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Determination of Antioxidant and Anticancer Activities and Exopolysaccharides of Metabolites Synthesized by Probiotic *Levilactobacillus brevis* Strains Newly Isolated from Breast Milk

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Abstract: In our study, the probiotic, exopolysaccharide, metabolite contents, antioxidant activities, and anticancer activities of metabolites on HT-29 and Caco-2 cell lines of four *Levilactobacillus brevis* (YAAS1, YAAS2, YAAS3, and YAAS4) strains isolated from breast milk in Türkiye and identified by MALDI-TOF MS were investigated. It was determined that the strains exhibited strong probiotic character by surviving in pH+pepsin, bile and pancreatin medium; although, they decreased depending on time and concentration. The strains synthesized the most acetic acid but could not synthesize tartaric, pyruvic, malic, and fumaric acids. It was determined qualitatively and quantitatively that the strains synthesized compounds similarly in terms of exopolysaccharide amount. The strains exhibited lower DPPH activity than standard antioxidants. However, ABTS activities were similar to standard antioxidants. Bacterial metabolites induced notable anticancer effects in the HT-29 cell line at low concentrations, while eliciting comparable effects in the Caco-2 cell line at high concentrations. When all the results were considered in general, it was determined that the strains showed identical properties to each other but *L. brevis* YAAS4 strain exhibited a better character structure. Given the scarcity of studies on *Lactobacillus brevis* strains newly isolated from human breast milk, this research significantly advances current understanding. Characterizing the specific properties identified in our study through future research will be crucial for determining the potential of these isolates as effective probiotics for industrial and health applications.

Keywords: *Levilactobacillus brevis*, MALDI-TOF MS, HT-29, Caco-2

Anne Sütünden Yeni İzole Edilen Probiyotik *Levilactobacillus brevis* Suşlarının Sentezlediği Metabolitlerin Antioksidan ve Antikanser Aktiviteleri ile Ekzopolisakkarit Tayini

Öz: Çalışmamızda, Türkiye'deki anne sütünden yeni izole edilen ve MALDI-TOF MS ile tanımlanan dört *Levilactobacillus brevis* (YAAS1, YAAS2, YAAS3 ve YAAS4) suşunun probiyotik, ekzopolisakkarit, metabolit içerikleri, antioksidan aktiviteleri ve metabolitlerinin HT-29 ve Caco-2 hücre hatları üzerindeki antikanser aktiviteleri araştırıldı. Suşların pH+pepsin, safra ve pankreatin ortamında canlılığını sürdürerek güçlü probiyotik karakter sergilediği, ancak bu özelliğinin zamana ve konsantrasyona bağlı olarak azaldığı belirlendi. Suşlar en fazla asetik asit sentezlediği, ancak tartarik, pirüvik, malik ve fumarik asitleri sentezlemedikleri belirlendi. Ekzopolisakkarit miktarı bakımından suşlar birbirlerine benzer şekilde sentezlediği nitel ve nicel olarak belirlendi. Suşlar standart antioksidanlardan daha düşük DPPH aktivitesi gösterdi. Ancak ABTS aktiviteleri standart antioksidanlara benzerdi. Bakteriyel metabolitler düşük konsantrasyonlarda HT-29 hücre hattına karşı iyi antikanser etki gösterirken, yüksek konsantrasyonlarda Caco-2 hücre hattına karşı benzer etki gösterdi. Tüm sonuçlar genel olarak değerlendirildiğinde suşların birbirlerine benzer özellikler gösterdiği, ancak *L. brevis* YAAS4 suşunun daha iyi bir karakter yapısı sergilediği belirlendi. Sonuç olarak insan sütünden yeni izole edilen *L. brevis* suşları üzerinde sınırlı sayıda çalışma olması nedeniyle sonuçlarımız literatüre önemli katkılar sunacaktır. Aynı zamanda *L. brevis* suşlarımızın sergilediği özellikler dikkate alınarak yapılacak yeni araştırmalar ile iyi karakterdeki suşların endüstri ve sağlık alanında iyi bir probiyotik bakteri kaynağı olmasına katkı sağlayacaktır.

Anahtar kelimeler: *Levilactobacillus brevis*, MALDI-TOF MS, HT-29, Caco-2

1. Introduction

Breast milk represents the optimal biological fluid for neonatal nutrition and provides comprehensive support for both nutritional and immunological needs. Notably, breast milk serves as a reservoir for a diverse bacterial community that plays a crucial role in establishing the infant's intestinal microbiota and modulating their innate immune responses (Martín et al., 2003; Lara-Villoslada et al., 2007). The presence of approximately 10^3-10^4 colony-forming units (CFU)/mL of commensal microorganisms in the breast milk of healthy women underscores its significance as a continuous source of probiotics for infants (Jeurink et al., 2013).

Probiotics are live bacteria that enhance intestinal microbial balance and immunological function, providing health advantages to the host when given in sufficient quantities (Hemarajata et al., 2012; FAO/WHO, 2023). However, the functional properties of probiotic strains are

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highly strain- and species-specific, and interspecies interactions can significantly influence their activity (Pino et al., 2019; Kim et al., 2020). Given their human origin, safe consumption history, consistent presence in neonates, and inherent adaptability to the gastrointestinal environment, probiotics isolated from human breast milk possess several desirable characteristics to be used as therapeutic agents (Duraisamy et al., 2022). Furthermore, isolating bacteria with beneficial properties from human sources offers a promising approach for developing autochthonous probiotics, i.e., probiotics derived from the host itself (Rajoka et al., 2017). The composition of the human milk microbiota exhibits considerable diversity, influenced by a range of maternal and environmental factors, including breastfeeding practices, maternal behaviors, milk components, genetics, geographic location, ethnicity, and sociodemographic characteristics (Anjum et al., 2020).

Probiotic studies on the *Levilactobacillus* genus, recently separated from *Lactobacillus*, are still limited but represent a promising area for potential research (Zheng et al., 2020). Research has shown that when it comes to species-level identification of *Lactobacillus* species, MALDI-TOF MS outperforms PCR techniques (Dušková et al., 2012). Furthermore, existing literature supports the applicability and accuracy of MALDI-TOF MS for the identification of *Lactobacillus* species (Dušková et al., 2012; Nacef et al., 2016; Treven et al., 2019). According to reports, *Levilactobacillus* species among lactic acid bacteria (LAB) are crucial in preventing a variety of metabolic ailments, including cancer, aging, immunological deficiencies, and cardiovascular issues (Rodríguez-Pastén et al., 2022). Major enzymatic and non-enzymatic antioxidants are also known to be released by LAB and to be supported in their production (Noureen et al., 2019). At the same time, LAB protects direct and indirect pathways. It is estimated that they produce metabolites with different effects such as bacteriocins, butyrate, lactic acid, exopolysaccharide (EPS), folate, hydrogen peroxide, and glutathione for indirect protection. These metabolites contribute to activities such as antimicrobial, antioxidant, immune strengthening, anticancer, antihistamine, and anticholesterol (Rajoka et al., 2017).

The probiotic, metabolite amount, antioxidant and anticancer activities of the metabolites and the presence of EPSs of bacterial strains show differences specific to the source, strain, and species. Considering the lack of information in the literature about *Levilactobacillus brevis* strain isolated from the milk of breastfeeding mothers in Turkey and local isolates, this study was designed for the isolation and characterization of these bacteria by MALDI-TOF MS technique. In this study, the probiotic (pH, pepsin, pancreatin, and bile) properties of the strains, their metabolite contents, qualitative and quantitative determination of EPS, antioxidant activities of the metabolites and their anticancer activity potential against HT-29 and Caco-2 were studied. For this purpose, the qualitative and quantitative determination of EPS synthesized by strains that are likely to be probiotic, the number of metabolites, and the antioxidant and anticancer activities of these metabolites against colon cancer cell lines were investigated. The results obtained are expected to make significant contributions to the selection of new human-derived probiotic isolates with potential applications in food and health.

2. Material and Method

2.1. Breast Milk Samples and Ethics Approval

Thirty healthy lactating mothers were recruited from the Neonatal Unit of Van Training and Research Hospital, affiliated with the University of Health Sciences, during the year 2018. Breast milk samples were collected from these participants with the primary objective of isolating LAB. Before the initiation of data collection, formal ethical approval was secured from the relevant Institutional Review Board on September 28, 2017 (approval number: 2017/7). Following collection, each milk sample was aseptically transferred into sterile containers and immediately transported to the laboratory under refrigerated conditions at 4°C. Upon arrival at the laboratory, the isolation procedures commenced without delay.

2.2. *L. brevis* Isolation and Standard Strain

Isolation of LAB from breast milk was performed with modifications to the methodology previously described by Kao et al. (2006). *Lactobacillus acidophilus* STD strain, obtained from Maysa Gıda-Lab (Istanbul), served as a standard control for all analyses, with the exception of *L. brevis* identification.

2.3. MALDI-TOF MS Analysis

For species identification, isolates were submitted to the Plant Health Clinic Application and Research Center at Hatay Mustafa Kemal University for analysis using MALDI-TOF MS. At the center, the proteins obtained by ethanol formic acid extraction method in the MALDI-TOF MS device were scanned in the form of protein fingerprint matching library and the identification of microorganisms (bacteria, yeast, and fungi) was revealed. The spectra obtained with the flex control software program of the device (Biotyper 3.0; Microflex LT; Bruker Daltonics GmbH, Bremen, Germany) were compared with the Maldi Biotyper Real-Time Classification (RTC) software (version.9) and genus and species identification was performed. As a result of the analysis, the data determined as yellow/green between 1700-3000 were accepted as reliable score values (Uysul et al., 2019).

2.4. Determination of Probiotic Properties

To evaluate the survival of *L. brevis* and *L. acidophilus* STD strains under simulated gastrointestinal conditions, their resistance to simulated gastric juice (pH + pepsin), pancreatin, and bile salts were tested. Isolates were incubated in de Man, Rogosa and Sharpe (MRS) medium at 37±2°C for 24 hours before being suspended. Bacterial suspensions were prepared by centrifuging 1 mL of activated cultures at 12,000 x g for 5 minutes at 4°C. The cell pellets were then washed twice with sterile PBS. A bacterial suspension was prepared from the bacterial strains at a density of 0.5 McFarland. This suspension was adjusted to a concentration of approximately 6.0–7.0 Log CFU/mL.

pH+pepsin solution was used to test the ability of the strains to pass through the stomach environment and reach the small intestine. For this purpose, 0.3% pepsin (SIGMA-Life science, USA) was added to 100 mL of

sterilized PBS and a stomach environment solution adjusted to pH 2.0 and pH 3.0 was prepared. The prepared starter culture suspensions were transferred to PBS (pH 2.0 and pH 3.0) buffer medium containing pepsin at a rate of 1% and incubated at 37˚C for three hours. While the incubation process continued, serial dilutions of the samples taken from each strain were prepared at 0, 1, 2, and 3 hours. To determine the resistance properties of the isolates against pancreatin (SIGMA-Life science, USA), the suspended strains were transferred to PBS buffer medium containing 1% pancreatin and incubated at 37˚C for 4 hours. PBS (pH 7.4) was used as a control. Following incubation, samples were taken from bacterial samples at the end of 0 and 4 hours and serial dilutions were prepared. To determine the resistance property of the *L. acidophilus* STD and *L. brevis* strains prepared by suspension against bile salts (SIGMA-Life science, USA), they were transferred to 1% PBS containing 0.3%, 0.5%, and 1% bile salt and incubated at 37˚C for 4 hours. Following incubation, samples were taken from bacterial samples at the end of 0 and 4 hours and serial dilutions were prepared. After *L. acidophilus* STD and *L. brevis* strains were treated with pH+pepsin, pancreatin, and bile medium, the dilutions prepared were cultured on MRS agar medium in three parallels using the drop culture method. At the end of the 24-48 hour incubation of the samples at 37 ˚C, the colonies in the control group and the experimental group were counted and the values were calculated as Log CFU/mL (Alan et al., 2025).

2.5. Extraction of Bacterial Metabolites (BM)

To obtain the extract containing bacterial metabolites (BM), bacterial cells activated by incubation at 37°C for 24 hours in cultured MRS medium were centrifuged at 5000 g for 5 minutes. Supernatants were filtered using a 0.22 µm filter (Kim et al., 2008). The obtained BMs were used for metabolite analysis, antioxidant properties, and cell culture.

2.6. Analysis of Metabolites

Quantitative analysis of metabolites produced by *L. brevis* strains was conducted in triplicate using an HPLC system (Agilent, USA). The analytical method employed was adapted from Alan (2024). A calibration curve was generated using the following commercially available standards: Acetoin, 2,3-butanediol, acetic, citric, malic, maleic, succinic, fumaric, and tartaric (Sigma, USA), and pyruvic acids (Chemservice, USA). In order to prepare stock standards, standards were weighed to be 1 mg/mL and dissolved in 0.03 M sulfuric acid (H_2SO_4) in 15 mL falcon tubes. Stock standards were prepared in 8 different dilutions (5, 10, 25, 50, 100, 200, 300 and 400 ppm) and loaded onto the HPLC device, and a calibration curve was drawn. For HPLC analysis, 0.2 mL of the active bacterial culture prepared by incubating at 37±2°C for 24 hours was added to 1.8 mL of 0.03 M H_2SO_4. The mixture was centrifuged at 5000 *g* for 5 minutes. 100 µL of the supernatant was added to 900 µL of 0.03 M H_2SO_4. The mixture was passed through filters with a pore diameter of 0.45 µm and approximately 0.5 mL. Samples were loaded onto the HPLC device to determine the postbiotic metabolites in the obtained mixture. Analyses were performed in 3 replicates. The configuration and components used in HPLC are summarized as: 1260 RID detector (G7162A), 1260 Quat Pump VL pump (0.6 mL/min), 1260 Vialsampler (20 µL), and G7130A column oven (65°C). The column used for the analysis is Agilent Hi-Plex H (7.7 × 300 mm, 8 µm).

2.7. Qualitative-Quantitative Determination of EPS

For EPS screening, the test tube method used by Christensen and colleagues was modified and used. The strains were added to the MRS medium in 6-well polystyrene plates at a rate of 1% and incubated at 37°C for 24 hours. The biofilms that developed on the bottom and side surfaces of the plates were stained with crystal violet. The spots on the plate were washed with phosphate buffered saline (PBS) and removed. The film seen on the bottom and sides of the plate was evaluated as biofilm formation (Christensen et al., 1985).

In terms of EPS production, the strains were cultured in Congo Red Agar (CRA) medium containing 37 g/L Brain Heart Infusion (BHI) agar, 0.8 g/L Congo Red, and 36 g/L sucrose and screened (Freeman et al., 1989). The strains cultivated on CRA medium by the streaking method were incubated at 37°C for 24-48 hours to distinguish between those producing EPS and those not. In terms of EPS production, strains that formed colonies with black and dry crystal texture were determined as positive, while colonies that remained pink were determined as negative (DuBois et al., 1956).

Pure cultures of *L. brevis* strains were obtained and EPS production amounts were determined (Marshall & Rawson, 1999). Pure cultures were activated by incubating them in MRS broth at 37±1°C for 24-48 hours. After the pellets obtained were dissolved in 1 ml of sterile pure water, the phenol sulfuric acid method was applied (Torino et al., 2001). The EPS content was determined by measuring the absorbance at 490 nm. A standard curve was created using glucose concentrations between 0 and 100 mg/L, following the phenol-sulfuric acid method.

A microtiter plate assay was performed to quantify biofilm formation (Stepanović et al., 2007). Briefly, 0.5 McFarland standard (approximately 1×10^8 CFU/mL) of each *L. brevis* strain was inoculated into 96-well microplates containing Brain Heart Infusion (BHI) broth supplemented with 1% (w/v) glucose. Plates were incubated at 37°C for 24 hours. After biofilms were stained with crystal violet, the staining was removed using methanol after washing with PBS. Measurement was made at 590 nm absorbance in a spectrophotometer (Shimadzu 1800, JAPAN). BHI broth and 1% glucose medium were used as negative controls.

2.8. Antioxidant Properties

2.8.1. DPPH radical scavenging activity

Stock solutions of bacterial metabolites and standard antioxidants were prepared by dissolving them in ethanol. Serial dilutions were carried out to achieve final concentrations of 10, 20, and 30 µg/mL, with a total volume of 800 µL per sample. Then, 200 µL of freshly prepared 0.1 mM DPPH radical solution was added to each sample. The mixtures were vortexed to ensure proper mixing and incubated at room temperature in the dark for 30 minutes. Following incubation, the absorbance of the reaction mixture was measured at 517 nm using a spectrophotometer (Shimadzu 1800, JAPAN) (Savcı et al.,

2020).

DPPH radical scavenging percentages of the extracts and standards were calculated using the following equation:

DPPH radical scavenging capacity (%) = [1-(A sample) / (A control)] X 100

Absorbance: The absorbance of the samples, A control: Represents the absorbance value of the control.

2.8.2. ABTS radical scavenging activity

The ABTS radical cation (ABTS•+) was generated by mixing 7 mM ABTS with 2.45 mM potassium persulfate. The mixture was kept in the dark at room temperature for 12-16 hours to allow the reaction to proceed. Afterward, the resulting ABTS•+ solution was diluted with a buffer to achieve an initial absorbance of 0.7 ± 0.01 at 734 nm. To this diluted solution, 1 mL of bacterial metabolite or fraction was added. After a 1-minute incubation, the absorbance of the reaction mixture was measured at 734 nm (Savcı et al., 2020).

ABTS radical scavenging percentages of the extracts and standards were calculated using the following equation:

ABTS radical scavenging capacity (%) = [(1-(A sample) / (A control)] X 100

Absorbance: The absorbance of the samples, A control: Represents the absorbance value of the control.

2.9. Cell Culture

BMs were added to the culture medium at final concentrations of 20 µL, 10 µL, 5 µL, and 2.5 µL. The culture medium for Caco-2 and HT-29 cell lines consisted of Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were cultured in a humidified incubator at 37°C with 5% CO_2 (Memmert, Germany). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was applied to determine the cytotoxicity of BMs prepared at different concentrations (Assaf et al., 2013). For the MTT test, $5x10^3$ cells were counted in 100 µL medium in each well of a 96 well plate and seeded with a cell counter. The seeded cells were incubated in a 5% CO_2 incubator for 24-48 hours. 100 µL of BMs diluted in medium was added to the cell lines. Only 100 µL of medium was added to the cells in the control wells. The samples were incubated 24 hours. After incubation, the medium in the wells was removed from the medium with the help of a vacuum pump. 10 µL of MTT solution and 90 µL of medium were added to each well and left in an incubator containing 5% CO_2 at 37ºC for 4 hours. After 4 hours, the medium containing MTT was removed from the medium. 100 µL of Dimethylsulfoxide (DMSO) was added to each well and the optical density (OD) was measured with a spectrophotometer (Thermo Scientific Multiskan GO, Finland) at a wavelength of 540 nm. The cell line medium without sample was used as the control group. The absorbance of the control group (cells grown without BM) was taken as 100% viability. The percentage of cell viability in the presence of BM was calculated relative to the control group. The inhibitory effect of BM on cell proliferation was determined by comparing the cell viability in the presence of BM to the control, and the percentage of inhibition was calculated following the method outlined by Alan et al. (2022).

2.10. Statistical Analysis

The data are expressed as the mean ± standard error of the mean (SEM) and are visually represented in graphs. All experiments were carried out in triplicate. Statistical comparisons were made between the results for each *L. brevis* strain and the *L. acidophilus* STD strain. For statistical analysis, one-way ANOVA was performed followed by Dunnett's multiple comparison test. A p-value of less than 0.05 was deemed statistically significant. The following symbols were used to represent the significance levels: **** ($p<0.0001$), *** ($p<0.001$), ** ($p<0.01$), * ($p<0.05$), and ns (not significant).

3. Results and Discussion

3.1. Identification of *Levilactobacillus brevis* with MALDI-TOF MS

A total of 50 LAB isolates were obtained from 30 distinct breast milk samples. 27 (54%) of the isolates identified with MALDI-TOF MS analysis were evaluated as reliable score values with data determined as yellow/green between 1.700-3.000. It was determined that 7 isolates with score values between 2.469-1.895 could be *Levilactobacillus brevis* strains (Table 1). Studies were conducted on the first four strains with the highest score values between 2.469-2.110. The strains were named as *Levilactobacillus brevis* YAAS1 (AS-15(1)), *Levilactobacillus brevis* YAAS2 (AS-23), *Levilactobacillus brevis* YAAS3 (AS-13(2)), and *Levilactobacillus brevis* YAAS4 (M-21).

Table 1. MALDI-TOF MS results of LAB isolates.

Isolates	Organism (best match)	Score Value	Score Value
1- AS-15(1)	*L. brevis*	2.469	2.458
2- AS-23	*L. brevis*	2.394	2.339
3- AS-13(2)	*L. brevis*	2.25	2.11
4- M-21	*L. brevis*	2.229	2.124
5- M-16	*L. brevis*	2.106	2.042
6- AS-13(1)	*L. brevis*	2.005	1.891
7- AS-8(1)	*L. brevis*	1.95	1.895

MALDI-TOF mass spectrometry was employed for the identification of *Lactobacillus plantarum*, *Lactobacillus zeae*, *Lactobacillus fermentum*, *Lactobacillus gasseri*, *Lactobacillus brevis*, *Lactobacillus reuteri*, *Lactobacillus paracasei*, and *Lactobacillus rhamnosus* in samples of breast milk and infant feces (Asenova et al., 2024). Previous studies have reported the presence of various *Lactobacillus* species in human breast milk, including *Lactobacillus paracasei*, *Lactobacillus oris*, *Lactobacillus gasseri*, *Lactobacillus salivarius*, *Lactobacillus rhamnosus*, *Lactobacillus casei*, *Lactobacillus fermentum*, and *Lactobacillus plantarum* (Ding et al., 2019; Zhang et al., 2020a). Similarly, the predominant *Lactobacillus* species isolated from infant feces include *Lactobacillus plantarum*, *Lactobacillus ruminis*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus mucosae*, *Lactobacillus fermentum*, and *Lactobacillus brevis* (Jost et al., 2014; Murphy et al., 2017). Albesharat et al. (2011) also showed that *Lactobacillus plantarum*, *Lactobacillus*

fermentum, and *Lactobacillus brevis*, which are key members of the Lactobacillaceae family, are commonly found not only in Syrian breast milk but also in the feces of infants and mothers as well as in locally fermented foods. The detection of L. brevis in breast milk samples using MALDI-TOF MS in this study aligns with previous findings reported in the literature.

3.2. Identical ABTS activity

The probiotic potentials of four *L. brevis* strains identified by MALDI-TOF MS and *L. acidophilus* STD in pH+pepsin, bile, and pancreatin environments are given in Figure 1. In the pH3+%0.3 pepsin containing medium, *L. brevis* YAAS3 and *L. brevis* YAAS4 showed more resistance than *L. acidophilus* STD strain in the 1st hour, *L. brevis* YAAS3 in the 2nd hour, and *L. brevis* YAAS4 in the 3rd hour and showed a very significant difference (P<0.01). When all times and strains were evaluated statistically, it was determined that *L. brevis* YAAS1 and *L. brevis* YAAS4 strains showed a significant difference against *L. acidophilus* STD (P<0.05) (Fig. 2a).

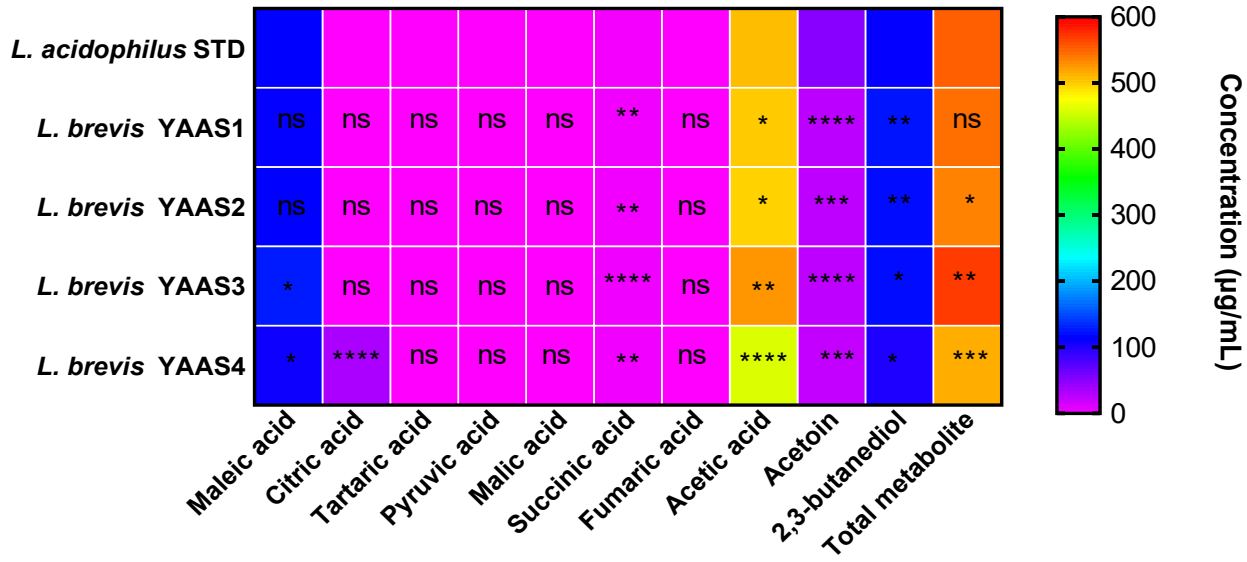

Figure 1. HPLC metabolite amounts of *L. brevis* and *L. acidophilus* STD strains. P>0.05; not significant (ns), P<0.001 and P<0.0001; highly significant (***), P<0.01; quite significant (**) and P<0.05; significant (*).

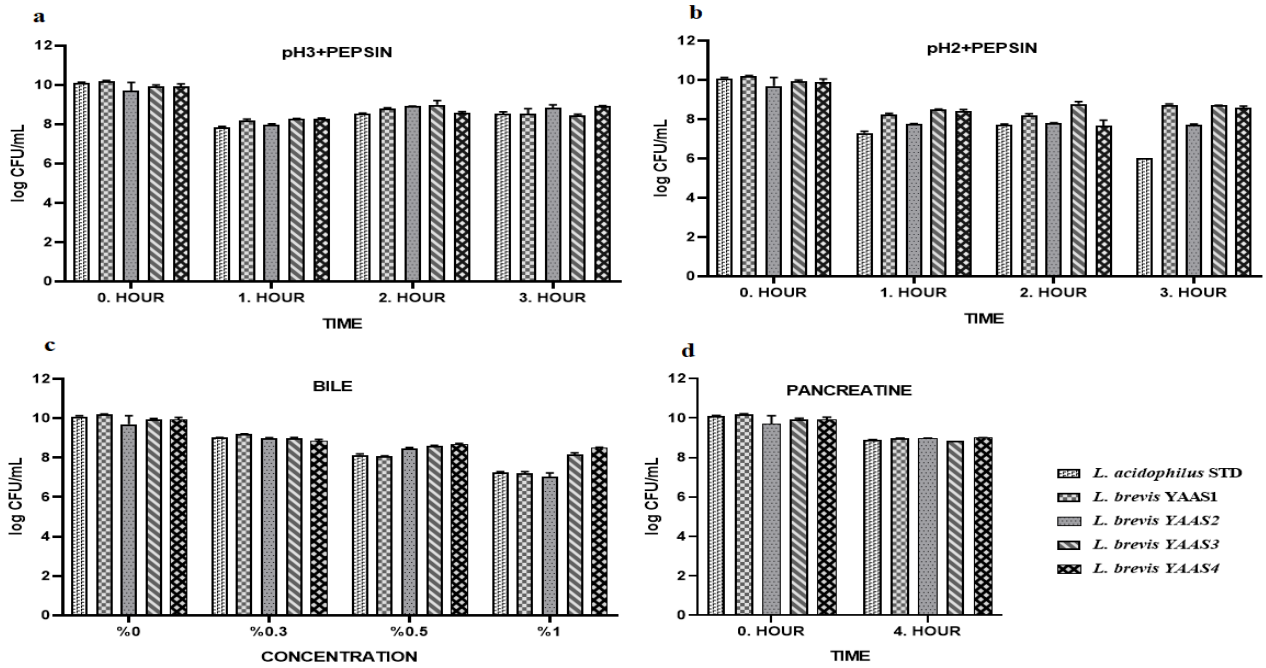

Figure 2. Viability rates demonstrating the resistance of *L. brevis* and *L. acidophilus* STD strains to conditions of pH, pepsin, bile, and pancreatin. pH3+pepsin (a); pH2+pepsin (b); bile (c) and pancreatin (d).

In the pH2+%0.3 pepsin containing medium, it was determined that all strains except *L. brevis* YAAS2 showed high resistance in the 1st hour, *L. brevis* YAAS4 strain in the 2nd hour, and all strains in the 3rd hour and showed a highly significant difference against *L. acidophilus* STD strain (P<0.0001). In addition, when all times and strains were evaluated statistically, it was determined that all strains showed a highly significant difference against *L.*

acidophilus STD (P<0.0001) (Fig. 2b). In the medium containing 1% bile, *L. brevis* YAAS3 and *L. brevis* YAAS4 strains and in the medium containing 0.5% bile, *L. brevis* YAAS4 strains were found to exhibit more resistance than *L. acidophilus* STD and showed a highly significant difference (P<0.0001). In addition, when all concentrations and strains were evaluated statistically in general, it was determined that *L. brevis* YAAS3 and *L. brevis* YAAS4 strains showed a highly significant difference against *L. acidophilus* STD (P<0.0001) (Fig. 2c). At the end of the 4th hour, it was observed that *L. brevis* strains in the medium containing pancreatin exhibited similar resistance to *L. acidophilus* STD (Fig. 2d).

The survival of LAB is significantly influenced by environmental pH (Yasmin et al., 2020). Gastric acid, primarily composed of hydrochloric acid, can degrade vital cellular components such as fatty acids, proteins, and DNA (Sahadeva et al., 2011). Studies have demonstrated a substantial reduction in bacterial viability upon exposure to gastric acid at pH 2 (Mandal et al., 2014). Furthermore, resistance to bile salts is crucial for probiotic survival within the human gastrointestinal tract (GIT) (Alameri et al., 2022). Previous research has shown that *L. brevis*, *L. paracasei* and *L. plantarum* exhibit resistance to 0.3% bile, while *L. brevis*, *L. acidophilus* and *L. gasseri* maintain viability in the presence of 1% bile (Kim et al., 2006; Jamaly et al., 2011). Another critical factor for probiotic selection is the ability to withstand the action of pancreatic enzymes. Several studies have demonstrated the growth of *Lactobacillus* strains in the presence of 0.5% pancreatin (Maragkoudakis et al., 2006; Khagwal et al., 2014). While the probiotic characteristics varied among the strains investigated in this study, the overall findings suggest that these strains possess probiotic potential, consistent with previously reported literature.

3.3. Metabolite Amounts

Figure 2 presents the concentrations of 2,3-butanediol, acetoin, fumaric, acetic, tartaric, succinic, pyruvic, malic, citric, and maleic acids in the cell-free supernatants of four *L. brevis* strains (YAAS1-4) and *L. acidophilus* STD after 24 hours of incubation. The analysis revealed that the *L. brevis* YAAS4 strain exhibited a significantly higher level of citric acid synthesis compared to the *L. acidophilus* STD strain (P<0.0001). Similarly, the *L. brevis* YAAS3 strain demonstrated a significantly greater production of succinic acid than the *L. acidophilus* STD strain (P<0.0001). Notably, citric acid synthesis was exclusively observed in the *L. brevis* YAAS4 strain. Furthermore, the *L. brevis* YAAS3 strain synthesized a significantly larger total quantity of metabolites compared to the *L. acidophilus* STD strain (P<0.01). Conversely, none of the tested strains exhibited the synthesis of tartaric acid, pyruvic acid, malic acid, or fumaric acid. In addition, the highest concentration of acetic acid and the lowest concentration of succinic acid were quantified. LAB have important biological activity functions due to the production of certain metabolites such as organic acids (Axel et al., 2015; Barcenilla et al., 2022; Choi et al., 2021). 14 different metabolites obtained as a result of *L. rhamnosus*, *L. brevis,* and *L. plantarum* metabolism were determined with HPLC-UV/DAD method and differences were determined between the strains (Vougiouklaki et al., 2022). In our study, as in literature, different amounts of metabolites were detected between the strains. The reason for the differences in metabolite content and amounts between the strains may be related to the metabolic and functional phenotype of the strains (Wegh et al., 2019).

3.4. Qualitative-Quantitative Determination of EPS

Biofilm formation by the strains was assessed using qualitative and quantitative methods. The tube method, a qualitative assessment, revealed increased biofilm formation by the strains compared to the control. This was evident by the enhanced crystal violet staining observed in the wells containing the strains, indicating increased EPS production (Fig. 3A). Furthermore, Congo red agar plate assays confirmed EPS production by both *L. brevis* and *L. acidophilus* STD as evidenced by the formation of black and dry crystal colonies after 72 hours of incubation (Fig. 3B). The OD value obtained in biofilm production of *L. brevis* and *L. acidophilus* STD strains using a microtiter plate test confirms the EPS amounts (Fig. 4A). Quantitative analysis using the microtiter plate method demonstrated variations in EPS production among the strains. *L. brevis* YAAS4 exhibited the highest EPS production (259.90 ± 1.06 mg/L), while *L. acidophilus* STD produced the least (233.70 ± 1.52 mg/L). Except *L. brevis* YAAS1, all strains produced significantly higher amounts of EPS compared to *L. acidophilus* STD (P<0.0001) (Fig. 4B).

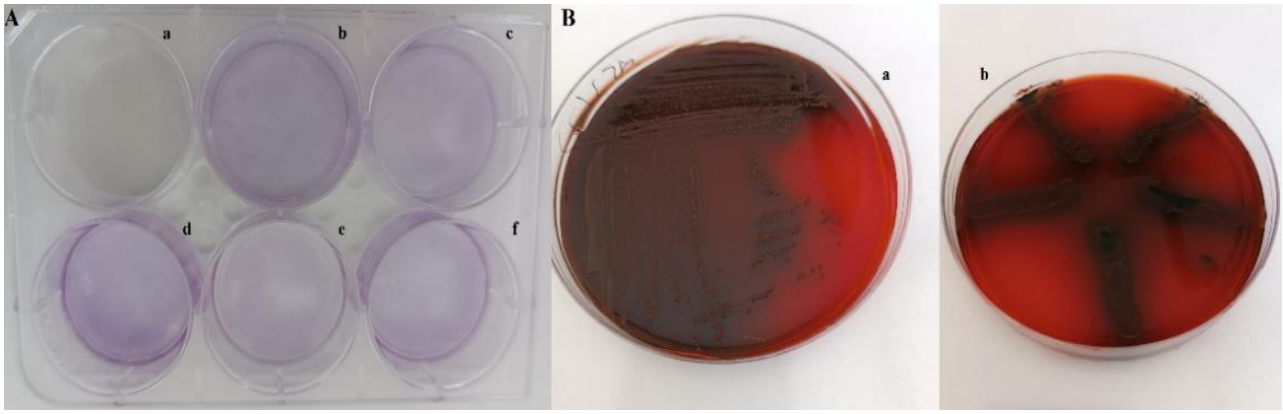

Figure 3. A: The tube method was employed to identify biofilm-forming *L. brevis* and *L. acidophilus* STD, as indicated by the formation of a visible film. (a) Control, (b) YAAS1, (c) YAAS2, (d) YAAS3, (e) YAAS4, (f) STD. B: Image of EPSs produced by the strains on Congo red agar. (a) YAAS4, (b) *L. brevis* and *L. acidophilus* STD.

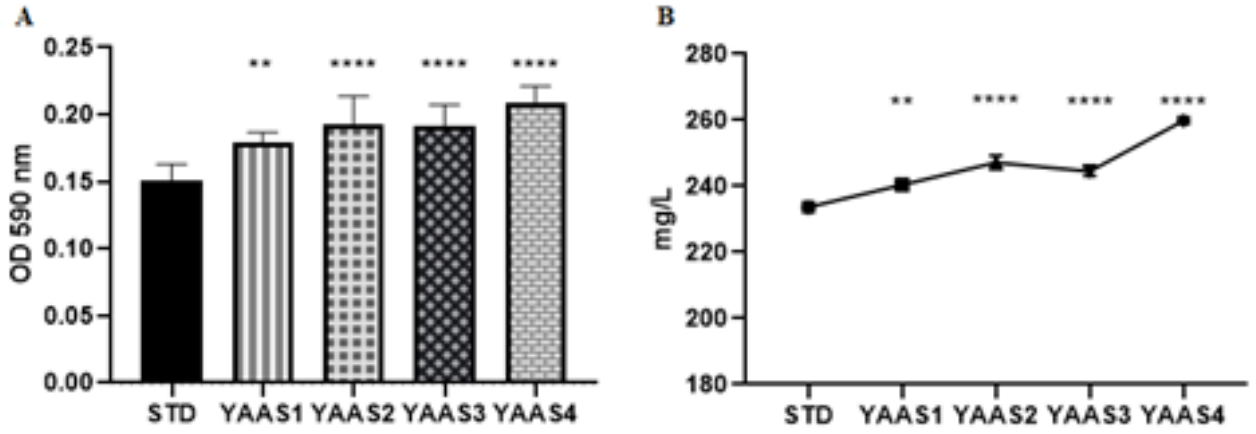

Figure 4. A: Biofilm production of *L. brevis and L. acidophilus* STD strains using a microtiter plate test. B: EPS amounts of *L. brevis* and *L. acidophilus* STD strains. P>0.05; not significant (ns), P<0.001 and P<0.0001; highly significant (***), P<0.01; quite significant (**) and P<0.05; significant (*).

The production of EPS by lactic acid bacteria (LAB), such as *L. brevis* and *L. acidophilus* STD, was confirmed using both quantitative and qualitative analytical techniques in line with previous research (Murugu et al., 2024). LABs are recognized as Generally Recognized As Safe (GRAS) microorganisms, exhibiting a diverse range of characteristics and possessing the capacity to synthesize EPS (Surayot et al., 2014). *L. brevis* is known to produce water-soluble EPS primarily composed of glucose and sucrose (Pourjafar et al., 2023; Angelin & Kavitha, 2020). In this study, the *L. brevis* strains demonstrated comparable levels of EPS production. It has been reported that LAB synthesize EPS yields ranging from 75 to 397 mg/L (Zhang et al., 2020b). Among our isolates, *L. brevis* YAAS4 (259.90 ± 1.06 mg/L) produced the most EPS, while *L. brevis* YAAS1 (240.50 ± 1.40 mg/L) produced the least EPS. It was also determined that the isolates generally synthesized similar amounts of EPS.

3.5. Antioxidant Properties

The antioxidant activities of four *L. brevis* and *L. acidophilus* STD strains were determined using DPPH and ABTS methods. According to the DPPH results obtained, it was determined that BHA and AA standard antioxidants showed better activity than the strains. The DPPH radical scavenging activities of the strains were determined as YAAS4>STD>YAAS1>YAAS2>YAAS3. The *L. brevis* YAAS4 strain exhibited a significantly higher scavenging activity compared to *L. acidophilus* STD, with a highly significant difference observed (P<0.0001). Based on the ABTS results, it was found that the strains exhibited activities comparable to those of standard antioxidants. The ABTS activities of probiotics were determined as YAAS3>YAAS1>YAAS4>STD>YAAS2. Since standards and all strains exhibited similar activity in ABTS radical scavenging activity, no significant difference was detected (Table 2).

Table 2. DPPH and ABTS activities of *L. brevis* and *L. acidophilus* STD strains and standard antioxidants (BHA and AA).

	ISOLATES AND STANDARDS						
	YAAS1	YAAS2	YAAS3	YAAS4	STD	BHA	AA
DPPH (IC_{50})	89.09±0.22	91.44±0.22	102.56±0.22	38.76±0.23	84.75±0.43	13.70±0.10	13.05±0.13
ABTS (IC_{50})	14.98±0.01	15.33±0.01	14.96±0.04	15.17±0.02	15.20±0.06	14.98±0.04	15.05±0.03

In the studies, free radical scavenging, chelating, and lipid peroxidation inhibitory activities were used to evaluate the antioxidant effects of LAB strains. It was stated that there was high DPPH radical scavenging activity in species belonging to the *Lactobacillus* genus (Das et al., 2015; Yang et al., 2020). It was reported that the extracts obtained from the *L. brevis* R4 strain had ABTS radical scavenging activity (Han et al., 2017). It was determined that the DPPH radical scavenging activities of the isolates were lower than the standards. However, the isolates exhibited similar activities to the standards in ABTS radical scavenging activity. These findings suggest that antioxidant activity is largely dependent on the strain and is not affected by the species' origin.

3.6. Anticancer activities of bacterial metabolites

The anti-proliferative effects BMs from four *L. brevis* strains and *L. acidophilus* STD were evaluated against Caco-2 and HT-29 cell lines (Fig. 5). For Caco-2 cells, the percentage inhibition of cell proliferation by BMs ranged from 3.64 ± 0.49% to 87.80 ± 0.75% across different concentrations (20 µL, 10 µL, 5 µL and 2.5 µL). With the exception of *L. brevis* YAAS2 BM at the 10 µL concentration, all other BM concentrations and strains exhibited significantly higher inhibitory effects on Caco-2 cell proliferation compared to *L. acidophilus* STD metabolite (p<0.0001) (Fig. 5a). Similarly, for HT-29 cells, the percentage inhibition of cell proliferation by BMs varied from 24.93 ± 0.80% to 74.24 ± 0.76%. At all tested concentrations, the inhibitory effects of BMs from all *L. brevis* strains on HT-29 cell proliferation were significantly higher than that of *L. acidophilus* STD metabolite (p<0.0001) (Fig. 5b).

Research has demonstrated that cell-free metabolites of *L. brevis* can exhibit anti-cancer effects and promote

apoptosis in MCF-7 cells (Peter et al., 2023). Additionally, metabolites from *L. brevis* OPK-3 have shown potential in inhibiting leukemia cell proliferation and inducing apoptosis in cancer cells (Zhao et al., 2019; Diguţă et al., 2020). Both *L. paracasei* and *L. brevis* metabolites have been found to have anticancer effects on HT-29 cancer cells. Based on MTT and apoptosis assays, it can be concluded that the induction of apoptosis by these metabolites is dependent on the strain, dose, and time (Mojibi et al., 2018). Other studies also support the various positive effects and anticancer properties of these metabolites (Son et al., 2018). In our study, cell-free metabolites obtained against Caco-2 and HT-29 cell lines showed positive effects as in the literature by showing varying and increasing anti-proliferative activity depending on the strain and dose.

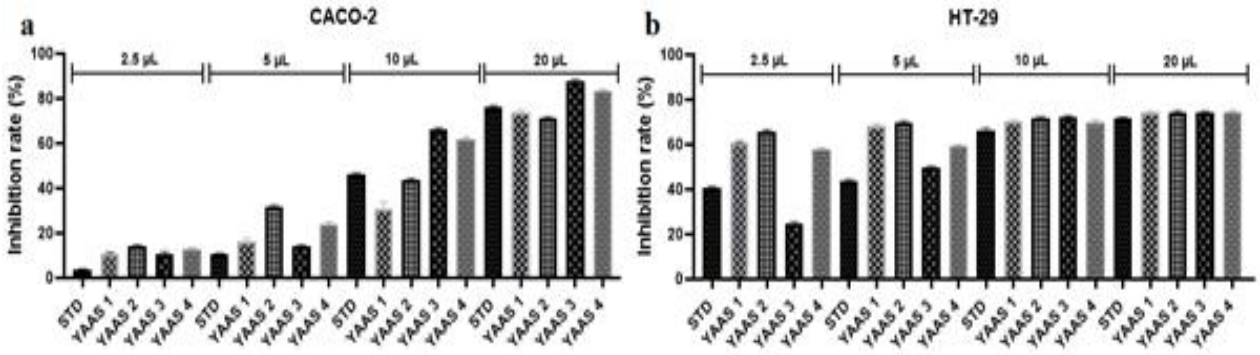

Figure 5. % inhibition effects of *L. brevis* and *L. acidophilus* STD strains on BMs of Caco-2 (a) and HT-29 (b) cell lines.

4. Conclusion

Four newly obtained *L. brevis* strains and an *L. acidophilus* STD strain used as a control, originating from breast milk, manifested pronounced probiotic characteristics as indicated by their substantial resistance to simulated gastric and intestinal milieus. Additionally, it was observed that all strains produced the highest levels of acetic acid and the lowest levels of succinic acid. EPSs synthesized by the strains in similar amounts were determined qualitatively and quantitatively. Despite the strains displaying ABTS activity commensurate with standard antioxidants, their DPPH activity was determined to be low. While BMs showed better activity against Caco-2 cell line depending on the strain and increasing concentration, they exhibited good activity against HT-29 cell line even at low concentrations. Overall, the strains exhibited similar properties, however the *L. brevis* YAAS4 strain demonstrated superior characteristics. Since there are limited studies on human-derived *L. brevis* strains in literature, our study is important. In addition, considering the probiotic, EPS, metabolites, antioxidant and anticancer data of our *L. brevis* strains, elucidation of the interaction mechanisms of these strains, and studies on how their probiotic properties will perform *in vivo* will prove that they may have high potential to be important probiotic candidates.

Ethics committee approval: This study was conducted in accordance with the ethical standards of animal experimentation. Legal research ethics committee approval permissions for the study were obtained from the Van Training and Research Hospital Clinical Research Ethics Committee (No: 2017/7).

Conflict of interest: The authors declare that there is no conflict of interest.

Author Contributions: Conception – Y.A.; Design – Y.A.; Supervision – Y.A.; Fund – Y.A., M.E.; Materials – Y.A., M.E.; Data Collection and Processing – Y.A., M.E.; Analysis Interpretation – Y.A., M.E.; Literature Review – Y.A., M.E.; Writing – Y.A., M.E.; Critical Review – Y.A., M.E.

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