ISSN: 2347-467X, Vol. 06, No. (2) 2018, Pg. 500-508

Current Research in Nutrition and Food Science

Journal Website:www.foodandnutritionjournal.org

Isolation and Characterization of Lactic Acid Bacteria in Philippine Fermented Milkfish *Chanos chanos*-Rice Mixture (Burong Bangus)

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Abstract

Isolation and characterization of bacteria in food products are important to determine and distinguish the beneficial or harmful effects of microbiota in certain samples. Lactic acid bacteria in foods had long been associated with good factors as food preservatives and with added fermentation metabolites. This study isolated and characterized lactic acid bacteria from burong bangus. The culture and purification process of bacteria isolation resulted in 4 strains of lactic acid bacteria namely *Enterococcus faecalis, Tetragenococcus muriaticus, Lactobacillus delbrueckii* subp. *delbrueckii* and *Carnobacterium divergens*. High enzymatic activity was observed with *E. faecalis* particularly on lipase and protease assay. While *C. divergens* have no enzymatic activity against lipase, protease, amylase and cellulase. The antimicrobial property of *L. delbrueckii* is only susceptible to amoxicillin unlike the other three bacteria isolates. No antagonistic activities observed with the four bacterial strains against *Bacillus subtilis, Staphylococcus aureus* and *Escherichia coli.* The result of this study showed promising benefits to the food industry especially in developing countries like the Philippines because population is not yet so aware of these organisms and the benefits that can be derived through their consumption.

Article History

Received: 18 April 2018
Accepted: 08 August 2018

Keywords

Burong isdang bangus, Bacteria isolation, Bacteria characterization, Lactic acid bacteria.

Introduction

Fermented fish and fishery products find an important place in the dietary lists of the Southeast Asian countries[1]. These are widely distributed in rural and urban markets and fondly consumed daily as popular products[2]. Philippine fermented products are divided into two groups: the first contains a high salt concentration of about 15-20 percent like bagoong and patis and second with low salt concentration mixed with rice like burong isda and burong hipon.

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Doi: http://dx.doi.org/10.12944/CRNFSJ.6.2.24

Several variations of burong isda are available in markets and its name depends on the fish used such as burong ayungin, *Therapon plumbeus*; burong gurami, *Osphronemus goramy*; burong tilapia, *Tilapia nilotica*; burong dalag, *Ophicephalus striatus*; burong hito, *Clarias batrachus*; burong kanduli, *Arias manillensis* and burong bangus, *Chanos chanos*[3]. Milkfish or commonly known as bangus are among the most favored fish in the country noted for its distinct characteristic and flavor. It contains a large quantity of omega-3 fatty acids, DHA and other important nutrients[4].

Lactic acid bacteria (LAB) are a group of related bacteria that produce lactic acid as a result of carbohydrate fermentation[5]. They are gram-positive, fastidious, acid tolerant, generally non-sporulating, catalase negative and non-respiring rod or cocci that are associated by their common metabolic and physiological characteristics to produce lactic acid as fermentation metabolites[6,7]. They are nonpathogenic organism reputed as GRAS or Generally Recognized as Safe status[8]. Lactobacilli are ubiquitous bacteria in the human and animal micro flora[9]. LAB has been used for fermentation of food products since ancient days and until today their major applications are still in food industry[10]. Their presence in food is a type of biopreservation system and it does not only add flavor to foods but also control pathogenic and spoilage microorganism through various ways such as the production of peroxidases, organic acids and bacteriocins[11]. Its various industrial applications include preservative, acidulant and flavor in food, textile and pharmaceutical industries[12].

This study aimed to isolate bacterial strains from fermented rice-milkfish (burong bangus) and subjected to biochemical testing for characterization. The isolates were further analyzed for qualitative bacterial enzyme assay and test for antagonistic and antimicrobial activity.

Materials and Methods

Sample Preparation

Fresh milkfish at approximately 700 g was purchased in the local market of Miagao, Iloilo and brought to the processing laboratory in an ice box. The samples were washed, eviscerated, filleted and meat chopped into small sizes. The final weight was 350 g chopped milkfish. Rice and salt used for the fermentation process was also purchased from the said local market. Salt was subjected to analysis to determine its purity. Prior to the experiment, glass bottles for packaging container of the fermented product were sterilized in boiling water for 15 minutes.

The ingredients used for the fermentation of burong is da includes: 29% chopped milkfish; 69% cooked rice and 2% rock salt[13]. All the ingredients were mixed in a bowl and placed in the glass bottles. Then it was stored at room temperature in the processing laboratory.

Bacterial Isolation

Total viable count was analyzed by spread plating the sample in Nutrient Agar and MRS (deMan Rogosa Sharpe) Agar with 2% sodium chloride. Ten grams of homogenized sample was inoculated in 90 ml of peptone water then serially diluted from 10^{-2} to 10^{-6} dilution. Three replicates per dilution were spread plate for both Nutrient Agar and MRS Agar. The plates were incubated at 37^0C for 48 hours then total viable counts were determined. The MRS plates were kept for isolation of bacterial samples. The plates with the ideal colony were chosen for further process. Ideal colonies were streak plate in MRS Agar with 2% sodium chloride and incubated for 24 hours at 37^0C. The isolates were further purified by zigzag plating the next day in MRS Agar with 2% sodium chloride then slant after 24 hours incubation. The slants were used for biochemical test analysis of the isolates[14].

Biochemical Testing

All the biochemical test utilized 24 hours fresh bacterial culture for inoculation, except for spore staining which required a week old culture for analysis; and all the media used for testing were added with 2% sodium chloride[15].

Qualitative Enzymatic Activity

The activity of the protease, cellulase, lipase and amylase of the isolates were qualitatively determined in an enzyme specific agar medium[16].Bacterial isolates extracellular protease, lipase, cellulase and amylase secretion were determined using gelatin peptone agar, tributyrin agar, carboxymethylcellulose agar and starch agar respectively. Enzyme activity of the isolates is expressed by the formation of the clearing or halo zone around the bacterial colony.

Antagonistic and Antimicrobial Assay

This test determines the antagonistic ability of the isolates against other pathogenic bacteria like *Escherichia coli, Bacillus subtilis* and *Staphylococcus aureus*. While the antimicrobial ability was determined against amoxicillin, cephalexin, chloramphenicol and erythromycin.

Materials such as a sterile swab, forceps, paper disk (Whattman filter paper No. 1), saline solution and Mueller- Hinton agar plates were prepared prior to the experiment and fresh 24 hours culture bacteria were used for the assay. Bacterial isolates and pathogenic bacteria were inoculated into the saline broth and turbidity was confirmed using the McFarland No. 5 turbidity standard. Paper disk was dropped with 10µl of saline broth inoculated with bacterial isolates and set aside. Saline broth with *E. coli, B. subtilis* and *S. aureus* were swab on the agar plates and dried paper disk drop with bacterial isolates was placed on the plates for antagonistic activity[17].

Saline broth with bacterial isolates was swab on the agar plates and 0.6 mm antimicrobial disk of amoxicillin, cephalexin, chloramphenicol and erythromycin were placed on the plates to determine antimicrobial activity of the isolates. All plates were incubated at 37^{0}C for 24 hours. Then, the zones of inhibition were measured and its susceptibility to microbial drugs was determined based on the Table of Standards for Antimicrobial Susceptibility Testing[18].

Results and Discussion

Aerobic plate count of burong isda in Nutrient Agar and MRS Agar is presented in Table 1. The total aerobic plate count of Nutrient Agar is 3.478 x 10^3 (3.54 log CFU/g) and for MRS agar is 2.34 x 10^5 (5.36 log CFU/g).

The aerobic plate count is an indicator commonly used in the food industry to determine the level of microorganism in a certain products[19]. Fresh fish and fishery products often have an APC of 10^4 -10^5 (5 log CFU/g), while some seafoods have APC of 10^6 –10^8 (8 log CFU/g) without any quality changes[20]. The result from this study was expected lower than the fresh fishes due to the fermentation process with the aid of salt addition and subsequently pH reduction[21]. Study on fermented tuna viscera concluded that during the fermentation period, counts decreased together with the increasing salt concentration[22].

Table 1: Aerobic plate count in Nutrient and MRS Agar

Dilutions	MRS Count	Total Plate Count
10^{-2}	TNTC	62
10^{-3}	471	6
10^{-4}	70	1
10^{-5}	9	0
10^{-6}	1	0
APC (CFU/ml)	$2.34x10^5$	$3.478x10^3$
Log count	5.36	3.54

Biochemical test results of bacterial strains from burong isda with presumptive identification were presented in Table 2. Different test was conducted to characterize the five isolates. Based on the tests result and further confirmation with the Bergey's Manual of Systematic Bacteriology, the five isolates from burong isda were summarized into four lactic acid bacteria species. Isolates 2 and 4 were identified as one species only.

Enterococcus faecalis of the *Enteroccocci* family are ancient members of the animal microbiome date back in the Devonian period about 412 million years ago. They are known to thrive in the nutrient-rich, oxygen-depleted environment of the intestinal tract found in a wide variety of host including humans[23]. They are a member of the lactic acid bacteria which consist of Gram-positive, cocci or rods that produce mainly lactic acid from the fermentation of carbohydrates. They are also known to exhibit important probiotic activity and applications in the food industry as starter culture and potential biopreservatives[24]. *Tetragenococcus* are halophilic histamine forming lactic acid bacteria first isolated from fermented squid liver sauce. They are abundantly present in the gut and internal organs of their host[25,26]. *Lactobacillus delbrueckii* are the important member of lactic acid bacteria commonly used as the starter for the production of fermented milk products such as yogurt and cheese[27]. They

are recognized probiotics that do not adhere well to the human intestine[28]. *Carnobacteria* is a dominant genus in the gastrointestinal tract of various seafood products, non-aciduric with low spoiling potential[29]. They are lactic acid bacteria utilized as a bacterial starter culture in the manufacture of fermented meat products[30]. *Carnobacterium divergens* isolated from seafoods have known to possess promising inhibition capacity to maintain a low level of *Listeria monocytogenes* in cold smoke salmon lower than 50 CFU/g during four weeks of storage at 4^0 and 8^0C[31].

Table 2: Biochemical test result of bacterial strains from burong isda with presumptive ID

BIOCHEMICAL TESTS	ISOLATES				
	B1	**B2**	**B3**	**B4**	**B5**
Bacterial shape	Doubled, quartered ovoid cocci	Small, singled, doubled, chained, tetrad cocci	Short, clustered, rods	Small, single, chained, doubled, tetrad cocci	Short, clustered, slendered, rods
Oxygen requirement	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe	Facultative anaerobe
Gram staining	+	+	+	+	+
Spore formation	-	-	-	-	-
Catalase	+	+	-	+	-
Oxidase	-	-	-	-	-
Glucose	-	-	+	-	+
Sorbitol	-	-	-	-	-
Glycerol	-	-	-	-	-
Mannitol	-	-	-	-	-
Arabinose	-	-	-	-	-
Lactose	-	-	-	-	-
Citrate	-	-	-	-	-
H2S	-	-	-	-	-
Indole	-	-	-	-	-
Methyl red	+	+	+	+	+
Voges-Praskauer	-	-	-	-	-
Gelatine	-	-	-	-	-
Starch	-	-	-	-	-
Casein	+	+	+	+	+
Arginine dihydrolase	+	-	-	-	+
Lecithinase	-	-	-	-	-
Motility	-	-	-	-	-
Presumptive Identification	*Enterococcus faecalis*	*Tetragenococcus muriaticus*	*Lactobacillus delbrueckii* subp. *delbrueckii*	*Tetragenococcus muriaticus*	*Carnobacterium divergens*

Legend: +, Positive; -, Negative

Table 3 and Fig. 1 presents the zone of activity in millimetre of qualitative enzyme assay of the bacterial isolates. As can be observed, isolate B1 had an enzyme activity both on lipase and protease assay with a value of 9.67 mm and 4.0mm, respectively. The second isolate had activity on protease assay

only with 2.86 mm zone of activity. The third isolate had activity on amylase assay only with the value of 5.0 mm zone of activity value. The fifth isolate had no activity for all the enzyme assay.

Isolate B1, *Enterococcus faecalis* are known to possess proteolyic system and lipolytic activity that contributes to the development of special flavour once incorporated in the manufacturing of cheese[24]. *Tetragenococcus muriaticus* isolated from fermented products had been found to contribute tolowering pH and the risk of putrefaction to hasten the degradation of the fermented fish product with the aid of the endogenous enzyme present in the fish[26,29]. *Lactobacillus delbrueckii* commonly use in dairy products that aid as thickener, stabilizer and gelling agent produced exopolysaccharide for that function thus; they have amylase activity[28]. *Carnobacterium* spp. is well known to produce tyramine a natural derivative of amino acid tyrosine[31].

Table 3: Zone of activity from enzyme assay of burong isda isolates

Isolate	Enzymes							
	Lipase		Protease		Amylase		Cellulase	
	Zone of actvity (mm)	Enzyme activity	Zone of actvity (mm)	Enzyme activity	Zone of actvity (mm)	Enzyme activity	Zone of actvity (mm)	Enzyme activity
B1	9.67±0.58*	+	4.0±1.0**	+		-		-
B2		-	2.86±0.58**	+		-		-
B3		-		-	5.0±2.83***	+		-
B5		-		-		-		-

Legend: +, Positive; -, Negative;
* Tributyrin agar medium; ** Peptone gelatine agar medium; *** Starch agar medium

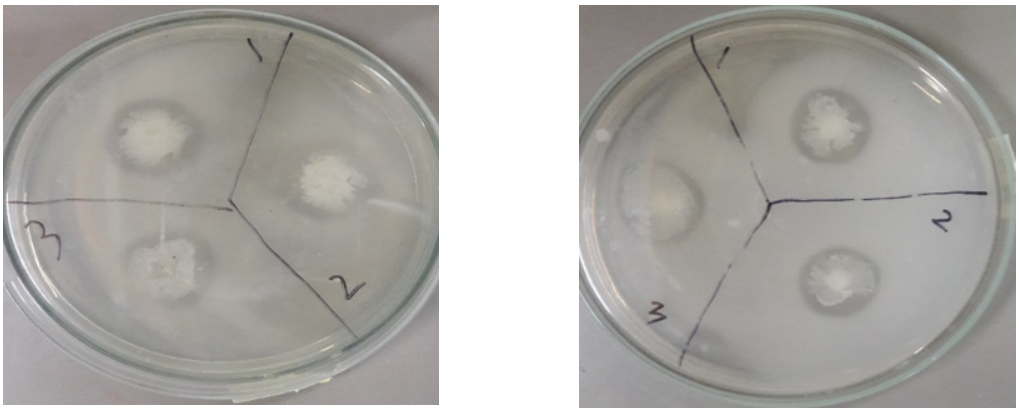

Fig. 1: Isolates B1 (left) and B2 (right) clearing zone in Gelatin Peptone Agar after 24 hours of incubation at 37˚C

Table 4 and Fig. 3 shows the antimicrobial activity and zone of inhibition of bacterial isolates against amoxicillin, chloramphenicol, cephalexin and erythromycin. Susceptibility of the isolates against the antibiotic agents were confirmed using the Clinical Laboratory Standards Institute Manual for Antimicrobial Susceptibility Testing. For amoxicillin, all the four isolates were susceptible with the zone

of activity of 44 mm for the first isolate, 33 mm for the second isolate, 35 mm for the third isolate and 28 mm for the fourth isolate. For chloramphenicol, three isolates were susceptible and one had no reaction with the zone of activity of 30 mm for the first isolate, 34 mm for the second isolate and 30 mm for the fifth isolate. For cephalexin, only two isolates were susceptible and two others had no reaction, the zone of activity for the first isolate was 35 mm and 38 mm for the second isolate. For erythromycin, only the first isolate was susceptible with 21 mm zone of activity while the other three isolates had no reaction.

Table 4: Antimicrobial activity and zone of inhibition of bacterial isolates against amoxicillin, chloramphenicol, cephalexin and erythromycin

Isolate	Antibiotics							
	Amoxicillin		Chloramphenicol		Cephalexin		Erythromycin	
	Zone of activity (mm)	Antimicrobial activity	Zone of activity (mm)	Antimicrobial activity	Zone of activity (mm)	Antimicrobial activity	Zone of activity (mm)	Antimicrobial activity
B1	44mm	S	30mm	S	35mm	S	21mm	S
B2	33mm	S	34mm	S	38mm	S	-	-
B3	35mm	S	-	-	-	-	-	-
B5	28mm	S	30mm	S	-	-	-	-

Legend: S, susceptible

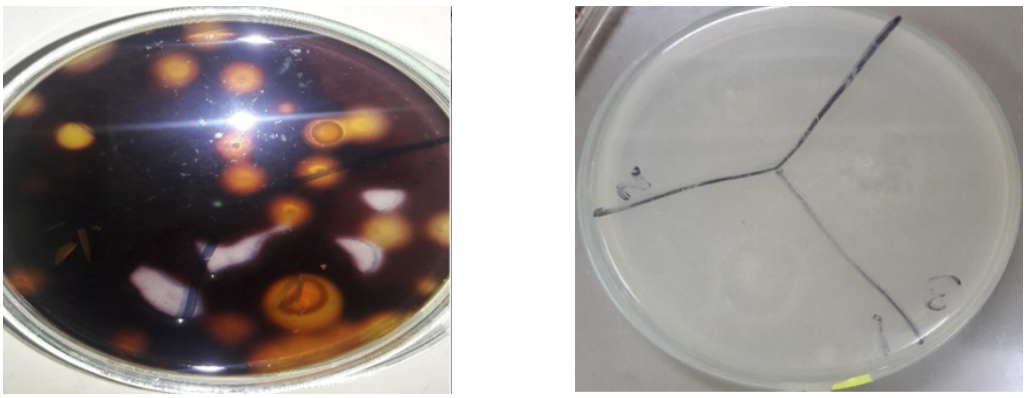

Fig. 2: Isolate B3 clearing zone in starch agar plate (left) and Isolate B4 lipase activity in tributyrin agar plate (right)

As can be observed in Fig. 4, all the isolates against *Escherichia coli, Bacillus subtilis* and *Staphylococcus aurues* had no inhibition zone. It had only reacted against amoxicillin that serve as the control against the three pathogenic microorganisms. Lactic acid bacteria are known to produce the variety of antimicrobial compounds such as ethanol, formic acid, acetone, hydrogen peroxide, diacetyl and bacteriocins that contributes for its ability to preserve foods and are naturally competitive known to overcome other microorganisms sharing the same niche[5]. The antagonistic and antimicrobial properties of lactic acid bacteria are due to the competition for nutrients and the production of one or more active

metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide and antimicrobial peptides such as bacteriocins[8].

Isolation and characterization of bacteria are important in determining the microbial flora of food products it further distinguishes the beneficial and harmful effects of microbiota in certain samples. This test requires a lot of time and effort to end up with the essential result, thus it is important to properly choose the exact test required for characterization of certain organisms. Lactic acid bacteria in food products had long been associated with good factors as food preservatives and with added fermentation metabolites. They only require the right environmental factors to grow and proliferate in food products. Bacterial isolation of this organisms is very much promising industry especially in developing countries like the Philippines because the population is not yet so aware of these organisms and the benefits that can be derived through their consumption.

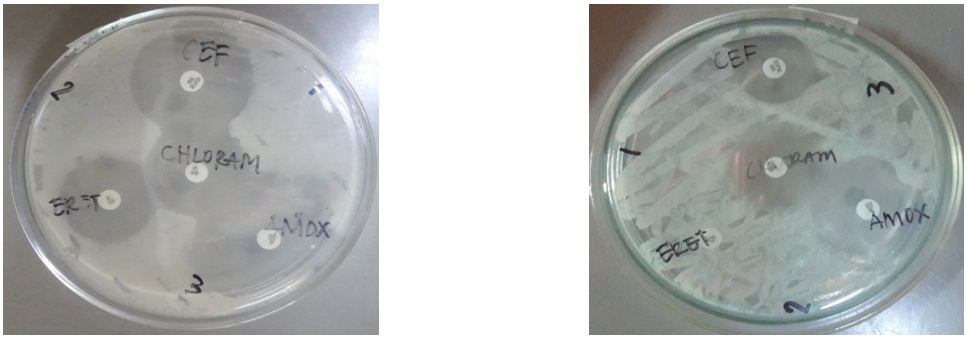

Fig. 3: Zone of Inhibition of Isolate B1 (left) and Isolate B2 (right) in antimicrobial disks.

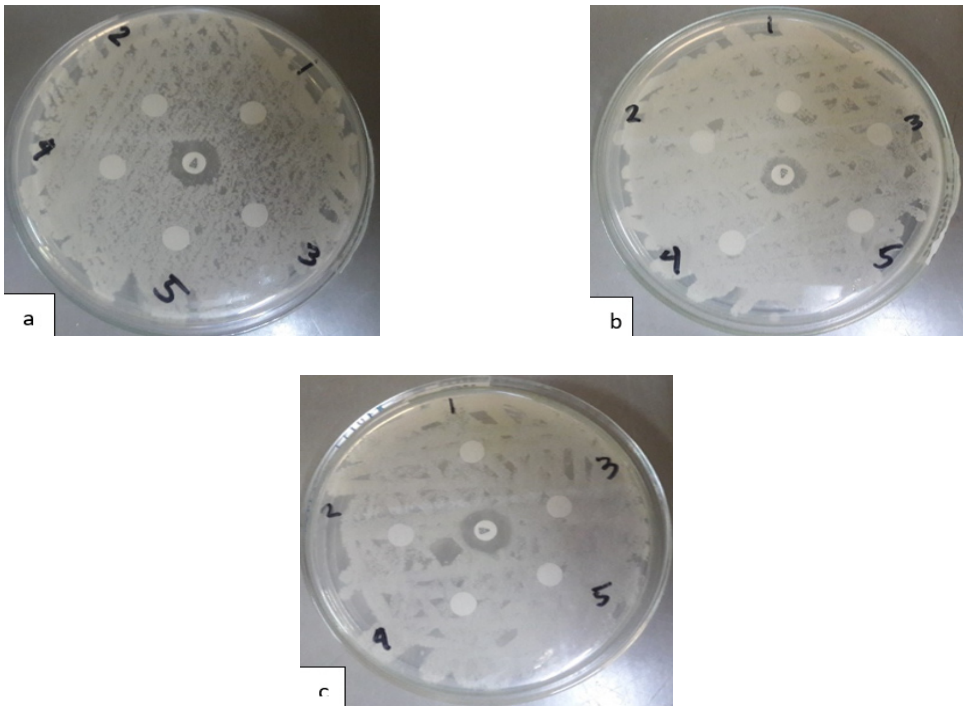

Fig. 4: Antagonistic activity of LAB isolates (1-5 coding corresponds to B1-B5) to Bacillus subtilis (a), Staphylococcus aureus (b) and Escherichia coli (c) in the MH agar medium

Acknowledgments

The authors would like to acknowledge Department of Science and Technology-Accelerated Science and Technology Human Resources Development Program (DOST-ASTHRDP) and University of the Philippines Visayas-College of Fisheries and Marine Sciences for supporting this study.

Conflict of Interest

The authors declare no conflict of interest.

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