Isolation, identification and characterization of thermoduric psychrotrophic bacteria from market samples of pasteurized milk collected from Mannuthy, Kerala State, India

ABSTRACT

Aims: To isolate, identify and characterize thermoduric psychrotrophic bacteria from locally available market samples of pasteurized milk.

Study design: Market samples of pasteurized milk were collected, subjected to laboratory pasteurization and thermoduric psychrotrophic bacteria were enumerated after 10 days of storage at 7ºC.The isolates obtained were identified by 16S rRNA sequencing and subsequent blasting.The isolates were characterized in terms of haemolytic, lipolytic and proteolytic activities. Selected isolates were also assessed for their decimal reduction time at 63ºC.

Place and Duration of Study: Verghese Kurien Institute of Dairy and Food Technology, (Formerly College of Dairy Science and Technology), Mannuthy, Thrissur, Kerala between September, 2019 to March, 2021.

Methodology: A total of 42 market samples of pasteurized milk were assessed for their thermoduric psychrotrophic bacterial count. The thermoduric psychrotrophic bacterial isolates were identified by 16S rRNA sequencing and the sequences obtained were searched with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) for their closest relatives/reference strains with a homology of over or equal to 99 per cent.Haemolytic, Proteolytic and Lipolytic Activities of the isolates were determined by streaking them on blood agar, skim milk agar (SMA) and tributyrin agar respectively and grading them based on the presence or absence of zone of clearance around the colonies developed. Decimal reduction time at 63°C of two selected isolates was also determined.

Results: Six thermoduric psychrotrophic bacteria were isolated from the 42 market samples of pasteurized milki.e., only 14.3 % of the tested samples had thermoduric psychrotrophic pbacterial population. 16S rRNA sequencing and subsequent blasting identified the isolates as *Aeromonas caviae*, *Moraxella osloensis* ,*Delftia tsuruhatensis*, *Staphylococcus arlettae* and two strains of *Carnobacterium maltaromaticum.* On assessing the haemolytic activity of the isolates, *A.caviae* DMV01 exhibited α haemolytic activity whereas *Staphylococcus arlettae* DMV02, *M. osloensis* DMV03, *C. maltaromaticum* DMV05 and *C. maltaromaticum* DMV06 exhibited ϒ haemolytic activities. *A. caviae* DMV01 was found to be lipolytic and the two *Carnobacterium* strains exhibited proteolytic activities. *Staphylococcus arlettae* DMV02 was found to be both proteolytic and lipolytic. *Aeromonas caviae* DMV01 exhibited D_{63} value of 4 minutes 38 seconds whereas for *Moraxella osloensis* DMV03 it was 25 minutes 18 seconds.

Conclusion: Exhibition of heat resistance by microorganisms capable of low temperature growth presents quite a challenging situation in terms of quality assurance and shelf life extension of pasteurized milk. So it is high time that dairy industry takes up initiatives to remain updated with the changes happening in pasteurized milk microflora in order to address the challenges such microorganisms may pose in the future.

Keywords: *Thermoduric Psychrotrophs, Aeromonas caviae, Staphylococcus arlettae, Carnobacterium maltaromaticum, Delftia tsuruhatensis*

INTRODUCTION

Microbiology of milk is an area warranting continuous research interest as it includes a multifaceted and highly flexible microbial community comprising of pathogens, spoilage and beneficial microorganisms. While the beneficial bacteria are explored for their industrial applications, the pathogenic and spoilage organisms are closely watched for their implications on the product quality and safety. Foodborne illnesses being a major threat to public health and safety, significant concerns

still occur with respect to the quality management of milk and milk products. Considering the highly perishable nature of milk, the dairy industry uses different techniques to assure the safety and quality of its products. Refrigeration is one of those food preservation techniques used either alone or in combination with other methods. A typical combination dairy industry adopts is pasteurization and subsequent chilled storage of pasteurized milk. Pasteurization is the process of heating every particle of milk to at least 63^{o}C for 30 min (Low Temperature Long Time, LTLT) or 72^{o}C for 15 sec (High Temperature Short Time, HTST) or to any temperature-time combination which is equally efficient. Pasteurization of milk typically reduces psychrotrophic and mesophilic populations, leaving only two main groups in pasteurized milk; the heat resistant bacteria, which survive pasteurization (thermoduric microorganisms) and the bacteria introduced through post-pasteurization contamination [1]. As low temperature storage of less than 8°C [2] is the recommended storage condition for pasteurized milk, microorganisms capable of growing under refrigeration play a critical role in determining the shelf life of these products. The trend towards higher pasteurization temperatures and extended refrigerated storage of milk prior to consumption has brought to attention a selected group of microorganisms ,the 'thermoduric psychrotrophs';the organisms capable of surviving pasteurization and growing under refrigeration conditions. Contamination with these types of microorganisms, factors like raw milk microbial load, extent of hygienic practices adopted, and climatic constraints are the major reasons for the poor shelf life of pasteurized milk in developing countries. Commonly reported pasteurization surviving psychrotrophs are members of the genera *Bacillus, Arthrobacter, Microbacterium, Enterococcus, Streptococcus, Lactobacillus* and *Corynebacterium* [3,4]. The psychrotrophic thermodurics along with the *Bacillus cereus* group and the sulphite-reducing clostridia (SRC) are reported as the major bacterial groups of concern to the dairy industry [5].Presence of thermoduric psychrotophs in 40% of the raw milk in the region of Castro in Parana, Brazil was reported by Ribeiro Junior *et al*.[6]. Despite the significance of this unique category microbial group in pasteurized milk not many studies are conducted in this subject area. As milk microbiota is highly dependent on several host and environmental factors, significant locale specific variations like those reported for human milk by Kumar *et al*.[7] can exist in bovine milk also. Regional wise studies will be helpful to understand the microenvironment of milk and milk products produced and marketed in each area.Taking into consideration these aspects,an attempt was made to isolate, identify and characterize thermoduric psychrotrophic bacteria from locally available market samples of pasteurized milk.

MATERIALS AND METHODS

Enumeration and isolation of thermoduric psychrotrophs:A total of 42 High Temperature Short Time (HTST) pasteurized milk samples in Low/High Density Polyethylene (LDPE/HDPE) sachets were collected from the local market (Mannuthy, Kerala, India) over a time period of three months from September, 2019. Samples were immediately transported to research laboratory at 4°C in ice boxes packed with ice packs. In order to avoid the chances of isolation of any post pasteurization contaminant psychrotroph, all the samples were subjected to laboratory pasteurization as per Bureau of Indian Standards stipulated procedure [8]For this,five ml uniformly mixed representative sample was transferred aseptically into sterile test tubes and were placed in a water bath ensuring that the level of milk was below the water level of the water bath. The sample was heated at 63°C for 30 minutes making sure that the milk attained the specified temperature in less than five minutes. To accurately monitor the heating process, a control tube was also kept along with the samples. At the end of heating, the tubes were immediately transferred to ice bath, chilled to 7°C and incubated at 7°C for 10 days[3]. Under aseptic conditions appropriate dilutions of these samples were pour plated using Plate Count Agar (PCA, HiMedia Laboratories Pvt.Ltd., Mumbai) and incubated at 7°C for 10 days for enumerating the thermoduric psychrotrophic organisms [9] colonies with distinct colony morphologies were picked, streaked to purity and subcultured in nutrient broth. Their thermoduric nature was reconfirmed through two more separate laboratory pasteurization trials.

Identification of the isolates: For primary identification, the isolates were subjected to Gram staining, catalase and oxidase tests [10] Scanning electron microscopy (SEM) was performed using the Tescan Vega 3 (Czech Republic) scanning electron microscope operated at 12kV and different magnifications. Genotypic identification of the isolate was carried out by 16S ribosomal RNA (16Sr RNA) sequencing. 16S-RS-F (CAGGCCTAACACATGCAAGTC) and 16S-RS-R (GGGCGGWGTGTACAAGGC) were used as forward and reverse primers respectively. The sequences obtained were searched with the NCBI BLAST program (http://www.ncbi.nlm.nih.gov) for their closest relatives/reference strains with a homology of over or equal to 99 per cent. The sequences were deposited in Genbank using BankIt (https://www.ncbi.nlm.nih.gov/WebSub/)

program. With the help of MEGA software, a phylogenetic tree was constructed using neighbour joining method to compare the evolutionary distances between their closest relatives.

Assessment of Haemolytic, Proteolytic and Lipolytic Activities of the isolates: For testing the haemolytic activity, active culture of the isolates were streaked on to blood agar plates, incubated and based on the type of clear zones developed around the colonies the isolates were graded [11]. Proteolytic .activity of the isolates was determined by streaking on skim milk agar (SMA) and subsequent incubation at 37°C for 24 hours. Similarly, for assessing the lipolytic activity, activated cultures of the isolates were streaked on tributyrin agar (TBA, HiMedia Laboratories Pvt. Ltd., Mumbai) and incubated at 37°C for 24 hours .In both cases, formation of a zone of clearance around the growth was considered as positive for enzyme production.

Determination of D_{63} value of selected isolates: Considering that the isolates were obtained from laboratory pasteurized milk their decimal reduction time at 63°C was determined as per Peng *et al*.[12]For this, 0.5 McFarland density adjusted cell suspensions were inoculated at the rate of one per cent into sterilized whole milk tubes. The inoculated tubes were mixed well and were placed along with the temperature control tube in a water bath and after the specified period of heating (0, 5, 10, 15, 20, 25 and 30 minutes) at 63°C, the corresponding sample tube was removed from the water bath. Immediately chilled to below 7°C, appropriately diluted with normal saline, pour plated using PCA and incubated at 37°C for 48 hours. Thermal reduction time (*D* -value) was calculated from the slope of the best-fit line graphically determined by plotting the $\log_{10}$ of values of the surviving cells per millilitre against the time of heat exposure at that temperature [13].

RESULTS AND DISCUSSION

Enumeration and isolation of thermoduric psychrotrophs: Thermoduric psychrotrophs were found in six out of the 42 samples tested. Thermoduric psychrotrophic count of laboratory pasteurized market samples after 10 days of refrigerated storage ranged from 0.3 Log_{10} CFU/ml in pasteurized milk sample -3 (PM-3) to 2.76 Log_{10} CFU/ml in PM-38 (Fig.1). Presence of thermoduric psychrotrophs in 14.3% of the pasteurized milk sample is much lower than the 27.2 % and 40% reported in raw milk samples by Johnston and Bruce [14]and *Ribeiro* Júnior*et al.*[6] respectively. As 86% of the tested samples were devoid of thermoduric psychrotrophs, psychrotrophic microorganisms entering as post-pasteurization contaminants could be the major determinants of shelf life of pasteurized milk

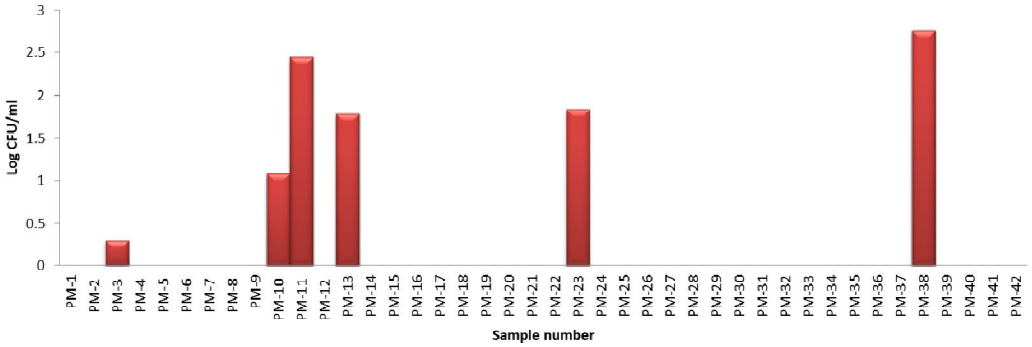
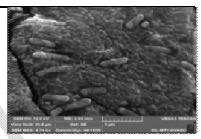
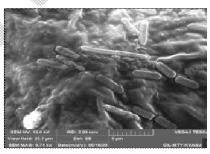
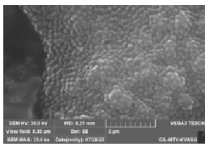
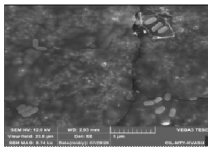
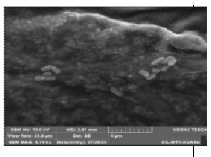


Fig.1.Thermoduric psychrotrophic count of laboratory pasteurized market samples of pasteurized milk after 10 days of refrigerated storage. PM-1 to PM-42 = Pasteurized milk samples with sample numbers

Identification of the isolates: The thermoduric psychrotrophic isolates obtained from six different samples were labeled Is1, Is2, Is3, Is4 Is5 and Is6. Preliminary identification revealed three of the isolates (Is1, Is2, Is3) as Gram negative, catalase and oxidase positive rods and the other three as Gram positive and oxidase negative(Table 1).Among the latter three,Is4 was a catalase positive cocci and the other two, Is5 and Is6 were catalase negative rods.As observed in our study some previous studies have also reported the occurrence of non spore forming thermoduric psychrotrophic bacterial

isolates from milk and milk products [15, ,3,16,6].In the current study, despite the development of colonies of members of the genus *Bacillus* in the thermoduric count Petri dishes no such colonies were developed on thermoduric psychrotrophic count Petri dishes . Absence of members of the genus *Bacillus* could be due to the additional 10 days of incubation at 7°C and subsequent enumeration carried out at low temperature (7°C). It can be inferred that these low temperature incubations might have lead to the selective isolation of these atypical isolates. Based on this observation it can be inferred that psychrotrophic B*acillus* spp. are not that prevalent in pasteurized milk, at least in the locality and during the period of sample collection.Reports of heat resistant Gram negative bacteria in milk are quite scanty. But considering the opinion of Quigley *et al*. [1] that the microbes, usually recognized as heat sensitive may have the potential to survive commercial pasteurization, isolation of Gram negative organisms from refrigerated samples of laboratory re-pasteurized pasteurized milk in this study merits closer attention. The isolates Is1, Is2, Is3, Is4 Is5, Is6 were identified as *Aeromonas caviae*, *Moraxella osloensis,Delftia tsuruhatensis, Staphylococcus arlettae* and two different strains of the species *Carnobacterium maltaromaticum* respectively. The nucleotide sequences were deposited in the NCBI database with accession numbers MT071634, MT158663, MT158670, MT158662 MT158664 and MT158665 as *A. caviae* DMV01, *M. osloensis* DMV03,*D.tsuruhatensis* DMV04, *S. arlettae* DMV02,*C.maltaromaticum*DMV05 and *C.maltaromaticum*DMV06 respectively.These isolates are not typically found in pasteurized milk and considering their natural habitats it can be inferred that these bacteria might have entered from the production or processing environment.Isolation of atypical organisms like *Aneurininbacillus migulanus*, *Bacillus shackletonii, Brevundimonas vesicularus* and *Moraxella osloensis* from pasteurized milk was reported by Tattersall, 2020 [17]. Though isolation of *Aeromonas* spp. from raw milk and various milk products are reported widely, there are not many reports of their isolation from pasteurized milk. Though many researchers [18,19,20, 21,22] have attempted isolation of *Aeromonas* spp. from pasteurized milk sample only some studies [21,22] have reported the isolation of *Aeromonas* spp. from pasteurized milk. But they were of the opinion that *Aeromonas* spp. do not survive pasteurization and their presence in pasteurized milk is due to post-processing contamination. Gennari *et al.* [23] reported the isolation of *Moraxella* like bacteria from 40 per cent of the 126 samples of fresh and spoiled meat and dairy products tested. Isolation of one psychrotrophic strain of *M. osolensis* from raw milk was reported by Hantsis-Zacharov and Halpern [24].In agreement with the observation of the current study, isolation of *M. osolensis* from pasteurized milk and heat exchanger plates within the cooling section was reported by Tattersall [17]. Presence of *Carnobacterium maltaromaticum* is reported in a variety of French soft-ripened or red-smear cheeses made from cow, sheep, or goat milks and are perceived as the dominant organism in the psychrotrophic LAB flora of these cheeses [25]. Also it is the most common species of *Carnobacteria* found in milk [26]. Presence of cold-active beta-galactosidase reported in *C. maltaromaticum* (formerly *Carnobacterium piscicola*) could be one of the contributors towards its psychrotrophic nature [27]. As isolation of *S. arlettae* from dairy animals is already reported [28, 29] it could be inferred that the *Staphylococcus arlettae* strain DMV02 isolated in this study could have entered the milk from the farm environment. *Delftia tsuruhatensis,* a member of the *Comamonadaceae* family, was first isolated from sludge in Japan in 2003[30].It is established as an opportunistic emergent healthcare-associated pathogen that can be easily misidentified[31].Though the isolate *Delftia tsuruhatensis* DMV 04 could not be studied further, considering that the bacteria of genus *Delftia* are environmental microorganisms it could be conceived that hygienic practices adopted at farm level are very vital in preventing the ingress of these types of microorganisms in the pasteurized milk production chain. So it is important that effective cleaning and handling of the equipments, maintenance of healthy animals and prevention of contaminations from water, soil and feed are to be given due emphasis to prevent their entry at farm level. To the best of our knowledge, isolates obtained in this study are unique in that thermoduric psychrotrophic strains of *A. caviae, M. osloensis, D. tsuruhatensis, S. arlettae* and *C. maltaromaticum* are being reported for the first time. Observations of the current study are noteworthy additions to the existing knowledge for redesigning production practices and processing methodologies for ensuring safe and quality products. Phylogenetic trees of the isolates were constructed with other closely related strains available in Genbank and are presented in Figures2- 5. . The isolate *A, caviae* DMV01 was found closer to the strains *Aeromonas caviae* strain AC2 MN737498, a wastewater isolate from Poland and *Aeromonas caviae* strain Til4 MT384382 , a Nile Tilapia isolate from Cochin , Kerala, India. Phylogenetic tree revealed strain SR3-3, an isolate from secondary peat swamp soil from a forest in Thailand as the close relative of *Moraxella osloensis* DMV03. Both the *Carnobacterium maltaromaticum* isolates were distantly related to each other. The phylogenetic tree revealed Staphylococcus arlettae strain ISP142A isolated from marine sponge of the Bay of Bengal as the closest relative *of Staphylococcus arlettae* DMV02.

Table 1: Details of the isolates obtained

Sl No.	Isolate	Gram's staining	Catalase	Oxidase	Identified as	Isolate Strain and Accession Numbers	SEM images of the isolates
1	Is1	Gram negative	Positive	Positive	*Aeromonas caviae*	DMV01 MT071634	
2	Is2	Gram negative	Positive	Positive	*Moraxella osloensis*	DMV03 MT158663	
3	Is3	Gram negative	Positive	Positive	*Delftia tsuruhatensis**	DMV04, MT158670	
4	Is4	Gram positive	Positive	Negative	*Staphylococcus arlettae*	DMV02, MT158662	
5	Is5	Gram positive	Negative	Negative	*Carnobacteriumm altaromaticum*	DMV05 MT158664	
6	Is6	Gram positive	Negative	Negative	*Carnobacteriumm altaromaticum*	DMV06 MT158665	

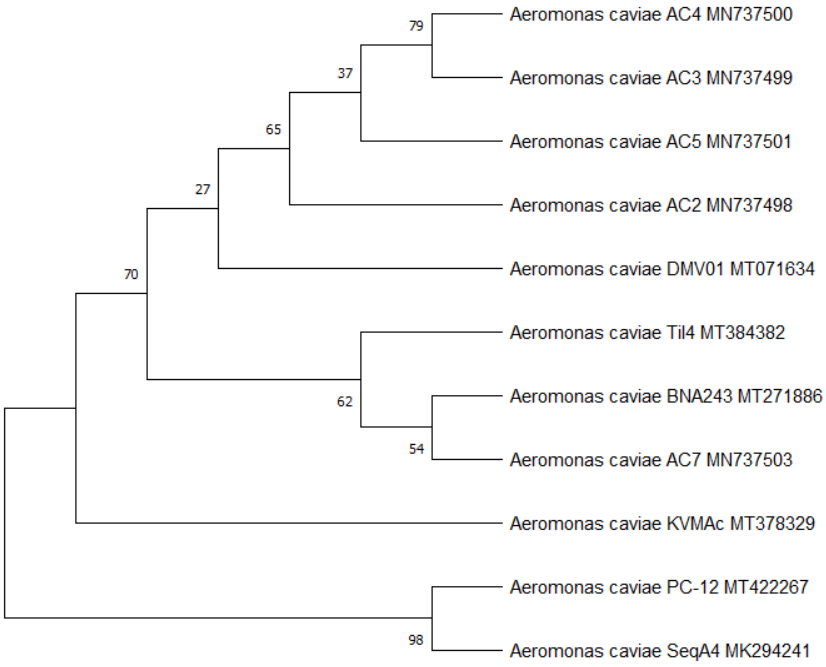


Fig. 2. Phylogenetic tree of *Aeromonas caviae* DMV01 (MT071634)

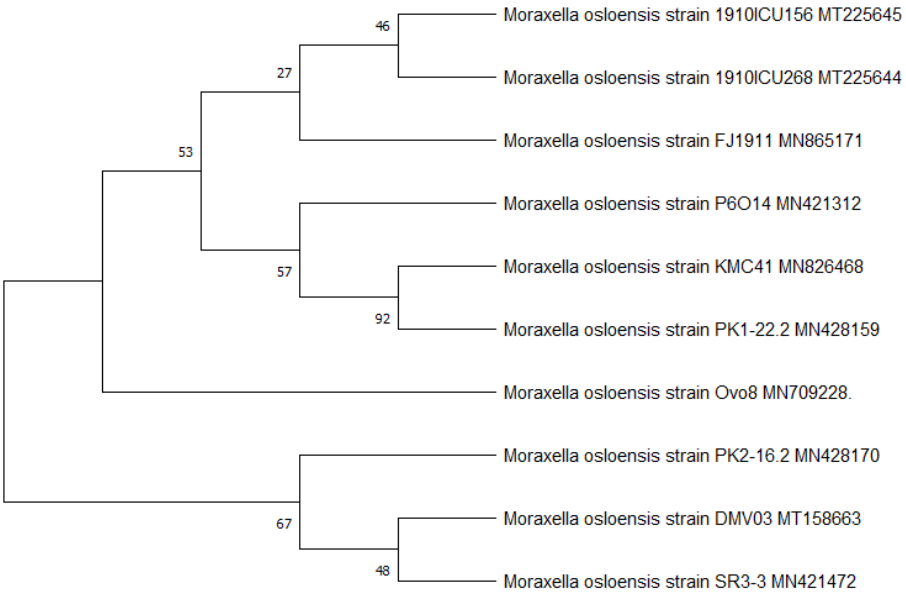


Fig. 3. Phylogenetic tree of *Moraxella osloensis* DMV03 (MT158663)

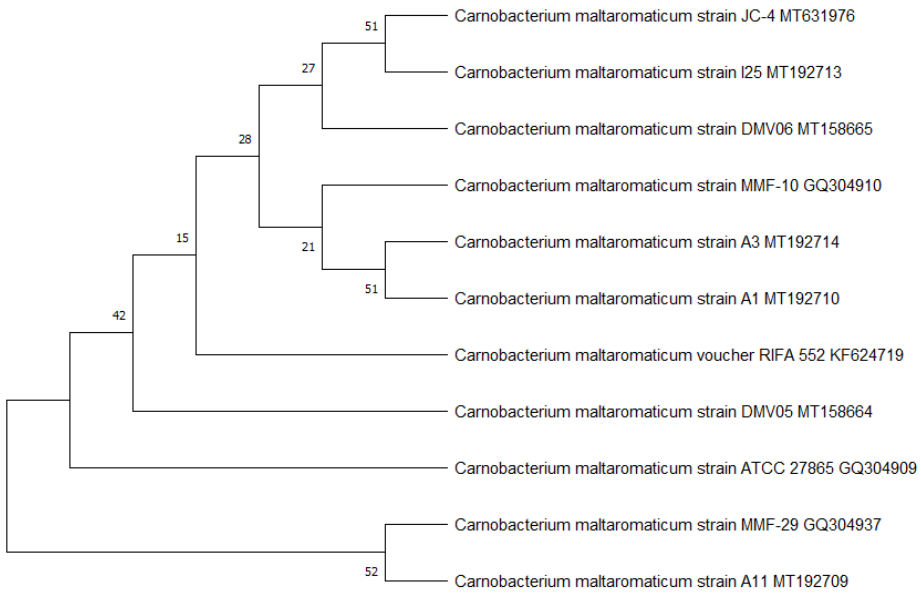


Fig. 4. Phylogenetic tree of *Carnobacterium maltaromaticum* DMV05 (MT158664) and DMV06 (MT158665)

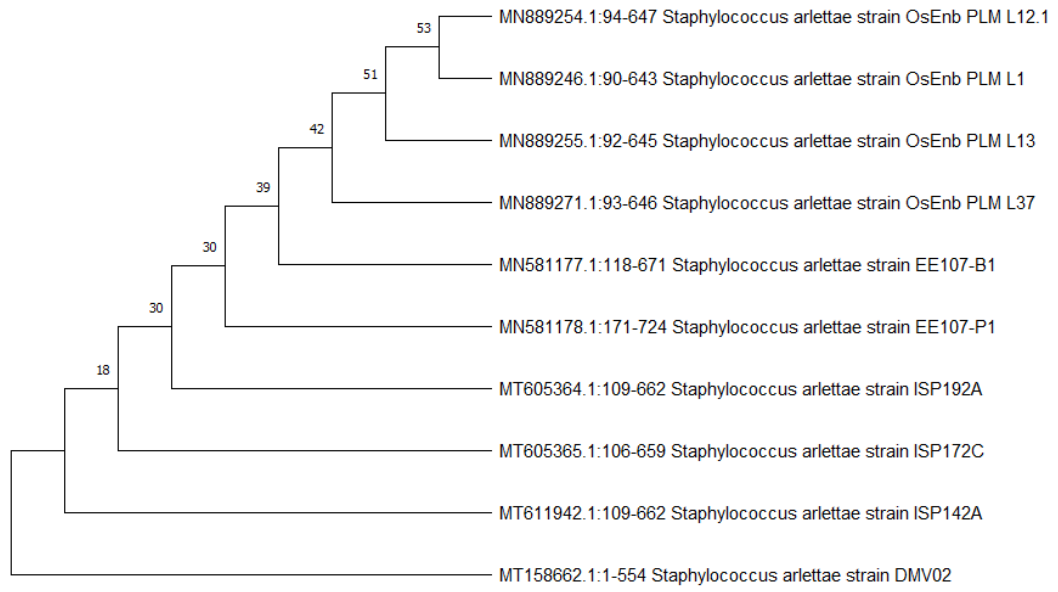


Fig. 5. Phylogenetic tree of *Staphylococcus arlettae* DMV02(MT 158662)

Assessment of haemolytic,**proteolytic and lipolytic activities of the isolates:** *A.caviae* DMV01 exhibited α haemolytic activity whereas the other four isolates exhibited haemolytic activity (Table 2) . Some of the earlier studies have reported β-haemolytic[32,33,34,35] α-haemolytic[36] and even non haemolytic strains of *Aeromonas caviae*. Altogether, these observations suggest strain wise

variations in this trait. According to Castro-Escarpulli *et al*. [37], β-haemolytic activity is significantly more frequent in clinical isolates than that in environmental isolates of *Aeromonas* spp. This could explain the α-haemolytic activity exhibited by the *Aeromonas caviae* strain isolated in the current study. However exhibition of α-haemolytic activity by an organism isolated from pasteurized milk invites special attention as both α and β-haemolytic phenotypes being considered as virulence associated determinants [38]. Contrary to the observation of the current study, α-haemolytic activity of *Carnobacterium* strains was reported by Mogrovejo *et al*.[39] whereas lack of haemolytic activity of *C. maltaromaticum* strains isolated from diseased fish was reported by Hammes and Hertel [40]. The □-haemolytic activity exhibited by *Moraxella osloensis* DMV03 is very much in agreement with the non-haemolytic characteristic of this isolate reported in Manual of clinical microbiology [41]. Non exhibition of haemolytic activity by *S. arlettae* is of relevance as these coagulase Negative Staphylococci (CoNS) are becoming increasingly recognized as an important cause of human and animal infections. Differences were observed in the proteolytic and lipolytic activities of the isolates.*Aeromonas caviae* exhibited lipolytic activity and the two *Carnobacterium* strains exhibited proteolytic activities (Table 2). *Staphylococcus arlettae* was found to be positive for both the proteolytic and lipolytic activities. Ability to secrete wide variety of enzymes associated with pathogenicity and environmental adaptability is considered as a hallmark characteristic of *Aeromonas* spp. [42]. However, the *Aeromonas caviae* isolate obtained in this study failed to elicit any proteolytic activity differing from the earlier report of synthesis of both intracellular and extracellular proteases by the strain *Aeromonas caviae* [43] but agreeing with the lack of caseinolytic activity of strains of *A. caviae*[33,44]. These authors concluded that large and significant variations in extracellular caseinolytic activity exist among *Aeromonas* isolates. Manna *et al.* [35] also reported variations in caseinase production potential of *Aeromonas caviae* strains isolated from meat, milk and fish in Kolkata, India. An extracellular thermo stable lipase producing *Aeromonas caviae* reported by Velu *et al.* [45] concurs with the isolation of a thermoduric *Aeromonas caviae* strain in this study. As in the case of *Aeromonas* species, variations are reported in the proteolytic activity of *Carnobacterium* species also. Casaburi *et al.* [46] reported absence of proteolytic activity at 4°C and 20°C for *Carnobacterium maltaromaticum* isolated from raw meat. Proteolytic and lipolytic activities of *Carnobacterium* strains isolated from gastrointestinal tract of coastal fish were reported by Sahnouni *et al.* [47]. Agreeing with the current report, lack of proteolytic and lipolytic activities by the psychrotrophic strain of *Moraxella osloensis*(Accession no: EF204255) isolated from raw milk was reported by Hantsis-Zacharov and Halpern [48]. Chauhan *et al*. [49]reported the suitability of *S arlettae* JPBW-1 lipase for the development of environmentally friendly detergent formulations. Exhibition of proteolytic and lipolytic activities by *S. arlettae* isolated from Indian ethnic fermented fish product was reported by Majumdar and Gupta [50].This is very much in agreement with the lipolytic and proteolytic activities exhibited by the *S. arlettae* isolate obtained in the current study.

Table 2. Haemolytic, proteolytic and lipolytic activities of the isolates

Sl No.	Isolate	Haemolytic activity	Proteolytic activity	Lipolytic activity
1.	*Aeromonas caviae* DMV01	α-haemolysis	-	+
2.	*Moraxella osloensis* DMV03	ϒ-haemolysis	-	-
3.	*Staphylococcus*	ϒ-haemolysis	+	+

	arlettae DMV02			
4.	*Carnobacterium maltaromaticum* DMV05	ϒ-haemolysis	+	-
5.	*Carnobacterium maltaromaticum* DMV06	ϒ-haemolysis	+	-

Determination of D_{63} value of the selected isolates: Considering the food safety issues [51,52,53] and uniqueness of being Gram negative thermoduric psychrotrophs, *A. caviae* DMV01 and *M. osloensis* DMV03 were selected for the D_{63}value determination. On assessing their survival at 63^{o}C for 30 minutes a subpopulation of heat resistant metabolically active cells intact enough to develop into colonies was observed (Fig.5, 6). This observation is in concurrence with the report of a heat resistant subpopulation of clinical and food isolates of *Aeromonas hydrophila* [54].The D-value of *A. caviae*DMV01 at 63^{o}C was found to be 4 minutes 38 seconds whereas for *M. osloensis*DMV03, it was 25 minutes 18 seconds (Figures 6 and 7). Both the observed values are higher than the D-values reported for other Gram negative organisms like *Salmonella* serotype cocktail at 62°C [55], *Campylobacter* spp., *Yersinia enterocolitica*, *Cronobacter sakazakii*at 60°C [56]and *E. coli* at 62.5°C [57]. Heat resistance studies of *Aeromonas* spp. found that their heat resistance in both broth and foods is lower than *S.typhimurium*, *S.aureus* and *E. coli* [58,59]. On analysing the D-values of 4 strains of *A.hydrophila* in liquid whole egg Schuman *et al*. [60]reported that they ranged from 3.62 to 9.43 min (at 48°C) to 0.026 to 0.040min (at 60°C). Compared to these reports, the *Aeromonas* isolate obtained in this study exhibited remarkably high heat resistance. To the best of our knowledge no studies are reported on the D-value of *Moraxella* spp., but there are reports on the irradiation D-values of highly radiation-resistant *Moraxella* spp. from beef [61,62.] Heat resistance of microorganisms is dependent not only on inherent genetic factors, but also on many environmental factors like the heating process [63]. Mercer *et al*. [64] categorized *E. coli,* another Gram negative bacterium into different heat resistance groups based on $D_{60°C}$ value. Accordingly, the isolates obtained in this study can be classified as highly heat resistant.

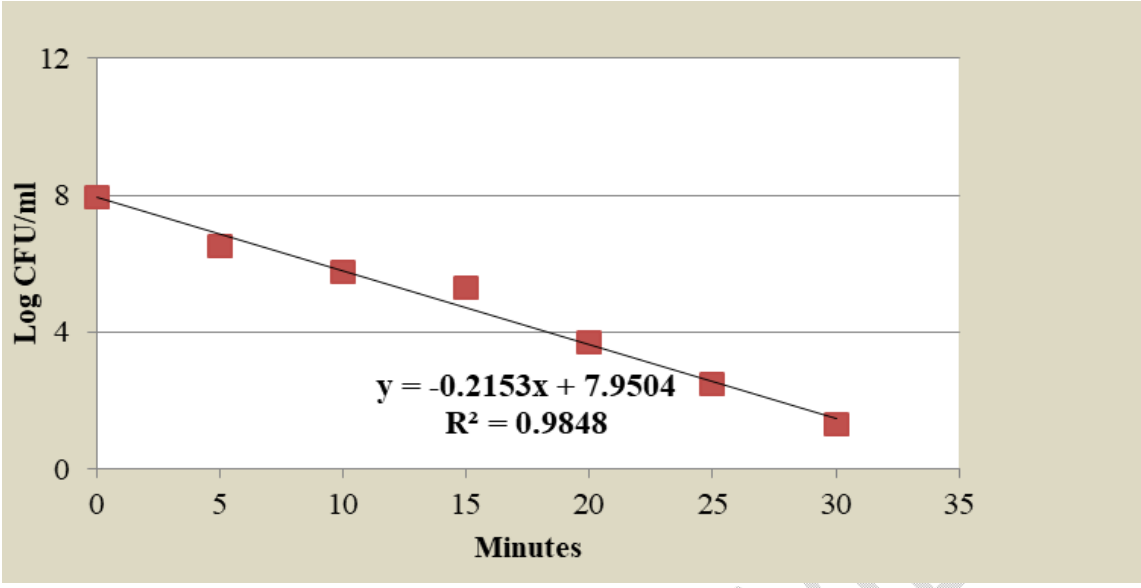


Fig. 6. D $_{63}$value determination graph of *Aeromonas caviae*DMV01

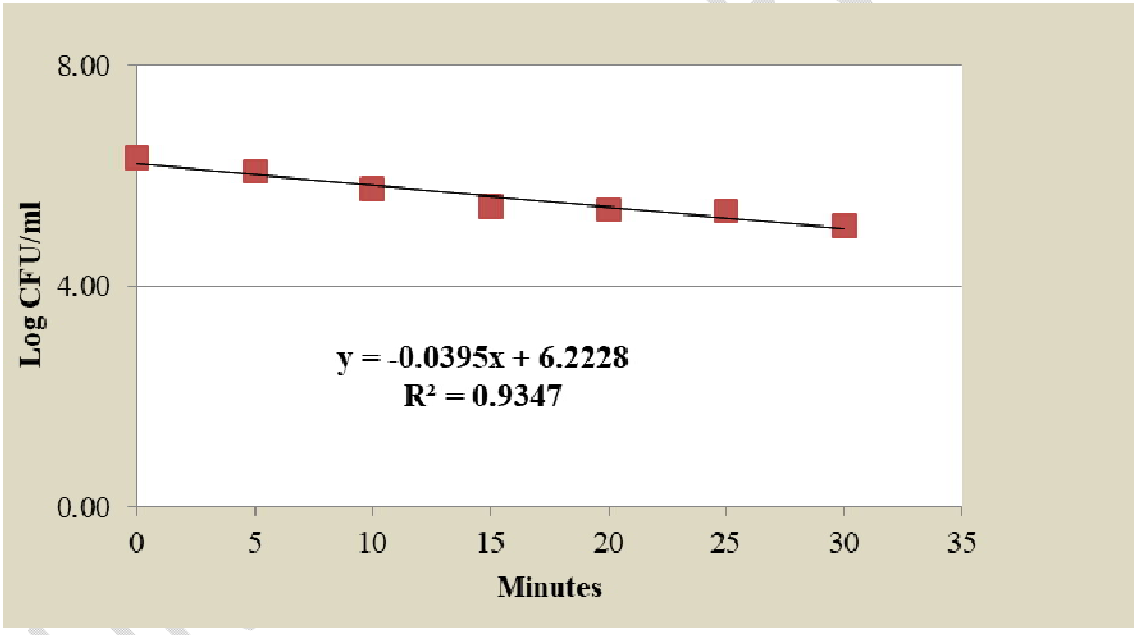


Fig. 7.D $_{63}$value determination graph of *Moraxella osloensis DMV03*

CONCLUSIONS

Isolation of thermoduric psychrotrophic bacteria from market samples of pasteurized milk, assessment of their enzymatic activities and D_{63} value determination of selected isolates were performed in this study. Three each of Gram negative and Gram positive thermoduric psychrotrophic bacteria were isolated. As most of these isolates belonged to bacterial species not typically isolated from pasteurized milk it can be opined that pasteurized milk microenvironment is an area warranting further studies.Ability of these isolates to grow at low temperature is of special concern as even a single surviving cell can grow into sufficient numbers and pose a potent threat in refrigerator stored pasteurized milk.These psychrotrophic organisms due to their ability to elicit enzymatic activities can have a negative impact on the

shelf life and quality of dairy products. The remarkably high heat resistance and psychrotrophic traits exhibited by these isolates can synergistically contribute towards increased persistence, co-selection and possible transfer of these resistance factors in the dairy processing environment. Observation of this study also highlight the need of large-scale regional specific research explorations on 'pasteurization surviving psychrotrophic' dairy associated microorganisms in order to remain prepared to address the ever-increasing quality and safety challenges evolving out in the dairy industry.

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