

Antibacterial activity of black cumin, clove, and ginger essential oils against specific spoilage organisms of ready-to-eat chilli shrimp paste

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Abstract

Ready-to-eat (RTE) chilli shrimp paste has a short shelf life; thus, chemical additives are usually added to extend it. However, certain additives have potential health implications. In this regard, plant essential oils (EOs) which exhibit antibacterial properties can be used as an alternative for extending the shelf life of RTE chilli shrimp paste. The present work intended to (1) establish the microbial profile of RTE chilli shrimp paste, (2) determine the antibacterial activities of black cumin, clove, and ginger EOs against the isolated specific spoilage organisms (SSO) of RTE chilli shrimp paste, and (3) determine their effect on the shelf life of RTE chilli shrimp paste. Biochemical tests and 16S rRNA gene sequencing were used to identify the SSO in RTE chilli shrimp paste. Disc diffusion assay was performed for antibacterial analysis of EOs. To monitor the shelf life of the paste, total plate count (TPC) and yeast and mould count (YMC) were carried out for five days. The results showed that the SSO of RTE chilli shrimp paste were dominated by *Staphylococcus* spp. followed by *Klebsiella aerogenes* and *Enterobacter tabaci*. EOs of black cumin, clove, and ginger showed inhibitory effects against the SSO with the highest inhibition by ginger EO of 27.50 ± 9.19 to 58.00 ± 7.21 mm. Application of ginger EO in RTE chilli shrimp paste showed a 3-log reduction of bacterial population and 4-log reduction for fungal population. It was concluded that ginger EO can be a highly potential candidate to be added into RTE chilli shrimp paste as a natural additive to extend its shelf life.

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Keywords

chili shrimp paste,
essential oil,
antibacterial activity,
specific spoilage
organisms,
ready-to-eat

Introduction

Chilli shrimp paste, a favourite spicy condiment in Malaysia, can enhance the palate while giving an appetising effect during a meal (Abdul Rashid *et al.*, 2008; Karim *et al.*, 2011). It is considered a heritage in the Malaysian food culture, and is also popular in the Southeast Asian countries (Cheok *et al.*, 2017). The main ingredients of ready-to-eat (RTE) chilli shrimp paste such as fresh chillies, toasted fermented shrimp paste (*belacan*), and calamansi juice contain natural microflora such as moulds, yeasts, and bacteria (Steinkraus, 1996; Saraya *et al.*, 2009; Karim *et al.*, 2011; Sobhi *et al.*, 2012). Generally, RTE chilli shrimp paste is uncooked, thus can easily spoil over time (Babak *et al.*, 2010). Previous studies have reported that the paste can be safely consumed without deterioration for three days when refrigerated (Passmore, 1991). This proves that RTE chilli shrimp paste has a short shelf life. To prevent spoilage, chemical preservatives are used to extend its shelf life; however, preservatives such as butylated hydroxytoluene (BHT) can harm human health (Raesi *et al.*, 2016).

Saeed *et al.* (2019) reported that synthetic preservatives could lead to asthma, allergic reactions, and various types of cancers (Saeed *et al.*, 2019). Apart from the health issues, the consumers' demand for more 'green food' has become a major concern in the food industry nowadays since the community is more aware of the importance of a healthy lifestyle (Rana and Paul, 2017).

To extend the shelf life of the paste, plant essential oils can be used. Essential oils are composed of many kinds or classes of molecules such as terpenoids, phenolics, aromatics, cyclic and acyclic compounds, acetones, and sulphur- and nitrogen-containing compounds (Tongnuanchan and Benjakul, 2014). The molecules have the ability to act as an antibacterial, antifungal, and insecticidal agents (Burt, 2004; Bakkali *et al.*, 2008; Raut and Karuppaiyil, 2014). Previous studies have found that essential oils of extracted plants such as ginger could effectively inhibit *Staphylococcus aureus* and *Escherichia coli* (da Silva *et al.*, 2018). The EO of black cumin has the ability to target the bacterial cell envelope, thus damaging the cell and leads to bacterial lysis (Sufya *et al.*, 2014). Cava *et al.* (2007) reported that clove EO

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has inhibitive properties through the inhibition of protease and amylase production, inhibition of glucose uptake, and interference with the proton motive force, electron flow, and active transport. The hydrophobicity of EO is essential as it allows them to penetrate into the lipids of the cell membrane of bacteria, thus distracting the structure, and making it more permeable to disrupt the cell growth (Dhifi *et al.*, 2016). Therefore, the application of EOs in RTE chilli shrimp paste is believed to delay the spoilage and improve the safety of the product.

The present work thus aimed to improve the shelf life of RTE chilli shrimp paste by using natural preservatives, specifically black cumin, clove, and ginger EOs. The main objectives of the present work were to establish the microbial profile and SSO of RTE chilli shrimp paste, to determine the antibacterial activities of black cumin, clove, and ginger EOs against the isolated SSO of RTE chilli shrimp paste, and to monitor the shelf life of RTE chilli shrimp paste upon the application of the EOs.

Materials and methods

Raw materials

The ingredients of RTE chilli shrimp paste were purchased from Tesco Extra (Cheras, Selangor, Malaysia); fresh red chillies (*Capsicum annum*), fermented shrimp paste (*belacan*), calamansi (*Citrus microcarpa*) juice as a source of acid, bird's eye chili (*Cap. frutescens*), sugar, and salt.

Sample preparation

The RTE chilli shrimp paste was prepared as described by Nadia *et al.* (2010). Firstly, the stems of the chillies were removed before washing. Next, the chillies were drained for 10 min to remove excess water. Then, the wet shrimp paste was chopped into smaller pieces and heated in the oven at 180°C for 25 min until dry. The washed chillies, dried shrimp paste, calamansi juice, sugar, and salt were mixed for 45 s under sterile conditions using a kitchen blender. The RTE chilli shrimp paste prepared was left at room temperature respectively for 5 d, and was observed daily. Three independent batches of RTE chilli shrimp paste were prepared, and three samples per batch were analysed.

Enumeration and isolation of spoilage bacteria in RTE chilli shrimp paste

The RTE chilli shrimp paste was subjected to total plate count (TPC) and yeast and mould count (YMC) daily for 5 d. For TPC, 1 g of RTE chilli shrimp paste was added to 9 mL of peptone water, giving a

dilution of 1:10. A series of dilutions was carried out, and 0.1 mL aliquot of each dilution was plated onto the Total Plate Count agar (Oxoid, UK). Inoculated plates were then incubated at 35°C for 48 h. The number of colonies was counted and expressed in CFU/g. For YMC, 1 g of RTE chilli shrimp paste was subjected to the same dilution series, and plated onto Potato Dextrose agar (Oxoid, UK). Inoculated plates were then incubated at 30°C for 120 h. The number of colonies was counted and expressed in CFU/g.

At the same time, the isolated colonies were classified based on their morphology. A single isolate from each morphotype was selected for identification and further analysis. They were purified by streaking method on Nutrient agar (Oxoid, UK) for 24 h at 37°C, and preserved as stock culture for subsequent uses (Ruangpan and Tendecia, 2004).

Phenotypic characteristic of isolated SSO

The isolated presumptive SSO was subjected to Gram-staining reaction and biochemical tests for identification (Rath and Bera, 2014).

Gram-staining

A smear was prepared, and the slide was flooded with a crystal violet stain, and left for 1 min. The crystal violet stain was then rinsed with water. Next, the slide was flooded with Gram's iodine solution for 1 min, and rinsed with water. The slide was held slanted and flooded with 95% alcohol for decolourisation, and rinsed with water to stop the decolourisation, and counterstained with safranin red for 1 min. The slide was then rinsed and blotted dry before viewed under a microscope.

Biochemical tests

The methods for confirmation included citrate, carbohydrate utilisation, catalase, oxidase, coagulase, urease, and motility tests, and were carried out as described by Andrews (1992). The Simmon's citrate agar was used to observe the citrate utilisation. Carbohydrate utilisation was carried out with glucose, lactose, sucrose, and mannitol peptone's solution. Slide technique with a drop of 3% H₂O₂ was used to perform the catalase test. Oxidase reagent was implemented for the oxidase test. The coagulase test was carried out by emulsifying the colony into coagulase plasma. Next, Christensen's agar slant was inoculated with the colony to perform the urease test. Finally, the stab line test was performed as a motility test.

16S rRNA gene sequencing for bacterial identification

The molecular identification was carried out as described by Fguiri *et al.* (2015) for further confirmation of isolated bacteria from the RTE chilli shrimp paste. The genomic DNA of isolated SSO of chilli shrimp paste was extracted using the DNA extraction and purification kit BigDye® Terminator v3.1 Cycle (Applied Biosystems) according to the manufacturer instructions. The bacterial 16S rDNA, full-length 1.5 kb, was amplified using universal primers 27F (5' AGAGTTTGATCMTGGCTCAG 3') and 1492R (5' TACGGYTACCTTGT-TACGACTT 3'). The total reaction volume of 25 µL contained gDNA purified using an in-house extraction method; 0.3 pmol of each primer, deoxynucleotides triphosphates (dNTPs, 400 µM each), 0.5 U DNA polymerase, supplied PCR buffer, and water. The PCR was performed as follows: one cycle (94°C for 2 min) for initial denaturation, and 25 cycles (98°C for 10 s; 53°C for 30 s; 68°C for 1 min) for annealing and extension of the amplified DNA. The PCR products were purified by standard methods, and directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The obtained nucleotide sequences were analysed using the blast tool of the NCBI site to obtain the identity percentages with the sequences present in the database.

Evaluation of antibacterial activities of EO on SSO of RTE chilli shrimp paste (model system)

The Kirby-Bauer disc diffusion method was used to measure the inhibitory effect of EOs. The isolated overnight bacteria were suspended into 5 mL sterile phosphate buffered saline solution, and adjusted to 0.5 McFarland standards (1.5×10^8 CFU/mL). 0.1 mL of diluted inoculum were uniformly spread on Mueller Hinton agar (Oxoid, UK) plates using a sterile cotton swab. Sterile paper discs (Whatman AA discs, 6 mm in diameter) were used to impregnate 100% of 10 µL of three essential oil samples black cumin (*Nigella sativa*), ginger (*Zingiber officinale*), and clove (*Syzygium aromaticum*) (commercial EOs, FSTM, UPM).

Gentamicin discs (10 µg) (Oxoid, UK) for Gram-negative bacteria and penicillin discs (10 µg) (Oxoid, UK) for Gram-positive bacteria were used as positive controls. The negative control used was 10 µL of sterile distilled water pipetted on a blank disc. All plates were incubated at 37°C for 24 h, and the diameters of zones of inhibition (mm) were measured.

Application of EO on RTE chilli shrimp paste (food system)

The RTE chilli shrimp paste was

supplemented with 10 µL (v/w) of the best inhibitory actions of EO based on the disc diffusion assay to give a final concentration of 0.01% (Fazlara *et al.*, 2008). The growth was monitored in comparison with the control that contained no EO. The TPC and YMC were recorded daily for 5 d during storage at room temperature (27°C).

Statistical analysis

One-way ANOVA and Tukey's test were used to tests significant effects ($p < 0.05$) of the addition of EO into RTE chilli shrimp paste.

Results and discussion

Identification of SSO of chilli shrimp paste

The phenotypic characteristics of isolates A to J from RTE chilli shrimp paste are shown in Table 1. The results showed that all isolates had different reactions towards the biochemical tests. Isolates A and B were Gram-negative bacteria, while isolates C-J were Gram-positive. Isolates C, D, G, and J tested positive for coagulase, glucose, lactose, and sucrose tests; and negative for motility test, thus could be identified as *Staphylococcus* spp. Throughout the analysis, six genera were found in the RTE chilli shrimp paste, and presumed as SSO. They were *Klebsiella* spp., *Enterobacter* spp., *Staphylococcus* spp., *Micrococcus* spp., *Streptococcus* spp., and *Enterococcus* spp.

The presumptive SSO was further confirmed by using 16S rDNA technique, and five genera were present in the RTE chilli shrimp paste (Figure 1). The RTE chilli shrimp paste was dominated by *Staphylococcus* spp. (80%), followed by *Klebsiella aerogenes* (10%), and *Enterobacter tabaci* (10%). *Staphylococcus gallinarum*, *S. kloosii*, *S. hominis*, *Klebsiella aerogenes*, and *Enterobacter tabaci* yielded 99 to 100% similarity to species existing in the GenBank (Table 2). Some of these bacteria were also mentioned by Steinkraus (1996) to be present in the ingredients of chilli shrimp paste, with the exception of *Klebsiella* spp. *Klebsiella*, *Enterobacter*, *Micrococcus*, *Staphylococcus*, and *Streptococcus* are expected to invade the paste from the raw ingredients as reported in Saraya *et al.* (2009). For *Staphylococcus* spp. and *Enterobacter* spp., there can be several factors involved such as cross-contamination, inadequate cleaning of processing equipment, utensils, and storage in a contaminated area (Bennett *et al.*, 2018). Meanwhile, the presence of *Klebsiella* in RTE chilli shrimp paste could be due to the poor standard when washing the red chillies which could not significantly diminish the attachment of bacteria

Table 1. Phenotypic characteristic of bacterial strains isolated from ready-to-eat chilli shrimp paste.

Biochemical test	Bacterial strain									
	A	B	C	D	E	F	G	H	I	J
Gram reaction	-rod	-rod	+cocci	+cocci	+cocci	+cocci	+cocci	+cocci	+cocci	+cocci
Catalase test	+	-	-	+	+	-	+	-	-	+
Citrate test	+	+	-	-	-	-	+	-	-	-
Oxidase test	-	-	-	-	-	-	-	-	-	-
Coagulase test	-	-	+	+	-	-	+	-	-	+
Urease test	-	-	-	+	+	+	+	-	-	-
Glucose test	-	+	+	+	+	+	+	+	+	+
Lactose test	+	+	-	-	+	+	-	-	-	-
Sucrose test	+	+	+	+	+	-	+	+	+	+
Mannitol test	+	+	-	+	+	+	+	+	+	+
Motility test	+	+	-	-	+	+	+	+	+	-

(+) = Positive reaction, and (-) = Negative reaction. Presumptive bacteria: A = *Klebsiella* spp., B = *Enterobacter* spp., C = *Staphylococcus* spp., D = *Staphylococcus* spp., E = *Micrococcus* spp., F = *Enterococcus* spp., G = *Staphylococcus* spp., H = *Streptococcus* spp., I = *Streptococcus* spp. and J = *Staphylococcus* spp.

Table 2. Molecular identification of bacterial isolates of specific spoilage organisms from ready-to-eat chili shrimp paste during 5-day storage using 16S rDNA gene sequencing technique.

Bacterial strain	Existing strain in the GenBank	Similarity (%)	NCBI gene bank accession number
A	<i>Klebsiella aerogenes</i>	99	NR_114737.1
B	<i>Enterobacter tabaci</i>	99	NR_146667.2
C	<i>Staphylococcus gallinarum</i>	100	NR_036903.1
D	<i>Staphylococcus gallinarum</i>	100	NR_036903.1
E	<i>Staphylococcus kloosii</i>	99	NR_024667.1
F	<i>Staphylococcus kloosii</i>	99	NR_024667.1
G	<i>Staphylococcus gallinarum</i>	100	NR_036903.1
H	<i>Staphylococcus kloosii</i>	99	NR_024667.1
I	<i>Staphylococcus hominis</i> subsp. <i>novobiosepticus</i>	99	NR_036956.1
J	<i>Staphylococcus gallinarum</i>	100	NR_036903.1

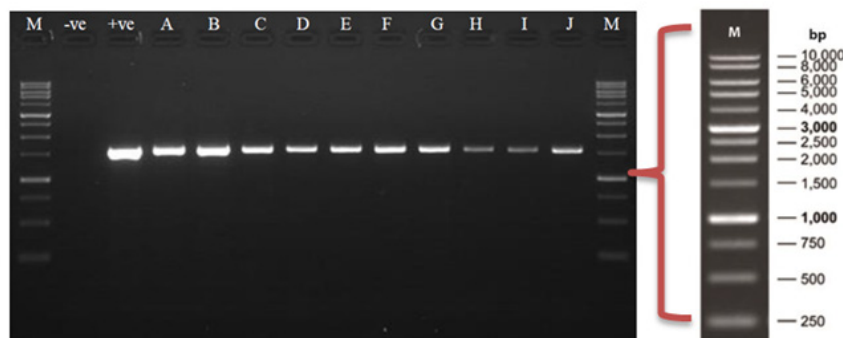


Figure 1. Isolated DNA bands of presumptive specific spoilage organisms from ready-to-eat chilli shrimp paste. Lane: M = Marker, A = *Klebsiella* spp., B = *Enterobacter* spp., C = *Staphylococcus* spp., D = *Staphylococcus* spp., E = *Micrococcus* spp., F = *Enterococcus* spp., G = *Staphylococcus* spp., H = *Streptococcus* spp., I = *Streptococcus* spp. and J = *Staphylococcus* spp.

on the fruit surfaces (Podschun and Ullmann, 1998).

Antibacterial activity of EO against SSO of RTE chilli shrimp paste

The antibacterial activity of black cumin, clove, and ginger EO against the SSO of RTE chilli shrimp paste is presented in Table 3. In general, all three EOs showed an inhibitory effect against the growth of the SSO of RTE chilli shrimp paste. This agrees with Paster *et al.* (1990) and Mardafkan *et al.* (2015) where they concluded that both Gram-positive and Gram-negative bacteria are sensitive to EOs. All the values from disc diffusion assay (DDA) were found to be significantly different ($p < 0.05$). EOs generate compounds that are responsible for the disruption of the cytoplasmic membrane, the driving force of protons, electron flow, active transport, and coagulation of cell contents (Canillac and Mourney, 2001; Marino *et al.*, 2001).

For Gram-positive isolates, the inhibitory effect of ginger EO was the most effective as compared to black cumin and clove EO with an inhibition zone of 27.50 ± 9.19 to 58.00 ± 7.21 mm. For Gram-negative isolates, clove EO showed the greatest inhibitory effect with an inhibition zone of 12.67 ± 0.58 mm. Black cumin EO was found to be ineffective against *Enterobacter* spp. The observed inhibitory effects were even greater than penicillin (positive control) with an inhibition zone of 14.67 ± 0.58 to 29.67 ± 0.58 mm. Azhar *et al.* (2010) also

reported that ginger extract presented higher diameters of inhibition zones for *Streptococcus* spp. as compared to ciprofloxacin, cefotaxime, cefalotin, cephalixin, and cephaloridine. This scenario may be possible because ginger EO possesses 30 major organic compounds dominated by α -zingiberene which acts as an antibacterial agent (Noori *et al.*, 2018). Furthermore, Burt (2004) reported that the inhibition of microorganisms by ginger EO occurred both *in vitro* and *in vivo*. The ineffectiveness of black cumin EO inhibition against *Enterobacter* spp. was also found in a previous study (Bakathir and Abbas, 2011).

Based on Table 3, Gram-positive isolates were more susceptible to the effects of EOs as compared to Gram-negative. This is because the cell wall of Gram-positive bacteria is less complex and lacks the natural sieve effect against large molecules due to the small pores in their cell envelope (Fagere and Al Magbou, 2016). Meanwhile, the outer membrane of Gram-negative bacteria cell wall restricts the diffusion of hydrophobic compounds through its lipopolysaccharide layer, thus rendering it less susceptible to EOs (Dhifi *et al.*, 2016).

The effect of ginger EO on RTE chilli shrimp paste for TPC

From the DDA, ginger EO showed the highest inhibition towards SSO of RTE chilli shrimp paste, and was selected to be added into RTE chilli

Table 3. Antibacterial activity of essential oils against specific spoilage organisms of ready-to-eat chilli shrimp paste.

Bacterial isolates	Essential oils (zone of inhibition in mm)				
	Positive control	Negative control	Black cumin	Ginger	Clove
<i>Klebsiella aerogenes</i>	17.67 ± 0.58^a	-	7.67 ± 0.58^c	6.67 ± 0.58^c	12.67 ± 0.58^b
<i>Enterobacter tabaci</i>	17.00 ± 1.00^a	-	-	8.00 ± 0.00^c	12.67 ± 0.58^b
<i>Staphylococcus gallinarum</i>	22.00 ± 0.00^b	-	6.50 ± 0.71^d	27.50 ± 9.19^a	15.00 ± 0.00^c
<i>Staphylococcus gallinarum</i>	23.00 ± 1.00^b	-	7.00 ± 0.00^c	31.33 ± 1.53^a	19.67 ± 0.58^{bc}
<i>Staphylococcus kloosii</i>	26.00 ± 0.00^b	-	8.67 ± 0.58^d	41.33 ± 10.26^a	13.33 ± 0.58^c
<i>Staphylococcus kloosii</i>	18.33 ± 1.15^b	-	7.00 ± 0.00^d	41.33 ± 7.57^a	14.00 ± 1.00^b
<i>Staphylococcus gallinarum</i>	23.33 ± 0.58^b	-	8.00 ± 0.00^d	31.67 ± 0.58^a	12.33 ± 0.58^c
<i>Staphylococcus kloosii</i>	14.67 ± 0.58^b	-	8.33 ± 0.58^b	58.00 ± 7.21^a	15.67 ± 0.58^b
<i>Staphylococcus hominis</i> subsp. <i>novobiosepticus</i>	29.67 ± 0.58^b	-	10.67 ± 0.58^c	56.00 ± 6.00^a	18.33 ± 0.58^c
<i>Staphylococcus gallinarum</i>	23.33 ± 0.58^b	-	8.33 ± 0.58^d	33.67 ± 0.58^a	13.33 ± 0.58^c

(-) = Diameter of the inhibitory zone, < 6 mm considered as no antibacterial activity. Values are mean diameter of inhibition zone (mm) \pm SD of three replicates. Different superscript letters in a column are significantly different ($p < 0.05$). Positive control = penicillin and gentamicin; negative control = sterile distilled water.

shrimp paste as a food model system. Figure 2 presents the TPC in RTE chilli shrimp paste during 5 d of storage at room temperature (27°C) which show considerable inhibition of 3-log reduction in TPC with the addition of ginger EO. The total population of bacteria of RTE chilli shrimp paste treated with ginger EO was less than in control (without ginger EO).

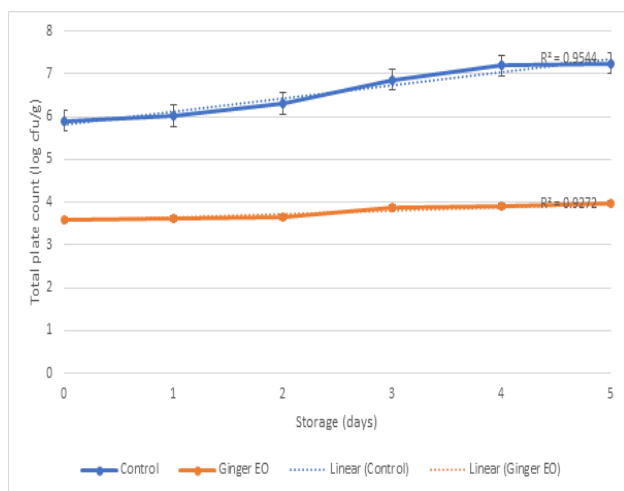


Figure 2. Total plate count of ready-to-eat chilli shrimp paste stored at room temperature for five days. Error bars indicate the standard deviation.

The observed antibacterial potency of ginger EO is due to the considerable amount of phenolic compounds such as eugenol, shogaols, zingerone, gingerdiols, and gingerols. Besides, ginger EO, which is rich in zingiberene (31.79%), a sesquiterpene compound, shows a relatively wide spectrum of antimicrobial activity. Burt (2004) and Sa-Nguanpuag *et al.* (2011) reported that the inhibition of microorganisms by ginger EO occurred both *in vitro* and *in vivo*. They also recommended ginger EO to be

added to fresh produce or minimally processed products to reduce the population of spoilage microorganisms. This indicated that ginger EO could be able to preserve the quality and extend the shelf life of RTE chilli shrimp paste.

The effect of ginger EO on RTE chilli shrimp paste for YMC

The conditions of food such as low pH, low water activity, or high carbohydrate content are usually unfavourable for the growth of bacteria with some exception; but, yeasts and moulds can still grow under these conditions and cause deterioration of various products. Figure 3 shows the YMC of RTE chilli shrimp paste during 5 d of storage at room temperature. The YMC of RTE chilli shrimp paste added with ginger EO was lower as compared to the control (without the addition of ginger EO), with a 4-log reduction after 5 d of storage. In this regard, Ponce *et al.* (2003) reported that EOs containing active compounds have shown antifungal and antibacterial properties. Ginger EO containing non-phenolic compounds has also been found to show high toxicity against yeasts (Krisch *et al.*, 2011). Krisch *et al.* (2011) suggested that the antifungal effect of EOs is affected by environmental factors such as water activity, where the higher the water activity, the higher the inhibition effect of EOs.

Conclusion

The present work demonstrated that RTE chilli shrimp paste was contaminated by SSO dominated by *Staphylococcus* spp. In order to control the spoilage, EO of ginger can be applied since it has been proven to reduce the SSO loads of the paste during the 5-day storage. Therefore, ginger EO is

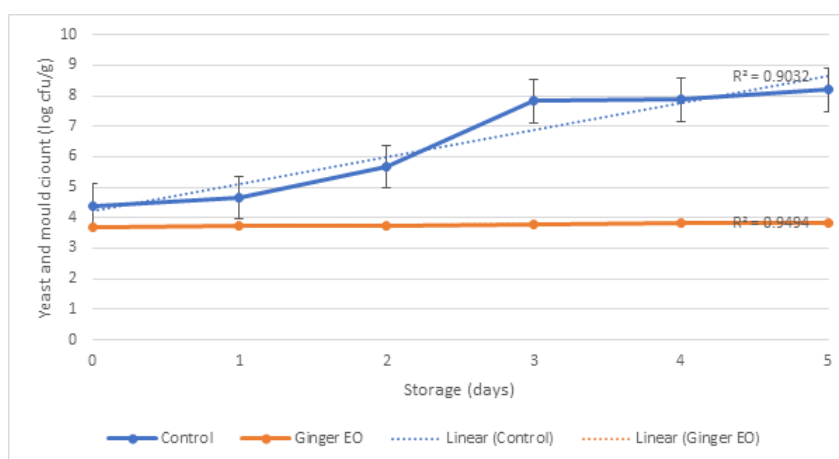


Figure 3. Yeast and mould count of ready-to-eat chilli shrimp paste stored at room temperature for five days. Error bars indicate the standard deviation.

recommended as a natural food additive and preservative since it exhibits moderate to significant antimicrobial properties which are a new application of food technology.

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