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Effects of ultrasound on antioxidant properties of postbiotics derived from pellets and supernatants *Enterococcus faecium* culture

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ABSTRACT

Accumulating studies have highlighted the great potential of postbiotics in protecting host health. Compared with traditional functional foods, postbiotics have the advantages of high physiological activity, long shelf life, easy absorption, etc. In this study, the effects of ultrasound treatment on the antioxidant activity of postbiotics derived from Enterococcus faecium bacteria were evaluated. This bacterium is alpha-hemolytic or non-hemolytic and is a symbiont of the human intestine. The microorganism was subjected to 6 different ultrasonic treatments (5, 10, 15, 20, 25, and 30 minutes). ABTS, DPPH, MIC and HRS tests were performed to investigate antioxidant activity at the pellet and supernatant levels. The results of one-way analysis of variance showed that the effect of ultrasound on the antioxidant properties of postbiotics was significant at different times at the pellet and supernatant levels. postbiotics exhibited higher antioxidant activity at the supernatant level with DPPH at 20 min, ABTS control and MIC at 20 min and HRS control. At the pellet level, higher antioxidant activity was observed with DPPH control, ABTS control and MIC at 20 min, as well as HRS at 30 min. In addition, samples treated for 5 minutes demonstrated the lowest antioxidant activity (0 to 36 percent) across all experiments at both pellet and supernatant levels, with the exception of the MIC control experiment (0 percent). The antioxidant property of postbiotics extracted at the supernatant level was higher than the pellet in all experiments except for ABTS. We suggest that postbiotics extracted from E. faecium be used as antioxidants in the food and pharmaceutical industries.

1- INTRODUCTION

According to the World Health Organization (WHO) and the Food and Agriculture Organization (FAO), probiotics are live, non-pathogenic microorganisms that confer beneficial health effects and, when consumed in adequate doses, can prevent or treat diseases such as cancer, cardiovascular diseases, diabetes, gastrointestinal cancers, and others [1, 2]. However, not all probiotic bacteria are considered safe. The risks associated with live probiotic bacteria, including systemic infections, harmful metabolic activities, excessive immune system stimulation, gene transfer, and adverse gastrointestinal effects, have been documented in case reports, clinical trials, and experimental models [3]. In recent years, researchers have proposed various solutions to address these challenges, and one of the most effective and practical approaches has been the use of the inanimate bacterial form (postbiotics) as an alternative to probiotics [4]. Postbiotics include any substance released from or produced through the metabolic activity of probiotics, which exerts a direct or indirect beneficial effect on the host [5]. Moreover, they readily bind to target sites and are easily accessible, standardizable, transportable, and storable [6]. Notably, they are non-pathogenic, non-toxic, and exhibit high resistance to hydrolysis [7]. Optimization of fermentation conditions can enable postbiotics to exert their maximum probiotic effects [8]. The production of postbiotics involves several steps. The first step is the fermentation of the substrate and culture by bacteria [9]. The second step is the inactivation of the probiotic strain through thermal methods such as pasteurization, sterilization, and ohmic heating, or non-thermal methods such as pulsed electric fields, ultrasound, irradiation, and supercritical carbon dioxide. The third step involves obtaining cell-free supernatant or postbiotic materials via centrifugation or filtration of the inactivated fermentations [9]. Currently, the most common method for microorganism inactivation is heat treatment, as it is simple and cost-effective. Nevertheless, heat treatment may cause irreversible damage to the heat-sensitive physiologically active substances of microorganisms, particularly proteins and polypeptides. Heat primarily damages the cell membrane, cleaves nucleic acids, inactivates essential enzymes, denatures proteins, and polymerizes ribosomes. From this perspective, non-thermal inactivation of microorganisms will have greater applicability in future research. Ultrasound increases extracellular enzymes, mainly beta-galactosidase, and facilitates the effective extraction of antioxidants and bioactive compounds such as phenols, flavonoids, and anthocyanins from probiotics [10, 11]. Ultrasound can be applied after fermentation to inactivate bacteria and before fermentation to improve stability, reduce processing time, and enhance quality [12].

Scientific evidence indicates that postbiotics possess diverse functional properties, including antimicrobial, antioxidant, anticancer, and immunomodulatory activities [13]. Antioxidants are substances that effectively prevent or delay unwanted oxidation events through distinct mechanisms [14]. Antioxidants have numerous applications in the pharmaceutical, cosmetic, hygiene, food, petrochemical, and polymer industries [15]. The endogenous antioxidant system in the human body constantly requires antioxidant compounds [16]. Therefore, there is currently growing interest in antioxidants, particularly those derived from microbial sources [17]. The use of probiotics and their metabolites (postbiotics), as well as their widespread application as food additives, has increased

significantly [18]. The antioxidant properties of postbiotics derived from *Lactobacillus plantarum* have been demonstrated in numerous studies [5, 19, 20]. However, the antioxidant activity of postbiotics can be influenced by the type of culture and substrate, as well as the method used for their production [21]. Various methods can be employed to determine the antioxidant activity of food substances. ABTS and DPPH are examples of proton radical scavenging assays. The ABTS method is highly sensitive; it detects a wide range of antioxidant compounds and can be applied in both hydrophilic and lipophilic systems, making it suitable for various sample types. The DPPH method is considered more reproducible and simpler; however, it is recommended only for hydrophilic samples, rendering it less versatile than ABTS. Metal-chelating activity (MIC), as one of the antioxidant mechanisms, reduces the concentration of the catalytic transition metal in lipid peroxidation [22]. HRS measures the hydroxyl radical scavenging activity, which is the most reactive radical associated with lipid peroxidation and cellular biomolecules [23]. Therefore, this study aimed to evaluate the antioxidant activity (ABTS, DPPH, MIC, and HRS) of postbiotics obtained from *Enterococcus faecium* (in both pellet and supernatant forms) using ultrasound at a fixed frequency and different time intervals (5, 10, 15, 20, 25, and 30 minutes), and to compare them with postbiotics not subjected to the ultrasound process.

2- Materials and Methods

2-1- Bacterial Preparation and Primary Culture

Enterococcus faecium PTCC 1957 was purchased as lyophilized ampoules from the Iranian Regional Collection of Fungi and Industrial Bacteria for experimental purposes. Bacterial preparation and primary culture were carried out according to the guidelines of the Iranian Regional Collection of Fungi and Industrial Bacteria. Slant stock and glycerol stock were prepared for bacterial preservation.

2-2- Preparation of Bacterial Suspensions

First, under aseptic conditions, one loopful of the desired slant stock was transferred into 5 mL of sterile specific culture medium for the strain and incubated at the required temperature (37°C) for 24 hours in a shaking incubator. Then, 100 µL of the suspension was transferred into a tube containing 10 mL of sterile specific liquid culture medium for each strain and incubated for 24 hours in a shaking incubator (at the appropriate temperature for the bacteria and agitation at 100 rpm). If a larger volume of suspension was needed, additional tubes were used (Erlenmeyer flasks with larger volumes of culture medium and initial bacterial suspension could also be used).

2-3- Preparation of Postbiotics

One hundred microliters of the probiotic bacterium *Enterococcus faecium* was grown in BHI broth (10 mL) for 24 hours at 37°C. Postbiotic cells were separated by centrifugation at 6000 × g for 10 minutes at 4°C. The pellet was washed twice with sterile distilled water by centrifugation at 4500 × g for 10 minutes, and then sterile phosphate-buffered saline (PBS) was added to the pellet (10 times the weight), and the mixture was vortexed until completely homogenized. The average cell weight after centrifugation was 0.11 g per tube (10 mL of culture medium). The supernatant was kept as the postbiotic, and the pellet (along with PBS) was kept as the live probiotic bacterial cells. Subsequently, postbiotics were prepared using the following two different methods:

- The pellet and supernatant without any processing (as control samples)

were filtered through a 0.22 µm sterile filter.

- The supernatant and pellet were placed in an ultrasonic bath at a fixed frequency of 37 kHz and an input power of 280 W for 5, 10, 15, 20, 25, and 30 minutes. The water level was adjusted to match the level of the Erlenmeyer flask contents. The Erlenmeyer flasks were placed in the center of the bath, and the temperature was maintained at 25°C. The pellet and supernatant were then filtered through a 0.22 µm sterile filter.

2-4- Determination of Antioxidant Activity

The antioxidant activity of the postbiotic samples at both supernatant and pellet levels was evaluated using the following methods.

2-4-1- DPPH Radical Scavenging Activity Assay

The DPPH radical scavenging activity was determined using a freshly prepared DPPH solution (0.1 mM) in pure methanol. For antioxidant activity assessment, 100 µL of the postbiotic solution was mixed with 3.90 mL of DPPH solution. After incubation at room temperature in the dark for 30 minutes, the absorbance of the samples was measured at 517 nm. For the blank samples, sterile BHI culture medium (for supernatant) or PBS (for pellet) was used instead of the postbiotic. The results were expressed as radical scavenging activity using the following equation [24]:

$$\text{Radical scavenging activity (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

2-4-2- ABTS Radical Scavenging Activity Assay

A stock solution of ABTS (4.7 mM) was prepared in pure methanol. This solution was then mixed with an equal volume of an aqueous potassium persulfate solution (6.2 mM). After storage at room temperature in the dark for 12–16 hours, the mixture was diluted with methanol to an absorbance of 0.7 (at 734 nm). Twenty microliters of postbiotic in methanol was mixed with 2 mL of the ABTS solution. After incubation at room temperature in the dark for 6 minutes, the absorbance of the analyzed samples was measured spectrophotometrically at 734 nm. For the blank samples, sterile BHI culture medium (for supernatant) or PBS (for pellet) was used instead of the postbiotic. The results were expressed as radical scavenging activity using the following equation [24]:

$$\text{Radical scavenging activity (\%)} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100$$

2-4-3- Metal Ion Chelating Activity Assay (MIC)

Three hundred microliters of the sample (postbiotic) or control sample (water) was mixed with 5 µL of iron (II) chloride (2 mM) and 10 µL of ferrozine (5 mM). After 10 minutes of incubation (in the dark at room temperature), the absorbance was measured at 522 nm [25].

$$\text{MIC (\%)} = [(A_{control} - A_{sample}) / A_{control}] \times 100$$

2-4-4- Hydroxyl Radical Scavenging Activity Assay (HRS)

To evaluate HRS activity, 250 µL of each sample was added to 250 µL of 2.5 mM 1,10-phenanthroline, 250 µL of PBS (0.1 M, pH 7.4), and 250 µL of 2.5 mM $FeSO_4$, before mixing with 250 µL of 20 mM H_2O_2. The test solution was incubated at 37°C for

90 minutes, and the absorbance was measured using a UV spectrophotometer at 536 nm. HRS activity was determined using the following equation [20]:

$$\text{HRS activity (\%)} = [(A_s - A_c) / (A_b - A_c)] \times 100$$

where A_s is the absorbance of the sample, A_c is the absorbance of the control sample (BHI culture medium for supernatant and PBS for pellet), and A_b is the absorbance of the solution without the sample and H_2O_2. All samples were tested in triplicate.

2-5- Statistical Analysis

Data analysis was performed using one-way analysis of variance (ANOVA), and means were compared using Duncan's test at a significance level of 0.05. Subsequently, a t-test was used to compare the antioxidant activity between the postbiotics produced at the pellet and supernatant levels. All analyses were conducted using SPSS software (version 23).

3- Results and Discussion

Postbiotics derived from probiotic bacteria have recently attracted researchers' attention due to their favorable effects and minimal side effects. These compounds are natural and pose no risk of bacteremia for immunocompromised individuals [26]. Therefore, they have high potential for industrial applications related to food and pharmaceutical products. In the present study, using DPPH, ABTS, MIC, and HRS assay methods, it was observed that postbiotics prepared from *Enterococcus faecium* (supernatant and pellet) exhibited significant antioxidant activity. Furthermore, the duration of ultrasound treatment significantly affected the antioxidant properties of the postbiotics. In confirmation of the present results, other researchers have extensively reported the antioxidant activity of postbiotics derived from bacteria [21, 27]. In a study by Noori et al. (2023), postbiotics produced from *Lactobacillus casei* exhibited high antioxidant activity as measured by the DPPH method [28]. Additionally, in the research conducted by Ageeli and Mohamed (2025), exopolysaccharides produced from *Lactiplantibacillus plantarum* B7 exhibited DPPH and ABTS radical scavenging activity, as well as HRS and superoxide scavenging activity [29].

3-1- Evaluation of Antioxidant Properties of Postbiotics Produced Using Ultrasound Treatment at Pellet and Supernatant Levels

The results of one-way analysis of variance (ANOVA) indicate that the effect of ultrasound on the antioxidant properties of postbiotics at different time intervals (0, 5, 10, 15, 20, 25, and 30 minutes) was significant at both the pellet and supernatant levels (Table 1 and Table 2). In confirmation of these results, high antioxidant properties of postbiotics prepared from *Lacticaseibacillus paracasei* using the ultrasound method were observed by Dong et al. (2024) through reducing power, DPPH radical scavenging activity, and HRS activity assays [30]. Moreover, according to the findings of Aguilar-Toalá et al. (2019), postbiotics produced from *L. casei* CRL 431 and *B. coagulans* GBI-30 strains exhibited approximately 79% oxidation inhibitory activity using the metal ion chelating assay [25]. In accordance with the results of Çelik et al. (2024), antioxidant activity was observed in the cell-free supernatant and exopolysaccharide of *S. salivarius* KC27L using DPPH radical scavenging, Fe^{2+} chelating activity, and superoxide anion radical scavenging activity assays [31].

Table 1: Results of one-way analysis of variance (ANOVA) for antioxidant properties of postbiotics produced using ultrasound treatment at different times on the Supernatant surface.

P-value	*F-value*	Mean squares	d.f	Parameters
0.000**	229.35	110.47	6	**DPPH**
0.000**	3841.32	1049.05	6	**ABTS**
0.000**	288.11	548.31	6	**MIC**
0.000**	251	1470.14	6	**HRS**

**: Indicates significance at the 1 percent level. *: Indicates significance at the 5 percent level. ns: Indicates no significance.

Table 2: Results of one-way analysis of variance (ANOVA) for antioxidant properties of postbiotics produced using ultrasound treatment at different times on the pellet surface.

P-value	*F-value*	Mean squares	d.f	Parameters
0.000**	34.79	6.64	6	**DPPH**
0.000**	702.59	405.12	6	**ABTS**
0.000**	886.67	307.65	6	**MIC**
0.000**	392.61	2244.34	6	**HRS**

**: Indicates significance at the 1 percent level. *: Indicates significance at the 5 percent level. ns: Indicates no significance.

Comparison of the means showed that the DPPH activity of postbiotics at the supernatant level at 20 minutes (20-S) was significantly higher than at other time points, while the 5-minute treatment exhibited the lowest activity. Additionally, the pellet control sample (0-P) showed higher DPPH activity compared to other postbiotics at the pellet level (Figure 1). The DPPH free radical possesses an unpaired electron on one of its nitrogen bridge atoms, which leads to a significant reduction in proton radical exposure. Moreover, it exerts a destructive and potent effect on body organs [32]. In the present study, the DPPH radical scavenging activity of the pellet ranged from 0.5% (corresponding to the 10-P sample) to 2.5% and 3.6% (corresponding to the 20-P and 0-P samples, respectively). The DPPH radical scavenging activity of the supernatant ranged from 0.1% (5-S) to 16.8% (20-S). The above results indicated that the 20-minute ultrasound treatment had a greater effect on the antioxidant activity (DPPH) of postbiotics at both the pellet and supernatant levels compared to other treatment times. However, the DPPH radical scavenging activity of postbiotics produced at the pellet level was considerably lower than that of the control postbiotic.

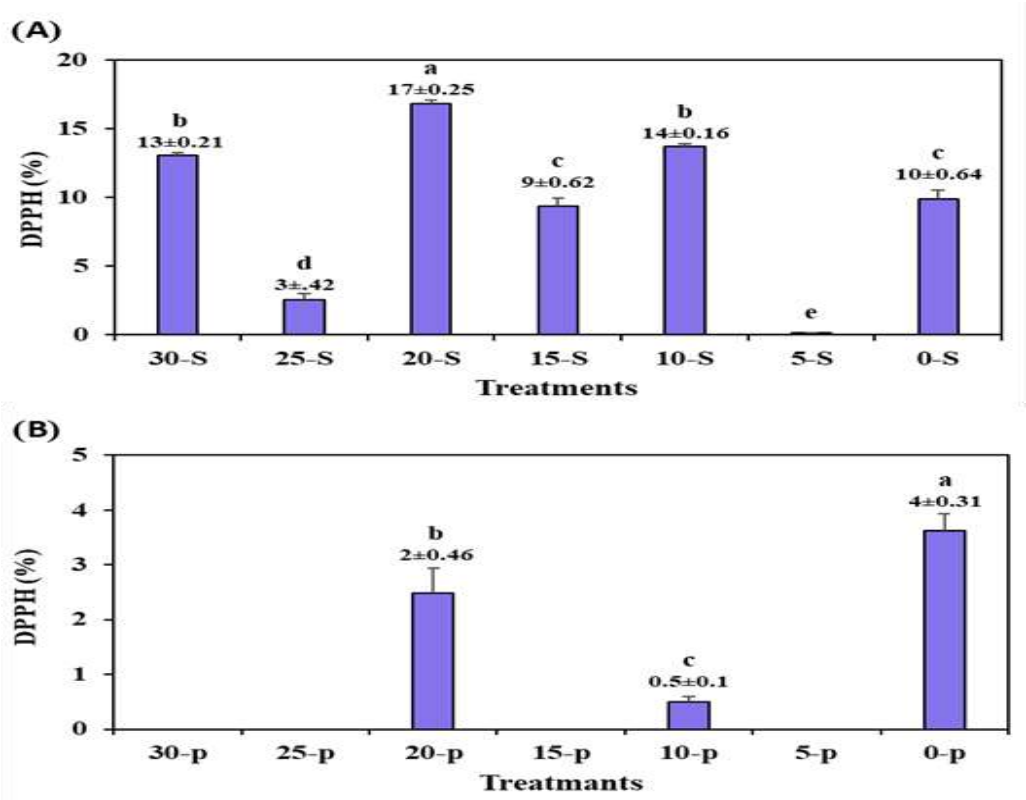

Figure 1: Comparison of the average antioxidant properties of postbiotics produced using ultrasound treatment at different times on the supernatant (A) and pellet (B) surfaces using the DPPH test. Data values represent the mean ± standard error. Different lower-case letters above the columns indicate statistically significant differences between the treatments at a = 0.05 based on the Duncan test (n=3). 0-P: Pellet 0 min (control), 5-P: Pellet 5 min, 15-P: Pellet 15 min, 20-P: Pellet 20 min, 25-P: Pellet 25 min, 30-P: Pellet 30 min 0-S: Supernatant 0 min (control), 5-S: Supernatant 5 min, 15-S: Supernatant 15 min, 20-S: Supernatant 20 min, 25-S: Supernatant 25 min, 30-S: Supernatant 30 min. The numbers above the columns indicate the mean ± standard error.

Regarding ABTS activity in the supernatant, the control postbiotic exhibited the highest level of activity, while the other samples showed no antioxidant activity at all (Figure 2). Similarly, ABTS antioxidant activity at the pellet level was highest in the control postbiotic, and the 5-minute treatment showed no antioxidant activity (Figure 2). The ABTS radical scavenging activity of the pellet in this study ranged from 0.2% (corresponding to the 15-P sample) to 31.7% (corresponding to the control sample). These results indicated that the samples produced by ultrasound had lower ABTS radical scavenging activity compared to the control samples. The ABTS radical scavenging activity of postbiotics produced by ultrasound was significantly reduced compared to the control sample, such that no ABTS activity was observed in any of the treated samples, whereas the control sample exhibited 49.5% ABTS radical scavenging activity. The above results suggest that ultrasound can have a negative effect on the ABTS activity of the produced postbiotics.

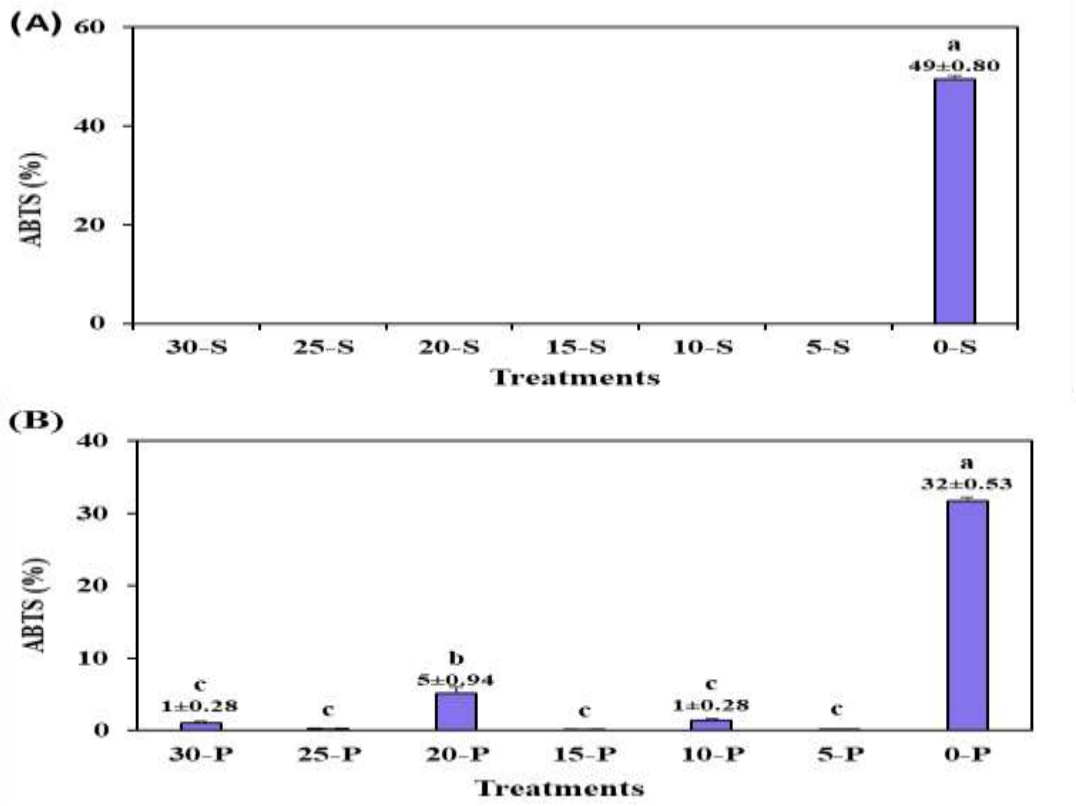

Figure 2: Comparison of the average antioxidant properties of postbiotics produced using ultrasound treatment at different times on the supernatant (A) and pellet (B) surfaces using the ABTS test. Data values represent the mean ± standard error. Different lower-case letters above the columns indicate statistically significant differences between the treatments at a = 0.05 based on the Duncan test (n=3). 0-P: Pellet 0 min (control), 5-P: Pellet 5 min, 15-P: Pellet 15 min, 20-P: Pellet 20 min, 25-P: Pellet 25 min, 30-P: Pellet 30 min 0-S: Supernatant 0 min (control), 5-S: Supernatant 5 min, 15-S: Supernatant 15 min, 20-S: Supernatant 20 min, 25-S: Supernatant 25 min, 30-S: Supernatant 30 min. The numbers above the columns indicate the mean ± standard error.

Similar to DPPH, at the supernatant level, the MIC activity of postbiotics at 20 minutes (20-S) was significantly higher than at other time points, while the 5-minute treatment showed the lowest value. Regarding the pellet, the MIC activity of postbiotics at 20 minutes was the highest, whereas the 0-P sample exhibited no MIC activity at all (Figure 3). Metal-chelating activity (MIC), as one of the antioxidant mechanisms, reduces the concentration of the catalytic transition metal in lipid peroxidation. Iron is an essential pro-oxidant for lipid oxidation among transition metals due to its high reactivity [22]. The MIC activity of the pellet produced by the ultrasound method ranged from 0% (P-0) to 43% (20-P). In the pellet samples, MIC activity was higher than in the control sample. The MIC percentage of the supernatant ranged from 12% (corresponding to the 5-S sample) to 54% (corresponding to the 20-S sample). Min et al. (2019) reported that the iron-chelating activities of exopolysaccharides increased to a maximum value of 69.7% when their concentration reached 10 mg/mL [33]. In another study, exopolysaccharide at a concentration of 4 mg/mL exhibited iron-chelating activity (27.8%) [22]. This property may be attributed to certain antioxidants and antioxidant enzymes present in postbiotics that possess metal-chelating abilities, such that they can inhibit metalloenzymes like angiotensin-converting enzyme [34]. However, in some studies, the percentage of antioxidant activity of the supernatant was lower than

the values obtained in the present study. For example, in the study by Çelik et al. (2024), the Fe^{2+} chelating activity of the cell-free supernatant of *S. salivarius* KC27L was 19.3% [31].

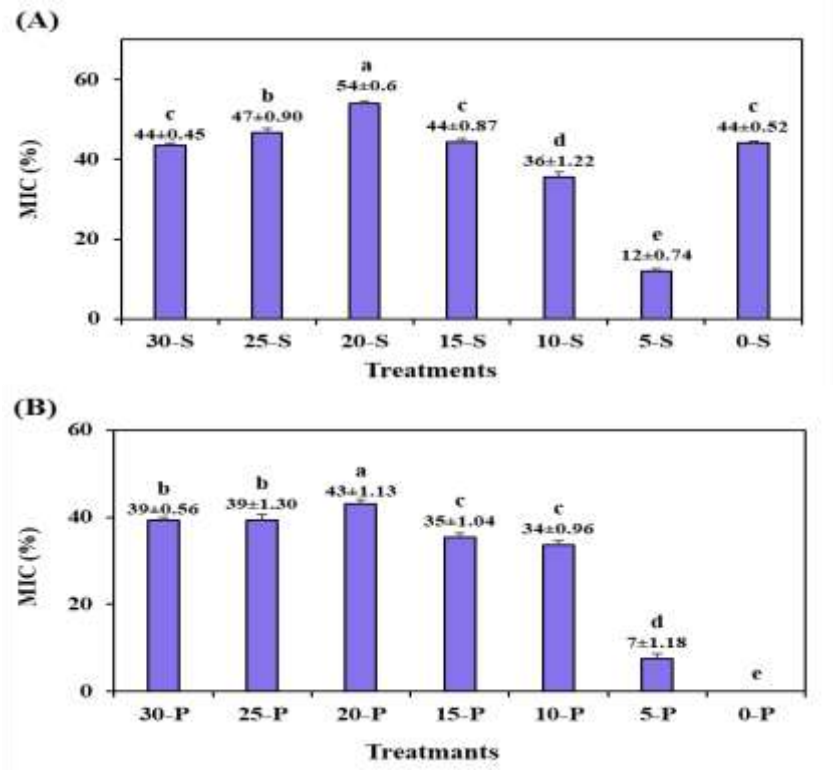

Figure 3: Comparison of the average antioxidant properties of postbiotics produced using ultrasound treatment at different times on the supernatant (A) and pellet (B) surfaces using the MIC test. Data values represent the mean ± standard error. Different lower-case letters above the columns indicate statistically significant differences between the treatments at a = 0.05 based on Duncan test (n=3). 0-P: Pellet 0 min (control), 5-P: Pellet 5 min, 15-P: Pellet 15 min, 20-P: Pellet 20 min, 25-P: Pellet 25 min, 30-P: Pellet 30 min 0-S: Supernatant 0 min (control), 5-S: Supernatant 5 min, 15-S: Supernatant 15 min, 20-S: Supernatant 20 min, 25-S: Supernatant 25 min, 30-S: Supernatant 30 min. The numbers above the columns indicate the mean ± standard error.

The control postbiotic at the supernatant level (0-S) exhibited the highest HRS activity, while the 5-minute treatment showed the lowest value. Furthermore, the HRS activity of the samples at the supernatant level at 20 and 25 minutes (20-S and 25-S) was not significantly different from that of 0-S. At the pellet level, the highest HRS activity was observed at 30 minutes (30-P). Moreover, the HRS activity of postbiotics prepared at 15 and 20 minutes at the pellet level (15-P and 20-P) was not significantly different from that of 30-P (Figure 4). Hydroxyl radicals readily and rapidly react with biomolecules such as DNA, lipids, and proteins, thereby causing severe oxidative damage to biomolecules within living cells, which can lead to cancer [32]. Hydroxyl radical scavenging activity (HRS) measures the most reactive radical, which is associated with lipid peroxidation and cellular biomolecules [23]. In this study, HRS activity of the pellet ranged from 26.5% (5-P) to 83.43% (corresponding to the 30-P sample). Additionally, there was no significant difference in HRS activity percentage among the 30-S, 20-S, and 15-S samples. HRS activity in the supernatant ranged from 36.44% (5-S) to 97.79% (0-S). Furthermore, the 0-S, 20-S, and 25-S samples showed no significant differences in HRS activity percentage. The HRS activity of the postbiotics in this study can be attributed to exopolysaccharides. It appears that the optimal hydroxyl radical

scavenging capacity of exopolysaccharides is due to the active transfer of hydrogen atoms from the hydroxyl groups present in the exopolysaccharides [29]. The HRS activity of postbiotics in this study was higher than the HRS activity results reported in the studies mentioned below. For example, the results of the study by Ageeli and Mohamed (2025) showed that the highest HRS activity percentages ranged between 65% and 78% [29]. Similar studies have reported HRS activity in exopolysaccharides from *L. helveticus* MB2-1, *L. plantarum* C88, and *L. plantarum* YW32, with inhibition percentages of 62.93%, 85.21%, and 77.5%, respectively [32, 35]. Notably, the samples produced at 5 minutes in all assays and at both pellet and supernatant levels (5-S and 5-P) exhibited the lowest antioxidant activity.

Overall, the results of the present study demonstrated the high antioxidant effects of the produced postbiotics, and differences in antioxidant activity among the postbiotics were also observable. The 20-minute ultrasound treatment exhibited the highest antioxidant properties compared to other treatment times, while the 5-minute treatment showed the lowest antioxidant properties among the various ultrasound treatment durations. This difference depends on mechanisms such as metal ion-chelating ability, the antioxidant enzyme system, and the antioxidant metabolites present in the postbiotics [35]. Furthermore, the results of this study indicated that the postbiotic production method can also have a direct impact on its antioxidant properties. The ultrasound method may induce chemical, mechanical, and biochemical effects due to the generation and collapse of cavitation bubbles [36]. Bacteria treated with relatively low-intensity ultrasound proliferate during the logarithmic phase. Moreover, this non-thermal technology increases extracellular enzymes, mainly beta-galactosidase, which can effectively extract antioxidants and bioactive compounds such as phenols, flavonoids, and anthocyanins. Accordingly, it can be expected that after applying ultrasound under appropriate conditions, better functional and physicochemical properties will be achieved in probiotic-based foods [37]. In some of the assay methods used in the present study, ultrasound had a reducing effect on the antioxidant properties of the postbiotics, which may be attributed to the induction of free radical formation that potentially reduces overall antioxidant activity [38, 39]. Additionally, increasing the frequency and duration of sonication may sometimes cause a slight reduction in soluble proteins, leading to changes in antioxidant capacity [39, 40]. In confirmation of this, Chávez-Alzaga et al. (2024) evaluated the effect of substrate treatments (whey, whole milk, and skim milk) and ultrasonic treatments (thermosonication and high-intensity ultrasound) on the antioxidant activity of water-soluble kefir postbiotics (WSKPs). They found that WSKP from whey without any processing exhibited higher antioxidant activity than ultrasonicated samples [21].

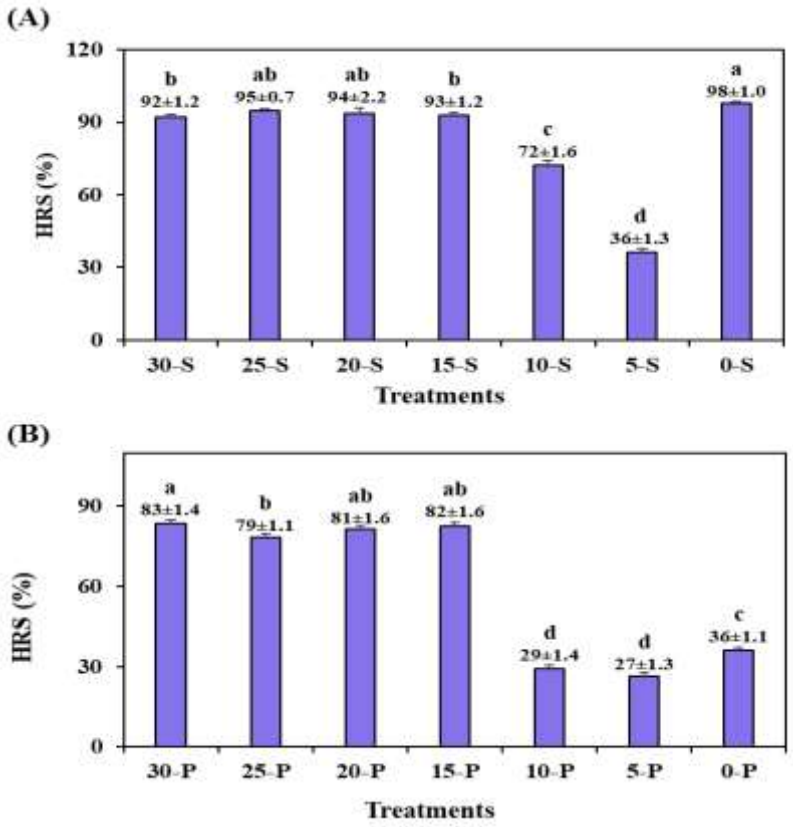

Figure 4: Comparison of the average antioxidant properties of postbiotics produced using ultrasound treatment at different times on the supernatant (A) and pellet (B) surfaces using the HRS test. Data values represent the mean ± standard error. Different lower-case letters above the columns indicate statistically significant differences between the treatments at a = 0.05 based on Duncan test (n=3). 0-P: Pellet 0 min (control), 5-P: Pellet 5 min, 15-P: Pellet 15 min, 20-P: Pellet 20 min, 25-P: Pellet 25 min, 30-P: Pellet 30 min 0-S: Supernatant 0 min (control), 5-S: Supernatant 5 min, 15-S: Supernatant 15 min, 20-S: Supernatant 20 min, 25-S: Supernatant 25 min, 30-S: Supernatant 30 min. The numbers above the columns indicate the mean ± standard error.

3-2- Comparison of Antioxidant Activity Between Postbiotics Produced at Pellet and Supernatant Levels

The results of the t-test showed that there was a significant difference between the antioxidant properties of postbiotics in the pellet and supernatant groups for the DPPH, MIC, and HRS methods, whereas no significant difference was observed between the antioxidant activity of the pellet and supernatant for the ABTS method (Table 3).

Table 3: T-test results of comparison antioxidant properties of postbiotics in the two pellet and supernatant groups

P-value	*t-value*	d.f	Method
0.000^{**}	-46.06	40	**DPPH**
0.763^{ns}	-0.30	40	**ABTS**
0.014^{*}	-3.18	40	**MIC**
0.003^{**}	-5.36	40	**HRS**

**: Indicates significance at the 1 percent level. *: Indicates significance at the 5 percent level. ns: Indicates no significance.

3-2- Comparison of Antioxidant Activity Between Postbiotics Produced at Pellet and Supernatant Levels

The results of the comparison of mean antioxidant activity between the two groups of postbiotics produced at the pellet and supernatant levels at 6 different time points using the t-test showed that the antioxidant activity of the supernatant was significantly higher than that of the pellet in the DPPH, MIC, and HRS methods. On the other hand, no significant difference was observed between the antioxidant activity of the pellet and supernatant samples in the ABTS method (Figure 5). This may be attributed to the metabolites produced by the postbiotics. In confirmation of these results, Çelik et al. (2024) showed that the DPPH activity of the cell-free supernatant of KC27L was 75.7% (approximately similar to the present study) [31]. The results of the present study are consistent with those of Almahbashi and Gunes Altuntas (2025), who compared three methods of postbiotic preparation (including cell-free supernatant, heat treatment, and enzymatic treatment with ultrasonication) using lactic acid bacteria *Lactiplantibacillus plantarum* F2, *Leuconostoc mesenteroides* AF1, and *Pediococcus pentosaceus* 50 [41]. They found that cell-free supernatant and heat treatment were the most effective methods. Various biologically active metabolites are produced during microbial growth, which can be separated from microbial cells by centrifugation and are rich in antioxidants, phenols, flavonoids, and antimicrobial and anticancer compounds [42]. This difference can also be attributed to changes in certain physicochemical properties, including molecular mass and the presence of specific functional groups [43]. Another reason why cell-free supernatant exhibits better biological effects is that various biomolecules act in cooperation with one another [44]. Nevertheless, the results of the present study differed from the observations of Sun et al. (2023), who found that the DPPH scavenging ability of the cell-free supernatant was significantly lower than that of heat-killed cells *of Lacticaseibacillus paracasei* and *Bifidobacterium lactis* [45].

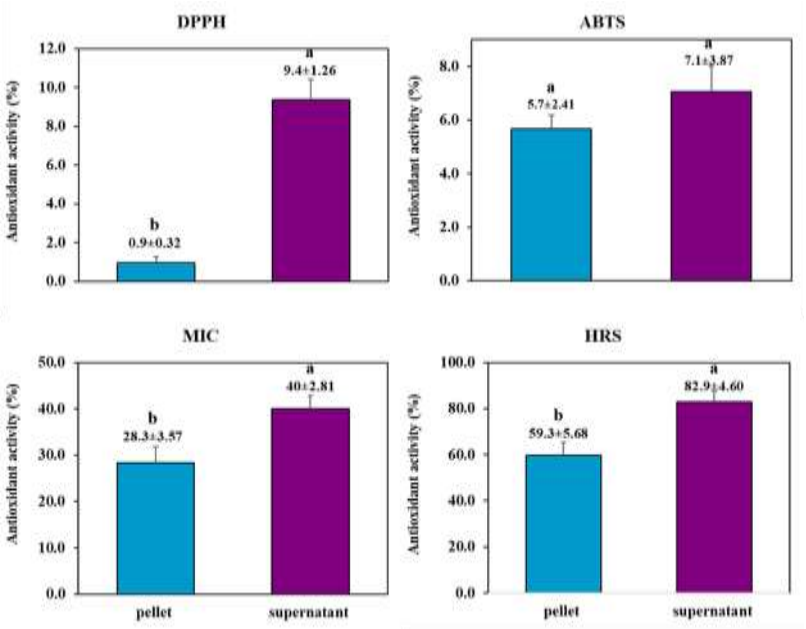

Figure 5: Comparison of the average antioxidant properties of postbiotics produced at the pellet and supernatant levels by different methods using the t-test. The numbers above the columns indicate the mean ± standard error.

4- Conclusion

This study provides useful information regarding the benefits of using postbiotics derived from *Enterococcus faecium*. In this research, we aimed to pave the way for the development of innovative and efficient natural therapeutics for various diseases by gaining a better understanding of the characteristics and potential health benefits of these postbiotics. According to the findings of this study, postbiotics derived from *Enterococcus faecium*, due to their significant antioxidant capacity, can exert multiple health-enhancing effects. Overall, it can be concluded that postbiotics prepared at the supernatant level exhibited higher antioxidant activity compared to those at the pellet level. Furthermore, the ultrasound treatment time was effective on the antioxidant properties of the postbiotics, with the 20-minute treatment having the greatest impact on their antioxidant activity. Therefore, it can be suggested that postbiotics produced by ultrasound should be increasingly utilized in human food, animal feed, and pharmaceutical sectors. Nevertheless, to further demonstrate the positive benefits of postbiotics, more scientific research on lactic acid bacteria is required, using established and modified methods for extraction, validation, characterization, and metabolomic and proteomic investigations to obtain postbiotics with significant efficacy.

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Author Contributions

All activities were carried out by the author.

Conflict of Interest

The author confirms that there are no financial conflicts of interest or competing interests in this study.

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مقاله علمی_پژوهشی

اثرات اولتراسوند بر خواص آنتی‌اکسیدانی پست‌بیوتیک‌های مشتق شده از پلت سلولی و سوپرناتانت کشت *Enterococcus faecium*

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چکیده

مطالعات فزاینده، پتانسیل بالای پست‌بیوتیک‌ها را در محافظت از سلامت میزبان برجسته کرده‌اند. در مقایسه با غذاهای فراسودمند سنتی، پست‌بیوتیک‌ها مزایایی مانند فعالیت فیزیولوژیکی بالا، ماندگاری طولانی، جذب آسان و غیره را دارند. این تحقیق به مطالعه اثرات تیمار اولتراسوند بر فعالیت آنتی‌اکسیدانی پست‌بیوتیک‌های مشتق شده از باکتری *Enterococcus faecium* پرداخته است. این باکتری، آلفا همولیتیک یا غیر همولیتیک و همزیست روده انسان است. میکروارگانیسم تحت ٦ عملیات زمانی فراصوت مختلف (۵، ۱۰، ۱۵، ۲۰، ۲۵ و ۳۰ دقیقه) قرار گرفت. آزمایش‌های ABTS، DPPH، MIC و HRS به‌منظور بررسی فعالیت آنتی‌اکسیدانی در سطح پلت و سوپرناتانت انجام شدند. نتایج تجزیه واریانس یک‌طرفه نشان داد که اثر اولتراسوند روی خواص آنتی‌اکسیدانی پست‌بیوتیک‌ها طی زمان‌های مختلف در سطوح پلت و سوپرناتانت معنی‌دار بوده است. پست‌بیوتیک‌ها در سطح سوپرناتانت فعالیت آنتی‌اکسیدانی بالاتری با DPPH در زمان ۲۰ دقیقه، ABTS کنترل و MIC در زمان ۲۰ دقیقه و HRS کنترل داشتند. در سطح پلت فعالیت آنتی‌اکسیدانی بالاتری با DPPH کنترل، ABTS کنترل و MIC در زمان ۲۰ دقیقه و HRS زمان ۳۰ دقیقه مشاهده شد. به‌علاوه، نمونه‌های تولید شده در زمان ۵ دقیقه در تمام آزمایشات و در هر دو سطح پلت و سوپرناتانت کمترین فعالیت آنتی‌اکسیدانی (۰ تا ۳٦ درصد) را نشان دادند و تنها در آزمایش MIC کنترل (۰ درصد) پایین‌ترین مقدار بود. خاصیت آنتی‌اکسیدانی پست‌بیوتیک‌های تولید شده در سطح سوپرناتانت در تمام آزمایش‌ها به جز ABTS نسبت به پلت بیشتر بود. پیشنهاد می‌شود که پست‌بیوتیک‌های استخراج شده از *E. faecium* به‌عنوان آنتی‌اکسیدان، در صنایع غذایی و دارویی مورد استفاده قرار گیرند.