Combined Antilisterial Effects of Barberry Extract, Cinnamaldehyde and Nisin

Soghra Valizadeh*a, Javad Aliakbarlu*a

*aDepartment of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

*Correspondence should be addressed to Ms Soghra Valizadeh. Email: soghravalizadeh@gmail.com

Background & Aims of the Study: Foodborne illnesses, resulting from consumption of contaminated foods, represent a substantial public health threat. Listeria monocytogenes is a foodborne pathogen bacteria and can cause serious problems in human. Because of this reason, Nowadays various compounds are being used to control these bacteria in foods. The aim of this study was to evaluate antilisterial potential of barberry extract, cinnamaldehyde (CA) and nisin, also to discover synergistic effects of dual combination of these compounds against Listeria monocytogenes in culture medium.

Material and methods: The antilisterial activity of barberry extract, cinnamaldehyde (CA) and nisin was evaluated using agar well diffusion method and minimal inhibitory concentration (MIC), also Combined antilisterial activity was examined by fractional inhibitory concentration (FIC) and time-kill assays.

Results: In agar well diffusion method, nisin (400 IU/ml) and CA (40 µl/ml) produced the largest inhibition zones (20.75±0.25 and 32.0±1, respectively). MIC values of nisin, CA and barberry extract were 25 IU/ml, 0.312 µl/ml and 37.5 mg/ml, respectively. In time kill assay, the combination of nisin with CA in MIC concentration was found the best combination against L. monocytogenes and a 6 log reduction in bacterial count was obtained.

Conclusion: The results of this study revealed that nisin and CA have convenient antilisterial activity and combination of these two compounds in MIC concentration showed synergistic effects against Listeria monocytogenes.

Background

Contaminated food can result in more than 200 human diseases, and many factors influence on the occurrence of foodborne diseases. For example, increased need for food due to increase in the world population, increased demand for eating out, and diversity in the microbial genomic and selection of resistant strains of pathogens resulted in the rate of foodborne diseases (1). Using antibacterial combination can result in a broader spectrum, prevention of the development of resistant mutants and synergistic antibacterial effect (2).

Listeria monocytogenes is considered as one of the most important food borne pathogens which is a causative agent of a serious and dangerous illness, listeriosis (3). The bacterium was isolated from various food products including raw vegetable, raw meat, dairy products and ready-to-eat meals (4). L. monocytogenes can grow at refrigeration temperatures, so ready-to-eat meals are important infection source for listeriosis and many outbreaks are caused by the consumption of these meals (5,6). Furthermore, the bacterium may result in severe problems in susceptible human hosts such as abortion and meningitis (5). Due to increasing trend of listeriosis in the last years, the development of...
new methods for the elimination of the bacterium from food products is necessary. Cinnamaldehyde (CA), a phenolic compound from cinnamon, has been studied for its antimicrobial activity (7-10). Antimicrobial activity of CA was reported against a wide range of microorganisms. Because of antibacterial activity and safety of CA, previous studies were suggested that CA may be applied in the food industry (11). Recently, Alves et al, investigate the antibacterial activities of four phenolic compounds, carvacrol, thymol, eugenol and cinnamaldehyde combined with nisin against Staphylococcus aureus and L. monocytogenes in cow milk and broth medium. A significant reduction in L. monocytogenes count was found compared with the control group (8). Another study indicated the synergistic interactions of nisin in combination with cinnamaldehyde against Staphylococcus aureus in pasteurized milk (9).

Antimicrobial peptides can promote elimination and reduction of microorganisms from food products and increase shelf-life of the products (12,13). Nisin is a bacteriocin compound and produced by Lactococcus lactis ssp. Lactis. Antimicrobial activity of nisin against L. monocytogenes was evaluated in previous studies (9,14). Application of nisin in the food industry as a preservative has been approved by the US Food and Drug Administration, also recognized a safe compound (15-17).

Spices have been applied as food additive since ancient times. In addition to improvement of organoleptic properties of food, spices can increase the shelf life of food by decreasing bacterial count and retarding lipid oxidation (18). Barberry (Berberis vulgaris L.) is extensively cultivated in Southern Khorasan Province (Northeast of Iran) and it is a popular condiment between Iranians (19). Previous works have proven the antioxidant and antibacterial properties of barberry extract (20, 21).

Aims of the study:

Antibacterial effects of nisin, CA and barberry extract have been separately reported in several studies. Then, the objective of present study was to investigate the combined antilisterial activity of CA, nisin and barberry extract in dual combinations to discover possible synergistic effects.

Materials & Methods

Chemical reagents:
Nisin and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich. Cinnamaldehyde (CA) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). BHI broth and agar were obtained from QUELAB (manufactured by European division, UK).

Preparation of water extract of barberry:
Barberry (Berberis vulgaris L.) fruit was purchased from a local market in Tabriz city. Ground fruits (100 g) were added to distilled water (1 L) and heated at 100°C for 60 min (21). The obtained extract was filtered through Whatman filter paper (Sigma-Aldrich, St. Louis, MO) and the filtrate was concentrated on a rotary evaporator (Heidolph, Laborata 4003, Schwabach, Germany) and then lyophilized. The lyophilized extract was placed in sealed bottles and stored at 4°C.

Bacterial culture:
Listeria monocytogenes (ATCC 19115) was obtained from the culture collection of Department of Food Hygiene and Quality Control, Faculty of Veterinary Medicine, Urmia University, Iran. The inoculum was prepared from the culture grown at 37 °C in BHI broth for 21 h.

Agar well diffusion method:
The antibacterial activity of nisin, CA and barberry extract on listeria monocytogenes was examined by agar well diffusion method (22). Barberry extract was dissolved in sterile distilled water to a final concentration of 600 mg/ml and sterilized by filtration through 0.45 mm membrane filter (Millipore, Bedford, MA). The
stock solution was used to prepare 500, 400 and 300 mg/ml of barberry extract. Stock of CA (40 µl/ml) was prepared by mixing 400 µl of pure compound with 600 µl of DMSO and then 9 mL of DMSO (10%) was added to the mixture to prepare a final concentration of 40 µl/ml. The stock solution of CA was used to provide other concentrations (30, 20 and 10 µl/ml). Stock nisin solution (105 IU/ml) was prepared by dissolving nisin in 0.02 mol/L HCl, which was then filter sterilized through a 0.22 µm pore filter (23). Stock nisin solution (400 IU/ml) was used to prepare other concentrations (300, 200 and 100 IU/ml). A 100 µl portion of bacterial suspension (106 cfu/ml) was spread over the surface of BHI agar plate and allowed to dry. The wells (6 mm in diameter) were cut from the agar with a sterile borer and 50 µL of each antibacterial solution was delivered into each well. Ampicillin (10µg/disk) and erythromycin (15µg/disk) disks used as positive controls. The plates were incubated for 24 h at 37°C. After incubation, the diameter of the inhibition zone was measured in mm (22).

**Determination of minimal inhibitory concentration (MIC):**

This method was performed according to the Clinical and Laboratory Standards Institute protocol (24) in 96 well micro-plates. Stock solutions of nisin, CA and barberry extract were 200 IU/ml, 2.5 µL/ml and 300 mg/ml, respectively. Different concentrations of nisin, CA and barberry extract were prepared by serial two fold dilution method. In each well, 95 µl of BHI broth, 100 µl of antibacterial compound and 5 µl of prepared bacterial suspension (106 cfu/ml) were added. The micro-plates were incubated for 24 h at 37°C. The MIC value of each compound was recorded after adding 50 µL of 0.01% resazurin to the respective wells and then assessing the color change. The bacterial growth was confirmed by color changes from blue to pink. MIC was defined as the lowest concentration of the compound that prevented resazurin color change from blue to pink.

**Determination of minimum bactericidal concentration (MBC):**

For Determination of MBC, The wells indicating complete absence of growth (blue wells) were identified and 5 µL of each well were spread on BHI agar plates. The plates were incubated for 24 h at 37°C. After incubation period, the lowest concentration with no growth (no colony) was defined as MBC (21). Assays were performed in triplicate.

**Determination of fractional inhibitory concentration (FIC):**

The fractional inhibitory concentration (FIC) of nisin and CA, nisin and barberry extract and CA and barberry extract against L. monocytogenes was determine using the checkerboard test. The method was performed using a 96 well microtiter plate with serial dilutions of the nisin, barberry extract and CA (25). The dilutions of compound A were prepared in horizontal rows while the dilutions of compound B were provided in vertical columns. The antimicrobial concentrations ranged from the upper of MIC value to seven serial twofold dilutions. The FIC index was calculated employing the minimal inhibitory concentrations (MIC) of the antimicrobial compounds alone and the respective MIC when the compounds were combined. The FIC index (FICI) was determined using the following equation:

\[ FICI = FIC_A + FIC_B \]

\[ FIC_A = \frac{MIC \text{ of } A \text{ in combination}}{MIC \text{ of } A \text{ alone}} \]

\[ FIC_B = \frac{MIC \text{ of } B \text{ in combination}}{MIC \text{ of } B \text{ alone}} \]

The interpretation of FICI was as follows: FICI \( \leq 0.5 \) indicated synergistic effect, FICI=0.5-2 represented additional effect, FICI=2-4 indicate indifference and FICI>4 represented antagonistic effect.

**Time-Kill synergy testing:**

The time-kill synergy assay was performed in test tubes containing an initial inoculum of 106 CFU/mL in BHI broth with a single or a combination of two compounds. Changes in
bacterial count during exposure to the antimicrobials were monitored. The bacterial counts were determined after predetermined time points (0, 2, 4, 6 and 24 h) of incubation by spreading 100 µl of appropriate dilutions on BHI agar plates. The plates were incubated at 37 °C for 24 h. The number of viable cells in each tube was estimated after counting bacterial colonies on plates and by multiplying by the appropriate dilution factor (26). In these experiments, all the compounds were used at MIC concentration. All experiments were conducted in duplicate, and mean values were calculated. The results were expressed as logarithms with corresponding standard errors (mean±SE). If bacterial count (CFU/ml) reduced by ≥2 log10 for the combination in comparison to the more active individual compound, as well as to the initial inoculum count, the interaction was regarded as synergistic (9).

Statistical analysis:
Statistical analyses were conducted using SPSS 18. One-way ANOVA and Tukey’s test were used to determine differences in the populations of *L. monocytogenes* in time kill synergy testing. Significant differences were considered at the 95% confidence level (P< 0.05).

Results

Agar well diffusion test was carried out to investigate the antilisterial activity of nisin, CA and barberry extract (Table 1). Barberry extract, CA and nisin with different concentrations displayed a variable degree of antibacterial activity. The inhibition zones of the highest concentration of nisin (400 IU/ml), CA (40 µl/ml) and barberry extract (600 mg/ml) were 20.75± 0.25, 32.0± 1 and 13±0.5, respectively. Among three compounds, barberry extract showed the weakest antilisterial activity (Table 1).

Table 2 shows MIC, MBC, and FIC values of CA, barberry extract and nisin against *L. monocytogenes*. In the present study, nisin and CA showed significant antilisterial activity and MIC value was 25 IU/ml and 0.312 µl/ml respectively.

In FIC assay, the FICI values were calculated to find the type of interaction between two compounds. The FICI values for combination of nisin and CA, nisin and barberry extract as well as barberry extract and CA against *L. monocytogenes* were 1, 2 and 2, respectively (Table 2).

The results of time kill assay are shown in Figures 1, 2 and 3. It was demonstrated that the combined antilisterial effect of nisin and CA was better than their individual activity and a 6 log reduction in bacterial count was obtained.
Figure 2) Time-kill synergy testing of barberry extract combined with nisin. Different small letters in each time indicate statistical significant difference (P<0.05) among treatments.

Figure 3) Time-kill synergy testing of nisin combined with cinnamaldehyde (CA). Different small letters in each time indicate statistical significant difference (P<0.05) among treatments.

**TABLE 1** Inhibitory zone (mm) of nisin (IU/ml), CA (µl/ml) and barberry extract (mg/ml) in agar well diffusion method.

| Compounds        | Concentrations | Nisin(IU/ml) | CA(µl/ml) | Barberry extract (mg/ml) |
|------------------|----------------|-------------|-----------|--------------------------|
|                  |                | 400 | 300 | 200 | 100 | 600 | 500 | 400 | 300 |
| Inhibitory zone  |                | 20.75± 0.25 | 19.25± 0.75 | 18.25± 0.25 | 15.5± 0.5 | 13±0.5 | 12±0.25 | 9±0.5 | - |

**TABLE 2.** Minimum inhibitory concentrations (MIC) and combined effects of nisin, CA and barberry extract against *Listeria monocytogenes*.

| Compounds                          | MIC | MBC | MIC in combination | MIC in combination | FIC | FICI |
|------------------------------------|-----|-----|--------------------|--------------------|-----|------|
|                                    | A   | B   | A                  | B                  |     |      |
| CA                                 | 0.312 | 37.5 | 1.25 | 150 | 0.312 | 37.5 | 1 | 1 | 2 |
| Barberry extract                    |     |     |                    |                    |     |      |
| Nisin                              | 37.5 | 25  | 150 | 200 | 37.5 | 25  | 1 | 1 | 2 |
| Erythromycin                       | 8   | -   |                    |                    |     |      |
| CA                                 |     |     |                    |                    |     |      |
| Barberry extract                    | 25  | 0.312 | 200 | 1.25 | 12.5 | 0.156 | 0.5 | 0.5 | 1 |
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Discussions

Studies suggested that the application of a combination of nisin and other antimicrobial compounds can enhance antibacterial activity and increase antibacterial spectrum of nisin (27). For this reason, in this study we investigate the combined antilisterial activity of CA, nisin and barberry extract in dual combinations to discover possible synergistic effects. Researchers investigated antibacterial activity of nisin and reported 125 µg/ml as MIC value of nisin against L. monocytogenes which is in agreement with the result of our study (8). The antibacterial effects of nisin were indicated against gram-positive food borne bacteria such as Staphylococcus aureus and L. monocytogenes (28). Antimicrobial effects of nisin on different strains of L. monocytogenes vary greatly (29,30). On the other hand, some cells can be found in sensitive populations of the bacteria that are resistant to certain concentrations of nisin, and survive and multiply in the presence of nisin (31). However, environmental conditions such as temperature and pH, composition and natural microbiota of food can influence antimicrobial effects of nisin (32).

For CA, the MIC value against L. monocytogenes was 0.312 µl/ml. In another study, MIC value of CA against L. monocytogenes was 65 µg/ml (8). Other researchers indicated that MIC value of CA against S. aureus was 0.31 mg/ml (33), and 1.56 µl/ml (~0.26 mg/ml) (11), which were similar to that calculated in the present study. It has been shown that reactive aldehyde groups in CA are able to create covalent cross-link between DNA and proteins through amine groups of Alyclobacillus acidoterrestris (34). It is also demonstrated that CA may cause a rapid decline in cellular levels of ATP in L. monocytogenes (8).

Similar to other compounds that were applied in this study, barberry showed antilisterial activity. The MIC of barberry extract against L. monocytogenes was 37.5 mg/ml in our study which is similar to finding of Aliakbarlu et al who reported MIC value of 36 mg/ml for barberry extract. They reported that among 10 examined extracts, barberry extract had the lowest MIC value after sumac extract (21).

Combination of CA with nisin resulted in decreased MIC values of both compounds. However, FICi values of other combinations indicated additional effects between CA and barberry extract and between barberry extract and nisin. Mechanistic interactions between two compounds are usually measured with the broth dilution checkerboard assay (35). In this assay, the FICi values were calculated to find the type of interaction between two compounds. The FICi values for combination of nisin and CA, nisin and barberry extract as well as barberry extract and CA against L. monocytogenes were 1, 2 and 2, respectively. Combination of CA with nisin resulted in decreased MIC values of both compounds. However, FICi values of other combinations indicated additional effects between CA and barberry extract as well as between barberry extract and nisin.

To evaluate and better understanding the interactions between nisin and CA, CA and barberry extract as well as barberry extract and nisin, we performed the time-kill assay. It was demonstrated that the combined antilisterial effect of nisin and CA was better than their individual activity. This combination was also better than combination of barberry extract and CA or barberry extract and nisin. The results of study shows a significant reduction (6 log) in L. monocytogenes count after 24 h incubation. Then, it can be concluded that there was a synergistic effect between nisin and CA. Inhibitory effects of nisin combined with carvacrol, thymol, eugenol and cinnamaldehyde against Staphylococcus aureus and L. monocytogenes has been reported. The synergistic effects were found against both bacteria assayed (8). The synergistic antibacterial effect of nisin and CA against...
Staphylococcus aureus has also been documented (9).

**Conclusion**

In conclusion, the results of this study showed that the combination of nisin and CA had synergistic antibacterial activity against L. monocytogenes. Then, in combination use of the antimicrobial compounds, lower concentrations are needed. The combination of nisin and CA should be examined against other foodborne pathogen bacteria.

**Footnotes**

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**Conflict of Interest:**
The authors declared no conflict of interest.

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