CHARACTERIZATION OF PSYCHROTROPHS SURVIVING PASTEURIZATION

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DECLARATION

I hereby declare that this thesis entitled "**Characterization of Psychrotrophs Surviving Pasteurization**" is a bonafide record of research done by me during the course of research and the thesis has not previously formed the basis of the award of any degree, diploma, fellowship or other similar title, of any other university or society.

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Vishnu Suresh

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ABBREVIATIONS

Sl. No	Abbreviations
1.	% - Percentage
2.	˚C - Degree centigrade
3.	µm – Micrometer
4.	AR – Antibiotic resistance
5.	BMC - Bulk milk cooler
6.	Bu - Butter
7.	CFU - colony forming unit
8.	CIP - Cleaning in place
9.	CLSI - Clinical and Laboratory Standards Institute
10.	cm – Centimeter
11.	CRA - Congo red agar
12.	Da - Dahi
13.	EPS - Exopolysaccharide
14.	ESL - Extended shelf life
15.	FSSAI -Food Safety Standards Authority of India
16.	g - Generation time
17.	h- Hour
18.	HTST - High temperature short time
19.	IC - Ice cream
20.	Is - Isolate
21.	ISO - International oragnisation for standardization
22.	LPS - Lipopolysaccahride

23.	LTLT - Low temperature long time
24.	MBRT - Methylene blue reduction time
25.	min – Minutes
26.	ml – Millilitre
27.	nm – Nanometre
28.	OD - Optical density
29.	PC - Psychrotrophic count
30.	PCA - Plate count agar
31.	PM - Pasteurized milk
32.	RM - Raw milk
33.	rpm - Revolution per minute
34.	S rRNA – Small subunit ribosomal RNA
35.	SE- Standard error
36.	SEM – Scanning electron microscopy
37.	SMA - Skim milk agar
38.	SPC - Standard plate count
39.	spp. - Species
40.	SPSS- Statistical Product and Service Solution
41.	SS - Stainless steel
42.	Sw - Swab
43.	TBA - Tributyrin agar
44.	TC - Thermoduric count
45.	TPC - Thermoduric psychrotrophic count
46.	TTC - 2,3,5- triphenyltetrazolium chloride

Introduction

1. INTRODUCTION

Dairy industry, being involved with a highly perishable product, relies on different preservation techniques to assure the safety and quality of its products. Pasteurization, the process of heating every particle of milk to at least 63^{o}C for 30min or 72^{o}C for 15sec or to any temperature-time combination which is equally efficient in approved and properly operated equipment is one of the major thermal preservation processes adopted by the dairy industry. Though this process is considered as an efficient treatment for addressing the safety hazards associated with milk, there are chances that even the pasteurized milk can become a potential food safety issue due to various reasons like equipment failure, temperature fluctuations, presence of heat resistant organisms, spores and post pasteurization contaminations. The heat resistant organisms capable of surviving pasteurization are called the thermoduric organisms. If these survivors are 'psychrotrophs', organisms capable of growing under refrigerated conditions the situation becomes more serious as refrigerated storage is the most widely recommended storage environment for milk and milk products. Further, with the on-going changes in lifestyle and demographics, 'convenience aspects' have evolved out as the major factor deciding the purchase pattern of daily need products. In this fast-paced world, consumers try to save their precious time by reducing purchase frequency. All these paved way to the introduction of extended shelf life products and prolonged storage of daily consumables. These changes, that have occurred in production, processing and storage resulted in a major shift in the nature of pasteurized milk microflora to a specific group of organisms called the 'thermoduric psychrotrophs'; the organisms capable of surviving pasteurization and grow under refrigeration conditions. Modifications occurred in the design of industrial food processing facilities also contributed towards the predominance of this type of microflora in dairy processing environment as well as in products. This shift can curtail the efficacy of processing and can negatively affect both product quality and safety. So it is necessary to study the presence and prevalence

of these pasteurization surviving psychrotrophs in milk and milk products so that effective measures can be developed to control and avoid them in food supply chain. In this context, this study to isolate, identify and characterize predominant pasteurization resistant psychrotrophs in the dairy environment was carried out with the following objectives.

1. To isolate and identify major pasteurization resistant psychrotrophs of dairy origin.

2. To assess the cross adaptation potential of selected isolates.

3. To understand the factors contributing to their persistence in the dairy environment.

Review of Literature

2. REVIEW OF LITERATURE

2.1 PASTEURIZATION - A BOON TO DAIRY INDUSTRY

Milk due to its high water activity, almost neutral pH and availability of essential nutrients makes it a perfect medium that supports the growth of several microorganisms. Though it is secreted as a sterile fluid into alveoli of udder, microbial contamination occurs at different stages beyond this stage of production (Solomon *et al.,* 2013). As milk allows the growth of copious and heterogeneous number of microorganisms, diverse and numerous microorganisms originating from the teat canal, udder skin, milking machines, tanks, and containers might enter and grow in it. Several studies have suggested that bacteria in milk not only originate from external colonization but also from an endogenous route of bacterial transmission (Addis *et al*., 2016).

The microorganisms present in milk can be spoilage organisms, pathogenic organisms, conditionally beneficial organisms and some other which have not been associated with beneficial or detrimental effects on human health or product quality (Boor *et al.,* 2017). As the types of organisms present in raw milk are influenced by temperatures, time of storage, methods of handling during and after milking they can also be categorized into different bacterial groups like psychrophiles, psychrotrophs, mesophiles, thermodurics and thermophiles based on their growth preferences. So the dairy industry relies on different heat treatment methods to minimize both the possible health hazards arising from pathogenic microorganisms and the spoilage issues associated with spoilage microorganisms. Among these thermal processes, pasteurization, the process of heating milk for a predetermined time at a predetermined temperature to destroy pathogens is the most widely adopted one.

Pasteurization is named after Louis Pasteur who discovered that spoilage organisms could be inactivated in wine by applying heat at temperature below its boiling points. This process was later applied to milk and remains the most important operation in the processing of milk (Namminga, 1999). Food Safety and

Standards Authority of India (FSSAI) defines pasteurization as a microbicidal heat treatment aimed at reducing the number of any pathogenic micro-organisms in milk and liquid milk products, if present, to a level at which they do not constitute a significant health hazard. Pasteurization conditions shall be designed to effectively destroy the organisms *Mycobacterium tuberculosis* and *Coxiella burnetii (*FSSAI, 2020). The two basic methods of pasteurization are the holding process commonly known as the "Batch" or "Low Temperature Long Time" (LTLT) pasteurization method and the "High Temperature Short Time" (HTST) pasteurization method. Pasteurization of milk typically reduces psychrotrophic and mesophilic populations, leaving only two main groups; the heat resistant bacteria, which survive pasteurization (thermoduric microorganisms) and the bacteria introduced through post-pasteurization contamination (Quigley *et al.*, 2013).

2.2 PASTEURIZATION RESISTANT ORGANISMS (THERMODURICS)

Thermodurics are those microorganisms which can survive pasteurization and successively grow in the pasteurized milk. The initial microbial load of freshly pasteurized milk usually consists of Gram-positive thermoduric organisms present in the raw milk. Gram-negative bacteria usually do not withstand pasteurization (Cousin, 1982), unless the total number of bacteria in the milk is greater than the thermal destruction capacity of the pasteurization process. Thermoduric bacteria isolated from processed milk products include *Arthrobacter, Streptococcus, Microbacterium*, *Enterococcus, Micrococcus*, *Lactobacillus* and spore-formers such as *Bacillus*, *Clostridium* and *Paenibacillus* (Olson and Mocquat, 1980; Martin, 1981; Hull *et al*., 1992; Kikuchi *et al*., 1996; Ralyea *et al*., 1998). Raw milk is generally considered to be a primary source of thermoduric micro-organisms in pasteurized milk (Te Giffel *et al.*, 1997). *Microbacterium lacticum* and some strains of *Enterococc*us, *Streptococcus, Lactobacillus* and *Coryneform* bacteria are reported to survive high- temperature, short-time (HTST) pasteurization (72-74°C for 15s) (Varnam and Sutherland, 2001). As hygienically produced milk should not contain more than a small

number of tens of thermoduric organisms per ml, counts higher than this in raw milk are a strong indicator of poor udder hygiene and poor temperature control at farm level (Burgess,2010). In the case of pasteurized milk, poor hygiene in dairy plant processing systems wherein contamination can occur before, during or after the pasteurization process are found to contribute thermoduric organisms (Murphy, 2007). Studies of Jayarao *et al.* (2004) and Pantoja *et al.* (2009) reported correlations between the total bacterial count and thermoduric count of raw milk. The thermoduric bacteria are reported as one of the bacterial groups that can have the most important adverse effect on milk quality (Burgess, 2010). Thermoduric strains usually related with dairy products and dairy processing lines include spore forming organisms like *Bacillus, Clostridium* and species of *Streptococci, Staphylococci* and *Enterococci* (De Garnica *et al*., 2010). The spore forming thermodurics can easily persist in dairy and can cause several defects in milk and milk products. Thermoduric strains of *Microbacterium phyllosphaerae*, *Rothia aeria* and *Streptococcus mitis* were isolated for the first time from a dairy produce by Walsh *et al.* (2012).

The thermodurics can be divided into different categories like thermophilic thermodurics, mesophilic thermodurics and psychrotrophic thermodurics depending on their physiological characteristics (Gleeson *et al.*, 2013). The most common aerobic thermoduric spore-formers identified across dairy manufacturing plants, and dairy products, belong to the *Bacillus* and *Geobacillus* genera, with *B. licheniformis, B. coagulans, B. cereus, B. pumilus,* and *Geobacillus* spp. being the predominant ones (Gopal *et al.,* 2015). Ribeiro Junior (2018) reported a mean thermoduric count of 3.2 $\pm 4.7 \times 10^2$ CFU/ml (2.1 per cent of the total bacterial count) for pasteurized samples of refrigerated raw milk samples collected from different Brazilian dairy farms. They also reported the prevalence of endospore-forming bacteria (50 per cent) with *Bacillus licheniformis* as the most common (34.1 per cent) species in the thermoduric isolates obtained.

2.3 PASTEURIZATION RESISTANT (THERMODURIC) PSYCHROTROPHS

Occurrence of psychrotrophic spore forming bacteria in raw and pasteurized milk was first reported by Grosskopf and Harper (1974). They reported that the quality loss and unpalatability of pasteurized milk (stored at 4°C for four weeks) was attributed to the proliferation of *Bacillus coagulans*. Since this first report in the United States, many researchers have isolated thermoduric psychorotrophs from a variety of liquid dairy products. Thermoduric psychrotrophs are usually Gram-positive rods and cocci which belong to the genera *Arthrobacter, Bacillus, Clostridium, Corynebacterium, Lactobacillus, Microbacterium, Micrococcus* or *Streptococcus* (Stadhouders,1975). Thermoduric psychrotrophs identified as *Bacillus subtilis, Lactobacillus casei, Micrococcus jlavus* and *Streptococcus faecalis* were isolated from laboratory-pasteurized milk (Tinuoye and Harmon, 1975). Non sporeforming, heat resistant bacteria that belonged to the genera *Arthrobacter, Microbacterium, Streptococcus* and *Corynebacterium* were isolated from pasteurized milk products in Oklahoma by Washam *et al.* (1977). Collins (1981) opined that the major control measure for heat resistant psychrotrophs in milk is to keep the number of psychorotrophic heat-resistant bacteria in raw milk as low as possible by ensuring healthy cows, clean equipment, and good (rapid) cooling. Johnston and Bruce (1982) reported that 27.2 per cent of milk samples collected from 1040 farms situated in the west of Scotland contained thermoduric psychrotrophs and the majority of them were identified as belonging to *Bacillus* group. Thermoduric psychrotrophs that survive pasteurization and grow in chilled milk may affect the keeping quality of milk and produce bitter, fruity, rancid, sour, yeasty, putrid and unclean off-flavours in milk (Cousin, 1982).

The persistence of thermoduric psychrotrophs in food industry due to the adoption of high temperature short time pasteurization and extended refrigerated storage has intensified their significance in the dairy food industry. It has been estimated that 25 per cent of all shelf life issues associated with conventionally pasteurized milk and cream products in Unites States are linked to thermoduric

psychrotrophs (Meer *et al.,* 1991). Most thermoduric psychrotrophs, particularly the spore formers, tend to grow slower and/or later in milk products. Therefore they generally cause quality concerns later in shelf-life and only become predominant in the absence of faster growing post-pasteurization contaminants such as *Pseudomonas* spp. (Murphy, 2007). Mane and Gandhi (2010) reported the isolation of 36 thermoduric psychrotrophic organisms from twenty five different samples of milk and fermented milk products. Samarzija *et al*.(2012) observed that immediately after the heat treatment of milk, spores of the thermo resistant psychrotrophic aerobic *Bacillus* spp. present in it are activated into their vegetative forms. Gleeson *et al.* (2013) opined that psychrotrophic thermodurics along with the *Bacillus cereus* group and the sulphite-reducing clostridia (SRC) are the major bacterial groups of concern to the dairy industry. Ribeiro Júnior *et al*. (2017) reported the isolation of thermoduric psychrotrophic strains of one bacteria and ten fungi from refrigerated raw milk.

2.4 INDUSTRIALLY RELEVANT CHARACTERISTICS OF THERMODURIC AND/OR PSYCHROTROPHIC DAIRY ISOLATES

2.4.1 Heat Resistance

Heat treatment is one of the major techniques used in the food industry to reduce bacterial load. Considering the essentiality of closely monitoring and assessing the development of heat resistance in industrial bacterial isolates many studies are being conducted in this area. Evans *et al.* (1970) determined the thermal resistance of organisms important to milk pasteurization viz, strains of *E.coli, Staphylococcus aureus, Streptococcus pyogenes, Corynebacterium diphtheriae, Salmonella typhosa,* and *Shigella dysenteriae* and found that a temperature of 82.2°C would adequately pasteurize milk. Batish *et al.* (1988) compared BHI broth with full cream milk and reported that growth medium specific variations in the D_{63} -values of *E. faecalis* and *E. faecium*. Throughout the history, pasteurization conditions have been modified as new information about the heat resistance of target microorganisms has been identified (Holsinger *et al.,*

1997). Many studies indicated that higher temperatures are necessary to eliminate more heat-resistant pathogens in raw milk and it is important that legislative bodies and food processors keep abreast of new information as they are available. Dumalisile *et al*.(2005) assessed the impact of different pasteurization temperatures on the survival of microbial contaminants isolated from pasteurized milk and found that only *B. cereus* (S4) strain survived pasteurization in the LTLT and the HTST pasteurization treatments, whereas the other bacterial and yeast strains did not survive. Isolation of resistant variants of *Listeria monocytogenes* after a heat inactivation at 72°C in milk, and the expression of high resistance to standard pasteurization conditions by a fraction of these variants was reported by Van Boeijen *et al*. (2011). On assessing 61 isolates of thermoduric enterococci isolated from laboratory pasteurized milk from silos in six dairy facilities McAuley *et al.* (2012) found *E. durans* (z values 8.7 and 8.8°C), *E. faecium* (z value 9.0 °C) and *E. hirae* (z values 8.5 and 9.8°C) as the most heat resistant isolates. In a study conducted by Boll *et al.* (2017), it was identified that a locus of heat resistance confer heat resistance to a variety of Enterobacteriaceae, including *Escherichia coli* isolates from food production surroundings.

2.4.2 Enzymatic Activity

The psychrotrophic thermoduric flora of milk is able to survive pasteurization, can subsequently grow in product during storage and also have the extracellular enzyme activity necessary to induce spoilage (Muir, 2011). Some studies are being reported on the enzymatic activity of the thermoduric psychrotrophic isolates. Psychrotrophic *Bacillus* spp. are found to secrete heat-resistant extracellular proteinases, lipases and phospholipases (lecithinases) that are of comparable heat resistance to those of pseudomonads (Sorhaug & Stepaniak, 1997). Mane and Gandhi (2010) observed varying extents of proteolytic activity by all the three thermoduric psycrotrophic isolates tested. Ribeiro Júnior *et al.* (2017) reported that all the thermoduric psychrotrophic organisms they have isolated from refrigerated raw milk were proteolytic in nature. Among the thermoduric bacteria isolated from refrigerated raw milk 32.6

per cent were proteolytic and lipolytic simultaneously, 31 per cent were exclusively proteolytic, and 48 (36.4 per cent) were only lipolytic (Ribeiro Júnior *et al*., 2018).

2.4.3 Antibiotic Resistance

Lopes *et al*. (2005) assessed the antimicrobial resistance (AR) profiles of dairy and clinical isolates and type strains of enterococci and reported that clusters containing only dairy isolates were susceptible to the majority of antimicrobials tested, as opposed to clusters constituted only by clinical enterococcal isolates. Munsch-Alatossava and Alatossava (2007) reported that 60 per cent of the 60 psychrotrophic isolates retrieved from farms, trucks, and silos harboured multi-resistant traits against a panel of 17 β-lactams and non-β-lactams antibiotics. Munsch-Alatossava *et al*. (2012) analysed the antibiotic resistance to four antibiotics in mesophilic and psychrotrophic bacterial populations isolated from 18 raw milk samples and reported that different psychrotrophic communities with varying AR levels dominated over time on storage at 4°C. Gautam *et al.* (2015) reported varying degrees of response against different antibiotics by *E. coli*, *S. aureus*, *S. typhi* and *B. subtilis* isolated from raw milk and different dairy products.

On determining the antibiotic susceptibility of 636 dairy enterococci isolates Terzić-Vidojević *et al*. (2015) found that 29.1 per cent of the isolates were susceptible to all 13 of the antibiotics tested. Multidrug resistance by 11.4 per cent and resistance to at least one antimicrobial agent by 20.5 per cent of the heat-resistant strains of *E. coli* isolated from raw milk cheeses were reported by Marti *et al*. (2016). James *et al*. (2018) reported varying degrees of resistance to different antibiotics by HTST pasteuruzation surviving *S. aureus* isolates. Hasan *et al.* (2018) observed that most of the *E. coli* isolates obtained from raw milk samples exhibited resistance against ampicillin and cefotaxime. Popović *et al*. (2018) assessed the antibiotic susceptibility of 56 dairy enterococci isolates and found that 23 out of 56 strains were antibiotic susceptible. Meng *et al*. (2020)

evaluated the resistance patterns of psychrotrophic *Pseudomonas* spp. isolated from bulk-tank milk and opined that antibiotic resistance of *Pseudomonas* spp. in raw milk should be a concern. Machado *et al*. (2020) reported the isolation of antibiotic resistant *M. caseolyticus, S. epidermidis*, and *K. varians* isolates, especially multiple drug resistance ones' from Brazilian pasteurized milk.

2.4.4 Biofilm Formation Potential

A biofilm is defined as a sessile microbial community characterized by adhesion to a solid surface and by production of a matrix that surrounds the bacterial cells and includes extracellular polysaccharides (EPS), proteins and DNA (Landini *et al*., 2010). Numerous studies are reported on biofilm formation potential of dairy bacterial isolates as well as on biofilm formation in dairy environment. Presence of biofilms of pathogenic microflora, including coagulase-positive *S. aureus* and enterics as well as spoilage organisms was observed in different segments of pasteurization lines in dairy plants (Sharma and Anand, 2002). Microorganisms originating from rinsing water (especially *Pseudomonas*, *Aeromonas,* and *Legionella* spp.) are reported to form biofilms that are difficult to eradicate (Momba *et al.,* 2000). They harbour other microorganisms thus increasing the chances of pathogen survival and further propagation during milk processing (Lomander, 2004). *Bacillus* species are often involved in biofilm formation and contamination of dairy products (Ryu and Beuchat, 2005a). Capacity to form biofilms allows *Bacillus* species to flourish in the dairy associated environment as it enables their dispersal and survivability (Marchand *et al.,* 2012). Biofilms on the surfaces of milk transport lines, milking containers and accessories are identified as major sources of contamination of dairy products in dairy industries (Srey *et al*., 2013).

Studies of different simulated conditions showed that the *Bacillus cereus* cell count in the biofilm developed on the surface of stainless steel chilling tanks can reach up to 10^6 CFU/cm^2 if inadequately cleaned tanker is left to stand empty at room temperature (Kumari and Sarkar, 2014). In another study *Bacillus*

licheniformis was identified as the predominant biofilm forming thermo tolerant bacteria from several isolates obtained from 80 per cent whey protein concentrate (MdZain *et al.,* 2015). Sadiq *et al*. (2017) explored the biofilm forming potential of 148 spore-forming dairy isolates, comprising of mesophilic and thermophilic bacteria and reported that only *Bacillus licheniformis*, *Anoxybacillus flavithermus* and *Geobacillus* species formed good biofilms on stainless steel in milk. On assessing the biofilm formation potential of 30 heat-resistant and six heat-sensitive *Escherichia coli* dairy isolates Marti *et al*. (2017) found that the heat-resistant, multidrug-resistant (MDR) strain FAM21845 as the only strain that formed significant biofilms on stainless steel under conditions relevant to the dairy industry. Popovic *et al*. (2018) assessed biofilm formation in dairy enterococci isolates and 30 out of the 75 strains (40 per cent) were found to form biofilm.

On comparing the biofilm-forming capabilities of different genotypes of *Staphylococcus aureus* dairy isolates, Thiran *et al*. (2018) reported the importance of growth conditions for the expression of biofilm-related genes. Ostrov *et al.* (2019a) sequenced the genomes of milk and non-milk-derived *Bacillus* strains and concluded that, unlike non-dairy *Bacillus* isolates, the dairy-associated *Bacillus* strains are capable of forming of robust biofilm during growth in milk. Elegbeleye and Buys (2020) reported moderate to strong biofilm formation by thermophilic psychrotolerant *Bacillus subtilis* complex group isolated from raw, pasteurized, and packaged extended shelf-life (ESL) milk samples. On assessing the biofilm production in 33 catalase-positive Gram-positive cocci isolated from Brazilian pasteurized milk, Machado *et al.* (2020) detected it in only five isolates of *Kocuria varians* and one isolate of *S. epidermidis.*

2.5 EFFECT OF CLEANING AGENTS AND CIP REGIMES ON BIOFILMS

One of the main strategies to maintain the optimal level of hygiene in dairy processing facilities is regular cleaning and disinfection. Chemical cleaning of the production and processing lines is the major method the dairy industry

adopts to control biofilms. Though prior to the 1960s, manual cleaning methods were used, later cleaning in place (CIP) systems were developed to improve cleaning efficiency (Stewart and Seiberling, 1996). However the efficiency of CIP procedures to completely eliminate biofilm forming bacteria remains uncertain and depends on different factors. The existence of biofilms even after CIP and sanitization treatment in different segments of pasteurization lines was reported by Sharma and Anand (2002).

Bremer *et al*. (2002) determined the effect of chlorine on mixed bacterial biofilms on stainless steel (SS) and conveyor belt surfaces and reported that effectiveness of chlorine is dependent on its concentration, solution pH, exposure time, the nature of the surface and the microbial species present. On using caustic and acid cleaning method for biofilm removal Parkar *et al.* (2003) found that it is very important to use the right concentrations of agents and the recommended temperatures. Dufour *et al.* (2004) developed a laboratory scale CIP system to study the effectiveness of chlorine and alternative sanitizers in reducing the number of viable bacteria attached to stainless steel surfaces and found that the effectiveness of combinations of nisin, lauricidin, and the LPS was similar to that of chlorine. *Bacillus* species spores to a lesser extent than vegetative cells embedded in biofilms were found to be protected against disinfectants such as chlorine, chlorine dioxide, and peroxyacetic-acid-based sanitizer (Ryu and Beuchat, 2005a).

Upon studying the resistance of three *Escherichi*a coli O157:H7 strains to chlorine, a sanitizer commonly used in the food industry, Ryu and Beuchat (2005b) reported that production of EPS and curli increases the resistance of *E. coli* O157:H7 to chlorine. Standard CIP regime (water rinse, one per cent sodium hydroxide at 65°C for 10 min, water rinse, one per cent nitric acid at 65°C for 10 min, water rinse) was studied against different alternative cleaning chemicals and it was reported that the standard clean-in-place (CIP) regime did not reproducibly ensure the removal of viable bacteria attached to stainless steel (SS) surfaces (Bremer *et al*., 2006). Ostrov *et al.* (2019b) evaluated the susceptibility of strong biofilm-forming dairy *Bacillus* isolates towards industrial cleaning methods using

two differently designed model systems and proved that the dairy-associated *Bacillus* isolates revealed a higher resistance to CIP procedures, compared to the non-dairy strain of *B. subtilis*. Kang *et al*. (2020) investigated the antimicrobial effect of hot water with citric acid against *Escherichia coli* O157:H7 biofilm on stainless steel (SS) and reported synergistic inactivation effect against *E. coli* O157:H7 biofilm. They also reported that *E. coli* O157:H7 biofilm showed more resistance than planktonic cells.

Materials and Methods

3. MATERIALS AND METHODS

3.1 SAMPLE COLLECTION

A total of 57 samples comprising of pasteurized milk, raw milk, milk product (ice cream, butter and dahi) samples and swab samples of dairy equipment (bulk milk silo's, cream outlet of cream separator, valves after pasteurization from two plants, valve before pasteurization and CIP rinse water valve) collected from different sources over a three months period from September, 2019 were used in this study. Pasteurized milk and milk products were collected from the local market (In and around Mannuthy, Thrissur) and swab samples were taken from the equipment of two organized dairy plants as detailed below. Raw milk samples were collected from bulk milk coolers (BMC's) of a nearby cooperative society. Pasteurized milk samples of the same brands were collected periodically at an interval of one week. All the samples were transported aseptically under cold conditions and analysed within 1h.

Table 1. Details of the samples collected

Sl no.	**Sample**	**Sample code***
1.	Raw milk	RM1,2
2.	Swab	Sw1,2,3,4,5,6
3.	Butter	Bu-A1
4.	Dahi	Da-B1,Da-C1, Da-D1, Da-E1
5.	Ice cream	IC-F1,IC-G1
6.	Pasteurized milk	PM-H 1,2,3,4,5, 6,7,8
		PM-I 1,2,3,4,5, 6,7,8
		PM-J 1,2,3,4,5, 6,7,8
		PM-K 1,2,3,4,5, 6,7,8
		PM-L 1,2,3,4,5, 6,7,8
		PM-N 1
		PM-O 1

*Bu-Butter, Da- Dahi, IC- Ice cream, PM- Pasteurized milk, RM- Raw milk, Sw- Swab
A,B,C,D,E,F,G,H,I,J,K,L,N,O - Brand code
1,2,3,4,5,6,7,8- Number of sample

3.1.1 Collection of Swab Samples of Dairy Equipment

Swabs were collected from the equipment by swabbing with sterile cotton swabs (HiMedia Laboratories Pvt. Ltd., Mumbai) and the tips were swirled in 0.1 per cent 10ml peptone water (ISO 18593, 2004; Dufour *at al.,* 2004). This peptone water was used as sample and further dilutions were made using normal saline.

3.2 MICROBIOLOGICAL ANALYSIS OF THE SAMPLES

For the microbiological analysis the samples were appropriately diluted in normal saline (0.85 per cent) and used for subsequent analysis. In the case of packed samples special care was taken to ensure that the samples were cut open under aseptic conditions.

3.2.1 Standard Plate Count (SPC)

Appropriate dilutions were pour plated using plate count agar (PCA, HiMedia Laboratories Pvt. Ltd., Mumbai), incubated at 37°C for 48h and the colonies developed were counted and the result was expressed as CFU/ml (IS:1479, 1960). In the case of swab samples, the count was calculated as CFU/ml of the solution used.

3.2.2 Psychrotrophic Count (PC)

Appropriately diluted samples were pour plated using PCA, incubated at 7°C and after 10 days the colonies developed were counted (Frank and Yousef, 2004). Care was taken to avoid the temperature rising above 7°C, in order to prevent the growth of non-psychrotrophic organisms.

3.2.3 Thermoduric Count (TC)

All the samples were subjected to laboratory pasteurization by aseptically transferring 5ml of the samples directly into sterile test tubes without touching the sides while transferring. The test tubes were closed tightly using cotton plugs and heated them in a water bath at 63°C for 30min ensuring that the milk level in the

tubes were below the water level of the water bath throughout the treatment period. An inoculated tube containing milk at the same level was also treated in the same way to serve as the temperature control tube. It was ensured that the temperature of milk reached 63°C within 5min. At the end of heating, the tubes were immediately transferred to ice bath and chilled to 10°C (IS:1479-3, 1977). Appropriate dilutions were made using normal saline and the dilutions were plated using PCA. The plates were incubated at 37°C for 48h and the colonies developed were counted.

3.2.4 Pasteurization Surviving Psychrotrophic Bacteria (Thermoduric Psychrotrophic Bacteria) (TPC)

Pasteurization surviving psychrotrophic bacteria were enumerated based on the method of Washam *et al*. (1977) with some modifications in the refrigerated per-incubation period of laboratory pasteurized milk. The samples were subjected to laboratory pasteurization as detailed above and at the end of heating; the tubes were transferred immediately to an ice water bath, chilled to 7°C and stored at 7°C. After 10 days, the samples were appropriately diluted with sterile normal saline (0.85 per cent) and subjected to pour plating using PCA (HiMedia Laboratories Pvt. Ltd., Mumbai). The plates were incubated at 7°C for 10 days and the colonies developed were counted and the result was expressed as CFU/ml.

3.3 ISOLATION OF PASTEURIZATION SURVIVING PSYCHROTROPHIC BACTERIA

Predominant colonies with distinct colony morphologies developed on TPC Petri-dishes (obtained as per 3.2.4) were selected as pasteurization surviving psychrotrophic bacteria and were streaked to purity. Morphology of the selected colonies was noted in terms of form, elevation, margin, pigmentation and size.

3.3.1 Maintenance of the Isolates

The isolates were maintained in nutrient broth and nutrient agar plates stored at 4°C. Prior to use the cultures were activated by inoculation to nutrient broth and subsequent incubation.

3.4 IDENTIFICATION OF THE ISOLATES

3.4.1 Preliminary Identification Tests

Preliminary identification of the isolates was done by subjecting them to Gram's staining, catalase test and oxidase tests. Gram's staining technique was done using the standard procedure (Cappuccino and Sherman, 2005). Catalase test was performed by immersing a loopful of bacterial colony in a loopful of three per cent H_2O_2 (Nice Chemicals, Kochi, Kerala) placed on a slide and checking for bubble formation, which indicated a positive reaction (Harrigan, 1998). For oxidase test a well isolated colony of the isolate was gently smeared on the oxidase disc (HiMedia Laboratories Pvt. Ltd., Mumbai) aseptically and observed for the development of purple colour within 5-10 seconds. Absence of development of purple colour was considered as a negative result.

3.4.2 Genotypic Identification of the Isolates

Samples were outsourced to Rajiv Gandhi Centre for Biotechnology, Trivandrum for 16S ribosomal RNA (16S rRNA) sequencing to identify the bacterial isolates. The sequences obtained were searched with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) for their closest relatives/reference strains with a homology of over or equal to 99 per cent. The sequences were deposited in Genbank using BankIt (https://www.ncbi.nlm.nih.gov/WebSub/) program. With the help of MEGA software, phylogenetic trees were constructed using neighbour joining method to compare the evolutionary distances between the closely related strains available in Genbank.

3.4.3 SEM Imaging of the Isolate

For performing the scanning electron microscope (SEM) imaging of the isolates, one ml each of broth cultures of isolates were taken in small centrifuge vials, centrifuged at 10000rpm for 10min and after draining the supernatant, the pellet was suspended using 0.5ml molecular grade water (HiMedia Laboratories Pvt. Ltd., Mumbai). The suspension was spread over the aluminium stubs, vacuum dried, sputter coated with gold and observed using Tescan Vega 3 (Czech Republic) scanning electron microscope.

3.5 CHARACTERIZATION OF THE ISOLATES

3.5.1 Assessment of Growth at Different Temperatures

Ability of the isolates to grow at different temperatures was tested by incubating nutrient broth tubes inoculated at the rate of two per cent with cultures equivalent to 0.5 McFarland turbidity at 7°C, 20°C, room temperature (27°C) and 37°C for 24h or till the development of visually perceivable growth. A nutrient broth tube without inoculum was kept along with the samples to serve as the negative control. Growth in each tube was assessed qualitatively by comparing with the respective control nutrient broth tube. Based on the observations the optimum growth temperature of each of the isolates was finalized. Hereafter these optimal growth temperatures are referred as their respective growth temperatures.

3.5.2 Motility of the Isolates

3.5.2.1 Semi Solid Agar – Triphenyl Tetrazolium Chloride Method

Motility of the isolates was tested as per Ball and Sellers (1966) using the dye 2,3,5- triphenyltetrazolium chloride (TTC). In brief, broth cultures of the isolates were stabbed into each of the TTC incorporated 5ml soft nutrient agar (with 0.8 per cent agar) and incubated at respective temperatures for 24h. Spreading of growth from the line of stab was considered as indicative of the motile nature of the isolate.

3.5.3 Assessment of Haemolytic Activity

Cultures incubated at respective temperatures for 24h were streaked on to blood agar Petri-dishes (Polyclinic, Thrissur), and incubated at their respective temperatures for 24h. Based on the type of clear zones formed around the colonies the isolates were graded as gamma-haemolytic (no clearance zone), alpha-haemolytic (green hued zones around the colonies) or beta-haemolytic (complete clearance) (Buxton, 2005).

3.5.4 Assessment of Biofilm Formation Ability

3.5.4.1 Congo Red Agar Method

Biofilm formation potential of the isolates was determined by streaking a single colony on Congo red agar (CRA) and subsequent incubation at respective temperature for 24h (Freeman *et al*., 1989). Development of black colonies was considered as an indication of their biofilm formation potential.

3.5.4.2 Tube Method

Tube method as described by Christensen *et al*. (1982) was performed by inoculating a loopful of culture (turbidity equivalent to 0.5 McFarland solution) into 10ml trypticase soy broth with one per cent glucose. The tubes were incubated at 37°C for 24h. After that the tubes were decanted, washed with phosphate buffer saline of pH 7.3 and dried. Tubes were then stained with crystal violet and rinsed with deionized water to remove the excess stain. Tubes were dried in inverted position and observed for the appearance of a line of violet colour on the sides and bottom of the test tubes indicating their biofilm formation potential.

3.6 ASSESSMENT OF SPOILAGE POTENTIAL

3.6.1 Enzymatic Activities

3.6.1.1 Proteolytic Activity

Proteolytic activity of the isolates was determined by streaking them on skim milk agar (SMA) and subsequent incubation at 37°C for 24h. Formation of

zone of clearance around the growth was considered as positive for proteolytic activity (Harrigan, 1998). In a similar way their proteolytic activities were also assessed at 7°C for seven days.

3.6.1.2 Lipolytic Activity

Lipolytic activity was determined using the tributyrin agar (TBA, HiMedia Laboratories Pvt.Ltd., Mumbai) Petri-dishes. For this the isolates were streaked on TBA agar, incubated at 37°C and 7°C for 24h and 7 days respectively. The plates were observed for development of zone of clearance around the growth. As in the case of proteolytic activity formation of zone of clearance was considered as a positive result (Harrigan, 1998).

3.6.2 Hydrogen Sulfide Production

Hydrogen sulphide production ability of the isolates was determined by the lead acetate paper strip test (Harrigan, 1998). For this, cultures incubated at 37°C for 24h were inoculated in five ml of peptone water, a lead acetate paper strip (HiMedia Laboratories Pvt.Ltd., Mumbai) was inserted between the plug and the test tube in such a way that it didn't touch the media and incubated at 37°C for 24h. Blackening of the lead acetate paper was considered as the positive test.

3.6.3 Gelatin Liquefaction Test

For this test, Gelatin agar (HiMedia Laboratories Pvt.Ltd., Mumbai) slants stabbed with the isolates were incubated at 37°C for 24h and observed after incubation. As gelatin is in liquid state at temperatures above 28°C in order to avoid any faulty observation, prior to examination the tubes were refrigerated for three hours. (Sahu *et al.,* 2019).

3.6.4 Evaluation of Defect Development

Ability of the isolates to form defects in milk was determined by the method of Washam *et al.* (1977). For this 50ml autoclaved and cooled whole milk inoculated with the cultures at two per cent rate, was incubated at refrigerated

(7°C) and room (27°C) temperatures and observed for any visual defects daily for seven days. An uninoculated milk sample was treated in the same way to serve as the control. Upon spoilage of the sample its pH was determined to have an idea regarding the extent of acid production. Samples without visual defects were subjected to clot on boiling test.

3.7 ASSESSMENT OF ANTIBIOTIC SUSCEPTIBILITY

Isolates were tested for their antibiotic susceptibility by disc diffusion assay as per Clinical and Laboratory Standards Institute (CLSI) guidelines (Bauer *et al*., 1966; CLSI, 2015). The antibiotics were selected on the basis of the extent of their current use, mode of action and CLSI guidelines, such that representatives from all the major antibiotic categories are included. Suspensions of the isolate colonies in nutrient broth, equivalent to the turbidity of 0.5 McFarland solution were spread onto Mueller Hinton agar plates. For each of the isolates two plates were prepared such that each plate can accommodate four antibiotic discs. Based on the identity of isolate antibiotics to be used were selected from the following antibiotics; amoxicillin (30µg), amoxyclav (30µg), ampicillin (10µg), azithromycin (15µg), cefixime (5µg), chloramphenicol (30µg), ciprofloxacin (30µg), co-trimoxazole (25µg), enrofloxacin (10µg), erythromycin (10µg), gentamycin (10µg), ofloxacin (5µg), pencillin (1 unit), streptomycin (10µg) and tetracycline (30µg). Selected antibiotics were placed over the surface of the inoculated Mueller Hinton Petri-dishes and the Petri-dishes were incubated at 37°C for 24h. The zone of clearance developed around each of the antibiotics was measured. The isolates were categorized as susceptible, intermediate, or resistant, based on CLSI standards (CLSI, 2015) or Vlkova *et al.* (2006).

3.8 ASSESSMENT OF CROSS ADAPTATION POTENTIAL

Based on the observations of the experiments conducted, some isolates were selected and were subjected to further studies.

3.8.1 Heat Resistance at 63°C

Heat resistance of the isolates was determined as per Peng *et al.* (2013) but at a temperature of 63°C. For this, cultures with a turbidity equivalent to 0.5 McFarland solution were inoculated into sterilised whole milk tubes at a rate of two per cent and

labelled with respective holding times ie; 0, 5, 10, 15, 20, 25 and 30min. The inoculated tubes along with the temperature control tube were placed in a water bath adjusted at 63°C such that the sample levels in the test tubes were well below the water level in the water bath and after the specified period of heating the samples were removed from the water bath. The heat treated samples were immediately chilled to below 5°C, appropriately diluted and pour plated using PCA and incubated at 37°C for 48h. Thermal reduction time (D-values) was calculated from the slope of the best-fit line graphically determined by plotting the $\log_{10}$ of values of the surviving cells per millilitre against the time of heat exposure (Sung and Collins, 1998).

3.8.2 Resistance to boiling process

25ml of sterilised whole milk inoculated at the rate of one per cent was exposed to direct heat and boiled for 5 seconds. Appropriate dilutions were made with normal saline and the surviving cells were enumerated as detailed in 3.8.1.

3.8.3 Resistance to pH

To assess the acid resistance, the isolates with a turbidity equivalent to 0.5 McFarland tubes were inoculated at the rate of two per cent in nutrient broth tubes adjusted to pH levels of 7.5, 6.5, 5.5, 4.5, 3.5 and incubated at 37°C for 24h. The incubation was followed by the optical density measurements at 600nm against uninoculated tube (Palumbo *et al.*, 1985).

3.9 ATTRIBUTES CONTRIBUTING TOWARDS PRESENCE IN DAIRY ENVIRONMENT

3.9.1 Ability to grow in Milk under Room and Refrigerated Storage

3.9.1.1 Qualitative Method -Methylene Blue Reduction Time

Methylene blue reduction test is commonly used to qualitatively evaluate the microbial load of milk. An improvised version of this test was used to study the rate of growth of the isolates in milk. For this one ml methylene blue dye

(0.005 per cent) was added to 10ml sterile skim milk (10 per cent) previously inoculated at two per cent rate with the isolates adjusted to a turbidity equivalent to 0.5 McFarland solution. Uninoculated sterile skim milk sample with one ml dye served as the negative control. The tubes were sealed properly and inverted three times to mix well. The tubes were placed in a water bath adjusted at 37°C and observed at regular intervals for any change in colour. The methylene blue reduction time (MBRT), the time taken for the methylene blue solutions to become completely colourless (IS:1479, 1960) was noted.

3.9.1.2 Quantitative method – Determination of Generation Time

Growth rate of the isolates at 7°C and 27°C was estimated in reconstituted sterile whole milk (10 per cent). For comparison purposes growth rate in nutrient broth was also estimated. For this, cultures with a turbidity equivalent to 0.5 McFarland solution were inoculated into 10ml growth media at the rate of two per cent and the tubes were incubated at 7°C and 27°C. Samples were withdrawn after 0, 2, 4, 6, 8, 10 and 24h, appropriate dilutions were prepared, plated using PCA and incubated at 37°C for 48h. Number of colonies developed in each Petri-dish was counted, growth curve was plotted, exponential phase was identified and the growth rate constant during the exponential phase was calculated using the following formula (Prescott *et al*., 1993).

$$\mu = \frac{\log N_t - \log N_0}{0.301t}$$

Where μ = Mean growth rate

N_t =Population at time t

$N_{0=}$ Population at time 0

t= Time interval

The generation time was calculated for each of the isolates as per the following formula

$$g = \frac{1}{\mu}$$

Where g=Mean generation time

3.9.2 Biofilm Forming Potential

3.9.2.1 Assessment of Swarming Motility

Swarming motility agar was prepared using one per cent tryptone, 0.5 per cent sodium chloride, and 0.25 per cent agar (HiMedia). A portion from the colony of the isolate developed on a streak plate incubated at 37°C for 24h was transferred with a sterile toothpick to the centre of the agar plate, and the plates were incubated face up at 37°C for 16 to 24h. Migration of the bacteria from the point of inoculation to the periphery of the plate creating a zone like appearance was considered indicative of its swarming motility (Rabaan *et al*., 2001).

3.9.2.2 Assessment of Biofilm Formation Potential of the Isolates on Glass Strips by Scanning Electron Microscopy

Sterilized 10ml trypticase soy broth (TSB, HiMedia Laboratories Pvt. Ltd., Mumbai) tubes were inoculated with 100µL (10^6 CFU/ml) isolates. Cover glass slips of less than one cm diameter were inserted into the inoculated test tubes and the tubes were incubated at 37°C for 32 h. After incubation, the broth was drained out from the test tubes and the glass slips were washed three times with sterile normal saline and air dried at room temperature (Korres *et al.,* 2013). Glass slip of a non-biofilm forming isolate was also prepared in the same way to serve as the negative control. Glass slips were mounted on aluminium stubs, sputter coated with gold and observed using Tescan Vega 3 (Czech Republic) scanning electron microscope operated at 5kV and different magnifications.

3.9.3 Resistance to Cleaning-In-Place (CIP) Procedure

Four stainless steel (SS) strips each of size 1cm×2.5cm were dipped in four 10ml TSB tubes inoculated with the isolate at two per cent level (Turbidity equivalent to 0.5 McFarland solution) and incubated for 72h at 37°C for allowing the formation of biofilm, if any (Angeles-Morales *et al.,* 2012). After incubation the broth was decanted and the strips were collected using a sterile forceps. To

obtain the initial count, one face of one of the strips was swabbed using a cotton swab, the swab was then dipped and mixed thoroughly in 10ml of 0.1 per cent peptone water (HiMedia Laboratories Pvt. Ltd., Mumbai). Appropriate dilutions were made from this and plated using PCA and incubated at 37°C for 24h. To simulate the industrial CIP conditions in laboratory, a standard CIP regimen of 5min cold water rinse, a 10min wash with NaOH at 65°C, a 2min cold water rinse, a 10min wash with nitric acid ($HNO_{3)}$ at 65°C, and a final 5min cold water rinse was selected (Dufour *et al.*,2004). Two of the remaining three strips were used for assessing the effect of two different concentrations (0.5 and 1 per cent) of CIP solutions (NaOH and HNO_3) (Bremer *et al.,* 2006). For this, each of the strips were placed into 250ml bottles, the bottles were filled manually with 100ml each of the solutions mentioned in the CIP regimen in a sequential manner and simultaneously incubated in a shaking incubator at 250rpm for the time period specified for each of the solutions (Ostrov *et al.,* 2019a). The fourth strip was also treated in the same way except that the CIP solutions were replaced with sterile water in order to serve as the control. After the CIP procedure, swabs were taken from one side of each of these three SS strips, dipped and mixed well in 10ml of 0.1 per cent sterile peptone water. Appropriate dilutions were made from this and pour plated using PCA and incubated at 37°C for 48h. The colonies developed were counted and the result was expressed as CFU/cm^2 of the stainless steel strip.

3.10 STATISTICAL ANALYSIS

For statistical analysis, all the counts were transformed to log CFU/ml and analysed using SPSS, version 26 (IBM, New York, US). The SPC, PC, TC and TPC of the samples were subjected to correlation coefficient analysis. Summary statistics and cumulative frequency distribution of the counts were analysed. The pasteurized milk sample counts were subjected to one way ANOVA and correlation coefficient analysis to find significant differences in between the brands and the relationship between the counts respectively. All the experiments were performed in triplicates unless otherwise mentioned.

Results

4. RESULTS

4.1 DETAILS OF SAMPLES COLLECTED

The 57 samples collected for this study were constituted by 42 pasteurized milk samples belonging to seven different brands, two samples each of ice cream and raw milk, six equipment swabs, one sample of butter and four samples of dahi.

4.2 MICROBIOLOGICAL ANALYSIS

Standard plate, thermoduric, psychrotrophic and pasteurization surviving (thermoduric) psychrotrophic counts of the samples are summarised in Table 2. Most of the samples exhibited thermoduric and psychrotrophic counts while the pasteurization surviving psychrotrophic organisms were found in six samples of the pasteurized milk only. Thermoduric organisms were present in all the pasteurized milk samples but absent in the butter and ice cream samples tested. Psychrotrophic organisms were not detected in nine out of the 57 samples of which 5 samples were pasteurized milk.

Table 2. Standard plate, thermoduric, psychrotrophic and pasteurization surviving (thermoduric) psychrotrophic counts (Log CFU/ml) of the samples.

No.	**Sample**	**SPC**	**Psychrotrophic count (PC)**	**Thermoduric count (TC)**	**Thermoduric psychrotroph Count(TPC)**
1.	RM1	8.04	5.95	4.00	0.00
2.	RM2	8.18	5.52	3.78	0.00
3.	Sw1	9.95	1.08	2.00	0.00
4.	Sw2	9.26	0.00	2.95	0.00
5.	Sw3	9.36	1.48	3.86	0.00
6.	Sw4	10.75	1.34	1.60	0.00
7.	Sw5	6.32	0.00	3.08	0.00
8.	Sw6	7.56	1.30	2.30	0.00
9.	Bu-A1	2.40	0.00	0.00	0.00

Table 2 continued

No.	Sample	SPC	Psychrotrophic count (PC)	Thermoduric count (TC)	Thermoduric psychrotroph Count(TPC)
10.	Da-B1		3.89	4.38	0.00
11.	Da-C1		2.08	3.95	0.00
12.	Da-D1		1.48	2.04	0.00
13.	Da-E1		2.30	3.28	0.00
14.	IC-F1	3.30	0.00	0.00	0.00
15.	IC-G1	3.65	1.90	0.00	0.00
16.	PM-H1	3.78	0.00	2.08	0.00
17.	PM-H2	3.90	2.26	2.00	0.00
18.	PM-H3	4.23	2.43	3.51	0.30
19.	PM-H4	2.30	0.00	1.70	0.00
20.	PM-H5	4.04	1.40	3.20	0.00
21.	PM-H6	3.88	2.16	2.41	0.00
22.	PM-H7	4.04	2.64	3.50	0.00
23.	PM-H8	3.70	0.00	2.20	0.00
24.	PM-I1	6.11	4.08	4.32	0.00
25.	PM-I2	5.48	4.28	2.95	1.08
26.	PM-I3	7.52	5.54	3.46	2.45
27.	PM-I4	6.11	4.20	3.46	0.00
28.	PM-I5	6.51	4.32	3.68	1.78
29.	PM-I6	5.61	3.89	2.51	0.00
30.	PM-I7	5.46	4.56	3.62	0.00
31.	PM-I8	6.26	3.38	3.27	0.00
32.	PM-J1	3.30	0.00	2.48	0.00
33.	PM-J2	4.15	0.00	2.00	0.00

Table 2 continued

No.	Sample	SPC	Psychrotrophic count (PC)	Thermoduric count (TC)	Thermoduric psychrotroph Count(TPC)
34.	PM-J3	4.61	2.58	2.60	0.00
35.	PM-J4	4.58	2.83	2.00	0.00
36.	PM-J5	3.93	2.28	2.90	0.00
37.	PM-J6	4.28	2.73	2.43	0.00
38.	PM-J7	4.41	3.15	1.90	1.83
39.	PM-J8	4.15	2.90	2.64	0.00
40.	PM-K1	3.48	2.30	2.20	0.00
41.	PM-K2	3.90	2.52	3.28	0.00
42.	PM-K3	4.32	2.32	3.53	0.00
43.	PM-K4	5.57	3.51	3.32	0.00
44.	PM-K5	4.28	2.53	3.04	0.00
45.	PM-K6	4.11	2.24	2.53	0.00
46.	PM-K7	5.49	3.46	3.08	0.00
47.	PM-K8	4.32	2.23	2.52	0.00
48.	PM-L1	3.78	2.41	2.32	0.00
49.	PM-L2	3.85	2.26	3.51	0.00
50.	PM-L3	6.37	2.78	3.58	0.00
51.	PM-L4	4.68	3.28	3.76	0.00
52.	PM-L5	4.58	2.26	3.36	0.00
53.	PM-L6	5.26	4.08	3.40	2.76
54.	PM-L7	4.15	2.92	2.45	0.00
55.	PM-L8	5.16	2.52	3.38	0.00
56.	PM-M1	5.45	3.56	3.85	0.00
57.	PM-N1	5.32	3.27	2.56	0.00

Summary statistics and cumulative frequency distribution of the pasteurized milk sample data are shown in Table 3. Out of the 42 pasteurized milk samples tested, standard plate counts (SPC, aerobic plate counts) of 19 samples were found to be higher than the 'm' value of 30,000 CFU/ml (4.5 log CFU/ml) in which 15 samples were higher than the 'M' value of 50,000 log CFU/ml (4.7 log CFU/ml), i.e. 35.7 per cent of the pasteurized milk samples tested were not meeting the FSSAI stipulated aerobic plate count standard. Ninety percentile of the pasteurized milk samples were found devoid of pasteurization surviving psychrotrophic organisms whereas 90 percentile of the samples exhibited psychrotrophic count (Table 3).

In order to identify the brands having increased bacterial counts, it was defined using values corresponding to 75^{th} percentile of their frequency distribution as the threshold values. Only one brand, namely brand 'I' had its mean count higher than the thresholds (SPC $\geq$ 5.47, PC $\geq$ 3.47,TC $\geq$ 3.47 and TPC $\geq$ 0) in the case of SPC and PC, none of the brands exhibited TC counts beyond the threshold and all the brands except brand K showed TPC counts beyond the threshold value (Table 4).

The statistical analysis also revealed significant differences ($p \leq 0.01$) in between the mean values of standard plate, psychrotrophic and thermoduric counts of the different brands of pasteurized milk samples tested. However there were no significant ($p > 0.01$) differences between the thermoduric psychrotrophic counts of the different brands. The brand 'I' which gave thermoduric psychrotrophic count in three out of the eight samples tested, was found to have the highest SPC, TC PC and TPC which were found to be significantly higher ($p \leq 0.01$) than that of the other brands.

Upon analysing correlation coefficients of the whole data (n=57) as well as pasteurized milk samples alone (n=42) positive correlation coefficients were found between SPC & TC, SPC & PC and PC & TC (Table 5). Additionally, in the case of pasteurized milk there was positive correlation between SPC and TPC also. However, on analysing the brand wise data, high positive correlation for both PC and TC with SPC was evident only in the brand 'H' (Table 6).

Table 3. Summary statistics and cumulative frequency distribution of Standard Plate Count (SPC), Psychrotrophic count (PC), Thermoduric count (TC), Thermoduric psychrotrophic count (TPC) (Log CFU/ml) of pasteurized milk samples

Item	**SPC**	**PC**	**TC**	**TPC**
Percentile				
10	3.72	0.00	2.00	0.00
20	3.89	2.24	2.27	0.00
25	3.96	2.26	2.43	0.00
30	4.04	2.28	2.48	0.00
40	4.17	2.45	2.57	0.00
50	4.32	2.61	3.00	0.00
60	4.60	2.89	3.28	0.00
70	5.33	3.29	3.41	0.00
75	5.47	3.47	3.47	0.00
80	5.52	3.69	3.51	0.00
90	6.22	4.26	3.66	1.57
N	42	42	42	42
Mean	4.67	2.66	2.91	0.24
SD	1.03	1.28	0.64	0.67
Minimum	2.30	0.00	1.70	0.00
Maximum	7.52	5.54	4.32	2.76

Table 4. Brand wise mean values of the microbiological parameters of pasteurized milk samples

Brand		**SPC**	**PC**	**TC**	**TPC**
H	Mean	3.73^{c}	1.36^{c}	2.57^{ab}	0.037^{a}
	SD	0.6	1.18	0.72	0.1
I	Mean	6.13^{a}	4.28^{a}	3.40^{a}	0.66^{a}
	SD	0.67	0.61	0.53	0.98
J	Mean	4.17^{ab}	2.05^{ab}	2.36^{c}	0.22^{a}
	SD	0.42	1.29	0.36	0.64
K	Mean	4.43^{ab}	2.63^{b}	2.93^{abc}	0^{a}
	SD	0.73	0.53	0.46	0
L	Mean	4.72^{b}	2.81^{b}	3.22^{bc}	0.34^{a}
	SD	0.86	0.62	0.53	0.97
Total	Mean	4.64	2.63	2.90	0.25
	SD	1.04	1.3	0.64	0.69
F value		14.331^{*}	11.376^{*}	5.225^{*}	1.224^{ns}
Sig		0	0	0.002	0.319

*Statistically significant ($p \leq 0.01$)

$^{a\text{-}e}$ Means with different superscripts vary significantly within the column

Table 5. Correlation coefficients between SPC and TC, PC and TPC for all the samples tested

Samples		**TC**	**PC**	**TPC**
n=57	SPC	0.489*	0.463*	0.176
	TC		0.583*	0.122
	PC			0.373*
Pasteurized milk (n=42)	SPC	0.577*	0.838*	0.327*
	TC		0.530*	0.161
	PC			0.424*

*Statistically significant ($p \leq 0.05$)

Table 6. Correlation coefficients between SPC, TC, PC and TPC of different brands of pasteurized milk samples tested

Brand		**TC**	**PC**	**TPC**
H	SPC	0.826*	0.834*	0.581
	TC		0.659	0.577
	PC			0.423
I	SPC	0.247	0.108	0.549
	TC		0.359	0.629
	PC			0.682
J	SPC	-0.392	0.536	0.249
	TC		-0.259	-0.581
	PC			0.581
K	SPC	0.527	0.515	-
	TC		0.619	-
	PC			-
L	SPC	0.571	0.551	0.412
	TC		0.323	0.082
	PC			0.581

*Statistically significant ($p \leq 0.05$)

4.3 ISOLATION OF PASTEURIZATION SURVIVING PSYCHROTROPHIC BACTERIA

All the six TPC Petri-dishes yielded only one type of colony, which were different from each other, resulting into the isolation of six thermoduric psychrotrophic isolates. The isolates thus obtained were labelled Is1, Is2, Is3, Is4,

Is5 and Is6. The colony morphologies of these colonies on nutrient agar noted in terms of form, elevation, margin, pigmentation and size are shown in Table 7.

Table 7. Colony morphology of the isolates

Isolate	Source	Form	Elevation	Margin	Pigmentation	Size
Is1	Pasteurized milk (H)	Circular	Convex	Entire	Milky	Pinpoint
Is2	Pasteurized milk (L)	Circular	Flat	Undulate	Milky	Pinpoint
Is3	Pasteurized milk (J)	Circular	Flat	Entire	Opaque	Pinpoint
Is4	Pasteurized milk (I)	Circular	Flat	Entire	Milky	Pinpoint
Is5	Pasteurized milk (I)	Circular/ spindle	Raised	Entire	Milky	Pinpoint
Is6	Pasteurized milk (I)	Circular/ spindle	Raised	Entire	Milky	Pinpoint

4.4 IDENTIFICATION OF THE ISOLATES

4.4.1 Preliminary Identification Tests

The results of preliminary identification tests; Gram's staining, catalase test and oxidase tests are shown in Table 8.

Table 8. Gram's staining, catalase and oxidase test results of the isolates

Sl No.	Isolate	Gram's staining	Catalase	Oxidase
1	Is1	Gram negative	Positive	Positive
2	Is2	Gram negative	Positive	Positive
3	Is3	Gram negative	Positive	Positive
4	Is4	Gram positive	Positive	Negative
5	Is5	Gram positive	Negative	Negative
6	Is6	Gram positive	Negative	Negative

After the preliminary identification studies, it was decided to proceed further with two isolates each from the Gram positive and Gram negative category and thus the isolates Is1, Is2 from Gram negative isolates and Is5, Is6 from Gram positive isolates were selected for genomic level identification and further studies.

4.4.2 Genotypic Identification of The Isolates

The isolates Is1, Is2 were identified genotypically as *Aeromonas caviae*, *Moraxella osloensis* respectively and Is5, Is6 as different strains of the species *Carnobacterium maltaromaticum*. The nucleotide sequences were deposited in the NCBI database under accession numbers MT071634, MT158663, MT158664 and MT158665 as *Aeromonas caviae* DMV01, *Moraxella osloensis* DMV03, *Carnobacterium maltaromaticum* DMV05 and *Carnobacterium maltaromaticum* DMV06 respectively (Table 9).

Table 9. Accession numbers and 16s rRNA partial sequences of the isolates

SL.No	**Isolate**	**Identified as**	**Strain and Accession Number**	**16s rRNA Partial sequence**
1	Is1	*Aeromonas caviae*	DMV01 MT071634	CTTGCTACTTTTGCCGGCGA GCGGCGGACGGGTGAGTAAT GCCTGGGAAATTGCCCAGTC GAGGGGGATAACAGTTGGA AACGACTGCTAATACCGCAT ACGCCCTACGGGGGAAAGCA GGGGACCTTCGGGCCTTGCG CGATTGGATATGCCCAGGTG GGATTAGCTAGTTGGTGAGG TAATGGCTCACCAAGGCGAC GATCCCTAGCTGGTCTGAGA GGATGATCAGCCACACTGGA ACTGAGACACGGTCCAGACT

				CCTACGGGAGGCAGCAGTGG GGAATATTGCACAATGGGGG AAACCCTGATGCAGCCATGC CGCGTGTGTGAAGAAGGCCT TCGGGTTGTAAAGCACTTTC ATCGAGGAGGAAAGGTCACT AGCTAAGATCTGCTGGCTGT GACGTT
2	Is2	*Moraxella osloensis*	DMV03 MT158663	TGGCGGACGGGTGAGTAACA TTTAGGAATCTGCCTAGTAG TGGGGGATAGCTCGGGGAAA CTCGAATTAATACCGCATAC GACCTACGGGTGAAAGGGG GCGCAAGCTCTTGCTATTAG ATGAGCCTAAATCAGATTAG CTAGTTGGTGGGGTAAAGGC CCACCAAGGCGACGATCTGT AACTGGTCTGAGAGGATGAT CAGTCACACCGGAACTGAGA CACGGTCCGGACTCCTACGG GAGGCAGCAGTGGGGAATAT TGGACAATGGGGGCAACCCT GATCCAGCCATGCCGCGTGT GTGAAGAAGGCCTTTTGGTT GTAAAGCACTTTAAGCAGGG AGGAGAGGCTAATGGTTAAT ACCCATTAGATTAGACGTTA CCTGCAGAATAAGCACCGGC TAACTCTGTGCCAGCAGCCG CGGTAATACAGAGGGTGCGA GCGTTAATCGGAATTACTGG GCGTAAAGCGAGTGTAGGTG GCTCATTAAGTCACATGTGA AATCCCCGGGCTTAACCTGG GAACTGCATGTGATACTGGT GGTGCTAGAATATGTGAGAG

				GGAAGTAGAATTCCAGGTGT AGCGGTGAAATGCGTAGAGA TCTGGAGGAA
3	Is5	*Carnobacterium maltaromaticum*	DMV05 MT158664	AGTGAGTGGCGGACGGGTGA GTAACACGTGGGTAACCTGC CCATTAGAGGGGGATAACAT TCGGAAACGGATGCTAATAC CGCATAGTTTCAGGAATCGC ATGATTCTTGAAGGAAAGGT GGCTTCGGCTACCACTAATG GATGGACCCGCGGCGTATTA GCTAGTTGGTGAGGTAATGG CTCACCAAGGCAATGATACG TAGCCGACCTGAGAGGGTGA TCGGCCACACTGGGACTGAG ACACGGCCCAGACTCCTACG GGAGGCAGCAGTAGGGAAT CTTCCGCAATGGACGAAAGT CTGACGGAGCAACGCCGCGT GAGTGAAGAAGGTTTTCGGA TCGTAAAACTCTGTTGTTAA AGAAGAACAAGGATGAGAG TAACTGCTCATCCCCTGACG G
4	Is6	*Carnobacterium maltaromaticum*	DMV06 MT158665	TGCTCTTTACCAAGTGAGTG GCGGACGGGTGAGTAACACG TGGGTAACCTGCCCATTAGA GGGGGATAACATTCGGAAAC GGATGCTAATACCGCATAGT TTCAGGAATCGCATGATTCT TGAAGGAAAGGTGGCTTCGG CTACCACTAATGGATGGACC CGCGGCGTATTAGCTAGTTG GTGAGGTAATGGCTCACCAA GGCAATGATACGTAGCCGAC CTGAGAGGGTGATCGGCCAC

				ACTGGGACTGAGACACGGCC CAGACTCCTACGGGAGGCAG CAGTAGGGAATCTTCCGCAA TGGACGAAAGTCTGACGGAG CAACGCCGCGTGAGTGAAGA AGGTTTTCGGATCGTAAAAC TCTGTTGTTAAAGAAGAACA AGGATGAGAGTAACTGCTCA TCCCCTGACG

Phylogenetic trees constructed with the help of MEGA software, using neighbour joining method are shown as Figures 1-4.

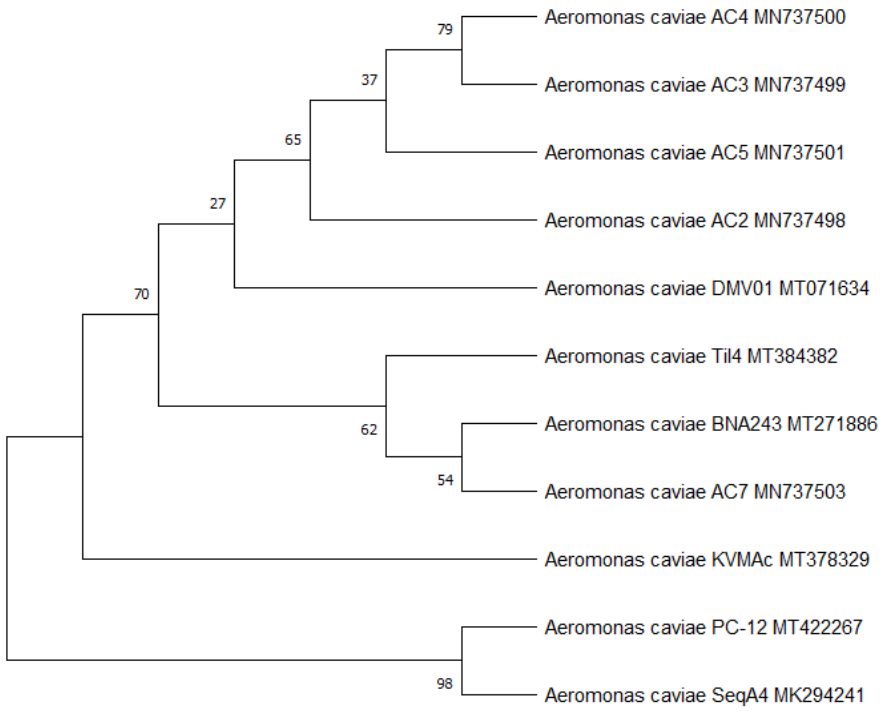


Fig. 1. Phylogenetic tree of *Aeromonas caviae* DMV01 (MT071634)

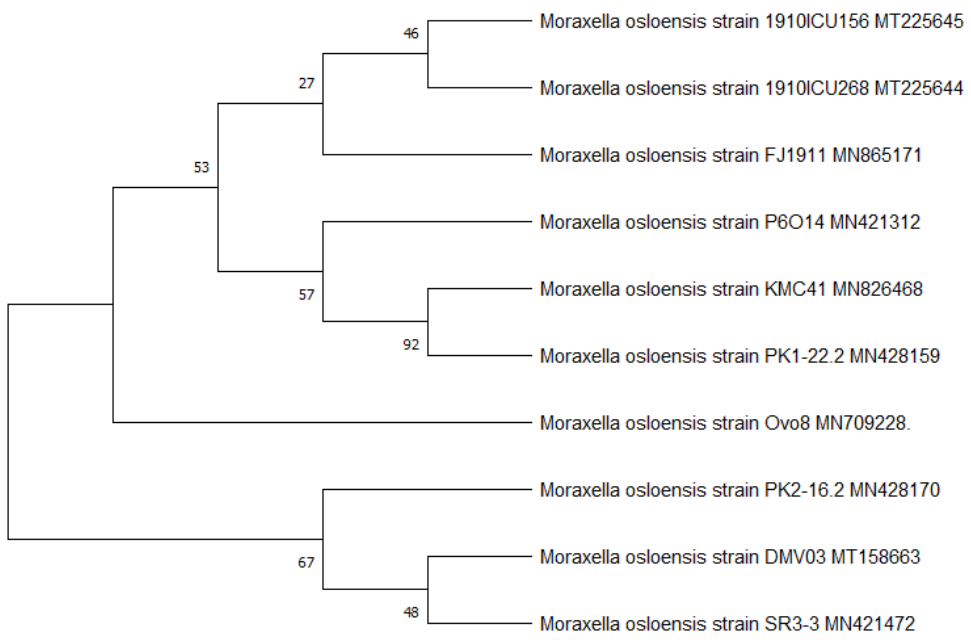


Fig. 2. Phylogenetic tree of *Moraxella osloensis* DMV03 (MT158663)

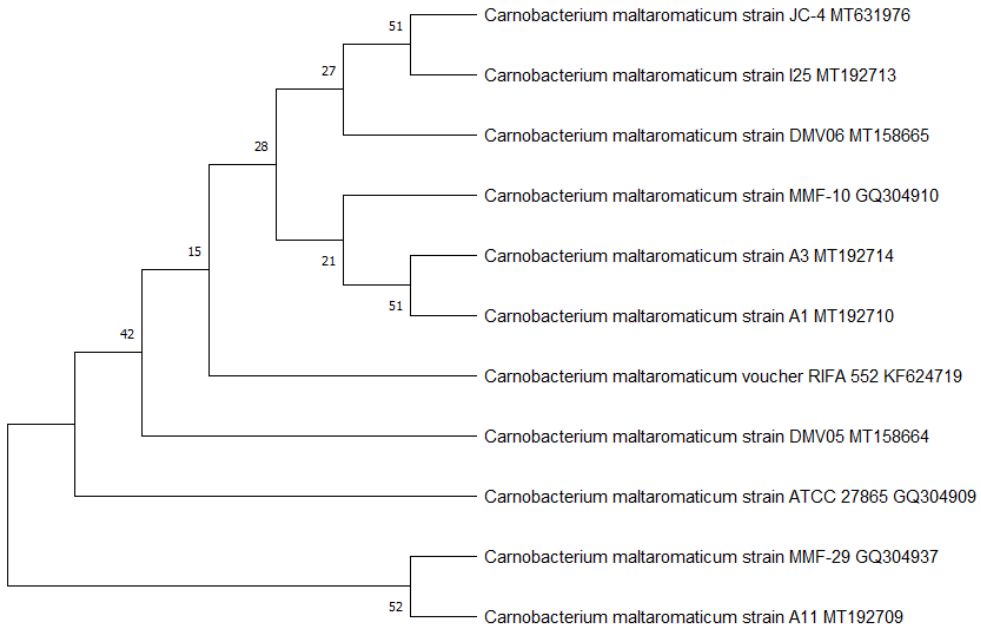


Fig. 3. Phylogenetic tree of *Carnobacterium maltaromaticum* DMV05 (MT158664) and DMV06 (MT158665)

4.4.3 SEM Imaging of the Isolates

Scanning electron and bright field microscopy images of the isolates are shown in the Fig.4. All the isolates were found to be rod shaped.

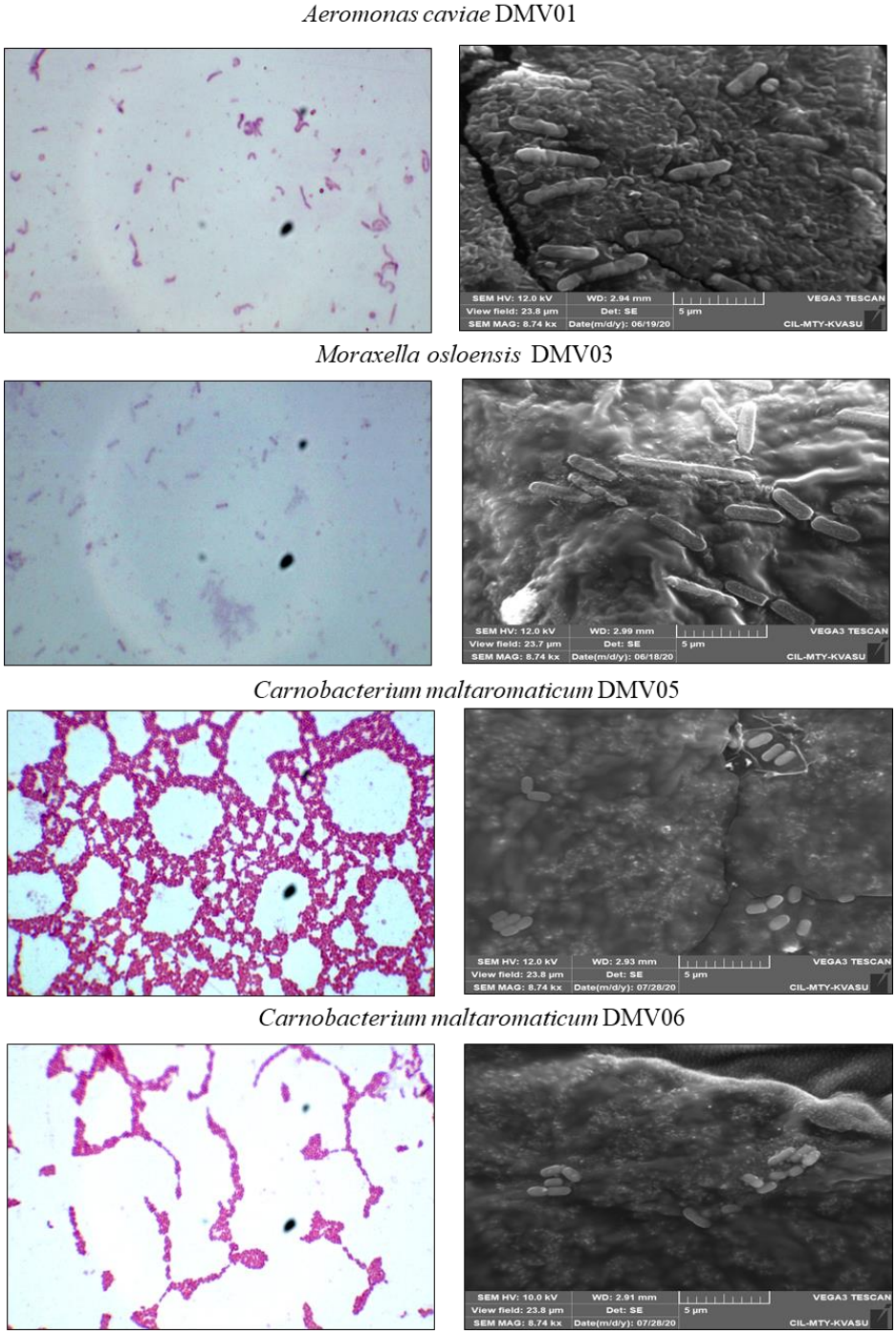


Fig. 4. Bright field and SEM images of the isolates

4.5 CHARACTERIZATION OF THE ISOLATES

4.5.1 Assessment of Growth at Different Temperatures

Ability of the isolates to grow at different temperatures was visually assessed and depending upon the extent of growth, the optimum temperature for each of the isolates was decided. Two isolates, namely *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 exhibited highest growth at 37°C and the other two isolates, the two strains of *Carnobacterium maltaromaticum* exhibited highest growth at 20°C (Table 10). In agreement with their mesophilic optimum growth range all the isolates took longer time to exhibit visually perceivable growth at 7°C (a minimum of two days to achieve 0.5 McFarland turbidity).

Table 10. Growth of the isolates at different temperatures

Temperature	***Aeromonas caviae* DMV01**	***Moraxella osloensis* DMV03**	***Carnobacterium maltaromaticum* DMV05**	***Carnobacterium maltaromaticum* DMV06**
7°C	-	-	-	-
20°C	+	+	+++	+++
Room temperature (27°C)	+++	++	++	++
37°C	+++	+++	+	+
Time taken to achieve 0.5 McFarland turbidity at 7°C	2 days	3 days	2 days	2 days
Time temperature combinations decided for the isolates attain 0.5 McFarland turbidity	2.5h at 37°C	16h at 37°C	24h at 20°C	24h at 20°C

- No growth, + Low, ++ Medium, +++ Good

4.5.2 Motility of the Isolates

4.5.2.1 Semi Solid agar –TTC Method

Among the isolates, only *Aeromonas caviae* DMV01 was found growing away from the line of stab and thus motile (Fig.5).

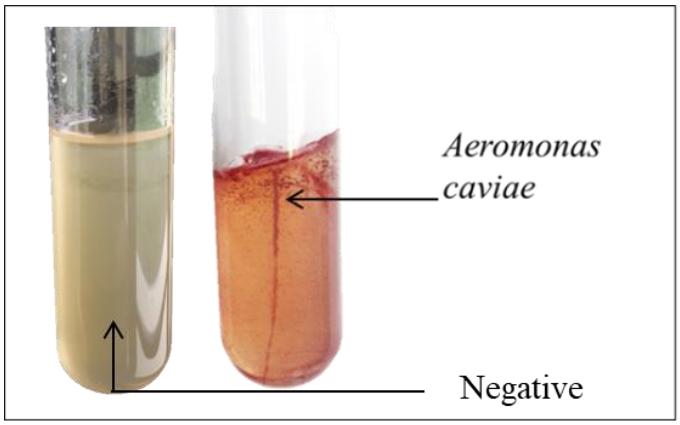


Fig. 5. Motility test of the isolate

4.5.3 Assessment of Haemolytic Activity

On assessing the haemolytic activity of the isolates on blood agar it was observed that *Aeromonas caviae* DMV01 (Is1) exhibited α-haemolysis and all the other isolates exhibited γ-haemolysis (Fig.6).

Fig. 6. Haemolytic activity of the isolates on blood agar; Is1 (*Aeromonas caviae* DMV01), Is2 (*Moraxella osloensis* DMV03), Is5 (*Carnobacterium maltaromaticum* DMV05), Is6 (*Carnobacterium maltaromaticum* DMV06).

4.5.4 Biofilm Forming Ability of the Isolates

4.5.4.1 Congo Red Agar Method

Aeromonas caviae DMV01 and *Carnobacterium maltaromaticum* DMV05 formed black colonies on Congo red agar indicating their biofilm formation potential. The other two isolates were found to be non-biofilm formers (Fig.7).

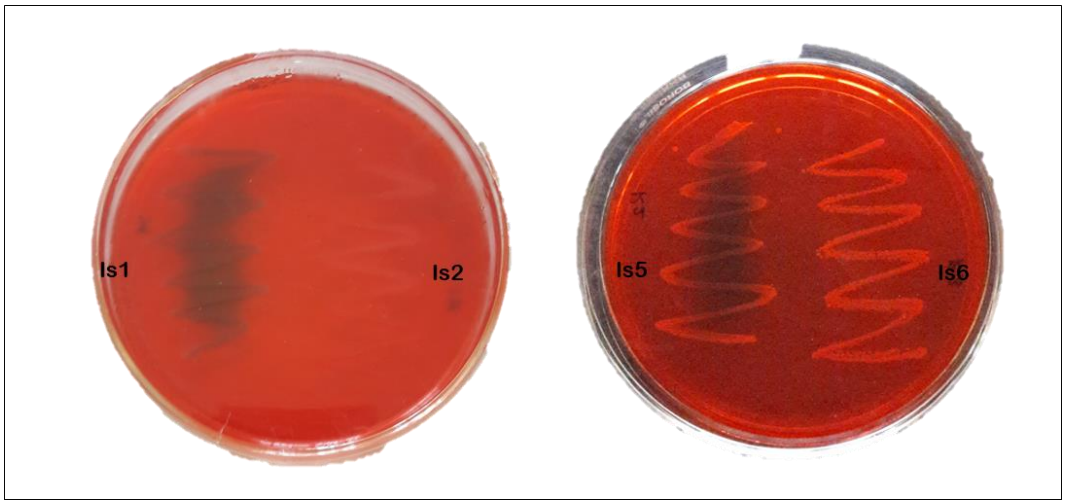


Fig. 7. Growth of the isolates on Congo red agar plates; Is1 (*Aeromonas caviae* DMV01), Is2 (*Moraxella osloensis* DMV03), Is5 (*Carnobacterium maltaromaticum* DMV05), Is6 (*Carnobacterium maltaromaticum* DMV06).

4.5.4.2 Tube Method

On assessing the retention of violet colour on the sides and bottom of the test tubes *Aeromonas caviae* DMV01 exhibited the highest retention followed by *Carnobacterium maltaromaticum* DMV05 (Fig.8) substantiating the observations obtained in the Congo red agar method.

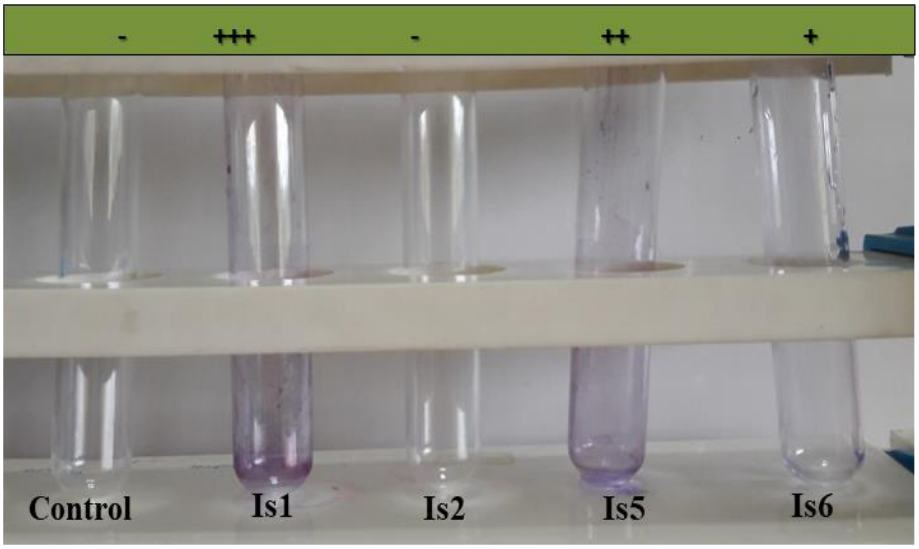


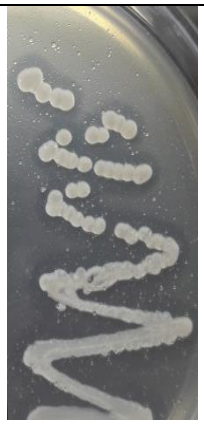
Fig. 8. Tube method for assessment of biofilm formation potential.

4.6 ASSESSMENT OF SPOILAGE POTENTIAL

4.6.1 Enzymatic Activities

On incubation at 37°C, both the strains of *Carnobacterium maltaromaticum* exhibited proteolytic activity on skim milk agar whereas lipolytic activity was exhibited by *Aeromonas caviae* only. *Moraxella osloensis* DMV03 did not exhibit any of these activities. However, all the isolates failed to exhibit any proteolytic activity at 7°C (Table 11).

Table 11. Enzymatic activities of the isolates*

Sl No.	**Isolate**	**Proteolytic activity at 37°C**	**Lipolytic activity at 37°C**	**Proteolytic activity at 7°C**
1	*Aeromonas caviae* DMV01	 No	 Yes	No
2	*Moraxella osloensis* DMV03	 No	 No	No

Sl No.	Isolate	Proteolytic activity at 37°C	Lipolytic activity at 37°C	Proteolytic activity at 7°C
3	*Carnobacterium maltaromaticum* DMV05	 Yes	 No	No
4	*Carnobacterium maltaromaticum* DMV06	 Yes	 No	No

4.6.2 Hydrogen Sulphide Production

All the isolates failed to produce hydrogen sulphide, an indicator of deteriorative nature (Table 12).

4.6.3 Gelatin Liquefaction

All the isolates failed to liquefy gelatin disclosing the inability of the isolates to produce extra cellular proteolytic enzymes called gelatinases (Table 12).

4.6.4 Evaluation of Defect Development

Ability of the isolates to form defects in milk was assessed at refrigerated (7°C) and room (27°C) temperatures. On room temperature storage, *Aeromonas caviae* DMV01 and both the *Carnobacterium maltaromaticum* isolates were found to cause visually detectable defects within 24h and 48h of inoculation respectively. However under the same conditions, *Moraxella osloensis* DMV03 elicited such defect only on the 7^{th} day of storage. It is worth noting that as in the case of biofilm formation, the difference in between the *Carnobacterium maltaromaticum* strains was clearly evident in their pH lowering ability at room and refrigerated conditions. At 7°C storage, isolates didn't elicit any visible defects in the milk even after seven days of storage (Table 13); however the refrigerated samples inoculated with *A. caviae* and *C. maltaromaticum* DMV05 gave positive clot on boiling test after seven days.

Table 12. Hydrogen sulphide production, gelatin liquefaction and details of defect development

Attributes	***Aeromonas caviae* DMV01**	***Moraxella osloensis* DMV03**	***Carnobacterium maltaromaticum* DMV05**	***Carnobacterium maltaromaticum* DMV06**
Hydrogen sulphide production	-	-	-	-
Gelatin liquefaction	-	-	-	-

Table 12 continued

Attributes	***Aeromonas caviae* DMV01**	***Moraxella osloensis* DMV03**	***Carnobacterium maltaromaticum* DMV05**	***Carnobacterium maltaromaticum* DMV06**
pH changes noticed for 7 days.				
Day 0	6.6±0.05	6.6±0.05	6.6±0.05	6.6±0.05
Day1	4.9±0.1	6.6±0.05	5.8±0.15	6±0.11
Day 2	-	6.6±0.05	5±0.05	5.5±0.01
Day 3	-	6.4±0.05	-	-
Day 4	-	6.1±0.05	-	-
Day 5	-	6±0.11	-	-
Day 6	-	5.8±0.05	-	-
Day 7	-	5.6±0	-	-
Days taken for defect appearance	1	7	2	2
Defect Noticed	Settled as a mass with wheying off	Appearance of small curd particles	Curdling (uniform coagulum) with wheying off	Curdling (non-uniform coagulum) with wheying off
Appearance of spoiled samples				

*Values shown are Mean ± S.E. of three experiments

-: Negative, +: Positive

Table 13. Defect development in isolate inoculated milk stored at refrigerated temperature (7°C)

Isolate	**pH* of the product in refrigerated condition (7 °C) for seven days**							**Clot on boiling test on 7th day**
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	
Aeromonas caviae DMV01	6.6±0	6.6±0.05	6.4±0.05	6.4±0.05	6.3±0.1	6.1±0.1	6.0±0.05	+
Moraxella osloensis DMV03	6.6±0	6.6±0.00	6.6±0.05	6.5±0	6.5±0	6.5±0	6.4±0.05	-
Carnobacterium maltaromaticum DMV05	6.6±0	6.6±0.05	6.5±0.05	6.5±0.1	6.3±0.1	6.1±0.1	5.9±0.05	+
Carnobacterium maltaromaticum DMV06	6.6±0	6.6±0.05	6.5±0.05	6.5±0.1	6.4±0.05	6.2±0.1	6.1±0.05	-

-: Negative, +: Positive

4.7. ANTIBIOTIC SUSCEPTIBILITY

Upon assessing the response pattern of the isolates to various antibiotics selected based on the identity of isolates they were found to be susceptible to majority of the antibiotics tested (Tables 14 to 17). *Aeromonas caviae* was found

susceptible to six out of the eight antibiotics used. *Moraxella osloensis* exhibited sensitivity to seven of the eight antibiotics, the resistance being towards amoxyclav only. *Carnobacterium* isolates exhibited strain wise difference in their antibiotic response pattern also with the DMV05 strain being resistant and strain DMV06 being moderately resistant to streptomycin.

Table 14. Antibiotic response pattern of *Aeromonas caviae* DMV01

Sl No.	**Antibiotic**	**Zone of clearance (mm inclusive of disc diameter 6mm)** [#]	**Interpretation***
1	Amoxyclav	11±0.5	Resistant
2	Amoxycyllin	12±0.5	Resistant
3	Chloramphenicol	29±1.5	Sensitive
4	Ciprofloxacin	27±0.5	Sensitive
5	Enrofloxacin	24±0.5	Sensitive
6	Gentamycin	21±0.5	Sensitive
7	Streptomycin	21±0.5	Sensitive
8	Tetracycline	27±1.5	Sensitive

* CLSI standards, 2015

[#]Values shown are Mean ± S.E. of three experiments

Table 15. Antibiotic response pattern of *Moraxella osloensis* DMV03

Sl No.	**Antibiotic**	**Zone of clearance (mm inclusive of disc diameter 6mm)** [#]	**Interpretation***
1	Amoxyclav	10±0.5	Resistant
2	Cefixime	31±0.5	Sensitive

Table 15 continued

Sl No.	Antibiotic	Zone of clearance (mm inclusive of disc diameter 6mm) #	Interpretation*
3	Chloramphenicol	33±0.5	Sensitive
4	Ciprofloxacin	>40	Sensitive
5	Co-Trimoxazole	36±1.5	Sensitive
6	Erythromycin	33±0.5	Sensitive
7	Oflaxacin	>40	Sensitive
8	Tetracycline	>40	Sensitive

* CLSI standards, 2015

#Values shown are Mean ± S.E. of three experiments

Table 16. Antibiotic response pattern of *Carnobacterium maltaromaticum* DMV05

Sl No.	Antibiotic	Zone of clearance (mm inclusive of disc diameter 6mm) #	Interpretation*
1	Amoxyclav	24±0.5	Sensitive
2	Amoxycyllin	26±0.5	Sensitive
3	Chloramphenicol	29±0.5	Sensitive
4	Ciprofloxacin	39±1.5	Sensitive
5	Enrofloxacin	>40	Sensitive
6	Gentamycin	30±0.5	Sensitive
7	Streptomycin	15±0	Resistant
8	Tetracycline	>40	Sensitive

* Vlkova *et al.* (2006)

#Values shown are Mean ± S.E. of three experiments

Table 17. Antibiotic response pattern of *Carnobacterium maltaromaticum* DMV06

Sl No.	**Antibiotic**	**Zone of clearance (mm inclusive of disc diameter)** [#]	**Interpretation***
1	Amoxyclav	29±0.5	Sensitive
2	Amoxycyllin	33±0.5	Sensitive
3	Chloramphenicol	30±0.5	Sensitive
4	Ciprofloxacin	>40	Sensitive
5	Enrofloxacin	>40	Sensitive
6	Gentamycin	27±0.5	Sensitive
7	Streptomycin	16±0.5	Moderate
8	Tetracycline	39±1.5	Sensitive

* Vlkova *et al.* (2006)

[#]Values shown are Mean ± S.E. of three experiments

4.8 ASSESSMENT OF CROSS ADAPTATION POTENTIAL

Considering food safety aspects, it was decided to proceed further with the two Gram negative isolates namely *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03.

4.8.1 Heat Resistance at 63°C

Marked differences were observed in the thermal resistance pattern of the isolates. Though both of them could survive the LTLT pasteurization time temperature combination the log reduction exhibited by *Moraxella osloensis* (1.23) was far lower than that exhibited by *Aeromonas caviae* (6.65) (Table 18).

Decimal reduction time at the LTLT pasteurization temperature of 63°C was found to be 4 minutes 38 seconds for *Aeromonas caviae* DMV01 (Fig.9) and 25 minutes 18 seconds for *Moraxella osloensis* (Fig.10).

Table 18. Survivability of the isolates to different periods of exposure at 63°C

Time of exposure (Minutes)	***Aeromonas caviae* DMV01**		***Moraxella osloensis* DMV03**	
	Survivors (Log CFU/ml)*	**Log reduction**	**Survivors (Log CFU/ml)***	**Log reduction**
0	7.95±0.02	-	6.32±0.32	-
5	6.53±0.26	1.42	6.07±0.46	0.25
10	5.77±0.21	2.18	5.76±0.32	0.56
15	5.30±0.26	2.65	5.43±0.26	0.89
20	3.69±0.24	4.26	5.38±0.31	0.94
25	2.47±0.42	5.48	5.34±0.33	0.98
30	1.30±0.35	6.65	5.09±0.36	1.23

*Values shown are Mean ± S.E. of three experiments. Significantly different from initial Log CFU/ml at five per cent level ($p \leq 0.05$)

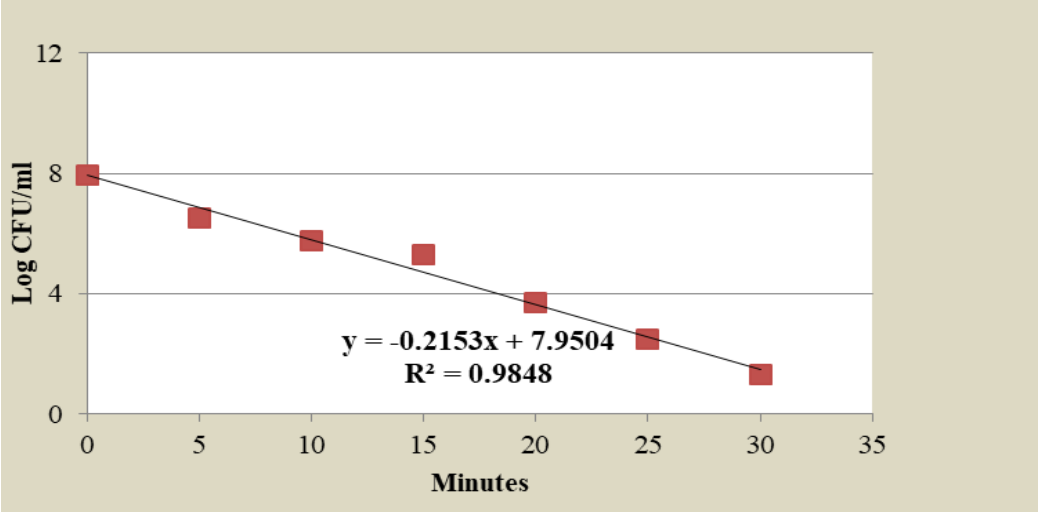


Fig. 9. D $_{63}$value determination graph of *Aeromonas caviae* DMV01

D $_{63}$value of *Aeromonas caviae* = -1/-0.2153 = 4 minutes 38 seconds

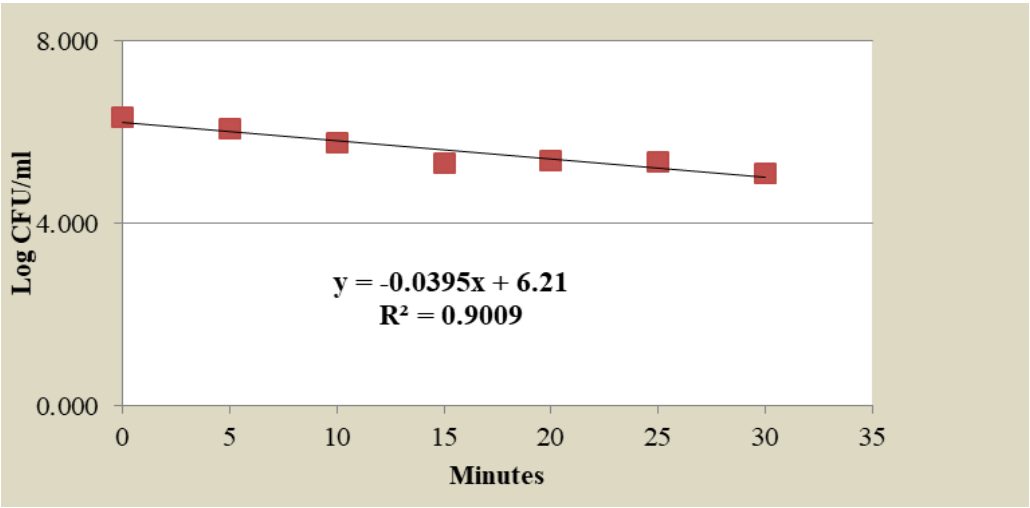


Fig. 10. D $_{63}$value determination graph of *Moraxella osloensis* DMV03

D $_{63}$value of *Moraxella osloensis* = -1/-0.0395 = 25 minutes 18 seconds

4.8.2 Resistance to Boiling Process

Both the isolates failed to survive the boiling point even for 1 second (Table 19).

Table 19. Survivability of the isolates to boiling process

Organism	Initial count (Log CFU/ml)	Final count (CFU/ml) after 1s	Final count (CFU/ml) after 5s
Aeromonas caviae DMV01	7.38	0	0
Moraxella osloensis DMV03	6.11	0	0

4.8.3 Resistance to pH

On assessing the resistance of the isolates at different pH levels, marked reduction in optical densities were observed (Table 20). While *Moraxella osloensis* failed to grow at and below a pH of 5.5 *Aeromonas caviae* could grow at 5.5 but not at 4.5 and below.

Table 20. Optical density (OD) of Growth at different pH levels

pH	***Aeromonas caviae*** DMV01		***Moraxella osloensis*** DMV03	
	OD*	**Grading**	**OD***	**Grading**
7.5	0.77±0.01	+++	0.82±0.03	+++
6.5	0.30±0.02	++	0.27±0.02	++
5.5	0.23±0.01	+	0.01±0	-
4.5	0.01±0	-	0.01±0	-
3.5	0.01±0	-	0.01±0	-

- No growth, + Poor growth, ++ Medium growth, +++ Good growth

*Results shown are average of two experiments

4.9 ATTRIBUTES CONTRIBUTING TOWARDS THEIR PRESENCE IN DAIRY ENVIRONMENT

4.9.1 Ability to Grow in Milk under Room and Refrigerated Storage

4.9.1.1 Qualitative Method -Methylene Blue Reduction Time

Aeromonas caviae DMV01 exhibited a methylene blue reduction time of 156.25±7.5min (2h 36min) whereas it was 362.5±4.33 min (6h 2min) for *Moraxella osloensis* DMV03 (Table 21).

Table 21. Methylene blue reduction time of *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03

Isolate	**Dye reduction time (Minutes)***
Aeromonas caviae	156.25±7.5
Moraxella osloensis	362.5±4.33

*Values shown are Mean ± S.E. of four experiments

4.9.1.2 Quantitative Method – Determination of Generation Time

Growth rate of the isolates was calculated by plotting $\log_{10}$ count of the organism against time. The graph revealed that the exponential phase of *Aeromonas caviae* in milk at room temperature (27°C) was for four hours (from the second hour to the sixth hour of incubation). In the same medium at 7°C the growth rate of the organism was lower and the exponential phase started from the fourth hour of incubation and extended up to 12^{th} hour. The growth rate and generation time of this organism at 27°C and 7°C were 0.05 generations/min, 22 minutes and 0.01 generations/min, 69 minutes respectively (Table 22). On using nutrient broth at 27°C as the growth medium, the growth rate became higher (0.06 generations/min) with shorter generation time (18 minutes 16 seconds) than that of milk and the exponential phase was from the second hour to fourth hour.

Moraxella osloensis also exhibited the same trend, a higher growth rate (0.027 generations/min) in milk at 27°C than at 7°C (0.01 generations/min, Table 21). The generation time was 37 minutes and 76 minutes respectively. On replacing broth as a growth medium, the growth rate and generation time were found to be 0.029 generations/min and 34 minutes respectively at room temperature (27°C).

It is interesting to note that *Aeromonas caviae* exhibited higher increment in microbial population(3.95 log CFU/ml) and count (10.15 log CFU/ml) in milk than in broth (3.29 and 9.88 log CFU/ml respectively) (Fig.11). But the reverse trend was observed in the case of *Moraxella osloensis* with increment in microbial population (5.01 log CFU/ml) and count (8.7 log CFU/ml) being higher in broth than in milk (4.36 log CFU/ml and 8.34 log CFU/ml respectively) (Fig.12).

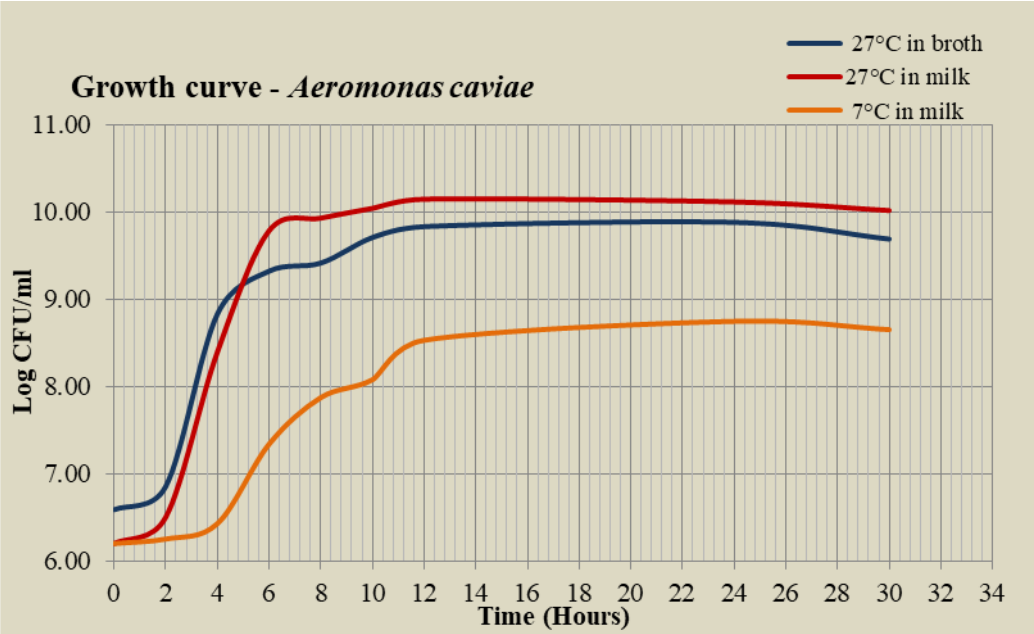


Fig. 11. Growth curve of *Aeromonas caviae* in milk at room (27°C) and refrigerated (7°C) and in broth at room temperatures. Counts shown are the average of three independent experiments

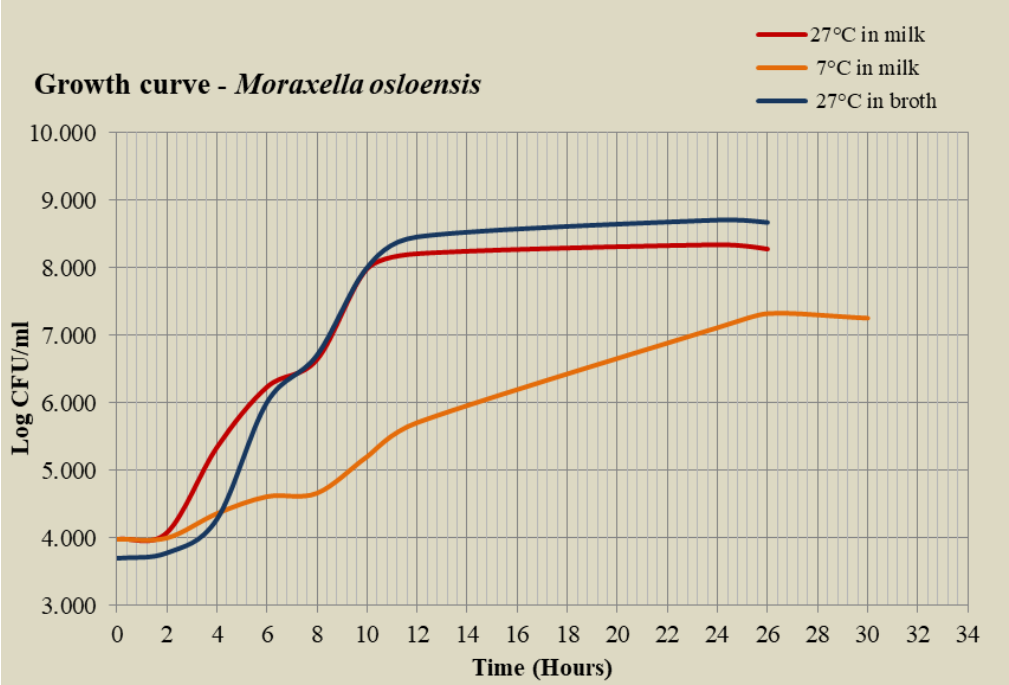


Fig. 12. Growth curve of *Moraxella osloensis* in milk at room (27°C) and refrigerated (7°C) and in broth at room temperatures. Counts shown are the average of three independent experiments

Table 22. Growth rate and generation time of *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 in milk at room (27°C) and refrigerated (7°C) and in broth at room temperature (27°C)

Parameter	***Aeromonas caviae* DMV01**			***Moraxella osloensis* DMV03**		
	Milk at 7°C	Milk at 27°C	Broth at 27°C	Milk at 7°C	Milk at 27°C	Broth at 27°C
Growth rate (Generations/min)	0.01	0.05	0.06	0.01	0.027	0.029
Generation Time (minutes)	69	22	18.27	76	37	34
Exponential growth period (h)	4^{th} -12^{th}	2^{nd} -6^{th}	2^{nd} -4^{th}	2^{nd} –26^{th}	2^{nd} -10^{th}	2^{nd} – 10^{th}

Table 22 continued.

Parameter	***Aeromonas caviae* DMV01**			***Moraxella osloensis* DMV03**		
	Milk at 7°C	Milk at 27°C	Broth at 27°C	Milk at 7°C	Milk at 27°C	Broth at 27°C
Lag period (h)	0 – 4th	0 – 2nd	0 - 2nd	0 – 2nd	0 – 2nd	0 – 2nd
Stationary phase starting hour	12th h	6th h	4th h	26th h	10th h	10th h

Among the two isolates assessed for their ability to grow in milk under room and refrigerated storage *A. caviae* DMV01 exhibited higher growth rate in milk and also exhibited biofilm forming potential. So it was decided to assess this isolate for its biofim formation potential and resistance to CIP procedures.

4.9.2 Biofilm Formation Potential

4.9.2.1 *Assessment of Swarming Motility*

Swarmer cells move in a group parallel to their long axis and maintain close contact with other cells. *Aeromonas caviae* showed clear surface movement on swarm agar plates. A consistent growth circle was visible on the swarm agar plates indicating the swarming motility of the isolate *Aeromonas caviae* (Fig.13).

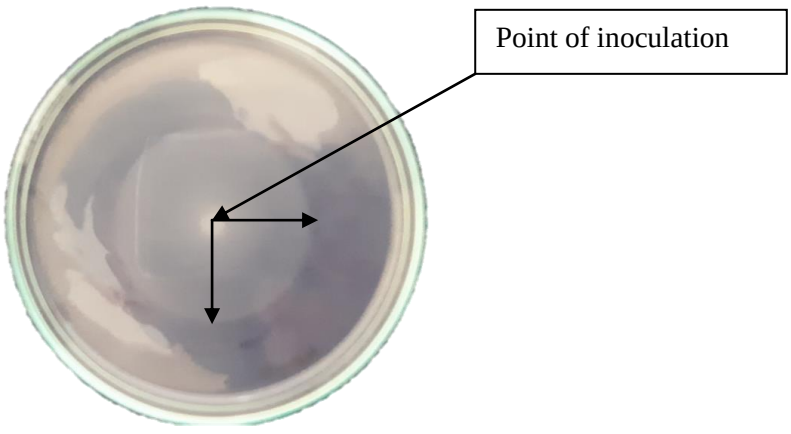


Fig.13. Growth around the axis from point of inoculation by the isolate *Aeromonas caviae* on swarm motility agar

4.9.2.2 Assessment of Biofilm Formation Potential of the Isolates on Glass Strips Using Scanning Electron Microscopy

High vacuum scanning electron microscopy at a magnification of 5000x revealed that *Aeromonas caviae* DMV01 is capable of forming biofilms on the glass surface (Fig. 14A). Numerous cells of *Aeromonas caviae* DMV01 appeared as a thick layer on the glass surface, whereas only a few cells were present in the case of *Moraxella osloensis*, the negative control (Fig. 14B). At higher magnification, the presence of some extracellular protrusions and slime like substances were visible in between the rod-shaped cells (Fig. 14C).

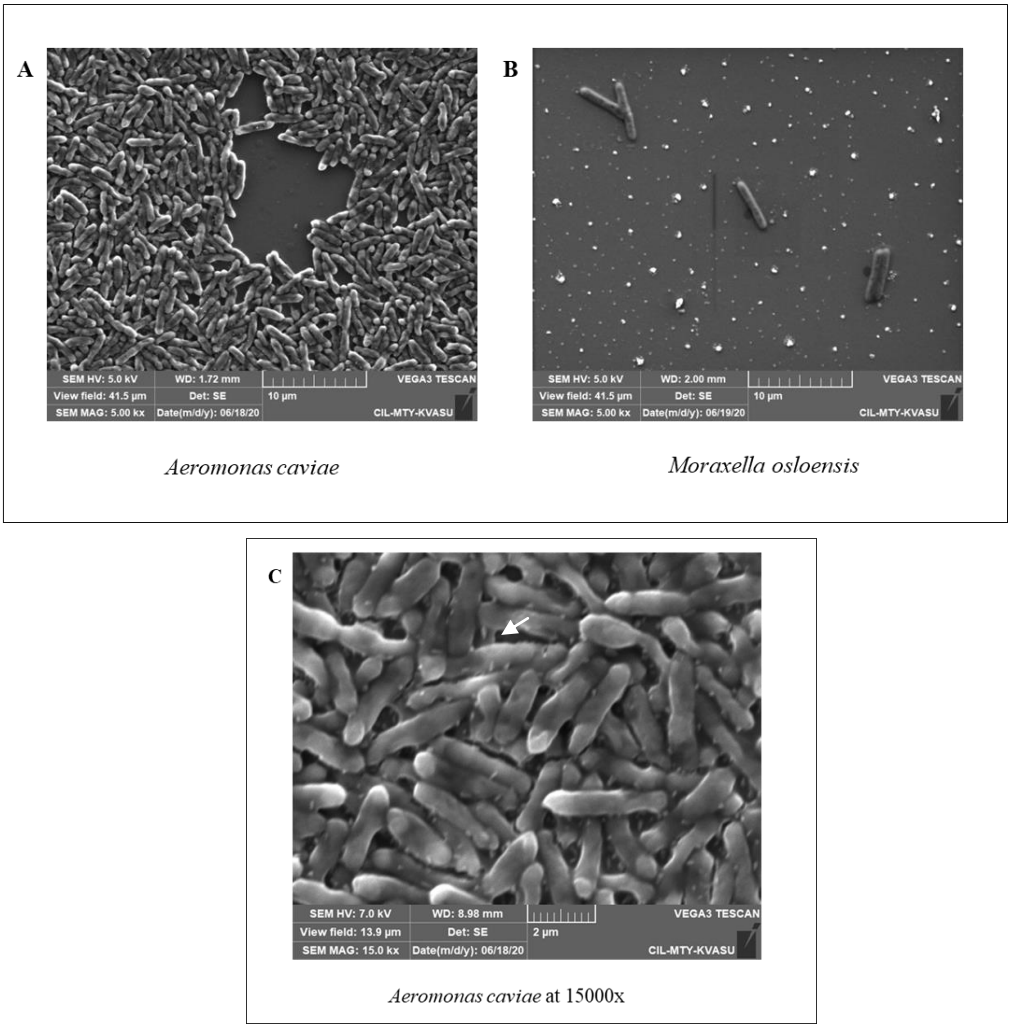


Fig. 14. SEM images (A) *Aeromonas caviae* on glass surface at 5000x (B) *Moraxella osloensis* on glass surface at 5000x (C) *Aeromonas caviae* on glass surface at 15000x

4.9.3 Resistance to Cleaning-in-place (CIP) Procedure

Despite its biofilm forming potential *Aeromonas caviae* DMV01 was found to be highly sensitive to the CIP regimen to the extent that use of CIP solutions at one per cent level resulted in complete removal whereas only very low number of cells remained when 0.5 per cent was used (a reduction of 3.2 log, Table 23). It is interesting to note that adoption of CIP regimen with sterile water alone without the CIP solutions resulted in 1.88 log reductions in the *Aeromonas caviae* DMV01 count.

Table 23. Count (Log CFU/cm^2) of biofilm forming *Aeromonas caviae* DMV01 on stainless steel after different treatments

	Prior to CIP	**Control (CIP with sterile water)**	**Treatment 1 (CIP regimen with 0.5 % CIP solutions)**	**Treatment 2 (CIP regimen with 1 % CIP solutions)**
Log CFU/cm^2	3.87±0.06	1.99±0.04	0.67±0.14	0

Values shown are Mean ± S.E. of three experiments

Discussion

5. DISCUSSION

5.1 MICROBIOLOGICAL ANALYSIS

Microbiological quality of pasteurized milk and milk products relies greatly on several factors like the quality of raw milk, time-temperature combination of pasteurization, process hygiene during filling and packing, post-process contamination, the temperature during transportation and storage (Ranieri *et al.,* 2009). To ensure the quality of milk reaching consumers, Food Safety and Standards Authority of India has stipulated a set of standards to be followed by food business operators. In this study, 57 samples collected from different sources comprising of pasteurized milk, raw milk, milk product (ice cream, butter and curd) samples and swab samples of dairy equipment (bulk milk silo, cream separator out, pasteurizer out's from two plants, valve before pasteurization and CIP water rinse valve) over three months period from September 2019 were assessed for in terms of standard plate, thermoduric, psychrotrophic and thermoduric psychrotrophic counts in an attempt to isolate pasteurization surviving psychrotrophic organisms. The mean values of SPC, PC, TC and TPC of the tested samples were found to be 5.19, 2.46, 2.80 and 0.18 (log CFU/ml) respectively (Table 2). It is interesting to note that despite the presence of thermoduric and psychrotrophic microorganisms in many of the samples tested, pasteurization surviving psychrotrophic organisms were found only in 14.3 per cent of the pasteurized milk samples. It can be derived that pasteurization and subsequent cold storage are effective hurdles in checking the microbial growth. With this clear observation that only a minute proportion of thermoduric organisms are able to grow at low temperatures, it can be inferred that the shelf life extension offered by cold storage is effective. Agreeing with the previous reports of *Bacillus* spp. as the predominant thermodurics in milk and milk products this study also observed prevalence of spreading type colonies indicative of *Bacillus* spp. in the TC Petri-dishes. Appearance of Gram positive rod shaped cells with spores on Gram staining of representative colonies very well substantiated this observation. *Bacillus* spp. is well known for their ability to

survive pasteurization and also being reported as thermoduric psychrotrophs in several studies (Johnston and Bruce, 1982; Meer *et al.,* 1991). Despite the presence of the large number of spreading type colonies in TC Petri-dishes, no such colonies developed on corresponding thermoduric psychrotrophic Petri-dishes indicating the absence of psychrotrophic *Bacillus* spp. in the tested samples.

The presence of thermoduric psychrotrophic organisms were found in 10.5 per cent of dairy samples and 14.3 per cent of pasteurized milk samples in the current study is much lower than the report of 40 per cent of the milk samples yielding thermoduric psychrotrophs by Ribeiro Júnior *et al*. (2017). An interesting observation is that five out of six samples which yielded thermoduric psychrotrophs had psychrotrophic counts higher than their thermoduric counts. The mean values of SPC, PC, TC and TPC of pasteurized milk samples were found to be 4.67±1.03, 2.66±1.28, 2.91±0.64 and 0.24±0.67 (log CFU/ml) (Table 3). Due to the resuscitation period provided the thermoduric psychrotrophic counts (TPC) obtained could be higher than the number actually present immediately after the pasteurization. But these counts give an idea on the extent of growth the thermoduric psychrotrophs can attain during refrigerated storage of pasteurized milk. Presence of large population of non-thermoduric organisms or the post pasteurization contaminants in the tested pasteurized samples calls for large scale attention towards addressing this issue through identification of their source of entry and implementation of corrective measures.

The FSSAI stipulated ‘m’ and ’M’ values of aerobic plate counts of pasteurized milk are 4.5 log CFU/ml ($3x10^4$ CFU/ml) and 4.7 log CFU/ml ($5x10^4$ CFU/ml, unsatisfactory) grading the product as of ‘satisfactory’ and ‘unsatisfactory’ quality respectively. Values in between ‘m’ and ‘M’ are considered as ‘marginally acceptable’ subjected to the sampling plan. Out of the 42 samples tested 23 (54.8%) were having counts lower than the stipulated ‘m’ value whereas for 15 (35.7 %) samples the counts were more than the ‘M’ value and for four samples it was in between ‘m’ and ‘M’. So 23 samples were of 'satisfactory' grade whereas 15 were 'unsatisfactory' in terms of aerobic plate

count. Non-conformance to the existing national standards is a major concern in the case of pasteurized milk all over the world. In concurrence with the observations of the current study pasteurized milk samples not meeting the stipulated national standards are reported in many other studies also (Breurec *et al.,* 2010; Kamana *et al.*, 2014; Koushki *et al.,*2016; Jamal *et al.,* 2018; Agarwal *et al.,* 2020). On contrast, some of the researchers (Chatterjee *et al.,* 2006; Banik *et al.,* 2014) reported strict adherence of market samples of pasteurized milk to the national standards. Considerable variations in the bacterial counts were observed in between pasteurized milk brands (Table 4). Only three brands exhibited mean SPC value of lower than 4.5 log CFU/ml maintaining the SPC within the 'm' limits prescribed by the FSSAI. Brand 'I' had highest count in all the microbiological tests executed. Obviously, the highest number of isolates were obtained from this brand.

On considering the entire samples tested, positive correlations ($p \leq 0.05$) were found between SPC and TC; SPC and PC; PC and TC; PC and TPC (Table 5). Additional to these correlations, statistically high ($p \leq 0.05$) positive correlation was observed between SPC and TPC in the case of pasteurized milk samples. Among the different correlation coefficients obtained the highest was observed between SPC and PC ($r=0.838$) of pasteurized milk samples. It could be well attributed to the ability of the psychrotrophic organisms to grow in both mesophilic and psychrophilic range which allows them to contribute equally to both the SPC and PC. The correlation studies in pasteurized milk are scarce, however, observations of the current study corroborate well with the already reported statistically significant correlation between psychrotrophic bacteria and SPC (Vyletelova, 1999; Prakash *et al.,* 2006; Cempirkova, 2012); SPC and thermoduric count (Jayarao *et al.,* 2004; Pantoja *et al.,* 2009) in raw milk. Though overall correlation coefficients of the total and pasteurized milk samples established some positive correlations in between some of the parameters tested such correlations were absent in all the brands except one (Table 6).

5.2 ISOLATION OF PASTEURIZATION SURVIVING PSYCHROTROPHIC BACTERIA

Concurrent possession of both the psychrotrophic and thermoduric traits in microorganisms confers significant growth advantage to them in dairy industry. Contrary to the expectations of isolating large number of thermoduric psychrotrophic

isolates this study yielded only six such isolates from the 57 samples assessed (Table 7). Colony morphologies of the isolates clearly indicated differences in between the isolates except the last two (Is5 and Is6), which were found very similar to each other. It is worth mentioning that only one type of colony developed on each of the six TPC Petri-dishes suggesting that the thermoduric psychrotrophs are a rarely encountered population in milk and milk products at least in this area of sample collection and their presence in these products is sporadic in nature. Isolation of three thermoduric psychrotrophs from the same brand over the three months study period invites special attention and needs to be further assessed through field level production and processing line studies. Observations of the current study are noteworthy additions to the existing knowledge on the isolation of thermoduric psychrotrophic microorganisms from milk and milk products (Tinuoye and Harmon, 1975; Washam *et al,*. 1977; Johnston and Bruce, 1982; Mane and Gandhi, 2010; Ribeiro Júnior *et al.,* 2017).

5.3 IDENTIFICATION OF THE ISOLATES

5.3.1 Preliminary Identification Tests

Out of the six thermoduric psychrotrophic isolates obtained, three of them were Gram negative and five of them were rod shaped. Unlike Gram-positive bacteria, Gram-negative bacteria are considered to be susceptible to heat, and their presence in heat treated products is indicative of post process contamination (Walsh *et al.,* 2012; Quigley *et al.,* 2013). Isolation of Gram negative and non-spore forming Gram positive pasteurization surviving psychrotrophic bacteria in the current study rather than the commonly reported Gram positive spore forming bacteria clearly indicate that there is still a lot to learn about the microbial profile of pasteurized milk. Though most of the studies have reported the prevalence of *Bacillus* spp., the spore formers in dairy associated thermoduric psychrotrophs population, some of the studies have reported non sporeforming thermoduric psychrotrophic bacterial isolates from milk and milk products (Tinuoye and Harmon, 1975; Washam *et al.,* 1977; Mane and Gandhi, 2010; Ribeiro Júnior *et*

al., 2017*).* However none of these studies reported the isolation of Gram negative thermoduric psychrotrophic bacterial isolates. But considering the suggested potential of the usually considered heat sensitive microbes to survive commercial pasteurization (Quigley *et al*., 2013), isolation of Gram negative organisms from laboratory re-pasteurized pasteurized milk samples in the present study merits closer attention.

5.3.2 Genotypic Identification of the Isolates

16S rRNA partial sequencing and blasting of the four isolates selected for further studies revealed them as members of three different genera (Table 9). Is5 and Is6 having almost the same colony morphology were identified as two different strains of the same species; *Carnobacterium maltaromaticum.* Phylogenetic tree revealed aquatic environments isolates as the close relatives of *Aeromonas caviae* DMV01 (Fig.1) .*Moraxella osloensis* DMV03 was showing a close similarity to the strain SR3-3, an isolate from secondary peat swamp soil from a forest in Thailand (Fig.2). Both the *Carnobacterium maltaromaticum* isolates were distantly related to each other. *Carnobacterium* species are mainly isolated from fish, meat, permafrost and Antarctic ice lakes.

The isolates obtained in this study were unique in many aspects. It is the first study reporting thermoduric psychrotrophic dairy isolates of *Aeromonas caviae*, *Moraxella osloensis* and *Carnobacterium maltaromaticum.* Though isolation of *Aeromonas* spp. from raw milk and various milk products are reported widely, there are not many reports of their isolation from pasteurized milk. Many researchers (Melas *et al*., 1999; Kirove *et al.,* 2006; Ahmed *et al.,* 2014) attempted to isolate *Aeromonas* spp. from pasteurized milk samples but they failed. Only two studies (Freitas *et al.,* 1993; Yucel *et al.,* 2005) have so far reported the isolation of *Aeromonas* spp. from pasteurized milk. But they were of the opinion that *Aeromonas* spp. do not survive pasteurization and their presence in pasteurized milk is due to post-processing contamination.

The genus *Moraxella*, named after the Swiss ophthalmologist Victor Morax, is composed of gram negative aerobic oxidase-positive coccobacilli. Moraxellae represent a minor constituent of the psychrotrophic flora found in milk and certain dairy products and are relatively rare in milk and dairy products (Santos *et al.*, 1999). Gennari *et al*. (1992) reported the isolation of *Moraxella* like bacteria from 40 per cent of the samples of fresh and spoiled meat and dairy products tested. *Moraxella* spp. have been reported in bulk-tank milk (Jayarao and Wang, 1999) and in an ice cream processing facility (Gunduz and Tuncel, 2006). *M. osloensis* have been detected in fresh cheeses, butter and ewes' milk (Santos *et al.*, 1999).Isolation of *Moraxella osolensis* from raw milk (Hantsis-Zacharov and Halpern, 2007), biofilms formed in milking machines (Weber *et al*., 2019), pasteurized milk and heat exchangers plates (Tattersall, 2020) are also being reported.

Carnobacterium maltaromaticum was first isolated from milk that had developed a distinct malt or chocolate-like flavour and aroma due to the presence of aldehydes formed by the organism (Cailliez-Grimal *et al*., 2014). Presence of *Carnobacterium maltaromaticum* is reported in a variety of French soft-ripened or red-smear cheeses made from cow, sheep, or goat milks and are perceived as the dominant organism in the psychrotrophic LAB flora of these cheeses (Cailliez-Grimal *et al*., 2014). Also it is the most common species of *Carnobacteria* found in milk (Issa and Tahergorabi, 2019).

5.3.3 SEM Imaging of the Isolates

SEM imaging revealed *Carnobacterium maltaromaticum* isolates as the shortest (1-2µm length and 1µm width) among the isolates obtained (Fig. 4). *Aeromonas caviae* and *Moraxella osloensis* were found to be bigger rods measuring 4-5µm, 3-4µm in lengths and 0.75µm, 1µm in breadths respectively.

5.4 CHARACTERIZATION OF THE ISOLATES

5.4.1 Assessment of Growth at Different Temperatures

Marked differences were observed among the isolates in their extent of growth at different temperatures (Table 10). *Aeromonas* caviae grew equally well at 37°C and 27°C but at a lower extent at 20°C. This is in agreement with the report of

Callister and Agger (1986) that all the *Aeromonas caviae* strains isolated from grocery store produce were able to grow at 35°C and 22°C in one day and at a considerably slow rate at 12°C. Highest growth was observed for *Moraxella osloensis* DMV03 at 37°C which was followed by that at 27°C and 20°C, an observation similar to that of Bovre and Henriksen (1967). Different from the other two isolates a opposite trend of preference to lower temperature was evident for *Carnobacterium maltaromaticum*. This observation corroborates with the study of Casaburi *et al.* (2011) that 78 per cent of the raw meat isolates of *C. maltaromaticum* were able to grow at 4, 10, and 20°C but not at 40°C.

5.4.2 Motility of the Isolates

Among the isolates, only *Aeromonas caviae* exhibited motility (Fig.5). This observation is very much in agreement with the earlier reports of motile nature of *Aeromonas caviae* (Kirov *et al.*, 2001; Rabaan *et al.*, 2001), non-motile nature of *Moraxella* spp. (Ulrich and Meredith, 2013) and *Carnobacterium maltaromaticum* (Cailliez-Grimal *et al*., 2014). *Aeromonas caviae* is included in the second group of aeromonads which consists of motile, mesophilic aeromonads (Hoel *et al.,* 2017). Motility confers important advantage to microorganisms in terms of availability of new nutrients and also considered as a key feature for their persistence in food environment (Zhang et al., 2018). Exhibition of this trait by one of the thermoduric psychrotrophic isolates obtained from dairy environment is of high relevance as consumption of contaminated water and food are considered as the main routes of transmission of this species (Krovacek *et al*., 1995; Granum *et al*., 1998; Zhang *et al*., 2012).

5.4.3 Assessment of Haemolytic activity

Haemolysins, considered as an important virulence factor are compounds produced by a variety of bacterial species. *A. caviae* behaved differently (α) than the other three isolates (γ) in the case of haemolytic activity also (Fig. 6). Some of the earlier studies have reported β-haemolytic (Karunakaran and Devi, 1993; Santos *et al*., 1996; Hatha *et al*., 2005; Manna *et al*., 2013), α-haemolytic (Suhet

et al., 2011) and even non haemolytic strains of *Aeromonas caviae*. Altogether, these observations suggest strain wise variations in this trait. According to Chacón *et al*. (2003), β-haemolytic activity is significantly more frequent in clinical isolates than that in environmental isolates of *Aeromonas* spp. This could explain the α-haemolytic activity exhibited by the *Aeromonas caviae* strain isolated in the current study. However exhibition of α-haemolytic activity by an organism isolated from pasteurized milk invites special attention as both α and β-haemolytic phenotypes being considered as virulence associated determinants and are of clinical relevance when assessing the significance of the haemolytic environmental species or strains (Ramachandran, 2013). Contrary to the observation of the current study, α-haemolytic activity of *Carnobacterium* strains was reported by Mogrovejo *et al*. (2020) whereas lack of haemolytic activity of *C. maltaromaticum* strains isolated from diseased fish was reported by Hammes and Hertel (2003). The γ-haemolytic activity exhibited by *Moraxella osloensis* is very much in agreement with the non-haemolytic characteristic of this isolate reported in Manual of clinical microbiology (Versalovic, 2011).

5.4.4 Assessment of Biofilm Forming Ability

Agreeing with the common observation of important roles of bacterial motility mechanisms in biofilm formation (Merritt *et al.,* 2007), *Aeromonas caviae* DMV01, the only motile isolate obtained in this study was identified as a biofilm producer (Figures 7 and 8). Additionally another isolate *Carnobacterium maltaromaticum* DMV05 also exhibited biofilm formation in spite of its lack of motility. Ability of members of the genera *Aeromonas* to produce biofilm is well reported (Bechet and Blondeau, 2003; Angeles-Morales *et al.,* 2012; Talagrand-Reboul *et al.,* 2017). The critical role which polar flagellum of *Aeromonas* spp. plays in biofilm formation and colonization of surfaces was well demonstrated for *Aeromonas caviae* strain Sch3 by Kirov *et al*. (2004). Ability of *Carnobacterium maltaromaticum* strains to produce biofilms was reported by Visvalingam *et al*. (2019).

It is interesting to note that out of the two *Carnobacterium maltaromaticum* isolates obtained from the same brand of pasteurized milk, only one exhibited biofilm

forming potential hinting the strain specific nature of this trait. The biofilm formation potential exhibited by the first isolated *Carnobacterium maltaromaticum* strain and subsequent isolation of another *Carnobacterium maltaromaticum* isolate needs to be researched further as it indicates the possibility of the existence of this isolate as a biofilm in the processing or production line of this particular brand. From the dairy industry point of view, biofilm forming microorganisms are of high public health and economic relevance as these microorganisms can get easily established in milk storage tanks, processing lines resulting in contamination of processed products. Such contaminated products can also act as potential carriers of biofilm detached microorganisms, which is of special relevance in the case of potentially pathogenic strains.

5.5 ASSESSMENT OF SPOILAGE POTENTIAL

5.5.1 Enzymatic Activities

Sensory defects resulting from enzymatic activities have a direct effect on overall milk quality as well as consumer acceptance (Santos *et al.,* 2003). Ability of psychrotrophic organisms to produce extracellular or intracellular proteases and lipases is considered as the major factor contributing towards their spoilage potential. Differences were observed in the enzymatic activity of the isolates; after 24h of incubation at 37°C proteolytic activity was exhibited by the two *Carnobacterium* strains whereas the lipolytic activity was exhibited by *Aeromonas caviae* alone (Table 11). However the non-exhibition of these activities under refrigerated condition can allow these pasteurization surviving organisms to grow unnoticed during refrigerated storage of pasteurized milk.

Display of differential enzymatic potential by thermoduric psychrotrophic bacterial isolates reported by Mane and Gandhi (2010) is in support of the observations of the current study. Ability to secrete wide variety of enzymes associated with pathogenicity and environmental adaptability is considered as a hallmark characteristic of *Aeromonas* spp. (Pemberton *et al.,* 1997). However, the *Aeromonas caviae* isolate obtained in this study failed to elicit any proteolytic activity differing from the earlier report of synthesis of both intracellular and extracellular

proteases by the strain *Aeromonas caviae* (Karunakaran and Devi, 1995) but agreeing with the lack of caseinolytic activity of strains of *A. caviae* (Sangtos *et al.,* 1996*;* Zacaria *et al.,* 2010). These authors concluded that large and significant variations in extracellular caseinolytic activity exist among *Aeromonas* isolates. Manna *et al.* (2013) also reported variations in caseinase production potential of *Aeromonas caviae* strains isolated from meat, milk and fish in Kolkata, India. An extracellular thermo stable lipase producing *Aeromonas caviae* reported by Velu *et al.* (2012) concurs with the isolation of a thermoduric *Aeromonas caviae* strain in this study. As in the case of *Aeromonas* species, variations are reported in the proteolytic activity of *Carnobacterium* species also. Casaburi *et al.* (2011) reported absence of proteolytic activity at 4°C and 20°C for *Carnobacterium maltaromaticum* isolated from raw meat. Proteolytic and lipolytic activities of *Carnobacterium* strains isolated from gastrointestinal tract of coastal fish were reported by Sahnouni *et al.* (2016). Agreeing with the current report, lack of proteolytic and lipolytic activities by the psychrotrophic strain of *Moraxella osloensis* (Accession no: EF204255) isolated from raw milk was reported by Hantsis-Zacharov and Halpern (2007).

5.5.2 Hydrogen Sulphide Production and Gelatin Liquefaction

Inability of the isolates to produce hydrogen sulphide and liquefy gelatin indicates their failure to reduce sulphur containing compounds to hydrogen sulphide and produce gelatinase respectively (Table 12). Absence of hydrogen sulphide production by majority of the *Aeromonas caviae* isolates was reported by Minana - Galbis *et al*. (2002).

5.5.3 Evaluation of defect development

The lipolytic *Aeromonas caviae* DMV01 and the proteolytic *Carnobacterium maltaromaticum* isolates DMV05, DMV06 induced visually perceivable defects in milk stored at room temperature within 24h and 48h respectively (Table 12). The non-proteolytic and non-lipolytic *Moraxella osloensis* isolate showed obvious defects only on the 7^{th} day, which could be attributed to the lowering of pH as it got reduced from 6.6 to 5.6 over this period. So it could be concluded that the delayed induction of spoilage by this isolate might be due to the absence of enzymatic activity and that

this trait acted synergistically with pH reduction for the other isolates which elicited faster spoilage. Though there was no visually perceivable defect, the milk inoculated with *Aeromonas caviae* DMV01 and *Carnobacterium maltaromaticum* DMV05 gave positive clot on boiling test after seven days of refrigerated storage reinforcing their psychrotrophic nature and ability to spoil refrigerated milk (Table 13). Absence of a perceivable defect can be due to lack of enzyme production or the inactivity of the produced enzymes at lower temperature. The observation of Yuan *et al.* (2018) that higher enzymatic activities of psychrotrophic organisms at optimal temperature than at 7°C are similar with the observations of the current study. In contrast, Mane and Gandhi, (2010) reported that all their thermoduric psychrotrophic isolates hydrolysed milk proteins at 7°C. It is interesting to note that the milk inoculated with the other *Carnobacterium maltaromaticum* isolate, despite of having a pH value (6.1), not much lower than the COB positive milk samples turned out COB negative along with the *Moraxella osloensis* inoculated milk having a pH of 6.4. It is noteworthy that these isolates could grow without any visible effect even up to seven days in refrigerated temperature and that for two isolates it could not be detected even on boiling. These observations warn about the presence of such rare unnoticed and undetected microbial populations in dairy environment.

5.6 ANTIBIOTIC SUSCEPTIBILITY

Aeromonas caviae DMV01 was found resistant to 25 per cent of the antibiotics tested (Table 14), interestingly they were all beta lactam antibiotics. This is in agreement with the earlier reports of their high levels of resistance to amoxicillin–clavulanic acid combination (Yucel and Erdogan, 2010; Ghenghesh *et al*., 2013). However, the isolate was found to be susceptible to all the protein synthesis inhibiting antibiotics tested; chloramphenicol, gentamicin and tetracycline. Similar observations of susceptibility among *Aeromonas* spp. to these antibiotics are reported by other researchers (Aravena-Román *et al*., 2012; Igbinosa and Okoh, 2012; Igbinosa, 2014; Zhou *et al*., 2019). Fluoroquinolones are reported as the first choice treatment for *Aeromonas* infections (Vila *et al*.,

2002). In concurrence with the reports of the susceptibility of *Aeromonas* isolates to ciprofloxacin, a fluoroquinolone (Vila *et al*., 2002; Zhou *et al*., 2019) this isolate was also found to be sensitive to this antibiotic.

Moraxella osloensis was found resistant to amoxyclav (Table 15), a beta lactam antibiotic. But Han *et al.* (2004) reported that they were found negative for beta lactamase through cefinase test. This difference is to be noted as therapy with penicillin and other cell wall active antibiotics are generally used to cure the infection caused by these organisms. *Carnobacterium* being a member of lactic acid bacteria, antibiotic studies of the organism are scanty. In the present study, both the *Carnobacterium* strains showed similar antibiotic sensitivity, but supporting the minor changes in their characteristics as seen earlier, one of the strains was moderately resistant to streptomycin while the other being fully resistant to it (Tables 16 and 17). These two isolates were found sensitive to gentamycin and tetracycline. A bit different to this, Kim and Austin (2008) reported a broad spectrum antibiotic resistant strain of *Carnobacterium maltaromaticum* which was resistant to streptomycin and gentamycin but sensitive to tetracycline. When we consider the antibiotics tested in this study common to all the isolates, both the Gram negative and Gram positive bacteria were susceptible to ciprofloxacin, chloramphenicol and tetracycline, while the Gram negative bacteria were found resistant to amoxyclav.

Considering the food safety aspects reported (Lim *et al.,* 2009, ShuHui *et al*., 2014; Skwor and Králová, 2019), the two Gram negative organisms namely *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 were selected to study further.

5.7 ASSESSMENT OF CROSS ADAPTATION POTENTIAL

5.7.1 Heat Resistance at 63°C

When the two Gram negative isolates where studied for their heat resistance, marked difference was observed in their resistance pattern. Though

both of them survived the laboratory pasteurization, there was a marked difference in the number of survivors (Table 18). Differences were also observed in the extent at which the reductions occurred at each time interval. The same standard procedure implemented in this study has been utilised by many researchers for the study of pasteurization survivors (Gunasekhara *et al.,* 2002; Gao *et al.,* 2002; Dumalisile *et al.,* 2005; Ribeiro Junior *et al.,* 2017). *Aeromonas caviae* DMV01 exhibited a gradual decrease in the number of cells with a six log reduction after 30 minutes, whereas *Moraxella osloensis* DMV03 had only one log reduction exhibiting a somewhat stable population from 10 minutes of heat treatment onwards (Table 18). Van Boeijen *et al*. (2011) reported that only 6 log reductions happened for heat resistant strains of *Listeria monocytogenes* when subjected to 72°C/15s. The fact that similar 6 log reduction was observed when *Aeromonas caviae* subjected to 63°C/30min, an intense heat treatment than that of HTST, reaffirms its thermal resistance.

It is interesting to note that the *Moraxella* isolate which was negative for many of the previously tested attributes was found to be a highly heat resistant Gram negative psychrotrophic strain. Not many studies are reported on the heat resistance of *Moraxella* spp. The heat sensitive nature of *Moraxella osloensis* strains isolated from ground beef reported by Welch and Maxcy (1975) is contradictory to the markedly high heat resistance exhibited by *Moraxella osloensis* DMV03. Gunashekara *et al.* (2002) demonstrated that a substantial portion of cells rendered incapable of forming colonies by heat treatment. But those cells are found to be metabolically active suggesting that a significant subpopulation surviving the pasteurization process could be viable but non culturable (VBNC). This observation indicate serious implications for food safety as these heat resistant strains can recover during subsequent storage and distribution and raise quality and safety issues.

The D-value of *Aeromonas caviae* DMV01 at 63^{o}C was found to be 4 minutes 38 seconds which is much lower than that of *Moraxella osloensis* DMV03 ie; 25 minutes 18 seconds (Figures 9 and 10). Both the values are higher

than the D-values reported for Gram negative organisms like *Salmonella* serotype cocktail at 62°C (Juneja *et al*., 2001), *Campylobacter* spp., *Yersinia enterocolitica*, *Cronobacter sakazakii* at 60°C (Arroyo *et al*., 2009) and *E coli* at 62.5°C (Peng *et al*., 2013). Heat resistance studies of *Aeromonas* spp. reported that their heat resistance in both broth and foods is lower than *Salmonella typhimurium*, *Staphylococcus aureus* and *Escherichia coli* (Palumbo and Buchanan, 1988; Nishikawa *et al*., 1993). On analysing the D-values of 4 strains of *Aeromonas hydrophila* in liquid whole egg Schuman *et al*. (1996) reported that they ranged from 3.62 to 9.43min (at 48°C) to 0.026 to 0.040min (at 60°C). However contradictory to these reports the *Aeromonas* isolate obtained in this study exhibited remarkably high heat resistance. To the best of our knowledge no studies are reported on the D-value of *Moraxella* spp., but there are reports on the irradiation D-values of highly radiation-resistant *Moraxella* spp. from beef (Maxcy, 1977; Bruns and Maxcy, 1978). Development of *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 colonies from the inoculated milk heat treated at 63°C for 30 minutes without any resuscitation step further aggravates the criticality of their presence in pasteurized milk.

In general, the heat resistance of any given microorganism is known to be affected not only by inherent genetic factors, but also by many environmental factors during heating (Juneja *et al.,* 2001). Although it is inappropriate to compare the D-value obtained in one medium with that obtained in another media, on comparing with the D-values reported for the microorganisms claimed as heat resistant, isolates in the current study can be regarded as remarkably heat resistant. In line with the D_{60}-value based heat resistance categorization provided by Mercer *et al*. (2015) for *E. coli*, another Gram negative bacterium, these isolates could be classified as highly heat resistant expecting that the D_{60}-value of these isolates would be more than 6 minutes.

5.7.2 Resistance to Boiling Process

On using the same initial population as that used for D_{63}-value determination, the isolates could not survive the boiling process even for one second, obviously not for five seconds (Table 19). This finding rules out the possibility of this being a

safety concern in house hold practices, but the practice of using packet milks in the preparation of milk shakes and sherbets in the local markets is a concern.

5.7.3 Resistance to pH

Despite their remarkable heat resistance both the isolates were found to be sensitive to low pH levels. Marked reduction in optical densities were observed on reducing the pH level from 7.5 to the extent that the O.D. was stagnant at 0.01 below 4.5 and 5.5 for *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 respectively (Table 20). This observation well corroborates with that the reports of no or delayed growth of *Aeromonas hydrophila* strains at pH 4.5 (Palumbo *et al.,* 1985) and the resistance of *Aeromonas* to pH ranges from 4.5 to 9 (Isonhood and Drake, 2002). These authors also reported that no data on pH tolerance exist for other *Aeromonas* species. Similar to the observations of the present study, ability of all the strains of *Moraxella* species isolated from marine fishery sources to grow in the pH range of 6.9 to 8.8 and the ability of pH below 4.9 or above 10.4 to limit their growth was reported by Vernon (1978). Ponce De Leon *et al.* (1993) also reported the sensitivity of *Moraxella* spp. to 0.05 per cent of citric acid (pH values below 5.5).

pH reduction and thermal treatment are two strategies widely adopted in food processing and food preservation. Some studies have reported that thermal resistance of microorganisms can be influenced by their responses to other physiological stresses (Bearson *et al.*, 1997; Buchanan and Edelson, 1999). However no such cross-protective effects were evident in the case of the isolates of the current study as *Moraxella osloensis* DMV03, the more thermal resistant was found to be less pH resistant than *Aeromonas caviae* DMV01. Moreover the thermal resistance exhibited by both the isolates was not reflected in their response to pH. On collating earlier reports and the results of current study no such thermal resistance imparted pH resistance (or vice versa) could be established for the isolates of the current study. Additionally the antibiotic

response pattern exhibited by the isolates also precludes the possibility of any such cross protection effect.

5.8 ATTRIBUTES CONTRIBUTING TOWARDS THEIR PRESENCE IN DAIRY ENVIRONMENT

5.8.1 Ability to Grow in Milk under Room and Refrigerated Storage

Aeromonas caviae elicited faster reduction of methylene blue than *Moraxella osloensis* indicating its comparatively better ability than latter to grow in milk (Table 21). Underpinning this observation *Aeromonas caviae* outperformed *Moraxella osloensis* in terms of generation time both in milk and nutrient broth at room and refrigerated temperatures (Figures 11 and 12). Similar to the growth curves obtained by Peng *et al.* (2005) for *Moraxella catarrhalis* and Goswami *et al.* (2014) for *Aeromonas hydrophila* at 27°C in BHI broth, log phases of *Aeromonas caviae* and *Moraxella osloensis* started at two hours on incubation at room temperature (27°C) in both the growth media assessed. At this temperature of growth, both the organisms exhibited higher growth rate in nutrient broth than in milk. It is interesting to note that *Moraxella osloensis* DMV03 exhibited the same period of lag phase at 7°C and 27°C indicating its potential to withstand cold environment.

Underlining their psychrotrophic nature, both the organisms grew at 7°C at a lower growth rate than at 27°C. Ability of these organisms to grow at low temperature was reported by Hantsis-Zacharov and Halpern (2007) for *Moraxella osloensis* H36, and Park *et al.* (2014); Lian *et al.* (2020) for *Aeromonas* spp. In spite of its longer adaptation period (lag phase of 4h) and shorter log phase (8h) during refrigerated storage in milk, the *Aeromonas* isolate exhibited higher growth rate than *Moraxella osloensis* DMV03 which exhibited 2 hours of lag phase and 24 hours of log phase. On collating the growth patterns of both the organisms with their growth rates it can be concluded that milk is a good medium for growth of these organisms. Additionally, ability of these organisms to grow at refrigerated

conditions without any visible changes points to the criticality of the situation as refrigeration is one of the most widely utilised storage methods in dairy industry.

5.8.2 Biofilm Formation Potential

5.8.2.1 Assessment of Swarming Motility

Swarming motility is a flagellum dependent behaviour that allows bacteria to move over the solid surfaces (Kirov *et al.,* 2002). These movements are made with the aid of lateral flagella, which enables bacteria to migrate over surfaces by rotated movements and the formation of side by side cell groups called rafts (Gavin *et al.,* 2002; Kirov *et al.,* 2002) The swarming motility exhibited by the *Aeromonas caviae* DMV01 isolate (Fig. 13) is in agreement with the reports of Gavin *et al.* (2002), Kirov *et al.* (2002) and Santos *et al.* (2011). *A. caviae* is found to produce inducible lateral flagella which they use in the phenomenon associated with the expansion of the area of colonization on surfaces, biomass production and biofilm formation (Gavin *et al*., 2003). These authors concluded that the presence of lateral flagella and swarming motility are clear pathogenic factors for mesophilic *Aeromonas* strains. This is an observation of great relevance considering that Aeromonads are well recognized for their colonizing capacity on surfaces and as important constituents of bacterial biofilms in water distribution systems, food processing systems and in the gastrointestinal tract (Scoaris *et al.,* 2008). Since many studies (Gavin *et al.,* 2002; Bechet and Blondeau, 2003; Gavin *et al.,* 2003; Santos *et al.,* 2011) revealed that the lateral flagella is required for increased cell adherence, invasion and biofilm formation by *A. caviae,* the biofilm potential exhibited by the isolate *A. caviae* DMV01 can be considered in conjunction with its swarming motility. Li *et al.* (2009) reported a high correlation between the occurrence of flagella and biofilm formation in bacteria and identified swarming motility and exopolysaccharides as the major factors associated with biofilm formation. Studies have shown that approximately 60 per cent of mesophilic *Aeromonas* strains possess lateral flagellar genes and are able to swarm on surfaces (Kirov *et al.,* 2003). Considering the fact that this

isolate with swarming motility was obtained from a pasteurized dairy product, aspects like its ability to form biofilms in dairy processing environment and response to cleaning regime need to be studied through *in situ*.

5.8.2.2 Assessment of Biofilm Formation Potential of the Isolates on Glass Strips Using Scanning Electron Microscopy

Agreeing with the earlier understanding that members of the genera *Aeromonas* are biofilm producers (Talagrand-Reboul *et al*., 2017), biofilm formation on the glass surface by the *Aeromonas caviae* DMV01 was clearly evident from the scanning electron microscopy (SEM) images (Fig. 14). Ability of certain strains of *Aeromonas caviae* to form biofilm on different surfaces are reported in other studies also (Bechet and Blondeau, 2003; Angeles-Morales *et al.,* 2012). After 32h at 37°C, the *A. caviae* DMV01 cells appeared like a lawn on the glass surface (Fig. 14A) different from the arrangement of cells of *Moraxella osloensis* (Fig. 14B), a non-biofilm former. Appearance of large numbers of the isolate with smooth appearance and extracellular vesicular material adhering to the surface (Fig. 14C) after 32 hours of incubation at 37°C is comparable with that obtained for *Aeromonas caviae* strain Sch3 cells after 48 hours of incubation at 28°C (Angeles-Morales *et al.,* 2012). The appearance of short vesicular material on cell surfaces might be the initial steps of biofilm formation as it is observed that in biofilm formation after the first step of colonization, the irreversibly attached bacteria start to express adhesion proteins like curli or fimbriae to produce intercellular connections and polymeric matrix (Donlan, 2002).

It is reported that as Aeromonads utilize biofilms to persist for several years and to emerge later in favourable conditions. The biofilms are considered critical in food industry as they act as long lasting microbial reservoirs (Kuhn *et al.,* 1997). Ability of *Aeromonas* strains to persist in the same source over years has been reported in earlier researches. Villari *et al.* (2003) observed the persistence of *A. caviae* isolates of the same clone over a three year period in natural mineral fresh water. Presence of these biofilm formers even in chlorinated water supply (Fernandez *et al.,* 2000) adds to the criticality of the situation as it

shows their capability to survive the disinfection treatments. Resistance of biofilm producing *A. hydrophila* than planktonic cells to disinfectants (Jahid and Ha, 2014) further questions the efficacy of the current sanitation practices dairy industry banks on from years.

5.8.3 Resistance to Cleaning-In-Place (CIP) Procedure

Cleaning of the production lines by CIP is considered as one of the simplest ways to control biofilms in dairy industry (Bremer *et al*., 2009). The observation of removal of *Aeromonas caviae* DMV01 cells from the biofilms formed on stainless steel strips on treatment with 0.5 and one per cent CIP solutions used in a laboratory level simulation (Table 23) is comparable to the report of Parkar *et al.* (2004). As in the current study these authors also reported a decrease in the effectiveness of standard cleaning in place regimen to remove the biofilms formed on stainless steel coupons by *Bacillus flavothermus,* a thermophilic spore-former on decreasing the strength of the solutions. A complete removal of the cells from the surface on using one per cent CIP solutions in combination with the turbulence offered by 250rpm agitation indicates that the *A. caviae* isolate is not resistant to the cleaning in procedure adopted in this study. Additionally, the very short age of the tested biofilm could also have contributed to its vulnerability to the CIP simulation adopted. In contrast, resistance to cleaning procedures are reported for many biofilm formers. Jahid and Ha (2014) observed higher resistance to disinfectants by *Aeromonas hydrophila* biofilms than planktonic cells. Several studies have reported the survival of *Bacillus cereus* and its biofilms even after sanitization (Zhou *et al.,* 2008; Shi and Zhu, 2009; Ostrov *et al.,* 2016; Silva *et al.,* 2018).

However cent per cent removal from plane surfaces does not guarantee the same effect on biofilms formed on pipelines with bents, T-joints and rubber gaskets, especially if lower CIP solution concentrations are used. *In situ* studies covering these aspects will be meaningful.

Summary

6. SUMMARY

Milk, well recognized as an ideal media for microbial growth possesses a multifaceted and highly flexible microbial community comprising of both the beneficial ones and the harmful ones. Pasteurization and subsequent refrigeration is one of the methods dairy industries widely utilise to control the aforesaid harmful organisms. As pasteurization is not capable of destroying the entire microbial population present, some organisms known as thermoduric organisms might be surviving this process. Among these pasteurization survivors some might be capable of growing under refrigeration conditions, the pasteurization surviving psychrotrophs also termed as thermoduric psychrotrophs. The unique combination of heat resistance and psychrotrophic properties existing in these organisms exacerbates the criticality of their presence in dairy industry. In this study, attempts were made to isolate pasteurization surviving psychrotrophic organisms from pasteurized milk and dairy associated samples and characterise them.

A total of 57 samples constituted by 42 samples of pasteurized milk, seven samples of milk products, two raw milk samples from BMC and six dairy equipment swabs were assessed in this study. Out of the 42 pasteurized milk samples tested, 19 samples exhibited aerobic plate counts higher than the ‘m’ value of 4.5 log CFU/ml stipulated by FSSAI and out of these samples 15 had aerobic plate counts higher than the ‘M’ value of 4.7 log CFU/ml. So it can be derived that 35.7 per cent of the pasteurized milk samples tested were not meeting the FSSAI stipulated aerobic plate count standard. Most of the samples exhibited thermoduric and psychrotrophic counts while the pasteurization surviving psychrotrophic organisms were found in six samples of the pasteurized milk only. Among the different brands of pasteurized milk samples assessed, one particular brand exhibited highest counts for all the parameters tested. Interestingly the same brand yielded thermoduric psychrotroph counts in three out of the eight samples tested. Though *Bacillus* like spreading colonies were formed on thermoduric

Petri-dishes, such colonies were not developed on thermoduric psychrotrophic Petri-dishes indicating the absence of pasteurization surviving psychrotrophic *Bacillus* spp. in the samples tested.

The six thermoduric psychrotrophs obtained were subjected to preliminary identification and three of them were found to be Gram negative. Two isolates both from the Gram positive and Gram negative category were selected for further studies. Molecular level identification of the isolates through 16s rRNA partial sequencing revealed them as *Aeromonas caviae, Moraxella osloensis,* and two different strains of *Carnobacterium maltaromaticum*. The obtained nucleotide sequences were deposited in the NCBI database under accession numbers MT071634, MT158663, MT158664 and MT158665 with strain numbers as DMV01, DMV03, DMV05 and DMV06 respectively.

On assessing these isolates for their motility, haemolytic activity, biofilm forming, defect causing abilities and antibiotic susceptibilities, wide variations were observed between the isolates. *Aeromonas caviae* DMV01 exhibited swarming motility, haemolytic, lipolytic activities, quick defect induction (first day) and resistance to two (beta lactam antibiotics) out of the eight antibiotics tested. Proteolytic activities, defect induction on the second day and moderate resistance or full resistance to one (Streptomycin) out of the eight antibiotics tested were exhibited by *Carnobacterium maltaromaticum* strains DMV05 and DMV06. Lack of proteolytic and lipolytic abilities and hence the delayed defect induction (seventh day) and resistance to one (Amoxyclav) out of the eight antibiotics tested was shown by *Moraxella osloensis* DMV03. Despite the pasteurization resistance and psychrotrophic nature exhibited by all the isolates and the biofilm forming ability and motility exhibited by some of the isolates, the same organism was not isolated a second time from the same source over the three months period of study. This overrules the prospects for their persistence in the dairy environment concerned except in the case of *Carnobacterium maltaromaticum*, in which case also it was not isolated from subsequent samples. Taking into consideration the earlier reports of pathogenicity of the *Aeromonas*

caviae and *Moraxella osloensis* isolates and the beneficial effects of *Carnobacterium maltaromaticum*, a member of the LAB group, it was decided to proceed further with the two Gram negative isolates namely *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03.

On assessing these two Gram negative isolates for their heat resistance, marked difference was observed in their resistance pattern though both of them could survive the normal LTLT pasteurization process. However clear difference was observed between the two isolates with the log reduction of *Moraxella osloensis* DMV03 markedly lower than that of *Aeromonas caviae* DMV01. Both the organisms could not survive boiling even for one second. However, despite their high heat resistance both of them were found to be sensitive to exposure to low pH with *Aeromonas caviae* DMV01 being comparatively more resistant than *Moraxella osloensis* DMV03. Considering the sensitivity *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 exhibited against antibiotics and low pH, it could be inferred that the high heat resistance mechanisms adopted by them did not impart any cross resistance against these stresses.

In order to understand the additional features that contribute towards their presence in dairy environment *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03 were compared for their ability to grow in milk and in a laboratory growth medium at room (27^0C) as well as refrigerated (7°C) temperatures. The growth curves clearly depicted the ability of these organisms to grow at low temperatures. The finding that both organisms grew well at room temperature and at a reduced rate in refrigerated conditions has done justice to their mesophilic and psychrotrophic nature. A clear demarcation with regard to growth rate was evident in the growth curve of *Aeromonas caviae* DMV01 and *Moraxella osloensis* DMV03. Considering its faster growth rate and biofilm forming potential *Aeromonas caviae* DMV01 was assessed further for its ability to form biofilms on glass surface and stainless steel strips. High vacuum scanning electron microscopy at a magnification of 15000x showed biofilm formation on glass surfaces and presence of extracellular vesicular materials, a characteristic of

biofilm producers. On subjecting the *Aeromonas caviae* DMV01 biofilms formed on stainless steel strips to a laboratory level simulation of CIP regime, 17 per cent of the cells survived on using the CIP solutions at 0.5 per cent whereas none of the cells survived CIP with 1 per cent solutions.

Based on the observations of this study, it could be concluded that pasteurization surviving psychrotrophic organisms, though at a low per cent are present in milk and milk products. While further researches are required on food quality and safety issues implicated by these pasteurization surviving psychrotrophic isolates, observations of this study indicate the need to study further the microenvironment of pasteurized milk. These psychrotrophic organisms due to their ability to elicit enzymatic activities and grow unnoticed under refrigerated conditions can have a negative impact on the shelf life and quality of dairy products. Observation of this study also highlight the need of large-scale region wise research explorations on 'pasteurization surviving psychrotrophic' dairy associated microorganisms in order to remain prepared to address the ever-increasing quality and safety challenges faced by the dairy industry.

Reference

7. REFERENCE

1. Addis, M.F., Tanca, A., Uzzau, S., Oikonomou, G., Bicalho, R.C. and Moroni, P. 2016. The bovine milk microbiota: Insights and perspectives from-omics studies. *Mol. Biosystems*. **12**:2359–2372.
2. Agarwal, A., Awasthi, A., Dua, A., Ganguly, S., Garg, V. and Marwaha. S.S. 2020. Microbiological profile of milk: Impact of household practices. *Indian J. Public Hlth*. **56**:86-94.
3. Angeles-Morales, B.E., Mondragón-Flores, R., Luna-Arias, J.P, Enríquez-Nieto, T.C., Parra-Ortega, B. and Castro-Escarpulli, G. 2012. Evaluation of morphological changes of *Aeromonas caviae* sch3 biofilm formation under optimal conditions. *Adv. Microbiol*. **2**(04):552–560.
4. Aravena-Román, M., Inglis, T.J.J., Henderson, B., Riley, T.V. and Chang, B.J. 2012. Antimicrobial susceptibilities of *Aeromonas* strains isolated from clinical and environmental sources to 26 antimicrobial agents. *Antimicrob. Agents Chemother*. **56**: 1110–1112.
5. Arroyo, C., Condón, S., and Pagán, R. 2009. Thermobacteriological characterization of *Enterobacter sakazakii*. *Int. J. Food Microbiol*. **136:**110–118.
6. Ball.R.J. and Sellers.W. 1966. Improved motility medium. *Appl. Microbiol.* **14**:670-673.
7. Banik, S.K., Das, K.K. and Uddin, M.A. 2015. Microbiological quality analysis of raw, pasteurized, UHT milk samples collected from different locations in Bangladesh. *Stamford J. Microbiol*. **4:**5–8
8. Batish, V.K., Chander, H.A. and Ranganathan, B. 1988. Heat resistance of some selected toxigenic *Enterococci* in milk and other suspending media. *J. Food Sci*. **53:**665–666.
9. Bauer, A.W., Kirby, W.M., Sherris, J.C. and Turck, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. of Clin. Pathology*. **45**: 493–496.
10. Bearson, S., Bearson, B. and Foster, J.W. 2006. Acid stress responses in enterobacteria. *FEMS Microbiol. Lett.* **147**(2):173–180.
11. Bechet, M. and Blondeau, R. 2003. Factors associated with adherence and biofilm formation by *Aeromonas caviae* on glass surfaces. *J. of Appl. Microbiol*. **94**:1072-78

12. Boll, E.J., Marti, R., Hasman, H., Overballe-Petersen, S., Stegger, M., Ng, K., Knøchel, S., Krogfelt, K. A., Hummerjohann, J. and Struve, C. 2017. Turn up the heat—food and clinical *Escherichia coli* isolates feature two transferrable loci of heat resistance. *Frontiers Microbiol.* **8**:28-36.
13. Boor, K.J., Wiedmann, M., Murphy, S. and Alcaine, S. 2017. A 100-year review: microbiology and safety of milk handling. *J.of Dairy Sci*. **100**(12):9933–9951.
14. Bovre, K. and Henriksen, S.D. 1967. A new *Moraxella* species, *Moraxella osloensis*, and a revised description of *Moraxella nonliquefaciens*. *Int. J. Syst. Bact.* **17**(2):127–135.
15. Bremer, P.J., Fillery, S. and McQuillan, AJ. 2006. Laboratory scale Clean-In-Place (CIP) studies on the effectiveness of different caustic and acid wash steps on the removal of dairy biofilms. *Int. J. of Food Microbiol*. **106**(3):254–262.
16. Bremer, P.J., Monk, I. and Butler, R. 2002. Inactivation of *Listeria monocytogenes*/*Flavobacterium* spp. biofilms using chlorine: impact of substrate, pH, time and concentration. *Lett. Appl. Microbiol*. **35**:321-325.
17. Breurec, S., Poueme, R., Fall, C., Tall, A., Diawara, A., Bada-Alambedji, R., Broutin, C., Leclercq, A. and Garin, B. 2010. Microbiological quality of milk from small processing units in Senegal. *Foodborne Pathogens Dis*. **7**(5):601–604.
18. Bruns, M.A. and Maxcy, R.B. 1978. Effect of selected solutes on growth and recovery of a radiation-resistant *Moraxella* spp. *J. Food Sci.* **43**(5):1386–1389.
19. Buchanan, R.L. and Edelson, S.G. 1999. pH-Dependent stationary-phase acid resistance response of enterohemorrhagic *Escherichia coli* in the presence of various acidulants. *J. Food Prot*. **62**(3):211–218.
20. Burgess, K. 2010. Key requirements for milk quality and safety: a processor's perspective in improving the safety and quality of milk: milk production and processing. Woodhead Publishing Series, Cambridge. pp.64-84.
21. Buxton. R 2005. *Blood agar plates and hemolysis protocols*. American society for microbiology, Washington.
22. Cailliez-Grimal, M.I., Afzal, A.M. and Revol-Junelles. 2014. Carnobacterium. In: Batt, C.A. and Tortorello (ed.), M.U. *Encyclopedia of Food Microbiology* (2nd Ed), Academic Press, Cambridge, pp. 379-383.
23. Callister, S.M. and Agger, W.A. 1987. Enumeration and characterization of *Aeromonas hydrophila* and *Aeromonas caviae* isolated from grocery store produce. *Appl. Environ. Microbiol.* **53**(2):249–253.

24. Cappuccino, J.C. and Sherman, N. 2004. *Microbiology-A Laboratory Manual*. (6th Ed.). Pearson Education Publication, 528p.
25. Casaburi, A., Nasi, A., Ferrocino, I., Di Monaco, R., Mauriello, G., Villani, F.and Ercolini, D. 2011. Spoilage-related activity of *Carnobacterium maltaromaticum* strains in air-stored and vacuum-packed meat. *Appl. Environ. Microbiol.* **77**(20):7382–7393.
26. Cempírková, R. 2012. Psychrotrophic vs. Total bacterial counts in bulk milk samples. *Veterinární Medicína*. **47**(8):227–233
27. Chacón, M. R. 2003. Characterisation of *Aeromonas* spp. isolated from frozen fish intended for human consumption in Mexico. *Int. J. Food Microbiol*. **84**:41-49.
28. Chatterjee, S.N., Bhattacharjee, I., Chatterjee, S.K and Chandra G.L. 2006. Microbiological examination of milk in Tarakeswar, India with special reference to coliforms. *Afr. J. Biotech*. **5**:1383-1385.
29. Christensen, G.D., Simpson, W.A., Bisno, A.L. and Beachey, E.H. 1982. Adherence of slime producing strains of *Staphylococcus epidermidis* to smooth surfaces. *Infect. Immun*. **37**:318-326.
30. Clinical and Laboratory Standards Institute 2015. *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria* (3rd Ed.). Clinical and Laboratory Standards Institute, Wayne.
31. Collins, E.B. 1981. Heat resistant psychrotrophic microorganisms. *J. of Dairy Sci*. **64**(1):157–160
32. Cousin, M.A. 1982. Presence and activity of psychrotrophic microorganisms in milk and dairy products. *J. Food Prot*. **45**(2):172–207.
33. De Garnica, M.L., Santos, J.A. and Gonzalo, C. 2010. Influence of storage and preservation on microbiological quality of silo ovine milk. *J. Dairy Sci*. **94**(4):1922–1927.
34. Donnelly, C.W. and Briggs, E.H. 1986. Psychrotrophic growth and thermal inactivation of *Listeria monocytogenes* as a function of milk composition. *J. Food Prot*. **49**(12):994–998.
35. Dufour, M., Simmonds, R.S. and Bremer, P.J. 2004. Development of a laboratory scale clean-in-place system to test the effectiveness of "natural" antimicrobials against dairy biofilms. *J. Food Prot*. **67**:1438–1443.

36. Dumalisile, P., Witthuhn, R.C. and Britz, T.J. 2005. Impact of different pasteurization temperatures on the survival of microbial contaminants isolated from pasteurized milk. *Int. J. Dairy Technol*. **58**(2):74–82.
37. Elegbeleye, J.A. and Buys, E.M. 2020. Molecular characterization and biofilm formation potential of *Bacillus subtilis* and *Bacillus velezensis* in extended shelf-life milk processing line. *J. Dairy Sci*. **103**(6):4991–5002.
38. Evans, D.A., Hankinson, D.J. and Litsky, W. 1970 Heat resistance of certain pathogenic bacteria in milk using a commercial plate heat exchanger. *J. Dairy Sci.* **53**:1659-1665.
39. Fernandez, M.C., Giampaolo, B.N., Ibañez, S.B., Guagliardo, M.V., Esnaola, M., Conca, L., Valdivia, P., Stagnaro, S. M. and Chiale, C. 2000. *Aeromonas hydrophila* and its relation with drinking water indicators of microbiological quality in Argentine. *Genetica*. **108**:35-40
40. Frank, J.F. and Yousef, A.E.2004. Tests for groups of microorganisms In: Wehr, H.M. and Frank, J.F. (ed.), *Standard methods for the examination of dairy products*. (17th Ed.). American Public Health Association., Washington, 227p.
41. Freeman,D.J., Falkiner,F.R. and Keane,C.T.1989. New method for detecting slime production by coagulase negative *Staphylococci. J.Clin.Pathol*. **42**:872-874.
42. Freitas, A.C., Nunes, M.P., Milhomem, A.M. and Ricciardi, I.D. 1993. Occurrence and characterization of *Aeromonas* species in pasteurized milk and white cheese in Rio de janeiro, Brazil. *J. Food Prot.* **56**(1):62–65.
43. FSSAI. 2020. The Food Safety and Standards Authority of India. Professional Book Publishers, New Delhi.
44. Gao, A., Mutharia, L., Chen, S., Rahn, K. and Odumeru, J. 2002. Effect of pasteurization on survival of *Mycobacterium paratuberculosis* in milk. *J. Dairy Sci*. **85**(12):3198–3205.
45. Gautam, A., Shukla, S., Ramteke, P.W. and Chandra, R. 2015. Studies on incidence and antibiotic susceptibility pattern of bacterial pathogens in dairy products. *Pharma Innovation J.* **4:**69-72.
46. Gavin, R., Merino, S., Altarriba, M., Canals, R., Shaw, J. G. and Tom J. M. 2003. Lateral flagella are required for increased cell adherence, invasion and biofilm formation by *Aeromonas* spp. *FEMS Microbiol. Lett.* **224**(1):77–83.

47. Gavín, R., Rabaan, A.A., Merino, S., Tomás, J.M., Gryllos, I. and Shaw, J.G. 2002. Lateral flagella of *Aeromonas* species are essential for epithelial cell adherence and biofilm formation: *Aeromonas* lateral flagellin locus. *Mol. Microbiol* **43**:383–397.

48. Gennari, M., Parini, M.,Volpon, D. and Serio,M.1992.Isolation and characterization by conventional methods and genetic transformation of Psychrobacter and Acinetobacter from fresh and spoiled meat, milk and cheese.*Int. J. Food Microbiol*.**15**:61-75.

49. Ghenghesh, K.S., Rahouma, A., Tawil, K., Zorgani, A. and Franka, E. 2013. Antimicrobial resistance in Libya. *Libyan J. Med.***8**:1–8.

50. Gleeson, D., O'Connell, A., and Jordan, K. 2013. Review of potential sources and control of thermoduric bacteria in bulk-tank milk. *Ir. J. Agric. Food Res*. **52**(2):11.

51. Gopal, N., Hill, C., Ross, P.R., Beresford, T.P., Fenelon, M.A. and Cotter, P.D. 2015. The prevalence and control of *Bacillus* and related spore-forming bacteria in the dairy industry. *Frontiers Microbiol*. **6:**112-119.

52. Goswami, U., Brenes, J.A., Punjabi, G.V., LeClaire, M.M. and Williams, D.N. 2014. Associations and outcomes of septic pulmonary embolism. *Open Respiratory Med. J*. **8**(1):28–33

53. Granum, P.E., O'Sullivan, K., Tomas, J.M., and Ormen, O. 1998. Possible virulence factors of *Aeromonas* spp. from food and water. *FEMS Immunol. Med. Microbiol.* **21**:131–137.

54. Grosskopf, J.C. and Harper, W.J. 1974 Isolation and identification of psychrotrophic sporeformers in milk. *Milchwissenschaft.* **29**:467-470.

55. Gunasekera, T.S., Sørensen, A., Attfield, P.V., Sørensen, S.J. and Veal, D.A. 2002. Inducible gene expression by nonculturable bacteria in milk after pasteurization. *Appl. Environ. Microbiol*. **68**(4):1988–1993.

56. Gunduz, G. T. and Tuncel, G. 2006. Biofilm formation in an ice cream plant. *Antonie Van Leeuwenhoek* **89**:329-336.

57. Hammes, W.P. and Hertel, C. 2003. The genera *Lactobacillus* and *Carnobacterium*. In: Dworkin, M. (ed.), *The Procaryotes: An Evolving Electronic Resource for the Microbiological Community*. Springer-Verlag, New York, pp.320–403.

58. Han, X.Y., and Tarrand, J.J. 2004. *Moraxella osloensis* Blood and catheter infections during anti-cancer chemotherapy: Clinical and microbiologic studies of 10 cases. *Am. J. Clin. Path*. **121**(4):581–587.
59. Hantsis-Zacharov, E. and Halpern, M. 2007. Culturable psychrotrophic bacterial communities in raw milk and their proteolytic and lipolytic traits. *Appl. Environ. Microbiol*. **73**(22):7162–7168.
60. Harrigan, W. F. 1998. *Laboratory methods in food microbiology*. (3rd Ed.). Academic press, London.
61. Hasan, A., Reza, A., Rume, F.I., Meher, M.M., Parvej, M.S. and Anower, A.M. 2018. Prevalence and antibiotic resistance pattern of *Escherichia coli* isolated from raw dairy milk. *Turkish J. Agri. Food Sci. Tech.*. **6**(9):1108.
62. Hatha, M., Vivekanandhan, A.A., Joice, J.G. and Christol. 2005. Antibiotic resistance pattern of motile aeromonads from farm raised fresh water fish. *Int. J. of Food Microbiol.* **98**(2):131–134.
63. Hoel, S., Vadstein, O. and Jakobsen, A.N. 2017. Species distribution and prevalence of putative virulence factors in mesophilic *Aeromonas* spp. isolated from fresh retail sushi. *Frontiers Microbiol*. **8**:931.
64. Holsinger, V.H., Rajkowski, K.T. and Stabel, J.R. 1997. Milk pasteurisation and safety: A brief history. *Rev. Sci. Tech. Off. Int. Epizooties.* **16:**441-451.
65. Hull, R.R.; Toyne, S.; Haynes, I.N. and Lehmann, F.L. 1992. Thermoduric bacteria: A re-emerging problem in cheese making. *Australian J. of Dairy Tech.* **47**:91-94.
66. Igbinosa, I.H. 2014. Antibiogram profiling and pathogenic status of *Aeromonas* species recovered from Chicken. *Saudi J. of Biological Sci*. **21**(5):481–485.
67. Igbinosa, I.H. and Okoh, A.I. 2012. Antibiotic susceptibility profile of *Aeromonas* species isolated from wastewater treatment plant. *Scient. Wld. J*. **9:**1–6.
68. IS: 1479 (Part 1). 1960. *Methods of test for dairy industry, Part 1- Rapid examination of milk*. Indian Standards Institution, Manak bhavan, New delhi-1
69. IS: 5402. 1969. *Method for Plate Count of Bacteria in Foodstuffs.* Indian Standards Institution, Manak bhavan, New delhi-1
70. ISO 18593. 2004 *Microbiology of food and animal feeding stuffs — Horizontal methods for sampling techniques from surfaces using contact plates and swabs.* International organization for standardization, Geneva, Switzerland.

71. Isonhood, J and Drake. M 2002. Stress response of *Aeromonas hydrophila* following environmental challenges. *Food Microbiol*. **19**(4):285–293.

72. Issa, A.T. and Tahergorabi, R. 2019. Milk Bacteria and Gastrointestinal Tract: Microbial Composition of Milk. In: Watson, R.R. and Preedy, V.R. (ed.). *Dietary Interventions in Gastrointestinal Diseases*. Academic Press, Cambridge, pp. 265-275.

73. Jahid, I.K., and Ha, S.D. 2014. Inactivation kinetics of various chemical disinfectants on *Aeromonas hydrophila* planktonic cells and biofilms. *Foodborne Pathogens Dis*. **11**(5):346–353.

74. Jamal, J.B., Akter, S. and Uddin, M.A. 2019. Microbiological quality determination of pasteurized, UHT and flavoured milk sold in Dhaka, Bangladesh. *Stamford J. Microbiol*. **8**(1):1–6.

75. James, L., Beena, A.K., Sudhakaran. A.V and Praseeda. K.S. 2018. Isolation and assessment of antibiotic response pattern of heat resistant *Staphylococcus aureus* from milk. *Int. J. Curr. Microbiol. App. Sci*. **7**(5):3322-3333

76. Jayarao, B.M., Pillai, S.R., Sawant, A.A., Wolfgang, D.R. and Hegde, N.V. 2004. Guidelines for monitoring bulk tank milk somatic cell and bacterial counts. *J. Dairy Sci*. **87**(10):3561–3573.

77. Jayarao, B. M. and Wang, L. 1999. A study on the prevalence of gram-negative bacteria in bulk tank milk. *J. Dairy Sci*. **82**:2620-2624. https://doi.org/10.3168/jds.S0022- 0302(99)75518-9.

78. Johnston, D.W. and Bruce, J. 1982. Incidence of thermoduric psychrotrophs in milk produced in the west of Scotland. *J. Appl. Bact*. **52**(3):333–337.

79. Juneja, V.K., Eblen, B.S. and Ransom, G.M. 2001. Thermal inactivation of *salmonella* spp. in chicken broth, beef, pork, turkey, and chicken: determination of D- and Z-values. *J. Food Sci*. **66**(1):146–152.

80. Kamana, O., Ceuppens, S., Jacxsens, L., Kimonyo, A. and Uyttendaele, M. 2014. Microbiological quality and safety assessment of the Rwandan milk and dairy chain. *J. Food Prot*. **77**(2):299–307.

81. Kang, J.W., Lee, H.Y. and Kang, D.H. 2020. Synergistic bactericidal effect of hot water with citric acid against *Escherichia coli* O157:H7 biofilm formed on stainless steel. *Food Microbiol*. **217**:85-93

82. Karunakaran, T. and Devi, B.G. 1994. Characterisation of haemolytic activity from *Aeromonas caviae*. *Epidemiology and Infection*. **112**(2):291–298.

83. Kikuchi, M., Matsumoto, Y., Sun XueMei, and Takao, S. 1996. Incidence and significance of thermoduric bacteria in farm milk supplies and commercial pasteurized milk. *Anim. Sci. Technol*. **67**(3):265-272.
84. Kim, D.H. and Austin, B. 2008. Characterization of probiotic *Carnobacteria* isolated from rainbow trout (*Oncorhynchus mykiss*) intestine. *Lett. Appl. Microbiol*. **47**(3):141–147.
85. Kirov, S.M., Castrisios, M. and Shaw, J.G. 2004. *Aeromonas* flagella (polar and lateral) are enterocyte adhesins that contribute to biofilm formation on surfaces. *Infect. Immun*. **72**:1939–1945.
86. Kirov, S.M., Tassell, B.C., Semmler, A. B.T., O'Donovan, L.A., Rabaan, A.A. and Shaw, J.G. 2002. Lateral flagella and swarming motility in *Aeromonas* species. *J. Bact*. **184**(2):547–555
87. Korres, N A.M., Aquije, G.M.F.V., Buss, D.S., Ventura, J.A., Fernandes, P.M.B. and Fernandes, A.A.R. 2013. Comparison of biofilm and attachment mechanisms of a phytopathological and clinical isolate of *Klebsiella pneumoniae* spp. *Pneumoniae*. *Scient. Wld. J*. **13:**1–6.
88. Koushki, M., Koohy-Kamaly, P., Azizkhani, M. and Hadinia, N. 2016. Microbiological quality of pasteurized milk on expiration date in Tehran, Iran. *J. Dairy Sci*. **99**(3):1796–1801.
89. Krovacek, K., Dumontet, S., Eriksson, E. and Baloda, S.B. 1995. Isolation, and virulence profiles, of *Aeromonas hydrophila* implicated in an outbreak of food poisoning in Sweden. *Microbiol. Immunol.* **39**:655–661
90. Kühn, I., Albert, M.J., Ansaruzzaman, M., Bhuiyan, N.A., Alabi, S.A., Islam, M.S., Neogi, P.K., Huys, G., Janssen, P., Kersters, K. and Möllby, R. 1997. Characterization of *Aeromonas* spp. isolated from humans with diarrhea, from healthy controls, and from surface water in Bangladesh. *J. Clin. Microbiol*. **35**(2):369–373.
91. Kumari, S. and Sarkar, P.K. 2014. In vitro model study for biofilm formation by Bacillus cereus in dairy chilling tanks and optimization of clean-in-place (CIP) regimes using response surface methodology. *Food Control*. **36**(1):153–158.
92. Landini, P., Antoniani, D., Burgess, J.G. and Nijland, R. 2010. Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Appl. Microbiol. Biotech*. **86**(3):813–823.

93. Li, M.Y., Zhang, J., Lu, P., Xu, J.L. and Li, S.P. 2009. Evaluation of biological characteristics contributing to biofilm formation. *Pedosphere*. **19**(5):554-561.

94. Lian, Z., Bai, J., Hu, X., Lü, A., Sun, J., Guo, Y. and Song, Y. 2020. Detection and characterization of *Aeromonas salmonicida* spp. salmonicida infection in crucian carp *Carassius auratus*. *Vet. Res. Commun*. **44**(2):61–72.

95. Lim, S., Yun, H., Joe, M. and Kim, D. 2009. Development of the radiation-resistant strain of *Moraxella osloensis* and effect of penicillin G on its growth. *Radiation Phys. Chem*. **78**:437–440.

96. Lomander, A. 2004. Evaluation of chlorines' impact on biofilms on scratched stainless steel surfaces. *Bioresource Tech*. **94**(3):275–283.

97. Lopes, M., Ribeiro, T., Abrantes, M., Figueiredo Marques, J.J., Tenreiro, R. and Crespo, M.T.B. 2005. Antimicrobial resistance profiles of dairy and clinical isolates and type strains of enterococci. *Int. J. Food Microbiol*. **103**(2), 191–198.

98. Machado, M.A.A., Ribeiro, W.A., Toledo, V.S., Ramos, G.L.P.A., Vigoder, H.C. and Nascimento, J.S. 2020. Antibiotic resistance and biofilm production in catalase-positive gram-positive cocci isolated from Brazilian pasteurized milk. *J. Food Quality Hazards Control*. **7:**67-74

99. Mahari, T. and Gashe, B.A. 1990. A survey of the microflora of raw and pasteurized milk and the sources of contamination in a milk processing plant in Addis Ababa, Ethiopia. *J. Dairy Res*. **57**(2):233–238.

100. Mane, N.V. and Gandhi, M.B. 2010. Studies on proteolytic thermoduric psychrotrophic bacteria in milk and fermented milk products. *J. Env. Res. Dev*. **5**:384-392

101. Manna, S.K., Maurye, P., Dutta, C. and Samanta, G. 2013. Occurrence and virulence characteristics of *Aeromonas* species in meat, milk and fish in India. *J. Food Safety*. **33**:461-469

102. Marchand, S., De-Block, J., De-Jonghe, V., Coorevits, A., Heyndrickx, M. and Herman, L. 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. *Comprehensive Rev. Food Sci. Food Safety*. **11**(2):133–147.

103. Marti, R., Muniesa, M., Schmid, M., Ahrens, C.H., Naskova, J. and Hummerjohann, J. 2016. Heat-resistant *Escherichia coli* as potential persistent reservoir of extended-spectrum β-lactamases and Shiga toxin-encoding phages in dairy. *J. Dairy Sci*. **99**(11):8622–8632.

104. Marti, R., Schmid, M., Kulli, S., Schneeberger, K., Naskova, J., Knøchel, S., Ahrens, C.H. and Hummerjohann, J. 2017. Biofilm formation potential of heat-resistant *Escherichia coli* dairy isolates and the complete genome of multidrug-resistant, heat-resistant strain fam21845. *Appl. Environ. Microbiol*. **83**(15).
105. Martin, J.H. 1981. Heat resistant mesophilic microorganisms. *J. Dairy Sci*. **64**(1):149–156.
106. Maxcy, R.B. 1977. Comparative viability of unirradiated and gamma irradiated bacterial cells. *J. Dairy Sci*. **42**(4):1056–1059.
107. McAuley, C.M., Gobius, K.S., Britz, M.L. and Craven, H.M. 2012. Heat resistance of thermoduric enterococci isolated from milk. *Int. J. Food Microbiol.* **154**:162–168.
108. Md Zain., Norbaizura, S., Flint., Steve., Bennett., Rod., Tay. and Hong-Soon. 2015. Biofilm formation by *B. licheniformis* isolated from whey protein concentrate 80 powder using three different media and two surfaces. In: *New Zealand Microbiological Society Conference*, Rotorua, New Zealand. Volume: 60
109. Meer, R.R., Baker, J., Bodyfelt, F.W. and Griffiths, M.W. 1991. Psychrotrophic *Bacillus* spp. in fluid milk products. *J. Food Prot*. **54**:11.
110. Melas, D.S., Papageorgiou, D.K. and Mantis, A.I. 1999. Enumeration and Confirmation of *Aeromonas hydrophila*, *Aeromonas caviae*, and *Aeromonas sobria* isolated from raw milk and other milk products in Northern Greece. *J. Food Prot*. **62**(5):463–466.
111. Meng, L., Liu, H., Lan, T., Dong, L., Hu, H., Zhao, S., Zhang, Y., Zheng, N. and Wang, J. 2020. Antibiotic resistance patterns of *Pseudomonas* spp. isolated from raw milk revealed by whole genome sequencing. *Frontiers Microbiol*. **11**:1005.
112. Mercer, R.G., Zheng, J., Garcia-Hernandez, R., Ruan, L., Gänzle, M.G. and McMullen, L.M. 2015. Genetic determinants of heat resistance in *Escherichia coli. Frontiers Microbiol*. **6**:130-138
113. Meredith, T.A. and Ulrich, J.N. 2013. Infectious Endophthalmitis. In: Ryan, S.J., Sadda, S.R., Hinton, D.R., Schachat, A.P., Wilkinson, C.P., Wiedemann, P., Schachat, A.P. (eds.), *Retina* (5th Ed.).W.B. Saunders, Philadelphia, pp. 2019-2039

114. Merritt, P.M., Danhorn, T. and Fuqua, C. 2007. Motility and chemotaxis in *Agrobacterium tumefaciens* surface attachment and biofilm formation. *J. Bact.* **7:**8005–8014

115. Miñana-Galbis, D., Farfán, M., Lorén, J.G. and Fusté, M.C. 2002. Biochemical identification and numerical taxonomy of *Aeromonas* spp. isolated from environmental and clinical samples in Spain. *J. Appl. Microbiol*. **93:**363-519.

116. Mogrovejo, D.C., Perini, L., Gostinčar, C., Sepčić, K., Turk, M., Ambrožič-Avguštin, J.,Brill, F.H.H. and Gunde-Cimerman, N. 2020. Prevalence of antimicrobial resistance and hemolytic phenotypes in culturable arctic bacteria. *Frontiers Microbiol*. **11**:570.

117. Momba, M.N.B., Kfir, R., Venter, S.N and Cloete. 2000. An overview of biofilm formation in distribution systems and its impact on the deterioration of water quality. *Wat. S.A.* **26**:59-66

118. Muir, D.D. 2011. *The stability and shelf life of milk and milk products*. Woodhead Publishing Series, Cambridge, pp. 755-778

119. Munsch-Alatossava, P., and Alatossava, T. 2007. Antibiotic resistance of raw-milk-associated psychrotrophic bacteria. *Microbiol. Res*. **162**(2):115–123.

120. Munsch-Alatossava, P., Gauchi, J.P., Chamlagain, B., and Alatossava, T. 2012. Trends of antibiotic resistance in mesophilic and psychrotrophic bacterial populations during cold storage of raw milk. *ISRN Microbiol*. **12**:1–13.

121. Murphy, S.C. (2007). Trouble-shooting sources and causes of high bacteria counts in raw milk. Dairy Food Environ. Sanit.. 20. 606-611.

122. Nagwa I., Ahmed L., Salah F.A, Aal, A.E, Ayoub, M.A., Magdy, S. and El Sayed . 2014. Enumeration and characterization of *Aeromonas* spp. isolated from milk and some dairy products in Sharkia Governorate, Egypt. *Alexandria J. Vet. Sci*. **40**:52-64

123. Namminga, K. 1999. Health risks of drinking raw (unpasteurized) milk. *PhD Thesis*. South Dakota State University, Brooking, SD.

124. Nishikawa, Y., Ogasawara, J. and Kimura, T. 1993. Heat and acid sensitivity of motile *Aeromonas*: A comparison with other food-poisoning bacteria. *Int. J. Food Microbiol*. **18**(4):271–278.

125. Olson, J.C., and Mocquat, G. 1980. Milk and milk products. In: Silliker , J.H., Elliot, R.P., Baird, A.C., Bryan, F.L., Christion, J.H., Clark, D.S., Olson,

J.C., Roberts, T.A. (eds.), *Microbial Ecol. Foods*. Academic Press, New York, NY,470p.

126. Ostrov, I., Paz, T. and Shemesh, M. 2019a. Robust biofilm-forming *Bacillus* isolates from the dairy environment demonstrate an enhanced resistance to cleaning-in-place procedures. *Foods* **8**(4):14-26.
127. Ostrov, I., Sela, N., Belausov, E., Steinberg, D. and Shemesh, M. 2019b. Adaptation of *Bacillus* species to dairy associated environment facilitates their biofilm forming ability. *Food Microbiol*. **82**:316–324.
128. Palumbo, S.A. and Buchanan, R.L. 1988. Factors affecting growth or survival of *Aeromonas hydrophila* in foods. *J. Food Safety*. **9**(1):37–51.
129. Palumbo, S.A., Morgan, D.R. and Buchanan, R.L. 1985. Influence of temperature, NaCl, and pH on the growth of *Aeromonas hydrophila*. *J. Food Sci*. **50**(5):1417–1421.
130. Pantoja, J.C.F., Reinemann, D.J. and Ruegg, P.L. 2009. Associations among milk quality indicators in raw bulk milk. *J. Dairy Sci*. **92**(10):4978–4987.
131. Park, S.Y., and Ha, S.D. 2014. Effect of temperature on the growth kinetics and predictive growth model of *Aeromonas hydrophila* on squid (*Sepioteuthis sepioidea*). *Food Sci. Biotech*. **23**(1):307–312.
132. Parkar, S.G., Flint, S.H. and Brooks, J.D. 2004. Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *Journal of Appl. Microbiol*. **96**(1):110–116.
133. Peng, S., Hummerjohann, J., Stephan, R. and Hammer, P. 2013. Heat resistance of *Escherichia coli* strains in raw milk at different subpasteurization conditions. *J. Dairy Sci*. **96**(6):3543–3546.
134. Ponce De Leon, S., Inoue, N. and Shinano, H. 1993. Effect of acetic and citric acids on the growth and activity (VB-N) of *Pseudomonas* sp. and *Moraxella* sp. *Bull. Fac. Fish. Hokkaido Univ*.**44**:80-85
135. Popović, N., Dinić, M., Tolinački, M., Mihajlović, S., Terzić-Vidojević, A., Bojić, S., Djokić, J., Golić, N. and Veljović, K. 2018. New insight into biofilm formation ability, the presence of virulence genes and probiotic potential of *Enterococcus* sp. dairy isolates. *Frontiers Microbiol*. **9**:78.
136. Prakash, M., Karthikeyan, V. and Karmegam, N. 2006. Determination of total counts of psychrotrophic bacteria and coliforms in milk samples. *J. Ecotoxicology Environ. Monitoring*. **16**:291-296

137. Prescott, L.M., Harley,J.P.and Klein,D.A. 1993.*Microbiology* (2nd Ed.).Wm.C.Brown Communications ,Inc. Dubuque, Iowa, 912p.

138. Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F. and Cotter, P.D. 2013. The complex microbiota of raw milk. *FEMS Microbiol. Rev.* **37**(5):664–698.

139. Rabaan, A.A., Gryllos, I., Tomás, J.M. and Shaw, J.G. 2001. Motility and the polar flagellum are required for *Aeromonas caviae* adherence to Hep-2 cells. *Infect. Immun.* **69**(7):4257–4267.

140. Ralyea, R.D., Wiedmann, M. and Boor, K.J. 1998. Bacterial tracking in a dairy system using phenotypic and ribotyping methods. *J. Food Prot*. **61**:1336-40.

141. Ramachandran, G. 2014. Gram-positive and Gram-negative bacterial toxins in sepsis. *Virulence*. **5**(1):213–218.

142. Ranieri, M.L., Huck, J.R., Sonnen, M., Barbano, D.M. and Boor, K.J. 2009. HTST pasteurization temperatures inversely affect bacterial numbers during refrigerated storage of pasteurized fluid milk. *J. Dairy Sci*. **92**(10):4823–4832.

143. Ribeiro Junior, J.C., Beloti, V., Massi, F.P. and Fungaro, M.H.P. 2017. Thermoduric psychrotrophic proteolytic microbiota from refrigerated raw milk. *Semina: Ciências Agrárias*. **38**(1):267.

144. Ribeiro Júnior, J.C., Tamanini, R., de Oliveira, A.L.M., Alfieri, A.A. and Beloti, V. 2018. Genetic diversity of thermoduric spoilage microorganisms of milk from Brazilian dairy farms. *J. Dairy Sci*. **101**(8).

145. Ryu, J.H. and Beuchat, L.R 2005a. Biofilm formation and sporulation by *Bacillus cereus* on a stainless steel surface and subsequent resistance of vegetative cells and spores to chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer. *J. Food Prot*. **68**(12):2614-2622

146. Ryu, J.H. and Beuchat, L.R. 2005b. Biofilm formation by *Escherichia coli* O157:H7 on stainless steel: Effect of exopolysaccharide and curli production on its resistance to chlorine. *Appl. Environ. Microbiol*. **71**(1):247–254.

147. Sadiq, F.A., Flint, S., Yuan, L., Li, Y., Liu, T. and He, G. 2017. Propensity for biofilm formation by aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders, *Int. J. Food Microbiol*. **262**:89-98.

148. Sadiq, F.A., Flint, S., Yuan, L., Li,Y., Liu, T. and He, G. 2017. Propensity for biofilm formation by aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *Int. J. Food Microbiol.* **262**:89–98.

149. Sahnouni, F., Ringø, E., Maizi, A., Belmaghnia, S., Matallah-Boutiba, A., Chemlal, D. and Boutiba, Z. 2016. Biochemical and antibacterial potential of autochthonous *Carnobacterium* and *Lactobacillus* species isolated from gastrointestinal tract of coastal fish. *J. Anim. Plant Sci*. **26**(4):1146-1155

150. Sahu, K.A., Nemani, R., Sinha, R. and Pradhan, P. 2019. Isolation and characterization of probiotic from fermented rice, idly and dosa batter and screening of antimicrobial activity. *Int. J. Chem. Tech. Res*. **12**(4):52-58.

151. Samaržija, D., Zamberlin, Š. and Pogačić, T. 2012. Psychrotrophic bacteria and milk and dairy products quality. *Mljekarstvo*. **62**(2):77-95

152. Santos, J.A, López-Díaz, T.M., García-Fernández, M.C., García-López, M.L. and Villalón, O.A. 1996. Fresh ewe's milk Spanish cheese, as a source of potentially pathogenic *Aeromonas* strains. *J. Food Prot.* **59**(12):1288-1291.

153. Santos, J.A., García-López, M.L. and Otero, A. 1999. *Moraxella*. In:Robinson R.K.(ed.) *Encyclopedia of Food Microbiology*, Elsevier,pp. 1487-1492.

154. Santos, P.G., Santos, P.A., Bello, A.R. and Freitas-Almeida, A.C. 2011. Association of *Aeromonas caviae* polar and lateral flagella with biofilm formation: *Aeromonas caviae* flagella and biofilm. *Lett. Appl. Microbiol*. **52**(1):49–55.

155. Schuman, J.D., Sheldon, B.W. and Foegeding, P.M. 1997. Thermal resistance of *Aeromonas hydrophila* in liquid whole egg. *J. Food Prot*. **60**(3):231–236.

156. Scoaris, D.D.O, Colacite, J., Nakamura, C.V., Nakamura, T.U, Filho, B.A.A, Filho, B.P.D. 2008. Virulence and antibiotic susceptibility of *Aeromonas* spp. isolated from drinking water. *Antonie Leeuwenhoek.* **93**:111–122

157. Sharma, M. and Anand, S.K. 2002. Characterization of constitutive microflora of biofilms in dairy processing lines. *Food Microbiol*. **19**(6):627–636.

158. Shi, X. and Zhu, X. 2009. Biofilm formation and food safety in food industries. *Trends Food Sci. Technol.* **20**:407–413.

159. ShuHui, Z., YongFu, H., LiZhi, F., KeFei, S. and DengFeng, X. 2014. Molecular classification and analysis of Moraxella osloensis from goats. *Chinese J. Zoonoses.* **30**(1):12-16.

160. Silva, H.O., Lima, J. A.S., Aguilar, C.E.G., Rossi, G.A.M., Mathias, L.A. and Vidal, A.M.C. 2018. Efficiency of different disinfectants on *Bacillus cereus* biofilms on stainless-steel surfaces in contact with milk. *Frontiers Microbiol.* **9**:2934.

161. Skwor, T. and Králová, S. 2019. *Aeromonas*. In: Doyle, M.P., Diez-Gonzalez, F. and Hill, C. (eds.), *Food Microbiology.* ASM Press, pp. 415–435.

162. Solomon, M., Mulisa, M., Yibeltal, M., Desalegn, G. and Simenew, K. 2013. Bacteriological quality of bovine raw milk at selected dairy farms in DebreZeit town, Ethiopia. *Comprehensive J. Food Sci. Technol*. **1**(1):1-8.

163. Sørhaug, T. and Stepaniak, L. 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. *Trends Food Sci. Tech*. **8**(2):35–41.

164. Srey, S., Jahid, I.K. and Ha, S.D. 2013. Biofilm formation in food industries: A food safety concern. *Food Control*. **31**(2):572–585.

165. Stadhouders, J. 1975. *Lipolysis in hard cheese made from pasteurized milk.* International Dairy Federation, Brussels, Belgium.

166. Stewart, J.C. and Seiberling, D.A. 1996. Clean in place. *Chem. Eng*. **103**(1):72–79.

167. Suhet, M.P., Schocken-iturrino, R.P. and Amaral, L.A. 2011. Hemolytic activity and resistance to antimicrobials by *Aeromonas* species isolated from intensive rearing of nile tilapia (*Oreochromis niloticus*). *Ars Veterinaria*. **27**:36-44.

168. Sung, N., and Collins, M.T. 1998. Thermal tolerance of *Mycobacterium paratuberculosis*. *Appl. Environ. Microbiol*. **64**:999–1005.

169. Talagrand-Reboul, E., Jumas-Bilak, E. and Lamy, B. 2017. The social life of *Aeromonas* through biofilm and quorum sensing. *Frontiers Microbiol.* **8**. 154-168

170. Tattersall,B. 2020. Effect of long pasteurization run times on bacterial numbers in milk. All Graduate Theses and Dissertations. 7910.

171. Te Giffel., Beumer, R.R. and André, G. 1997. Effect of pasturing on the incidence of *Bacillus cereus* spores in raw milk. *Int. Dairy J*. **7**:201–205.

172. Terzić-Vidojević, A., Veljović, K., Begović, J., Filipić, B., Popović, D., Tolinački, M., Miljković, M., Kojić, M. and Golić, N. 2015. Diversity and antibiotic susceptibility of autochthonous dairy enterococci isolates: Are they safe candidates for autochthonous starter cultures? *Frontiers Microbiol*. **6**:1-10

173. Thiran, E., Di Ciccio, P.A., Graber, H.U., Zanardi, E., Ianieri, A. and Hummerjohann, J. 2018. Biofilm formation of *Staphylococcus aureus* dairy isolates representing different genotypes. *J. Dairy Sci*. **101**(2):1000–1012.

174. Tinuoye, O.L. and Harmon, L.G. 1975. Growth of thermoduric psychrotrophic bacteria in refrigerated milk. *Am. Dairy Rev*. **12**:26-30

175. Van Boeijen, I.K.H., Francke, C., Moezelaar, R., Abee, T. and Zwietering, M.H. 2011. Isolation of highly heat-resistant *L. monocytogenes* variants by use of a kinetic modeling-based sampling scheme. *Appl. Env. Microbiol.* **77**:2617–24.

176. Varnam, A.H. and Sutherland, J.P. 2001 *Milk and Milk Products – Technology, Chemistry and Microbiology*, Aspen Publishers, Gaithersburg, MD.

177. Velu, N., Divakar, K., Nandhinidevi, G. and Gautam, P. 2012. Lipase from *Aeromonas caviae* AU04: Isolation, purification and protein aggregation. *Biocatalysis Agri. Biotech*. **1**(1):45–50.

178. Vernon, S.M. 1978. Nutritional and biochemical characterization of *Moraxella* species isolated from fishery sources. *PhD thesis*, Oregon State University, U.S.

179. Versalovic, J. 2011. *Manual of clinical microbiology* . ASM Press, Washington.

180. Vila, J., Marco, F., Soler, L., Chacon, M., and Figueras, M.J. 2002. In vitro antimicrobial susceptibility of clinical isolates of *Aeromonas caviae, Aeromonas hydrophila* and *Aeromonas veronii* biotype *sobria*. *J. Antimicrob. Chemother*. **49**(4):701–702.

181. Villari, P., Crispino, M., Montuori, P. and Boccia, S. 2003. Molecular typing of *Aeromonas* isolates in natural mineral waters. *Appl. Environ. Microbiol*. **69**(1):697–701.

182. Visvalingam, J., Zhang, P., Ells, T.C. and Yang, X. 2019. Dynamics of biofilm formation by *Salmonella typhimurium* and beef processing plant bacteria in mono- and dual-species cultures. *Microbiol. Ecol.* **78**:375–387.

183. Vlková, E., Rada, V., Popelářová, P., Trojanová, I. and Killer, J. 2006. Antimicrobial susceptibility of *Bifidobacteria* isolated from gastrointestinal tract of calves. *Livestock Sci*. **105:**253–259.

184. Vyletelova, M., Hanus, O., Urbanova, E. and Kopunecz, P. 1999. The occurrence and identification of psychrotrophic bacteria with proteolytic and lipolytic activity in bulk milk samples in primary production conditions. *Zivocisna Vyroba.* **45**:373–383.

185. Walsh, C., Meade, J., Mcgill, K. and Fanning, S. 2012. The biodiversity of thermoduric bacteria isolated from whey: Thermotolerant bacteria in whey. *J. Food Safety*. **32**(2):255–261.

186. Washam, C.J., Olson, H.C. and Vedamuthu, E.R. 1977. Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. *J. Food Prot.* **40**(2):101–

108. Johnston, D.W. and Bruce, J. 1982. Incidence of thermoduric psychrotrophs in milk produced in the west of Scotland. *J. Appl. Bacteriol*. **52**(3):333–337.

187. Weber, M., Liedtke, J., Plattes, S. and Lipski, A. 2019. Bacterial community composition of biofilms in milking machines of two dairy farms assessed by a combination of culture-dependent and–independent methods. *PLoS ONE* **14**(9):e0222238.

188. Welch, A.B. and Maxcy, R.B. 1975. Characterization of radiation-resistant vegetative bacteria in beef. *Appl. Microbiol*. **30**(2):242–250.

189. Yuan, L., Sadiq, F.A., Liu, T.J., Li, Y., Gu, J.S., Yang, H.Y. and He, G.Q. 2018. Spoilage potential of psychrotrophic bacteria isolated from raw milk and the thermo-stability of their enzymes. *J. Zhejiang Uni. Sci.* **19**(8):630–642.

190. Yucel, N., Erdem, B. and Kaya, D. 2005. Some virulence properties and characterization of motile *Aeromonas* species from milk and white cheese. *Int. J. Dairy Tech*. **58**(2):106–110

191. Zacaria, J., Delamare, A.P.L., Costa, S.O.P. and Echeverrigaray, S. 2010.Diversity of extracellular proteases among *Aeromonas* determined by zymogram analysis. *J. Appl. Microbiol*. **507**:212-219.

192. Zhang, H., Hu, Y., Zhou, C., Yang, Z., Wu, L., Zhu, M., Bao, H., Zhou, Y., Pang, M., Wang, R. and Zhou, X. 2018. Stress resistance, motility and biofilm formation mediated by a 25kb plasmid pLMSZ08 in *Listeria monocytogenes*. *Food Control*. **94**:345–352.

193. Zhang, Q., Shi, G.Q., Tiang, G.P., Zou, Z.T., Yao, G.H. and Zeng, G. 2012. A foodborne outbreak of *Aeromonas hydrophila* in a college, Xingyi City, Guizhou, China, 2012. *W. Pacif. Surveillance Response J*. **3**(4):39–43.

194. Zhou, G., Liu, H., He, J., Yuan, Y. and Yuan, Z. 2008. The occurrence of *Bacillus cereus*, *B. thuringiensis* and *B. mycoides* in Chinese pasteurized full fat milk. *Int. J. Food Microbiol.* **121**:195–200.

195. Zhou, Y., Yu, L., Nan, Z., Zhang, P., Kan, B., Yan, D. and Su, J. 2019. Taxonomy, virulence genes and antimicrobial resistance of *Aeromonas* isolated from extra-intestinal and intestinal infections. *BMC Infect. Dis*. **19**(1):158.

Acad Form No. 19

KERALA VETERINARY AND ANIMAL SCIENCES UNIVERSITY

COLLEGE OF DAIRY SCIENCE AND TECHNOLOGY

PROGRAMME OF RESEARCH WORK FOR MASTERS DEGREE THESIS

1. Title of thesis

Characterization of Psychrotrophs Surviving Pasteurization

2a. Title of the departmental/KVASU research project of which this forms a part

NIL

2b. Code No. if any, and order by which departmental/KVASU research project is approved

NIL

3a. Name of the student

Vishnu Suresh

3b. Admission No

2018-MDM-05

4a.Name of the Major Advisor (Guide)

Dr. Ligimol James

4b. Designation

Assistant Professor

Department of Dairy Microbiology

College of Dairy Science and Technology

Mannuthy, Thrissur- 680651

5. Objectives of the study

1. To isolate and identify major pasteurization resistant psychrotrophs of dairy origin
2. To assess the cross adaptation potential of selected isolates
3. To understand the factors contributing towards their persistence in dairy environment

6. Practical/Scientific Utility

Pasteurization, the process of heating every particle of milk to at least 63^{o}C for 30min or 72^{o}C for 15 sec or to any temperature-time combination which is equally efficient in approved and properly operated equipment is one of the major thermal processes adopted by the dairy industry.

As pasteurization is intended to keep the product microbiologically safe, any microorganism surviving this process; are of high public health and economic relevance. If the survivors are ‘psychrotrophic’ i.e., capable of growing under refrigerated conditions the situation becomes more serious as refrigerated storage is the most widely recom-

mended storage environment for milk and milk products. Ability of microorganisms to evolve rapidly with the environment is crucial for their survival and it is quite possible that the innate microflora of milk may adopt some adaptation mechanisms upon frequent exposure to this regime of heating and subsequent refrigerated storage. Some of the studies carried out in the department points towards this direction i.e., a shift in dairy associated microflora towards a heat resistant psychrotrophic one. Such a definite change in the microbial pattern could be the result of numerous factors like, society level bulk milk cooler storage of milk, silo storage of raw milk at the processing plant and adoption of higher pasteurization. Modifications occurring in the design of industrial food processing facilities and the increasing international trade may also contribute towards the predominance of this type of microflora in dairy processing environment as well as in products. Such a change can curtail the efficacy of processing and can negatively affect both product quality and safety. In addition, the various adaptation mechanisms adopted by microorganisms against a particular stress may impart cross protection against a range of apparently unrelated challenges. In this context this study is proposed to isolate, identify and characterize predominant pasteurization resistant psychrotrophs in the dairy environment. It is also envisaged to assess the cross adaptation potential of the selected isolates and determine the major features contributing towards their adaptation and persistence in the dairy environment.

7. Important publications on which study is based

Development of stress resistance in *Staphylococcus aureus* after exposure to sub lethal environmental conditions were studied by Cebrian *et al.* (2010) and found that some stress agents induced resistance in bacteria to other stresses in addition.

McAuley *et al.* (2012) evaluated the heat resistance of thermoduric enterococci isolated from milk by screening the $\log_{10}$ reduction incurred after pasteurization at 63°C/30minutes.

In a ten year survey of thermophilic spore-forming bacteria isolated from spoiled canned food, Andre *et al.* (2013) concluded that the spoilage bacteria involved are highly heat-resistant,

thermophilic and non-pathogenic with two species representing 69% of spoilage cases.

On assessing the heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders Sadiq *et al.*(2016) found out that spores of obligate thermophiles are more heat resistant than most of the spores of mesophilic isolates.

Martinez *et al.*(2017) isolated and identified spore-formers associated with concentrated-milk processing in Nebraska and found that among thermophilic strains, the most common one associated with concentrated milk was *B. licheniformis.*

Boll *et al.* (2017) characterized in detail the presence of a variant of LHR (Locus of heat resistance) and demonstrated its presence on a plasmid in the highly heat resistant dairy *E. coli* isolate FAM21805.

Ostrov *et al.* (2019) isolated robust bio-film forming *Bacillus* isolates and demonstrated their enhanced resistance to cleaning in place (CIP). According to their results, the dairy-associated *Bacillus* isolates demonstrate a higher resistance to CIP procedures, compared to the non-dairy strain of *Bacillus subtilis.*

8. Outline of technical programme

Milk, milk products (Ice cream, Cheese, Butter, Curd) as well as samples from dairy processing environment will be collected. All samples will be subjected to laboratory pasteurization at 63°C for 30 minutes followed by refrigerated storage at 7°C for 14 days. Appropriate samples will be plated in plate count agar (PCA) and the Petri-dishes will be incubated at 7±1°C for 10 days (Frank and Yousef, 2004).

Predominant colonies with distinct colony morphologies will be selected, sub-cultured to purity and identified. Spoilage potential of isolates will be assessed by inoculating to sterile milk and storing at 7°C. Samples will be examined at fixed intervals until a defect is noticed.

Isolates will be assessed for their resistance to antibiotics (Decimo *et al*., 2016). Resistance towards CIP cleaning solutions (Commercial detergents used in dairy sector, sodium hydroxide and nitric acid) will be analysed under laboratory conditions (50^{o}C/ 250 rpm/10 min) as per Ostrov *et al*. (2019a).

The isolates will be evaluated for their proteolytic, lipolytic activities and biofilm forming ability (Ostrov *et al.*, 2019b).

9. Main items of observation to be made

- Identification of predominant pasteurization resistant psychrotrophic microorganisms of dairy origin.
- Assessment of the cross adaptation potential of selected isolates in terms of resistance to antibiotics and CIP reagents.
- Major factors contributing towards adaptation and persistence of selected isolates: enzymatic and biofilm formation abilities.

10. Facilities

10a. **Existing**: Laboratory facilities in the Department of Dairy Microbiology and allied departments in College of Dairy Science and Technology will be utilized for the study.

10b. **Additional facilities required:**

- Facilities for molecular level study

11. Duration of study

Four semesters

12. Financial estimate

Cost of chemicals	:10,000/-
Contingencies	:10.000/-
Documentation	:5,000/-
Total	**: ₹ 25,000/-**

Signature of the Student:

Signature of the Major advisor:

Place: Mannuthy

Date: 06/09/2019

Name and signature of members of the Advisory Committee

Chairman

Dr. Ligimol James
Assistant Professor
Department of Dairy Microbiology
College of Dairy Science and Technology
Mannuthy.

Members

Dr. A.K Beena
Professor and Head
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Dr. Aparna Sudhakaran.V
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APPENDIX I

References

André, S., Zuber, F. and Remize, F. 2013. Thermophilic spore-forming bacteria isolated from spoiled canned food and their heat resistance. Results of a French ten-year survey. *Int. J. Food Microbiol*. **165**: 134–143.

Boll, E.J., Marti, R., Hasman, H., Petersen, S., Stegger, M., Ng, K., Knøchel, S., Krogfelt, K.A., Hummerjohann, H. and Struve, C. 2017. Turn up the heat—food and clinical *Escherichia coli* isolates feature two transferrable loci of heat resistance. *Front. Microbiol*. **8**: 1-14.

Cebrián, G., Sagarzazu, N., Pagán, R., Condón, S. and Mañas, P. 2010. Development of stress resistance in *Staphylococcus aureus* after exposure to sub lethal environmental conditions. *Int. J. Food Microbiol.* **140:** 26–33.

Decimo, M., Silvetti, T. and Brasca, M. 2016. Antibiotic resistance patterns of gram-negative psychrotrophic bacteria from bulk tank milk. *J. Food Sci.* **81**(4): M9 44-51.

Frank, J.F. and Yousef, A.E.2004. Tests for groups of microorganisms In: Wehr, H.M. and Frank, J.F. (ed.), *Standard methods for the examination of dairy products*. (17th Ed.). American Public Health Association., Washington, 227p.

McAuley, C.M., Gobius, K.S., Britz, M.L. and Craven, H.M. 2012. Heat resistance of thermoduric enterococci isolated from milk, *Int. J. Food Microbiol*. **154**: 162–168.

Martinez, B.A., Stratton, J. and Bianchini, A. 2017. Isolation and genetic identification of spore formers associated with concentrated milk processing in Nebraska. *J. Dairy Sci.* **100**(2): 1-14.

Ostrov, I., Paz, T. and Shemesh, M. 2019a. Robust biofilm-forming *Bacillus* isolates from the dairy environment

demonstrate an enhanced resistance to cleaning-in-place procedures. *Foods*. **8** (134): 5-14.

Ostrov, I., Selab, N., Belausov, E., Steinberg, D. and Shemesh, M. 2019b. Adaptation of *Bacillus* species to dairy associated environment facilitates their biofilm forming ability. *Food Microbiol.* **82**: 316–324.

Sadiq, F.A., Li, Y., Liu, T., Flint, S., Zhang, G., Yuan, L., Pei, Z. and He, G. 2016. The heat resistance and spoilage potential of aerobic mesophilic and thermophilic spore forming bacteria isolated from Chinese milk powders. *Int. J. Food Microbiol*. **238**: 193–201.

Washam, C.J., Olson, H.C. and Vedamuthu, E.R. Heat-resistant psychrotrophic bacteria isolated from pasteurized milk. *J. Food Prot.* **4**(2): 101-108.

APPENDIX II

Time frame of work

Semester I

1. Collection of literature
2. Planning of research programme

Semester II

1. Collection of literature
2. Preparation of synopsis

Semester III

1. Isolation, characterization and identification of organisms
2. Screening of the organisms

Semester IV

1. Analysing characteristics of the cultures
2. Interpreting results
3. Writing thesis
4. Submission of thesis

CHARACTERIZATION OF PSYCHROTROPHS SURVIVING PASTEURIZATION

Mr. VISHNU SURESH

(2018-MDM-05)

ABSTRACT OF THESIS

Submitted in partial fulfillment of the requirement for the degree of

MASTER OF TECHNOLOGY

(Dairy Microbiology)

2021

Faculty of Dairy Science

Kerala Veterinary and Animal Sciences University

DEPARTMENT OF DAIRY MICROBIOLOGY

COLLEGE OF DAIRY SCIENCE AND TECHNOLOGY

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ABSTRACT

Pasteurization is the major thermal preservation process the dairy industry adopts to address the safety hazards associated with milk and milk products. Microorganisms capable of surviving this process are called the thermodurics. Among these thermodurics, some might be capable of growing under refrigeration conditions. These pasteurization surviving psychrotrophs also termed thermoduric psychrotrophs are of high of high public health and economic significance as refrigerated storage is the most widely recommended storage environment for milk and milk products. In an attempt to understand the thermoduric psychrotrophic profile of local dairy environment with special emphasis to pasteurized milk, a total of 57 samples constituted by 42 samples of pasteurized milk, seven samples of milk products, two raw milk samples from BMC and six dairy equipment swabs were assessed in this study. Thermoduric psychrotrophic organisms were found only in 10.5 per cent of the total samples and 14.3 per cent of pasteurized milk samples tested yielding six thermoduric psychrotrophic isolates. From these isolates, four were selected and genotypically identified as *Aeromonas caviae, Moraxella osloensis,* and two different strains of *Carnobacterium maltaromaticum.* Their nucleotide sequences were deposited in the NCBI database under accession numbers MT071634, MT158663, MT158664 and MT158665 with strain numbers as DMV01, DMV03, DMV05 and DMV06 respectively. On assessing these isolates for motility, haemolytic activity, biofilm forming, defect causing abilities and antibiotic susceptibilities, wide variations were observed in between them. Out of the six isolates, two Gram negative isolates namely *A. caviae* and *M. osloensis* were assessed further for heat resistance at 63^{o}C, resistances to boiling process, pH, ability to grow in milk under room and refrigerated storage. Though both of them could survive the normal LTLT pasteurization process, marked difference was evident in their resistance pattern. The D-value of *A. caviae* DMV01 at 63^{o}C was found to be 4min

38s, much lower than that of *M. osloensis* DMV03 (25min 18s). Both these organisms could not survive boiling even for one second. Despite their high heat resistance both of them were found to be sensitive to exposure to low pH. On assessing the generation time, a clear demarcation was evident in the growth curve of *A. caviae* DMV01 and *M. osloensis* DMV03. Considering the higher growth rate and biofilm forming potential of *A. caviae* DMV01, it was further assessed for its ability to form biofilms on glass surface and stainless steel strips. On subjecting the *A. caviae* DMV01 biofilms formed on stainless steel strips to a laboratory level simulation of CIP regime, 17 per cent of the cells survived on using the CIP solutions at 0.5 per cent whereas none of the cells survived CIP with 1 per cent solutions.

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