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Muş yöresinin doğal yoğurtlarındaki probiyotik *Lactiplantibacillus plantarum*'ların postbiyotik metabolitleri ve kolon kanser hücre hattı üzerindeki etkileri

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ÖZ

Probiyotik Laktik Asit Bakterileri (LAB) izole edildiği kaynağa bağlı olarak farklı karakterde olabildikleri gibi, postbiyotik metabolit miktarı ve antikanser aktivite bakımından farklı potansiyellere sahip olabilirler. Bu nedenle yoğurttan izole edilmiş 125 LAB izolatının MALDI-TOF MS ile 22 izolatın *Lactiplantibacillus plantarum*'a benzerlik gösterdikleri belirlendi. *L. plantarum* izolatları farklı pH, pepsin, pankreatin ve safra tuzuna karşı probiyotik dirençleri araştırıldığında, bütün izolatların iyi aktivite gösterdiği belirlendi. İyi probiyotik özellik gösteren altı *L. plantarum* (3, 7, 13, 25, 26 ve 29) suşu ile *L. plantarum* STD'in postbiyotik metabolit miktarı HPLC ile belirlendi. Bu izolatların tartarik asit, pürivik asit ve malik asit sentezi tespit edilmedi. Bu izolatların en fazla asetik acid, en az süksinik acid sentezledikleri belirlendi. Ayrıca bu izolatların kolon kanseri hücre hatları (HT-29 ve Caco-2) üzerinde konsantrasyon artışına bağlı olarak daha iyi antikanser aktivite sergiledikleri gözlemlendi. *L. plantarum* izolatlarının yeni *in vivo* çalışmalar ile desteklenerek gıda ve sağlık endüstrisine katkı sağlayacağı düşünülmektedir.

Anahtar Kelimeler: Antikanser, LAB, *Lactiplantibacillus plantarum*, Postbiyotik metabolit, Probiyotik.

Postbiotic metabolites of probiotic *Lactiplantibacillus plantarum* in natural yoghurts from Muş region and their effects on colon cancer cell lines

ABSTRACT

Probiotic Lactic Acid Bacteria (LAB) can exhibit different characteristics depending on their source of isolation, and they may also possess varying potentials in terms of postbiotic metabolite amounts and anticancer activity. Therefore, 22 of 125 LAB isolates isolated from yogurt were determined to be like *Lactiplantibacillus plantarum* using MALDI-TOF MS. When the probiotic resistance of *L. plantarum* isolates to different pH, pepsin, pancreatin, and bile salts was investigated, all isolates were found to show good activity. The amount of postbiotic metabolites of six *L. plantarum* strains (3, 7, 13, 25, 26, and 29) and L. plantarum STD, which showed good probiotic properties, was determined by HPLC. Tartaric acid, pyruvic acid, and malic acid synthesis were not detected in these isolates. It was determined that these isolates synthesized the most acetic acid and the least succinic acid. Furthermore, it was observed that these isolates exhibited improved anticancer activity on colon cancer cell lines (HT-29 and Caco-2), depending on increasing concentrations. It is believed that *L. plantarum* isolates will contribute to the food and health industries with further support from new *in vivo* studies.

Keywords: Anticancer, LAB, *Lactiplantibacillus plantarum*, Postbiotic metabolite, Probiotic.

GİRİŞ

Fermentation is one of the oldest techniques used to preserve food, relying on the activity of microorganisms to enhance shelf life and safety [1]. Yogurt, a popular fermented dairy product, is known for its appealing taste, smooth texture, and health-promoting effects such as supporting the immune system and improving gut health [2]. This functional food is produced through the lactic acid fermentation of milk, primarily by bacteria like *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, under specific temperature and time conditions [3]. Other strains from the *Lactobacillus* genus, including *L. acidophilus*, *L. rhamnosus*, *L. casei*, and *L. plantarum*, may also be used in the fermented milk-making process [4]. These bacteria break down lactose in milk to produce lactic acid, along with compounds like carbon dioxide, acetic acid, dactyls, and acetaldehyde, which contribute to yogurt's distinct flavor and aroma [5]. Research indicates that probiotics

in fermented dairy products can survive in the human gut and offer several health advantages [6]. The most commonly used probiotic bacteria belong to the group of lactic acid bacteria (LAB), which are taxonomically divided into six main genera: *Enterococcus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Bifidobacterium*, and *Lactobacillus* [7]. To enhance health and reduce disease risk, it is recommended to include such beneficial microbes in the diet. In animal nutrition, LAB strains like *L. plantarum*, *Streptococcus thermophilus*, *L. casei*, *L. delbrueckii subsp. bulgaricus*, *L. reuteri*, and *L. acidophilus* are commonly used as feed additives to improve product quality [8]. Compared to polymerase chain reaction (PCR), the matrix-assisted laser desorption/ionization (MALDI), and the mass analyzer is time-of-flight (TOF) analyzer technique has demonstrated greater accuracy and efficiency in identifying *Lactobacillus* species at the species level, according to existing studies [9-11].

Postbiotics are bioactive compounds that are either secreted by live cells—whether probiotic or not—or released following the breakdown of those cells, and they can exert beneficial effects on the host organism [12]. They are also described as metabolic by-products produced by lactic acid bacteria (LAB) in environments conducive to microbial activity [13]. These metabolites include substances such as organic acids, ethanol, diacetyl, acetaldehyde, short-chain fatty acids, microbial cell components, extracellular polysaccharides, and functional proteins [14,15]. Numerous clinical investigations have examined the role of probiotics in preventing, managing, and treating various forms of cancer, particularly colorectal cancer. Some LAB strains, categorized as probiotics, are believed to help reduce the risk of colorectal cancer through immune system modulation [16]. Probiotic features can differ among isolates, depending on their source. Since each individual's gut microbiota is influenced by their diet and the types of food consumed, it becomes possible to isolate probiotic strains from people of different backgrounds that may have distinct anticancer properties [17]. The levels of probiotic and postbiotic compounds, along with their potential anticancer effects, can vary significantly based on bacterial species, strains, and their original sources. In this context, 125 LAB strains were isolated from 117 traditionally made yogurt samples collected in the Muş region and identified using MALDI-TOF MS technology. The study focused on assessing the probiotic characteristics (such as resistance to pH, pepsin, pancreatin, and bile) of *Lactobacillus plantarum* strains identified through MALDI-TOF MS, analyzing their postbiotic profiles using HPLC, and evaluating the anticancer effects of these metabolites on colon cancer cell lines (HT-29 and Caco-2). Based on the findings, it is anticipated that well-performing *L. plantarum* strains may be incorporated into yogurt production, offering both direct benefits to the food sector and indirect advantages to human health.

MATERIAL and METHODS

Analysis of *L. plantarum* by MALDI-TOF MS

In a previous study, 125 LAB strains were isolated from 117 yogurt samples that were randomly obtained from households in various villages of the Muş region, where yogurt is traditionally prepared [18]. The identification of these LAB strains at the species level was carried out using MALDI-TOF MS technology, with the analysis outsourced to the Plant Health Clinic Application and Research Center at Hatay Mustafa Kemal University. In this facility, proteins extracted through the ethanol-formic acid method were analyzed using the MALDI-TOF MS system. These protein profiles were matched against a reference library to identify microorganisms, including bacteria, fungi, and yeasts, based on their unique protein fingerprints. The spectra, generated via the device's flexControl software (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany), were compared using the MALDI Biotyper Real-Time Classification (RTC) software (version 9) for genus and species-level identification. Identification results with score values shown in yellow or green within the 1700–3000 range were considered reliable according to the system's standards [19].

Preparation of Bacterial Suspension

The isolates were incubated at 37±2°C for 24 hours before being suspended. The prepared active bacterial culture was centrifuged at 5000 g for 5 minutes at 4°C to remove the supernatant. Then, it was washed twice with sterile phosphate buffer (PBS; pH 7.4). Bacterial suspensions were prepared from bacterial strains at a density of 0.5 McFarland [20]. This suspension was adjusted to a concentration of approximately 6.0–7.0 Log CFU/mL. The prepared bacterial suspensions were used in all experimental stages. The *L. plantarum* STD strain obtained from Maysa Food-Lab. (Tuzla, Istanbul) was used as the standard in all analyses except for *L. plantarum* MALDI-TOF MS identification.

Probiotic Studies

Assessment of Isolate Resistance to Varying pH Levels and Pepsin

To determine whether the starter cultures could survive passage through the stomach and reach the small intestine, a simulated gastric environment was used. This artificial gastric fluid was prepared by dissolving 0.3% pepsin (SIGMA-Life Science, USA) in 100 mL of sterilized phosphate-buffered saline (PBS), adjusted to pH levels of 2.0 and 3.0. Culture suspensions (6.0–7.0 log CFU/mL) were then mixed with the pepsin-containing PBS solutions at both pH values and incubated at 37°C for a duration of three hours. During incubation, samples from each bacterial strain were collected at 0, 1, 2, and 3 hours, and serial dilutions were performed. Following exposure to the simulated gastric conditions, *L.*

plantarum samples were plated on MRS agar using the dilution method, with three replicates per condition. After incubating the plates at 37°C for 24 to 48 hours, viable colony counts from both the control and treated samples were expressed as Log CFU/mL to evaluate bacterial survival [21,22].

Evaluation of Isolate Tolerance to Pancreatin and Bile Salts

To assess the isolates' resistance to pancreatin, bacterial suspensions were added to a phosphate-buffered saline (PBS) solution containing 1% pancreatin and incubated at 37°C for four hours. PBS at pH 8.0 served as the control condition. Samples were collected at the beginning (0 hours) and after 4 hours of incubation, followed by serial dilutions of each. The *L. plantarum* strains exposed to the pancreatin solution were plated on MRS agar using the dilution method, with three replicates for each condition. After incubating the cultures at 37°C for 24 to 48 hours, the number of colonies formed in both control and test samples was counted, and results were expressed as Log CFU/mL [21,22].

To evaluate the tolerance of suspended *L. plantarum* strains to bile salts, the cultures were introduced into PBS solutions containing 0.3%, 0.5%, and 1% (pH 8.0) concentrations of bile salts and incubated at 37°C for four hours. Samples were collected at the start (0 hour) and after 4 hours of incubation, and serial dilutions were performed. These diluted samples were then spread onto MRS agar plates in triplicate for each condition. Following incubation at 37°C for 24 to 48 hours, the resulting colonies from both control and treated groups were counted, and the results were reported as Log CFU/mL [21,22].

Quantification of Postbiotic Metabolites

For the determination of postbiotic metabolites, standard compounds such as maleic acid, citric acid, tartaric acid, malic acid, succinic acid, fumaric acid, acetic acid, pyruvic acid, acetoin, and 2,3-butanediol were used. Stock solutions of these standards were prepared at a concentration of 1 mg/mL by dissolving the compounds in 0.03 M sulfuric acid (H_2SO_4) in 15 mL Falcon tubes. These stock solutions were subsequently serially diluted into eight concentrations (5, 10, 25, 50, 100, 200, 300, and 400 ppm) and injected into the HPLC system to create calibration curves [23]. For the analysis, 0.2 mL of bacterial culture, incubated at 37±2°C for 24 hours, was combined with 1.8 mL of 0.03 M H_2SO_4. The mixture was centrifuged at 5000 g for 5 minutes. From the resulting supernatant, 100 µL was mixed with 900 µL of the same sulfuric acid solution, filtered through a 0.45 µm membrane, and approximately 0.5 mL was transferred into HPLC vials. The samples were then processed in the HPLC system to measure the postbiotic metabolites, with each sample analyzed in triplicate.

Cell Culture

Postbiotic Extract Preparation

To obtain extracts containing postbiotic compounds, bacterial cultures were first centrifuged at 5000 g for 5 minutes. The resulting supernatants were then filtered using a 0.22 µm membrane to remove any remaining cells. These postbiotic-rich filtrates were used in varying volumes 20, 10, 5, and 2.5 µL for testing on cell cultures [24]. The prepared extracts were stored at +4°C until use in cell line assays.

Treatment of Cell Lines with Extracts

Caco-2 and HT-29 human colon cancer cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM), enriched with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were incubated at 37°C in an atmosphere of 5% CO_2 and 95% humidity. To evaluate the cytotoxic effects of the postbiotic metabolites, the MTT assay was utilized [25]. In this procedure, 5×10^3 cells in 100 µL of medium were seeded into each well of a 96-well plate using an automated cell counter. After 24–48 hours to allow the cells to adhere, 100 µL of the postbiotic extracts, diluted to the desired concentrations, were added to the treatment wells. The control wells received only 100 µL of medium without the extract. After 24 hours of exposure, the medium was removed by a vacuum pump, and each well received 10 µL of MTT reagent and 90 µL of fresh medium. The plate was then incubated for an additional 4 hours.

Following this incubation, the MTT solution was carefully removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. Absorbance was measured at 540 nm using a microplate reader. The control wells (cells not exposed to postbiotics) served as the baseline, and their average absorbance represented 100% cell viability. The absorbance values from the treated wells were compared to the control group to assess cell viability, and inhibition percentages were calculated based on the decrease in metabolic activity.

% inhibition=1-(OD_Sample/OD_Control)x100

OD_Sample: Optical density of the sample, OD_Control: Optical density of the control.

Statistical Evaluation

All findings from the probiotic, postbiotic, and in vitro cell culture experiments were presented as mean values accompanied by their standard error of the mean (mean ± SEM), depending on the experimental design. Graphs using these data were prepared using GraphPad Prism 8 software. Each experiment was conducted in a minimum of three replicates. The outcomes of the *L. plantarum* isolates were statistically compared to those of the reference *L. plantarum* standard strain. One-way analysis of variance (ANOVA) followed by Dunnett's post hoc

test was utilized to determine statistical significance. A p-value of less than 0.05 was considered significant. Statistical significance levels were denoted by asterisks as follows: $P < 0.05$ (), $P < 0.01$ (), $P < 0.001$ () and $P < 0.0001$ (****). Results with $P > 0.05$ were interpreted as not statistically significant (ns).

RESULTS and DISCUSSION

Identification of *L. plantarum* by MALDI-TOF MS

A total of 125 LAB isolates obtained from 117 traditionally produced yogurt samples randomly collected from households in various villages across the Muş region were identified using the MALDI-TOF MS technique. Identification scores ranging from 1.700 to 3.000 and labeled yellow/green were considered reliable. Among these, 22 isolates (17.6%) with scores between 1.733 and 2.302 were identified as potential *Lactobacillus plantarum* strains (see Table 1). MALDI-TOF MS has gained popularity in recent years for microbial identification due to its rapid analysis, affordability, precision, and reliability [26]. This method is increasingly viewed as a strong alternative to conventional biochemical and molecular techniques [27,28]. In prior research using this technique, bacteria found in dairy products like yogurt and cheese were classified as *L. lactis* (15), *L. garvieae* (8), *L. plantarum* (7), *Enterococcus faecium* (3), *Leuconostoc citreum* (2), and *L. casei* (1) [29]. Another study reported the identification of 2 *L. helveticus*, 2 *L. plantarum*, 3 *L. fermentum*, 1 *E. faecalis*, and 76 *L. delbrueckii* isolates out of 84 using the MALDI-TOF MS Biotyper system [30]. Similarly, a separate investigation using yogurt samples revealed 27 *L. delbrueckii* and 42 *S. thermophilus* strains, along with smaller numbers of other LAB strains, including 3 *L. plantarum* [31]. Compared to these findings, the identification of 22 *L. plantarum* strains in this study represents a notably higher proportion. This difference may be attributed to regional factors such as the specific origin of the yogurt samples, the feeding practices of livestock, and unique local environmental conditions.

Table 1. MALDI-TOF MS Results

Isolates	Organism (best match)	Score Value	Organism (second best match)	Score Value
1	*L. plantarum*	1.973	*L. plantarum*	1.927
2	*L. plantarum*	2.111	*L. plantarum*	2.09
3	*L. plantarum*	1.961	*L. plantarum*	1.954
7	*L. plantarum*	1.824	*L. plantarum*	1.788
8	*L. plantarum*	1.955	*L. plantarum*	1.915
9	*L. plantarum*	1.927	*L. plantarum*	1.925
13	*L. plantarum*	2.241	*L. plantarum*	2.194
14	*L. plantarum*	2.197	*L. plantarum*	2.11
15	*L. plantarum*	2.067	*L. plantarum*	2.021
16	*L. plantarum*	1.926	*L. plantarum*	1.924
20	*L. plantarum*	1.832	*L. plantarum*	1.733
22	*L. plantarum*	2.302	*L. plantarum*	2.27
24	*L. plantarum*	1.943	*L. plantarum*	1.933
25	*L. plantarum*	2.205	*L. plantarum*	2.199
26	*L. plantarum*	2.168	*L. plantarum*	2.118
27	*L. plantarum*	2.038	*L. plantarum*	2.008
29	*L. plantarum*	2.012	*L. plantarum*	1.949
31	*L. plantarum*	2.157	*L. plantarum*	2.15
32	*L. plantarum*	2.013	*L. plantarum*	1.99
33	*L. plantarum*	2.049	*L. plantarum*	2.042
35	*L. plantarum*	1.838	*L. plantarum*	1.833
36	*L. plantarum*	1.905	*L. plantarum*	1.764

Probiotic Properties of *L. plantarum*

For microorganisms to be considered probiotic, they must resist the stomach environment where pH is between 1-4, and considering the duration of food in the stomach, probiotic microorganisms must maintain their viability at low pH in the stomach for 3 hours [32,33]. The development of 22 *L. plantarum* isolates and standard strains identified according to MALDI-TOF MS

analysis results in pH2+pepsin and pH3+pepsin environments is given in Figure 1.

It was observed that the isolates grew in the range of 6.00±0.00-9.06±0.02 Log CFU/mL for 3 hours in the medium containing pH2 and pepsin. At the 1st hour of incubation, all isolates except 5 isolates (*L. plantarum* 7, 15, 20, 24 and 31), at the 2nd hour, all isolates except 3 isolates (*L. plantarum* 15, 20 and 31) and at the 3rd hour, all isolates showed better growth than *L. plantarum* STD, exhibiting a highly significant difference (P<0.0001). It was observed that the isolates generally did not show much change depending on time in the medium containing pH2 and pepsin, and they adapted to the medium.

It was found that the isolates grew in the range of 7.61±0.06-8.85±0.02 Log CFU/mL for 3 hours in the presence of pH3 and pepsin. At the 1st hour of incubation, 12 isolates (*L. plantarum* 3, 9, 15, 16, 20, 24, 26, 29, 31, 32, 35 and 36) and at the 2nd hour, 4 isolates (*L. plantarum* 7, 13, 26 and 36) showed better activity than *L. plantarum* STD with a highly significant difference (P<0.0001). At the end of the 3rd hour, some of the isolates showed activity like the standard, while it was observed that the activities of some isolates decreased with time.

It was determined that *L. plantarum* isolates could survive in a pH 2.5 pepsin environment. It was observed that there was no serious decrease in colony formation due to the extension of the incubation period [34]. In the study conducted by Miray et al., it was determined that 10 isolates showed growth in a pH 3 environment on 39 isolates, and 30 isolates were observed to survive in a pepsin environment [35]. In the study conducted by Tokatlı et al. in 2015, it was observed that 4 isolates out of 21 isolates lost their viability in the pepsin environment, and 17 isolates maintained their viability [36]. In the study on *L. plantarum* strains isolated from traditional pickles in 2020, it was observed that the isolates did not show any activity in pH 1, and the isolates became active in pH 2 and pH 3 environments as time progressed [37]. In our study, it was observed that our isolates could remain viable, similar to the literature data, and there was no serious decrease depending on time.

One of the obstacles that microorganisms that pass through the low pH and pepsin barrier of the stomach must overcome after reaching the small intestine is the pancreatin enzyme, which has a complex structure that negatively affects microbial growth [38]. In our study with *L. plantarum* strains, 1 mg/ml pancreatin medium, adjusted to pH 8.0 in PBS buffer, was used to determine the resistance property against pancreatin. The time interval was 4 hours, and the results in pH 8.0 medium were given (Figure 1). It was observed that the isolates grew in the range of 6.00±0.00-9.21±0.02 Log CFU/mL in the 4th hour in the medium containing pancreatin. It was determined that, except for 5 of the isolates (*L. plantarum* 7, 8, 15, 20, and 36), the other isolates showed a highly significant difference by growing better than *L. plantarum* STD (P<0.0001). It was determined that *L. plantarum* isolates 7 and 16 showed similar properties to the standard.

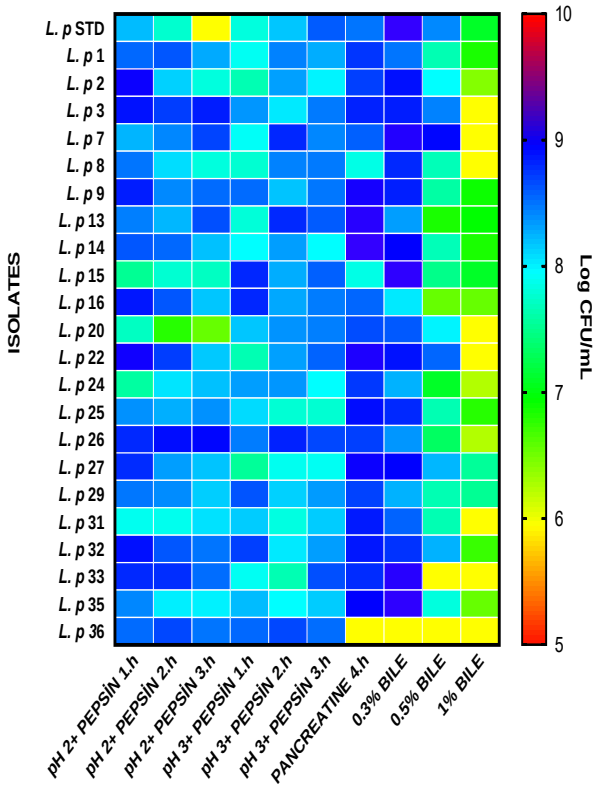

Figure 1. Viability rates of *L. plantarum* and *L. plantarum* STD strains showing resistance to pH, pepsin, bile and pancreatin conditions

In a study conducted in 2021, it was determined that 14 isolates colonized against pancreatin after 4 hours, and the *L. plantarum* MH13 isolate was seen to be at the highest level [39]. In a study conducted by Tokatlı et al. On 21 isolates in 2015, it was determined that 12 isolates formed colonies, 9 of which could not maintain their viability, and there was a significant decrease in the number of isolates forming colonies [36]. In a study conducted in 2022, it was observed that 23 out of 39 isolates maintained their viability after 6 hours of incubation [35]. It can be said that most of our isolates adapted to the environment and showed good growth.

One of the most important criteria sought for microorganisms to be accepted as probiotics is their ability to maintain their viability and colonization in the small intestine, which is a large part of the digestive system and where bile secretion is intense [38]. In scientific studies, 0.3% bile salt concentration is accepted as the critical value, and the determination of resistance properties of cultures is based on this value [40,41]. In this study, the viability of isolates in 0.3%, 0.5%, and 1% bile media was determined (Table 3). It was observed that the isolates grew in the range of 6.00±0.00-9.20±0.01 Log CFU/ml in the media containing 0.3%, 0.5%, and 1% bile salt. 6 isolates in 0.3% media (*L. plantarum* 7, 14, 15, 27, 33 and 35), 4 isolates in 0.5% media (*L.*

plantarum 3, 22, 27 and 32), and. In 1% medium, 8 isolates (*L. plantarum* 1, 9, 13, 14, 15, 16, 19, and 32) showed activity similar to *L. plantarum* STD.

In a study conducted by Zheng et al. in 2020, it was determined that the *L. plantarum* E680 isolate showed the best resistance to bile salts and low acidity [42]. In another study, it was observed that K3, K4, E41 isolates maintained their viability in 0.3% medium, and their numbers decreased in the 0.27-3.4 log range as a result of the increase in incubation time [43]. In a study conducted by Miray et al., according to the data obtained in 0.3% and 1% bile medium, it was observed that all isolates maintained their viability, and as a result of the observations, colonization was faster in 0.3% bile medium [35]. In a study conducted in 2021, in the presence of 0.3%, 0.5%, and 1% bile salts, it was observed that 14 isolates maintained their viability, and the *L. plantarum* MH10 isolate had the highest viability rate in 0.3% medium [39]. Considering the data in this study, it was observed that the activity of the isolates decreased as the bile concentration increased.

Postbiotic Metabolite Amount

Postbiotic metabolites are metabolic compounds produced by probiotic LAB [44]. Considering the activities in simulated stomach (pH+ pepsin) and intestine (bile and pancreatin) environment, the postbiotic substance contents of *L. plantarum* strains 3, 7, 13, 25, 26, and 29 and *L. plantarum* STD showing high and low activity among the isolates were determined by HPLC.

The concentrations of maleic acid, citric acid, tartaric acid, pyruvic acid, malic acid, succinic acid, fumaric acid, acetoin, 2,3-butanediol, and acetic acid in the cell-free supernatants of *Lactobacillus plantarum* and *L. plantarum* STD strains after 24 hours of incubation are presented in Table 2. The metabolite analysis revealed that tartaric acid, pyruvic acid, and malic acid were absent in the strains. Among the metabolites detected, acetic acid was present in the highest amounts across all isolates, while succinic acid was found in the lowest concentrations. Maleic acid was not detected in the *L. plantarum* STD and *L. plantarum* 26 strains, citric acid was absent in the *L. plantarum* 25 strain, and acetoin was missing in both the *L. plantarum* 25 and 26 strains. Additionally, fumaric acid was only present in the *L. plantarum* 26 strain. When evaluating the overall data and considering the total quantity of postbiotics, the strains contained higher levels of postbiotic metabolites compared to *L. plantarum* STD, with a highly significant difference observed ($P < 0.0001$).

Table 2. Quantitative analysis results of postbiotic metabolites

COMPOUNDS (µg/mL)	ISOLATES						
	***L. plantarum* STD**	***L. plantarum* 3**	***L. plantarum* 7**	***L. plantarum* 13**	***L. plantarum* 25**	***L. plantarum* 26**	***L. plantarum* 29**
Maleic acid	0.00±0.00	118.20±3.72^{e}	117.10±1.66^{e}	112.10±2.64^{e}	132.90±3.79^{e}	0.00±0.00^{a}	193.70±3.96^{e}
Citric acid	17,78±0,16	114.50±6.25^{e}	82.69±1.08^{e}	49.40±1.47^{e}	0.00±.0.00^{d}	17.39±0.52^{a}	35.55±0.63^{d}
Tartaric acid	0.00±0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Pyruvic acid	0.00±0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Malic acid	0.00±0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
Succinic acid	3.75±0.14	4.72±0.25^{c}	4.50±0.24^{b}	3.58±0.08^{a}	2.78±0.13^{c}	4.65±0.18^{c}	6.99±0.10^{e}
Fumaric acid	0.00±0.00	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	21.58±0.65^{e}	0.00±0.00^{a}
Acetic acid	67.83±0.18	455.90±7.48^{e}	464.00±18.58^{e}	426.90±7.51^{e}	513.30±4.21^{e}	464.50±6.17^{e}	473.20±5.96^{e}
Acetoin	11.16±0.14	60.66±1.56^{e}	54.61±0.92^{e}	17.53±0.54^{d}	0.00±0.00^{e}	0.00±0.00^{e}	0.00±0.00^{e}
2,3- Butanediol	46.10±1.17	103.90±2.75^{e}	99.89±1.44^{e}	81.54±1.78^{e}	84.14±7.27^{e}	76.82±1.23^{d}	102.30±1.91^{e}
TOTAL	163.1±4.03	857.90±22.01^{e}	822.80±23.92^{e}	691.10±14.03^{e}	733.10±15.41^{e}	585.00±8.75^{e}	811.80±12.56^{e}

a: ns. b: *. c: **. d: ***. e: ****

The production of acetic and lactic acid through postbiotics generated by *L. plantarum* strains has been previously documented [45]. Variations in organic acid concentrations within the postbiotics produced by different *L. plantarum* strains may be influenced by the fermentation medium, which can affect heterofermentative pathways, including citrate metabolism [46]. Additionally, a separate study noted that the production of acetic acid by *L. plantarum* strains was lower than that of lactic acid [47]. A review of the literature revealed that studies focused on postbiotic metabolites from *L. plantarum* are relatively limited. Based on the findings of this study, it was observed that, in general, the total amount of metabolites produced by the strains exceeded that of the standard strain, with the *L. plantarum* 3 isolate generating the highest level of postbiotics.

Anticancer Activity

Probiotic bacteria are recognized for their numerous health benefits to living organisms [48]. Recently, the anticancer potential of these probiotics, especially in relation to their positive impact on the digestive system, has become a significant area of research [49,50]. In this study, the inhibitory effects of postbiotic metabolites from *L. plantarum* strains on Caco-2 and HT-29 cell lines were examined (Figure 2).

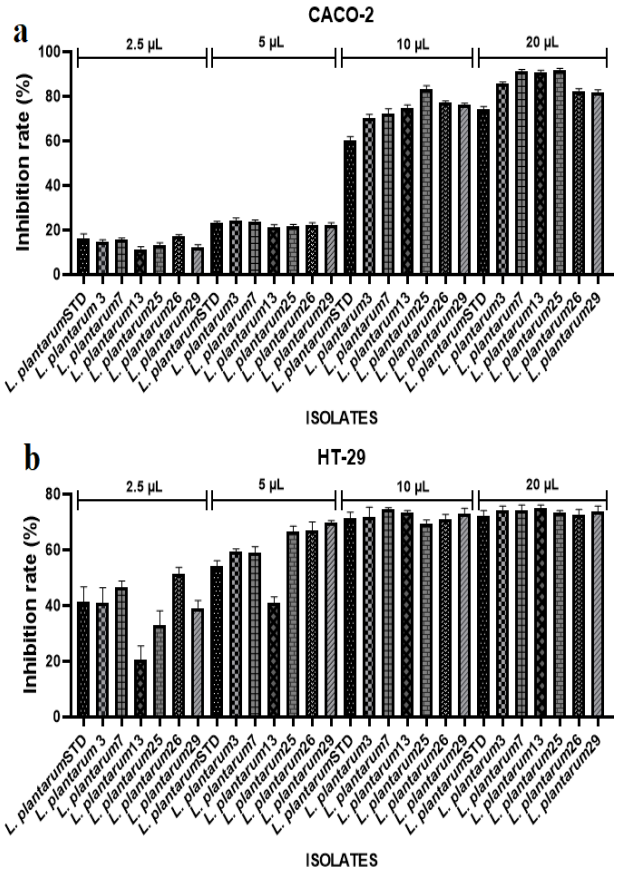

Figure 2. % inhibition effects of postbiotic metabolites of *L. plantarum* and *L. plantarum* STD strains on Caco-2 (a) and HT-29 (b) cell lines.

When evaluating the postbiotic metabolite effects at various concentrations on the Caco-2 cell line, the % inhibition values ranged from 11.43±1.10% to 91.50±1.05%. It was found that postbiotic metabolites from all strains at 10 and 20 µL concentrations exhibited significantly better activity than the postbiotic metabolite from *L. plantarum* STD ($P<0.0001$). In the case of the HT-29 cell line, the % inhibition values from postbiotic metabolites at different concentrations varied between 20.61±4.91% and 75.02±1.19%. For the HT-29 cell line, postbiotic metabolites from three *L. plantarum* strains (L. plantarum 25, 26, and 29) at a 5 µL concentration showed highly significant inhibition ($P<0.0001$), outperforming the postbiotic metabolite of *L. plantarum* STD. Additionally, postbiotic metabolites from all strains at 10 and 20 µL concentrations showed similar effects to *L. plantarum* STD.

Previous research has shown that LAB isolates can have varying inhibitory effects on different cell lines. One study reported cytotoxic effects of 37%-68.5% on Caco-2 cells and 45.7%-48% on HRT cells [51]. Another study found that LAB cell extracts inhibited the Caco-2 cell line by 40.7%, the HT-29 cell line by 50%, and the L-929 cell line by 46.7%. This study also highlighted that the inhibitory effects of cell-free extracts from LAB isolates were concentration-dependent across all cell lines [52]. Such concentration-dependent inhibition has been observed in other studies as well [53,54]. For instance, LAB extracts showed 31.15%-69.83% inhibitory effects on the HCT-116 cell line [53], and cytotoxic effects ranging from 58.28%-86.80% on Caco-2 cells and 78.50%-86.30% on HT-29 cells in another study [55]. In this research, we found that the impact of postbiotic metabolites on cell lines varied based on the bacterial strain, likely due to differences in the metabolites produced by each strain. Overall, the higher concentrations (10 and 20 µL) of postbiotic metabolites from all strains demonstrated superior inhibitory activity against the Caco-2 cell line compared to *L. plantarum* STD, and showed similar effects on the HT-29 cell line. These findings align with the results from other studies, but additional research is needed to fully understand the underlying mechanisms of the antitumor activity.

CONCLUSION

Out of the 125 LAB strains isolated from 117 yogurt samples randomly collected from families making traditional yogurt in various villages of the Muş region, 22 strains were identified as *L. plantarum* using MALDI-TOF MS. These isolates, along with the *L. plantarum* STD strains, demonstrated excellent probiotic characteristics, showing significant resistance in simulated stomach and intestinal conditions. It was found that all strains produced the highest levels of acetic acid and the lowest levels of succinic acid. Furthermore, at higher concentrations (10 and 20 µL), the postbiotic metabolites from all strains showed stronger inhibitory effects on the Caco-2 cell line compared to *L. plantarum* STD. Similar activity was observed for the postbiotic metabolites of both strains and *L. plantarum* STD against the HT-29 cell line at these higher concentrations. Given the probiotic properties, postbiotic metabolite production, and anticancer activity of these *L. plantarum* strains, further research is needed to clarify the interaction mechanisms involved and to investigate how these properties perform *in vivo*. Such studies will help demonstrate the potential of these strains as promising probiotic candidates.

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