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ORIGINAL ARTICLE

Chokeberry Extract: Inhibitory Activity on Some Lactic Acid Bacteria and the Growth Stimulative Effect on Limosilactobacillus Fermentum MA-7

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ABSTRACT

Protecting and balancing the intestinal flora is important for protection body against many diseases. It is important to develop probiotics, which are an important part of the flora balance, and prebiotic agents that encourage the development of probiotics. The study aimed to determine the potential use of the ethanol extract obtained from Chokeberry fruits with probiotic candidate *Limosilactobacillus fermentum* MA-7 as natural additive agents in the pharmaceutical and food industries.The biological activity of the extract was determined against probiotic candidate lactic acid bacteria (LAB) strains isolated from human breast milk. First, the inhibitory activity of the extract was determined using the disc diffusion method against five different LABs. Then, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the extract on the LABs were determined using the micro-dilution method. Finally, the biological activity of the extract on *L. fermentum* MA-7 at concentrations of 50 and 100 mg/ml was determined using the macro-dilution method.The extract showed antibacterial activity only against *Streptococcus thermophilus* MAS-1, with an inhibition zone diameter of 6.43 mm. MIC and MBC values of the extract were found as 12.5 mg/ml - >50 mg/ml and 25 mg/ml - >50 mg/ml, respectively. The stimulatory activity of the extract on *L. fermentum* MA-7 was obtained using macro-dilution method. The number of viable cells was determined after 0, 24 and 48 hours. A concentration of 50 mg/ml extract promoted the growth of *L. fermentum* MA-7 at 24 and 48 hours. 100 mg/ml extract concentration inhibited the growth of *L. fermentum* MA-7 after 24 hours but increased its growth after 48 hour compared to 24th hour. It has been shown that Chokeberry fruit ethanol extract at appropriate concentrations can be an alternative as a natural stimulant for *L. fermentum* MA-7 to support its development in the pharmaceutical industry and as a natural additive in the food industries.

Keywords: Antimicrobial activity; Aronia Melanocarpa; Extract; Lactic acid bacteria; Living cell

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INTRODUCTION

Gastrointestinal microflora (GIM) is an important factor affecting human health. GIM is in a variable and dynamic balance[16,10]. Healthy GIM includes many species of lactic acid bacteria (LAB) that have a variety of benefits[4].

Probiotic bacteria are defined as microorganisms that provide benefit when applied to the host in sufficient quantities' [25]. LABs consist of various Gram-positive bacteria and are used in the pharmaceutical and food industries due to their probiotic properties. LAB strains have Generally Recognized as Safe (GRAS) status and are considered safe for humans [2,7,20]. *Lactobacillus* has an important role in maintaining homeostasis in the human intestine[27]. Lack of *Lactobacillus* in the intestine causes the host to encounter specific health problems. Therefore, administering *Lactobacillus* species to humans as probiotic supplements helps balance abnormal intestinal microbiota and protects the host[21]. *L. fermentum* has many therapeutic effects such as protection against pathogens, antioxidation and immunomodulation. The addition of *L. fermentum* strains preserves foods and increases their nutritional properties [1].

Plants have been widely used since ancient times in the treatment of various diseases[6]. Chokeberry (*Aronia melanocarpa*) is native to North America and is a shrub belonging to the Rosaceae family[5]. Chokeberry has high hypotensive, antimicrobial, antiplatelet, antiviral, anti-inflammatory and antidiabetic properties [12,14,15,23]. As the demand for healthy nutrition increases today, the use of Chokeberry as a functional food is increasing in Türkiye and the world [13]. Chokeberry cultivation, which can be considered new for our country, has been increasing in recent years. Chokeberry first started to be grown in Yalova and Kırklareli in 2014. Nowadays, it is grown in almost all regions of Türkiye, including Istanbul, Çanakkale, Bursa, Samsun, Ordu, Ankara, Manisa, Bayburt, Antalya and Tekirdağ[24]. Chokeberry has a dark purple color thanks to the anthocyanins it contains. Anticyanins is a water-soluble natural pigment and can be used safely in food and pharmaceutical industries [18]. Chokeberry has become the center of attention in recent years with its many features that improve human health .

The purpose of the study was to ascertain whether ethanol extract from domestically farmed chokeberries and probiotics might be used in the food and pharmaceutical sectors. Therefore, firstly the inhibitory activity of the extract against probiotic candidate lactic acid bacteria (LAB) strains isolated from breast milk was determined. Afterwards, the stimulatory activity of the extract on the growth of *L. fermentum* MA-7 was investigated.

Aim of study

METHODS

1. Materials and Chemicals

Waring blender (Waring-8011ES, USA), Sonicator device (Hielscher UP100H, Germany), 0.22 µm syringe filter (ISO-LAB, Germany), Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, Japan), M17-Agar and De Man-Rogosa and Sharpe (MRS)-Agar (Merck-KGaA, Germany).

2. Preparation of Extract

Chokeberry berries were purchased as fresh fruit from Chokeberry Food and Health I.C (Yalova-Türkiye). First, tap water was used to wash the fruits, and then purified water. The fruits were allowed to air dry without exposure to sunshine. A Waring blender was used to grind the dried fruits, and a sonicator equipment (Amplitude, 100%; Cycle, 1) was used to extract 99.9% ethanol from them. After extraction, the solvent was concentrated using a rotary evaporator (Heidolphch, Germany). The extract was dissolved using DMSO and sterilized with a 0.22 µm syringe filter (Imran Bashir et al., 2018).

3. Determination of Antimicrobial Activity

3.1. Test Microorganisms

Limosilactobacillus fermentum MA-7, *Lactobacillus delbrueckii* MA-9, *Limosilactobacillus vaginalis* MA-10, *Lactobacillus gasseri* MA-1 (MRS-Agar), and *Streptococcus thermophilus* MAS-1 (M17-Agar) were the probiotic candidate LABs that were extracted from breast milk. The 24-hour active cultures that were maintained at 37°C were utilized.

3.2. Disc Diffusion Assay

The inhibitory activity of chokeberry fruit ethanol extract on probiotic candidate LABs was determined using the disc diffusion assay. LABs prepared at 0.5 McFarland concentration was inoculated onto solid medium (100 µl). Sterile discs were placed on the agar medium and then 10 µl (1 mg/disc) of the extract was dropped onto the discs. At the end of 24 hours of incubation, the inhibition zones formed around the discs were measured with a caliper and recorded. The average of three replicates was recorded.

3.3. Micro-Dilution Method

The MIC (Minimum Inhibitory Concentration) and MBC (Minimum Bactericidal Concentration) values of chokeberry fruit ethanol extract on LABs were determined using the micro-dilution method. The bacterial suspension (adjusted to 0.5 McFarland), liquid medium and chokeberry fruit ethanol extract were added to the tubes and vortexed. After 24 h of incubation, the concentration at which no bacterial growth occurred in the tubes was recorded as the MIC value of the extract. Then, spot cultivation of the samples taken from the tubes was carried out on solid medium. At the end of the incubation, the concentration on the solid medium at which bacterial growth did not occur was recorded as the MBC value of the extract.

3.4. Macro-Dilution Method

The stimulatory effect of chokeberry ethanol extract on *L. fermentum* MA-7 was determined using macro-dilution assay. The viable colony count was obtained at the concentration of 50 and 100 mg/ml of the extract. *L. fermentum* MA-7 suspension prepared at 0.5 McFarland concentration was added to the mixture of chokeberry fruit ethanol extract and the growth medium. The control consisted of medium and bacterial suspension. After incubation at 37°C for 0, 24 and 48 h, the samples were diluted in physiological serum (PS) and spread onto agar medium. At the end of each incubation period, the viable cell counts were obtained and the values as expressed as Log_{10} CFU/ml.

3.5. Statistical Analysis

GNU SPSS (version 25.0) software was used to analyze the data, and Tukey's post-hoc test along with One-Way analysis of variance (ANOVA) was used to validate statistical significance. At the $p<0.05$ level, the difference between the film groups was deemed statistically significant.

RESULTS AND DISCUSSION

Plants are an alternative source of food supplements because they have a prebiotic effect and have improving effects on the activity of probiotic microorganisms [19, 22]. The biological

activity of chokeberry ethanol extract on probiotic candidate LABs isolated from breast milk was investigated. Furthermore, the extract's MIC and MBC values were ascertained. The inhibitory activity was not determined on *L. fermentum* MA-7, *L. delbrueckii* MA-9, *L. vaginalis* MA-10, *L. gasseri* MA-1. The only antibacterial activity of the extract was obtained against *S. thermophilus* MAS-1 with a zone diameter of 6.43 mm ($p<0.05$). MIC values ranged from 12.5 mg/ml to >50 mg/ml (Table 1). MBC values varied from 25 mg/ml to >50 mg/ml. The highest MIC and MBC values were determined against *L. fermentum* MA-7. The high MIC and MBC values of the extract and the fact that it does not create an inhibition zone mean that *L. fermentum* MA-7 may be used in food or pharmaceutical mixtures with Chokeberry ethanol extract. Additionally, this indicates that the extract may be a prebiotic candidate.

Table 1. Antimicrobial Activity of Chokeberry Ethanol Extract.

Microorganisms	**Chokeberry Ethanol Extract**		
	Inhibition Zone Diameter (mm)	**MIC (mg/ml)**	**MBC (mg/ml)**
L. fermentum MA-7	NA[a]	>50	>50
L. delbrueckii MA-9	NA[a]	25	50
L. vaginalis MA-10	NA[a]	25	50
L. gasseri MA-1	NA[a]	25	50
S. thermophilus MAS-1	6.43±0.07[b]	12.5	25

* NA: No-Activity, MIC: Minimum-Inhibitory-Concentration, MBC: Minimum-Bactericidal-Concentration
**Differing superscript values in the columns indicate that the one-way ANOVA and Tukey's post-hoc test were substantially different ($p<0.05$). F (20333.557) Sig. (0.000)

Probiotics strengthen the immune system to ensure intestinal integrity, increase defense activity against pathogens and have an antagonistic effect on pathogens. Prebiotics have a stimulating synergistic effect for the proliferation of beneficial microorganisms adapted to the intestinal system and provide the energy they need [17]. Active compounds found in plants have prebiotic and antimicrobial properties[8].Therefore, finding and developing natural compounds that promote the development of LABs, such as *L. fermentum*, which have an important role in the human body, is of great interest. According to disc diffusion assay results, the extract did not show antimicrobial activity on *L. fermentum* MA-7. Additionally, the values of MIC and MBC of *L. fermentum* MA-7 were found to be above the tested concentrations. To obtain the upper concentration of the extract that can be used together with *L. fermentum* MA-7 in determining the stimulating effect, the number of viable cells of *L. fermentum* MA-7 at 50 mg/ml and 100 mg/ml was determined by evaluating the MIC and MBC values. The results are given in Figure 1 and the images on the petri plate are given in Figure 2. Chokeberry ethanol extract at 50 mg/ml concentration significantly increased the growth of *L. fermentum* MA-7 after 24 and 48 hours compared to 0. hour (Figure 2-B) ($p<0.05$). In fact, *L. fermentum* MA-7 retained the same amount of viable cell after 48 h as after 24 h (Figure 2-A). Compared to the control group at 50 mg/ml extract concentration, while the number of viable cells was the same at 0. hour, it increased by 3 logarithmic units after 24 h and by approximately 4 logarithmic

units after 48 h. At the 24. hour, the test group containing 50 mg/ml extract increased the development of *L. fermentum* MA-7 to a greater extent than the other groups, and the data showed statistically significant difference ($p<0.05$). At 50 mg/ml extract concentration, the number of viable cells of *L. fermentum* MA-7 at 24 and 48 hours increased compared to the control group. At 100 mg/ml Chokeberry ethanol extract concentration, the viable cell count of *L. fermentum* MA-7 after 24 h decreased compared to the control and 50 mg/ml extract concentration groups (Figure 2-C). However, an increase in the count of viable cells of *L. fermentum* MA-7 was observed after 48 h compared to 24 h. This means that the increase in the extract concentration shows an inhibition effect after 24 h. Therefore, it can be said that appropriate concentrations of the extract may show prebiotic activity.

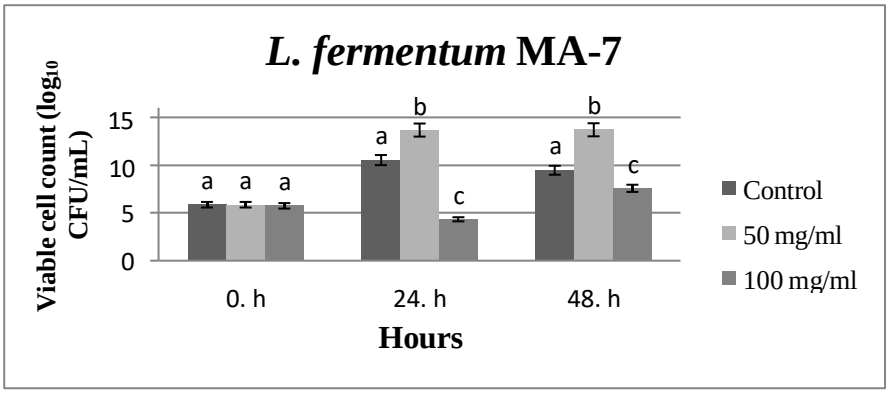

Figure 1. Prebiotic Activity of Chokeberry Ethanol Extract on *L. fermentum* MA-7.
*A one-way ANOVA followed by Tukey's post-hoc test revealed significant differences ($p<0.05$) between the various superscript values in the columns. At 0.hours, F (0.271) Sig.(0.771), 24.hours and 48.hours; F (117202.288) Sig.(0.000), and F (80.422) Sig.(0.000)

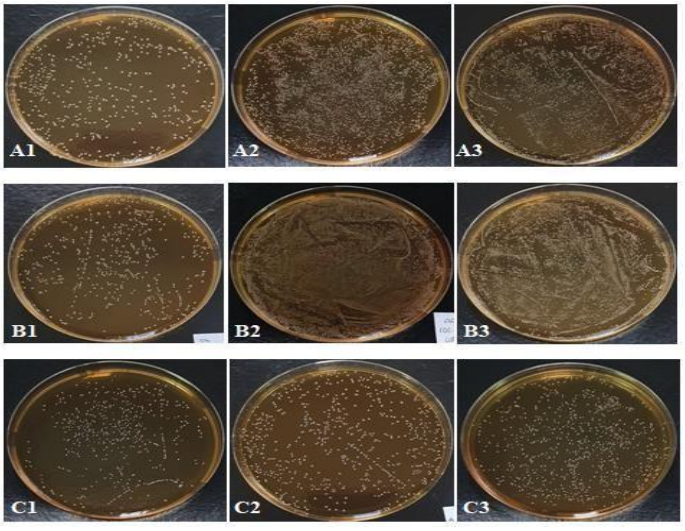

Figure 2. Activity of Chokeberry Ethanol Extract on *L. fermentum* MA-7 Control (A), 50 mg/ml (B) and 100 mg/ml (C) Concentrations at 0. (1), 24. (2) and 48. (3) hours.

In a previous study, it was determined the biological activity of extracts obtained from

Chokeberry fruits with ethanol and water on LABs (*Brochothrix thermosphacta*, *Leuconostoc mesenteroides*, *Weissella viridescens*). The results showed that LAB strains was sensitive to the extract. In addition, in frozen and ready-made foods with added ethanol and water extracts, a decrease in the count of live cells of pathogens that cause spoilage and an increase in the number of live cells of LABs was observed [26]. The increase in the viability of the tested LABs of Chokeberry extracts resulted similar to our study. Their results showed that Chokeberry extracts can be used in food preservation, as they inhibit the development of pathogens that cause food spoilage and increase the development of LAB. In another study, Chokeberry fruit fermented (4 weeks) using the probiotic microorganism *Lactobacillus paracasei* SP5 showed that the cell viability of *L. paracasei* SP5 increased during fermentation[3]. The increase in the number of living cells during fermentation of Chokeberry fruits showed that the fruit supports the development of *L. paracasei* SP5 and can be used as nutrients. Similar to the results in literature studies, our results showed that Chokeberry ethanol extract had growth stimulating activity on *L. fermentum* MA-7.

CONCLUSION

The current study provides new findings regarding the effect of ethanol extract from Chokeberry berries on the growth of lactic acid bacteria as a probiotic candidate isolated from breast milk. The ethanol extract had no biological activity on most tested LABs. Also, the extract with stimulatory effect promoted the growth of *L. fermentum* MA-7. Chokeberry ethanol extract with promising prebiotic potential may lead to the development of new products in food and pharmaceutical industries.

Abbreviations

CFU: Unit for Colony Formation
DMSO: Dimethyl Sulfoxide
GIM: Gastrointestinal Microflora
LAB: Lactic Acid Bacteria
MBC: Minimum Bactericidal Concentration
MIC: Minimum Inhibitory Concentration
NA: No Activity
PS: Physiological Serum

DECLARATIONS

1. Authors' contributions

All authors have equally contributed to the research.

2. Funding Statement

This research is self-funded

3. Conflict of Interest

The authors declares no conflict of interest

4. Ethical approvals

The work does not include any human or animal participants.

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