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Anticancer and probiotic activities of *Lacticaseibacillus paracasei* isolated from traditionally fermented pickles

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Abstract

Background: Probiotics are living organisms and play a pivotal role in the natural treatment of gastrointestinal disorders including diarrhea, constipation, lactose intolerance and some bowel cancers. Beneficial effects are mostly attributed to post-biotic metabolites produced by bacteria, and the unique biological characteristics may vary depending on their species and source. The present study focused on identifying unique *Lacticaseibacillus paracasei* strains with unique probiotic and anticancer effects originating from fermented pickles.

Results: Bacterial strains isolated from naturally fermented pickles were identified by 16S rRNA sequence analysis. Their probiotic properties were determined in simulated gastrointestinal environments created by pancreatin and pepsin. In addition, their adhesion capacities on L-929, HT-29 and Caco-2 cell lines were tested and then the cytotoxic effects of postbiotic metabolites and bacterial extracts obtained from bacterial isolates on cell lines were tested to determine their anticancer activities. In the overall results, isolates were found to be acceptable probiotics and to have anticancer activity.

Conclusion: In addition to the acceptable activities of all strains, by elucidating their interaction mechanisms and evaluating their probiotic, functional and antiproliferative effects under *in vivo* conditions, especially LP001 and LP002 could be true sources of probiotics with anticancer effects in food and healthcare fields.
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Keywords: Antioxidant; Anticancer; Bacterial adhesion; *Lacticaseibacillus paracasei*; Postbiotics

INTRODUCTION

In recent years, the increased prevalence of life-threatening diseases such as cancer has had a significant impact on people's dietary preferences by leading to a heightened preference for natural products.[1-4] In this perspective, natural pickles are one of the most preferred food products. Lactic acid bacteria (LAB), known as probiotics, are crucial factors in the production of natural pickles[5,6] and their adequate consumption is well known to protect and maintain host health.[7-9] Previous studies have highlighted that probiotics are recommended as intestinal regulators and offer some benefits in the treatment of various gastrointestinal disorders, including colorectal cancer,[9,10] and ameliorate various health concerns including irregular cholesterol levels, hypertension and oxidative stress.[7,11,12] They have also been reported to significantly delay the precursors of diabetes such as hyperglycemia and hyperinsulinemia.[13] Postbiotics, known as antioxidants, are secreted by probiotics and encompass a diverse array of organic acids and molecules, including enzymes, peptides, peptidoglycan and lactic acid,[1,14,15] and fermentation serves as one of the primary mechanisms for the production of many bioactive substances such as postbiotics. The beneficial effects of probiotics are ascribed to known impacts of postbiotics that extend to immunological stimulation, encompassing anti-inflammatory, immunomodulatory, anti-obesogenic, antihypertensive, antioxidant and hypocholesterolemia activities.[8,14,15] An imbalance in the intestinal microbiota was reported to cause the development of autoimmune or allergic diseases[16] and the probiotic content of the body plays an important role in the regulation of this balance by creating the desired microflora in the gastrointestinal tract (GIT).[17] Fermented foods are characterized by the enzymatic breakdown of carbohydrates by probiotics, and are highly favored due to their taste, flavor and color.[18] The diversity and quantitative composition of postbiotics are pivotal elements to obtaining unique probiotics. The ability to survive despite challenges within the GIT, including gastric acidity, bile salt exposure and pancreatin, is a known probiotic characteristic. Because of these features,

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probiotics may manifest their beneficial and unique biological effects on the intestinal flora.[10,15,18-20] *Lacticaseibacillus paracasei* species are generally used as starters in food fermentations and/or probiotics.[21] It has been determined that some *L. paracasei* strains have probiotic, technological, bioprotective, anti-inflammatory, simulated and live gastrointestinal digestion bypass, immunomodulatory, anti-proliferative and pro-apoptotic effects.[22-24] In addition, *L. paracasei* strains have been proven to be suitable for industrial processing to produce readymade probiotic foods.[25,26] In particular, some readymade foods enriched with the probiotic *L. paracasei* IMPC2.1 strain have been tested in human nutrition studies and have been found to show promising and successful health benefits.[27,28] Hence, the possible novel biological effects of probiotics are attracting the attention of researchers.

The aim of the research reported here was to obtain unique bacterial strains that can survive in gastric pH and have effective inhibition on cancer cell lines, with the data suggesting potential unique strains for novel research in the pharmaceutical and food industries. Bacterial identification was conducted by 16S rRNA sequence analysis. Different *in vitro* environments simulating the adverse conditions of the GIT were prepared using pancreatin, pepsin and bile. Subsequently, the probiotic potential of *L. paracasei* strains was ascertained by evaluating their viabilities in simulated GIT conditions. Additionally, their colonization capacities were assessed by determining their adhesive effects on healthy cell line L-929 and colon cancer cell lines HT-29 and Caco-2. Postbiotic metabolite (PM) samples and bacterial extract (BE) samples were prepared, and their inhibitory effects on cell lines were evaluated. To explain more clearly the probiotic and anticancer results, the quantitative amounts of some PMs were determined using high-performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Sample collection and bacterial isolation

Pickle samples were acquired from traditional fermented pickles producers in different regions of Turkey and subsequently stored at 4 °C in 50 mL sterile Falcon tubes (LABSOLUTE, Germany). The isolation was performed using the protocol described by Mandel and Higa.[29] *Lactiplantibacillus plantarum* (*Lp*), *Lactobacillus delbrueckii subsp bulgaricus* (*Lb*), *Lactobacillus reuteri* (*Lr*) and *Lacticaseibacillus casei* (*Lc*) obtained from Maysa food-lab (Tuzla-Istanbul, Turkey) were used as standards.

Strain selection and bacterial identification

Potential strains were chosen based on their morphological characteristics, catalase testing, and Gram-positive/negative classification. 16S rRNA sequence analyses were performed at the University and Industrial Cooperation Center (USKIM) in Kahramanmaraş Sütcü İmam University. The reverse and forward sequence results were combined based on chromatograms using the Codon Code Aligner V.6.0.2 program. 16S rRNA gene sequence (1282 nt) of strains was compared to sequences of type strains in GenBank databases.[30] The evolutionary tree was carried out using the maximum-likelihood[31] algorithm drawn from the MEGA X software package.[32] Evolutionary distance matrix was calculated using the model of Jukes and Cantor.[33] Cantor + Gama Distributed (JC + G)[34] topology of the resultant tree was evaluated by bootstrap analyses[35] based on 1000 resamplings. *Lactobacillus brevis* was used as the outgroup.

Preparation of bacterial suspensions

Bacteria were activated by incubating at 37 °C for 24 h and centrifuged at 5000 × *g* for 5 min at 4 °C. Supernatants were removed and washed twice with sterile phosphate-buffered saline (PBS; pH 7.4). Bacterial suspensions were prepared according to 0.5 McFarland density[36] and their concentrations were adjusted to approximately 6.0–7.0 log CFU mL^{-1}. The suspensions were employed in the determination of the biological activities of the bacterial isolates.

Resazurin microtiter test

Resazurin microtiter test was performed using a slight modification of the protocol proposed by Jung *et al*.[37] Microtiter test was performed at 590 nm using a microplate reader (Thermo Scientific Multiskan Go, Finland). The viability of the isolates was calculated using Eqn (1) whose abbreviations are as follows: ALRM: Ambient (A), LAB (L), Resazurin (R), Medium (M); ARM: Ambient (A), Resazurin (R), Medium (M); LRM: LAB (L), Resazurin (R), Medium (M); RM: Resazurin (R), Medium (M):

$$\text{Viability}\,(\%) = \left(\frac{\text{ALRM}-\text{ARM}}{\text{LRM}-\text{RM}}\right)\times 100 \quad (1)$$

Determination of probiotic properties

Determination of viability at simulated gastric pH

Simulated gastric pH media (pH of 2.0 and 3.0) were created by adding 3% pepsin (Sigma-Life Science, USA) in standard PBS solution and isolate viability rates were tested in gastric pH using the method of Elcioglu and Kunduhoglu.[38] Standard PBS solution with pepsin was employed to evaluate the viabilities of strains at gastric pH. Bacterial isolates were dissolved in PBS and incubated at 37 °C for 3 h. While this study was ongoing, bacterial viabilities were measured in 10^{-5} dilutions prepared from the samples collected at the end of hours 1, 2 and 3. The results were calculated as viability rates using Eqn (1).

Determination of viability in simulated intestinal tract

Following the incubation of the isolates in simulated PBS medium including 1 mg mL^{-1} pancreatin (pH 8), viability rates were determined using the method of Tokatli *et al*.[39] The bile resistances of the isolates were determined after incubation in MRS broth nutrient medium including 0.3%, 0.5% and 1% of bile salt according to the method of Soliman *et al*.[40] Following the incubation period for 4 h, isolate viabilities were tested and the viability rates were calculated using Eqn (1).

Quantitative measurement of postbiotic metabolites

Postbiotic metabolite levels were quantitatively determined by measuring in triplicate using an Agilent Technologies 1260 Infinity II HPLC (Agilent, USA) according to the method of Ball and Lloyd.[41] The standards used in the study and their respective suppliers are as follows: maleic acid, citric acid, tartaric acid, malic acid, succinic acid, fumaric acid, acetic acid (Sigma-Aldrich, USA), pyruvic acid (Chemservice, USA), acetoin (Toronto Research Chemical Inc., Canada) and 2,3-butanediol (Dr Ehrenstofer GmbH, Germany). The configuration and components used for HPLC were as follows: 1260 RID detector (G7162A), 1260 Quat Pump VL pump (0.6 mL min^{-1}), 1260 Vialsampler (20 μL injected) and G7130A column furnace (65 °C). The column used for analysis was an Agilent Hi-Plex H (7.7 × 300 mm, 8 μm).

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Cell culture tests

Bacterial adhesion tests on cell lines

The adhesion capacities of isolates on cell lines were evaluated by modifying the protocol recommended by Mellor *et al.*[42] L-929 as healthy cell line and HT-29 and Caco-2 as colon cancer cell lines were utilized and the cell lines were procured from Muş Alparslan University, Central Research Laboratory. After measurement at 590 nm, the results were calculated as adhesion effects using Eqn (1).

Preparation of PM and BE samples

Following bacterial activation by overnight incubation, the BE and PM extracts were prepared according to the protocol recommended by Kim *et al.*[43] The extracts prepared at 2.5 and 5 mg mL^{-1} were filtered using a 0.22 μm filter and subsequently stored at +4 °C until the experimental stage.

Determination of inhibition effects

The inhibitory effects of PM and BE samples on healthy (L-929) and colon cancer (HT-29 and Caco-2) cell lines were assessed using the protocol recommended by Assaf *et al.*[44] The tests were performed using a microplate reader (Thermo Scientific Multiskan Go, Finland) at 540 nm. Inhibition rates of the cell lines were then calculated using Eqn (2). The plain nutrient medium served as the control group and the average absorbance values of the control group were considered as 100% viability.

$$\text{Inhibition rate} = \left(1 - \frac{OD_{\text{Sample}}}{OD_{\text{Control}}}\right) \times 100 \quad (2)$$

Statistical analysis

All tests were performed using at least three replicates. By using the data obtained from the tests, the mean and the standard error of mean were calculated and graphed. These data were compared statistically using Dunnet's multiple comparison test, following one way analysis of variance. $P < 0.05$ was considered statistically significant. When compared to the standards, statistically significant positive effects were shown as (+) and negative effects were shown as (−). The expressions of the symbols according to the significant levels of APA are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); $P > 0.05$, not significant (ns).

RESULTS

Identification of bacterial strains

16S rRNA gene sequence (1282 nt) of strains was compared with the sequences of type strains in GenBank databases.[30] The constriction of the evolutionary tree was performed using the maximum-likelihood[31] algorithm drawn from the MEGAX software package program.[32] According to the 16S rRNA gene region, all isolates were diagnosed as *L. paracasei* with a 99% similarity. The evolutionary distance matrix was calculated using the model of Jukes and Cantor[33] Cantor + Gama Distributed (JC + G)[34] and topology of the resultant tree evaluated by bootstrap analyses[35] based on 1000 resamplings. *L. brevis* was used as the outgroup. A close homology was observed in the phylogenetic tree between the sample isolates and the reference isolates. In the results observed in the phylogenetic tree derived from 16S rRNA sequence, it is noteworthy that the bacterial isolates primarily cluster within themselves and are highly similar to each other. Moreover, it is apparent that *L. paracasei* (OQ449697) and *L. paracasei* (ON430603) exhibit the highest similarity to LP001, LP002 and LP003 strains (Fig. 1).

Viability results in simulated GIT

Isolate viability rates in simulated gastric pH

Isolates were exposed to simulated gastric pH for 3 h and their viability rates were determined at 1 h intervals (Fig. 2). A general

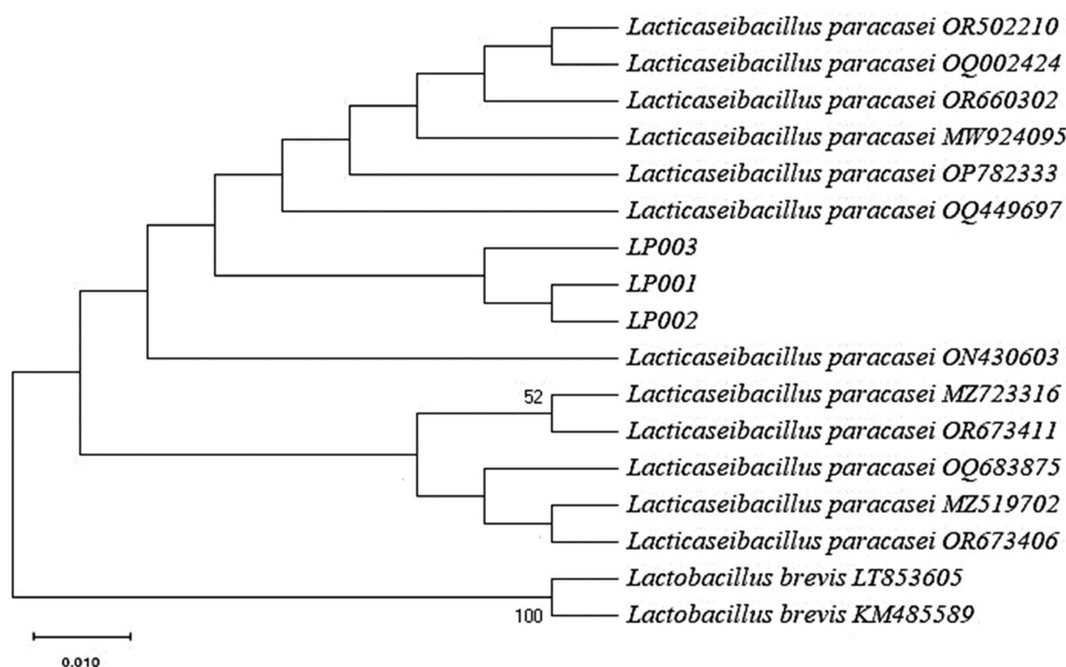

Figure 1. Phylogenetic tree of *L. paracasei* strains identified by 16S rRNA sequence. The phylogenetic relationship was inferred using the maximum likelihood method. Bootstrap values expressed as a percentage of 1000 replications are given at the branching points. Only values ≥50% are shown. LP001, LP002, LP003 are the symbols of bacteria isolated from pickle samples.

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decrease was detected in bacterial viability at pH 2 (Fig. 2(a)). Standard viabilities increased at the second hour of pH 3 and decreased again at the third hour (Fig. 2(b)). Differently, the viability of the isolates increased depending on the time at pH 3. LP001 showed similar pH resistance to the standards or higher than them and also exhibited the highest viability rate at the third hour in simulated gastric pH 3.

Viability rates in simulated intestinal tract

Following 4 h of incubation in simulated pancreatin medium, isolate viabilities were evaluated and it was observed that they remained viable at similar rates to the standards. It was especially observed that LP001 had similar viability to the standards, and higher than *Lb* viability (Fig. 3(a)). The viabilities were evaluated following the incubation in simulated intestinal tract created using three different bile concentrations, and approximately similar viabilities to the standards were recorded (Fig. 3(b)). A general decrease in viability was detected based on increasing bile concentration. LP001 viability was mostly similar to that of the standards at all concentrations and higher than standards even at highest concentration (Fig. 3(b)).

Bacterial effects on cell lines

Bacterial adhesion effects on cell lines

The adhesive capacities of the isolates on cell lines were tested and it was observed that all isolates adhered to L-929 more effectively than standards. LP002 and LP003 showed similar or higher adhesion effects on HT-29 than standards. While LP001 and LP002 showed mostly higher adhesion effects on the Caco-2 cell line compared to the standards, LP003 exhibited a similar adhesion to the standards (Fig. 4). In the overall examination, it can be said that especially LP002 adhered better on colon cancer cell lines than standards and the isolates also adhered relatively better on HT-29 and Caco-2 than on L-929.

Suppressive effects of PMs

PMs were prepared at 2.5 and 5 mg mL^{-1}, and their suppressive effects on cell lines were evaluated. Results were presented as percentages of viability and inhibition rates (Fig. 5). For the effects of 5 mg mL^{-1} PM on L-929 viability, all isolates exhibited similar inhibitory effects to the standards. However, more pronounced fluctuations were observed in 2.5 mg mL^{-1} PM results and L-929 viability was suppressed less by LP001. Compared to LP001, the other PMs suppressed the L-929 cell line better by showing similar effects to standards (Fig. 5(a)). According to the effects of PMs on HT-29, 5 mg mL^{-1} PMs suppressed the HT-29 viability relatively better than *Lp* and *Lr* standards. In contrast, 5 mg mL^{-1} isolate PMs suppressed HT-29 viability at similar rates to *Lb* and *Lc* standards. For the effects of 2.5 mg mL^{-1} PMs on HT-29, the PMs of LP002 and LP003 suppressed HT-29 viability at similar rate to *Lb* and were recorded as lower than those of the other standard PMs (Fig. 5(b)). For the suppressive results of PMs on Caco-2 viability, overall, isolate PMs diminished the Caco-2 viability to levels below 15% across all concentrations and exhibited a relatively

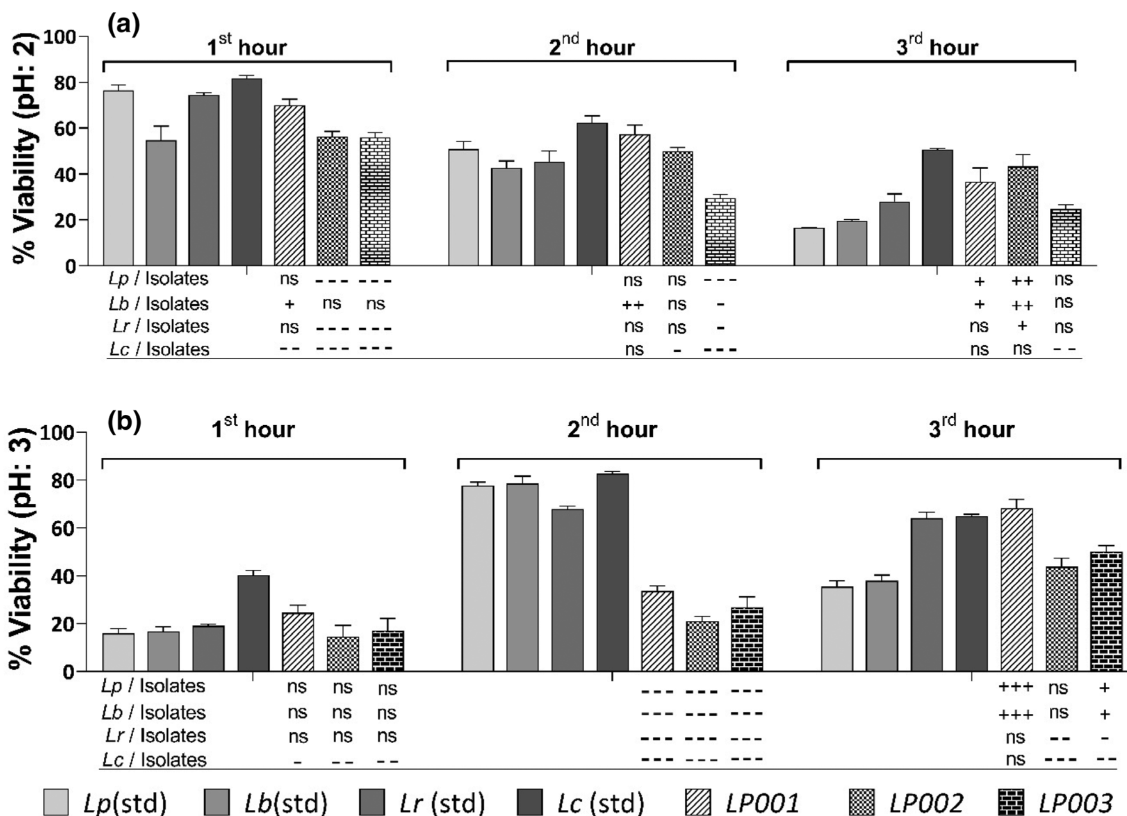

Figure 2. Viability percentages showing the bacterial resistance to simulated gastric pH 2 (a) and pH 3 (b). $P < 0.05$ was considered statistically significant. When compared to the standards, increased viability rates were symbolized as (+) and decreased viability rates were symbolized as (−). Accordingly, significance levels are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); and $P > 0.05$, not significant (ns). The abbreviations are as follows. Standards: *L. plantarum* (*Lp*), *L. bulgaricus* (*Lb*), *L. reuteri* (*Lr*), *L. casei* (*Lc*) and *L. paracasei* strains: LP001, LP002 and LP003.

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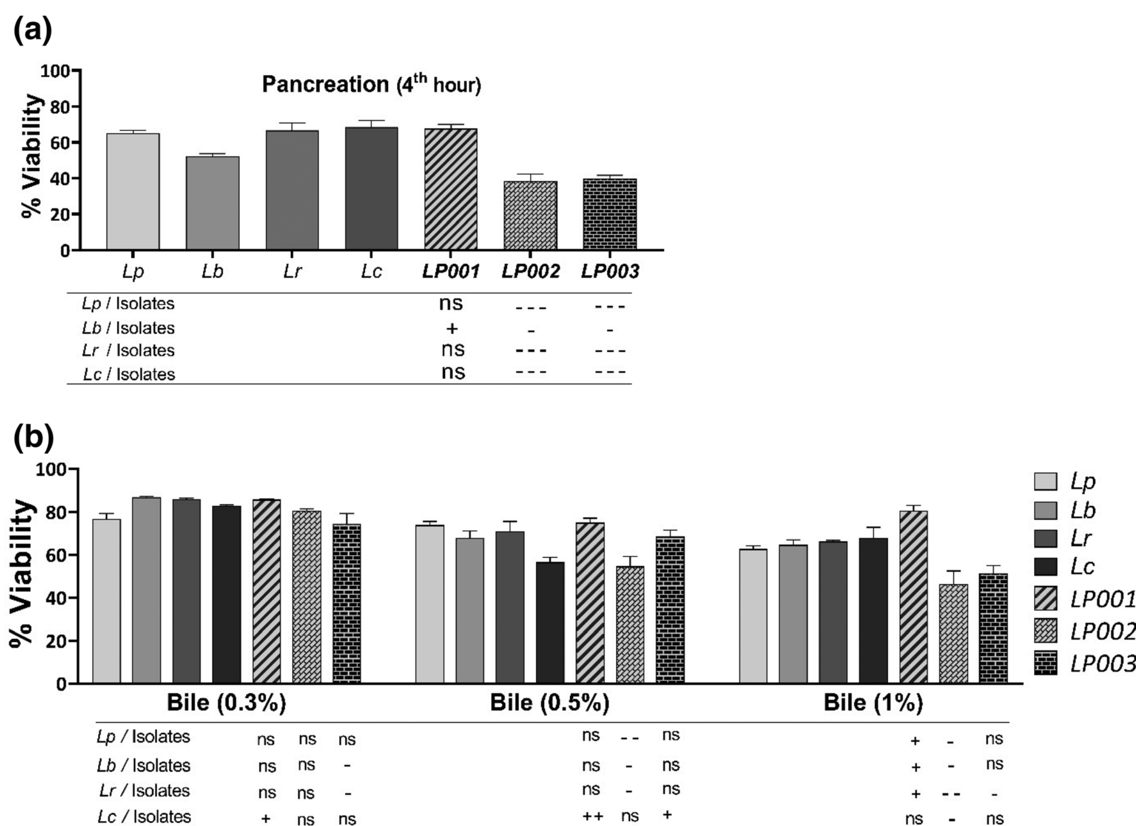

Figure 3. Viability rates showing the bacterial resistance to simulated intestinal tract. (a) Bacterial viability rates following incubation for 4 h in simulated pancreatin medium. (b) Bacterial viability rates in simulated media with different bile concentrations. When compared statistically to standards, increasing viability rates were shown as (+) and decreasing viability rates were shown as (−). Accordingly, significance levels are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); and $P > 0.05$, not significant (ns). The abbreviations are as follows. Standards: *L. plantarum* (*Lp*), *L. bulgaricus* (*Lb*), *L. reuteri* (*Lr*), *L. casei* (*Lc*) and *L. paracasei* strains: LP001, LP002 and LP003.

better suppressive effect on Caco-2 viability compared to the HT-29 and L-929 viabilities. For the suppression results on Caco-2, isolate PMs suppressed Caco-2 viability at statistically similar rates to the standards (Fig. 5(c)).

Suppressive effects of BEs

Bacterial extracts (BE) were prepared at 2.5 and 5 mg mL^{-1} concentrations and their suppressive effects on cell lines were assessed. The results were presented as viabilities and inhibition rates (Fig. 6). For the effects on L-929, the suppressive effect of the BEs demonstrates a proportional increase depending on concentration. For the suppressive results of 5 mg mL^{-1} BE, the BEs of isolate were observed to have similar suppressive effects to *Lb* and *Lc* standards. The 2.5 mg mL^{-1} isolate BEs exhibited a lower inhibition effect on L-929 than the BE of *Lp* and no statistically significant difference was observed in the effects of the BEs from other isolates and standards (Fig. 6(a)). For the results of 5 mg mL^{-1} BEs on HT-29, the BEs from LP002 and LP003 inhibited better HT-29 viability than the BEs of *Lp* and *Lb* (26% and 25%, respectively). Similar inhibitory effects were noticed in the results of the isolate BEs and standard BEs (Fig. 6(b)). The suppressive effects of BEs were also tested on Caco-2 viability. The 5 mg mL^{-1} BEs from LP002 and LP003 inhibited Caco-2 viability at the same rates as standards and higher than the BE of *Lb*. The 2.5 mg mL^{-1} BEs from LP002 and LP003 were able to inhibit Caco-2 viability better than the BEs of *Lp*, *Lb* and *Lc* (Fig. 6(c)).

Quantitative PM results

Quantitative amounts of PM were investigated to reveal more precisely the reason behind the probiotic and anticancer results. Some PM components such as maleic acid, tartaric acid and fumaric acid were not detected. Except for *Lc* and LP003, it is noteworthy that succinic acid was detected at the lowest quantity in the other isolates. Contrary to this, acetic acid was detected as the most abundant component in the other isolates, except for LP002. Finally, LP001 and LP002 isolates were found to have two highest PM concentrations (Table 1).

DISCUSSION

Surviving at low pH and being able to manipulate the intestinal flora through their unique effects are known characteristics of probiotics.[19] Bacterial properties are well known to vary contingent upon the isolation source and provide unusual properties in the background.[45] Therefore, this study aimed to obtain *Lactobacillus* strains with unique biological activity. Dietary probiotics are recognized for their ability to withstand gastric acidity and bile exposure, and for their ability to colonize and proliferate within the digestive tract.[13] It is known that food remains in the stomach for approximately 3 h.[46] Therefore, the isolates were incubated in simulated gastric pH (pH 2 and pH 3) for 3 h and the viability rate was measured systematically at 1 h intervals for 3 h. Observations revealed a gradual decline in the viability of all strains over the experiment time at pH 2. It is noteworthy that LP001 and LP003

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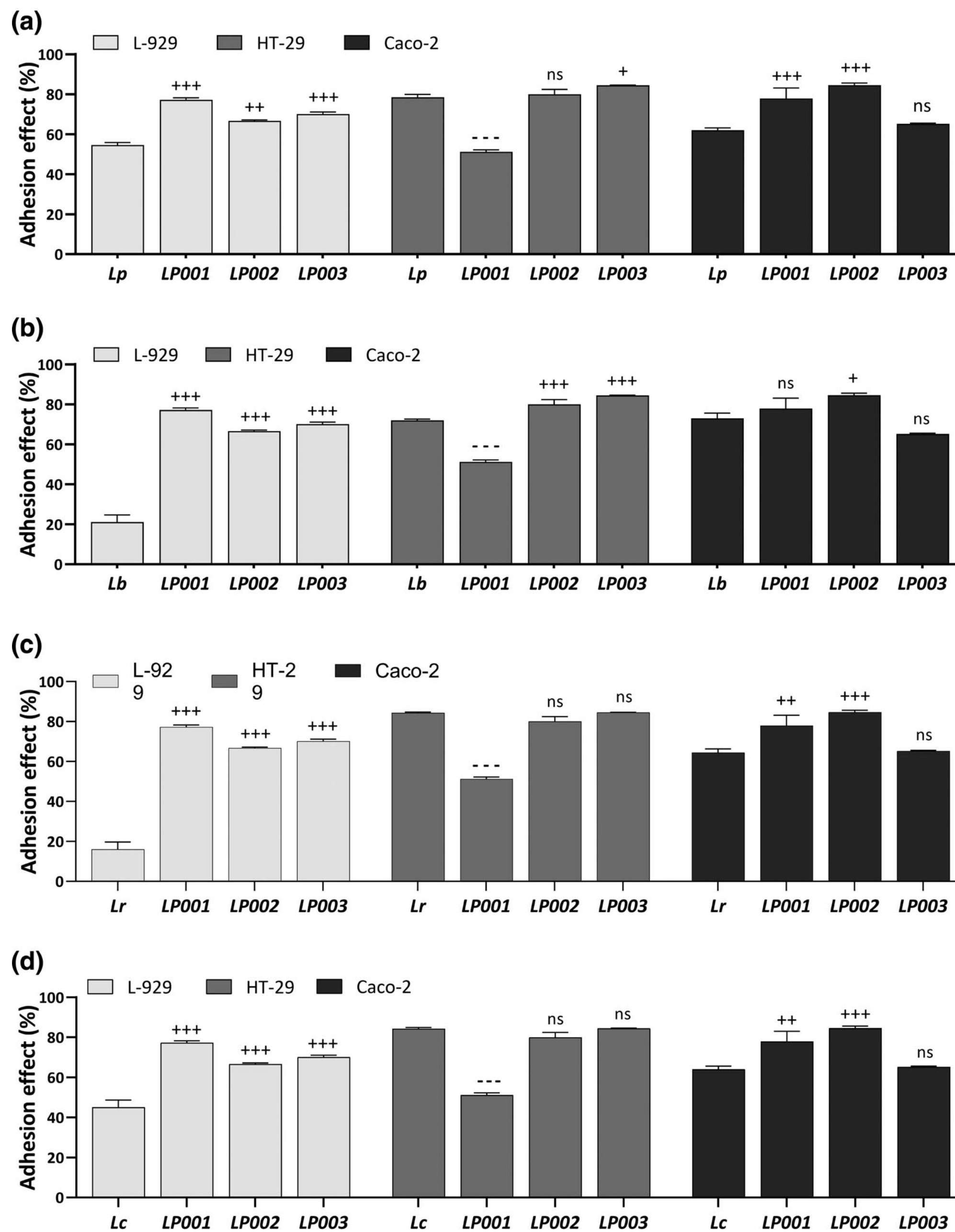

Figure 4. Adhesion percentages of bacterial isolates on cell lines. *Lp*, *Lb*, *Lr* and *Lc* were used as standards. (a) Adhesion effects of isolates compared to *Lp*, (b) adhesion effects of isolates compared to *Lb*, (c) adhesion effects of isolates compared to *Lr* and (d) adhesion effects of isolates compared to *Lc*. When compared statistically to standards, increasing adhesive effects were shown as (+) and decreasing adhesive effects were shown as (−). Accordingly, significance levels are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); and $P > 0.05$; not significant (ns). The abbreviations are as follows. Standards: *L. plantarum* (*Lp*), *L. bulgaricus* (*Lb*), *L. reuteri* (*Lr*), *L. casei* (*Lc*) and *L. paracasei* strains: LP001, LP002 and LP003.

exhibited higher viability at pH 3 compared to *Lp* and *Lb* standards. This observation highlights the potential of LP001 and LP003 as acceptable probiotic candidates. An increase in the second hour and a noteworthy decrease in the ongoing third hour were noted in the viability of the standards at pH 3. However, the regular increases in the viabilities of isolate are crucial results at pH 3. These data are a clue that the samples show a better probiotic effect at pH 3 than at pH 2. Previous research indicates that bacterial viability was adversely impacted by the gastric environment over time.[47] Particularly, the present findings are in this line at pH 2 and the acceptable viability of the isolates at both pH values contributes to their probiotic potential. Increasing viability rates over 3 h at pH 3 also confirm the probiotic potential of the samples (Fig. 2). Consequently, LP001 exhibited similar resistance to *Lp*, *Lb* and *Lr* at almost all times, showing high viability at pH 2 and pH 3. This result is of significance for LP001 as a promising probiotic candidate. Following the exposure to simulated medium with pancreatin and bile salt for 4 h, the viability rates of the isolates were determined (Fig. 3). The pancreatin resistance of LP001 was better than that of *Lb*, in contrast to the findings observed for LP002 and LP003, and was also similar to that of *Lp*, *Lr* and *Lc* standards. This compatibility with pH results confirms

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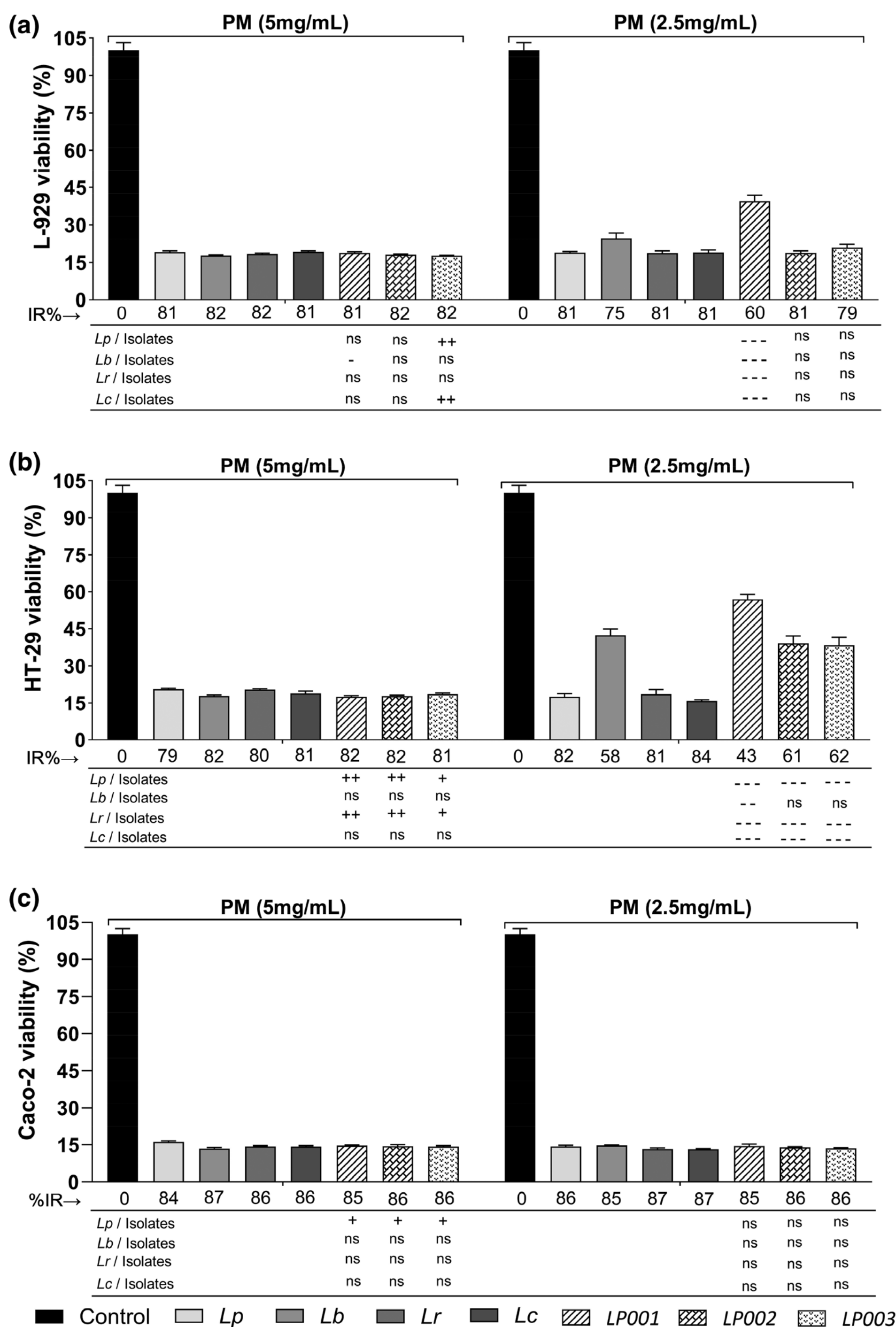

Figure 5. Suppressive effects of PM on cell lines. (a) Viability rates of L-929, (b) viability rates of HT-29 and (c) viability rates of Caco-2. IR% shows inhibition rates in the viability of cell lines. APA significance level was used for statistical significance rating. When compared statistically to the standards, desired inhibition effects were expressed as (+), and undesirable inhibition effects were expressed as (−). Accordingly, significance levels are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); and $P > 0.05$, not significant (ns). The abbreviations are as follows. Standards: *L. plantarum* (*Lp*), *L. bulgaricus* (*Lb*), *L. reuteri* (*Lr*), *L. casei* (*Lc*) and *L. paracasei* strains: LP001, LP002 and LP003.

the probiotic properties of LP001. Although the viability of all isolates decreased with increasing bile concentration, the viabilities of LP002 and LP003 were similar to or less than those of the standards at all concentrations. Contrary to this, it is noteworthy that the viability of LP001 was similar to or higher than that of the standards at all concentrations. This result, in agreement with the LP001 viability in simulated pancreatin medium, confirms its probiotic potential.

Activities such as the regulation of the GIT, flora manipulation and strengthening the immune system are dependent on permanence in the intestines. Some members of the flora are recognized for their pivotal role in colon cancer prevention by

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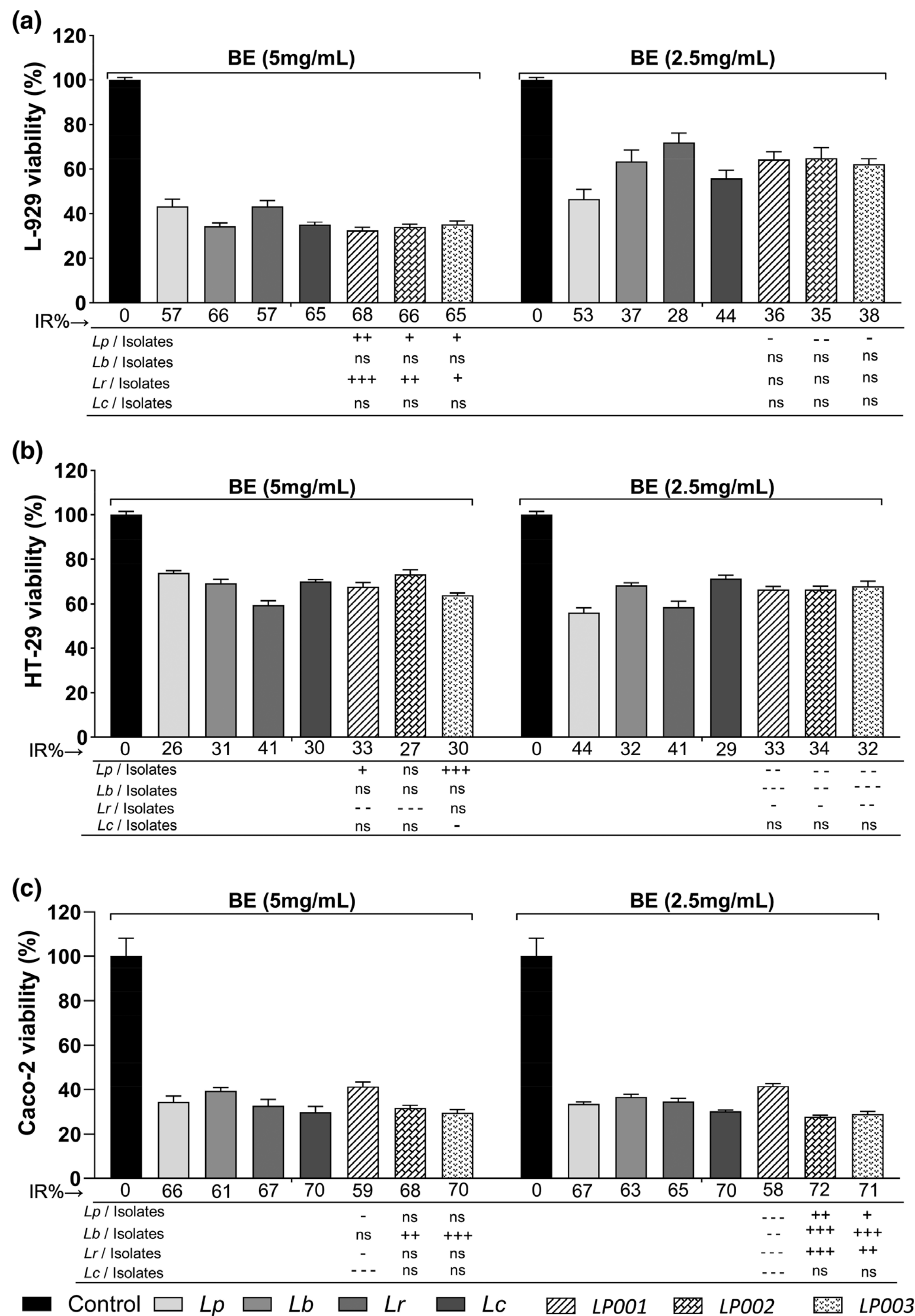

Figure 6. Suppressive effects of BEs on cell lines. (a) Viability rates of L-929, (b) viability rates of HT-29 and (c) viability rates of Caco-2. IR% shows inhibition rates of isolates on cell lines. APA significance level was used for statistical significance rating. When compared statistically to the standards, desired inhibition effects were expressed as (+) and undesirable inhibition effects were expressed as (−). Accordingly, significance levels are as follows: $P < 0.05$, significant (+ or −); $P < 0.01$, very significant (+ + or − −); $P < 0.001$ and $P < 0.0001$, extremely significant (+ + + or − − −); and $P > 0.05$, not significant (ns). The abbreviations are as follows. Standards: *L. plantarum* (*Lp*), *L. bulgaricus* (*Lb*), *L. reuteri* (*Lr*), *L. casei* (*Lc*) and *L. paracasei* strains: LP001, LP002 and LP003.

increasing their persistence in the GIT.[19] In general, the isolates could adhere to L-929 more effectively than the standards. The adhesion effects of LP002 and LP003 on HT-29 were observed to be mostly similar to the standards. Moreover, the adhesion effect of LP001 and LP002 isolates on Caco-2 was recorded as mostly higher than the standards. The adhesion results on HT-29 and Caco-2 cell lines indicate that LP002 has high permanence potential in the intestines, which contributes to its anticancer potential. Compared to the adhesion capacity on L-929, the high adhesion capacities of LP002 and LP003 on HT-29 and the adhesion capacity of LP002 on Caco-2 are notable anticancer signs as L-929 are healthy cells.

The molecules produced by lactobacilli, including peptidoglycans, surface proteins, cell wall polysaccharides, secreted proteins,

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Table 1. Quantitative postbiotic metabolite concentrations (μg mL^{-1}). The highest and lowest values are in bold

		Lp	*Lb*	*Lr*	*Lc*	LP001	LP002	LP003
Postbiotic Metabolites	Maleic acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Citric acid	17.89 ± 0.19	23.12 ± 0.10	6.77 ± 0.00	11.22 ± 0.03	16.11 ± 0.02	17.16 ± 0.19	19.14 ± 0.02
	Tartaric acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Pyruvic acid	0.00 ± 0.00	4.50 ± 0.01	0.00 ± 0.00	0.00 ± 0.00	4.91 ± 0.02	4.77 ± 0.02	0.00 ± 0.00
	Malic acid	9.50 ± 0.26	8.11 ± 0.41	6.04 ± 0.29	**6.68 ± 0.33**	11.47 ± 0.17	9.88 ± 0.02	9.45 ± 0.01
	Succinic acid	**3.75 ± 0.22**	**0.55 ± 0.03**	**5.35 ± 0.19**	9.44 ± 0.17	**0.60 ± 0.07**	**0.29 ± 0.03**	0.00 ± 0.00
	Fumaric acid	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
	Acetic acid	**67.81 ± 2.53**	**55.36 ± 0.91**	**44.92 ± 3.28**	**70.97 ± 0.03**	**58.95 ± 0.09**	54.34 ± 0.53	**52.26 ± 0.24**
	Acetoin	11.04 ± 0.05	4.95 ± 0.45	9.96 ± 0.09	14.72 ± 1.08	17.16 ± 2.93	19.29 ± 0.60	**8.66 ± 1.81**
	2,3-Butanediol	46.08 ± 2.04	38.16 ± 0.45	0.00 ± 0.00	52.88 ± 4.33	61.98 ± 1.00	**73.36 ± 3.55**	37.71 ± 1.28
Total PM content		**156.07**	**134.75**	**72.94**	**165.91**	**171.18**	**179.09**	**127.22**

Lp, *L. plantarum*; *Lb*, *L. bulgaricus*; *Lr*, *L. reuteri*; *Lc*, *L. casei*; LP001, LP002, LP003, *L. paracasei* isolates.

bacteriocins and organic acids, are known as immunomodulatory and antitumor molecules[15] and have an undeniable ability to reduce the side effects of conventional cancer treatment.[48] In addition to bacterial adhesion, their suppressive and cytotoxic effects on cancer cell lines are also important for sustainable specific anticancer effects. PM and BE samples were prepared at 2.5 and 5 mg mL^{-1} and their suppressive effects on cell lines were investigated (Fig. 5). It is possible to say that PM samples are more effective than BE samples in suppressing cell viability. For the results of 2.5 mg mL^{-1} PM on L-929, the inhibition effect of PM from LP001 was observed to be lower than other samples and standards. Since L-929 is a healthy cell line, the low suppressive effect of PM from LP001 is a positive result. The effects of 5 mg mL^{-1} PM on the L-929 cell line were observed to be mostly similar. In addition, the similar effects of the other isolates to the standards at both concentrations are useful results for their anticancer potential. In the inhibition tests of HT-29, the sample and standards had close inhibition effects at 5 mg mL^{-1} concentration, while 2.5 mg mL^{-1} PM effects revealed more significant inhibition differences. Although the suppressive effects of 2.5 mg mL^{-1} PMs are similar to or lower than those of the standards, the 5 mg mL^{-1} of PMs suppressed HT-29 viability at the same rates as standards, which contributes to their anticancer potentials. For the inhibition test results of Caco-2, PMs exhibited a robust inhibitory effect by reducing the Caco-2 viability to less than 15% at both concentrations, which also support the anticancer potentials of PMs. The suppressive effects of BEs prepared at 2.5 and 5 mg mL^{-1} were tested on cell lines. For the results of inhibition of L-929 viability, the suppressive effect of 5 mg mL^{-1} BE was recorded as higher than that of 2.5 mg mL^{-1} (Fig. 6(a)). Additionally, the suppressive effects of BEs were noted to be mostly equivalent to those of standards at both concentrations. Particularly, 2.5 mg mL^{-1} BEs suppressed L-929 less than the *Lp* standard. This makes more valuable the specific effect of the isolates on cancer cell lines. More pronounced fluctuations were detected in the results of 2.5 mg mL^{-1} BE on L-929 compared to the results of 5 mg mL^{-1} BEs. For the inhibition results of BEs on HT-29, HT-29 viability was mostly not affected by BEs at both concentrations compared to the standards. Although 5 mg mL^{-1} BE from LP001 and LP003 suppressed HT-29 viability relatively better than the BE from *Lp*, they mostly exhibited similar suppressive effects to those of the standards (Fig. 6(b)). This similarity supports their useful potential as anticancer agents. For the inhibition results of BEs on Caco-2, BEs suppressed the Caco-2 viability better compared to the HT-29 viability and the suppression levels were not significantly affected by the concentration change of BEs (Fig. 6(c)). As a notable result confirming the anticancer potential of the samples, BEs from LP002 and LP003 better suppressed Caco-2 viability compared to the standards. Compared to BEs, the higher suppressive results of PMs may be associated with the antioxidant effect or the immune system modulation supported by PMs.

PMs are important molecules behind the beneficial effects of probiotics, and fermentation is an important mediator in the production of PMs[7] and bioactive nutraceuticals such as organic acid and bacteriocins.[19] In this study, the quantitative level of PMs was determined using HPLC. Previous research has highlighted differences in quantitative PM levels and specific metabolic properties depending on species and isolation source.[45] Considering the studies emphasizing the unique biological properties of each strain, it is clear that LP001 and LP002 have the highest quantitative PMs, consistent with probiotic and anticancer effects.

CONCLUSION

The resistance of LP001 to simulated gastric pH (pH 3), pancreatin and bile makes it an acceptable probiotic candidate. The adhesion effects of LP002 and LP003 on HT-29 and Caco-2 are important criteria for their anticancer potential. Furthermore, PMs showed superior inhibitory effects on cell lines compared to BEs. Particularly, 5 mg mL^{-1} PM suggests that the strains may serve as indirect suppressors in colon cancer formation. Since simulated environments created in *in vitro* conditions are not an exact equivalent of the GIT, high bacterial viability may be observed or may carry the potential for death due to the vulnerability of cells

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compared to living tissues. However, the study plays an important role in obtaining *L. paracasei* strains with novel and unique biological activity by comparing their effects with known standards under the same conditions. In addition, the results observed independently of the real GIT environment are of great importance for understanding the real effects of intestinal flora. Therefore, all isolates are important for the health of the intestinal flora. Particularly, the probiotic effect of LP001 and the anticancer effect of LP002 are prominent and these are likely to be a real source of probiotic bacteria with anticancer properties for food and health applications as a result of elucidating the interaction mechanisms and investigating how their probiotic, functional and antiproliferative activities will perform *in vivo*.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

CONFLICTS OF INTEREST

The authors declare that they have no known conflicts of interest that could have appeared to influence the work reported in this paper.

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