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CHARACTERIZATION, *IN-VITRO* EVALUATION OF PROBIOTIC POTENTIAL AND ANTAGONISTIC ACTIVITY OF SELECTED LACTIC ACID BACTERIA STRAINS ISOLATED FROM NATURAL ORIGIN AGAINST SOME HUMAN PATHOGENS

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Probiotic bacteria have recently become popular for their health-related beneficial effects. In this study, 19 lactic acid bacteria were isolated from breast milk, dairy products, infant stool, vaginal swabs, fermented beverages, and fermented grapes. These isolates were evaluated for their tolerance to gastrointestinal conditions, hydrophobicity, auto-aggregation, and antimicrobial activity. Five isolates, belonging to Pediococcus spp. and Lactobacillus spp., showed potential probiotic features. These isolates were tested for the presence of virulence enzymes and for susceptibility to various antibiotics. The isolates were found to be non-hemolytic and lacked gelatinase and deoxyribonuclease enzymes, however, some antibiotic resistance genes were detected. To ensure the safety of the used probiotics, cell-free supernatants (CFS) of the promising candidates were prepared and its antimicrobial activity before and after neutralization was assessed against standard strains of Candida albicans, Gram-positive, and Gram-negative bacteria. The neutralization of the CFS significantly diminished the antibacterial and antifungal activities of probiotics. Using the time-kill assay, the combination of CFS of Lactiplantibacillus plantarum with gentamicin and ceftazidime was tested against Staphylococcus aureus and Escherichia coli clinical isolates. A significant reduction in log number of survivors was obtained with gentamicin against E. coli and with ceftazidime against both isolates. The anti-biofilm ability of the CFS of the selected probiotics was tested as well. Neutralized CFS caused 26-52% inhibition of S. aureus isolate biofilm formation compared to the control. In conclusion, the five selected isolates could be considered promising probiotic candidates that can be used as biotherapeutics in case of bacterial infection.

INTRODUCTION

In the last decade, probiotic bacteria have become popular for their human health-related beneficial effects. Owing to the unceasingly expanding scientific research directing attention to probiotics' valuable health impact, the demand for new probiotic dietary supplements, as well as probiotic foodstuffs and beverages, has increased[1&2].

Probiotics are live micro-organisms that can exist as single or mixed cultures. They can confer advantageous health effects for the host if they are administered in adequate amounts[3]. A great number of the previously identified probiotic bacteria, including *Lactococcus* spp., *Lactobacillus* spp., *Streptococcus* spp., *Bifidobacteria* spp., *Enterococcus* spp. and *Pediococcus* spp., originally belong to the lactic acid bacteria (LAB) genera. However, the most promising probiotic candidates are *Lactobacillus* spp. and *Bifidobacteria* spp.[3].

Probiotic strains can be successfully isolated from various sources. Amongst the common sources of probiotics are dairy and dairy-related products, breast milk[4], and human vagina[5]. Numerous probiotic strains have been isolated from the human gut, as well as from

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the fecal samples of healthy adults and infants. Besides, non-dairy fermented substrates, such as fermented meat and fruits, represent a valuable source of probiotics[4].

Many studies highlighted the significant effect of probiotics on human health. It is believed that they might help in reducing the incidence of cancer, as well as preventing coronary heart disease by controlling blood pressure and reducing the levels of serum cholesterol[1]. Probiotics are also capable of ameliorating bacterial vaginosis by supporting the normal vaginal lactobacilli microbiota[1]. Moreover, they are reported to play a considerable role in augmenting the immune response, improving the functionality of the intestine, enhancing lactose metabolism, as well as diminishing the severity of rheumatoid arthritis, inflammatory bowel disease, and irritable bowel syndrome[1&3].

A real probiotic strain should possess specific desirable properties. Such properties include tolerance to bile toxicity and gastric acidity, proper adhesion to epithelial and mucosal surfaces, the capability of improving the host immune response, as well as the potential antimicrobial activity against various pathogens[1&6]. This activity has currently become one of the most significant features of promising probiotic candidates due to the widespread of antibiotic-resistant pathogens and the misuse of antibiotics. Thus, special attention has been paid to the utilization of probiotics and their antimicrobial metabolites, either alone or in combination with different antibiotics, for the treatment and prevention of various microbial infections as an alternative antimicrobial strategy[2&7].

In view of the current data, this study aimed at the isolation, identification and characterization of some LAB strains isolated from various natural sources, including dairy products, fermented grapes, breast milk, infant feces, vaginal swabs, and fermented beverages, to assess their potential as promising probiotic candidates. Moreover, the antimicrobial activity of such candidates, alone or in combination with antibiotics, against different pathogens was tested. The anti-biofilm ability of selected candidates to prevent formation of biofilms during the growth of pathogenic bacteria was evaluated as well.

MATERIALS AND METHODS

Collection and isolation of microorganisms from natural sources

A total of 47 samples were collected from different natural sources. Twenty-four samples were collected aseptically from healthy women's breast milk within 4-6 months of giving birth to healthy babies. The nipple and mammary areola of the breast were washed and wiped with sterile saline and about 5 mL of milk was collected in a sterile falcon manually or using a sterile breast pump[2]. Thirteen samples were taken from dairy products that were purchased from local vendors (3 curd samples, 7 yogurt samples, 2 household milk samples, and 1 soft cheese (Karish) sample). Three vaginal samples from healthy women of childbearing age, and 5 samples from fermented beverages (3 fermented sugarcane juice and 2 fermented carob juice samples) were obtained. One sample was taken from fermented grapes where 500 g of washed fresh grapes were fermented in 300 mL sterile distilled water and 100 g glucose in 1 L jar for 30 days in a relatively warm place. The jar was shaken for 30 s and left to stand for 5 mins at room temperature, then the supernatant was collected from which the sample was taken. One sample was obtained from the feces of a healthy breastfed 4-month-old infant. The feces sample was collected in a sterile falcon, then approximately 1 g of the sample was homogenized with 5 mL sterile saline[2].

All the obtained samples were 10-fold serially diluted in sterile saline then 40 µL from different dilutions were spread over deMan, Rogosa and Sharpe (MRS) agar plate and incubated aerobically at 37°C for 24-48 hrs for the isolation of LAB.

Characterization and identification of LAB isolates

The tested isolates were streaked onto the surface of MRS agar plates containing 1% $CaCO_3$ and incubated aerobically at 37°C for 24-48 hrs. Formation of a clear zone surrounding the colonies indicated the presence of acid producing bacteria[8]. LAB isolates were confirmed by being Gram-positive, catalase negative, non-endospore forming and non-motile bacteria[9]. Promising isolates were further identified using the 16s rRNA

sequencing[10]. The sequencing was conducted at GATC Biotech DNA sequence company, Germany. Primers used are listed in table 1.

Survival of LAB isolates under conditions simulating the human gastrointestinal tract (GIT)

Bacterial cells from 5 mL overnight cultures were harvested by centrifugation at 6000 g for 10 mins at 4°C. For the acid and pepsin tolerance tests, bacterial pellets were washed twice with phosphate buffer saline (PBS), pH 7.2, then resuspended in 1 mL PBS solution, pH 3, and/or pH 2, either alone or containing pepsin (3 mg/mL) then incubated for 3 hrs at 37°C[11]. For the bile and pancreatin tolerance tests**,** the pellets were resuspended in 1 mL PBS solution, pH 8, containing 0.3% ox-bile or 1 mg/mL pancreatin, respectively, then incubated at 37°C for 4 hrs[11&12]. To simulate the *in-vivo* conditions of gastric digestion, a volume of reconstituted skimmed milk (RSM) (11% solids, w/v) was added to PBS solution, pH 2 to reach a final pH of ca.3 and then pepsin (3 mg/mL) was added. After washing bacterial pellets twice, they were suspended in RSM and incubated at 37°C for 3 hrs[13]. For each test, bacterial viable count was done, and the resistant strains were defined as those whose initial counts did not decrease by more than one log after the indicated incubation period[14].

Cell surface hydrophobicity

Bacterial cultures were harvested by centrifugation followed by pellet resuspension in PBS pH 7 to an OD_{630} of approximately 0.6. An aliquot of 1 mL of xylene was added to test tubes containing 3 mL of washed cells. Tubes were vortexed for 2 mins and the suspensions were left for 30 mins before measuring the OD_{630} of the aqueous phase. Hydrophobicity was calculated as follows:

$$\text{Hydrophobicity \%} = [(\text{initial } OD_{630} - OD_{630} \text{ with xylene}) / (\text{initial } OD_{630})] \times 100.$$

The strains were classified as: those with low hydrophobicity (0-35%), moderate hydrophobicity (36-70%), and high hydrophobicity (71-100%)[15]. Data were expressed as means ± S.D.

Auto-aggregation

Bacterial cultures, in MRS broth, were centrifuged and the pellets were resuspended in PBS pH 7 to approximately 10^8 CFU/mL. Each suspension was vortexed for 10 s and incubated for 5 hrs at room temperature. A volume of 200 µL of the upper part of each suspension was withdrawn at 0 and 5 hrs intervals to measure the absorbance at 630 nm. The percentage of auto-aggregation was calculated as follows:

$$\text{Auto-aggregation } (\%) = 1 - (A_t/A_0) \times 100$$

Where A_0 and A_t are the absorbance values at t = 0 hrs and 5 hrs, respectively[16]. Data were expressed as means ± S.D.

Antimicrobial activity of LAB isolates using the agar overlay technique

The antimicrobial activity of LAB isolates was determined against the standard strains: *Staphylococcus aureus* ATCC 6538, *Escherichia coli* NCTC 10418, *Listeria monocytogenes* EGD-e (serotype 1/2a), *Salmonella enterica subsp. enterica serovar Typhimurium* ATCC 14028 and *Candida albicans* ATCC 231. Two µL of overnight culture of each LAB strain were spotted on MRS agar plates and incubated aerobically at 37°C for 24-48 hrs. The plates were then overlaid with 10 mL of soft (0.6% w/v agar) Müller-Hinton medium, or Sabouraud Dextrose medium (for *C. albicans*) seeded with 1% v/v of an overnight culture of the tested pathogen (final count of 10^6 CFU/mL). After 24-48 hrs, inhibition zones around the LAB spots were measured. Strains showing inhibition zone diameters of more than 20 mm, 10 to 20 mm, and less than 10 mm were considered as strong, intermediate, and low inhibitors of microbial growth, respectively[2].

Phenotypic characterization of some virulence factors among selected probiotic candidates

To test for gelatinase activity, overnight cultures of the tested isolates were spot inoculated on gelatin agar plates and incubated for 48 hrs, then, flooded with 10 mL saturated ammonium sulfate. The presence of a clear zone around the colonies showed gelatinase enzyme production[17]. For the detection of DNase activity, the overnight cultures of isolates were spot inoculated on DNase agar

plates and incubated for 48 hrs. The cultures were flooded with 10 mL 1N HCl. The appearance of clear zones around the colonies confirmed DNase production[17]. Isolates were also tested for hemolytic activity by streaking on blood agar plates. After incubation for 48 hrs, the plates were examined for α-, β- or γ-hemolysis[12]. In all virulence tests, *Staphylococcus aureus* ATCC 6538 was used as a positive control.

Determination of the minimum inhibitory concentrations (MICs) of some antibiotics against selected probiotic candidates using the broth microdilution technique

Overnight cultures of selected LAB isolates were centrifuged at 7000 rpm for 10 mins. Cell pellets were adjusted to OD_{600} of 0.2-0.3, then diluted 100-fold in double strength MRS broth. A volume of 100 µL of diluted LAB inoculum was added to 100 µL of two-fold serial dilutions of selected antibiotics in a sterile 96-well microtiter plate to reach a final count of $5x10^5$ CFU/mL. Negative and positive controls were included in the test. After aerobic incubation at 37°C for 48 hrs, results were recorded using microtiter plate reader at OD_{630} nm (Biotek ELx800, USA)[12&18]. Results were interpreted according to the EFSA guidelines (2018)[19].

Detection of genes conferring antibiotic resistance

DNA extraction from the tested LAB isolates was done as previously described[20]. PCR was employed to detect seven antibiotic resistance genes: *erm*B, *aac(6')-aph(2"), aph(3")-III, bla, blaZ, van*X, and *gyr*A[21-23]. All primers used are listed in table 1. The PCR products were separated by electrophoresis (Hoefer Scientific, USA) on a 1% agarose gel containing 0.2 µg/mL ethidium bromide for 25 mins in 1X TBE buffer. The sizes of the PCR products were determined by comparison with a molecular-sized standard (100 bp DNA ladder, Thermo Fisher Scientific, UK). The bands were visualized under UV light using a gel documentation system (High-Performance UV transilluminator, UVP, USA).

Table 1: Primers used for the amplification of 16s rRNA and genes encoding for resistance to different antibiotics among selected potential probiotic candidates.

Target gene	Nucleotide sequence (5'→ 3')	Amplicon size	Reference
16s rRNA	F: (5'- AGAGTTTGATCMTGGCTCAG -3') R: (5'- TACGGYTACCTTGTTACGACTT -3')	1530 bp.	10
*erm*B	F: (5'- CATTTAACGACGAAACTGGC -3') R: (5'GGAACATCTGTGGTATGGCG -3')	425 bp.	21
aac(6')-aph(2'')	F: (5'- CCAAGAGCAATAAGGGCATA -3') R: (5'- CACTATCATAACCACTACCG -3')′	220 bp.	21
aph(3'')-III	F: (5'- GCCGATGTGGATTGCGAAAA -3') R: (5'- GCTTGATCCCCAGTAAGTCA -3')	292 bp	21
bla	F: (5'- CATARTTCCGATAATASMGCC -3') R: (5'- CGTSTTTAACTAAGTATSGY -3')	297 bp.	22
blaZ	F: (5'- ACTTCAACACCTGCTGCTTTC -3') R: (5'- TAGGTTCAGATTGGCCCTTAG -3')	240 bp.	23
*van*X	F: (5'- TCGCGGTAGTCCCACCATTCGTT -3') R: (5'- AAATCATCGTTGACCTGCGTTAT -3')	454 bp.	22
*gyr*A	F: (5'- CAMCGKCGKATTCTTTACGGAATG-3') R: (5'- TTRTTGATATCRCGBAGCATTTC-3')	286 bp	22

Assessment of the mechanism of antimicrobial activity of potential probiotic candidates against different standard strains

The mechanism of the antimicrobial activity of selected LAB isolates was determined against the previously mentioned standard strains. The overnight culture of each LAB isolate was centrifuged, and the supernatant was filtered using a syringe filter (0.2-µm pore size)[24]. The prepared CFS was further neutralized (nCFS) to pH 6.5 using 5 M NaOH. In sterile 96-well microtiter plate, 100 µL of CFS before and after neutralization were added to 100 µL of bacterial suspension inoculated in double strength Luria-Bertani broth (or Sabouraud Dextrose broth in case of *C. albicans*) to obtain a final inoculum of approximately 10^6 CFU per well. Positive and negative controls were included in the experiment. The plates were incubated at 37°C for 24 hrs, and the OD_{630} was measured. The total percent inhibition of bacterial growth was calculated as follows:

% inhibition = [(OD of positive control - OD of sample)/OD of positive control] x 100

Data were expressed as means ± S.D.[25].

Evaluation of the antibacterial activity of *L. plantarum* in combination with other antibiotics against pathogenic clinical isolates using time-kill assay

The antibacterial activity of *L.* plantarum, isolated from fermented grapes, was assessed in combination with gentamicin or ceftazidime, against uropathogenic S. aureus or *E. coli* isolates. First, the MIC values of the antibiotics or the CFS of *L. plantarum* against the selected isolates were determined using the broth microdilution technique. Then, 5 mL of double strength nutrient broth containing ¼ MIC of the tested antibiotic with ¼ MIC of the CFS of L. plantarum were added to 5 mL of overnight culture of either *S. aureus* or *E. coli* so that its final count was approximately 10^6 CFU/mL. Samples were kept in shaking incubator at 150 rpm, 37°C and a volume of 100 µL was aseptically withdrawn at 0, 3, 6 and 24 hrs, ten-fold serially diluted then dropped onto nutrient agar plates. Average number of colonies/sectors was counted after 24 hrs incubation period and the log number of survivors per mL of each clinical isolate, before and after treatment, was plotted against killing time. The inhibitory effect of the antibiotic or CFS of *L. plantarum* on *S. aureus* or *E. coli* isolates, at the same used concentrations, were also tested and plotted. The results were interpreted as previously described by Chambers and Sande[26].

Assessment of the anti-biofilm activity of the nCFS of different probiotic candidates on *S. aureus* biofilm formation

CFS of each of the tested probiotics was prepared as discussed earlier using Tween 80-free MRS broth then it was neutralized by 5 M NAOH to pH 6.5[24]. The tested *S. aureus* clinical isolate was cultured in sterile Tryptone soya broth supplemented with 0.5% (w/v) glucose. Then, 100 µL of the diluted culture were added to 96-well microtiter plate containing 100 µL of nCFS of the probiotic so that the final count of *S. aureus* was ca. 10^6 CFU/mL. After incubation for 24 hrs at 37°C, the medium was discarded, and planktonic cells were removed by gentle washing with sterile PBS. The biofilms were fixed with 200 µL methanol for 15 mins, stained with 200 µL of 1% crystal violet for 20 mins, and rinsed thrice with water. After dissolving crystal violet with 200 µL of 33% acetic acid, absorbance at 630 nm was measured and the percentage inhibition of biofilm formation was calculated as:

Percentage inhibition = 100 - [(OD of wells in the presence of CFS X 100) / OD of control wells].

Data were expressed as means ± S.D.[27].

RESULTS AND DISCUSSION

Results

Collection and isolation of microorganisms from natural sources

Different LAB strains were collected from various natural sources and the results are illustrated in table 2. The table showed that, out of 47 samples collected, nineteen LAB isolates were obtained; 7 from dairy products (D1-D7), 6 from fermented beverages (F1-F6), 3 from breast milk (B1-B3) while only one isolate was obtained from either a vaginal swab, infant stool or fermented grapes, and were given the codes V1, S1 and G1, respectively. All these isolates were confirmed to be acid producing bacteria as manifested by the presence of clear zones around the colonies in $CaCO_3$ test.

Table 2: Identification and morphology of different lactic acid bacteria obtained from natural origins and their designated codes.

Isolate source	Designated code	Morphology	Identification[a]
Breast milk	B1	Cocci	*Pediococcus acidilactici*
	B2	Cocci	*Enterococcus faecalis*
	B3	Rods	*Limosilactobacillus fermentum*
Vaginal swab	V1	Rods	*Ligilactobacillus salivarius*
Infant stool	S1	Cocci	*Enterococcus avium*
Dairy products	D1	Rods	*Lactobacillus delbrueckii*
	D2	Cocci	*Lactococcus lactis*
	D3	Cocci	*Enterococcus faecalis*
	D4	Cocci	*Lactococcus garvieae*
	D5	Cocci	*Lactococcus lactis*
	D6	Cocci	*Enterococcus faecalis*
	D7	Cocci	*Lactococcus lactis*
Fermented beverages	F1	Cocci	*Leuconostoc holzapfelii*
	F2	Rods	*Lacticaseibacillus paracasei*
	F3	Rods	*Lactobacillus hilgardii*
	F4	Coccoid	*Weissella confuse*
	F5	Coccoid	*Weissella cibaria*
	F6	Cocci	*Leuconostoc mesenteroides*
Fermented grapes	G1	Rods	*Lactiplantibacillus plantarum*

[a]*L. fermentum, L. salivarius, L. paracasei, and L. plantarum* changed into *Limosilactobacillus fermentum, Ligilactobacillus salivarius, Lacticaseibacillus paracasei, Lactiplantibacillus plantarum,* respectively[28].

Characterization and identification of LAB isolates

According to biochemical and 16s rRNA sequencing results, the collected LAB isolates' codes, sources, morphology, and identification are summarized in table 2.

Six isolates (31.6%) belonged to the *Lactobacillus* spp.; F2: *Lacticaseibacillus paracasei* (formerly known as *Lactobacillus paracasei*), F3: *Lactobacillus hilgardii*, B3: *Limosilactobacillus fermentum* (formerly known as *Lactobacillus fermentum*), V1: *Ligilactobacillus salivarius* (formerly known as *Lactobacillus salivarius*), D1: *Lactobacillus delbrueckii* and G1: *Lactiplantibacillus plantarum* (formerly known as *Lactobacillus plantarum*)[28]. Four isolates were identified as *Enterococcus* spp.: 3 *Enterococcus faecalis* isolates B2, D3 and D6 and a single *Enterococcus avium* isolate S1. The obtained *Lactococcus* spp. (4 isolates) were isolated from dairy products; 3 *Lactococcus lactis* isolates: D2, D5 and D7 and one *Lactococcus garvieae* isolate: D4. From fermented beverages, two isolates belonged to *Leuconostoc* spp. were obtained, (F1: *Leuconostoc holzapfelii* and F6: *Leuconostoc mesenteroides*) and two other isolates were identified as *Weissella* spp.; F4: *Weissella confusa* and F5: *Weissella cibaria*. Only one isolate from breast milk, B1, was identified as *Pediococcus acidilactici.*

Survival of LAB isolates under conditions simulating human GIT

Most of the tested isolates showed less than one log reduction in number of survivors when present in a saline media of pH 3. However, addition of pepsin to such media caused no change in bacterial count among the majority of the bacteria tested as shown in table 3. None of the tested LAB isolates were able to tolerate pepsin or even to grow at pH 2, but upon addition of RSM, 7 isolates showed acceptable tolerance (<1 log reduction). These isolates were B1, V1, F3, D1, D3, D6 and G1. Upon exposing the isolates to 0.3% ox-bile at pH 8 for 4 hrs, most of isolates (73.7%) showed more than one log reduction in survivors. On the contrary, they had a good tolerance to pancreatin (Table 3).

Table 3: Number of survivors in colony forming units of different lactic acid bacteria strains under different stress conditions in the GIT.

Code	Viable cell count (Log CFU/mL)[a]											
	pH 3		pH3 + pepsin		pH2 + pepsin		RSM[b]		Bile		Pancreatin	
	0 hrs	3 hrs	0 hrs	3 hrs	0 hrs	3 hrs	0 hrs	3 hrs	0 hrs	4 hrs	0 hrs	4 hrs
B1	10.34	9.74	10.11	10.11	9.83	0	10.19	9.54	9.97	9.8	9.7	9.7
B2	9.43	9.43	9.3	9.3	9.23	7	9.51	6.18	9.32	6.51	9.6	9.6
B3	9.8	9.18	9.52	8.82	9.78	7	9.85	8.76	8.94	3.3	10	10
V1	9.56	9.48	9.38	9.38	9.74	0	9.88	9.47	9.72	6.22	9.6	9.6
S1	9.49	8.78	9.4	9.4	9.28	0	9.38	7.89	8.98	5.72	9.51	9.51
D1	9.17	8.67	9.17	9.17	9.26	0	9.24	9.24	9.02	8.63	9.47	9.47
D2	9.04	8.7	9.22	9.13	9.01	0	9.41	4.86	9.14	5.72	9.48	9.32
D3	8.8	7.41	9.28	9.2	9.24	0	8.89	8.44	8.68	3.7	9.08	8.93
D4	7.76	7.32	8.42	8.42	8.24	0	8.29	6.54	8.32	6.95	8.6	7.9
D5	9.6	8.8	9.65	9.65	9.58	4	9.45	8.15	9.16	7.57	9.74	9.74
D6	9.08	9.08	9.43	9.43	9.43	5	9.5	9.18	9.33	7.48	9.44	9.44
D7	9.3	7.75	9.65	9.54	9.47	0	9.78	6.26	8.11	6.65	9.3	9.3
F1	9.18	7.46	9.02	9.02	9.34	4	8.94`	6.4	8.51	6.01	8.65	8.34
F2	9.45	9.45	9.5	9.5	9.65	7	9.88	7.51	9.81	9.3	9.7	9.7
F3	9.57	9.57	9.48	9.33	9.37	0	8.88	8.17	9.51	9.18	9.88	9.54
F4	6.7	6.61	6.18	6.03	9.34	0	8.68	7.4	9.27	3.3	7.54	7.54
F5	9.51	9.11	8.66	7.92	9.16	0	9.57	7.36	9.44	5.25	8.02	8.02
F6	9.52	9.52	9.6	9.6	9.37	0	9.48	4.19	9.63	7.81	9.4	8.9
G1	10.33	10.21	10.04	10.04	9.68	5	10.06	10.06	10	9.37	9.88	9.88

[a]values are means with standard deviation ranging from 0-0.9.
[b]Reconstituted Skimmed Milk.

Cell surface hydrophobicity and auto-aggregation

Figure 1 clearly demonstrated the wide discrepancies between the bacteria tested either in their cell hydrophobicity characteristics or their autoaggregation capabilities. About 31.6% of the isolates showed high cell surface hydrophobicity ranging between 93 and 76%. Only one isolate, *W. confusa* F4, showed medium hydrophobicity of 40%. The rest of the isolates possessed low hydrophobicity (Fig. 1). The percentage of auto-aggregation ranged between 78 and 26%. The highest % of auto-aggregation was detected in case of *L. garvieae*: D4 (78%) while the lowest auto-aggregation ability was observed in case of *L. lactis* D7 and *E. faecalis* B2 which showed only 26% of auto-aggregation (Fig. 1).

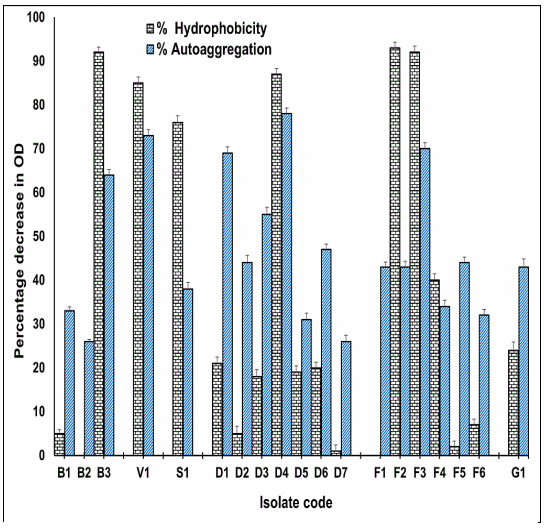

Fig. 1: Percentage of cell surface hydrophobicity and auto-aggregation among the collected LAB isolates.

Antimicrobial activity of LAB isolates

The antimicrobial effect of the probiotics studied using the agar overlay method against common standard strains is illustrated in figure 2. Most of the LAB isolates tested were strong inhibitors of the growth of either *E. coli* (63.2%) or *S. enterica* (84.2%), while the rest intermediately inhibited their growth. However, their inhibitory effect against *L. monocytogenes* was almost equally divided between strong and intermediate. On the other hand, most of the LAB isolates intermediately inhibited the growth of *S. aureus* except *W. cibaria* F5, that caused strong inhibition. None of the tested LAB isolates showed any inhibition activity against *C. albicans* (Fig. 2).

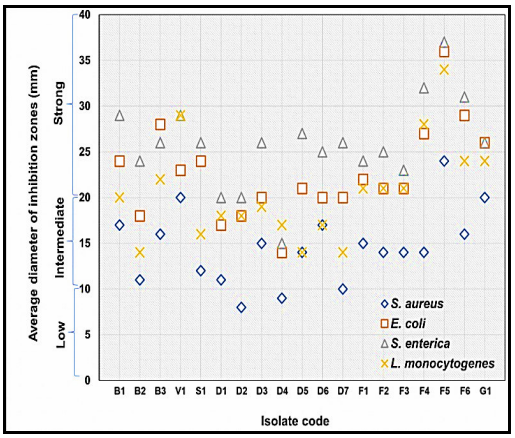

Fig. 2: Antibacterial activity of LAB isolates against selected standard strains using the agar overlay technique.

Based on the previous results, five isolates were considered to be promising probiotic candidates and were included in the rest of the experiments. These isolates were: *P. acidilactici* B1, *L. delbrueckii* D1, *L. paracasei* F2, *L. hilgardii* F3, and *L. plantarum* G1.

Screening for some virulence factors and antibiotic susceptibility of the chosen probiotic candidates

All tested isolates were non hemolytic and lacked both gelatinase and deoxyribonuclease enzymes. The MICs of each of the tested antibiotics against the LAB isolates are listed in table 4. All the tested strains were resistant to kanamycin, gentamicin, ciprofloxacin, and vancomycin with MIC ranges of (512 - >1024 µg/mL), (32 - >512 µg/mL), (8 - >512 µg/mL), and (4 - >256 µg/mL), respectively. Only D1 and F2 showed susceptibility to ampicillin (MIC= 2 µg/mL). In case of erythromycin, D1 and F3 were sensitive (MIC= 0.5 and 1 µg/mL, respectively). Among the seven tested antibiotic resistance genes, 4 genes (*aac(6')-aph(2"), gyr*A, *erm*B and *bla*Z were detected in all of the tested isolates. *aph(3")-III* gene was detected in B1, F2 and G1 while *van*X gene was detected in B1, D1, F2, and G1. On the contrary, *bla* gene was not detected in any of the isolates (Table 4).

Table 4: Minimum inhibitory concentration (µg/mL) and genes of resistance to some selected antibiotics among lactic acid bacteria isolates using polymerase chain reaction.

Antibiotic	Genes of resistance	Isolate name MIC (µg/mL), resistance pattern[a]				
		P. acidilactici	*L. delbrueckii*	*L. paracasei*	*L. hilgardii*	*L. plantarum*
Ampicillin	*bla*	8, R	2, S	2, S	4, R	4, R
	blaZ	-	-	-	-	-
		+	+	+	+	+
Kanamycin	*aph(3'')-III*	>1024, R	512, R	1024, R	>1024, R	>1024, R
		+	-	+	-	+
Gentamycin	*aac(6')-aph(2'')*	512, R	64, R	128, R	32, R	>512, R
		+	+	+	+	+
Ciprofloxacin	*gyr*A	256, R	256, R	8, R	128, R	>512, R
		+	+	+	+	+
Vancomycin	*van*X	>256, R	4, R	>256, R	>256, R	>256, R
		+	+	+	-	+
Erythromycin	*erm*B	2, R	0.5, S	2, R	1, S	2, R
		+	+	+	+	+

[a]The MICs are interpreted according to European food safety authority (EFSA) 2018[19]. S: sensitive, R: resistant.

Assessment of the mechanism of the antimicrobial activity of the chosen probiotics against standard strains

To assess the cause of the antibacterial activity of the probiotics tested against the previously mentioned isolates, the inhibitory effect of their CFS was plotted in figure 3 before and after neutralization with NaOH. Generally, the non-neutralized CFS of all the tested probiotics showed more than 90% growth inhibition against the clinical isolates tested. However, upon neutralization, the antibacterial activity of both D1 and G1 was abolished against *S. aureus* while that of B1, F2, and F3 was decreased by 4.2, 3.7, and 3.8 folds, respectively (Fig. 3).

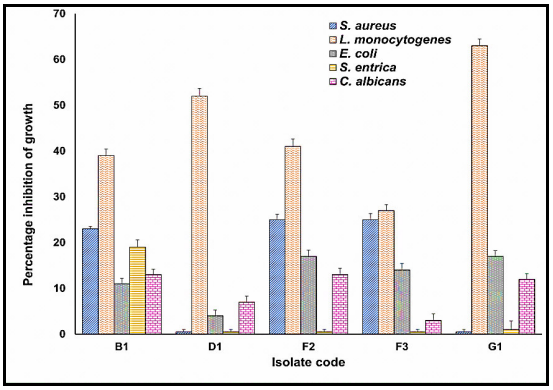

Fig. 3: Comparative antimicrobial activity of neutralized cell-free supernatant of potential probiotics against selected Gram-positive, Gram-negative, and *Candida albicans* standard strains.

Neutralization of the CFS of D1 resulted in 26.1-fold reduction in growth of *E. coli*. Such effect was less obvious with the rest of probiotics against the same isolate. The neutralization of the CFS of D1, F2, and F3 completely eliminated the antibacterial activity against *S. enterica*, while nCFS of G1 and B1 led to 70.8 and 4.9-fold reduction, respectively against salmonella (Fig. 3).

Against *L. monocytogenes,* percentage inhibition of growth, after neutralization of CFS of probiotics, ranged between 27% in F3 and 63% in G1. Similarly, in case of *C. albicans*, the neutralization of the CFS significantly diminished the antifungal activity of the tested probiotics. The percentage of inhibition of growth ranged between 70.8 and 44.3 in case of non-neutralized CFS, while it ranged between 12.9 to 2.9 in case of nCFS (Fig. 3).

Time-kill curve of the CFS of *L. plantarum* combined with antibiotics

Figures 4A-4D illustrate the effect of CFS of *L. plantarum* G1 either alone or combined with an antibiotic against either *E. coli* or *S. aureus* clinical isolates causing urinary tract infections. When tested against *E. coli* clinical isolate, the combination of G1 with ceftazidime showed a synergistic effect after 24 hrs with about 2.43 log reduction in survivors compared to the most active single component (Fig. 4A). Similarly, another synergistic effect was noticed with the combination of G1 with gentamicin, after 24 hrs, with 2.06 log reduction in survivors (Fig. 4B). The most promising combination tested against *S. aureus* isolate was that of G1 with ceftazidime showing a synergistic effect after 24 hrs with 3.63 log reduction in survivors (Fig. 4C). The combination of G1 with gentamicin just resulted in an additive effect after 24 hrs with about 0.47 log reduction in survivors (Fig. 4D).

The anti-biofilm effect of the nCFS of probiotics on uropathogenic *S. aureus* biofilm formation

The effect of the nCFS of the chosen probiotic candidates on the formation of *S. aureus* clinical isolate biofilm was shown in figure 5. About 50% reduction in biofilm formation resulted upon incubation of nCFS of G1 and F2 with *S. aureus* growing media. A slightly lower percentage of biofilm inhibition was observed in case of B1 and F3 (45 and 43%, respectively). However, the least effect was noticed in case of D1 (only 26% inhibition of biofilm formation).

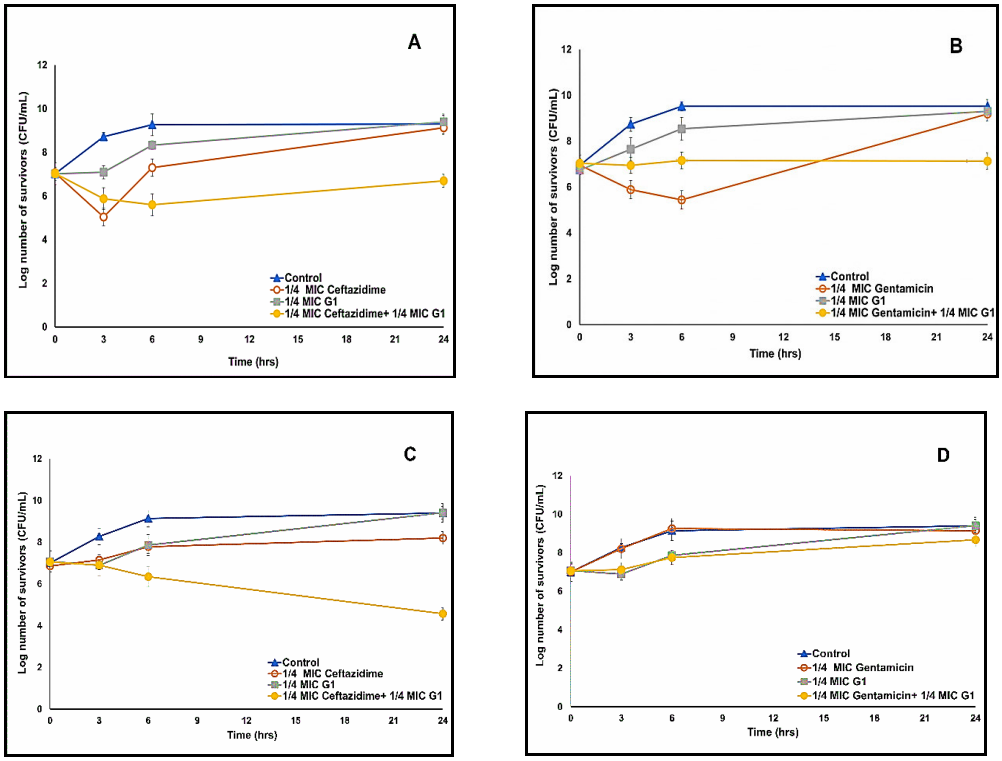

Fig. 4: Time-kill kinetics assay of the cell-free supernatant of *L. plantarum* against urinary tract pathogens *E. coli* and *S. aureus* combined with different antibiotics. Fig. 4A and 4B represent CFS of *L. plantarum* combined with ceftazidime and gentamicin, respectively, against *E. coli*. Fig. 4C and 4D represent CFS of *L. plantarum* combined with ceftazidime and gentamicin, respectively, against *S. aureus*.

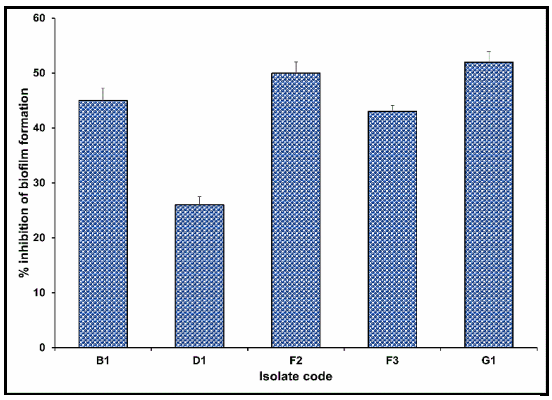

Fig. 5: Effect of the neutralized cell-free supernatant of potential probiotic candidates on biofilm formation of uropathogenic *S. aureus* isolate.

Discussion

Recently, consumption of probiotics obtained from "unconventional sources" has markedly increased. Current trends are now focusing on getting probiotics from sources different from milk products to be used for lactose intolerant people. Such sources include non-dairy fermented beverages and foods, human breast milk, as well as feces of breast-fed infants[29]. In our study, 19 isolates were obtained from various natural sources. Among our isolates, one *L. salivarius* strain (V1) was isolated from a vaginal swab. In a study conducted by Pino *et al.*, *L. salivarius* represented 20% of the lactobacilli isolated from the vagina of healthy women[30]. Some LAB, including *Lactobacillus* and *Leuconostoc* genera, are reported to be used as starters for homemade foods and beverages. Also, *Weissella* spp. have been commonly isolated from various fermented foods[31]. Similarly, our isolates that were isolated from fermented beverages belonged to *Leuconostoc* spp. (F1 and F6), *Lactobacillus* spp. (F2 and F3), as well as *Weissella* spp. (F4 and F5). Bacteria commonly associated with dairy products are related to various genera including *Lactobacillus* and *Lactococcus*[32]. In

addition, the isolation of *E. faecalis* from dairy products has been previously reported[33]. In our study, D1 belonged to *Lactobacillus* spp., D2, D4, D5 and D7 belonged to *Lactococcus* spp, while D3 and D6 were identified as *E. faecalis*.

Probiotic candidates intended to be orally ingested should show resistance to stress conditions encountered in GIT to ensure their beneficial activity[34]. Among the tested isolates, 84.2% tolerated presence at pH 3 environment and all the isolates tolerated pepsin at pH 3. It is thought that pepsin might help in maintaining the pH homeostasis of LAB, as well as supporting the role of H^+-ATPase in the protection of bacteria[35]. None of the tested isolates tolerated pepsin (3 mg/mL) at pH 2. However, upon addition of RSM, 36.8% of the isolates showed acceptable tolerance. This could be attributed to the increase in pH value due to the addition of RSM or the direct protective effect of the food matrix on the bacterial cells[13].

In our study, B1, D1, G1, F2 and F3 tolerated 0.3% ox-bile. The most significant bile resistance mechanisms among *Lactobacillus* spp. were reported to be due to hydrolysis of bile salt, active efflux of bile salts, as well as modifications in the architecture of cell membrane and cell wall[36]. In addition, tolerance of *P. acidilactici* to bile salt has been previously reported[37]. All the tested isolates tolerated pancreatin. Also, Ruiz-Moyano *et al*. found that 46 out of 51 tested LAB strains could survive after 3 hrs of exposure to 1.9 mg/mL of pancreatic enzymes[38].

LAB strains having hydrophobic cell surface and showing good aggregation capacity could achieve better adherence to the intestinal cells to exert their beneficial effects[39]. Among the tested LAB isolates in this study, the highest hydrophobicity was detected in case of *L. paracasei* F2 (93%). However, this value was more than 3 times greater than what was reported by Xu *et al*.[40]. The wide variation of auto-aggregation ability among our tested isolates could be explained according to Krausova *et al*. who illustrated that auto-aggregation assays done among probiotic bacteria revealed strain-specific variations irrespective of their taxonomic group or origin[41]. LAB isolates under study showed inhibitory activity against both Gram-negative and Gram-positive bacteria. Such inhibition is commonly associated with the production of various antimicrobial substances, including bacteriocins, organic acids, ethanol, hydrogen peroxide, and acetaldehyde by the probiotic isolates[42]. Pino *et al.* reported that *L. salivarius* probiotic strain isolated from healthy women vagina showed inhibitory activity against *E. coli*, *S. aureus* and *L. monocytogenes*[30]. In addition, Birri *et al.* found that *E. avium*, from infant feces, showed noticeable inhibition of *L. monocytogenes* due to the production of a bacteriocin termed avicin A[43]. In Egypt, Bassyouni *et al.* showed that LAB isolates obtained from Egyptian dairy product exerted a promising antibacterial effect against *E. coli* and *Salmonella typhimurium*[44]. Also, Tadesse *et al*. showed that *Lactobacillus* and *Leuconostoc* isolates, from traditional Ethiopian fermented beverages, showed antibacterial activity against *S. aureus*, *Salmonella* spp. and *E. coli* O157:H7[45]. All the tested isolates were non-hemolytic. None of them showed DNase or gelatinase activities. Similarly, Abouloifa *et al.* had previously reported that the *Lactobacillus* strains isolated from traditional fermented Moroccan green olives lacked important virulence determinants and that assured the safety of their intake[17].

On the other hand, all the tested strains were resistant to kanamycin, gentamicin, ciprofloxacin, and vancomycin. Generally, resistance of lactobacilli to aminoglycosides is often high[46]. Also, it is well known that most nucleic acid synthesis inhibitors show low inhibition against the majority of *Lactobacillus* spp.[46]. Hummel *et al.* had previously reported that the resistance to aminoglycoside (including gentamicin) and ciprofloxacin among the tested LAB strains was greater than 70%[47]. Commonly, lactobacilli have been reported to show susceptibility to penicillin[46]. However, among our isolates, only 2 isolates were sensitive to ampicillin. Ali *et al.* found that *L. plantarum* and *L. paracasei* strains, isolated from fermented dairy products, showed resistance to vancomycin and ciprofloxacin, but contradictory to our findings, they reported the susceptibility of these strains to gentamicin and erythromycin[48].

Among the seven tested antibiotic resistance genes, 4 genes (*aac(6')-aph(2"),*

*gyr*A, *erm*B and blaZ) were detected in all of the tested isolates. *aph(3")-III* gene was detected in B1, F2 and G1 while *vanX* gene was detected in B1, D1, F2 and G1. Lactobacilli are commonly recognized to be intrinsically resistant to the glycopeptide vancomycin where this resistance is attributed to the vanX gene encoding the enzyme D-ala-D-ala dipeptidase[49]. Moreover, erythromycin resistance genes have been found in various *Lactobacillus* spp. where the *erm*B gene, encoding a rRNA methylase that acts on the 23S ribosomal subunit, is the most commonly detected[46]. *aac*(6')-*aph(2"), aph(3')-IIIa* and *bla*Z have been reported to exist much less frequently among lactobacilli[46]. However, among our tested *Lactobacillus* isolates, each of *aac(6')-aph(2")* and *bla*Z were detected among all the isolates, while *aph(3')-IIIa* was detected in F2 and G1.

A great concern has been raised regarding the possible transfer of antibiotic resistance genes from potential probiotic candidates of LAB to other pathogenic bacteria that might exist in GIT[50]. However, possessing intrinsic antibiotic resistance genes in LAB with low horizontal transfer potential to other organisms was considered acceptable for probiotic selection[51]. In addition, Guo *et al*. reported the failure of the transfer of *gyr*A from *Lactobacillus* isolates[49]. The lack of transferability of antimicrobial resistance genes from LAB to pathogenic bacteria has been previously reported. Besides, the threat of lactobacillemia associated with the probiotic *Lactobacillus* was regarded to be "unequivocally negligible"[17]. To ensure the safety of the used probiotics, CFS of each of the promising probiotic candidates in our study was prepared and utilized for the rest of the experiments.

To elucidate the mechanism of the antibacterial and antifungal activities of probiotics, CFS of the selected probiotics were compared before and after neutralization against the tested standard strains. The inhibitory activity of the non-neutralized CFS was markedly greater than that of the neutralized ones. Similarly, it has been reported that the neutralization of CFS of *Lactobacillus* strains with alkali had diminished their antibacterial activity. This finding suggested that the main inhibitory mechanisms might be due to the produced organic acids from glucose fermentation[52].

To the best of our knowledge, the kinetics of *in-vitro* combinations of CFS of *L. plantarum* with gentamicin or ceftazidime against *S. aureus* and *E. coli*, using the time-kill assay, have not been widely tested. Our results showed that the combinations of G1 with each of ceftazidime and gentamicin showed a synergistic effect against *E. coli* while synergism was only obtained in case of G1/ceftazidime combination against *S. aureus*. The chief bacterial inhibitory essentials produced by lactobacilli include the organic acids and bacteriocins[53]. In their study, Chen *et al.* showed that the antibacterial activity of *Lactobacillus* strains was mainly dependent on the production of organic acids that act as permeabilizers of the outer membrane of Gram-negative pathogens thus enhancing the activity of antimicrobial metabolites[54]. In addition, bacteriocin/antimicrobial combinations might have prodigious value in the reduction of the possibility of the development of resistance and reducing the required antibiotic concentration for fruitful treatments[55].

The nCFS of all the tested probiotic candidates showed inhibition of *S. aureus* biofilm formation with a percentage of inhibition ranging between 26 and 52. Similarly, Barzegari *et al.* stated that biosurfactants of both *L. plantarum* and *P. acidilactici* have been reported to inhibit the biofilm formation of *S. aureus* chiefly through affecting the expression of various biofilm-related genes, as well as hindering the release of the signaling molecules in quorum sensing systems[56].

In conclusion, the current study showed that breast milk, dairy products, fermented beverages, and fermented grapes were good sources of promising probiotic candidates belonging to both *Pediococcus* and *Lactobacillus* spp. In addition, the obtained results highlighted the significance of *L. hilgardii*, as a potential probiotic candidate, although it was not thoroughly investigated in previous studies. This study suggested the utilization of such probiotic candidates to formulate novel probiotics that might act as biotherapeutic agents preventing bacterial infection and biofilm formation. However, *in-vivo* trials are crucial to ensure the safety and

efficacy of these probiotics for human health benefit.

Author disclosure statemnt

No conflict of interest exists with this publication. All authors have contributed substantially to the manuscript and have approved the final submission.

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نشرة العلوم الصيدليـــة
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فحص إمكانات عزلات مختارة من سلالات حمض اللبنيك المعزولة من اصول طبيعية كبروبيوتيك وتوصيفها وتقييمها مختبريا للنشاط المضاد للميكروبات ضد بعض مسببات الأمراض البشرية

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أصبحت بكتيريا البروبيوتيك شائعة مؤخرًا لآثارها المفيدة المتعلقة بالصحة. في هذه الدراسة تم عزل ١٩ بكتيريا حمض اللاكتيك من لبن الأم ومنتجات الألبان وبراز الأطفال والمسحات المهبلية والمشروبات المخمرة والعنب المخمر. تم تقييم هذه العزلات من حيث تحملها لظروف الجهاز الهضمي ، ونفور الماء ، والتجمع الذاتي ، والنشاط المضاد للميكروبات. تم فصل خمسة عزلات تنتمي إلى فصيله البيديوكوكس وفصيلة اللاكتوباسيليس كانت لديها ميزات بروبيوتيك محتملة. تم اختبار هذه العزلات لوجود إنزيمات الفوعة والتأثر بالمضادات الحيوية المختلفة. تبين أن العزلات ليس لديها قدرات لتحلل كرات الدم الحمراء وتفتقر إلى إنزيمات الجيلاتيناز وإنزيمات الديوكسي ريبونوكلياز ، ومع ذلك تم الكشف عن بعض الجينات المقاومة للمضادات الحيوية. لضمان سلامة البروبيوتيك المستخدمة ، تم تحضير المواد الطافية الخالية من الخلايا (CFS) للمرشحين الواعدين وتم تقييم نشاطها المضاد للميكروبات قبل وبعد التحييد الحمضي ضد السلالات القياسية من بكتيريا المبيضات البيضاء والبكتيريا إيجابية الجرام وسالبة الجرام. أدى تحييد حمض ال CFS إلى تقليل الأنشطة المضادة للبكتيريا والفطريات للبروبيوتيك بشكل كبير. باستخدام فحص معدل القضاء علي البكتيريا ، تم اختبار مزيج CFS من اللاكتوبلانتيباسيليس بلانتارم مع الجنتاميسين والسيفتازيديم ضد العزلات السريرية من الستافيلوكوكس اوريوس والايشريشيا كولاي تم الحصول على انخفاض ملحوظ في عدد اللوغاريتمات للناجين باستخدام الجنتاميسين ضد الاشريشيا كولاي والسيفتازيديم ضد كلا العزلتين. تم أيضًا اختبار القدرة المضادة للفوعة لـ CFS للبروبيوتيك المختار. تسببت CFS المتعادلة في تثبيط ٢٦-٥٢٪ من تكوين البيوفيلم لعزلة الاستافيلوكوكس اوريوس مقارنةً بالمجموعة الضابطة. في الختام ، يمكن اعتبار العزلات الخمس المختارة كسلالات بروبيوتيك واعدة يمكن استخدامها كعلاج حيوي في حالة العدوى البكتيرية ولمنع تكون الأغشية الحيوية.