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Effect of spices on *Vibrio parahaemolyticus* survival and growth

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ABSTRACT

In this study, the antibacterial activity of a total of 16 spices at a final concentration of 2.5 % against *V. parahaemolyticus* at two different temperatures, 5 and 37 ºC, was tested. Anise seed, chili, cloves, cinnamon, coriander seed, cumin, curry, garlic, ginger, oregano, paprika, black and white pepper, rosemary, thyme and turmeric, were collected from a retail store from the same producer. Prior to antibacterial screening, the spices were analyzed using standard microbiological procedures for: aerobic spore forming bacteria, sulfite-reducing clostridia, yeast and molds, *Enterobacteriaceae, Escherichia coli*, coagulase-positive staphylococci, and the presence of *Salmonella* spp. and *Listeria monocytogenes*. For detection of anti-*Vibrio* activity, the same amount of *V. parahaemolyticus* culture in NaCl-BHI broth, at 10^4 cfu/mL, was added to the 5 % suspension of spices, incubated for 24 h and then inoculated onto TCBS agar. After screening, minimal inhibitory concentrations were determined for spices, which showed strong antimicrobial activity. *Salmonella* spp., *Listeria monocytogenes, E. coli* and coagulase-positive staphylococci were not detected in any of the spice samples. Aerobic spore forming bacteria were present in 93.7 %, sulfite reducing clostridia in 43.7 %, yeasts in 12.5 %, molds in 62.5 % and *Enterobacteriaceae* in 18.7 % of the spice samples. At 5 °C, all spices except anise and coriander seed showed antibacterial activity against *V. parahaemolyticus*, with viable count reduced by at least 1 log; strong antibacterial activities at this temperature were found for oregano, garlic, thyme, cloves, cinnamon, curry, rosemary, ginger and turmeric. Oregano, garlic, thyme, cloves and cinnamon showed strong antibacterial activity at 37 °C. The lowest minimal inhibitory concentration at 37 °C was 0.078 % in cloves, and at 5 °C was 0.0012 % in turmeric. The effect of the accompanying microflora of the spices on the number of *V. parahaemolyticus* was not observed. This result showed that some spices have potential for reducing the risk of contaminating *V. parahaemolyticus* in seafood, combined with low temperature.

Key words: *Vibrio parahaemolyticus*, spices, antibacterial activity

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Introduction

Spices have been used in food preparation since ancient times, not only for improving the flavor, smell, appearance and digestibility of food, but for food preservation and also as folk medicine (SHAN, 2007). The antimicrobial activities of spices have been reported against various microorganisms, including foodborne pathogens (FILIPOVIĆ et al., 2014; NEDOROSTOVA et al., 2009; ZHANG et al., 2009; SHAN et al., 2007; SOUZA et al., 2007; YANO et al., 2006; MOREIRA et al., 2005; DEL CAMPO et al., 2000; ARORA and KAUR, 1999). Research concerning the utilization of spices and their extracts and essential oils, as natural bio-preservatives *in vitro* and in food systems, as well as their incorporation into packaging materials, has increased since the 1990s (TAJKARIMI et al., 2010; BURT, 2004).

Nowadays consumers are questioning the safety of food containing synthetic preservatives and additives, so there is growing interest in using spices as natural antibacterial substances (compounds) for preservation of food (elimination of undesirable pathogens and/or delay microbial spoilage), and also for improving food sensory qualities, as well as the health of consumers.

Vibrio parahaemolyticus is a foodborne pathogen which causes mild gastroenteritis in humans after consummation of raw or insufficiently treated seafood. As the Japanese style of cuisine of eating raw and lightly cooked seafood is increasingly popular, there has been a growing number of cases of food borne disease caused by *V. parahaemolyticus*, not only in Asia and Oceania, but in the USA and Europe (MIKUŠ et al., 2010; YANO et al., 2006). Food contamination, as an enormous public health problem, could be controlled by the use of natural preservatives, such as spices and their essential oils (ARORA and KAUR 1999).

Thus, the aim of this research was to test the antibacterial activity of spices and herbs against *V. parahaemolyticus,* and their potential for use during culinary preparation of seafood in the catering industry and households, to reduce the risk of this pathogen.

Materials and methods

Dry spices and herbs. Spices and herbs were collected from a retail store, from the same producer. All spices were bought ground. These spices were: anise seed, chili, cloves, cinnamon, coriander seed, cumin, curry, garlic, ginger, oregano, paprika, black pepper and white pepper, rosemary, thyme and turmeric.

Microbiological analyses. The spices were subjected to microbiological analysis and the following parameters were monitored: aerobic spore forming bacteria, sulfite-reducing clostridia, yeast and molds, *Enterobacteriaceae, Escherichia coli*, coagulase-positive staphylococci, and the presence of *Salmonella* spp. and *Listeria monocytogenes*.

Enumeration of these microorganisms was performed after preparing initial suspensions under aseptic conditions, by diluting 10 g of the test sample (spice) in 90 mL of Buffered Peptone Water (BPW, Merck, Darmstadt, Germany). After homogenization of the initial suspension for 2 min (Stomacher 400 Circulator, Seward, UK), serial dilutions were made. Appropriate dilution samples (1 or 0.1) mL were poured or spread onto agar plates. Sulfite-reducing clostridia were determined on Sulfite Polimixin Sulphadiazine (SPS) agar, anaerobically 72 h at 37 °C; yeasts and molds on Yeast extract Glucose Chloramphenicol (YGC, Merck, Germany) agar at 25 °C, 5 - 7 days; *Enterobacteriaceae* on Violet Bile Red Glucose (VRBG, Merck, Germany) agar 24 h at 37 °C; *E. coli* on Coli-ID agar (bioMérieux, Marcy l'Etoile, France) at 37 °C 24 h, coagulase-positive staphylococci on Baird-Parker (BP, Merck, Germany) agar 48 h at 30 °C. For the enumeration of the total count of aerobic spore forming bacteria, the initial suspension was heated for 10 min at 80 °C in a water bath, then quickly cooled and serially diluted. Initial and serial dilutions (1 mL) were poured onto Plate Count Agar (PCA; Merck, Germany) and incubated for 72 h at 30 °C.

For *Salmonella* spp. detection 25 g of spices was homogenized in 90 mL Buffered Peptone Water (BPW, Merck, Germany) and incubated 18 h at 37 °C. Then, 0.1 mL of pre-enrichment was transferred into 10 mL of selective enrichment broth, according to Rappaport and Vassiliadis (RVS, Merck, Germany) and incubated for 24 h at 41.5 °C. After incubation the cultures were streaked onto xylose lysine brilliant-green phenol red (XLD, Merck, Germany) and brilliant-green phenol-red lactose sucrose agar (BPLS, Merck, Germany), and incubated for 24 h at 37 °C.

For detection of *Listeria monocytogenes* 10 g of spices was homogenized in 90 mL of Half Fraser (HF; Merck, Darmstadt, Germany) broth and incubated at 30 °C for 24 h. Then 0.1 mL of pre-enrichment culture was transferred into Fraser broth and incubated at 37 °C for 48 h. After the incubation period, the enrichment culture was streaked onto Palcam (Merck, Germany) and *Listeria* Selective agar acc. Ottaviani and Agosti (ALOA, Merck, Germany) and incubated at 37 °C for 48 h.

Indicator microorganism. As the indicator microorganism, *Vibrio parahaemolyticus* NCTC 10885 strain (TCS Biosciences Ltd, Buckingham, UK) isolated from purified oyster was used. A loopful of the working culture *V. parahaemolyticus* NCTC 10885 was transferred to 3 mL of Brain Heart Infusion Broth (BHI, Oxoid, Basingstoke, UK) with 3 % NaCl (NaCl-BHI broth) and incubated at 37 ºC for 18 h. The overnight culture was used for experiments.

Anti-Vibrio activity screening of spices. Screening was done by the method of YANO et al. (2006). Suspension (5 %) of spices was made in distillated water. 100 µL aliquot of the suspension of each spice was put into the wells of two micro-titer plates, and incubated at 5 and 37 ºC for 30 min. The overnight culture of *V. parahaemolyticus* was diluted with

fresh NaCl-BHI broth to a level of 10^4 cfu/mL, from which 100 µL were inoculated onto each well with spices, and then the plates were incubated at 5 and 37 ºC for 24 h. After incubation, 100 µL aliquot of the mixed suspension was spread onto Thiosulfate Citrate Bile Sucrose (TCBS, Merck, Germany) agar plates and incubated at 37 ºC for 18 h. For negative control, instead of spices, distilled water was used.

Determination of minimal inhibitory concentrations (MICs). To determine the minimal inhibitory concentration of spices, a modified method of YANO et al. (2006) was used. Briefly, serial dilution by a dilution factor of 2 was prepared for each spice. Each well in a row (12 wells) of the micro-titer plate was filled with 100 µL of distilled water, then in the first well in a column, 100 µL of 5 % suspension of the spice or herb to be tested was pipetted. After mixing by pipetting 100 µL of the mixture was transferred to the second column of wells, in a process of serial dilution of 1:1, until the 12 column. The further process was the same as for screening of 5 % suspension of spices for herbs: Micro-titer plates were incubated first at 5 and 37 ºC µL for 30 min, before bacterial inoculation. Each well was inoculated with 100 µL prepared bacterial inoculum (overnight culture of *V. parahaemolyticus* was diluted to a level of 10^4 cfu/mL). The micro-titer plates were then incubated at 5 and 37 ºC for 24 h. After incubation, 100 µL of the mixture from each well was spread onto TCBS and incubated at 37 ºC for 24 h. The concentration of spices and herbs in the lowest serial dilution at which growth did not occur was recorded as MIC.

Results

Microbiological analyses. All samples were free from *Salmonella* spp. and *Listeria monocytogenes*, while *Escherichia coli* and coagulase-positive staphylococci were below the detection limit of the methods used (<1 log and < 2 log cfu/g, respectively). In all but one sample, aerobic spore forming bacteria were present (93.7 %) in the range of 1 log (black pepper) to 4.50 log cfu/g, with relatively high levels (>5 log cfu/g) found in curry and turmeric (Table 1). Sulfite-reducing clostridia were detected in 7 of 16 spice samples (43.7 %), ranging from 1.1 log (garlic) to 2.3 log cfu/g (thyme), with levels higher than 2 log cfu/g only in coriander (2.26 log) and thyme (Table 1). Yeasts were present only in coriander (2.53 log cfu/g) and oregano (3.33 log cfu/g), while molds were present in 62.5 % (10 of 16) of spice samples, in the range of 2.1 log (cumin and garlic) to 4.52 log cfu/g (oregano) (Table 1). *Enterobacteriaceae* were present in 18.7 % (3 of 16) of the spices: anise seed (1.88 log cfu/g), curry (1.94 log cfu/g) and oregano (2 log cfu/g) (Table 1).

Table 1. Results of microbiological analyses of spices

Spice	Aerobic spore forming bacteria	Sulfite-reducing clostridia	Yeasts	Molds	*Enterobacteriaceae*
Anise seed	1.80 ± 0.08	<1	<2	3.13 ± 0.06	1.88 ± 0.03
Chili	4.51 ± 0.08	<1	<2	<2	<1
Cloves	<1	<1	<2	<2	<1
Cinnamon	3.26 ± 0.11	1.58 ± 0.17	<2	3.27 ± 0.05	<1
Coriander seed	4.45 ± 0.08	2.26 ± 0.07	2.53 ± 0.21	3.49 ± 0.04	<1
Cumin	2.92 ± 0.02	<1	<2	2.1 ± 0.17	<1
Curry	5.85 ± 0.05	1.69 ± 0.09	<2	2.89 ± 0.10	1.94 ± 0.03
Garlic	4.03 ± 0.07	1.1 ± 0.17	<2	2.1 ± 0.17	<1
Ginger	4.78 ± 0.03	1.95 ± 0.05	<2	<2	<1
Oregano	2.89 ± 0.11	1.26 ± 0.24	3.33 ± 0.15	4.52 ± 0.04	2 ± 0.00
Paprika	1.72 ± 0.05	<1	<2	<2	<1
Black pepper	1.1 ± 0.17	<1	<2	<2	<1
White pepper	3.95 ± 0.02	<1	<2	3.14 ± 0.13	<1
Rosemary	4.33 ± 0.05	<1	<2	3.01 ± 0.07	<1
Thyme	4.38 ± 0.08	2.30 ± 0.07	<2	3.65 ± 0.05	<1
Turmeric	5.96 ± 0.01	<1	<2	<2	<1

Anti-Vibrio activity screening of spices. After 24 h of incubation at 5 °C *V. parahaemolyticus* count in the negative control decreased to 10^3 cfu/mL, while after incubation at 37 °C the count increased to 10^8 cfu/mL. All the spices, except anise and coriander seed, showed antibacterial activity (viable count reduced for at least 1 log cfu/mL) at 5 °C (Table 2). Chili, cumin, paprika, black and white paper reduced number of *V. parahaemolyticus* for 1 log cfu/mL, while cloves, cinnamon, curry, garlic, ginger, oregano, rosemary, thyme and turmeric showed antibacterial activity with no growth of viable *V. parahaemolyticus*. Oregano, garlic, thyme, cloves and cinnamon showed antibacterial activity against *V. parahaemolyticus* at 37 °C; oregano, garlic, cloves and cinnamon with no growth of viable bacterium, while thyme reduced number of *V. parahaemolyticus* for 5 log cfu/mL (Table 2).

Determination of minimal inhibitory concentrations (MICs). Results for MIC are shown in Table 3. The lowest MIC at temperature of 5 °C was 0.0012 % in turmeric, then 0.019 % in cinnamon and 0.078 % in cloves and curry. In garlic, and thyme MIC was 0.312 %; in ginger and oregano 0.625 %. At 37 °C lowest MIC was in cloves (0.078 %) and then in cinnamon, garlic, and oregano (0.312 %). Generally, MIC at 5 °C were lower than at 37 °C in all spices, except in cloves and garlic where MIC was the same at both temperatures.

Table 2. Results of screening antimicrobial properties of spices

Spice	Incubation at 5 ºC	Incubation at 37 ºC
Anise seed	-	-
Chili	+	-
Cloves	++	++
Cinnamon	++	++
Coriander seed	-	-
Cumin	+	-
Curry	++	-
Garlic	++	++
Ginger	++	-
Oregano	++	++
Paprika	+	-
Black pepper	+	-
White pepper	+	-
Rosemary	++	-
Thyme	++	+*
Turmeric	++	-

++ no growth of *V. parahaemolyticus;* + growth reduced for about 1 log cfu/mL compared to negative control; +* growth reduced for about 5 log cfu/mL compared to negative control; - number of bacteria is not reduced compared to negative control

Table 3. Minimal inhibitory concentrations (MIC) of spices against *Vibrio parahaemolyticus*

Spice	Incubation at 37 ºC	Incubation at 5 ºC
Cinnamon	0.312 %	0.019 %
Cloves	0.078 %	0.078 %
Curry	-	0.078 %
Garlic	0.312 %	0.312 %
Ginger	-	0.625 %
Oregano	0.312 %	0.625 %
Rosemary	-	2.5 %
Thyme	2.5 %	0.312 %
Turmeric	-	0.0012 %

- MIC was not determined, since 2.5 % suspension of spices did not show antibacterial effect

Discussion

In our study *Salmonella* spp., *Listeria monocytogenes*, *E. coli* and coagulase-positive staphylococci were not present in any of the spice samples. Similar results

were obtained by other researchers, indicating that coagulase-positive staphylococci are not typically present in dry spices (WITKOWSKA et al., 2011; ABOU DONIA, 2008; KNEIFEL and BERGER, 1994), while the occurrence of *Listeria* spp. (WITKOWSKA et al., 2011; KNEIFEL and BERGER, 1994), *Salmonella* spp. (WITKOWSKA et al., 2011; ABOU DONIA, 2008; GARCIA et al., 2001; KNEIFEL and BERGER, 1994) and *E. coli* (BANERJEE and SARKAR, 2003; GARCIA et al., 2001) is quite rare. Our results showed that the majority of the spices were contaminated with aerobic spore formers, as they were present in 93.7 % of all the spices, at levels ranging from 1.1 log - 5.96 log cfu/g (Table 1). This finding is in agreement with other reports, which suggests that aerobic spore formers are the predominant bacterial population within herbs and spices, since spores may survive processing, drying and storage for long periods of time, in contrast to vegetative cells (WITKOWSKA et al., 2011; BANERJEE and SARKAR, 2003; KNEIFEL and BERGER, 1994; KOVACS-DOMJAN, 1988). The finding of sulfite reducing clostridia which were detected in 43.7 % of samples at levels less than 3 log cfu/g and only in two samples higher than 2 log cfu/g, was in accordance with results of EISGRUBER and REUTER (1987). Yeasts were detected in 12.5 %, while molds were present in 62.5 % of spices (Table 1). Our findings are in agreement with other studies, which state that yeasts are present in spices in low numbers, while contamination by molds is much more frequent (WITKOWSKA et al., 2011; KNEIFEL and BERGER, 1994). Enterobacteria were present in only three spices at levels of ≤2 log cfu/g (Table 1) which is in accordance with findings of KNEIFEL and BERGER (1994). Cloves were not contaminated with any of analyzed microorganisms; they were followed by black pepper and paprika, in which only aerobic spore forming bacteria were detected (1.1 log and 1.72 log cfu/g, respectively). In turmeric we also found only aerobic spore forming bacteria, but at a level of 5.96 log cfu/g.

From the 16 spices screened in our study, cinnamon, cloves, garlic, oregano and thyme showed strong antimicrobial activity against *V. parahaemolyticus* at both temperatures, while at 5 °C strong antibacterial activity was also found for curry, ginger, rosemary and turmeric. Weak antibacterial activity was found at 5 °C for chili, cumin, paprika, black and white pepper. The lowest MIC at 37 °C was 0.078 % (cloves), and at 5 °C 0.0012 % (turmeric). Generally, MIC at 5 °C were lower than at 37 °C. Since bacteria are sensitive to low storage temperatures (FERNANDEZ-PIQUER et al., 2011) it is understandable that more spices were effective and that MIC were generally lower at this temperature. Previous *in vitro* studies have also reported antibacterial activities of cinnamon (CHANG, 2001), cloves (YANO et al., 2006), garlic (VUDDHAKUL et al., 2007; YANO et al. 2006), ginger (YANO et al., 2006), oregano (YANO et al., 2006), rosemary (YANO et al., 2006; BEUCHAT, 1976), thyme (YANO et al., 2006; BEUCHAT, 1976) and turmeric (YANO et al., 2006) against *V. parahaemolyticus*. The antibacterial effect of cinnamon essential oil on *V. parahaemolyticus* was also confirmed in an in-food system study by ARANCIBIA et al. (2014) which proved that the release of cinnamon essential oil from polysaccharide bilayer

films was effective for *V. parahaemolyticus* inhibition in chilled shrimps. Our results are in accordance with the results of YANO at al. (2006), who investigated the effect of 2.5 % suspension of spices and herbs on *V. parahaemolyticus* at two temperatures (5 and 30 °C). In their study the spices investigated were not all the same as in our study, but those which were (anise and coriander seed, cloves, cumin, garlic, ginger, oregano, rosemary, thyme and turmeric) showed the same trend, and also the antimicrobial activity at 30 °C was in accordance with our results at 37 °C. The lowest MIC at 5 °C in this investigation was also for turmeric (0.004 %), after marjoram (which we did not test). Values for MIC were not exactly the same as in our study. Differences in MICs may be attributed to the fact that in our study MICs were determined from serial dilutions prepared with distilled water, while YANO et al. (2006) used sterile natural seawater (as a nutrient poor medium) and also Na-HI broth (nutrient rich medium) to determine MIC.

Spices with low accompanying microbial count at 37 °C showed strong anti-*Vibrio* activity (cloves), as well as the spices with a higher microbial count (cinnamon, garlic, oregano, thyme); and some spices with low microbial count (black pepper, paprika, cumin) did not show anti-*Vibrio* activity at 37 °C, nor those with a higher count (coriander, rosemary, turmeric, ginger). Coriander seed (aerobic spore formers - 4.45 log, sulfite-reducing clostridia - 2.26 log, yeasts - 2.53 log, molds - 3.49 log cfu/g), also did not show anti-*Vibrio* activity either at 5 °C. Based on the given results we may conclude that the accompanying microflora did not affect the number of *Vibrio*. In the study conducted by ABHIROSH et al. (2009), there was no effect of the autochthonous bacteria (*Enterobacteriaceae*, *Alcaligenes*, *Aeromonas*, *Pseudomonas*, *Moraxella*, *Bacillus*, *Micrococcus*, *Actinomycetes*) on the survival of *V. parahaemolyticus* in estuarine water.

As seafood, which is the main source of food borne disease caused by *V. parahaemolyticus*, is generally stored at refrigerator temperatures, our results (spices reduced the survival of the bacterium at low temperatures) lead to the conclusion that spices have potential for reducing the risk of contamination by *V. parahaemolyticus* in seafood, and may be used in hurdle technology with low temperatures.

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SAŽETAK

U ovom istraživanju pretražena je antibakterijska aktivnost ukupno 16 začina finalne koncentracije 2,5 % prema *V. parahaemolyticus*, na temperaturi od 5 i 37 ºC. Anis, čili, klinčić, cimet, korijander, kumin, curry, češnjak, đumbir, origano, paprika, crni i bijeli papar, ružmarin, timijan i kurkuma, kupljeni su u maloprodaji od istog proizvođača. Prije pretraživanja antibakterijske aktivnosti, začini su pretraženi standardnim mikrobiološkim postupcima na: aerobne sporogene bakterije, sulfitreducirajuće klostridije, kvasce i plijesni, *Enterobacteriaceae, Escherichia coli*, koagulaza pozitivne stafilokoke, te prisutnost bakterija *Salmonella* spp. i *Listeria monocytogenes*. Za utvrđivanje anti-vibrio aktivnosti, 5 % otopini začina dodana je kultura *V. parahaemolyticus* u NaCl-BHI bujonu u broju 10^4 cfu/mL, te je uslijedila inkubacija 24 sata, a zatim nacjepljivanje na TCBS agar. Nakon utvrđivanja antibakterijske aktivnosti, utvrđena je i minimalna inhibicijska koncentracija za začine koji su pokazali jaku aktivnost. *Salmonella* spp., *Listeria monocytogenes, E. coli* i koagulaza-pozitivni stafilokoki nisu nađeni niti u jednom uzorku začina. Aerobne sporogene bakterije utvrđene su u 93,7 %, sulfitreducirajuće klostridije u 43,7 %, kvasci u 12,5 %, plijesni u 62,5 % i *Enterobacteriaceae* u 18,7 % uzoraka začina. Na temperaturi od 5 °C, svi začini osim anisa i korijandera pokazali su antibakterijsku aktivnost protiv *V. parahaemolyticus* smanjivši ukupan broj bakterija za najmanje 1 log cfu/mL, a jaku antibakterijsku aktivnost na toj temperaturi pokazali su origano, češnjak, timijan, klinčić, cimet, curry, ružmarin, đumbir i kurkuma. Na 37 °C, origano, češnjak, timijan, klinčić i cimet su pokazali antibakterijsku aktivnost, i to jaku. Minimalna inhibicijska koncentracija na 37 °C bila je 0,078 % za klinčić, i 0,0012 % za kurkumu na 5 °C. Nije primijećen utjecaj prateće mikroflore začina na broj *V. parahaemolyticus*. Ovi rezultati su pokazali da neki začini imaju potencijal za smanjenje rizika od *V. parahaemolyticus* u proizvodima ribarstva, a u kombinaciji s niskom temperaturom.

Ključne riječi: *Vibrio parahaemolyticus*, začini, antibakterijska aktivnost