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Evaluation of the Antimicrobial Activity of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Human Intestine against Pathogenic Microorganisms

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ABSTRACT

Currently, it is crucial to screen efficient, safe, and accessible therapies from a variety of prospective antimicrobial agents due to the rapid development of microbial resistance against chemotherapeutic drugs (mainly antibiotics). Bacteriocins are a type of antimicrobial peptide created by bacteria that are ribosomally synthesized. Bacteriocins have evolved into one of the tools used to combat bacteria because of their distinctive traits. Therefore, bacteriocins may replace antibiotics to treat multiple drugs resistance pathogens. Accordingly, the purpose of this study was to investigate the antibacterial effects of secondary metabolites from two bacteriocin-producing strains (*Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232)) on 24 pathogenic and spoilage microorganisms either alone or in combination. The 50% inhibitory concentration, minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), or minimum fungicidal concentration (MFC) were determined. The fractional inhibitory concentration (FIC) and its effect were also defined. Results showed that all the 12 bacterial and ten fungal strains were inhibited by both bacteriocin-producing strains and only four fungal strains were not affected by all studied 12 strains. It was found that the bactericidal activity of both bacteriocin-producing strains against *S. aureus*, *L. monocytogenes*, and the fungi strains *M. phaseolina* was the highest among all the tested strains. It was also noticed that a combination of (LA102) and (LC232) gives a 20% synergistic effect and 40 % additive relationship and indifferent relationship without any microbes showing an antagonistic relationship. Moreover, to screen for better functional and bacteriocin-producing strains, this study offers a practical, thorough, and shared profile of newly developed antimicrobial agents.

Keywords: Probiotics, Bacteriocin, Lactic Acid Bacteria, LAB, Antimicrobial, IC_{50}, MIC, MBC, FIC.

Introduction

Probiotics use in the prevention and treatment of illnesses first came to light around the turn of the twentieth century, when Elie Metchnikoff published his findings in 1907, who stated that harmful bacteria in our intestine could be replaced with a beneficial bacterium upon ingesting fermented dairy foods in his famous book "The Prolongation of Life".[1] Lilly and Stillwell coined the phrase "microbially derived factors that stimulate the growth of other organisms" in 1965. [1, 2] Years later, it has been emphasized that in addition to being necessary for viability, they also have a positive impact on the host.[2] It has been argued that probiotics are "living microorganisms, which, upon ingestion in certain numbers, exert health benefits beyond intrinsic nutrition" (a widely accepted definition of probiotics). [3] When consumed in appropriate numbers, probiotics are generally believed to offer several health benefits for both humans and animals. [4]

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Probiotics are members of the lactic acid bacteria family (LAB), primarily *Bifidobacteria*, *Lactobacilli*, and *Enterococcus*. *Pediococcus*, *Bacillus*, and several yeasts have also been identified as acceptable candidates. [3, 5] Gram-positive, non-spore-forming, catalase-negative, non-motile rods and cocci bacteria comprise the LAB. The species now employed in probiotic formulations are diverse, although the majority contain *Bifidobacterium* (*B.*) and *Lactobacillus* (*L.*) spp.[6-8] The growing public interest in probiotic products and their health benefits has necessitated the hunt for new probiotic species with greater beneficial actions.[7, 9] Probiotics are thought to offer numerous health benefits in both people and animals when consumed in sufficient quantities. [3, 4] These health benefits include improved gastrointestinal tract health and immune system modulation, anti-carcinogenic and anti-diarrheal properties, and cholesterol-lowering properties.[10] Other partially confirmed research is currently being undertaken to ascertain the health claims of probiotics against a variety of ailments such as autism, allergies, and oral health. [11, 12] Other novel health benefits discovered in the last few decades include improved immune system response, reduced postmenopausal symptoms, and improved skin health. [13, 14] In general, probiotic organisms are selected based on specific physiological and biochemical criteria that ensure their viability and maximum efficacy, such as their ability to produce lactase and vitamins, antioxidative properties, cholesterol assimilation, and the ability to withstand process and storage conditions, as well as the production of antimicrobial substances against pathogenic bacteria. [7, 15-16]

Many lactic acid bacteria strains are effective probiotics that treat various diseases by acting in multiple ways. These bacteria produce bacteriocins, antimicrobial peptides that either suppress or eliminate

other harmful bacteria including *Listeria*, *Clostridium*, *Salmonella*, and different closely related strains. Bacteriocins are cationic peptides that cause cell death by creating pores in the target cells and releasing the contents of the cytoplasm. Bacteriocins are also known to modify the host's immune system and native microbiota, which can impact several of the host's processes that support health.[17, 18]

According to the WHO, multidrug-resistant pathogens are an important global public health issue. [19] Common antibiotics are no longer as effective due to the fast spread of those pathogens.[20,21] Thus, there is a specific need to search for new antimicrobial substances, particularly those targeted at multidrug-resistant pathogens.[20] Accordingly, Bacteriocin is the most important class of antimicrobial peptides with uses in human health. It is generally known that relevant pathogenic microorganisms, particularly multidrug-resistant pathogens, can be killed or inhibited by bacteriocins *in vitro*.[22]

In the present study, the antimicrobial activity of bacteriocin-produced by two probiotic strains, *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) isolated from the intestine of healthy, breastfed, newborn Jordanian infants, screened and evaluated against a wide range of pathogenic and spoilage microorganisms, including 12 strains of bacteria and 12 strains of fungi.

Materials and Methods

Probiotic Bacteria: Source and Culture Conditions

Two probiotic bacteriocin-producing strains, *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232), were poised from the culture collection of the Department of Nutrition and Food Technology, Al-Balqa Applied University (Salt, Jordan). The strains were chosen based on a prior study published in 2016 that found promising anticancer activity. They are also known for their ability to produce bacteriocin.[18, 24] These bacterial strains also were previously shown to have good probiotic qualities such as high acid and bile resistance, good adhesion properties, antibacterial, antioxidant, and robust *in vivo* hypocholesterolemic activity.[6,18] Previously, these strains were identified from healthy, breastfed newborn Jordanian infants. Isolates were cultivated in modified MRS (M-MRS) broth to eliminate the generation of any chemicals other than bacteriocins, such as Hydrogen peroxide (H_2O_2) and lactic acid which are produced by LAB strains, isolates were grown in modified MRS (M-MRS) broth. M-MRS broth had been modified by the addition of phosphate buffer (pH: 7.0) to prevent pH decrease owing to lactic acid production. The medium was additionally supplemented with 0.2% glucose and 0.02% L-Cysteine (Oxoid Ltd., Basingstoke, UK) and stored at 4°C between transfers. [6, 18]

Probiotic Extracts: Preparation and Characterization.

The two isolates *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) were grown on MRS broth (Oxoid, UK) and incubated for 24 hours at 37°C using anaerobic kits (Gas generation kit, Carbon Dioxide system, 120 Oxoid Ltd, Basingstoke, UK). [24] The LAB cultures were then centrifuged (5000 g/15 min/27°C) and sonicated four times at 14-second intervals with four-minute freezing cycles using a Sonicator (ultrasonic cleaner, Jeiotech UC-10; LAB COMPANION). With 0.1M sodium hydroxide (NaOH), the pH of the cell-free supernatant was brought down to 7.0. The cell-free supernatant was then concentrated using a rotational vacuum concentrator (RVC 2-18 CD, CHARIST), filter-sterilized with a micro-filter (0.22 µm; Millipore Ltd., Hertfordshire, England), and kept at 2°C until used.[18,23]

Antimicrobial activity

Microbial Strains and culture conditions

Standardized pure cultures of bacterial and fungal strains were obtained from the University of Jordan Hospital Laboratory in Amman, and the Faculty of Agriculture Department of Biotechnology, Al-Balqa Applied University (Table 1, Table 2). Isolates were maintained separately at 20°C in Brain Heart Infusion (BHI, Difco, MD, USA) for bacteria and Potato Dextrose (PDA, Oxoid) with 20% glycerol for fungi. Bacterial strains were identified before the experiment using the Gram stain, oxidase, and catalase assays, as stated in (Table 1). Each culture was revived three times before being transferred separately to BHI broth for bacteria and saline solution (0.85%) supplemented with (0.2%) Tween-80 for fungal strains, respectively. They were grown at 37°C and 27°C for 24 and 48 hours, for bacteria and fungi, to reach the stationary phase. [21, 25]

Table 1: Bacterial strains were used in this study

No.	Microbe name	Origin	Properties	Catalase test	Oxidase test
1.	*Listeria monocytogenes*	ATCC 19111	Gram-positive, rod-shaped, non-spore-forming, facultative anaerobic	Positive	Positive
2.	*Listeria monocytogenes*	ATCC 15313			
3.	*Escherichia coli*	ATCC 25922	Gram-negative, rod-shaped, non-spore-forming, flagellated, facultatively anaerobic.	Positive	Negative
4.	*Escherichia coli (O157:H7)*	ATCC 35150			
5.	*Salmonella typhimurum*	ATCC 14028	Gram-negative, motile by peritrichous flagella, non-spore-forming, motile, aerobic to facultative anaerobic	Positive	Negative
6.	*Salmonella typhimurum*	ATCC 13311			
7.	*Staphylococcus aureus*	ATCC 25923	Gram-positive cocci, grow in clusters, nonmotile, non-spore-forming	Positive	Negative
8.	*Staphylococcus aureus*	ATCC 13567			
9.	*Geobacillus steriothermophilus*	ATCC 12980	Gram-positive, rod-shaped, spore-forming	Positive	Positive
10.	*Pseudomonas aeruginosa*	ATCC 27853	Gram-negative, rod-shaped, motile, non-spore-forming, facultative aerobes	Positive	Positive
11.	*Clostridium botulinum*	ATCC 3502	Gram-positive, rod-shaped, anaerobic, spore-forming, motile	Negative	Negative

Table 2: Fungal strains were used in this study

No.	Microbe name	Origin
1.	*Fusarium oxysporum f. sp. lini*	Plant origin
2.	*Fusarium oxysporum f. sp. lycopersici*	MTCC. 10270
3.	*Aspergillus niger*	MTCC 872
4.	*Aspergillus flavus*	MTCC 13062
5.	*Aspergillus fumigatus*	MTCC 2550
6.	*Beauveria bassiana*	Plant origin
7.	*Rhizopus stolonifer.*	Plant origin
8.	*Fusarium fujikuroi*	MTCC 9930
9.	*Candida albicans*	MTCC 3017
10.	*Cephalosporium aphidicola*	Plant origin
11.	*Macrophomina phaseolina*	MTCC 2165
12.	*Curvularia lunata*	Plant origin

Screening of Antimicrobial activity

To achieve an inoculum of about (10^5 CFU/ml), an 18-hour culture was diluted with a sterile physiological saline solution of 0.85% (w/v) sodium chloride supplemented with (0.2%) Tween 80, which was chosen after procedure optimization. Bacterial inoculum (100µl) was inoculated on the surface of pre-dried Tryptic Soy Agar plates (TSA; Oxoid) and allowed to fully dry. Mold inoculums, on the other hand, were cultivated on Potato Dextrose Agar (PDA; Oxoid) using the pour plate technique. The antibacterial activity was determined using the agar well diffusion assay (AWDA). Using capillary pasture pipettes (Borosilicate glass, Fisher Scientific Company), wells (5mm) were formed on each plate, and 25µl of each bacteriocin-producing bacterial extract, either alone or in combination, was added. To rule out any impact of other ingredients used in dissolving or preparing the extract on the investigated bacteria, un-inoculated M-MRS broth was used as a negative control. Standard antibiotic discs were used also as a positive control against bacterial strains whereas Amphotericin B (AB) 10µg/ml was used against fungus strains for comparison purposes. After allowing the extract to diffuse across the surface of petri-dishes for 20 minutes at room temperature, bacteria plates were incubated at 37°C for 24 hours and mold plates were incubated at 27°C for 48 hours. The inhibitory zone was measured using a caliper (in millimeters), and the assay was repeated three times in two independent experiments. [21, 24-25]

Determination of Minimum Inhibitory Concentration (MIC) Minimum Bactericidal Concentration (MBC) and/or Minimum Fungicidal Concentration (MFC)

Each probiotic bacteriocin-producing strain (*Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232)) either separately or together, had their MIC values determined. The 96-well micro-dilution method was used to calculate MIC values.[6,21,28] The corresponding wells received 100µl of an overnight culture containing 6.0 log10 CFU/ml of each bacteria and mold. Each extract was made as a two-fold serial dilution using Dimethyl sulfoxide (DMSO) at concentrations ranging from 0.08 µl/ml to 100 µl/ml. Each well was then filled with 100 µl of each serial dilution, resulting in a total volume of 200 µl. The plates were then sealed and incubated at 37°C and 27°C for 24 and 48 hours, respectively, for bacteria and mold plates. Using a microplate reader (ELX 800, Biotek, Highland Park, VT, USA), absorbance (Abs) was measured at 600 nm. Additionally, negative controls were made with un-inoculated M-MRS broth and 0.05% DMSO. By using the pour plate technique; 20 µl aliquots from the clear wells were cultured to calculate both the MBC and MFC. [6, 21, 28]

Determination of fractional inhibitory concentration index (FIC)

The inhibitory concentration of the antimicrobial combination divided by the inhibitory concentration of the individual antibacterial component is known as the fractional inhibitory concentration index (FIC).[28] The MICs of all drug formulae, both alone and in combination, were used to construct the FIC index for the combination of two different antimicrobial agents. The FIC was calculated for each combination using the following formula:

FIC drug (A) = MIC of a drug (A) when tested in combination with drug (B) / MIC of drug (A) alone

FIC drug (B) = MIC of the drug (B) when tested in combination with drug (A) / MIC of the drug (B) alone

FIC = FIC drug (A) + FIC drug (B). [28]

The FIC results were interpreted as follows: ≤ 0.5: synergistic activity, 0.5-1: additive activity, 1-4: indifference, > 4: antagonism.[28]

Statistical analysis

GraphPad Prism, ANOVA test was performed to find any statistical differences between the control group and treatment groups. Then Dunnett's post hoc analysis was applied. A p-value of 0.05 or less was recognized as statistical significance in all studies. Data were expressed as the means ± the standard error of means (SEM) values of two independent experiments.

Results and Discussion

This paper aimed to screen for efficient, secure, and accessible therapies from prospective antimicrobial agents due to the rapid development of microbial resistance against chemotherapeutic drugs. Bacteriocins produced by lactic acid bacteria are considered convenient, comprehensive, superior, and functional compounds with antimicrobial activity. The key outcomes of screening for antibacterial and antifungal activity of various bacteriocin-producing LAB extracts and their combinations against various bacterial and fungal stains are shown in Tables 3 and 4, respectively. Probiotic extracts were shown to have significant inhibitory activity (6–13 mm) against all of the tested bacterial strains, whilst their antifungal activity ranged from negative effects against four of the tested fungal strains to strong inhibition activity (2–10 mm) against *B. bassiana* and *G. fujikuroi*. Both bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) had inhibition zones ranging from 18.78 to 10 mm in diameter against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Staphylococcus aureus*, *Geobacillus steriothermophilus*, and *Pseudomonas aeruginosa*. Bacteriocin-producing strain *Lacticaseibacillus casei* (LC232) had low inhibitory activity against both strains of *C. botulinum* (ATCC 3502) and (ATCC 19397), scoring (6.24 ± 0.045 mm) and (6.57 ± 0.012 mm), respectively. On the other hand; bacteriocin-producing strain *Lactobacillus acidophilus* (LA102) showed a mild inhibition activity (9.76 ± 0.066 mm) and (9.15 ± 0.057 mm), respectively. There was a huge variation in inhibition between bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) against *P. aeruginosa* (18.78 ± 0.035 mm) and (11.35 ± 0.066 mm), respectively. On the other hand, combined treatment scored (17.00± 0.024 mm). *S. typhimurium* (ATCC 14028) was inhibited with both bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) more than the *S. typhimurium* (ATCC 13311) ((15.57± 0.045 mm) and (11.78 ± 0.057 mm)) and ((611.75 ± 0.033 mm) and (6.57 ± 0.012 mm))

Probiotics are thought to be a strong compound against some diseases and have a well-known antibacterial action. Probiotic extracts contain a variety of substances with various hypothesized modes of action, such as organic acids, hydrogen peroxide, bacteriocins, diacetyl, and numerous other inhibitory substances. By cultivating the lactic acid bacteria strains on modified media (M-MRS), our study attempted to reduce the impact of these organic acids and hydrogen peroxides produced by these bacteria, leaving the inhibitory effect to come entirely from bacteriocins. Toxic substances known as bacteriocins are effective against many pathogenic strains. [24, 28-29] Recent research shows that probiotic extracts have a weak inhibitory effect on most of the mold strains, as just 4 of the 12 investigated mold strains were not inhibited by them. Those findings that *Lactobacillus acidophilus* produced chemical compounds that were efficient against both Gram-positive and Gram-negative bacteria, such as *S. typhimurium*, *S. aureus*, *L. monocytogenes*, and *E. coli*, were supported by several investigations. [30-32] Bacteriocin is known to be more effective against Gram-positive

bacteria; Gram-negative bacteria may be resistant to such powerful substances since they have an additional outer membrane that serves as a barrier for those microorganisms.[33]

Compared to positive controls, bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) had lower antimicrobial activity in both gram-positive, gram-negative, and fungi strains. For example; using (10 µg\ml) of Amphotericin B (AB) inhibits the growth of *Fusarium oxysporum f. sp. Lini, Fusarium fujikuroi*, and *Aspergillus flavus* up to 18.0 mm in diameter (Table 4).

Using the bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232), our results showed that treatments with these extracts had variable antimicrobial activities against various types of pathogenic microorganisms, including both Gram-negative and Gram-positive bacteria. The IC_{50} ranged from 75.9 to 29.2 and from 77.3 to 33.21, respectively (Table 5). Whereas the IC_{50} against fungal strains ranged from 57.128 to 37.028 and from 75.032 to 43.057 using Bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) respectively (Table 6). The development of LAB was shown to produce antifungals, mycotoxin synthesis inhibitors, and mycotoxin detoxifying agents. These organisms have been shown to produce fatty acids, cyclic dipeptides, proteinaceous substances, organic acids, and bacteriocins with potent antifungal effects; however, in some instances, the mechanism of action—which is still unknown in others—seems to include damage to the fungal membrane.[33-34]

These findings are consistent with published reports that the genus *Pediococcus* a lactic acid bacteria- bacteriocin-producing strain; is capable of producing antifungal compounds.[34] For example, *Pediococcus acidilactici* LAB isolated from meat was able to restrict the growth of food- and foodborne molds as well as plant-pathogenic fungi. *P. pentosaceus* (L006) isolated from maize leaves was able to control the growth of mycotoxigenic molds such as a fungus that produces fumonisin.[34-35]

The growth of *L. monocytogenes*, *E. coli*, *S. aureus, S. typhimurium*, and G. *steriothermophillus* was all considerably suppressed by a therapy combining the bacteriocin-producing strains *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232). Numerous research that support our findings revealed that combinations of therapies were more successful. [35] It has been shown that using a combination of substances boosts the effectiveness of the extracts because of their various biochemical properties, which result in protein dislocation, protein transport inhibition, phosphorylation, and inhibition of some enzymatic action by causing membrane disruption and cell membrane destruction, which kills the microorganism.[35-36]

Table 3: The inhibitory zone in millimeters (mm) generated through tested extracts against various microbial strains that were measured by caliper

No.	Microbe name	Origin	Bacteriocin-producing LAB strain (LC232) (1,2,3)	Bacteriocin-producing LAB strain (LA102) (1,2,3)	Combined treatment of both stains (LC232 + LA102) (1,2,3)	Control positive (Antimicrobial agent) (1,2,4)
1.	*L. monocytogenes*	ATCC 19111	16.45 $^{b(a)}$ ± 0.066	14.92$^{d(f)}$ ± 1.120	14.56 $^{c(e)}$ ± 0.045	17.00 ^{a} ± 0.066 (STR)
2.	*L. monocytogenes*	ATCC 15313	12.66 $^{d(cde)}$ ± 0.005	15.65$^{cb)}$ ± 0.066	15.89 $^{b(d)}$ ± 0.023	16.00 ^{a} ± 0.035 (STR)
3.	*E. coli*	ATCC 25922	12.76 $^{d(c)}$ ± 0.045	15.57$^{c(bc)}$ ± 0.067	16.32$^{b(c)}$ ± 0.037	19.00 ^{a} ± 0.032 (AMP)
4.	*E. coli*	ATCC 35150	14.15 $^{c(b)}$ ± 0.035	10.86$^{d(j)}$ ± 0.033	13.22 $^{b(h)}$ ± 0.066	20.00 ^{a} ± 0.033 (AMP)
5.	*S. typhimurum*	ATCC 14028	11.78 $^{c(f)}$ ± 0.057	15.57 $^{b(bed)}$ ± 0.045	17.20 $^{b(a)}$ ± 0.057	18.00 ^{a} ± 0.005 (AMP)
6.	*S. typhimurum*	ATCC 13311	8.97 $^{d(j)}$ ± 0.066	11.75 $^{c(i)}$ ± 0.033	14.00 $^{b(g)}$ ± 0.005	22.00 ^{a} ± 0.045 (AMP)
7.	*S. aureus*	ATCC 25923	10.86 $^{d(i)}$ ± 0.096	12.66 $^{c(g)}$ ± 0.036	12.32 $^{b(i)}$ ± 0.066	15.00 ^{a} ± 0.024 (P)
8.	*S. aureus*	ATCC 13567	11.46 $^{d(g)}$ ± 1.120	14.45 $^{bc(e)}$ ± 1.185	14.22 $^{b(f)}$ ± 0.005	15.00 ^{a} ± 0.033 (P)
9.	*G. steriothermophilus*	ATCC 12980	12.67 $^{c(cd)}$ ± 0.005	12.57 $^{bc\ (gh)}$ ± 0.035	12.03 $^{d(j)}$ ± 0.032	16.00 ^{a} ± 0.045 (van)
10.	*P. aeruginosa*	ATCC 27853	11.35 $^{d(h)}$ ± 0.066	18.78 $^{c(a)}$ ± 0.035	17.00 $^{b(b)}$ ± 0.024	23.00 ^{a} ± 0.024 (AMK)
11.	*C. botulinum*	ATCC 3502	6.24 $^{d(l)}$ ± 0.045	9.76 $^{d(h)}$ ± 0.066	9.98 $^{b(l)}$ ± 0.001	12.00 ^{a} ± 0.066 (Van)
12.	*C. botulinum*	ATCC 19397	6.57 $^{d(k)}$ ± 0.012	9.15 $^{c(k)}$ ± 0.057	11.01 $^{b(k)}$ ± 0.005	12.00 ^{a} ± 0.032 (Van)

(1): Results are Mean ± standard error of the mean (SEM) of three determinations of two independent experiments.
(2): Results with different letters in the same row are significantly different ($p<0.05$).
(3): Results with different letters between brackets in the same column are significantly different ($p<0.05$).
(4): Streptomycin (STR), Vancomycin (V), Ampicillin (AMP), Penicillin (P), and Amikacin (AMK) were used as positive controls for bacterial strains

Table 4: The inhibitory zone in millimeters (mm) generated through tested extracts against various microbial strains that were measured by caliper

No.	Microbe name	Origin	Bacteriocin-producing LAB strain (LC232) (1,2,3)	Bacteriocin-producing LAB strain (LA102) (1,2,3)	Combined treatment of both stains (LC232 + LA102) (1,2,3)	(Amphotericin B (AB) 10 µg\ml) (1,2,4)
1.	*F. lini*	Plant origin	4.57 $^{d(f)}$ ± 0.032	6.55 $^{c(g)}$ ± 0.033	8.15 $^{b(f)}$ ± 0.015	18.00 ^{a} ± 0.005
2.	*F. lycopersici*	MTCC. 10270	6.58 $^{d(e)}$ ± 0.012	8.22 $^{c(e)}$ ± 0.057	8.80 $^{b(e)}$ ± 0.023	16.00 ^{a} ± 0.066
3.	*A. niger*	MTCC 872	6.76 $^{d(d)}$ ± 0.033	9.15 $^{c(d)}$ ± 0.076	10.32$^{b(b)}$ ± 0.005	14.00 ^{a} ± 0.032
4.	*A. flavus*	MTCC 13062	2.45 $^{d(h)}$ ± 0.066	3.67 $^{c(h)}$ ± 0.035	6.22 $^{b(h)}$ ± 0.066	18.00 ^{a} ± 0.022
5.	*A. fumigatus*	MTCC 2550	8.35 $^{d(c)}$ ± 0.012	12.24 $^{b(b)}$ ± 0.066	10.15 $^{c(d)}$ ± 0.007	15.00 ^{a} ± 0.024
6.	*B. bassiana*	Plant origin	-	-	-	16.00 ± 0.033
7.	*Rh. stolonifer.*	Plant origin	-	-	-	20.00 ± 0.024
8.	*F. fujikuroi*	MTCC 9930	8.45 $^{d(b)}$ ± 1.120	10.22 $^{bc(bc)}$ ± 1.120	10.22 $^{b(c)}$ ± 0.005	18.00 ^{a} ± 0.005
9.	*C. albicans*	MTCC 3017	4.45 $^{d(g)}$ ± 0.012	6.78 $^{c(f)}$ ± 0.005	8.00 $^{b(g)}$ ± 0.022	20.00 ^{a} ± 0.022
10.	*C. aphidicola*	Plant origin	-	-	-	22.00 ± 0.045
11.	*M. phaseolina*	MTCC 2165	10.18$^{d(a)}$ ± 0.450	12.35 $^{c(a)}$ ± 0.570	14.22 $^{b(a)}$ ± 0.003	18.00 ^{a} ± 0.022
12.	*C. lunata*	Plant origin	-	-	-	14.00 ± 0.022

(1 The results reflect the mean± standard error of the mean (SEM) of three determinations from two separate experiments.
(2): Results with different letters in the same row are significantly different ($p<0.05$).
(3): Results with different letters between brackets in the same column are significantly different ($p<0.05$).
(4): Amphotericin B (AB) was used as a control positive.
(-) : have no effect

Table 5: IC_{50} values of drugs used against different bacterial strains data were screened using the wells diffusion method

No.	Microbe name	Origin	Bacteriocin-producing LAB strain (*LC232*) (1,2)	Bacteriocin-producing LAB strain (*LA102*) (1,2)
1	*L. monocytogenes*	ATCC 19111	46.550^{c} ± 0.001	36.2270 ^{b}i± 0.032
2	*L. monocytogenes*	ATCC 15313	48.652^{b} ± 0.032	34.056 ^{k} ± 0.128
3	*E. coli*	ATCC 25922	35.36 ^{h} ± 0.005	54.466 ^{e} ± 0.006
4	*E. coli*	ATCC 35150	38.223 ^{f} ± 0.055	33.214 ^{l} ± 0.006
5	*S. typhimurum*	ATCC 14028	35.360 ^{hi} ± 0.065	55.416 ^{d} ± 0.002
6	*S. typhimurum*	ATCC 13311	29.542 ^{j} ± 0.128	47.426 ^{f} ± 0.001
7	*S. aureus*	ATCC 25923	26.210 ^{l} ± 0.032	37.873 ^{h} ± 0.055
8	*S. aureus*	ATCC 13567	28.340 ^{k} ± 0.032	44.221 ^{g} ± 0.012
9	*G. steriothermophilus*	ATCC 12980	57.930 ^{a} ± 0.012	35.360 ^{j} ± 0.032
10	*P. aeruginosa*	ATCC 27853	37.955 ^{g} ± 0.055	77.360 ^{a} ± 0.012
11	*C. botulinum*	ATCC 3502	41.420 ^{e} ± 0.065	66.960 ^{b} ± 0.005
12	*C. botulinum*	ATCC 19397	44.230 ^{d} ± 0.012	65.802 ^{c} ± 0.002

(1): The results reflect the mean± standard error of the mean (SEM) of three determinations from two separate experiments.
(2): Results with different letters in the same row are significantly different ($p<0.05$)

When treated with *Lacticaseibacillus casei* (LC232) alone, the MIC values for *L. monocytogenes*, S. *typhimurium, G. steriothermophillus*, and *P. aeruginosa* were 10 µg/ml for all ATCC strains. Additionally, 10 µg/ml of *Lactobacillus acidophilus* (LA102) treatment was required to stop their proliferation (Table 7). It was found that the MIC values were (1.25, 2.50, and 5 µg/ml), respectively, against those strains when a combined treatment were used from both extracts (Table 7), demonstrating that both extracts exhibit a very powerful bactericidal action when added to one another and even better than being alone. A combined treatment made from *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) demonstrated low MIC values (10 µg/ml) against both strains of *C. botulinum*.

Natural antimicrobial peptides are regarded as the most rudimentary and ancient form of immunity among all types of organisms. Prokaryotes create bacteriocins, which are gene-coded, ribosomally manufactured peptides that only affect a small subset of sensitive bacteria.[38] One of the promising groups of antibiotics, these cationic small peptides may replace traditional antimicrobial medicines in the treatment of infectious disorders and help counteract the ongoing development of antibiotic resistance against dangerous bacteria. [30, 38]

When *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) were used as independent treatments, the MBC of *L. monocytogenes*, *E. coli*, *S. aureus*, *S. typhimurium*, and *G. steriothermophillus* was 20 g/ml for all strains, while the MBC value using a combined treatment was reduced to half of this value (10 g/ml) (Table 7). Both probiotic bacteria extracts (*Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232)) exhibit a high fungicidal action, as evidenced by low MIC values against *A. flavus* and *F. fujikuroi* (5.0 g/ml and 10.0 g/ml), respectively (Table 8). Those findings were supported by MBC values of (10.0 g/ml and 20.0 g/ml) and (5.0 g/ml and 10.0 g/ml), respectively (Table 8). A combination of

Lactobacillus acidophilus (LA102) and *Lacticaseibacillus casei* (LC232) has a high fungicidal activity, as evidenced by low MIC values against the identical strains (*A. flavus* and *F. fujikuroi*) (5.0g/ml and 10.0g/ml, respectively) (Table 11). The MFC for the combination therapy was (10.0 g/ml vs. 5.0 g/ml) (Table 8).

The fractional inhibitory concentration index (FIC) was calculated for the combination of various two antimicrobial agents utilizing the MICs of all medication formulae determined alone and in combination. (Table 9, Table 10, and Table 11), the FIC was calculated for the bacteriocin-producing strains combination and the FIC results were interpreted as follows: ≤ 0.5: synergistic activity, 0.5-1: additive activity, 1-4: indifference, > 4: antagonism. [28] For all microbial isolates, the percentage of isolates for which the corresponding combination generated an antagonistic, indifferent, additive, or synergistic effect was computed. A combination treatment of *Lacticaseibacillus casei* (LC232) and *Lactobacillus acidophilus* (LA102) results in a 20% synergistic effect, 40% additive relationship, and 40% indifference in their interaction, with no microorganisms demonstrating antagonistic association (Table 11).

A combined treatment of *Lactobacillus acidophilus* (LA102) and *Lacticaseibacillus casei* (LC232) results in a 20% synergistic effect, 40% additive relationship, and 40% indifference in their relationship with no microorganisms demonstrating antagonistic association (Table 11).

It was also shown that a combination of *Lactobacillus acidophilus* (LA102) with *Lacticaseibacillus casei* (LC232) was more effective against both strains of *L. monocytogenes* and *E. coli*, demonstrating a synergistic effect. On the other hand, a mixture of *Lactobacillus acidophilus* (LA102) with *Lacticaseibacillus casei* (LC232) shows an additive activity.

Table 6: IC_{50} values of drugs used against different fungal strains data were screened using the wells diffusion method

No.	Microbe name	Origin	Bacteriocin-producing LAB strain (*LC232*) (1,2)	Bacteriocin-producing LAB strain (*LA102*) (1,2)
1	*F. lini*	Plant origin	55.360 ^{c} ± 0.006	56.232 ^{d} ± 0.025
2	*F. lycopersici*	MTCC. 10270	44.033 ^{g} ± 0.007	55.128 ^{e} ± 0.026
3	*A. niger*	MTCC 872	67.128^{a} ± 0.032	66.554 ^{c} ± 0.008
4	*A. flavus*	MTCC 13062	37.028 ^{h} ± 0.033	46.004 ^{g} ± 0.009
5	*A. fumigatus*	MTCC 2550	44.146 ^{f} ± 0.034	43.057 ^{h} ± 0.010
6	*B. bassiana*	Plant origin	-	-
7	*Rh. stolonifer.*	Plant origin	-	-
8	*F. fujikuroi*	MTCC 9930	49.244 ^{d} ± 0.027	75.032^{a} ± 0.075
9	*C. albicans*	MTCC 3017	65.023^{b} ± 0.032	55.000 ^{ef} ± 0.064
10	*C. aphidicola*	Plant origin	-	-
11	*M. phaseolina*	MTCC 2165	47.128 ^{e} ± 0.034	75.000 ^{ab} ± 0.066
12	*C. lunata*	Plant origin	-	-

(1): results are Mean ± standard error of the mean (SEM) of three determinations of two independent experiments
(2): Results with different letters in the same row are significantly different ($p<0.05$).
(-) : have no effect

Table 7: MIC and MBC of bacteriocin were used against different bacterial strains. Values were screened using the wells micro-dilution method by using 96-well plates. The MIC values represented the lowest drug dilution at which the growth is absent, and MBC values were obtained by plating 20µl aliquots from the clear wells

No.	Microbe name	Origin	Bacteriocin-producing LAB strain (LC232)		Bacteriocin-producing LAB strain (LA102)		Combined treatment of both stains (LC232 + LA102)	
			(MIC / MBC (µg/ml))		(MIC / MBC (µg/ml))		(MIC / MBC (µg/ml))	
1.	*L. monocytogenes* (1,2)	ATCC 19111	10	20	10	20	1.25	10
2.	*L. monocytogenes* (1,2)	ATCC 15313	10	20	10	20	1.25	10
3.	*E. coli*(1,2)	ATCC 25922	10	20	5	10	0.625	10
4.	*E. coli (O157:H7)* (1,2)	ATCC 35150	5	10	10	20	0.625	10
5.	*S. typhimurum*(1,2)	ATCC 14028	10	20	5	10	2.5	10
6.	*S. typhimurum*(1,2)	ATCC 13311	10	20	5	10	2.5	10
7.	*S. aureus*(1,2)	ATCC 25923	2.5	10	2.5	5	1.25	2.5
8.	*S. aureus*(1,2)	ATCC 13567	2.5	10	2.5	5	1.25	2.5
9.	*G. steriothermophilus*(1,2)	ATCC 12980	10	20	12.5	25	5	10
10.	*P. aeruginosa*(1,2)	ATCC 27853	10	20	12.5	25	5	10
11.	*C. botulinum*(1,2)	ATCC 3502	12.5	25	15	30	10	20
12.	*C. botulinum*(1,2)	ATCC 19397	12.5	25	15	30	10	20

(1): Microbial strains with MIC values.
(2): Microbial strains with MBC values.

S. aureus, *S. typhimurum*, and *G. steriothermophillus* (Table 9) and against *A. fumigatus*, and *M. phaseolina* (Table 10). Synergistic treatment combinations may successively disrupt a microbial metabolic pathway, with one acting as a cell wall inhibitor and enhancing the entry of the active ingredient of the other treatment into the bacteria, resulting in synergistic effects.[40] As in the instance of employing probiotic bacteria extracts against Gram-negative bacteria, one treatment may change the cell membrane and allow the second treatment to enter. Another potential reason for synergism is that therapy may prevent the second treatment from being inactivated by microbial enzymes, or they may simply block the microbial metabolic pathway.[28,39,40]

Conclusion

It was found that both bacteriocin-producing strains inhibited all the 12 bacterial and 10 fungal strains and only four fungal strains were not affected by all studied 12 strains. Moreover, this study provides a convenient, comprehensive, and shareable profile for screening superior functional and bacteriocin-producing LAB strains.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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Table 8: MIC and MFC of bacteriocin used against different fungal strains. Values were screened using the wells micro-dilution method by using 96-well plates. The MIC values represented the lowest drug dilution at which the growth is absent, and MBC values were obtained by plating 20µl aliquots from the clear wells

No.	Microbe Name	Origin	Bacteriocin-producing LAB strain (LC232) (MIC / MFC (µg/ml))		Bacteriocin-producing LAB strain (LA102) (MIC / MFC (µg/ml))		Combined treatment of both stains (LC232 + LA102) (MIC / MFC (µg/ml))	
1.	*F. Lini* (1,2)	Plant origin	10	20	10	20	12.5	25
2.	*F. lycopersici* (1,2)	MTCC. 10270	10	20	10	20	10	20
3.	*A. niger* (1,2)	MTCC 872	10	20	12.5	25	10	20
4.	*A. flavus* (1,2)	MTCC 13062	5	10	10	20	5	10
5.	*A. fumigatus* (1,2)	MTCC 2550	5	10	5	10	2.5	5
6.	*B. bassiana*	Plant origin	NI[3]	NI	NI	NI	NI	NI
7.	*Rh. stolonifera*	Plant origin	NDI	NI	NI	NI	NI	NI
8.	*F. fujikuroi* (1,2)	MTCC 9930	2.5	5	2.5	5	2.5	5
9.	*C. albicans* (1,2)	MTCC 3017	10	20	10	20	12.5	25
10.	*C. aphidicola*	Plant origin	NI	NI	NI	NI	NI	NI
11.	*M. phaseolina* (1,2)	MTCC 2165	10	20	10	20	5	10
12.	*Curvularia lunata*	Plant origin	NI	NI	NI	NI	NI	NI

(1): Microbial strains with MIC values.
(2): Microbial strains with MFC values.
(3): NI: Not Identified.

Table 9: Fractional inhibitory concentration index (FIC), results for the antimicrobial combination against each bacterial isolate

No.	Microbe Name	Origin	FIC Index*	Effect of combined treatment against microbe**
1.	*L. monocytogenes*	ATCC 19111	0.25	synergistic activity

2.	*L. monocytogenes*	ATCC 15313	0.25	synergistic activity
3.	*E. coli*	ATCC 25922	0.1875	synergistic activity
4.	*E. coli*	ATCC 35150	0.1875	synergistic activity
5.	*S. typhimurum*	ATCC 14028	0.75	additive activity
6.	*S. typhimurum*	ATCC 13311	0.75	additive activity
7.	*S. aureus*	ATCC 25923	1	additive activity
8.	*S. aureus*	ATCC 13567	1	additive activity
9.	*G. steriothermophilus*	ATCC 12980	0.9	additive activity
10.	*P. aeruginosa*	ATCC 27853	0.9	additive activity
11.	*C. botulinum*	ATCC 3502	1.466667	Indifference
12.	*C. botulinum*	ATCC 19397	1.466667	Indifference

*: Each isolate's FIC was determined in triplicate.
**: The percentage of isolates that had an antagonistic, indifferent, additive, or synergistic effect as a result of the corresponding combination is shown.

Table 10: Fractional inhibitory concentration index (FIC), results for the antimicrobial combination against each isolate

No.	Microbe Name	Origin	FIC Index*	Effect of Combined Treatment Against Microbe**
1.	*F. lini*	Plant origin	2.5	indifference
2.	*F. lycopersici*	MTCC. 10270	2	indifference
3.	*A. niger*	MTCC 872	1.8	indifference
4.	*A. flavus*	MTCC 13062	1.5	indifference
5.	*A. fumigatus*	MTCC 2550	1	additive activity
6.	*B. bassiana*	Plant origin	NI[1]	NI
7.	*Rh. stolonifer.*	Plant origin	NI	NI
8.	*F. fujikuroi*	MTCC 9930	2	indifference
9.	*C. albicans*	MTCC 3017	2.5	indifference
10.	*C. aphidicola*	Plant origin	NI	NI
11	***M. phaseolina***	MTCC 2165	1	additive activity
12	***C. lunata***	Plant origin	NI	NI

(1): NI: Not Identified.
*: Each isolate's FIC was determined in triplicate.
**: The percentage of isolates that had an antagonistic, indifferent, additive, or synergistic effect as a result of the corresponding combination is shown.

Table 11: Results for the antibacterial combination against all isolates according to the fractional inhibitory concentration index (FIC)

Tested extracts	Additive (%)	Indifference (%)	Synergic (%)	Antagonism (%)	Mean+ SD**	Min/Max
*LC232 + LA102**	40%	40%	20 %	0%	0.56 $\pm$ 0.33	0.1875/2.5

*: The percentage of isolates that had an antagonistic, indifferent, additive, or synergistic effect as a result of the corresponding combination is shown.
**: Each antimicrobial combination's mean $\pm$ SD, lowest and maximum FIC against all microbial isolates is presented.

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