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RESEARCH ARTICLE

STUDIES ON BACTERIOCIN PRODUCING FUNCTIONAL STRAINS OF *Streptococcus thermophilus* WITH PROBIOTIC ATTRIBUTES

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ABSTRACT

Consumers' demand and market potential of fermented food and dairy products calls for innovation in food and dairy processing and needs novel starter strains to boom food industry. Nine strains of bacteriocin producing *Streptococcus thermophilus* with sound technological properties isolated from milk and milk products were evaluated for *in-vitro* acid and bile tolerance in simulated gastro-intestinal environment, adherence to cell surface and antibiotic sensitivity profile. All the nine strains were found to be acid and bile tolerant with some degree of variability. The percent hydrophobicity of studied strain was ranging from 5% to 33.5% with all the tested hydrocarbons. All the strains of *S. thermophilus* were sensitive to antibiotic ofloxacin, vancomycin, polymixin B and ciprofloxacin while showed variable behavior towards remaining 19 antibiotics. Three strains namely PMD9, PMK20 and PMl31 were found to possess appreciable probiotic properties in all respect and could be novel agents for their exploitation as functional dairy starters. Further use of these products could be for fortifying these products in yoghurts for health benefits, or countering antibiotic resistance.

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INTRODUCTION

Research during the past two decades has revealed that *Streptococcus thermophilus* has properties that make it one of the most commercially important lactic acid bacteria with massive use in various fermentations and is considered as the second most important industrial dairy starter after *Lactococcus lactis* (Chausson and Maurisson, 2002).World Health Organization (WHO) has defined probiotics as live microorganisms which when administered in adequate amounts confer a health benefit on the host (Gilliland *et al.*, 2001). These organisms favorably alter the intestinal microflora balance, promote intestinal integrity and mobility, inhibit the growth of harmful bacteria and increase resistance to infection (Veldman, 1992) and should possess the properties like survival in the gastrointestinal (GI) tract, Persistence in the host, and proven safety for consumer (Tuomola *et al.*, 2001; De-Vries *et al.*, 2006). Among the other mechanisms by which probiotic bacteria cause beneficial effects on the host, modulation of immune responses which has been particularly studied and extensively reviewed (Borchers *et al.,* 2009; Yan and Polk, 2011). Recent study suggests that dietary supplementation with probiotic strains *S. thermophilus* FP4 and *B. breve* BR03 attenuates performance decrements and muscle tension in the days following muscle-damaging exercise, especially in sports personnel (Ralf Jager *et al*., 2016). Novel Functional starters can play an important role in meeting the needs of commercial dairy industry, in this regard as they possess at least one inherent functional property and can contribute to food safety and/or one or more organoleptic, technological, nutritional, or health advantages. The use of such carefully selected strains as starter cultures or co-cultures in fermentation processes can help to achieve *insitu* expression of the desired property, maintaining a perfectly natural and healthy product. The next generation of value added dairy products could definitely meet the emerging needs of consumers and simultaneously address to their concerns and beliefs. Although *S. thermophilus* has not been well studied as a probiotic, this bacterium has many potential health benefits. The objective of present research was to investigate the

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probiotic attributes in a simulated gastric environment of nine bacteriocinogenic strains of *Streptococcus thermophilus* with broad spectrum antibacterial activity and sound technological properties with the aim to evaluate the possibility of *Streptococcus thermophilus* strains as functional dairy starter, as much information is not available on these strains. The scope of these strains for beneficial puposes appears to be enormous, given its predicted role in health benefits.

MATERIALS AND METHODS

Selection of bacteriocinogenic *Streptococcus thermophilus* strain

In this study, nine strains of *Streptococcus thermophilus* namely PML3, PMD7, PMD9, PMM15, PMK20, PMC25, PML31, PMD36 and PMM43 were used. These strains were isolated from a variety of milk and indigenous milk products. The strains were tested for bacteriocin production by agar spot assay and spot on agar assay and genetically identified by species specific PCR with lacZ gene of *S. thermophilus* (Lick *et al.*, 1996). The strains were found to be active against a large no. of Gram positive lactic, non-lactic and pathogenic bacteria. The strains were technologically characterized and selected for assessing starter activity on the basis of good acidifying activity, positive galactose utilization and negative urease activity. Strains were stored at −80˚C in M17 broth, with 20% glycerolasacryo protective agent. Before use, the strains were reactivated by incubation in M17 brothat 37˚C

Preparation of gastric and intestinal juice

Simulated gastrointestinal juice was prepared as described by Kos *et al.* (2000). Pepsin (3g/L) was suspended in a sterile sodium chloride solution (0.5%) and the pH was adjusted to 1.0, 2.0 and 3.0 with concentrated HCI. Pepsin (from porcine stomach mucosa) was obtained from Sigma Chemical Co, St. Louis, USA. Simulated small intestinal juice was prepared by suspending pancreatin (1 g/L) and bile salts (1%, 2% and 3%) in a sterile NaCl solution (0.5%, pH 8) in a sterile sodium chloride solution (0.5%) and adjusting the pH to 8.0 with 0.1mol/l NaOH. Pancreatin (from hog pancreas,) was obtained from Fluka Biochemica.

Survival in gastric and intestinal juice

Procedure used to check survival of the test cultures in simulated gastric and intestinal juice was as per method given by Kos *et al.* (2000) with slight modifications. The selected isolates of *Streptococcus thermophilus* were tested for their tolerance in simulated gastric juice and the cell counts were taken at 0, 1 and 2 h of incubation at 37^0C by pour plate method. After exposure in simulated gastric juice, the cells were centrifuged and resuspended in simulated intestinal juice and cell counts were taken at intervals of 0, 1, 2 and 3 h of incubation at 37˚C by pour plate method and evaluation of tolerance/ survival in the gastrointestinal tract was observed. Control studies were made by taking sterile sodium chloride solution (0.5%) with pH 6.5 and without any bile salt.

Cell Surface Hydrophobicity and antibiotic susceptibility

Adhesion to hydrocarbons namely N-hexadecane, N-octane and xylem was carried out to assess the cell surface hydrophobicity of selected isolates (Doyle and Rosenberg, 1995). The adherence of cells was expressed in terms of percent hydrophobicity. For determining the antibiotic susceptibility/resistance of bacteriocin producing *Streptococcus thermophilus* isolates against different antibiotics, the cultures were screened for antibiotic susceptibility by disc diffusion method (NCCL, 1999). Diameter (mm) of zone of inhibition was measured using antibiotic zone scale and results were expressed in terms of resistance, moderate susceptibility or susceptibility after comparing with the interperative zone diameters given by Charteris *et al.* (1998) for disc diffusion antibiotic susceptibility test. All the tests were performed at least in triplicates on different days.

RESULTS

In vitro tolerance to gastric acidic environment

It has been observed that all strains of *Streptococcus thermo philus* showed a good survival in acidic condition and are capable to tolerate acidity of gastric environment. However, none of them are capable to show any growth under acidic stress. Amongst all the isolates PMD9, PMK 20 and PML31 were relatively more resistant to acidic conditions than other isolates in the selected pH range. The acid tolerance pattern of these isolates namely PMD9, PMK 20 and PML31 is presented in Figure 1.

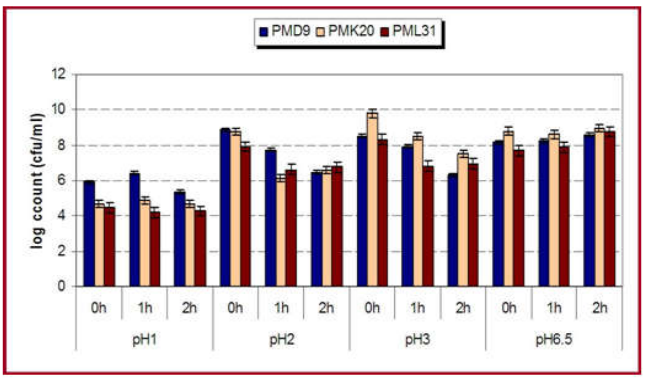

Figure 1. Effect of different pH on log count of bacteriocinogenic *S. thermophilus* strains

It has been observed that all the isolates showed a count in the range of 6-8 log cfu /ml at pH 6.5. All isolates showed growth of one log cycle at pH 6.5 (control) within three hours of incubation. At pH 1.0 isolates PML25 and PMD9 showed a two log cycle reduction. However, only one log cycle reduction was observed in isolates PMD36, PMM43 and PML3 even after 2 h of exposure. The log cfu/ml of isolate PML3 was found to be least at pH 1.0after 2h of incubation. All remaining strains showed a survival of 4-3 log cfu/ml at pH 1.0. Isolate PMD9, PMK 20 and PML31 showed almost constant growth pattern during 2h of incubation at pH 1.0 and appeared to be the most acid tolerant isolates among the selected group and showed good survival of 4-5 log cfu/ml (Figure 1). Further, it was observed that all isolates showed better survival at pH2 with only 1-2 log cycle reduction. Among all the isolates, PML31 was observed with maximum log count after 2h of incubation. PMD9, PMK 20 and PML31 also showed better count of 6 log cfu/ml after 2h interval (Figure 1) followed by the isolates PMK20 and PMD9, respectively. At pH 3, isolate PMD7 showed an abrupt

reduction of 4 log count after 2h of incubation. Out of nine isolates, six isolates namely PMD9, PMM15, PML25, PML31, PMD36 and PMM43 were observed with cell count of 6 log cfu/ ml. However, the isolate PMK20showed maximum log cfu/mlafter 2h of incubation (Figure 1).

In vitrotolerance to intestinal environment

In the present study, a good bile tolerance was observed among *S. thermophilus*strains. However, amongst all the strains PMD9, PMK20 and PML31 showed constant growth and good tolerance at all selected pH and bile combination during 0-3 h of incubation. The results for bile tolerance of the selected bacteriocinogenic *S. thermophilus* strains PMD9, PMK20 and PML31 are presented in Fig 2 to 4. The results for control (pH 6.5 and 0% bile) have been shown in Fig. 5.

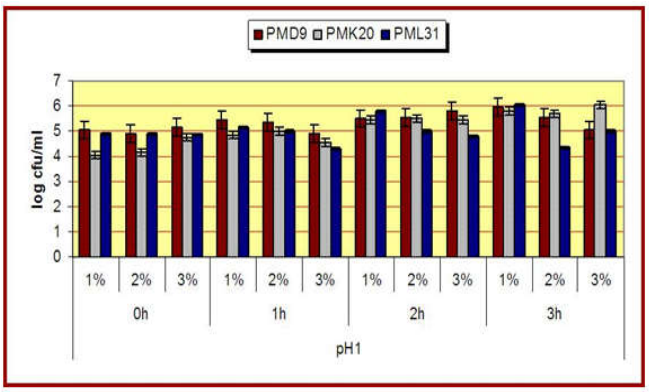

Figure 2. Effect of different bile concentration on selected strains after exposure to pH 1

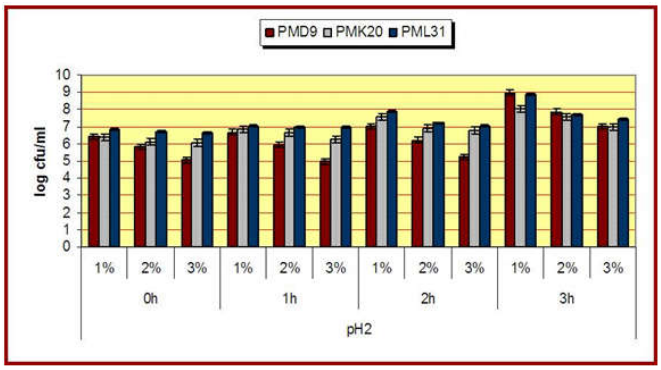

Figure 3. Effect of different bile concentration on selected strains after exposure to pH 2

Among the survivors of pH 1 with 1% bile concentration *S. thermophilus* PML31 showed maximum log count with a growth of 2 log cycle while PMK20 showed one log cycle increment (Figure 2) followed by PMD7. PMD 9 and PML25 were observed to show constant growth in all selected time periods at this combination. Though loss of one log cycle was observed in the strains PMM15 and PML 3. After surviving pH 1 and 2% bile salt concentration, only one isolate, PMK20 showed one log cycle increment. Maximum log count at pH 1 and 2% bile salt concentration has observed in case of PMD9 after 3h of incubation (Figure 2), whereas, minimum count was noticed in PML3. A reduction of 2 log cycle was observed in PMM 43 after 3h of incubation for this treatment. All other isolates showed almost constant growth pattern. The treatment with pH 1 followed by 3% bile salt concentration can be considered as the most hostile environment for the survival and growth of any microorganism as there is a severe exposure to dual stress. Almost constant log count was observed in PMD9, PML25 and PMD7. However, strains PMD36, PMM43 and PML3 showed two log cycle reductions under this condition. Strain PMD36 and PML 3 showed drastic reduction after 3h of incubation. Increment of 1 and 2 log cycle was found in PML31 and PMK 20, respectively. Isolate PMK20 showed best survival at pH 1 and 3% bile salt concentration followed by PMD9 and PML 31 (Figure 2).

Survival of probiotic cells in pH 2 followed by 1% bile combination is supposed to be the most optimal condition of simulated environment. At pH2 and 1% bile salt concentration, all isolates showed constant growth except PML3 and PMM43. At pH2 and 1% bile salt concentration, strain PMD9 showed maximum log count among all strains (Figure 3). It has been found that after pH 2 and in 2% bile salt concentration, all isolates showed increase in cell count up to 2 log cycles. However, strain PMM43 and PML3 showed 1-2 log cycle reduction after 3h of incubation under same circumstances. Maximum log count was found in isolate PMD9 followed by PML31 and PMK 20 (Figure 3).After exposure to pH 2 with 3% bile salt concentration, three strains namely PMD36, PMM43 and PML3 showed maximum reduction whereas, all other strains showed gradual growth during the entire span of incubation. Two log cycle increments have been observed in case of PMD9. Maximum log count was observed in *S. thermophilus* PML31 followed by PMK20, after 3h of incubation (Figure 3). Gradual reduction was noticed in case of PMM43 and PML 3 at all selected range of bile concentration after surviving pH 2. The tested strains showed good tolerance and growth in all the selected bile concentration after treatment at pH 3. Only one strain PMM43 showed gradual reduction of 1 to 3 log cycle with increase of bile from 1% to 3% respectively. All other isolates showed gradual increment in log cycle at pH3 with all bile salt concentration. The results for best three strains are presented in Figure 4. The selected strains PMD9, PMK20 and PML31 showed growth of 6-9 log cfu/ml at control conditions i.e. pH6.5 with 0% bile concentration (Figure 5).

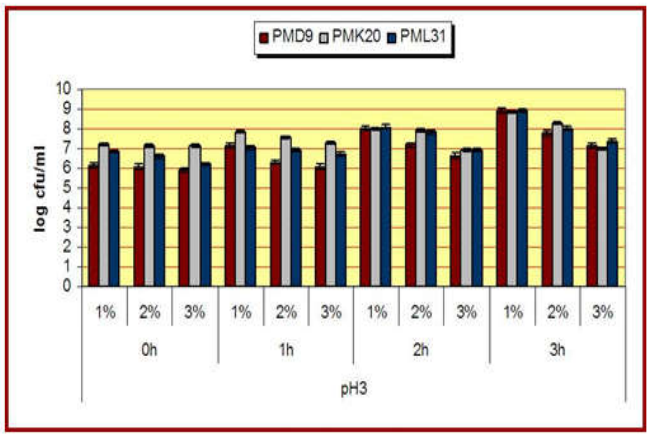

Figure 4. Effect of different bile concentration on selected strains after exposure to pH 3

Cell surface hydrophobicity

All the nine strains of *S. thermophilus* were evaluated for their hydrophobicity towards three different hydrocarbons i.e. n-hexadecane, n-octane and xylene, which may reflect the

colonization potential of the organism to intestinal lumen. The results pertaining to the surface hydrophobicity of the three selected isolates are given in Table 1.

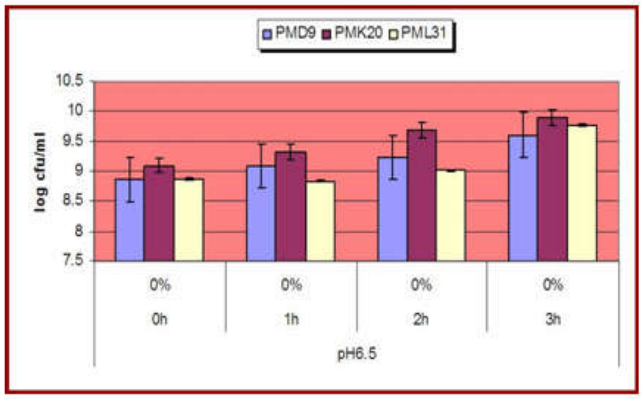

Figure 5. Control test for simulated acid bile tolerance with pH 6.5 and 0% bile

Table 1. Cell surface hydrophobicities of bacteriocinogenic *S. thermophilus* strains with different hydrocarbons

S. No.	Isolates	% Hydrophobicity (mean + SEM)		
		(Xylene)	(n-Octane)	(n-Hexadecane)
1	PML3	15.33 ± 0.735	18.58±1.071	12.71±1.325
2	PMD7	9.30 ± 0.713	11.47±0.715	7.04±1.256
3	PMD9	33.77 ± 0.855	37.75 ± 3.122	26.37 ± 1.032
4	PMM15	28.34 ± 0.324	34.77±0.685	21.49±1.133
5	PMK20	12.47 ± 0.837	16.33±0.157	9.66±0.647
6	PML25	14.42 ± 1.022	17.64±1.233	11.43±0.768
7	PML31	21.68 ± 1.225	22.35±0.316	17.21±1.017
8	PMD36	18.25 ± 0.542	26.27±1.084	13.56±0.685
9	PMM43	7.89 ± 0.347	12.42±1.225	5.16±0.572

It may observed from Table 1 that the cell surface hydrophobicity of *S. thermophilus* strains was not very high and thus the *Streptococcus thermophilus* strains may be designated as a transient probiotics. It was observed that the strains showed maximum adherence towards n-Octane while lowest towards n-Hexadecane. The *S. thermophilus* strain PMD9 showed maximum adherence with all the three test hydrocarbons whereas PMM43 showed minimum adherence level. PMM15 strain which showed a medium level of acid and bile tolerance had a comparatively good adherence. Whereas, on the contrary, the strains PMK20 and PML31 showed relatively lower adherence level in spite of showing good acid and bile tolerance.

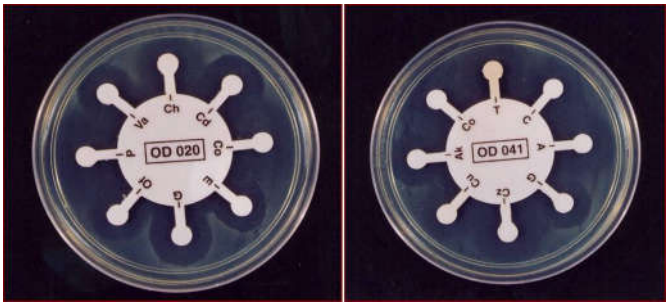

Plate 1. Antibiotic susceptibility profile of S. thermophilus PMD9

Antibiotic sensitivity

The antibiogram of nine technologically sound bacteriocinogenic *S. thermophilus* strains was investigated using Disc Diffusion Assay on Mueller Hinton Agar No. 2 for a total number of twenty three clinically important antibiotics according to NCCLS (2000). The results of antibiotic resistance tests are shown in Figures 6, 7 and plate 1. All the nine strains of *S. thermophilus* possessing bacteriocin production trait were sensitive to antibiotic ofloxacin, vancomycin, polymixin B and ciprofloxacin whereas 88.8% of the strains were sensitive to cefazolin.

However, 55% of the stains were resistant to erythromycin and 33% to tetracycline and gentamicin. More than eighty-five percent of the bacteriocinogenic *S. thermophilus* strains were observed to be resistant to streptomycin. None of the isolates were found to be resistant to few antibiotics like bacitracin, amoxicillin and novobiocin however, 33% of the strains were found to be moderately sensitive to them (Figures 6 and 7). *S.thermophilus* PMD9 and *S. thermophilus* PML3 were sensitive to all the antibiotics tested except streptomycin and showed moderate sensitivity to two antibiotics (Figures 6 and 7).

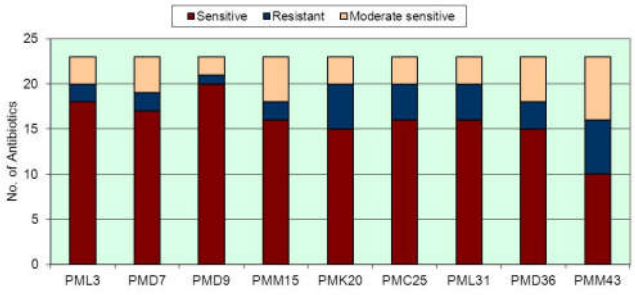

Figure 6. Antibiotic sensitivity of tested bacteriocinogenic *S. thermophilus* strains

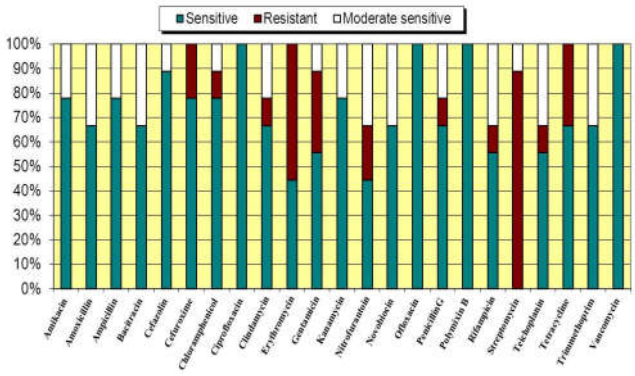

Figure 7. Percent Inhibitory activity of different antibiotics used for sensitivity testing

DISCUSSION

Probiotics represent one of the largest functional food markets. Most of the available products are some form of dairy, such as milk, ice cream, yogurt, cheese, and frozen desserts, despite the continuously growth of the nondairy sector, with products like soy-based drinks, fruit-based foods, and other cereal-based products (Granato *et al,* 2010).Apart from this, the human trials also confirmed that the enrichment of probiotics in vegetables such as artichokes and olives, represents a way to achieve the target "functional diet" (Valerio *et al.,* 2011; Riezzo *et al.,* 2012; Sistoand Lavermicocca, 2012). Development of almost actual (simulated) conditions of gastrointestinal tract under *in-vitro* set up with sequential exposure to acid and bile may lead to better assessment of

selected cultures as probiotics in comparison to traditional acid and bile tolerance tests and help in adjudging these cultures as preferably acid and bile tolerant and establishing them as potential probiotics. The survival of ingested organism through the passage of gastrointestinal (GI) tract of human beings is an important criterion for exerting probiotic effects. About 2.5 liters of gastric juice is secreted each day having pH of approximately 2.0 and bile salt content not less than 0.5% w/v (Hill, 1990). In contrast, about 0.7 liter of pancreatic juice is secreted into the proximal small intestine each day having pH of about 8 with same percentage of bile content as found in gastric juice (Keele and Neil, 1965). These secretions are responsible for a wide range of pH with different bile salt concentration in GI tract. The ability of probiotic organisms to tolerate acid and bile during passage through GI tract with enzymes pepsin and pancreatin ensures that the organism could survive within the gut. It is speculated by several workers that bacteria in general are sensitive to low pH values in the stomach (Berrada *et al.,* 1990; Hammes *et al.,* 1997; Erkkila and Petaja, 2000) and it is, therefore, most important that the probiotics are consumed within food matrix. Milk has been shown to be an excellent vehicle for probiotic bacteria probably due to its high buffering capacity (Huang *et al.*, 2004). Thus, our strains *S. thermophilus* PMD9, PMK20 and PML31 would show better survival through stomach, if consumed along with milk or within a food matrix. According to Martins *et al.* (2007), the microorganisms used as probiotics must confront to a variety of simultaneous or sequential adverse conditions such as mild heat shock (internal body temperature), acidic gastric juice, alkaline pancreatic juice and presence of bile salts. This problem is particularly important when probiotics are not originally from the digestive tract of mammals, as is the case of *Streptococcus thermophilus* strains. The reports concerning the *in vitro* probiotic characteristics of lactobacilli are unlimited while those dealing with *in vitro* probiotic characteristics of *Streptococcus thermophilus*, especially the bile tolerance, are too less (Huang *et al.*, 2004). Data obtained in these investigations suggested that the interplay between gastric and intestinal stresses may aid in the development of better probiotics as a biological alternative to pharmaceutical interventions.

Once bacteria reach the small intestinal tract, their ability to survive depends on their resistance to bile (Gilliland, 1984). Bile entering the duodenal section of the small intestine has been reported to reduce the survival of bacteria. This is probably due to the fact that all bacteria have cell membranes consisting of lipids and fatty acids which are very susceptible to destruction by bile salts which have detergent characteristics. Hence, the success of a probiotic organism also depends on the selected strain possessing bile-tolerance characteristics (Jin *et al.,* 1998). Adhesion to hydrocarbons is considered as a biochemical marker for adherence to the epithelial cells in the gut. Attachment of the organism to intestinal mucosa is an essential feature to impart beneficial effects to host. Hydrophobicity test is based on the interaction of the cell surface with phagocytes, adherence to non- wetable solid surfaces, partitioning at liquid: liquid and liquid: air interface. Hence the adhesion ability of probiotics to intestinal epithelial cell is considered as one of the most important selection criteria (Brassert *et al.,* 1994). Hydrophobicity for different hydrocarbons has been established as the *in vitro* biochemical marker to assess the colonization potential of organisms (Rosenberg *et al.,* 1980).

The hydrophobic nature of the outermost surface of various microbial cells has been implicated in such biological phenomena as interactions between bacteria and phagocytes (Cunningham *et al.,* 1975) and attachment of bacteria to the host tissues (Smyth *et al.,* 1978). Hydrophobicity is directly related to concentration of carbon in hydrocarbon form and inversely related to oxygen concentration or to the nitrogen/phosphate ratio (Mozes *et al.,* 1988). Smyth *et al.* (1978) has reported that cells with greater hydrophobicity values show greater attractive forces and therefore, higher levels of adhesion. The hydrophobicity was expressed as the coefficient of applied cell suspension that has been excluded from the aqueous phase. As a result of this assay, hydrophobicity values were found in the range of 5.16% to 37.75% (Table 1). It clearly depicts that each and every probiotic strain should be judged by its own merits and that extrapolation from related strain is not acceptable (Ouwehand *et al*., 1999). Greater surface hydrophobicity of bacterial cells results in greater attractive forces and higher level of adhesion, whereas smaller electro-kinetic potentials of cells and lower level of ionic structure results in greater repulsive electrostatic interaction and lower levels of adhesion (Prakash *et al*., 1997). Higher cell surface hydrophobicity may favor the colonization of mucosal surfaces and play a role in the adhesion of bacteria to epithelial cells and ECM proteins (Schillinger *et al*., 2005). Adhesion to the intestinal mucosa is thought to be an important property for colonization by preventing wash-out (Wadstrom *et al*., 1987) especially in the small intestine where flow rates are relatively higher. The determination of microbial adhesion to hexadecane as a way to estimate the ability of a strain to adhere to epithelial cells is a valid qualitative phenomenological approach (Kiely and Olson, 2000). The EU Scientific Committee on Animal Nutrition (SCAN) has provided guidelines to regulate bacteria used in feeds (i.e. probiotic bacteria, starter bacteria) and according to SCAN guidelines; these should not contain any acquired antibiotic resistances (SCAN, 2002).

Examples of acquired resistance through exchange of resistance encoding genes are resistance to tetracycline, chloramphenicol, glycopeptides, and vancomycin. In this study, majority of strains were found to be sensitive to these antibiotics. EU has not yet established similar guidelines for human probiotics but realistically, only those strains may have potential in the development of future probiotics that are safe from antibiotic resistance point of view. *S. thermophilus* strains are generally known to be highly sensitive to various antibiotics and hence used as test organism for detection of antibiotic residues in milk. Among resistances to antibiotics, vancomycin resistance is of major concern. Some LAB including strains of *L. casei*, *L. rhamnosus*, *L. plantarum*, *Pediococcus* spp. and *Leuconostoc* spp. have been reported to be resistant to vancomycin. Such a resistance is usually intrinsic, that is, chromosomally encoded and non-transmissible (Handwerger *et al*., 1994; Klein *et al*., 1998). However, in the present study none of the isolates were found to be vancomycin resistant. Although *S. thermophilus* strains are widely used as starter cultures, there are only very few reports regarding their resistance to antibiotics. Only sketchy information is available in a few reports depicting studies limited to disc diffusion tests and detailed studies have not been carried out. Sozzi and Smiley (1980) screened 15 strains of *S. thermophilus* for resistance against 35 antimicrobial agents. In a study by Aslim and Beyatli (2004), 34

Streptococcus thermophilus strains isolated from Turkish yoghurts were examined for their antibiotic resistance patterns. In the present study, we observed more prevalence of sensitivity and the antibiotic sensitivity of the strains compared well with the one reported by these workers. Sozzi and Smiley (1980) reported resistance to gentamicin, streptomycin, and sulphadiazine. It is of interest to note that out of the total nine strains tested, five strains exhibited resistance to erythromycin. Aslim and Beyatli (2004) reported that 14 strains out of total 34 strains screened by them were resistant to erythromycin. Contrary to this, Sozzi and Smiley (1980) reported all 15 strains to be sensitive to erythromycin. Since only limited studies are available and the methods and media used are different, a valid comparison of results is not possible. However, it is important to note that many probiotics may not benefit immunocompromised individuals, people with organ failure, and dysfunctional gut barrier, where they may cause infection. Anyway the beneficial effects of *S.thermophilus* probiotic strains, are reported to confer colon health, support the immune system besides protecting the small intestine irritation and countering antibiotic associated diarrhea.

Conclusion

The present investigation evaluated the possibilities of bacteriocinogenic strains of *S. thermophilus* isolated from various dairy products on probiotic grounds for their role as possible functional starters giving an edge to starter technology. Since such technologically favorable starters may offer exciting opportunities for their exploitation either as functional starters/ protective cultures in food and dairy industry or as animal and human probiotics. Further these strains were found to be sensitive to several antibiotics. The judicious use of these beneficial microorganisms could bring revolutionary and exciting changes in the present realm of functional foods and nutraceuticals and a few probiotics could counter antibiotic resistance.

Conflict of Interest: All the authors declare that they have no conflict of interest.

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