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Isolation and Identification of Bacteria from Raw Materials Contaminated by Rope-Producing Bacteria

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Ropey bread is a bacterial spoilage condition of bread. The spoilage involved in ropey bread is primarily due to *Bacillus subtilis*. Several studies have shown that rope spoilage can be controlled using antimicrobial agents such as acetic acid, lactic acid, and quaternary ammonium cations (QACs/quats). This research consisted of five main steps: isolation, identification, confirmation, and molecular characterization of bacteria from raw materials contaminated by rope-producing bacteria, and antimicrobial test against rope-producing bacteria. The confirmation test was done in order to determine the rope-producing ability of isolates suspected as *Bacillus* sp. There were two different treatments in this test. In the first treatment, the inoculums were mixed with bread dough. In the second treatment, each slice of loaf was placed into a petri dish, uniformly soaked with inoculums. The first treatment did not show rope spoilage for all of the loaves, however, 6 of 11 loaves in the second treatment developed rope. The largest inhibition halos for antimicrobial test were produced by quats. This means quats is the strongest antimicrobial agent against rope-producing bacteria. The molecular characterization showed that all of the suspected isolates had 98-99% similarity to *B. subtilis*.

Key words: antimicrobial agents, *Bacillus subtilis*, identification, isolation, ropey bread

Ropey bread merupakan kerusakan pada roti yang disebabkan oleh bakteri. Bakteri yang terlibat dalam kerusakan *ropiness* berasal dari *genus Bacillus*, terutama *Bacillus subtilis*. Beberapa studi menunjukkan bahwa kerusakan *ropiness* dapat dikontrol menggunakan agen antimikrob seperti asam asetat, asam laktat, dan *quaternary ammonium cations* (QACs/quats). Penelitian ini dibagi menjadi lima tahapan, yaitu isolasi, identifikasi, konfirmasi, dan karakterisasi molekuler bakteri dari bahan-bahan mentah yang terkontaminasi bakteri penyebab *ropey bread*, serta uji antimikrob terhadap bakteri penyebab *ropey bread*. Konfirmasi bakteri yang dilakukan ini dibagi menjadi dua perlakuan. Pada perlakuan pertama, inokulum dicampurkan dengan adonan roti. Pada perlakuan kedua, tiap-tiap potongan roti ditempatkan dalam cawan petri steril yang telah berisi inokulum. Perlakuan pertama tidak menunjukkan kerusakan *ropiness* pada semua potongan roti, tetapi 6 dari 11 potongan roti pada perlakuan kedua memberikan hasil yang positif terjadinya kerusakan. Zona bening terbesar pada uji antimikrob diproduksi oleh quats. Dengan demikian, quats merupakan agen antimikrob terkuat terhadap bakteri penyebab *ropey bread*. Identifikasi bakteri penyebab *ropey bread* secara molekuler menunjukkan bahwa seluruh isolat yang dipilih memiliki kemiripan 98-99% dengan *B. subtilis*.

Kata kunci: agen antimikrob, *Bacillus subtilis,* identifikasi, isolasi, *ropey bread*

Ropey bread is a bacterial spoilage condition of bread. The spoilage is characterized by a sweet fruity odor, similar to that of over-ripe melons or pineapples. Subsequently enzymatic degradation result in patchy discoloration and the bread eventually becomes very soft and sticky to the touch due to breakdown of starch and proteins by microbial amylases and proteases, and by the production of extracellular slimy polysaccharides (Yibar *et al*. 2012).

In the bread making process, the raw materials such as flour, yeast starter, butter, water, salt, sugar, and egg, are mixed and knead until the dough becomes smooth. The dough is then rounded and left to rise by fermentation of the yeast starter. The loaf is subsequently baked at 160 °C and thereafter the loaf is cooled before slicing or wrapping. If the raw materials contaminated by rope-producing bacteria, the high temperature used in the baking process will cause spore formation. Furthermore, the moist environment after the baking process will enhance spore germination and growth of vegetative cells of the bacteria (Yibar *et al*. 2012).

The bacteria involved in ropiness are from genus *Bacillus*, primarily *Bacillus subtilis* (formerly referred to as *B. mesentericus*). However, *B. licheniformis*, *B. megaterium* (Sorokulova *et al*. 2003), *B. pumilus*, *B. cereus* (Valerio *et al*. 2012), and *B. subtilis* can also be the causative agents of ropey bread (Rumeus and Turtoi 2013). *Bacillus* species are aerobic, Gram-positive, and able to form endospore that are resistant to high temperature. These bacteria are common soil inhabitants and may contaminate raw materials or

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bakery equipments used. The raw materials commonly used are flour, yeast, butter, and water. All types of flour were contaminated with *Bacillus* species, although flour is generally considered as microbiologically safe product as it is a low water-activity commodity (Yibar *et al*. 2012).

Bacterial spores cause major problems in the food industry due to their stress resistance. Spores that have retained their viability during baking can germinate and cause ropiness if they are exposed to a warm and moist environment. The water-activity (a_w), pH, and temperature during storage may also play important roles in spore germination and growth of vegetative cells of *Bacillus* species (Yibar *et al*. 2012). Ropiness occurs particularly when warm (25-30 °C) and humid (water-activity, ≥ 0.95) environmental conditions allow spore germination. Moreover, rope-producing bacteria are also characterized by faster development and enhanced proteases and amylases production during growth in the bread crumb (Pepe *et al*. 2003).

Rope-producing bacteria have the potential to cause disease in humans. *B. subtilis* can cause conjunctivitis (Alloyna 2011). *B. pumilus* are capable of causing food borne illness (Matarante *et al*. 2004). *B. licheniformis* causes bacteremia, peritonitis, gastrointestinal disease, and eye infection (Haydushka *et al*. 2012). *B. megaterium* produces enterotoxin that is also important in the symptoms of food borne illness (Lopez & Alippi 2010). *B. cereus* produces two types of toxins that have been associated with diarrheic (heat-labile) and emetic (heat-stable) toxins (Yibar *et al*. 2012).

The usual recommended control procedures such as sanitation of bakery equipments, stringent temperature control during baking, and testing of raw materials may reduce the initial spore counts in dough, but do not prevent germination and growth of *Bacillus* spores in non-acidified bread without added preservatives (antimicrobial). Several studies were made for inhibition of ropiness including usage of antimicrobial agents such as acetic acid, lactic acid, and quaternary ammonium cations (QACs) (Erem *et al*. 2009). However, acetic acid, used singly, has a negative effect on the organoleptic qualities of the baked product. Reducing the concentration of acetic acid, and using it in combination with other antimicrobial agents such as lactic acid, may thus be an alternative approach. Lactic acid is commonly used as flavor enhancers and humectants in meat products, but has not been evaluated as bread preservatives (Pattison *et al*. 2004).

Quaternary ammonium cations, frequently called the quats are used most frequently as antimicrobial agents. Quats with low concentration are able to inhibit bacteria growth, but do not kill the bacteria. Previous study (Malek and Malek 2012) showed that Quats were capable used as antimicrobial agents against *B. subtilis* that is one of rope-producing bacteria.

The aims of the present study were to isolate and identify the bacteria from raw materials contaminated by rope-producing bacteria, and to test antimicrobial agents against rope-producing bacteria.

MATERIALS AND METHODS

Samples. The raw materials of bread such as flour, yeast starter, water, and butter were collected from a local bakery that produced ropey bread in Jakarta.

Isolation of Bacteria from Raw Materials Contaminated by Rope-producing Bacteria. To isolate the bacteria, 1 g of each sample (1 mL water) were aseptically weighed and homogenized with 9 mL sterile aquades. Ten-fold serial diution of the suspensions (10^0, 10^{-1}, 10^{-2}, and 10^{-3} g mL^{-1}) were prepared with the same diluent, and 100 µL aliquot of each dilution was inoculated on nutrient agar (NA) using spreader and incubated at 37 °C for 24 h. The colonies were counted (CFU mL^{-1}). As many as 6-8 chosen colonies were streaked on NA, and incubated at 37 °C for 24 h in order to obtain pure isolates (Erem *et al*. 2009). The contaminated raw materials in this study were also compared to raw materials that were sold in the market. Therefore, flour sample from local market was collected to isolate the suspected bacteria and the chosen isolates were subsequently treated in conjunction with the other suspected isolates from contaminated raw materials.

Identification of Rope-producing Bacteria. Identification was performed using biochemical tests: Gram staining, spore staining, microscopy observation (phase contrast), protease, amylase, formation of indole, catalase, oxidase, Voges-Proskauer test, growth at 50 °C, and anaerobic growth (Erem *et al*. 2009).

Confirmation Test for the Determination of Rope-producing Bacteria. There were two different treatments in this test. In the first treatment, *Bacillus* species were grown at 37 °C in nutrient broth (NB) for 72 h diluted to an OD_{600} of 0.350, and the inoculums were mixed with bread dough and cooked at 160 °C for 15 min. In the second treatment, each slice of loaf (cooked at 160 °C for 15 min) was placed into a petri dish, uniformly soaked with 1 mL of inoculums. After cooling, all treated loaves were stored at 30 °C for 96 h

and examined for rope production (Thompson *et al*. 1998).

Molecular Characterization of Rope-producing Bacteria. Genomic DNA extraction was conducted using colony pick PCR. A single colony of each isolate was picked and dissolved in 10 µL ddH_2O in order to be amplified. The PCR conditions used with 2 µL DNA template, 2 µL primer 63f and 1387r, 25 µL MyTaq HS Red Mix (Bioline) 2X, and 19 µL ddH_2O. The PCR was performed with the following optimized thermal profile: initial denaturation at 95 °C for 120 s followed by 40 cycles with 60 s of denaturation at 95 °C, 60 s of primer annealing at 55 °C and 90 s of primer extension at 72 °C and as last step final elongation at 72 °C for 600 s. Amplification size of about 1300 bp was checked by electrophoresis on agarose gel (1%) at 80 V for 70 m. A 1 kb DNA ladder was used as the molecular weight marker (Ryzinska-Paier *et al*. 2011). The restriction enzyme *Pst* I was used for digestion in ARDRA (amplified ribosomal DNA restriction analysis) assay in order to distinguish the band patterns of isolates. These isolates with different profiles were sent to Genetika Science, Malaysia for sequencing. Data analysis nucleotide sequence was analyzed using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI) for identification.

Antimicrobial Test Against Rope-producing Bacteria. The antimicrobial test was carried out by agar well diffusion method. Mueller Hinton agar (MHA) was swabbed (sterile cotton swab) with 24 h old-broth culture of *Bacillus* species (diluted to an OD_{600} of 0.132). Wells (10 mm) were made in MHA using sterile cork borer. Ten-fold serial dilution of antimicrobial agents (10^0, 10^{-1}, 10^{-2}, and 10^{-3} mg mL^{-1}) such as acetic acid, lactic acid, and quats were prepared with the same diluent. About 50 µL aliquot of each dilution was added into the wells and allowed to diffuse at room temperature for 2 h. The plates were incubated at 37 °C for 24 h. The diameter of the inhibition zone was measured.

RESULTS

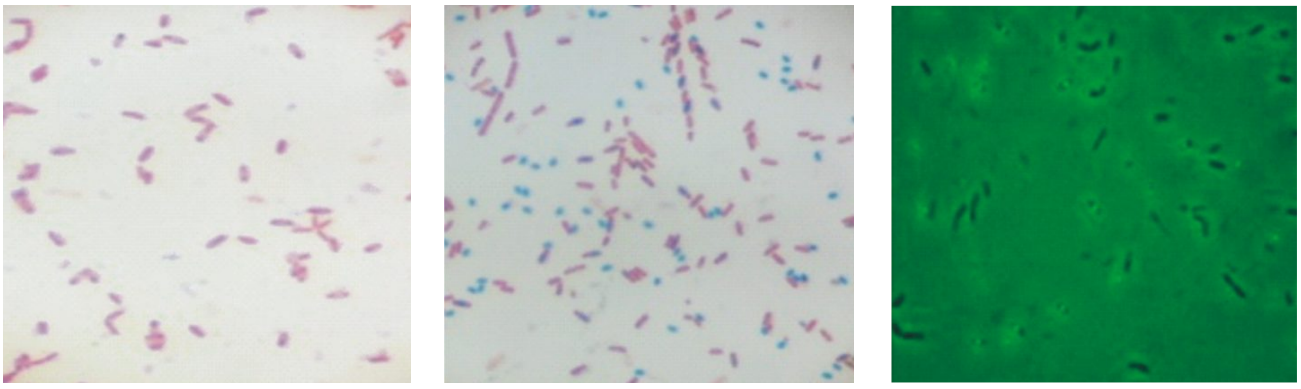
Isolation and Identification of Rope-producing Bacteria. The selected isolates were chosen based on Gram staining, spore staining, and microscopy observation (phase contrast) that showed characteristics of *Bacillus* species, Gram-positive, and endospore forming (Fig 1).

The biochemical test results in this study are summarized in Table 1. As many as 11 isolates were suspected as *Bacillus* species. The most widely isolates were obtained from water. The various results between biochemical tests were shown in protease test, amylase test, and growth at 50 °C. High protease activities were shown by isolate GK5 and MP7, whereas high amylase activities were shown by GK1, A2, and A3.

However, there were no significant differences between all isolates based on protease and amylase activity, as well as the capability of the isolates to grow at 50 °C.

Confirmation Test for the Determination of Rope-Producing Bacteria. The first treatment of confirmation test did not show rope spoilage for all of the loaves, however, 6 of 11 loaves in the second treatment developed rope (Fig 2). The rope spoilage was shown by discoloration and sweet fruity odor from the loaves. The other five loaves did not show negative results. The fifth loaves also showed ropiness, however the discolorations did not show significant results.

Molecular Characterization of Rope-producing Bacteria. All suspected isolates showed the same band patterns according to ARDRA assay with *Pst*I restriction

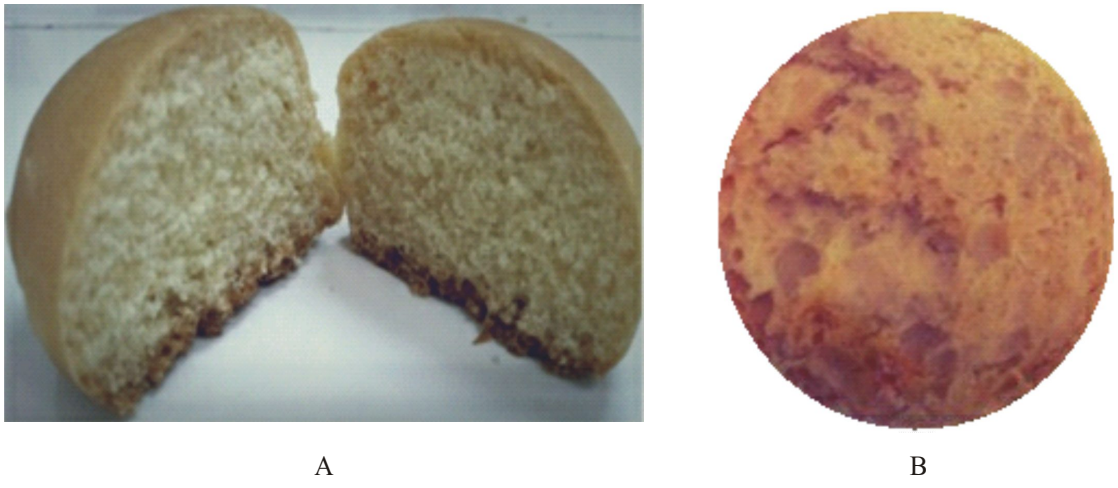


A B C

Fig 1 Gram staining (A), spore staining (B), and microscopy observation results for suspected species (C).

Table 1 Biochemical test results of the suspected isolates

Source	Code	Gram	Spore	Proteas	Amylase	Indole	Catalase	Oxidase	VP	Growth at 50 °C	Anaerobic growth
Flour	GK1	+	+	13[a]	9[b]	-	+	+	-	+++	-
Flour	GK3	+	+	13	8	-	+	+	-	+++	-
Flour	GK5	+	+	14	8	-	+	+	-	+++	-
Flour	GK6	+	+	12	5	-	+	+	-	+++	-
Yeast Starter	MP7	+	+	14	8	-	+	+	-	+++	-
Water	A2	+	+	12	9	-	+	+	-	+++	-
Water	A3	+	+	13	9	-	+	+	-	+++	-
Water	A4	+	+	13	5	-	+	+	-	+++	-
Water	A5	+	+	13	5	-	+	+	-	+++	-
Water	A6	+	+	12	6	-	+	+	-	++	-
Butter	M2	+	+	13	5	-	+	+	-	++	-



A B

Fig 2 Confirmation test result with first treatment (A) which showed negative results and second treatment (B) which showed positive results.

enzyme (Fig 3). This means all of the suspected isolates belonged to genus *Bacillus* with the same species. Since the results of ARDRA assay showed the same profiles, the molecular characterization only used two isolates (GK1 and GK3) for representation.

The suspected isolates were determined as *B. subtilis* (Table 2). The suspected isolate (BS1) from flour sample (raw materials) was also identified based on molecular characterization and the result was it had also similarity profile to *B. subtilis*.

Antimicrobial Test Against Rope-Producing Bacteria. The antimicrobial test results in this study are summarized in Table 3. The largest inhibition halos (diameters, cm) were produced by quats due to the capability to inhibit until the highest dilution (10^{-2}) and have the largest inhibition halos, depicted by isolate M2 and Gk3.

DISCUSSION

Microbial spoilage is the major problem causing economic losses as well as possible food borne illness risks. Rope spoilage is one of the frequent problems, occurring most frequently in baking industry and usually caused by *Bacillus* species. The objectives of this study were to isolate and to identify the bacteria from raw materials contaminated with rope-producing bacteria, and to test antimicrobial agents against rope-

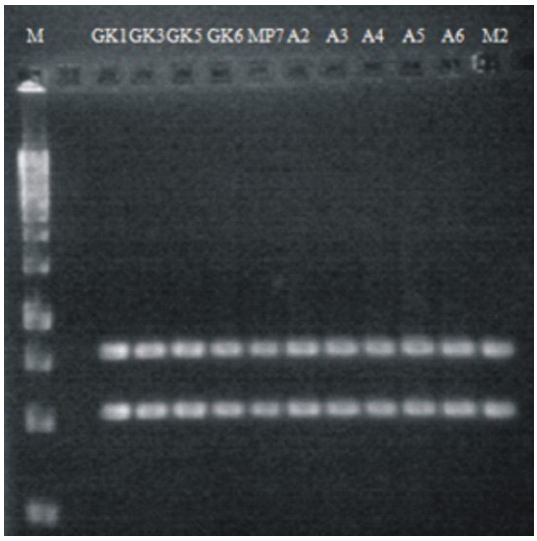


Fig 3 ARDRA assay results.

Table 2 DNA sequencing analysis of 16S rRNA gene of suspected isolates

Isolate code	Species	Similarity	Accession number
GK1	*Bacillus subtilis*	99%	JX944823.1
GK3	*Bacillus subtilis*	98%	AB905422.1
BS1	*Bacillus subtilis*	98%	JF262038.1

Table 3 Antimicrobial test results of the suspected isolates. NA-No activity; a-Values, including diameter of well (10 mm), are means of two replicates; b± Standard deviation

Code	Diameters of inhibition halos (cm[a])									Distilled water
	Acetic acid (AA)			Lactic acid (LA)			Quats (Q)			
	10^{0}	10^{-1}	10^{-2}	10^{0}	10^{-1}	10^{-2}	10^{0}	10^{-1}	10^{-2}	
GK1	2.4 ± 0.0 [b]	0.9 ± 0.1	NA	2.9 ± 0.7	1.6 ± 0.3	0.2 ± 0.2	2.5 ± 0.9	1.9 ± 1.3	1.2 ± 1.1	NA
GK3	3.2 ± 0.2	1.0 ± 0.1	NA	3.1 ± 0.1	0.8 ± 1.1	0.1 ± 0.1	3.4 ± 0.4	3.2 ± 0.6	2.2 ± 0.3	NA
GK5	2.7 ± 0.2	0.5 ± 0.6	NA	3.3 ± 0.9	1.6 ± 0.1	0.3 ± 0.4	2.6 ± 1.0	1.8 ± 1.6	1.5 ± 1.3	NA
GK6	1.7 ± 0.7	0.3 ± 0.4	NA	2.3 ± 1.3	1.4 ± 0.6	0.2 ± 0.2	2.7 ± 1.3	2.0 ± 1.5	1.3 ± 1.8	NA
MP7	3.8 ± 0.0	0.9 ± 0.0	NA	3.4 ± 0.1	2.0 ± 0.6	NA	3.3 ± 0.0	3.1 ± 0.1	2.0 ± 0.2	NA
A2	3.0 ± 0.0	0.2 ± 0.0	NA	3.7 ± 0.2	2.0 ± 0.3	0.5 ± 0.1	1.8 ± 0.0	1.0 ± 0.0	0.4 ± 0.0	NA
A3	2.8 ± 0.4	1.1 ± 0.4	NA	3.2 ± 0.1	1.9 ± 0.4	0.2 ± 0.3	3.1 ± 0.1	2.8 ± 0.3	2.1 ± 0.3	NA
A4	4.0 ± 0.0	1.3 ± 0.0	NA	3.6 ± 0.0	3.2 ± 0.0	NA	3.3 ± 0.1	3.0 ± 0.1	1.2 ± 1.6	NA
A5	2.9 ± 1.0	1.0 ± 0.4	NA	3.2 ± 0.1	2.3 ± 1.1	0.2 ± 0.2	3.1 ± 0.1	2.8 ± 0.1	1.9 ± 0.0	NA
A6	2.8 ± 0.0	1.1 ± 0.0	NA	3.2 ± 0.0	2.2 ± 0.0	0.4 ± 0.0	3.2 ± 0.0	3.0 ± 0.0	2.0 ± 0.0	NA
M2	3.8 ± 0.0	1.1 ± 0.0	NA	3.2 ± 0.0	2.3 ± 0.7	0.5 ± 0.1	3.9 ± 0.8	3.2 ± 0.3	2.5 ± 0.1	NA

producing bacteria.

In this study, water was the source of the most widely isolates that were obtained (2.31×10^6 CFU mL^{-1}) followed by flour (2.33×10^5 CFU mL^{-1}), whereas yeast starter and butter had almost no isolates. Moreover, only 4-7 colonies were obtained from the isolation of bacteria from raw flour sample. Water and flour have been contaminated largely by rope-producing bacteria because *Bacillus* species are soil inhabitants. Although flour is generally considered as microbiologically safe product as it has low water-activity commodity. All of these bacteria were not removed completely in spite of thorough cleaning during milling process of the flour (NPCS 2011).

The purpose of the confirmation test was to confirm the isolates from raw materials were surely the causative agents of ropey bread by using two treatments, the mixing inoculation of bread dough and the direct inoculation of loaf slices (Fig 2). A problem with this approach was the presence of very low numbers of *Bacillus* spores within the purchased flour. This was indicated by the absence of ropiness in the mixing inoculated treatment (Fig 2a). However, ropiness was detected in the direct inoculation of loaf slice (Fig 2b). In this study, raw flour sample also contained *B. subtilis* (Table 2). *B. subtilis* is one of the causative agents of ropey bread.

It was expected that the mixing inoculation treatment (first treatment) would have similar spore contamination levels with the direct inoculation treatment (second treatment) because the two treatments used similar raw materials. However, the loaves in the first treatment did not develop rope, whereas the second treatment showed positive results. There were several factors that enhanced the rope development. Besides temperature and pH, water-activity or humidity can also enhanced the spore germination and vegetative cells growth of rope-producing bacteria. In this study, the temperature and the storage condition had been attempted to resemble the conditions of ropey bread production. However, humidity cannot be managed in this study. Therefore, spore formation which is caused by temperature of baking process was not enhanced by humidity and thus spore germination and growth of vegetative cell were disrupted. The positive results of the second treatments showed that rope spoilage can be caused from environment or the condition after the baking process since inoculation were carried out in direct manner (NPCS 2011).

The results of molecular characterization method of all of the isolates were *B. subtilis*, which belonged to be one of species associated with ropiness in bread. All of the bacteria isolated were also characterized to determine their amylase and protease activities, and heat resistance. The hydrolysis of starch and proteins by microbial amylases and proteases encourage rope formation (Pepe *et al*. 2003).

The antimicrobial test revealed that acetic acid, lactic acid, and quats were effective inhibitors of *B. subtilis* strains examined. Lactic acid was more effective inhibitor compared to acetic acid due to the concentration of the solution. The lactic acid had higher concentration (80-90%, almost pure) than acetic acid (25%, about the concentration of domestic vinegar) that was used in this study. Even though, Pundir and Jain (2011) showed acetic acid was more effective than lactic acid due to the amount of undissociated acid present. This means that the effectiveness of organic acids as antimicrobials differ widely based on concentration and the amounts of undissociated acids. Other than that, pH and molarity are also capable to affect the effectiveness of antimicrobial agents.

Acetic acid and lactic acid are bactericidal agents that can lower the pH of outer surface by releasing the proton causing membrane disruption, enzyme denaturation, and changing the permeability of the cells. These acids can be toxic for molds and bacteria due to inability of the affected organisms to metabolize these acids. However, in mammals, acetic acid and lactic acid are metabolized in a manner similar to that of other fatty acids, and it has not been shown to cause any toxic effects at the levels utilized. Both of these antimicrobial agents are organic acids that contain carboxyl functional group. The undissociated forms of these carboxylic acid antimicrobial agents are active, and the range of effectiveness extends up to pH 5.0 in most applications (Pundir and Jain 2011).

Between three antimicrobial used in this study, quats was found to be best antimicrobial agents. Quats are positive compounds that are good penetrants and thus have value for porous surfaces. Quats are membrane active agents with target site predominantly at the cytoplasmic (inner) membrane in bacteria (Carmona-Ribeiro and Carrasco 2013). Quats are often used to disinfect and sterilize medical tools or used in the foodservice industry as sanitizing agents (Ohta *et al*. 2008).

Quats are generally more effective in the alkaline pH range, whereas carboxylic acids are most ideally performed in the pH range of below 3. Carboxylic acids

are food grade that can be mixed with the bread dough in baking industry. However, these acids are also able to be used as sanitizing agents and acid rinse. Carboxylic acid sanitizers on baking equipments are noncorrosive to stainless steel, provide a good shelf life, and are cost effective. Unlike carboxylic acids, quats are not food grade and thus they can only used as antiseptics, disinfectants, or sanitizers on food-handling and food-processing equipments (Marriott & Gravani 2006).

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