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Antibacterial agents produced by lactic acid bacteria isolated from Threadfin Salmon and Grass Shrimp

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Abstract

Lactic acid bacteria (LAB) are non-pathogenic bacteria that have an important role in human daily life. LAB produce antimicrobial agents, such as bacteriocins, diacetyl and hydrogen peroxide which help to extend the shelf life of food products. In this study, LAB were isolated from selected seafood; threadfin salmon and grass shrimp. Antibacterial activity of LAB extracts against *Salmonella typhimurium*, *Listeria monocytogenes*, *Bacillus cereus* and *Escherichia coli* were determined using the disc diffusion method. Three strains of LAB were selected for the characterization of antibacterial agents produced by LAB. The parameters such as pH, heat, incubation period and medium, were analyzed in this experiment. Changes in environmental parameters affected the activity of antibacterial agents. The antibacterial agents produced by LAB were generally heat stable and stable in a wide range of pH levels. However, the inhibition activity of LAB was destroyed with a heat treatment of 121°C, and the antibacterial effect was reduced at a pH of 12, which occurs in most strains. The medium containing NaCl enhanced the antibacterial activity of P1S1 and P3S3 strains against *S. typhimurium*, *L. monocytogenes* and *E. coli*. Moreover, the antibacterial agents exhibited the greatest inhibition activity at incubation times between 24 and 72 h. The antibacterial activity was reduced after an incubation time of 96 h. The characterization of antibacterial agents aids in the improvement of food products safety.

Introduction

Lactic acid bacteria (LAB) have been widely used in various fermented food products (Buckenhüskes, 1993). Mainly, LAB consist of the *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Melissococcus*, *Carnobacterium*, *Oenococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* genera (Stiles and Holzapfel, 1997; Ercolini *et al*., 2001; Holzapfel *et al*., 2001). LAB are Gram-positive bacteria, non-motile, non-spore forming and non-respiring bacteria (Nair and Surendran, 2005). Generally, LAB are characterized base on rods or coccus shape and their negative catalase activity (Françoise, 2010). They have similar morphological, metabolic and physiological characteristics. LAB ferment carbohydrates and produce lactic acid as the major end product. Thus, they are given the name lactic acid bacteria. LAB are divided into the following two major groups according to the metabolic pathways used to ferment carbohydrates: homofermentative or heterofermentative (McDonald *et al*., 1987). The LAB groups that have important roles in foods normally belong to the *Lactococcus*, *Leuconostoc*, *Lactobacillus* and *Streptococcus* genera (Nair, 2000).

LAB can produce antimicrobial agents that exert strong antagonistic activity against many microorganisms, including pathogenic and food spoilage microorganisms. Organic acids, hydrogen peroxide, diacetyl and bacteriocins are examples of antimicrobial agents produced by LAB (Daeschel, 1989). A preservation method using microbial antagonism activity, which is the oldest method of preservation, has been observed in food products. Pure cultures of LAB have been used since the beginning of the twentieth century as starter cultures in fermented food products, such as yogurts, fermented milk, preserved meats and preserved fish (Analie and Bennie, 2001; Liu, 2003). Starter cultures consisting of LAB produce bacteriocins and other antimicrobial

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substances that aid in extending the shelf life of food, modifying food sensory attributes and improving texture (Leroy and Vuyst, 2004; Herreros *et al*., 2005). Currently, natural and minimally processed foods are greatly demanded by consumers. Thus, there is an increased interest in using naturally produced antimicrobial agents, such as bacteriocins (Cleveland *et al*., 2001).

There is a high consumer demand for fish and prawn, particularly in south-east Asia because of their delicious taste and nutritional value. Grass shrimp (*Penaeus monodon*), also known as black tiger, is an important aquatic shellfish in south-east Asia and also an important export commodity in China (Jiang *et al*., 1991). Marine fish such as threadfin salmon is preferred by consumer compared to freshwater fish which rich in earthy odour and flavour (Guttman and Jaap van Rijn', 2008). The most important role of lactic acid fermentation in Asian diets is the preservation of perishable seafood in sanitary and safe condition (Lee, 1997), but the use of LAB in the marine industry is not extensively developed, except in Asia for preparation of fish sauces and traditional food with fermented mixture of seafood and vegetable (Françoise, 2010). Hence it is very important to exploit the bioprotective potential of endogenous LAB to control the quality and safety of marine products.

Previous antibacterial activity studies have been performed by isolating LAB from fermented products. According to Assefa *et al*., (2008) 12 strains of LAB isolated from Ergo (traditional Ethiopian fermented milk) exhibit antibacterial activity against *S. typhi*, *S. flexneri*, *S. aureus* (ATCC-25923) and *E. coli* (ATCC-25922). However, studies based on LAB isolated from fresh seafood have not been intensively exploited. In this research, LAB were isolated from fresh fish and prawn samples, and the isolated LAB used in this study may be further used as natural biopreservatives in foods. Moreover, characterization of antibacterial agents produced by LAB is required to improve the safety of food products. Antibacterial agents are effective over a wide range of pH values and temperatures (Fekadu *et al*., 1998). However, the essential parameters affecting the optimal conditions of antibacterial activity should be studied to allow the LAB that produce antibacterial agents to be fully utilized as potent biopreservatives in food. Using this approach, the effect of some environmental parameters, such as pH, heat treatment, incubation period and nutrient composition, on the level of antibacterial activity were evaluated. The objectives of this study were to screen and determine the antibacterial properties of LAB isolated from selected seafood and to characterize the antibacterial agents produced.

Materials and Methods

Sample and media preparation

Three batches of fish (threadfin salmon) and prawn (grass shrimp) samples were obtained from a wholesale wet market in Seri Kembangan, Selangor, Malaysia. The following solutions were prepared: de Man-Rogosa-Sharpe (MRS) broth (Merck, Darmstadt, Germany), MRS-A (MRS + 1.5% of bacterial agar), 0.85% saline water, methylene blue, crystal violet, safranin, Gram's iodine, acetone (95%), 3% hydrogen peroxide solution, NaCl (2, 4 and 6.5%), Mueller-Hinton (MH) broth (Merck, Darmstadt, Germany), MHA (MH + 1.5% of bacterial agar), tryptone (1.0%), yeast extract (1.0%), beef extract (1.0%), NaCl (1.0%), glucose (1.0%), 1 M NaOH and 1 M HCl. Four indicator microorganisms were used as controls in this study. Two of the indicator microorganisms were Gram-positive (*L. monocytogenes* ATCC 19115 and B. cereus ATCC 14579) and the other two indicator microorganisms were Gram-negative (*S. typhimurium* ATCC 13311 and *E. coli* ATCC 25922).

LAB isolation and enumeration

LAB were isolated according to methods of Nair and Surendran (2005) and Adnan and Tan (2007) with slight modifications. Briefly, seafood samples (10 g) were homogenized with 90 ml of 0.85% saline water in a stomacher blender. Isolation of bacteria was carried out on de Mann-Rogosa-Sharp (MRS) agar from appropriate 10-fold dilutions (10^{-2} and 10^{-3}). The MRS plates were incubated 48 h at 30°C anaerobically. Colonies were selected at random from plates and the isolates purified by repeated streaking onto MRS agar. Purified strains of LAB were inoculated into MRS broth (pH 6.5) and incubated 24 h at 30°C. All purified strains were kept in MRS broth containing 20% glycerol at -70°C.

The following characteristics were investigated for each isolate: Gram staining, catalase activity, gas production from glucose, growth at different temperatures and NaCl concentrations, and pH. Arginine hydrolysis test was performed on LAB that was identified as rod-shaped bacteria (Plessis *et al*., 2004). Arginine hydrolysis test was performed in MRS broth without glucose and meat extract containing 0.3% arginine and 0.2% sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent (Schillinger and Lücke, 1987).

Antibacterial activity assay

The modified methods of Savadogo *et al*. (2004) and Girum *et al*. (2005) were used to determine the antibacterial activities of the isolates. Five single isolated colonies were selected from MRS agar plates and transferred to grow in sterile MRS broth. The broth culture was incubated anaerobically at 35°C for 24 h. After incubation, the culture was centrifuged at 10 000 rpm for 20 min at 4°C to obtain the culture supernatant. Un-inoculated MRS broth was used as a control. The indicator microorganisms (*S. typhimurium* ATCC 13311, *L. monocytogenes* ATCC 19115, B. cereus ATCC 14579 and E. coli ATCC 25922) were grown in Tryptone Soy broth (TSB) for 24 h at 35°C. A sterile cotton swab was dipped into the culture of the indicator microorganisms and rotated several times, and the swab was then pressed firmly on the inside wall of the tube above the fluid level to remove excess inoculums. The dried surface of Mueller-Hinton agar (MHA) was inoculated by streaking the swab over the entire agar surface. This procedure was repeated by streaking two or more times while rotating the plate each time to ensure an even distribution of inoculums. For the bioassay, the sterile filter disc was dipped into the culture supernatant and touched to the side of container to remove excess liquid, and it was then placed on a MHA plate (Branen *et al*., 1975). After 24 h of anaerobic incubation, each plate was evaluated, and the diameters of the inhibition zones, including the diameter of the disc, were measured using a transparent ruler (Assefa *et al*., 2008).

Effect of heat treatment and pH on antibacterial activity produced by LAB

The effect of heat and pH treatment on the production of antibacterial activity produced by LAB were measured following method of Assefa *et al*. (2008) with slight modifications. Briefly, the culture supernatants of the antibacterial agent producing strains were grown in MRS broth for 24 h followed by exposure to various heat treatments. The culture supernatants were incubated anaerobically for 30 min at 30, 60 and 80°C or for 15 min at 121°C. For the effect of pH on antibacterial agents, cultured supernatants were exposed to various pH levels (3.0, 6.0, 9.0 and 12.0), by adjusting the pH of MRS broth using 1 M NaOH or 1 M HCl (Hernadez *et al*., 2005). After incubation for 4 h, the pH was readjusted to 6.5, and the antibacterial activity was determined.

Effect of medium component and incubation period on antibacterial activity produced by LAB

The effect of medium ingredients on antibacterial production was evaluated using different medium components according to Rebecca *et al*. (2008) with slight modifications. The supplements tested included tryptone (1.0%), yeast extract (1.0%), beef extract (1.0%) and NaCl (1.0%). LAB were grown in MRS broth containing the specific medium component. The antibacterial activity was determined as mentioned earlier. The effect of incubation period on antibacterial agents produced by LAB was determined following the method of Balasubramanyam and Varadaraj (1998) and Ponce *et al*. (2008) with slight modification. LAB

Table 1. Preliminary identification of LAB isolated from fish and prawn samples

Sample	Strains	Catalase test	Gram stain; shape	Growth at different temperatures (° C)			Growth at different pH levels			Growth at different NaCl concentrations (%)			Gas production	Arginine hydrolysis	Genera of LAB
				10	40	45	3.9	4.4	9.6	2.0	4.0	6.5			
Fish	F1S2	-	+; cocci	+	+	+	-	-	-	+	+	+	-	ND	*Enterococcus*
	F1S5	-	+; cocci	+	+	+	-	-	+	+	+	+	-	ND	*Enterococcus*
	F2S1	-	+; cocci	-	+	-	-	-	+	+	+	-	-	ND	*Streptococcus*
	F3S2	-	+; cocci	+	-	-	-	-	+	+	+	+	-	ND	*Lactococcus*
	F3S4	-	+; cocci	-	-	-	-	-	+	+	+	+	-	ND	*Streptococcus*
	F3S5	-	+; cocci	+	-	-	-	-	+	+	+	+	-	ND	*Lactococcus*
Prawn	P1S1	-	+; cocci	+	+	-	-	+	-	-	-	-	-	ND	*Lactococcus*
	P1S3	-	+; cocci	+	+	-	-	-	+	+	+	+	-	ND	*Lactococcus*
	P1S4	-	+; cocci	+	+	-	-	+	+	+	+	-	-	ND	*Lactococcus*
	P2S2	-	+; cocci	-	+	-	-	-	+	+	+	-	-	ND	*Streptococcus*
	P2S3	-	+; rod	+	+	+	-	+	+	+	+	+	+	+	*Lactobacillus*
	PSS4	-	+; rod	+	+	-	-	+	+	+	+	+	+	+	*Lactobacillus*
	P3S3	-	+; cocci	-	+	+	-	+	+	+	-	-	-	ND	*Streptococcus*

"ND" represents not determined

were grown in MRS broth and incubated at 37°C for 24, 48, 72 and 96 h. The antibacterial activity of the cell supernatants was then determined.

Results and Discussion

Enumeration of LAB

A total of 30 isolates were isolated from both the fish (threadfin salmon) and prawn (grass shrimp) samples. For the morphological identification, 13 Gram-positive and catalase-negative isolates were selected for further identification based on the following parameters: cell morphology; gas production from glucose; arginine test; and, growth at different pH levels, temperatures and NaCl concentrations (Schillinger and Lücke, 1987). The preliminary LAB identification results are shown in Table 1, and the distribution of different LAB genera in the fish and prawn samples are shown in Table 2. The strains isolated from the fish samples were classified as *Lactococcus* (33.3%), *Streptococcus* (33.3%) and *Enterococcus* (33.3%). The absence of Lactobacillus in fish samples was probably due to sample preparation which discarded gastrointestinal tract of the fish samples since *Lactobacillus* are commonly found in those part of fish (Ringø and Strøm, 1994; Ringø *et al*., 1997). The strains isolated from the prawn samples were classified as *Lactococcus* (42.9%), *Streptococcus* (28.6%) and *Lactobacillus* (28.6%). The absence of *Enterococcus* in prawn samples indicated that prawn samples were in good quality because high numbers of *Enterococcus* in fresh prawn become a quality issue due to products not iced immediately after harvest (Lalitha and Surendran, 2006).

Antibacterial activity assay

The LAB antibacterial activity was determined using the disc diffusion method, and the results are shown in Table 3. Generally, the strains isolated from the fish and prawn samples had antagonistic activity against *E. coli*, *S. typhimurium*, *B. cereus* and *L. monocytogenes*. However, these results excluded the following strains: F1S2, F2S1 and F3S4 strains against *E.coli*; F1S5, F2S1, F3S4 and P2S4 strains against *S. typhimurium*; F1S5, P1S3 and P2S3 strains against *B. cereus*; and F2S1, P2S2 and P3S3 strains against *L. monocytogenes*. These strains did not show inhibition activity towards the specific indicator microorganisms. The P3S3 strain had the broadest spectrum of inhibition as it inhibited the growth of all four indicator microorganisms with the highest inhibition zone diameters. According to Ammor *et al*., (2006) LAB produce a wide range of products from low molecular mass compounds, such as hydrogen peroxide, carbon dioxide and diacetyl, to high molecular mass compounds, such as bacteriocins. Organic acid produced by LAB leads to a reduction in pH levels and increases the production of hydrogen peroxide (Ponce *et al*., 2008). These products exhibit antibacterial activity against various pathogenic microorganisms, including Gram-positive and Gram-negative bacteria (Maragkuodakis *et al*., 2009).

Table 2. Percentage distribution of different LAB genera in fish and prawn samples

Sample	Lactic Acid Bacteria (%)				
	Lactococcus	*Streptococcus*	*Lactobacillus*	*Enterococcus*	Total (%)
Fish	33.3	33.3	-	33.3	100
Prawn	42.9	28.6	28.6	-	100

Table 3. Antibacterial activity of 13 isolates of LAB obtained from fish and prawn samples against different indicator microorganisms

Sample	Strains	Average diameter of inhibition zone (mm) ± S.D			
		E. coli ATCC 25922	*S. typhimurium* ATCC 13311	*B. cereus* ATCC 14579	*L. monocytogenes* ATCC 19115
Fish	F1S2	-	6.20 ± 0.03	6.30 ± 0.03	6.20 ± 0.03
	F1S5	6.30 ± 0.03	-	-	6.20 ± 0.03
	F2S1	-	-	6.20 ± 0.03	-
	F3S2	6.50 ± 0.00	6.30 ± 0.03	7.70 ± 0.06	6.80 ± 0.03
	F3S4	-	-	6.50 ± 0.05	6.30 ± 0.03
	F3S5	6.20 ± 0.03	6.70 ± 0.03	6.20 ± 0.03	6.30 ± 0.03
Prawn	P1S1	7.30 ± 0.06	9.00 ± 0.00	6.80 ± 0.03	7.20 ± 0.03
	P1S3	6.70 ± 0.03	6.80 ± 0.03	-	6.20 ± 0.03
	P1S4	7.30 ± 0.08	6.80 ± 0.03	6.30 ± 0.03	6.50 ± 0.05
	P2S2	7.00 ± 0.09	6.70 ± 0.06	6.20 ± 0.03	-
	P2S3	6.70 ± 0.06	6.50 ± 0.00	-	-
	P2S4	6.30 ± 0.03	-	6.30 ± 0.03	6.30 ± 0.03
	P3S3	7.70 ± 0.12	7.70 ± 0.03	7.80 ± 0.10	8.20 ± 0.03

Values are means ± standard deviations of triplicates.
Diameter of inhibition zone (mm) includes the disc diameter of 6.00 mm.
"-" represents no inhibition.

Effect of heat treatment and pH on antibacterial agents produced by LAB

From the above results, the three strains (P1S1, P3S3 and F3S2) with the highest inhibition zone diameters were selected for the characterization of produced antibacterial agents. The effects of heat treatment, pH, nutritional composition and incubation time on the antibacterial agents were analyzed. The effect of heat treatment on the antibacterial activity of produced antibacterial agents is shown in Table 4. The antibacterial substances were stable within wide range of temperatures (30 to 60°C) as shown by the three isolates (P1S1, P3S3 and F3S2) against *E. coli*, *S. typhimurium*, *B. cereus* and *L. monocytogenes*. There were exceptions in the case of the P1S1 strain. This strain did not inhibit *E. coli* at a temperature of 60°C, and it was not effective against *B. cereus* at temperatures of 60 and 80°C. The P1S1 strain was also inactive against *L. monocytogenes* at a temperature of 30°C. The antagonistic activity toward the four

Table 4. Effect of heat treatment and pH on the antimicrobial activity of the P1S1, P3S3 and F3S2 strains against different indicator microorganisms

Factors affecting the antimicrobial activity		Strains	Average diameter of inhibition zone (mm) ± S.D			
			E. coli ATCC 25922	*S. typhimurium* ATCC 13311	*B. cereus* ATCC 14579	*L. monocytogenes* ATCC 19115
Heat treatment	30°C	P1S1	6.30 ± 0.03	6.80 ± 0.03	6.30 ± 0.03	-
		P3S3	6.20 ± 0.03	6.30 ± 0.03	6.80 ± 0.03	6.30 ± 0.03
		F3S2	6.20 ± 0.03	6.20 ± 0.03	6.20 ± 0.03	6.30 ± 0.06
	60°C	P1S1	-	6.30 ± 0.03	-	6.20 ± 0.03
		P3S3	6.30 ± 0.03	7.80 ± 0.03	7.80 ± 0.03	6.80 ± 0.03
		F3S2	6.50 ± 0.00	6.20 ± 0.03	6.30 ± 0.03	6.20 ±0.03
	80°C	P1S1	6.30 ± 0.06	6.30 ± 0.03	-	6.20 ± 0.03
		P3S3	8.00 ± 0.10	7.80 ± 0.08	9.00 ± 0.20	8.50 ± 0.05
		F3S2	6.30 ± 0.06	6.30 ± 0.03	-	-
	121°C	P1S1	-	-	-	-
		P3S3	-	-	-	-
		F3S2	-	-	-	-
pH	3	P1S1	7.80 ± 0.08	10.00 ± 0.10	10.70 ± 0.15	6.50 ± 0.05
		P3S3	7.80 ± 0.03	8.30 ± 0.12	9.50 ± 0.09	7.70 ± 0.15
		F3S2	6.50 ± 0.05	8.30 ± 0.06	10.20 ± 0.10	7.80 ± 0.03
	6	P1S1	9.00 ± 0.10	7.70 ± 0.13	6.20 ± 0.03	6.20 ± 0.03
		P3S3	9.00 ± 0.10	8.70 ± 0.03	9.80 ± 0.13	8.00 ± 0.10
		F3S2	8.00 ± 0.10	9.00 ± 0.17	6.20 ± 0.03	7.00 ± 0.10
	9	P1S1	6.20 ± 0.03	6.50 ± 0.05	6.20 ± 0.03	6.20 ± 0.03
		P3S3	8.70 ± 0.06	8.30 ± 0.06	10.50 ± 0.09	7.50 ± 0.05
		F3S2	7.20 ± 0.03	8.30 ± 0.12	7.50 ± 0.05	-
	12	P1S1	7.80 ± 0.03	7.70 ± 0.03	6.50 ± 0.05	-
		P3S3	8.20 ± 0.10	8.70 ± 0.06	8.70 ± 0.12	6.20 ± 0.03
		F3S2	6.50 ± 0.05	7.70 ± 0.12	8.70 ± 0.15	-

Values are means ± standard deviations of triplicates.
Diameter of inhibition zone (mm) includes the disc diameter of 6.00 mm.
"-" represents no inhibition.

Table 5. Effect of medium components and incubation period on the antimicrobial activity of the P1S1, P3S3 and F3S2 strains against different indicator microorganisms

Factors affecting the antimicrobial activity		Strains	Average diameter of inhibition zone (mm) ± S.D			
			E. coli ATCC 25922	*S. typhimurium* ATCC 13311	*B. cereus* ATCC 14579	*L. monocytogenes* ATCC 19115
Medium component	Beef extract	P1S1	6.80 ± 0.06	6.20 ± 0.03	6.30 ± 0.03	6.50 ± 0.00
		P3S3	7.30 ± 0.06	6.30 ± 0.03	7.30 ± 0.06	7.50 ± 0.05
		F3S2	6.50 ± 0.05	7.50 ± 0.05	6.20 ± 0.03	6.20 ± 0.03
	Yeast extract	P1S1	6.20 ± 0.03	7.80 ± 0.08	6.20 ± 0.03	6.20 ± 0.03
		P3S3	6.70 ± 0.06	6.50 ± 0.05	6.30 ± 0.06	6.70 ± 0.06
		F3S2	7.00 ± 0.00	7.70 ± 0.06	-	6.30 ± 0.03
	NaCl	P1S1	7.30 ± 0.03	6.30 ± 0.06	6.30 ± 0.03	6.70 ± 0.06
		P3S3	8.00 ± 0.10	8.70 ± 0.06	7.20 ± 0.03	8.20 ± 0.03
		F3S2	6.80 ± 0.08	6.50 ± 0.00	6.70 ± 0.03	6.20 ± 0.03
	Tryptone	P1S1	7.00 ± 0.10	6.70 ± 0.03	-	-
		P3S3	6.80 ± 0.08	8.20 ± 0.08	6.20 ± 0.03	7.30 ± 0.06
		F3S2	6.20 ± 0.03	7.50 ± 0.05	8.00 ± 0.10	-
Incubation period (h)	24	P1S1	6.70 ± 0.03	6.50 ± 0.05	6.50 ± 0.05	6.30 ± 0.03
		P3S3	7.20 ± 0.03	7.50 ± 0.05	8.00 ± 0.10	6.50 ± 0.05
		F3S2	6.70 ± 0.03	6.30 ± 0.03	6.30 ± 0.03	-
	48	P1S1	6.70 ± 0.03	6.50 ± 0.05	6.70 ± 0.03	6.20 ± 0.03
		P3S3	6.50 ± 0.05	7.00 ± 0.00	6.80 ± 0.03	6.30 ± 0.03
		F3S2	-	6.30 ± 0.03	6.20 ± 0.03	-
	72	P1S1	6.50 ± 0.05	-	6.30 ± 0.03	-
		P3S3	7.20 ± 0.08	6.70 ± 0.03	7.30 ± 0.10	6.80 ± 0.03
		F3S2	-	6.80 ± 0.03	6.30 ± 0.03	6.30 ± 0.03
	96	P1S1	6.30 ± 0.03	6.50 ± 0.05	6.20 ± 0.03	-
		P3S3	6.70 ± 0.06	6.70 ± 0.06	6.70 ± 0.06	6.50 ± 0.05
		F3S2	6.50 ± 0.05	6.30 ± 0.03	6.30 ± 0.03	-

Values are means ± standard deviations of triplicates.
Diameter of inhibition zone (mm) includes the disc diameter of 6.00 mm.
"-" represents no inhibition.

indicator microorganisms was completely destroyed at 121°C. According to Hernandez *et al*., (2005) the antibacterial activity of the substances produced by *L. plantarum* TF711 remains 75% even after heat treatment at 100°C for 30 min. However, sterilization (121°C) completely inactivates the antibacterial effect. Moreover, Assefa *et al*., (2008) demonstrated that produced antibacterial agents are stable with heat treatment but that sterilization at 121°C for 15 min completely destroys the antagonistic activity. Assefa *et al*., (2008) and Castro *et al*., (2011) found that heat treatment at 70 °C/15 min, 85 °C/30 min or 100 °C/15 min does not affect the antibacterial activity of the cell-free supernatant against either *L. innocua* or *S. aureus*.

Generally, the antibacterial agents were stable within wide range of pH values from 3.0 to 9.0 (Table 4). The inhibition effects were more obvious in the range of pH values from 3.0 to 6.0. Bacteriocin is one of the antibacterial agents produced by LAB, and its activity is high at pH values ranging from 5.8 to 6.5 (Nilsen *et al*., 1998). At a pH of 12, the antibacterial activity against *B. cereus* and *L. monocytogenes* was reduced in the P1S1, P3S3 and F3S2 strains, and the average inhibition zone diameter was also reduced. According to Ponce *et al*., (2008) the supernatants produced by *L. lactis*, *E. hirae* and *E. canis* have antagonistic effects against three types of microorganisms in a pH range of 2.0 to 9.0. Luo *et al*., (2011) reported that the effect of pH on produced antibacterial agents is not strong in three out of five supernatant strains, showing inhibition in pH range of 3.0 to 9.0. Assefa *et al*., (2008) demonstrated that the antibacterial agents produced by LAB isolated from Ergo exhibit stability within a broad range of pH values (ranging from 2 to 10) but that the antibacterial activity decreases at a pH of 12. According to Castro *et al*., (2011) *L. sakei* has antibacterial activity against *L. innocua* and *S. aureus* within a wide range of pH values, with maximal inhibition activity achieved at a pH of 3.5; however the antibacterial activity is ineffective at a pH of 6.50.

Influence of medium components and incubation period on antibacterial agents produced by LAB

Table 5 shows the results for the effect of medium components on the antibacterial agents. Beef extract, yeast extract, NaCl and tryptone supplements increased the antibacterial effect of LAB as shown by the increased average inhibition zone diameter. Medium supplemented with NaCl significantly increased the antibacterial activity as shown by the P1S1 and P3S3 strains against *E. coli*, *S. typhimurium* and *L. monocytogenes*. According to Leroy and Vuyst (1999) and Settani *et al*., (2008) the presence of a moderate amount of NaCl (1.0-2.0%) improves LAB growth and increases bacteriocin activity. According

to Settanni *et al*., (2008) the presence of nitrogen and carbohydrate sources increases the production of bacterion-like inhibitory substances by *E. mundtii* strains, but the presence of Tween 80, triammonium citrate, magnesium sulfate and manganese sulfate in growth medium does not affect the synthesis of bacterion-like inhibitory substances. A larger amount of antibacterial agents are synthesized when the growth medium is supplemented with NaCl (1-2.0%), yeast extract (2-3.0%), glucose (1.0%) and Tween 80 (0.5%), but the addition of sodium acetate, triammonium citrate, potassium phosphate and magnesium sulfate does not have an effect on the quantity of antibacterial agents produced according to Rebecca *et al*., (2008). Nutrients are required in the production of antibacterial agents, and different supplement types added to growth medium at different concentrations affect the amounts of antibacterial agents being synthesized.

The effect of incubation time on the antibacterial agents production of LAB is shown in Table 5. The F3S2 strain did not inhibit the growth of *E. coli* at incubation times of 48 and 72 h, and the inhibitory effect of P1S1 strain against *L. monocytogenes* was inactivated after 72 h of incubation. The F3S2 isolate only showed antibacterial activity against *L. monocytogenes* after 72 h of incubation time. In general, the three isolates demonstrated optimal inhibition activity against the test microorganisms at incubation times between 24 and 72 h. The antagonistic activity of isolates was decreased after 96 h of incubation time. Balasubramanyam and Varadaraj (1998) found that the antibacterial activity of the culture filtrate from *L. delbruecki* ssp. bulgaricus against *B. cereus* occurs at an incubation time of 24 h and that the inhibition activity increase until 48 h. Bacteriocin is one of the antimicrobial agents optimally produced between 48 and 60 h (Ogunbanwo *et al*., 2003). According to Campos *et al*., (2006) the maximum production of bacteriocin from the selected LAB strains is observed in the stationary phase of growth, which generally ranges from 21 to 72 h. The results in the present study were similar to those reported by Campos *et al*., (2006).

Conclusions

In this study, the LAB strains isolated from the fish and prawn samples exhibited inhibitory effects against Gram-positive and Gram-negative bacteria. LAB extracts inhibit the growth of four indicator microorganisms, namely *S. typhimurium*, *L. monocytogenes*, *E. coli* and *B. cereus*. The antagonistic activity may have been due to the presence of organic acid, hydrogen peroxide and bacteriocin, which act as antibacterial substances. Changes in the environmental parameters affected the activity of the antibacterial agents. The antibacterial activity was enhanced when medium containing NaCl (1.0%) was used as shown with the P1S1 and P3S3 strains against *S. typhimurium*, *L. monocytogenes* and *E. coli*. The antibacterial agents exhibited the greatest inhibition activity at incubation times between 24 and 72 h. After 72 h, the antibacterial activity diminished. Characterization of antibacterial agents is important because these compounds inhibit the growth of food borne pathogens. The optimal conditions for antagonistic activity should be evaluated to allow LAB to be utilized as potent food biopreservatives. Further studies will be necessary to elucidate the types of antibacterial agents that are produced by LAB. Moreover, LAB should be carefully evaluated before being utilized as biopreservatives and the mechanisms of action of the antibacterial agents that they produce should be further studied.

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