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Evidence for probiotic potential of a capsular-producing *Streptococcus thermophilus* CHCC 3534 strain

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The purpose of this research was to evaluate the probiotic potential of a capsulated *Streptococcus thermophilus* CHCC 3534 strain. The strain proved to tolerate 0.4% oxgall and was sufficiently resistant to pH as low as 2.5 for 3 h of exposure. The strain demonstrated high adherence to human intestinal mucus, and showed a unique resistance to different antibiotics. Crude extracts of *S. thermophilus* CHCC 3534 contained a diffusible antimicrobial compound "bacteriocin" with a broad spectrum that inhibited the growth of closely related lactic acid bacteria and a number of food spoilage bacteria, including *Salmonella typhimurium* and *Staphylococcus aureus*. The bacteriocin was heat stable, resistant to pH, inactivated by proteolytic enzymes and resistant to -amylase and lipase. SDS-PAGE analysis of the partially purified bacteriocin revealed one peptide with a molecular weight ranging from 14.4 to 18.4 kDa. The strain may have an industrial significance and represents an interesting candidate for use in food biopreservation, probiotic formulations and in the control of spoilage caused by food-borne pathogens.

Key words: Streptococcus thermophilus, probiotic, acid tolerance, adhesion, bacteriocin, bile.

INTRODUCTION

Commercial lactic acid bacteria (LAB) products have been one of the major health-related foods in the world. Evaluation of the probiotic function for LAB strains from these products is important for the public. A strain is considered probiotic if it proves to be safe and posses some basic characteristics, including acid and bile tolerance, ability to adhere to intestinal epithelium and antagonistic activity against pathogenic bacteria (Lin et al., 2006). Throughout the past two decades, probiotic microorganisms have been increasingly included into comercial dairy products (de Souza et al., 2008), a response to the consumer demand for healthy food options that improve overall health, intestinal function and digestion (Luckow and Delahunty, 2004). *Streptococcus thermophilus* is one of the most important LAB used for food industry. It is traditionally used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* in the manufacture of yogurt, cooked cheese (parmigiano, grana, gruyere, emmental, etc.) and mozzarella cheese (Delcour et al., 1996; Parente and Cogan, 2004). A recent review proposed that fresh yoghurt preparations could be considered probiotic products because they confer measurable health benefits to the host, as compared with products with heat-killed bacteria (del Campo et al., 2005; Guarner et al., 2005). Despite of that, focus has long been on the incorporation of selected strains of *Lactobacillus* spp. into milk and fermented milk products (Patrignani et al., 2006) due to the extensive studies performed on their probiotic properties compared to the limited research and scarce and unconvincing data concerning *S. thermophilus* strains. Moreover, far less is known about the certainty of the health promoting effects of several members belonging to *S. thermophilus* (Holzapfel et al., 2001; Abbot, 2004) in addition to the significant controversy regarding the survival of their cells after passage through the human gastrointestinal tract (Mater et al., 2005). Recently,

Table 1. LAB and pathogenic strains used in the study.

Strains	Incubation temperature (°C)	Source
S . thermophilus CHCC 3534	37	Chris Hansen Co., Denmark
Bacillus sp.	30	Faculty of Medicine
E.coli	30	Faculty of Medicine
Klebsiella sp.	30	Faculty of Medicine
Pseudomonas sp.	30	Faculty of Medicine
Salmonella typhimurium	30	Faculty of Medicine
Shigella sp.	30	Faculty of Medicine
Staph.aureus	30	Faculty of Medicine
Lb. delbrueckii subsp. *lactis* 643 LMB	42	Dairy culture collection (Fac. Agriculture)
Lb. delbrueckii subsp. *lactis* 651 LMB	42	Dairy culture collection (Fac. Agriculture)
Lb. delbrueckii subsp. *lactis* 1441 LMB	42	Dairy culture collection (Fac. Agriculture)
Lb. delbrueckii subsp. *lactis* 642 LMB	42	Dairy culture collection (Fac. Agriculture)
Lb. delbrueckii subsp. *bulgaricus* 645 LMB	42	Dairy culture collection (Fac. Agriculture)
Enterococcus 1442 LMB	42	Dairy culture collection (Fac. Agriculture)
Enterococus 1443 LMB	42	Dairy culture collection (Fac. Agriculture)
Lactococcus lactis subsp. *lactis* 1444 LMB	30	Dairy culture collection (Fac. Agriculture)
Lb. plantarum P1	37	Dairy culture collection (Fac. Agriculture)
Lb. plantarum P164	37	Dairy culture collection (Fac. Agriculture)
Lb. pentosus P191	37	Dairy culture collection (Fac. Agriculture)
Lb. fermentum P10	37	Dairy culture collection (Fac. Agriculture)
Lb. fermentum P 193	37	Dairy culture collection (Fac. Agriculture)
Bifidobacterium longum B1	37	Dairy culture collection (Fac. Agriculture)

biopreservation using natural microflora and their antimicrobial products has become a topic of interest and an alternative technique to chemical additives for enhancing food safety (Kabuki et al., 2007). The ability of *S. Thermophilus* strains to produce bacteriocin has been studied in the past however, most recent studies in the literature deal with bacteriocins of various lactobacilli, lactococci, pediococci, and leuconostoc strains and relatively limited data is known about purified and sequenced bacteriocins from *S. thermophilus* species (Kabuki et al., 2007). The growing need for new strains of LAB with functions that improve the well-being of human nutrition concurrent with reducing the risk of diseases has prompted the work described here.

The main objective of this work was to verify the relevant probiotic characteristics of a capsulated *S. Thermophilus* CHCC 3534 strain for providing the evidence whether the strain qualifies for the notion according to the current concept of probiotics. The parameters examined to assess the probiotic features included: tolerance to low pH and bile salts, adhesion to human intestinal mucus, antibiotic susceptibility and production of antimicrobial compounds. Due to the little research carried out on *S. thermophilus* bacteriocins, another aim in the study arose which was the partial characterization of the inhibitory compound produced by the strain and the evaluation of its effectiveness against food- spoilage microorganisms. Partial characterization studies involved the investigation of the enzyme, pH and heat susceptibilities, antimicrobial spectrum, as well as molecular weight determination of the produced bacteriocin. The production of capsular polysaccharides (CPS) by *S. thermophilus* CHCC 3534, based on previous extensive studies (Khalil et al., 2007a), was the motive for selecting the strain for the present investigation. The ability of the strain to impart desired rheological properties due to the production of CPS is an advantageous property that could be consi-dered an important selection criterion for starter cultures (Patrignani et al., 2006), in addition to the primary probiotic properties. To our knowledge, this is the first report to provide evidence of interesting probiotic properties of a capsule-producing *S. thermophilus* strain.

MATERIAL AND METHODS

Microorganisms

LAB and pathogenic strains employed in the study, their sources and growth conditions are listed in Table 1. The capsule-producing strain was provided by Chris Hansen Co., Denmark. Other LAB strains were provided by the Dairy culture collection at the Laboratory of Microbial Biochemistry of Dairy Microorganisms, Department of Dairy Science and Technology, Faculty of Agriculture,

Alexandria University, Egypt and were used in the antimicrobial spectrum and adhesion assays. Pathogenic strains were provided by the Microbiology Department, Faculty of Medicine, Alexandria University, Egypt and were used in the antimicrobial spectrum assay.

Probiotic properties

Acid and bile tolerance: Aliquots (10 ml) of M17 broth were adjusted to pH values from 1.5 to 5 (with 0.5 increment increase) with 1 N NaOH or HCl. Tubes were inoculated (1% [v/v]) with overnight M17 broth culture and incubated aerobically at 37°C for 3 h. Cultures turbidity were hourly monitored at 600 nm. Initial and final cultures OD_{600} values were measured against uninoculated M17 medium and plotted against the pH values tested (Khalil et al., 2007b). This experiment was performed in triplicate.

Aliquots (10 ml) of M17 broth supplemented with different concentrations of oxgall (Bronadica, Hispan Lab, SA) (0.15 - 0.4% final concentration) were inoculated (1% [v/v]) with overnight M17 broth culture and incubated aerobically at 37°C for 3 h. The bile tolerance of the strain was determined as described above (Khalil et al., 2007b).

***In vitro* adhesion assay to intestinal mucus:** Prior to adhesion, *S. thermophilus* CHCC 3534 and the positive control strains (*Lactobacillus plantarum*, *Lactobacillus fermentum*, *Lactobacillus. Pentosus and Bifidobacterium longum*), as reported by Khalil et al. (2007b), were propagated in MRS broth overnight at 37°C. Bacteria were harvested by centrifugation (10 000 x g, for 10 min at 4°C) and washed twice with phosphate buffer saline (PBS: 10 mM KH_2PO_4, 150 mM NaCl, pH 7.2). The optical density of the bacterial suspensions at 600 nm was adjusted with PBS to 0.5 ± 0.02, giving a count that varied between 10^6 and 10^8 CFU/ml. Human intestinal mucus was isolated from faeces of healthy new-borns (3 - 6 months), according to the method of Ouwehand et al. (2001) and Khalil et al. (2007b). For the adherence assay, the crystal violet method devised by Vesterlund et al. (2005) was used. Each determination was triplicated.

Antibiotic susceptibility: The disk susceptibility test was done according to the Bauer-Kirby method. The test strain was screened for possible resistance against 14 selected antibiotics (Difco laboratories, MI, USA), belonging to the different antibiotic classes and included: streptomycin (10 g), ampicillin (20 g), amoxicillin (30 g), clavulinic acid (20 g), vancomycin (30 g), erthromycin (15, and 300 g), tetracycline (20 g), unasyn (20 g), sulfamethoxazole/trimethoprim (25 g), levofloxacin (10 g), ofloxacin (10 g), furadantin (300 g), ciprofloxacin (10 g) and metronidazole (10 g). The assay was carried out using multiple discs on the same plate to eliminate differential effects from growth time and tempe-rature.

Antagonistic activity and characterization of the inhibitory compound bacteriocin: Cell-free supernatant (CFS) of *S. thermophilus* CHCC 3534 culture was prepared by growing the strain in M17 broth at 37°C until the early stationary phase (8 - 10 h). Cells were separated by centrifugation (10 000 × g for 10 min at 4°C), the supernatant was neutralized to pH 6 with 1 M NaOH andfilter-sterilized with disposable bacterial filters (0.2 m; Fischer chemi-cals, UK). For the detection of antimicrobial substances in the resulting CFS, the agar well-diffusion (AWD) assay devised by Kabuki et al. (2007) was used.

Sensitivity of bacteriocin to proteolytic and other enzymes: Samples of the crude bacteriocin were examined for susceptibility to proteolytic and other enzymes. The following enzymes (Oxford laboratory reagents) and respective buffers were employed: papain, pepsin, and trypsin in 0.05 M sodium phosphate (pH 7), 0.002 M HCl (pH 7) and 40 mM Tris-HCl (pH 8.2), respectively. Non-proteolytic enzymes such as lipase and -amylase in 0.1 M potassium phosphate (pH 6) and 0.1 M potassium phosphate (pH 7), respecttively, were also examined. Enzyme solutions were filter sterilized and mixed in equal aliquots with the crude bacteriocin to a final concentration of 1 mg/ml, incubated at 37°C for 2 h and then heat-inactivated at 100°C for 15 min. M17 broth containing the enzyme-treated bacteriocin was inoculated (1% [v/v]) with *Salmonella. Typhimurium* early exponential phase culture and incubated 30°C, where growth was monitored spectrophotometrically at 600 nm for up to 12 h. The bacteriocin activity was determined using the optical density measurement (ODM) method by Vinderola et al. (2002).

Bacteriocin sensitivity to heat treatment and effect of pH: The thermal stability of the bacteriocin was assessed by exposing aliquots of *S. thermophilus* CHCC 3534 crude bacteriocin to different temperatures (-4, 0, 3, 40, 50, 60, 70, 80, 90, 100, and 121°C) for 15 min before being tested for antimicrobial activity. Heated aliquots were cooled in ice water. Bacteriocin activity of the samples and controls were determined using the ODM method, as described above. To test the effect of pH, *S. thermophilus* CHCC 3534 filter-sterilized CFSs were adjusted to pHs from 2 to 12 (at increment of one pH unit) with sterile 1 N NaOH or 1 N HCl (Albano et al., 2007). Samples were incubated at room temperature (25°C) for 1 h before being tested for antimicrobial activity by the ODM method, as described above.

Partial purification and molecular weight determination: The test strain was grown in M17 broth for 10 h at 37°C. Cells were harvested by centrifugation (10 000 x g for 20 min at 4°C) and the bacteriocin was precipitated from the CFS with 45% saturated ammonium sulfate (Akyptis et al., 1998). The molecular weight of the bacteriocin was estimated according to the method of Sambrook and Russell (2001). The apparent molecular mass of the sample was calculated by comparison with the mobility of the standard markers (Bio-RAD, Germany).

Statistical analysis of data: Data were expressed as mean ± standard deviation. Statistical significance was determined using the student's t-test. $P < 0.05$ was considered significant.

RESULTS

Acid and bile tolerance, adhesion to intestinal mucus and antibiotic susceptibility

S. thermophilus CHCC 3534 cells failed to grow at the lowest pH value (pH 1.5) but survived with no significant change in culture turbidity at pH 2 (Figure 1). The strain exhibited better survival at pH 2.5, as confirmed by the doubling of the final culture turbidity value compared to the initial one. pH 3 and above were not inhibitory to the growth of the cells, which significantly ($P < 0.01$) survived the acidity after 3 h of exposure. The maximal growth was distinguished in M17 supplemented with 0.2% oxgall (Figure 2). In the presence of 0.25 to 0.35% oxgall, an insignificant drop ($P > 0.05$) in final culture turbidity was observed. At 0.4% oxgall, the final culture OD_{600} reading exceeded that of the initial by more than 0.4 units, con-

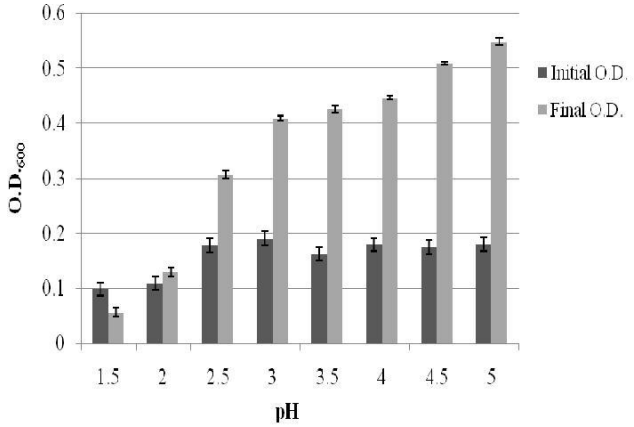

Figure 1. Acid tolerance of *S. thermophilus* CHCC 3543 grown for 3 h at 37°C in M17 broth adjusted to different pH values. Bars represent the standard error of the mean values of the OD_{600} measurements of three independent experiments (*n* = 3).

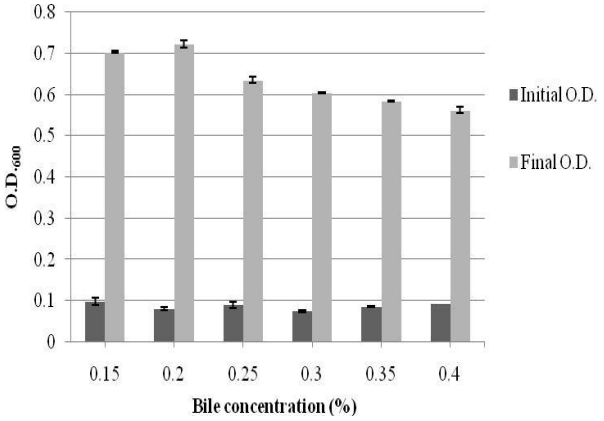

Figure 2. Survival of *S. thermophilus* CHCC 3543 in M17 broth supplemented with different concentrations of oxgall, as determined by the cultures turbidity after 3 h of exposure. Bars represent the standard error of the mean values of the OD_{600} measurements of three independent experiments (*n* = 3).

firming the interesting feature of bile tolerance of the strain. The strongest *in vitro* adhesion was recorded for *S. thermophilus* CHCC 3534 (11.54%) in comparison to the low level of adhesion (5.66%) of the reference strain *B. longum* (Figure 3). The strain was resistant to all antibiotic representatives used in the study, except to Unasyn (20 g), where an inhibition zone of size 1.5 mm was distinguished (data not shown).

Bacteriocin antimicrobial spectrum

The agar well-diffusion (AWD) assays results (Table 2)

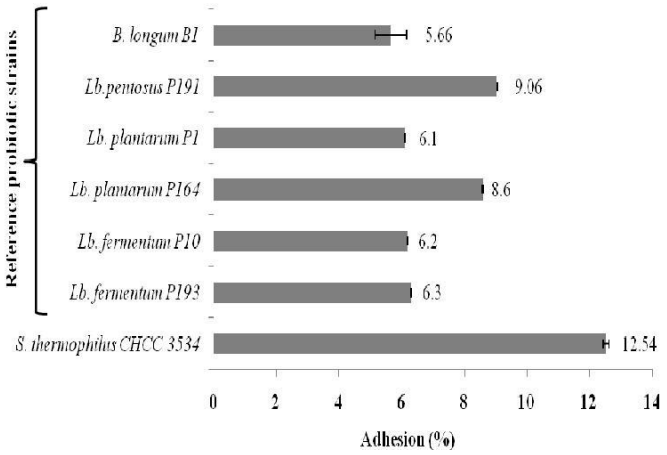

Figure 3. Adhesion of *S. thermophilus* CHCC 3534 to intestinal mucus isolated from infant faecal samples in comparison to reference probiotic strains. Results are expressed as the % of adhering bacteria. Bars represent the mean ± standard deviation of triplicates OD_{600} values recorded for each strain.

showed that the crude bacteriocin had a broad antimicrobial spectrum against closely related LAB strains and other pathogenic strains. The activity was slight to moderate against all pathogens (inhibition zones< 10 mm) with an exception to *S. aureus* and *S. typhimurium*, where they were strongly inhibited (10 mm inhibition zone size). No activity was detected against the thermophilic *L. delbrueckii* subsp*. bulgaricus*, *Lactococcus* or the enterococci strains.

Effect of enzymes, temperature and pH on bacteriocin activity

S. thermophilus CHCC 3534 bacteriocin activity was not modified by the action of either -amylase or lipase, where maximal growth reductions to *S. typhimurium* were recorded (87 and 68% growth reduction, respectively). Treatment with proteolytic enzymes resulted in the reduction of the bacteriocin activity to 50% or more as compared to the non- proteolytic enzymes (Figure 4). Exposure to 80°C and above resulted in loss in bacteriocin activity against *S. aureus*, and increase in activity against *S. typhimurium* (Figure 5). The bacteriocin resisted the autoclaving temperature for 15 min, but recorded a low activity against the latter indicator strain. It is worth noting that the bacteriocin was stored at -4°C for at least 2 months without detectable loss of activity (data not shown) . The bacteriocin activity against *S. typhimurium* and *S. aureus* indicator strains was found to be stable at pH values between 2 and 6. Maximal bacteriocin activity against both indicator strains was recorded (43% and 15% growth reduction, respectively) at pH 7, whereas the former activity was reduced to almost half (24% growth

Table 2. Antimicrobial spectrum of *S. thermophilus* CHCC 3534 bacteriocin against pathogenic strains and closely related LAB strains, as determined by the AWD method.

Target strains	Inhibition zone (mm)
Bacillus spp	2
E.coli	4
Klebsiella spp	5
Pseudomonas sp	4
Sal. typhimurium	10
Shigella spp	4
Staph. Aureus	10
Lb. delbrueckii subsp. *lactis* 643LMB	> 15
Lb. delbrueckii subsp. *lactis* 651 LMB	> 15
Lb. delbrueckii subsp. *lactis* 1441 LMB	> 15
Lb. delbrueckii subsp. *lactis* 642 LMB	> 15
Lb. delbrueckii subsp. *bulgaricus* 645 LMB	0
Enterococcus 1442 LMB	0
Enterococcus 1443 LMB	0
Lactococcus lactis subsp. *lactis* 1444 LMB	0

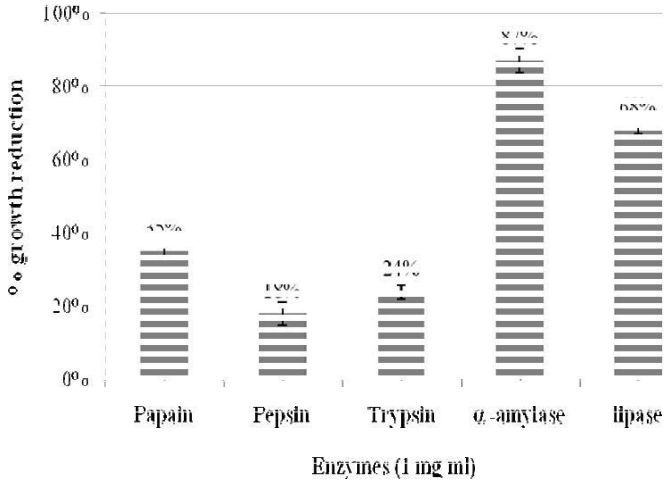

Figure 4. Effect of enzyme treatment on the activity of *S. thermophilus* CHCC 3534 bacteriocin against *S. typhimurium*. Results are expressed as % of mean values of activity (n = 3) ± standard deviations.

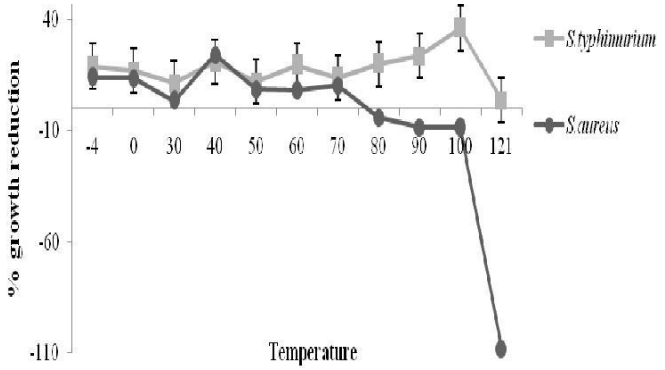

Figure 5. Effect of heat treatment on the activity of *S. thermophilus* CHCC 3534 bacteriocin. Activity is expressed as the % of growth reduction against ( ) *S. typhimurium* and ( ) *S. aureus*.

reduction) at pH 8. The bacteriocin activity was lost at pH 9 and above (Figure 6).

Determination of bacteriocin molecular weight

SDS-PAGE of partially purified bacteriocin revealed that it had an apparent molecular weight ranging from 14.4 – 18.4 kDa, as determined by SDS-PAGE, where only single protein band was detected (Figure 7).

DISCUSSION

The characterization of the probiotic potential of a strain is important in its selection, as this will provide an indication of its presumed public safety and enable the possibility of its integration in dairy products and health-related foods. Viability and the survival of probiotic bacteria are the most important parameters in order to provide therapeutic functions to the product (Succi et al., 2005). Hence, the survival of *S. thermophilus* CHCC 3534 under *in vitro* conditions that mimic the physico-chemical events occurring in the gastrointestinal tract was studied. The incubation time chosen for acid and bile tolerance tests was 3 h, simulating the residence time in the human stomach (Olejnik et al., 2005). Survival at pH

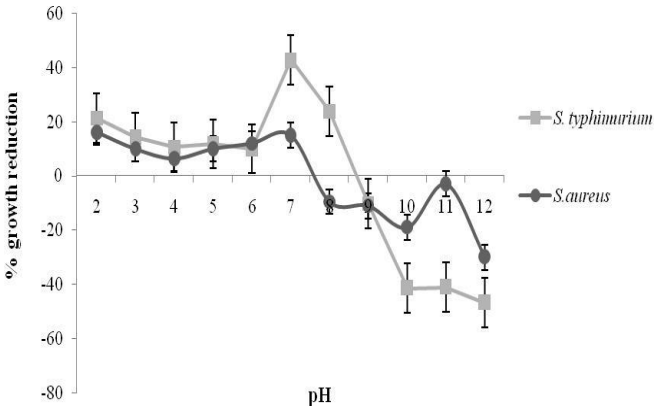

Figure 6. Effect of pH on the activity of *S. thermophilus* CHCC 3534 bacteriocin. Activity is expressed as the % of growth reduction against ( ) *S. typhimurium* and ( ) *S. aureus*.

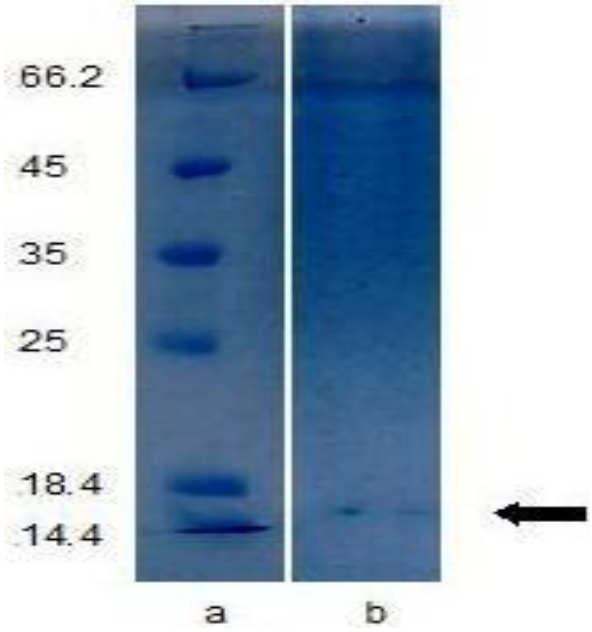

Figure 7. SDS-polyacrylamide gel containing the molecular size markers stained with Coomassie Brilliant blue (lane a) and the partially purified bacteriocin of *S. thermophilus* CHCC 3534 from gel chromatography (lane b). The arrow indicates the position of the single peptide band. Sizes on the left are indicated in kDa.

1.5 represented a lethal environment to *S. thermophilus* CHCC 3534. This finding was supported by studies of Haller et al. (2001), indicating the death of all experimental strains at pH 1.5. The strain remarkably resisted pH as low as 2 and 2.5 and maintained its viability, which was in accordance with the work reported by Maura and Meriem (2008), elucidating the high survival percentages of *L. plantarum* strains after 2h, 4h, and 6 h of incubation at pH 2. The presence of bile salts in the environment of bacterial cultures is much more detrimental than the effect of low pH (Olejnik et al., 2005). *S. thermophilus* CHCC 3534 showed great resistance to detrimental actions of bile salts where the cells survived all bile treatments starting from a concentration of 0.15%. This behavior allowed us to predict the potentiality of this strain as a probiotic microorganism, since it survived a bile concentration (0.4% oxgall solution) equivalent to the physiological concentration in the duodenum (Brashears et al., 2003), and at the same time higher than the concentration (0.3% oxgall) that have previously been applied by other investigators (Fernández et al., 2003; Kuhle et al., 2005).

Adhesion ability of pathogenic bacteria is an important concern, although, in contrast, adhesion of probiotic strains is a desirable feature (Perelmuter et al., 2008). Reference strains used as adhesion controls displayed lower adhesion than that of *S. thermophilus* CHCC 3534 inspite of their documented probiotic properties (Khalil et al., 2007b). These observations were explained on the basis of the existing relationship between high cell surface hydrophobicity and hence, surface capsular polysaccharides and the stimulation of adhesion to human intestinal mucus (Ruas-Madiedo et al., 2006; Tallon et al., 2007). The inability of the cells of the reference strains to produce extracellular polysaccharides as determinants for adhesion might account for their low affinity to the intestinal mucus. This data can be of industrial signifycance, as the possibility of modifying the concentration of CPS may affect the level of bacterial adhesion of *S. thermophilus* CHCC 3534. Administration of antibioitics often causes disturbances in the normal intestinal microbiota (Lindberg et al., 2004). *S. thermophilus* CHCC 3534 was significantly ($P > 0.05$) insensitive to the antibiotics employed regardless of their concentration or mode of action (D'Aimmo et al., 2007). The susceptibility of our strain to 20 µg/ml of Unasyn might be attributed to the synergistic effect of sulbactam and ampicillin being the major components of the antibiotic (Bayer et al., 1980). The antibiotic resistance of the test strain provides a competitive advantage over the antibiotic sensitive strains enabling their survival (Plummer et al., 2005). Crude *S. thermophilus* CHCC 3534 extracts contained a diffusible antimicrobial compound with a broad spectrum. This pronounced activity has not been demonstrated in some strains such as *S. thermophilus* SBT1277 or *S. Thermophilus* ST110 (Kabuki et al., 2007). The inability of the bacteriocin to inhibit the growth of the thermophilic *L. delbrueckii* subsp*. bulgaricus* indicator strain supports the hypothesis that it may be used in thermophilic starter for hard cheese making, because it is not active against thermophilic lactobacilli (Mathot et al., 2003).

The inactivation of the bacteriocin by treatment with -amylase and lipase enzymes suggests that activity was not dependent on the presence of either a carbohydrate or lipid moiety (Maurad and Meriem, 2008). Resistance to -amylase was contradictory to several reports showing the inactivation of thermophilins by -amylase treatment, indicating the requirement of a glycosidic moiety for full activity (Gilbreth and Somkuti, 2005). The antimicrobial compound was heat and pH stable, which was consistent to the characteristics of many bacteriocins produced by LAB (Kabuki et al., 2007). Heat stability of the bacteriocin was maintained for 15 min at temperatures ranging from -4°C to 100°C, with a decrease in activity at 121°C, which reinforces this study's suggestion of the possibility of use of this bacteriocin in the manufacture of hard cheese making. Contradictory observations were made for *S. thermophilus* 580 bacteriocin, where its heat instability was due to a heat labile peptide (Mathot et al., 2003). SDS-PAGE revealed that the bacteriocin had an apparent molecular weight for its single component ranging from 14.4 and 18.4 kDa. Data reported on the molecular sizes of *S. thermophilus* bacteriocins is highly versatile. *S. thermophilus* 580 bacteriocin was found to be more than 100 kDa in size (Mathot et al., 2003), while the two bacteriocin components of *S. thermophlius* ST110 were estimated to be 4.0 and 4.5 kDa respectively (Gilbreth and Somkuti, 2005).

In conclusion, results obtained in the present work provide adequate evidence for the probiotic potential of *S. thermophilus* CHCC 3534 strain. The use of a pure probiotic starter culture such as the strain under investtigation, endowed with appropriate probiotic and other desirable characteristics, could represent a practicable alternative to the use of mixed cultures consisting of both probiotic and starter strains. In view of the interesting inhibition spectrum of the bacteriocin of the *S. thermophilus* CHCC 3534 strain and its technological properties (good pH and heat tolerance), it is concluded that this bacteriocin have a potential application as a biopreservative, and may represent a promising candidate as starter culture for probiotic fermented dairy foods. Future research using *in vivo* studies should be undertaken on this interesting strain to determine its health-promoting benefits.

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