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ORIGINAL PAPER

Evidence for Probiotic Potential of a Capsular-Producing *Streptococcus thermophilus* CHCC 3534 Strain

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Abstract

The purpose of this research was to evaluate the probiotic potential of an capsulated *Streptococcus thermophilus* CHCC 3534 strain. The strain tolerates 0.4% oxgall (bile) and was sufficiently resistant to pH as low as 2.5 for 3 hours of exposure. The strain demonstrated high adherence to human intestinal mucus, and showed 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 á-amylase and lipase. A SDS-PAGE analysis of the partially purified bacteriocin revealed one component with a molecular weight ranging from 14.4 to 18.4 kDa. The strain may have industrial significance and represents an interesting candidate for use in biopreservation, probiotic food formulations and in the control of spoilage caused by food borne pathogens.

Key words: *Streptococcus thermophilus*, acid tolerance, adhesion, bacteriocin, bile

Introduction

Throughout the past two decades, probiotic lactic acid bacteria (LAB) have been increasingly included into commercial dairy products (de Souza *et al.*, 2008), as a response to the consumer demand for healthy food options that improve overall health. *Streptococcus thermophilus* is one of the most important LAB used in food industry. In spite of that, focus has long been on the incorporation of selected strains of *Lactobacillus* species into milk and fermented milk products (Patrignani *et al.*, 2006), due to the extensive studies performed on their probiotic properties compared to the scarce and unconvincing data concerning *S. thermophilus* strains. In addition, much less is known about the certainty of the health promoting effects of several members belonging to *S. thermophilus* and the survival of their cells after passage through the human gastrointestinal tract (Mater *et al.*, 2005; Holzapfel *et al.*, 2001). The growing need for new strains of LAB with functions that improve the well-being of human nutrition has prompted the work described here. The main objective of this work was to verify the relevant technological probiotic characteristics of the *S. thermophilus* CHCC 3534 strain selected for the study on the basis of its interesting feature of capsular polysaccharide production (Khalil, 2004). Due to little research carried out on *S. thermophilus* bacteriocins, we aimed also to partially characterize the inhibitory compound produced, and evaluate its effectiveness against food spoilage microorganisms.

Published studies deal with bacteriocins of various lactobacilli, lactococci, pediococci, and leuconostoc strains, and relatively limited data is known about purified bacteriocins from *S. thermophilus* species (Kabuki *et al.*, 2007).

Experimental

Materials and Methods

Microorganisms. LAB and pathogenic strains used in the study, their sources, and growth conditions are listed in Table I.

Probiotic and technological properties

Acid and bile tolerance. 10 ml aliquots of M17 broth were adjusted to pH values from 1.5 to 5 (with

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Table I
LAB and pathogenic strains used in the study

Strains	Incubation temperature
S. thermophilus CHCC 3534*	37°C
Bacillus sp.**	30°C
*E. coli***	30°C
Klebsiella sp.**	30°C
Pseudomonas sp.**	30°C
*Salmonella typhimurium***	30°C
Shigella sp.**	30°C
*Staphylococcus aureus***	30°C
Lactobacillus delbruekii subsp. *lactis* 643 LMB***	42°C
Lactobacillus delbruekii subsp. *lactis* 651 LMB***	42°C
Lactobacillus delbruekii subsp. *lactis* 1441 LMB***	42°C
Lactobacillus. delbruekii subsp. *lactis* 642 LMB***	42°C
Lactobacillus. delbruekii subsp. *bulgaricus* 645 LMB***	42°C
Enterococcus 1442 LMB***	42°C
Enterococus 1443 LMB***	42C
Lactococcus lactis subsp. *lactis* 1444 LMB***	30°C
Lactobacillus plantarum P1***	37°C
Lactobacillus plantarum P164***	37°C
Lactobacillus pentosus P191***	37°C
Lactobacillus fermentum P10***	37°C
Lactobacillus fermentum P 193***	37°C
Bifidobacterium longum B1***	37°C

* Capsule producing study strain provided by Chris Hansen Co., Denmark. ** Pathogenic strains provided by the Microbiology Department, Faculty of Medicine, Alexandria University, used in the antimicrobial spectrum assay. *** LAB strains 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, used in the antimicrobial spectrum and adhesion assays.

0.5 increment increase) with 1 N NaOH or HCl or oxagell (0.15–0.4 % final concentration). Tubes were inoculated (1% vol/vol) with overnight M17 broth culture, incubated aerobically at 37°C for 3 hours. Cultures turbidity was hourly monitored at 600 nm. Initial and final OD_{600} values were measured against uninoculated M17 medium, and plotted against the tested pH values. The experiments were performed in triplicate.

***In vitro* adhesion assay to intestinal mucus.** Prior to adhesion assay, *S. thermophilus* CHCC 3534 and the positive control strains (*Lactobacillus plantarum*, *Lb. fermentum*, *Lb. pentosus, and Bifidobacterium longum*) (Table I) were treated according to the method reported by Khalil *et al.* (2007). 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*. (2007). For the adherence assay, the crystal violet method devised by Vesterlund *et al.* (2005) was used. Experiments were performed in triplicate.

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

Antagonistic activity and bacteriocin antimicrobial spectrum. Cell-free supernatant (CFS) of the *S. thermophilus* CHCC 3534 culture was prepared by growing the strain in 100 ml of M17 broth at 37°C till the early stationary phase (8–10 hours). Cells were separated by centrifugation (10 000×g, for 10 minutes at 4°C), the supernatant was neutralized to pH 6 with 1 M NaOH, and filter-sterilized with disposable bacterial filters (0.2 µm; Fischer chemicals, 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, other non-proteolytic enzymes such as lipase and α-amylase in 0.1 M potassium phosphate (pH 6), and 0.1 M potassium phosphate (pH 7) respectively. Enzyme solutions were filter sterilized and mixed in equal volumes with the crude bacteriocin to a final concentration of 1 mg/ml, incubated at 37°C for 2 hours, then heat-inactivated at 100°C for 15 minutes. M17 broth containing the enzyme treated bacteriocin was inoculated (1% vol/vol) with the *S. typhimurium* in early exponential phase culture and incubated 30°C, where growth was monitored spectrophotometrically at 600 nm for up to 12 hours. 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 the *S. thermophilus* CHCC 3534 crude bacteriocin to different temperatures (–4, 0, 3, 40, 50, 60, 70, 80, 90, 100, and 121°C) for 15 minutes before they were tested for antimicrobial activity. Heated aliquots were cooled in ice water prior the addition of bacteria. The bacteriocin activity of the tested samples and controls was determined using the ODM method as described above. To test the effect of pH on *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 hour before test for antimicrobial activity by the ODM method as described above.

Partial purification and molecular weight determination. The tested strain was grown in M17 broth for 10 hours at 37°C. The cells were harvested by centrifugation (10 000×g for 20 minutes at 4°C) and the bacteriocin was precipitated from the CFS with 45% saturated ammonium sulfate. 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 was expressed as mean ± standard deviation. The statistical significance was determined using the student's t-test. $P<0.05$ was considered significant.

Results

Growth and adhesion properties *S. thermophilus*. The *S. thermophilus* CHCC 3534 cells failed to grow at the lowest pH value (1.5), but survived but with no significant change in the culture turbidity at pH 2 (Fig. 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. The pH 3 and above values were not inhibitory to the growth of the cells which significantly ($p<0.001$) survived the acidity after 3 hours of exposure. The maximum growth was selected in M17 supplemented with 0.2% oxgall (Fig. 2). In the presence of 0.25 to 0.35% oxgall, an insignificant drop in the final culture turbidity was observed. At 0.4% oxgall, the final culture

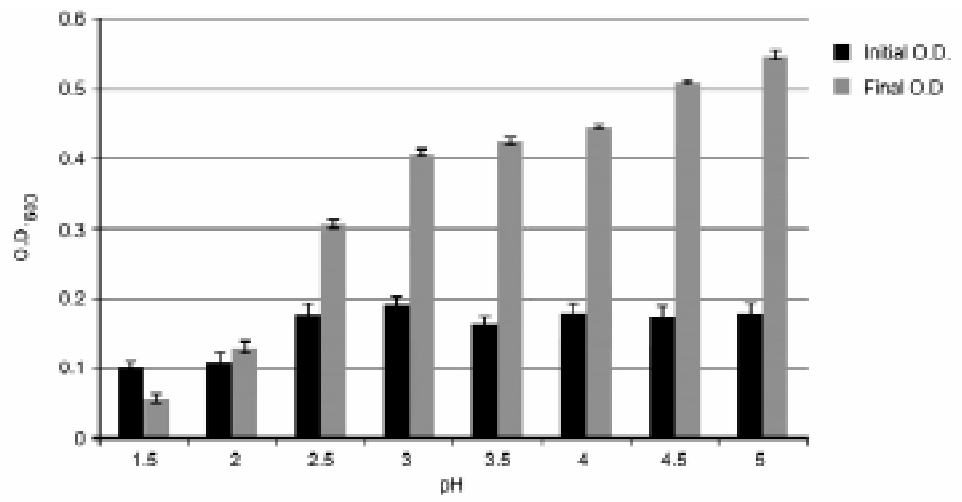

Fig. 1. Acid tolerance of *S.thermophilus* CHCC 3543 grown for 3 hours at 37ºC in M17 broth adjusted to different pH values.

Bars represent the standard error of the mean values of the OD_{600} measurements of three independent experiments (n = 3). Black and grey bars represent initial and final OD_{600} readings respectively.

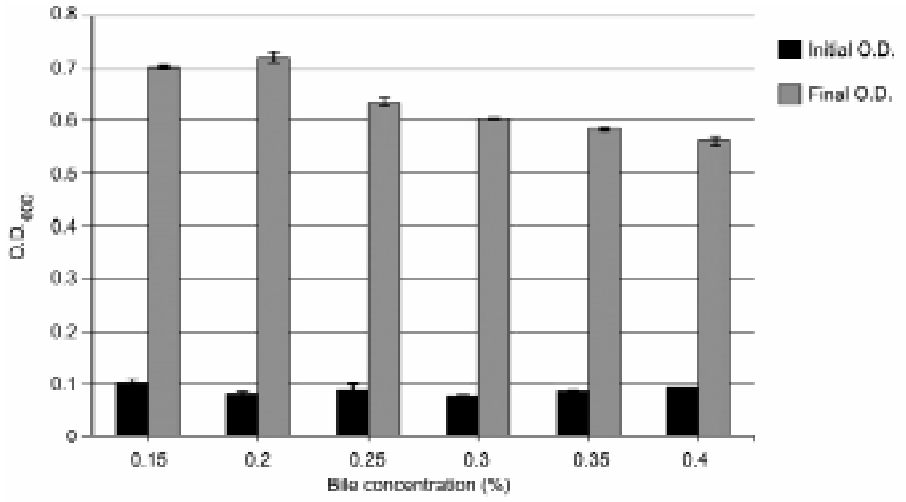

Fig. 2. Survival of *S.thermophilus* CHCC 3543 in M17 broth supplemented with different concentrations of oxgall, as determined by the cultures turbidity after 3 hours of exposure at 37°C.
Bars represent the standard error of the mean values of the OD_{600} measurements of three independent experiments (n = 3).
Black and grey bars represent initial and final OD_{600} readings respectively.

OD_{600} reading exceeded that of the initial by more than 0.4 units, confirming 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* (data not shown). The strain was resistant to all antibiotic representatives used in the study except for Unasyn (20 µg), where an inhibition zone of 1.5 mm in diameter was distinguished (data not shown).

Bacteriocin antimicrobial spectrum. Results bacteriocin spectrum tests (Table II) show that the crude bacteriocin had a broad antimicrobial spectrum against closely related LAB strains as well as other pathogenic strains. The activity was low to moderate against all pathogens (inhibition zones <10 mm) with an exception of *S. aureus* and *S. typhimurium*, that were strongly inhibited (10 mm inhibition zone size). No activity was detected against thermophilic *Lb. delbruekii* subsp. *bulgaricus*, *Lactococcus*, or enterococcae strains.

Table II
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 sp.	2
E. coli	4
Klebsiella sp.	5
Pseudomonas sp.	4
S. typhimurium	10
Shigella sp.	4
S. aureus	10
Lb. delbruekii subsp. *lactis* 643LMB	>15
Lb. delbruekii subsp. *lactis* 651 LMB	>15
Lb. delbruekii subsp. *lactis* 1441 LMB	>15
Lb. delbruekii subsp. *lactis* 642 LMB	>15
Lb. delbruekii subsp. *bulgaricus* 645 LMB	0
Enterococcus 1442 LMB	0
Enterococcus 1443 LMB	0
Lactococcus lactis subsp. *lactis* 1444 LMB	0

Effect of enzymes, temperature and pH on bacteriocin activity. The *S. thermophilus* CHCC 3534 bacteriocin activity was not modified by the action of either α-amylase or lipase, where maximal growth reductions of *S. typhimurium* were recorded (87% and 68% growth reduction respectively). The treatment with proteolytic enzymes resulted in the reduction of the bacteriocin activity to 50% or more as compared to the non-proteolytic enzymes (Table III). Exposure to 80°C and above resulted in a loss in bacteriocin activity against *S. aureus*, and an increase in the activity against the *S. typhimurium* (Fig. 3). The bacteriocin survived the autoclaving temperature for 15 minutes, but its activity against the *S. typhimurium* indicator strain was low. The bacteriocin was stored at –4°C for at least 2 months without any detectable loss of

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

Enzyme	Bacteriocin activity
Papain	35 ± 0.02
Pepsin	18 ± 0.03
Trypsin	24 ± 0.02
α-amylase	87 ± 0.03
Lipase	68 ± 0.01

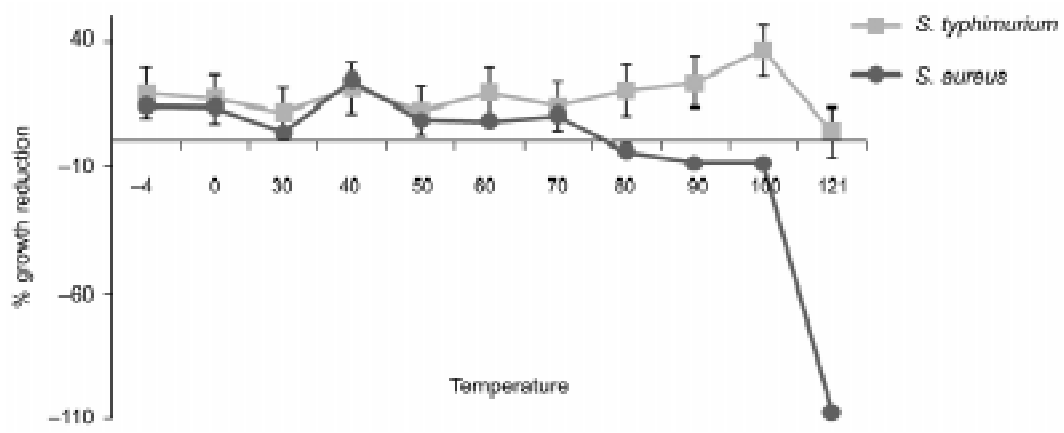

Fig. 3. Effect of heat treatment on the activity of *S. thermophilus* CHCC 3534 bacteriocin.
Activity is expressed as the % of growth reduction against (■) *S.typhimurium* and (●) *S. aureus*.

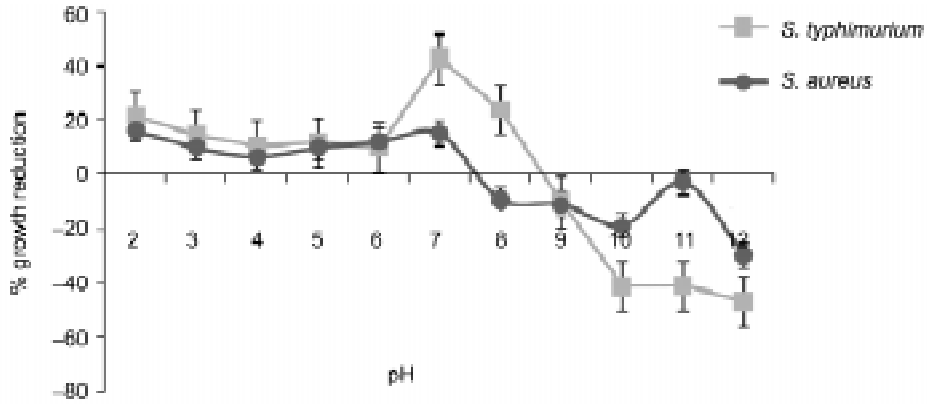

Fig. 4. Effect of pH on the activity of *S. thermophilus* CHCC 3534 bacteriocin.
Activity is expressed as the % of growth reduction against (■) *S.typhimurium* and (●) *S. aureus*

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. The 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 a half (24% growth reduction) at pH 8. The bacteriocin activity was lost at pH 9 and above (Fig. 4).

Determination of bacteriocin molecular weight. SDS-PAGE of the 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 a single protein band was detected (Fig. 5).

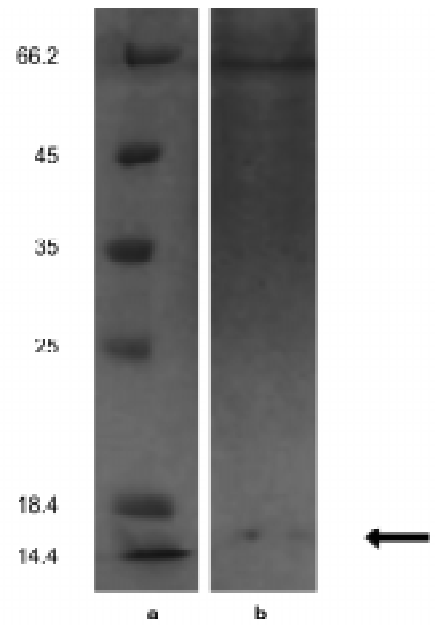

Fig. 5. SDS-polyacrylamide gel containing the molecular size markers stained with Coomassie Brilliant blue (lane a) and the partially purified bacteriocin of *S.thermophilus* CHCC 3534 from gel chromatography (lane b).The arrow indicates the position of the single peptide band. Sizes on the left are indicated in kDa.

Discussion

The survival of *S. thermophilus* CHCC 3534 *in vitro* conditions that mimic the physico-chemical events occurring in the gastrointestinal tract was studied. Incubation time chosen for acid and bile tolerance tests was 3 hours, simulating the residence time in the stomach (Olejnik *et al.*, 2005). pH 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 *Lb. plantarum* strains after 2, 4, 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 action of bile salts, the cells survived all bile treatments starting from a concentration of 0.15%. This behaviour suggests potential of this strain as a probiotic, since it survived a bile concentration (0.4% oxgall solution) equivalent to the physiological concentration in the duodenum (Brashears *et al.*, 2003). Reference strains used as adhesion controls displayed lower adhesion than that of *S. thermophilus* CHCC 3534 in spite of their documented probiotic properties (Khalil *et al*., 2007). 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 (Tallon *et al.*, 2007). *S. thermophilus* CHCC 3534 was significantly insensitive to the antibiotics employed regardless of their concentration or mode of action (D'Aimmo *et al.*, 2007). The susceptibility of tested strains 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). Crude *S. thermophilus* CHCC 3534 extracts contained a diffusible antimicrobial compound with a broad activity 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 *Lb. delbruekii* subsp. *bulgaricus* indicator strain supports the hypothesis that it may be used in thermophilic starter for hard cheese making since it is not active against thermophilic lactobacilli (Mathot *et al.*, 2003). The lack of inactivation of the bacteriocin by treatment with α-amylase and lipase enzymes suggests that the activity was not dependent on the presence of either a carbohydrate or lipid moiety (Maurad and Meriem, 2008). Resistance to á-amylase was contradictory to several reports showing the inactivation of thermophilins by α-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 with the characteristics of many bacteriocins produced by LAB (Kabuki *et al.*, 2007). Heat stability of the bacteriocin was maintained for 15 minutes at temperatures ranging from –4 to 100°C, with a decrease in the activity at 121°C, which reinforces our suggestion that this bacteriocin may be possibly used 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. Reported molecular sizes of *S. thermophilus* bacteriocins was 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 kDa and 4.5 kDa respectively (Gilbreth and Somkuti, 2005). In conclusion, the results obtained in the present work provide strong evidence for the probiotic potential of the *S. thermophilus* CHCC 3534 strain. The use of a pure probiotic starter culture such as the strain under investigation endowed with appropriate probiotic properties coupled with other desirable characteristics, may represent an alternative to the use of mixed cultures consisting of probiotic and starter strains.

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