

**ANTIBIOTIC RESISTANCE OF
ENTEROCOCCUS SPECIES ISOLATED
FROM MARKET SAMPLES OF DAHI**



**THESIS SUBMITTED TO THE
NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
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IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

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IN
DAIRYING
(DAIRY MICROBIOLOGY)**

**BY
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(B.Tech. Dairy Technology)**

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*Dedicated to my beloved
Sisters & Brothers*

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MASTER OF SCIENCE IN DAIRYING (DAIRY MICROBIOLOGY)


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Dated: 13 June, 2005


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ABSTRACT

Microbial resistance to antibiotics is a worldwide problem. Multi-resistant enterococci are emerging as leading nosocomial pathogens. Intestinal tract of humans and animals is the natural habitats of these organisms. Prevalence and antibiotic resistance profile of enterococci has been studied in various foods of animal origin like meat, poultry, pig, chicken and cheese. In view of the lack of information an attempt has been made to determine the prevalence and antibiotic resistance profile of enterococci in Dahi samples, in this study.

Thirty-eight Dahi sample comprising of 22 samples from local Karnal market including sweet shops and small scale dairy units and 16 samples from villages near about Karnal were taken for the isolation of enterococci using Modified Citrate Azide agar. A total of 31 samples (81.6%) were found to be positive for enterococci, out of which a total of 79 colonies were picked up for identification. Among 79 isolates 53 showed characteristics of enterococci and the study focused on their characterization & antibiotic resistance. Based on the physiological and biochemical tests the isolates were assigned to different species. *E. faecalis* was found to be the most prevalent (24 strains, 45%) followed by *E. faecium* (12 strains, 23%), *E. durans* (6 strains, 11%) and *E. avium* (4 strains, 8%). Seven strains could not be assigned to any particular species as they exhibited atypical sugar fermentation profile.

The resistance of the isolates to 9 different antibiotics was determined by Disc diffusion agar overlay method. All isolates were found to be sensitive to teicoplanin and chloramphenicol, whereas all the strains showed resistance to streptomycin and gentamicin. Resistance to ciprofloxacin was found in 29.2% of *E. faecalis* isolates, 41.7% of *E. faecium*, 83.3% of *E. durans* and 25% of *E. avium* isolates. Most of the isolates were sensitive to vancomycin and tetracycline. Resistance to erythromycin was found in most of the isolates. The MIC of the isolates ranges from 0.25 µg/ml (erythromycin) to > 512 µg/ml (streptomycin). Multiple drug resistance was prevalent in the isolates as all the cultures were resistant to streptomycin and gentamicin; 19 strains showed resistance against Gen. Str. Ery. & Cip.; 25 strains were resistant to Str. Gen. & Ery.; 1 strain was resistant to Str. Gen. & Cip. Horizontal transfer of AR genes and/or infection by such strains can be a cause for concern. Present study showed that enterococci from these samples are sensitive to glycopeptides i.e. vancomycin & teicoplanin. However, MDR strains can be a cause of concern to public safety. Data generated in the present study can be used to elucidate the role of Dahi in the spread of antibiotic resistance to human population in subsequent risk assessment exercises. This study has led to the establishment of a well characterized collection of 53 AR enterococcal strains.

सारांश

सूक्ष्मजीवों का एंटीबायोटिक्स के लिए प्रतिरोध पूरे विश्व की समस्या है। बहु एंटीबायोटिक प्रतिरोधी एंटीरोकाक्साई हस्पतालों से संक्रमण का कारण बनते जा रहे हैं। ये जीवाणु मनुष्य तथा अन्य जानवरों की आंत में सामान्यतः पाए जाते हैं। गीट तथा जानवरों से मिलने वाले भोज्य पदार्थों में एंटीरोकाक्साई की उपस्थिति व एंटीबायोटिक प्रतिरोध विषय पर अध्ययन किया जा रहा है। इस विषय में दही पर समुचित ज्ञान के अभाव में यह अध्ययन दही में एंटीरोकाक्साई की उपस्थिति व एंटीबायोटिक प्रतिरोध को जानने का एक प्रभाव है।

दही के कुल 38 नमूनों की जांच की गई जिसमें 22 नमूने करनाल शहर की दुकानों से लिए गए तथा 16 नमूने आस-पास के गांव से लिए गए। इन नमूनों से एंटीरोकाक्साई की गणना व कल्चर प्राप्त करने के लिए रूपांतरित सिट्रेट एजाइड अगार का प्रयोग किया गया। कुल 31 नमूनों में (81.6%) एंटीरोकाक्साई पाए गए जिनमें मुख्यतः इ.फिकेलिस, इ.फिसियम, इ. डयूरनस व इ.एवियम प्रजातियों की पहचान की जा सकी। कुल 53 कल्चर प्राप्त करके उनका एंटीबायोटिक प्रतिरोध के लिए डिस्क डिफ्यूजन द्वारा किया अध्ययन गया। कुल 9 एंटीबायोटिक्स इस अध्ययन के लिए प्रयुक्त किए गए। सभी एंटीरोकाक्साई टीकोपलैनिन, बैंकोमाइसिन व क्लोरमफेनिकल से संवेदनशील पाए गए। इसके विपरीत सभी एंटीरोकाक्साई स्ट्रेप्टोमाइसिन व जैंटामाइसिन से प्रतिरोधी पाए गए। सिप्रोकलोकससिन व इरीथरोमाइसिन के लिए इ. फिकेलिस (29.2%) इ. फिसियम (41.7%), इ. डयूरनस (83.3%) व इ.एवियम (25%) प्रतिरोधी स्ट्रेन पाए गए। प्रतिरोधी स्ट्रेनज की एम.आई.सी. 0.25 माईक्रोग्राम/मिली से ~ 512 माईक्रोग्राम/मिली के बीच पाई गई। बहुप्रतिरोधक भी भारी मात्रा में व्याप्त पाया गया। 19 स्ट्रेन

1. INTRODUCTION

Microbial resistance to antibiotics is a world-wide problem in human and veterinary medicine. Extensive use of antibiotic has led to the emergence and dissemination of resistant bacteria and resistance genes in animals and humans. In both populations antibiotics are used for therapy and prophylaxis of infectious diseases.

Enterococci are widely distributed in nature. Intestinal tract of humans and animals is the natural habitat of this organism. They gain entry into raw material and foodstuffs through the water supply, food animals, or unhygienic conditions of the production and handling. Enterococci survive in environmental conditions that destroy other organisms of sanitary significance. Contrary to other faecal bacteria that are released into the environment, the enterococci can survive for a long time also outside their natural intestinal hosts. Due to their resistance to freezing, low pH and moderate heat treatment; the enterococci have been suggested as indicator of faecal contamination in food products (Banwart, 1989).

Even though the enterococci are not regarded as highly pathogenic organisms, they are among the most common organisms encountered in nosocomial infections (Lukasova and Sustackova, 2003). These bacteria are responsible for infections as endocarditis, urinary tract infections and hospital acquired bacteremia. In the human intestine *E. faecalis* and *E. faecium* are the most prevalent species. In production animals like poultry, cattle and pigs *E. faecium* is a predominant species, but other species also occur in high numbers like *E. faecalis*, *E. gallinarum*, *E. durans* and *E. avium* are encountered less frequently.

A range of natural and acquired antibiotic resistance is characteristic of enterococci. The natural resistance includes cephalosporines, aminoglycosides (low level type), polymixins, lyncomycin and clindamycin (mostly). Additionally,

enterococci often possess resistance or intermediate susceptibility to quinolones (Lukasova and Sustackova, 2003). They are able to acquire resistances to macrolides, tetracyclines, chloramphenicol, trimethoprim, rifampicin, aminoglycosides (high level type) and ampicillin. In addition to selection in hospitals, another potential source of acquired resistance genes is the therapeutic use of antimicrobial agents in veterinary medicine and animal feeding.

Enterococci are found as a component of the natural microflora of certain foods, where they may have beneficial effects. In certain cheeses they are significant in ripening and development of flavor (Centeno *et al.*, 1996; Wessels *et al.*, 1990). Some enterococci also produce bacteriocin (Girrafa, 1995). Enterococci are also used as starter cultures and probiotics to improve the microbial balance of the intestine (Aguirre & Collins, 1993; Gasser, 1994; Bellomo *et al.*, 1980; Lewenstein *et al.*, 1979). Enterococci enter into foods through faecal contamination and may be implicated in foodborne illnesses. The implications of enterococci for food safety have been reviewed by Franz *et al.* (1999).

Triggered by the apparent duality between their beneficial and harmful properties, a lot of research has focused on the potential role of food enterococci as reservoirs and/or vehicles of antibiotic resistance (AR) in many commodities (Klare *et al.*, 2003; Klein, 2003). Prevalence and antibiotic resistance patterns of enterococci has been studied in different foods like cheese (Huys *et al.*, 2004), sausages, minced meat & cheese (Peters *et al.*, 2003), pigs and broiler (Aarestrup *et al.*, 2000b, 2001), retail meats (Hayes *et al.*, 2003), chicken & turkey (Simjee *et al.*, 2002) and many more. However information from India is rather scanty. Batish (1980) investigated occurrence of enterococci and their virulence factors in kulfi, cream, butter and skim milk powder. Mathur *et al.* (2003) reported antibiotic resistance of isolates of enterococci from clinical samples at AIIMS, New Delhi. There is a general paucity of information on

fermented milks which are consumed in large quantity in India. Dahi is the most popular fermented milk in India it is prepared by almost every housewife by traditional method of back sloping. Dahi is consumed without heat treatment and the microflora enters our intestinal tract and interacts with the intestinal bacteria. This study was undertaken to determine the prevalence of enterococci in Dahi samples from local Karnal market and adjoining villages; and their pattern of antibiotic resistance.

2. Review of Literature

The history of the enterococci began when Thiercelin (1899) first used the term to indicate the intestinal origin of a gram positive diplococcus. The new genus *Enterococcus* was proposed by Thiercelin and Janhaud (1903). Later on, Andrews and Horder (1906) renamed Thiercelin's "enterocoque" as *Streptococcus faecalis*. It was assumed that the strain, isolated from a patient with endocarditis, originated from the human intestine. Based on the serological typing system for streptococci developed by Lancefield (1933), enterococci react with group D antisera. This observation is in agreement with the classification suggested by Sherman (1937) who divided the streptococci into four groups, enterococci, lactic, viridans and pyogenic. The terms faecal streptococci, enterococci and group D streptococci have often been used simultaneously. Finally the genus *Enterococcus* was officially established when Schleifer and Kilpper-Balz (1984) proposed that enterococci should be separated from the genus *Streptococcus*.

According to the physiological criteria described by Sherman (1937), enterococci are able to grow at 10 and 45⁰C, survive for at least 30 min at 60⁰C, and grow at a PH 9.6 and in the presence of 6.5% NaCl (w/v). Because of their ability to ferment carbohydrates to L-lactic acid, enterococci are well known as typical homofermentative lactic acid bacteria (LAB). Basically enterococci are facultatively anaerobic, gram positive bacteria which occur ubiquitously. They can be found in soil, on plants, in milk products and other foods. They are part of normal microflora in the gastro-intestinal tract as well as in the faeces of vertebrates. Their ability to survive in the environment, their pronounced heat resistance and the fact that they can dominate the microbial population of heat treated foods, implies that enterococci can be used as indicator for faecal contamination of water (Stiles and Holzapfel, 1997).

Enterococcus species are ubiquitous, commensal inhabitants of the gastrointestinal tract of humans and animals. They are frequently isolated from environmental sources such as soil, surface water and raw plant and animal products where their intrinsic ruggedness allow them to persist and spread in the environment (Johnston and Jaykus, 2004). Because of their high prevalence in the gastrointestinal tracts of many food animals, it is often unavoidable that these organisms enter the human food chain via contamination of raw meat and raw milk. They are even found as a component of the natural flora of certain foods where they may have beneficial effects. In certain cheeses they are significant in ripening and development of flavour (Wessels *et al.*, 1990; Centeno *et al.*, 1996).

Enterococci may cause infections of the urinary tract, bloodstream, endocardium, abdomen, biliary tract, and burn wounds (Jett *et al.*, 1994). Besides the problems of increasing resistance of enterococci to antibiotics, several virulence factors have been discovered (Murray 1990; Jett *et al.*, 1994; Morrison *et al.*, 1997; Hardie and Whiley, 1997; Witte *et al.*, 1999; Mundy *et al.*, 2000; Eaton and Gasson, 2001; Franz *et al.*, 2001; Girrafa, 2002).

No phenotypic criteria are available for clearly distinguishing the genus *Enterococcus* unequivocally from others, since there are no particular criteria, which are typical of all enterococci. This means that identification of the genus level necessarily is followed by species identification: e.g. when a strain shows the characteristics of an enterococcal species, it can be presumed that the strain is an *Enterococcus* (Devriese *et al.*, 1995).

2.1 Isolation and Enumeration of Enterococci

Owing to the importance of enterococci in different foods, feeds, and clinical and environmental samples, a diversity of media has been described and proposed. Commonly two culture media are applied, the (membrane filter) *Enterococcus* selective (SB) agar according to Slanetz and Bartley (1957) and the Kanamycin Aesculin Azide (KAA) agar. These media usually form the basis

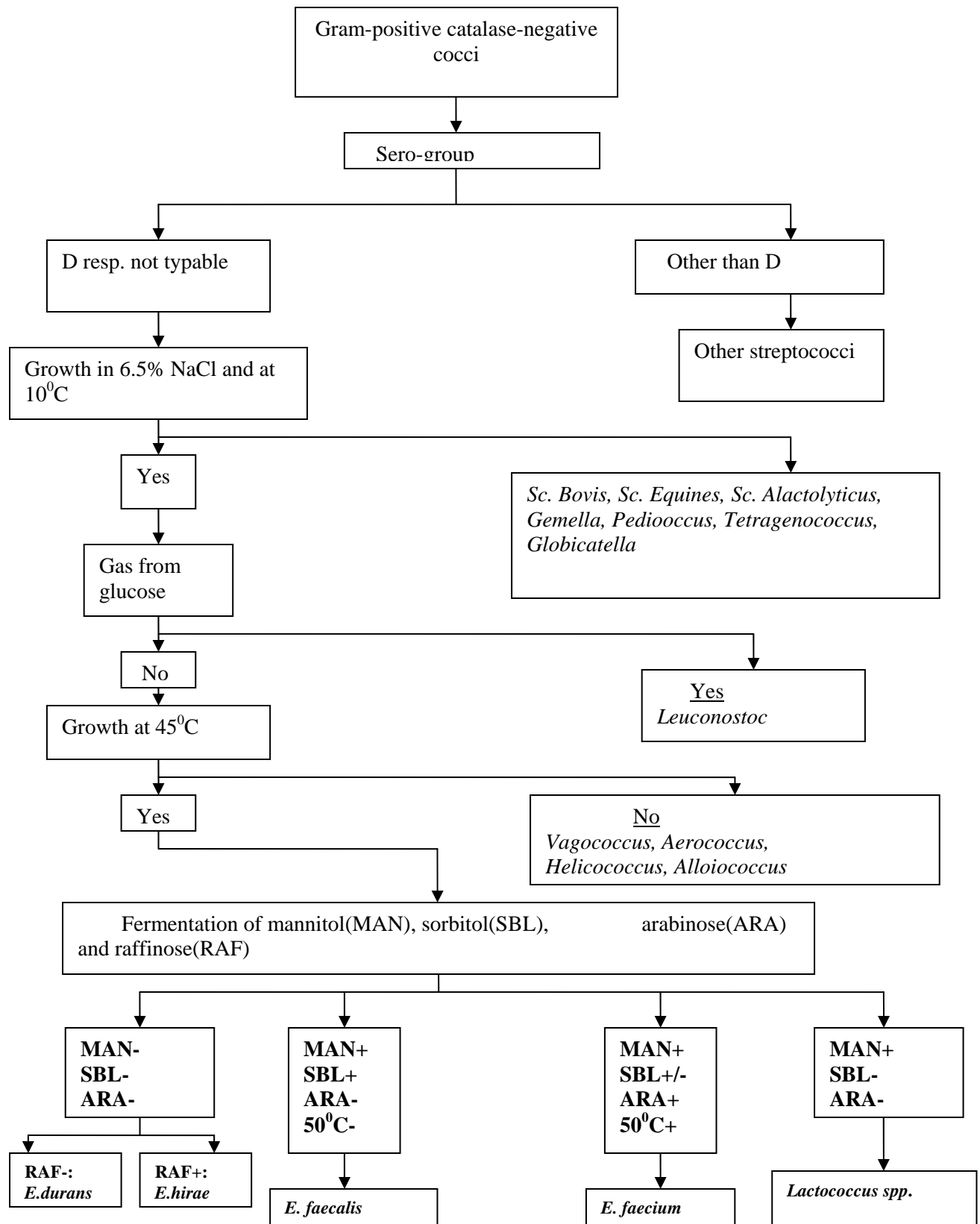


Fig.2.1: Differentiation of enterococci from other Gram-positive, catalase-negative cocci (modified according to Facklam and Elliott, 1995.)

for the estimation of enterococcal counts in water, food, feeds and clinical specimens. In addition to these media, other substrates such as MRS (De Man *et al.*, 1960) or Rogosa agar (Devriese *et al.*, 1991) have been frequently used. However, there are only useful if enterococci are the only microbial component in the product. In the case of selective enumeration as single components, the media described above are advantageously applied. However a much more complicated situation exists if samples containing a mixed microflora have to be examined. For these purpose, the use of media containing either selective chromogenic dyes or selectively inhibitory substances (e.g., antibiotics) may enable some differential bacteriological enumeration (Kneifel *et al.*, 1994).

Because of their requirements for several vitamins and amino acids, enterococci can not be grown easily in synthetic media. Profuse and rapid growth is only achieved if rich complex media such as Brain Heart Infusion (BHI) broth or Trypticase Soy (TS) broth are used (Devriese *et al.*, 1995). Like other members of the LAB, enterococci are often found associated with a microflora of considerable diversity. Therefore, quantitative and selective isolation methods or, in some cases, elective media are needed (Reuter, 1985). A number of selective agents, incubation conditions, and combinations thereof have been reported. Most of these media lack sufficient selectivity, which is necessary to clearly distinguish enterococci from the accompanying microflora. Comparisons of media suggested for the enumeration of enterococci have been published by Mallnam and Seligann (1950) and Barnes (1959). For the isolation of faecal streptococci, various selective and differential agents have been used in numerous media (Hartman *et al.*, 1966; Barnes, 1976).

For the identification of enterococci it is of primary importance to differentiate them from other Gram-positive, catalase-negative cocci. This is possible for the differentiation from the genus *Streptococcus* (*Sc.*) by confirming the serological group D for enterococci according to Lancefield. Only a few species of streptococci also belong to serogroup D: *Sc. Bovis*, *Sc. Alactolyticus*

and *Sc. Equines*. These streptococci can be differentiated from enterococci by the lack of growth in 6.5% NaCl and at 10⁰C.

More difficult is the differentiation from other cocci such as *Pediococcus*, *Latococcus*, or *Tetragenococcus*, if serotyping is not possible. A considerable number of strains (depending on the species 10-70%) are not typable (Reuter, 1995). A simplified testing scheme with reactions to be considered is given in **Fig. 2.1** modified according to Facklam and Elliott (1995).

2.2 Media used for the Examination of Enterococci in Various Kinds of Foods

Due to the heterogeneity in the composition of the media, it is impossible to recommend one universal medium, which meet all requirements. Several authors have published reviews dealing with media for the enumeration and isolation of enterococci (Barnes, 1959, 1976; Hartman *et al.*, 1966; Sabbaj *et al.*, 1971; Pavlova *et al.*, 1972; Switzer and Evans, 1974; Pagel and Hardy, 1980; Reuter, 1985, 1992). Basically, the choice of a particular medium depends on whether enterococci are to be counted in total and whether the habitat is highly contaminated or not

(Reuter, 1985, 1992). Garg and Mital (1991) have reviewed the several media for the isolation, enumeration and identification of enterococci from food and milk products. They concluded that there is no ideal media available for the isolation of enterococci from foods, because most media display drawbacks in terms of selectivity and recovery. KF agar is frequently used for the isolation of enterococci in nondairy foods, whereas Citrate Azide agar is recommended for dairy products. The parallel use of two media, one highly, the other moderately selective might be a reasonable way to obtain acceptable results from a food habitat (Reuter, 1985). Media for the examination of enterococci are usually incubated at 35-37⁰C. However, when examining enterococci in dairy products, a

higher incubation temperature (45°C) is necessary to suppress the growth of the background microflora (Deibel and Hartman, 1984).

Several authors (Knudtson and Hartman, 1993; Klein *et al.*, 1998; Hayes *et al.*, 2003) have reviewed the enumeration of enterococci in meat and meat products. Efthymiou *et al.* (1974) have dealt with the selective isolation and enumeration of enterococci with regard to cheese. Media for the detection of enterococci in milk and milk products have also been compared by other authors (Batish *et al.*, 1984; Batish and Ranganathan, 1984; Neaves *et al.*, 1988; Garg and Mital, 1991).

2.3 Incidence of Enterococci in Milk Products

Because of their ubiquitous nature, enterococci have been encountered more or less in several milk products. The occurrence of *S. faecalis* and *S. faecalis var. liquefaciens* in market samples of Dahi in India has been reported by Laximinarayana *et al.* (1952). Derwinora (1965) examined samples of ice cream at different stages of manufacture, recovered heavy loads of enterococci from the above samples. Bacteriological analysis of ice cream sold in Bombay market also showed *S. faecalis* to be a frequent contaminating organism (Mokashi *et al.*, 1970). Janossy (1970) found that out of 445 samples of milk and milk products and flour confectionary made with whipped cream, 186 samples (41.7%) contained *S. faecalis subsp. zymogenes* or *subsp. liquefaciens*, the highest counts occurring in cultured cream (63.2% of 174 samples).

Cantomi *et al.* (1974) analyzed 20 butter samples and found that Group D streptococci count averaged 10^5 CFU/g, out of 280 isolates of *S. faecalis*, 3% were of human origin, 17% of animal and 80% of plant origin. Batish (1980) found that the incidence of enterococci in milk and milk products was 80.3%, the number being maximum (100%) in sweet chese, *kulfi*, *kulfi* mix, and ice cream

and minimum (59.2%) in non fat dry milk samples; *S. faecalis var. faecalis* was the most predominant type (33.8%) in milk and milk products.

Enterococci occur and grow in a variety of cheeses, especially artisanal cheeses produced in Southern Europe (Portugal, Spain, Italy and Greece) from raw or pasteurized goat, ewe's, water-buffalo or bovine milk. High levels of contaminating enterococci usually result from poor hygienic practices during cheese manufacture (Thompson and Marth, 1986; Litopoulou-Tzanetaki, 1990; Lopez-Diaz *et al.*, 1995) and lead to deterioration of sensory properties in some cheeses (Thompson and Marth, 1986; Lopez-Diaz *et al.*, 1995). Levels of enterococci in cheese curds range from 10^4 to 10^6 CFU/g, and in the fully ripened cheeses from 10^5 to 10^7 CFU/g. Numbers vary with cheese type and production season and ranged from 10^4 to 10^6 CFU/g for Emmental cheese during a 15 years survey, and from 10^4 to 10^7 CFU/g for Appenzeller cheese (Teuber *et al.*, 1996). *E. faecalis* and *E. faecium* were the dominant enterococcal isolates in these cheeses. Suzzi *et al.* (2000) reported that enterococci were isolated from an artisanal Italian caprine cheese and that the most common species were *E. faecalis* and *E. faecium*, followed by *E. durans*. Wessels *et al.* (1988) also showed that *E. faecalis* (73.4% of isolates) was the prevalent species in cottage cheese, while Rao *et al.* (1986), found that *E. faecalis* was dominant in all dairy products except dried milk in which *E. faecium* dominated; *E. faecalis* is also amongst the predominant microorganisms found in raw milk (Devriese *et al.*, 1995). On the other hand Gelsomino *et al.* (2003) found that *E. casseliflavus* is predominant strain in cheddar-type cheese and the incidence of enterococci in this cheese is 3.2×10^4 CFU/g. Citak *et al.* (2004), isolated enterococci from Turkish white cheese; among the 101 isolates of enterococci 62 were *E. faecalis*, 25 were *E. faecium*, 7 were *E. durans*, 5 were *E. mundtii* and 2 were *E. hirae*.

2.4 Incidence of Enterococci in Meats & other Animal Foods

Enterococci may enter into raw material and food products from primary habitats such as intestines of animals and humans and from sources associated with unsanitary conditions of the production and handling with foods. The resistant enterococci can be potentially transferred from food animals to human via food chain (Mateu and Martin, 2001). Quednau *et al.* (1998) isolated 279 strains of *Enterococcus sp.* from the retailed chicken and pork. Seventy-three per cent of *Enterococcus* isolates from Swedish chicken were resistant to one or more of the tested antibiotics. The corresponding values for Swedish pork, Danish chicken and pork were 9%, 55% and 14%, respectively. Enterococci isolated from sausages and raw milk exhibited resistance to tetracycline, chloramphenicol, gentamicin, and erythromycin; several *E. faecium* and *E. faecalis* strains were shown to transfer tetracycline resistance genes into chromosome of *Lactococcus lactis* subs. *lactis* var. *diacetylactis* (Perreten, 1996). Ten strains of genus *Enterococcus* were isolated from the Egyptian Dominati cheese. All strains were resistant against oxacillin and cephazolin and sensitive against ampicillin, amoxicillin, and cefaclor (Hemati *et al.*, 1997). All enterococci isolated from raw milk cheese showed resistance to 1 of 12 antibiotics. The resistance genes for tetracycline, chloramphenicol and erythromycin were found to be identical to genes found in resistant enterococci from human infections (Perreten and Teuber, 1995).

According to the study of Sorensen *et al.* (2001) the ingestion of resistant *E. faecium* of animal origin leads to detectable concentrations of the resistant strains in stool of the health volunteers for up to 14 days after ingestion and therefore food animals appear to be an important reservoir of the transferable antibiotic resistance. Many studies deal with the occurrence of vancomycin-resistant enterococci in foodstuffs and with the possibility of animal-human transfer of vancomycin resistance. Pavia *et al.* (2000) investigated antimicrobial resistance of enterococci in samples of meat sold in retail outlets of Catanzaro. Enterococci were isolated from 45% of the samples, mostly from the chicken meat. Overall, 29% of the samples were contaminated with VRE. 10-12% of *E.*

faecalis isolated from the Danish chicken and pork but none of the Swedish isolates were resistant to vancomycin (Ahrne *et al.*, 1996). 242 (79%) of the 305 retail chicken products from the Netherlands were contaminated with VRE. The highest homology between VRE from chicken products and human strains was 60% (Braak *et al.*, 1998). In another study, Simonsen *et al.* (1998) examined faecal VRE strains from poultry farmers and their broilers at five avoparcine exposed Norwegian farms. Animal and human *E. faecium* strains at one farm were genetically closely related with indistinguishable VanA elements. The results indicate that the transmission of VanA glycopeptide resistance in enterococci between humans and animals can occur. Jensen *et al.* (1998) examined 38 high-level vancomycin resistant *E. faecium* isolates of human and animal origin from Europe and the United States. Their results were similar and indicated either horizontal gene transfer between *E. faecium* organisms of human and animal origin or the existence of a common reservoir for glycopeptide resistance. On the other hand the interesting opinion has been demonstrated by Lemcke and Bütte (2000). Out of 1643 enterococcal isolates from 115 poultry and 50 pork samples, 420 isolates could be identified as vancomycin resistant. Comparing VanA-positive food isolates with those from different human sources by means of the pulsed field gel electrophoresis it could clearly be demonstrated that they do not show homologous fingerprints according to the source of origin. It is therefore unlikely that there is a close genetic relationship between isolates from foodstuffs and humans. In production animals like poultry, cattle and pigs, *E. faecium* is a frequent species, but other species occur also in higher numbers like *E. faecalis* and *E. cecorum*, and less frequently *E. gallinarum* and *E. durans* or *E. avium* (**Table 2.1**). In a study of enterococci from raw meat products, *E. faecalis* was the predominant species from beef and pork cuts (Stiles *et al.*, 1978). *E. faecium* has also been frequently isolated from bologna, a processed meat sausage containing pork (Stiles *et al.*, 1978). Pig carcasses from three different slaughter plants contained mean log counts of 10^4 to 10^8 enterococci per 100 cm^2 of carcass surface throughout processing, and *E. faecalis* and *E.*

Table 2.1: Ecology of Enterococci in food

Species	Cheese	Fish/ Crustaceae	Meat	Cheese-meat combination	Pork carcasses	Sausage			Minced beef	Minced pork
						Fresh	Expired	Spoiled		
<i>E. faecalis</i>	(+)	+	+	(+)	++	++	+	(+)	++	++
<i>E. faecium</i>	++	(+)	++	+	(+)	-	-	++	(+)	(+)
<i>E. durans/hirae</i>	(+)	-	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
<i>E. gallinarum</i>	-	(+)	(+)	(+)	ND	ND	ND	ND	(+)	(+)
<i>E. casseliflavus</i>	-	-	(+)	-	ND	ND	ND	ND	-	(+)
<i>E. mundtii</i>	-	(+)	(+)	-	ND	ND	ND	ND	ND	ND
<i>E. avium</i>	ND	ND	ND	ND	ND	ND	ND	ND	-	(+)
<i>E. malodoratus</i>	ND	ND	ND	ND	(+)	(+)	(+)	-	ND	ND
<i>E. pseudoavium</i>	ND	ND	ND	ND	(+)	-	+	-	ND	ND
<i>E. raffinosus</i>	ND	ND	ND	ND	(+)	-	(+)	-	ND	ND

++: usual; +: frequent; (+): occasional; -: not mentioned; ND: not investigated

faecium were the most predominant *Enterococcus* sp. isolated (Kundston and Hartmann, 1993). *E. faecalis* predominated the Gram-positive enterococcal species isolated from chicken samples collected at poultry abattoirs (Turtura and Lorenzelli, 1994). The fermented meat products salami and landager were found to contain enterococci at numbers ranging from 100 to 2.6×10^5 CFU/g (Teuber *et al.*, 1996). Klein *et al.* (1998) isolated enterococci from minced meat; the counts were at concentrations between 0.5×10^1 and 7.1×10^2 CFU/g and *E. faecalis* was the most predominant species.

In a recent study Hayes *et al.* (2003) isolated 1357 enterococcal strains from variety of raw meat samples (chicken, turkey, pork and beef). *E. faecium* was the predominant species recovered (61%), followed by *E. faecalis* (29%) and *E. hirae* (5.7%).

2.5 Genus *Enterococcus* and Animals

Enterococci form an essential part of the autochthonous microflora of humans and animals. The species distribution shows some characteristics (Table 2.2) in the human intestine *E. faecium* and *E. faecalis* are the most frequent species.

Table 2.2: Occurrence of Enterococci in the Gastro-intestinal Tract

Species	Human	Cattle	Pig	Fowl
<i>E. faecalis</i>	++	(+)	+	++
<i>E. faecium</i>	++	++	+	++
<i>E. durans/hirae</i>	(+)	-	(+)	(+)
<i>E. gallinarum</i>	(+)	-	-	(+)
<i>E. casseliflavus</i>	(+)	-	-	-
<i>E. cecorum/columbae</i>	-	+	+	++

++: usual; +: frequent; (+): occasional; -: not mentioned. (Reuter, 1992; Leclerc *et al.*, 1996; Manero and Blanch, 1999).

Naturally enterococci occur in large numbers in the intestines of mammals, birds, reptiles and insects. Devriese *et al.* (1992) found in faeces of nonruminating calves several species of enterococci: *E. avium*, *E. cecorum*, *E. durans*, *E. faecalis*, *E. faecium*, and *E. hierae*. *E. faecalis* were most frequent. Enterococci were infrequent in dairy cows. The same authors (Devriese *et al.*, 1991) identified intestinal enterococcal flora of poultry. Many authors confirmed the occurrence of antibiotic resistant strains of *Enterococcus* sp. isolated from animals. One important reason is the amount of anti-microbial agent used in animals. According to FEDESA, the European Federation of Animal Health, the sales of anti-microbials in the EU in 1997 were 3949 tonnes for animal health and 1599 tonnes as growth promoters (Ungemach, 2000). Another critical issue is the use of in-feed or in water medication with prophylactic purposes. Widespread resistance to chloramphenicol, macrolides, kanamycin, streptomycin and tetracycline was found among isolates of *E. faecalis* and *E. faecium* isolated from humans, broilers and pigs (Aarestrup *et al.*, 2000a).

Resistance of *Enterococcus* sp. from the mammary gland was tested to penicillin, cloxacilin, cephalosporin, ceftiofur, novobiocin, enrofloxacin, erythromycin and pirlimycin. The MIC for 90% isolates were 4, 64, 32, 64, 4, 1, 4, and 4 (Wats *et al.*, 1995). Epidemiological study of Aarestrup *et al.* (2000b) showed a statistically significant association between the use of avilamycin for growth promotion and occurrence of avilamycin-resistant *E. faecium* on broiler farms. On the other hand Davis and Roberts (1999) did not find any evidence that the feeding of this growth promoter caused selection of enterococci resistant to tylosin and avilamycin. Two main classes of antimicrobial agents have been the target of the most studies: fluoroquinolones and glycopeptides. Both types of compounds are first-line drugs for the treatment of many life-threatening infections in humans. Fluoroquinolones were introduced for veterinary use in the early 1990s (Mateu and Martin 2001). Glycopeptides like avoparcin have been used extensively as growth promoters in the Europe and several authors have suggested them as being responsible for the development of vancomycin-

resistant enterococci in animals (Borgen *et al.*, 2000). Bogaard *et al.* (2000b) showed that in Netherlands, within 2 years of stopping the use of avoparcin, the prevalence and numbers of VRE have decreased significantly, not only in the faecal flora of food animals but also in the endogenous flora of healthy humans. Resistant bacteria from animals can infect or reach the human population by direct contact, and also via food products of animal origin. The resistant bacteria can colonize humans and/or transfer their resistance genes to other bacteria belonging to the endogenous flora of humans. The genes encoding the resistance can be transferred to pathogenic bacteria, and disseminated into the environment from animals to foods of animal origin. Some authors paid attention to the transmission of the resistant enterococci from animals to humans. Bogaard *et al.* (1997) found that VRE could be detected in 50% of faecal samples from turkeys and 39% of faecal samples from turkey farmers. Furthermore VRE were isolated from 20% of faecal samples from turkey slaughterers and 14% from urban residents from the same area. Similar results obtained Aarestrup *et al.* (2000a) who compared antimicrobial resistance phenotypes and resistance genes in enterococci from humans, broilers and pigs. Enterococci can be spread by faecal-oral transmission, contact with infected body fluids or contact with contaminated surfaces. Noskin *et al.* (2000) found that enterococci are capable of prolonged survival (at least one week) on fabric seat cushions and can be transferred to hands.

2.6 Genus *Enterococcus* and Humans

In recent years enterococci have emerged as pathogens in association with serious nosocomial infections. A major problem in enterococcal infections is vancomycin resistance (Rice, 2001). Vancomycin is a powerful antibiotic that is often the drug of the last resort. It is generally limited to be used against gram positive bacteria that are resistant to β -lactam-antibiotics and other ones (Mayhall, 1996). The risk factor for the appearance of VRE in hospitalized patients is heavy use of vancomycin, third-generation cephalosporines and

similarly active β -lactams (Edmond *et al.*, 1995). There may be a link among the use of glycopeptide avoparcin as growth promotor for farm animals, the selection for vancomycin resistant enterococci and the colonization (infection) of humans by these bacteria through the food chain. The route of the transmission of VRE from livestock to human has not been proven, but if VRE colonization occurs in a community without hospital exposure, this possibility should be considered (Aarestrup *et al.*, 2000a). The relationship between VRE colonization of the food animals and VRE colonization of humans was suggested by Bates *et al.* (1994), who recovered VRE with identical ribotypes from retail chicken carcasses and humans.

Reinert *et al.* (1999) found resistance to vancomycin in 1.5% from 730 enterococcal isolates in Germany. In Sweden VRE were found only sporadically (Hallgren *et al.*, 2000). The occurrence of VRE in Czech Republic was first described in 1997 (Kolar *et al.*, 1997). Their frequency reached 3.7%. Sievert *et al.* (2002) described the first clinical isolate of *Staphylococcus aureus* that was fully resistant to vancomycin. The presence of *vanA* in this VRSA suggests that the resistance determinant might have been acquired through exchange of genetic material from the vancomycin-resistant *Enterococcus* also isolated from the same clinical material. VRE possess a serious threat to certain high-risk patients. It is necessary to control the spread of these dangerous organisms and judicious use of vancomycin should be promoted.

2.7 Antibiotic Resistance of Enterococci

Enterococci have been known to be resistant to most antibiotics used in clinical practice. They are naturally resistant to cephalosporins, aminoglycosides and clindamycin and may also be resistant to tetracyclines and erythromycin. They are intermediate sensitive to penicillin and ampicillin and glycopeptides. The strains that produce β -lactamase are rare. Enterococci are known to acquire antibiotic resistance with relative ease and to be able to spread these resistance

genes to other species (Kuhn *et al.*, 2000). *Enterococcus faecalis* has been reported to transfer plasmids harboring antibiotic-resistance traits to other enterococci and to *Listeria monocytogenes* in water treatment plants (Marcinek *et al.*, 1998). *Enterococcus faecium* conjugative transposons can be transferred from animal bacteria to human ones. Such conjugative transposons can (Knudston and Hartman, 1993; Devriese *et al.*, 1995; Dazo, 1996; Klein *et al.*, 1998). also transfer vancomycin resistance to *Staphylococcus aureus*, streptococci and lactobacilli. Multidrug-resistant and vancomycin-resistant enterococci are commonly isolated from humans, sewage, aquatic habitats, agricultural run-off and animal sources, which indicates their ability to enter human food chain (Rice *et al.*, 1995).

2.7.1 Vancomycin Resistance

Most surprising in recent years has been the emergence of acquired resistance to vancomycin among enterococci. Vancomycin resistant enterococci (VRE) threatens to compromise effective treatment of infections caused by these multi-resistant bacteria particularly in seriously ill patients who may need treatment with vancomycin where other antibiotics have failed.

VRE were first isolated from sewage treatment plants in England and a small town in Germany and later from manure samples from pig and poultry farm (McDonald *et al.*, 1997). Bates *et al.*, (1993) recovered VRE from livestock faeces and from uncooked chicken samples purchased from retail outlets. Klare *et al.* (1995) suggested a possible relationship between the recovery of these microorganisms and the use of avoparcin, a glycopeptide antimicrobial drug used as livestock feed additive in many European countries.

Avoparcin is an antibiotic that acts in the same way as vancomycin, except that it is used solely for veterinary practice. VRE are cross resistant to avoparcin and to teicoplanin. There may be a link among the use of avoparcin,

the selection for VRE and the colonization of humans by these bacteria through the food chain (Khachatourians, 1998).

Three phenotypes of vancomycin resistance (types A, B, and C) are now well described; a fourth, type D, has been recently reported (Perichon *et al.*, 1997). VanA-type strains are typically highly resistant to vancomycin and moderately to highly resistant to teicoplanin. This phenotype is often plasmid or transposon mediated and is inducible i.e., exposure of bacteria to vancomycin results in the induction of the synthesis of several proteins that together confer resistance (Derlot *et al.*, 1991).

2.7.2 Aminoglycosides Resistance

Enterococci exhibit also inherent low-level aminoglycoside resistance. The MICs are between 2 and 16 µg/ml (Murray, 1990). Some of enterococcal strains have acquired high level aminoglycoside resistance (HLAR). The MICs are ≥2000 µg/ml (Sahm *et al.* 1991). Rice *et al.* (1995) investigated 248 environmental isolates of enterococci for HLAR. The highest percentage of the resistance was seen for kanamycin, closely followed by tobramycin and to a lesser degree by streptomycin and gentamicin. Multiple antibiotic resistance patterns were observed in 95% of HLAR isolates. The most frequently occurring multiple resistance patterns was HLAR to both kanamycin and tobramycin, followed by multiple resistances to streptomycin, kanamycin and tobramycin. All isolates that exhibited gentamicin resistance were also resistant to kanamycin and tobramycin, but not to streptomycin. These environmental enterococci may contribute to the dissemination of HLAR strains to the human population. Resistance to high level aminoglycosides was also prevalent across all species recovered from retail meats (Hayes *et al.*, 2003). Aside from the single isolate of *E. avium* that was resistant, the observed frequency of resistance to any of the three tested aminoglycosides (streptomycin, kanamycin, gentamicin) was highest among isolates of *E. casseliflavus* (86%), followed by those of *E. faecium* (58%),

E. gallinarum (56%), *E. durans* (38%), *E. faecalis* (17%), and *E. hirae* (12%). The patterns of susceptibility to high-level aminoglycosides were interesting in that resistance to kanamycin was the most prevalent, followed by resistance to streptomycin and resistance to gentamicin. The enterococci isolated from minced beef and pork in Germany (Klein *et al.*, 1998) showed low level resistance to aminoglycosides. Among the vancomycin resistant enterococci (VRE) 34.2% were resistant to streptomycin and none of these were gentamicin resistant. Citak *et al.*, (2004) isolated the *Enterococcus* species from Turkish white cheese and observed that maximum number of isolates was resistant to streptomycin, oxacillin and erythromycin than to the other antibiotics; the corresponding values being 89.1%, 88.1% and 93% respectively. The most effective antimicrobials were ampicillin (69.3% of isolates were susceptible), imipenem (67.3% susceptible) and ciprofloxacin (63.5% susceptible). Similar results have been reported by Pavia *et al.* (2000); while Teuber *et al.* (1996) indicated that enterococci isolated from fermented sausage were frequently resistant to streptomycin; isolates from Emmental and Appenzeller cheeses showed a high frequency of resistance to erythromycin, gentamicin, vancomycin and tetracycline. Similarly, Batish and Ranganathan (1986) found that about 80% of *Enterococcus* isolates from milk samples showed varying degree of resistance towards chloramphenicol, erythromycin, streptomycin, tetracycline and gentamicin.

Plasmid and transposon-encoded genes, besides those necessary for replication and transfer, typically confer traits that provide survival advantages to organisms in unusual environments (Eberhard, 1989.). Such traits include antibiotic or heavy metal resistance, bacteriocin activity, metabolism of unusual substrates, and virulence factors. Many putative enterococcal virulence factors reside on conjugative plasmids. The ease with which these determinants spread horizontally between strains in a natural environment, such as the gastrointestinal tract, likely determines the rate at which adaptive and pathogenic traits are disseminated to potentially less virulent endogenous flora. As such,

naturally occurring mechanisms of gene exchange are fundamentally an expression of virulence.

2.8 Exchange of Genetic Material

Enterococci possess potent and unique abilities to exchange genetic material among themselves and with other genera (Clewell, 1990; Schaberg *et al.*, 1986). At least three conjugative systems exist by which enterococci naturally transfer genetic elements. First, narrow-host-range, pheromone-responsive plasmids unique to enterococci have been described (Clewell, 1993). These plasmids transfer at high frequencies on solid surfaces, in broth, and in vivo (Huycke *et al.*, 1992; Clewell, 1993). Second, many plasmids with a broad host range (e.g., pAMB1) readily transfer at low frequency among enterococci, *Streptococcus spp.*, *Staphylococcus aureus*, *Lactobacillus sp.*, *Bacillus subtilis*, and other species (Schaberg *et al.*, 1986; Eberhard, 1989). Coexisting pheromone responsive plasmids can greatly increase the transfer frequency of these plasmids (Clewell, 1993). Transfer requires contact between donor and recipient cells on a solid surface and can occur in vivo (Morelli *et al.*, 1988). Finally, conjugative gene exchange occurs through highly promiscuous transposons found in gram-positive and gram-negative bacteria (Scott, 1992.). One transposon, Tn1545, has been shown to transfer at low frequency from *E. faecalis* to *Listeria monocytogenes* in the intestinal tracts of gnotobiotic mice (Doucet-Populaire *et al.*, 1991). Tn916 is the most thoroughly characterized conjugative transposon and has been used extensively in molecular genetic studies (Frank *et al.*, 1981; Scott, 1992). The only other known mechanism of natural gene exchange for enterococci is through infection with bacteriophages (Natkin, 1967; Caprioli *et al.*, 1975). Enterococcal bacteriophages have been isolated from stool (Rogers *et al.*, 1963) and have a narrow host range rendering them of potential use for strain typing (Kuhnen *et al.*, 1988). The ability of enterococcal bacteriophages to spread virulence traits or antibiotic resistance determinants is largely unknown.

3. MATERIALS AND METHODS

3.1 Isolation of enterococci

3.1.1 Sample collection

A total number of 38 Dahi samples were collected. Among them some from sweet shops, small scale dairy units of different cities of Haryana and others from villages of Karnal.

3.1.2 Culture Medium

Modified Citrate Azide Agar (Saraswat *et al.*, 1963) was employed for the isolation of enterococcal cultures from different Dahi samples. Composition of the medium is given below:

Component	Quantity
Yeast extract	10 g
Pancreatic digest of Casein	10 g
Sodium Citrate	20 g
Agar	15 g
Distilled water	1000 ml

The ingredients were heated to dissolve and 100 ml aliquots of the medium base were autoclaved at 121°C for 20 min. one ml of 0.1% sterile (121°C for 20 min) Triphenyl Tetrazolium Chloride and 0.5 ml of 4% sterile (121°C for 20 min) aqueous Sodium Azide were added to each aliquot of autoclaved (100 ml) basal medium tempered to 48-50°C.

3.1.3 Procedure

Aseptically weighed 10 g of Dahi samples were transferred to 90 ml of sterile sodium citrate solution at 45° to 50°C. Serial dilutions were subsequently prepared in sterile normal saline (0.85%). Appropriate dilutions of the samples were plated out on Modified Citrate Azide Agar; with a thin layer of the same medium overlaid and plates were incubated at 37°C for 48 to 72 hrs. Typical colonies showing characteristic red coloration were counted and representative colonies were picked up randomly and transferred to MRS broth (de Man *et al.*,

1960) in test tubes and incubated at 37⁰C for 24-48 hrs for microscopic examination.

The above isolates were purified by repeated streaking on the selective medium (Modified Citrate Azide Agar) to obtain well isolated colonies.

3.2 Composition of Different Culture Media

3.2.1 MRS medium (de Man *et al.*, 1960)

Component	Quantity
Yeast extract	5.0 g
Beef extract	10.0 g
Peptone	10.0 g
Dextrose	20.0 g
K ₂ HPO ₄	2.0 g
Sodium Acetate	5.0 g
Tri Ammonium Citrate	2.0 g
Magnesium Sulphate (MgSO ₄ , 7H ₂ O)	0.1 g
Manganese Sulphate (MnSO ₄ , 4H ₂ O)	0.05 g
Tween 80	1.0 ml
Distilled water	1000 ml
pH: 6.5±0.2	

3.2.2 Tellurite Agar

Component	Quantity
Peptone	10g
Lactose	5.0g
NaCl	5.0g
KH ₂ PO ₄ (dibasic)	2.0g
KH ₂ PO ₄ (monobasic)	2.0g
Agar	15.0g
Distilled water	1000 ml
K-Tellurite	0.4 ml

3.2.3 Arginine Broth

Component	Quantity
Tryptone	5.0 g
Yeast extract	5.0 g
K ₂ HPO ₄	5.0 g
Glucose	0.5 g
Arginine monohydrate	3.0 g
Distilled water	1000 ml
pH: 7.0 ±0.2	

3.2.4 TND Medium

Component	Quantity
Tryptone	17.0 g
Neopeptone	3.0 g
Dextrose	2.5 g
NaCl	5.0 g
K ₂ HPO ₄	2.5 g
Distilled water	1000 ml
Final pH: 7.2	
The solid medium contained 1.5% agar	

3.2.5 CHL Medium

Component	Quantity
Polypeptone	10.0 g
Yeast extract	15.0 g
Tween 80	1.0 ml
K ₂ HPO ₄	2.0 g
Sodium acetate	5.0 g
Diammonium Citrate	2.0 g
MgSO ₄ , 7H ₂ O	0.2 g
MnSO ₄ , 4H ₂ O	0.05 g
Bromocresol purple	0.17 g
Distilled Water	1000 ml
pH: 6.9	

3.2.6 Blood Agar

Component	Quantity
Beef extract	10.0 g
Peptone	10.0 g
NaCl	5.0 g
Agar	15.0 g
Bovine blood, defibrinated	50 ml

3.2.7 Bile Aesculin Agar

Component	Quantity
Peptone	8.0 g
Bile salt	20.0 g
Ferric citrate	0.5 g
Aesculin	1.0 g
Agar	15.0 g
pH: 7.1±0.2	

3.3 Preliminary Tests for the Identification of Enterococci

The isolates were subjected to the following physiological and biochemical tests to confirm them as enterococci.

3.3.1 Microscopic Examination

The isolates were examined for shape, size and arrangement of cells as well as for Gram's reaction.

3.3.2 Action on Litmus Milk

Litmus milk tubes were inoculated with the test culture and incubated at 37°C. The sequence of reduction of litmus and clotting or proteolysis of milk were noted after incubation for 48 hrs.

3.3.3 Oxidase Test

A portion of growth from a colony was removed and rubbed over a filter paper strip (Whatman No: 1) streaked in freshly prepared solution of phenylene-diamine-dihydrochloride. Positive reaction was indicated by an intense deep purple blue color, which appeared within 5-10 seconds.

3.3.4 Catalase Test

A portion of growth from a colony was transferred to a clean glass slide and one drop of 3% H₂O₂ was added. The absence of effervescence indicated the purity of isolates, since enterococci are Catalase negative.

3.3.5 Growth in 6.5% NaCl

TND broth (Batish, 1980) with 6.5% NaCl was inoculated with the test culture and incubated at 37⁰C for 24-48 hrs. Appearance of turbidity indicated growth.

3.3.6 Growth at pH 9.6

TND broth (Batish, 1980) was adjusted to pH9.6 and inoculated with the test culture and incubated at 37⁰C for 24 hrs. Appearance of turbidity indicated growth.

3.3.7 Growth at 10⁰C and 45⁰C

The cultures were inoculated into TND broth and incubated at 10⁰C and 45⁰C and ability of the organisms to grow at these two temperatures was assessed on the basis of development of turbidity.

3.3.8 Resistance to 60⁰C for 30 Minutes

TND broth tubes were inoculated with the test culture. The broth culture was subjected to a heat treatment at 60⁰C for 30 min in a constant temperature water bath (60⁰C). Soon after heat treatment, the cultures were cooled to 30⁰C and transferred to an incubator at 37⁰C for 48 hrs. Appearance of turbidity indicated growth.

3.3.9 Growth in 0.1% Methylene Blue Milk

The test culture was inoculated in sterilized reconstituted non fat dry cow's milk to which 0.1% methylene blue was added. The culture tubes were incubated at 37⁰C for 96 hrs. Reduction of methylene blue indicated positive reaction.

3.3.10 Arginine Hydrolysis

The test culture was inoculated in arginine broth and incubated at 37⁰C for 24 hrs. Nessler's reagent (0.25 ml) was then added to the culture. Development of a brown color in the culture tube indicated a positive reaction for arginine hydrolysis.

3.3.11 Aesculin Hydrolysis

Bile Aesculin Agar petriplates were spot inoculated with the test culture. The petriplates were incubated at 37⁰C for 24 hrs. Appearance of black color around the colonies indicated hydrolysis of aesculin. Bile tolerant colonies which did not hydrolyse aesculin were whitish.

3.3.12 Growth in K-Tellurite

The test culture was streaked on 0.04% K-Tellurite agar and incubated at 37⁰C for 48 hrs. Appearance of black colonies indicates a positive reaction.

3.3.13 Tetrazolium Reduction at pH 6.0

The TND agar was adjusted at pH 6 and autoclaved (121⁰C for 20 min). To the tempered agar (48⁰C) 1 ml of 0.1% tetrazolium blue was added and mixed properly. The above mixture was then poured in sterile petriplates and allowed to solidify. A loopful of test culture was then streaked on the agar and incubated at 37⁰C for 5 days. Development of pink color along the streaked line was considered as positive reaction.

3.3.14 Fermentation of Sugars

Small test tubes (12x75 mm) were used for production of acid from different sugars. CHL medium was used as the basal medium. Four ml of the medium was taken in each tube and sterilized by autoclaving. One sugar disc (Hi-Media) was aseptically added to each tube. Each sugar tube was inoculated with 0.1 ml of inoculum.

3.3.15 Hemolytic Activity

The test was conducted by using Blood Agar base with 5% bovine blood supplement and streaking the cultures on surface of the medium as recommended by Batish (1982). The medium was prepared by adding and properly mixing 5 ml of bovine blood to 95 ml of sterile melted and tempered blood agar base. The blood agar prepared above was poured into petriplates and the surface of the plates were dried by keeping the plates overnight at 37⁰C in an incubator. Loopful of broth culture was streaked on it and the plates were incubated up to 120 hrs at 37⁰C. Nature of hemolysis, if any, result was noticed after this period of incubation.

3.3.16 Liquefaction of Gelatin

The test was carried out using TND agar containing 0.4% gelatin. Petriplates containing the medium were spot inoculated with the test culture and incubated at 37°C for 3 days. The petriplates were then flooded with saturated ammonium sulphate solution. Developments of clear zones around the spot inoculation against an opaque background indicate a positive reaction.

3.4 Antibiotic Susceptibility Test

Antibiotic susceptibility was determined semiquantitatively by disc diffusion using modification of the agar overlay diffusion method.(Charteris *et al.*, 1998) MRS agar was used for susceptibility testing. Petriplates (9 cm), containing 15 ml of agar were overlaid with 4 ml of soft agar seeded with 200 µl of an active culture at 37°C. This provides a moderately heavy inoculum of about 10⁶ to 10⁷ viable cell per ml of agar overlay. Petriplates were allowed to stand at room temperature for 15 min prior to dispensing antibiotic containing discs with the help of forceps. Plates were incubated at 37°C for 24 hrs. without pre-diffusion of pre-incubation. Inhibition zone diameters were measured using antibiotic zone scale and results were expressed in terms of resistance, moderate susceptibility or susceptibility.

Table 3.1: Antimicrobial agents and associated interperative zone diameters for Disc Diffusion Antibiotic Susceptibility testing (Charteris *et al.*, 1998)

Group	Antibiotic		Interperative zone diameters (mm)		
	Name	Disc conc. (µg)	R	MS	S
β-lactam	Ampicillin	10	≤ 12	13-15	≥ 16
Glycopeptides	Vancomycin	30	≤ 14	15-16	≥ 17
	Teicoplanin	30	≤ 10	11-13	≥ 14
Aminoglycosides	Gentamicin	10	≤ 12	-	≥ 13
	Streptomycin	10	≤ 11	12-14	≥ 15
Tetracyclines	Tetracycline	30	≤ 14	15-18	≥ 19
Amphenicol	Chloramphenicol	30	≤ 13	14-17	≥ 18
Macrolides	Erythromycin	15	≤ 13	14-17	≥ 18
Quinolones	Ciprofloxacin	5	≤ 13	14-18	≥ 19

R (Resistant), MS (Moderately susceptible), or S (Susceptible)

3.5 Minimum Inhibitory Concentration: (Andrews, 2001)

3.5.1 Preparation of the McFarland 0.5 standard

A half millilitre of 0.048 M BaCl₂ (1.175% w/v BaCl₂, H₂O) was added to 99.5 ml of 0.36N H₂SO₄ (1% v/v). It was then mixed with magnetic stirrer. 1-5 ml (depending upon the volume usually used in the tests) of the standard was distributed into screw cap of the same size as those used in growing of the broth cultures. The tubes were sealed tightly to prevent loss by evaporation. It was then stored in the dark at room temperature. This turbidity standard was vigorously agitated on a mechanical vortex mixer just before use.

3.5.2 Preparation of Bacterial Inoculum

The top four to five colonies of a single type was touched and inoculated into suitable broth (MRS broth). The tubes were incubated with shaking at 35⁰-37⁰C until it is visibly turbid (approximately 4-6 hr). The density of the culture was adjusted to equal that of a McFarland standard. Add sterile distilled water or saline and compare against a white background with contrasting black lines to aid the visual comparison. Cultures adjusted to the McFarland 0.5 standard contain approximately 10⁸ CFU/ml. The adjusted suspension was diluted to 1:10 (in sterile broth, distilled water, saline) to obtain a desired inoculum of 10⁷ CFU/ml. The suspension was further diluted in sterile broth or saline to produce an inoculum suitable for Disc Diffusion Test or for Broth Dilution MICs.

Table 3.2: Suggested Ranges for MIC Determination (mg/l)

Antibiotic	Enterococci
Ampicillin	0.12-128
Chloramphenicol	1.0-128
Erythromycin	0.25-128
Gentamicin	0.5-2048
Teicoplanin	0.5-2048
Tetracycline	-
Vancomycin	0.12-128
Streptomycin	-
Ciprofloxacin	0.25-128

3.5.3 Preparation of Antibiotic Dilution Range

It is generally accepted that MICs should normally be determined in doubling dilution step based on the unit of 1 mg/l, going up (2, 4, 8, 16 mg/l etc.) and down (0.5, 0.25, 0.125 mg/l etc.) as required. However, in special cases a closer range of concentrations may be needed e.g. 1, 2, 3, 4, 5, etc. Weigh 0.1 g antibiotic powder and dissolve in 10 ml solvent (water, alcohol or other solvent; depending on the group of antibiotic) to prepare 10000 mg/l stock solution.

Table 3.3: Storage of Antibiotic solutions

Antibiotic	Diluent	Storage of solution			Storage of powder
		+4 ^o C	-20 ^o C	-70 ^o c	
Gentamicin (sulphate)	water	6 months	NR	NR	Unopened vials 3 yrs. at room temperature
Erythromycin	Alcohol ^a and water	1 week	-	-	+5 ^o C stable 3 yrs.; protect from moisture and light
Streptomycin (sulphate)	Water	-	-	-	+4 ^o C; protect from light and moisture
Ciprofloxacin (hydrochloride monohydrate)	water	2 weeks	3 months	3 months	Below 25 ^o C for 3 yrs.; protect from light

NR= not recommended, a= enough to dissolve the antimicrobial powder.

Method:

I. From the initial solution (10,000 mg/l) it has been prepared further working stock solutions of 1000 and 100 mg/l as follows:

1 ml 10,000 mg/l +9 ml water	= 1000 mg/l
100 µl 10,000 mg/l + 9.9 ml water	= 100 mg/l
Working stock solution A	= 10,000 mg/l
B	= 1000 mg/l
C	= 100 mg/l

II. Preparation of dilution range of 0.25 – 128 mg/l as follows:

a. Eleven sterile test tubes were labeled as follows:

128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.

b. The following amount was dispensed from stock solution (A) with a variable micro- pipette.

256 µl into bottle labeled 128

128 µl into bottle labeled 64

64 µl into bottle labeled 32

32 µl into bottle labeled 16

c. The following amount was dispensed from stock solution (B)

160 µl into bottle labeled 8

80 µl into bottle labeled 4

40 µl into bottle labeled 2

d. The following amount was dispensed from stock solution (C)

200 µl into bottle labeled 1

100 µl into bottle labeled 0.5

50 µl into bottle labeled 0.25

III. 20 ml broth was added to each bottle, including the antibiotic-free control (bottle labeled 0). The medium was allowed to cool to 55⁰C before addition.

IV. The broth and antimicrobial agent were thoroughly mixed before plates poured or before broth is dispensed into smaller volumes.

V. Also included a sterility check of each broth dilution in each batch of tests so that the introduction of a contaminant can be detected.

3.5.4 Broth Macrodilution

i. **Table 3.2** (Suggested ranges for MIC determination) has been consulted for this method.

ii. Stock solution of antibiotics was prepared by the following recommendations cited in **Table 3.3** (Storage of antibiotic solutions)

- iii. Working solution was prepared as shown in section 3.5.3 (preparation of antibiotic dilution range).
- iv. 3X1/2" sterile capped tubes was arranged in two rows for each antibiotics, to cover the chosen range of dilutions in duplicate.
- v. 1 ml volumes of antibiotic solutions at working dilution were transferred in broth to tubes.
- vi. After that inocula of test organisms were prepared to contain a 1×10^5 CFU/ml (section 3.5.2).
- vii. 1 ml aliquots of test organisms were added to both row of tubes and mixed the contents of each tube thoroughly.
- viii. Inoculated and uninoculated controlled tubes were also set up which contained all ingredients except antibiotic.
- ix. All the tubes were incubated at 35° to 37° C for 16-18 hrs.
- x. After 16-18 hrs of incubation the MIC end point was read out. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth.

4. RESULTS AND DISCUSSION

A total of 38 samples of Dahi were collected comprising of 22 samples from different from different sweet shops, small scale dairy units of Karnal market and 16 samples from villages around Karnal. The titratable acidity of the collected samples ranged from 0.72% to 1.08% Lactic acid. The samples were pour plated on Modified Citrate Azide agar to enumerate and isolate enterococci (Saraswat *et al.*, 1963). Out of 38 samples, 31 samples were found to be positive for enterococci. Typical pink colonies obtained after 48-72 hrs. of incubation at 37⁰C were counted. The enterococcal counts ranged from 2.84 log CFU/g to 4.14 log CFU/g as given in **Table 4.1**. The typical representative colonies (maximum 3 from each plate) were randomly picked up with the help of sterile tooth picks and inoculated in MRS broth and incubated for 24 hrs at 37⁰C. The cultures thus obtained were further purified by streaking on Modified Citrate Azide Agar. Streaking was repeated three times to ensure proper purification of the isolates. A total number of seventy nine such isolates were examined microscopically after Gram's staining to ascertain their morphology & purity. The Gram- positive cocci in short chains/pairs and the absence of any rods confirmed the purity of the culture.

Enterococci have generally been isolated from Dahi samples where they grow along with starter lactic acid bacteria (LAB). They grow under condition similar to those for the growth of lactic acid bacteria (Laximinarayana *et al.*, 1952).

4.1 Biochemical Characterization of Enterococcal Isolates and their tentative Identification

Various physiological and biochemical characteristics were studied to identify the isolates. During characterization 26 isolates did not display the biochemical characteristics of *Enterococcus* genus and were excluded from

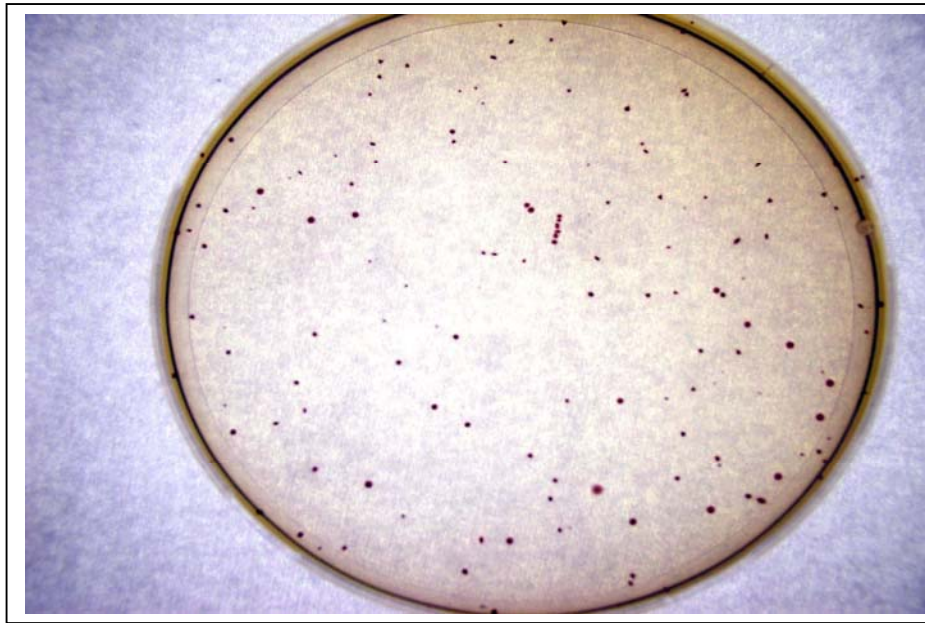


Plate 4.1: Isolation of enterococci on Modified Citrate Azide Agar

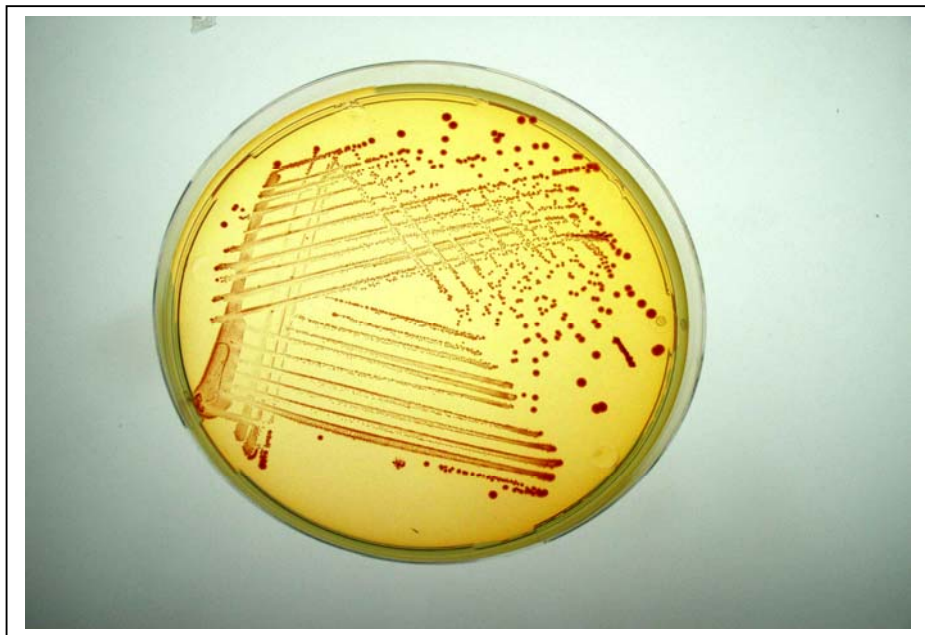


Plate 4.2: Purification of enterococci by streaking on Modified Citrate Azide Agar

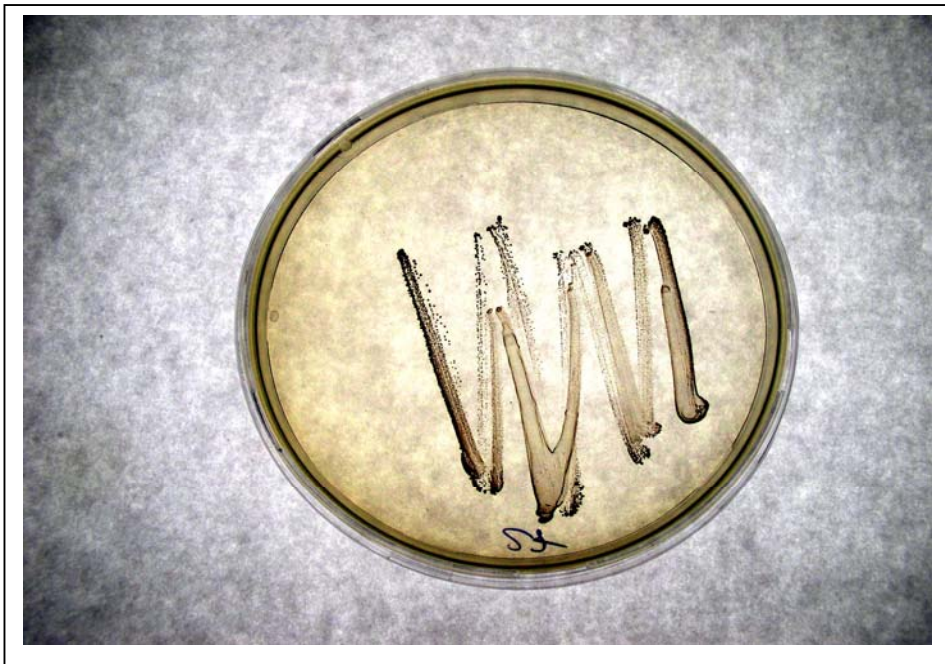


Plate 4.3: Streaking on K-Tellurite Agar

the study. Finally 53 enterococcal isolates were characterized at species level.

All the isolates were found to be gram-positive cocci with cells being arranged in short chains or pairs and fulfilled the Sherman's criteria of ability to grow at 10 & 45⁰C, at pH 9.6, in the presence of 6.5% NaCl, and the ability to reduce 0.1% methylene blue (**Table 4.2**).

All the isolates were found to be Oxidase positive, Catalase negative, Aesculine positive. All isolates hydrolyzed arginine but did not liquefy gelatin. For further differentiation into species, guidelines given by given by Facklam and Elliott (1995) and Manero and Blanch (1999) were followed. The fermentation of four sugar namely mannitol, sorbitol, arabinose, and raffinose were the key parameter for identification of enterococci to different species.

Fermentation of L-arabinose and growth in 0.04% K-Tellurite were useful criteria for the differentiation of two species of enterococci namely *E. faecalis* and *E. faecium*. As shown in **Table 4.2**, 24 isolates could be assigned as *E. faecalis* as they fermented mannitol and sorbitol but did not ferment arabinose. Out of these 24 isolates 2 isolates (i.e. 15 and 18) did not grow in presence of 0.04% K-Tellurite.

These isolates were also able to reduce tetrazolium at pH 6 as indicated by the pink coloration developed along the line of growth on the Tryptone Neopeptone Dextrose agar plates fortified with 0.001% tetrazolium blue. The isolates which fermented sugar L-arabinose but unable to grow in the presence of 0.04% tellurite, were identified as the strains of *E. faecium*. The number of such isolates was twelve. These isolates were also unable to ferment xylose. The strains *E. durans* were identified on the basis of their inability to ferment majority of sugars such as mannitol, sorbitol, L-arabinose, xylose, and raffinose but they were able to ferment sucrose, melibiose, galactose and salicin. The number of such isolates was six. The isolates which were able to ferment almost all sugar except raffinose were identified as *E. avium*. The numbers of such isolates was four. The remaining seven out

of 53 isolates could not match for any enterococcal species hence unable to identify on the basis of above tests.

Table 4.1: No. of sample collected, their source, acidity and Enterococcal counts

Sample No.	Source	%Acidity (L.A)	Enterococcal Count	
			x10 ² CFU/g	log(CFU/g)
1	M A R K E T	0.72	58	3.76
2		0.78	17	3.23
3		0.82	91	3.96
4		0.84	43	3.63
5		0.73	23	3.36
6		0.80	15	3.17
7		0.89	0.0	0.0
8		0.76	65	3.81
9		0.76	61	3.78
10		0.88	31	3.49
11		0.74	22	3.34
12		0.82	0.0	0.0
13		0.89	105	4.02
14		0.76	141	4.14
15		0.72	14	3.14
16		0.81	0.0	0.0
17		0.90	7	2.84
18		0.90	19	3.27
19		0.81	45	3.65
20		1.08	51	3.70
21		0.81	11	3.04
22		0.99	56	3.74
23	V I L L A G E	0.72	13	3.11
24		0.78	76	3.88
25		0.73	0.0	0.0
26		0.83	81	3.90
27		0.92	62	3.79
28		0.73	9	2.95
29		0.76	19	3.27
30		0.94	0.0	0.0
31		0.81	0.0	0.0
32		0.87	42	3.62
33		0.81	26	3.41
34		0.94	52	3.71
35		0.76	29	3.46
36		0.84	54	3.73
37		0.76	0.0	0.0
38		0.94	83	3.92

Table 4.2: Identification of Enterococcal isolates

Physiological and Biochemical Tests:	Isolate No.							
	1	2	3	4	5	6	7	8
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+
Growth at 45 ⁰ C	+	+	+	+	+	+	+	+
Growth at 10 ⁰ C	+	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+	+
Resistance to 60 ⁰ C for 30 min	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+	+
Growth in k-Tellurite	-	-	-	-	-	-	+	-
Liquefaction of gelatin	-	-	-	-	-	-	-	-
Hemolysis	non	non	non	alpha	non	non	alpha	non
sugar fermentation:								
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	-	-	-	-	+/-	+/-	+	+
Sucrose	+	+	+	+	+	+	+	+
Arabinose	+	+	+	+	+	+	-	+
Xylose	-	-	-	-	-	-	-	-
Melibiose	+	+	+	+	-	-	-	+
Cellobiose	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-
Galactose	+/-	+/-	+/-	+/-	+/-	+	+	+
Salicin	+	+	+	+	+	+	+	-
Presumptive identification	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecium</i>	<i>E. faecalis</i>	<i>E. faecium</i>

P: pair SC: Short chain, +/-: variable

Continued...

Physiological and Biochemical Tests:	Isolate No.							
	9	10	11	12	13	14	15	16
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+
Growth at 45 ⁰ C	+	+	+	+	+	+	+	+
Growth at 10 ⁰ C	+	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+	+
Resistance to 60 ⁰ C for 30 min	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+	+
Growth in k-Tellurite	+	-	+	+	-	+	+	+
Liquefaction of gelatin	-	-	-	-	-	-	-	-
Hemolysis	non	beta	non	non	beta	alpha	alpha	non
sugar fermentation:								
Mannitol	+	+	+	+	-	+/-	+	+
Sorbitol	+	+/-	+	+/-	-	-	+	+
Sucrose	+	+	+	+	+	+	+	+
Arabinose	-	+	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-
Melibiose	+/-	-	-	-	+	+/-	+/-	-
Cellobiose	+	+	+	+	+	+	+	+
Raffinose	-	+/-	-	-	-	-	-	-
Galactose	+	+	+	+/-	+/-	+/-	+	+
Salicin	-	+/-	+/-	+	+	+	+	+
Presumptive identification	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. durans</i>	<i>E. durans</i>	<i>E. faecalis</i>	<i>E. faecalis</i>

Continued...

	Isolate No.							
Physiological and Biochemical Tests:	17	18	19	20	21	22	23	24
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+	+
Growth at 10°C	+	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+	+
Resistance to 60°C for 30 min	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+	+
Growth in k-Tellurite	+	+	+	+	+	+	+	+
Liquefaction of gelatin	-	-	-	-	-	-	-	-
Hemolysis	non	non	non	non	non	alpha	alpha	Beta
sugar fermentation:								
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	+
Sucrose	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-	-	-
Xylose	-	-	-	-	-	-	-	-
Melibiose	-	-	-	-	-	-	-	-
Cellobiose	+	+	+	+	+	+	+	+
Raffinose	-	-	-	+	-	-	-	-
Galactose	+	+	+	+	+	+	+	+
salicin	+	+	+	+	+	+	+	+
Presumptive identification	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>

Continued...

Physiological and Biochemical Tests:	Isolate No.							
	25	26	27	28	29	30	31	32
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+	+
sOxidase Test	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+
Growth at 45 ⁰ C	+	+	+	+	+	+	+	+
Growth at 10 ⁰ C	+	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+	+
Resistance to 60 ⁰ C for 30 min	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+	+
Growth in k-Tellurite	+	+	+	+	+	+	+	-
Liquefaction of gelatin	-	-	-	-	-	-	-	-
Hemolysis	alpha	non	alpha	non	non	non	beta	non
Sugar fermentation								
Mannitol	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	-	-	+
Sucrose	+	+	+	+	+	+	+	+
Arabinose	-	+	+	+	-	+	-	+/-
Xylose	-	+	+	+	-	+	-	-
Melibiose	-	+	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	+	-	+/-
Galactose	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	-	+	-	+
Presumptive identification	<i>E. faecalis</i>	<i>E. avium</i>	<i>E. avium</i>	<i>E. avium</i>	Atypical	<i>E. faecium</i>	Atypical	<i>E. faecium</i>

Continued...

	Isolate No.							
Physiological and Biochemical Tests:	33	34	35	36	37	38	39	40
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+	+
Growth at 45 ⁰ C	+	+	+	+	+	+	+	+
Growth at 10 ⁰ C	+	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+	+
Resistance to 60 ⁰ C for 30 min	+	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+	+
Growth in k-Tellurite	+	-	+	+	+	-	+	+
Liquefaction of gelatin	-	-	-	-	-	-	-	-
Hemolysis	alpha	alpha	non	beta	alpha	non	alpha	alpha
Sugar fermentation								
Mannitol	+	+	+	+	+	+	+	-
Sorbitol	+	-	+	-	+	+	+/-	+/-
Sucrose	+	+	+	+	+	+	+	+
Arabinose	-	-	-	-	-	+	-	-
Xylose	-	-	-	-	-	-	-	-
Melibiose	-	-	+	-	+	+	+	+
Cellobiose	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	+/-	-	-
Galactose	+	+	+	+	+	+	+	+
Salicin	+	+	+	+	+	+	+	+
Presumptive identification	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecium</i>	<i>E. avium</i>	<i>E. durans</i>

Continued...

	Isolate No.						
Physiological and Biochemical Tests:	41	42	43	44	45	46	47
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+	+
Growth at 45°C	+	+	+	+	+	+	+
Growth at 10°C	+	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+	+
Resistance to 60°C for 30 min	+	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+	+
Growth in k-Tellurite	+	+	+	+	+	-	+
Liquefaction of gelatin	-	-	-	-	-	-	-
Hemolysis	non	non	Non	non	non	non	non
sugar fermentation:							
Mannitol	-	-	+	-	-	+	+
Sorbitol	+/-	+	+	-	-	+	+
Sucrose	+	+	+	+	+/-	+	+
Arabinose	-	+	+	-	-	-	-
Xylose	-	-	-	-	-	-	-
Melibiose	+	+	+	+/-	+/-	+	-
Cellobiose	+	+	+	+	+	+	+
Raffinose	-	+	+	-	-	-	-
Galactose	+	+	+	+/-	+/-	+	+
Salicin	+	+	+	-	+	+/-	+
Presumptive identification	<i>E. durans</i>	Atypical	Atypical	<i>E. durans</i>	<i>E. durans</i>	Atypical	<i>E. faecalis</i>

Continued...

Physiological and Biochemical Tests:	Isolate No.					
	48	49	50	51	52	53
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Arrangement of cells	P & SC	P & SC	P & SC	P & SC	P & SC	P & SC
Gram's reaction	+	+	+	+	+	+
Oxidase Test	+	+	+	+	+	+
Catalase test	-	-	-	-	-	-
Growth in PH 9.6	+	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+	+
Growth at 45 ⁰ C	+	+	+	+	+	+
Growth at 10 ⁰ C	+	+	+	+	+	+
Growth in 0.1% methylene blue milk	+	+	+	+	+	+
Resistance to 60 ⁰ C for 30 min	+	+	+	+	+	+
Aesculin hydrolysis	+	+	+	+	+	+
Tetrazolium reduction at PH 6	+	+	+	+	+	+
Arginin hydrolysis	+	+	+	+	+	+
Growth in k-Tellurite	+	+	+	+	+	+
Liquefaction of gelatin	-	-	-	-	-	-
Hemolysis	non	alpha	beta	alpha	alpha	Alpha
sugar fermentation:						
Mannitol	+	+	+	+	+	+
Sorbitol	+	-	-	-	-	+
Sucrose	+	+	+	+	+	+
Arabinose	-	-	-	-	-	-
Xylose	-	-	-	-	-	-
Melibiose	-	+	+	+/-	+/-	+/-
Cellobiose	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-
Galactose	+	+	+	+	+	+/-
Salicin	+	-	+	+	+	+
Presumptive identification	<i>E. faecalis</i>	Atypical	Atypical	<i>E. faecalis</i>	<i>E. faecalis</i>	<i>E. faecalis</i>

4.2 Distribution of Enterococci

From 31 samples, 53 strains of enterococci were isolated which belonged to the following species: 24 isolates were *E. faecalis* strains (45%), 12 were *E. faecium* (23%), 6 were *E. durans* (11%), 4 were *E. avium* (7%). Remaining 7 isolates (13%) could not be assigned to particular species (**Fig.4.1**). *E. faecalis* was the predominant species isolated in these samples of Dahi. Laximinarayana *et al.* (1952) also reported predominance of *E. faecalis* in Dahi. Wessels *et al.* (1988) showed that *E. faecalis* (73.4% of isolates) was the prevalent species in cottage cheese, while Rao *et al.* (1986) working in India, found that *E. faecalis* was dominant in all dairy products except dried milk in which *E. faecium* dominated. *E. faecalis* was also among the predominant microorganisms found in raw milk (Devriese *et al.*, 1995). Suzzi *et al.* (2000) reported that enterococci were isolated from an artisanal Italian caprine cheese and that the most common species were *E. faecalis* and *E. faecium* followed by *E. durans*.

The above 53 isolates were further examined for their hemolytic activity by streaking the fresh test cultures on the blood agar. Out of 53 isolates 17 were found to be α -hemolytic as indicated by the slightly dark zone along the line of growth on streaked blood agar plate and six were found to be β -hemolytic as indicated by their clear zone along the line of growth on the streaked. Remaining thirty isolates were non-hemolytic (γ). It is well known that hemolytic activity has been associated with the virulence properties of enterococci although β -hemolysis is only a rough guide to potential pathogenicity (Moellering, 1992).

4.3 Antibiotic Resistance

Antibiotic susceptibility test was carried out by Disc Diffusion

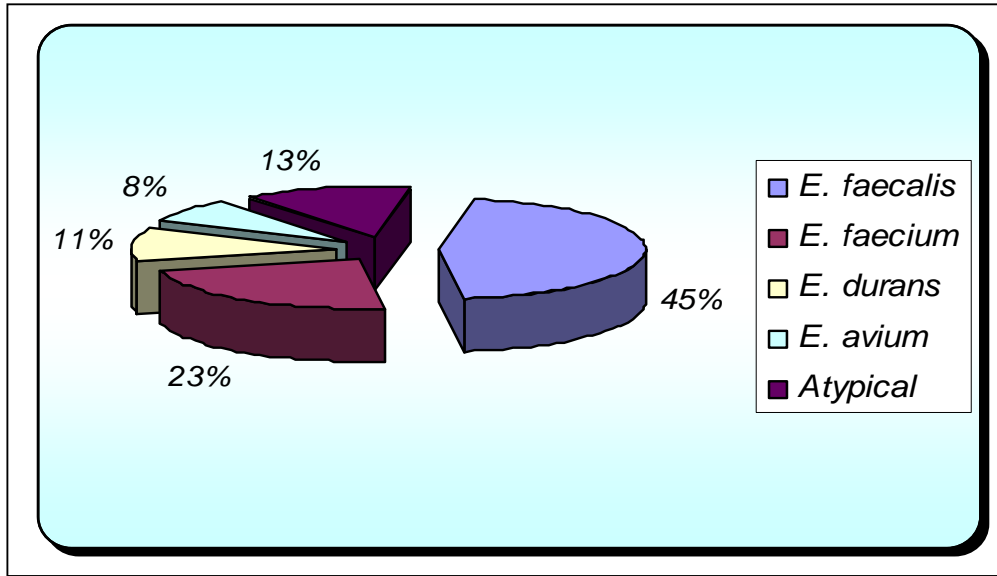


Fig. 4.1: Distribution of Enterococci in Dahi

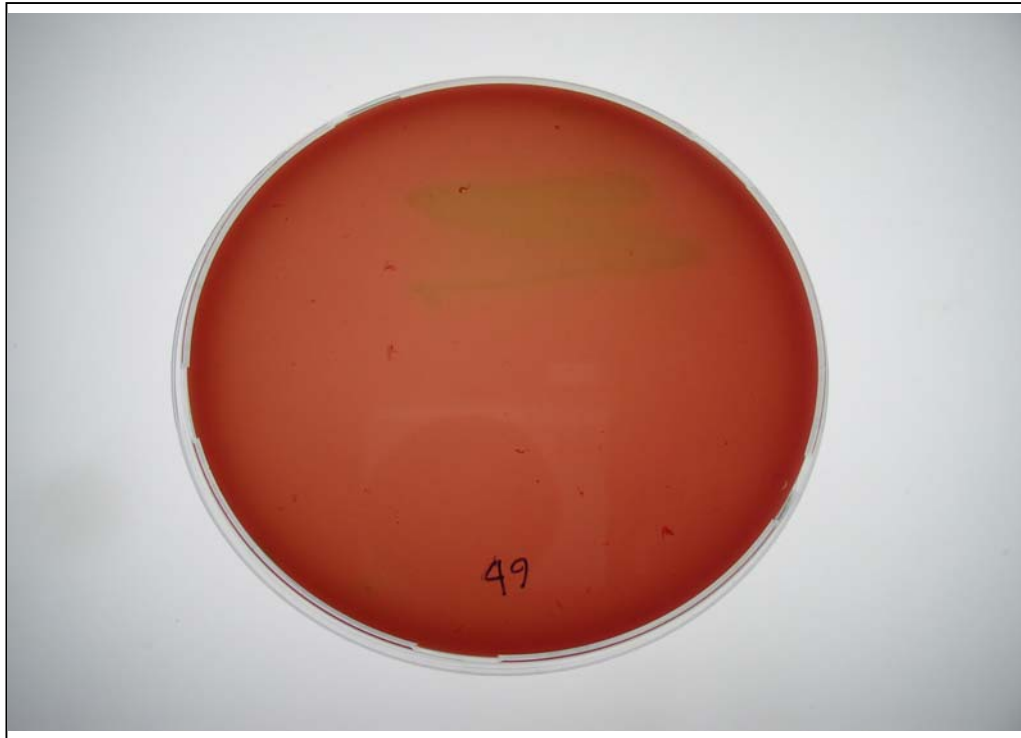


Plate 4.4: α -hemolysis on Blood Agar (Isolate No. 49)

Soft Agar Overlay method. MRS agar and MRS soft agar were used for this purpose. A total of nine antibiotics were taken in different concentrations (**Table 3.2**). All the isolates showed low level resistance to gentamicin and low to medium level resistance to streptomycin. None of the enterococci isolates exhibited resistance to ampicillin, vancomycin, teicoplanin, tetracycline and chloramphenicol. Among the 53 isolates, 44 were resistant to erythromycin and 20 isolates were found to be resistant to ciprofloxacin. Only one isolate was moderately susceptible to ampicillin. The range of zone diameter of all antibiotics against all the isolates are presented in **Table 4.3**. Klein *et al.* (1998) recovered 555 isolates of enterococci from raw minced beef and pork in Germany and found that most of the strains were susceptible to streptomycin and gentamicin in contrast to our findings of 100% resistance towards gentamicin and streptomycin. However most of the strains screened by them were sensitive to vancomycin, teicoplanin, chloramphenicol, tetracycline and ampicillin as observed in the present study also. Teuber *et al.* (1996) also reported that enterococci isolated from fermented sausage were frequently resistant to streptomycin. In a recent study Hays *et al.* (2003) reported that resistance to high level aminoglycosides was prevalent across all species recovered from retail meats. Whereas Batish and Ranganathan (1986) found that about 80% of *Enterococcus* isolates from milk samples showed varying degrees of resistance towards chloramphenicol, erythromycin, streptomycin, tetracycline and gentamicin.

4.4 Antibiotic resistance of *Enterococcus faecalis* isolates

All the 24 *Enterococcus faecalis* isolates were found to be resistant to streptomycin and gentamicin. None of the isolates of this species were resistant to ampicillin, vancomycin, chloramphenicol and tetracycline (**Table 4.4**). Only one strain was moderately susceptible to vancomycin. Ranjan, (2002) also similar results against vancomycin.



Plate 4.5: Antibiotic sensitivity/resistance of Isolate No. 38



Plate 4.6: Antibiotic sensitivity/resistance of Isolate No. 38

Table 4.3 Diameter of inhibition Zone of all Antibiotics by Disc Diffusion Method

Antibiotic Disc	Disc concentration (µg)	Range of zone diameter (mm)
Ampicillin	10	14-30
Vancomycin	30	15-20
Chloramphenicol	30	19-32
Tetracycline	30	16-37
Teicoplanin	30	14-20
Gentamicin	10	without zone to maximum 10
Erythromycin	15	without zone to maximum 16
Ciprofloxacin	5	9.5-20
Streptomycin	10	No zone

Among the 24 isolates of *E. faecalis*, 20 isolates were found to be resistant to erythromycin and remaining 4 isolates were moderately susceptible to this antibiotic. So none of the isolates was sensitive to erythromycin. Most of the isolates of this species were found to be moderately susceptible to ciprofloxacin. Among the total isolates, 7 were found to be resistant, 15 moderately susceptible and two isolates were sensitive to ciprofloxacin. Citak *et al.* (2004) also reported 33.8% *E. faecalis* strains to be resistant to ciprofloxacin which were isolated from Turkish white cheese.

Table 4.4: Antibiotic Resistance patterns of *Enterococcus faecalis* Isolates

Antibiotic	% <i>Enterococcus faecalis</i> isolates (n=24)		
	Resistant	Moderate Susceptible	Susceptible
Ampicillin	-	-	100
Vancomycin	-	4.20	95.83
Teicoplanin	-	-	100
Gentamicin	100	-	-
Streptomycin	100	-	-
Tetracycline	-	-	100
Chloramphenicol	-	-	100
Erythromycin	83.33	16.66	-
Ciprofloxacin	29.16	62.50	8.33

4.5 Antibiotic resistance of *Enterococcus faecium* isolates

All of the *E. faecium* isolates were found to be susceptible to vancomycin, tetracycline, chloramphenicol, teicoplanin and ampicillin and one strain which was moderately susceptible to ampicillin (**Table 4.5**). All 12 isolates were resistant to streptomycin and gentamicin. Among the 12 isolates 2 (16.66%) were found to be moderately susceptible to vancomycin. All the isolates were resistant to erythromycin except one strain. Among the 12 isolates 5 (41.67%) were found to be resistant to ciprofloxacin, 4 (33.33%) were intermediate resistant and remaining 3 (25%) isolates were sensitive. Hayes *et al.* (2003), reported that 39-87% strains of *E. faecium* to be resistant to tetracycline, erythromycin, and ciprofloxacin of enterococcal isolates from meat.

Table 4.5: Antibiotic resistance patterns of *Enterococcus faecium* isolates

Antibiotic	% <i>Enterococcus faecium</i> isolates (n=12)		
	Resistant	Moderate Susceptible	Susceptible
Ampicillin	-	8.33	91.66
Vancomycin	-	16.66	83.33
Teicoplanin	-	-	100
Gentamicin	100	-	-
Streptomycin	100	-	-
Tetracycline	-	16.66	83.33
Chloramphenicol	-	-	100
Erythromycin	91.66	8.33	-
Ciprofloxacin	41.67	33.33	25.0

4.6 Antibiotic resistance of *Enterococcus durans* isolates

All the *E. durans* isolates were found to be sensitive to ampicillin, tetracycline and chloramphenicol. Among 6 isolates 2 (33.33%) were intermediate resistant to vancomycin and remaining 4 (66.66%) isolates were sensitive (**Table 4.6**). All of *E. durans* isolates were resistant to erythromycin, gentamicin and streptomycin. Most of the isolates were found to be resistant to ciprofloxacin (83.33%) and no such isolates were there which were sensitive to ciprofloxacin

antibiotic. Citak *et al.* (2004) also reported that all *E. durans* strains to be resistant to streptomycin and erythromycin whereas 71% as resistant to gentamicin. However, Citak *et al.* (2004) reported all strains as resistant to chloramphenicol in contrast to 100% susceptibility as observed in present study.

4.7 Antibiotic resistance of *Enterococcus avium* isolates

From the 53 isolates , 4 were identified as *E. avium* strains. All the 4 strains were found to be sensitive to ampicillin, vancomycin, teicoplanin, and chloramphenicol (**Table4.7**). Single strain among these 4 was found to be resistant to erythromycin and rest (75%) were intermediate resistant. No isolates were found to be either resistant or sensitive to ciprofloxacin.

Table 4.6: Antibiotic resistance patterns of *Enterococcus durans* isolates

Antibiotic	% <i>Enterococcus durans</i> isolates (n=6)		
	Resistant	Moderate Susceptible	Susceptible
Ampicillin	-	-	100
Vancomycin	-	33.33	66.66
Teicoplanin	-	-	100
Gentamicin	100	-	-
Streptomycin	100	-	-
Tetracycline	-	-	100
Chloramphenicol	-	-	100
Erythromycin	100	-	-
Ciprofloxacin	83.33	16.66	-

Table 4.7: Antibiotic resistance patterns of *Enterococcus avium* isolates

Antibiotic	% <i>Enterococcus avium</i> isolates (n=4)		
	Resistant	Moderate Susceptible	Susceptible
Ampicillin	-	-	100
Vancomycin	-	-	100
Teicoplanin	-	-	100
Gentamicin	100	-	-
Streptomycin	100	-	-
Tetracycline	-	-	100
Chloramphenicol	-	-	100
Erythromycin	25.00	75.00	-
Ciprofloxacin	-	100	-

4.8 Antibiotic resistance of Atypical isolates

The number of atypical isolates was 7 which could not be assigned as species level. All such isolates were found to be sensitive to ampicillin, vancomycin, teicoplanin, tetracycline and chloramphenicol (Table 4.8). Most of the isolates (85.71%) were found to be resistant to erythromycin and remaining 14.29% were intermediate resistant. Coming to the ciprofloxacin resistance pattern, 3 (42.85%) isolates were found to be resistant and remaining 4 (57.14%) isolates were intermediate resistant, no isolates were sensitive to ciprofloxacin.

Table 4.8: Antibiotic Resistance patterns of Atypical Isolates

Antibiotic	% Atypical isolates (n=7)		
	Resistant	Moderate Susceptible	Susceptible
Ampicillin	-	-	100
Vancomycin	-	-	100
Teicoplanin	-	-	100
Gentamicin	100	-	-
Streptomycin	100	-	-
Tetracycline	-	-	100
Chloramphenicol	-	-	100
Erythromycin	85.71	14.29	-
Ciprofloxacin	42.85	57.14	-

4.9 MIC range of Enterococcal isolates

MIC may be defined as the minimum concentration of antibiotic solution that can inhibit the growth of organism at specified temperature. In this study MIC was determined for the selected organisms which were found to be resistant by Disc Diffusion Soft Agar Overlay method. For MIC determination 4 µg/ml to 512 µg/ml antibiotic solution was used for gentamicin, 0.25 µg/ml to 64 µg/ml for erythromycin, 16 µg/ml to 512 µg/ml for streptomycin and 0.25 µg/ml to 32 µg/ml for ciprofloxacin. . Since all the isolates were resistant to gentamicin and streptomycin, MIC was determined for all the enterococcal isolates. The MIC for gentamicin ranged between 64 µg/ml to >256 µg/ml. Among the total isolates three isolates (Isolate

No. 28, 31, & 40) were exhibited MIC > 256 µg/ml. Whereas the MIC range for streptomycin was 128 µg/ml to > 512 µg/ml. Coming to the erythromycin resistance, one isolate (Isolate No. 8) showed minimum inhibitory concentration of 64 µg/ml. The MIC for the other isolates ranged between 0.25 µg/ml to 32 µg/ml. In case of ciprofloxacin the range of MIC was 2 µg/ml to 16 µg/ml. Among the total resistant isolates two isolate (Isolate No. 13 and 40) showed MIC of 16 µg/ml.

Among the *E. faecalis* isolates no strains were found to exhibit MIC > 256 µg/ml for gentamicin, all were ranged from 64 µg/ml to 256 µg/ml. The *E. faecalis* strains also showed low to medium level resistance to streptomycin; eight isolates (33.3%) were found to exhibit MIC > 512 µg/ml. But none of the isolates of this species showed MIC 16 of µg/ml for ciprofloxacin.

Most of the isolates of *E. faecium* strains showed MIC 128 µg/ml for gentamicin, one isolate (Isolate No. 6) was there which showed MIC of 256 µg/ml. The MIC for streptomycin ranged from 256 µg/ml to > 512 µg/ml, 50% of the *E. faecium* isolates exhibited MIC of 256 µg/ml. The MIC for erythromycin ranged from 0.25 µg/ml to 64 µg/ml, whereas for ciprofloxacin it ranged from 2 µg/ml to 8 µg/ml.

All the *E. durans* strains showed MIC > 512 µg/ml for streptomycin except one strain (Isolate No. 13) which showed MIC 256 µg/ml. The MIC for gentamicin ranged from 64 µg/ml to > 256 µg/ml. Coming to the erythromycin resistance, the MIC ranged from 0.5 µg/ml to 32 µg/ml and for ciprofloxacin it ranged from 2 µg/ml to 8 µg/ml.

Among the four *E. avium* isolates no such strains were there which were found to be resistant to ciprofloxacin. Three strains exhibited MIC

>512 for streptomycin and MIC for gentamicin ranged from 64 µg/ml to > 256 µg/ml. There was no *E. avium* isolates which showed MIC > 8 µg/ml.

4.10 MIC range for Gentamicin

Since all the 53 isolates showed resistance to gentamicin, MIC for each isolates was performed by Broth Macrodilution method. The dilution of antibiotic ranged between 0.25 µg/ml to 512 µg/ml. Most of the isolates showed MIC in the range of 64-128 µg/ml (Fig. 4.2). There was no strains which exhibited MIC < 64 µg/ml, nine enterococcal isolates showed MIC in the range of 256-512 µg/ml.

4.11 MIC range for Erythromycin

Most of the enterococcal isolates were resistant to erythromycin. The number of such isolates was highest in the range of 1-2 µg/ml followed by 4-8 µg/ml (Fig.4.3). Thirteen isolates exhibited erythromycin resistance at very high level of 16-32 µg/ml and one strain at >32 µg/ml. Most of the *E. faecalis* and *E. avium* strains were found to be in the range of 1-2 µg/ml whereas majority of *E. faecium* strains had MIC in the range of 4-8 µg/ml.

4.12 MIC range for Streptomycin

All the enterococcal isolates exhibited low to medium level resistance to streptomycin. It is known that enterococci has low level intrinsic resistance to streptomycin and gentamicin and also able to acquire resistance gene from other species. Most of the enterococcal isolates were found to be in the range of 256-512 µg/ml followed by > 512 µg/ml (Fig.4.4). Only two strains (*E. faecalis* and *E. avium*) were found to be in the range of 64-128 µg/ml.



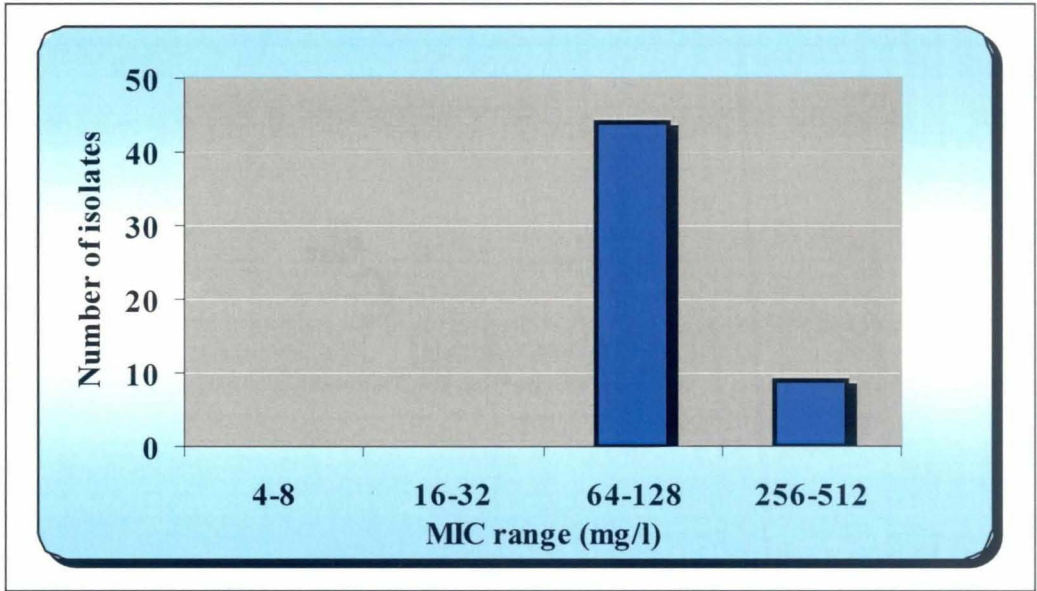


Fig. 4.2: MIC range for Gentamicin

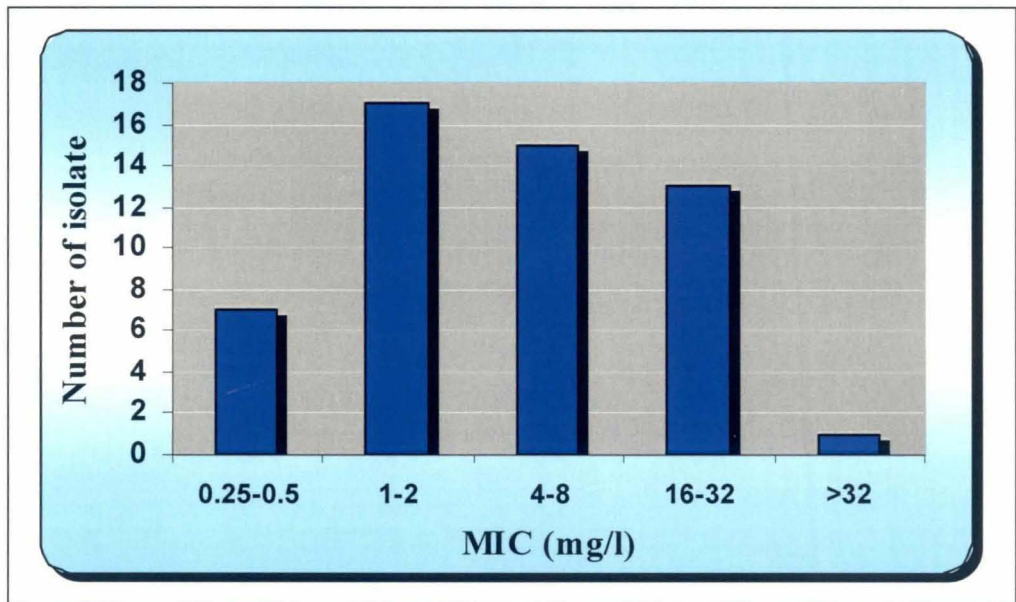


Fig. 4.3: MIC range for Erythromycin

4.13 MIC range for Ciprofloxacin

Maximum number of enterococcal isolates exhibited MIC in the range of 4-8 µg/ml for ciprofloxacin followed by 1-2 µg/ml and 6-32 µg/ml (Fig. 4.5). There were no such strains which showed MIC less than 1 µg/ml. Most of the *E. faecalis*, *E. durans* and *E. faecium* strains were found to be in the range of 4-8 µg/ml.

4.14 Multiple Drug Resistance patterns

Out of the 53 isolates 35.8% of strains exhibited co-resistance to four different antibiotics i.e. streptomycin, gentamicin, erythromycin and ciprofloxacin. Co-resistance to aminoglycosides (gentamicin + streptomycin) and erythromycin was found prevalent in 47.2% of the isolates. Only one isolate showed co-resistance to aminoglycosides and ciprofloxacin. Hayes *et al.* (2004) also reported co-resistance to four different types of antimicrobials in 52.7% of enterococcal isolates from poultry production environments.

Table 4.10: Multiple drug resistance patterns of *Enterococcus* isolates

Multi drug combination	No. of <i>Enterococcus</i> isolates(n=53)	% <i>Enterococcus</i> isolates
Gen. + Ery. + Str. + Cip.	19	35.84
Gen. + Ery. + Str.	25	47.2
Gen. +Str. + Cip.	1	1.8
Gen. + Str.	8	15.1

Gen.: Gentamicin, Ery.: Erythromycin, Str.: Streptomycin. Cip.: Ciprofloxacin

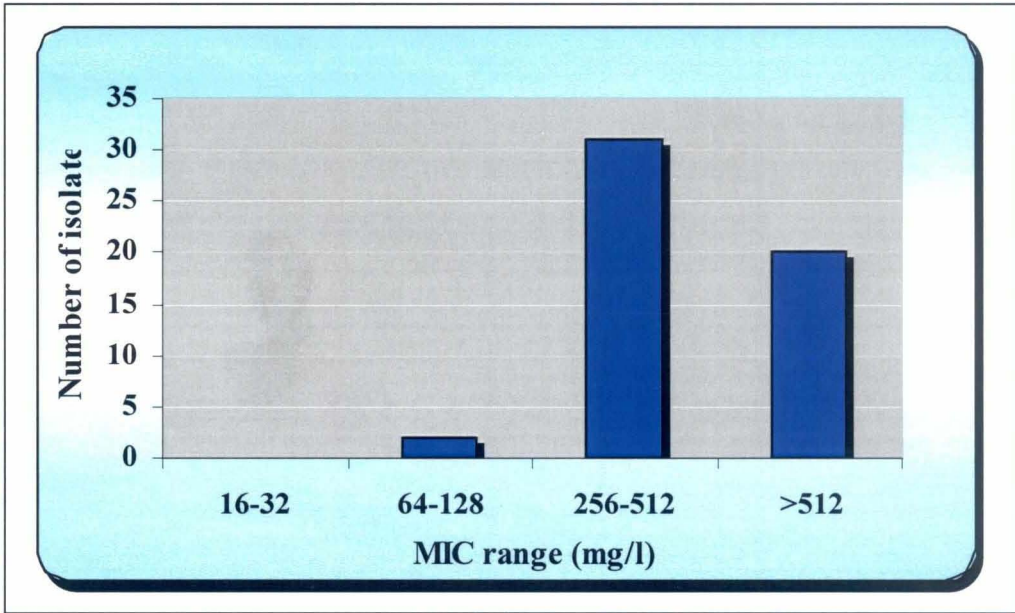


Fig. 4.4: MIC range for Streptomycin

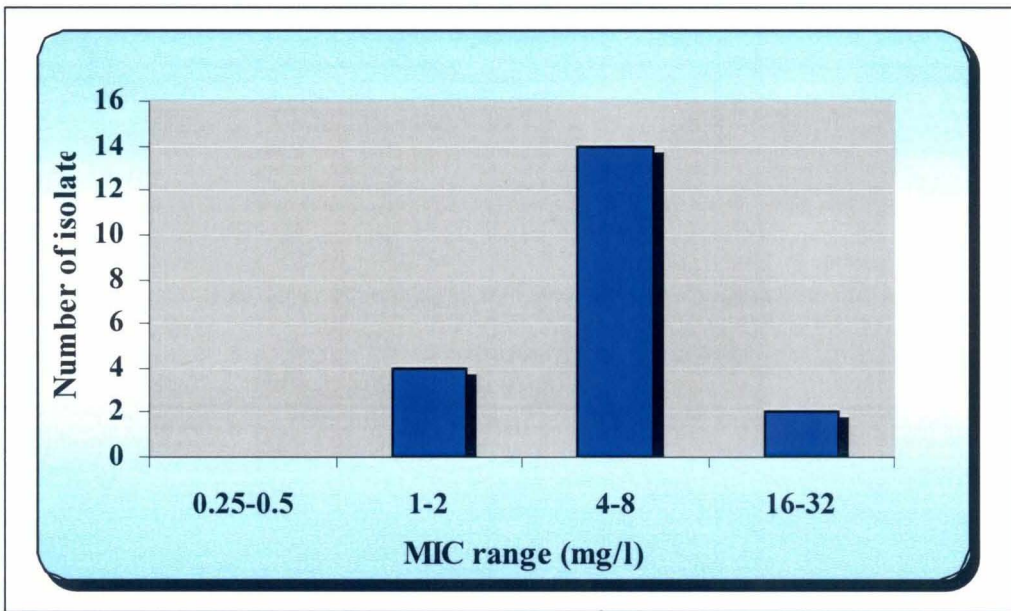


Fig. 4.5 MIC range for Ciprofloxacin

***Enterococcus faecalis* strains**

Among twenty four isolate, seven strains (29.2%) showed resistance to all the four antibiotics, thirteen strains (54.2%) showed resistance to gentamicin, erythromycin and streptomycin simultaneously. Remaining four strains were co-resistant to gentamicin and streptomycin.

Table 4.11: Multiple drug resistance patterns of *Enterococcus faecalis* strain

Multi drug combination	No. of <i>E. faecalis</i> isolates (n=24)	% <i>E. faecalis</i> isolates
Gen. + Ery. + Str. + Cip.	7	29.2
Gen. + Ery. + Str.	13	54.2
Gen. + Str. + Cip.	Nil	Nil
Gen. + Str.	4	16.6

***Enterococcus faecium* strains**

Five isolates (41.6%), among the twelve isolates showed co-resistance to four antibiotics i.e. gentamicin, erythromycin, streptomycin and ciprofloxacin. Six isolates (50%) exhibited multi resistance to gentamicin, erythromycin and streptomycin. Like *E. faecalis* strains no such *E. faecium* isolates were present which showed multi drug resistant to gentamicin, streptomycin and ciprofloxacin combination. The remaining one isolate of *E. faecium* showed co-resistance to gentamicin and streptomycin only. Hayes *et al.* (2004) screened 104 isolates of *E. faecium* and reported 32% to be co-resistant to lincosamide, penicillin, streptomycin and tetracycline (LPST), whereas 14% were co-resistant to lincosamide, streptomycin and tetracycline (LST). They reported wide diversity of co-resistance in *E. faecium* strains. In the present study only 12 isolates have been studied.

***Enterococcus durans* strains**

All the *E. durans* isolates exhibited multiple resistances to gentamicin, erythromycin, streptomycin and ciprofloxacin. Hayes *et al.* (2004) screened 5 isolates of *E. durans* and reported 4 strains to be

Table 4.12: Multiple drug resistance patterns of *Enterococcus faecium* strains

Multi drug combination	No. of <i>E.faecium</i> isolates (n=12)	% <i>E.faecium</i> isolates
Gen. + Ery. + Str. + Cip.	5	41.6
Gen. + Ery. + Str.	6	50
Gen. +Str. + Cip.	Nil	Nil
Gen. + Str.	1	8.3

co-resistant to lincosamide, macrolide and tetracycline (LMT) and the fifth strain was co-resistant to five antimicrobials i.e. lincosamide, penicillin, macrolide, streptomycin and tetracycline (LMPST). The diversity of resistance pattern appears to be limited in *E. durans* although the total number of isolates is rather small.

Table 4.13: Multiple Drug resistance patterns of *Enterococcus durans* strains

Multi drug combination	No. of <i>E. durans</i> isolates (n=6)	% <i>E. durans</i> isolates
Gen. + Ery. + Str. + Cip.	6	100
Gen. + Ery. + Str.	Nil	Nil
Gen. +Str. + Cip.	Nil	Nil
Gen. + Str.	Nil	Nil

***Enterococcus avium* strains**

Among the four E. avium isolates three showed multiple resistance to gentamicin and streptomycin combination and remaining one was resistant to gentamicin, erythromycin and streptomycin

combination. No isolates showed multiple resistance to all four antibiotics. Hayes *et al.* (2004) reported one isolate of *E. avium* to be resistant to lincosamide, macrolide & tetracycline.

Table 4.14: Multiple Drug resistance patterns of *Enterococcus avium* strains

Multi drug combination	No. of <i>E. avium</i> isolates (n=4)	% <i>E. avium</i> isolates
Gen. + Ery. + Str. + Cip.	Nil	Nil
Gen. + Ery. + Str.	1	25
Gen. +Str. + Cip.	Nil	Nil
Gen. + Str.	3	75

Atypical isolates

Seven strains that could not be assigned to particular species are put into the atypical group. Out of this group a majority of 5 strains showed co- resistance to gentamicin, erythromycin, & streptomycin. Whereas one isolates showed co-resistance to gentamicin, erythromycin, streptomycin & ciprofloxacin and the other one to gentamicin, streptomycin, & ciprofloxacin combination.

Table 4.15: Multiple Drug resistance patterns of Atypical isolates

Multi drug combination	No. of atypical isolates (n=7)	%Atypical isolates
Gen. + Ery. + Str. + Cip.	1	14.3
Gen. + Ery. + Str.	5	71.4
Gen. +Str. + Cip.	1	14.3
Gen. + Str.	Nil	Nil

The present study has shown prevalence of antibiotic resistant enterococci in Dahi sample, *E. faecalis* was most prevalent, followed by *E. faecium*, *E. durans* and *E. avium*. Similar trend has been observed in other foods many previous workers (Suzzi *et al.*, 2000). While *Enterococcus* resistance to glycopeptides is among current

clinical concerns, vancomycin-resistant enterococci could not be detected in the present study. Citak *et al.* (2004) have reported vancomycin resistant enterococci from cheese samples. All the enterococci in this study are showing resistance to aminoglycosides (streptomycin and gentamicin), which may be natural resistance as reported earlier (Lukasova and Sustackova, 2003). Multiple drug resistance was detected in large number of isolates which showed co-resistance to gen.+ery.+strep. and also to four antibiotics i.e. Gen.+strep.+cip.+ery. which may be a cause for concern. This study provides evidence that can be used in subsequent risk management exercises to elucidate the role of Dahi prepared by traditional method in the dissemination of antibiotic resistance to human populations. The findings indicate that Dahi samples harbour strains of enterococci that are resistant to many commonly used antibiotics. As Dahi consumed without any heat treatment these resistant bacteria become all the more important. The present study should be helpful in identification of future study topics and initiatives aimed at reducing the public health burden of antibiotic resistant pathogens.

5. SUMMARY AND CONCLUSION

- A total of thirty eight samples of Dahi were collected for isolation of enterococci.
- Modified Citrate Azide Agar medium was used for enumeration and isolation of enterococci.
- Out of which thirty one samples were found to harbor ^{of} enterococci ranging from 7×10^2 to 141×10^2 CFU/g.
- The isolates were screened for various characteristics and fifty three isolates were found to be enterococci.
- On the basis of morphological, physiological and biochemical tests, twenty four strains were tentatively identified as *E. faecalis*; twelve as *E. faecium*; six as *E. durans*; four as *E. avium* and seven strains could not be assigned to any species due to their atypical sugar fermentation pattern.
- *Enterococcus faecalis* was the predominant species (45%), followed by *E. faecium* (23%), *E. durans* (11%) and *E. avium* (8%). The atypical isolates accounted for 13% of the isolated strains.
- All the fifty three isolates were tested for Gelatin liquefaction and none of these isolates liquefied gelatin.
- All the enterococcal isolates were also tested for hemolytic activity and out of these, thirty were found to be non (γ) hemolytic, seventeen were found to be α hemolytic and only six were found to be β hemolytic.
- All the isolates were tested for antibiotic resistance by Disc Diffusion Soft Agar Overlay method against nine antibiotics namely, ampicillin, vancomycin, teicoplanin, streptomycin, gentamicin, chloramphenicol, erythromycin, tetracycline and ciprofloxacin.
- All the isolates showed resistance to streptomycin as none of them had a zone of clearance by Disc Diffusion method.

- All the isolates were found to be sensitive to ampicillin, vancomycin, chloramphenicol, tetracycline and teicoplanin.
- All the enterococcal isolates were found to be resistant to gentamicin as only small zone of clearance could be noticed in few cases.
- Twenty isolates were found to be resistant to ciprofloxacin and forty four were resistant to erythromycin.
- Minimum inhibitory concentration was determined for four antibiotics i.e. gentamicin, streptomycin, erythromycin and ciprofloxacin for the resistant strains by using Broth macrodilution method.
- MIC ranges for the resistant isolates were: erythromycin = 0.25-64 µg/ml, streptomycin= 128- >512 µg/ml, gentamicin= 64- >256 µg/ml and ciprofloxacin= 2-16 µg/ml.
- One strain which exhibited MIC at 64 µg/ml for erythromycin. Majority of the strains were almost evenly distributed showing MIC 1-2 µg/ml (17), 4-8 µg/ml (15) and 16-32 µg/ml (13).
- Most of the strains (44) showed MIC for gentamicin between 64-128 µg/ml, whereas nine strains showed MIC at 256-512 µg/ml.
- For streptomycin, majority of strains showed MIC at 256-512 µg/ml whereas twenty had MIC at >512 µg/ml. Two strains showed MIC at 64-128 µg/ml.
- For ciprofloxacin, twenty isolates showed resistance and the MIC ranged between 1-2 µg/ml for four isolates, 4-8 µg/ml for fourteen isolates and 16-32 µg/ml for two isolates.
- *E. faecalis* isolates showed varying MIC range for erythromycin (0.25-16 µg/ml). No isolates of this species were found to exhibit MIC >256 µg/ml for gentamicin, all were ranged from 64 µg/ml to 256 µg/ml. The *E. faecalis* strains also showed low to medium level resistance to streptomycin. None of the isolates of this species showed MIC at 16 µg/ml for ciprofloxacin. Among twenty four isolates of this species seven (29.2%) showed co-resistance to gentamicin, erythromycin, streptomycin and ciprofloxacin and most of the isolates (54.2%)

exhibited multiple resistance to gentamicin, erythromycin and streptomycin.

- Most of the isolates of *E. faecium* strains showed MIC of 128 µg/ml for gentamicin. The MIC for erythromycin ranged from 0.25 µg/ml to 64 µg/ml and for ciprofloxacin it ranged from 2 µg/ml to 8 µg/ml. Most of the *E. faecium* isolates exhibited MIC of 256 µg/ml. Five (41.6%), among the twelve *E. faecium* isolates showed co-resistance to four antibiotics i.e. gentamicin, erythromycin, streptomycin and ciprofloxacin. Six (50%) exhibited multiple resistance to gentamicin, erythromycin and streptomycin.
- All the *E. durans* strains were found to be resistant to ciprofloxacin and most of the isolates of this species showed MIC >512 µg/ml for streptomycin. The MIC for gentamicin ranged from 64 µg/ml to > 256 µg/ml and for erythromycin it ranged between 0.5 µg/ml and 32 µg/ml. All the *E. durans* isolates exhibited co-resistance to gentamicin, erythromycin, streptomycin and ciprofloxacin.
- Among the *E. avium* species there were not a single strains which exhibited ciprofloxacin resistance. Like *E. durans* species most of the strains of this species showed MIC >512 µg/ml for streptomycin and the MIC for gentamicin ranged from 64 µg/ml to > 256 µg/ml. Three among the four isolates showed multiple resistance to gentamicin and streptomycin and remaining one showed co-resistance to gentamicin, erythromycin and streptomycin combination.
- The present study has shown prevalence of antibiotic resistant enterococci in Dahi sample, *E. faecalis* was most prevalent, followed by *E. faecium*, *E. durans* and *E. avium*.
- While Enterococcus resistance to glycopeptides is among current clinical concerns, vancomycin-resistant enterococci could not be detected in the present study.
- Multiple drug resistance was detected in large number of isolates which showed co-resistance to genta+erythro+strepto. and also to four antibiotics i.e. Gen.+strep.+cip.+ery. which may be a cause for concern.

- This study provides evidence that can be used in subsequent risk management exercises to elucidate the role of Dahi prepared by traditional method in the dissemination of antibiotic resistance to human populations.
- The findings indicate that Dahi samples harbour strains of enterococci that are resistant to many commonly used antibiotics. As Dahi consumed without any heat treatment these resistant bacteria become all the more important.
- The present study should be helpful in identification of future study topics and initiatives aimed at reducing the public health burden of antibiotic resistant pathogens.

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APPENDIX

Table 1: Diameter of inhibition Zone of all Antibiotics

Isolate No.	Ampicillin	Vancomycin	Gentamicin	Chloramphenicol	Streptomycin	Tetracyclin	Teicoplanin	Erythromycin	Ciprofloxacin
1	14	18	8	23	6	29	14	8	13.5
2	18	17	8	24	6	28	15	9	13
3	19.5	18	6	24	6	29.5	14	9	13
4	18	16	6	24.5	6	28	14	8	11
5	21	18.5	6	20	6	24	15	8	11.5
6	22.5	17	6	20	6	23.5	14.5	8	12
7	21.5	17	6	21.5	6	25	14.5	7.5	11
8	20	16	6	24.5	6	29	14	6	17
9	19.5	17	6	22.5	6	28	15	6	11.5
10	18	17	6	20.5	6	26	14	6	12
11	21.5	16.5	6	19.5	6	23	14	8	9.5
12	21	15.5	6	20.5	6	26	14.5	6	12.5
13	24.5	15	8	19	6	27	15	9.5	11
14	19.5	19.5	6	22	6	25	15	14	12.5
15	29	18.5	6	25	6	30.5	20	12.5	17
16	29.5	19	6	23	6	29	17	9.5	14.5
17	30	17	6	25	6	29.5	17	13	13.5
18	30	17.5	6	25	6	26.5	17.5	9.5	20
19	29.5	19	6	27	6	30	17.5	14	16.5
20	31	17	6	32	6	36	18	14	18
21	30	18.5	6	29	6	36.5	18	16	17.5
22	36	18	6	27	6	37	17.5	13	16
23	31	18.5	6	31	6	33	17.5	12	17.5
24	30.5	18.5	6	30	6	33	18	12	15
25	31	18	6	28	6	32	17.5	13.5	16.5
26	30.5	17.5	6	29	6	32.5	18.5	16	16
27	28	20	6	30	6	31.5	19	14	17

Continue...

Isolate No.	Ampicillin	Vancomycin	Gentamicin	Chloramphenicol	Streptomycin	Tetracyclin	Teicoplanin	Erythromycin	Ciprofloxacin
28	29.5	18.5	7.5	27.5	6	31.5	20	14.5	18
29	25	18	6	24.5	6	26	15	6	13.5
30	29	17	6	25	6	17	17.5	13	15
31	24.5	17	6	22	6	24.5	16	6	13
32	24.5	17.5	6	22	6	16	17	10.5	13
33	20	18	6	21	6	25.5	15	13	13
34	19.5	18	6	19	6	23.5	14	13.5	12.5
35	27.5	18.5	6	27.5	6	29	16	6	13.5
36	24.5	18.5	7	24	6	7	16	16	14
37	24.5	18.5	7	24.5	6	28.5	16.5	6	12.5
38	29	19	9.5	25	6	28.5	17	15	14
39	24.5	18.5	7	28	6	27.5	16.5	6	14
40	27	16	6	24	6	23.5	15.5	9.5	12
41	32.5	18	6	27.5	6	31.5	18.5	12.5	15
42	30	16.5	6	26.5	6	31	18	11.5	15
43	27	17.5	6	25	6	27	17	12.5	13.5
44	26.5	19	6	24.5	6	27.5	16	6	12.5
45	25	17.5	6	26	6	27	15.5	6	11
46	26	17.5	9	26.5	6	31	17.5	13.5	17.5
47	27.5	18	6	24	6	29.5	18	12.5	19
48	23	17	9.6	24.5	6	26	18	14	15.5
49	24	19	6	25	6	26	15.5	8.5	12.5
50	23	18	6	25.5	6	26	17	17	13
51	25	17	6	21	6	27.5	16	12.5	13.5
52	25.5	17.5	7	25	6	27	18	9	12.5
53	23.5	18	10	24	6	26	15.5	10.5	12

Table 2: Minimum inhibitory concentrations of antibiotic resistant Enterococcal isolates

Isolate No.	Gentamicin	Erythromycin	Streptomycin	Ciprofloxacin
1	128	8	256	-
2	128	8	256	-
3	128	8	256	-
4	64	16	256	-
5	128	4	512	8
6	256	8	256	8
7	128	16	>512	8
8	64	64	512	-
9	64	32	256	2
10	128	32	256	2
11	256	4	>512	8
12	128	8	256	8
13	64	16	>512	16
14	128	4	>512	2
15	128	0.5	256	-
16	128	2	256	-
17	256	4	256	-
18	128	16	>512	-
19	128	-	256	-
20	256	-	>512	-
21	64	-	256	-
22	128	1	512	-
23	128	2	>512	-
24	64	0.5	512	-
25	64	1	256	-
26	64	-	>512	-
27	128	-	>512	-
28	>256	-	>512	-
29	128	1	>512	-
30	128	2	>512	-
31	>256	32	>512	-
32	128	16	512	4
33	64	0.25	>512	2
34	64	0.25	>512	4
35	128	32	256	-
36	64	4	256	-
37	64	1	128	4
38	64	1	>512	-
39	128	8	128	-
40	>256	8	256	16
41	256	16	256	-
42	128	16	>512	-
43	256	4	256	-
44	64	8	256	4
45	128	32	256	4
46	64	1	>512	-
47	128	2	>512	-
48	64	-	>512	-
49	256	8	256	4
50	64	-	256	4
51	128	2	256	-
52	64	2	256	8
53	64	1	256	4