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Original article

ANTIBACTERIAL ACTIVITY OF *ZIZIPHORA CLINOPODIOIDES* ESSENTIAL OIL AND NISIN AGAINST *BACILLUS SUBTILIS* AND *SALMONELLA* TYPHIMURIUM IN COMMERCIAL BARLEY SOUP

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Summary

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The objective of the current study was to evaluate the antibacterial activity of nisin (250 and 500 IU/mL), *Ziziphora clinopodioides* essential oil (0.1 and 0.2%) and their combination against *Bacillus subtilis* and *Salmonella* Typhimurium in commercial barley soup during refrigerated storage. Based on our findings, the population of pathogens was reduced with the addition of the essential oil and nisin, increased concentration of the added antibacterial agents and the longer storage time of commercial barley soup. The group treated with the essential oil at 0.2% and nisin at 500 IU/mL showed the most rapid decrease in the number of *S.* Typhimurium and *B. subtilis*. By the end of seven and five days, populations of *S.* Typhimurium and *B. subtilis* in overall concentrations of the essential oil with nisin were totally inhibited, respectively. Our findings suggest the possibility of utilising commercial barley soup with a mixture of *Z. clinopodioides* essential oil and nisin for the reduction of *B. subtilis* and *S.* Typhimurium.

Key words: antibacterial activity, *Bacillus subtilis*, barley soup, nisin, *Salmonella* Typhimurium, *Ziziphora clinopodioides* essential oil

INTRODUCTION

Barley soup is one of the most commonly consumed foods in many Mediterranean countries such as Iran. It is prepared by boiling different meat such as chicken, beef or lamb with barley, adding a variety of vegetables and a little salt (Moosavy *et al*., 2008). It is, due to special and different compounds such as onion, meat, carrot, parsley and barley, a rich source of high quality protein, vitamins and minerals and make appropriate medium for growth of food-borne pathogens and

spoilage microorganisms (Pajohi *et al.*, 2011). The major bacterial food-borne pathogens in soup include *Salmonella* spp., *Staphylococcus aureus,* some *Escherichia coli* especially *E. coli* O157:H7, *Bacillus cereus* and *Bacillus subtilis* (Siddiqua *et al.*, 2015). The genus *Salmonella* has caused a large proportion of food-borne outbreaks in different geographic areas. Some serovars like *S*. Typhimurium became a main cause of animal and human infection (Bajpai *et al.*, 2012). Some foods such as meat, unpasteurised milk and dairy products and commercial barley soup contaminated with *Salmonella* bacteria were found to play a great role in increasing food poisoning outbreaks in people, in particular those with weakened immune systems and children (Humphrey & Jorgensen, 2006; Bajpai *et al.*, 2012). On the other hands, *B. subtilis,* a rod shape spore forming Gram-positive bacterium, can produce emetic toxin and then cause intoxication in humans. The vehicles include meat, seafood dishes, commercial barley soup, rice and bread. The symptoms are vomiting, diarrhoea, abdominal pain, cramps, nausea, headaches with duration of 2–7 h (Riemann & Cliver, 2013).

A lot of studies have been done to make new natural preservation methods in an attempt to control food-borne pathogens while keeping a high organoleptical and nutritional quality of the food products (Devlieghere *et al.*, 2004). In this way, attempts have been focused on the potential applications of essential oils of various plants. *Ziziphora clinopodioides* is a plant belonging to the *Lamiaceae* family that widely distributed in Turkey, Iraq and west of Iran (Schulz *et al.*, 2005; Behravan *et al.*, 2007; Ozturk & Ercisli, 2007). This plant with a Persian name of Kakouti Kouhi is traditionally used to treat diarrhoea, intestinal gas, nausea and vomiting. In addition, it used as flavour ingredient in a wide variety of foods especially meat and soup (Behravan *et al.*, 2007; Shahbazi *et al.*, 2015a). The major constituents of the essential oil of this plant are thymol, carvacrol, *p*-cymene and limonene (Shahbazi *et al.*, 2015a,b).

To demonstrate the potential application of natural antimicrobial agents in food industries, they must be examined separately and in combination with other antimicrobial preservative agents such as nisin (Shahbazi *et al.*, 2015a). Nisin (E234) is classified as a class-Ia bacteriocin or lantibiotic and produced by strains of *Lactococcus lactis* subsp. *lactis* or *Streptococcus uberis.* This compound affects the wide spectrum of Gram-positive bacteria and some of Gram-negative bacteria by damaging the layer membrane of them (Govaris *et al.*, 2010). Therefore, the aim of the present study was to investigate the antibacterial activity of nisin, *Z. clinopodioides* essential oil and their combination against *B. subtilis* and *S.* Typhimurium in commercial barley soup during refrigerated storage.

MATERIALS AND METHODS

Plant material

Three samples of fresh leaves (200 g each) of *Z. clinopodioides* plant were gathered from same location at full flowering stage in March-July 2014 from Zagros Mountain ranges (Gilane Gharb city, Kermanshah province, west of Iran). Authentication of the plant was conducted by Dr. Seyed Mohammad Masoumi (Faculty of Agriculture, Razi University, Kermanshah, Iran) and a representative voucher specimen (No. 6816) has been placed in the herbarium of the Research Center of Natural Resources of Tehran, Iran.

Isolation of essential oil

The air-dried samples (100 g) of *Z. clinopodioides* leaves were hydro-distilled for 3.5 h in an all-glass Clevenger-type apparatus in accordance with the method described in the European Pharmacopoeia (Council of Europe, 1999). Heat was supplied to the heating mantle (50 ºC) and the essential oil was extracted with 500 mL of water for 3.5 h (until no more essential oil was recovered). The essential oil was collected, dried over anhydrous sodium sulfate (Na_2SO_4) (Merck, Darmstadt, Germany), stored in darkness in an amber vial and kept at low temperature (4±1 °C).

Preparation of nisin

Pure nisin powder was purchased from Sigma-Aldrich Company (UK). A stock suspension was prepared by dissolving appropriate amount of nisin in 0.02 M HCl, then the stock solution was centrifuged at 1500×*g* for 20 min, filtered by 0.22 µm pore filter (Sigma-Aldrich, USA) and kept at –20 °C until use (Shahbazi, 2015a).

Test microorganisms

Bacillus subtilis (ATCC 6633) and *Salmonella* Typhimurium (ATCC 14028) were obtained from the culture collection of the Iranian Research Organization for Science and Technology (IROST), Tehran, Iran. The cultures were stored frozen (–20 °C) in Brain Heart Infusion broth (BHI; Merck, Darmstadt, Germany) medium containing 17% glycerol and reactivated by subculture twice before testing. The optical densities of the 20 hour-old bacterial cultures were evaluated by using a spectrophotometer at 600 nm. A loopful of each stock culture was transferred to 10 mL BHI broth and incubated for 24 h at 37 °C. An inoculum with a population of pathogen cells of 5 log CFU/mL was used for the inoculation of soup samples.

Experimental design

Barley soup was prepared by adding commercial barley powder soup purchased from a market of Kermanshah city, west of Iran to the 400 mL distilled water

Table 1. Concentrations of *Z. clinopodioides* essential oil and nisin in the different batches of commercial barley soup

Batch	Concentrations (v/v)
Control	–
A	0.1% *Z. clinopodioides* essential oil
B	0.2% *Z. clinopodioides* essential oil
C	250 IU/mL nisin
D	500 IU/mL nisin
E	0.1% *Z. clinopodioides* essential oil+ 250 IU/mL nisin
F	0.1% *Z. clinopodioides* essential oil+ 500 IU/mL nisin
G	0.2% *Z. clinopodioides* essential oil+ 250 IU/mL nisin
H	0.2% *Z. clinopodioides* essential oil+ 500 IU/mL nisin

into 500 mL conical flask according to producer manual and then sterilised at 121 °C for 15 min. After cooling, *Z. clinopodioides* essential oil (0.1 and 0.2%) and nisin (250 and 500 IU/mL) separately and in combination were added. Therefore, for each experiment, nine batches (Batch A-Batch H) were designed as shown in Table 1. Then, approximately 5 log CFU/mL of *B. subtilis* or *S.* Typhimurium separately were inoculated. The samples were kept at 4±1 °C for 9 days. The samples were withdrawn at 2-day interval to check the level of contamination by plate method using plate count agar in triplicate. The colonies grown after 24 h of incubation at 37 °C were counted and count was expressed as log CFU/mL.

Statistical analysis

SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) software package was used for data analyses. Mean and standard deviations of each experiment were calculated and then were subjected to analysis of variance. Tukey's test and 2-way ANOVA at 95% confidence interval was used to determine mean differences among the treatments.

RESULTS

The results of the antimicrobial activity of *Z. clinopodioides* essential oil, nisin and their combination against *B. subtilis* and *S.* Typhimurium in commercial barley soup during storage at 4 ºC are shown in Table 2 and 3. During storage of the control sample at refrigerated temperature, *B. subtilis* and *S.* Typhimurium populations decreased steadily with storage time and reached values of 4.01 log CFU/mL and 3.78 log CFU/mL at 9 days, respectively. Based on our findings, the population of pathogens decreased with the addition of the essential oil and nisin, increased concentration of the added antibacterial agents and the longer storage time of commercial barley soup. With respect to *S.* Typhimurium, the groups treated with nisin at concentration of 250 and 500 IU/mL (Batches C and D) had insignificantly ($P>0.05$) higher microbial counts. The group treated with the essential oil at 0.2% and nisin at 500 IU/mL (Batch H) showed the most rapid decrease in the number of *S.* Typhimurium. A similar result was found about *B. subtilis.* In the case of *B. subtilis*, both concentrations of nisin, had inhibitory effect on the growth of this bacterium. By the end of seven and five days, populations of *S.* Typhimurium and *B. subtilis* in overall concentrations of the essential oil with nisin were totally inhibited, respectively. With respect to samples treated with the essential oil at 0.2% and nisin at 500 IU/mL (Batch H), the difference between this group and other groups was larger than that of samples treated with 0.2% essential oil and nisin at 250 IU/mL (Batch H). However, no significant differences were found ($P>0.05$). The antibacterial activity of the essential oil and nisin against growth of two selected pathogens was the most effective on *B. subtilis.*

DISCUSSION

In order to control and eliminate foodborne pathogens, various chemicals and synthetic compounds such as antibiotics as preservative have been used. In recent years, due to increasing concern of consumers related to safety of foods containing synthetic additives, many attempts have been made to replace these compounds with natural antimicrobial agents such as herbal extracts and essential oils and also

Table 2. Effect of *Z. clinopodioides* essential oil, nisin and their combination on *Salmonella* Typhimurium (log CFU/mL) in commercial barley soup stored at 4°C. Data are presented as mean± SD

Soup batch	Days of storage					
	0	1	3	5	7	9
Control	5.00±0.00	5.00±0.00	4.89±0.01	4.80±0.00	3.84±0.02	3.78±0.01
0.1% *Z. clinopodioides* essential oil	5.00±0.00	3.91+0.01	3.53±0.00	2.60±0.01	ND	ND
0.2% *Z. clinopodioides* essential oil	5.00±0.00	3.16±0.01	3.08±0.01	2.17±0.00	ND	ND
250 IU/mL nisin	5.00±0.00	4.87±0.01	4.00±0.00	3.06±0.00	3.88±0.01	1.44±0.01
500 IU/mL nisin	5.00±0.00	3.95±0.01	3.99±0.01	2.44±0.01	2.77±0.00	0.85±0.05
0.1% *Z. clinopodioides* essential oil+ 250 IU/mL nisin	5.00±0.00	3.00±0.00	2.03±0.00	0.99 ±0.05	ND	ND
0.1% *Z. clinopodioides* essential oil+ 500 IU/mL nisin	5.00±0.00	2.88±0.01	1.44±0.04	0.36±0.00	ND	ND
0.2% *Z. clinopodioides* essential oil+ 250 IU/mL nisin	5.00±0.00	2.41±0.01	1.10±0.01	ND	ND	ND
0.2% *Z. clinopodioides* essential oil+ 500 IU/mL nisin	5.00±0.00	2.30±0.01	0.49±0.01	ND	ND	ND

ND: not detected.

Table 3. Effect of *Z. Clinopodioides* essential oil, nisin and their combination in *Bacillus subtilis* (log CFU/mL) in commercial barley soup stored at 4°C. Data are presented as mean± SD

Soup batch	Days of storage					
	0	1	3	5	7	9
Control	5.00±0.00	4.84±0.01	4.86±0.00	4.63±0.01	4.30±0.01	4.01±0.01
0.1% *Z. clinopodioides* essential oil	5.00±0.00	3.80±0.01	1.88±0.01	0.48±0.02	ND	ND
0.2% *Z. clinopodioides* essential oil	5.00±0.00	3.78±0.00	1.63±0.00	0.19±0.03	ND	ND
250 IU/mL nisin	5.00±0.00	4.30±0.02	2.69±0.01	1.33±0.01	ND	ND
500 IU/mL nisin	5.00±0.00	4.32±0.04	2.30±0.01	1.01±0.03	ND	ND
0.1% *Z. clinopodioides* essential oil+ 250 IU/mL nisin	5.00±0.00	3.11±0.00	1.23±0.01	ND	ND	ND
0.1% *Z. clinopodioides* essential oil+ 500 IU/mL nisin	5.00±0.00	3.00±0.05	1.11±0.03	ND	ND	ND
0.2% *Z. clinopodioides* essential oil+ 250 IU/mL nisin	5.00±0.00	2.23±0.01	0.38±0.01	ND	ND	ND
0.2% *Z. clinopodioides* essential oil+ 500 IU/mL nisin	5.00±0.00	2.18±0.02	0.29 ±0.01	ND	ND	ND

ND: not detected.

several bacteriocins particularly nisin (Tajkarimi *et al.,* 2010; Shahbazi, 2015c). In the present study, the antibacterial activity of nisin, *Z. clinopodioides* essential oil and their combination against *B. subtilis* and *S.* Typhimurium in commercial barley soup were investigated during refrigerated storage.

As previously described, the antibacterial activity of the essential oil and nisin against both selected pathogens was the most effective on *B. subtilis*. The differences in the inhibitory effects between *S.* Typhimurium and *B. subtilis* are related to their different cell wall structures. Indeed, generally the cell wall of Gram-positive bacteria consists of a single layer, whereas the Gram-negative cell wall has a multilayered structure bounded by an outer cell membrane (Kim *et al.*, 2013). The antibacterial properties of *Z. clinopodioides* essential oil have been reported by other authors (Behravan *et al.*, 2007; Ozturk & Ercisli, 2007; Aghajani *et al.*, 2008). The most important reason of the strong antibacterial activities of carvacrol and thymol is the acidic nature of their hydroxyl groups and involvement in the formation of hydrogen bonds (Cavar *et al.*, 2008).

The results of the present study are in agreement with Moosavy *et al.* (2008) and Pajohi *et al.* (2011). Pajohi *et al.* (2011) found that the synergistic effect of the essential oil of *Cuminum cyminum* L. seed in combination with nisin at 0.25 µg/ml against *B. subtilis* in commercial barley soup. Moreover, Moosavy *et al.* (2008) reported significant inhibitory effect of *Zataria multiflora* Boiss. essential oil on *S.* Typhimurium and *S. aureus* in commercial barley soup. Except the described two earlier studies, several researches have reported the synergistic effect of nisin with various essential oils against common food-borne pathogenic bacteria such as *Listeria monocytogenes* (Yamazaki *et al.*, 2004, Shahbazi, 2015a), *E. coli* O157:H7 (Solomakos *et al.*, 2008; Saldaña *et al.*, 2012; Shahbazi *et al.*, 2015a), *B. cereus* (Periago & Moezelaar, 2001; Rajkovic *et al.*, 2005; Misaghi & Basti, 2007), *B. subtilis* (Rajkovic *et al.*, 2005) *Salmonella* spp. (Govaris et al., 2010; Saldaña *et al.*, 2012) and *S. aureus* (Shahbazi *et al.*, 2015a). It seems that essential oils enhance the effect of nisin by increasing the number of pores in the phospholipid bilayer membrane structure by nisin and also by increasing the size of the pores formed (Tajkarimi *et al.*, 2010; Shahbazi *et al.*, 2015b).

The results of the present study, for the first time, indicated that *Z. clinopodioides* essential oil in combination with nisin have inhibitory effects against *B. subtilis* and *S.* Typhimurium inoculated in commercial barley soup. Our findings suggest the possibility of utilising commercial barley soup with a mixture of *Z. clinopodioides* essential oil and nisin for the reduction of *B. subtilis* and *S.* Typhimurium.

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