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Antibacterial Activity of Spice Extracts against Food-related Bacteria

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Abstract The present study was designed to identify spices and herbs that possess antibacterial activity and to apply for control of microorganisms in food. Alcoholic extracts of seventeen spices and five herbs were prepared and examined for growth inhibition of several kinds of food-related bacteria in culture media. *Bacillus stearothermophilus*, which produces heat-resistant spores, was highly sensitive to most of the spices tested. Both germination of spores and outgrowth of vegetative cells of this organism were inhibited by most of the spices tested. The MICs (minimal inhibitory concentrations) were 0.005% for paprika, 0.01% for anise and 0.02% for mace both on agar and in liquid media. The inhibition of this organism by effective spices was influenced by pH and NaCl concentration of the basal medium. When the pH and NaCl were 6.0 and 1.5%, respectively, the MICs decreased to 10−20%. No synergistic effect of spice and sodium lactate was observed except for mace. At 0.05%, nutmeg, sage, and white pepper perfectly inhibited the growth of this organism. Garlic showed no antibacterial effect and turmeric was also less effective. In general, antibacterial activity of herbs such as chamomile was comparatively low. Against *B. coagulans*, sage and rosemary inhibited the growth and the MICs were 0.05% and 0.2%, respectively. Both *Escherichia coli* and *Salmonella* were not inhibited by 0.2% concentration of all the spices and herbs tested. Sage showed the inhibitory effects against *B. subtilis*, *B. cereus* and *Staphylococcus aureus*. These results suggest that control of the thermophilic bacteria, especially *B. stearothermophilus*, in canned foods is possible by some spices.

Key words: Antibacterial activity, *Bacillus stearothermophilus*, inhibition, flat sour, food poisoning bacteria, spice

INTRODUCTION

Microbiological food safety has been of great concern in every nation irrespective of developed or underdeveloped. From the earliest times, man has devised ways for preserving foods. In general, foods were preserved by use of heat, cold, drying, salting and fermentation. In recent years, chemicals such as food additives are extensively used and some advanced technology for long-term storage are being developed. At the present time, the "Hazard Analysis Critical Control Point" (HACCP) approach to quality assurance is introduced in routine surveillance during food processing (KOKUBO, 1994).

Among above-mentioned means for controlling microorganisms in foods, some of chemical food additives have been questioned because of increased awareness of safety and the increased ability to evaluate safety (BRANEN, 1993). Thus there is currently great interest in antibacterial compounds naturally present in foods and food ingredients (BEUCHAT and GOLDEN, 1989). For example, allicin in garlic and onion (CAVALLITO and BAILEY 1944; KYUNG et al., 1996), lysozyme in eggs (HUGHEY and JOHNSON, 1987) and several minor components in milk (ASHTON and BUSTA, 1968; REITER, 1978) are studied for enhanced microbial stability of some foods. Among them the antimicrobial activity of plant extracts such as spices and herbs has become a subject of considerable interest.

Spices have been widely consumed in the human diet from remote antiquity not only as flavoring substances but as antimicrobial agents. Recently spices have received attention also in their useful physiological functions and antioxidant activity. There are a lot of reports about antimicrobial activity of spice extracts and its essential oils (BEUCHAT, 1976; HUHTANEN, 1980; CONNER and BEUCHAT, 1984). However, the available informations are for a small group of microorganisms and they are tested at high concentrations which are no practical use. More research is required on antimicrobial effects to food-related bacteria such as food spoilage bacteria and foodborne bacterial pathogens. The objective of this study was to evaluate the antibacterial activty of seventeen spices and five herbs to several bacteria which are important in control of foods. Combined effects of pH, NaCl concentration and addition of sodium lactate on the antibacterial activity were also determined.

MATERIALS AND METHODS

Test bacterium

Stock culture of *Staphylococcus aureus* 209P, *Escherichia coli* IFO 3301, *Bacillus cereus* IFO3457 and *Salmonella* Typhimurium IFO12529, and *Vibrio cholerae* non-O1 isolated from aquatic environments were tested for sensitivity test. *B. subtilis* (BFB0011) and *B. cereus* var *mycoides* (BFB0034) isolated from vegetables, and *B. coagulans* (BFB0039 and BFB0098) and *B. stearothermophilus* (BFB0081 and BFB0082) isolated at the research and development center of Aohata Co., Ltd., from canned food causing flat sour spoilage were also used. D_{121} values (min) of spores of *B. coagulans* and *B. stearothermophilus* were 2.5−2.8 and 2.7−4.1 respectively.

Inoculum preparation

Spores and vegetative cells were used as inocula for *Bacillus* spp. Overnight culture in nutrient broth (NB) at 35°C were used as vegetative cells except for *B. stearothermophilus* which was cultured in tripticase soy broth (TSB) at 50°C. A spore suspension of *Bacillus* spp. was prepared by the following methods. *B. stearothermophilus* was incubated in TSB at 50°C, *B. subtilis* and *B. cereus* in NB and *B. coagulans* in nutrient agar (NA) with 50% soil extract (CLAUS and BERKELEY, 1986) at 35°C for 7 days. The spores were harvested by centrifugation, washed twice and resuspended in distilled water. After heat treatment at 80°C for 15 min, viable spores were enumerated by pour-plate method. The spore suspension was diluted to give 1.0×10^6 cfu/ml and stocked at 4°C until use.

Preparation of spice extracts

The following seventeen spices and five herbs were obtained from local retail shops:

mace (*Myristica fragrans*, external coat), bay leaf (*Laurus nobilis*), nutmeg (*Myristica fragrans, seed*), garlic (*Allium sativum*), sage (*Salvia officinalis*), cinnamon (*Cinnamonum zeylanicum*), thyme (*Thymus vulgaris*), paprika (*Capsicum annuum*), oregano (*Oreganum vulgare*), anise (*Pimpinella anisum*), turmeric (*Circuma longa*), cardamon (*Elettania cardamomum*), white pepper (*Pipper nigrum*, water soaked), black pepper (*Pipper nigrum*, dried fruit), allspice (*Pimenta officinalis*), rosemary (*Rosmarinus officinalis*), clove (*Eugenia caryophyllata*), german chamomile (*Matricaria chamomilla*), lavender (*Lavandula officinalis*), peppermint (*Mentha piperita*), hibiscus and rose hip. Alcoholic extracts of spices and herbs were made by steeping 10g of powder or crushed pieces in 90 ml of ethanol for 2 days at room temperature with stirring. The mixture was centrifuged at 10,000 rpm for 15 min and filtered by membrane filter (0.22μm). The filtrate was regarded as 10% concentration of spice extract.

Screening of spices for inhibitory activity

The antibacterial activity of spices was screened using agar dilution assay. As basal medium, tripticase soy agar (TSA) was used for *B. coagulans* and *B. stearothermophilus* and nutrient agar for other bacteria. Appropriate amount of 10% spice extract was added to sterile melted basal medium kept at 50°C to obtain the concentration of 0.1%, 0.2% and 0.5% and agar plates were prepared. The overnight culture (vegetative cells) or spore suspension was then streaked onto the agar plates. Results for these assays were determined by comparing the occurrence of colonies on control plate after 3 day's incubation at 35°C or 50°C. The extent of the inhibition was divided into four degrees: perfect inhibition (no colony), strong inhibition, weak inhibition, and no inhibition.

Determination of MIC

Based on the results of the screening test, the MIC was determined both by agar dilution assay and broth dilution assay. In the broth dilution assay, the spice extract was serially diluted and distributed in basal medium (TSB) for *B. coagulans* and *B. stearothermophilus*, and these bacterial strains were then inoculated with one loop (*ca* 2 μℓ) of the culture or spore suspension. The MIC was defined as the lowest concentration at which no growth was observed after 3 day's incubation. The sensitivity tests stated above were repeated at least three times.

Effects of pH, NaCl and sodium lactate on MICs of spices

MICs of four spices (mace, sage, paprika, anise) which showed strong antibacterial activity against *B. stearothermophilus* were examined on media with different pH (6.5 and 6.0) and concentrations of NaCl (1.0% and 1.5%). Synergistic effect of spice and sodium lactate (0.5% and 1.0%) was also tested.

RESULTS

Antibacterial activity of spices

The 17 spices and 5 herbs were screened for their inhibitory activities against *S. aureus*, *E. coli*, *Salmonella* and *V. cholerae* (Table 1). *E. coli* and *Salmonella* showed no sensitivity to most of the spices tested. The growth of *V. cholerae* was inhibited by extracts of sage, white pepper and clove at the concentration of 0.2%. Only sage and bay leaf at 0.2% inhibited *S. aureus*. The antibacterial activity of spice extracts against *B. stearother-*

Table 1 Inhibition of foodborne bacteria by spices and herbs

Spice	*S.aureus*			*E.coli*			*Salmonella*			*V.cholerae*		
	0.5*	0.2	0.1	0.5	0.2	0.1	0.5	0.2	0.1	0.5	0.2	0.1
Mace	++	—	—	—	—	—	+w	—	—	+w	—	—
Bay leaf	++	++	—	—	—	—	—	—	—	—	—	—
Nutmeg	++	+w	—	—	—	—	+w	—	—	—	—	—
Garlic	+w	—	—	—	—	—	—	—	—	—	—	—
Sage	++	++	+	—	—	—	—	—	—	++	++	—
Cinnamon	++	—	—	+	—	—	++	—	—	++	+	—
Thyme	++	—	—	—	—	—	—	—	—	—	—	—
Paprika	—	—	—	—	—	—	—	—	—	—	—	—
Oregano	++	—	—	—	—	—	+w	—	—	—	—	—
Anise	+	—	—	—	—	—	—	—	—	—	—	—
Turmeric	++	—	—	—	—	—	—	—	—	—	—	—
Cardamon	+	—	—	—	—	—	—	—	—	—	—	—
White pepper	++	+	—	—	—	—	—	—	—	++	++	+w
Black pepper	++	+	—	—	—	—	—	—	—	++	+	—
Allspice	+	—	—	—	—	—	—	—	—	+	+w	—
Rosemary	++	+w	—	—	—	—	—	—	—	++	+	+w
Clove	++	+w	—	+	—	—	++	—	—	++	++	—
Chamomile	+w	—	—	—	—	—	—	—	—	—	—	—
Lavender	++	—	—	—	—	—	—	—	—	+	+w	—
Peppermint	+w	—	—	—	—	—	—	—	—	—	—	—
Hibiscus	+w	—	—	+w	—	—	+w	—	—	+w	—	—
Rose hip	—	—	—	—	—	—	—	—	—	—	—	—

++ : perfect inhibition
+ : strong inhibition
+w : weak inhibition
— : no inhibition
* Tested concentration (%)

mophilus was generally high (Table 2). All the tested spices, except garlic, inhibited the growth of this organism at 0.1%. *B. cerus* var *mycoides* was comparatively sensitive to spices and completely inhibited by mace and sage at 0.1%, and thyme, paprika, oregano, black pepper, rosemary and clove at 0.2%. On the contrary, *B. coagulans* and *B. subtilis* were tolerant to most of the spices. Only rosemary and sage showed inhibitory activity against *B. coagulans*. Antibacterial effects of herbs observed in this study were very low.

MICs of spices against Bacillus *spp.*

Since sage showed perfect inhibition of *Bacillus*, MICs of sage against *Bacillus* spp. were examined by broth dilution assay (Table 3). The MIC for two strains of *B. stearothermophilus* and one strain (BFB0040) of *B. coagulans* was 0.05% at which the other *Bacillus* spp. could grow. The inhibition of *B. stearothermophilus* by spice extracts is shown in Table 4. The most inhibitory spice was paprika with a MIC of 0.005% (50 ppm). It was also observed that this spice suppressed the growth at even as low as 0.002% on agar plate.

Table 2 Inhibition of *Bacillus* spp. by spices and herbs

Spice	B1			B2			B3			B4		
	0.5*	0.2	0.1	0.5	0.2	0.1	0.5	0.2	0.1	0.5	0.2	0.1
Mace	++	++	++	++	+w	—	++	—	—	++	++	++
Bay leaf	++	++	++	++	+w	—	++	—	—	++	+	—
Nutmeg	++	++	++	++	+w	—	++	—	—	++	—	—
Garlic	++	—	—	—	—	—	—	—	—	++	—	—
Sage	++	++	++	++	++	++	++	++	++	++	++	++
Cinnamon	++	++	++	++	—	—	+	—	—	++	+w	—
Thyme	++	++	++	++	—	—	++	—	—	++	++	+w
Paprika	++	++	++	++	—	—	—	—	—	++	+	+w
Oregano	++	++	++	++	—	—	++	—	—	++	+w	+w
Anise	++	++	++	+	—	—	—	—	—	+w	+w	—
Turmeric	++	++	++	++	—	—	++	—	—	++	—	—
Cardamon	++	++	++	++	—	—	—	—	—	—	—	—
White pepper	++	++	++	++	—	—	+	—	—	++	+	+w
Black pepper	++	++	++	++	+w	—	+	—	—	++	++	—
Allspice	++	++	++	++	—	—	—	—	—	++	+	—
Rosemary	++	++	++	++	++	+w	++	—	—	++	++	—
Clove	++	++	++	++	+w	—	++	—	—	++	++	+w
Chamomile	++	++	+w	++	—	—	—	—	—	++	—	—
Lavender	++	+	—	++	—	—	+w	—	—	++	—	—
Peppermint	++	++	+w	++	—	—	—	—	—	+	—	—
Hibiscus	++	++	—	—	—	—	+w	—	—	++	—	—
Rose hip	++	—	—	—	—	—	—	—	—	—	—	—

++ : perfect inhibition
\+ : strong inhibition
+w : weak inhibition
— : no inhibition
* Tested concentration (%)
B1 : *B.stearothermophilus* (BFB0082)
B2 : *B.coagulans* (BFB0039)
B3 : *B.subtilis*
B4 : *B.cereus* var *mycoides*

Anise and mace were also quite active with MICs of 0.01−0.02%. The delay of growth and weak turbidity were observed in broth at 0.005% concentration of these two spices. Nutmeg and white/black pepper showed moderate antibacterial activity with MIC of 0.05−0.1%. As observed for the spore inoculum (Table 3 and 4), similar results were also noticed for the vegetative cells. No spore germination was observed in broth at the MIC by microscopic examination. Though the MIC was 0.1% for all the other spices tested, low growth inhibitions were observed on agar plate at the concentration of 0.01−0.05%.

Effects of pH, NaCl and sodium lactate on the MIC of spices

MICs (ppm) of spices to *B. stearothermophilus* were monitored when pH and NaCl concentration of basal medium were changed or sodium lactate was added (Table 5). Original pH and NaCl concentration of TSB are 7.3 and 0.5%, respectively. Antibacterial activ

Table 3 Antibacterial activity of sage extract against *Bacillus*

Species	Conc. of sage extract (%)					MIC(%)
	0.2	0.1	0.05	0.02	0.01	
B.stearothermophilus (BFB0081)	++	++	++	—	—	0.05
B.stearothermophilus (BFB0082)	++	++	++	+w	—	0.05
B.coagulans (BFB0039)	++	++	—	—	—	0.1
B.coagulans (BFB0040)	++	++	++	—	—	0.05
B.subtilis (BFB0011)	++	++	—	—	—	0.1
B.cereus var *mycoides* (BFB0034)	++	++	+w	—	—	0.1
B.cereus (IFO3457)	++	++	—	—	—	0.1

++ : perfect inhibition
\+ : strong inhibition
+w : weak inhibition
— : no inhibition

Table 4 Antibacterial activity of spice extracts against *B.stearothermophilus*

Spice	Agar dilution assay (%)				Broth dilution assay (%)				MIC(%)
	0.05	0.02	0.01	0.005	0.05	0.02	0.01	0.005	
Mace	++	++	+	—	++	++	+	+w	0.02
Bay leaf	+w	+w	+w	—	—	—	—	ND	0.1
Nutmeg	++	+	+w	—	+	+	—	ND	0.05—0.1
Cinnamon	+w	+w	+w	—	—	—	—	ND	0.1
Thyme	+w	+w	+w	—	—	—	—	ND	0.1
Paprika	++	++	++	++	++	++	++	++	0.005
Oregano	+w	+w	+w	—	—	—	—	ND	0.1
Anise	++	++	++	+	++	++	++	+	0.01
Turmeric	+w	+w	+w	—	—	—	—	ND	0.1
Cardamon	+w	+w	+w	—	—	—	—	ND	0.1
White pepper	+	+w	+w	—	++	+w	—	ND	0.05—0.1
Black pepper	+	+w	+w	—	+	+w	—	ND	0.1
Allspice	+w	+w	+w	—	+w	—	—	ND	0.1
Rosemary	+w	+w	+w	—	—	—	—	ND	0.1
Clove	+w	+w	+w	—	—	—	—	ND	0.1

++ : perfect inhibition
\+ : strong inhibition
+w : weak inhibition
— : no inhibition

ity of spice was enhanced at slight acidic or salted condition. MIC of mace decreased from 100 to 20 ppm with increasing concentration of NaCl (0.5% to 1.5%). The decrease in pH was found to be less effective on the MIC. As observed for paprika, MIC of anise at pH 6.5 or in 1.0−1.5% NaCl changed to 20 ppm. When pH and NaCl of basal medium were 6.0 and 1.5%, no growth was observed in broth containing 5 ppm of paprika or anise. Under this condition, however, slight inhibition was noticed in the control medium that had no extract of spice. Since 1.5% sodium lactate inhibited the growth of *B. stearothermophilus*

Table 5 Effects of pH, NaCl and sodium lactate on MIC of spices against *B.stearothermophilus*

pH	NaCl (%)	Na-Lac (%)	MIC (ppm) Mace	Sage	Paprika	Anise
7.3	0.5	—	100—200	500	50	50—100
7.3	1.0	—	50	500	20	20
7.3	1.5	—	20	200	20	20
6.5	0.5	—	50	200	20	20
6.5	1.0	—	20	200	10	20
6.0	0.5	—	50	100	20	20
6.0	1.5	—	20	50	5	5
7.3	0.5	0.5	100	500	50	50
7.3	0.5	1.0	20	500	50	50

without spice, MICs of spices were determined at 0.5% and 1.0%. MIC of mace was 20 ppm in 1.0% sodium lactate. In other cases, no synergistic effect of spice and sodium lactate was observed.

DISCUSSION

Of the 17 spices screened in culture media at the concentration of 0.1−0.5%, sage demonstrated antibacterial effects to gram-positive spore former, *Bacillus* spp. It is well-known that some spices have comparatively broad spectrum of antibacterial effects, whereas others may be specific to certain species. The inhibition of spice is dependent upon Gram type and species. There are a few informations on the mechanism of inhibition of bacteria by spices. Some potential modes of action were reported; interference with the phospholipid bilayer of the membrane may cause increased permeability and loss of cellular constituents or impairment of a variety of enzyme system may affect the production of cellular energy and synthesis of structural components (Wendakoon and Sakaguchi, 1995). Kim *et al.* (1995) also suggested the destruction or inactivation of genetic materials as the mode of inhibition. In general, gram-positive bacteria are more sensitive to spices than gram-negative bacteria (Farag *et al.*, 1989). Also in this study, gram-negative bacteria, *E. coli* and *Salmonella*, were less sensitive than gram-positive bacteria. Outer membrane or some excretory system of gram-negative bacteria might protect the action of spice. In gram-positive bacteria, spore-forming bacteria were more sensitive than non-spore former, *S. aureus*. From the results of this study (Table 4), it is understood that the inhibition of outgrowth of *B. stearothermophilus* by spices occurred at the same level compared to that of germination of spores. It is not clear whether the spice has bactericidal action or just bacteriostatic action at MIC level. *B. stearothermophilus* was highly sensitive to most of the extract of spices tested. This organism is thermophilic and can grow at 70°C. The spores of *B. stearothermophilus* and *B. coagulans* are of paticular interest to the canning food industry because of their extra-high heat resistance. The spores of *B. stearothermophilus* was reported to survive at 100°C for up to 20 hr with an initial population of 10^5-10^6 cells/g (Banwart, 1989). The flat sour spoilage of low-acid canned food was sometimes happened by these organisms (Matsuda *et al.*, 1985). The strains used in this study were isolated from meat sauce which caused flat sour spoilage in can. If the materials are contaminated with the spores of this organism and the can or pouch products, paticularly the large size, are stored at elevated temperature, the outbreak of unusual can food by flat sour spoilage may be possible. Meat sauce already contains several kinds of spice. Though antibacterial activity of spice was influenced by the surrounding condition such as pH and salts, it may be difficult to control microorganisms in foods only by spice. There is currently great interest in inhibitory activity of sodium lactate against various food microorganism (MAAS *et al.*, 1989; HOUYAMA, 1994). Though sodium lactate compound was not effective with spice to *B. stearothermophilus* (Table 5), it is worthwhile to examine the synergistic effect of spice and harmless known bacteriostatic chemicals at low level. Some spices were reported to cause an increased sensitivity of the spores to heat (BLANK *et al.*, 1988). In contrast with antibacterial function, some spices are reported to stimulate the acid production of fermented meat starter culture (ZAIKA and KISSINGER, 1979). These phenomena are worthy of further study.

The use of spices as antimicrobial agents in foods is generally limitted because of flavor consideration. Parika, anise and mace inhibited this thermophile at the level of 0.005−0.02% which may not exceed the organoleptically acceptable levels. Though garlic and its effective component identified as allicin probably have long been recognized and studied most extensively for their antimicrobial activity (DEWIT *et al.*, 1979; SALEEM and AL-DELAIMY, 1982), no effects were observed to all the bacteria tested in this study. It may be due to the use of dry garlic powder in the present study. Herbs had less effective to food-borne bacteria (Table 1 and 2). Since herbs, unlike spices, can be used at high concentration, it seems to have potential use for safety in foods. From the results of this study, it can be suggested that control of thermophilic spore-formers in foods may be possible. Because foods contain a lot of ingredients which may decline the antibacterial activity of spice, further works are needed in various foods.

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食品細菌に対する香辛料エキスの抗菌性

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食品の微生物制御で重要となる数種の細菌を対象として，17種の香辛料と5種のハーブの抗菌性を試験した。試料にはエタノールで抽出したエキスを用いた。香辛料の抗菌性は全般的にグラム陰性菌よりもグラム陽性菌に対して強かった。とくに，高い耐熱性の芽胞を形成して缶詰のフラットサワー原因菌として問題となる *Bacillus stearothermophilus* は大部分の香辛料に感受性を示した。なかでも，パプリカはMIC(最小発育阻止濃度)が0.005%と最も強い抗菌性を示し，アニス(MIC:0.01%)，メース(MIC:0.02%)がそれに次いだ。これらの抗菌性は基礎培地のpHを弱酸性にしたり，塩分濃度を少し高めることによって強くなり，pH 6.0および 1.5%NaCl 存在下でMICは約1オーダー低下した。しかし，乳酸ナトリウムとの相乗効果はほとんど認められなかった。以上の結果は，寒天希釈法と液体希釈法，接種物として栄養細胞と芽胞，いずれを用いた場合も差はみられなかった。セージは0.1%濃度で *Bacillus* 属細菌 (*B. subtilis*，*B. cereus*，*B. coagulans*)の発育を完全に阻止した。カモミールなどのハーブの抗菌性は比較的低かった。グラム陰性菌について *Escherichia coli* と *Samonella* はいずれの香辛料でも0.2%濃度で発育阻害はみられず，*Vibrio cholerae* non-O1がセージ，ホワイトペッパーおよびクローブに感受性を示したのみであった。以上，食品中での耐熱性芽胞形成菌の制御に一部の香辛料が有効である可能性を明らかにした。

キーワード：抗菌性，香辛料，最小発育阻止濃度，食中毒細菌，耐熱性芽胞形成菌，フラットサワー