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Original Article

Antibacterial effects of Persian shallot extract, *Lactobacillus acidophilus,* and sodium nitrite on *Listeria monocytogenes* and *Salmonella* Typhimurium

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Abstract

In recent years, the negative side effects of extensive use of chemical preservatives have become a major health concern. Therefore, it is necessary to find the safe and natural biological preservatives with health promotion effects. This study aimed to investigate the stimulatory effect of Persian shallot extract (PSE) on *Lactobacillus acidophilus* growth, and evaluate the individual and combined antibacterial effects of PSE, *L. acidophilus* and sodium nitrite against *Listeria monocytogenes* and *Salmonella* Typhimurium. The minimum inhibitory concentration values of PSE were 2, 4, and 8 mg/mL against *L. monocytogenes, S.* Typhimurium*, L. acidophilus,* respectively. The results revealed that PSE (0.05 mg/mL) significantly increased (up to 1.3 $\log_{10}$ CFU/mL) *L. acidophilus* growth compared to control group. The treatments of *L. acidophilus* and PSE as well as the treatment of *L. acidophilus* with nitrite and PSE prevent the growth of *L. monocytogenes* completely. Also, the combined treatment of *L. acidophilus* and PSE showed the greatest antibacterial effect against *S.* Typhimurium, and caused 4.27 $\log_{10}$ CFU/mL reduction compared to control treatment. However, the combined treatment of *L. acidophilus,* PSE and sodium nitrite caused only 0.92 $\log_{10}$ CFU/mL reduction in *S.* Typhimurium count. It can be concluded that PSE not only could increase the growth of *L. acidophilus* but also had strong antibacterial properties, and combined use of PSE, *L. acidophilus*, and sodium nitrite showed significant anti-listeria activity.

Keywords: Foodborne pathogen, Natural antibacterial, Probiotic, Shallot extract, Sodium nitrite.

Introduction

Humans are always looking for ways to preserve food from chemical changes, microbial deterioration, and the growth of foodborne pathogens. Different preservation methods such as cooling, drying, cooking, fermentation, and chemical preservatives have been used for many years (Moradi et al., 2019). However, the use of chemical preservatives has always been a controversial issue that does not completely fulfill the purpose (Slavov et al., 2019.); so that the food safety problems caused by the extensive use of chemical preservatives have become a major health concern (Wang et al., 2020). Considering the adverse side effects of chemical compounds, the demand for natural additives and functional probiotics has been increased greatly (Maghsoudi et al., 2018).

The Food Agriculture Organization (FAO) and World Health Organization (WHO) define probiotics as

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"Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Morelli et al., 2012). The most common and industrial probiotic microorganisms are lactic acid bacteria (LAB) (Cizeikiene et al., 2021). *Lactobacillus acidophilus* is one of the most popular probiotics that is used as a biological preservative in different types of foods (Kamali et al., 2024). Antimicrobial activity and production of bacteriocins by LAB can increase their capability to control foodborne pathogens and food spoilage microorganisms (Hossain et al., 2020). On the other hand, the inhibitory effects of probiotics, especially *L. acidophilus*, on foodborne pathogens such as *Salmonella* and *Listeria* have been confirmed previously (Fernández et al., 2003).

L. monocytogenes is a Gram-positive, foodborne pathogen that causes a potentially fatal disease called listeriosis (Masebe et al., 2022), and it mostly occurs due to consuming milk and dairy products (Mahmoudi et al., 2012). *Salmonella* Typhimurium, a Gram-negative bacillus, is one of the main causes of foodborne diseases (Maghsoudi et al., 2018). This bacterium is associated with disease outbreaks caused mainly by contaminated meat and eggs (Mnayer et al., 2014).

From many years ago, medicinal plants have been used for the treatment of different infections (Zeynali Aghdam et al., 2019). Plant essential oils and extracts with antimicrobial, anticancer, antioxidant, and free radical scavenging activity have a very high potential as new natural preservative in the protection of raw and processed foods (Imaz et al., 2023). One of the medicinal plants that is native to Iran and has different therapeutic applications is Persian shallot (*Allium hirtifolium*). Persian shallot has bulbs, short stems with meaty leaves. This plant has strong antioxidant and antibacterial activity due to its organosulfur and flavonoids compounds (Mehdizadeh et al., 2021; Vahdat et al., 2024).

Sodium nitrite is used to retard the oxidation of lipids and prevent the growth of microorganisms in meat products (Sindelar et al., 2012). The use of sodium nitrite in meat products induces the unique color, texture and flavor, and improves the food safety and extends the shelf-life (Shiravani et al., 2024). Due to potentially harmful effects of chemical compounds, researchers are trying to find the suitable, healthy and natural alternatives. In this study, we used PSE and *L. acidophilus* to reduce sodium nitrite. Therefore, the present study aimed to investigate the combined antibacterial effects of PSE, *L. acidophilus,* and sodium nitrite against *L. monocytogenes* and *S.* Typhimurium.

Materials and Methods

Preparation of Persian shallot extract (PSE)

Fresh shallots were purchased from the market of Urmia (Iran), peeled, sliced, and dried away from sunlight. The thin sheets were powdered using a grinder (Moulinex blender, France) and some shallot powder was mixed with distilled water at a ratio of 1:5, Then, the mixture was shaken for 24 h at room temperature (25 °C) using a shaker incubator (Memmert, Germany) at 125 rpm. After that, the resulting solution was filtered using a filter paper (Merck, Germany) and concentrated using a rotary device (Heidolph, Germany) at 45 °C for 3-5 hours (Mohammadiani et al., 2021). Then, the extract was dried using a freeze dryer (Zist Farayand Tajhiz Sahand, Tabriz, Iran; freezing temperature: – 50 °C), and stored at a refrigerator until use.

Preparation of PSE solution

To prepare PSE solution, 400 mg of PSE was dissolved in 2 mL of distilled water, and then sterilized by passing through a 0.45 µm filter (Jetbiofil, Malasia). Next, 1 mL of the solution was serially added to three tubes containing 1 mL of sterile distilled water. Then, the concentrations of 200, 100, 50 and 25 mg/mL were obtained.

Preparation of sodium nitrite solution

First, 1 g of sodium nitrite (Merck, Germany) was dissolved in 10 mL of sterile distilled water, and sterilized by passing through a 0.22 µm filter (Jetbiofil, Malasia). Then, this stock solution was serially diluted in a series of tubes to obtain different concentrations of 0.195, 0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, and 100 mg/mL (Karim et al., 2021).

Preparation of the bacterial suspension

L. monocytogenes ATCC 19115, *Salmonella* Typhimurium ATCC 14028, and *L. acidophilus* LA5 were obtained from Department of Food Hygiene

and Quality Control, Urmia University. The bacteria were sub-cultured two times consecutively before use. A characteristic colony of the bacteria was transferred from the plate count agar to 10 mL of brain heart infusion (BHI) broth (Merck, Germany), and incubated at 37 °C for 24 hours. It should be noted that the de Man, Rogosa and Sharpe (MRS) culture (Quelab, Canada) medium was used to sub-cultured *L. acidophilus* (Dogan et al., 2004).

Investigating the growth stimulatory effect of PSE on *L. acidophilus*

L. acidophilus was cultured in MRS broth and incubated at 37 °C for 24 hours, and then its count was adjusted to 10^6 CFU/mL using a spectrophotometer (Novaspec II, Pharmacia, Muttenz, Switzerland) at 600 nm. Then, 100 µL of *L. acidophilus* and 100 µL of PSE with final concentrations of 0.5, 1, 2, and 4 mg/mL were added in 4 tubes, each containing 4.8 mL of broth. After 24 h of incubation at 37 °C, the samples were diluted in 0.1% peptone water (Quelab, Canada), and cultured on MRS agar. After 48 h of incubation at 37 °C, the colonies were counted, and results were expressed as log CFU/mL (China et al., 2012).

Determining the minimum inhibitory concentration (MIC)

The MIC of PSE and sodium nitrite against *L. acidophilus*, *L. monocytogenes,* and *S.* Typhimurium were determined by microdilution method using the 96-well microplates (Sarstedt, Montreal, QC, Canada). The bacterial concentration was adjusted to 10^6 CFU/mL, and each well was filled with 95 µL of BHI broth, 100 µL of different concentrations of PSE (0.25- 16 mg/ml) or sodium nitrite (0.195-100 mg/mL), and 5 µL of the bacterial suspension. Some wells were considered as sterility control (broth containing PSE or sodium nitrite) and growth control (broth containing bacteria). The microplates were incubated at 37 °C for 24 hours. The MIC was determined visually, and the lowest concentration without turbidity was considered as MIC value (Güllüce et al., 2004).

Investigating the antibacterial effect of PSE against *L. monocytogenes* and *S.* Typhimurium

L. monocytogenes and *S.* Typhimurium were cultured in BHI broth, and after 24 hours of incubation, their counts were adjusted to 10^6 CFU/mL. Then, 100 µL of the bacteria and 100 µL of PSE with different concentrations (0, 0.5, 1, 2, and 4 mg/mL) were added into tubes containing 4.8 mL of broth. After 24 hours of incubation at 37 °C, the tubes were diluted in 0.1% peptone water, and cultured on the plate count agar (Sigma- Aldrich, USA) and the colonies were counted after 48 hours of incubation at 37 °C. Finally, the results were reported as $\log_{10}$ CFU/mL (Kim et al., 2011).

Investigating the combined effect of *L. acidophilus*, PSE and sodium nitrite against *L. monocytogenes* and *S.* Typhimurium

To perform this experiment, tubes containing 5 mL of BHI broth were used, and the effect of different treatments (**Table 1**) including the effect of *L. acidophilus*, *L. acidophilus* + PSE, *L. acidophilus* + sodium nitrite, and *L. acidophilus* + SPE + sodium nitrite on *L. monocytogenes* and *Salmonella* Typhimurium was investigated. After incubation at 37 °C for 24 h, the tubes were diluted in 0.1% peptone water and the desired dilutions were cultured on Palcam and XLD agar (liofilchem, Italy) for enumeration of *L. monocytogenes* and *S.* Typhimurium, respectively. Finally, the colonies were counted after 48 hours incubation at 37 °C, and the results were reported as $\log_{10}$ CFU/mL (Mahmoudzadeh et al., 2022).

Statistical Analysis

The statistical analysis of data was performed using SPSS software (version 18) (IBM Corporation, Armonk, NY, USA. Analysis of variance (ANOVA) procedure and Duncan's multiple range test were used to compare the means at significance level of $P<0.05$.

Results and Discussion

Growth stimulatory effect of PSE

The growth stimulatory effect of PSE on *L. acidophilus* is shown in **Table 2**. The concentration of 0.5 mg/mL had the greatest growth stimulatory

Table 1. The individual and combined antibacterial treatments *of L. acidophilus*, PSE, and sodium nitrite used to inhibit *L. monocytogenes* and *S.* Typhimurium.

***L. monocytogenes* treatments**	***S.* Typhimurium treatments**
L. acidophilus control (10^9) *	*L. acidophilus* control (10^9) *
L. monocytogenes control (10^7) *	*S.* Typhimurium control (10^7) *
L. acidophilus (10^9) * +*L. monocytogenes* (10^7) *	*L. acidophilus* (10^9) * + *S.* Typhimurium (10^7) *
L. acidophilus (10^9) * + PSE (2) ** + *L. monocytogenes* (10^7) *	*L. acidophilus* (10^9) * + PSE (2) ** + *S.* Typhimurium (10^7) *
L. acidophilus (10^9) * + sodium nitrite (0.1) ** + *L. monocytogenes* (10^7) *	*L. acidophilus* (10^9) * + sodium nitrite (0.1)**+ *S.* Typhimurium (10^7) *
L. acidophilus (10^9) * + PSE (2) ** + sodium nitrite (0.1) **+ *L. monocytogenes* (10^7) *	*L. acidophilus* (10^9) *+ PSE (2) ** + sodium nitrite (0.1)**+ *S.* Typhimurium (10^7) *

* Unit of measurement is CFU/mL; ** Unit of measurement is mg/mL.

effect on *L. acidophilus* compared to other concentrations, so that it significantly ($p<0.05$) increased *L. acidophilus* growth compared to the control.

Table 2. The growth stimulatory effect of PSE on *L. acidophilus*.

PSE (mg/mL)	Log_{10} CFU/mL
0	$8.96 \pm 0.04^{b,c}$
0.5	9.09 ± 0.05^{a}
1	8.99 ± 0.04^{b}
2	8.90 ± 0.04^{c}

Different small letters indicate significant statistically differences among the concentrations.

MIC of PSE and sodium nitrite

Table 3 shows the MIC values of PSE and sodium nitrite against *L. acidophilus*, *L. monocytogenes,* and *S.* Typhimurium. The MIC values of PSE were 2, 4, and 8 mg/mL against *L. monocytogenes, S.* Typhimurium, and *L. acidophilus,* respectively. The results indicated that *L. acidophilus* had the lowest susceptibility to PSE. Meanwhile, *L. monocytogenes* was the most sensitive bacterium to PSE, while *S.* Typhimurium showed the highest resistance to sodium nitrite (MIC=12.5 mg/mL).

Antibacterial activity of PSE, *L. acidophilus*, and sodium nitrite

The results of antibacterial effect of PSE against *L. monocytogenes* and *S.* Typhimurium are given in **Table 4**. PSE at concentration of 2 mg/mL was able to completely inhibit the growth of both bacteria.

Table 5 shows the individual and combined antibacterial effects of *L. acidophilus*, PSE and sodium nitrite against *L. monocytogenes* and *S.* Typhimurium. The results showed that the combination of *L. acidophilus* and PSE, as well as the combination of *L. acidophilus* along with PSE and sodium nitrite were able to prevent the growth of *L. monocytogenes* completely. In the case of *S.* Typhimurium, the combined treatment of *L. acidophilus* and PSE had the highest antibacterial effect and induced a 4.27 log_{10} cycles reduction compared to control treatment.

This study showed that PSE had a growth stimulatory effect on *L. acidophilus* (Moldovan et al., 2022). Various studies have been conducted in the field of shallot growth stimulatory effects on lactobacillus and probiotics. In this regard, a study evaluated the physicochemical and antioxidative properties of shallot probiotic yogurt containing inulin, and the results showed that shallot inulin concentration, and storage period had significant effects on the survival of *L. acidophilus.* At the end of the storage period, the highest and lowest number of probiotic bacteria was related to 1.5% inulin and 0.6% shallot treatment and 0.3% shallot treatment, respectively (Farahbaksh, 2021). Another study also investigated the effect of shallot on probiotic bacteria viability in low-fat stirred yogurt, and the results showed that on the first day, the number of probiotic bacteria in the sample containing 0.4% shallot was higher than the sample containing 0.3%, but with the increase of the storage period, the viability of these bacteria increases significantly in the samples without shallot compared to the samples containing it. They suggested that this result may be due to shallot's antimicrobial properties (Ramezani., 2019). Similarly, it was reported that the

water extract of shallot has a higher inhibitory effect than its ethanolic extract on the growth of *Lactobacillus bulgaricus* and *Streptococcus thermophiles*. However, both starter bacteria in shallot yogurt were alive and active up to 14 days of storage at refrigerator (Ashrafian, 2022). Plant species have several functional factors for probiotic growth. Functional components such as phenolic, antioxidants and minerals compounds enhance the bacterial growth. Meanwhile, some species of the probiotics are able to metabolize phenolic compounds (Alberto et al., 2001).

Table 3. The MIC (mg/mL) of PSE and sodium nitrite against *L. monocytogenes*, *S.* Typhimurium, and *L. acidophilus.*

Bacteria	PSE	Sodium nitrite
L. monocytogenes	2	3.12
***S.* Typhimurium**	4	12.5
L. acidophilus	8	6.25

The results of the antibacterial effect of PSE against *L. monocytogenes* and *S.* Typhimurium showed that all concentrations of PSE could inhibit the growth of *L. monocytogenes* and *S.* Typhimurium, and the concentration of 2 mg/mL was able to completely inhibit the growth of both tested bacteria.

The compounds found in shallot have inhibitory activity against a wide range of microorganisms, such as bacteria, viruses, fungi and parasites. Allium-derived antimicrobial compounds inhibit microorganisms by reacting with the sulfhydryl (SH) groups in target proteins (Moldovan et al., 2022). Shallot extract can inhibit both Gram-positive and Gram-negative bacteria (Moldovan et al., 2022).

Previous studies have shown that different shallot extracts have antibacterial activity against a variety of pathogenic bacteria (Mekvimol et al., 2021; Moldovan et al., 2022). A study examined *in vitro* antioxidant and antibacterial properties of shallot and scallion against four food-borne pathogenic bacteria, *L. monocytogenes*, *S*. Typhimurium, *E. coli* O157:H7, and *S. aureus*, and the results showed that 10 mM of shallot extract could decrease *L. monocytogenes* and *S*. Typhimurium count by 0.61 and 1.43 log, respectively (Yin et al., 2003). In another study, it was also shown that the crude aqueous extract of shallot, unlike onions and garlic after autoclaving, exhibited antimicrobial activity against *L. monocytogenes* and *S.* Typhi with a MIC in the range of 5 to 20 µg/mL (Moradi et al., 2013). In this regard, the studies by Negi et al. (2012) and Mnayer et al. (2014) showed the potential of shallot extract to inhibit *L. monocytogenes* and *S.* Typhimurium, respectively. However, it was reported that shallot extract has no inhibitory effect on *E. coli* strains compared to 12 other *Allium* species (Maidment et al., 2001). The studies conducted on the antibacterial properties of shallot showed that these effects are caused by organosulfur compounds such as diallyl disulfide and diallyl sulfide as well as other compounds such as Allicin, Ajoene and polyphenol derivatives (Mozin., 2015) The presence of a large amount of active phenolic compounds in shallots, including flavonoids, has antimicrobial activities that can inhibit the bacterial growth (Mekvimol et al., 2021). The quercetin is the main active substance of the flavonoids obtained from these plant extracts, which destroys the bacterial cell membrane by inhibiting the activity of extracellular proteins, and plays an important role in inhibiting Gram-positive and Gram-negative bacteria (Adam et al., 2021).

The present study showed that *L. monocytogenes* is more sensitive to both substances (PSE and sodium nitrite) compared to other two bacteria (Adam et al., 2021; Shan et al., 2007) The higher sensitivity of Gram-positive is due to the absence of a lipopolysaccharide cell wall, which may prevent active compounds from entering the cytoplasmic membrane (Bozin et al., 2007). On the other hand, the resistance of Gram-negative bacteria to antibacterial substances is related to the hydrophobic surface of the outer membrane, which is rich in lipopolysaccharide molecules which creates a barrier against the penetration of different antibiotic molecules, and is also associated with the enzymes of the periplasmic space, which are able to break the molecules introduced from outside (Mozin et al., 2015; Shan et al., 2007).

Table 4. The antibacterial effect of PSE against *L. monocytogenes* and *S.* Typhimurium.

PSE (mg/mL)	*L. monocytogenes* (Log_{10} CFU/mL)	*S.* Typhimurium (Log_{10} CFU/mL)
0	9.47 ± 0.06[a]	9.41 ± 0.02[a]
0.5	9.34 ± 0.03[b]	9.34 ± 0.03[b]
1	9.19 ± 0.05[c]	9.09 ± 0.06[c]
2	0 ± 0[d]	0 ± 0[d]

Different small letters indicate significant statistically differences among the concentrations.

The results of present study showed that the treatments of *L. acidophilus* and PSE and the treatment of *L. acidophilus* with sodium nitrite and PSE, compared to the treatments without PSE, prevent the growth of *L. monocytogenes* completely. Therefore, this complete inhibitory effect can be attributed to PSE. Also, the combined treatment of *L. acidophilus* and PSE had the greatest anti-*salmonella* effect, while the treatment containing *L. acidophilus* and PSE and sodium nitrite had less effect. It is possible that there is an antagonistic effect between nitrite and PSE compounds, because the presence of sodium nitrite reduced the effect of shallot (Amin et al., 2009; Zeynali Aghdam et al., 2019). A study showed that shallot by-products inhibited the growth of *E. coli* and *Salmonella* species, while increased the growth of *Lactobacillus* species in the digestive tract of chickens. (Abdollahzadeh et al., 2014; Raeisi et al., 2015; Mozin et al., 2015).

Conclusion

This study evaluated the growth stimulatory effect of PSE on *L. acidophilus,* and antibacterial activity of PSE, alone and in combination with sodium nitrite and *L. acidophilus,* against *L. monocytogenes* and *S.* Typhimurium. The results indicated the growth stimulatory effect of PSE. MIC results showed that *L. monocytogenes* was the most sensitive bacterium to PSE and sodium nitrite, while *S.* Typhimurium showed the highest resistance to sodium nitrite. The combined use of *L. acidophilus* and PSE completely inhibited *L. monocytogenes,* and induced a significant antibacterial effect against *S.* Typhimurium. Further studies are needed to investigate the antibacterial mechanism of action of PSE.

Acknowledgments

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Conflicts of Interest

The authors declared no conflict of interest.

Table 5. The individual and combined antibacterial effects of *L. acidophilus*, PSE, and sodium nitrite against *L. monocytogenes* and *S.* Typhimurium.

L. monocytogenes	Log CFU/mL	*S.* Typhimurium	Log CFU/mL
L. monocytogenes (control)	8.44 ± 0.04[b]	*S.* Typhimurium (control)	8.98 ± 0.02[a]
L. acidophilus (control)	8.98 ± 0.02[a]	*L. acidophilus* (control)	8.53 ± 0.05[c]
L. acidophilus + *L. monocytogenes*	7.90 ± 0.01[c]	*L. acidophilus* + *S.* Typhimurium	8.67 ± 0.03[b]
L. acidophilus + sodium nitrite + *L. monocytogenes*	7.71 ± 0.28[d]	*L. acidophilus* + sodium nitrite + *S.* Typhimurium	7.68 ± 0.01[e]
L. acidophilus + PSE + *L.* monocytogenes	0 ± 0[e]	*L. acidophilus* + PSE + *S.* Typhimurium	4.71 ± 0.01[f]
L. acidophilus + sodium nitrite + PSE + *L. monocytogenes*	0 ± 0[e]	*L. acidophilus* + sodium nitrite + PSE + *S.* Typhimurium	8.05 ± 0.02[d]

Different letters indicate significant statistically differences among the treatments.

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