Jalali *et al. AMB Express* (2024) 14:114
https://doi.org/10.1186/s13568-024-01768-3

AMB Express

ORIGINAL ARTICLE Open Access

Functional properties and safety traits of *L. rhamnosus* and *L. reuteri* postbiotic extracts

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Abstract
Postbiotics are the non-viable bacterial products or the low molecular weight metabolites produced by probiotics that have received considerable attention owing to their health promoting effects. The present study aimed to investigate the safety and antibacterial properties of postbiotic components of *Lacticaseibacillus rhamnosus (Lra)* and *Limosilactobacillus reuteri (Lre)* for their potential applications in food products. The freeze dried postbiotic metabolites (FD-P) from *Lra* and *Lre* were extensively analyzed for their physico-chemical properties and antibacterial actions against common food borne pathogens. Higher levels of total flavonoids (1971.79 ± 20 mg Qu/ g), total short-chain fatty acid (23 µg/g), sugar contents, CAT, and SOD anti-oxidative enzymes were detected in the *Lra* postbiotic, while GSH-px levels and riboflavin were higher in *Lre* postbiotics ($P < 0.01$). No significant differences were recorded in the total phenolic (2501 and 2518 mg GAE/ L) and crude protein contents (305. 58 and 296.23 µg/g) of the postbiotics ($p \geq 0.05$), respectively. Both FD-P samples showed enhanced activities against Gram-Positive pathogens compared to Gram-Negative pathogens ($p < 0.05$), while combining the two postbiotics further potentiated the antibacterial actions. Both FD-P samples were non-hemolytic to human erythrocyte cells, and exhibited low cytotoxicity in MRC 5 and IPEC-J2 cell lines at the highest used concentrations (150 mg/ml). In summary, the postbiotics derived from *Lra* and *Lre* are safe bioactive ingredients with enhanced antibacterial and antioxidant capabilities, having potential applications as a natural preservatives in food system, potentially enhancing safety and extending the shelf life of food products.

Keywords Postbiotic metabolites, Antibacterial actions, Antioxidant activity, Safety, Porcine cell lines

Introduction

Postbiotics are the active compounds produced during probiotic fermentation and are known to exert beneficial health effects on the host's body. The word postbiotics is derived from the Greek words, "post" referring to after, and "bios" referring to life (Vinderola et al. 2022). In 2021, the International Scientific Association for Probiotics and Prebiotics (ISAPP) defined postbiotics as "inanimate preparations of microbes and/or their components that exert health benefits on the host" (Salminen et al. 2021). Based on the documented definition, a postbiotic suspension might include (a) non-viable bacterial components such as cell wall fragments, peptidoglycan, teichoic acids, and, (b) metabolites that are the intermediate

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or end product of a bacterial metabolism (Liang and Xing 2023). These metabolites, also referred to as biogenic or cell-free supernatant, are the soluble molecules secreted by living bacteria or released during bacterial lysis and include short-chain fatty acids (SCFA), bioactive proteins or bacteriocins, hydrogen peroxide, exopolysaccharides, proteins, lipids, vitamins, amino acids, etc. (Aggarwal et al. 2022). Postbiotics have several functional properties, including antibacterial, antioxidant, immunomodulatory, anti-proliferative, and anti-inflammatory potential (Compare et al. 2017; Liang and Xing 2023). Reports have further indicated that the dead probiotic cells and their metabolites could mimic the actions of their viable parent strains (Green et al. 2020), showing antibacterial actions and providing beneficial health effects (Mohammadi et al. 2022). However, postbiotics from different bacteria show differences in antibacterial and other physico-chemical characteristics (Selvam et al. 2024), that might depend on factors such as the bacterial strain, culture medium, and the inactivation methods used for postbiotic preparations (Moradi et al. 2021; Incili et al. 2022).

Among a number of metabolites produced by probiotics, short chain-fatty acids (SCFA) are the most important and widely studied secondary metabolite that has gasped wide attention owing to their different therapeutic applications (Markowiak-Kopeć and Śliżewska 2020). Some of the most important SCFAs, like propionate, butyrate, acetate, and lactate, perform several vital functions in the host when present in adequate amounts (Hati et al. 2019). Apart from these, other bioactive proteins with antibacterial actions, (bacteriocins) have been studied in a wide number of bacteria. These peptides, produced by the ribosome have antibacterial actions and have been used as bio-preservatives in a number of dairy products.

In recent years, postbiotic metabolites from probiotic microbes have been highly acknowledged for their preservative role in the food industry (Letizia et al. 2022). These metabolites have promising effects in food preservations and food packaging where they can control the growth of pathogens, inhibit microbial biofilms, and biodegrade harmful chemical contaminants like mycotoxins, pesticides, and heavy metals (Yilmaz et al. 2022). In food matrix, postbiotics might provide higher safety, better processability, and enhanced stability over a wide range of temperatures, pH, and water activities (Siciliano et al. 2021). Additionally, utilizing postbiotics unlike probiotics could have less or no interactions with other food components (Barros et al. 2021), and are known to enhance the flavor and aroma of the food (Kim et al. 2020). An essential advantage of using postbiotic metabolites particularly during the industrial production of food products, instead of their live parent culture, especially where survival and viability of probiotics are suspected (Thorakkattu et al. 2022). Owing to the importance and health benefits of metabolites (postbiotic), in this study we characterized and identified the functions of the metabolites derived from the two indigenous LAB strains, namely *L. rhamnosus* (*Lra*) and *L. reuteri* (*Lre*), Understanding the biochemical composition and safety parameters of the intracellular postbiotics, can enhance their potential application as functional ingredients in food, pharmaceuticals, and other health related products.

Materials and methods

Bacterial growth media and culture conditions

Lacticasibacillus rhamnosus LRH-B2 and *Limosilactobacillus reuteri* LRE-B16 were obtained from a local probiotic manufacturer (Biorun. Co. Karaj, Iran). The two probiotic strains were grown in De Man Rogosa and Sharpe (MRS, Merck, Germany) broth at 37 °C under anaerobic conditions (5% CO_2) for 18–24 h. For antibacterial assay, the indicator cultures including *Escherichia coli* ATCC25922 (*Ec*), *Listeria monocytogenes* ATCC 13,932 (*Lm*), *Salmonella enterica* serovar Typhimurium ATCC 14,028 (*St*), and *Staphylococcus aureus* ATCC 64,542 (*Sa*) were grown in Brain Heart and Infusion (BHI, Oxoid UK) and or Trypticase Soya Broth (TSB, Difco, BD, USA), at 37 °C for 18 h under aerobic conditions.

Preparations of postbiotic fractions

Freshly grown cultures of *Lra* and *Lre* in MRS broth, were centrifuged (8000 x *g*, for15 min at 4 °C) and the collected pellets were washed twice with sterile 0.85% (w/v) sodium chloride (NaCl, Merck, Darmstadt, Germany) solution. The bacterial cell counts were adjusted to approximately 10^9 colony forming unit (CFU) per mL and considered as 10% (v/v) inoculum. The intracellular cell free extracts were prepared according to the method of Osman et al. 2021. The cell cultures (10^9 CFU/mL) were treated briefly with lysozyme at the concentrations of 1 mg/mL for 30 min at 37 °C and followed by ultrasonic disruption. The suspension was sonicated on an ice bath at 5 min intervals. The cell debris was removed via centrifugations at 8000 x *g*, for 15 min at 4 °C, and filter sterilized using 0.22 µM Millipore membranes (Sartorius Minisart, Germany). The sterilized suspensions were freeze dried as described by Moradi et al. (2021). After performing the quality control of the lyophilized powder (FD-P), the microbe free samples were stored at -20 °C for further use.

Likewise, the combined postbiotic samples (MFD-P) were prepared by mixing the two FDP samples in equal proportions (1:1). For all analysis, a suspension of the FD-P and MFD-P fractions was made by dissolving 1 mg of the respective samples in 1 mL of the sterile phosphate buffer solution (0.01 M, pH 7.0±2.0).

Characterizations of FD-P postbiotics

1) Quantifications of antioxidant activity

Free radical scavenging potential of the postbiotic extracts was determined by 1,1-Diphenyl-2-picrylhydrazyl (DPPH) assay using the published method (Aydin et al. 2021). Different concentrations of. 100 µL (5, 10, 15, 20, 25, and 50 mg/mL) of FD-P samples were mixed with equal amounts of freshly prepared ethanol solution of DPPH radical (0.1 mM) and incubated at room temperatures for 30 min in dark. Absorbance (OD_{517nm}) was recorded by spectrophotometer (Shimadzu UV-1800, Japan), and percent DPPH radical scavenging activity was estimated using the equation:

$$\text{DPPH scavenging activity (inhibition \%)} = [(\text{A control} - \text{B sample})/\text{A control}] \times 100$$

Where, control was the absorbance of the control (A) and sample (B) was the absorbance of the reaction mixture.

2) Quantifications of total phenolic contents (TPC)

Folin-Ciocalteu's method with slight modifications was used for the determination of the total phenolic contents of the postbiotic (Kocabey et al. 2016). Total phenolic contents were estimated based on the calibration curve of Gallic acid standard (mg GAE/ 100 g dry weight).

3) Quantifications of total flavonoid content (TFC)

TFC was performed using aluminum chloride colorimetric technique as recommended by de Souza et al. (2014). Quercetin was used as the standard for plotting the calibration curve and the data was expressed as quercetin equivalent (QE) per g of the samples.

4) Quantifications of Lactic acid and SCFAs contents

Lactic acid concentrations in g/L was determined by the method of Borshchevskaya et al. (2016). 2.0 mL of 0.2% iron (III) chloride was added to the postbiotic suspensions and mixed thoroughly. The absorbance was read immediately after mixing, at 390 nm using UV visible spectrophotometer. Different lactic acid (Sigma Co, USA) concentrations ranging from 0 to 80 mg/L was used to plot standard curve.

Similarly, the SCFAs including acetic acid, butyric acid, and propionic acid in the individual postbiotic samples were quantified using High Performance Liquid chromatography (HPLC) method according to the modified protocols (LeBlanc et al. 2017). The HPLC system (Hewlett-Packard HP1050 series, Model 1050 UV, Agilent Technologies, USA) was equipped with a UV-Vis detector and diode array detector and absorbance was measured at 210±5 nm. Postbiotic sample (100 µL) was injected through a pre-packed column (Rezex ROA-organic acid H + (8%), 150×7.80 mm, Phenomenex, CA, USA) attached to an ion-exclusion microguard refill cartridge. The two mobile phases H_2SO_4 (0.05 M) and acetonitrile (2%) were pumped through the column with an isocratic gradient (0.8−0.7 mL/min, 35 °C). Different concentrations (1, 10, 100, 500 and 1000 ppm) of acetic acid, propionic acid, and butyric acids were used to prepare a standard solution. The concentrations of SCFAs were calculated from the standard curves and their respective linear regression equations (R2≥0.99).

5) Quantifications of total protein and sugar content

The crude protein in the postbiotic preparations were determined using Pierce™ Bicinchoninic acid protein assay (BCA assay) kit (Thermo Fisher Scientific), in accordance with the manufacturer's instructions.

The phenol-sulfuric acid method was used to determine total soluble sugar contents in the postbiotic preparations as described earlier (BeMiller 2017).

6) Quantification of antioxidant enzymes

The activities of catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GSH-px) in the extracted postbiotic preparations were quantified using the standard assay kits (Abcam, UK), following the manufacturer's instructions.

7) Quantification of riboflavin

The amount of riboflavin in the prepared postbiotic suspensions was determined using the enzyme-linked immunosorbent assay (ELISA) kit for vitamin B2 (Abbexa, UK) in accordance with the manufacturer's instructions. Sterile BHI broth medium was used as negative control.

8) Evaluation of D-lactate production

After de-proteinizing the samples by the precipitation of 6 N trichloroacetic acid (TCA) 10% (w/v), the presence of D-lactate was evaluated using the colorimetric D-lactate Assay Kit (Abcam, UK), in accordance with the manufacturer's instructions.

Antagonistic activity of FD-P and MFD-P samples

Antibacterial activity in the FD-P and MFD-P suspensions against the mentioned bacterial pathogens was determined using microtiter plate method as reported

earlier (Tong et al. 2023). BHI broth was used as test quality control, while positive control included bacterial suspensions in BHI broth. After incubation of plates at 37 °C, the turbidity was measured at OD_{600} using ELISA reader and the antibacterial activity calculated by the given Equation:

$$\text{Inhibition}\% = (P_1 - P_0) - (T_1 - T_0)/(P_1 - P_0) \times 100$$

Where, P_0 and T_0 are the turbidity values in positive controls and test samples at 0 h, while, P_1 and T_1 is the turbidity change in positive controls and the test samples after 24 h of incubations at 37 °C.

Minimum inhibitory and bactericidal concentrations of FD-P and MFD-P samples

The MIC and MBC of FD-P and MFD-P samples against the selected pathogens were determined according to the Clinical and Laboratory Standards Institute (CLSI 2020). 20 µL of different concentrations of the FD-P and MFD-P suspensions were added into the 96-well microtiter plates which contained 180 µL of the pathogens (0.5 McFarland). Negative controls (only BHI broth) and positive control (bacterial inoculum) was used. All plates were incubated at 37 °C for 48 h and observed the turbidity.

The MBC was determined by taking the clear well (no turbidity during MIC) and spot on the agar plates. After 48 h of incubations at 37 °C, the plates were observed for MBC. All experiments were performed in triplicate.

Survival assay of pathogens in the presence of MFD-P

Percent survival of the respective pathogens in the presence of different concentrations of MFD-P (1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg/mL) was determined by microdilution method. Briefly: 50 µL of the selected pathogens suspensions (10^6 CFU/mL) was added to 96-wells plate which contained different concentrations (1, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0 mg/mL) of the MP. The plates were incubated under aerobic conditions at 37 °C and for 18 h and the number of viable cells was determined. Positive control included pathogens in BHI broth without MFD-P, likewise, negative control included BHI broth without pathogens. Percent survival was determined using the formula.

$$\text{Survival percentage}\,(\%) = \log\ \text{CFU}\ N_t / \log\ \text{CFU}\ N_c \times 100$$

Where, Nc is the number of viable bacteria in control group and Nt is the number of viable bacteria in treatment groups.

Partial characterization of the antagonistic compounds

Thermal resistance of the antibacterial substance was determined by exposing the FD-P samples (15 mg/mL) to 100 °C at 5, 10, 15, 20, 25 and 30 min and the remaining activity determined by agar well diffusion assay.

The antagonistic compound in the prepared postbiotic preparations (FD-P) was tested for their sensitivity to proteolytic, lipolytic, glycolytic and catalase enzymes (Sigma-Aldrich, Germany) as described earlier (Noroozi et al. 2019).

Safety of postbiotic preparations

1) MTT assay

Safety of the postbiotic was determined by evaluating the viability of the intestinal porcine epithelial (IPEC-J2) and normal cell line (MRC5 ATCC® CCL171™), after exposing to different concentrations of the postbiotic preparations, as described by others (Noroozi et al. 2019; Tong et al. 2023). The cell lines (10^4cells/well) were seeded in 96-well plate and after overnight incubations FD-P samples (i.e. 5, 10, 20, 30, 40, and 50 mg/mL) were added into the wells. MRS broth and MTT without the FD-P was used as control. After incubations for 48 h, 20 µL of 5 mg/mL MTT (Sigma, St. Louis, MO) was aseptically added into the wells, and incubated at 37 °C for 3 h. Later, the media was removed carefully and 150 µL of MTT solvent was added to the wells, and the plates were covered with tinfoil and agitated for 15 min on orbital shaker. The absorbance (OD_{570}) was read and the cellular viability was estimated as the percent of survival, relative to the control cells, using on the following formula:

$$\%\text{Viability} = [(OD_{570}\,\text{of treated} - OD_{570}\,\text{of blank})]/ [OD_{570}\,\text{of control} - OD_{570}\,\text{of blank})] * 100$$

2) Hemolytic inhibition assay

The safety of the postbiotic was evaluated by exposing its different concentrations to the human erythrocyte cells as described by Chuah et al. (2019). Briefly, peripheral blood was withdrawn from healthy volunteers after their consent and approval from RVSRI ethics committee. The RBS was washed and incubated at 37 °C for 30 min in the presence of different concentrations of the postbiotics (5 to 50 mg/mL). The control included erythrocyte suspensions with 0.9% NaCl (0% hemolysis), while total hemolysis (100% hemolysis) was induced by incubations of the erythrocyte suspensions in deionized water. Hemolysis potential was determined after 0, 8, 12, 24 and 48 h, by taking aliquots, diluted in saline and centrifuged at 400 x *g* for 10 min. The absorbance of the supernatant was

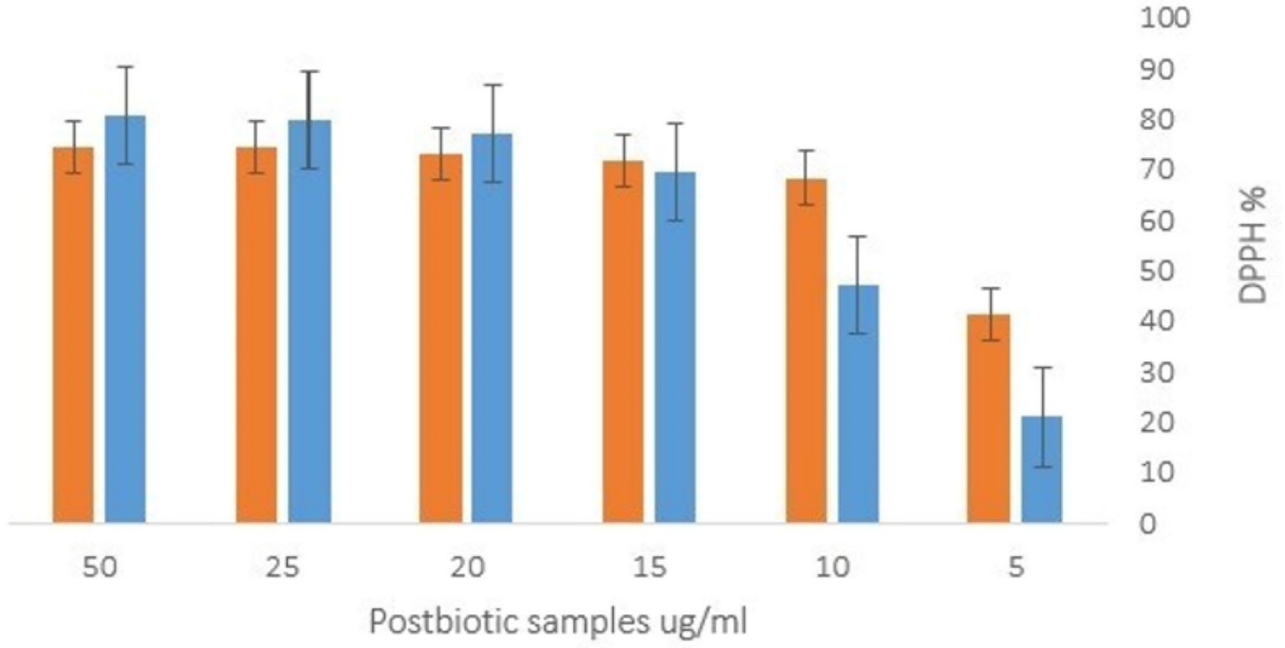

Fig. 1 Antioxidant activity of different concentrations of *L. rhamnosus* (*Lra*) and *L. reuteri* (*Lre*) postbiotic as detected by DPPH assay. Three independent biological replicates with two technical replicates each were performed. Mean ± standard deviation was shown and ANOVA for independent data followed by Tukey HSD test

measured at 540 nm and the percentage hemolysis was determined using the mentioned formula.

$$A/B \times 100$$

Where, A is the sample absorbance and B is the total hemolysis.

Statistical analysis

All experiments were performed in triplicate. The experimental data were analyzed with one-way ANOVA, performed in a completely randomized simple design using SPSS v.20 software (SPSS Inc., Chicago, IL). Statistical differences were determined by the Tukey HSD test at the 95% confidence interval ($\alpha = 0.05$).

Results

Antioxidant activity of postbiotic preparations (FD-P)

The antioxidant capacity of different concentrations of the *Lra* and *Lre* extracted postbiotic samples is shown in Fig. 1. *Lra* postbiotics antioxidant activity was significantly higher (58.53%) at the concentrations of 10 µg/mL, compared to that of *Lre* (47.35%) with significant differences ($p < 0.05$). However, at higher concentrations (≥15 µg/ mL) these differences became insignificant ($p \geq 0.05$). The antioxidant activity of the two postbiotics were lower compared to the positive control.

Table 1 Bioactive components evaluated in the postbiotics (FD-P) of *L. Rhamnosus* and *L. Reuteri*

Bioactive compounds	*L. rhamnosus*	*L. reuteri*
Total phenolic (mg GA/100 g)	2601.07 ± 0.512	2597.81 ± 0.443
Total Flavonoids (mg Qu/100 g)	1971.79 ± 20.67	1635.24 ± 20.421
Total protein (µg/ g)	305. 58 ± 1.651	296.23 ± 1.456
Total soluble sugar (mg/g)	76.543 ± 1.683	69.894 ± 1.352
CAT (U/g)	7835 ± 179.41	5364 ± 146.32
SOD (U/g)	1567 ± 24.22	1266 ± 29.14
GSH-px (U/g)	477 ± 17.08	548 ± 18.05
Riboflavin (ng/ml)	143.65 ± 22.03	267.44 ± 19.67
D-lactate (nmol/ml)	49.32 ± 1.73	27.71 ± 2.15

Bioactive Components in prepared postbiotics

Bioactive contents of the tested postbiotics are depicted in Table 1. Based on the obtained results, total flavonoids were significantly higher in *Lra* postbiotics compared to *Lre* postbiotics ($p < 0.05$). While, no significant differences were recorded in the total phenolic (2501 and 2518 mg GAE/ L) and crude protein contents (305. 58 and 296.23 µg/ g) of the postbiotics ($p \geq 0.05$), respectively. Total soluble sugar contents estimated in *Lra* (76.54) were higher compared to *Lre* (69.89) with insignificant ($p \geq 0.05$) differences.

Significant amount of the antioxidant enzymes including CAT, SOD and GSH-px were recorded in the tested FD-P samples. However, the levels of CAT and SOD were higher in *Lra* postbiotics compared to *Lre* postbiotics ($p < 0.05$). While, GSH-px levels and riboflavin was higher in *Lre* postbiotics ($P < 0.01$) compared to *Lra* postbiotics

(Table 2). Low amounts of D-lactate were also detected in both the FD-P samples that was higher in *Lra* compared to *Lre* ($P<0.001$).

The lactic acid and short chain fatty acid concentrations in FD-P samples is shown in Fig. 2. Acetic acid appeared to be the major acid in both postbiotic extracts, while, least amount of butyric acid was recorded in the extracts. Concentrations of lactic acid were slightly higher in *Lre* postbiotic extracts (3.72 µg/ mL) compared to *Lra* (2.98 µg/ mL), while the differences were insignificant ($p\geq0.05$). However, total SCFA levels were higher in *Lra* postbiotic preparations (20.23 µg/ mL) compared to *Lre* (17.95 µg/ mL), with significant differences ($P\leq0.05$).

Thermal resistance of the postbiotics were determined by exposing them to different temperatures. FD-P samples from *Lre* resisted 100 °C for 25 min and autoclaving temperatures 121 °C for 15 min (data not shown). In contrast, *Lra* metabolites resisted 100 °C for only 5 min and lost their activity at autoclaving temperatures. The tested proteolytic enzymes resulted in complete loss of the activity of both postbiotic extracts. While, catalase, lipolytic and glycolytic enzyme had no effect on the inhibitory activity of both the extracts.

Table 2 Inhibition percentages of the pathogens after exposure to FD-P and MFD-P samples after 24 h of incubations at 37 °C

Postbiotics	Pathogens			
	Ec	*Lm*	*Sa*	*ST*
FD-P (*Lra*)	69.84 ± 2.01	89.88 ± 1.82	71. 73 ± 1.35	53.11 ± 1.28
FD-P (*Lre*)	67.19 ± 1.45	85.54 ± 1.61	72.66 ± 1.25	52.47 ± 1.67
MFD-P	73.45 ± 1.56	93.46 ± 1.52	83.24 ± 1.74	73.43 ± 1.55

FD-P (*Lra*): postbiotic preparations from *L. rhamnosus*, FD-P (*Lre*): postbiotic preparations from *L. reuteri*

MFD-P: mixture of *Lra* and *Lre* postbiotics

Antagonistic activity

The antibacterial activity of FD-P samples of (*Lra*) and (*Lre*) revealed their effectiveness against the tested pathogens (Table 2). Both the postbiotics inhibited the tested pathogens, while, *Lr*a postbiotics showed more effectiveness compared to *Lre* postbiotics. Among the tested pathogens, *Lm* appeared the most sensitive pathogen and approximately 80% of their viability was lost after treatment with the postbiotic samples. In contrast, *S*T was the most resistant pathogen and only 50% of their growth was inhibited by the respective postbiotics. No significant difference was seen in the sensitivity of *Sa* and *Ec* against the two postbiotics. While, synergistic antibacterial actions were seen when the two postbiotics were mixed together (MFD-P) and their inhibitory actions against all tested pathogens was significantly enhanced ($p<0.05$). In the presence of MFD-P, ≥ 90% of the *Lm* was inhibited while, *Sa*, *Ec*, and *S*T were inhibited at 83.24, 73.45 and 73. 38%, respectively.

MIC and MBC concentrations of FD-P and MFD-P samples

While determining the MIC and MBC values (Fig. 3), FD-P fractions from *Lra* demonstrated stronger antagonistic actions compared to *Lre* fractions owing to their lower MIC values. *Lm* was highly sensitive to the actions of the *Lra* postbiotics and only 5.75 mg/ mL of the extract

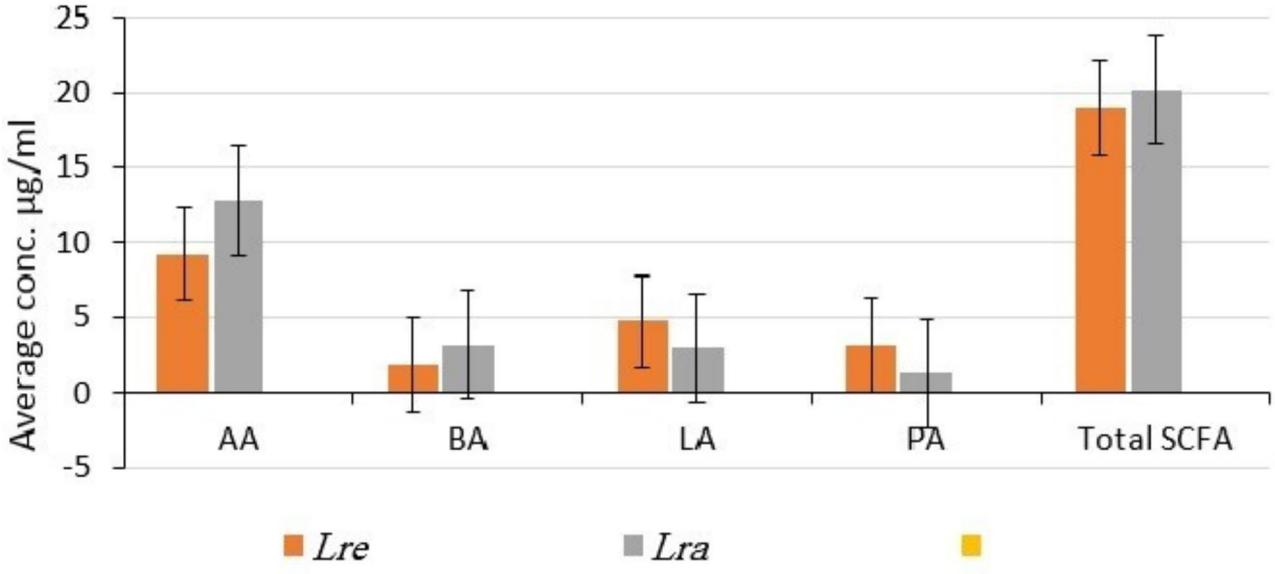

Fig. 2 Lactic Acid (LA) and short chain fatty acids (AA: Acetic acid; BA: Butyric acid; PA; propionic acid) concentrations in Postbiotics of *L. rhamnsous* (*Lra*) and *L. reuteri* (*Lre*). Three independent biological replicates with two technical replicates each were performed. Mean ± standard deviation were shown and ANOVA for independent data followed by Tukey HSD test

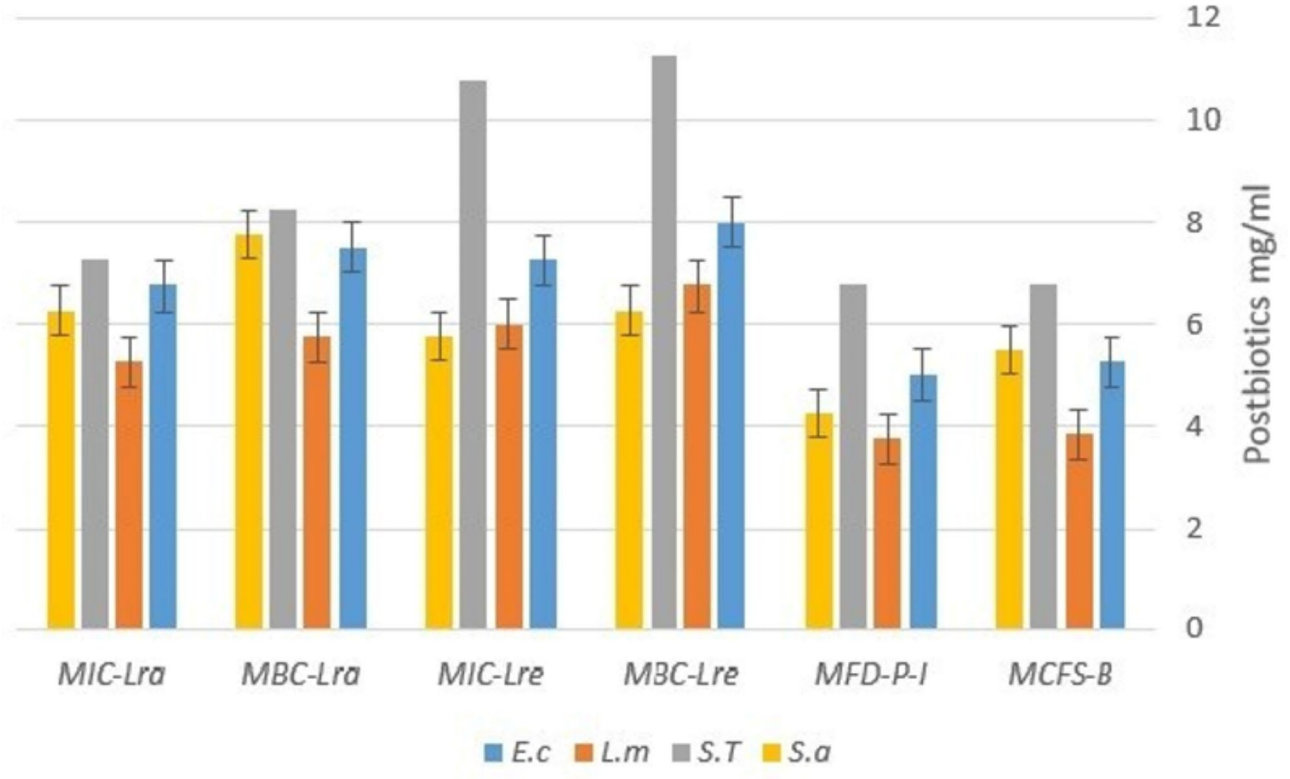

Fig. 3 MIC and MBC of *L. rhamnosus* (*Lra*) and *L. reuteri* (*Lre*) postbiotics against *E. coli (Ec)*, *L. monocytogenes (Lm)*, *S.* Typhimurium (ST) *and S. aureus (Sa)*. MIC-*Lra* and MBC-*Lra*: MIC and MBC values of *L. rhamnosus* postbiotic; MIC-*Lre* and MBC-*Lre*: MIC and MBC values of *L. reuteri* postbiotic; MFD-P-I and MFD-P-B: MIC and MBC values of mixed postbiotics, respectively

could inhibit its growth while, higher concentrations of the mentioned postbiotic (6.25, 6.75 and 7.25 mg/ mL) were required to inhibit the growth of *Sa*, *Ec*, and ST respectively. The antibacterial actions of *Lre* postbiotic against *Lm*, *Ec* and *S*T was weaker compared to *Lra* postbiotics ($p<0.05$), while, its actions against *Sa* appeared stronger than *Lra* postbiotic ($p<0.05$). *S.* Typhimurium appeared to be the most resistant pathogen, while, it was more resistant to *Lre* compared to *Lra* postbiotic. As predicted during in vitro antagonistic assay, combining the two postbiotic fractions (MFD-P) resulted in enhanced antibacterial activity and further decrease in the MIC and MBC values against the pathogens were recorded ($p<0.05$). Approximately 3.75 and 4.25 mg/ mL of MFD-P inhibited the growth of *Lm* and *Sa*, respectively, while, slightly higher MIC values (5.0 and 6.75 mg/ mL) were recorded against the tested Gram-negative pathogens (*Ec* and *ST)*, respectively.

Effect of postbiotics on the survival of pathogens

Survival percentages of the pathogens in the presence of different concentrations of MFD-P is shown in Fig. 4. A direct correlation was seen between the survival percentages of the pathogens and the concentrations of the MFD-P, and with increasing MFD-P concentrations lower survival rate of the respective pathogens ($p<0.05$) was seen. Highest decrease in the survival of *L. monocytogenes* was recorded in the presence of only 3 mg/mL of the MFD-P. In contrast, higher concentration of the MFD-P (9.5 mg/mL) was required to inhibit the growth of *S.* Typhimurium. Lower concentrations of the MFD-P were required to inhibit the growth of the tested Gram positive pathogens, than Gram negative.

Safety of the postbiotics

The cell viability of the intestinal porcine epithelial and MRC5 cell line was tested and result is summarized in (Fig. 5). The viability of both MRC5 and IPEC-J2 cells was not affected by postbiotic concentrations up to 60 mg/mL. However, higher concentrations (≥70 mg/ mL) showed slight cytotoxicity against normal MRC 5 cell lines and approximately 6.75 and 4.36% of the mentioned cell viability was lost after exposure to 70 mg/mL of *Lre* and *Lra* postbiotics, respectively. Comparatively, *Lre* postbiotics showed slightly higher cytotoxicity than *Lra* postbiotics, while MRC5 cell lines were more prone to these postbiotics compared to IPEC-J2 cell lines. However, the cytotoxicity of both postbiotics, even to the highest used concentrations of 150 mg/mL was below 10%.

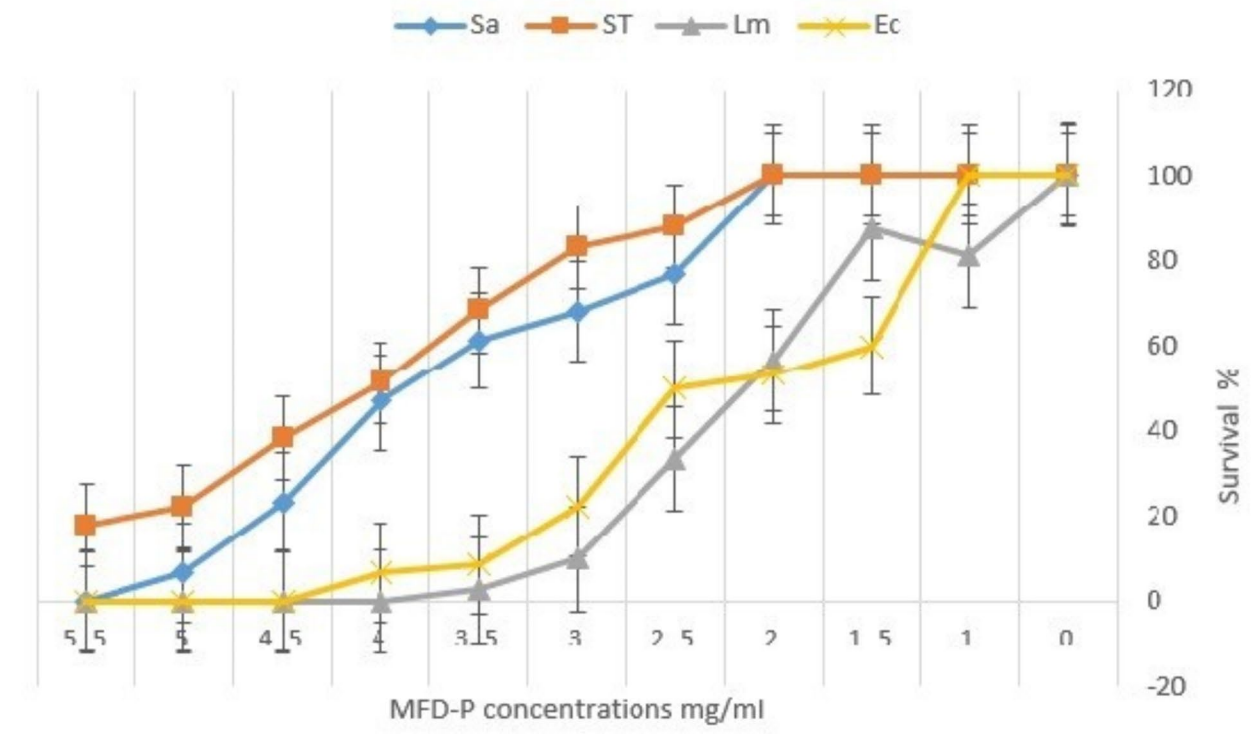

Fig. 4 Survival percentage of *Escherichia coli (Ec)*, *Listeria monocytogenes* (*Lm*), *Staphylococcus aureus* (*Sa*), and *Salmonella* Typhimurium (*ST*) in the presence of mixed postbiotics (MFD-P). Three independent biological replicates with two technical replicates each were performed. Mean ± standard deviation was shown and ANOVA for independent data followed by Tukey HSD test

Testing the hemolytic activity of the FD-P samples did not exhibit any inhibitory effects on the human erythrocytes. The data indicated that none of the used concentrations of FD-P samples results in hemolysis of red blood cells, except for *Lra* postbiotic that showed only 2% hemolysis at the highest used concentration of 100 mg/mL after 48 h (data not shown).

Discussion

Antioxidant activity is known to influence the occurrence of inflammation, cellular damage, and disease susceptibility in the body (Aguilar-Toalá et al. 2018). The studied postbiotics extracted from *Lra* and *Lre* exhibited significant DPPH scavenging activity, total phenolic and flavonoids. Our results align with previous reports (Aydin et al. 2021; Chang et al. 2021) showing that the higher antioxidant activity of *Lra* postbiotics might be attributed to the higher content of total flavonoids and phenolic compounds compared to *Lre* postbiotic. Likewise, presence of organic acids such as lactic acid and acetic acid being excellent electron donors owing to their hydroxyl groups, might also be responsible for the observed free radical scavenging activity (Wang et al. 2017). In contrast, Tong and his colleagues (2023), correlated the observed antioxidant properties of the postbiotics to the total soluble sugar contents.

Organic acids produced by probiotic bacteria act by decreasing the intracellular pH, resulting in dissipation of cell membrane potential generating an unfavorable microenvironment for pathogenic bacteria, with a consequent preservative effect in the food (Bangar et al. 2022). More interestingly, these acids contribute to the development of aroma and flavor in diverse fermented food products. The postbiotics in this study showed significant variations in the amount of SCFA produced including acetic acid, butyric, propionic acids and lactic acids, indicated that organic acid production is a strain dependent property. Similar to the reports of Kang and his colleagues (2021), the highest concentrations of acetic acid were seen in the tested postbiotic samples. Acetic acid is known to be among the main SCFA produced by a number of intestinal bacteria present in human gut. Adequate amounts of lactic acid were detected in the postbiotics that is known to have several health benefits. Acetic acid and lactic acid are highly recommended for their bio-preservative effects in fermented foods, due to their strong antimicrobial activity. While, other SCFA detected including propionic and butyric acid are known to possess anti-bacterial, anti-hypercholesterolemia and

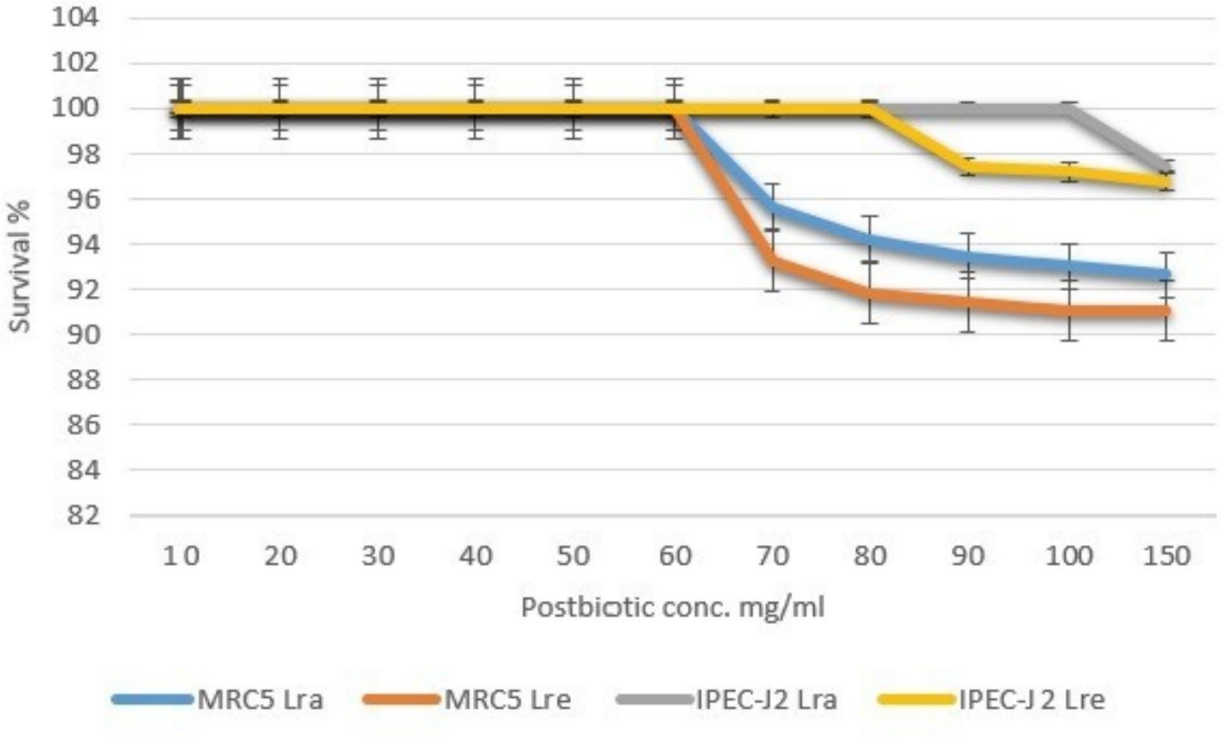

Fig. 5 Survival percentages of MRC5 and IPEC-J2 cell lines after exposure to different concentrations of postbiotic from *Lra* (*L. rhamnosus*) and *Lre* (*L. reuteri*)

anti-cancer effects (Nakkarach et al. 2021). Moreover, butyric acid and propionic acids had been shown to enhance aroma and play crucial role in cured cheese, or Dutch cheese aromas, respectively (Fox et al. 2017). Our results corroborated hardly any differences in the total amount of SCFA levels in both *Lra* and *Lre* the postbiotics. In agreement with the reports of Bangar and his colleagues (2022), no significant differences were seen among the FD-P samples of the two tested strains when comparing the total production of organic acids.

Lactate, a common by-product of anaerobic metabolism exists in two isoforms i.e. L-lactate and D-lactate. Blood D-lactate titers of ≥3 mM/L could cause D-lactic acidosis, a very rare neurological disorder that induces slurred speech, ataxia, and sometimes coma by impacting the central nervous system (Stuivenberg et al. 2022). Although controversial, but it is often recommended to limit administration of probiotic species to those that do not produce D-lactic acid (Rao et al. 2018). Owing to these health concerns, the concentrations of D-Lactate is evaluated in both samples. Although, the concentrations of this acid was higher in *Lra* compared to *Lre* postbiotics, but the levels were extremely lower than the range thought to be of health concern and hence might be considered safe for use.

CAT and SOD are crucial antioxidant enzymes produced by probiotic microorganisms. These enzymes are involved in hydrogen peroxide and superoxide dismutase radical scavenging, and are thought to help the host in reducing oxidative stress and several ROS-linked diseases (Wang et al. 2017). Our study showed that the tested postbiotics harbor CAT and SOD activity which indicated their possible role in alleviating ROS accumulations, consecutively preventing oxidative stress.

Riboflavin is involved in the metabolism of proteins, lipids, carbohydrates, and ketone bodies (Olfat et al. 2022). Inadequacy of Riboflavin could lead to a condition known as ariboflavinosis, which is responsible for anemia, hypertension, diabetes mellitus, etc. Both the postbiotic samples contained considerable amounts of Riboflavin. These results agree with previous findings that showed the ability of the probiotic strains to secrete vitamin B2 during growth (LeBlanc et al. 2017). Hence, addition of Riboflavin producing strains in a probiotic formulations might be a promising source for preventing ariboflavinosis.

The extracted postbiotic demonstrated variable level of antagonistic actions against both Gram-positive and Gram-negative pathogens. As studied earlier, related the observed antagonistic variations to the differences in the structure of the antimicrobial agents produced by

bacteria, like catabolic end-products including solvents (acetone, butanol, ethanol), and acids (lactic, acetic, formic, and butyric) and most importantly peptides, proteins and enzymes (Makras et al. 2006). While, Incilli and his colleagues (2022), related these differences in the antimicrobial actions to the resistance profile of the target pathogen. Compared to Gram-negative pathogens, the two postbiotics were more effective against Gram-positive pathogens such as *L. monocytogenes* and *S. aureus*. Postbiotic metabolites are known to exert their detrimental effect on the morphological characteristics of pathogenic bacteria thereby causing cell death and elimination. The synergistic antibacterial actions of the combined postbiotics (MFD-P) have been reported earlier (Serna-Cock et al. 2019). Consistent with these reports, we also report the enhancement of antibacterial activity of the combined postbiotics compared to mono-culture postbiotic. The enhanced activity of MFD-P compared to FD-P fractions, might be attributed to the presence of differences in the types and concentrations of the metabolic end products in the two metabolites that after combination are synergized and resulted in enhanced antibacterial activity (Noori et al. 2023).

Some of the major antimicrobial agents produced by majority of LABS includes organic acids, CO_2, hydrogen peroxide and low molecular weight proteins. During characterizations of the antagonistic agents, the antibacterial agent in both postbiotic extracts appeared sensitive to the tested proteolytic enzymes that might be preliminary indication of the presence of a protein or peptide inhibitory substance. Bacteriocins orbacteriocides like substances are antimicrobial peptides that are known to exert their actions on the target bacteria either by inhibiting bacterial cell wall synthesis or by acting on the cell membrane and creating pores on the surface of cell membrane that results in loss in the metabolites and simultaneous death (Anjana and Tiwari 2022). A number of bacteriocins producing *L. rhamnosus* strains has been reported to date (Chen et al. 2023). While, *L. reuteri* has shown to produces a potent low molecular weight antimicrobial protein termed reuterin, which is capable of inhibiting wide range of pathogenic microorganisms including viruses, bacteria, fungi and protozoa (Alam et al. 2022).

Thermal variations observed in the antagonistic agent produced by the two probiotic strains, indicated differences in their protein structure. Such differences in the nature of the antibacterial peptides has been reported earlier (Zhang et al. 2022). While, the two antibacterial agents appeared sensitive to the actions of glycolytic and lipolytic enzymes that might rule out the presence of carbohydrate and or lipid moiety associated with the bioactivity of the antibacterial peptides (Rahmeh et al. 2017).

Mechanical barrier of the intestine is an important component of the intestinal barrier and plays a crucial role in maintaining intestinal health. During the cell viability test, the studied postbiotics showed high cell safety in the intestinal porcine epithelial cell line (IPEC-J2) that was almost comparable to MRC5 normal cell lines. No toxic dose effect was seen as none of the tested postbiotics effected the viability of the tested cell lines up to the concentrations of ˂70 mg/mL, while higher concentrations had slight toxic effects especially on MRC 5 cell lines. The results might be indicative of the significance of appropriate doses for using postbiotics. However, it is worth noting that the cytotoxicity levels of the two postbiotics even at highest used concentrations (150 mg/mL) was below 10% and hence could be considered safe.

Erythrocytes are employed as a prime candidate for the determination of membranolytic or cytolytic activities. None of the tested FD-P concentrations was found hemolytic. These results are in agreement with the findings of Chuah et al. (2019), who showed that the non-hemolytic nature of the postbiotics might indicate the non-toxic nature of the postbiotics to the tested cellular model. These results imply that the studied postbiotics might not pose any adverse effects on the membrane permeability properties.

In conclusion, the study highlights the promising antibacterial effects of postbiotics from *Lra* and *Lre*, showcasing their potential as a promising natural strategy for the elimination of pathogenic bacteria especially in food products. Moreover, increase in the concentrations of the beneficial compounds achieved after mixing the two postbiotics appeared to have enhanced antibacterial effects, eliminating pathogens in shorter period of time. This research lays the groundwork for further exploration and development of postbiotic based strategies to ensure food safety, quality with potential health benefits. Further studies are recommended to explore the pharmacological efficacy of these postbiotics as therapeutic drugs and or as a functional food additive.

Received: 1 July 2024 / Accepted: 12 September 2024
Published online: 09 October 2024

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