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Evaluation of probiotic curd marketed in Pudukottai main market for the presence of contaminant bacteria

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Abstract
Curd is a traditional fermented food consumed by people all over India for its nutritive value and role in maintaining human gut microflora. Recently curd is being sold in many kinds of packs with and without brand names. The authenticity of these products is always questionable. In the present study, samples randomly collected from Pudukottai Main Market were analysed for the presence of LAB (Lactic Acid Bacteria) and contaminant bactyeria (*Staphylococcus* sp and *E. coli*). The microbial load of *L. acidophilus* was in general very low in all the samples ranging between 2.93 to 4.26 log values. But the microbial count of *Staphylococccus epidermis* is higher than *Lactobacillus acidophilus* both at 0 hr and 12 hrs. But *E. coli* was completely absent. Absence of *E. coli* confirmed that the products are not having any faecal matter contamination possibly entering either through water source or food handlers. Anyway the count of *Staphylococcus epidermidis* is alarmingly high in all the samples tested ranging between log value of 5.58 to 6.04, which could be prompted by poor handling during processing and packing. Earlier *S. epidermidis* and other coagulase-negative staphylococci (CoNS) have been considered nonpathogenic commensal organisms whereas nowadays they seem to be opportunistic pathogen. Hence the present study indicates that at most care must be taken from the manufacturer side for hygienic handling of the product throughout the commercialization.

Keywords: Probiotic, curd, LAB, *Lactobacillus acidophilus*, *Staphylococccus epidermis*, *E. coli*, CoNS, *Staphylococcus epidermidis*

Introduction
Curd (Yogurt) is one of the most popular fermented dairy products regularly used in India particularly in Tamilnadu. Fermented milk always help in the absorption of calcium and phosphorus and inhibits the undesirable bacterial flora of intestine which may lead to constipation, autointoxication and colitis. Because of this curd is recommended for sick and convalescent people (Khan *et al*., 2008) [1]. Dairy foods containing viable probiotic bacteria represent one of the functional foods, which when ingested in sufficient amounts, beneficially influence the health of the host by improving the composition of intestinal microflora (Neamah *et al*., 2006) [2]. Nowadays curd is available in many packages with and without brand names and descriptions. Selling such food items as street vended food could create major risk to public health, due to the unsanitary and unhygienic conditions, including poor infrastructure, improper storage temperature, and poor hygiene among the handlers during commercialization (Rodrigues *et al.*, 2016) [3].
In general, the dairy products are contaminated with Staphylococcal cells due to improper and unhygienic handling. Staphylococci are typical Gram-positive bacteria forming irregular clusters of cocci. Staphylococci are widespread in nature, although they are mainly found on the skin, skin glands and mucous membranes of mammals and birds, but can cause infection under certain circumstances. *S. aureus* is more pathogenic than the other common members of the genus, *S. epidermidis* and *S. saprophyticus*. *S. epidermidis* has been known to cause various hospital-acquired infections (such as prosthetic or indwelling devices)
Ahmad *et al*. (2013) [4] studied on quality assessment of yogurt produced at industrial level in Qena city, Egypt. They showed that out of 100 random samples purchased from various dairy shops, street vendors and supermarkets located in Qena city, Egypt,*S. aureus* were detected in 72% and 35% of small and large scale yogurt samples.
Enterotoxigenic *S. aureus* strains in dairy products possess a potential health hazard to consumers, and their presence is used as a part of hazard analysis and risk assessment of milk and milk products (Zouharova and Rysanek, 2008) [5]. Outbreaks of food poisoning by *S. aureus* are often associated with unsanitary handling of food at an inappropriate temperature for a prolonged period of time (Huang *et al*., 2001) [6]. Hence an attempt was made to study for

the presence of such contaminating *Staphylococcus* sp and *E. coli* in the curd samples that are marketed in the shops available in Pudukottai Main market.

Materials and Methods

Collection of Curd samples

The curd samples were randomly bought from the shops available in Pudukottai Main Market. Three branded samples (sample code A, B, C) and one unbranded sample (sample code D) were collected for experimentation. The branded samples had date of manufacture and other details printed on the pack but the unbranded sample was packed in pp cover without any details on it.

Biochemical characteristics of yoghurt samples

Determination of pH Value

It was performed using a digital pH meter (Infra Digi model). Three readings were recorded and the average was calculated

Isolation and Determination of Lactic acid bacterial load

For the isolation of lactic acid bacteria, MRS Agar was used (De man *et al*., 1960) [7]. Serial dilutions upto 10^{-4} were made by using standard serial 10-fold dilution in buffered peptone water and eventually transferred 10 µL for drop plating on MRS agar. The drops were absorbed to agar in less than 30 minutes. After the drops on the agar got absorbed, the plates were incubated in inverted positions at 35 ± 1 ˚C under anaerobic condition. Enumeration of *Lactobacillus* sp viable cells were done after 72 hrs incubation. Each dilution was plated in duplicate with four drops per plate and the viable cell counts were expressed as CFU mL^{-1}. The population count was taken by this drop plate technique at 0hr and 12 hrs.

Isolation and Determination of contaminant bacterial load

The same procedure was followed for enterobacters on EMB agar plate and incubated for 18-24 h at 35 °C ± 0.5 °C and examined for *E. coli* colonies, i.e., dark centered and flat, with or without metallic sheen. Using MSA agar plates samples were plated following the same protocol for detection of *Staphylococcal* colonies and **incubated** overnight at 37 °C

Characterisation of the isolated bacterial strains

Biochemical characterization of the isolated bacteria was performed according to their morphological, cultural and biochemical characteristics according to Bergey's manual of determinative of bacteriology (Holt *et al.,* 1994)[8] by performing Gram staining, motility test, catalase assay, Starch Hydrolysis, milk coagulation assay and NaCl and phenol tolerance test.

Gram staining test: The isolated bacteria were examined using gram staining kit (Collins *et al.,* 2004) [9], and was observed under Phase Contrast Microscope (Magnus, MLX) with a magnification of 1000x.

Motility test: It was done using hanging-drop wet method (MacFaddin, 2000) [10]. The slide was observed under a light microscope with 40x magnification to check the motility of the bacteria.

Catalase test: A loopful of bacterial colony was transferred to a surface of clean, dry glass slide using a loop and placed a drop of 3% H_2O_2 on to the slide and mixed together and observed for bubbling.

Coagulase test: Placed a small drop of distilled water on clean slide and one or two colonies of *Staphylococcus* from MSA agar plate were emulsified on each drop to make a smooth suspension. The test suspension was added with a drop of citrated plasma and mixed well with a needle and observed for clumping within 5-10 seconds.

Milk Coagulation Assay: For milk coagulation test, overnight culture of the bacteria was added into 10% sterile skim milk and incubated at 37 ºC for 48 hours in incubator (Chakraborty and Bhowal, 2015) [11].

Lactose Utilization test: Media for Lactose Utilization was prepared using (Peptone 10 gms, Nacl 15 gms, Phenol Red 0.018 gm, Lactose 5 gm in 1 litter distilled water, PH 7) and incubated at 35 ºC, for 48 hours in rotary incubator. Change of colour from yellow to red, was concluded as positive result (Pundir *et al.,* 2013) [12]

Tolerance of NaCl and Phenol: For observing the tolerance of the culture to NaCl, overnight culture of the bacteria incubated into MRS broth with 4% NaCl Conc for 24 hours and then observed their turbidity. Similar experiments were performed using 0.4% phenol as inhibitory substance.

Results and Discussion

Isolation, characterisation and confirmation of Lactic acid bacteria

The isolated single colonies in the MRS agar growth **medium** were circular with undulate margin, smooth surface, white or yellow colored. When observed under microscope, they were rod shaped, single or chain, gram positive, non spore forming. These results suggested that the isolated bacteria could be identified as Lactobacilli. Hanging-drop wet method showed that the isolated bacteria were non motile. The non motile behavior is a characteristic of *L. acidophilus*. While performing catalase test, no bubbles was observed indicating that the isolated bacterium is catalase negative as it could not mediate the decomposition of H_2O_2 to produce O_2. It is a known fact that *Lactobacillus acidophilus* is catalase negative as per Bergey's. The positive results in Milk Coagulation Assay, Lactose Utilization, Phenol (0.4%) test & 4% NaCl test and their inability to hydrolyse starch, further confirmed that the isolates are *Lactobacillus acidophilus.* The morphological and biochemical characters are tabulated in Table 1 & 2.

Table 1: Morphological characterisation of *Lactobacillus acidophilus*

Morphological characters	Observation
Form	Circular
Margin	Undulate
Surface	Smooth mucoid
Elevation	Flat
Microscopic observation	
Shape	Rod, Single or chain
Gram Reaction	+ve
Spore staining	-ve
Motility	Non motile

Table 2: Biochemical characterisation of *Lactobacillus acidophilus*

Biochemical characters	Observation
Catalase test	-ve
Lactose Utilization	+ve
Starch Hydrolysis	-ve
Milk Coagulation Assay	+ve
Phenol (0.4%) test	+ve
4% Nacl test	+ve

Isolation, characterisation and confirmation of Contaminant bacteria

Bacterial colonies isolated in MSA plates were Gram-positive, cocci, arranged in clusters and non motile when observed under microscope and this suggested that they could be Staphylococcal colonies. They formed white, raised, colonies about 1–2 mm in diameter on MSA agar with pink zone around and were negative for coagulase and positive for catalase (Table 3). These characters further confirmed that the isolates were *Staphylococcus epidermis.* Negative reaction for coagulase confirmed that they are not the pathogenic *Staphylococcus aureus.*

Table 3: Morphological and Biochemical characterisation of the isolated *Staphylococcus epidermis*

Characters	Observation
Morphological characters	
Form	Circular
Margin	Entire
Surface	Smooth
Elevation	Raised
Colony color on Blood Agar	White
Colony color on MSA	White with pink zone
Microscopic observation	
Shape	Spherical, clusters
Gram Reaction	+ve
Motility	Non motile
Biochemical characters	
Catalase test	+ve
Coagulase test	-ve
Starch Hydrolysis	-ve

Determination of microbial load of *Lactobacillus acidophilus* in curd samples

The microbial load of *Lactobacillus acidophilus* was recorded by taking the sample at 0 hr and 12 hrs. There was 10 fold increases in the load at 12 hrs from 0 hr sample but the initial load was very much lower than the expected load of >6.0 log value. A log value of 4.26 was recorded in sample D followed by C with A and B statistically on par. This variation in load reflected in the reduction in pH of the curd after 12 hours. The pH of all the samples at 0 hr was in the range of 5.0 to 5.8 wherein the normal pH of curd lies between 4.2 to 4.5. This must be due to the highly low count of LAB in the samples. Curd is normally treated as a probiotic food and hence it should be rich in LAB count whereas both branded and unbranded samples recorded lower level of LAB at 0 hr with increase in count at 12 hrs. comparing to branded samples (A,B,C), unbranded sample (D) was having higher *L. acidophilus* log count both at 0 hr and 12 hrs (4.26 & 5.06). But the microbial count of *Staphylococccus epidermis* is higher than *Lactobacillus acidophilus* in all the samples at both sampling times. The count was statistically on par in 3 samples *viz.*, D, B and C followed by sample A. Significant correlation couldn't be observed between pH and *Staphylococccus epidermis* load. This was in accordance with the finding of McDonald *et al.*, (1986) [13] who observed acidification of spent PDS (peritoneal dialysis solutions) to less than pH 6.35 produced less rapid growth wherein it drops from 1.9 to 0.7 when the pH is reduced from 7.75 to 4.95. Korting *et al*., (1992) [14] also stated that *Staphylococcus epidermidis* resembled *Staphylococcus aureus* showing no major difference at pH 5.5 and 7.0.

Table 4: Microbial load of Lactic Acid Bacteria and Contaminant bacteria in curd samples

Sample code	pH		*Lactobacillus acidophilus*		*Staphylococccus epidermis*		*E. coli*	
	0 hr	12 hrs	0 hr	12 hrs	0 hr	12 hrs	0 hr	12 hrs
A (Branded)	5.6	5.2	$11.0x10^2$ $(3.04)^c$	$3.1x10^3$ $(3.49)^c$	$38x10^4$ $(5.58)^b$	$42x10^4$ $(5.62)^b$	0	0
B(Branded)	5.8	5.2	$8.6x10^2$ $(2.93)^c$	$2.5x10^3$ $(3.40)^c$	$102x10^4$ $(6.01)^a$	$86x10^4$ $(5.93)^a$	0	0
C (Branded)	5.8	5.3	$22.3x10^2$ $(3.35)^b$	$10.4x10^3$ $(4.02)^b$	$63x10^4$ $(5.80)^{ab}$	$52x10^4$ $(5.72)^{ab}$	0	0
D (Unbranded)	5.0	4.8	$18.3x10^3$ $(4.26)^a$	$11.6x10^4$ $(5.06)^a$	$110x10^4$ $(6.04)^a$	$95x10^4$ $(5.98)^a$	0	0
CD	NS	NS	0.29	0.46	0.28	0.24		

*Log values given in parentheses; Values followed by superscript letters within the column differ significantly

Determination of microbial load of contaminant bacteria in curd samples

Food Safety and Standards Authority of India has brought *E. coli* as Safety indicator organism and *Satphylococcus aureus* as Hygiene Indicator organism in defining Microbiological Requirements for Milk and Milk Products [15]. The permissible limit for both organisms is 10-100 CFU/g of yoghurt samples. But *Staphylococcus epidermis* is not

categorised under this standards. Plating of the samples on MSA and EMB agar proved that all the 4 samples had inocula of *Staphylococcus epidermis* but *E. coli* was completely absent (Fig 1,2). Absence of *E. coli* confirmed that the products are not having any faecal matter contamination possibly entering either through water source or food handlers. Anyway the count of *Staphylococcus epidermidis* is alarmingly high in all the samples tested.

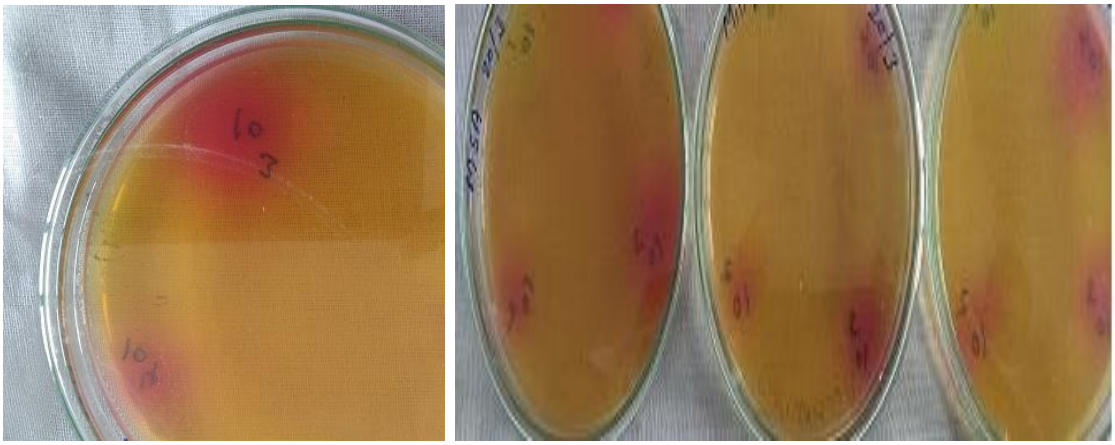

Fig 1: *Staphylococcus epidermis* colonies plated from sample D and A on MSA agar plates

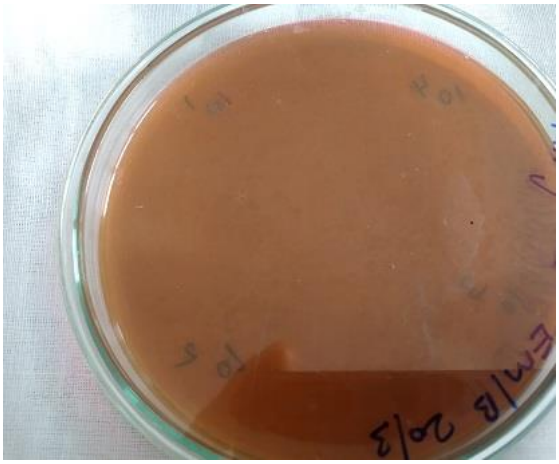

Fig 2: EMB agar plates showing no colonies of *E. coli* for the sample D

Historically, *S. epidermidis* and other coagulase-negative staphylococci (CoNS) have been considered nonpathogenic commensal organisms whereas nowadays they are seen as an important opportunistic pathogen (Otto, 2009) [16]. It is observed that *S. epidermidis* can cause opportunistic infections particularly biofilm-associated infections on indwelling medical devices (Uckay *et al*., 2009) [17]. These often can disseminate into the bloodstream; and in fact, *S. epidermidis* is the most frequent cause of nosocomial sepsis. Today, *S. epidermidis* is a major nosocomial pathogen posing significant medical and economic burdens (Nguyen *et al*., 2017) [18]. From the data of the present study, it is observed that there is increased load (upto 10^4) of *S. epidermidis* cells in both branded and unbranded curd sample. It shows that there is possibilities for the entry of *S. epidermidis* into the samples due to improper handling and unhygienic packing methods followed by the handlers, as the source of this bacteria is mainly skin, skin glands and mucous membranes of mammals. Since its presence is recorded in both branded and unbranded curd samples, it rings a bell for adoption of appropriate protocols in all kinds of manufacturing units (small & Large scale) and proper monitoring by the concerned Food Safety Authority.

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