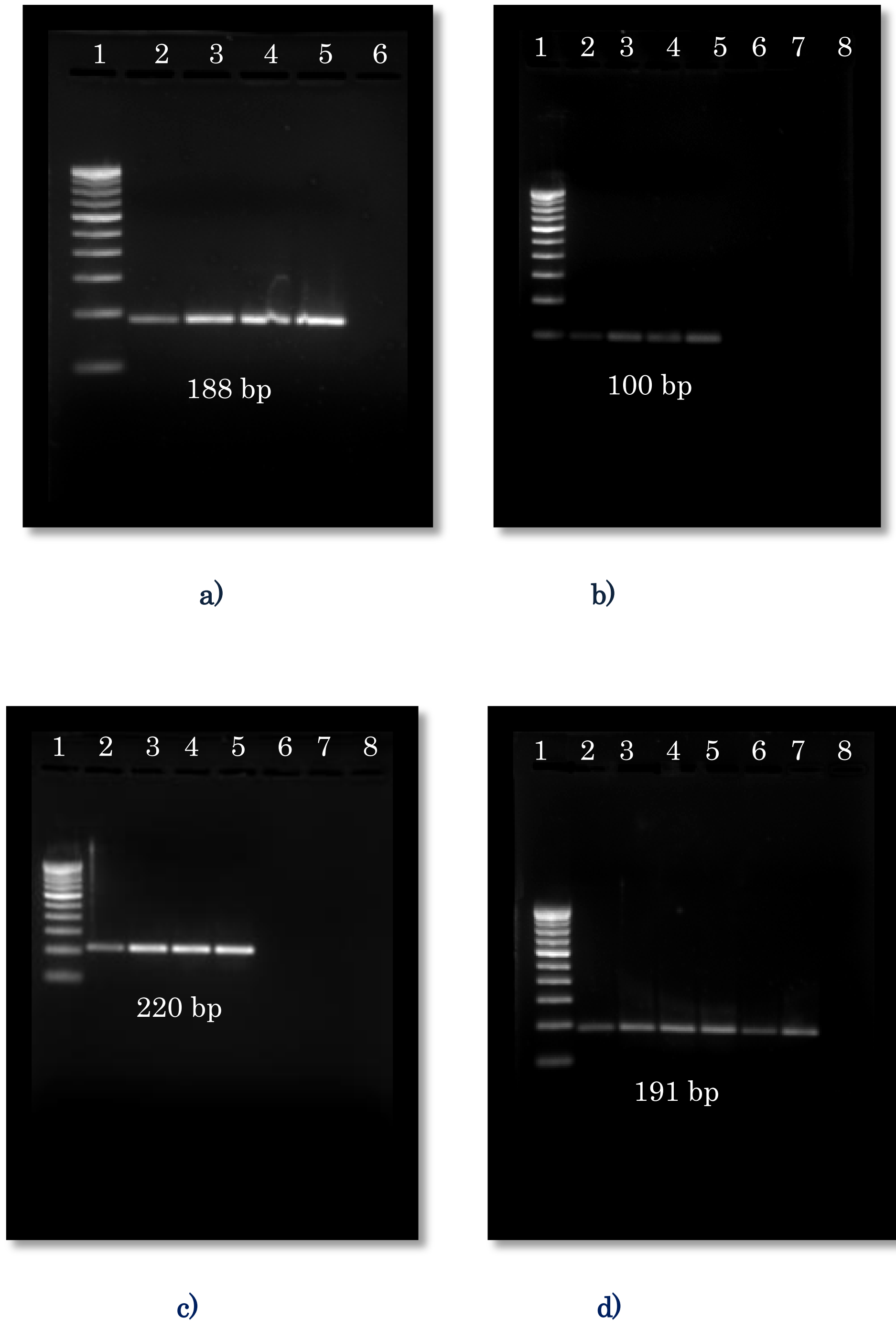


Plate 4.15. Electrophoretic profile of genes of *mpt* operon of *Enterococcus* spp. by PCR with primers targeted to a) σ^{54}, b) IIC, c) IID and d) *glpQ*

1: 100 bp ladder; 2: *E. faecium* (positive control); 3: Nisin resistant variant of *E. faecium*; 4: Pediocin 34 resistant variant of *E. faecium*; 5: Enterocin FH99 resistant variant of *E. faecium*; 6: Negative control

cultures. The difference between CT of the target gene and CT of the endogenous reference gene (GAPDH) was defined as the ΔCT.

$\Delta Ct = Ct_{target} - Ct_{reference\ gene}$

The ΔΔCT value describes the difference between the average ΔCT of the wild type strain and the resistant variant.

$\Delta\Delta Ct = (Ct_{target} - Ct_{reference\ gene})_{calibrator} - (Ct_{target} - Ct_{reference\ gene})_{sample}$

The difference in expression was calculated as $2^{\Delta\Delta CT}$, and a twofold difference (ΔΔCT = 1) was considered as significant

4.10.7.3.1 Expression of σ^{54}, IIC, IID and *glpQ* genes in *L. monocytogenes* ATCC 53135

In case of nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, down regulation of σ^{54}, IIC, IID and *glpQ* genes was observed (Fig. 4.62). A critical appraisal of data for nisin resistant variant of *L. monocytogenes* ATCC 53135 indicated 79.89, 71.17, 4.97 and 3296.45 fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively. Similarly decrease in σ^{54}, IIC, IID and *glpQ* gene expression relative to the wild type strain in case of both pediocin 34 and enterocin FH99 resistant variants was observed. Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed 79.89, 71.17, 4.97 and 3296.45 fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively. For pediocin 34 resistant variant of *L. monocytogenes* ATCC 53135, 2.53, 2.06, 2.24 and 2.13-fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively, was observed. For enterocin FH99 resistant variant of *L. monocytogenes* ATCC 53135, 105.17, 90.71, 5.41 and 4430.64-fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively, was observed.

4.10.7.3.2 Expression of σ^{54}, IIC, IID and *glpQ* genes in *L. monocytogenes* MTCC 657

In case of nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, down regulation of σ^{54}, IIC, IID and *glpQ* genes was

observed (Fig.4.63). Nisin resistant variant of *L. monocytogenes* MTCC 657 showed 71.01, 75.58, 1.52 and4973.34-fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively. For pediocin 34 resistant variant of *L. monocytogenes* MTCC 657, decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 77.97, 78.79, 1.82 and 4870.99-fold, respectively. For enterocin FH99 resistant variant of *L. monocytogenes* MTCC 657, decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 88.64, 92.41, 1.57 and 6539.66-fold, respectively.

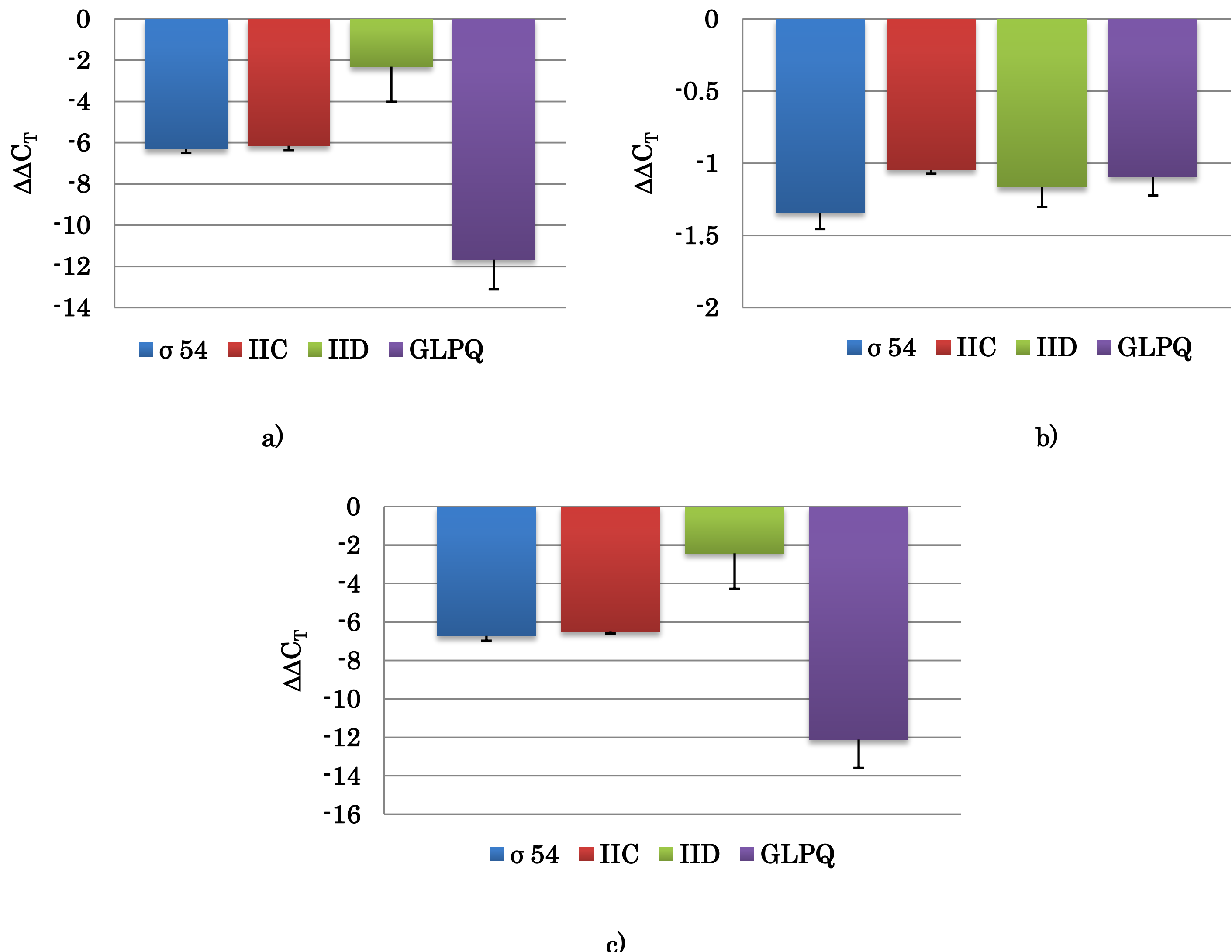
4.10.7.3.3 Expression of σ^{54}, IIC, IID and *glpQ* genes in *E. faecium* DSMZ 20477

In case nisin, pediocin 34 and enterocin FH99 resistant variants of *E. faecium* VRE, down-regulation of σ^{54}, IIC, II D and *glpQ* genes was observed (Fig. 4.64). Nisin resistant variant of *E. faecium* DSMZ 20477 showed 24.25, 26.53, 13.17 and 19.69-fold decrease in σ^{54}, IIC, II D and *glpQ* gene expression, respectively. In case of pediocin 34 resistant variant of *E. faecium* DSMZ 20477 the decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 108.38, 79.34, 52.34 and 65.34-fold, respectively, relative to the wild type strain. In case of enterocin FH99 resistant variant of *E. faecium* DSMZ 20477, the decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 254.23, 206.50, 259.57 and 229.12- fold, respectively, in contrast to the wild type strain.

4.10.7.3.4. Expression of σ^{54}, IIC, IID and *glpQ* genes in *E. faecium* VRE

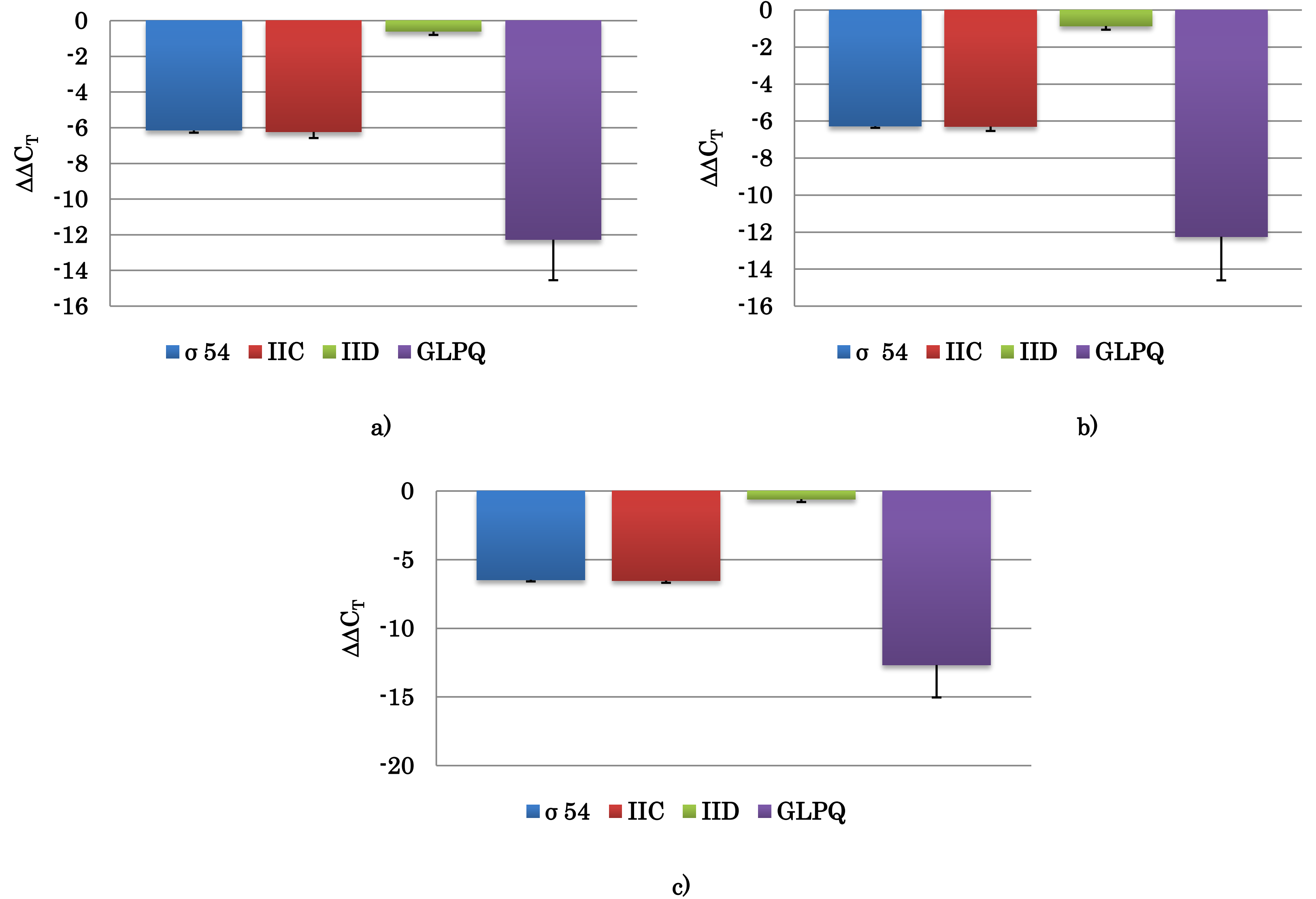
In case nisin, pediocin 34 and enterocin FH99 resistant variants of *E. faecium* VRE, down-regulation of σ^{54}, IIC, II D and *glpQ* genes was observed **(Fig. 4.65)**. Nisin resistant variant of *E. faecium* VRE showed 12.04, 6.63, 8.69, and 8.69 fold decrease in σ^{54}, IIC, II D and *glpQ* gene expression was, as compared to the wild type strain, respectively. In case of pediocin 34 resistant variant of *E. faecium* VRE, the fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 69, 25.99, 36.25 and 36.25, respectively relative to the wild type strain. In case of enterocin FH99 resistant variant of *E. faecium* VRE, the fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 73.51, 91.77, 97 and 97, respectively, relative to the wild type strain.

Fig. 4.62. Expression profile of σ^{54}, IIC, IID and *glpQ* genes in a) Nisin resistant, b) Pediocin 34 resistant and Enterocin FH99 resistant variant of *L. monocytogenes* ATCC 53135.



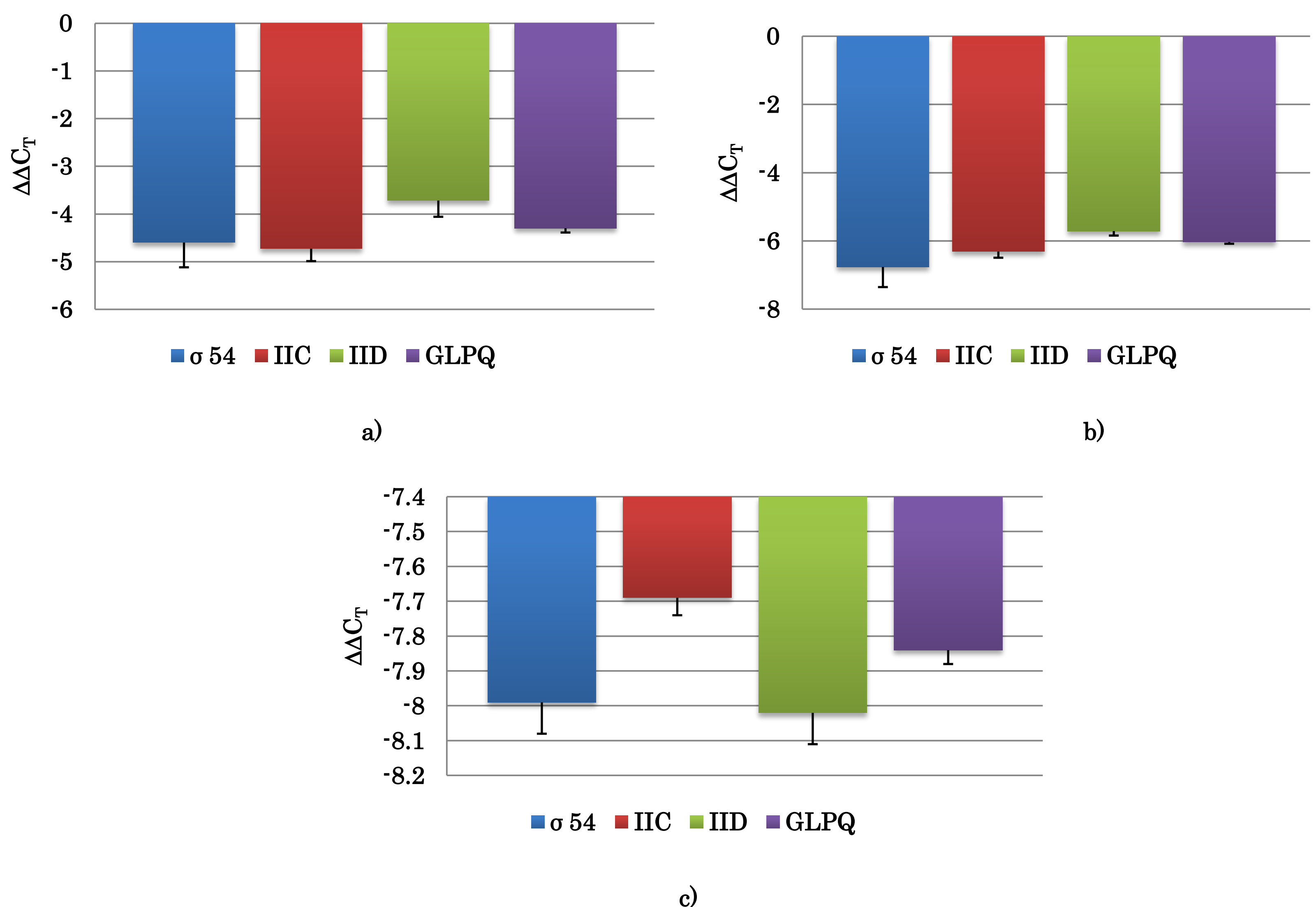
The results are from two experiments, with each individual experiment consisting of triplicate PCR reactions. The corresponding wild type strains have not been included since the $\Delta\Delta C_T$ value for the resistant strains is calculated relative to wild -type

Fig. 4.63. Expression profile of σ^{54}, IIC, IID and *glpQ* genes in a) Nisin resistant, b) Pediocin 34 resistant and Enterocin FH99 resistant variant of *L. monocytogenes* MTCC 657.



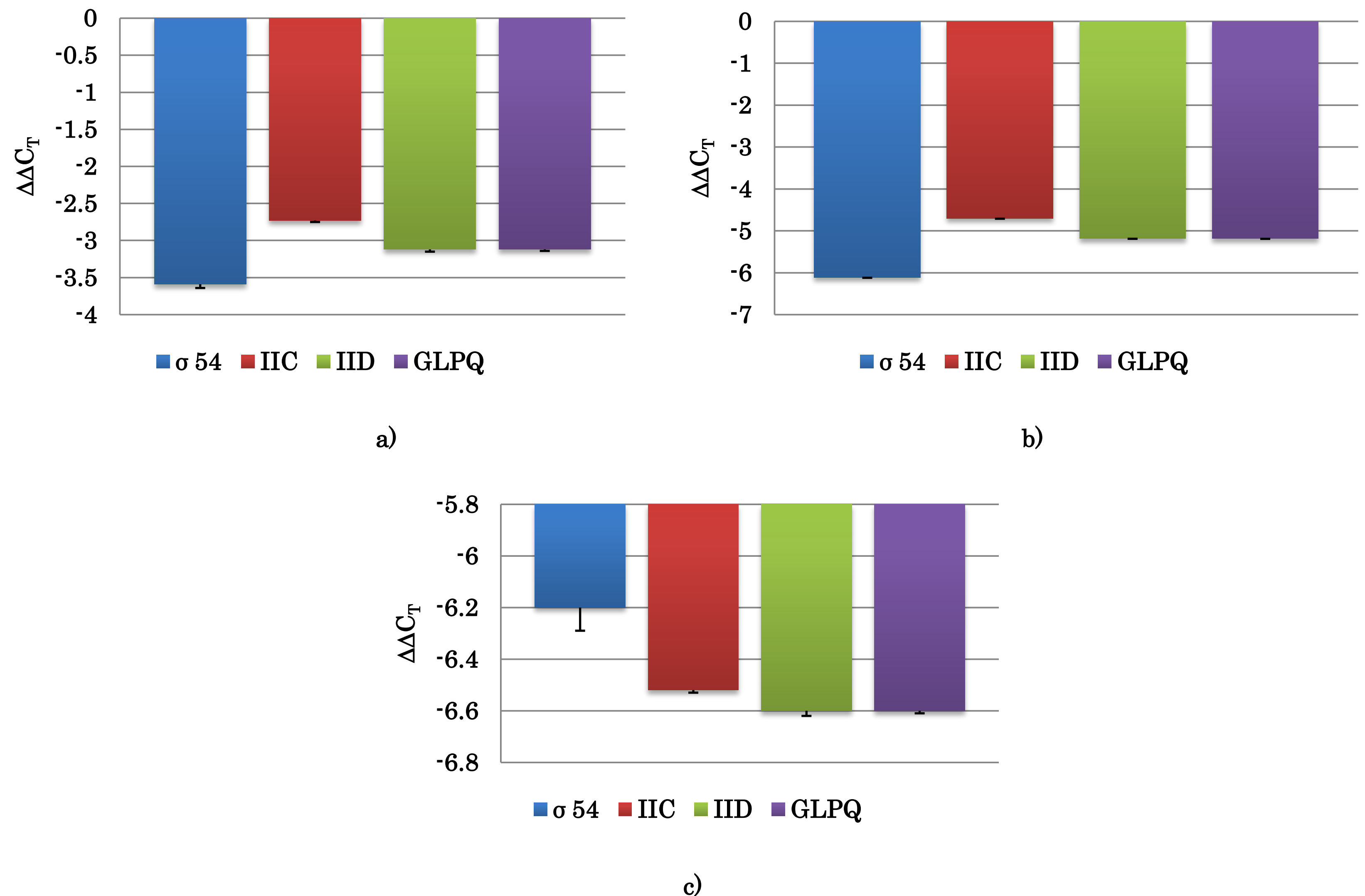
The results are from two experiments, with each individual experiment consisting of triplicate PCR reactions. The corresponding wild type strains have not been included since the $\Delta\Delta C_T$ value for the resistant strains is calculated relative to wild -type

Fig. 4.64. Expression profile of σ^{54}, IIC, IID and *glpQ* genes a) Nisin resistant, b) Pediocin 34 resistant and Enterocin FH99 resistant variant of *E. faecium* DSMZ 20477.



The results are from two experiments, with each individual experiment consisting of triplicate PCR reactions. The corresponding wild type strains have not been included since the$\Delta\Delta C_T$ value for the resistant strains is calculated relative to wild -type

Figure 4.65. Expression profile of σ^{54}, IIC, IID and *glpQ* genes in a) Nisin resistant, b) Pediocin 34 resistant and Enterocin FH99 resistant variant of *E. faecium* VRE.



The results are from two experiments, with each individual experiment consisting of triplicate PCR reactions. The corresponding wild type strains have not been included since the$\Delta\Delta C_T$ value for the resistant strains is calculated relative to wild -type

Most bacteriocins are believed to kill sensitive cells by disrupting the integrity of target membranes, which leads to dissipation of the proton motive force, depletion of intracellular solutes, and eventually cell death (Drider *et al.*, 2006). The fact that each bacteriocin displays a defined inhibitory spectrum strongly suggests that the individual bacteriocins recognize specific receptor molecules on target cells. In fact, it has been shown that several of the class I lantibiotics as well as the non-lantibiotic lactococcin 972 employ lipid II, a precursor in cell wall synthesis, as a docking molecule, and interaction between a bacteriocin and lipid II leads to inhibition of cell wall synthesis and/or pore formation, depending on the structure of the bacteriocin and its concentration (Brotz *et al.*, 1998; Wiedemann *et al.*, 2001; Martinez *et al.*, 2008). Similarly, some studies have shown that the mannose-phosphotransferase system (man-PTS) might serve as a receptor for some pediocin-like bacteriocins This is based on the observations that resistant mutants have an altered expression pattern of man-PTS and that heterologous expression of cloned man- PTS genes renders resistant cells sensitive (Ramnath *et al.*, 2000; Dalet *et al.*, 2001; He´chard *et al.*, 2001; Gravesen *et al.*, 2002; Ramnath *et al.*, 2004). More recently (Diep *et al.*, 2007), it has been conclusively demonstrated for several bacteriocins from subclass IIa and some from subclass IIc that the membrane components (ManM/PtnC and ManN/PtnD) of the man-PTS are directly involved as receptors, and that in bacteriocin producing cells, a cognate immunity protein tightly binds the receptor in a bacteriocin-dependent manner, to prevent killing by the bacteriocin

The man-PTS transporter family is responsible for the concomitant import and phosphorylation of carbohydrates such as mannose and glucose in bacteria (Postma *et al.*, 1993). A PTS transporter normally consists of three major components: enzyme I (EI), HPr and enzyme II (EII). The first two are cytoplasmic proteins involved in the transfer of a phosphoryl group to EII, which in turn relays the phosphoryl group to imported sugar molecules. EI and HPr serve as common phosphoryl group suppliers for different EIIs, while the individual EIIs are specific for each PTS family and are responsible for the sugar specificity. EII in man-PTS consists of four subunits, IIA, IIB, IIC and IID, in which the first two appear as one single (IIAB) or two separate (IIA and IIB) proteins, and the last two (IIC and IID)

normally are separate proteins. Subunits IIA and IIB are located in the cytoplasm, while IIC and IID together form a membrane-located complex through which the sugar entities enter the cell (Postma *et al*., 1993). Expression of the genes encoding these four subunits is coordinated, as they are commonly clustered in one operon (Deutscher *et al*., 2006). A few reports have linked sensitivity to class IIa bacteriocins to specific man-PTSs.

Therefore, it is evident from these results σ^{54} is involved in conferring resistance to nisin, pediocin 34 and enterocin FH99. σ^{54} is required for expression of a wide variety of genes but is not essential for growth. Most of the genes under σ^{54} control are involved in nitrogen metabolism, as well as in pilus production, dicarboxylic acid transport, and toluene and xylene catabolism (Thony & Hennecke, 1989; Merrick, 1993). Hence, it is likely that the bacteriocin could enter the cell by using a receptor normally used for other purposes. It should be emphasized that some class II bacteriocins (e.g., mesentericin Y105), as well as colicins, have been demonstrated to interact with a specific membrane protein (Van Belkum *et al.,* 1991; Chikindas *et al.,* 1993), in contrast to lantibiotics (e.g., nisin), which seem to act directly on the membrane.

Expression of *mptACD* is controlled by the σ^{54} transcription factor and the activator ManR (Dalet *et al*., 2001).For instance, the *mpt* operons in *Enterococcus faecalis* V583 and *L. monocytogenes* EGD-e have been shown to be required for sensitivity to mesentericin Y105 (Dalet *et al*., 2001; He´chard *et al*., 2001). Another man-PTS of *Li. monocytogenes* EGD-e, encoded by the mpo operon, has also been shown to be involved in sensitivity to class IIa bacteriocins via regulation of the *mpt* operon (Arous *et al*., 2004). Furthermore, in a recent study of bacteriocin sensitivity and immunity (Diep *et al*., 2007), it was shown that the man operon of *Lactobacillus sakei* 23K confers sensitivity to a number of class IIa bacteriocins, while the ptn operon of *Lactococcus lactis* IL1403 confers sensitivity to the class IIc bacteriocins lactococcin A and lactococcin B. Bacterial RNA polymerases are multisubunit enzymes formed by a core enzyme and a subunit that directs promoter recognition. Besides the major σ factor, σ^{70}, bacteria can use alternate subunits, such as σ^{54} (Buck

et al., 2000) that target specific promoters sharing consensus sequences, located at positions 224/212 upstream from the transcription start. To date, only two σ^{54} factors have been described in Gram positive bacteria, namely in *Bacillus subtilis* (Garden *et al.,* 1997) and *Listeria monocytogenes*. In the latter, lack of σ^{54} leads to resistance to mesentericin Y105, a subclass IIa bacteriocin (Dalet *et al.,* 2001)

In case of *L. monocytogenes* ATCC 53135*. L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE, σ54, IIC and IID genes were down-regulated. IIC and IID of *mpt* operon together form a membrane-located complex through which the sugar entities enter the cell (Postma *et al*., 1993). The distal gene of the *mpt* operon, *mptD*, seems to play a particular role in sensitivity. In conclusion, we propose that EII_t^{Man} could either influence the expression of an unknown molecule involved in sensitivity or, via its IICMan±IIDMan membrane complex, be a docking molecule or a receptor for pediocin 34 and enterocin FH99 and other subclass IIa bacteriocins. The distal gene of the *mpt* operon, *mptD*, seems to play a particular role in sensitivity since its up-regulation suggested that it led to resistance. Interestingly, it has been reported that MptD bears an additional domain compared to three other IIDMan subunits found in the *L. monocytogenes* EGDe genome and to most of IIDMan subunits described in the literature except *E. faecalis* (Hechard *et al*., 2001) and *Streptococcus salivarius* (Lortie *et al*., 2000) are found in GenBank. Among 22 bacterial sequenced genomes (finished or unfinished) found to possess at least one orthologue of IIDMan, only several Gram-positive bacteria (i.e. *E. faecalis*, *Streptococcus* spp., *Lactococcus lactis* and *Clostridium acetobutylicum*) have a IIDMan with an additional domain. Interestingly, these Gram positive bacteria have sometimes been described to be sensitive to subclass IIa bacteriocins. It has been earlier proposed that this domain could directly interact with bacteriocins or that its deletion could change the structure of the permease, leading to a lower affinity for the bacteriocins.

In *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE another gene which was down regulated was *glpQ* which encodes a putative glycerophosphoryl diester phosphoesterase (*glpQ*). GlpQ is an

exoprotein found to participate in fatty acid and phospholipid degradation in many bacteria (Antelmann *et al*., 2000). A hypothesis that could be drawn from our data is that the absence of *glpQ* activity in bacteriocin resistant variants leads to an intact fatty acid and phospholipid composition of the cell membrane, which should contribute to the resistant phenotype of this mutant. Moreover, the *glpQ* protein was originally suggested to belong to the Pho regulon in *Bacillus subtilis* (Antelmann *et al*., 2000), and to be governed by a pleiotropic two-component regulatory system PhoP-PhoR (Groisman, 2001). Interestingly, PhoP and Mg^{2+} also control the resistance of many Gram-negative bacteria to antimicrobial peptides, as supported by a number of lines of evidence (Groisman *et al*., 1992; Moss *et al*., 2001). Similarly, in Gram-positive bacteria, different studies have highlighted the role of a two-component regulatory system in the resistance of *L. monocytogenes* (Cotter *et al*., 2002), *Enterococcus faecalis* (Comenge *et al*., 2003) and *Staphylococcus aureus* (Kuroda *et al*., 2003) to inhibiting substances such as antimicrobial peptides and antibiotics.

In our study we observed that *mpt* operon was also involved in regulating the development of nisin resistance in the nisin variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE. These results are in contrast to the earlier reports which suggest that the acquisition of nisin resistance is a complex phenomenon and may differ among strains, as suggested by reports on alterations in fatty acid composition and cell wall changes in nisin-resistant *Listeria*. Nisin resistance may result from the presence of a nisin-degrading enzyme, nisinase, which has been reported in several Gram-positive bacteria (Galesloot, 1956; Alifax & Chevalier, 1962; Jarvis 1967; Lipinska, 1977; Hoover & Hurst, 1993). The nisinase from several *Bacillus* species specifically reduced the C-terminal dehydroalanyl lysine of nisin to alanyllysine (Jarvis, 1970; Jarvis & Farr, 1971). In 1998, Crandall & Montville (1998) put forward a model for the nisin resistance of *Listeria monocytogenes* ATCC 700302 which included three factors: (i) variation of peptidoglycan composition (Maisnier & Richard, 1996), which should make it possible to increase the binding of divalent cations that should interact with the cationic peptide; (ii) modification of the electric charge of the

membrane by phospholipids content changes, thereby preventing pore formation (Ming & Daeschel, 1993; Revol *et al.*, 1996; Crandall & Montville,1998); and (iii) increase in membrane rigidity, preventing peptide insertion and association (Ming & Daeschel, 1993). Resistance has been correlated with both an altered fatty acid composition (Ming & Daeschel, 1993; Mazzotta & Montville, 1997) and an altered phospholipids composition (Ming & Daeschel, 1995). Also, in case of nisin resistant variants of *L. monocytogenes* ATCC 53135, *E. faecium* DSMZ 20477 and *E. faecium* VRE the *glpQ* gene was down regulated *glpQ* is an exoprotein found to participate in fatty acid and phospholipid degradation in many bacteria (Antelmann *et al.*, 2000). A hypothesis that could be drawn from our data is that the absence of *glpQ* activity in bacteriocin resistant variant leads to an intact fatty acid and phospholipid composition of the cell membrane, which should contribute to the resistant phenotype of this mutant.

4.11 EPILOGUE

The present investigation was aimed at the determination of Minimum Inhibitory Concentrations (MIC) of different bacteriocins against strains of *L. monocytogenes, E. faecium and E. faecalis*, isolation and molecular & biochemical characterization of bacteriocin resistant variants of *L. monocytogenes, E. faecium and E. faecalis*, to study the effect of mild heat treatment, pH and combination of bacteriocins on the susceptibility of resistant variants. The results of the present study indicate that treatment with a combination of two or more different bacteriocins has an advantage of protection against many spoilage and pathogenic bacteria because of synergistic and/or additive effects. This study showed that resistance to a bacteriocin may extend to other bacteriocins within the same class. Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage and among food borne pathogens. The development of bacteriocin resistance may hinder further application of bacteriocins in food preservation and it also raises concerns about the extensive use of bacteriocins in food regarding the cross resistance in food

borne pathogens towards other bacteriocins and towards clinically used antibiotics. The nisin, pediocin 34 and enterocin FH99 resistant strains did not show intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In no case were the bacteriocin resistant *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 variants examined, resistant to inhibitors than the parental strains. The heat resistance of wild type and the nisin resistant, pediocin 34 and enterocin FH99 variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 was assayed at 45, 50 and 55°C. The results suggested that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of target organisms. Together, these findings suggest that rational design of multiple-hurdle preservation systems incorporating bacteriocins can improve food safety without being undermined by resistance-related phenomena. Results showed that bacteriocin resistance in *Listeria monocytogenes* and *E. faecium* is a complex phenomenon involving alterations in both the cytoplasmic membrane and the cell wall and a requirement for divalent cations. Results also showed that mannose, glucose, fructose and cellobiose have an effect on sensitivity of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to nisin, pediocin 34 and enterocin FH99 suggesting that the level of sensitivity is tightly linked to sugar availability, suggesting that sugars (mannose, glucose, fructose and cellobiose) directly cause expression of a molecule responsible for sensitivity to nisin, pediocin 34 and enterocin FH99. From Real Time PCR studies, it is evident that σ54 is involved in conferring resistance to nisin, pediocin 34 and enterocin FH99. In case of strains of *L. monocytogenes* and *E. faecium* and *E. feacalis*, IIC and IID genes were down-regulated. GlpQ which encodes a putative glycerophosphoryl diester phosphoesterase was also down regulated.

5. SUMMARY AND CONCLUSIONS

5.1 SUMMARY

The salient findings of the present investigation encompassing the determination of Minimum Inhibitory Concentrations (MIC) of different bacteriocins against strains of *L. monocytogenes, E. faecium and E. faecalis*, isolation and molecular & biochemical characterization of bacteriocin resistant variants of *L. monocytogenes, E. faecium and E. faecalis*, to study the effect of mild heat treatment, pH and combination of bacteriocins on the susceptibility of resistant variants are summarized in this section.

- The selected bacteriocinegenic strains *E. faecium* FH99 and *Pedioccoccus pentosaceous* 34 were found to be pure and showed inhibitory activity against *Pediococcus acidilactici* LB 42 in spot-on-lawn assay.
- The Gram-positive bacterial species studied in this work differed considerably in their sensitivity to Nisin, Enterocin FH99, and Pediocin 34 indicating genus, species and strain differences in the degree of inhibition.
- *E. faecalis* ATCC 29212, *Bacillus cereus* ATCC 13061, *B. cereus* NCDC 66*, S. aureus* NCDC 110 were sensitive to Nisin only. *E. faecium* DSMZ 20477, *E. faecium* (VRE), *L. monocytogenes* ATCC 53135 and *L. monocytogenes* MTCC 657 were sensitive to all the three bacteriocins used in the study.
- Nisin was most effective in inhibiting the *E. faecium* DSMZ 20477 followed by Enterocin FH99. Pediocin 34 was least effective in inhibiting *E. faecium* DSMZ 20477. However, in case of *E. faecium* (VRE), Nisin, followed by Pediocin 34, were observed to be more effective than Enterocin FH 99.
- Nisin was also observed to be most effective in inhibiting the *Listeria monocytogenes* ATCC 53135, followed by Pediocin 34 and Enterocin FH99. Pediocin 34 proved to be more effective than Nisin and Enterocin FH99 in inhibiting *L. monocytogenes* MTCC 657.
- Combinations of different bacteriocins produced a more effective antibacterial effect against *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes*

ATCC 53135 and *L. monocytogenes* MTCC 657 in comparison to the bacteriocins used alone. Also synergistic effect was observed between the bacteriocins.

- The number of survivors increased considerably after 24h incubation with all the three bacteriocins.
- The nisin resistant strains of *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) were more susceptible to most of the antibiotics tested than their wild type counterparts.
- Pediocin 34 resistant strain of *L. monocytogenes* MTCC 657 and Nisin resistant strain of *E. faecalis* ATCC 29212 were observed to be resistant to gentamicin.
- Pediocin 34 resistant variant of *L. monocytogenes* MTCC 657 displayed cross resistance to Enterocin FH99 but not to nisin. On the other hand its Enterocin FH99 resistant variant was sensitive to both Nisin as well as Pediocin 34.
- Nisin resistance in *E. faecium* DSMZ 20477 conferred cross resistance to both Pediocin34 as well as Enterocin FH99. Enterocin FH99 resistant variant displayed cross resistance to Pediocin 34 and Pediocin resistant variant of *E. faecium* DSMZ 20477 showed cross resistance to Enterocin FH99.
- *L. monocytogenes* ATCC 53135 showed sensitivity to Nisin, Pediocin 34 and Enterocin FH99. However, Pediocin 34 resistant variant appeared to be sensitive to Nisin but resistant to Enterocin FH99, indicating that the development of resistance to one of these bacteriocins may show resistance to the other bacteriocins. Nisin resistant variant retained the sensitivity to Pediocin as well as Enterocin FH99.
- Nisin, pediocin 34 and enterocin FH99 resistant strains did not have any intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In no case were the bacteriocin resistant *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 variants examined, resistant to inhibitors than the parental strains

- Resistance of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to Nisin, Pediocin 34 and Enterocin FH99 does not alter their heat sensitivity.

- Nisin, Pediocin 34 and Enterocin FH99 resistant cells of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 were further sensitized to nisin, pediocin 34 and enterocin FH99 by sub-lethal heat.

- Addition of divalent cations significantly reduced the inhibitory activity of Nisin, Pediocin 34 and Enterocin FH99 against cells of resistant variants of test culture *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212. The addition of EDTA however restored this activity.

- The impact of divalent cations on bactericidal activity of nisin, pediocin 34 and enterocin FH99 revealed that Mg^{2+} Mn^{2+} and Ca^{3+} cations were able to reduce the binding of antimicrobial peptide to the cell membrane. Divalent cations seem to affect the initial electrostatic interaction between the positively charged bacteriocin and the negatively charged phospholipids of the membrane.

- When the cell wall was removed, the wild type strain and the nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477*, E. faecium (*VRE*) and E. faecalis* ATCC 29212 showed identical sensitivity to the three bacteriocins.

- Although the bacteriocin resistant variants appeared to lose their acquired resistance towards Nisin, Pediocin 34 and Enterocin FH99, the protoplasts of the resistant variants appeared to be more resistant to bacteriocins than the protoplasts of their wild type counterpart.

- According to the hydrophobicity measurements significant differences ($p < 0.001$) were observed between wild type and their nisin resistant, pediocin 34 resistant and enterocin FH99 resistant counterparts, respectively.

- An increase in amino group containing phospholipids in the nisin resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, pediocin 34 resistant variant and enterocin FH99 resistant variant of *E. faecium* DSMZ 20477, pediocin 34 resistant variant and enterocin FH99 resistant variant of *E. faecium* VRE was observed.

- Results showed that mannose, glucose, fructose and cellulose have a effect on sensitivity of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 sensitivity to nisin, pediocin 34 and enterocin FH99. It underlines that the level of sensitivity is tightly linked to sugar availability, suggesting that sugars (mannose, glucose, fructose and cellulose) directly causes expression of a molecule responsible for sensitivity to nisin, pediocin 34 and enterocin FH99.

- In case of nisin, pediocin 34 and enterocin FH99 resistant variants of *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE down regulation of σ^{54}, IIC, IID and *glpQ* genes was observed.

- From Real Time PCR studies, it was evident that σ^{54} is involved in conferring resistance to nisin, pediocin 34 and enterocin FH99.

- Nisin resistant variant of *L. monocytogenes* ATCC 53135 showed 79.89, 71.17, 4.97 and 3296.45 fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively.

- For pediocin 34 resistant variant of *L. monocytogenes* ATCC 53135, 2.53, 2.06, 2.24 and 2.13-fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively, was observed.

- For enterocin FH99 resistant variant of *L. monocytogenes* ATCC 53135, 105.17, 90.71, 5.41 and 4430.64-fold decrease in ^{54}, IIC, IID and *glpQ* gene expression, respectively, was observed.

- Nisin resistant variant of *L. monocytogenes* MTCC 657 showed 71.01, 75.58, 1.52 and4973.34-fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression, respectively.

- For pediocin 34 resistant variant of *L. monocytogenes* MTCC 657, decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 77.97, 78.79, 1.82 and 4870.99-fold, respectively.

- For enterocin FH99 resistant variant of *L. monocytogenes* MTCC 657, decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 88.64, 92.41, 1.57 and 6539.66-fold, respectively.

- Nisin resistant variant of *E. faecium* DSMZ 20477 showed 24.25, 26.53, 13.17 and 19.69-fold decrease in σ^{54}, IIC, II D and *glpQ* gene expression, respectively.

- In case of pediocin 34 resistant variant of *E. faecium* DSMZ 20477 the decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 108.38, 79.34, 52.34 and 65.34-fold, respectively, relative to the wild type strain.

- In case of enterocin FH99 resistant variant of *E. faecium* DSMZ 20477, the decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 254.23, 206.50, 259.57 and 229.12- fold, respectively, in contrast to the wild type strain.

- Nisin resistant variant of *E. faecium* VRE showed 12.04, 6.63, 8.69, and 8.69 fold decrease in σ^{54}, IIC, II D and *glpQ* gene expression was, as compared to the wild type strain, respectively.

- In case of pediocin 34 resistant variant of *E. faecium* VRE, the fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 69, 25.99, 36.25 and 36.25, respectively relative to the wild type strain.

- In case of enterocin FH99 resistant variant of *E. faecium* VRE, the fold decrease in σ^{54}, IIC, IID and *glpQ* gene expression was 73.51, 91.77, 97 and 97, respectively, relative to the wild type strain.

- It was observed that *mpt* operon was also involved in regulating the development of nisin resistance in the nisin variants of *L. monocytogenes* ATCC 53135, *E. faecium* DSMZ 20477 and *E. faecium* VRE. These results are in contrast to the earlier reports which suggest that the acquisition of nisin resistance is due to(i) variation of peptidoglycan which should make it possible to increase the binding of divalent cations that should interact with the cationic peptide; (ii) modification of the electric charge of the membrane by phospholipids content changes, thereby preventing pore formation and (iii) increase in membrane rigidity, preventing peptide insertion and association

5.2 CONCLUSIONS

Food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. To inactivate food-borne pathogens, various novel technologies such as biopreservation systems have been studied. Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced by different groups of bacteria but the bacteriocins produced by many lactic acid bacteria (LAB) offer potential applications in food preservation. The use of bacteriocins in the food industry can help to reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods which are more naturally preserved. However the development of highly tolerant and/or resistant strains may decrease the efficiency of bacteriocins as biopreservatives. Several mechanisms of bacteriocin resistance development have been proposed among various food-borne pathogens. The acquiring of resistance to bacteriocins can significantly affect physiological activity profile of bacteria, alter cell-envelope lipid composition and also modify the antibiotic susceptibility/resistance profile of bacteria. The results of the present study indicate that treatment with a combination of two or more different bacteriocins has an advantage of protection against many spoilage and pathogenic bacteria because of synergistic and/or additive effects. The results of this study also show that although the use of Nisin is permitted in

a number of countries in a variety of foods, other bacteriocins with different and/or more effective antimicrobial activity may be considered as new biopreservatives. This study showed that resistance to a bacteriocin may extend to other bacteriocins within the same class. Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage and among food borne pathogens. The development of bacteriocin resistance may hinder further application of bacteriocins in food preservation and it also raises concerns about the extensive use of bacteriocins in food regarding the cross resistance in food borne pathogens towards other bacteriocins and towards clinically used antibiotics. The nisin, pediocin 34 and enterocin FH99 resistant strains did not show intrinsic resistance to low pH, sodium chloride, potassium sorbate, or sodium nitrite. In no case were the bacteriocin resistant *L. monocytogenes* ATCC 53135, *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 variants examined, resistant to inhibitors than the parental strains. The heat resistance of wild type and the nisin resistant, pediocin 34 and enterocin FH99 variants of *Listeria monocytogenes* ATCC 53135, *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 was assayed at 45, 50 and 55°C. The results suggested that nisin, pediocin 34 and enterocin FH99 resistance does not alter the heat sensitivity of target organisms. Together, these findings suggest that rational design of multiple-hurdle preservation systems incorporating bacteriocins can improve food safety without being undermined by resistance-related phenomena.

Further insight into the mechanism of action of nisin, pediocin 34 and enterocin FH99 suggested that bacteriocin resistance in *Listeria monocytogenes* and *E. faecium* is a complex phenomenon involving alterations in both the cytoplasmic membrane and the cell wall and a requirement for divalent cations. Results also showed that mannose, glucose, fructose and cellobiose have an effect on sensitivity of *Listeria monocytogenes* ATCC 53135, *Listeria*

monocytogenes MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 to nisin, pediocin 34 and enterocin FH99. It underlines that the level of sensitivity is tightly linked to sugar availability, suggesting that sugars (mannose, glucose, fructose and cellulose) directly cause expression of a molecule responsible for sensitivity to nisin, pediocin 34 and enterocin FH99. Further, our results also indicate that resistance to nisin, pediocin 34 and enterocin FH99 may be associated with the loss of a mannose-specific PTS protein.

5.3 FUTURE PROSPECTS

- More studies are needed to determine the distribution of bacteriocin resistance phenomenon among microorganisms that cause food spoilage.
- Knowledge of the characteristics of bacteriocin resistant variants & the conditions that prevent their emergence will help in determining the optimal conditions for application of bacteriocins in foods and minimize the incidence of resistance.
- Bacteriocin resistance among starter culture should be studied Analysis of end products of glucose metabolism in lactic acid bacteria starter cultures should be carried out. Subsequent changes in end product would profoundly influence technological as well as organoleptic qualities of the fermented food product.

BIBLIOGRAPHY

Abee, T. 1995. Pore-forming bacteriocins of gram-positive bacteria and self-protection mechanisms of producer organisms. *FEMS Microbiol Lett.* **129:**1–10.

Abee, T., Rombouts, F.M., Hugenholtz, J., Guihard, G., Letellier, L. 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. *Appl Environ Microbiol.* **60**:1962–1968.

Abriouel, H., Valdivia, E., Gálvez, A., Maqueda, M. 1998. Response of *Salmonella choleraesuis* LT2 spheroplasts and permeabilized cells to the bacteriocin AS-48. *Appl Environ Microbiol.* **64:**4623–4626.

Achemchem, F., Abrini, J., Mart´ınez-Bueno, M., Valdivia, E., Maqueda, M. 2006. Control of *Listeria monocytogenes* in goat's milk and goat's jben by the bacteriocinogenic *Enterococcus faecium* F58 strain. *J Food Prot.* **69**:2370–2376.

Alifax, R., Chevalier, R. 1962. Etude de la nisin produite par *Streptococcus thermophihs. J Dairy Res.* **29** : 233-240.

Allende, A., Mart´ınez, B., Selma, V., Gil, M.I., Su´arez, J.E., Rodr´ıguez, A. 2007. Growth and bacteriocin production by lactic acid bacteria in vegetable broth and their effectiveness at reducing *Listeria monocytogenes* in vitro and in fresh-cut lettuce. *Food Microbiol.* **24**:759–766.

Amezquita, A., Brashears, M.M. 2002. Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. *J Food Prot.* **65**:316–325.

Ananou, S., Gálvez, A., Martínez-Bueno, M., Maqueda, M., Valdivia, E. 2005a. Synergistic effect of enterocin AS-48 in combination with outer membrane permeabilizing treatments against *Escherichia coli* O157:H7. *J Appl Microbiol* **99:**1364–1372.

Ananou, S., Garriga, M., Hugas, M., Maqueda, M., Mart´ınez-Bueno, M., G´alvez, A., Valdivia, E. 2005b. Control of *Listeria monocytogenes* in model sausages by enterocin AS-48. *Int J Food Microbiol.* **103**:179–190.

Ananou, S., Maqueda, M., Mart´ınez-Bueno, M., G´alvez, A., Valdivia, E. 2005c. Control of *Staphylococcus aureus* in sausages by enterocin AS-48. *Meat Sci.* **71**:549–576.

Ananou, S., Valdivia, E., Martínez Bueno, M., Gálvez, A., Maqueda, M. 2004. Effect of combined physico-chemical preservatives on enterocin AS-48 activity

against the enterotoxigenic *Staphylococcus aureus* CECT 976 strain. *J Appl Microbiol.* **97:**48–56.

Annette, V., Russell, N.J., Van T Hof, R., Rombouts, F.M., Abee, T. 1997. Modifications of memrane Phospholipid Composition in Nisin Resistant *Listeria monocytogenes* Scott A. *Appl Environ Microbiol.* **63(9)**:3451-3457.

Antara, N.S., Sujaya, I.N., Yokota, A., Asano, K., Tomita, F. 2004. Effects of indigenous starter cultures on the microbial and physicochemical characteristics of Urutan, a Balinese fermented sausage. *J Biosci Bioeng.* **98**:92–98.

Antelmann, H., Scharf, C., Hecker, M. 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis:* Proteomics and transcriptional analysis. *J Bacteriol.* **182**:4478-4490.

Arous, S., Dalet, K., He´ chard, Y. 2004. Involvement of the mpo operon in resistance to class IIa bacteriocins in *Listeria monocytogenes*. *FEMS Microbiol Lett.* **238:**37–41.

Arqu´es, J.L., Rodr´ıguez, E., Gaya, P., Medina, M., Guamis, B., Nu˜nez, M. 2005a. Inactivation of *Staphylococcus aureus* in raw milk cheese by combinations of high-pressure treatments and bacteriocinproducing lactic acid bacteria. *J Appl Microbiol.* **98**:254–260.

Arqu´es, J.L., Rodr´ıguez, E., Gaya, P., Medina, M., Nu˜nez, M. 2005b. Effect of combinations of high-pressure treatment and bacteriocin-producing lactic acid bacteria on the survival of *Listeria monocytogenes* in raw milk cheese. *Int Dairy J.* **15**:893–900.

Aymerich, M.T., Garriga, M., Costa, S., Monfort, J.M., Hugas, M. 2002. Prevention of ropiness in cooked pork by bacteriocinogenic cultures. *Int Dairy J.* **12**:239–246.

Aymerich, T., Holo, H., Havarstein, L.S., Hugas, M., Garriaga, M., Nes, I.F. 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium,* a new antilisterial bacteriocin in the pediocin family of bacteriocins. *Appl Environ Microbiol.* **62:**1676-1682.

Baccus-Taylor, G., Glass, K.A., Luchanski, J.B., Murer, A.J. 1993. Fate of *Listeria monocytogenes* and pediococcal starter cultures during the manufacture of chicken summer sausage. *Poultry Sci.* **72**:1772–1778.

Bakes, S.H., Kitis, F.Y.E., Quattlebaum, R.G., Barefoot, S.F. 2004. Sensitization of Gram-negative and Gram-positive bacteria to jenseniin G by sublethal injury. *J Food Protect.* **67:**1009–1013.

Bankerroum, N., Sandine, W.E. 1988. Inhibitory action of nisin against *Listeria monocytogenes*. *J Dairy Sci.* **71**:3237–3245.

Bari, M.L., Ukuku, D.O., Kawasaki, T., Inatsu, Y., Isshiki, K., Kawamoto, S. 2005. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. *J Food Prot.* **68**:1381–1387.

Beard, B.M., Sheldon, B.W., Foegeding, P.M. 1999. Thermal resistance of bacterial spores in milk-based beverages supplemented with nisin. *J Food Protect.* **62:**484–491.

Benech, R.O., Kheadr, E.E., Lacroix, C., Fliss, I. 2002a. Antibacterial activities of nisin Z encapsulated in liposomes or produced *in situ* by mixed culture during cheddar cheese ripening. *Appl Environ Microbiol.* **68**:5607–5619.

Benech, R.O., Kheadr, E.E., Laridi, R., Lacroix, C., Fliss, I. 2002b. Inhibition of *Listeria innocua* in cheddar cheese by addition of nisin Z in liposomes or in situ production by mixed culture. *Appl Environ Microbiol.* **68**:3683–3690.

Bennik, M.H.J., van Overbeek, W., Smid, E.J., Gorris, L.G.M. 1999. Biopreservation in modified atmosphere stored mungbean sprouts: the use of vegetable-associated bacteriocinogenic lactic acid bacteria to control the growth of *Listeria monocytogenes*. *Lett Appl Microbiol.* **28**:226–232.

Berg´ere, J.L., Lenoir, J. 2000. Cheese manufacturing accidents and cheese defects. In Eck, A., and Gillis, J.C. (Eds) *Cheesemaking: From Science to Quality Assurance,* 2nd ed. Intercept Ltd., London. 477–518.

Berry, E., Liewen, M., Mandigo, R., Hutkins, R. 1990. Inhibition of *Listeria monocytogenes* by bacteriocin producing *Pediococcus* during the manufacture of fermented semidry sausage. *J Food Prot.* **53**:194–197.

Biswas, S.R., Ray, P., Johnson, M.C., Ray, B. 1991. Influence of growth conditions on the production of a bacteriocin AcH by *Pediococcus acidilactici* H. *Appl Environ Microbiol.* **5**:1265–1267.

Black, E.P., Kelly, A.L., Fitzgerald, G.F. 2005. The combined effect of high pressure and nisin on inactivation of microorganisms in milk. *Inn Food Sci Emerg Technol.* **6**:286–292.

Bligh, E.G., Dyer, W.J. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem Physiol.* **37:**911–917.

Borch, E., Kant-Muermans, M.L., Blixt,Y. 1996. Bacterial spoilage of meat and cured meat products. *Int J Food Microbiol.* **33**:103–120.

Bouttefroy, A., Mansour, M., Linder, M., Milliere, J.B. 2000. Inhibitory combinations of nisin, sodium chloride, and pH on *Listeria monocytogenes* ATCC 15313 in broth by an experimental design approach. *Int J Food Microbiol.* **54:** 09–115.

Bower, C.K., Daeschel, M.A. 1999. Resistance responses of microorganisms in food environments. *Int J Food Microbiol.* **50**:33-44.

Boziaris, I.S., Humpheson, L., Adams, M.R. 1998. Effect of nisin on heat injury and inactivation of *Salmonella enteritidis* PT4. *Int J Food Microbiol.* **43**:7–13.

Breuer, B., Radler, F. 1996. Inducible resistance against nisin in *Lactobacillus casei. Arch Microbiol.* **65**:114–118.

Brotz, H., Josten, M., Wiedemann, I., Schneider, U., Go¨ tz, F., Bierbaum, G., Sahl, H.G. 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. *Mol Microbiol.* **30:**317–327

Bruno, M.E.C., Montville, T.J. 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. *Appl Environ Microbiol.* **59**:3003– 3010.

Buchanan, R.L., Bagi, L.K. 1999. Microbial competition: effect of *Pseudomonas fluorescens* on the growth of *Listeria monocytogenes. Food Microbiol.* **16**:523–529.

Buck, M., Gallegos, M.T., Studholme, D.J., Guo, Y., Gralla, J.D. 2000. The Bacterial Enhancer-Dependent σ54 (σN) Transcription Factor. *J Bacteriol.* **182 (15):**4129–4136.

Budu-Amoako, E., Ablett, R.F., Harris, J., Delves-Broughton, J. 1999. Combined effect of heat and moderate heat on destruction of *Listeria monocytogenes* on Cold-Pack lobster meat. *J Food Protect.* **62:**46-50.

Cai, Y., Ng, L.K., Farber, J.M. 1997. Isolation and characterization of nisin-producing *Lactococcus lactis* subsp. *lactis* from bean sprouts. *J Appl Microbiol.* **83**:499–507.

Calder´on-Miranda, M.L., Barbosa-C´anovas, G.V., Swanson, B.G. 1999. Inactivation of *Listeria innocua* in skim milk by pulsed electric fields and nisin. *Int J Food Microbiol.* **51**:19–30.

Callewaert, R., Hugas, M., De Vuyst, L. 2000. Competitiveness and bacteriocin production of enterococci in the production of Spanish-style dry fermented sausages. *Int. J Food Microbiol.* **57**:33–42.

Calvez, S., Kohler, A., Pre´vost, H., Møretrø, T., Drider, D. 2010. Physiological and Structural Differences Between *Enterococcus faecalis* JH2-2 and Mutant Strains Resistant to (P)-Divercin RV41. *Probiotics & Antimicro Prot.* **2**:226–232.

Calvez, S., Prevost, H., Drider, D. 2008a. Identification of a New Molecular Target of Class IIa Bacteriocins in *Listeria monocytogenes* EGDe. *Folia Microbiol.* **53 (5)**:417-422.

Calvez, S., Prevost, H., Drider, D. 2008b. Relative expression of genes involved in the resistance/ sensitivity of *Enterococcus faecalis* JH2-2 to recombinant divercin RV41. *Bioctechnol Lett.* **30**:1795-1800.

Calvez, S., Rince´, A., Auffray, Y., Pre´ vost, H., Djamel, D. 2007. Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. *Microbiol.* **153**:1609–1618.

Campanini, M., Pedrazzoni, I., Barbuti, S., Baldini, P. 1993. Behaviour of *Listeria monocytogenes* during the maturation of naturally and artificially contaminated salami: effect of lactic acid bacteria starter cultures. *Int J Food Microbiol.* **20**:169–175.

Capellas, M., Mor-Mur, M., Gervilla, R., Yuste, J., Guamis, B. 2000. Effect of high pressure combined with mild heat or nisin on inoculated bacteria and mesophiles of goats' milk fresh cheese. *Food Microbiol.* **17**:633–641.

Carlin, F., Nguyen-The, C., Morris, C. E. 1996. Influence of background microflora on *Listeria monocytogenes* on minimally processed fresh broad-leaved endive (*Cichoriumendivia* var. latifolia). *J Food Prot.* **59**:698–703.

Chen, H., Hoover, D.G. 2003. Bacteriocins and their food applications. Comprehensive Rev *Food Sci Food Safety.* **2**:82–100.

Chen, Y., Ludescher, R.D., Montville, T.J. 1997a. Electrostatic interactions, but not the YGNGV consensus motif, govern the binding of pediocin PA-1 and its fragments to phospholipid vesicles. *Appl Environ Microbiol.* **63**:4770–4777.

Chen, Y., Shapira, R., Eisenstein, M., Montville, T.J. 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. *Appl Environ Microbiol.* **63**:524–531

Chen, Y., Shapira, R., Eisenstein, M., Montville, T.J. 1997b. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. *Appl Environ Microbiol.* **63**:524–531.

Chikindas, M.L., Garcia-Garcera, M.J., Driessen, A.J., Ledeboer, A.M., Nissen-Meyer, J., Nes, I.F., Abee, T., Konings, W.N., Venema, G. 1993. Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl Environ Microbiol.* **59**:3577–3584.

Chung, K.T., Dickson, J.S., Crouse, J.D. 1989. Effects of nisin on growth of bacteria attached to meat. *Appl Environ Microbiol.* **55**:1329–1333.

Cintas, L.M., Casaus, P., Fernandez, M.F., Herna´ndez, P.E. 1998. Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A, and lactocin S against spoilage and foodborne pathogenic bacteria. *Food Microbiol.* **15**:289–298.

Cleveland, J., Montville, T.J., Nes, I.F., Chikindas, M.L. 2001. Bacteriocins: safe, natural anti-microbials for food preservation. *Int. J Food Microbiol.* **71**:1-20.

Clinical and Laboratory Standards Institute (CLSI). (2007). Performance Standards 636 for Antimicrobial Disk Susceptibility tests, 27 (1).

Cobo Molinos, A., Abriouel, H., Ben Omar, N., Valdivia, E., Lucas, R., Maqueda, M., Mart´ınez Ca˜namero, M., G´alvez, A. 2005. Effect of immersion solutions containing enterocin AS-48 on *Listeria monocytogenes* in vegetable foods. *Appl Environ Microbiol.* **71**:7781–7787.

Comenge, Y.R., Quintiliani, R., Jr Li, L., Dubost, L., Brouard, J.P., Hugonnet, J.E., Arthur, M. 2003. The CroRS two component regulatory system is required for intrinsic beta-lactam resistance in *Enterococcus faecalis*. *J Bacteriol.* **185**:7184–7192.

Corsetti, A., Gobbetti, M., Smacchi, E. 1996. Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance from *Lactobacillus sanfrancisco* C57. *Food Microbiol.* **13**:447-456.

Cotter, P.D., Guinane, C.M., Hill, C. 2002. The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. *Antimicrob Agents Chemother.* **46**:2784–2790.

Cotter, P.D., Hill, C., Ross, R.P. 2005. Bacteriocins: developing innate immunity for food. *Nat Rev Microbiol.* **3**:777–788.

Crandall, A.D., Montville, T.J. 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. *Appl Environ Microbiol.* **64**:231–237.

Daeschel, M.A., Jung, D.S., Watson, B.T. 1991. Controlling wine malolactic fermentation with nisin and nisin-resistant strains of *Leuconostoc oenos*. *Appl Environ Microbiol.* **57**:601–603.

Dalet, K., Briand, C., Cenatiempo, Y., He´chard, Y. 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. *Curr Microbiol.* **41**:441–443.

Dalet, K., Cenatiempo, Y., Cossart, P. He' chard, Y. 2001. A σ54-dependent dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. *Microbiol.* **147**:3263-3269.

Davies, E.A., Delves-Broughton, J. 1999. Nisin. In Robinson, R., Batt, C., and Patel, P., (Ed.). *Encyclopedia of Food Microbiology,* Academic Press, London. 191–198.

Davies, E.A., Adams, M.R. 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. *Int J Food Microbiol*. **21**:341–347.

Davies, E.A., Falahee, M.B., Adams, M.R. 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. *J Appl Bacteriol*. **81**:139–146.

De Man, J.C., Rogosa, M., Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. *J Appl Bacteriol.* **2**:130–135.

De Martinis, E.C.P., Crandall, A.D., Mazzotta, A.S., Montville, T.J. 1997. Influence of pH, salt, and temperature on nisin resistance in *Listeria monocytogenes*. *J Food Prot*. **60**:420-423.

De Martinis, E.C.P., Franco, B.D.G.M. 1998. Inhibition of *Listeria monocytogenes* in a pork product by a *Lactobacillus sake* strain. *Int J Food Microbiol.* **42**:119–126.

De Vugst, L., Vandamme, E.J. 1994. Bacteriocins of lactic acid bacteria. Microbiol. Genet Appl. London: Blackie Acad and professional ISBND-75, 140174-9.

Deegan, L.H., Cotter, P.D., Hill, C., Ross, P. 2006. Bacteriocins: biological tools for bio-preservation and shelf-life extension. *Int Dairy J*. **16**:1058–1071.

Degnan, A.J., Byong, N., Luchansky, J.B. 1993. Antilisterial activity of pediocin AcH in model food systems in the presence of an emulsifier or encapsulated within liposomes. *Int J Food Microbiol.* **18**:127–138.

Degnan, A.J., Yousef, A.E., Luchansky, J.B. 1992. Use of *Pediococcus acidilactici* to control *Listeria monocytogenes* in temperature abused vacuum-packaged wieners. *J Food Prot.* **55**:98– 103.

Delves-Broughton, J., Blackburn, P., Evans, R. J., Hugenholtz, J. 1996. Applications of the bacteriocin, nisin. *Antonie Van Leeuwen.* **69**:193–202.

Delves-Broughton, J., Williams, G.C., Wilkinson, S. 1992. The use of bacteriocin, nisin, as a preservative in pasteurized liquid whole egg. *Lett Appl Microbiol.* **15**:133–136.

Demel, R.A., Peelen, T., Siezen, R.J., de Kruijff, B., Kuipers, O.P. 1996. Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity—a monolayer study. *Eur J Biochem*. **235**:267–274.

Desroche, N., Beltramo, C., Guzzo, J. 2005. Determination of an internal control to apply reverse transcription quantitative PCR to study stress response in the lactic acid bacterium *Oenococcus oeni*. *J Microbiol Methods.* **60**:325–333.

Deutscher, J., Francke, C., Postma, P.W. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev*. **70:**939–1031.

Dicks, L.M.T., Mellett, F.D., Hoffman, L.C. 2004. Use of bacteriocin-producing starter cultures of *Lactobacillus plantarum* and *Lactobacillus curvatus* in production of ostrich meat salami. *Meat Sci.* **66**:703–708.

Diep, D.B., Godager, L., Brede, D., Nes, I.F. 2006. Data mining and characterization of a novel pediocin-like bacteriocin system from the genome of *Pediococcus pentosaceus* ATCC 25745. *Microbiol*. **152:**1649–1659.

Diep, D.B., Skaugen, M., Salehian, Z., Holo, H., Nes, I.F. 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc Natl Acad Sci* U S A. **104**:2384–2389.

Drider, D., Fimland, G., H´ehard, Y., McMullen, L.M., Pr´evost, H. 2006. The continuing story of class IIa bacteriocins. *Microbiol Mol Biol Rev.* **70**:564–582.

Driessen, A.J.M., van den Hooven, H.W., Kuiper, W., van der Kamp, M., Sahl, H.G., Konings, R.N., Konings, W.N. 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. *Biochem.* **34**:1606–1614.

Drosinos, E.H., Mataragas, M., Veskovic-Moracanin, S., Gasparik- Reichardt, J., Hadziosmanovic, M., Alagic, D. 2006. Quantifying nonthermal inactivation of *Listeria monocytogenes* in European fermented sausages using bacteriocinogenic lactic acid bacteria or their bacteriocins: a case study for risk assessment. *J Food Prot.* **69**:2648–2663.

Duffes, F., Jenoe, P., Boyaval, P. 2000. Use of two-dimensional electrophoresis to study differential protein expression in divercin V41 resistant and wild-type strains of *Listeria monocytogenes*. *Appl Environ Microbiol*. **66**:4318–4324.

Dykes, G,A., Hastings, J.W. 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. *Lett Appl Microbiol.* **26**:5-8.

Eijsink, V.H., Axelsson, L., Diep, D.B., Havarstein, L.S., Holo, H., Nes, I.F. 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. *Anton van Leeuwen.* **81**:639–654

Ennahar, S., Sashihara, Y., Sonomoto, K., Ishizaki, A. 2000. Class IIa bacteriocins: biosynthesis, structure and activity. *FEMS Microbiol Rev.* **24**:85-106.

Ennahar, S., Sonomoto, K., Ishizaki, A. 1999. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. *J Biosci Bioeng.* **87**:705–716.

Far´ıas, M.E., Nu˜nez de Kairuz, M., Sesma, F., Palacios, J., de Ruiz Holgado, A.P., Oliver, G. 1999. Inhibition of *Listeria monocytogenes* by the bacteriocin enterocin CRL35 during goat cheese making. *Milchwissen.* **54**:30–32.

Farber, J.M., Daley, E., Holley, R., Usborne, W.R. 1993. Survival of *Listeria monocytogenes* during the production of uncooked German, American and Italian-style sausages. *Food Microbiol.* **10**:123–132.

Fimland, G., Johnsen, L., Dalhus, B., Nissen-Meyer, J. 2005. Pediocin-like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure, and mode of action. *J Peptide Sci.* **11**:688–696.

Foegeding, P., Thomas, A., Pilkington, D., Klaenhammer, T. 1992. Enhanced control of *Listeria monocytogenes* by in situ-produced pediocin during dry fermented sausage production. *Appl Environ Microbiol.* **58**:884–890.

Folli, C., Ramazzina, I., Arcidiaco, P., Stoppini, M., Berni, R. 2003. Purification of bacteriocin AS-48 from an *Enterococcus faecium* strain and analysis of the gene cluster involved in its production. *FEMS Microbiol Lett.* **221**:143–149.

Foulqui´e Moreno, M.R., Rea, M.C., Cogan, T.M., De Vuyst, L. 2002. Applicability of a bacteriocin-producing *Enterococcus faecium* as a co-culture in cheddar cheese manufacture. *Int J Food Microbiol.* **81**:73–84.

Foulqui´e Moreno, M.R., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L. 2006. The role and application of enterococci in food and health. *Int J Food Microbiol.* **106**:1–24.

Franz, C.M.A.P., Holzapfel, W.H., Stiles, M.E. 1999a. Enterococci at the crossroads of food safety. *Int J Food Microbiol.* **47**:1-24.

Franz, C.M.A.P., Stiles, M.E., Schleifer, K.H., Holzapfel, W.H. 2003. Enterococci in foods – a conundrum for food safety. *Int J Food Microbiol.* **88**:105-122.

Franz, C.M.A.P., van Belkum, M.J., Holzapfel, W.H., Abriouel H., G´alvez, A. 2007. Diversity of enterococcal bacteriocins and their grouping into a new classification scheme. *FEMS Microbiol Rev.* **31**:293–310.

Galesloot, T.E. 1956. Melkuurbacterien die het antibioticum van *S. lactis* (nisine) onwerkzaam maken. *Nederlands Melken Zuiveltijdschrzft.* **10**:143-155.

Galvez, A., Abriouel, H., Lopez, R.L., Omar, N.B. 2007. Bacteriocin-based strategies for food biopreservation. *Int J Food Microbiol.* **120**: 51-70.

Galvez, A., Gimenez-Gallego, G., Maqueda, M., Valdivia, E. 1989. Purification and amino acid composition of peptide antibiotic AS-48 produced by *Streptococcus Enterococcus faecalis subsp. liquefaciens* S-48. *Antimicrob Agents Chemother.* **33:**437-441.

Galvez, A., L´opez, R.L., Abriouel, H. 2008. Application of Bacteriocins in the Control of Foodborne Pathogenic and Spoilage Bacteria. *Crit Rev Biotech.* **28**:125–152.

Garc´ıa-Graells, C., Masschalck, B., Michiels, C.W. 1999. Inactivation of *Escherichia coli* in milk by high-hydrostatic-pressure treatment in combination with antimicrobial peptides. *J Food Prot.* **62**:1248–1254.

Garcı´a Garcera´, M.J., Elferink, M.G.L., Driessen, A.J.M., Konings, W.N. 1993. *In vitro* pore-forming activity of the lantibiotic nisin. *Eur J Biochem.* **212**:417–422.

Gardan, R., Rapoport, G., Debarbouille., M. 1997. Role of the transcriptional activator RocR in the arginine-degradation pathway of *Bacillus subtilis*. *Mol Microbiol.* **24:**825–837.

Garneau, S., Martin, N.I., Vederas, J.C. 2002. Two-peptide bacteriocins produced by lactic acid bacteria. *Biochem.* **84:**577-592.

Garriga, M., Aymerich, M.T., Costa, S., Monfort, J.M., Hugas, M. 2002. Bactericidal synergism through bacteriocins and high pressure in a meat model system during storage. *Food Microbiol.* **19**:509– 518.

Ghosh, H.K., Murray, R.G.E. 1967. Fine structure of *Listeria monocytogenes* in relation to protoplast formation. *J Bacteriol.* **93:**41 1-426.

Giraffa, G. 1995. Enterococcal bacteriocins: their potential as anti- *Listeria* factors in dairy technology. *Food Microbiol.* **12**:291–299.

Giraffa, G. 2002. Enterococci from foods. *FEMS Microbiol Rev.* **26:**163-171.

Giraffa, G., Carminati, D., Neviani, E. 1997. Enterococci isolated from dairy products: a review of risks and potential technological use. *J Food Prot.* **60:**732-738.

Glaser, P., Frangeul, L., Buchrieser, C., 52 other authors. 2001. Comparative genomics of *Listeria* species. *Sci.* **294**:849-852.

Goff, J.H., Bhunia, A.K., Johnson, M.G. 1996. Complete inhibition of low levels of *Listeria monocytogenes* on refrigerated chicken meat with pediocin AcH bound to heat-killed *Pediococcus acidilactici* cells. *J Food Prot.* **59**:1187–1192.

Gram, L., Ravn, L., Rasch, M., Bruhn, J.B., Christensen, A.B., Givskov, M. 2002. Food spoilage—interactions between food spoilage bacteria. *Int J Food Microbiol.* **78**:79-97.

Grande, M.J., Lucas, R., Abriouel, H., Ben Omar, N., Maqueda, M., Mart´ınez-Bueno, M., Mart´ınez-Ca˜namero, M., Valdivia, E., G´alvez, A. 2005a. Control of *Alicyclobacillus acidoterrestris* in fruit juices by enterocin AS-48. *Int J Food Microbiol.* **104**:289– 297.

Grande, M.J., Lucas, R., Abriouel, H., Valdivia, E., Ben Omar, N., Maqueda, M., Mart´ınez-Ca˜namero, M., G´alvez, A. 2006b. Inhibition of *Bacillus licheniformis* LMG 19409 from ropy cider by enterocin AS-48. *J Appl Microbiol*. **101**:422– 428.

Grande, M.J., Lucas, R., Abriouel, H., Valdivia, E., Ben Omar, N., Maqueda, M., Martínez-Bueno, M., Martínez-Cañamero, M., Gálvez, A., 2006a. Inhibition of toxicogenic *Bacillus cereus* in rice-based foods by enterocin AS-48. *Int J Food Microbiol*. **106**:185–194.

Grande, M.J., Lucas, R., Valdivia, E., Abriouel, H., Maqueda, M., Mart´ınez-Ca˜namero, M., Ben Omar, N., G´alvez, A. 2005b. Stability of enterocin AS-48 in fruit and vegetable juices. *J Food Prot.* **68**:2085–2094.

Gravesen, A., Kallipolitis, B., Holmstrøm, K., Høiby, P.E., Ramnath, M., Knøchel, S. 2004. *pbp2229*- Mediated Nisin Resistance Mechanism in *Listeria monocytogenes* Confers Cross-Protection to Class IIa Bacteriocins and Affects Virulence Gene Expression. *Appl Environ Microbiol*. **70 (3)**:1669–1679.

Gravesen, A., Ramnath, M., Rechinger, K.B., Andersen, N., Jansch, L., He´chard, Y., Hastings, J.W., Knøchel, S. 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes. Microbiol*. **148**:2361–2369.

Gravesen, A., Ramnath, M., Rechinger, K.B., Andersen, N., Jansch, L., He´ chard, Y., Hastings, J.W., Kno¨ chel, S. 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in Listeria monocytogenes. *Microbiol*. **148:**2361–2369.

Gravesen, A., Sorensen, K., Aarestrup, F.M., Knochel, S. 2001. Spontaneous nisin-resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. *Microbial Drug Res*. **7**:127–135.

Gravesen, A., Warthoe, P., Knochel, S., Thirstrup, K. 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative ß-glucoside-specific PTS system. *Microbiol*. **146**:1381-1389.

Groisman, E.A. 2001. The pleiotropic two componenet regulatory system PhoP-PhoQ. *J Bacteriol*. **183**:1835-1842.

Groisman, E.A., Heffron, F., Solomon, F. 1992. Molecular genetic analysis of the *Escherichia coli phoP* locus. *J Bacteriol*. **174**:486-491.

Gupta, H., Malik, R.K., De, S., Kaushik, J.K. 2010. Purification and Characterization of Enterocin FH 99 Produced by a Faecal Isolate *Enterococcus faecium* FH 99. *Indian J Microbiol.* **50**:145–155.

Hancock, L.E., Perego, M. 2004. Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. *J Bacteriol*. **186**:7951–7958.

Hanlin, M.B., Kalchayanand, N., Ray, P., Ray, B. 1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. *J Food Prot*. **56**:252–255.

Harris, L.J., Fleming, H.P., Klaenhammer, T.R. 1991. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL500 to nisin. *J Food Prot.* **54**:836–840.

Harris, L.J., Fleming, H.P., Klaenhammer, T.R. 1992. Developments in nisin research. *Food Res Int*. **25**:57-66.

Harwood, J.L., Russell, N.J. 1984. Lipids in plants and microbes. George Allen and Unwin, London, United Kingdom.

He´ chard, Y., Pelletier, C., Cenatiempo, Y., Fre` re, J. 2001. Analysis of s54-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EIIMan) is involved in sensitivity to a bacteriocin, mesentericin Y105. *Microbiol*. **147**:1575–1580.

He´ chard, Y., Sahl, H.G. 2002. Mode of action of modified and unmodified bacteriocins from Gram-positive bacteria. *Biochimie* **84**:545–557.

Henderson, J.T., Chopko, A.L., Vanwassenaar, P.D. 1992. Purification and primary structure of Pediocin PA-1 produced by *Pediococcus acidilactici* PAC 1.0. *Arch Biochem Biophys*. **295**:5–12

Heng, N.C.K., Tagg, J.R. 2006. What's in a name? Class distinction for bacteriocins. Nat Rev Microbiol. 4 doi: 10.1038/ nrmicro1273-c1

Heng, N.C.K., Wescombe, P.A., Burton, J.P., Jack, R.W., Tagg, J.R. 2007. The diversity of bacteriocins produced by Gram-positive bacteria. Bacteriocins – Ecology and Evolution (Riley MA & Chavan MA, eds), Springer, Heidelberg, Germany. 45–92.

Hirsch, A., Grinsted, E., Chapman, H.R., Mattick, A.T.R. 1951. A note on the inhibition of an anaerobic spore former in Swiss-type cheese by a nisin-producing *Streptococcus*. *J Dairy Res.* **18**:205– 206.

Hoover, D.G., Hurst, A. 1993. Nisin. In: Antimicrobials in Foods (ed), 2nd edn. Marcel Dekker, N.Y. 369-394

Huang, J., Lacroix, C., Daba, H., Simard, R.E. 1994. Growth of *Listeria monocytogenes* in milk and its control by pediocin 5 produced by *Pediococcus acidilactici* UL5. *Int Dairy J.* **4**:429–443.

Hugas, M., Garriga, M., Aymerich, M.T., Monfort, J.M. 1995. Inhibition of *Listeria* in dry fermented sausages by the bacteriocinogenic *Lactobacillus sakei* CTC 494. *J Appl Bacteriol.* **79**:322–330.

Hugas, M., Garriga, M., Aymerich, T. 2003. Functionality of enterococci in meat products. *Int J Food Microbiol.* **88**:223–233.

Hugas, M., Garriga, M., Monfort, J.M. 2002. New mild technologies in meat processing: high pressure as a model technology. *Meat Sci.* **62**:359–371.

Hugas, M., Neumeyer, B., Pag´es, F., Garriga, M., Hammes, W.P. 1996. Comparison of bacteriocin-producing lactobacilli on *Listeria* growth in fermented sausages. *Fleischwirtschaft* **76**:649– 652.

Hugas, M., Pag´es, F., Garriga, M., Monfort, J.M. 1998. Application of the bacteriocinogenic *Lactobacillus sakei* CTC 494 to prevent growth of *Listeria* in fresh and cooked meat products packaged with different atmospheres. *Food Microbiol.* **15**:639–650.

Ibrahim, H.R., Aoki, T., Pellegrini, A. 2002. Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. *Curr Pharm Des.* **8**:671–693.

Jack, R.W., Tagg, J.R., Ray, B. 1995. Bacteriocins of gram positive bacteria. *Microbiol Rev.* **59**:171-200.

Jamuna, M., Babusha, S.T., Jeevaratnam, K. 2005. Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates againast food spoilage and pathogenic organisms in model and food systems. *Food Microbiol.* **22**:449-454.

Jamuna, M., Jeevaratnam, K. 2009. Antibacterial efficacy of nisin and pediocins against food spoilage and pathogenic organisms in broth and a vegetarian food. *J Food Sci Technol.* **46(6)**:563-568.

Jarvis, B. 1967. Resistance to nisin and production of nisin-inactivating enzymes by several *Bacillus* species. *J Gen Microbiol.* **7**:33 – 48.

Jarvis, R. 1970. Enzymic reduction of the C-terminal dehydroalanyl- lysine sequence in nisin. *Proceedings Biochem Soci.* **119**:56.

Jarvis, U., Far, J. 1971. Partial purification, specificity and mechanism of action of the nisin-inactivating enzyme from *Bacilliis cereus. Biochimica et Biophysica Acta*. **227**:232-240

Jones, L.W. 1974. Effect of butterfat on inhibition of *Staphylococcus aureus* by nisin. *Can. J Microbiol.* **20**:1257-1260.

Kalchayanand, N. 1990. Extension of shelf-life of vacuum-packaged refrigerated fresh beef by bacteriocins of lactic acid bacteria, PhD thesis, University of Wyoming, Laramie, WY.

Kalchayanand, N., Dunne, C.P., Sikes, A., Ray, B. 2003. Inactivation of bacterial spores by combined action of hydrostatic pressure and bacteriocins in roast beef. *J Food Safety* **23**:219–231.

Kalchayanand, N., Hanlin, M., Ray, B. 1992. Sublethal injury makes Gram-negative and resistant Gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. *Lett Appl Microbiol.* **15**:239-243.

Kawai, Y., Kemperman, R., Kok, J., Saito, T. 2004. The circular bacteriocins gassericin A and circularin A. *Curr Prot Pept Sci.* **5**:393–398.

Kayser, F.H. 2003. Safety aspects of enterococci from the medical point of view, *Int J Food Microbiol.* **88:**255–262.

Kemperman, R., Jonker, M., Nauta, A., Kuipers, O.P., Kok, J. 2003. Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. *Appl Environ Microbiol.* **69**:5839–5848.

Killian, J.A., Koorengevel, M.C., Bouwstra, J.A., Gooris, G., Dowhan, W., de Kruiff, B. 1994. Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930. *Biochim Biophys Acta*. **1189**:225–232.

Klaenhammer, T.R. 1993. Genetics of bacteriocins produced by lactic acid bacteria. *FEMS Microbiol Rev.* **12**:39-85.

Knight, K.P., Bartlett, F.M., McKellar, R.C., Harris, L.J. 1999. Nisin reduces the thermal resistance of *Listeria monocytogenes* Scott A in liquid whole egg. *J Food Prot.* **62**:999–1003.

Komitopoulou, E., Boziaris, I.S., Davies, E.A., Delves-Broughton, J., Adams, M.R. 1999. *Alicyclobacillus acidoterrestris* in fruit juices and its control by nisin. *Int J Food Sci Technol.* **34**:81-85.

Kuroda, M., Kuroda, H., Oshima, T., Takeuchi, F., Mori, H., Hiramatsu, K. 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. *Mol Microbiol.* **49**:807–821.

L´opez-Pedemonte, T., Roig-Sagu´es, A.X., Trujillo, A.J., Capellas, M., Guamis, B. 2003. Inactivation of spores of *Bacillus cereus* in cheese by high hydrostatic pressure with the addition of nisin or lysozyme. *J Dairy Sci.* **86**:3075–3081.

Lahti, E., Johansson, T., Honkanen-Buzalski, T., Hill, P., Nurmi, E. 2001. Survival and detection of *Escherichia coli* O157:H7 and *Listeria monocytogenes* during the manufacture of dry sausage using two different starter cultures. *Food Microbiol.* **18**:75–85.

Laukov´a, A., Czikkov´a, S. 2001. Antagonistic effect of enterocin CCM 4231 from *Enterococcus faecium* on "bryndza", a traditional Slovak dairy product from sheep milk. *Microbiol Res.* **156**:31–34.

Laukov´a, A., Czikkov´a, S., Burdova, O. 1999a. Anti-staphylococcal effect of enterocin in Sunar and yogurt. *Folia Microbiol. (Praha).* **44**:707–711.

Laukov´a, A., Czikkov´a, S., D´obransky, T., Burdova, O. 1999b. Inhibition of *Listeria monocytogenes* and *Staphylococcus aureus* by enterocin CCM4231 in milk products. *Food Microbiol.* **16**:93–99.

Laukov´a, A., Czikkova, S. 1999. The use of enterocin CCM 4231 in soy milk to control the growth of *Listeria monocytogenes* and *Staphylococcus aureus*. *J Appl Microbiol.* **87**:182–186.

Laukov´a, A., Vlaemynick, G., Czikkov´a, S. 2001. Effect of enterocin CCM 4231 on *Listeria monocytogenes* in Saint-Paulin cheese. *Folia Microbiol.* **46**:157–160.

Lavermicocca, P., Valeria, F., Evidente, A., Lazzaroni, S., Corsetti, A., Gobbetti, M. 2000. Purification and characterization of novel antifungal compounds by sourdough *Lactobacillus plantarum* 21 B. *Appl Environ Microbiol.* **66**:4084-4090.

Le Breton, Y., Boe¨ l, G., Benachour, A., Pre´ vost, H., Auffray, Y., Rince´, A. 2003. Molecular characterization of *Enterococcus faecalis* two-component signal

transduction pathways related to environmental stresses. *Environ Microbiol.* **5**:329–337.

Leistner, L. 1978. Hurdle effect and energy saving. In: Downey, W.K. (Ed.). Food Quality and Nutrition. Applied Science Publishers, London, UK. 553–557.

Leistner, L. 2000. Basic aspects of food preservation by hurdle technology. *Int J Food Microbiol.* **55:**181–186.

Leistner, L., Gorris, L.G.M. 1995. Food preservation by hurdle technology. *Trends Food Sci Technol.* **6**:41–46.

Leroy, F., Foulqui´e Moreno, M.R., De Vuyst, L. 2003. *Enterococcus faecium* RZS C5, an interesting bacteriocin producer to be used as a co-culture in food fermentation. *Int J Food Microbiol.* **88**:235–240.

Leroy, F., Lievens, K., De Vuyst, L. 2005a. Interactions of meat associated bacteriocin-producing Lactobacilli with *Listeria innocua* under stringent sausage fermentation conditions. *J Food Prot.* **68**:2078–2084.

Leroy, F., Lievens, K., De Vuyst, L. 2005b. Modeling bacteriocin resistance and inactivation of *Listeria innocua* LMG 13568 by *Lactobacillus sakei* CTC 494 under sausage fermentation conditions. *Appl Environ Microbiol.* **71**:7567–7570.

Leroy, F., Verluyten, J., De Vuyst, L. 2006. Functional meat starter cultures for improved sausage fermentation. *Int J Food Microbiol.* **106**:270–285.

Liang, Z., Mittal, G.S., Griffiths, M.W. 2002. Inactivation of *Salmonella typhimurium* in orange juice containing antimicrobial agents by pulsed electric field. *J Food Prot.* **65**:1081–1087.

Liao, C.H.S., Fett, W.F. 2001. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. *J Food Prot.* **64**:1110–1115.

Limonet, M., Revol-Junelles, A., Cailliez-Grimal, C., Milliere, J. 2004. Synergistic mode of action of mesenterocins 52A and 52B produced by *Leuconstoc mesenteroides* subsp. *mesenteroides* FR 52. *Curr Microbiol.* **48**:204-207.

Lipinska, E. 1977. Nisin and its applications. In *Anribiotics and Antibiosis in Agrrcutlurr* ed. Woodbine, M London: Butterworths.103-130

Liserre, A.M., Landgraf, M., Destro, M.T., Franco, B.D.G.M. 2002. Inhibition of *Listeria monocytogenes* by a bacteriocinogenic *Lactobacillus sake* strain in modified atmosphere-packaged Brazilian sausage. *Meat Sci.* **61**:449–455.

Lortie, L. A., Pelletier, M., Vadeboncoeur, C., Frenette, M. 2000. The gene encoding IIABManL in *Streptococcus salivarius* is part of a tetracistronic operon encoding a phosphoenol- pyruvate:mannose glucose phosphotransferase system. *Microbiol.* **146**:677-685.

Lucas, R., Grande, M.J., Abriouel, H., Maqueda, M., Ben Omar, N., Valdivia, E., Mart´ınez-Ca˜namero, M., G´alvez, A. 2006. Application of the broad-spectrum bacteriocin enterocin AS-48 to inhibit *Bacillus coagulans* in low-pH canned fruit and vegetable foods. *Food Chem Toxicol.* **44**:1774–1781.

Mahadeo, M., Tatini, S.R. 1994. The potential of nisin to control *Listeria monocytogenes* in poultry. *Lett Appl Microbiol.* **18:**323-326.

Maisnier-Patin, S., Deschamps, N., Tatini, S.R., Richard, J. 1992. Inhibition of *Listeria monocytogenes* in Camembert cheese made with a nisin-producing starter. *Lait* **72**:249–263.

Maisnier-Patin, S., Richard, J. 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentration. *FEMS Microbiol Lett.* **140**:29–35.

Maisnier-Patin, S., Tatini, S.R., Richard, J. 1995. Combined effect of nisin and moderate heat on destruction of *Listeria monocytogenes* in milk. *Lait.* **75:**81-91.

Mantovani, H.C., Russell, J.B. 2001. Nisin Resistance of *Streptococcus bovis. Appl Environ Microbiol.* **67(2)**:808–813.

Maqueda, M., G´alvez, A., S´anchez-Barrena, M.J., Gonz´alez, C., Albert, A., Rico, M., Valdivia, E. 2004. Peptide AS-48: prototype of a new class of cyclic bacteriocins. *Curr Prot Pept Sci.* **5**:399–416.

Martin, I., Ruysschaert, J.M., Sanders, D., Giffard, C.J. 1996. Interaction of nisin with membranes revealed by fluorescence quenching of an introduced tryptophan. *Eur J Biochem.* **239**:156–164.

Martınez , B., Rodrıguez, A. 2005. Antimicrobial susceptibility of nisin resistant *Listeria monocytogenes* of dairy origin. *FEMS Microbiol Lett.* **252**:67–72.

Martinez, B., Bottiger, T., Schneider, T., Rodriguez, A., Sahl, H.G., Wiedemann, I. (2008). Specific interaction of the unmodified bacteriocin lactococcin 972 with the cell wall precursor lipid II. *Appl Environ Microbiol.* **74**:4666–4670.

Mazzotta, A.S., Crandall, A.D., Montville, T.J. 1997. Nisin resistance in *Clostridium botulinum* spores and vegetative cells. *Appl Environ Microbiol.* **63**:2654-2659.

Mazzotta, A.S., Montville, T.J. 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. *J Appl Microbiol.* **82**:32–38.

Mazzotta, A.S., Montville, T.J. 1999. Characterization of fatty acid composition, germination and thermal resistance in a nisin-resistant mutant of *Clostridium botulinum* 169B and the wild-type strain. *Appl Environ Microbiol.* **65**:659-664.

McAuliffe, O., Ross, R.P., Hill, C. 2001. Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbial Rev.* **25:**285-308.

Mehla, J., Sood, S.K. 2011. Substantiation in *Enterococcus faecalis* of Dose-Dependent Resistance and Cross-Resistance to Pore-Forming Antimicrobial Peptides by Use of a Polydiacetylene-Based Colorimetric Assay. *Appl Environ Microbiol.* **77(3)**:786-793.

Menssens, W., De Vugst, L. 2002. Inhibitory substances produced by Lactobacilli isolated from sourdoughs- a review. *Int J Food Microbiol.* **72**:31-43.

Merrick, M.J. 1993. In a class of its own—the RNA polymerase σ factor 54 (N). *Mol Microbiol.* **10:**903–909.

Ming, X., Daeschel, M.A. 1993. Nisin resistance of food-borne bacteria and the specific responses of *Listeria monocytogenes* Scott A. *J Food Prot.* **56**:944–948.

Ming, X., Daeschel, M.A. 1995. Correlation of cellular phospholipids content with nisin resistance of *Listeria monocytogenes* Scott A. *J Food Prot.* **58**:416-420.

Modi, K.D. 1999. Sensitivity of nisin-resistant *Listeria monocytogenes* Scott A to heat and preservatives [M.S. thesis]. New Brunswick: Rutgers, the State University of New Jersey. 100 p. Available from: Rutgers University Library of Science and Medicine.

Modi, K.D., Chikindas, M.L., Montville, T.J., 2000. Sensitivity of nisin-resistant Listeria monocytogenes to heat and the synergistic action of heat and nisin. *Lett Appl Microbiol.* **30:**249–253.

Moll, G.N., Konings, W.N., Driessen, A.J.M. 1999. Bacteriocins: mechanism of membrane insertion and pore formation. *Anton van Leeuwen.* **76:**185–198.

Montville, T.J., Bruno, M.E. 1994. Evidence that dissipation of proton motive force is a common mechanism of action for bacteriocins and other antimicrobial proteins. *Int J Food Microbiol.* **24**:53-74.

Montville, T.J., Chen, Y. 1998. Mechanistic action of pediocin and nisin: recent progress and unresolved questions. *Appl Microbiol Biotechnol.* **50:**511–519.

Montville, T.J., Winkowski, K., Ludescherer, R.D. 1995. Models and mechanisms for bacteriocin action and application. *Int Dairy J.* **5**:797–814.

Morett, E., Segovia, L. 1993. The sigma 54 bacterial enhancer binding protein family: mechanism of action and phylogenetic relationship of their functional domains. *J Bacteriol.* **175**:6067-6074.

Moss, J.E., Fisher, P.E., Vick, B., Groisman, E.A., Zychilinsky, A. 2001. The regulatory protein PhoP controls the resolution of *Shigella flexneri* infections. *Cell Microbiol.* **2**:443–452.

Motlagh, A.M., Holla, S., Johnson, M.C., Ray, B., Field, R.A. 1992. Inhibition of *Listeria* spp. in sterile food systems by pediocin AcH, a bacteriocin produced by *Pediococcus acidilactici* H. *J Food Prot.* **55**:337–343.

Mu˜noz, A., Ananou, S., G´alvez, A., Mart´ınez-Bueno, M., Rodr´ıguez, A., Maqueda, M., Valdivia, E. 2007. Inhibition of *Staphylococcus aureus* in dairy products by enterocin AS-48 produced in situ and ex situ: bactericidal synergism through heat and AS-48. *Int Dairy J.* **17**:760–769.

Mu˜noz, A., Maqueda, M., G´alvez, A., Mart´ınez-Bueno, M., Rodriguez, A., Valdivia, E. 2004. Biocontrol of psychrotrophic enterotoxigenic *Bacillus cereus* in a non fat hard type cheese by an enterococcal strain-producing enterocin AS-48. *J Food Prot.* **67**:1517–1521.

Mullet-Powell, N., Lacoste-Armynot, A.M., Vin˜as, M., Simeon de Buochberg, M. 1998. Interactions between pairs of bacteriocins from lactic acid bacteria. *J Food Prot*. **61**:1210–1212.

Muriana, P.M. 1996. Bacteriocins for control of*Listeria* spp. in food. *J Food Prot.* **59**:S54–S63.

Murray, B.E. 1990. The life and times of the *Enterococcus. Clin Microbiol Rev*. **3**:46-65.

N´u˜nez, M., Rodr´ıguez, J.L., Garc´ıa, E., Gaya, P., Medina, M. 1997. Inhibition of *Listeria monocytogenes* by enterocin 4 during the manufacture and ripening of Manchego cheese. *J Appl Microbiol.* **83**:671–677.

Naghmouchi, K., Drider, D., Kheadr, E., Lacroix, C., Pre´ vost, H., Fliss, I. 2006. Multiple characterizations of Listeria monocytogenes sensitive and insensitive variants to divergicin M35, a new pediocin like bacteriocin. *J Appl Microbiol*. **100**:29–39.

Nes, I.F., Diep, D.B., Havarstein, L.S., Brurberg, M.B., Eijsink V., Holo, H. 1996. Biosynthesis of bacteriocins in lactic acid bacteria. *Anton van Leewen.* **70**:113-128.

Nes, I.F., Diep, D.B., Holo, H. 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J Bacteriol.* **189**:1189-1198.

Nes, I.F., Holo, H. 2000. Class II antimicrobial peptides from lactic acid bacteria. *Biopolymers.* **55**:50-61.

Nieto-Lozano, J.C., Reguera-Useros, J.I., Pel´aez-Mart´ınez, M.C., Hardisson de la Torre, A. 2006. Effect of a bacteriocin produced by *Pediococcus acidilactici* against *Listeria monocytogenes* and *Clostridium perfringens* on Spanish raw meat. *Meat Sci.* **72**:57–61.

Nissen, H., Holo, H., Axelsson, L., Blom, H. 2001. Characterization and growth of *Bacillus* spp. in heat-treated cream with and without nisin. *J Appl Microbiol.* **90**:530–534.

Okereke, A., Montville, T.J. 1991. Bacteriocin-mediated inhibition of *Clostridium botulinum* spores by lactic acid bacteria at refrigeration and abuse temperatures. *Appl Environ Microbiol.* **57**:3423-3428.

O'Sullivan, L., Ross, R.P., Hill, C., 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie* **84**:593–604.

Parente, E., Hill, C. 1992. Inhibition of *Listeria* in buffer, broth, and milk by enterocin 1146, a bacteriocin produced by *Enterococcus faecium. J Food Prot.* **55**:503–508.

Pawar, D.D., Malik, S.V.S., Bhilegaonkar, K.N., Barbuddhe, S.B. 2000. Effect of nisin and its combination with sodium chloride on the survival of *Listeria monocytogenes* added to raw buffalo meat mince. *Meat Sci.* **56**:215–219.

Peschel, A., Otto, M., Jack, R.W., Kalbacher, H., Jung, G., Gotz, F. 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. *J Biol Chem.* **274**:8405–8410.

Pol, I.E., Mastwijk, H.C., Slump, R.A., Popa, M.E., Smith, E.J. 2001. Influence of food matrix on inactivation of *Bacillus cereus* by combinations of nisin, pulsed electric field treatment, and carvacrol. *J Food Prot.* **64**:1012–1018.

Pospiech, A., Neumann, B. 1995. A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Gene.* **11**:217-218.

Postma, P.W., Lengeler, J.W., Jacobson, G.R. 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev.* **57**:543-594.

Pucci, M.J., Vedamuthu, R.E.R., Kunka, B.S., Vandenbergh, P.A. 1988. Inhibition of *Listeria monocytogenes* by using bacteriocin PA-1 produced by *Pediococcus acidilactici* PAC11.0. *Appl Environ Microbiol.* **54**:2349–2353.

Raetz, C.H.R. 1986. Molecular genetics of membrane phospholipid synthesis in *Escherichia coli*. *Annu Rev Genet.* **42**:614–659.

Ramnath, M., Arous, S., Gravesen, A., Hastings, J.W., He´ chard, Y. 2004. Expression of *mptC* of *Listeria monocytogenes* induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. *Microbiol.* **150**:2663–2668.

Ramnath, M., Beukes, M., Tamura, K., Hastings, J.W. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl Environ Microbiol.* **66**:3098-3101.

Ramnath, M., Beukes, M., Tamura, K., Hastings, J.W. 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Appl Environ Microbiol.* **66**:3098–3101.

Rayman, K., Malik, N., Hurst, A. 1983. Failure of nisin to inhibit outgrowth of *Clostridium botulinum* in a model cured meat system. *Appl Environ Microbiol.* **46**:1450–1452.

Rayman, M.K., Aris, B., Hurst, A. 1981. Nisin: a possible alternative or adjunct to nitrite in the preservation of meats. *Appl Environ. Microbiol.* **41**:375-380.

Rehkiff, N., Atrih, A., Lefebvre, G. 1994. Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. *Curr Microbiol.* **28**:237–241.

Revol-Junelles, A.M., Mathis, R., Krier, F., Fleury, Y., Delfour, A., Lefebvre, G. 1996. *Leuconostoc mesenteroides* subsp. *mesenteroides* FR52 synthesizes two distinct bacteriocins. *Lett Appl Microbiol.* **23**:120–124.

Riley, M.A., Wertz, J.E. 2002. Bacteriocins: evolution, ecology, and application. *Annu Rev Microbiol.* **56**:117–137.

Roberts, C.M., Hoover, D.G. 1996. Sensitivity of *Bacillus coagulans* spores to combinations of high hydrostatic pressure, heat, acidity and nisin. *J Appl Bacteriol.* **81**:363-368.

Robichon, D., Gouin, E., Debarbouille, M., Cossart, P., Cenatiempo, Y., He´chard, Y. 1997. The *rpoN* (σ54) gene from *Listeria monocytogenes* is involved in

resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides. J Bacteriol.* **179**:7591–7594.

Rocourt, J., Cossart, P. 1997. *Listeria monocytogenes*. In: Doyle, M.P., Buechat, L.R., Montville, T.J. (Eds.), Food Microbiology — Fundamentals and Frontiers. American Society for Microbiology (ASM) press, Washington DC. 337–352.

Rodr´ıguez, E., Gaya, P., N´u˜nez, M., Medina, M. 1998. Inhibitory activity of a nisin-producing starter culture on *Listeria innocua* in raw ewe's milk Manchego cheese. *Int J Food Microbiol.* **39**:129– 132.

Rodr´ıguez, J.L., Gaya, P., Medina, M., N´u˜nez, M. 1997. Bactericidal effect of enterocin 4 on *Listeria monocytogenes* in a model dairy system. *J Food Prot.* **60**:28–32.

Rodri´ıguez, E., Arque´es, J.L., Gaya, P., Tomillo, J., N´un˜nez, M., Medina, M. 2000. Behaviour of *Staphylococcus aureus* in semi-hard cheese made from raw milk with nisin-producing starter cultures. *Milchwissen.* **55**:633–635.

Rodriguez, J.M., Martinez, M.I., Horn, N., Dodd, H.M. 2003. Heterologous production of bacteriocins by lactic acid bacteria. *Int J Food Microbiol.* **80**:101-116.

Rogers, A.M., Montville, T.J. 1994. Quantification of factors which influence nisin's inhibition of *Clostridium botulinum* 56A in a model food system. *J Food Sc.* **59**:663-669.

Rose, N.L., Sporns, P., Stiles, M.E., McMullen, L.M. 1999. Inactivation of nisin by glutathione in fresh meat. *J Food Sci.* **64**:759–762.

Rosenberg, M., Gutnick, D., Rosenberg, E. 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. *FEMS Microbiol Lett.* **9**:29-33.

Ross, A.I.V., Griffiths, M.W., Mittal, G.S., Deeth, H.C. 2003. Combining nonthermal technologies to control foodborne microorganisms. *Int J Food Microbiol.* **89**:125–138.

Ross, R.P., Galvin, M., McAuliffe, O., Morgan, S., Ryan, M., Twomey, D.P., Meaney, W.J., Hill, C. 1999. Developing applications for lactococcal bacteriocins. *Anton van Leeuwen.* **76**:337–346.

Ross, R.P., Morgan, S., Hill, C. 2002. Preservation and fermentation: past, present and future. *Int J Food Microbiol.* **79**:3–16

Russell, A.D. 1991. Mechanisms of bacterial resistance to non-antibiotics: Food additives and food and pharmaceutical preservatives. *J Appl Bacteriol.* **71**:191-201.

Sabia, C., de Niederh¨ausern, S., Messi, P., Manicardi, G., Bondi, M. 2003. Bacteriocin-producing *Enterococcus casseliflavus* IM 416K1, a natural antagonist for control of *Listeria monocytogenes* in Italian sausages ("cacciatore"). *Int J Food Microbiol.* **87**:173–179.

Saier, M.H., Jr Reizer, J. 1994. The bacterial phosphotransferase system: new frontiers 30 years later. *Mol Microbiol.* **13**:755-764.

Sambrook, J., Fritsch, E.F., Maniatis, T.. 1989. Molecular cloning, 2nd ed. *CSH Laboratory Press*, New York.

Sarantinopoulos, P., Leroy, F., Leontopoulou, E., Georgalaki, M.D., Kalantzopoulos, G., Tsakalidou, E., De Vuyst, L. 2002. Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek feta cheese making. *Int J Food Microbiol.* **72**:125–136.

Scannell, A.G.M., Hill, C., Ross, R.P., Marx, S., Hartmeier, W., Arendt, E.K. 2000. Development of bioactive food packaging materials using immobilised bacteriocins lacticin 3147 and Nisaplin. *Int J Food Microbiol.* **60**:241–249.

Schillinger, U., Chung, H.S., Keppler, K., Holzapfel, W.H. 1998. Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. *J Appl Microbiol*. **85**:657–663.

Schillinger, U., Kaya, M., L¨ucke, F.K. 1991. Behaviour of *Listeria monocytogenes* in meat and its control by a bacteriocin-producing strain of *Lactobacillus sakei*. *J Appl Bacteriol.* **70**:473–478.

Schuman, J.D., Sheldon, B.W. 2003. Inhibition of *Listeria monocytogenes* in pH-adjusted pasteurized liquid whole egg. *J Food Prot.* **66**:999–1006.

Schved, F., Lalazar, A., Linder, P., Juven, B.J. 1994. Interaction of the bacteriocin produced by *Pediococcus acidilactici* SJ-1 with the cell envelope of *Lactobacillus* spp. *Lett Appl Microbiol.* **19**:281-283.

Scott, V.N., Taylor, S.L. 1981a. Effect of nisin on the outgrowth of *Clostridium botulinum* spores. *J Food Sc*. **46**:117-120.

Scott, V.N., Taylor, S.L. 1981b. Temperature, pH and spore load effects on the ability of nisin to prevent the outgrowth of *Clostridium botulinum* spores. *J Food Sc*. **46**:121-126.

Shingler, V. 1996. Signal sensing by sigma 54-dependent regulators: derepression as a control mechanism. *Mol Microbiol*. **19**:409-416.

Simonetta, A.C., Moragues de Velasco, L.G., Frison, L.N. 1997. Antibacterial activity of enterococci strains against *Vibrio cholera*. *Lett Appl Microbiol.* **24**:139-143.

Smid, E.J., Gorris, L.G.M. 1999. Natural antimicrobials for food preservation. In: M.S. Rahman, Editor, *Handbook of Food Preservation*, Marcel Dekker, New York. 285–308.

Sobrino-L´opez, A., Mart´ın Belloso, O. 2006. Enhancing inactivation of *Staphylococcus aureus* in skim milk by combining highintensity pulsed electric fields and nisin. *J Food Prot.* **69**:345– 353.

Solomakos, N., Govaris, A., Koidis, P., Botsoglou, N. 2008. The antimicrobial effect of the essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. *Food Microbiol.* **25**:120–127.

Somers, E.B., Taylor, S.L. 1981. Further studies on the antibotulinal effectiveness of nisin in acidic media. *J Food Sc.* **46**:1972-1973.

Song, H.J., Richard, J. 1997. Antilisterial activity of three bacteriocins used at sub-minimal inhibitory concentrations and cross-resistance of the survivors. *Int J Food Microbiol.* **36**:155–161.

Stergiou, V.A., Thomas, L.V., Adams, M.R. 2006. Interactions of nisin with glutathione in a model protein system and meat. *J Food Prot.* **69**:951–956.

Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. *Anton van Leeuwen.* **70:**331–345.

Sulzer, G., Busse, M. 1991. Growth inhibition of *Listeria* spp. on Camembert cheese by bacteria producing inhibitory substances. *Int J Food Microbiol.* **14**:287–296.

Taalat, E., Yousef, A.E., Ockerman, H.W. 1993. Inactivation and attachment of *Listeria monocytogenes* on beef muscle treated with lactic acid and selected bacteriocins. *J Food Prot.* **56**:29–33.

Taylor, S.L., Cann, D.D., Welch, B.J. 1990. Antibotulinal properties of nisin in fresh fish packaged in an atmosphere of carbon dioxide. *J Food Prot.* **53**:953-957.

Taylor, S.L., Somers, E.B., Krueger, L.A. 1985. Antibotulinal effectiveness of nisin-nitrite combinations in culture medium and chicken frankfurter emulsions. *J Food Prot.* **48**:234-239.

Terebiznik, M.R., Jagus, R.J., Cerrutti, P., De Huergo, M., Pilosof, A.M. 2002. Inactivation of *Escherichia coli* by a combination of nisin, pulsed electric fields, and water activity reduction by sodium chloride. *J Food Prot.* **65**:1253–1258.

Tessema, G.T., Møretrø, T., Kohler, A., Axelsson, L., Naterstad, K. 2009. Complex phenotypic and genotypic responses of *Listeria monocytogenes* strains

exposed to the class IIa bacteriocin sakacin P. *Appl Environ Microbiol.* **75**:6973–6980.

Thomas, L.V., Clarkson, M.R, Delves-Broughton, J., 2000. Nisin. In: Naidu, A.S. (Ed.), Natural food antimicrobial systems. CRC Press, Boca-Raton, FL, pp. 463–524.

Thomas, L.V., Delves-Broughton, J. 2001. New advances in the application of the food preservative nisin. *Adv Food Sci.* **2**:11–22.

Thomas, L.V., Ingram, R.E., Bevis, H.E., Davies, E.A., Milne, C.F., Delves-Broughton, J. 2002. Effective use of nisin to control *Bacillus* and *Clostridium* spoilage of a pasteurized mashed potato product. *J Food Prot.* **65**:1580–1585.

Thony, B., Hennecke, H.1989. The 224/212 promoter comes of age. *FEMS Microbiol Rev.* **63:**341–358.

Tomita, H., Fujimoto, S., Tanimoto, K., Ike, Y. 1997. Cloning and genetic organization of the bacteriocin 31 determinant encoded on the *Enterococcus faecalis* pheromone-responsive conjugative plasmid pPD1. *J Bacteriol.* **179:**7843-7855.

Torri Tarelli, G., Carminati, D., Giraffa, G. 1994. Production of bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci. *Food Microbiol.* **11**:243–252.

Ueckert, J.E., ter Steeg, P.F., Coote, P.J. 1998. Synergistic anti- bacterial action of heat in combination with nisin and magainin II amide. *J App Microbiol.* **85:**487-494.

Ukuku, D.O., Shelef, L.A. 1997. Sensitivity of six strains of *L. monocytogenes* to nisin. *J Food Prot.* **60**:867–869.

Ulhman, U., Schillinger, U., Rupnow, J.R., Holzapfel, W.H. 1992. Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. *Int J Food Microbiol.* **16**:141-15.

Urso, R., Rantsiou, K., Cantoni, C., Comi, G., Cocolin, L. 2006. Technological characterization of a bacteriocin-producing *Lactobacillus sakei* and its use in fermented sausages production. *Int J Food Microbiol.* **110**:232–239.

Vadyvaloo, V., Hastings, J.W., van der Merwe, M.J., Rautenbach, M. 2002. Membranes of Class IIa Bacteriocin-Resistant *Listeria monocytogenes* Cells Contain Increased Levels of desaturated and Short-Acyl-Chain Phosphatidylglycerols. *Appl Environ Microbiol.* **68(11)**:5223-5230.

Vadyvaloo, V., Snoep, J.L., Hastings, J.W., Rautenbach, M. 2004. Physiological implications of class IIa bacteriocins resistance in *Listeria monocytogenes*. *Microbiol.* **150**:335–340.

Van Belkum, M.J., Kok, J., Venema, J., Holo, H., Nes, I.F., Konings, W.N., Abee, T. 1991. The bacteriocin lactococcin A specifically increases the permeability of lactococcal cytoplasmic membranes in a voltage-independent, protein-mediated manner. *J Bacteriol.* **173**:7934–7941.

Verheul, A., Russell, N.J., Van, T.H.R., Rombouts, F.M., Abee, T. 1997. Modifications of membrane phospholipid composition in nisin-resistant *Listeria monocytogenes* Scott A. *Appl Environ Microbiol.* **63**:3451–3457.

Vescovo, M., Torriani, S., Orsi, C., Macciarolo, F., Scolari, G. 1996. Application of antimicrobial-producing lactic acid bacteria to control pathogens in ready-to-use vegetables. *J Appl Microbiol.* **81**:113–119.

Vignolo, G., Palacios, J., Farias, M.E., Sesma, F., Schillinger, U., Holzapfel, W., Oliver, G. 2000. Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. *Curr Microbiol.* **41**:410–416.

Villani, F., Salzano, G., Sorrentino, E., Pepe, O., Marino, P., Coppola, S. 1993. Enterocin 226 NWC, a bacteriocin produced by *Enterococcus faecalis* 226, active against *Listeria monocytogenes*. *J Appl Bacteriol.* **74**:380–387.

Vogel, R., Pohle, B., Tichaczek, P., Hammes, W. 1993. The competitive advantage of *Lactobacillus curvatus* LTH 1174 in sausage fermentations is caused by formation of curvacin A. *Syst Appl Microbiol.* **16**:457–462.

Wan, J., Harmark, K., Davidson, B.E., Hillier, A.J., Gordon, J.B., Wilcock, A., Hickey, M.W., Coventry, M.J. 1997. Inhibition of *Listeria monocytogenes* by piscicolin 126 in milk and Camembert cheese manufactured with a thermophilic starter. *J Appl Microbiol.* **82**:273– 280.

Wandling, L.R., Sheldon, B.W., Foegeding, P.M. 1999. Nisin in milk sensitizes Bacillus spores to heat and prevents recovery of survivors. *J Food Protect.* **62**:492–498.

Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O.P., Bierbaum, G., de Kruijff, B., Sahl, H.G. 2001. Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. *J Biol Chem.* **276**:1772–1779.

Wilderdyke, M.R., Smith, D.A., Brashears, M.M. 2004. Isolation, identification, and selection of lactic acid bacteria from alfalfa sprouts for competitive inhibition of foodborne pathogens. *J Food Prot.* **67**:947–951.

Yamazaki, K., Murami M., Kawai Y., Inoue N., Matsuda, T. 2000. Use of nisin for inhibition of *Alicyclobacillus acidoterrestris* in acidic drinks. *Food Microbiol.* **17**:315–210

Yan, L.Z., Gibbs, A.C., Stiles, M.E., Wishart, D.S., Vederas, J.C. 2000. Analogues of bacteriocins: antimicrobial specificity and interactions of leucocin A with its enantiomer, carnobacteriocin B2, and truncated derivatives. *J Med Chem* **43:**4579–4581.

Yuste, J., Fung, D.Y. 2004. Inactivation of *Salmonella* Typhimurium and *Escherichia coli* O157:H7 in apple juice by a combination of nisin and cinnamon. *J Food Prot.* **67**:371–377.

Zajdel, J.A., Ceglowski, P., Dobrzanski, W.I. 1985**.** Mechanism of action of lactostrepsin **5,** a bacteriocin produced by *Streptococcus cremoris* 202. A*ppl Environ Microbiol.* **49**:969-974.

APPENDIX I

Composition of different types of Media and Buffers, used in the study, are given below. Sterilization of media and buffers were performed at 121 °C for 20 min under 15 psi pressure.

COMPOSITION OF DIFFERENT CULTURE MEDIA AND BUFFERS

1. TRYPTOSE GLUCOSE EXTRACT AGAR (TGE)

Tryptone	10.0 g
Glucose	10.0 g
Yeast extract	10.0 g
Magnesium sulfate	0.05 g
Manganese sulfate	0.05 g
Tween 80	2.0 ml
Agar	15.0 g
Distilled water	1000 ml

pH 6.5 $\pm$ 0.2

For soft agar 7 g agar was added instead of 15 g per 1000 ml medium.

2. BRAIN HEART INFUSION MEDIUM (BHI)

Infusion from Beef heart	5.0 g
Infusion from Calf brain	12.50 g
Dextrose	2.0 g
Dipotassium hydrogen phosphate	2.5 g
Sodium chloride	5.0 g
Peptone	10.0 g
Distilled water	1000 ml

pH 7.4 $\pm$ 0.2

For soft agar 7 g agar was added instead of 15 g per 1000 ml medium

3. BRAIN HEART INFUSION BROTH (BHI)

Brain Heart Infusion Powder	8.75
Dipotassium hydrogen phosphate	2.5 g
Sodium chloride	5.0 g
Peptone	10.0 g
Distilled water	1000 ml

pH 7.4 $\pm$ 0.2

The broth was supplemented with sugars viz. glucose, mannose, fructose and cellobiose at concentrations of 5mM, 10mM, 15mM, 20mM and 25mM.

4. PHOSPHATE UREA MAGNESIUM SULPHATE (PUM) BUFFER

K_2HPO_4	22.2 g
KH_2PO_4	7.26 g
Urea	1.8 g
$MgSO_4.7H_2O$	0.2 g
Distilled water	1000 ml

pH 7.1

5. PHOSPHATE BUFFERED SALINE (PBS)

NaCl	8.00 g
KCl	0.20 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.20 g
Distilled Water	1000 ml

pH 7.0

6. PEPTONE WATER

Peptone	1g
Distilled Water	1000 ml

pH 7.0

7. GLYCEROL STOCK MEDIUM

Glycerol	50.0 ml
Distilled Water	50.0 ml

Sterilized by autoclaving. Glycerol stock added in equal amount to the culture in each cryovial, at the time of preservation

APPENDIX II

IIa. REAGENTS FOR ISOLATION OF GENOMIC DNA

1.1. SET Buffer (Solution I)

6.7% Sucrose	6.70 g
50 mM Tris	0.606 g
1mM EDTA (Di-Sodium EDTA)	0.0372 g
Deionized Water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6N NaOH and volume was made to 100 ml. Buffer was autoclaved (121 ºC /15 min) after preparation.

1.2. SDS Solution (10%)

SDS	10 g
Deionized water	100 ml

Solution was autoclaved before use

1.3. 5M NaCl

NaCl	29.22 g
Deionized water	100 ml

1.4. Chloroform: Isoamyl alcohol (24:1)

Isoamyl Alcohol	4.0 ml
Chloroform	96.0 ml

1.5. TE Buffer

Tris (10mM)	0.1212 g
Di-Sodium EDTA (1mM)	0.0372 g
Deionized water	100 ml

All the components were dissolved in 80 ml of water and pH was adjusted to 8.0 with 6N NaOH and volume was made to 100 ml. Buffer was autoclaved (121 ºC /15 min) after preparation.

IIb. REAGENTS FOR AGAROSE GEL ELECTROPHORESIS OF DNA

2.1. TAE Buffer (50 X) (Stock Solution)

Tris	24.20 g
0.5 M Di-Sodium EDTA (pH 8.0)	10.00 ml
Glacial Acetic Acid	5.71 ml
Deionized Water	100 ml

pH of this solution was not adjusted. The solution was autoclaved at 121 ºC /15min

2.1.1. Working Solution (1X)

Working solution was prepared by diluting 1 ml of stock solution to 50 ml with distilled water.

2.2. Gel Loading Solution

Bromophenol Blue	0.05 g
Sucrose	40.00 g
Di-Sodium EDTA	3.72 g
SDS	0.50 g
Deionized Water	100 ml

2.3. Staining Solution

2.3.1. Stock Solution (10 mg ml^{-1}):

Ethidium bromide (EtBr) was prepared as a stock solution of 10 mg/ml in water, stored at room temperature in screw cap tubes wrapped in aluminium foil.

2.3.2. Working Solution (0.5 μg ml^{-1}):

The working solution was prepared by adding 50 µl of stock solution to 1l of distilled water.

APPENDIX III

III REAGENTS FOR RNA GEL ELECTROPHORESIS

3.1 10X FA gel buffers

3 N-[morpholino] propanesulfonic acid (MOPS)(free acid)	200mM
EDTA	10mM
Sodium acetate	50mM

pH to 7.0 with NaOH

3.2. 1X FA gel Running Buffer

10X FA gel buffer	100ml
37% (12.3 M) Formaldehyde	20ml
RNase free water	880ml

3.3. 5X RNA Loading Buffer

Saturated aqueous bromophenol blue solution	16 µl
500mM EDTA, pH 8.0	80 µl
37% (12.3 M) Formaldehyde	720 µl
100% Glycerol	2ml
Formamide	3.084ml
10X FA gel buffer	4ml

RNase free water to 10ml

Stability: Approximately 3 months at 4°C

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MECHANISMS

Nisin and Class IIa Bacteriocin Resistance Among *Listeria* and Other Food-Borne Pathogens and Spoilage Bacteria

Gurpreet Kaur, Ravinder Kumar Malik, Santosh Kumar Mishra, Tejinder Pal Singh,
Arun Bhardwaj, Garima Singroha, Shilpa Vij, and Naresh Kumar

Food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. To inactivate food-borne pathogens, various novel technologies such as biopreservation systems have been studied. Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity produced by different groups of bacteria, but the bacteriocins produced by many lactic acid bacteria offer potential applications in food preservation. The use of bacteriocins in the food industry can help reduce the addition of chemical preservatives as well as the intensity of heat treatments, resulting in foods that are more naturally preserved. However, the development of highly tolerant and/or resistant strains may decrease the efficiency of bacteriocins as biopreservatives. Several mechanisms of bacteriocin resistance development have been proposed among various food-borne pathogens. The acquiring of resistance to bacteriocins can significantly affect physiological activity profile of bacteria, alter cell-envelope lipid composition, and also modify the antibiotic susceptibility/resistance profile of bacteria. This article presents a brief review on the scientific research about the various possible mechanisms involved in the development of resistance to nisin and Class IIa bacteriocins among the food-borne pathogens.

Introduction

For the past few decades, food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. To inactivate food-borne pathogens, various novel technologies such as biopreservation systems have been studied. Biopreservation of foods offers an alternative control measure for improving the stability and safety of mildly processed food products and has, therefore, been the focus of increased attention in the last few years. Biopreservation reduces the amount of chemical preservatives to be added to the food as well as the intensity of heat treatments, both of which can otherwise negatively affect the food quality.[36,57] Lactic acid bacteria (LAB) are critical for the production of a great multitude of fermented foods. The most important contribution of these bacteria to fermented products is to preserve the nutritive qualities of the raw material and inhibit the growth of spoilage and pathogenic bacteria. Protection of food from spoilage and pathogenic microorganisms by LAB is through the production of organic acids, hydrogen peroxide, diacetyl,[73] and antifungal compounds such as fatty acids[16] or phenullactic acid[63] and/or bacteriocins.[26]

Bacteriocins produced by LAB have been the subject of considerable research and industrial interest due to their potential as food biopreservatives. Although bacteriocins may be found in many Gram-positive and Gram-negative bacteria,[88] those produced by LAB have received particular attention in recent years due to their potential application in the food industry as natural preservatives.[34] Compared with classical peptide antibiotics, which are synthesized through enzymatic condensation of free amino acids, the bacteriocins are proteinaceous antibacterial compounds that constitute a heterologous subgroup of ribosomally synthesized antimicrobial peptides with antimicrobial activity against closely related bacteria.[26] In general, these substances are cationic peptides that display hydrophobic or amphiphilic properties, and the bacterial membrane is, in most cases, the target for their activity.[76] Bacteriocins of LAB are a heterogeneous group of peptides and proteins that were earlier classified into five main classes bacteriocins.[61,80] The bacteriocins were later on grouped into two major classes, which display great diversity with regard to their mode of action, structure, genetics, mode of secretion, and choice of target organisms[79]: class I: lantibiotics; which are small, post-translationally modified peptides that contain unusual amino acids such as lanthionine. The lantibiotics have been divided into two subgroups: type A, elongated molecules with a flexible structure in solution, and type B, which tend to have a more rigid, globular structure (although there are some exceptions) and

Microbial Metabolites Laboratory, Dairy Microbiology Division, National Dairy Research Institute, Karnal, India.

class II: the heat-stable nonlantibiotics. Lack of consensus also exists in the distinction between subgroups of the nonlantibiotics belonging to class II, which include a large number of small peptides that only have in common their marked thermostability and a lack of modified residues.[14,17,29,33,90] In the new classification scheme, Nes *et al.*[79] subdivided class II into four subclasses: IIa (antilisteria pediocin-like bacteriocins), IIb (two-peptide bacteriocins), IIc (leaderless peptide bacteriocins), and class IId (circular bacteriocins). A third class of bacteriocins has been suggested that includes secreted heat-labile, cell-wall degrading enzymes, but the classification of such enzymes as being bacteriocins has recently been thrown into doubt.[17] In contrast to this, Heng *et al.*[52] proposed to retain this class for large bacteriocins, which are subdivided into IIIa (bacteriolysins) and IIIb (nonlytic proteins). Nevertheless, according to the recommendations of several research groups,[51,52,58,59,69] the circular, post-translationally modified bacteriocins deserve to be upgraded to a new class IV.

Although the use of bacteriocins for preservation (biopreservation) is a novel approach to eliminating or controlling pathogens in food, the development of highly tolerant or resistant strains remains the main concern and decreases the efficiency of bacteriocins as biopreservatives. Resistant foodborne pathogens are posing a global problem that is further facilitated by international trade of raw and processed foods.[5] In foods with a long shelf life, even a small number of these resistant cells can multiply to very high number and, thus, may lead to food-borne outbreaks and food spoilage. Among bacteriocins produced by LAB, nisin, a class I bacteriocin, has demonstrated antilisterial activity. It was the first bacteriocin to be characterized and is the only one approved for use in food applications.[13] Therefore, most research in this area has focused on specific bacteriocins, such as nisin and some of the class IIa peptides. The emergence of nisin-resistant *Listeria monocytogenes* mutants has already been reported.[25,40] In the current report, extensive study is presented to review the proposed mechanisms of nisin resistance and also class II bacteriocin resistance developed among the food-borne pathogens.

Emergence Of Nisin Resistance

The resistance responses of microorganisms to antimicrobials may be innate, apparent, or acquired. Innate resistance is a chromosomally controlled property that is naturally associated with a microorganism. Differences in resistance to antimicrobials occurring among different types, genera, species, and strains of microorganisms under identical environmental conditions and antimicrobial concentrations are most likely controlled innately. Mechanisms of innate resistance may include cellular barriers preventing entry of the antimicrobial (*e.g.*, the outer membrane of Gram-negative bacteria and teichoic acids contained within Gram-positive bacteria), cellular efflux (*i.e.*, mechanisms that pump compounds out of the cell), lack of a biochemical target for antimicrobial attachment or microbial inactivation, and inactivation of antimicrobials by microbial enzymes.[5] Apparent resistance is related to assay or application conditions. As with any preservation technique, susceptibility to antimicrobials is dependent on the conditions of the application.[23] The presence of interacting stress conditions (*e.g.*, low pH, high temperatures, and high pressure) may increase or decrease the measured resistance of a microorganism. Acquired resistance results from genetic changes in the microbial cell through mutation or acquisition of genetic material from plasmids.[23,91]

The emergence of nisin resistant mutants, which are generated when nisin-sensitive cells are exposed to relatively high nisin concentrations, has been described for several *Lactobacillus* spp., *Streptococcus* spp., *Leuconostoc* spp., *Bacillus* spp., and *Clostridium* spp. and for *Staphylococcus aureus*, and *Li. monocytogenes*.[6,20,25,74,75] Harris *et al.*[47] detected mutant strains of *Li. monocytogenes*, at frequencies of 10^{-6} and 10^{-8}, which were able to grow at nisin concentrations 5 to 10 times higher than was the original population, indicating the potential for nisin resistant variants to arise from widespread application of the lantibiotic.

Proposed Mechanisms For Development Of Nisin Resistance

The acquisition of nisin resistance is reported to be a complex and may differ among strains, as suggested by reports on alterations in fatty acid composition and cell wall changes in nisin-resistant *Listeria*. Nisin resistance may result from the presence of a nisin-degrading enzyme, nisinase, which has been reported in several Gram-positive bacteria.[2,35,53,54,66] The nisinase from several *Bacillus* species specifically reduced the C-terminal dehydroalanyl lysine of nisin to alanyllysine.[55,56] In 1998, Crandall and Montville[19] put forward a model for the nisin resistance of *Li. monocytogenes* ATCC 700302, which included three factors: (i) variation of peptidoglycan composition,[67] which should make it possible to increase the binding of divalent cations that interact with the cationic peptide; (ii) modification of the electric charge of the membrane by phospholipids content changes, thereby preventing pore formation;[19,74,87] and (iii) increase in membrane rigidity, preventing peptide insertion and association.[74] Resistance has been correlated with both an altered fatty acid composition[72,74] and an altered phospholipids composition.[75]

Nisin Resistance Through Cell Wall Modification

Crandall and Montville[19] studied the changes in the cell wall of the nisin resistant strain by evaluating the strain's sensitivity to cell wall-acting compounds. The nisin resistant strain was reported to be more resistant than the wild type to lysozyme, which catalyzes the hydrolysis of the β-1, 4 glycosidic bond between *N*-acetylmuramicglucosamine and *N*-acetylglucosamine of cell wall peptidoglycan, and more sensitive to the cell wall-acting antibiotics benzyl penicillin and ampicillin, which block the cross-linking reaction of peptidoglycan synthesis. These altered sensitivities suggested compositional changes in the cell wall of the nisin resistant strain.

The cell wall has also been implicated in nisin-resistant *Listeria innocua*, in which altered sensitivities to cell wall-acting antibiotics and enzymes and a thickened cell wall were observed.[67] In addition, removal of the cell wall from nisin-resistant *Li. monocytogenes* F6861 resulted in the loss of nisin resistance, suggesting that differences in the cell wall were responsible for resistance in this strain.[25] Similarly a *Sta. aureus* mutant reported to be defective in D-alanine

incorporation to lipoteichoic acids was more sensitive to nisin than wild-type cells, and it was suggested that the positively charged D-alanine residue was excluding the positively charged nisin molecule.[81] Experiments with *Streptococcus bovis* indicated that the nisin-resistant cells had an increased positive (*i.e.*, less negative) charge than nisin-sensitive cells. Nisin-resistant cells (i) bound less cytochrome *c* than nisin-sensitive cells, (ii) were more lysozyme resistant, and (iii) were less hydrophobic.[68]

Also, it has been reported that several of the class I lantibiotics as well as nonlantibiotic lactococcin 972 employ lipid II, a precursor in cell wall synthesis as a docking molecule and interaction between a bacteriocin and lipid II leads to inhibition of cell wall synthesis and/or pore formation, depending on the structure of the bacteriocin and its concentration.[7,71,99]

Role Of Divalent Cations In Bacterial Resistance Against The Inhibitory Activity Of Bacteriocins

Divalent cations are required to sufficiently stabilize the altered nisin resistant cell's cytoplasmic membrane against disruption by nisin.[1,19] This stabilization might involve interfering with nisin's binding or a specific interaction of the cations with membrane components or some combination of the two. Divalent cations have been suggested to interact extensively with cell wall components, especially the negatively charged teichoic acids.[19] Perhaps the change in the cell wall of the nisin resistant strain requires additional divalent cations to either stabilize extra negative charge or prevent nisin from binding to anionic sites. In the presence of divalent cations, a tighter packing of the acyl chains is introduced,[48,60] which can hinder nisin insertion.

Abee *et al*.[1] found that di- and trivalent cations (Mg^{2+}, Ca^{2+}, and Gd^{3+}) decreased the nisin Z-induced rate of K^{+} efflux from whole cells of *Li. monocytogenes* Scott A. Alternatively or additionally, the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane. The nisin resistant strain of *Li. monocytogenes* was reported to require divalent cations to resist the inhibitory effect of nisin and this effect was shown to be dependent on the concentration of divalent cations.[19] Crandall and Montville[19] suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipid head groups.

Nisin Resistance Through Modifications Of Membrane Phospholipid Composition

The development of nisin resistance of the target cells has also been correlated with both an altered fatty acid composition[72,74] and an altered phospholipids composition.[75] Ming and Daeschel[75] reported that the stable nisin-resistant mutant of *Li. monocytogenes* contained a greater proportion of straight chain fatty, acids whereas the parent strain contained more branched chain fatty acids; no changes in unsaturation of lipid acyl chains were reported.

In a study conducted by Verheul *et al*.,[96] it was shown that higher nisin concentrations were required to dissipate the proton motive force in a nisin-resistant variant of *Li. monocytogenes* Scott A compared with its parent wild-type strain, which correlated with the nisin sensitive status of the strains in brain heart infusion broth. This suggested a possible role for the energy transducing cytoplasmic membrane in the acquisition of resistance in *Li. monocytogenes* Scott A. The results demonstrated that phospholipid head group alterations, particularly in the content of diphosphatidylglycerol (DPG), were the basis of a nisin-resistant variant of *Li. monocytogenes* Scott A. They also reported that the nisin-resistant strains produce relatively more phosphatidylglycerol (PG) and less DPG than does the wild-type strain. It was suggested that this could be due to the decreased activity of the enzyme DPG synthetase, which forms DPG by condensation of two PG molecules.[83] In a study conducted by Demel *et al*.,[27] it has been demonstrated that nisin penetrates more deeply into monolayers of DPG than into those of other lipids including PG, phosphatidylcholine, phosphatidylethanolamine, monogalactosyl-diacylglycerol, and digalactosyldiacylglycerol.

Similar changes in membrane fatty acid composition, including increased long-chain fatty acids, decreased short-chain acids, and a lower C15/C17 ratio, were found for nisin-resistant *Li. monocytogenes* ATCC 700302.[72] Both the fatty acid composition change and the alteration in membrane phospholipids were observed only when nisin-resistant *Li. monocytogenes* ATCC 700302 was grown in the presence of nisin, indicating that nisin induced these changes.[72] Crandall and Montville[19] confirmed that the major phospholipids in the nisin-resistant strain of *Li. monocytogenes* ATCC 700302 contained significantly more zwitterionic phosphatidylethanolamine and less anionic PG and cardiolipin. Given the role that anionic phospholipids play in nisin's interaction with membranes,[30,37,70] a decrease in the net negative charge of the lipid bilayer was proposed to hinder nisin's ability to bind and interact with the membrane. Also, resistance to sakacin P has been reported to introduced distinct changes mainly in the polysaccharide, fatty acid, and protein regions, which represented the major cell membrane components in *Li. monocytogenes* strains.[93]

Resistance To Class Iia Bacteriocins From The Loss Of *Mpt* Expression

It has been suggested that resistance to class IIa bacteriocins occurs at either a low (two- to fourfold increase in minimum inhibitory concentration *i.e.*, MIC) or a high level (1,000-fold increase in MIC).[79] High-level resistance of *Enterococcus faecalis*, *Li. Monocytogenes*, and some other Gram-positive bacteria to class IIa bacteriocins has been reported to result from the loss of expression of mannose-specific phosphotransferase system (*mpt*), either in defined mutants or in spontaneous resistant strains.[41] The *mpt* operon encodes a mannose permease (EII_t^{Man}) that belongs to the mpt, and the mpt–phosphotransferase system (PTS) transporter family is responsible for the import and phosphorylation of carbohydrates such as mannose and glucose in bacteria.[82] A phosphate moiety is transferred from phosphoenolpyruvate to the transported sugar via the PTS proteins EI, HPr, and EII. The latter is a complex permease made of three or four subunits that can be fused: IIA and IIB are cytoplasmic subunits responsible for phosphorylation, whereas IIC is an integral membrane subunit involved in sugar transport. A IID membrane subunit, associated with IIC, is found

specifically in permeases of the mannose family. Sigma 54 (σ54) has been described to be involved in expression of mpt.[100] Sigma (σ) factors are subunits of the RNA polymerase holoenzyme involved in the initial step of transcription. Among them, σ54 is unique, as it targets conserved -24/-12 promoter sequences and requires an activator protein for transcription initiation, referred to as σ54-associated activator.[77,92] Also, since the genes encoding these four subunits are clustered in one operon, their expression has been reported to be coordinated.[78]

Based on the observations that resistant mutants have an altered expression pattern of mannose-PTS (man-PTS) and that heterologous expression of cloned man-PTS genes renders resistant cells sensitive, some studies have shown that the man-PTS might serve as a receptor for some pediocin-like bacteriocins.[22,41,49,84,85] Also, it has been demonstrated that the membrane components (ManM/PtnC and ManN/PtnD) of the man-PTS are directly involved as receptors for several bacteriocins from subclass IIa and some from subclass IIc and that in bacteriocin-producing cells, a cognate immunity protein tightly binds the receptor in a bacteriocin-dependent manner, to prevent killing by the bacteriocin.[28] Also, it has been suggested that the *man* operon of *Lactobacillus sakei* 23K confers sensitivity to a number of class IIa bacteriocins, and the *ptn* operon of *Lactococcus lactis* IL1403 confers sensitivity to the class IIc bacteriocins lactococcin A and lactococcin B.[28]

The level of mpt expression has been shown to correlate with the level of bacteriocin sensitivity,[22,49] which implies that EII_t^{Man} permease might be a target molecule for class IIa bacteriocins. Two bacteriocin-resistant spontaneous mutants of *Li. monocytogenes* were linked to PTSs. In the first mutant, resistant to leucocin A, a two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis protein analysis revealed absence of a IIAB subunit of a PTS permease.[84] In the second mutant, resistant to pediocin PA-1, a β-glucoside PTS permease was described to be over-expressed.[42] Finally, Dalet e*t al.*[22] described that interruption of genes encoding a σ54- associated activator (MptR) and a σ54-dependent PTS permease of the mannose family (termed EII_t^{Man}) led to resistance of *E. faecalis* to Mesentericin Y105. To find a link between σ54 and sensitivity of *Li. monocytogenes* to Mesentericin Y105, Dalet *et al.*[22] searched for σ54-associated activators and σ54dependent genes in the *Li. monocytogenes* EGDe genome. They found an activator and a PTS permease of the mannose family that are required for sensitivity of *Li. monocytogenes* to Mesentericin Y105. Dalet *et al.*[22] also hypothesized that the transcriptional factor σ54 (encoded by *rpoN*) could direct the expression of a receptor, as *rpoN* mutants of *Li. monocytogenes*[89] and *E. faecalis*[21] were shown to be resistant to mesentericin Y105 and related subclass IIa bacteriocins.

Attempts were made to unravel the resistance of *Li. monocytogenes* to DivercinV41 (DvnV41) that involved a proteomic approach, comparing protein profiles of *Li. monocytogenes* wild-type and a mutant resistant to DvnV41. *Li. monocytogenes* with a DvnV41-resistant phenotype displayed differential protein synthesis.[31] This mutant strain was reported to lack at least nine protein spots, two of which were shown to have a molecular mass and pI matching those of the *mptA* cluster, which is controlled by the σ54 transcription factor in coordination with the ManR regulator.[22] It was subsequently proposed that high level of resistance to class IIa bacteriocins was acquired through one general mechanism: the lack of EII_t^{Man} expression.[41]

Vadyvaloo *et al*.[94] reported that the presence of glucose or mannose induced the sensitivity of *Li. monocytogenes* and *E. faecalis* to Mesentericin Y105. These sugars also induced expression of the *mpt* operon of *Li. monocytogenes*, indicating that EII_t^{Man} transports glucose and mannose in accordance with previous observations showing a specific inducible effect of the transported sugar on PTS permease expression.[82] These correlated results suggested that the level of EII_t^{Man} expression is directly linked to sensitivity of *Li. monocytogenes* to Mesentericin Y105 (MesY105). Vadyvaloo *et al.*[94] investigated the effect of the missing *MptA* subunit of the EII_t^{Man} on glucose metabolism in Class IIa bacteriocin-resistant *Li. monocytogenes* strains. They focused on glucose consumption rates and analysis of the end products of glucose metabolism. The observation that the specific growth rate in Class IIa bacteriocin-resistant strains, B73-MR1 and EGDe-mptA, on media containing glucose was lower than that of the corresponding wild-type strains has also been described for another class IIa bacteriocin-resistant *Li. monocytogenes* strain, 412P, also showing loss of MptA expression.[41] The decreased growth rate in 412P, and in other class IIa bacteriocin-resistant B73 strains, has been interpreted as a fitness cost associated with class IIa bacteriocin resistance.[32] This fitness cost was thought to be due to energy expensive metabolic pathways in resistant strains.[32] By taking a closer look at the physiology of these resistant strains, one can suggest an explanation for the reduction in specific growth rate, namely the reduced consumption rate of glucose. It was reported that EII_t^{Man} may be the major transporter of glucose for *Li. monocytogenes* considering the greater than 50% decrease in glucose consumption rate observed for the resistant strains lacking MptA and evidence for the existence of only the glucose-specific enzyme IIA component and no other functional components of the glucose specific PTS in *Li. monocytogenes* EGDe.[38] Further, Vadyvaloo *et al.*[94] suggested that the lower activity of glucose transporting enzymes causing extensive decrease in the glucose consumption rate was responsible for the decrease in specific growth rate. In contrast to the results obtained in media containing glucose, they observed an increased specific growth rate for the resistant strains compared with the wild-type strains in the absence of glucose. They suggested that it might due to the missing glucose transporter that an up-regulation of metabolic routes for other substrates has occurred which gives these cells an advantage in the absence of glucose. During the physiological characterization Vadyvaloo *et al.*[94] noted that, in addition to the apparent disadvantage of a lower growth rate in the presence of glucose, the resistant strains had a higher biomass yield on glucose. A product analysis revealed that the resistant strains had more of a mixed-acid type of fermentation as compared with the homolactic fermentation in the wild-type strains. In a homolactic fermentation, 2 mol ATP is formed per mole of glucose fermented and in a pure mixed acid fermentation (*i.e.*, no lactate formed and acetate and ethanol formed in a 1: 1 ratio), 3 mol ATP is formed per mole of glucose fermented. Their results indicated that physiological responses, related to the absence of MptA in class IIa bacteriocin-resistant strains, could further compromise the potential use of class IIa bacteriocins as biopreservatives. Although resistant strains, in the presence of glucose,

showed a lower specific growth rate than the wild-type strains, it was reported that the biomass yield on glucose (and potentially other energy sources) was significantly increased. Vadyvaloo *et al*.[94] also reported that together with the inactivation of the MptA, a shift in metabolism occurs that could significantly alter the final concentrations of the fermentation products, thus suggesting a strong possibility that the end product of metabolism in LAB starter cultures could change as a result of acquiring this type of resistance to class IIa bacteriocins. The shift in metabolism and subsequent change in the end product would profoundly influence both the organoleptic qualities and spoilage potential of the food product.

Besides the high-resistance strains, two spontaneous mutants of *Li. monocytogenes*, B73-V1 and B73- V2, were also described to have an intermediate resistance level (2–4 times the IC50 for wild-type sensitive strain) to leucocin A, a class IIa bacteriocin.[95] Some modifications of membrane phospholipids were associated to this intermediate resistance phenotype, although the authors suggested that other mechanisms are likely to be involved. To characterize the intermediate resistance, Arous *et al*.[4] studied a new intermediate resistant strain that was interrupted in a man-PTS operon similar to mpt, namely *mpo.* The mpo operon is composed of four open reading frames, *mpoA, mpoB, mpoC, and mpoD*. They putatively encode four proteins, MpoA (124 aa), MpoB (161 aa), MpoC (295 aa), and MpoD (292 aa), which show greatest similarities with PTS permease subunits of the mannose family. Mpo is located just downstream of the gene encoding *ManR*, the σ54-dependent activator that is responsible for *mptACD* expression. While characterizing the intermediate resistance mechanism at the molecular level, the expressions of both *mptACD* and *mpoABCD* were followed by real-time polymerase chain reaction in each mutant strain compared with the wild type as a reference. The highly resistant strains, namely EGK54 and B73-MR1, were reported to exhibit an induced *mpo* expression (11.3- and 3.6-fold, respectively), whereas no significant changes were observed in strains B73-V1 and B73-V2. The highly resistant strains were dramatically affected in EII_t^{Man} t expression, suggesting that *mpo* was induced by the loss of EII_t^{Man} expression. A similar mechanism of regulation has been described for a β-glucoside permease gene, which is highly expressed in these mutants[42] as well as in a *rpoN* mutant.[4] Arous *et al*.[4] also hypothesized that *mpo* is regulated by carbon catabolite regulation, as a putative catabolite responsive element sequence was located upstream from *mpoABCD*.

Given that the IIC and IID subunits of *mpt* operon are probably present in the membrane, these two subunits were hypothesized to be the potential targets of class IIa bacteriocins. The *mptACD* operon of *Li. monocytogenes* heterologously expressed in an insensitive species, such as *Lactoc. lactis*,[85] rendered this strain sensitive to various class IIa bacteriocins. Each gene of the *mptACD* operon was expressed independently in *Lactoc. Lactis*, and the expression of *mptC* alone was found to be sufficient to confer sensitivity. The IIC subunit was, therefore, proposed as the target molecule of the class IIa bacteriocin.[85] Enhanced nisin resistance in some mutants of *Li. monocytogenes* has also been associated with increased expression of three genes, *pbp2229*, *hpk1021*, and *lmo2487*, encoding a penicillin-binding protein, a histidine kinase, and a protein of unknown function, respectively.[39]

Calvez *et al.*[9] also identified three genes associated with the resistance of *E. faecalis* JH2-2 to DvnV41. The first one was the *rpoN* gene, which encodes the σ54 factor. It has been postulated that *rpoN* is involved in the expression of a target molecule for class IIa bacteriocins, loss of whose expression leads to resistance.[29] The second gene identified encoded a putative glycerophosphoryl diester phosphoesterase (GlpQ), an exoprotein found to participate in fatty acid and phospholipids degradation in many bacteria.[3] A hypothesis that could be drawn from data given by Calvez *et al.*[9] was that the absence of GlpQ activity in mutant 36H4 led to an intact fatty acid and phospholipids composition of the cell membrane, which contributed to the resistant phenotype of the mutant. Moreover, the GlpQ protein was originally suggested to belong to the Pho regulon in *Bacillus subtilis*[3] and to be governed by a pleiotropic two-component regulatory system PhoP-PhoR.[43] Interestingly, PhoP and Mg^{2+} were also reported to control the resistance of many Gram-negative bacteria to antimicrobial peptides, as supported by a number of lines of evidence.[44,78] Similarly, in Gram-positive bacteria, different studies highlighted the role of a two-component regulatory system in the resistance of *Li. monocytogenes*,[18] *E. faecalis*,[15] and *Sta. aureus*[62] to antimicrobial peptides and antibiotics. To gain a more comprehensive view of the role of a two-component signal transduction system pathway in resistance to pediocin-like bacteriocins, Calvez *et al.*[9] examined the resistance of each insertional mutant characterized so far in *E. faecalis* JH2[64] and *E. faecalis* V583.[45] The results obtained rejected any relationship between a two-component regulatory system and resistance to DvnV41. Finally, the third gene identified encoded a putative phosphoesterase (PDE). Overall, it was reported that the *rpoN* gene was associated with the high-level resistance and the newly identified genes with the intermediate resistance of *E. faecalis* JH2-2 to DvnV41, MesY105, and Ped PA 1/Ach.

Listeria orthologs of enterococcal *pde* and *glpQ* genes, that is, two ORFs named *lmo0052* and *lmo1292*, exhibiting the highest similarity (65.4% and 60.7%) to enterococcal *pde* and *glpQ* gene, respectively, were identified in the genome of *Li. monocytogenes* EGDe.[10] Also, the relative expression of *mptABCD* operon, *glpQ, pde, rpoN, mptR*, and *gap*-1 was studied by reverse transcription combined with the real-time polymerase chain reaction to understand the role of each gene in resistance/sensitivity of *E. faecalis* JH-2 to class IIa bacteriocins such as recombinant DvnV41 (DvnRV41).[11] DvnRV41 was reported to up-regulate the expression of *gap*-1 gene, which codes for glyceraldehyde 3-phosphate dehydrogenase, an enzyme involved in glycolysis and gluconeogenesis. The up-regulation of *mptC* and *gap*-1 genes indicated that *E. faecalis* JH2-2 could activate sugar metabolism. The down-regulation of *glpQ* gene by DvnRV41 was not expected and resulted in contradictory to the former,[9] which ruled out that resistant phenotype of *E. faecalis* JH2-2 to DvnV41 was acquired after inactivation of glpQ gene. Finally, the down regulation of *glpQ* gene was attributed to interplay and/or energy compensation between down- and up-regulated genes. Also, it was suggested that the expression of glpQ could depend on the growth conditions, amount of DvnV41, and growth phase.[11]

Cross Resistance To Bacteriocins

Resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *Li. monocytogenes* was shown to be cross resistant to the class IIa bacteriocin, pediocin PA-1, and the Class IV leuconocin S.[19] Pediocin PA-1 is a 44-amino acid peptide whose sequence has been determined[50] and modeled into a three-dimensional structure,[13] which predicts that initial binding to membranes is through electrostatic interactions. Leuconocin S is a small (molecular weight, 10,000) glycoprotein.[65] Both leuconocin S and pediocin PA-1, similar to nisin, act against *Li. monocytogenes* by depleting the Proton Motive Force.[8] *Li. monocytogenes* mutants resistant to mesentericin 52, curvaticin 13, and plantaricin were also reported to be cross-resistant to the other bacteriocins.[86] In addition, piscicolin 126-resistant mutants of *Li. monocytogenes* that emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02.[98] These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy[46] might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the other bacteriocins.

Cross-resistance between bacteriocins has been observed when the sensitivity of *Listeria* variants to lactocin 705, enterocin CRL35, and nisin was tested.[97] Similar results were obtained by Rekhif *et al.*,[86] who reported that mutants of *Li. monocytogenes* ATCC 15313 resistant to one of three bacteriocins tested (mesenterocin 52, curvaticin 13, and plantaricin C19) displayed more resistance to the two others but not to nisin. Insensitivity of a variant to lactocin 705 and enterocin CRL35 while retaining sensitivity to nisin, and vice versa, may be associated with the mechanism by which a bacteriocin enters the cell after binding to the cell surface, as well as with the ability to form pores in bacterial membranes.

Conclusion

The use of bacteriocins in biopreservation is a novel approach in eliminating or controlling *Li. monocytogenes* and other pathogens in food while the development of resistant variants of the organism remains the main concern and limits this use. There have been systematic studies to investigate bacteriocin resistance in bacterial pathogens. Most data exist on *Listeria* spp. whereas the number of reports on development of bacteriocin resistance among other pathogens or food spoilage bacteria is limited. The acquirement of resistance to bacteriocins can significantly affect physiological activity, alter cell-envelope lipid composition, and modify the antibiotic susceptibility/resistance profile of bacteria. Improved control of the target microorganisms and inhibition of bacteriocin-resistant strains and species can be obtained by using a combination of one or more bacteriocins. However, resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes.

Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage. Among the food-borne pathogens, knowledge of the characteristics of bacteriocin-resistant variants and the conditions that prevent their emergence will help in determining the optimal conditions for application of bacteriocins in foods and minimize the incidence of resistance.

Disclosure Statement

No competing financial interests exist.

References

1. **Abee, T., F.M. Rombouts, J. Hugenholtz, G. Guihard, and L. Letellier.** 1994. Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. Appl. Environ. Microbiol. **60:**1962–1968.
2. **Alifax, R., and R. Chevalier.** 1962. Etude de la nisin produite par *Streptococcus thermophihs*. J. Dairy Res. **29:**233–240.
3. **Antelmann, H., C. Scharf, and M. Hecker.** 2000. Phosphate starvation-inducible proteins of *Bacillus subtilis:* proteomics and transcriptional analysis. J. Bacteriol. **182:**4478–4490.
4. **Arous, S., K. Dalet, and Y. Hechard.** 2004. Involvement of the *mpo* operon in resistance to class IIa bacteriocins in *Listeria monocytogenes.* FEMS Microbiol. Lett. **238:**37–41.
5. **Bower, C.K., and M.A. Daeschel.** 1999. Resistance responses of microorganisms in food environments. Int. J. Food Microbiol. **50:**33–44.
6. **Breuer, B., and F. Radler.** 1996. Inducible resistance against nisin in *Lactobacillus casei*. Arch. Microbiol. **65:**114–118.
7. **Brotz, H., M. Josten, I. Wiedemann, U. Schneider, F. Götz, G. Bierbaum, and H.G. Sahl.** 1998. Role of lipid-bound peptidoglycan precursors in the formation of pores by nisin, epidermin and other lantibiotics. Mol. Microbiol. **30:**317–327.
8. **Bruno, M.E.C., and T.J. Montville.** 1993. Common mechanistic action of bacteriocins from lactic acid bacteria. Appl. Environ. Microbiol. **59:**3003–3010.
9. **Calvez, S., A. Rincé, Y. Auffray, H. Pré vost, and D. Drider.** 2007. Identification of new genes associated with intermediate resistance of *Enterococcus faecalis* to divercin V41, a pediocin-like bacteriocin. Microbiology **153:**1609–1618.
10. **Calvez, S., H. Prevost, and D. Drider.** 2008. Identification of a new molecular target of class IIa bacteriocins in *Listeria monocytogenes* EGDe. Folia Microbiol. **53:**417–422.
11. **Calvez, S., H. Prevost, and D. Drider.** 2008. Relative expression of genes involved in the resistance/sensitivity of Enterococcus faecalis JH2-2 to recombinant divercin RV41. Bioctechnol. Lett. **30:**1795–1800.
12. **Chen, Y., R. Shapira, M. Eisenstein, and T.J. Montville.** 1997. Functional characterization of pediocin PA-1 binding to liposomes in the absence of a protein receptor and its relationship to a predicted tertiary structure. Appl. Environ. Microbiol. **63:**524–531. ◀AU1
13. **Chung, K.T., J.S. Dickson, and J.D. Crouse.** 1989. Effects of nisin on growth of bacteria attached to meat. Appl. Environ. Microbiol. **55:**1329–1333.
14. **Cleveland, J., T.J. Montville, I.F. Nes, and M.L. Chikindas.** 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbial. **71:**1–20.
15. **Comenge, Y.R., R. Quintiliani, L. Li, Jr., L. Dubost, J.P. Brouard, J.E. Hugonnet, and M. Arthur.** 2003. The CroRS two component regulatory system is required for intrinsic beta-lactam resistance in *Enterococcus faecalis.* J. Bacteriol. **185:**7184–7192.
16. **Corsetti, A., M. Gobbetti, and E. Smacchi.** 1996. Antibacterial activity of sourdough lactic acid bacteria: isolation of a bacteriocin-like inhibitory substance from *Lactobacillus sanfrancisco* C57. Food Microbiol. **13:**447–456.

17. **Cotter, P.D., C. Hill, and R.P. Ross.** 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3:**777–788.
18. **Cotter, P.D., C.M. Guinane, and C Hill.** 2002. The LisRK signal transduction system determines the sensitivity of *Listeria monocytogenes* to nisin and cephalosporins. Antimicrob. Agents Chemother. **46:**2784–2790.
19. **Crandall, A. D., and T.J. Montville.** 1998. Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl. Environ. Microbiol. **64:**231–237.
20. **Daeschel, M.A., D.S. Jung, and B.T. Watson.** 1991. Controlling wine malolactic fermentation with nisin and nisin-resistant strains of *Leuconostoc oenos*. Appl. Environ. Microbiol. **57:**601–603.
21. **Dalet, K., C. Briand, Y. Cenatiempo, and Y. Héchard.** 2000. The *rpoN* gene of *Enterococcus faecalis* directs sensitivity to subclass IIa bacteriocins. Curr. Microbiol. **41:**441–443.
22. **Dalet, K., Y. Cenatiempo, P. Cossart, and Y. Héchard.** 2001. A σ54-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. Microbiology **147:**3263–3269.
23. **Davidson, P.M., and M.A. Harrison.** 2002. Resistance and adaptation to Food antimicrobials, sanitizers, and other process controls. Food Tech. **56:**68–78.
24. **Davies, E.A., M.B. Falahee, and M.R. Adams.** 1996. Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J. Appl. Bacteriol. **81:**139–146.
25. **Davies, E.A., and M.R. Adams.** 1994. Resistance of *Listeria monocytogenes* to the bacteriocin nisin. Int. J. Food Microbiol. **21:**341–347.
26. **De Vugst, L., and E.J. Vandamme.** 1994. Bacteriocins of Lactic Acid Bacteria: Microbiology Genetics and Applications. Blackie Acad and professional, London. ISBND-75 140174-9.
27. **Demel, R.A., T. Peelen, R.J. Siezen, B. de Kruijff, and O.P. Kuipers.** 1996. Nisin Z, mutant nisin Z and lacticin 481 interactions with anionic lipids correlate with antimicrobial activity—a monolayer study. Eur. J. Biochem. **235:**267–274.
28. **Diep, D.B., M. Skaugen, Z. Salehian, H. Holo, and I.F. Nes.** 2007. Common mechanisms of target cell recognition and immunity for class II bacteriocins. Proc. Natl. Acad. Sci. U. S. A. **104:**2384–2389.
29. **Drider, D., G. Fimland, Y. Héhard, L.M. McMullen, and H. Prévost.** 2006. The continuing story of class IIa bacteriocins. Microbiol. Mol. Biol. Rev. **70:**564–582.
30. **Driessen, A.J.M., H.W. van den Hooven,W. Kuiper, M. van der Kamp, H.G. Sahl, R.N. Konings, and W.N. Konings.** 1995. Mechanistic studies of lantibiotic-induced permeabilization of phospholipid vesicles. Biochemistry **34:**1606–1614.
31. **Duffes, F., P. Jenoe, and P. Boyaval.** 2000. Use of two-dimensional electrophoresis to study differential protein expression in divercin V41 resistant and wild-type strains of *Listeria monocytogenes*. Appl. Environ. Microbiol. **66:**4318–4324.
32. **Dykes, G.A., and J.W. Hastings.** 1998. Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett. Appl. Microbiol. **26:**5–8.
33. **Eijsink, V.H., L. Axelsson, D.B. Diep, L.S. Havarstein, H. Holo, and I.F. Nes.** 2002. Production of class II bacteriocins by lactic acid bacteria; an example of biological warfare and communication. Antonie Van Leeuwenhoek **81:**639–654.
34. **Ennahar, S., K. Sonomoto, and A. Ishizaki.** 1999. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. J. Biosci. Bioeng. **87:**705–716.
35. **Galesloot, T.E.** 1956. Melkuurbacterien die het antibioticum van *S. lactis* (nisine) onwerkzaam maken. Nederlands Melken Zuiveltijdschrzft. **10:**143–155.
36. **Galvez, A., H. Abriouel, R.L. Lopez, and N.B. Omar.** 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. **120:**51–70.
37. **García Garcerá, M.J., M.G.L. Elferink, A.J.M. Driessen, and W.N. Konings.** 1993. *In vitro* pore-forming activity of the lantibiotic nisin. Eur. J. Biochem. **212:**417–422.
38. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend , F. Baquero F,** et al. 2001. Comparative genomics of *Listeria* species. Science **294:**849–852.
39. **Gravesen, A., B. Kallipolitis, K. Holmstrøm, P.E. Høiby, M. Ramnath, and S. Knøchel.** 2004. *pbp2229*-mediated nisin resistance mechanism in *Listeria monocytogenes* confers cross-protection to class IIa bacteriocins and affects virulence gene expression. Appl. Environ. Microbiol. **70:**1669–1679.
40. **Gravesen, A., K. Sorensen, F.M. Aarestrup, and S. Knochel.** 2001. Spontaneous nisin-resistant Listeria monocytogenes mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. Microbial. Drug Res. **7:**127–135.
41. **Gravesen, A., M. Ramnath, K.B. Rechinger, N. Andersen, L. Jansch, Y. Héchard, J.W. Hastings, and S. Knøchel.** 2002. High-level resistance to class IIa bacteriocins is associated with one general mechanism in *Listeria monocytogenes.* Microbiology **148:**2361–2369.
42. **Gravesen, A., P. Warthoe, S. Knochel, and K. Thirstrup.** 2000. Restriction fragment differential display of pediocin-resistant *Listeria monocytogenes* 412 mutants shows consistent overexpression of a putative β-glucoside-specific PTS system. Microbiology **146:**1381–1389.
43. **Groisman, E.A.** 2001. The pleiotropic two componenet regulatory system PhoP- PhoQ. J. Bacteriol. **183:**1835–1842.
44. **Groisman, E.A., F. Heffron, and F. Solomon.** 1992. Molecular genetic analysis of the *Escherichia coli phoP* locus. J. Bacteriol. **174:**486–491.
45. **Hancock, L.E., and M. Perego.** 2004. Systematic inactivation and phenotypic characterization of two-component signal transduction systems of *Enterococcus faecalis* V583. J. Bacteriol. **186:**7951–7958.
46. **Hanlin, M.B., N. Kalchayanand, P. Ray, and B. Ray.** 1993. Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. J. Food Prot. **56:**252–255.
47. **Harris, L.J., H.P. Fleming, and T.R. Klaenhammer.** 1991. Sensitivity and resistance of *Listeria monocytogenes* ATCC 19115, Scott A and UAL500 to nisin. J. Food Prot. **54:**836–840.
48. **Harwood, J.L., and N.J. Russell. 1984.** Lipids in Plants and Microbes. George Allen and Unwin, London, United Kingdom.
49. **Héchard, Y., C. Pelletier, Y. Cenatiempo, and J. Frére.** 2001. Analysis of σ54-dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII^{Man}) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology **147:**1575–1580.
50. **Henderson, J.T., A.L. Chopko, and P.D. Vanwassenaar.** 1992. Purification and primary structure of Pediocin PA-1 produced by Pediococcus acidilactici PAC-1.0. Arch. Biochem. Biophys. **295:**5–12.

51. **Heng, N.C.K., and J.R. Tagg.** 2006. What's in a name? Class distinction for bacteriocins. Nat. Rev. Microbiol. **4**:160.
52. **Heng, N.C.K., P.A. Wescombe, J.P. Burton, R.W. Jack, and J.R. Tagg.** 2007. The diversity of bacteriocins produced by Gram-positive bacteria. *In* M.A. Riley and M.A. Chavan, (eds.), Bacteriocins–Ecology and Evolution. Springer, Heidelberg, Germany, pp. 45–92.
AU2 ▶ 53. **Hoover, D.G., and A. Hurst**. 1993. Nisin. *In* (ed.), Antimicrobials in Foods, 2nd edition. Marcel Dekker, NY, pp. 369–394.
54. **Jarvis, B.** 1967. Resistance to nisin and production of nisin-inactivating enzymes by several *Bacillus* species. J. Gen. Microbiol. **7**:33–48.
55. **Jarvis, R.** 1970. Enzymic reduction of the C-terminal dehydroalanyl- lysine sequence in nisin. Proc. Biochem. Soc. **119:**56.
56. **Jarvis, U., and J. Far.** 1971. Partial purification, specificity and mechanism of action of the nisin-inactivating enzyme from *Bacilliis cereus.* Biochimica et Biophysica Acta **227:**232–240.
57. **Kalchayanand, N., M. Hanlin, and B. Ray.** 1992. Sublethal injury makes Gram-negative and resistant Gram-positive bacteria sensitive to the bacteriocins, pediocin AcH and nisin. Lett. Appl. Microbiol. **15:**239–243.
58. **Kawai, Y., R. Kemperman, J. Kok, and T. Saito.** 2004. The circular bacteriocins gassericin A and circularin A. Curr. Protein Pept. Sci. **5**:393–398.
59. **Kemperman, R., M. Jonker, A. Nauta, O.P. Kuipers, and J. Kok.** 2003. Functional analysis of the gene cluster involved in production of the bacteriocin circularin A by *Clostridium beijerinckii* ATCC 25752. Appl. Environ. Microbiol. **69:**5839–5848.
60. **Killian, J.A., M.C. Koorengevel, J.A. Bouwstra, G. Gooris, W. Dowhan, and B. de Kruiff.** 1994. Effect of divalent cations on lipid organization of cardiolipin isolated from *Escherichia coli* strain AH930. Biochim. Biophys. Acta. **1189:**225–232.
61. **Klaenhammer, T.R.** 1993. Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12:**39–85.
62. **Kuroda, M., H. Kuroda, T. Oshima, F. Takeuchi, H. Mori, and K. Hiramatsu.** 2003. Two-component system VraSR positively modulates the regulation of cell-wall biosynthesis pathway in *Staphylococcus aureus*. Mol. Microbiol. **49:**807–821.
63. **Lavermicocca, P., F. Valeria, A. Evidente, S. Lazzaroni, A. Corsetti, and M. Gobbetti.** 2000. Purification and characterization of novel antifungal compounds by sourdough *Lactobacillus plantarum* 21 B. Appl. Environ. Microbiol. **66:**4084–4090.
64. **Le Breton, Y., G. Boeö l, A. Benachour, H. Pré vost, Y. Auffray, and A. Rincé**. 2003. Molecular characterization of *Enterococcus faecalis* two-component signal transduction pathways related to environmental stresses. Environ. Microbiol. **5**:329–337.
65. **Lewus, C.B., S. Sun, and T.J. Montville.** 1992. Production of an amylasesensitive bacteriocin by an atypical *Leuconostoc paramesenteroides* strain. Appl. Environ. Microbiol. **58:**143–149.
66. **Lipinska, E.** 1977. Nisin and its applications. *In* M. Woodbine (ed.), Anribiotics and Antibiosis in Agricutlure. Butterworths, London, pp. 103–130.
67. **Maisnier-Patin, S., and J. Richard.** 1996. Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentration. FEMS Microbiol. Lett. **140:**29–35.
68. **Mantovani, H.C., and J.B. Russell.** 2001. Nisin Resistance of *Streptococcus bovis*. Appl. Environ. Microbiol. **67:**808–813.
69. **Maqueda, M., A. Gálvez, M.J. Sánchez-Barrena, C. González, A. Albert, M. Rico, and E. Valdivia.** 2004. Peptide AS-48: prototype of a new class of cyclic bacteriocins. Curr. Protein Pept. Sci. **5**:399–416.
70. **Martin, I., J.M. Ruysschaert, D. Sanders, and C.J. Giffard.** 1996. Interaction of nisin with membranes revealed by fluorescence quenching of an introduced tryptophan. Eur. J. Biochem. **239:**156–164.
71. **Martinez, B., T. Bottiger, T. Schneider, A. Rodriguez, H.G. Sahl, and I. Wiedemann.** 2008. Specific interaction of the unmodified bacteriocin lactococcin 972 with the cell wall precursor lipid II. Appl. Environ. Microbiol. **74:**4666–4670.
72. **Mazzotta, A. S., and T.J. Montville.** 1997. Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10°C and 30°C. J. Appl. Microbiol. **82:**32–38.
73. **Menssens, W., and L. De Vugst.** 2002. Inhibitory substances produced by Lactobacilli isolated from sourdougts—a review. Int. J. Food Microbiol. **72:**31–43.
74. **Ming, X., and M.A. Daeschel.** 1993. Nisin resistance of food borne bacteria and the specific responses of *Listeria monocytogenes* Scott A. J. Food Prot. **56:**944–948.
75. **Ming, X., and M.A. Daeschel.** 1995. Correlation of cellular phospholipids content with nisin resistance of *Listeria monocytoges* Scott A. J. Food Prot. **58:**416–420.
76. **Moll, G.N., Konings, W.N., and Driessen, A.J.M.** 1999. Bacteriocins: mechanism of membrane insertion and pore formation. Antonie Van Leeuwenhoek **76:**185–198.
77. **Morett, E., and L. Segovia.** 1993. The sigma 54 bacterial enhancer binding protein family: mechanism of action and phylogenetic relationship of their functional domains. J. Bacteriol. **175:**6067–6074.
78. **Moss, J.E., P.E. Fisher, B. Vick, E.A. Groisman, and A. Zychilinsky.** 2001. The regulatory protein PhoP controls the resolution of *Shigella flexneri* infections. Cell Microbiol. **2:**443–452.
79. **Nes, I.F., D.B. Diep, and H. Holo.** 2007. Bacteriocin diversity in *Streptococcus* and *Enterococcus*. J. Bacteriol. **189:**1189–1198.
80. **Nes, I.F., D.B. Diep, L.S. Håvarstein, M.B. Brurberg, V. Eijsink, and H. Holo.** 1996. Biosynthesis of bacteriocins in lactic acid bacteria. Antonie Van Leeuwenhoek **70:**113–128.
81. **Peschel, A., M. Otto, R.W. Jack, H. Kalbacher, G. Jung, and F. Gotz.** 1999. Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins and other antimicrobial peptides. J. Biol. Chem. **274:**8405–8410.
82. **Postma, P.W., J.W. Lengeler, and G.R. Jacobson.** 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol. Rev. **57:**543–594.
83. **Raetz, C.H.R.** 1986. Molecular genetics of membrane phospholipid synthesis in *Escherichia coli*. Annu. Rev. Genet. **42:**614–659.
84. **Ramnath, M., M. Beukes, K. Tamura, and J.W. Hastings.** 2000. Absence of a putative mannose-specific phosphotransferase system enzyme IIAB component in a leucocin A-resistant strain of *Listeria monocytogenes*, as shown by two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Appl. Environ. Microbiol. **66:**3098–3101.
85. **Ramnath, M., S. Arous, A. Gravesen, J.W. Hastings, and Y. Hé chard.** 2004. Expression of mptC of Listeria mono-

cytogenes induces sensitivity to class IIa bacteriocins in *Lactococcus lactis*. Microbiology **150:**2663–2668.

86. **Rekhif, N., A. Atrih, and G. Lefebvre.** 1994. Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr. Microbiol. **28:**237–241.
87. **Revol-Junelles, A.M., R. Mathis, F. Krier, Y. Fleury, A. Delfour, and G. Lefebvre.** 1996. *Leuconostoc mesenteroides* subsp. mesenteroides FR52 synthesizes two distinct bacteriocins. Lett. Appl. Microbiol. **23:**120–124.
88. **Riley, M.A., and J.E. Wertz.** 2002. Bacteriocins: evolution, ecology, and application. Annu. Rev. Microbiol. **56:**117–137.
89. **Robichon, D., E. Gouin, M. Debarbouille, P. Cossart, Y. Cenatiempo, and Y. Héchard.** 1997. The *rpoN* (σ54) gene from *Listeria monocytogenes* is involved in resistance to mesentericin Y105, an antibacterial peptide from *Leuconostoc mesenteroides*. J. Bacteriol. **179:**7591–7594.
90. **Ross, R.P., S. Morgan, and C. Hill.** 2002. Preservation and fermentation: past, present and future. Int. J. Food Microbiol. **79:**3–16.
91. **Russell, A.D.** 1991. Mechanisms of bacterial resistance to non-antibiotics: Food additives and food and pharmaceutical preservatives. J. Appl. Bacteriol. **71:**191–201.
92. **Shingler, V.** 1996. Signal sensing by sigma 54-dependent regulators: derepression as a control mechanism. Mol Microbiol. **19:**409–416.
93. **Tessema, G.T., T. Møretrø, A. Kohler, L. Axelsson, and K. Naterstad.** 2009. Complex phenotypic and genotypic responses of *Listeria monocytogenes* strains exposed to the class IIa bacteriocin sakacin P. Appl. Environ. Microbiol. **75:**6973–6980.
94. **Vadyvaloo, V., J.L. Snoep, J.W. Hastings, and M. Rautenbach.** 2004. Physiological implications of class IIa bacteriocins resistance in *Listeria monocytogenes*. Microbiology **150:**335–340.
95. **Vadyvaloo, V., J.W. Hastings, M.J. van der Merwe, and M. Rautenbach.** 2002. Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl. Environ. Microbiol. **68:**5223–5230.
96. **Verheul, A., N.J. Russell, R. Van T Hof, F.M. Rombouts, and T. Abee.** 1997. Modifications of membrane Phospholipid Composition in Nisin Resistant *Listeria monocytogenes* Scott A. Appl. Environ. Microbiol. **63:**3451–3457.
97. **Vignolo, G., J. Palacios, M.E. Farias, F. Sesma, U. Schillinger, W. Holzapfel, and G. Oliver.** 2000. Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. Curr. Microbiol. **41:**410–416.
98. **Wan J., K. Harmark, B.E. Davidson, A.J. Hillier, J.B. Gordon, A. Wilcock, M.A. Hickey, and M.J. Coventry.** 1997. Inhibition of *Listeria monocytogenes* by piscicolin 126 in milk and Camembert cheese manufactured with a thermophilic starter. J. Appl. Microbiol. **82:**273–280.
99. **Wiedemann, I., E. Breukink, C. van Kraaij, O.P. Kuipers, G. Bierbaum, B. de Kruijff, and H.G. Sahl.** 2001. Specific binding of nisin to peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276:**1772–1779.
100. **Xue, J., and K.W. Mille.** 2007. Regulation of the Mannose Permease Two (*mpt*) Operon in *Listeria innocua* by the ManR Protein. Appl. Environ. Microbiol. **73:**5648–5652.

Address correspondence to:
Ravinder Kumar Malik, Ph.D.
Dairy Microbiology Division
National Dairy Research Institute
Karnal 132 001
India

E-mail: rkm.micro@gmail.com

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ORIGINAL ARTICLE

Antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 against *L. monocytogenes*, *E. faecium* and *E. faecalis* and bacteriocin cross resistance and antibiotic susceptibility of their bacteriocin resistant variants

Gurpreet Kaur · Tejinder Pal Singh · Ravinder Kumar Malik · Arun Bhardwaj · Sachinandan De

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Abstract The bacteriocin susceptibility of *Listeria monocytogenes* MTCC 657, *Enterococcus faecium* DSMZ 20477, *E. faecium* VRE, *and E. faecalis* ATCC 29212 and their corresponding bacteriocin resistant variants was assessed. The single and combined effect of nisin and pediocin 34 and enterocin FH99 bacteriocins produced by *Pediococcus pentosaceus* 34, *and E. faecium* FH99, respectively, was determined. Pediocin34 proved to be more effective in inhibiting *L. monocytogenes* MTCC 657. A greater antibacterial effect was observed against *E. faecium* DSMZ 20477 and *E. faecium* (VRE) when the a combination of nisin, pediocin 34 and enterocin FH99 were used whereas in case of *L. monocytogenes* MTCC 657 a combination of pediocin 34 and enterocin FH99 was more effective in reducing the survival of pathogen. Bacteriocin cross-resistance and the antibiotic susceptibility of wild type and their corresponding resistant variants were assessed and results showed that resistance to a bacteriocin may extend to other bacteriocins within the same class and also the acquired resistance to bacteriocins can modify the antibiotic susceptibility/resistance profile of the bacterial species used in the study. According to the hydrophobicity nisin resistant variant of *L. monocytogenes* was more hydrophobic ($p<0.001$), whereas the pediocin 34 and enterocin FH99 resistant variants were less hydrophobic than the wild type strain**.** Nisin, pediocin 34 and enterocin FH99 resistant variants of *E. faecium* DSMZ 20477 and *E. faecium* VRE were less hydrophobic than their wild type counterparts. Nisin resistant *E. faecalis* ATCC 29212 was less hydrophobic than its wild type counterpart.

Keywords Nisin · Pediocin 34 · Enterocin FH99 · *Enterococcus* · *Listeria*

G. Kaur · T. P. Singh · R. K. Malik (✉)
Dairy Microbiology Division, National Dairy Research Institute,
Karnal 132001, India
e-mail: rkm.micro@gmail.com

A. Bhardwaj
Mitchell Cancer Institute, University of South Alabama Mobile,
Mobile, AL, USA

S. De
Animal Biotechnology Centre, National Dairy Research Institute,
Karnal 132001, India

Introduction

For the past few decades, food safety has been an important issue globally due to increasing food-borne diseases and change in food habits. Illness caused due to the consumption of contaminated foods has a wide economic and public health impact worldwide. Therefore, the need to avoid economic losses due to microbial spoilage of raw materials and food products, the preservation of foods by natural, biological methods may be a satisfactory approach to solve many of the current food-related issues. Bacteriocins are ribosomally-synthesized peptides or proteins with antimicrobial activity, produced mainly by lactic acid bacteria (LAB). Several LAB bacteriocins with broad spectra of inhibitory activity offer potential applications in food biopreservation (Galvez et al. 2008). Nisin is a well known broad spectrum bacteriocin active against Gram-positive pathogens associated with food. Its use as food biopreser-

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vative is limited by its effectiveness against Gram-negative bacteria. Although the use of bacteriocins for preservation (biopreservation) is a novel approach to eliminating or controlling pathogens in food, the development of highly tolerant or resistant strains remains the main concern and decreases the efficiency of bacteriocins as biopreservatives. The various possible mechanisms involved in the development of resistance to nisin and Class IIa bacteriocins among the food-borne pathogens have been reviewed by Kaur et al. (2010).

Food application of pediocins and enterocins can provide a good alternative, besides nisin, in protecting food against food borne pathogens. As products of lactic acid bacteria, they provide natural means of preservation and can be accepted by the consumers in the way nisin became accepted. As the trend of consumption of minimal processed and preserved foods is increasing, use of pediocins by the food industry could offer solutions and provide alternatives to conventional preservation means. Importantly, enterocins also show a strong activity against *Listeria*, which can be of practical use in the food industry (Giraffa 1995; Galvez et al. 1998; Nunez et al. 1997). Application of enterococcal bacteriocins on dairy foods has been the focus of many investigations (Foulique Moreno et al. 2006; Giraffa 1995). *Enterococcus* strains displaying a limited inhibitory spectrum due to the production of enterocins targeted towards *Listeria* and/or *Clostridium* (Franz et al. 1996; Giraffa 1995; Torri Tarelli et al. 1994) would be interesting as protective cultures for cheese manufacture, given their very limited antagonistic activity towards dairy starter cultures such as *Lactococcus* and *Streptococcus* (Foulique Moreno et al. 2006; Sarantinopoulos et al. 2002). Recently, *Lactobacillus brevis* FPTLB3 isolated from freshwater fish has been reported to produce bacteriocin that had broad spectrum of inhibition (3200 AU/ml) against *Escherichia coli* MTCC 1563, *Enterococcus faecalis* MTCC 2729, *Lactobacillus casei* MTCC 1423, *Lactobacillus sakei* ATCC 15521 and *Staphylococcus aureus* ATCC 25923 (Banerjee et al. 2011).

The objective of our study was to evaluate the antibacterial efficacy of nisin, pediocin 34 (produced by *Pediococcus pentosaceous* 34) and enterocin FH99 (produced by *Enterococcus faecium* FH99) either alone or in combinations against Gram-positive bacteria i.e. *E. faecium* DSMZ 20477, *E. faecium* (VRE), *E. faecalis* ATCC 29212 and *L. monocytogenes* MTCC 657. The cross resistance of the bacteriocin resistant variants to various antibiotics and three bacteriocins viz, nisin, pediocin 34 and enterocin FH99, was investigated. Also in this study the surface properties such as surface hydrophobicity, which could impair the interaction of the antimicrobial peptides with the cytoplasmic membrane, were compared between the wild type and the resistant variants.

Materials and methods

Bacterial strains and culture conditions

E. faecium FH99, bacteriocinogenic strain was an isolate from human feces (Gupta et al. 2010). *Pediococcus pentosaceous* 34 (Rao and Malik 2003), a bacteriocinogenic strain was an isolate from cheddar cheese, *Pediococcus acidilactici* LB 42 (a sensitive strain used for detection of bacteriocin producers), was obtained from , Department of Animal Science, University of Wyoming, Laramie Wyoming, USA. *E. faecalis* DSMZ 20477 was obtained from , Institute of Microbiology and Toxicology, Federal Research Centre for Nutrition, Karlsruhe, Germany. *E. faecium* VRE (a vancomycin resistant strain isolated from human feces). *E. faecalis* 29212 was procured from American Type Culture Collection (ATCC) while *L. monocytogenes* MTCC 657 was procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. Table 1 shows the culture conditions and the culture medium used for the bacterial cultures.

Bacteriocin solutions preparation

One hundred milliliter aliquots of MRS broth (De Man et al. 1960) (pH 6.5) (HiMedia, Mumbai) were inoculated with active culture of *E. faecium* FH99 and *Pediococcus pentosaceus* 34 was inoculated (1%) and incubated at 37 °C for 24 h. Cell free culture supernatant (CFCS) were prepared by centrifugation of the cultures in refrigerated centrifuge at 10,000 rpm for 10 min. The supernatant was filter sterilized by passing through a 0.2 μm (Millipore), 45 mm diameter membrane filter and used for partial purification after neutralization. Crude enterocin FH99 and pediocin 34 were precipitated from broth media by 60% ammonium sulphate precipitation and the precipitates were dissolved in sterilized Milli Q water. The bacteriocins were purified by the method earlier described by Gupta et al. (2010). Nisin A (Nisaplin ®) was procured from Danisco (Gurgaon, India). Nisin stock solutions were prepared from pure nisin in 0.02 N HCl and autoclaved (Crandall and Montville 1998).

Antimicrobial activity assays

Measurement of activity units (AU/ml)

The antibacterial activity of nisin, pediocin 34 and enterocin FH99 was obtained using the spot on lawn assay as described by Ulhman et al. (1992), against *Pediococcus acidilactici* LB 42. Five microlitres of serial dilutions of the partially purified bacteriocin of *Enterococcus faecium* FH99 and *Pediococcus pentosaceus* 34 grown in MRS broth (De

Table 1 Bacterial strains used in the study and culture conditions

Bacteria	Strains	Culture Conditions
Pediococcus pentosaceous 34	Bacteriocinogenic strain ; Lab. isolate	37 °C, MRS
Enterococcus faecium FH99	Bacteriocinogenic strain ; Lab. isolate	37 °C, MRS
Pediococcus acidilactici	LB 42 (Indicator strain)	37 °C, MRS
Enterococcus feacalis	ATCC 29212	37 °C, BHI
Listeria monocytogenes	MTCC 657	37 °C, BHI
Enterococcus faecium	DSMZ 20477	37 °C, BHI
Enterococcus faecium VRE	Vancomycin Resistant Strain (VRE) Lab. isolate	37 °C, BHI

DSMZ Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures)

MTCC Microbial Type Culture Collection (Chandigarh, India)

ATCC American Type Culture Collection

BHI Brain Heart Infusion Broth

MRS de Man Rogosa and Shrape

Man et al. 1960) were spotted on the Tryptone Glucose Yeast Extract (TGE) agar plates (Biswas et al. 1991) (1.5% agar). Before spotting, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism. Plates were kept undisturbed for 3–4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. Three independent replicates of experiment were done. The activity units of the culture broth were calculated using the following formula and expressed as activity units per ml:

$$\text{Activity Units per ml(AU/ml)} = 200 \times \text{Reciprocal of highest dilution that gave a clear zone}$$

Bacteriocin susceptibility test

The bacteriocin susceptibility & MICs of the target strains i.e. *Listeria monocytogenes* MTCC 657*, Enterococcus faecium* DSMZ 20477*, E. faecium* VRE*,* and *E. faecalis* ATCC 29212 was tested by the spot on lawn assay. For MIC determinations, 5 μl of a 1:2 dilution series of a bacteriocin solution was placed in wells. The Minimum Inhibitory Concentration (MIC) value was interpreted as the lowest concentration of bacteriocin that resulted in a clear inhibition halo after 18 h incubation at 37 °C. The MIC was defined as the lowest concentration of bacteriocin that induced an inhibition zone.

Kinetics of cell growth inhibition by bacteriocins

Overnight cultures of *E. faecium* DSMZ 20477, *E. faecium* (VRE), *E. faecalis* ATCC 29212 and *L. monocytogenes* MTCC 657 were inoculated into fresh BHI broth tubes (1%) containing either pediocin 34 and enterocin FH99. These bacteriocins were used individually or in combination; the bacteriocin amounts used in the mixtures correspond to the calculated MICs of each bacteriocin. Additionally, the efficacy of nisin, pediocin 34 and enterocin FH99 in combination (half the concentration of MICs for each bacteriocin) was also evaluated. At different time intervals (1, 2, 4, 6 and 24 h) the survivors were enumerated on Brain heart infusion (BHI) agar medium after appropriate dilutions in saline solutions, and colonies were counted after 24–48 h of incubation at 37 °C. Three independent replicates of experiment were done.

Isolation of spontaneous bacteriocin resistant variants

Spontaneous resistant mutants of strains *E. faecalis* ATCC 29212, *E. faecium* DSMZ 20477, *E. faecium* VRE and *L. monocytogenes* MTCC 657 to nisin, pediocin 34 and enterocin FH99 were isolated after sequential exposure to a bacteriocin concentration 10-fold higher the MIC. Only nisin resistant variant for *E. faecalis* ATCC 29212 was developed since it was already resistant to pediocin 34 and enterocin FH99. The stability of these resistances in cultures without bacteriocins was checked and determined by MICs.

Bacteriocin cross-resistance by agar diffusion method

The sensitivity of *E. faecium* DSMZ 20477*, E. faecium* (VRE*), E. faecalis* ATCC 29212 *and L. monocytogenes* MTCC 657 and their resistant variants to nisin, pediocin 34 and enterocin FH99 were qualitatively determined by the agar well diffusion method (Barefoot and Klaenhammer 1983). Briefly, 5 ml of molten TGE agar (Biswas et al. 1991) containing 0.75% (w/v) agar medium were cooled at 47 °C and seeded with 1% (v/v) overnight culture of *Listeria monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 or

their nisin, pediocin 34 or enterocin FH99 resistant variants. Seeded agar was then poured onto TGE agar plate and allowed to solidify at room temperature. Wells (8 mm) were cut in the solidified agar using a sterile metal cork borer and filled with 80 μl of sample. The plates were left at 5 °C for 2 h to allow diffusion of the tested aliquot and then incubated for 18 h at 37 °C. Absence or presence of inhibition zones as well as their diameters were recorded.

Antibiotic disk diffusion susceptibility test

Pattern of resistance/susceptibility to antibiotics of wild type and bacteriocin resistant variants isolates was studied by disc diffusion method as recommended by National Committee for Clinical Laboratory Standards (CLSI; Wayne, PA, USA). Antibiotic discs containing ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), penicillin G (10 units), novobiocin (30 μg), bacitracin (10 μg), streptomycin (10 μg), tetracycline (30 μg), vancomycin (30 μg), rifampicin (5 μg), nalidixic acid and kanamycin (30 μg) were obtained from HiMedia. All the measurements were done in triplicate.

Cell surface hydrophobicity

Cell surface hydrophobicity of wild type *E. faecium* DSMZ 20477*, E. faecium* (VRE)*, E. faecalis* ATCC 29212 *and L. monocytogenes* MTCC 657 and their bacteriocin resistant variants was determined according to the method described by Rosenberg et al. (1980) with slight modification using n-Hexadecane. Cultures of the strains were grown in BHI broth overnight at 37 °C. The cells (8 log_{10} cfu/ml) were harvested by centrifugation at 12,000 x g for 5 min at 5 °C, washed twice and resuspended in 5 ml phosphate urea magnesium (PUM) buffer (K_2HPO_4: 22.2 g; KH_2PO_4: 7.26 g; Urea: 1.8 g; $MgSO_4.7H_2O$: 0.2 g; in 1000 ml of distilled water; pH 7.1) and the cell suspension was adjusted to an absorbance value (A_{610}) of approx. 0.8–1.0. Three ml of the bacterial suspension were put in contact with 1 ml of each of n-Hexadecane. The cells were pre-incubated at 37 °C for 10 min and then vortexed for 120 s. The suspension was then kept undisturbed at 37 °C for 1 h to allow phase separation and the hydrocarbon layer was allowed to rise completely. After 1 h, aqueous phase was removed carefully with a Pasteur pipette and the absorbance (A_{610}) was measured using Spectrophotometer (Jenway Geneva, Jenway Ltd. Gransmore Green, Felsted, Dunmow, UK). The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity (%Hydrophobicity) calculated with the equation:

$$\{\mathrm{ODinitial} - \mathrm{ODfinal}/\mathrm{ODinitial}\} \times 100$$

Where $OD_{initial}$ and OD_{final} are the absorbance (at 610 nm) before and after extraction with the n-Hexadecane. Three independent replicates of experiment were done.

Statistical analysis

Results were expressed as mean±SE of triplicates for each sample. Calculation of mean, standard Error (SE) was performed by subjecting data to various statistical analyses as and when needed, using SYSTAT 6.0.1., Statistical Software Package, 1996, 'SPSS, Inc. (Richmond, CA, USA)', Microsoft R excel 2000 Software Package, Microsoft Corporation,(Redmond, WA,USA). A two-way ANOVA was performed for the data on the evaluation of antibacterial efficacy of the bacteriocins individually and in combination against the indicator organisms and the significance ($P<0.05$) was evaluated by Duncan's multiple range test.

Results and discussion

Bacteriocin sensitivity profile of *E. faecalis*, *E. faecium* and *L. monocytogenes* strains

Antibacterial efficiency of three bacteriocins nisin, pediocin 34 (bacteriocin produced by *Pediococcus pentosaceous* 34) and enterocin FH99 (bacteriocin produced by *Enterococcus faecium* FH99), was evaluated against Gram positive food spoilage and pathogenic bacteria. Table 2 depicts the susceptibility of the target strains to nisin, pediocin 34 and enterocin FH99 bacteriocins. The Gram positive bacterial species studied in this work differed considerably in their sensitivity to nisin, pediocin 34 and enterocin FH99 as also reported by Bankerroum and Sandine (1988) and Ukuku and Shelef (1997) indicating important genus, species and strain differences in the degree of inhibition. *E. faecalis* ATCC 29212 was sensitive to nisin only. *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* MTCC 657 were sensitive to all the bacteriocins used in the study. Table 3 shows the MICs of the wild type strains *E. faecalis* ATCC 29212, *E. faecalis* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* MTCC 657 as determined by the spot-on-lawn assay. Pediocin 34 was more effective than nisin & enterocin FH99 in inhibiting *L. monocytogenes* MTCC 657. The MICs of the developed bacteriocin resistant variants are shown in Table 3.

Kinetics of cell growth inhibition by bacteriocins

In the present study, the antibacterial efficacy of nisin, pediocin 34 and enterocin FH99 was evaluated singly as well as in different combinations against several Gram-positive

Table 2 Susceptibility of wild type strains and resistant variants to nisin, pediocin 34 and enterocin FH99 bacteriocins

Culture	Strain	Nisin	Pedicin 34	Enterocin FH99
L. monocytogenes ATCC 53135	WT	+	+	+
	Nr	−	+	+
	Pr	+	−	−
	Er	+	+	−
L. monocytogenes MTCC 657	WT	+	+	+
	Nr	−	+	+
	Pr	+	−	−
	Er	+	+	−
E. faecium DSMZ 20477	WT	+	+	+
	Nr	−	−	−
	Pr	+	−	−
	Er	+	−	−
E. faecium VRE	WT	+	+	+
	Nr	−	−	−
	Pr	+	−	−
	Er	+	−	−
E. feacalis ATCC 29212	WT	+	−	−
	Nr	−	−	−

WT wild type, *Nr* nisin resistant, *Pr* Pedioicn 34 resistant, *Er* Enterocin FH99 resistant

With Halo production (+), without halo production (−)

bacteria i.e. *E. faecalis* ATCC 29212, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* MTCC 657 in BHI broth. The calculated MICs of the bacteriocins were used to evaluate the antibacterial effect of bacteriocins alone (Table 3). In order to evaluate additive and synergistic effect of bacteriocins the different combinations and the concentrations of bacteriocins used against the target organisms are shown in Table 4. To evaluate the additive effect the concentrations of the bacteriocins in each combination used correspond to the MICs of the target organisms, whereas in order to evaluate the synergistic effect, the bacteriocins were used at half the concentrations of the MICs of the target organisms.

The bactericidal effectiveness of nisin against *E. faecalis*, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* MTCC 657 is shown in Table 5. In case of *E. faecalis* ATCC 29212, maximum viability loss of 4.3 log cycles was observed after 2 h incubation with nisin. Nisin was most effective in inhibiting the *E. faecium* DSMZ 20477 followed by enterocin FH99. Pediocin 34 was least effective in inhibiting *E. faecium* DSMZ 20477. In case of *E. faecium* (VRE), nisin followed by enterocin FH99 were observed to be more effective than pediocin 34. The viable cell count (log cfu/ml) of target organisms after treatment with nisin, pediocin 34 and enterocin FH99 alone and in different combinations is presented in Table 5. Pediocin 34 proved to be more effective than nisin and enterocin FH99 in inhibiting *L. monocytogenes* MTCC 657. Cintas et al. (1998) also reported pediocin to be more effective than nisin against some food borne pathogens such as *L. monocytogenes*. In case of all the bacterial strains it was observed that even when the bacteriocins displayed the most rapid inhibitory activity at 1 h, the survivors resumed growth, reaching the highest cell counts at 24 h. Similar observations were also made by Schillinger et al. (1998), who reported a regrowth of survivors of *L. monocytogenes* Scott A after exposure to nisin concentrations between 10 and 500 IU/ml as well as with those of Song and Richard (1997), who observed that survivors of *L. innocua* resumed growth after the addition of nisin, pediocin AcH, and enterococcin EFS2 into TSBYE broth. According to Muriana (1996), several studies indicated the immediate decrease of target cells by one to three log cycles cfu/ml when a bacteriocin was added, with none or little effect on future inoculations.

Table 3 MICs of nisin, pediocin 34 and enterocin FH99 bacteriocins

Pathogen	Nisin (IU/ml)	P ediocin 34 (AU/ml)	Enterocin FH99 (AU/ml)
E. faecalis ATCC 29212	26.5	ND	ND
Listeria monocytogenes MTCC 657	50	600	700
E. faecium DSMZ 20477	53.5	70	937.5
E. faecium VRE	14	2187.5	3750

ND Not Determined

E.faecalis ATCC 29212 was inherently resistant to Pediocin 34 and Enterocin FH99

Table 4 Concentrations of nisin, Pediocin 34 and Enterocin FH99 used alone and in different combinations to evaluate cell growth inhibition by bacteriocins

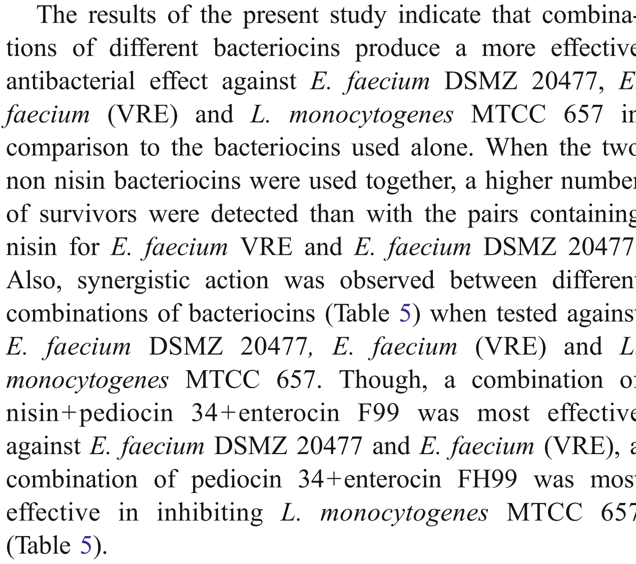
Pathogen	ADDITIVE[a]				SYNERGISTIC[b]			
	N+P	N+E	E+P	N+P+E	N+P	N+E	E+P	N+P+E
Listeria monocytogenes MTCC 657	50+600	50+700	600+700	50+600+700	25+300	25+350	300+350	25+300+350
E. faecium DSMZ 20477	53.5+70	53.5+937.5	937.5+70	53.5+70+937.5	26.25+35	26.25+468.75	468.75+35	26.25+35+468.75
E. faecium VRE	14+2187.5	14+3750	2187.5+3750	14+2187.5+3750	7+1093	7+1875	1093+1875	7+1093+1875

N Nisin, *P* Pediocin 34, *E* Enterocin FH99

Units for concentrations: Nisin = IU/ml, Pediocin 34=AU/ml, Enterocin FH99 = AU/ml

[a] Combination of bacteriocins corresponded to MICs of the target organisms

[b] Combination of bacteriocins correspond to Half the concentration of MICs of target organisms



The results of the present study indicate that combinations of different bacteriocins produce a more effective antibacterial effect against *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *L. monocytogenes* MTCC 657 in comparison to the bacteriocins used alone. When the two non nisin bacteriocins were used together, a higher number of survivors were detected than with the pairs containing nisin for *E. faecium* VRE and *E. faecium* DSMZ 20477. Also, synergistic action was observed between different combinations of bacteriocins (Table 5) when tested against *E. faecium* DSMZ 20477*, E. faecium* (VRE) and *L. monocytogenes* MTCC 657. Though, a combination of nisin+pediocin 34+enterocin F99 was most effective against *E. faecium* DSMZ 20477 and *E. faecium* (VRE), a combination of pediocin 34+enterocin FH99 was most effective in inhibiting *L. monocytogenes* MTCC 657 (Table 5).

Similar results were reported by Hanlin et al. (1993) who while studying the antibacterial efficiency of pediocin AcH and nisin against several Gram-positive bacterial strains, assumed that a mixture containing more than one bacteriocin would have greater bactericidal effect to a sensitive population, since cells resistant to one bacteriocin might be killed by the other bacteriocin. Moreover synergistic effects were reported when the interactions between pairs of bacteriocins from lactic acid bacteria were tested which are in accordance with the results obtained by Mullet-Powell et al. (1998) One possible explanation for the different effectiveness of bacteriocin pairs would be that the bacteriocins used in this study belonged to different classes, which vary considerably in the nature and sequence of amino acid residues as earlier suggested by Moll et al. (1999). The synergistic action of combinations of two different bacteriocins with different structures produced by the same strain has also been reported in agar medium by Limonet et al. (2004). Similar results have been reported by Jamuna et al. (2005) who showed that the bacteriocins from *L. acidophilus* and *L. casei* have a better antibacterial activity in combination with Nisin than when used alone against food spoilage and pathogenic organisms in liquid and food systems. Vignolo et al. (2000) also reported that the combined effect of lactocin 705, enterocin CRL35, and nisin against *L. monocytogenes* FBUNT in meat slurry showed no viable counts after incubation for 3 h. Jamuna and Jeevaratnam (2009) have also reported the synergistic effect of nisin and bacteriocin from *Pediococcus acidilactici* to be more effective in inhibiting the growth of *L. monocytogenes* and *S. aureus* in sealed pouches of vegetable pulav. When pediocin 34 and enterocin FH99 were used alone or in combination, a higher number of survivors were detected than with the pairs containing nisin for the strains of *E. faecium*. Cross-resistance between bacteriocins has also been observed when the sensitivity of

Table 5 Viable cell count (log cfu/ml) of *L. monocytogenes* and *Enterococcus* strains after treatment with nisin (N), pediocin 34 (P) and enterocin FH99 (E) alone and in different combinations

Culture	Incubation Time	Control	Antibacterial effect of Bacteriocins when used alone			ADDITIVE				SYNERGISTIC			
			N	P	E	N+P	N+E	E+P	N+P+E	N+P	N+E	E+P	N+P+E
E. faecalis ATCC *29212*	1 h	$8.4\pm0.01^{a,m}$	$4.6\pm0.06^{a,n}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	2 h	$8.4\pm0.03^{b,m}$	$4.0\pm0.01^{b,n}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	4 h	$8.2\pm0.07^{c,m}$	$5.9\pm0.03^{c,n}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	6 h	$8.3\pm0.01^{d,m}$	$5.8\pm0.09^{d,n}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
	24 h	$8.2\pm0.07^{e,m}$	$2.3\pm0.03^{e,n}$	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
L. monocytogenes MTCC 657	1 h	$7.6\pm0.09^{a,m}$	$6.9\pm0.09^{a,n}$	$5.7\pm0.06^{a,o}$	$6.8\pm0.07^{a,p}$	$5.3\pm0.04^{a,q}$	$4.8\pm0.11^{a,r}$	$3.0\pm0.05^{a,s}$	$6.0\pm0.10^{a,t}$	$3.8\pm0.04^{a,u}$	$3.4\pm0.05^{a,v}$	$2.3\pm0.03^{a,w}$	$3.4\pm0.05^{a,x}$
	2 h	$7.5\pm0.07^{b,m}$	$6.9\pm0.07^{b,n}$	$5.9\pm0.04^{b,o}$	$6.5\pm0.07^{b,p}$	$5.2\pm0.03^{b,q}$	$5.2\pm0.09^{b,r}$	$3.6\pm0.22^{b,s}$	$5.0\pm0.12^{b,t}$	$3.9\pm0.08^{b,u}$	$4.3\pm0.09^{b,v}$	$2.3\pm0.09^{b,w}$	$3.5\pm0.07^{b,x}$
	4 h	$7.6\pm0.07^{c,m}$	$7.0\pm0.07^{c,n}$	$5.3\pm0.03^{c,o}$	$7.3\pm0.09^{c,p}$	$5.7\pm0.11^{c,q}$	$5.3\pm0.12^{c,r}$	$3.9\pm0.05^{c,s}$	$7.0\pm0.14^{c,t}$	$3.6\pm0.09^{c,u}$	$4.0\pm0.09^{c,v}$	$2.4\pm0.09^{c,w}$	$4.1\pm0.05^{c,x}$
	6 h	$8.6\pm0.09^{d,m}$	$6.8\pm0.09^{d,n}$	$6.1\pm0.03^{d,o}$	$7.5\pm0.07^{d,p}$	$6.0\pm0.10^{d,q}$	$4.0\pm0.13^{d,r}$	$3.8\pm0.09^{d,s}$	$7.8\pm0.16^{d,t}$	$4.3\pm0.03^{d,u}$	$2.9\pm0.08^{d,v}$	$2.1\pm0.05^{d,w}$	$4.5\pm0.01^{d,x}$
	24 h	$7.1\pm0.15^{e,m}$	$6.8\pm0.15^{e,m}$	$5.6\pm0.03^{e,o}$	$6.4\pm0.05^{e,p}$	$5.1\pm0.15^{e,q}$	$4.2\pm0.21^{e,r}$	$2.4\pm0.18^{e,s}$	$4.3\pm0.18^{e,t}$	$4.5\pm0.04^{e,u}$	$4.5\pm0.09^{e,v}$	$2.0\pm0.02^{e,w}$	$3.9\pm0.09^{e,x}$
E. faecium DSMZ 20477	1 h	$6.9\pm0.04^{a,m}$	$5.8\pm0.09^{a,n}$	$6.7\pm0.02^{a,m}$	$6.1\pm0.04^{a,p}$	$6.8\pm0.05^{a,m}$	$6.2\pm0.09^{a,r}$	$6.5\pm0.02^{a,s}$	$5.5\pm0.015^{a,t}$	$3.0\pm0.09^{a,u}$	$4.6\pm0.09^{a,v}$	$4.6\pm0.05^{a,w}$	$2.3\pm0.03^{a,x}$
	2 h	$8.2\pm0.01^{b,m}$	$6.5\pm0.03^{b,n}$	$6.9\pm0.09^{b,o}$	$7.3\pm0.03^{b,p}$	$6.8\pm0.09^{b,q}$	$6.1\pm0.09^{b,r}$	$5.3\pm0.03^{b,s}$	$4.9\pm0.08^{b,t}$	$3.3\pm0.03^{b,u}$	$3.4\pm0.05^{b,v}$	$4.4\pm0.05^{b,w}$	$2.3\pm0.01^{b,x}$
	4 h	$7.6\pm0.08^{c,m}$	$7.4\pm0.07^{c,m}$	$7.6\pm0.09^{c,m}$	$7.3\pm0.03^{c,m}$	$7.0\pm0.02^{c,q}$	$6.9\pm0.09^{c,r}$	$6.0\pm0.09^{c,s}$	$5.3\pm0.03^{c,t}$	$3.0\pm0.05^{c,u}$	$3.3\pm0.09^{c,v}$	$5.4\pm0.09^{c,w}$	$2.2\pm0.05^{c,x}$
	6 h	$8.5\pm0.04^{d,m}$	$5.7\pm0.08^{d,n}$	$7.4\pm0.06^{d,o}$	$8.3\pm0.06^{d,p}$	$7.1\pm0.04^{d,q}$	$6.1\pm0.04^{d,r}$	$7.3\pm0.03^{d,s}$	$6.2\pm0.12^{d,t}$	$5.3\pm0.03^{d,u}$	$4.0\pm0.06^{d,v}$	$6.3\pm0.04^{d,w}$	$3.0\pm0.02^{d,x}$
	24 h	$8.9\pm0.09^{e,m}$	$8.0\pm0.03^{e,n}$	$8.4\pm0.03^{e,o}$	$8.5\pm0.02^{e,p}$	$8.3\pm0.03^{e,q}$	$8.3\pm0.09^{e,r}$	$8.2\pm0.02^{e,s}$	$7.3\pm0.03^{e,t}$	$5.3\pm0.03^{e,u}$	$5.0\pm0.05^{e,v}$	$7.6\pm0.05^{e,w}$	$2.3\pm0.06^{e,x}$
E. faecium VRE	1 h	$5.1\pm0.21^{a,m}$	$3.2\pm0.04^{a,n}$	$3.4\pm0.09^{a,o}$	$4.3\pm0.06^{a,p}$	$2.4\pm0.11^{a,q}$	$3.4\pm0.22^{a,r}$	$5.3\pm0.12^{a,s}$	$2.8\pm0.11^{a,t}$	$2.3\pm0.03^{a,u}$	$2.6\pm0.09^{a,v}$	$3.6\pm0.09^{a,w}$	$1.6\pm0.06^{a,x}$
	2 h	$6.8\pm0.03^{b,m}$	$2.4\pm0.02^{b,n}$	$5.6\pm0.03^{b,o}$	$5.3\pm0.04^{b,p}$	$2.7\pm0.22^{a,q}$	$2.1\pm0.14^{b,r}$	$4.6\pm0.08^{b,s}$	$1.6\pm0.10^{b,t}$	$2.8\pm0.09^{b,u}$	$2.3\pm0.01^{b,v}$	$2.4\pm0.09^{b,w}$	$1.4\pm0.06^{b,x}$
	4 h	$6.2\pm0.12^{c,m}$	$2.9\pm0.05^{c,n}$	$4.1\pm0.05^{c,o}$	$4.9\pm0.05^{c,p}$	$4.0\pm0.23^{a,q}$	$2.3\pm0.04^{c,r}$	$4.4\pm0.07^{c,s}$	$2.6\pm0.19^{c,t}$	$3.1\pm0.01^{c,u}$	$2.0\pm0.03^{c,v}$	$2.3\pm0.09^{c,w}$	$2.0\pm0.09^{c,x}$
	6 h	$9.2\pm0.02^{d,m}$	$5.0\pm0.03^{d,n}$	$4.4\pm0.09^{d,o}$	$7.0\pm0.12^{d,p}$	$4.5\pm0.31^{a,q}$	$4.1\pm0.13^{d,r}$	$4.3\pm0.12^{d,s}$	$3.1\pm0.02^{d,t}$	$3.5\pm0.02^{d,u}$	$2.2\pm0.03^{d,v}$	$2.7\pm0.02^{d,w}$	$2.1\pm0.02^{d,x}$
	24 h	$8.3\pm0.07^{e,m}$	$5.4\pm0.03^{e,n}$	$3.4\pm0.07^{e,o}$	$3.6\pm0.08^{e,p}$	$5.0\pm0.12^{a,q}$	$4.1\pm0.02^{d,r}$	$7.3\pm0.03^{e,s}$	$3.0\pm0.21^{e,t}$	$2.4\pm0.12^{e,u}$	$2.1\pm0.02^{e,v}$	$2.4\pm0.05^{e,w}$	$1.6\pm0.09^{e,x}$

Values are mean±SE of three independent determinations (n=3); *ND* Not detected. Values with different alphaphets, (a,b,c.d,e for incubation time and m,n,o,p,q,r,s,t,u,v,w,x for different bacteriocin treatments) are statistically significant (P<0.05)

Listeria variants to lactocin 705, enterocin CRL35, and nisin was tested and insensitivity of a variant to lactocin 705 and enterocin CRL35 while retaining sensitivity to nisin, and vice versa, was associated with the mechanism by which a bacteriocin enters the cell following binding to the cell surface, as well as with the ability to form pores in bacterial membranes Vignolo et al. (2000).

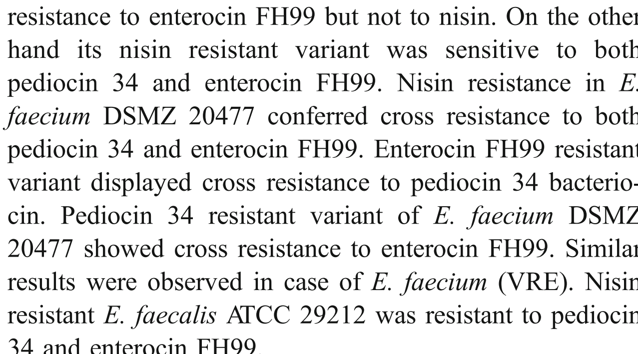
Stability of developed resistance

The stability of nisin, pediocin 34 and enterocin FH99 resistance was determined for the resistant variants of *E. faecium* DSMZ 20477*, E. faecium* (VRE)*, E. faecalis* ATCC 29212 *and L. monocytogenes* MTCC 657. We assessed the MICs (Table 3) of the developed bacteriocin resistant variants by the spot on lawn assay as described by Ulhman et al. (1992). Nisin resistant variants of *E. faecium* DSMZ 20477*, E. faecium* (VRE*), E. faecalis* ATCC 29212 and *L. monocytogenes* MTCC 657 were was 60, 300, 100 and 200 fold more resistant to nisin than their corresponding wild type strains, respectively (data not shown). Pediocin 34 resistant variants of *E. faecium* DSMZ 20477*, E. faecium* (VRE*)* and *L. monocytogenes* MTCC 657 were was 80, 2 and 6-fold more resistant to pediocin 34 than their corresponding wild type strains, respectively and enterocin FH99 resistant variants of *E. faecium* DSMZ 20477*, E. faecium* (VRE*)* and *L. monocytogenes* MTCC 657 were was 70, 2 and 5-fold more resistant to enterocin FH99 than their corresponding wild type strains. Harris et al. (1991) detected mutant strains of *L. monocytogenes*, at frequencies of 10^{-6} and 10^{-8}, which were able to grow at nisin concentrations 5 to 10 times higher than was the original population.

The nisin resistance phenotype in *L.monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* VRE and *E.faecalis* ATCC 29212 was stable during at 50, 40, 20 and 30 successive cultures, respectively, without nisin. The pediocin resistance phenotype in *L.monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE was stable during at 20, 30 and 10 successive cultures, respectively, without pediocin 34. The enterocin resistance phenotype in *L.monocytogenes* MTCC 657, *E. faecium* DSMZ 20477 and *E. faecium* VRE was stable during at 30, 30 and 20 successive cultures, respectively, without pediocin 34.

Bacteriocins cross resistance

The bacteriocin cross resistance profiles of wild type and their corresponding nisin, pediocin 34 and enterocin FH99 resistant variants is shown in Table 2. Pediocin 34 resistant variant of *L. monocytogenes* MTCC 657 displayed cross



resistance to enterocin FH99 but not to nisin. On the other hand its nisin resistant variant was sensitive to both pediocin 34 and enterocin FH99. Nisin resistance in *E. faecium* DSMZ 20477 conferred cross resistance to both pediocin 34 and enterocin FH99. Enterocin FH99 resistant variant displayed cross resistance to pediocin 34 bacteriocin. Pediocin 34 resistant variant of *E. faecium* DSMZ 20477 showed cross resistance to enterocin FH99. Similar results were observed in case of *E. faecium* (VRE). Nisin resistant *E. faecalis* ATCC 29212 was resistant to pediocin 34 and enterocin FH99.

Several reports suggest that resistance to a bacteriocin may extend to other bacteriocins within the same class or even in other classes. The nisin resistant strain of *L. monocytogenes* ATCC 700302 was observed to be showing cross resistance to the class IIa bacteriocin pediocin PA-1 and the class IV leuconocin S (Crandall and Montville 1998). *L. monocytogenes* mutants resistant to mesenterocin 52, curvaticin 13, and plantaricin were also reported to be cross-resistant to the other bacteriocins (Rekhif et al. 1994). In addition, piscicolin 126-resistant mutants of *L. monocytogenes* which emerged in cheese made from milk containing the bacteriocin were also resistant to pediocin P02 (Ukuku and Shelef 1997). These reports of cross-resistance indicate that the use of multiple bacteriocins to achieve greater antibacterial efficacy (Hanlin et al. 1993) might not be feasible. The development of resistance to one of the bacteriocins in the combination might render the organism resistant to the others too.

Antibiotic susceptibility

An undesirable consequence of an extended use of natural antimicrobials such as nisin in food might be cross-resistance to clinically used antibiotics in food-borne pathogens only few studies have comprehensively addressed this issue (Crandall and Montville 1998; Gravesen et al. 2001). In this work, we have analyzed the antibiotic susceptibility of *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) and *E. faecalis* ATCC 29212 along with their nisin, pediocin 34 and enterocin FH99 resistant counterparts as shown in Table 6.

The nisin resistant strains of *L. monocytogenes* MTCC 657, *E. faecium* DSMZ 20477, *E. faecium* (VRE) were more susceptible to most of the antibiotics tested than their wild type counterparts. This could be related to the fitness cost commonly associated to the development of the nisin resistant phenotype i.e. the changes conferring bacteriocin resistance could possibly reduce the growth potential of the cells or render them more sensitive to preservation parameters such as salt, low pH, or low temperature (Dykes and Hastings 1998). Only cross-resistance to the membrane

Table 6 Susceptibility[a] of wild type strains and their bacteriocin resistant counterparts to antibiotics

Bacterial culture & Strains		Antibiotics													
		T	V	B	S	K	E	G	Na	A	R	P	C	Nv	Pb
L. monocytogenes ATCC 53135	WT	S	M	M	M	M	M	M	R	M	S	M	S	M	M
	Nr	S	S	M	M	M	S	S	R	S	S	S	S	S	S
	Pr	S	M	M	M	M	M	M	R	M	S	M	S	M	M
	Er	S	M	M	M	M	M	M	S	M	S	M	S	M	R
L. monocytogenes MTCC 657	WT	S	S	S	S	S	S	S	R	S	S	S	S	R	S
	Nr	S	S	S	S	S	S	S	S	S	S	S	S	M	S
	Pr	S	S	S	M	M	R	R	R	S	S	M	S	R	S
	Er	S	S	S	S	S	S	S	R	S	S	S	S	S	S
E. faecium DSMZ 20477	WT	S	S	S	R	R	M	R	R	R	R	R	M	R	S
	Nr	S	S	S	R	R	M	R	R	S	S	S	S	S	S
	Pr	S	S	S	R	R	M	R	R	R	R	R	M	R	S
	Er	S	S	S	R	R	M	M	R	R	S	S	S	M	S
E. faecium VRE	WT	M	R	S	R	R	R	R	R	R	S	R	S	S	S
	Nr	S	S	S	R	R	R	R	R	R	S	S	S	S	R
	Pr	S	S	S	R	R	R	R	R	R	S	R	S	S	R
	Er	S	S	S	R	R	R	R	R	R	S	S	S	S	R
E. faecalis ATCC 29212	WT	S	S	S	R	M	R	S	S	S	S	S	S	S	R
	Nr	S	S	S	R	R	R	R	R	S	S	S	S	S	M

WT wild type, *Nr* nisin resistant, *Pr* Pedioicn 34 resistant, *Er* Enterocin FH99 resistant

T Tetracycline, *V* Vancomycin, *B* Bacitracin, *K* Kanamycin, *E* Erythromycin, *G* Gentamycin, *Na* Nalidixic acid, *A* Ampicillin, *R* Rifampicin, *P* PenicillinG, *C* Chloramphenicol, *Nv* Novobiocin, *Pb* Polymyxin B

S sensitive; *M* moderately sensitive; *R* resistant

[a] Zone of Inhibition calculated according to the table given by NCCLS (2001)

disturbing polymixin B was depicted by the three resistant variants of *E. faecium* (VRE). This result seems reasonable as nisin shares the same primary target with polymixin B: the cytoplasmic membrane. Also, the resistance has been partially correlated with changes in the membrane composition which potentially interfere with the pore forming ability of nisin in the cytoplasmic membrane (Crandall and Montville 1998; Mazzotta and Montville 1997). Moreover, changes in the cell envelope such as a thickened cell wall, polysaccharide production or a higher degree of D-alanine substitution in the teichoic acids were also described as resistance strategies to avoid killing by cationic antimicrobial peptides (Davies et al. 1996; Peschel et al. 1999). Basically, these mechanisms lower the net negative surface charge and restrict the accessibility of nisin and, hence, of other cationic drugs such as polymixin B and amino glycosides, to their targets.

Pediocin 34 resistant strain of *L. monocytogenes* MTCC 657 and nisin resistant strain of *E. faecalis* ATCC 29212 were observed to be resistant to gentamicin. Nisin resistant variant of *E. faecalis* ATCC 29212 was also resistant to nalidixic acid. The site of action of gentamicin is protein synthesis and nalidixic acid inhibits a subunit of DNA gyrase and induces formation of relaxation complex analogue. It also inhibits the nicking dosing activity on the subunit of DNA gyrase that release the positive binding stress on the supercoiled DNA. The increase in resistance to gentamicin and nalidixic acid might result from an alteration in the cell wall which prevents these compounds from reaching their targets (Crandall and Montville 1998).

Cell surface hydrophobicity

According to the hydrophobicity measurements significant differences ($p<0.001$) were observed between wild type *E. faecium* DSMZ 20477, *E. faecium* (VRE), *E. faecalis* ATCC 29212 *and L. monocytogenes* MTCC 657 and their nisin resistant, pediocin 34 resistant and enterocin FH99 resistant counterparts, respectively (Fig. 1).

Nisin resistant variant of *L. monocytogenes* was more hydrophobic than the corresponding wild type, whereas the pediocin 34 and enterocin FH99 resistant variants were less hydrophobic than the wild type strain Nisin, pediocin 34 and enterocin FH99 resistant variants of *E. faecium* DSMZ

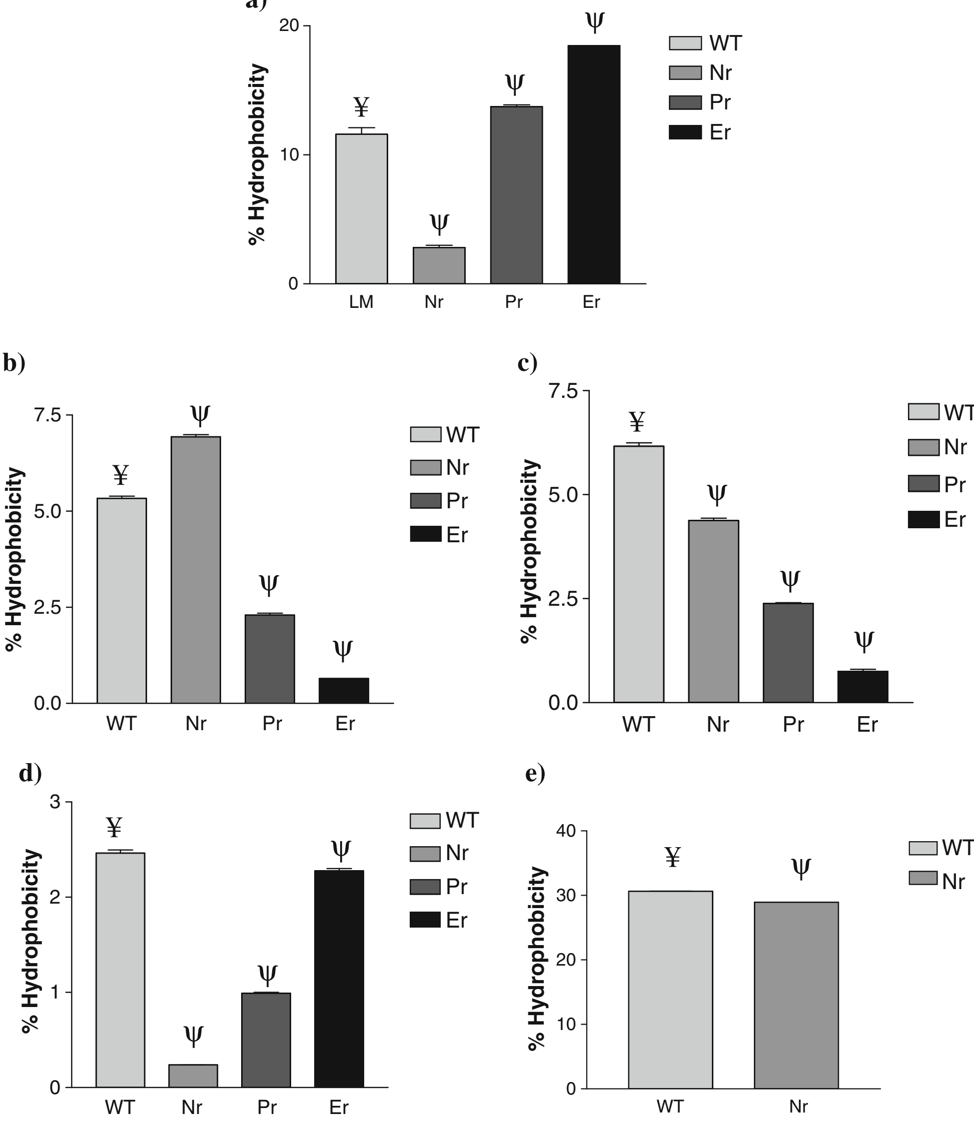


Fig. 1 Surface hydrophobicity * of the wild type (WT) (**a**) *Listeria monocytogenes* ATCC 53135, (**b**) *L. monocytogenes* MTCC 657 (**c**) *E. faecium* DSMZ 20477, (**d**) *E. faecium* VRE, (**e**) *E. faecalis* ATCC 29212 and their nisin resistant (Nr), Pediocin 34 resistant (Pr) and Enterocin FH99 resistant (Er) variants. *Values are presented as mean±SEM; n=3. ¥, ψ Values with different superscripts differ significantly at the level of $p<0.001$ between wild type and bacteriocin resistant strains

20477 and *E. faecium* VRE were less hydrophobic than their wild type counterparts. Also, nisin resistant *E. faecalis* 29212 was less hydrophobic than its wild type counterpart. This may be due to a substantial change in the surface architecture of the resistant variants which might involve a different protein display at the surface. These results are in accordance with those reported by Martinez and Rodriguez (2005).

Conclusion

The results of the present study indicate that treatment with a combination of two or more different bacteriocins has an advantage of protection against many spoilage and pathogenic bacteria because of synergistic or additive effect. The results of this study also show that although the use of Nisin is permitted in a number of countries in a variety of foods, other bacteriocins viz, pediocin and enterocin with different and/or more effective antimicrobial activity may be considered as new biopreservatives. This study showed that resistance to a bacteriocin may extend to other bacteriocins within the same class. The development of bacteriocin resistance may hinder further application of bacteriocins in food preservation and it also raises concerns about the extensive use of bacteriocins in food regarding the cross resistance in food borne pathogens towards other bacteriocins and towards clinically used antibiotics. Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage and among food borne pathogens

References

Banerjee SP, Dora KC, Chowdhury S (2011) Detection, partial purification and characterization of bacteriocin produced by Lactobacillus brevis FPTLB3 isolated from freshwater fish Bacteriocin from *Lb. brevis* FPTLB3. J Food Sci Technol. doi:10.1007/s13197-011-0240-4

Bankerroum N, Sandine WE (1988) Inhibitory action of nisin against *Listeria monocytogenes*. J Dairy Sci 71:3237–3245

Barefoot SF, Klaenhammer TR (1983) Detection and activity of Lactacin B, a bacteriocin produced by *Lactobacillus acidophilus*. Appl Environ Microbiol 45:1808–1815

Biswas SR, Ray P, Johnson MC, Ray B (1991) Influence of growth conditions on the production of a bacteriocin AcH by *Pediococcus acidilactici* H. Appl Environ Microbiol 5:1265–1267

Cintas LM, Casaus P, Fernandez MF, Hernández PE (1998) Comparative antimicrobial activity of enterocin L50, pediocin PA-1, nisin A, and lactocin S against spoilage and foodborne pathogenic bacteria. Food Microbiol 15:289–298

Crandall AD, Montville TJ (1998) Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl Environ Microbiol 64(231):237

Davies EA, Falahee MB, Adams MR (1996) Involvement of the cell envelope of *Listeria monocytogenes* in the acquisition of nisin resistance. J Appl Bacteriol 81:139–146

De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 2:130–135

Dykes GA, Hastings JW (1998) Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett Appl Microbiol 26:5–8

Foulique Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of enterococci in food and health. Intl J Food Microbiol 106:1–24

Franz CMAP, Schillinger U, Holzapfel WH (1996) Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. Intl J Food Microbiol 29:255–270

Galvez A, Valdivia E, Abriouel H, Camafeita E, Mendez E, Martinez-Bueno M, Maqueda M (1998) Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Arch Microbiol 171:59–65

Galvez A, López RL, Abriouel H (2008) Application of bacteriocins in the control of foodborne pathogenic and spoilage bacteria. Crit Rev Biotech 28:125–152

Giraffa G (1995) Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. Food Microbiol 12:291–299

Gravesen A, Sorensen K, Aarestrup FM, Knochel S (2001) Spontaneous nisin resistant *Listeria monocytogenes* mutants with increased expression of a putative penicillin-binding protein and their sensitivity to various antibiotics. Microb Drug Resis 7:127–135

Gupta H, Malik RK, De S, Kaushik JK (2010) Purification and Characterization of Enterocin FH 99 Produced by a Faecal Isolate *Enterococcus faecium* FH 99. Indian J Microbiol 50:145–155

Hanlin MB, Kalchayanand N, Ray P, Ray B (1993) Bacteriocins of lactic acid bacteria in combination have greater antibacterial activity. J Food Protect 56:252–255

Harris LJ, Fleming HP, Klaenhammer TR (1991) Sensitivity and resistance of Listeria monocytogenes ATCC 19115, Scott A and UAL500 to nisin. J Food Prot 54:836–840

Jamuna M, Jeevaratnam K (2009) Antibacterial efficacy of nisin and pediocins against food spoilage and pathogenic organisms in broth and a vegetarian food. J Food Sci Technol 46 (6):563–568

Jamuna M, Babusha ST, Jeevaratnam K (2005) Inhibitory efficacy of nisin and bacteriocins from *Lactobacillus* isolates against food spoilage and pathogenic organisms in model and food systems. Food Microbiol 22:449–454

Kaur G, Malik RK, Mishra SK, Singh TP, Bharadwaj A, Singroha G, Vij S, Kumar N (2010) Nisin and class IIa bacteriocin resistance among listeria and other food-borne pathogens and spoilage bacteria. Microb Drug Resis 17(2):197–205

Limonet M, Revol-Junelles A, Cailliez-Grimal C, Milliere J (2004) Synergistic mode of action of mesenterocins 52A and 52B produced by *Leuconstoc mesenteroides* subsp. *mesenteroides* FR 52. Curr Microbiol 48:204–207

Martinez B, Rodriguez A (2005) Antimicrobial susceptibility of nisin resistant *Listeria monocytogenes* of dairy origin. FEMS Microbiol Lett 252:67–72

Mazzotta AS, Montville TJ (1997) Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 °C and 30 °C. J Appl Microbiol 82:32–38

Moll GN, Konings WN, Driessen AJM (1999) Bacteriocins: mechanism of membrane insertion and pore formation. Anton Leeuwen 76:185–198

Mullet-Powell N, Lacoste-Armynot AM, Viñas M, Simeon de Buochberg M (1998) Interactions between pairs of bacteriocins from lactic acid bacteria. J Food Prot 61:1210–1212

Muriana PM (1996) Bacteriocins for control of *Listeria* spp. in food. J Food Prot (Suppl). 54–63

National Committee for Clinical Laboratory Standards (2001) Performance standards for antimicrobial susceptibility testing; eleventh international supplement. NCCLS document M100–S11. National Committee for Clinical Laboratory Standards. Wayne, Pa.

Nunez M, Rodrıguez JL, Garcıa E, Gaya P, Medina M (1997) Inhibition of *Listeria monocytogenes* by enterocin 4 during the manufacture and ripening of Manchego cheese. J Appl Microbiol 83:671–677

Peschel A, Ott M, Jack RW, Kalbacher H, Jung G, Gotz F (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274:8405–8410

Rao KN, Malik, RK (2003) Emerging biotechnologies in dairy and meat processing Chromosomal linked bacteriocin production in *Pediococcus pentosaceus*. In: Marwaha SS, Arora JK (eds.) Biotechnological Strategies in Agroprocessing. Asiatech Publications, New Delhi, pp. 230–257

Rekhif N, Atrih A, Lefebvre G (1994) Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr Microbiol 28:237–241

Rosenberg M, Gutnick D, Rosenberg E (1980) Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiol Lett 9:29–33

Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki MD, Kalantzopoulos G, Tsakalidou E, De Vuyst L (2002) Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek feta cheese making. Intl J Food Microbiol 72:125–136

Schillinger U, Chung HS, Keppler K, Holzapfel WH (1998) Use of bacteriocinogenic lactic acid bacteria to inhibit spontaneous nisin-resistant mutants of *Listeria monocytogenes* Scott A. J Appl Microbiol 85:657–663

Song HJ, Richard J (1997) Antilisterial activity of three bacteriocins used at sub-minimal inhibitory concentrations and cross-resistance of the survivors. Int J Food Microbiol 36:155–161

Torri Tarelli G, Carminati D, Giraffa G (1994) Production of bacteriocins active against *Listeria monocytogenes* and *Lis-*

teria innocua from dairy enterococci. Food Microbiol 11:243–252

Ukuku DO, Shelef LA (1997) Sensitivity of six strains of *L. monocytogenes* to nisin. J Food Prot 60:867–869

Ulhman U, Schillinger U, Rupnow JR, Holzapfel WH (1992) Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. Int J Food Microbiol 16:141–151

Vignolo G, Palacios J, Farias ME, Sesma F, Schillinger U, Holzapfel W, Oliver G (2000) Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. Curr Microbiol 41:410–416

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Mechanism of Nisin, Pediocin 34, and Enterocin FH99 Resistance in *Listeria monocytogenes*

Gurpreet Kaur · Tejinder Pal Singh · Ravinder Kumar Malik · Arun Bhardwaj

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Abstract Nisin-, pediocin 34-, and enterocin FH99-resistant variants of *Listeria monocytogenes* ATCC 53135 were developed. In an attempt to clarify the possible mechanisms underlying bacteriocin resistance in *L. monocytogenes* ATCC 53135, sensitivity of the resistant strains of *L. monocytogenes* ATCC 53135 to nisin, pediocin 34, and enterocin FH99 in the absence and presence of different divalent cations was assessed, and the results showed that the addition of divalent cations significantly reduced the inhibitory activity of nisin, pediocin 34, and enterocin FH99 against resistant variants of *L. monocytogenes* ATCC 53135. The addition of EDTA, however, restored this activity suggesting that the divalent cations seem to affect the initial electrostatic interaction between the positively charged bacteriocin and the negatively charged phospholipids of the membrane. Nisin-, pediocin 34-, and enterocin-resistant variants of *L. monocytogenes* ATCC 53135 were more resistant to lysozyme as compared to the wild-type strain both in the presence as well as absence of nisin, pediocin 34, and enterocin FH99. Ultra structural profiles of bacteriocin-sensitive *L. monocytogenes* and its bacteriocin-resistant counterparts revealed that the cells of wild-type strain of *L. monocytogenes* were maximally in pairs or short chains, whereas, its nisin-, pediocin 34-, and enterocin FH99-resistant variants tend to form aggregates. Results indicated that without a cell wall, the acquired nisin, pediocin 34, and enterocin FH99 resistance of the variants was lost. Although the bacteriocin-resistant variants appeared to lose their acquired resistance toward nisin, pediocin 34, and enterocin FH99, the protoplasts of the resistant variants appeared to be more resistant to bacteriocins than the protoplasts of their wild-type counterparts.

Keywords *L. monocytogenes* · Nisin · Pediocin 34 · Enterocin FH99 · Bacteriocin resistance

G. Kaur · T. P. Singh · R. K. Malik (✉)
Dairy Microbiology Division, National Dairy Research Institute, Karnal 132001, India
e-mail: rkm.micro@gmail.com

A. Bhardwaj
Mitchell Cancer Institute, University of South Alabama, Mobile, AL, USA

Introduction

For the past few decades, food safety has been an important issue globally due to increasing food-borne diseases and changes in food habits. Illness caused due to the consumption of contaminated foods has a wide economic and public health impact worldwide. *L. monocytogenes* can be found in a wide variety of raw and processed foods. Milk and dairy products and various meats and meat products have been associated with *Listeria* contamination [26]. Foods such as soft cheeses, hot dogs, and seafood have been implicated in several outbreaks of human listeriosis.

Among the wide spectrum of antibacterial products released by microorganisms, the bacteriocins, especially those produced by lactic acid bacteria (LAB), have attracted the greatest attention as tools for food biopreservation. Bacteriocins are ribosomally synthesized peptides or proteins with antimicrobial activity. Several LAB bacteriocins with inhibitory activity offer potential applications in food biopreservation [10]. Food application of pediocins and enterocins can also provide a good

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alternative means in protecting food against food-borne pathogens. As products of lactic acid bacteria, they provide natural means of preservation and can be accepted by the consumers in the way nisin became accepted. As the trend of consumption of minimally processed and preserved foods is increasing, use of pediocins by the food industry could offer solutions and provide alternatives to conventional preservation means. Importantly, enterocins also show a strong activity against *Listeria*, which can be of practical use in the food industry [11, 13, 22]. Application of enterococcal bacteriocins on dairy foods has been the focus of many investigations [8, 13]. *Enterococcus* strains displaying a limited inhibitory spectrum due to the production of enterocins targeted toward *Listeria* and/or *Clostridium* [9, 13, 29] would be interesting as protective cultures for cheese manufacturers, given their very limited antagonistic activity toward dairy starter cultures such as *Lactococcus* and *Streptococcus* [8, 27].

However, similar to the use of antibiotics, the concern with the use of bacteriocins is the development of resistance in food-borne pathogens. The resistance mechanism is complex and may be due to three major factors: (1) variation of peptidoglycan composition [17], which should make it possible to increase the binding of divalent cations that should interact with the cationic peptide; (2) modification of the electric charge of the membrane by changes in the phospholipid content, thereby preventing pore formation [4, 20, 25]; and (3) increase in membrane rigidity, preventing peptide insertion and association [20]. However, resistance to bacteriocins has also been correlated with an altered fatty acid composition [18, 20] and an altered phospholipid composition [21]. Studies aimed at characterizing the resistance mechanisms of bacterial targets have revealed the stability of this phenomenon [7, 24], which occurs at either a low or a high level. In *L. monocytogenes* and *E. faecalis*, low-level resistance has been attributed to alterations in membrane lipid composition [31, 32] and high-level resistance has been attributed to the inactivation of the *mptACD* operon, which encodes the EII^{t}_{Man} mannose permease of the phosphotransferase system (PTS) [5, 15].

In order to assess the utility of pediocin 34 and enterocin FH99 as a food preservative against *Listeria*, it was important to determine their cellular target(s) and potential mechanisms of resistance to these bacteriocins. In this communication, we report on the isolation and characterization of nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes*. We propose that nisin, pediocin 34, and enterocin FH99 resistance in these variants is mediated by changes in their cell wall architecture limiting the access of these bacteriocins to a potential target in the cytoplasmic membrane and/or in the cytoplasm of the bacterium.

Materials and Methods

Bacterial Strains and Culture Conditions

Enterococcus faecium FH99 bacteriocinogenic strain was an isolate from human feces [14]. *Pediococcus pentosaceous* 34, a bacteriocinogenic strain isolated from cheddar cheese and *Pediococcus acidilactici* LB 42 (a sensitive strain used for detection of bacteriocin producers), was obtained from Prof Bibek Ray, Department of Animal Science, University of Wyoming, Laramie, Wyoming, USA. *E. faecium* FH 99, *P. pentosaceus* 34, and *P. acidilactici* LB 42 were maintained in MRS broth at 37 °C for 24 h. *Listeria monocytogenes* ATCC 53135 were procured from American Type Culture Collection (ATCC) and cultivated in Brain Heart Infusion (BHI) broth at 37 °C for 24 h.

Bacteriocins

One liter aliquots of MRS broth (pH 6.5) (HiMedia, Mumbai) were inoculated with active cultures of *E. faecium* FH 99 [14] and *P. pentosaceus* 34 (1% v/v) and incubated at 37 °C for 24 h. Cell-free culture supernatants (CFCS) were prepared by centrifugation of the cultures in refrigerated centrifuge (HANIL, Supra-30 K) at 7,000*g* for 10 min, filter sterilized by passing through a 0.2 µm, 45 mm diameter membrane filter and used for partial purification after neutralization. To 1,000 mL of cell-free culture supernatant, solid ammonium sulfate was added slowly with constant stirring to achieve 60% saturation and stirring was continued for another 1 h in a cold room at 5–7 °C. The mixture was then kept overnight in the cold room. It was then centrifuged at 7,000*g* for 20 min, and the precipitates were recovered. The supernatant was subsequently adjusted to 80% saturation levels by further addition of solid ammonium sulfate. The pellet in each case was dissolved in sterile Milli Q water. Nisin A (Nisaplin®) was procured from Danisco (Gurgaon, India). Nisin stock solutions were prepared from Nisaplin in 0.02 N HCl and autoclaved.

Antimicrobial Activity Assays

Measurement of Activity Units (AU)

The inhibitory spectrum of activity was obtained using the spot-on-lawn assay as described by Ulhman et al. [30], against *P. acidilactici* LB 42. Five microliters of the partially purified bacteriocin of *E. faecium* FH99 and *P. pentosaceus* 34 grown in MRS broth [6] was spotted on the tryptone glucose yeast extract (TGE) agar plates [2] (1.5% agar). Before spotting, TGE agar plates were overlaid with TGE soft agar (0.75%) seeded with actively

growing cells of the test organism, *P. acidilactici* LB 42 (1% v/v). Plates were kept undisturbed for 3–4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. Three independent replicates of experiment were done, and samples were tested in triplicate in each assay. The activity units of the partially purified bacteriocins were calculated using the following formula and expressed as activity units/mL: activity units/mL (AU/mL) = 200 × reciprocal of highest dilution that gave a clear zone.

The inhibitory spectrum of activity was obtained using the spot-on-lawn assay as described by Ulhman et al. [30] against *L. monocytogenes* ATCC 53135. Five microliters of nisin and the partially purified bacteriocin from *E. faecium* FH 99 and *P. pentosaceus* 34 grown in MRS broth [6] were spotted on TGE agar plates (1.5% agar) overlaid with TGE soft agar (0.75%) seeded with actively growing cells of the test organism [2]. Plates were kept undisturbed for 3–4 h for diffusion of bacteriocin through agar and then incubated. The sensitivity of the strain in question was evaluated by checking for clear zones around the spots. For MIC determinations, 5 μL of a 1:2 dilution series of a bacteriocin solution was spotted. The MIC was defined as the lowest concentration of bacteriocin that induced an inhibition zone. Three independent replicates of experiment were done.

Isolation of Spontaneous Bacteriocin-Resistant Variants

To obtain resistant strains, the wild-type strain of *L. monocytogenes* ATCC 53135 was cultured in BHI broth with increasing concentrations of bacteriocin corresponding to one-, three-, six- and then to 10- and 100-fold the MIC. The stability of these resistances in cultures without nisin, pediocin 34, and enterocin FH99 was checked and determined by MICs.

Bacteriocin-Induced Changes in Bacterial Cell Morphology by Scanning Electron Microscopy

To visualize differences occurring in the morphology between wild-type *L. monocytogenes* ATCC 53135 and its nisin-, pediocin 34-, and enterocin FH99-resistant variants were grown overnight in BHI broth and incubated at 37 °C for 24 h. Bacterial cells were washed in 0.1 M phosphate-buffered saline (pH 7.0) and fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.0). After 2 h on a rotator (2 rpm) at room temperature, the samples were washed three times with the same buffer and postfixed with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.0). After 1 h at room temperature, the samples were washed three times in the same buffer and for dehydration of cells, graded series of ethanol water mixtures of 30, 50, 70% and absolute alcohol were used. After dehydration in absolute alcohol, these were subsequently dehydrated in propylene oxide for 15 min and allowed to dry. Dried samples were mounted on aluminum stubs with silver paint and sputter coated with gold at approximately 200 Å thickness. The samples were visualized with a Scanning Electron Microscope (SEM), Model 501, Philips-Holland with EDAX and EBIC attachments.

Divalent-Cation Requirement of Nisin-Resistant, Pediocin 34-Resistant, and Enterocin FH99-Resistant Variants

Divalent-cation requirement of nisin-resistant, pediocin 34-resistant, and enterocin FH99-resistant variants were determined by the method described by Crandall and Montville [4]. Overnight cultures of wild-type or nisin-, pediocin 34-, and enterocin FH99-resistant variants were harvested by centrifugation (7,000*g*; HANLIN, Supra-30 K), washed once with phosphate-buffered saline, and resuspended in 50 mM MES (morpholineethanesulfonic acid) buffer (pH 6.5) containing either divalent cations alone, divalent cations plus EDTA, EDTA alone, or none of these. The metal ions tested (final concentration of 10 mM) were $MgSO_4$, $MgCl_2$, $CaCl_2$, and $MnSO_4$ (Hi Media, Mumbai). The final concentration of EDTA was 20 mM. The cells were then treated with a final concentration of 50 IU of nisin, 500AU/mL of pediocin 34, and 120 AU/mL of enterocin FH99 for 20 min. Control samples were not treated with nisin, pediocin 34, or enterocin FH99. The cells were then diluted in saline and plated on BHI agar plates. The plates were incubated at 37 °C, and colonies were counted after 48 h. The results are reported as log reductions in cell viability relative to an untreated control. Three independent replicates of the experiment were done with each individual experiment consisting of triplicate reactions.

Lysozyme Sensitivity of Nisin-Resistant, Pediocin 34-Resistant, and Enterocin FH99-Resistant Variants

Overnight cultures of wild-type cells and nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes* ATCC 53135 were inoculated at 1% (v/v) into fresh BHI containing 2 mg of lysozyme (Sigma)/mL. The cultures were incubated at 37 °C, and samples were drawn at different time intervals (1, 2, 4, 6, and 24 h). The survivors at each time interval were enumerated on BHI agar medium after appropriate dilutions in saline, and colonies were counted after 24–48 h of incubation at 37 °C. Three independent replicates of experiment were done.

Role of the Cell Envelope of *L. monocytogenes* ATCC 53135 in the Acquisition of Bacteriocin Resistance

Protoplast Formation

Protoplasts of wild-type *L. monocytogenes* ATCC 53135 and its respective bacteriocin-resistant variants were prepared using the method of Ghosh and Murray [12]. *L. monocytogenes* ATCC 53135 and its nisin-, pediocin 34-, and enterocin FH99-resistant variants were grown in 100 mL BHI broth (pH 7.3) at 37 °C for 20 h to get 10^9 CFU/mL. Cell concentrations were determined using spread plates. Cells were harvested by centrifugation at 7,000*g* for 20 min (at 4 °C), washed with water, and weighed. The cell pellets were then resuspended in lysozyme incubation buffer (0.015 mol/L NaCl; 0.03 mol^{-1} Tris–HCl (pH 6.7) (Sigma) and 0.4 mol^{-1} sucrose at 20 mg/mL wet weight before addition of lysozyme (Sigma) solution (6 mg/mL) to give a final concentration of 600 μg/mL lysozyme. The samples were shaken for 15 min at 37 °C, and 1 mol/L $MgCl_2$ was added to give a final concentration of 0.02 mol/L $MgCl_2$. After a 45-min incubation at 37 °C without shaking, the samples were centrifuged at 1,500*g* for 15 min and the pellets washed in protoplast buffer (0.03 mol/L Tris–HCl (pH 6.7); 0.01 mol/L $MgCl_2$ and 0.5 mol/L sucrose). The pellets were then resuspended to their original volume (100 mL) with protoplast buffer and stored briefly on ice before use.

Determination of the Efficiency of Protoplast Formation

The total number of CFU/mL present in each protoplast preparation was determined using spread plates. All enumerations were conducted using protoplast buffer as a diluent and BHI that had been hydrated with protoplast buffer. To determine the percentage of protoplasts present, a small volume of each protoplast suspension was centrifuged (1,500*g* for 15 min) and resuspended in an equal volume of water. The cells were incubated at 30 °C for 30 min with constant shaking to ensure lysis. Control whole cells were treated in the same manner.

Inactivation of Whole Cells and Protoplasts by Nisin, Pediocin 34, and Enterocin FH99

Overnight cultures of *L. monocytogenes* ATCC 53135 and its nisin-resistant, pediocin 34-resistant, and enterocin FH99-resistant variants were grown to 10^9 CFU/mL in BHI broth (10 mL) at 30 °C. After centrifugation (1,500*g* for 15 min), they were washed and resuspended to their original volume in protoplast buffer (0.03 mol/L Tris–HCl (pH 6.7); 0.01 mol/L $MgCl_2$ and 0.5 mol/L sucrose). After 15 min equilibration at 30 °C, samples were removed and diluted in protoplast buffer, and cells were enumerated on BHI. Bacteriocin was added to the remaining cultures (MICs as shown in Table 1 were used) and samples taken for cell enumeration periodically over 3 h. Protoplasts were treated in exactly the same way as whole cells, except for the use of buffered diluents and agar. Three independent replicates were done.

Statistical Analysis

The data were subjected to various statistical analyses as and when needed, using SYSTAT 6.0.1 (Statistical Software Package, 1996, SPSS, Inc., USA), Microsoft R excel 2000 Software Package (Microsoft Corporation, USA) and GraphPad 3.02, 1999 (GraphPad Software Inc., San Diego CA).

Determination of Mean and Standard Error of the Mean (SEM)

The experimental data, as and when necessary, are presented as the mean and standard error of the mean (SEM) of different parameters studied in the present investigation. The mean and SEM were determined running Microsoft Excel 2000 Software Package, Microsoft Corporation, USA.

Graphical Presentation

The mean ± SEM of different parameters studied were graphically presented using Graph Pad 3.02, 1999, GraphPad Software Inc., San Diego CA.

Results

Table 1 shows the MICs of the wild-type strains *L. monocytogenes* ATCC 53135 as determined by the spot-on-lawn assay. The nisin, pediocin 34, and enterocin FH99 resistance phenotype in *L. monocytogenes* ATCC 53135 was stable during 60, 40, and 10 successive cultures without nisin, pediocin 34, and enterocin FH99, respectively. MICs of the wild-type and the nisin-, pediocin 34-, and enterocin FH99-resistant variants of *Listeria monocytogenes* ATCC 53135 were determined by the spot-on-lawn assay (data not shown). The MIC was determined as the minimal concentration giving a visible zone of inhibition after 24 h at 37 °C. Nisin-, pediocin 34-, and enterocin FH99-resistant variant of *L. monocytogenes* ATCC 53135 were 92, 64, and 1,250 fold more resistant to nisin,

Table 1 Minimum inhibitory concentration (MICs) of wild-type and the nisin-, pediocin 34-, and enterocin FH99-resistant variants of *Listeria monocytogenes* ATCC 53135

Culture	Strains	Nisin IU/mL	Pediocin 34 AU/mL	Enterocin FH99 AU/mL
L. monocytogenes ATCC 53135	WT	13.2	540	120
	Nr	1,250	540	120
	Pr	400	35,000	7,500
	Er	400	35,000	150,000

WT wild type, *Nr* nisin resistant, *Pr* pediocin 34 resistant, *Er* enterocin FH99 resistant

pediocin 34, and enterocin FH99, respectively, than its corresponding wild-type strain.

Bacteriocin-Induced Changes in Bacterial Cell Morphology by Electron Microscopy

In the present investigation, attempts were made to study ultra structural profiles of bacteriocin-sensitive *L. monocytogenes* ATCC 53135 and its bacteriocin-resistant counterparts. The cells of wild-type (bacteriocin sensitive) *L. monocytogenes* were maximally in pairs or short chains. On the other hand, nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes* ATCC 53135 tend to form aggregates (Fig. 1).

Divalent-Cation Requirement of Nisin-, Pediocin 34-, and Enterocin FH99-Resistant Variants

A major component of growth media, which could be required by the bacteriocin-resistant strain to resist bacteriocins, is divalent cations. Therefore, the nisin, pediocin 34, and enterocin FH99 sensitivity of the resistant strains of *L. monocytogenes* ATCC 53135 in the absence and presence of different divalent cations was assessed.

Figure 2 shows the effect of $MgSO_4$, $MgCl_2$, $CaCl_2$, and $MnSO_4$ on the sensitivity of nisin-, pediocin 34-, and enterocin FH99-resistant *L. monocytogenes* ATCC 53135 variants to nisin, pediocin 34, and enterocin FH99 in the presence or absence of EDTA. In case of *L. monocytogenes* ATCC 53135, the viability of the nisin-resistant cells suspended in MES buffer supplemented with 10 mM $MgSO_4$ and treated with 50 IU of nisin/mL was reduced by 0.50 log cycles only. The effect was confirmed to be due to the divalent cation by involving EDTA, a chelator of divalent cations. A significant difference ($P < 0.05$) in viable count of nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes* ATCC 53135 was observed when treated with nisin, pediocin 34, and enterocin FH99 alone or in combination with EDTA. Inclusion of 20 mM EDTA in the system containing $MgSO_4$ increased the lethality caused by nisin to a 2.51 log reduction. The inhibition of pediocin 34- and enterocin FH99-resistant variants was also affected by the addition of Mg^{2+} ions. In the presence of 10 mM $MgSO_4$, a reduction of 0.35 and 0.08 log cycles

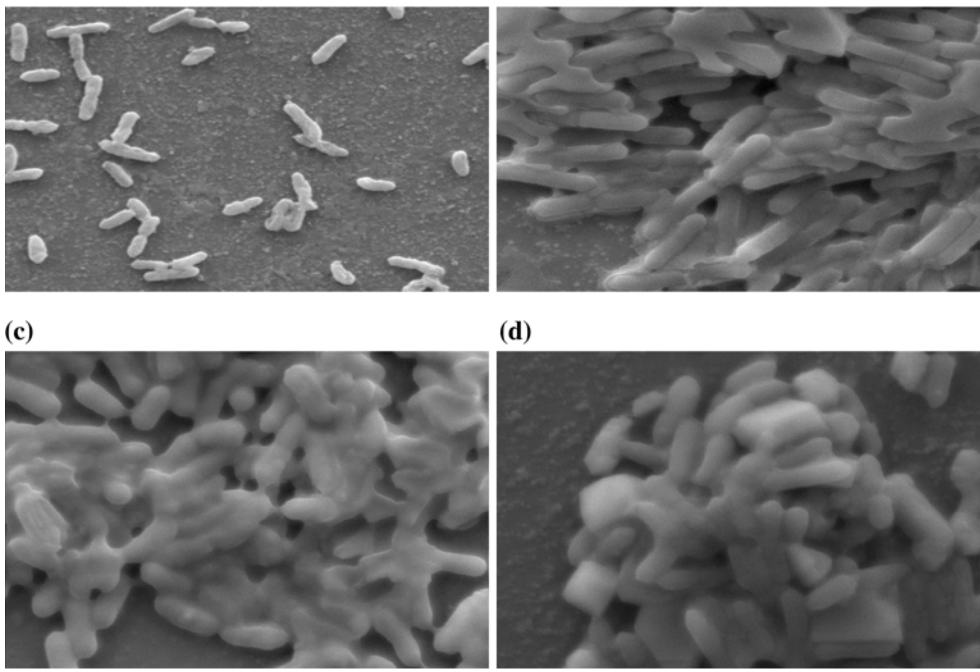

Fig. 1 Scanning electron micrographs of (**a**) wild-type *L. monocytogenes* ATCC 53135 & its (**b**) nisin-resistant variants, (**c**) pediocin 34-resistant variants and (**d**) enterocin FH99-resistant variants

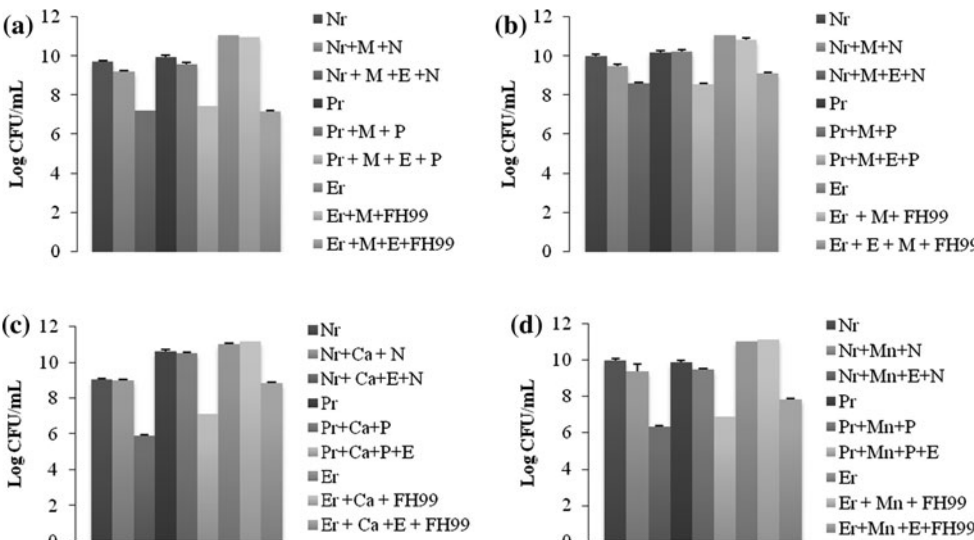

Fig. 2 Effect of (**a**) $MgSO_4$ (M), (**b**) $MgCl_2$ (M), (**c**) $CaCl_2$ (Ca), and (**d**) $MnSO_4$ (Mn) on sensitivity of nisin-resistant (Nr), pediocin 34 (Pr)-resistant, and enterocin FH99 (Er)-resistant *L. monocytogenes* ATCC 53135 to nisin (N), pediocin 34(P), and enterocin FH 99 (FH99) in presence or absence of EDTA (E)

was caused by pediocin 34 and enterocin FH99, but the inclusion of 20 mM EDTA increased the lethality caused by pediocin 34 by 2.52 log cycles and enterocin FH99 by 3.87 log cycles (Fig. 2a). For the nisin- and pediocin-resistant variants, in the presence of $MgCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 1.40 and 1.63 log cycles, respectively. However, for the enterocin-resistant variant, a reduction of about 1.94 log cycles was observed after inclusion of EDTA in the system (Fig. 2b).

For the nisin- and pediocin-resistant variants, in the presence of $CaCl_2$, inclusion of EDTA resulted in the increase of lethality caused by nisin and pediocin 34 to about 3.17 and 3.50 log cycles, respectively. However, for the enterocin-resistant variant, reduction of about 2.21 log cycles was observed after inclusion of EDTA in the system (Fig. 2c). In the presence of $MnSO_4$, inclusion of EDTA resulted in the increase of lethality by nisin and pediocin 34 to about 3.65 and 3.02 log cycles, respectively, for the nisin- and pediocin-resistant variants. However, for the enterocin-resistant variant, reduction of about 3.19 log cycles was observed after inclusion of EDTA in the experimental system (Fig. 2d).

Lysozyme Sensitivity of Nisin-, Pediocin 34-, and Enterocin FH99-Resistant Variants

In the presence of lysozyme, the nisin-resistant variant showed an increase of 0.62 log cycles after 4 h, whereas the wild-type strain showed a decrease of about 2.5, 2.9, and 2.08 log cycles after 4, 6, and 8 h, respectively. Pediocin 34-resistant variant of *L. monocytogenes* ATCC 53135 depicted a log reduction of 0.58, 2.88, and 2.04 log cycles after 4, 6, and 8 h, respectively. Enterocin FH99-resistant variant showed an increase of 0.44 log cycles after 4 h. However, after 6 and 8 h, it showed a decrease of approximately 0.57 and 0.82 log cycles, respectively (Fig. 3a).

The effect of lysozyme on the wild-type and nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes* ATCC 53135 in the presence as well as in the absence of nisin, pediocin 34, and enterocin FH99 was determined. A significant differences ($P < 0.05$) in viable count of wild-type *L. monocytogenes* and its nisin-, pediocin 34-, and enterocin FH99-resistant variants were observed when treated with nisin, pediocin 34, and enterocin FH99 alone and in combination with lysozyme. Wild-type *L. monocytogenes* ATCC 53135 showed a log reduction of 3.07 and 3.86 log cycles after 1- and 2-h and no growth after 2-h incubation with lysozyme and nisin. On the other hand, the nisin-resistant variant showed a log reduction of about 0.57, 1.09, and 1.64 log cycles after 2, 6, and 24 h, respectively, in the presence of lysozyme and nisin (Fig. 3b). In the presence of pediocin 34 and lysozyme, wild-type *L. monocytogenes* ATCC 53135 showed a log reduction of 1.9 and 3.6 log cycles after 4 and 8 h, respectively, and no growth was observed after 24 h, whereas, pediocin 34-resistant variant showed a log reduction of 3.11 after 6 h and no growth after 8 and 24 h (Fig. 3c). Wild-type *L. mononocytogenes* ATCC 53135 in the presence of both lysozyme and enterocin FH99 showed a log reduction of 0.35 and 4.0 log cycles after 4 and 8 h, respectively, and no growth after 24-h incubation. Enterocin-resistant variant showed an increase of approximately 0.89 log cycles after 6 h whereas a log reduction of 1.27, 1.23, and 0.09 log cycles was observed after 6-, 8-, and 24-h incubation (Fig. 3d). In conclusion, it was observed that *L. monocytogenes* ATCC 53135 nisin-, pediocin 34-, and

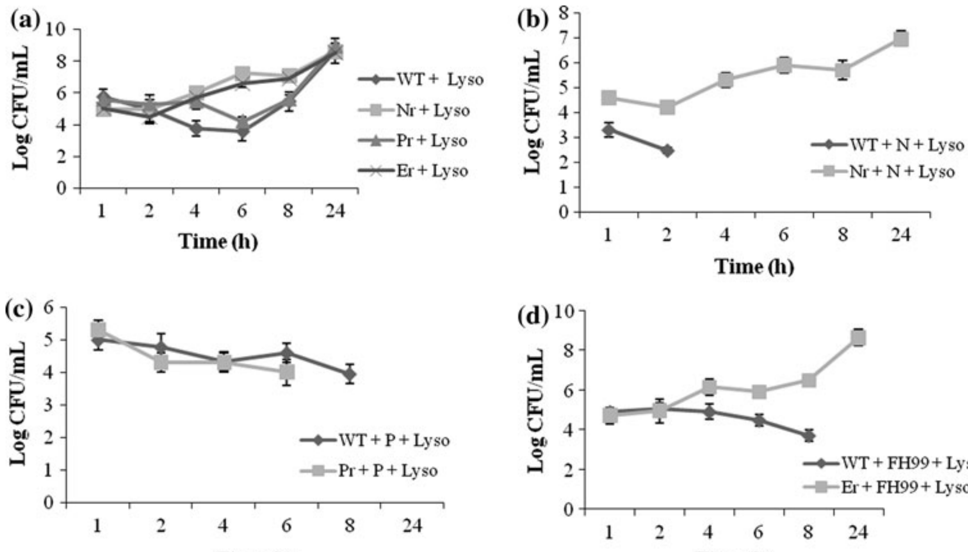

Fig. 3 Growth of wild-type (WT), nisin-resistant (Nr), pediocin 34-resistant (Pr), and enterocin FH99-resistant (Er) variants of *L. monocytogenes* ATCC 53135 in presence of (**a**) lysozyme, (**b**) growth of WT and Nr variant in presence of both lysozyme and nisin, (**c**) Growth of WT and Pr variant in presence of both lysozyme and pediocin 34 and (**d**) Growth of WT and Er variant in presence of both lysozyme and enterocin FH99

enterocin-resistant variants were more resistant to lysozyme when compared with the wild-type strain both in the presence as well as absence of either nisin, pediocin 34, and enterocin FH99.

Role of the Cell Envelope in the Acquisition of Bacteriocin Resistance

The role of the cell wall in the acquisition of bacteriocin resistance in strains of wild-type and resistant variants was investigated. For this, protoplasts were prepared using the method of Ghosh and Murray [12]. Efficiency of protoplast formation and inactivation of whole cells and protoplasts by bacteriocins was determined (Fig. 4). The efficiency of protoplast formation in those cells surviving lysozyme treatment as determined by lysis in water was high (>90%). Control cells were unaffected by suspension in water.

The inactivation of whole cells and protoplasts of *L. monocytogenes* ATCC 53135 and its nisin-, pediocin 34-, and enterocin FH99-resistant variants was studied using nisin, pediocin 34, and enterocin FH99 (MICs of each bacteriocin as given in Table 1 were used).

Significant differences ($P < 0.05$) in viable count of whole cells of wild-type *L. monocytogenes* and whole cells of its nisin-, pediocin 34-, and enterocin FH99-resistant variants were observed when treated with nisin, pediocin 34, and enterocin FH99, respectively. Also, significant differences ($P < 0.05$) in viable count of protoplasts of wild-type *L. monocytogenes* and protoplasts of its nisin-, pediocin 34-, and enterocin FH99-resistant variants were observed when treated with nisin, pediocin 34, and enterocin FH99, respectively. In the presence of nisin, the number of whole cells of wild-type *L. monocytogenes* ATCC 53135, decreased by 1.6 log cycles over 3 h compared with a decrease of 0.5 log in the nisin-resistant variant whole cell population. After a 3-h incubation with nisin, the nisin-resistant *L. monocytogenes* ATCC 53135 protoplasts had decreased in concentration by 3.09 log cycles compared with a 0.50 log cycle reduction of the nisin-resistant *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* ATCC 53135 wild-type protoplasts was about a 5.23 log cycles after 3 h (Fig. 4a). After a 3-h incubation with pediocin 34-, the pediocin-resistant *L. monocytogenes* ATCC 53135 protoplasts had decreased in concentration by 2.00 log cycles compared with a 0.44 log cycle reduction of the pediocin 34-resistant *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* ATCC 53135 wild-type protoplasts was about 4.55 log cycles compared to a reduction of 1.20 log cycles of wild-type whole cells after 3 h (Fig. 4b). After a 3-h incubation with enterocin FH99, the enterocin-resistant *L. monocytogenes* ATCC 53135 protoplasts decreased in concentration by 2.06 log cycles compared to only 0.24 log cycle reduction of the enterocin FH99-resistant *L. monocytogenes* ATCC 53135 whole cells. In contrast, the reduction in concentration of the *L. monocytogenes* ATCC 53135 wild-type protoplasts was about 4.85 log cycles compared to a reduction of 2.24 log cycles of wild-type whole cells after 3 h (Fig. 4c).

Discussion

Bacteriocin-Induced Changes in Bacterial Cell Morphology by Electron Microscopy

The formation of bacterial cell aggregates observed in resistant cells may be the prime mechanism of resistance because overall, a smaller cell surface in aggregated cells is

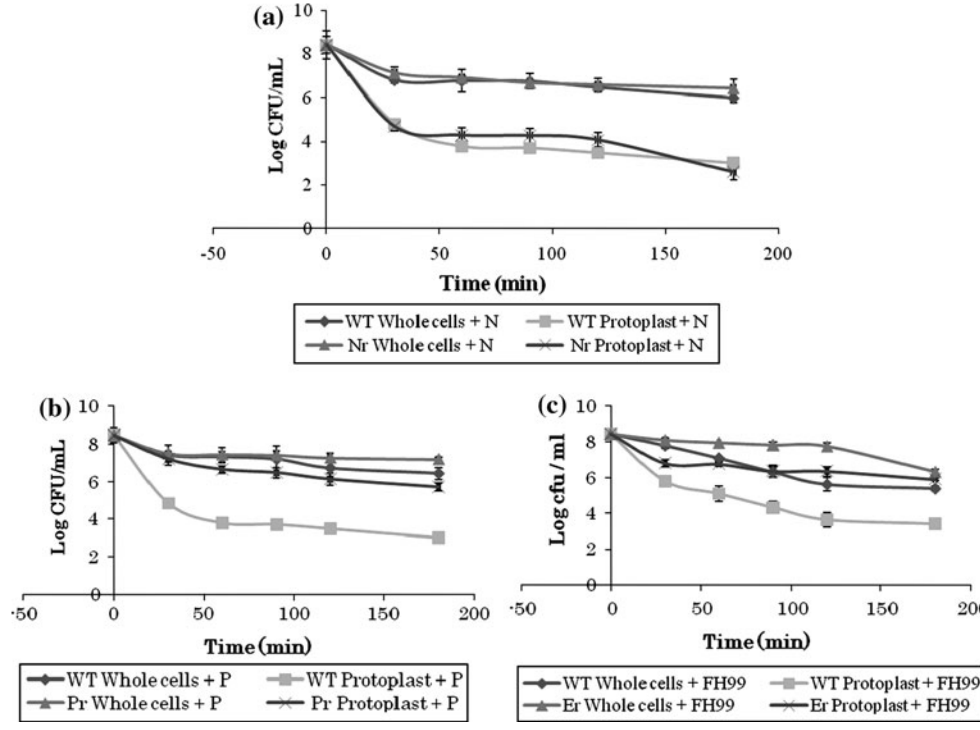

Fig. 4 The effect of (**a**) nisin (N) on whole cells and protoplast cells of WT and nisin-resistant variant cells (Nr), (**b**) pediocin 34 (P) on WT protoplast cells and protoplast of pediocin 34-resistant variant, (**c**) enterocin FH99 (FH99) on whole cells and protoplast cells of WT and enterocin FH99-resistant variant cells(Er). Mean and range of triplicate experiments indicated

exposed to bacteriocins. Similar results have been reported by Mehla and Sood [19].

Divalent-Cation Requirement of Nisin-, Pediocin 34-, and Enterocin FH99-Resistant Variants

Supplementation of 10 mM $MgSO_4$, $MgCl_2$, $CaCl_2$, and $MnSO_4$ reduced the lethality caused by nisin, pediocin 34, and enterocin FH99. In the absence of bacteriocins, the divalent cations had no effect on cell viability. The effect due to divalent cations was further confirmed by experiments involving EDTA, a chelator of divalent cations. Inclusion of 20 mM EDTA in any of the systems containing divalent cations increased the lethality caused by nisin, pediocin 34, and enterocin FH99. Divalent cations did not prevent nisin, pediocin 34, and enterocin FH99 from killing wild-type cells (data not shown). The EDTA, either alone or in combination with divalent cations, had no effect on the viability of nisin-, pediocin 34-, and enterocin FH99-resistant variants (data not shown).

The addition of divalent cations significantly reduced the inhibitory activity of nisin, pediocin 34, and enterocin FH99 against cells of resistant variants of test culture *L. monocytogenes* ATCC 53135. Similar results have earlier been reported by Abee et al. [1] who found that di- and trivalent cations (Mg^{2+}, Ca^{2+}, and Gd^{3+}) decreased the nisin Z-induced rate of K^{1+} efflux from whole cells of *L. monocytogenes* Scott A. They suggested that di- and trivalent cations might inhibit the electrostatic interactions between the positive charges on the nisin molecule and negatively charged phospholipids head groups. Alternatively or additionally, the neutralization of the negative head group charges may induce a condensation of these phospholipids, resulting in a more rigid membrane [1]. In conclusion, the impact of divalent cations on bactericidal activity of nisin, pediocin 34, and enterocin FH99 revealed that Mg^{2+}, Mn^{2+}, and Ca^{3+} cations were able to reduce the binding of antimicrobial peptide to the cell membrane. Divalent and trivalent cations seem to affect the initial electrostatic interaction between the positively charged bacteriocin and the negatively charged phospholipids of the membrane [23].

Lysozyme Sensitivity of Nisin-, Pediocin 34-, and Enterocin FH99-Resistant Variants

Lysozyme had two modes of action: (1) enzymatic lysis of the bacterial cell wall and (2) membrane perturbation inducing cell death [16]. The efficacy of lytic activity of lysozyme is preferentially directed to Gram-positive bacteria because of the cell wall composition (peptidoglycan, the target of lysozyme). Nisin-, pediocin 34-, and enterocin-resistant variants of *L. monocytogenes* ATCC 53135 were more resistant to lysozyme as compared to the wild-type strain both in the presence as well as absence of nisin, pediocin 34, and enterocin FH99. These results suggest that certain cell wall-associated modifications might have occurred in these resistant variants that made them resistant toward the lytic effect of lysozyme and bacteriocins. The results of our study are in contrast (no additive effect between lysozyme and bacteriocins was observed) to the

studies conducted by Calvez et al. [3] where they showed that the resistant variant to divercin RV41 did not confer any cross-resistance but exhibited an additive effect ascribed to the combined action of lysozyme and (P)-DvnRV41. This phenomenon could be due to the mode of action of each substance. The lysozyme, which causes cell wall disruption and stress in the cell, was unable to do so due to some modification in the cell wall architecture. Therefore, there might be a possibility that the access to IIC subunit encoded by the *mptC* gene of the mannose transport system might be hindered, which thereby might constitute a putative target of pediocin 34, and enterocin FH9 [3]. With wild-type cells, nisin passes through the cell wall, binds to the cytoplasmic membrane, probably via electrostatic interactions with the anionic phospholipids (phosphatidylglycerol and cardiolipin), and disrupts the membrane through the formation of pores. In the nisin-resistant variant, cell wall alterations may prevent nisin from interacting with the cytoplasmic membrane. Nisin, which does reach the membrane, interacts with it to induce the changes in fatty acid and phospholipid composition. Nisin's ability to bind to the cytoplasmic membrane of the nisin-resistant variant may be hindered by the decrease in the net negative charge of the membrane surface, and its ability to insert may be hampered by a decrease in membrane fluidity [4].

Role of the Cell Envelope in the Acquisition of Bacteriocin Resistance

Results indicated that without a cell wall, the acquired nisin, pediocin 34, and enterocin FH99 resistance of the variants were lost. When the cell wall was removed from the wild-type strain, the nisin-, pediocin 34-, and enterocin FH99-resistant variants of *L. monocytogenes* ATCC 53135 showed sensitivity to the three bacteriocins. Although the bacteriocin-resistant variants appeared to lose their acquired resistance toward nisin, pediocin 34, and enterocin FH99, the protoplasts of the resistant variants appeared to be more resistant to bacteriocins than the protoplasts of their wild-type counterparts. This may be due to non-specific adsorption of the bacteriocins on to freshly exposed hydrophobic sites on the protoplast. These results concur with Schved et al. [28] who attributed the resistance of *Lactobacillus plantarum* strains to pediocin SJ-1, to the barrier properties of the cell wall. However, our results are in contrast to the observation of Zajdel et al. [33] who in their study stated that the bacteriocin lactostrepsin (Las) 5 did not kill protoplasts prepared from either sensitive or resistant bacterial cells. The authors suggested that interaction with the cell wall is a condition necessary for Las 5 activity. The observations with protoplasts, however, indicate that the cell wall architecture plays a major role in the development of bacteriocin resistance.

Conclusions

Since bacteriocins are considered as potential tools for biopreservation, more study is needed to determine the distribution of bacteriocin-resistance phenomena among microorganisms that cause food spoilage. Among the food-borne pathogens, knowledge of the characteristics of bacteriocin-resistant variants and the conditions that prevent their emergence will help in determining the optimal conditions for application of bacteriocins in foods and minimize the incidence of resistance. Insight to the mechanism of action of nisin, pediocin 34, and enterocin FH99 suggests that nisin, pediocin 34, and enterocin FH99 resistance in *L. monocytogenes* is linked to a modification in the cell wall that might limit the diffusion of these bacteriocins into the cell. This, in turn, suggests that nisin, pediocin 34, and enterocin FH99 require access to the cytoplasmic membrane and/or the cytoplasm to exert their antimicrobial activity.

References

1. Abee T, Rombouts FM, Hugenholtz J, Guihard G, Letellier L (1994) Mode of action of nisin Z against *Listeria monocytogenes* Scott A grown at high and low temperatures. Appl Environ Microbiol 60:1962–1968
2. Biswas SR, Ray P, Johnson MC, Ray B (1991) Influence of growth conditions on the production of a bacteriocin AcH by *Pediococcus acidilactici* H. Appl Environ Microbiol 5:1265–1267
3. Calvez S, Kohler A, Prévost H, Møretrø T, Drider D (2010) Physiological and structural differences between enterococcus faecalis JH2–2 and mutant strains resistant to (P)-divercin RV41. Probiotics Antimicrob Prot 2:226–232
4. Crandall AD, Montville TJ (1998) Nisin resistance in *Listeria monocytogenes* ATCC 700302 is a complex phenotype. Appl Environ Microbiol 64:231–237
5. Dalet K, Cenatiempo Y, Cossart P, Héchard Y (2001) A σ54-dependent PTS permease of the mannose family is responsible for sensitivity of *Listeria monocytogenes* to mesentericin Y105. Microbiolgy 147:3263–3269
6. De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 2:130–135
7. Dykes GA, Hastings JW (1998) Fitness costs associated with class IIa bacteriocin resistance in *Listeria monocytogenes* B73. Lett Appl Microbiol 26:5–8
8. Foulquie Moreno MR, Sarantinopoulos P, Tsakalidou E, De Vuyst L (2006) The role and application of enterococci in food and health. Int J Food Microbiol 106:1–24
9. Franz CMAP, Schillinger U, Holzapfel WH (1996) Production and characterization of enterocin 900, a bacteriocin produced by *Enterococcus faecium* BFE 900 from black olives. Int J Food Microbiol 29:255–270
10. Galvez A, López RL, Abriouel H (2008) Application of bacteriocins in the control of food borne pathogenic and spoilage bacteria. Crit Rev Biotech 28:125–152
11. Galvez A, Valdivia E, Abriouel H, Camafeita E, Mendez E, Martinez-Bueno M, Maqueda M (1998) Isolation and characterization of enterocin EJ97, a bacteriocin produced by *Enterococcus faecalis* EJ97. Arch Microbiol 171:59–65

12. Ghosh HK, Murray RGE (1967) Fine structure of *Listeria monocytogenes* in relation to protoplast formation. J Bactreriol 93: 411–426
13. Giraffa G (1995) Enterococcal bacteriocins: their potential as anti-*Listeria* factors in dairy technology. Food Microbiol 12: 291–299
14. Gupta H, Malik RK, De S, Kaushik JK (2010) Purification and characterization of enterocin FH 99 produced by a faecal isolate *Enterococcus faecium* FH 99. Indian J Microbiol 50:145–155
15. Héchard Y, Pelletier C, Cenatiempo Y, Frére J (2001) Analysis of σ54 dependent genes in *Enterococcus faecalis*: a mannose PTS permease (EII^{Man}) is involved in sensitivity to a bacteriocin, mesentericin Y105. Microbiology 147:1575–1580
16. Ibrahim HR, Aoki T, Pellegrini A (2002) Strategies for new antimicrobial proteins and peptides: lysozyme and aprotinin as model molecules. Curr Pharm Des 8:671–693
17. Maisnier-Patin S, Richard J (1996) Cell wall changes in nisin-resistant variants of *Listeria innocua* grown in the presence of high nisin concentration. FEMS Microbiol Lett 140:29–35
18. Mazzotta AS, Montville TJ (1997) Nisin induces changes in membrane fatty acid composition of *Listeria monocytogenes* nisin-resistant strains at 10 °C and 30 °C. J Appl Microbiol 82: 32–38
19. Mehla J, Sood SK (2011) Substantiation in *Enterococcus faecalis* of dose-dependent resistance and cross-resistance to pore-forming antimicrobial peptides by use of a polydiacetylene-based colorimetric assay. Appl Environ Microbiol 77(3):786–793
20. Ming X, Daeschel MA (1993) Nisin resistance of food bore bacteria and the specific responses of *Listeria monocytogenes* Scott A. J Food Prot 56:944–948
21. Ming X, Daeschel MA (1995) Correlation of cellular phospholipids content with nisin resistance of *Listeria monocytoges* Scott A. J Food Prot 58:416–420
22. Núñez M, Rodríguez JL, García E, Gaya P, Medina M (1997) Inhibition of *Listeria monocytogenes* by enterocin 4 during the manufacture and ripening of Manchego cheese. J Appl Microbiol 83:671–677
23. Naghmouchi K, Drider D, Kheadr E, Lacroix C, Prévost H, Fliss I (2006) Multiple characterizations of *Listeria monocytogenes* sensitive and insensitive variants to divergicin M35, a new pediocin like bacteriocin. J Appl Microbiol 100:29–39
24. Rekhif N, Atrih A, Lefebvre G (1994) Selection and properties of spontaneous mutants of *Listeria monocytogenes* ATCC 15313 resistant to different bacteriocins produced by lactic acid bacteria strains. Curr Microbiol 28:237–241
25. Revol-Junelles AM, Mathis R, Krier F, Fleury Y, Delfour A, Lefebvre G (1996) *Leuconostoc mesenteroides* subsp. mesenteroides FR52 synthesizes two distinct bacteriocins. Lett Appl Microbiol 23:120–124
26. Rocourt J, Cossart P (1997) Listeria monocytogenes. In: Doyle MP, Buechat LR, Montville TJ (eds) Food microbiology: fundamentals and frontiers. American Society for Microbiology (ASM) press, Washington, DC, pp 337–352
27. Sarantinopoulos P, Leroy F, Leontopoulou E, Georgalaki MD, Kalantzopoulos G, Tsakalidou E, Vuyst LD (2002) Bacteriocin production by *Enterococcus faecium* FAIR-E 198 in view of its application as adjunct starter in Greek Feta cheese making. Int J Food Microbiol 72(1–2):125–136
28. Schved F, Lalazar A, Linder P, Juven BJ (1994) Interaction of the bacteriocin produced by *Pediococcus acidilactici* SJ-1 with the cell envelope of *Lactobacillus* spp. Lett Appl Microbiol 19:281–283
29. Torri Tarelli G, Carminati D, Giraffa G (1994) Production of bacteriocins active against *Listeria monocytogenes* and *Listeria innocua* from dairy enterococci. Food Microbiol 11:243–252
30. Ulhman U, Schillinger U, Rupnow JR, Holzapfel WH (1992) Identification and characterization of two bacteriocin-producing strains of *Lactococcus lactis* isolated from vegetables. Int J Food Microbiol 16:141–151
31. Vadyvaloo V, Hastings JW, van der Merwe MJ, Rautenbach M (2002) Membranes of class IIa bacteriocin-resistant *Listeria monocytogenes* cells contain increased levels of desaturated and short-acyl-chain phosphatidylglycerols. Appl Environ Microbiol 68(11):5223–5230
32. Vadyvaloo V, Snoep JL, Hastings JW, Rautenbach M (2004) Physiological implications of class IIa bacteriocins resistance in *Listeria monocytogenes*. Microbiology 150:335–340
33. Zajdel JA, Ceglowski P, Dobrzanski WI (1985) Mechanism of action of lactostrepsin *5*, a bacteriocin produced by *Streptococcus cremoris* 202. Appl Errviron Microbiol 49:969–974