

Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu

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ABSTRACT

Three isolates of lactic acid bacteria (LAB) from the fermented food product, Budu, were identified as genus *Lactobacillus* (*Lactobacillus casei* LA17, *Lactobacillus plantarum* LA22 and *L. paracasei* LA02), and the highest population was *Lb. paracasei* LA02. The antibacterial agent produced by the isolates inhibited the growth of a range of gram-positive and gram-negative microorganisms. Antimicrobial sensitivity test to 18 different types of antibiotic were evaluated using the disc diffusion method. Inhibition zone diameter was measured and calculated from the means of five determinations and expressed in terms of resistance or susceptibility. All the LAB isolates were resistant to colesticin sulphate, streptomycin, amikacin, norfloxacin, nalidixic acid, mecillinam, sulphanethoxazole/ trimethoprim, kanamycin, neomycin, bacitracin and gentamycin but susceptible to erythromycin, penicillin G, chloramphenicol, tetracycline, ampicillin and nitrofurantion.

Keywords: Lactic acid bacteria, antimicrobial activity, antibiotic sensitivity, fermented fish product, inhibition zone

INTRODUCTION

“Antimicrobial agent” is a general term used to refer to any compound which include antibiotics, food antimicrobial agents, sanitizers, disinfectants, and other substances that acts against microorganisms. Antibiotics, on the other hand are antimicrobial agents produced by bacteria, fungi or of synthetic in nature. Antibiotic resistance refers to the ability of microorganisms to withstand the bacteriostatic and bactericidal effects of antibiotics. It provides a survival benefit to the invading microorganisms and under such circumstances it is difficult to eliminate the infection caused by these microorganisms. The mechanisms by which microorganisms exhibit resistance to antibiotics include drug inactivation or modification, alteration of target site, alteration in the metabolic pathway, and reduced drug accumulation (Katzung, 2004). The development of antibiotic resistance in bacteria is of public concern in view of the fact that a patient could develop antibiotic resistance by contacting a resistant microorganism or the emergence of a microorganism in the patient's body when treatment with antibiotic begins (Nagulapally, 2007).

Antimicrobials have been used increasingly as a primary intervention for inhibition or inactivation of pathogenic microorganisms in foods (Davidson and Zivanovic, 2003). Generally, food antimicrobial agents are not used alone to control foodborne pathogens, but are

included as components of the multiple approaches to microbial control. Lactic acid bacteria (LAB) from fermented products may act as a reservoir of antimicrobial-resistance genes that could be transferred to pathogens, either in the food web or, more importantly, in the gastrointestinal tract of human and animal (Belén Flórez *et al.*, 2005). However, there are different views pertaining to the resistance susceptibility breakpoints of most antimicrobials in LAB (Charteris *et al.*, 2001; Danielsen and Wind, 2003). Distinguishing the intrinsic, nonspecific, and acquired resistance is difficult as it requires antimicrobial-resistance patterns of many LAB species (Teuber *et al.*, 1999).

The present study was undertaken to determine the antimicrobial activity and antibiotic susceptibility of three isolates of LAB from a Malaysian fermented fish product, known as Budu. Being delicacy to a sector of the Malaysian population, this fermented food product is commercially available and information obtained from this study could contribute to the potential use of these LAB isolates in the food and pharmaceutical industries.

MATERIALS AND METHODS

Source of sample

The fermented fish product, Budu, manufactured by a small scale domestic factory in Kota Bharu, Kelantan,

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Malaysia was used as the source for isolation. This fermented product was prepared from marine fries, *Stolephorus* genera. The fries were mixed with tamarind and coconut sugar in salt solution (15%) and then fermented at room temperature in large earthen ware pots. The ratio between fries and salt solution was 1:2. Samples for the isolation were taken directly from the pots after 6 months of fermentation. All samples were kept refrigerated, so that their microbial load did not change during storage, and processed between 2 to 8 h after the collection.

Isolation of lactic acid bacteria

At first, 25 mL of sample was mixed with 225 mL of buffered peptone water (BPW, Oxoid) to obtain a 1:10 dilution. Serial dilutions of the samples were prepared in 0.1% peptone water. MRS agar was supplemented with 0.01% sodium azide to inhibit the growth of gram-negative bacteria. The diluted sample was streaked on the MRS agar plates and then incubated anaerobically for 24 h.

The stock cultures of LAB and the indicator microorganisms were maintained in MRS broth or nutrient broth supplemented with 15% glycerol and stored at -80 °C. Working cultures were prepared as slants on MRS agar for LAB or nutrient agar supplement for the indicators, and stored at 4 °C. Prior to its use in experiments the LAB cultures were transferred twice into the appropriate medium. API 50CHL stripes were used to identify all isolates to species level. Bacterial cells were inoculated according to procedures as recommended by the manufacturer. The APIs were incubated at 37 °C and reactions were observed after 48 h. The API database (Biomérieux SA) and accompanying computer software were used to interpret the data obtained.

Morphological, physiological and biochemical examination of LAB

The morphological, physiological and biochemical examination of the isolates were determined by the standard procedure of gram staining, catalase test and gas production test.

Determination of antibiotic susceptibility

Pure culture colonies of LAB were inoculated in MRS broth at 37 °C for 24 h. A sterile cotton wool swab dipped into the bacterial suspension was spread evenly on the surface of the MRS agar plate. The inoculated plate was allowed to dry before placing the diffusion discs containing antibiotics.

Susceptibility of the three isolates to 18 types of antibiotics was performed by the disc diffusion method as described by Bauer *et al.* (1966). Using commercially available antibiotics disc (Oxide) containing penicillin G (2 Units), ampicillin (25 µg), erythromycin (10 µg), colestin sulphate (10 µg), streptomycin (10 µg), amikacin (30 µg), norfloxacin (10 µg), chloramphenicol (30 µg), tetracycline (10 µg), nalidixic acid (30 µg), gentamycin (30 µg),

mecillinam (25 µg), nitrofurantion (300 µg), sulphamethoxazole/ trimethoprim (25 µg), vancomycin (30 µg), bacitracin (10 µg), kanamycin (30 µg), neomycin (30 µg) were placed on the surface of the agar plates. Precaution was taken to ensure that there was uniform contact between the antibiotic disc and agar plate. The plates were then incubated at 37 °C for 24 h.

Inhibition zone diameters were measured inclusive of the diameter of the discs. Results were expressed as sensitive, S (≥ 21 mm); intermediate, I (16-20 mm) and resistant, R (≤ 15 mm), respectively according to that described by Vlková *et al.*, (2006).

Assay for antimicrobial agent

Cell-free culture supernatants for antibacterial assay was prepared by growing the isolates in MRS broth at 37 °C and centrifuged at 12,000 x *g* for 10 min at 4 °C. The antimicrobial activity of the cell-free culture supernatant was determined by the agar well diffusion assay according to the method by Tagg *et al.* (1976). Aliquots of supernatants (100 µL) were placed in wells (6 mm diameter) cut in cooled soft nutrient agar plates (25 mL) previously seeded (1% v/v) with the appropriate indicator strains. The plates were incubated under optimal conditions for growth of the target microorganisms. After 24 h, diameters of the growth inhibition zones were measured. The inhibitory spectrum of the antibacterial agent produced by isolates of lactobacilli against different gram-positive and gram-negative bacteria was determined by agar-well diffusion assay.

RESULTS

From the morphological examination, all isolates were gram positive and short chain bacilli. The isolates were catalase negative, facultative anaerobes, negative to the production of gas from glucose and growth was totally inhibited at 5 °C and 45 °C (Table 1). Based on these characteristics and on the basis of the API 50CHL carbohydrate fermentation profiles, the isolates were phenotypically identified as *Lb. casei* LA17, *Lb. plantarum* LA22 and *Lb. paracasei* LA02.

Results of the sensitivity studies of the LAB isolates tested against 18 different types of antimicrobial agents are shown in Table 2. All isolates were susceptible to β -lactam group of antibiotic which include penicillin G and ampicillin. The isolates were also susceptible to erythromycin, chloramphenicol, nitrofurantion and tetracycline. In addition, all isolates were also resistant to a large number of aminoglycosides, which include amikacin, kanamycin, neomycin, streptomycin and gentamycin. Furthermore, all isolates were also resistant to the following groups of antimicrobial agents: tetracyclines (tetracycline), quinolones (nalidixic acid and norfloxacin), sulfonamides (sulphamethoxazole), colestin sulphate and mecillinam. Results from this study also showed that *Lb. paracasei* LA02 was resistant to vancomycin while *Lb. plantarum* LA22 and *Lb. casei* LA17 were susceptible to vancomycin.

Table 1: Morphological, physiological and biochemical characteristics of the LAB isolated from fermented fish product, Budu

Characteristics	LAB isolates		
	<i>Lb. paracasei</i> LA02	<i>Lb. casei</i> LA17	<i>Lb. plantarum</i> LA22
Cell morphology	Bacilli		Short bacilli
Gram stain reaction	+		+
Colony morphology	Circle, creamish (0.5 to 1.8 mm)	white to (1mm)	Circle, creamish to whitish (0.3 to 0.5 mm)
Catalase activity	-	-	-
Glucose fermentation	+	+	+
Growth at extreme temperatures	5 °C 45 °C	-	-
Gas production	-	-	-

positive reaction (+), negative reaction (-).

Table 2: Assessment of three strains of LAB to 18 different types of antibiotics

Antimicrobial agents	<i>Lb. paracasei</i> LA02	<i>Lb. casei</i> LA17	<i>Lb. plantarum</i> LA22	Antimicrobial agents	<i>Lb. paracasei</i> LA02	<i>Lb. casei</i> LA17	<i>Lb. plantarum</i> LA22
<u>β-lactam</u>				<u>Aminoglycosides</u>			
<u>Penicillins</u>				amikacin	+++	+++	+++
penicillin G	+	+	+	kanamycin	+++	+++	+++
ampicillin	+	+	+	neomycin	+++	+++	++
<u>Peptides</u>				streptomycin	+++	+++	+++
<u>Glycopeptide</u>				gentamycin	+++	+++	++
vancomycin	+++	+	+	<u>Tetracyclines</u>			
<u>Polypeptides</u>				tetracycline	+	+	+
bacitracin	++	+++	+++	<u>Quinolones</u>			
<u>Macrolides</u>				nalidixic acid	+++	+++	+++
erythromycin	+	+	+	norfloxacin	+++	+++	+++
<u>Fenicols</u>				<u>Sulfonamides</u>			
chloramphenicol	+	+	+	sulphanethoxazole/ trimethoprim	+++	+++	+++
<u>Nitrofurantoin</u>				<u>Others</u>			
nitrofurantion	+	+	+	colestin sulphate	+++	+++	+++
				mecillinam	+++	+++	+++

(resistant: +++, intermediate: ++, susceptible: +)

Table 3: Inhibitory spectrum of selected strains of antibacterial-producing LAB on gram-positive and gram-negative bacteria

Indicator species	Strain	Source	Activities (mm)		
			<i>Lb. paracasei</i> LA02	<i>Lb. casei</i> LA17	<i>Lb. plantarum</i> LA22
<i>B. cereus</i>	11778	ATCC	+++	+++	+++
<i>S. aureus</i>	25923	ATCC	+++	+++	+++
<i>Salmonella enterica</i>	1331	ATCC	+++	+++	+++
<i>Listeria monocytogenes</i>	15313	ATCC	+++	+++	++
<i>E. coli</i>	-	UPM	+++	+++	++
<i>Lactococcus lactis</i>	11454	ATCC	+++	+++	+

Degree of inhibition: + = Moderate inhibition zone (6-9 mm); ++ = Strong inhibition zone (10-14mm)
+++ = Very strong inhibition zone (15-18mm); - = No inhibition zone

All isolates have ability to produce antibacterial agents (Table 3). *Lb. paracasei* LA02 and *Lb. casei* LA17 produced about the same amount of antibacterial agent, and higher than that produced by *Lb. plantarum* LA22. The inhibitory spectrum of the antibacterial agent produced by selected isolates of Lactobacilli against different gram-positive and gram-negative bacteria was determined by agar-well diffusion assay. Table 3 also shows the antagonistic effect of the antibacterial agent on the growth of other gram-positive and gram-negative pathogenic microorganisms. All isolates studied, demonstrated an inhibitory effect on *E. coli*, *Listeria monocytogenes*, *Salmonella enterica*, *Staphylococcus aureus* and *Bacillus cereus*. The antibacterial agents reported here are expected to be peptidic antibacterial agent such as bacteriocin. However, identification and characterization of these antibacterial agents are being carried out in our laboratory.

DISCUSSION

To date, knowledge on the susceptibility of LAB to antimicrobial agents is rather limited in view of the large numbers of genera and species encountered in this group of bacteria apart from variances in the resistance spectra (Ocana *et al.*, 2006). Transfer of resistance to antimicrobial agents is an essential mechanism if the LAB are to adapt and survive in specific environment (Herrerros *et al.*, 2005). Hence in view of the above, before a strain of LAB can be used as a feed additive it must undergo antibiotic resistance screening to ensure its safe application.

Relatively few microorganisms are able to grow in Budu due to reduced oxygen level, low pH and very high salt content. Reports on the isolation and identification of LAB from Budu are very limited in the literature. Ohhira (1990) reported that 14 strain of *Lactobacillus plantarum* and 1 strain of *Streptococcus faecalis* have been isolated from Budu. In this study, three LAB strains have been isolated from Budu. The susceptibility of these LAB isolates tested against 18 different types of antimicrobial agents in the present study showed that they are susceptible to the β -lactam group of antibiotics (penicillin G and ampicillin) and other principal types of antibiotics which include erythromycin, chloramphenicol, nitrofurantion and tetracycline. This finding is contrary to previous reports which stated that LAB are normally resistant to the principle types of antibiotics (Halami *et al.*, 2000). β -lactam which are bactericidal, became the most widely used therapeutic class of antimicrobial agent because of their broad antibacterial spectrum and excellent safety profile. They inhibit bacteria cell wall synthesis and have a lethal effect on gram-positive bacteria. Erythromycins belong to macrolides group and have similar range of action and efficacy similar to that of penicillin. This group of antibiotics binds to ribosome, blocks protein synthesis (bacteriostatic) and are effective against gram-positive microorganisms (selectively accumulated compared with gram negatives).

From the 18 antimicrobial agents tested against, the three LAB isolates were resistant to 11 different types of antimicrobial agents. Most evident was the resistant to the aminoglycosides, sulfanamides and quinolones groups of antimicrobial agents. Aminoglycosides work by impeding the protein formation of bacteria in invading cells and are effective against a wide variety of bacteria and other microorganisms and are believed to prevent the production of proteins in the invading bacterial cell in this group. Bacterial resistance to aminoglycosides can result from mutations affecting the ribosomes and from changes in cellular permeability, but the most important cause of resistance is enzymically catalyzed inactivation of the antibiotics (Franklin and Snow, 2005).

Unfortunately, bacterial resistance to sulfonamides often coexists with trimethoprim resistance. Sulfonamide resistance could be due either to mutation in the chromosomal gene that mediates dihydropteroate synthesis, or to the acquisition of plasmid-borne genes coding for sulfonamide-resistant forms of the enzyme (Franklin & Snow, 2005). Amongst the antibiotics, vancomycin, an inhibitor of cell wall synthesis is of major concern as it is one of the last antibiotics broadly effective against clinical infections caused by multi-drug resistance pathogens (Johnson *et al.*, 1990). *Lb. casei* LA17 and *Lb. plantarum* LA22 were vancomycin sensitive in contrast to *Lb. paracasei* LA02.

All LAB isolates in this study showed that they are resistant to gram-negative spectrum antibiotic (nalidixic acid) and aminoglycoside antibiotics (amikacin, kanneomycin, neomycin and streptomycin). These findings concur with those reported by Zhou *et al.*, (2005) and Termerman *et al.* (2003) who claimed that most *Lactobacillus*, *Enterococcus* and *Pediococcus* strains used as probiotic were resistant to gram-negative spectrum and aminoglycoside antibiotics. Resistance of these antibiotics is usually intrinsic meaning that it is not transferring the genes to pathogenic or bacterial flora (Ammor *et al.*, 2007).

Research on antimicrobial substances produced by LAB, has led to their potential use as natural preservatives. These antibacterial compounds may be used to combat the growth of pathogenic microorganisms in the food industry. Antimicrobial compounds can be applied either as purified chemical agents or as viable cultures in the case of fermented products (Barnby-Smith, 1992). Novel purified antimicrobial compounds require convincing data to substantiate their lack of toxicity to ensure their acceptance before use in various food processes.

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