Palestinian Medical and Pharmaceutical Journal (PMPJ). 2024; 9(3): 347-360

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

Nora Hamdaoui[1,2,], Ali Azghar[3], Chaymae Benkirane[1], Haytham Bouaamali[1], Mouncif Mohamed[2], Douaae Ou-yahia[4], Bouchra El Guerrouj[4], Abdeslam Asehraou[5], Adil Maleb[3], Belkheir Hammouti[6], Raed Alkowni[7,*], Shehdeh Jodeh[8] & Meziane Mustapha[1]*

[1]Laboratory of Improvement of Agricultural Production, Biotechnology and Environment, Department of Biology, Faculty of Sciences, Université Mohamed Premier, Oujda 60000, Morocco. [2]Process Engineering and Food Technologies Department, Institute of Agronomy and Veterinary medicine (IAV-Hassan II) BP 6202 Rabat, Morocco. [3]Laboratory of Microbiology, Mohammed VI University Hospital, Faculty of Medicine and Pharmacy, Mohammed First University, Oujda, Morocco. [4]Oriental Center for Water and Environmental Sciences and Technologies, Mohammed First University, Oujda 60000, Morocco. [5]Laboratory of Bioresources, Biotechnologies, Ethnopharmacology, and Health, Faculty of Sciences, University Mohamed Premier, Oujda, Morocco. [6]University of Euro-Mediterranean of Fez (UEMF), BP. 15, 30070 Fez, Morocco. [7]Department of Biology and Biotechnology, Faculty of Science, An-Najah National University, Nablus, P400, Palestine. [8]Department of Chemistry, Faculty of Science, An-Najah National University, P.O. Box 7, P400 Nablus, Palestine

*Corresponding authors: hamdaoui.nora@gmail.com; ralkowni@najah.edu

Received: (29/10/2023), Accepted: (26/11/2023), Published: (1/9/2024).
DOI: https://doi.org/10.59049/2790-0231.1223

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, Aminosides, Phenicols, 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. thermophiles*. 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), where 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 (1.02x 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 ± 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, Aminosides, Phenicols, 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 Antibiogram 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.

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.

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 *S. thermophilus (in mm)*			
	24h	**48h**	**72h**	**168h (1week)**
E. coli (ATCC 25922)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8 – 11
E. coli (ATCC 35218)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	8 – 11
S.aureus (ATCC29213)	8–10	9- 12	11 - 13	14 – 34
Bacterial suspension of *S. thermophilus (in mm)*				
E. coli (ATCC 25922)	10– 15	9– 12	7 – 8	0.00 ± 0.00
E. coli (ATCC 35218)	10– 15	9 – 12	7 – 8	0.00 ± 0.00
S. aureus (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 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).

Table (2): The inhibitory diameters recorded on cell free supernatants (CFS), neutralized CFS and enzyme (catalase and Proteinase k) against multi-resistant bacteria.

	Antibacterial activity (Inhibition zone, mm)											
	Neutralized (CFS)			Proteinase k			Catalase			Mix between (NaOH, Proteinase k, Catalase)		
MDR bacteria (ATCC)	*E. coli* 25922	*E. coli* 35218	*S. aureus* 29213	*E. coli* 25922	*E. coli* 35218	*S. aureus* 29213	*E. coli* 25922	*E. coli* 35218	*S. aureus* 29213	*E. coli* 25922	*E. coli* 35218	*S. aureus* 29213
All cell free supernatant of *S. thermo-philus*	6±0.6	6±0.7	9±0.5	6±0.0	6±0.3	8±0.5	8±0.7	7±0.3	10±1	NI	NI	NI

NI : No inhibition

Antioxidant Activity

The results of the DPPH scavenging activity of the ten studied bacterial isolates (expressed as inhibition percentage) and their bacterial load (expressed as CFU/mL) were shown in Table 3.

The statistical analysis showed that ST5 has the highest antioxidant value reaching 44.44%, followed by ST3 and ST2. On contrary, the ST9 bacterium presented the weakest antioxidant activity, with a value of 18.97%. According to the results, we can observe that the antioxidants present in the extracellular of *S. thermophilus* can neutralize the free radicals by donating the required number of electrons to stabilize them.

Table (3): DPPH scavenging activity of the ten studied *S. thermophilus* isolates (expressed as inhibition percentage) and their bacterial load (expressed as CFU/ml).

Bacterial isolates	DPPH inhibition (%)	bacterial load (CFU/mL)
ST1	30.58 ± 1.57 ^{b}	31 x 10^6
ST2	35.20 ± 3.22 ^{ab}	10.3 x 10^6
ST3	36.60 ± 1.57 ^{ab}	300 x 10^6
ST4	30.75 ± 0.47 ^{b}	60 x 10^6
ST5	44.44 ± 5.93 ^{a}	9.5 x 10^6
ST6	29.65 ± 6.48 ^{b}	38 x 10^6
ST7	30.84 ± 0.55 ^{b}	42 x 10^6
ST8	29.91 ± 0.55 ^{b}	11.2 x 10^6
ST9	18.97 ± 3.60 ^{c}	48 x 10^6
ST10	32.49 ± 0.34 ^{b}	10.1 x 10^6

ST: *S. thermophilus*

$^{(a,b,c)}$ Referred to statically differences ($p< 0.05$) between means.

Tolerance to Bile Salts

Table 4 shows the results of the resistance to bile salts. No survival was observed after 24h in two concentrations of bile salts. The notable remark was the ability of the *S. thermophilus* isolates to survive for 6 h of incubation at 37 °C with a concentration of 0.1% of bile salt.

Table (4): Effect of bile salts concentrations on the viability of twenty LAB isolates.

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 1	25± 0.9x10^2	<30	75±6 x10	<30	0.00±0.00	0.00±0.00
ST 2	27± 0.7x10^2	<30	112±5 x10	<30	0.00±0.00	0.00±0.00
ST 3	30± 0.2x10^2	<30	95±7 x10	<30	0.00±0.00	0.00±0.00

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

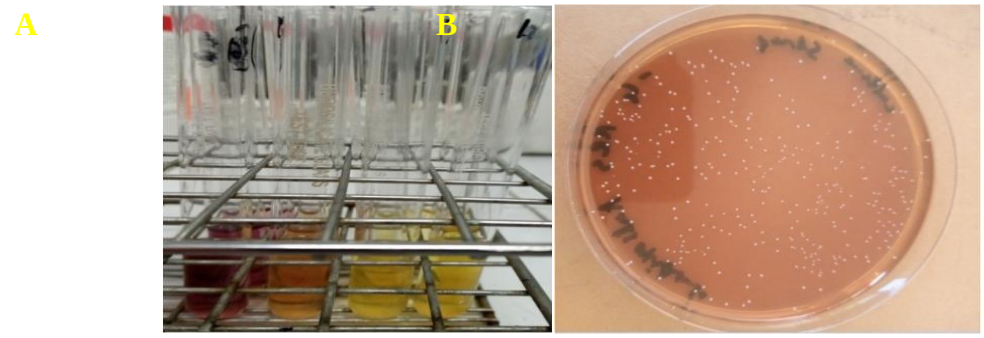
Antibiotic susceptibility test

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	
	Meropenem	-	-	41	S	
Glycopeptides	Teicoplanine	16	16	21	S	
	Vancomycine 5	15	15	18	S	Cell Wall Synthesis Inhibition
Clindamycine	Clindamycine	19	19	28/33	S	
Cotrimoxazole	Sulfamethoxazole-Trimethoprime	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	
Phenicols	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. thermophiles 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 ±1.4mm), 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 Table1. 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 10^6CFU/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 9.5x10^6CFU/ml), followed by ST2 (35.2% against 10.3x10^6CFU/ml) and ST10 (32.49% against 10.1x10^6CFU/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 de 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 freudenreichiissp. 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

explained by the fact that bacterial species adapted to the mammalian gut are able to endure the antibacterial activities of bile salts by multiple physiological adjustments that include remodeling of the cell envelope and activation of efflux systems and stress responses [22].Thus, the result of the optic density showed viability and proliferation in two concentrations 0.1% 0.3%for all the incubation periods. Furthermore, these isolates showed the ability to survive and grow in bile salt. These characteristics are necessary for probiotic bacteria to grow and survive in a gastric condition. The present study showed that *S. thermophilus* have the capacity for resistance to bile salt for6 hours in concentration at 0.1%.

The antibiotic-resistance genes present in LAB have been known to spread between different LAB species via horizontal gene transfer [10].

Aslim and Beyatli [23] carried out a study on antibiotic resistance and plasmid DNA content of *S. thermophilus*, and showed that most of the plasmid-containing isolates were resistant to antibiotics, but has also shown sensitivity to some antibiotics too. Thus, isolates lacking plasmid DNA were susceptible to most antibiotics, yet a strain was found lacking plasmid DNA while being resistant to some of the antibiotics. Many species of lactic acid bacteria include extra-genomic DNA in the form of plasmids. Some of these plasmids have important characteristics such as drug resistance, metabolic functions, or phage resistance. Resistance to different antibiotics may be coded by chromosomal genes, and different plasmids. In our study, it is clear that all isolates of *S. thermophilus* do not produce ß-lactamases because penicillin's are inactivated by ß-lactamases (penicillinases) produced by many lactic acid bacteria. The enzyme is coded by chromosomal or plasmid genes. Table 5 presented the antibiotic resistance of *S. thermophilus* isolates. All isolates were resistant to Cotrimoxazole (trimethoprim-sulfamethoxazole) and rifampicin. Antibiotic sensibility revealed an overall similar behavior between all isolates of *S. thermophiles* (Table 5). In this study, all isolates of *S. thermophilus* were susceptible to penicillin G, which is in total accordance with what have been recorded by Zhou et al. [35].

Akpinar et al. [28] reported that most of the *S. thermophilus* isolates isolated from homemade yogurt samples were not found to be resistant towards all the tested antibiotics. According to Aslim and Beyatli[23], the resistance of *S. thermophilus* to antibiotics was not coded by plasmid DNAs and the genes of resistance were in the chromosomal DNA part.

CONCLUSION

Ten *S. thermophilus* isolates from raw cow milk were studied for their antibiotic susceptibilities, bile salt resistance, and antibacterial and antioxidant activities. According to the results of the antibacterial activity test, *S. thermophilus* isolates had higher potentiality activity against *S. aureus* than they did against *E. coli*. Every isolate that was tested had the ability to produce antibacterial substances like proteins, acids, and hydrogen peroxide in addition to having antioxidant properties. *S. thermophilus* isolates (ST5), was found to have a high antioxidant activity. The ST2 isolate was revealed to have strong resistance to bile salt after six hours. Tests to determine the isolates' susceptibility to 20 different antibiotics revealed that they were resistant to rifampicin and trimethoprim-sulfamethoxazole. Due to these findings *S. thermophilus* bacteria could be advisable as an excellent antibacterials alternative that can be used as supplementary food and Nutritional enhancers.

ACKNOWLEDGMENTS

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

Author contributions

"N.H. and A.A. did the formal analysis. C.B. and H.B. made an investigation. M.M. did the methodology. D.O. and B.H. did resources. B.E.G. and M.M. did supervision. A.A. and A.M. validation. M.M. wrote the original draft. S.J. and R.A. did the writing, review, and editing. All authors read and approved the final manuscript."

FUNDING

This research received no funding from any association

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

Not applicable.

Competing interests

The authors declare that they have no competing interests.

REFERENCE

1] Perin LM, Nero LA. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant Lactococcuslactis. BMC Microbiol. 2014; 14, 36 (2014). https://doi.org/10.1186/1471-2180-14-36

2] Mokoena M P. Lactic acid bacteria and their bacteriocins: classification, biosynthesis and applications against uropathogens: a mini-review. Molecules. 2017; 22(8), 1255. https://doi.org/10.3390/molecules22081255

3] Ren D, Zhu J, Gong S, Liu H, Yu H. Antimicrobial characteristics of lactic acid bacteria isolated from homemade fermented foods. BioMed research international, 2018; https://doi.org/10.1155/2018/5416725

4] Beristain-Bauza S C, Mani-López E, Palou E, López-Malo A. Antimicrobial activity and physical properties of protein films added with cell-free supernatant of Lactobacillus rhamnosus. Food Control. 2016; 62: 44-51. https://doi.org/10.1016/j.foodcont.2015.10.007.

5] Sari M, Suryanto D, Yurnaliza. Antimicrobial activity of lactic acid bacteria isolated from bekasam against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922, and salmonella sp. IOP Conference Series: Earth and Environmental Science. 2018;130:012011. doi:https://doi.org/10.1088/1755-1315/130/1/012011

6] Yerlikaya O, Saygili D, Akpinar A. Evaluation of antimicrobial activity and antibiotic susceptibility profiles of Lactobacillus delbrueckii subsp. bulgaricus and Streptococcus thermophilus strains isolated from commercial yoghurt starter cultures. Food Science and Technology. 2021;41(2):418-425. doi:https://doi.org/10.1590/fst.03920

7] Feng T, Wang J. Oxidative stress tolerance and antioxidant capacity of lactic acid bacteria as probiotic: a systematic review. Gut Microbes. 2020; 9;12(1):1801944. doi: 10.1080/19490976.2020.1801944. PMID: 32795116; PMCID: PMC7524341.

8] Ajao O, Banwo K, Ogunremi O, Sanni A. Antimicrobial properties and probiotic potentials of lactic acid bacteria isolated from raw beef in Ibadan, Nigeria. Journal of Microbiology, Biotechnology and Food Sciences. 2021; 770-773. DOI:10.15414/jmbfs.2018.8.2.770-773.

9] Crowley S, Mahony J, van Sinderen D. Current perspectives on antifungal lactic acid bacteria as natural bio-preservatives.Trends in Food Science & Technology. 2013; 33(2), 93-109. http://dx.doi. org/10.1016/j.tifs.2013.07.004.

10] Li Y, Li L, Kromann S, Chen M, Shi L, Meng H. Antibiotic resistance of Lactobacillus spp. and Streptococcus thermophilus isolated from Chinese fermented milk products. Foodborne Pathogens and Disease. 2019; 16(3), 221-228.DOI: 10.1089/fpd.2018.2516.

11] Ohaegbu C G, Ngene A C, Asuquo U I, Coulthard O D, Nwachukwu E. Characterization and antimicrobial activities of lactic acid bacteria isolated from selected Nigerian traditional

fermented foods. African Journal of Biotechnology. 2022; 21(5), 218-236.DOI: 10.5897/AJB2021.17450.

12] Martinović A, Cocuzzi R, Arioli S, Mora D. Streptococcus thermophilus: To Survive, or Not to Survive the Gastrointestinal Tract, That Is the Question! Nutrients. 2020; Jul 22;12(8):2175. doi: 10.3390/nu12082175. PMID: 32708008; PMCID: PMC7468695

13] Iyer R, Tomar SK, Uma Maheswari T, Singh R. Streptococcus thermophilus strains: Multifunctional lactic acid bacteria. Int. Dairy J. 2010; 20 (3):133-141, 10.1016/j.idairyj.2009.10.005

14] Aslam M, Shahid M, Rehman FU, Naveed NH, Batool AI, Sharif S, Asia A. Purification and characterization of bacteriocin isolated from Streptococcus thermophilus. African Journal of Microbiology Research. 2011; 5(18):2642–2648. doi: 10.5897/AJMR11.225.

15] Mathot AG, Beliard E, Thuault D. Streptococcus thermophilus580 produces a bacteriocin potentially suitable for inhibition of Clostridium tyrobutyricumin hard cheese. J. Dairy Sci. 2003; 86: 3068-3074.

16] Hamdaoui N, Rokni Y, Mouncif M, Meziane M. Role of Lactic Acid Bacteria in Food and Human Health: A Review İstanbul International Modern Scientific Research Congress –III, May 06-08, Istanbul, Turkey. 2022a; 31.05.2022a. https://www.istanbulkongresi.org/_files/ugd/614b1f_3897e93dbe5e44a9b6b09034db94b9fc.pdf

17] İncili G K, Karatepe P, Akgöl M, Güngören A, Koluman A, İlhak O İ, Hayaloğlu A A. Characterization of lactic acid bacteria postbiotics, evaluation in-vitro antibacterial effect, microbial and chemical quality on chicken drumsticks. Food Microbiology. 2022; 104, 104001.https://doi.org/10.1016/j.fm.2022.104001

18] Kim S H, Kang K H, Kim S H, Lee S, Lee S H, Ha E S., Sung N J, Kim J G, Chung M J. Lactic acid bacteria directly degrade N-nitrosodimethylamine and increase the nitrite-scavenging ability in kimchi. Food Control. 2017; 71, 101-109. https://doi.org/10.1016/j.foodcont.2016.06.039.

19] Santos-Sánchez, N.F., Salas-Coronado, R., Villanueva-Cañongo, C., & Hernández-Carlos, B. (2019). Antioxidant Compounds and Their Antioxidant Mechanism. *Antioxidants*. 10, 1-29. DOI:http://dx.doi.org/10.5772/intechopen.85270

20] Chourasia R, Kumari R, Singh S P, Sahoo D, Rai A K. Characterization of native lactic acid bacteria from traditionally fermented chhurpi of Sikkim Himalayan region for the production of chhurpi cheese with enhanced antioxidant effect. LWT. 2022; 154, 112801. https://doi.org/10.1016/j.lwt.2021.112801

21] Succi M, Tremonte P, Reale A, et al. Bile salt and acid tolerance ofLactobacillusrhamnosusstrains isolated from Parmigiano Reggiano cheese. FEMS Microbiology Letters. 2005;244(1):129-137. doi:https://doi.org/10.1016/j.femsle.2005.01.037

22] Urdaneta V, Casadesús J. Interactions between Bacteria and Bile Salts in the Gastrointestinal and Hepatobiliary Tracts. Frontiers in Medicine. 2017;4. doi:https://doi.org/10.3389/fmed.2017.00163

23] Aslim B, Beyatli Y. Antibiotic Resistance and Plasmid DNA Contents of Streptococcus thermophilus Strains Isolated from Turkish Yogurts," Turkish Journal of Veterinary & Animal Sciences. 2004; 28: (2), https://journals.tubitak.gov.tr/veterinary/vol28/ iss2/2

24] Andrian D, Rizkinata D, Susanto T S R, Lucy J, Jan T T. Isolation of Streptococcus thermophilus and Lactobacillus delbrueckii as starter culture candidate originated from Indonesian cow's milk. Microbiology and Biotechnology Letters. 2018; 46(3), 201-209. https://doi.org/10.4014/mbl.1805.05011

25] Li K, Cui H, Duan Z. Complete Genome Sequence of Streptococcus thermophilus VHProbi R08, Isolated from Fermented Sour Porridge. Microbiology Resource Announcements. 2023; e01196-22. DOI: https://doi.org/10.1128/mra.01196-22

26] N'tcha C, Sina H, Kayodé A P P, Gbenou J D, Baba-Moussa L. Antimicrobial activity and chemical composition of "Kpètè-Kpètè": a starter of Benin traditional beer Tchoukoutou. BioMed Research International, 2017; https://doi.org/10.1155/2017/6582038

27] DehghaniChampiri I, Bamzadeh Z, Rahimi E, RouhiLIsolation and Identification of Lactobacillus brevis from Cottage Cheese of Bazoft City, Iran and Evaluation of Its Antimicrobial Activity Against Some Pathogenic Microorganisms. Iranian Journal of Medical Microbiology. 2022; 16(1), 17-34. DOI:10.30699/ijmm.16.1.17

28] Akpinar A, Yerlikaya O, Kilicß S. Antimicrobial activity and antibiotic resistance of Lactobacillus delbrueckiisspbulgaricus and Streptococcus thermophilus strain isolated from Turkish homemade yoghurts. Afr J Microbiol Res. 2011; 5(6): 675-682. DOI: 10.5897/AJMR10.835.

29] Mohamad N I, Manan M A, Sani N A. The Antibacterial Activity of Lactic Acid Bacteria from Pickled Spondiasdulcis (Ambarella) against Foodborne Pathogens. Trends in Sciences. 2022; 19(5), 2896. https://doi.org/10.48048/tis.2022.2896

30] Shirazi O U, Khattak M MA K, Shukri N A M, Nasyriq M N. Determination of total phenolic, flavonoid content and free radical scavenging activities of common herbs and spices. Journal of Pharmacognosy and Phytochemistry. 2014; 3(3), 104-108.

31] Abouloifa H, Rokni Y, Bellaouchi R, Ghabbour N, Karboune S, Brasca M, Asehraou A. Characterization of probiotic properties of antifungal Lactobacillus strains isolated from traditional fermenting green olives. Probiotics and antimicrobial proteins. 2020; 12(2), 683-696. https://doi.org/10.1007/s12602-019-09543-8.

32] Abubakr MAS, Hassan Z, Imdakim MMA, Sharifah NRS. Antioxidant activity of lactic acid bacteria (LAB) fermented skim milk as determined by 1,1-diphenyl-2- picrylhydrazyl (DPPH) and ferrous chelating activity (FCA). African Journal of Microbiology Research. 2012; 6, 6358–6364. https://doi.org/10.1007/s12602-019-09543-8.

33] Afify AEMM, Romeilah RM, Sultan SI, Hussein MM. Antioxidant activity and biological evaluations of probiotic bacteria strains. International Journal of Academic Research, 2012; 4(6).

34] Li M, Wang Y, Cui H, Li Y, Sun Y, Qiu H-J. Characterization of Lactic Acid Bacteria Isolated from the Gastrointestinal Tract of a Wild Boar as Potential Probiotics. Front. Vet. Sci. 2020; 7:49. doi:10.3389/fvets.2020.00049.

35] Zhou N, Zhang J X, Fan M T, Wang J, Guo G, Wei X Y. Antibiotic resistance of lactic acid bacteria isolated from Chinese yogurts. Journal of dairy science. 2012; 95(9), 4775-4783.http://dx.doi.org/10.3168/jds.2011-5271.

36] Aktaş H M, Erdoğan A. Characterization of technological properties of lactic acid bacteria isolated from Turkish Beyaz (white) cheese. Journal of Food Processing and Preservation. 2022. DOI: 10.1111/jfpp.16837.

37] Rokni Y, Abouloifa H, Bellaouchi R, et al. Characterization of β-glucosidase of Lactobacillus plantarum FSO1 and Candida pelliculosa L18 isolated from traditional fermented green olive. J Genet EngBiotechnol. 2021;19(1):117. Published 2021 Aug 9. doi:10.1186/s43141-021-00213-3

38] Zupanc M, Pandur Z, StepisnikPerdih T, Stopar D, Petkovsek M, Dular M. Effects of cavitation on different microorganisms: The current understanding of the mechanisms taking place behind the phenomenon. A review and proposals for further research,

Ultrason.Sonochem. 2019; 57 (2019) 147–165, https://doi.org/ 10.1016/j.ultsonch.2019.05.009.

39] Peshkovsky AS, Peshkovsky SL, Bystryak S. Scalable high-power ultrasonic technology for the production of translucent nanoemulsions, Chem. Eng. Process.ProcessIntensif. 2013; 69 (2013) 77–82. https://doi.org/10.1016/j.cep.2013.02.010.

40] Pandur Z, Dular M, Kostanjsek R, Stopar D. Bacterial cell wall material properties determine E. coli resistance to sonolysis. UltrasonicsSonochemistry. 2022; 83 (2022) 105919. https://doi.org/10.1016/j.ultsonch.2022.105919

41] Hassanzadazar H, Ehsani A, Mardani K, Hesari J. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. In Veterinary Research Forum. 2012; 3(3), p. 181. Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

42] Hamdaoui N, Rokni Y, Asehraou A, Mouncif M, Mennane Z, Omari A, Sellam A, Hammouti B, Meziane M. Technological Aptitude and Sensitivity of Lactic Acid Bacteria Leuconostoc Isolated from Raw Milk of Cows: From Step-by-Step Experimental Procedure to the Results. Indonesian Journal of Science and Technology. 2022b; 8(2), 157-170. doi:https://doi.org/10.17509/ijost.v8i2.53730

43] Ebner S, Smug LN, Kneifel W, Salminen SJ, Sanders ME. Probiotics in dietary guidelines and clinical recommendations outside the European Union. World J Gastroenterol. 2014 Nov 21;20(43):16095-100. doi: 10.3748/wjg.v20.i43.16095. PMID: 25473160; PMCID: PMC4239494.

44] Jaradat NA, Zaid AN, Abuzant A, Shawahna R. Investigation the efficiency of various methods of volatile oil extraction from Trichodesma africanum and their impact on the antioxidant and antimicrobial activities. J Intercult Ethnopharmacol. 2016 May 4;5(3):250-6. doi: 10.5455/jice.20160421065949. PMID: 27366351; PMCID: PMC4927130.

45] Jaradat, N; Al-Masri, M; Abu Shanab, B; Hussein, F; Qneibi, M; Nasser Eldin, A; Yassin, T; and Khawaja, M (2017) "Phytochemical and Antibacterial Assessment of Rhagadiolus Stellatus Plant in Jerusalem Area - Palestine," Palestinian Medical and Pharmaceutical Journal: 2 :(1) 4. Available at: https://doi.org/10.59049/2790-0231.1028

46] Jaradat, N. Qualitative, Quantitative, Cytotoxic, Free Radical Scavenging, and Antimicrobial Characteristics of Hypericum lanuginosum from Palestine. Molecules 2022, 27, 4574. https://doi.org/ 10.3390/molecules27144574

47] Jaradat N, Al-Lahham S, Abualhasan MN, Bakri A, Zaide H, Hammad J, Hussein F, Issa L, Mousa A, Speih R. Chemical Constituents, Antioxidant, Cyclooxygenase Inhibitor, and Cytotoxic Activities of Teucrium pruinosum Boiss. Essential Oil. Biomed Res Int. 2018 Jul 30;2018:4034689. doi: 10.1155/2018/4034689. PMID: 30151381; PMCID: PMC6091332.

48] Alqub, M and Jaradat, N (2023) "In vitro studies of Bituminaria bituminosa L. extracts from Palestine for their antioxidant, qualitative, and quantitative properties," Palestinian Medical and Pharmaceutical Journal. 8:(1)8. Available at: https://doi.org/10.59049/2790-0231.1144

49] Hassan AHA, Alkhalifah DHM, Al Yousef SA, Beemster GTS, Mousa ASM, Hozzein WN, AbdElgawad H. Salinity Stress Enhances the Antioxidant Capacity of Bacillus and Planococcus Species Isolated from Saline Lake Environment. Front Microbiol. 2020 Sep 14;11:561816. doi: 10.3389/fmicb.2020.561816. PMID: 33042068; PMCID: PMC7521018.