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The effect of nisin and garlic (*Allium sativum* L.) essential oil separately and in combination on the growth of *Listeria monocytogenes*

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ABSTRACT

In the present study, the anti-listerial activity of the nisin and garlic (*Allium sativum* L.) essential oils (GO) alone and in combination was investigated at different temperatures (20 and 30 °C), pH (6.8, 5.6 and 4.8) and NaCl concentrations (0, 0.5, 2.5 and 4.5 g/100 mL). Minimum inhibitory concentrations (MICs) against *Listeria monocytogenes* were assessed for the nisin and essential oil. Furthermore, for combinations of the antimicrobials, the Differences in Population (DP) method were used to determine their effect. Both essential oil and nisin possessed considerable antimicrobial effects on the microorganism. The MICs for nisin and GO were 12.5 IU/mL and 100 μg/mL, respectively. Regardless of NaCl concentration and temperature, the anti-listerial activity of both GO and nisin was strongly influenced by pH. Moreover, at the same temperature, and regardless of pH value, the growth of the organism was also affected by increasing NaCl concentration. In combination, the DP was related strongly to the agent's concentration and media pH. Meanwhile, among all combined systems, the effect of the combination (EC) of nisin with GO at 30 °C, pH 5.6 and 0 g/100 mL NaCl, showed significant anti-listerial activity ($P \leq 0.05$).

1. Introduction

Listeria monocytogenes is of concern to the food industry due to its high mortality rate and wide distribution in the environment (Valero, Carrasco, Pérez-Rodriguez, Garcia-Gimeno, & Zurera, 2006). It can be found in a wide variety of raw and processed foods. This Gram-positive pathogen has the ability to survive under a wide range of conditions such as refrigeration temperatures (2–4 °C), acidic pH and high salt concentrations (up to 10 g/100 g) in foods (Gandhi & Chikindas, 2007). Control of *L. monocytogenes* in various foods usually includes the application of 'hurdle' technology by combining factors such as low temperature, acidification, low a_w and chemical or natural preservatives (Boziaris, Skandamis, Anastasiadi, & Nychas, 2006; Hill, Cotter, Sleator, & Gahan, 2002; Valero et al., 2006).

In a constant attempt to improve food safety, "natural" materials have been sought that act as antibacterial, antioxidant, flavor and color enhancement agents for subsequent increase of organoleptic acceptability and shelf life of various foods. *Allium* is the largest and most important genus of the Alliaceae family and comprises 450 species, which were widely distributed in the northern hemisphere. Among them, onion (*Allium cepa* L.) and garlic (*Allium sativum* L.), are well known species being used in traditional medicine and food in many countries (Lanzotti, 2006). Garlic and its constituents have antimicrobial activity against some important foodborne pathogens (Benkeblia, 2004; Harris, Cottrell, Plummer, & Lloyd, 2001; Kumar, & Berwal, 1998; Sofia, Prasad, Vijay, & Srivastava, 2007; Yano, Satomi, & Oikawa, 2006; Yin & Cheng, 2003). It was indicated that the antibacterial activity of garlic resulted from thiosulfinates, particularly allicin, which is responsible for most of the antimicrobial activity, as well as its flavor and aroma (Amagase, Petesch, & Matsuura, 2001; Harris et al., 2001). Allicin is extremely unstable and further breaks down to produce some organosulfur compounds such as diallyl sulfide (DAS), diallyl disulfide (DADS), diallyl trisulfide, ajoenes, methyl allyl di- and trisulfides, vinyl dithiins, and other sulfur compounds, depending on how the garlic is prepared (Raghavan, 2006, pp. 113–116). Five types of garlic preparations are currently available on the market:

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garlic essential oils (GO), garlic oil macerate, garlic powder, aged garlic extract and fresh garlic (Helou & Harris, 2007). Among them, the antimicrobial activity of garlic oil (undiluted form) is 200 times greater than garlic powder and 900 times the strength of fresh garlic (Helou & Harris, 2007; Raghavan, 2006, pp. 113–116). Moreover, the oil and extract of garlic have been deemed generally recognized as safe (GRAS) for use in food by the FDA (Helou & Harris, 2007).

Nisin, a well known antimicrobial of the lantibiotic group, has GRAS status for use in food products in many countries (Boziaris & Nychas, 2006). This microbially-derived polypeptide has an inhibitory effect on Gram-positive bacteria. The anti-listerial properties of nisin have been well studied and applied in a variety of foods, including vegetables, dairy products and meats. However, some microorganisms may develop resistance to this compound and regrow under optimum conditions (Murdock, Cleveland, Matthews, & Chikindas, 2007).

In order to enhance antibacterial activity of nisin and expand its range of application, many approaches have been tried. Combination of nisin with other antibacterial substances is an alternative approach (He & Chen, 2006). Several studies have shown that anti-listerial activity of nisin can be enhanced by the lactoperoxidase system (Boussouel, 1999), lactoferrin (Murdock et al., 2007), thymol (Ettayebi, Yamani, & Rossi-Hassani, 2000), different plant essential oil (Pol & Smid, 1999; Solomakos, Govaris, Koidis, & Botsoglou, 2008; Yamazaki, Yamamoto, Kawai, & Inoue, 2004), carbon dioxide (Nilsson, Chen, Chikindas, Huss, Gram, & Montville, 2000), EDTA (Schved, Henis, & Juven, 1994), ethanol (Brewer, Adams, & Park, 2002), grape seed extract (Theivenran, Hettiarachy, & Johnson, 2006), garlic extract (Singh, Falahee, & Adams, 2001) and garlic shoot juice (Kim, Choi, Bajpai, & Kang, 2008). As well as the type of antimicrobial agents to be used, intrinsic and extrinsic factors such as pH, salt concentration and temperature need to be addressed when formulating a combination system (Razavi- Rohani & Griffiths, 1996). Therefore, the current study was performed to determine the effect of nisin and GO alone and in combination on *L. monocytogenes* at different temperatures (20 and 30 °C), pH (6.8, 5.8 and 4.8) and NaCl concentrations (0, 0.5, 2.5 and 4.5 g/100 mL) in brain heart infusion (BHI) broth.

2. Material and methods

2.1. Extraction of the essential oil

Garlic cloves (*Allium sativum L.)* were freshly purchased from local markets. The bulbs were pressed and squeezed by hand and homogenized in distilled water (1:5) and subjected to steam-distillation for 3 h using a Clevenger-type apparatus. Yellowish oil with an unpleasant strong odor was obtained (0.12 g oil/kg garlic bulb) and dried by adding anhydrous sodium sulfate, filter sterilized through a 0.22-μm filter and stored at 4 °C before being used.

2.2. Gas chromatography and mass spectrometry

The composition of the volatile fraction was determined using a Hewlett–Packard 6890 N gas chromatograph (Palo Alto, CA, USA) fitted with an HP-5MS column (30-m length × 0.25-mm i.d. × 0.25 μm film thickness). The column temperature program was adjusted as follows: initial temperature of the oven was 50 °C; holding at this temperature for 6 min; a final temperature of 240 °C; a heat gradient of 3 °C per min; temperature increased to 300 °C at a rate of 15 °C per min; and holding at this temperature for 3 min. Temperature of the injector port was 290 °C and the carrier gas was helium, with a flow rate of 1.5 mL/min. Column eluate was analyzed by mass spectrometry using a Hewlet-Packard 5973 N mass spectrometer (Palo Alto, CA, USA) with ionization voltage equal to 70 eV and in the EI-mode was used. The oven temperature was programmed from 50 °C to 265 °C at 2.5 °C/min. The temperature of the injector port was held at 250 °C, and the temperature of the detector was set at 250 °C. Constituents of the oil were identified and confirmed by comparing the experimental gas chromatographic retention indices and MS fragmentation pattern with those of the manufacturer's database (WILEY 2001 data software) and literature data.

2.3. Nisin preparation

A stock solution of nisin (10^4 IU/mL) was prepared by suspending 20 mg pure nisin (Sigma–Aldrich Darmstadt, Germany) in 1 mL of 0.02 mol/L HCl, (pH 2) (Elotmani & Assobhei, 2003). This solution was subsequently centrifuged at 1500xg for 20 min, sterilized by filtration through 0.22 μm filters and stored at 4 °C for up to four days.

2.4. Bacterial strain and preparation of working inoculums

L. monocytogenes ATCC 19118 was obtained from the culture collection of the Department of Food Hygiene and Quality Control at Urmia University. The bacterial culture was maintained at 4 °C on Brain Heart Infusion (BHI) agar and was reactivated by subculture twice before testing. For the evaluation of antibacterial activity, four or five well-isolated colonies were removed with a sterile wire loop and inoculated into a tube containing 10 mL of BHI broth and incubated at 35 °C for 20 h. The optical density of the 20 h old culture of the strain was determined spectrophotometrically at 600 nm and was standardized immediately prior to use to an OD_{600nm} of 0.15 (approximately 10^6 CFU/mL) using sterile BHI broth. Bacterial cells were enumerated by plating on BHI agar and counting viable cells after incubation for 24 h at 35 °C.

2.5. Determination of the minimum inhibitory concentration

The minimal inhibitory concentrations (MIC) of nisin and garlic essential oil (GO) were determined using a broth micro-dilution assay (Qaiyumi, 2007). Briefly, the GO was dissolved in dimethyl sulfoxide (DMSO) (10 g/100 mL) (Hayet et al., 2009; Sahin et al., 2004) and then in BHI broth to achieve a concentration of 6400 μg/mL. Serial twofold dilutions were made in a concentration range from 25 to 3200 μg/mL in sterile test tubes containing BHI broth. Geometric dilutions of nisin were also prepared from stock solution with concentrations of 3.12, 6.25, 12.5, 25, 50, 100, 200, 400 and 800 IU/mL. The 96-well sterile micro-dilution plates with U-bottom wells were prepared by dispensing into each well 160 μL of BHI broth and 20 μL of the bacterial inoculum. An aliquot (20 μL) of GO and nisin at the appropriate concentration was added into the wells. For every experiment two growth controls consisting of BHI broth without essential oil or nisin and BHI broth containing DMSO inoculated with the diluted medium culture and two sterility controls containing essential oil or nisin were run in each plate. The total volume in each well was 200 μL. Contents of each well were mixed on a plate shaker at 250 rpm for 20 s and incubated at 37 °C for 24 h. The lowest concentration of each agent showing visually no growth (by comparing with the first growth control) was taken as its minimal inhibitory concentration (MIC) and confirmed by plating 20 μL samples from clear wells onto BHI agar medium.

2.6. Garlic essential oil and nisin combination procedure

Two temperatures (20 and 30 °C), three pHs (6.8, 5.6 and 4.8), four NaCl concentrations (0, 0.5, 2.5 and 4.5 g/100 mL) along with