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Synergism between carvacrol or thymol increases the antimicrobial efficacy of soy sauce with no sensory impact

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ABSTRACT

Here, we examined the antimicrobial effects of soy sauce containing essential oils (EOs) against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* at 22 °C and 4 °C. To screen a variety of combined effects, soy sauce was mixed with six different EOs (carvacrol, thymol, eugenol, *trans*-cinnamaldehyde, β-resorcylic acid, and vanillin), each at a concentration of 1 mM for 10 min. None of the oils showed bactericidal activity when used alone. Soy sauce combined with carvacrol and thymol induced the greatest antibacterial activity against all tested bacteria; therefore, these oils were further tested at 0.25, 0.5, and 1 mM (0.0039%, 0.0078%, and 0.0157%) for 1, 5, and 10 min at 4 °C and 22 °C. In addition, sensory evaluation of soy sauce containing each EO at 0.25, 0.5, 1, and 2 mM was performed using the nine point hedonic test. Carvacrol or thymol (1 mM) eliminated all the test bacteria (initial population, 7.0–7.5 log CFU/ml) in 1–5 min at 22 °C and within 10 min at 4 °C. *L. monocytogenes* was slightly more tolerant at 4 °C, which may be attributable to the ability of the cell membrane to adapt to low temperatures. The sensory scores for soy sauce containing EOs were not significantly different from that of soy sauce without EOs ($P > 0.05$). The stability of EO efficacy in soy sauce was also verified. These results suggest that carvacrol and thymol act synergistically with other factors present in soy sauce to increase antimicrobial activity against major foodborne pathogens at both 4 °C and 22 °C. The synergism may be attributable to the combination of factors (mainly high salt concentration and low pH imparted by organic acids) present in soy sauce and the membrane attacking properties of carvacrol and thymol. This method will facilitate the production of microbiologically safe soy sauce, soy sauce-based marinades, and various marinated foods.

1. Introduction

Soy sauce, made by fermenting soy bean and wheat flour with molds or yeasts, is one of the popular condiments worldwide. Indeed, it is the third popular condiment in the USA (after mayonnaise and ketchup), with a market share worth 725 million dollars in 2014 (Ferdman and King, 2014). The soy sauce market in the USA has grown 40% since 2000 (Silva, 2014). In addition, because soy sauce contains much less sodium (307 mg per teaspoon) than salt (2325 mg per teaspoon), it is a potential salt replacement for those on a low sodium diet; this will only serve to increase market share even further (USDA, 2014a,b).

Soy sauce is often used to complement salads, meat products, sea foods, and fried rice (amongst other things). Because of its antimicrobial factors such as high concentration of salts (17%), low pH (4.6 by lactic acid), ethanol (2%), and preservatives (mainly sorbates or benzoates), soy sauce has been utilized to control food deterioration, and that is one of the reasons why soy sauce is used to preserve and season foods (Kataoka, 2005; Masuda et al., 1998). Unfortunately, contrary to expectation, foods containing soy sauce have caused several outbreaks of food poisoning (CDC, 1995, 1998; Mizuta et al., 1956). Furthermore, previous studies show that long exposure (6 h–7 days) to soy sauce merely inhibits the growth of foodborne pathogens such as *Escherichia coli*, *E. coli* O157:H7, *Salmonella* spp., *Shigella flexneri*, *Shigella sonnei*, and *Staphylococcus aureus*, rather than killing them or reducing their numbers to a safe limit, particularly at temperatures used for refrigeration (Kataoka, 2005; Masuda et al., 1998). The US Department of Agriculture (USDA) and the US Food and Drug Administration (USFDA) recommend that seasoned or marinated foods be stored at refrigeration temperatures; however, once soy sauce is contaminated with foodborne pathogens, it cannot inactivate the pathogens at these temperatures, and can even cross-contaminate foods seasoned with it (USDA, 2013; USFDA, 2014). However, combining soy sauce with other antimicrobial factors may improve the safety of various foods containing soy sauce.

There is a growing interest in "natural" food additives to guarantee food safety and to satisfy health conscious people (Kim and Rhee, 2013). Essential oils (EOs) are natural plant extracts that have antibacterial properties (Bakkali et al., 2008; Burt, 2004; Janssen et al., 1987;

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Kivanc and Akgäul, 1988). Although naturally occurring EOs are becoming popular with the food industry, there are several barriers to their use in foods; 1) they must be present at high concentrations to exhibit effective bactericidal activity (Burt, 2004; Gutierrez et al., 2008); 2) they are much more expensive than synthetic additives; 3) the strong smell of EOs is unacceptable from an organoleptic perspective (Angienda and Hill, 2011; Lambert et al., 2001). To overcome these barriers, hurdle technologies based on EOs have been developed, and several studies report the combined antibacterial effects of various EOs (Bevilacqua et al., 2010; Delaquis et al., 2002; Didry et al., 1993; Marino et al., 2001; Pei et al., 2009). Other studies report the combination of EOs with other food preservation methods, e.g., nisin, sodium chloride, sodium nitrite, chelators, organic acids, physical treatments, and fatty acids (Ait-Ouazzou et al., 2013; Choi et al., 2013; de Oliveira et al., 2010; Pol and Smid, 1999; Zhou et al., 2007). As mentioned above, studies show that the efficacy of EOs is increased when they are combined with other preservation methods (Angienda and Hill, 2011). Marination is such a method of food preservation; thus EOs may increase the antibacterial activity of marinades such as soy sauce.

The objectives of the present study were to improve the antimicrobial activity of soy sauce, thereby increasing the safety of soy sauce-based foods stored at both refrigeration and room temperatures. First, we screened six different EOs (carvacrol, thymol, eugenol, *trans*-cinnamaldehyde, β-resorcylic acid, and vanillin) and selected the most effective for further study in experiments with *E. coli* O157:H7, *S.* Typhimurium, and *Listeria monocytogenes*, which together cause the majority of foodborne diseases (CDC, 2013). Sensory changes (i.e., smell) were also evaluated.

2. Materials and methods

2.1. Bacterial strains

E. coli O157:H7 (ATCC 35150, 43890, and 43895), *S.* Typhimurium (ATCC 14028, 19585, and DT104 Killercow), and *L. monocytogenes* (ATCC 19111, 19115, and 19117) were obtained from the Korea University Food Microbiology Culture Collection. Stock cultures were stored at −20 °C in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, MD, USA) supplemented with 20% glycerol and sub-cultured every month.

2.2. Cell suspensions

Each strain was separately resuscitated in 10 ml fresh TSB (Difco) for 18 h at 37 °C. To obtain the three-strain cocktail, the culture suspensions of each bacterial strain were combined in a plastic 50-ml centrifuge tube (Difco), which was then centrifuged at 3000 rpm for 15 min (Centra-CL2; International Equipment Company, Needham Heights, MA, USA). After discarding the supernatant, the pellet was washed twice in 0.85% sterile saline solution. The final bacterial pellet was suspended in 10 ml of 0.85% sterile saline (approximately 9 log CFU/ml).

2.3. Antimicrobial efficacy of soy sauce containing essential oils

The antibacterial effects of soy sauce containing six different EOs were investigated. Soy sauce (Preservative-Free Kikkoman Soy Sauce, Kikkoman Corporation, Japan), was purchased from a local market and stored at room temperature. EO solutions (100 mM) were prepared immediately prior to use by dissolving the oil (carvacrol, thymol, eugenol, *trans*-cinnamaldehyde, β-resorcylic acid, or vanillin; Sigma-Aldrich, St. Louis, MO, USA) in 98% ethanol.

In case of the individual treatments, 9.9 ml of soy sauce was used, and an aliquot (0.1 ml) of each EO stock solution was added to 9.8 ml of 0.85% saline to yield a final concentration of 1 mM. For combined treatment, an aliquot (0.1 ml) of each EO stock solution was added to 9.8 ml of soy sauce. All samples were prepared in 22 °C incubator or in a 4 °C refrigerator prior to the addition of 0.1 ml of bacterial cell suspension. The microbial population in each soy sauce sample was then examined 10 min later. Soy sauce containing 1% ethanol was used as control. All experiments were repeated six times.

2.4. Antimicrobial efficacy of soy sauce containing carvacrol and thymol

Carvacrol and thymol were tested under different conditions: concentration, 0.25, 0.5, or 1 mM; time, 1, 5, or 10 min; temperature, 22 °C or 4 °C. To obtain the final concentrations of carvacrol and thymol in soy sauce, stock solutions of 25, 50, and 100 mM were prepared in 98% ethanol. An aliquot (0.1 ml) of each was then pipetted into 9.8 ml of soy sauce. The sauce samples were prepared in a 22 °C incubator or 4 °C refrigerator. Immediately after preparation, 0.1 ml of bacterial suspension was added. Soy sauce containing 1% ethanol was used as control. All the experiments were repeated six times.

2.5. Microbiological analysis

One ml of sample was serially diluted 10-fold in 9 ml of 0.85% saline until 10^{-4}. Next, an aliquot (0.1 ml) of each was plated in duplicate onto MacConkey Agar containing Sorbitol (SMAC; Difco (*E. coli* O157:H7)), Xylose-Lysine-Desoxycholate Agar (XLD; Difco (*S.* Typhimurium)), or Oxford Agar supplemented with *Listeria* selective supplement (Oxford; Difco (*L. monocytogenes*)). For samples containing low levels of bacteria, 0.2 ml of undiluted sample was spread onto five plates of each selective agar (detection limit, 1 CFU/ml). Colonies were counted after the plates were incubated at 37 °C for 18 h.

2.6. Validation of antimicrobial efficacy

The extent of recovery of injured cells (*E. coli* O157:H7, *S.* Typhimurium, and *L. monocytogenes*) was examined at the treatments where bacterial growth was not detected by plating method (Jang and Rhee, 2009; Rhee et al., 2003). One ml of treated sample was inoculated into 20 ml of TSB (Difco). Following incubating at 37 °C for 18 h, the enriched samples were streaked onto Tryptic Soy Agar (TSA; Difco). Whether the growth occurred was recorded as positive or negative, after the plates were incubated at 37 °C for 18 h. The experiments were performed in triplicate.

2.7. Stability of effects of carvacrol and thymol in soy sauce

To evaluate the stability of effects of carvacrol and thymol in soy sauce, the long time exposure test and the long time storage test were performed. (*1*) *Long time exposure test*. The experiment was performed as same as *2.4*, but the test time was 14 days (not 1, 5, 10 min). (*2*) *Long time storage test*. Soy sauce samples containing carvacrol and thymol made 14 days ago were used. The bacterial suspension was added in the stored samples, and the microbiological analysis was performed after 1, 5, 10 min.

2.8. Evaluation of pH

The pH of soy sauce and of soy sauce combined with EOs was measured at room temperature using a pH/Ion meter (S220 SevenCompact pH/Ion, Mettler-Toledo, Greifensee, Switzerland) calibrated with buffers at pH 4.0 and 7.0.

2.9. Sensory evaluation

The odor of soy sauce samples was evaluated by a panel of 30 untrained volunteers (13 males and 17 females) ranging in age from 19 to 30. Testing was based on the nine point hedonic test. Samples (10 ml) of soy sauce with or without EOs were placed in small paper cups. The panel members were then asked to sniff the samples and