รายงานการวิจัย

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เรื่อง

ผลของเครื่องเทศต่อการควบคุมเชื้อซาลโมเนลลาในสภาพที่มีกล้าเชื้อแบคทีเรีย แลคติก ในระหว่างการหมักเนื้อ

(Effect of spices on controlling *Salmonella* in the presence of starter culture during meat fermentation)

โดย

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บทคัดย่อ

การศึกษานี้ได้ทำการตรวจสอบฤทธิ์ต้านแบคทีเรียซาลโมเนลลาจำนวน 20 ซีโรไทป์ และเอนเทอโรแบคทีเรียชนิดอื่นอีก 5 ชนิดของสารสกัดจากเครื่องเทศ (ด้วยเอทธานอล) และน้ำมันหอมระเหยของเครื่องเทศไทย 14 ชนิดได้แก่ กระวาน อบเชย กานพลู ลูกผักชี ยี่หร่า กระเทียม ขิง กะเพรา ใบมะกรูด ผิวมะกรูด ตะไคร้ ดอกจันทน์ ลูกจันทน์ พริกไทยดำ พริกไทยขาว และขมิ้น ด้วยวิธี agar diffusion เพื่อการคัดเลือกในขั้นต้น ในจำนวนสารสกัดจากเครื่องเทศเหล่านี้ พบว่าสารสกัดจากเครื่องเทศ (ด้วยเอทธานอล) 9 ชนิดและน้ำมันหอมระเหยของเครื่องเทศ 11 ชนิด มีผลยับยั้งแบคทีเรียที่ทดสอบและได้ทำการคัดเลือกมาทดสอบหาความเข้มข้นต่ำสุดที่ให้ผลยับยั้งแบคทีเรีย (Minimum Inhibitory Concentration, MIC) ด้วยวิธี microbroth dilution ผลปรากฏว่าสารสกัดจากกานพลูด้วยเอทธานอลสามารถยับยั้งการเจริญของแบคทีเรียทั้งหมดที่ทดสอบได้ดีที่สุด น้ำมันกานพลูและน้ำมันยี่หร่ามีฤทธิ์ยับยั้งการเจริญของแบคทีเรียได้สูง โดยสามารถยับยั้งแบคทีเรียทุกสายพันธุ์ที่ทดสอบ มีค่า MIC เท่ากับ 4.2 ไมโครลิตรต่อมิลลิลิตร ส่วนน้ำมันกระวาน น้ำมันลูกผักชี และน้ำมันผิวมะกรูดก็มีฤทธิ์ยับยั้งแบคทีเรียสูงเช่นกัน โดยทั่วไปน้ำมันหอมระเหยของเครื่องเทศให้ผลยับยั้งแบคทีเรียได้ดีกว่าสารสกัดจากเครื่องเทศ (ด้วยเอทธานอล) เชื้อซาลโมเนลลาซีโรไทป์ที่ถูกยับยั้งด้วยสารสกัดจากเครื่องเทศได้ง่ายที่สุดคือ *Salmonella* Typhimurium ขณะที่เชื้อซาลโมเนลลาซีโรไทป์ที่ต้านทานการยับยั้งของสารสกัดจากเครื่องเทศได้ดีที่สุดคือ *S.* Derby และ *S.* Rissen ส่วนเชื้อ *Escherichia coli* เป็นเชื้อที่ถูกยับยั้งด้วยน้ำมันหอมระเหยของเครื่องเทศส่วนใหญ่ได้ง่ายกว่าเชื้อแบคทีเรียชนิดอื่นๆที่ไม่ใช่ซาลโมเนลลาที่นำมาทดสอบ

การศึกษาผลของน้ำมันหอมระเหยของเครื่องเทศ (น้ำมันกานพลู น้ำมันลูกผักชี และน้ำมันดอกจันทน์ ความเข้มข้น 0.02%) ต่อการควบคุมเชื้อ *Salmonella* Agona ในสภาพที่มีกล้าเชื้อแบคทีเรียแลคติก *Lactobacillus plantarum* และ *Pediococcus acidilactici* ในระหว่างการหมักแหนมเป็นเวลา 3 วัน ที่อุณหภูมิ 30°C พบว่า *S.* Agona ที่รอดชีวิตมีจำนวนลดลงในแหนมทุกทรีตเมนต์ในระหว่างการหมัก โดย *S.* Agona ในแหนมที่เติมน้ำมันเครื่องเทศทุกชนิดลดลงมากกว่า *S.* Agona ในแหนมชุดควบคุมหลังจากหมักนาน 3 วันแต่ไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติระหว่างจำนวน *S.* Agona ในแหนมที่เติมน้ำมันเครื่องเทศแต่ละชนิด ($P > 0.05$) เซลล์ของ *S.* Agona ยังคงเหลือรอดอยู่ในแหนมทุกทรีตเมนต์หลังจากหมักนาน 3 วัน โดยพบจำนวนเซลล์ที่รอดชีวิตอยู่ในช่วง 3.83-4.64 log CFU ต่อกรัมจากจำนวนเซลล์เริ่มต้น 6.0 log CFU ต่อกรัม

Abstract

Crude ethanolic extracts and essential oils of 14 Thai spices, including cardamom, cinnamon, clove, coriander, cumin, garlic, ginger, holy basil, kaffir lime's leaves and peels, lemongrass, mace, nutmeg, black and white pepper, and turmeric were examined for their antibacterial activity against 20 *Salmonella* serotypes and 5 other enterobacteria using disk diffusion method as preliminary screening. Of these, 9 crude ethanolic extracts and 11 essential oils showed antibacterial effects on the bacterial strains tested, and were selected to determine the minimum inhibitory concentration (MIC) using microbroth dilution test. The ethanolic extract of clove was the most inhibitory to the growth of all bacterial strains tested. Clove and cumin oils were potent inhibitors of bacterial growth, showing the broadest antibacterial activity against all test bacteria at the MIC of 4.2 μl/ml. The oils of cardamom, coriander, and kaffir lime's peels were also highly inhibitory. In general, inhibitory activity of spice oils was greater than that of their own ethanolic extracts. The most susceptible serotype of *Salmonella* to the ethanolic extracts and oils of spices was *S.* Typhimurium, while *S.* Derby and *S.* Rissen were the most resistant. *Escherichia coli* was more susceptible to most of spice oils than other non-salmonellae strains tested.

The effect of spice essential oils (0.02% of clove oil, coriander oil, and mace oil) on controlling the growth of *Salmonella* Agona in the presence of *Lactobacillus plantarum* and *Pediococcus acidilactici* as a starter culture was evaluated during nham fermentation for 3 days at 30°C. The survival number of *S.* Agona significantly declined during fermentation in all treatments. The number of viable *S.* Agona in nham with all types of essential oils decreased greater than those in control treatment after 3 day fermentation , but no significant differences ($P > 0.05$) were observed between the number of survivors in nham with each type of essential oil added. In all treatments, *S.* Agona cells in nham were not completely eliminated after 3 day fermentation, showing the survival number of 3.83-4.64 log CFU/g from the original number of 6.0 log CFU/g.

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1. Introduction

In recent years, food safety concerns have been focused on pathogens, such as *Salmonella* which is recognized as one of the leading causes of foodborne bacterial diseases. The problem of human salmonellosis following consumption of contaminated food has increased worldwide. Based on reports from 1973 to 1997, cases of salmonellosis other than typhoid have been reported almost each year. These outbreaks were epidermiologically linked to the consumption of several types of foods, including chocolate, egg drink, cuttlefish, mayonnaise, fruit soup, fresh fruits and vegetables, dairy products, and fermented meat products (D'Aoust, 1997; Moore, 2004; Sauer et al., 1997; Wallace et al., 2000). Fermented meat products, including salami, and Lebanon bologna have recently been linked with transmission of *Salmonella* (Pontello et al., 1998; Sauer et al., 1997). This pathogenic bacteria has also been isolated from Thai food products, such as, Thai fermented sausage, Thai fermented pork (nham), fermented fruits, papaya salad, fruit drink, ice cream, ground peanut, sugar-coated tamarind, and biscuit with filling (Nanasombat, 1996; Nanasombat et al., 2002). These reports indicate that *Salmonella* may adapt and survive in these products, and suggest the need to control *Salmonella*.

Traditional food preservation methods that inactivate foodborne pathogens include heating, chilling, freezing, drying, irradiation, fermentation, controlled atmosphere storage, and use of chemical preservatives. In addition, there are a number of novel preservation techniques being developed recently. These novel processes include ionizing radiation, microwave, ohmic heating, increased hydrostatic pressure, pulsed electric field, magnetic field, high-intensity light, uses of gases, natural antimicrobials, and biopreservatives (Sofos, 2002). Recently, the growing concern about safety of foods has led to the development of natural antimicrobials to control foodborne pathogens. Spice is one of the most commonly used natural antimicrobial agents in foods. Addition of spices in foods not only impart flavor and pungent stimuli, but also provide antioxidant and antimicrobial properties (Hirasa and Takemasa, 1998). Some researchers have studied the antibacterial activity of spice extracts against several types of bacteria (Alzoreky and Nakahara, 2002; Nevas et al., 2004). Natural antimicrobial compounds in spices were found to possess antimicrobial activity (Kim et al., 1995; Shelef, 1983). Gram-negative bacteria, including some species of enterobacteria are more

resistant to antimicrobial compounds in spices than gram-positive bacteria (Farag et al., 1989; Shelef et al., 1980).

Recent researches have been focused on the antimicrobial effects of spices in foods. Bagamboula et al.(2003) reported the reduction of *Shigella sonnei* in spaghetti sauce containing basil and thyme after 16 days of storage at 12°C. Nkanga and Uraih (1981) also described inhibitory effects of clove, red pepper, black pepper and onion on growth of *Staphylococcus aureus* in meat homogenate. Onion and black pepper were less inhibitory than clove and red pepper. Yuste and Fung (2004) reported that the addition of cinnamon and nisin in apple juice resulted in great inactivation of *S.* Typhimurium and *Escherichia coli* O157:H7. Although some researchers have studied the antibacterial effects of spices against *Salmonella*, very few serotypes of *Salmonella* have been tested, for example *S.* Typhimurium (Elgayyar et al., 2001; Kim et al., 1995; Lachowicz et al., 1998; Juven et al. 1994), *S.* Enteritidis (Tassou et al., 1995), *S.* Infantis (Alzoreky and Nakahara, 2002), and *S.* Anatum (Swetwiwathana et al., 1999). Antimicrobial activity of spices may differ with strains within the same genera of bacteria. The sensitivity of each serotype of *Salmonella* to different types of spices has not been reported. Although some spices have been shown to inhibit growth of *Salmonella*, not all of them may inhibit the growth of all serotypes, and may show different degrees of inhibition to different serotypes of *Salmonella*. In addition, the antimicrobial property of spices may differ depending on the forms of spices added, such as fresh, or dried, or extracted forms of spices. To be able to use spice extracts to control *Salmonella* in foods, the antibacterial effects of crude ethanolic extracts and essential oils of spices against several serotypes of *Salmonella* need to be investigated. Therefore, the aim of this study is to select some spice extracts with strong anti-*Salmonella* activity for use in Thai fermented pork.

2. Materials and methods

2.1 Bacterial strains

Twenty seven bacterial strains (20 of *Salmonella* serotypes, 5 of other enterobateria and 2 of lactic acid bacteria) were used in this study. Thirteen serotypes of *Salmonella*, including *S.* Typhimurium

(DMST 0562), *S.* Newport (DMST 7101), *S.* Choleraesuis ssp. Choleraesuis (DMST 8014), *S.* Anatum (DMST 7108), *S.* Virchow (DMST 10635), *S.* Weltevreden (DMST 10637), *S.* Agona (DMST 10338), *S.* London (DMST 4110), *S.* Lexington (DMST 4112), *S.* Rissen (DMST 7097), *S.* Derby (DMST 8535), *S.* Enteritidis (DMST 10633), *S.* Senftenberg (DMST 7113), and 6 species of other enterobacteria, such as *Citrobacter freundii* (DMST 1959), *Enterobacter aerogenes* (DMST 8841), *Escherichia coli* (DMST 4212), *Serratia marcescens* (DMST 4228), *Klebsiella pneumoniae* (DMST 8216), *Proteus vulgaris* (DMST 0557) were obtained from the culture collection of the Department of Medical Sciences, Ministry of Public Health, Thailand. An antibiotic- resistant strain of *Salmonella*, *S.* Typhimurium DT104 (8748A-1) was obtained from the Center of Food Safety, The University of Georgia, Griffin. Six serotypes of *Salmonella* used in this study are *S.* Panama (SAP 08904/02), isolated from nham (Thai fermented pork), *S.* Stanley (SAP 0896/02), *S.* Orion (SAP 08991/02), *S.* Amsterdam (SAP 08913/02), *S.* Schwarzengrund (SAP 08906/02), and *S.* Hardar (SAP 08907/02), isolated from fresh pork. Two species of lactic acid bacteria, *Lactobacillus plantarum* TISTR 050 and *Pediococcus acidilactici* TISTR 051 were obtained from Microbiological Resources Centre for Southeast Asian Region (Bangkok MIRCEN). Enterobacterial cultures were maintained on Nutrient Agar (NA) slopes (pH 6.8, Difco Laboratories). Lactic acid bacterial cultures were maintained on deMan Rogosa Sharpe Medium (MRS, Difco, pH 6.5±0.2). They were subcultured from slants and subsequently stored at $4^{\circ}C$.

2.2 Antibacterial activity testing of crude ethanolic extracts and essential oils of Thai Spices against Salmonellae and other enterobacteria

2.2.1 Culture preparation

A loopful of 24 h surface growth on a NA slope of each bacterial strain was transferred individually to 5 ml of Brain Heart Infusion (BHI) broth (pH 7.6, Difco). After incubation at $37^{\circ}C$ for 24 h, bacterial cells were collected by centrifugation at 3000 rpm for 15 min, washed twice and resuspended in 0.1% peptone water. Turbidity was adjusted to match that of a 5 McFarland standard (10^8 CFU/ml). Then, a 1:10 dilution of the cell suspension was performed to give an inoculum concentration of 10^7 CFU/ml.

2.2.2 Extractions of spices

Fourteen types of spices, including cardamom, cinnamon, clove, coriander, cumin, garlic, ginger, holy basil, kaffir lime's leaves and peels, lemongrass, mace, nutmeg, pepper (black and white), and turmeric were purchased at retail in Bangkok, Petchaburi and Burirum, Thailand. These spices were extracted by two methods: 1) ethanolic extraction and 2) steam distillation using the procedure outlined below.

Table 1 List of spices and their edible parts

Spices	Botanical name	Plant parts
Cardamom	*Amomum krervanh* Pierre	Seeds
Cinnamon	*Cinnamomum verum* J.S.Presel	Barks
Clove	*Eugenia caryophyllus* (Sprengel) Bullock & Harrison	Flower buds
Coriander	*Coriandrum sativum*	Fruits
Cumin	*Cuminum cyminum* Linn.	Seeds
Garlic	*Allium sativum* Linn.	Bulbs
Ginger	*Zingiber officinale* Vern. Adrak	Rhizomes
Holy basil	*Ocimum sanctum* Linn.	Leaves
Kaffir lime	*Citrus hystrix* DC.	Leaves and Fruits
Lemongrass	*Cymbopogon citratus* (DC.) Stapf.	Rhizomes
Mace	*Myristica fragrans* Houtt.	Seed coat
Nutmeg	*Myristica fragrans* Houtt.	Fruits
Pepper, black and white	*Piper nigrum* Linn.	Fruits
Turmeric	*Curcuma longa* Linn.	Rhizomes

2.2.2.1 Preparation of crude ethanolic extracts

The spice materials were cut into small pieces and 20 g of each was soaked in 100 ml of 95% ethanol and shaked at 150 rpm for 4 days at ambient temperature. The mixtures were then filtered using cheese cloth. The filtrates were evaporated using vacuum rotary evaporator (BÜCHI Rotavapor

R-200/205, Model R205V800) and frozen at -80°C before drying in freeze drier (Labconco, Model Lyph. Lock 6). Stock solutions of crude ethanolic extracts were prepared by diluting the dried extracts with 10% dimethyl sulphoxide (DMSO) solution to get a final concentration of 400 mg/ml.

2.2.2.2 Preparation of essential oils

The small pieces of each spice material (300 g) were placed in a flask (2 L) together with distilled water (1 L). After steam distillation, the 100% pure essential oils were collected, dispensed into dark bottles and stored at 4°C until used. The stock solutions of crude ethanolic extracts and essential oils were used in disk diffusion test and microbroth dilution test.

2.2.3 Screening of spice extracts using disk diffusion technique

The disk diffusion test was performed using the standard procedure as described by Jorgensen et al.(1999). The inoculum suspensions were swabbed on the entire surface of Mueller-Hinton agar (MHA, pH 7.3 ± 0.1, Difco). Sterile 6-mm filter paper discs (Schleicher & Schuell) were aseptically placed on MHA surfaces and immediately added with crude ethanolic extracts and essential oils at the volume of 20 µl and 15 µl, respectively. A 20-µl aliquot of 10% DMSO was also added to a sterile paper disc as a negative control, whereas a disc containing known amount of amoxycillin (10 µg/disc) was placed in one quadrant of the plate as a positive control. The plates were left at ambient temperature for 15 min to allow excess prediffusion of extracts prior to incubation at 37°C for 24 h. Diameters of inhibition zones were measured, and values of less than 6 mm were considered as nonactive extracts against the test bacteria. The experiment was done in duplicate.

2.2.4 Determination of the minimum inhibitory concentration using microbroth dilution test

The dilution test was performed to determine minimum inhibitory concentrations (MICs) using the standard procedure as described by Jorgensen et al.(1999). One hundred microliter of Mueller-Hinton broth (MHB) was added in each well of a microtiter plate. The 100-µl aliquot of stock solution of crude ethanolic extract (400 mg/ml) was added and subsequently two-fold serially diluted with MHB. The inoculum suspenions (20 µl) of the test organisms were then added in each well containing crude ethanolic extract and MHB. The final concentrations of the extract in each well were 181.8, 90.9, 45.5, 22.7, 11.4, 5.7, and 2.8 mg/ml. The antibacterial activity testing of essential oils were

performed using similar procedure. The different amounts of the oils (8, 6, 4, 2, 1, and 0.5 μl) was added to the broth cultures (120 μl) to get oil concentrations of 62.5, 47.6, 32.3, 16.4, 8.3, and 4.2 μl/ml. Negative and positive controls were also performed using 10% DMSO (without spice extracts) and penicillin G (starting with 50,000 unit/ml concentration and ten-fold serially diluted to 0.005 unit/ml), respectively. Duplicate wells were run for each spice extract or oil at each concentration. The plates were incubated at 37°C for 24 h, and the turbidity was measured at 620 nm using the microplate reader (iEMS Reader MF, Labsystems). The lowest concentration that inhibited visible growth of the test organisms was recorded as the MIC. Each experiment was done in duplicate.

2.3 Effects of spice essential oils on controlling of *Salmonella* Agona in the presence of starter culture during nham fermentation

In this study, survival of *S.* Agona (DMST 10338) in nham (Thai fermented pork) with and without spice essential oils during fermentation at 30°C in the presence of mixed starter culture of *L. plantarum* TISTR 050 and *P. acidilactici* TISTR 051 was studied .

2.3.1 *S.* Agona inoculum preparation

To prepare *S.* Agona inoculum, a loopful of 24 h surface growth of *S.* Agona in BHI slant was transferred to 10-ml BHI broth and subsequently incubated at 37°C for 24 h. Cells were centrifuged at 3000 ×*g* for 15 min, washed twice and resuspended in 0.1% peptone water. The turbidity was adjusted to match that of a 5 McFarland standard (10^8 CFU/ml). Then, the suspension was ready for inoculation in nham.

2.3.2 Starter culture preparation

A loopful of 24 h surface growth of each culture of *L. plantarum* TISTR 050 and *P. acidilactici* TISTR 051 on a MRS slope was individually transferred to 10 ml MRS broth and incubated at 37°C for 24 h. Cells were then collected by centrifugation at 3000 ×*g* for 15 min., washed twice with 0.1%

peptone water, and resuspended in 10 ml of the same solution. Sterile rice flour (40 g) in plastic bag was added with *L. plantarum* and *P. acidilactici* cell supension (2 ml each) and thoroughly mixed.

2.3.3 Inoculation and processing of nham mix

Fresh pork (57.1%), Pork skin (30.7%), cooked rice (5.3%), fresh garlic (4.4%), salt (2.2%), sodium tripolyphosphate (0.26%), ascorbic acid (0.05%), sodium nitrite (0.01%), sodium nitrate (0.04%) and the mixed starter culture (0.18%, *L. plantarum* TISTR 050 and *P. acidilactici* TISTR 051 in the ratio of 1:1) were mixed together, and split into four batches. Clove oil, coriander oil, and mace oil in the concentration of 0.02% were added in the first, the second and the third batch, respectively. The fourth batch was used as a negative control (without addition of essential oil). The cell suspension of *S.* Agona was inoculated into nham mix using the procedure as described by Jung and Beuchat (1999). Inoculated and uninoculated samples were tightly packed into sterile plastic bags. All sample bags were placed in the incubator at 30°C to ferment the products for three days. Viable cells of *S.* Agona in each sample were enumerated at 0, 1, and 3 days of fermentation by spread plating onto Xylose Lysine Deoxycholate (XLD) agar (pH 7.4±0.2 , Difco) and pH of the samples was also measured.

2.3.4 Statistical analysis

Data from two replications were analysed by using analysis of variance to determine if significant differences ($P \leq 0.05$) existed between mean values and using Duncan multiple range test to compare between treatment means (SAS Institute, 1990).

3. Results

3.1 Preliminary screening of spice extracts

The results of the inhibition zones of 20 *Salmonella* serotypes and 5 other enterobacteria indicated that crude ethanolic extracts of cardamom, cinnamon, clove, cumin, kaffir lime's leaves, kaffir lime's peels and lemongrass showed various degrees of inhibition, depending on the bacterial strains (Table

2). Clove, cumin, and kaffir lime's peel extracts showed the broadest antibacterial activity by inhibiting growth of all bacterial strains tested (the inhibition zone of 8-22 mm), while cardamom, cinnamon and kaffir lime's leave extracts inhibited the growth of almost all strains (7-12 mm), except *S.* Typhimurium, *S.* London and *S. marcescens*. Lemongrass extract was active against only 17 strains (7-11 mm). Of all spices tested, crude ethanolic extract of clove showed the highest antibacterial activity against some strains, such as *S.* Typhimurium (15 mm) and *S. marcescens* (22 mm). However, coriander, garlic, ginger, holy basil, mace, nutmeg, black pepper, white pepper and turmeric ethanolic extracts were inactive against all bacterial strains tested.

Essential oils of cardamom, clove, coriander, cumin, kaffir lime's peels, and nutmeg inhibited the growth of all bacterial strains tested (8-21 mm), but those of garlic, ginger, holy basil, kaffir lime's leaves, and mace inhibited growth of only some strains (Table 3). Compared to other spice oils, oils of clove and kaffir lime's peels showed greater antibacterial activity against all strains tested, with the inhibition zones of 12-19 mm and 12-21 mm, respectively. Oils of cinnamon, lemongrass, black pepper, white pepper, and turmeric were not extracted in this experiment because of very small amount of oils in plant materials. When the inhibition zone diameter of both forms of spice extracts was compared, inhibitory activity of essential oils was greater than that of ethanolic extracts, especially clove and kaffir lime's peels. Among *Salmonella* serotypes tested, the ethanolic extracts and oils exhibited slightly different degree of inhibition. *S.* Typhimurium is the most susceptible *Salmonella* serotype to both oils and extracts of clove and kaffir lime's peels. Of all enterobacteria tested, *S. marcescens* is the most susceptible strain to both oil and extract of clove.

Table 2 Antibacterial activity of crude ethanolic extracts of spices against *Salmonella* spp. and other enterobacteria using disk diffusion test

Crude ethanolic extracts of spices	Diameter of Inhibition Zone (mm)[a]																							
	S. Agona	*S.* Amsterdam	*S.* Anatum	*S.* Choleraesuis	*S.* Derby	*S.* Enteritidis	*S.* Hadar	*S.* Lexington	*S.* London	*S.* Newport	*S.* Orion	*S.* Panama	*S.* Rissen	*S.* Senftenberg	*S.* Schwarzengrund	*S.* Stanley	*S.* Virchow	*S.* Weltevreden	*S.* Typhimurium	*S.* Typhimurium DT104	*Citrobacter freundii*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*	*Serratia marcescens*
Cardamom	10	9	7	11	9	10	10	10	10	10	9	9	10	9	8	10	10	10	-	9	12	10	10	10	10
Cinnamon	9	7	9	9	9	10	8	8	-	9	8	8	8	10	7	8	9	10	8	9	9	8	8	8	9
Clove	10	9	11	11	10	10	10	12	11	11	11	10	10	11	9	10	11	11	15	10	12	10	10	10	22
Coriander	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cumin	9	9	11	11	10	10	10	9	10	10	9	9	10	11	9	9	10	11	10	8	9	10	10	10	9
Garlic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ginger	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Holy basil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Kaffir lime leaves	10	10	10	10	10	10	7	8	8	10	10	9	10	9	9	9	11	10	10	10	8	10	9	8	-
Kaffir lime peels	10	10	10	9	11	10	9	10	10	10	10	10	10	11	10	9	10	9	11	8	10	10	11	9	10
Lemongrass	-	10	10	10	10	10	-	10	10	10	-	-	10	10	-	10	11	10	11	10	-	-	7	7	-
Mace	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nutmeg	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pepper, Black	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pepper, White	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Turmeric	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
amoxycillin	26	22	27	26	-	22	21	23	23	27	-	-	20	8	-	-	25	25	29	-	16	-	17	-	12
10%DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Data are mean of two replications.
[b]No inhibition was observed (inhibition zone of <6 mm.).

Table 3 Antibacterial activity of essential oils of spices against *Salmonella* spp. and other enterobacteria using disk diffusion test

Essential oils of spices	Diameter of Inhibition Zone (mm)[a]																							
	S. Agona	*S.* Amsterdam	*S.* Anatum	*S.* Choleraesuis	*S.* Derby	*S.* Enteritidis	*S.* Hadar	*S.* Lexington	*S.* London	*S.* Newport	*S.* Orion	*S.* Panama	*S.* Rissen	*S.* Senftenberg	*S.* Schwarzengrund	*S.* Stanley	*S.* Virchow	*S.* Weltevreden	*S.* Typhimurium	*S.* Typhimurium DT104	*Citrobacter freundii*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*	*Serratia marcescens*
Cardamom	10	9	11	10	10	10	10	9	11	10	10	10	10	10	10	10	10	10	14	9	10	10	11	11	11
Cinnamon	N[b]	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Clove	15	12	15	15	13	13	15	15	15	15	13	13	12	15	12	12	15	14	16	11	15	13	14	13	19
Coriander	10	10	11	11	9	8	10	9	10	10	11	10	9	10	10	9	10	10	12	9	10	10	10	12	12
Cumin	10	9	8	10	9	8	11	8	10	9	10	9	9	10	10	9	9	8	10	9	12	9	11	13	13
Garlic	8	8	9	9	8	8	9	8	8	8	8	9	8	8	8	9	9	9	10	8	8	8	10	8	-
Ginger	7	-	8	8	8	8	8	-	-	8	8	-	8	8	-	-	7	-	10	8	8	-	9	10	12
Holy basil	-[c]	-	9	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	9	-	-	-	9	10	8
Kaffir lime leaves	7	9	8	8	8	8	8	8	7	-	-	7	-	8	7	7	9	8	10	8	8	-	8	10	9
Kaffir lime peels	15	14	15	16	15	15	14	15	15	16	16	15	12	15	15	16	15	14	21	14	16	14	16	16	15
Lemongrass	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Mace	-	-	9	-	8	-	9	-	9	8	-	-	-	7	-	-	9	7	9	7	9	-	-	7	10
Nutmeg	9	9	9	9	9	9	9	10	9	10	9	9	9	9	9	10	10	9	10	8	10	10	10	10	10
Pepper, Black	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Pepper, White	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
Turmeric	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
amoxycillin	26	22	27	26	-	22	21	23	23	27	-	-	20	8	-	-	25	25	29	-	16	-	17	-	12
10%DMSO	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Data are mean of two replications.
[b]No essential oils were extracted.
[c]No inhibition was observed (inhibition zone of <6 mm).

3.2 Determination of minimum inhibitory concentration

Nine serotypes of *Salmonella*, including *S.* Agona, *S.* Anatum, *S.* Choleraesuis, *S.* Derby, *S.* Enteritidis, *S.* Rissen, *S.* Senftenberg, *S.* Typhimurium and *S.* Typhimurium DT104 which are great potential pathogens and most commonly isolated from fresh and fermented meat, and four species of other enterobacteria, including *C. freundii*, *E. aerogenes*, *E. coli*, and *K. pneumonia* were selected as test organisms for MIC determination of spice oil and ethanolic extracts. The MIC values of crude ethanolic extracts of cardamom, cinnamon, clove, cumin, garlic, holy basil, kaffir lime's leaves and peels, and lemongrass indicated that clove had the highest antibacterial action against 13 bacterial strains tested, followed by kaffir lime's peels and holy basil (Table 4). Among *Salmonella* serotypes, *S.* Typhimurium was the most susceptible bacteria to most of the spice extracts tested, while *S.* Derby was the most resistant, followed by *S.* Rissen, *S.* Agona, and *S.* Typhimurium DT104. *E. aerogenes* and *E. coli* were also resistant to most of the spice extracts. Similarly, the results of the MIC determination of penicillin G against 13 bacterial strains as mentioned above indicated that *S.* Typhimurium was the most sensitive to Penicillin G (the MIC of 0.5 unit/ml; data not shown), whereas *S.* Derby, *S.* Typhimurium DT104, and *E. aerogenes* were the most resistant (the MIC of 5,000 unit/ml).

Oils of cardamom, clove, coriander, cumin,and kaffir lime's peels strongly inhibited the growth of almost all of the test bacteria (the MIC of 4.2 µl/ml), except *S.* Rissen which required higher MIC (Table 5). Oils of clove and cumin showed the broadest antibacterial action by distinctly inhibiting the growth of all test bacteria. The oils of cardamom, coriander, and kaffir lime's peels were also highly inhibitory, inhibiting almost all of bacterial strains tested. Very low concentration (4.2 µl/ml) of these oils was sufficient to inhibit the test bacteria. Nutmeg and mace oils exhibited slightly different degrees of inhibition. They inhibited almost all bacterial strains with the same MIC value, except *S.* Typhimurium and *E. aerogenes* which needed higher MIC to be inhibited by mace oil. Oils of holy basil and kaffir lime's leaves required higher concentration (>62.5 µl/ml) to inhibit most of the test organisms, except *S.* Typhimurium (4.2 µl/ml). Both oil and ethanolic extracts of kaffir lime's peels inhibited the bacteria tested more efficiently than those of kaffir lime's leaves. The MIC values of garlic and ginger oils varied depending on the bacterial strains. Ginger oil seemed to be a more

potent inhibitor to most bacterial strains than garlic oil. While ginger oil showed a high bactericidal effect to *S.* Choleraesuis, *S.* Senftenberg and *E. coli*, garlic oil exhibited the most potent inhibitory activity against *S.* Typhimurium. On the other hand, *S.* Derby, *S.* Enteritidis, *S.* Typhimurium DT104, *C. freundii* , *E. aerogenes*, and *K. pneumonia* exhibited a high resistance to ginger oil, while *S.* Derby, *S.* Rissen, *C. freundii*, and *E. aerogenes* were the most resistant microorganisms to garlic oil. Compared with other non-*Salmonella* strains tested, *E. coli* was the most sensitive to garlic and ginger oil. Among *Salmonella* serotypes tested, *S.* Typhimurium was the most susceptible serotype to most of the spice oils, while *S.* Rissen was the most resistant serotype, followed by *S.* Derby, *S.* Agona, *S.* Typhimurium DT104, and *S.* Senftenberg. Among the non-salmonellae strains tested, *E. coli* was more susceptible to most spice oils than *C. freundii*, *E. aerogenes*, and *K. pneumonia*.

Table 4 Minimum inhibitory concentrations of crude ethanolic extracts of spices against *Salmonella* spp. and other enterobacteria

Crude ethanolic extract of spices	Minimum inhibitory concentrations (mg/ml)												
	S. Agona	*S.* Anatum	*S.* Choleraesuis	*S.* Derby	*S.* Enteritidis	*S.* Rissen	*S.* Senftenberg	*S.* Typhimurium	*S.* Typhimurium DT104	*Citrobacter freundii*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*
Cardamom	90.9	90.9	45.5	>181.8	>181.8	>181.8	>181.8	22.7	90.9	45.5	>181.8	90.9	45.5
Cinnamon	181.8	90.9	181.8	181.8	181.8	>181.8	90.9	181.8	181.8	45.5	181.8	>181.8	90.9
Clove	5.7	2.8	2.8	22.7	2.8	5.7	5.7	2.8	2.8	11.4	11.4	2.8	45.5
Cumin	181.8	>181.8	45.5	>181.8	90.9	181.8	181.8	>181.8	181.8	>181.8	181.8	>181.8	45.5
Garlic	181.8	90.9	90.9	>181.8	90.9	181.8	90.9	45.5	90.9	90.9	>181.8	90.9	>181.8
Holy basil	45.5	22.7	45.5	90.9	90.9	45.5	90.9	45.5	45.5	45.5	90.9	90.9	11.4
Kaffir Lime leaves	181.8	>181.8	181.8	>181.8	181.8	181.8	>181.8	90.9	181.8	181.8	>181.8	181.8	5.7
Kaffir Lime peels	45.5	90.9	45.5	90.9	45.5	45.5	45.5	45.5	45.5	45.5	90.9	45.5	>181.8
Lemongrass	181.8	181.8	90.9	181.8	181.8	181.8	181.8	90.9	181.8	181.8	>181.8	181.8	>181.8

Table 5 Minimum inhibitory concentrations of essential oils of spices against *Salmonella* spp. and other enterobacteria

Essential oils of spices	Minimum inhibitory concentrations (µl/ml)												
	S. Agona	*S.* Anatum	*S.* Choleraesuis	*S.* Derby	*S.* Enteritidis	*S.* Rissen	*S.* Senftenberg	*S.* Typhimurium	*S.* Typhimurium DT104	*Citrobacter freundii*	*Enterobacter aerogenes*	*Escherichia coli*	*Klebsiella pneumonia*
Cardamom	4.2	4.2	4.2	4.2	4.2	8.3	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Clove	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Coriander	4.2	4.2	4.2	4.2	4.2	>62.5	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Cumin	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Garlic	62.5	47.6	47.6	>62.5	47.6	>62.5	47.6	16.4	62.5	>62.5	>62.5	47.6	62.5
Ginger	8.3	8.3	4.2	>62.5	>62.5	8.3	4.2	8.3	>62.5	>62.5	>62.5	4.2	>62.5
Holy basil	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	4.2	>62.5	>62.5	>62.5	>62.5	>62.5
Kaffir lime leaves	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	>62.5	4.2	>62.5	>62.5	>62.5	>62.5	>62.5
Kaffir lime peels	4.2	4.2	4.2	4.2	4.2	8.3	4.2	4.2	4.2	4.2	4.2	4.2	4.2
Mace	8.3	4.2	4.2	8.3	4.2	>62.5	8.3	8.3	4.2	4.2	47.6	4.2	8.3
Nutmeg	8.3	4.2	4.2	8.3	4.2	>62.5	8.3	4.2	4.2	4.2	4.2	4.2	4.2

3.3 Effect of spice essential oils on controlling of *Salmonella* Agona in Thai fermented pork

In all treatment, *S.* Agona numbers declined from 6.0 log CFU/g to 3.83-4.64 log CFU/g during fermentation, and the pH of all nham samples was reduced from 6.0-6.1 to 4.5-4.6 after three day fermentation (Figure 1 and Table 6). The number of viable *S.* Agona in nham with essential oils decreased greater than those in control treatment after fermentation to similar pH level. Fermentation to pH 4.5 yielded *S.* Agona viable cell reduction of 1.36 and 2.06-2.17 log cycles in nham without spice oil (control) and the other treatments (nham with spice oil), respectively. However, no significant differences were observed between the number of survivors in each treatment with essential oil added ($P > 0.05$). The significant difference of the number of survivors was found only at day 3 of fermentation between control treatment and the other three treatments with spice oils added. *S.* Agona survived in all treatment after 3 day fermentation, showing the survival number of 3.83-4.64 log CFU/g.

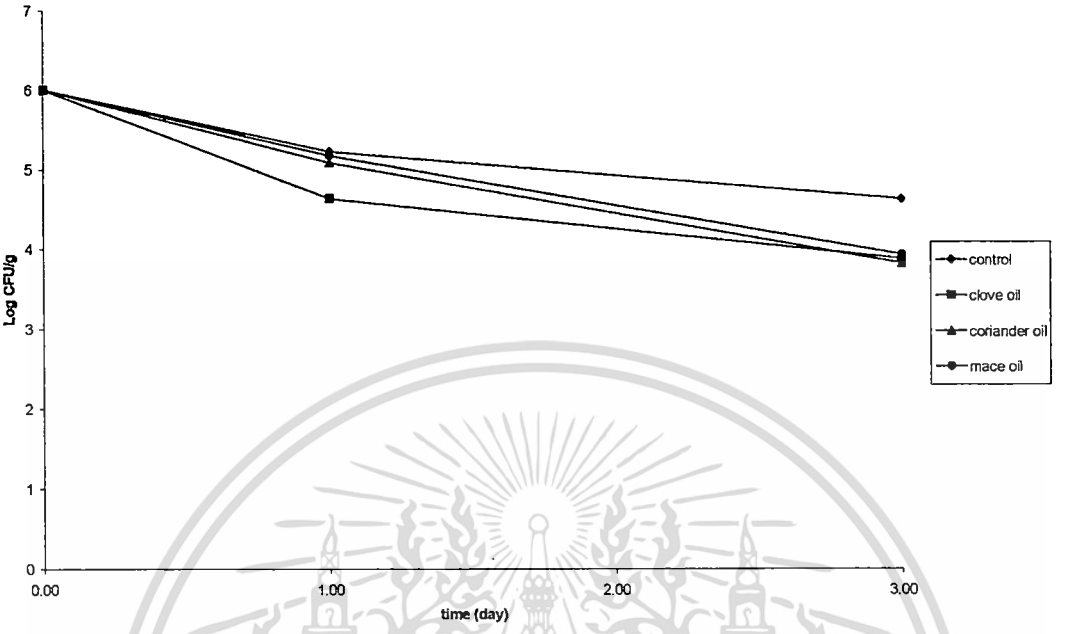

Figure 1 Survival of *Salmonella* Agona in nham during fermentation: ♦, nham without spice oil added (control); ■, nham with clove oil added; ▲, nham with coriander oil added; ●, nham with mace oil added

Table 6 Change in pH during nham fermentation

Time (days)	pH[a]			
	Nham without spice oil added (control)	Nham with clove oil added	Nham with coriander oil added	Nham with mace oil added
0	6.06	5.97	6.00	6.01
1	5.19	5.13	5.11	5.11
3	4.51	4.54	4.54	4.57

[a]Data are mean of two replication.

4. Discussion

In this study, the crude ethanolic extracts and essential oils of 14 Thai spices were screened for their antibacterial properties. The degree of antibacterial property of spices was considered from the MIC values against the enterobacteria strains. The low MIC value indicated the strong antibacterial activity of spices. Oka (1964) demonstrated that the adsorption of spice preservative on the bacterial cell was found to depend on its concentration. The results of the present study indicated that clove exhibited the strongest antibacterial activity in both forms of extracts, followed by kaffir lime's peels. The antibacterial activity of clove is attributed to eugenol (2-methoxy-4-allyl phenol). Clove bud oil contains high eugenol (70-90%) content (de Guzman and Siemonsma, 1999). This compound is an antimicrobial compound having wide spectra of antimicrobial effect (Beuchat and Golden, 1989; Kim et al., 1995) which may contribute to growth inhibition of *Salmonella* and other enterobacteria. High tannin (10-19%) content in clove provides additional antimicrobial activity (Shelef, 1983). Similar findings have been reported by other researchers (Suksringam, 1975; Farag et al., 1989). Kaffir lime's peels also contain antimicrobial compounds. The major constituents in essential oil of kaffir lime's peels are β-pinene (30.6%), limonene (29.2%), sabinene (22.6%) and other compounds, whereas the main compound in kaffir lime's leaves is citronellal (65.4%) (Lawrence et al., 1970). β-pinene and and limonene had greater inhibitory activity against *S.* Enteritidis than citronellal (Hirasa and Takemasa, 1998). Uribe et al. (1985) also reported that β-pinene inhibited the respiration of both intact cells of *S. cerevisiae* and its isolated mitochondria. This may be the reason why kaffir lime's peels showed greater antibacterial activity against all test bacteria than kaffir lime's leaves.

The oils of cumin, cardamom, and coriander also were also highly inhibitory to the test bacteria as they may contain potent antimicrobial compounds. The major constituents of these oils are as the following: cuminaldehyde (20-72%) and monoterpene hydrocarbons (e.g. β-pinene, γ-terpinene, p-cymene) in cumin oil; 1,8-cineole (20-60%) and α-terpinyl acetate (20-53%) in cardamom; linalool (74 %) and other components (small amounts of α-pinene, γ-terpinene, geranyl acetate, camphor and geraniol) in coriander oil (de Guzman and Siemonsma, 1999; Ashurst, 1991). Mace and nutmeg oil moderately inhibited the test bacteria, with similar degree of antibacterial action. Nutmeg oil is much resembling mace oil. Mutmeg oil contains monoterpene hydrocarbons (61-88% e. g. α-pinene, β-

pinene, sabinene), oxygenated monoterpenes (5-15%) and aromatic ethers (2-18% e.g. myristicin, elemicin, safrole) (de Guzman and Siemonsma, 1999), whereas mace oil consists of monoterpenes (87.5%), monoterpene alcohols (5.5%), and other aromatics (7.0%) (Farrell, 1990). Garlic and ginger oil possessed moderate antibacterial activity in this study. *E. coli* was sensitive to garlic extracts than *E. aerogenes* which is in agreement with previous observation (Arora and Kaur, 1999). The major antimicrobial compound in garlic is allicin (Conner, 1993). Garlic extracts were found to have antibacterial activity against several bacteria, including *S.* Typhimurium, *S.* Typhi, *E. coli*, *Bacillus cereus*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* (Arora and Kaur, 1999; Johnson and Vaughn, 1969; Saleem and Al-Delaimy, 1982). The major pungent components of ginger are gingerone and gingerol which have strong inhibitory activity against pathogenic bacteria (Hirasa and Takemasa, 1998).

In the present study, most of the spice essential oils exhibited stronger antibacterial activity than their own ethanolic extracts, with the exception of holy basil. The antimicrobial properties of spices have been shown to be attributed to the essential oil fraction (Chaisawadi et al., 2003). Dhar et al. (1968) also reported that essential oils of plants have a broad spectrum of antimicrobial effects, whereas other extracts possess specific antimicrobial activity toward certain groups of microorganisms. This is because of the fact that some essential oils contain active components which influence certain metabolic functions of microbial cells. As mentioned, most components of spice oils belong to the terpenoid family. There has been much speculation on the contribution to the terpene fraction to their antimicrobial activity in most of the recent researches on spices (Conner, 1993). Some researchers have demonstated the antimicrobial activity of the most common terpene compounds, such as thymol, carvacrol, linalool, eugenol, α-pinene, and β-pinene in spices against several microbial strains (Kim et al. 1995; Hirasa and Takemasa, 1998; Juven et al., 1994). Cyclic terpene compounds have been reported to cause loss of membrane integrity and dissipation of proton motive force (Sikkema et al., 1995). Wilkins et al. (1989) suggested that the antimicrobial action of spices is due to the impairment of a variety of enzyme systems involving in the production of energy or synthesis of structural components in microbial cells.

The results of the survival study of *S.* Agona in nham added with spice oils indicated that *S.* Agona in nham was not completely eliminated after 3 day fermentation. This is probably due to the

low concentration (0.02%) of the spice oils added and the decreasing of spice antimicrobial activity in the presence of food materials. Addition of higher concentration (MIC level) of spice oils to nham mixture was previously done, but the overall flavor of nham was not be acceptable because of the strong flavor of spice oils. Therefore, using very low concentration of spice oil can not control *Salmonella* in nham.

5. Conclusion

In conclusion, the degree of antibacterial properties of spices tested can be put in the following order: clove > kaffir lime's peels > cumin > cardamom > coriander > nutmeg > mace > ginger > garlic > holy basil > kaffir lime's leaves. These spices may be selected for use as powerful anti-*Salmonella* agents in fermented meat products and other foods, depending upon the desired flavor of the products. The oil fraction of these spices is recommended, with the exception of holy basil which should be used in the form of whole spice. Of all *Salmonella* serotypes tested, *S.* Typhimurium is the most vulnerable strain to crude ethanolic extracts and oils of spices, while *S.* Derby and *S.* Rissen were the most resistant. *Escherichia coli* was more sensitive to most of the spice oils than other non-salmonellae strains tested. Addition of 0.02% clove, coriander, or mace oil did not completely eliminate *S.* Agona cells in nham during fermentation at 30°C. There are some limitations in using spices in fermented meat: 1) The antimicrobial activity of spices may be diminished when they are added to food materials, containing protein, carbohydrate, fat, salt, etc; and 2) Flavor of some spices or spice extracts are too strong. The overall flavor of the products may not be acceptable if the large amount of spices is needed to add in the products in order to inhibit the pathogenic bacteria. The possible way is to use spices in combination with other preservatives (Shelef, 1983), such as acid, salt, sugar, and other chemical preservatives, or other food preservation systems (Leistner, 1999), such as thermal processing, cold storage, etc.

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