Synergistic of antimicrobial activities of lactic acid bacteria in fermented *Tilapia nicoliticus* incorporated with selected spices

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**Abstract**

Fermented tilapia (*Tilapia nicoliticus*) is one of the famous fermented food in Malaysia. Lactic acid bacteria (LAB) which well known as GRAS (Generally Regarded as Safe) are present in most fermented foods and they are well-known non-pathogenic bacteria that play an important role in everyday life. Apart from LAB, spices have also been used for centuries across different regions of the world to improve aroma, flavour and food preservative. This research was aimed to explore a potential natural food preservative using LAB isolated from fermented *Tilapia nicoliticus* incorporated with various spices (9% turmeric, 6% chilli and 9% black pepper) against foodborne pathogens. The isolation of LAB in different media (MRS, MRS+CaCO$_3$, M17 and Tomato Juice Agar) showed the highest LAB count on day-9 and day-15 during the fermentation period in fermented Tilapia incorporated with black pepper, turmeric and chilli. The highest antimicrobial activity by LAB against *Bacillus cereus* was observed in fermented tilapia incorporated with black pepper. On the other hand, fermented fish incorporated with chilli showed the highest antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enterica* serovar Typhimurium. Higher antimicrobial activity was detected in fermented Tilapia in the presence of LAB together with the spices, in comparison to the presence of LAB alone, suggesting synergistic effects between LAB in fermented fish with spices could enhance stronger antimicrobial activities against food pathogens and therefore, served as a natural food preservative.

1. Introduction

Food contamination has been the biggest concern for consumers for a long time. It has been shown that food contamination can either come from microbiological or chemical origins. For this reason, there has been intensive and continuous research to get knowledge about contamination sources and the way to prevent them. One of the food contamination sources is due to pathogenic bacteria. This type of contamination has become a global concern because it can affect human health (Rather *et al.*, 2017; Salleh *et al.*, 2017). Ninety per cent of foodborne illnesses are caused by contamination (Burbudhe *et al.*, 2003; Arul and Saravanan, 2011). Food products may transmit certain microorganisms and cause foodborne illnesses in several ways, including through infections, intoxications or toxicoinfections (Knechtges, 2012). An infection happens when a pathogenic bacterium present in food is ingested and then multiplies, as is true for *Salmonella*, *Campylobacter*, *Listeria* and some enteropathogenic *Escherichia coli*. Most foodborne illnesses are infections caused by bacteria, viruses and parasites.

The global demand for fish and fish-based products has increased and several changes such as vulnerability
and susceptibility to oxidation, and seasonal availability have resulted in the development of preservation methods (Zang et al., 2016; Yu et al., 2018). Among other preservation methods such as freezing, salting and smoking, fermentation, in particular, has been the most widely used (Panda et al., 2011). In modern terms, fermented fish is defined as fresh fish that has undergone a series of desirable biochemical changes through the action of microorganisms or enzymes. These changes include acidification (carbohydrate catabolism), gelation of myofibrillar and sarcoplasmic proteins of muscle, and degradation of proteins and lipids. Usually, all these fermented fish products were traditionally produced based on empirical knowledge without any knowledge of the microbes involved in the process until the development of modern microbiology (Mouritsen et al., 2017). There are different types of fermented fish products according to the final product appearance. Fermented fish can be divided into three groups: Fermented as a whole or in pieces such that the fish retains as much of its original structure as possible (Zeng et al., 2013), fermented fish pastes where the fish are converted into paste-like products (Giri et al., 2010) and fermented fish sauce where the fish are completely converted into liquid form (Lopetcharat et al. 2001).

Fermented fish has a long history in Europe and North Africa, but now Southeast and East Asian countries are the leaders in its production (Zang et al., 2020). In Malaysia, a type of fermented fish known as Pekasam, is a fermented whole fish product prepared by mixing freshwater fish with salt and ground roasted uncooked rice. A minimum amount of salt should also be used, and then the fish undergo different fermentation periods (2-3 weeks or 12 months) to make different products. A longer fermentation period can produce better products (Ezzat et al., 2015).

Lactic Acid Bacteria (LAB) have been isolated from many fermented foods to be used as probiotics and functional food materials (Solieri et al., 2014). The main LAB groups are Gram-positive, catalase-negative organisms and belong to genera Lactobacillus, Bifidobacterium, Lactococcus and Leuconostoc (Leroy and De Vuyst, 2004). LAB are able to produce acids, hydrogen peroxide and bacteriocins that possess great potential as food biopreservatives (Aslim et al., 2005). Probiotics are live microorganisms and non-pathogenic. If they are administered in adequate amounts, it will give balance and health benefits to the host (Food and Agriculture Organisation and World Health Organization, 2001). A study from Paludan-Muller et al. (2002) showed the potential of LAB that could be isolated from fermented fish.

Bacterial growth and lipid oxidation are the main factors that determine food quality loss and shelf-life reduction (Tajkarimi et al., 2010). To reduce these problems, chemical additives are commonly used in food products to extend their shelf life. However, consumers have growing concerns about health-related issues associated with the use of synthetic antimicrobial/antioxidant agents (Aziz and Karboune, 2016). In order to satisfy the consumers’ demands and restore their confidence in the safety of food products, the food industry was motivated to look for natural alternatives that exhibit strong antimicrobial and/or antioxidant properties (Fernandez-Lopez et al., 2005; Ahmad et al., 2019). There are a few natural sources that can be used, such as spices (cinnamon, clove, nutmeg, ginger, black pepper, garlic etc.) and plant extracts (tea, grape seed, cranberry, blueberry, strawberry and rosemary) that contain antioxidant components and can be used as natural antioxidant agents to inhibit lipid oxidation in food products (Brewer, 2011; Jiang et al., 2011). Some natural antioxidants/antimicrobials are able to extend the shelf life of food products (Salleh et al., 2021). Natural preservatives are easily accessible by the community and implemented sustainably through awareness and scientific merits and the ingredients also have been used as sustainable alternative to synthetic food preservatives.

Spices are normally used as flavouring agents, medicine, preservatives as well as colouring agents. Extract of spices hold preservatives and natural antioxidant properties, and also have antibacterial, antifungal and antiviral activities (Hernández et al., 2011). Spices stabilise food from microbial deterioration by making the microbial growth progressively slower and eventually totally suppressed (Souza et al., 2005). Most of the existing preservatives are based on synthetic chemicals. Anand and Sati (2013) reported that the application of some spices as preservatives in food are conducted to determine its efficiency since spices are natural sources and can be used to replace synthetic preservatives in food, such as nitrates, which have been claimed to possess negative effects on human health.

Tilapia fish was a common fish and widely used in Malaysian cuisine and was abundantly found all over the country. Tilapia has been identified as one of the main species for freshwater aquaculture in the Third National Agriculture Policy (DPN3) (Hamli et al., 2013). Based on the report by Awang (2001), fermented fish from freshwater fish is more popular than marine fish due to their flavour and texture. Small-sized tilapia of less than 350 g per fish is suitable for fermentation. The selection of tilapia in this study because this fish can survive in different culture systems, disease resistant and tolerate...
unfavourable conditions of water (Attalla, 2001), suggesting this type of fish is suitable for large scale commercial fermented fish.

However, the effect of LAB that is isolated from fermented *Tilapia nicoliticus* and added with a selected spice has never been reported. This research was conducted to observe a potential natural food preservative using LAB from fermented fish incorporated with various spices (turmeric, chilli and black pepper).

2. Materials and methods

2.1 Fermented Tilapia (*Tilapia nicoliticus*) preparation

Fermented tilapia was prepared and the recipe for ingredient was referred to Noor ‘Izzati (2013). The tilapia weighed between 300 g to 350 g was obtained alive from a local fresh market and placed directly in iced water. The fermented tilapia products were prepared according to the procedures of the fermented fish industry as stated by Awang (2001) but the salt used was reduced to 20%. The fermented fish was prepared according to the ingredients listed in Table 1. Fresh spices used in the study were purchased from a local Hock Kee Seng market in Gong Badak, Kuala Terengganu, Malaysia. Then, black pepper, chilli and turmeric in powder form were mixed with the fermented tilapia.

Table 1. The fermented tilapia fish ingredients and spices incorporated with fermented tilapia fish give the highest reduction of total plate count based on a study by Noor ‘Izzati (2013)

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>The percentage (%) (based on fish weight)</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>100</td>
<td>~300-350</td>
</tr>
<tr>
<td>Salt</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>Crushed roasted rice</td>
<td>20</td>
<td>65</td>
</tr>
<tr>
<td>Asam gelugur (Garcinia atroviridis)</td>
<td>5</td>
<td>16.25</td>
</tr>
<tr>
<td>Brown sugar</td>
<td>5</td>
<td>16.25</td>
</tr>
<tr>
<td>Spices</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black pepper</td>
<td>9</td>
<td>29.25</td>
</tr>
<tr>
<td>Chilli</td>
<td>6</td>
<td>19.5</td>
</tr>
<tr>
<td>Turmeric</td>
<td>9</td>
<td>29.25</td>
</tr>
</tbody>
</table>

Fermented tilapia without any spices were used as control. The spices had undergone UV treatment under a laminar airflow cabinet (ERLA-CFM SERIES, VFM-4, Malaysia) for 30 mins before being mixed with the fish in order to reduce microbial contamination (Nicorescu et al., 2013). After that, the tilapia with spices was placed in a sterile-labelled plastic container to undergo fermentation and fermented at ambient temperature (30 - 37°C) for 15 days. During the fermentation process, the samples were withdrawn (300 g to 350 g), then homogenised and placed into a sterile container on the predetermined day from day 1 until day 15. The samples of fermented tilapia were placed into a sterile rectangular plastic container with Lid 1000 mL (115 × 165 × 71 mm) and transported immediately to the laboratory for microbiological analysis.

2.2 Isolation of lactic acid bacteria (LAB) from fermented Tilapia nicoliticus

For isolation purposes, 10 g of sample was homogenised in 90 mL of sterile normal saline (0.85% NaCl) solution. The homogenates were aseptically prepared using a stomacher at high speed for 3 mins. Next, 1 mL of sample from the stomacher bag was added into 9 mL of MRS broth and the sample solution was incubated at 30°C for 48 hrs in anaerobic condition. Then, serial dilution was made in a normal saline solution from 10⁻¹ to 10⁻⁶. An amount of 0.1 mL of sample was spread on MRS agar (Merck, Germany), MRS agar with 0.8% CaCO₃, M17 agar (Oxoid, UK), and Tomato Juice Agar (TJA) (Oxoid, UK) following the method of Aween et al. (2012). Then, the plates were incubated anaerobically at 37°C for 48 hrs. Different morphology of the colony which dissolved CaCO₃ and formed a clear zone on the medium was purified by streaking on different plates to get a single colony. Then, the plates were incubated again at 37°C for 48 hrs under anaerobic condition using a CO₂ incubator (Memmerth INC 153, Germany). The different morphology of the colonies on the plates had undergone a confirmation test (modified from Panthavee et al., 2007).

2.3 Antimicrobial activity of cell free supernatant (CFS) of LAB by well diffusion method

The antimicrobial activity of the cell-free culture supernatant (CFS) was determined using agar well diffusion assay (Liassi et al., 2009). Firstly, one loop of the LAB was grown in 10 mL MRS broth in a centrifuge tube. CFS for the antibacterial assay was obtained after centrifugation at 8,000 rpm for 15 mins at 4°C by using a laboratory refrigerated centrifuge (Gyrozen, Korea). Well diffusion method used Mueller Hinton (Merck, Germany) as the medium for tested pathogens. The tested pathogens were prepared by mixing them with 0.85% NaCl and their concentration was adjusted to 0.5 MacFarland standard. The American Type Culture Collection (ATCC) test strain of bacteria was used for this assay which is *Escherichia coli* ATCC 11775, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579 and *Salmonella enterica* serovar Typhimurium ATCC 14128. After that, the bacterial suspensions were swabbed onto MHA by sterile cotton swabs. Then, the wells of 6 mm diameter were made using cork borers. One drop of molten Mueller Hinton
Agar (MHA) was added into each well for lining purposes. Approximately 50 µl of LAB supernatant were added into the wells and the plates were incubated at 37°C for 24 hrs. After 24 hrs of incubation, the diameters of inhibition zones were measured following the method by Liasi et al. (2009).

2.4 Statistical analysis

The data were expressed as mean ± standard error (SE). Results were analysed by multiple comparisons two-way analysis of variance (ANOVA) using Tukey’s test, Graph Pad Prism 5 where probability (p<0.05) considered statistically significant.

3. Results and discussion

3.1 Lactic acid bacteria (LAB) count of fermented tilapia fish against different periods of fermentation on various types of agar

The microbial count of LAB in fermented tilapia incorporated without spices (control), 9% black pepper, 6% chilli and 9% turmeric on MRS agar is shown in Figure 1.

Comparatively, the highest number of microbioc counts on the MRS with CaCO₃ agar was observed in fermented tilapia incorporated with turmeric on day 0, followed by chilli on day 3 and day 6 (Figure 2). However, from day 9 until day 15, the highest microbial count was observed in fermented tilapia incorporated with black pepper. On day 0 and day 9, the highest number of LAB count on MRS with CaCO₃ agar was observed in fermented tilapia incorporated with turmeric and black pepper, respectively.

Figure 2. Growth of LAB in fermented tilapia (log₁₀ CFU/g) incorporated without spices, 9% black pepper, 6% chilli and 9% turmeric on MRS agar. Data are mean of triplicated (n = 3) indicating standard error (SE)

3.2 Comparison of microbial counts of fermented tilapia fish against different periods of fermentation on M17 agar

Fermented tilapia with chilli showed the highest number of LAB during 6 days of the fermentation. Meanwhile, for day 9 and day 15, black pepper showed the highest LAB count for MRS agar. However, on day 12, chilli showed a slightly higher microbial count compared to black pepper and turmeric. The control showed the lowest microbial count from start until the end of the fermentation period. From Figure 1, the highest count of LAB microbial count was noticed on day 9 of fermentation with the black pepper’s fermented tilapia.

Comparatively, the highest number of microbial counts on the MRS with CaCO₃ agar was observed in fermented tilapia incorporated with turmeric on day 0, followed by chilli on day 3 and day 6 (Figure 2). However, from day 9 until day 15, the highest microbial count was observed in fermented tilapia incorporated with black pepper. On day 0 and day 9, the highest number of LAB count on MRS with CaCO₃ agar was observed in fermented tilapia incorporated with turmeric and black pepper, respectively.

Figure 3. Growth of LAB in fermented tilapia (log₁₀ CFU/g) incorporated without spices, 9% black pepper, 6% chilli and 9% turmeric on M17 agar. Data are mean of triplicated (n = 3) indicating standard error (SE)

Figure 4 shows the microbial count of fermented tilapia incorporated without spices, 9% black pepper, 6% chilli and 9% turmeric on M17 agar.

Figure 4. Growth of LAB in fermented tilapia (log₁₀ CFU/g) incorporated without spices, 9% black pepper, 6% chilli and 9% turmeric on M17 agar. Data are mean of triplicated (n = 3) indicating standard error (SE)
chilli and 9% turmeric on Tomato Juice agar.

From day 0 until day 3, fermented tilapia incorporated with turmeric showed the highest number of microbial counts. From day 6 until the end of the fermentation period, fermented tilapia incorporated with black pepper showed the highest number of microbial counts. LAB count can be observed to have the highest count in fermented tilapia incorporated with black pepper on day 15 of the fermentation on Tomato Juice agar. The LAB from fermented fish incorporated with black pepper showed the highest growth meanwhile most of the LAB growth was the highest at the end of the fermentation period (9 to 15 days of fermentation).

3.2 Screening for antimicrobial activity against various foodborne pathogens by well diffusion method

From the isolation procedure, 32 isolates of LAB which have been confirmed as Gram-positive and catalase and oxidase negative were further tested for their antimicrobial activity.

From Table 2, the antimicrobial activity of isolated LAB against Bacillus cereus. The highest antimicrobial activity recorded was 26.00±0.58 mm that recorded from LAB isolated from fermented fish incorporated with 9% black pepper on M17 agar. Meanwhile, there is no antimicrobial activity recorded from isolated LAB from fermented fish incorporated with 9% turmeric.

Table 3 shows the antimicrobial activity of isolated LAB against Staphylococcus aureus. The highest antimicrobial activity was 14.00±0.58 mm was from LAB isolated from fermented fish incorporated with 6% chilli on M17 agar. While the lowest (8.33±0.89 mm) was from control on MRS+CaCO₃ agar. LAB isolated from fermented fish incorporated with 9% turmeric showed no antimicrobial activity for all types of media.

In comparison, antimicrobial activity against Escherichia coli of LAB isolated from fermented fish without spices was the highest on M17 agar as shown in Table 4. Meanwhile, the lowest antimicrobial activity was observed on LAB isolated from fermented fish incorporated with 9% black pepper on M17 agar.

Finally, Table 5 shows the highest antimicrobial activity against Salmonella enterica serovar Typhimurium can be observed from LAB isolated from fermented tilapia incorporated without spices on MRS agar. Meanwhile, the lowest was from LAB isolated from fermented fish incorporated with 6% chilli on MRS with CaCO₃ agar. However, the LAB isolated from fermented fish incorporated with 9% turmeric shows no antimicrobial activity at all.

From the results obtained, it was proven that LAB was the dominant bacteria found in fermented food as

Table 2. The antimicrobial activity of isolated LAB against Bacillus cereus of day 15 of fermentation

<table>
<thead>
<tr>
<th>Media</th>
<th>Spices</th>
<th>control</th>
<th>9% black pepper</th>
<th>6% chilli</th>
<th>9% turmeric</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS</td>
<td></td>
<td>0</td>
<td>25.00±0.29</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M17</td>
<td></td>
<td>0</td>
<td>26.00±0.58</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>MRS+CaCO₃</td>
<td></td>
<td>25.00±0.29</td>
<td>0</td>
<td>18.00±0.29</td>
<td>0</td>
</tr>
<tr>
<td>Tomato Juice</td>
<td></td>
<td>17.00±0.58</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E (mm) of 3 replicates. Values with different lowercase superscript and uppercase superscript within the row and column, respectively are significantly different at P<0.05.

Table 3. The antimicrobial activity of isolated LAB against Staphylococcus aureus of day 15 of fermentation

<table>
<thead>
<tr>
<th>Media</th>
<th>Spices</th>
<th>control</th>
<th>9% black pepper</th>
<th>6% chilli</th>
<th>9% turmeric</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS</td>
<td></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>M17</td>
<td></td>
<td>8.37±0.88</td>
<td>11.50±0.29</td>
<td>14.00±0.58</td>
<td>0</td>
</tr>
<tr>
<td>MRS+CaCO₃</td>
<td></td>
<td>8.33±0.89</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tomato Juice</td>
<td></td>
<td>10.83±0.44</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E (mm) of 3 replicates. Values with different lowercase superscript and uppercase superscript within the row and column, respectively are significantly different at P<0.05.
agreed by Hwanhlem et al. (2011) and Grosu-Tudor et al. (2014) extensively report that LAB was a dominant bacteria found in fermented meat, fish, fruits, vegetables and dairy products. The fermented tilapia incorporated with black pepper showed the highest number of LAB, followed by chilli and turmeric. The LAB count was the highest at the end of the fermentation period and might be due to the production of lactic acid from LAB during the fermentation process. The lactic acid decreased the pH of the product thereby decreasing the number of other microbes. The typical acid-forming bacteria rapidly increased in number, becoming the predominant microbes after fermentation had started and reached their maximum density at the end of the fermentation (Hugas, 1998; Ngasotter et al., 2020).

The LAB produces a variety of antibacterial substances including bacteriocins to inhibit the growth of pathogenic bacteria (Ekhay et al., 2013; Yaseen et al., 2015). Moreover, as reported by Rohmah et al. (2012), the LAB (Lactobacillus casei and L. paracasei) isolated from fermented fish in Malaysia shows an antimicrobial effect against B. cereus, S. aureus, S. enterica, Listeria monocytogenes and E. coli.

All spices used in this study are well-known sources that can be used as antimicrobials. For example, turmeric is well-known as an antimicrobial and antiseptic agent. De et al. (1999) reported that the essential oil from turmeric has an antimicrobial property against pathogenic bacteria and fungi. Chilli has also been found to possess antimicrobial properties due to the capsaicin from chilli that acts as a potent inhibitor of Helicobacter pylori. Moreover, the essential oil of black pepper can inhibit the growth and germination of Clostridium botulinum (Hariri and Ghiasvand, 2016). Black pepper is also rich in glutathione peroxidase and glucose-6-phosphate dehydrogenase (Karthikeyan and Rani, 2003), and can damage the membrane of bacteria to avoid its growth (Karsha and Lakshmi, 2010).

Some spices including black pepper, chilli and turmeric in the form of powder, extract or extracted oil is known to control microbial spoilage of food (Karapinar and Aktug, 1987; Meena and Sethi, 1994; De and De, 2019). Previous studies also showed that a lot of different types of spices have antimicrobial effect. Clove, oregano and thyme showed inhibitory effect against E. coli (Moriera et al., 2007). Conner and Beuchat (1984) suggested that the antimicrobial activity of the essential oils of herbs and spices could be the result of damage to enzymatic cell systems, including those associated with energy production and synthesis of structural compounds.

The incorporation of spices and fermented fish do not affect the growth of LAB because our findings did not show any major inhibition growth of LAB during the 15 days of the fermentation period. The application of extremely high concentration of phenolic compounds could inhibit the LAB growth as previously reported by Stead (1993) but the report from Kittisakulnam et al. (2016) showed that appropriate amounts of spices added do not interfere with LAB production in meat products incorporated with spices.

The combination of the LAB from fermented tilapia with the spices (black pepper, chilli and turmeric) shows high antimicrobial activities against B. cereus, S. aureus, E. coli, and S. enterica ser. Typhimurium. However, the fermented fish without the spices also displayed a high antimicrobial effect in several stages of fermentation towards the foodborne bacteria. According to O’Bryan et al. (2015), antimicrobial compounds produced by LAB during fermentation also inhibited the growth of inactive

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**Table 4. The antimicrobial activity of isolated LAB against Escherichia coli of day 15 of fermentation**

<table>
<thead>
<tr>
<th>Media</th>
<th>Spices</th>
<th>control</th>
<th>9% black pepper</th>
<th>6% chilli</th>
<th>9% turmeric</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS</td>
<td>0</td>
<td>13.10±0.21&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>14.50±0.77&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>12.43±0.23&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>M17</td>
<td>16.43±0.23&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>12.10±0.21&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>0</td>
<td>15.43±0.30&lt;sup&gt;Bc&lt;/sup&gt;</td>
<td>0</td>
</tr>
<tr>
<td>MRS+CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>15.00±0.35&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>0</td>
<td>12.43±0.30&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tomato Juice</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E (mm) of 3 replicates. Values with different lowercase superscript and uppercase superscript within the row and column, respectively are significantly different at P<0.05.

**Table 5. The antimicrobial activity of isolated LAB against Salmonella enterica serovar Typhimurium of day 15 of fermentation**

<table>
<thead>
<tr>
<th>Media</th>
<th>Spices</th>
<th>control</th>
<th>9% black pepper</th>
<th>6% chilli</th>
<th>9% turmeric</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRS</td>
<td>19.87±0.19&lt;sup&gt;Ab&lt;/sup&gt;</td>
<td>14.93±0.23&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>14.20±0.15&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>M17</td>
<td>0</td>
<td>11.50±0.36&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>MRS+CaCO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>18.87±0.49&lt;sup&gt;Ba&lt;/sup&gt;</td>
<td>0</td>
<td>10.87±0.58&lt;sup&gt;Bb&lt;/sup&gt;</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Tomato Juice</td>
<td>12.50±0.23&lt;sup&gt;C&lt;/sup&gt;</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± S.E (mm) of 3 replicates. Values with different lowercase superscript and uppercase superscript within the row and column, respectively are significantly different at P<0.05.
spoilage and pathogenic microorganisms. Organic acids such as lactic, acetic and propionic acids provide an acidic environment and inhibit the growth of acid-sensitive spoilage microorganisms. Acids can directly act upon the cell walls of Gram-negative bacteria and cause the failure of proton motive forces, reducing the colonization of pathogenic bacteria (Dittoe et al., 2018). The salt content in fermented fish also helps to enhance the flavour by activating some of the enzymes inherent in the fish’s flesh or the microorganisms (Mariutti and Bragagnolo, 2017).

Fermented fish incorporated with spices is the best method to preserve fresh products due to the combination of both salt and spices that have dual effects which result in lowering the water activity level and having a specific inhibitory effect on the growth of some microorganisms. The spices have the capacity to migrate inside the fish, resulting in water oozing out of the fish and decreasing moisture (Horner, 1997; Alex and Eagappan, 2017). This decrease in moisture leads to an increase in spice content and consequently extends the shelf life of the product (Lopez, 1987; Itou and Akabane, 2000). Spices help slow down the microbial growth and eventually totally suppresses it (Souza et al., 2005; Torres et al., 2015). In agreement with previous study from Geremew et al. (2015), the combined effect of spices incorporated with the fermented goods can also be seen in the production of traditional cottage cheese (metata ayib) as LAB antimicrobial activity had reduced the risk of spoilage and pathogenesis. The use of spices would decrease the chances of food poisoning, food spoilage and increase food shelf life. Other than that, metata ayib also exhibited antimicrobial activity against pathogenic test strains.

Different selective media were used in this study in order to increase the chances of LAB species to grow in the fermented fish. Tomato juice agar is recommended for the cultivation and enumeration of Lactobacilli (MacFaddin, 1985). MRS in an improved media from Tomato Juice Agar which developed by de Man, Rogosa and Sharpe that designed to encourage the growth of the lactic acid bacteria which includes species of the following genera: Lactobacillus, Streptococcus, Pediococcus and Leuconostoc (Lankaputhra et al., 1996). The addition of calcium carbonate in MRS Agar as reported by Hwanhlem et al. (2011) and Aween et al. (2012) was an excellent indicator for acid-producing strains since it dissolved when interacting with acid then a clear zone is observed. Another media, M17 Agar was used as it is suitable for the growth and enumeration of lactic streptococci and their bacteriophages (Terzaghi and Sandine, 1975).

Other than acting as a very good antimicrobial agent for fermented fish, spices are also used for flavouring foods and bringing a distinguished flavour to each food style that gives culinary identity (Exploratorium, 2013; Torres et al., 2015). Flavour given by spices is due to a certain family of chemicals such as phenylpropanoids, monoterpenes and other phenol compounds (Peter and Shylaja, 2012; Torres et al., 2015)

4. Conclusion

The fermented tilapia incorporated with black pepper, chilli and turmeric show no significant effect on the growth of LAB and the highest growth of LAB was observed in the later stages of fermentation in relation to different media used. Only MRS, MRS with CaCO₃ and M17 agar show high microbial activity against the foodborne pathogens. Other than that, the incorporation of fermented tilapia fish with black pepper and chilli show antimicrobial activity, however, turmeric was not given an antimicrobial activity for pathogens except for E. coli. Although the antimicrobial effect of LAB without the addition of spices also can be observed when a combination of LAB and spices were used, the antimicrobial effect was the highest against B. cereus. Obtaining antimicrobials from natural sources such as spices is a good alternative for the preservation of fresh products and also give an extra taste to the fermented fish. Further research can be conducted to find out the antimicrobial activity of other Malaysian spices as well as the mixture and combination of different spices.

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