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Probiotic Properties, Antioxidant Potential, Bile Salts Tolerance and Antibiotic Susceptibility Assessment of Streptococcus Thermophilus Isolates

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Abstract

Streptococcus thermophilus was considered one of the probiotic bacterial strains found in milk, cheese, and yogurt that provided health benefits to humans. Due to their probiotic potential, *S. thermophilus* can be very helpful in replacing chemical preservatives. This study came out to investigate the probiotic activity of different ten *S. thermophilus* isolates obtained from raw cow milk in eastern Morocco. The antimicrobial tests against multidrug-resistant bacteria (MDR): *Staphylococcus aureus*, and *Escherichia coli* were evaluated using the disc diffusion technique. In addition, the antioxidant potential was tested using the DPPH free radical assay. The bile salt tolerance was evaluated using M17 broth containing variable amounts of bile salt (0% as control; 0.1% and 0.3% as tests) at 37 °C for 3, 6 and 24 hours. Then cell counts were enumerated after 48 hours of incubation on M17 agar. Antibiotic susceptibility of *S. thermophilus* isolates against 20 antibiotics from 8 families (Rifamycins, Aminoglycosides, Phenicol, Beta-lactam, Glycopeptides, Clindamycin, Cotrimoxazole, and Cyclines) were tested following a 24-hour incubation period using the antibiotic discs method. The diameters of the inhibition zones results were then compared to the recommendations made by the Antibiogram Committee of the French Microbiology Society of 2021. The results showed remarkable *in vitro* inhibitory ability of the *S. thermophilus* isolates against the tested multidrug-resistant bacteria *S. aureus*, confirming their potentiality as antibacterial agents. The mixtures of NaOH, catalase and proteinase K showed no inhibition zones against all tested multidrug-resistant bacteria, suggesting the main antibacterial effect of all isolates based on acids, hydrogen peroxide, and/or proteins. For the antioxidant activity, the DPPH inhibition values varied between 18.97% and 44.44% for the bacterial isolates ST9 and ST5, respectively. Notably, *S. thermophilus* isolates were able to survive up to 6 h of 37°C incubation with a bile salt concentration of 0.1%, where ST5 was the most promising isolate in terms of antioxidant activity. All isolates of *S. thermophilus* exhibited sensibility to all tested antibiotics except for rifampicin and trimethoprim-sulfamethoxazole compared to the European Committee on Antimicrobial Susceptibility Testing.

Keywords

Streptococcus thermophilus, Antioxidants, Antibacterial activity, Bile salts, Antibiotic

Cover Page Footnote

The authors would like to thank both An-Najah National University in Nablus, Palestine (www.najah.edu) and Mohammed Premier University in Oujda, Morocco for using their labs.

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Probiotic Properties, Antioxidant Potential, Bile Salts Tolerance and Antibiotic Susceptibility Assessment of *Streptococcus Thermophilus* Isolates

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ABSTRACT

Streptococcus thermophilus was considered one of the probiotic bacterial strains found in milk, cheese, and yogurt that provided health benefits to humans. Due to their probiotic potential, *S. thermophilus* can be very helpful in replacing chemical preservatives. This study came out to investigate the probiotic activity of different ten *S. thermophilus* isolates obtained from raw cow milk in eastern Morocco. The antimicrobial tests against multidrug-resistant bacteria (MDR): *Staphylococcus aureus*, and *Escherichia coli* were evaluated using the disc diffusion technique. In addition, the antioxidant potential was tested using the DPPH free radical assay. The bile salt tolerance was evaluated using M17 broth containing variable amounts of bile salt (0% as control; 0.1% and 0.3% as tests) at 37 °C for 3, 6 and 24 hours. Then cell counts were enumerated after 48 hours of incubation on M17 agar. Antibiotic susceptibility of *S. thermophilus* isolates against 20 antibiotics from 8 families (Rifamycins, Aminoglycosides, Phenicol, Beta-lactam, Glycopeptides, Clindamycin, Cotrimoxazole, and Cyclines) were tested following a 24-hour incubation period using the antibiotic discs method. The diameters of the inhibition zones results were then compared to the recommendations made by the Antibiogram Committee of the French Microbiology Society of 2021. The results showed remarkable *in vitro* inhibitory ability of the *S. thermophilus* isolates against the tested multidrug-resistant bacteria *S. aureus*, confirming their potentiality as antibacterial agents. The mixtures of NaOH, catalase and proteinase K showed no inhibition zones against all tested multidrug-resistant bacteria, suggesting the main antibacterial effect of all isolates based on acids, hydrogen peroxide, and/or proteins. For the antioxidant activity, the DPPH inhibition values varied between 18.97% and 44.44% for the bacterial isolates ST9 and ST5, respectively. Notably, *S. thermophilus* isolates were able to survive up to 6 h of 37°C incubation with a bile salt concentration of 0.1%, where ST5 was the most promising isolate in terms of antioxidant activity. All isolates of *S. thermophilus* exhibited sensibility to all tested antibiotics except for rifampicin and trimethoprim-sulfamethoxazole compared to the European Committee on Antimicrobial Susceptibility Testing.

Keywords: *Streptococcus thermophilus*, Antioxidants, Antibacterial activity, Bile salts, Antibiotic.

INTRODUCTION

Raw milk contains many essential nutrients and is characterized by a rich and diverse lactic microflora [1]. Lactic Acid

Bacteria (LAB) are natural and considered as important microorganisms used in various fields of health and industrial food fermentation [2]. Their metabolites are generally safe [3,4] and have been used as

probiotics as well as food preservatives [5,6,7]. In fact, LABs were used in food fermentation due to their potential for the production of wide range of metabolites that are responsible for improving the organoleptic quality of food and health. In addition to that, Lactic Acid Bacteria can be used as protective agents due to their capability to synthesize of antimicrobial metabolites [8]. Besides, certain strains of LABs gained much attention due to their health-promoting properties, what are called “probiotics”.

Lactic Acid Bacteria were classified into 17 genera: *Lactobacillus*, *Enterococcus*, *Melissococcus*, *Streptococcus*, *Lactosphaera*, *Alloiococcus*, *Lactococcus*, *Leuconostoc*, *Aerococcus*, *Dolosigranulum*, *Globicatella*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus*, *Carnobacterium*, and *Weissella* [9,6].

Several studies have reported that *S. thermophilus* is considered a probiotic, providing health benefits when consumed by humans [10]. *S. thermophilus* (formerly known as *Streptococcus salivarius* subsp. *thermophilus*) is gram-positive, anaerobic, aero-tolerant, catalase-negative, homo-fermentative, able to grow at high temperatures 45 °C. It is one of probiotic bacterial strain that found in milk, cheese, and yogurt [11]. Although, *S. thermophilus* is a food species, it has contradictory interspecific interactions (commensal and opportunistic species) [12]. Moreover, it resists biological barriers, such as gastric juice and bile salts [13]. In the dairy industry, *S. thermophilus* is used alone or in combination with several Lactobacilli and mesophilic starters for cheese-making, and is widely applied with *Lactobacillus delbrueckii ssp. bulgaricus* as a yogurt starter.

Despite the evolution of modern technological means of food processing, more than 9000 people die from foodborne infections very year worldwide. Researchers and food manufacturers are increasingly oriented to use bacteria as a prevention tool instead of physical treatments (high pressures, ionizing rays, pasteurization, sterilization, freezing, refrigeration, etc.) and chemicals (nitrites, sulfites) [3]. In fact, *S. thermophilus* produces bacteriocins; the heterogeneous

group of peptides with a broad spectrum of activity against bacteria [14], and thus it can be used as a broad inhibitory spectrum against several pathogenic bacteria, such as *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *Escherichia coli*, *Yersinia pseudotuberculosis*, and *Clostridium tyrobutyricum* [15].

Lactic Acid Bacteria’s antimicrobial substances include lactic acid, acetic acid, organic acids, hydrogen peroxide, formic acid, reuterin caproic acid, carbon dioxide, reuterocycline diacetyl, nisin, pediocin, ethanol, and bacteriocins or related substances [16,17]. According to Beristain-Bauza et al. [4], current trends in active food packaging include searching for natural antimicrobial compounds to achieve user-friendly packaging and reduce human consumption of synthetic preservatives. Antimicrobial packaging is important to enhance the shelf-life of dairy products, poultry, fish, meat, and vegetables.

Lactic acid bacteria (LAB) have antioxidant effects since they have the potential to chelate ferrous ions and degrade nitrite and cholesterol [18,3]. Antioxidants are natural and non-natural chemical substances that slow down or prevent the oxidation of other chemical substances when in contact with them. They are defined as reducing agents (such as thiols and polyphenols) and chemicals involved in oxide-reducing reactions [19]. Nowadays, the need for natural compounds as alternatives to synthetic antioxidants [19,20] is highly demanded. The production of antioxidant compounds could be achieved by organic synthesis, biotechnology methods, or determination of antioxidant activity.

Bile salts, an essential part of the digestion process, contain antibacterial compounds that reduce the survival of bacteria by destroying their cell membranes. Bile salts are synthesized in the liver and stored in the gallbladder, causing oxidative damage to the bacterial DNA. They can disrupt calcium, chelate iron, denature proteins, and control the expression of eukaryotic genes involved in host defense and immunity [21]. They play a role in intestinal homeostasis by controlling the size and composition of the intestinal

microbiota. Bile salts that are secreted in the small intestine as “primary bile salts”, can be transformed into “secondary bile salts” by the commensal bacteria. Resistance to bile salts is, therefore, considered as an important parameter for selecting probiotic strains [22].

Certain bacterial strains have genes responsible for various functions, such as sugar metabolism, bacteriocin production, and antibiotic resistance. Scientists observed a correlation between antibiotic resistance and the presence of plasmids in certain strains of *S. thermophilus*. For that reason, strains that have been found to be sensitive to most antibiotics carry only a single or no plasmid [23], as the antibiotic resistance genes are mainly encoded.

Due to the probiotic potentiality of *S. thermophilus*, that can be very helpful in replacing chemical preservatives, this study came out to investigate the probiotic activity of several *S. thermophilus* strains against multidrug-resistant bacteria. Besides their antioxidant potential, bile salts tolerance as well as their antibiotic susceptibility, were evaluated.

Material and methods

Preparation of Bacterial strains

Thirty-seven strains were isolated from raw cow milk obtained from the village of Tafoughalt located in eastern Morocco based on their specific phenotypic and biochemical characteristics. Ten of these isolates were identified as *S. thermophilus* at the Laboratory of Improvement of Agricultural Production, Biotechnology and Environment (Department of Biology/Faculty of Sciences, Mohamed Premier University, Oujda, Morocco). Three multidrug-resistant (MDR) bacteria: *S. aureus* (ATCC 29213), *Escherichia coli* (ATCC 25922, and ATCC 35218), were thankfully provided by the Laboratory of Microbiology (Faculty of Medicine and Pharmacy, Oujda, Morocco) and used in the antimicrobial tests.

The *S. thermophilus* isolates were cultured in (M17) media at 30°C for 24 h, and their identification is carried out by Galerie API 20. In the meanwhile, the multidrug-resistant (MDR) bacteria were cultured on Mueller Hinton Agar (MHA) and incubated at 37°C for 24 h.

Screening of the Antimicrobial Activity

Antibacterial activity

In this test, the effect of the incubation time of *S. thermophilus* cultures (after 24 h, 48 h, 72 h, and a week) on the inhibition of multidrug-resistant (MDR) bacteria was evaluated, and their pH was measured.

Both bacterial suspension and cell-free supernatant (CFS) of *S. thermophilus* isolates were tested against *E. coli* (ATCC 25922 and ATCC 35218), as well as *S. aureus* (ATCC 29213) by the disc diffusion techniques. The cultures of multidrug-resistant (MDR) bacteria were adjusted to densities of 0.5 McFarland by the BD PhoenixSpec™ nephelometer and were spread on MHA by cotton swabs. Thereafter, all the supernatants of *S. thermophilus* were precipitated via centrifugation at 13000 ×g for 20 min at 4°C. The antibacterial potentiality of *S. thermophilus* strains was screened with the disc diffusion method by adding 40 mL of the supernatant to the disk. The Petri dishes were kept in the refrigerator at 4°C for 2 hours, before being incubated at 37 °C for 24 h. The results were classified based on the diameter of the inhibition zones [36]:

Sign	Inhibition zone
–	no inhibition zone
+	>6 to <10 mm,
++	>11 to <16 mm
+++	>16 mm.

The experiment was performed in triplicate.

Assessing antibacterial inhibitory compounds of *S. thermophilus*

The cultures of *S. thermophilus* were centrifuged at 13000 rpm for 10 min. In order to eliminate the antibacterial activity due to the acidity, the pH of the CFS was adjusted to 7.0 with NaOH. Each indicator bacteria previously prepared were inoculated on Mueller Hinton Agar. In order to ensure the protein nature of the antibacterial compound, the supernatants were treated with a proteolytic enzyme as 40 µl of proteinase K (1 mg/ml) was added to 1 ml of CFS and incubated for 2 h at 37°C [3]. The inhibitory

activity of the hydrogen peroxide was ruled out by the addition of the catalase enzyme. The catalase (1 mg/ml)-treated CFS were incubated at 25 °C for 30 min [29]. The multidrug-resistant bacterial culture (0.5 McFarland, prepared as stated in the above section) was swabbed on the Agar plates surface were inoculated. Therefore 40 µl of each treated CFS were placed into the disc. The Petri dishes were incubated at 37°C for 24h. All of the solutions were filter sterilized through a 0.22 µm-pore-size filter. The presence of antibacterial activity in the *S. thermophilus* (CFS) was evaluated by formation of inhibition zone around the disc. Inhibition zones formed in the medium were measured in millimeters (mm).

Antioxidant Activity

The antioxidant activity of the bacteria was assessed using the DPPH (2,2-diphenyl-1-picryl-hydrazyl) free radical scavenging assay. The ten isolates of *S. thermophilus* were cultured in M17 medium and incubated for 6 days at 37°C [37]. After centrifugation for 10 min at 4000 rpm at 4°C, the pellets and the supernatants were collected to evaluate their antioxidant activity separately. In this study, the ultrasonic crusher noise isolation chamber; which is designed to apply a cavitation effect in a liquid through ultrasonic waves, was used to disintegrate bacterial cells [38, 39, 40]. For that, the pellet containing the bacteria was resuspended in water and subjected to sonication (ultrasonic cell crusher noise isolating chamber) to burst the bacterial cells and release their intracellular medium. The sonication was performed at 40 kHz during 2 min (alternating 30s of sonication and 30s of pause), in an ice bath, at a temperature not exceeding 40°C to avoid excessive sample heating. The antioxidant activity of the intracellular medium was compared to the supernatant representing the extracellular medium. For this reason, a volume of 60 µl of the cell free supernatant from each bacterium was mixed with 2ml of an ethanolic solution of DPPH (1.02×10^{-4} M). The mixture was vortexed and incubated for 30 min in darkness. After centrifugation, the absorbance was measured on a spectrophotometer (vis-7220G) at 517 nm against a blank containing the culture medium instead of the cell free

supernatant. The DPPH inhibition was then calculated using the following formula:

$$(Abs_{DPPH} - Abs_{ST}/Abs_{DPPH}) * 100$$

where Abs_{DPPH} is the absorbance of DPPH and Abs_{ST} is the absorbance of the supernatant of bacteria. The test was performed in triplicate and the results were expressed in means \pm standard deviation.

Tolerance to Bile Salts

The tolerance of *S. thermophilus* to bile salts was made according to Hassanzadazar et al. [41]. For that, two free bile salts were used: cholate (the most abundant primary bile salt in human bile) and sodium deoxycholate (secondary bile salt from cholate). Bacterial strains were grown in M17 broth media at 37 °C overnight, then the bacterial pellet was inoculated in M17 broth containing different concentrations of bile salt (0.1% and 0.3%), while the control was devoid of bile salt. The cultures were incubated at 37 °C for 3, 6 and 24 hours. The cell count (CFU/mL) of each culture was enumerated on M17 agar and incubated at 37 °C for 48 h. The optical density was measured at 540 nm using a UV spectrophotometer. All experiments were carried out in triplicates.

Antibiotic susceptibility test

This test of sensibility toward antibiotics was performed according to the method described by Andrianet al. [24] and Hamdaoui et al. [42], with some modifications. The isolates of *S. thermophilus* were examined against 20 antibiotics belonging to 8 families (Rifamycins, Aminoglycosides, Phenicol, Beta-lactam, Glycopeptides, Clindamycin, Cotrimoxazole, and Cyclines). The cultures of *S. thermophilus* were inoculated in M17 broth media and incubated overnight at 37°C. The turbidity was adjusted to 0.5 McFarland standard. The suspensions were swabbed on M17 plated media using sterile cotton swabs. After drying at room temperature for 15 min, the antibiotics discs were placed on the surface of the agar plates and were incubated at 37°C for 24 h.

The antimicrobial discs employed the following antibiotics: rifampicin, amoxicillin, ampicillin, vancomycin, oxacillin, cefoxitin, tiamulin, erythromycin, kanamycin,

neomycin, streptomycin, gentamicin, chloramphenicol, lincomycin, clindamycin, tetracycline, sulfonamides, nalidixic acid, ofloxacin and ciprofloxacin.

After 24 hours of incubation, the results were read by measuring the diameters of inhibition zones around the antibiotics discs, and compared to recommendation standards of Antibioqram Committee of the French Microbiology Society of 2021.

Statistical analysis

An analysis of variance (ANOVA) followed by a Post-hoc Tukey test were carried out to compare the means of some parameters among the ten tested bacterial isolates.

RESULTS

Antibacterial activity of *S. thermophilus*

Ten of *S. thermophilus* isolates were identified and tested for their antibacterial activity against multidrug-resistant bacteria (*E. coli* ATCC 25922, *E. coli* ATCC 35218, and *S. aureus* ATCC29213). The antibacterial activity of *S. thermophilus* isolates is presented in Table 1.

Table (1): Antibacterial effect of cell-free supernatants and bacterial suspension of *Streptococcus thermophilus* against multi drug resistant bacteria, with different incubation periods.

MDR bacteria	Supernatants of <i>S. thermophilus</i> (in mm)			
	24h	48h	72h	168h (1week)
<i>E. coli</i> (ATCC 25922)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8 – 11
<i>E. coli</i> (ATCC 35218)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8 – 11
<i>S. aureus</i> (ATCC29213)	8–10	9- 12	11 - 13	14 – 34
Bacterial suspension of <i>S. thermophilus</i> (in mm)				
<i>E. coli</i> (ATCC 25922)	10– 15	9– 12	7 – 8	0.00 ± 0.00
<i>E. coli</i> (ATCC 35218)	10– 15	9 – 12	7 – 8	0.00 ± 0.00
<i>S. aureus</i> (ATCC 29213)	26- 30	26 - 29	19 – 25	15 – 20

Effect of Enzymes on the Antibacterial Activity

In this study, untreated CFS, neutralized CFS, and both catalase and proteinase K-treated CFS were tested for antibacterial activity using the disk diffusion method. The mixtures of NaOH, catalase and proteinase K showed no inhibition zones against all tested

All cell-free supernatants (CFS) of *S. thermophilus* isolates showed no antibacterial effect against *E. coli* ATCC 25922 and *E. coli* ATCC 35218 after 24h, 48h, and 72h of incubation, while they showed low activity after one week (8-11mm). In contrary, the inhibition zone of the supernatants of *S. thermophilus* against *S. aureus* (ATCC29213) ranged between 8 to 10 mm after 24h of incubation, and reached 14 to 34mm after one week.

The suspensions of *S. thermophilus* against *E. coli* ATCC 25922 and *E. coli* ATCC 35218 showed medium antibacterial activity after 24h (10 to15mm). This later kept decreasing over the incubation time (7 to 8 mm after 72h, and no inhibition after one week).

The suspension of *S. thermophilus* against *S. aureus* showed the highest antibacterial activity (26-30mm) after 24h. Nevertheless, this important activity decreased gradually resulting on an inhibition zone of 15- 20 mm after one week.

multidrug-resistant bacteria. This finding suggests that the main antibacterial effect of all isolates is dependent on acids, hydrogen peroxide and proteins. The reduction of the inhibition zone by neutralized CFS indicated that the acidic condition of the CFS, due to the presence of organic acids, might have contributed to the antibacterial activity of the isolates (Table 2).

LAB isolates	Viable counts (CFU/mL)					
	3h		6h		24h	
	%Bile salts (w/v)					
	0.1%	0.3%	0.1%	0.3%	0.1%	0.3%
ST 4	29± 0.3x10 ²	<30	86±3 x10	<30	0.00±0.00	0.00±0.00
ST 5	26± 0.8x10 ²	<30	74±1 x10	<30	0.00±0.00	0.00±0.00
ST 6	29± 0.2x10 ²	<30	68±5 x10	<30	0.00±0.00	0.00±0.00
ST 7	28± 0.5x10 ²	<30	80±2 x10	<30	0.00±0.00	0.00±0.00
ST 8	24± 0.9x10 ²	<30	57± 8 x10	<30	0.00±0.00	0.00±0.00
ST 9	29± 0.5x10 ²	<30	59± 7 x10	<30	0.00±0.00	0.00±0.00
ST 10	28± 0.5x10 ²	<30	84±2 x10	<30	0.00±0.00	0.00±0.00

Antibiotic susceptibility test

Ten *S. thermophilus* isolates were assayed for susceptibility to 19 antibiotics. The results obtained (Table 5) were compared to the standards proposed by the European committee on antimicrobial susceptibility

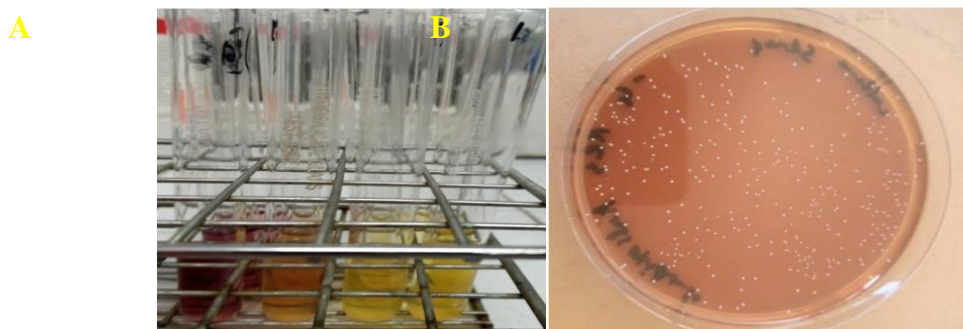
testing. All isolates tested were sensitive to all antibiotics except rifampicin and trimethoprim-sulfamethoxazole. These results are in disagreement with those reported by Yerlikaya et al. [6] exempted for trimethoprim-sulfamethoxazole.

Table (5): Antibiotic susceptibility of *S. thermophilus* isolates against antibiotics. S means susceptible, R means resistant.

		Critical diameter D-d (mm)		Inhibition diameter (mm)	S/R	Mode of action
		S>	R<			
β-lactam	Penicillin G	18	12	25	S	
	Ampicilline	21	15	30- 35	S	Cell Wall Synthesis Inhibition
	Amoxicilline	-	-	33	S	Cell Wall Synthesis Inhibition
	Cefotaxime	23	23	31	S	
	Ceftriaxone	27	27	35	S	
	Cefepime	25	25	34	S	
	Imipenem	-	-	43	S	
	Ertapenem	-	-	36	S	
Glycopeptides	Meropenem	-	-	41	S	
	Teicoplanine	16	16	21	S	
	Vancomycine 5	15	15	18	S	Cell Wall Synthesis Inhibition
Clindamycine	Clindamycine	19	19	28/33	S	
Cotrimoxazole	Sulfamethoxazole-Trimethoprim	19	16	0	R	
Aminosides	Gentamicine 30			22	S	ProteinSynthesis Inhibition
	Gentamicine 500	17	17	30	S	
	Streptomycin	19	19	26	S	
Rifamycines	Rifampicin	22	17	15	R	RNA Polymerase Inhibition
Cyclines	Tetracycline	23	21	35	S	
Phenicol	Chloramphenicol	23	23	32	S	



Supplementary (1): A: Zone of inhibition of an antibiotic. B: Antibacterial activity of cell-free supernatants of *S. thermophilus* isolates against *S. aureus* after 1-week incubation. C: Antibacterial activity of bacterial suspension of *S. thermophilus* isolates against *S. aureus* after 24hr incubation.



Supplementary (2): A: Antioxidant activity. B: enumeration of *S. thermophilus* colonies during bile salt-resistance test.

DISCUSSION

S. thermophilus is one of the predominant lactic acid bacteria, also classified among the genus of probiotic bacteria by their secretion of antimicrobial molecules and is an important component of many dairy starter cultures, specifically fermented milks [24, 25, 43].

The antibacterial activity of LAB has many reasons, which include: nutrient competition, bacteriocins, hydrogen peroxide production and pH lowering by the production of organic acids [11,26]. Today, researchers are interested in the search for safe, natural and accessible antimicrobials [27, 44, 45].

S. thermophilus isolates produced antibacterial substances that were degraded by distinct enzyme solutions, mainly by proteinase K, Catalase and NaOH. According to Aslam et al. [14], the bacteriocin activity was completely inhibited after treatment with proteinase K. *S. thermophilus* incubated for 24 to 72 h had the lowest activity against *E. coli* (8 ± 1.4 mm), all isolates of *S. thermophilus* incubated after 1 week had high effect against *E. coli* (8-11mm), and *S. aureus* ATCC 29213(14-34mm) (Table1). This activity may be due to the production of several antibacterial compounds produced during the

bacterial growth and excreted to the extracellular medium. Furthermore, the supernatant treated with mixed proteinase K, Catalase, and NaOH entirely lost its antibacterial activity.

NaOH, catalase, and proteinase K were added to rule out the inhibitory activity of the lactic acid, hydrogen peroxide H_2O_2 , and proteins, respectively. Thus, it is observed that the antioxidant activity in the cellular supernatant increases with the incubation time, unlike the bacterial suspension. Akpinar et al. [28] reported that two isolates among sixteen isolates of *S. thermophilus* had antibacterial activity against *S. aureus* and *E. coli*. However, the study conducted by Andrian et al. [24] showed that *S. thermophilus* had the lowest antibacterial activity against *E. coli* and *S. aureus*. The inhibitory ability of our isolates of *S. thermophilus* in vitro against the tested multidrug-resistant bacteria, such as *S. aureus* ATCC29213, *E. coli* ATCC 25922, and *E. coli* ATCC 35218, confirms the production of antibacterial agents and may be predictive of good probiotics. The result showed that all untreated CFS of *S. thermophilus* had an antibacterial effect against the multidrug-resistant bacteria, and the sizes of the

inhibition zones varied as summarized in Table 1. All isolated bacterial suspensions of *S. thermophilus* did not have an inhibitory effect on *E. coli* and *S. aureus* after 1 week, which means it has nutrient competition. Cell-free supernatant of *S. thermophilus* could be considered as a natural antibacterial with good acceptance.

The second part of this study was about the antibacterial properties of *S. thermophilus*. Enzyme treatment using catalase and Proteinase K have a significant effect on the antibacterial activity of all *S. thermophilus* isolates against all multidrug-resistant bacteria. This means that all isolates of *S. thermophilus* produced antibacterial substances, such as hydrogen peroxide and proteins. All isolates of *S. thermophilus* decreased the antibacterial effect of the inhibited bacteria after either NaOH, proteinase K or catalase treatment was administered. The mixtures of enzymes of NaOH, catalase, and proteinase K showed no inhibition zones against all tested multidrug-resistant bacteria (Table 2). This finding suggested that the main antibacterial effect of all isolates depend on acids, hydrogen peroxide and proteins. In this study, the ability of catalase-treated CFS with an amount of hydrogen peroxide to maintain decreased inhibition against multidrug-resistant bacteria confirmed that hydrogen peroxide contributes to the antibacterial activity of the isolates. The reduction in the amount of hydrogen peroxide in CFS caused by the catalase enzyme will break down the hydrogen peroxide compound into water and oxygen [29]. The ability of Proteinase K-treated CFS to maintain reduced inhibition compared to untreated CFS showed that the protein compound might be responsible for the antimicrobial activity of CFS of the *S. thermophilus*. Proteinase K will inactivate the proteinaceous compound when added to the CFS.

The high proportion of antioxidants is the reason for the various health benefits imparted [30]. DPPH-based assays are frequently used for quantifying the antioxidant activity in plant and bacterial extracts as they are quick, easy, and low-cost approach for assessing antioxidant capacity [46]. The DPPH inhibition for the ten studied bacterial isolates were varied from 18.97% to 44.44% (Table

3); suggesting that this considerable antioxidant activity of these bacterial isolates was by secreting antioxidant molecules in the culture medium, that is in perfect agreement with several previous studies.

It is noteworthy that the antioxidant power of bacteria is closely related to bacterial load. Selecting bacteria having a high antioxidant activity with a low bacterial load is advantageous. For example, although the ST3 bacterium has a relatively good activity (36.6%), its bacterial load is too high (300×10^6 CFU/mL), so this strain does not show a real important activity. According to the obtained results, the most promising bacteria in terms of antioxidant activity is the strain ST5 (44% against 9.5×10^6 CFU/ml), followed by ST2 (35.2% against 10.3×10^6 CFU/ml) and ST10 (32.49% against 10.1×10^6 CFU/ml).

In the literature, numerous studies have assessed the antioxidant power of several plants and bacterial isolates from different substrates [47, 48, 49]. A recent study evaluating the DPPH antioxidant potential of several *Lactobacillus* isolates isolated from traditional fermenting green olives, found values ranging from 43% to 52.99% [31]. Another study concerning lactic acid bacterial isolates in fermented skim milk reported values of DPPH antioxidant activity fluctuating between 14.7 and 50.8% [32], which are similar to our finding. In contrast, Afify and his colleagues have found an important DPPH antioxidant activity reaching a value of 97.75% for the cell free extract of *Propionibacterium freudenreichii* ssp. *Shermanii* (ATCC 1907) [33], which is higher according to our results.

The LAB resistant to bile salts may inspire novel therapeutic strategies for gastrointestinal and hepatobiliary diseases involving microbiome alteration, as well as novel schemes against bacterial infections. Bile salts play a vital role in intestinal homeostasis by controlling the composition and size of the intestinal microbiota [22]. Bile salts have been reported as the biggest obstacle to the survival of LAB in the gastrointestinal tract of the host [34].

In this study, all isolates were able to resist during 6h to the bile salt at two concentrations (Table 4), these findings can be

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Data availability

Adequate and clear descriptions of the applied materials and tools are provided in the materials and method section of manuscript. In addition, the obtained data is clearly justified by mentioning the figures and tables in the manuscript.

Declarations Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

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