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Chapter 24

# Lactic Acid Bacteria in Philippine Traditional Fermented Foods

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Additional information is available at the end of the chapter

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## 1. Introduction

The Philippine archipelago is home to a diverse array of ecosystems, organisms, peoples, and cultures. Filipino cuisine is no exception as distinct regional flavors stem from the unique food preparation techniques and culinary traditions of each region. Although Philippine indigenous foods are reminiscent of various foreign influences, local processes are adapted to indigenous ingredients and in accordance with local tastes. Pervasive throughout the numerous islands of the Philippines is the use of fermentation to enhance the organoleptic qualities as well as extend the shelf-life of food.

Traditional or indigenous fermented foods are part and parcel of Filipino culture since these are intimately entwined with the life of local people. The three main island-groups of the Philippines, namely – Luzon, Visayas, and Mindanao, each have their own fermented food products that cater to the local palate. Fermentation processes employed in the production of these indigenous fermented foods often rely entirely on natural microflora of the raw material and the surrounding environment; and procedures are handed down from one generation to the next as a village-art process. Because traditional food fermentation industries are commonly home-based and highly reliant on indigenous materials without the benefit of using commercial starter cultures, microbial assemblages are unique and highly variable per product and per region. Hence the possibility of discovering novel organisms, products, and interactions are likely.

Various microorganisms are involved in common food fermentation processes. In particular, lactic acid bacteria (LAB) in food is a type of biopreservation system. They not only contribute to the flavor of the food but LAB are also able to control pathogenic and spoilage microorganisms through various ways that include, but are not limited to, production of peroxidases, organic acids, and bacteriocins. Traditionally, identification of LAB in foods is largely dependent on culture-based methods; and properties of each isolate are evaluated

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under controlled conditions. However, with the advent of molecular techniques, the enumeration of microorganisms missed by culture-dependent methods is now possible. Also, as more LAB metabolites, such as bacteriocins, are being reported, a wider database for identification and comparison with potential novel products are now available.

As the production and consumption of traditional fermented food products become increasingly relevant in the face of rapidly increasing population and food insecurity, more research and development to ensure the safety and nutritional quality of these fermented products is warranted. For a more extensive discussion of the principles and technology of Philippine fermented foods, the readers are directed to Sanchez (2008). This book is a detailed reference based on decades of research. Some data from the book will be presented again here in addition to other data from more recent studies. It is not the intention of this present paper to repeat what has been presented in the book, especially regarding fermentation processes, but only to present, as complete as possible, the data that are available regarding LAB present in indigenous/traditional fermented foods.

This paper aims to briefly review the various lactic acid-fermented indigenous fermented specialties in the different regions of the Philippines. Majority of the discussion will focus on recent data gathered from bacteriocin research and metagenomics studies of Philippine fermented specialties. Lastly, the health applications of the different fermented food products and their development as functional foods will be evaluated.

## 2. Regional fermented specialties in the Philippines

There are various lactic acid-fermented indigenous food products in the Philippines. Table 1 gives a summary of these different fermented specialties found in the different regions. Although a particular product type can be seen throughout the whole country, the texture, taste, and appearance would vary depending on the local taste, materials used, and process employed. For example, bagoong is a common fermented fish paste found all over the Philippines but the characteristic of the product found in Luzon is different from that found in the Visayas and Mindanao regions. Bagoong also takes on different names; there is bagoong na isda, bagoong alamang, bagoong na sisi, and guinamos (Sanchez, 2008). A product that is processed in a similar manner is dayok; it is made of brined fish entrails. Research indicates that this is also a lactic acid-fermented food but the LAB involved have not been identified yet (Besas and Dizon, 2012). Longanisa is sausage made of beef, pork, or chicken. It also takes on many forms depending on where it is made. The more famous ones are Vigan Longanisa in Northern Luzon, Pampanga Longanisa in Central Luzon, Lucban Longanisa in Southern Luzon, and Cebu Longanisa in the Visayas. The tastes vary from spicy, garlicky, sour, to sweet.

In lactic acid-fermented foods, LAB are important in preventing the growth of spoilage organisms, and altering flavor, aroma, and texture of the product. Although LAB are initially present in low numbers in the raw materials used, they soon proliferate as other organisms are inhibited by the initial addition of salt and as the continuous growth of LAB decreases the pH of the food making it less conducive for growth of other organisms. Recent

studies, however, have shown that there are a lot more benefits that can be derived from LAB in traditional fermented foods.

| CATEGORY | PRODUCT NAME | REGION | MAJOR INGREDIENTS | LACTIC ACID BACTERIA INVOLVED (as determined from culture-based methods) | APPEARANCE AND/OR USAGE |
|---|---|---|---|---|---|
| Fermented vegetables, fruits | Burong mustasa | Luzon | Mustard leaves, cooked rice and/or rice washings | *Leuconostoc mesenteroides, Enterococcus faecalis, Lactobacillus plantarum* | Side dish |
| | Burong pipino | Whole Phil | cucumber | *Leu. mesenteroides, L. brevis, Pediococcus cerevisiae, L. plantarum* | Side dish |
| | Burong mangga | Whole Phil | Immature mango | *Leu. mesenteroides, L. brevis, P. cerevisiae, L. plantarum* | Side dish |
| | Atchara | Whole Phil | Immature papaya or chayote, or turnip (singkamas) | Unknown | Side dish |
| Cheese | Kesong puti | Luzon, Visayas | Cow or carabao milk | *Lactococcus lactis* | White soft cheese |
| Fermented fish and fishery products | Balao-balao | Luzon | Cooked rice, shrimp, salt | *Leu. mesenteroides, P. cerevisiae, L. plantarum* | Side dish, condiment |
| | Burong-isda | Luzon | Freshwater fish, rice, salt | *Leu. mesenteroides, E. faecalis, P. cerevisiae, L. plantarum, P. acidilactici, Leu. paramesenteroides* | Side dish, condiment |
| | Tinabal | Visayas | Parrot fish (for tinabal molmol) and frigate fish (for tinabal mangko), salt | *P. pentosaceus, S. equinus, Leuconostoc* sp., *Lactobacillus* sp. | Side dish, viand |
| | Burong talangka | Luzon | Small shore crabs (*Varuna litterata*) | *Leu. mesenteroides, E. faecalis, P. cerevisiae, L. plantarum* | Side dish, viand |
| | Patis | Whole Phil | Small fish, salt | *P. halophilus* (in mixed fermentation) | Fish sauce (patis), fish paste (bagoong), used as condiment, sauce, flavoring agent, viand |
| | Bagoong isda | Whole Phil | Small fish, salt | | |
| | Bagoong alamang | Whole Phil | Small shrimps, salt | | |
| | Bagoong na sisi | Visayas | Shell fish, salt | | |
| | Guinamos | Bagoong isda in Visayas, Mindanao | Salt water small fish (dilis/belabid – *Stolephorus* sp.), salt | | Condiment, viand, side dish |
| | Dayok | Visayas, Mindanao | Fish entrails, salt | Unidentified LAB | Condiment, viand, side dish |
| Fermented meat, sausages | Longanisa | Whole Phil | Ground pork, beef, or chicken meat, spices and preservatives | *P. acidilactici, Lactococcus lactis* (together with *Micrococcus aurantiacus*) | Viand |
| | Agos-os | Visayas | Sweet potato and ground pig's head | *E. faecalis* | Viand |
| | Burong kalabi | Luzon | Cooked rice, ground carabao meat | *L. plantarum* | Side dish, viand |
| | Burong babi | Luzon | Cooked rice, ground pork | *L. plantarum* | Side dish, viand |

| | | | | | |
|---|---|---|---|---|---|
| Fermented rice, cassava, sugar cane, coconut, soya | Puto | Whole Phil | Rice, sugar | *L. mesenteroides, E. faecalis, P. cerevisiae* (in mixed fermentation with *Saccharomyces cerevisiae*) | Steamed rice cake |
| | Bibingka | Whole Phil | Rice, sugar | | Baked rice cake |
| | Tapuy | Luzon | Rice, glutinous rice | *Leuconostoc, L. plantarum* (in mixed fermentation with molds and yeasts) | Wine; beer |
| | Pangasi | Mindanao | Rice | Unknown | Wine |
| | Landang | Visayas, Mindanao | Cassava, or buli palm flour | Unknown | Dried jelly pellets pellets, rice substitute |
| | Puto balanghoy | Mindanao | Cassava | Unknown | Steamed cake |
| | Basi | Luzon | Sugar cane | Unknown | Wine |
| | Suka | Whole Phil | Sugar cane juice (for sukang Iloco), palm inflorescence sap (for sukang tuba) | *Leuconostoc, Lactobacillus, Streptococcus* in the initial fermentation phase only | Vinegar, condiment, flavoring |
| | Sinamak | Luzon | Sugar cane juice, spices (chilies, onions, garlic) | Unknown | Spiced vinegar, condiment, flavoring |
| | Pinakurat | Visayas, Mindanao | Coconut sap, chilies, salt, various spices | Unknown | Spiced vinegar, condiment, flavoring |
| | Tuba | Whole Phil | Coconut sap | Unknown | Wine |
| | Lambanog | Whole Phil | Coconut sap | Unknown | Wine |
| | Toyo | Whole Phil | Soybeans | *P. halophilus, E. faecalis, L. delbrueckii* (in mixed fermentation with *Aspergillus sojae* and *Saccharomyces rouxii*) | Condiment, flavoring agent, seasoning |

(Sources: Banaay et al., 2004; Besas and Dizon, 2012; Lee, 1999; Olympia et al., 1995; Sanchez, 2008; Tan et al., 2007)

**Table 1.** Regional Lactic Acid-Fermented Specialties in the Philippines

## 3. Research initiatives on LAB from Philippine fermented foods

### 3.1. Bacteriocin research

Bacteriocins are antimicrobial proteins or peptides produced by certain bacterial strains. Unlike the peptide antibiotics they usually have a narrow spectrum of antimicrobial activity, usually inhibiting growth of closely related bacterial species or strains and lacking lethality to the producer strain (Riley and Wertz, 2002).

The bacteriocins of LAB are small, cationic, hydrophobic, or amphiphilic peptides or small proteins, composed of 20 to 60 amino acid residues (Chen & Hoover, 2003). The bactericidal mode of action and biochemical properties depend on the protein moiety that could be specific to a particular LAB strain, *i.e.* the N-terminal amino acids as determinant of receptors in the cell wall of the susceptible strains/species and C-terminal amino acids for the biochemical properties. LAB bacteriocin must have the following desirable properties: "(1) not active and nontoxic to eukaryotic cells, (2) become inactivated by digestive proteases, having little influence on the gut microbiota, (3) low pH and heat-tolerant, (4)

have a relatively broad antimicrobial spectrum, against many food-borne pathogenic and spoilage bacteria, (5) show a bactericidal mode of action, usually acting on the bacterial cytoplasmic membrane: no cross resistance with antibiotics, and (6) have genetic determinants that are usually plasmid-encoded, facilitating genetic manipulation" (Apaga, 2012 as cited from Abriouel et al., 2007).

LAB bacteriocins have attracted attention in recent years because of their generally regarded as safe (GRAS) status and good value as natural biopreservatives which can find applications in the food and cosmetic industries (Cleveland et al., 2001; Daeschel, 1993; Riley and Wertz, 2002). Nisin, produced by strains of *Lactococcus lactis*, has been used in over 50 countries as anti-listerial and anti-clostridium substance. LAB bacteriocins with selective inhibition on food pathogens such as *Listeria monocytogenes*, but no inhibition on important lactic acid bacterial inocula such as the noted probiotic *Lactobacillus paracasei* or *Lactobacillus rhamnosus*; and yogurt-producing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus thermophilus*, may provide advantage over those that have a wider spectrum of antimicrobial activity and would kill these beneficial organisms, including nisin (De Vos, 1993; Jack and Ray, 1995; Nielsen et al., 1990). Hence, efforts on the search for LAB bacteriocins and elucidation of their properties are actively being pursued by several research laboratories. The future holds a wide array of LAB bacteriocins available for various specific applications.

### 3.2. Isolation and identification of bacteriocin-producing LAB

Some efforts on the isolation of bacteriocin-producing LAB had been started for more than a decade now in two major research institutions in the country namely: University of the Philippines Los Banos (specifically, the National Institutes of Molecular Biology and Biotechnology or BIOTECH-UPLB and the Institute of Biological Sciences or IBS-UPLB) and the Philippine Root Crop Research and Training Center, Visayas State University (VSU). These two institutions branched out knowledge on bacteriocin research through affiliate tutorship, as thesis advisers and as trainors to students and staff from a few other academic institutions which also did bacteriocin researches like University of Santo Tomas (UST), University of the Philippines Manila (UPM), De La Salle University (DLSU) and Ateneo de Manila University (ADMU). BIOTECH-UPLB and IBS-UPLB jointly worked on bacteriocins of *Lactobacillus plantarum* or plantaricins and those of *Pediococcus acidilactici* or pediocins. On the other hand, VSU devoted some efforts on the enterocins of *Enterococcus* spp. (Tan et al., 2001). DLSU also tried isolation of bacteriocin-producing LAB for food applications. UST was able to isolate bacteriocin-like inhibitory substances against medically important pathogens like *K. pneumoniae* (Dedeles et al., 2011). UPM and ADMU worked on human and animal health applications of bacteriocins.

Various fermented food products with proteinaceous components were the major sources of isolated LAB for bacteriocin screening. Such fermented food products are home-grown or produced by small enterprises and are still commercially available from

public markets in Luzon, Philippines and some parts of the Visayas like Leyte island. Examples of Philippine indigenous fermented foods that were good sources of bacteriocin-producing LAB are fermented rice and shrimp (*balao-balao*), fermented rice and fish mixture (*burong kanin at isda*), fermented pork (*burong babi*) in Central Luzon (Elegado et al., 2003; Gervasio and Lim, 2007) and fermented pork and sweet potato (*agos-os*) in Eastern Visayan region (Samar and Leyte). On the other hand, pickled vegetables like mustard leaf (*burong mustasa*) and green papaya (*achara*), fermenting fruits like pickled green mango, *bignay* or mango wine (Samnang 2010), fermented salted fish (*bagoong*), spicy sausages (*longganisa*) may contain some LAB but often times they are not bacteriocinogenic (Gervasio and Lim, 2007). The obvious reasons are the presence of inhibitory substances like salt, spices, alcohol or acid and of course the dearth of proteinaceous materials in the food material.

In one of the first isolation studies for bacteriocinogenic LAB, various proteinaceous fermented foods native to Central and Southern, Philippines were screened for bacteriocin-producing bacterial isolates. Seventy one out of several hundreds of colony-forming unit isolated by agar plate streaking were found antagonistic to the indicator microorganism, *Lactobacillus plantarum* ATCC 14917, through direct assay. By "spot-on-lawn" assay by pH-neutralized culture supernatant, nine (9) isolates were confirmed to be bacteriocin producers (Elegado et al., 2003). Banaay et al. in 2004 also reported on the isolation of 1,100 putative LAB from indigenous fermented foods in Luzon, Philippines. A strain of *Lactobacillus plantarum* was selected as the best bacteriocin producer. In another study, out of the 160 putative LAB obtained from 19 fermented food products from public markets in Central Luzon, 32 LAB isolates were found to be bacteriocinogenic (Gervasio and Lim, 2007). Santiago et al. (2008) were also able to find two LAB isolates, *Lactobacillus fermentum* LBA-19 and *Lactobacillus casei* LTI-21, screened from among several LAB isolates from various fermented food products from different regions in the Philippines.

Being pleomorphic, identification of LAB is quite challenging. A combination of various microbiological and molecular biology tools would help in finding the real identity. Banaay et al. (2004) did a thorough identification of the bacteriocinogenic LAB isolate using conventional morphological, biochemical and physiological methods, chemotaxonomic methods, as well as molecular methods. This is especially relevant to the identification of *Lactobacillus plantarum* which is a known pleomorphic bacteria. Most other Philippine LAB researchers often times directly apply 16S rRNA gene sequencing and homology search for LAB purified through repeated agar streaking and putatively identified as LAB just after determining its acid–forming, Gram positive and catalase negative properties. (Elegado et al., 2003; Gervasio and Lim, 2007; Santiago et al., 2008). Aside from 16S rRNA genes, other conserved genes were used for identification such as phenylalanyl-tRNA synthase (*pheS*) gene (Dedeles et al., 2011). Detection of bacteriocin genes through PCR may also be helpful in confirming the identity of the bacteriocinogenic LAB as well as the probability of producing the bacteriocin (Table 2).

| ISOLATE/ STRAIN No. | IDENTIFICATION | (primer) HOMOLOGY to *P. acidilactici* type strain | REFERENCE | Bacteriocin gene by PCR; fingerprinting; HOMOLOGY |
|---|---|---|---|---|
| AA-5a | partial 16S rRNA gene ID: *P. acidilactici* | (1492R)98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Elegado et al. 2003 | ped $^{+}$; REP and RAPD |
| 4E2 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ |
| 4E4 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 97% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 98% *P. acidilactici* 8D2CCH01MX; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ |
| 4E5 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* DSM20284 | Laxamana et al. (2011) | ped$^{+}$ ; REP |
| 4E6 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284; (27F) 99% *P. acidilactici* 8D2CCH01MX ; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$;[ 99% *P. acidilactici* bacteriocin genes ; pSMB74 ] |
| 4E10 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 96% *P. acidilactici* UL5; 99% *P. lolii to* NGRI0510Q (27F) 99% *P. acidilactici* LAB 001 ; 99% *P. lolii* NGRI 0510Q | Apaga (2012) | ped$^{-}$ |
| 4BL7 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* 8D2CCH01MX; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ |
| 3G3 | API CHL50 ID: *Lactobacillus pentosus*(doubtful) partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 99% *P. acidilactici* IMAU20090 (27F) 98% *P. acidilactici* DSM20284 | Elegado and Perez (2012) | ped$^{+}$ ; REP; ped$^{+}$ |
| 3G8 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 99% *P. acidilactici* UL5 (27F) 98% *P. acidilactici* DSM20284 | Elegado and Perez (2012) | ped$^{+}$ |
| 3F3 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 95% *P. acidilactici* UL5 (27F) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ |
| 3F8 | partial 16S rRNA gene ID: *P.acidilactici* | (1492R) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ |
| 3F10 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 97% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 97% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ [99% *P. acidilactici* genomic scaffold]; |
| IG7 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 97% *P.acidilactici* UL5 99% *P. acidilactici* 8D2CCH01MX; (27F) 98% *P. acidilactici* DSM20284 | Apaga (2012) | ped$^{+}$ [100% pediocin operon;PSMB74]; |

| | | | | |
|---|---|---|---|---|
| $K_2A_2$-3 | API: *Pediococcus pentosaceus* (good) partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 97% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Villarante (2011); Elegado and Perez (2012) | $ped^+$ ; $plan^+$ $ped^+$ ; REP |
| $K_2A_2$-1 | API: *P. acidilactici* (doubtful) | - | Abuel (2007) | $ped^+$ ; $plan^+$ $ped^+$ |
| $K_2A_2$-5 | API: *P. acidilactici* (doubtful); partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 97% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 99% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Apaga (2012) | $ped^+$ [99% *P. acidilactici* genomic scaffold]; $plan^+$ |
| $K_2A_1$-1 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 99% *P. acidilactici* L94; 99% *P. acidilactici* DSM20284 (27F) 98% *P.acidilactici* JS-9-4; 99% *P. acidilactici* DSM20284 | Apaga (2012) | $ped^+$ |
| $K_3A_2$-2 | API: *Lactococcus lactis* (good) partial 16S rRNA gene ID: *P. pentosaceus* | - | | $ped^+$ ; $plan^+$; $ped^+$ |
| $K_3A_2$-3 | partial 16S rRNA gene ID: *P. acidilactici* | 100% *P. acidilactici* UL5 | Elegado and Perez (2012) | $ped^+$ |
| S3 | partial 16S rRNA gene ID: *P. acidilactici* | (1492R) 98% *P. acidilactici* UL5; 99% *P. acidilactici* DSM20284 (27F) 97% *P. acidilactici* LAB 001; 99% *P. acidilactici* DSM20284 | Apaga (2012) | $ped^+$ [99% pediocin operon; pSMB72]; |

**Table 2.** Identification and bacteriocin gene determination of putative *Pediococcus acidilactici* through 16S rRNA and pediocin gene PCR amplification and sequencing.

## 3.3. Purification and characterization of bacteriocins

Purification of bacteriocin peptides or small proteins into homogeneity is necessary in order to fully characterize them, particularly the determination of molecular mass, the primary structure or amino acid sequence and secondary structure. For pediocin, it was found that a simple and rapid method is effective for its purification. This method involves adsorption of pediocin onto the cell wall of the producer cell at pH 6 and 0.05 M NaCl and then subsequent desorption at pH 2.0 and 1 M NaCl (Elegado et al., 1997; Yang et al., 1992). This method seemed more applicable to pediocin but not with the lactococcin, nisin or plantaricin. The reason is not clear but it could be related to variation in cell wall properties. The pH-adsorption/desorption method was able to provide materials for pH and temperature tolerance assays, estimation of molecular mass through SDS-PAGE, residual activity determination after protease, amylase and other enzyme actions (Laxamana et al., 2011). Enough amount of semi-purified bacteriocin from pediococci using this method was obtained for further purification through preparative reverse phase HPLC for various characterization studies, including the determination of secondary structures by circular dichroism and confirmation of double bonds through trypsin digestion and electrospray mass spectrometry (Elegado and Kwon, 1998). Other preparative purification methods prior to reverse phase HPLC and spectrometry included ion exchange chromatography and gel

filtration chromatography (Elegado et al., 2003), and hydrophobic interaction chromatography (Villarante et al., 2011). This method could also be applied with bacteriocins of pediococci and lactobacilli. The properties obtained from well characterized bacteriocinogenic LAB are shown in Table 3.

| Isolate | Identity | Bacteriocin | Purification mode | Properties |
|---|---|---|---|---|
| AA5a | *Pediococcus acidilactici* | pediocin | pH adsorption/desorption<br>Reversed-phase HPLC | Tolerant to<br>pH 2-9 and 121 °C |
| BS25 | *Lactobacillus plantarum* | plantaricin | Gel filtration chromatography<br>Reversed-phase HPLC | MW = 3,830 Da |
| K2a2-3 | *Pediococcus acidilactici* | pediocin | Hydrophobic interaction and ion-exchange chromatographies<br>Reversed-phase HPLC | MW = 4,626 Da |
| K2a2-1 | *Pediococcus acidilactici* | pediocin | pH adsorption/desorption | Optimum pH = 5-7<br>Resistant to boiling but not to autoclaving |
| 4E5 | *Pediococcus acidilactici* | pediocin | pH adsorption/desorption | Tolerant to pH 2-9; slight loss of activity at 100 °C; loss of activity at 121 °C; tolerates high salt; est. MW = 6,500 Da by SDS-PAGE |

**Table 3.** List of purified and characterized bacteriocins from LAB isolated from Philippine indigenous fermented foods.

## 3.4. Optimization of bacteriocin production through fermentation kinetics

Bacteriocin production is largely dependent on the nutrients and nitrogen content of the fermentation medium. For instance, increased yeast extract concentration and polypeptone amount increases bacteriocin production. Molasses, raw sugar and sago hydrolyzates of amylase digestion were found to be good carbon sources. Other possible substrate base and supplements are cheese whey, coconut water and rice bran extract. Initial sugar concentration of usually 2 to 3% and inoculation rate of 3% by volume of at least $10^8$ cells/mL provides good bacteriocin production (Elegado et al., 2001).

Bacteriocin production is highly dependent on cell or biomass growth. LAB are microaerophilic and most are either mesophilic or slightly thermophilic. The following conditions are applicable to their production: pH= 5.5 to 6.0; temperature = 35 – 40 °C; agitation = 50 rpm; without aeration. Usually, bacteriocin is optimally produced or secreted in the culture broth during the early stationary phase of growth. For *Pediococcus acidilactici*, culturing at 40 °C promotes earlier optimum bacteriocin production of around 10-12 hours. At 37 °C, bacteriocin production is from 14-16 hours (Sagpao et al., 2007).

## 3.5. Applications

Pediocins and plantaricins are the commonly found bacteriocins in Philippine fermented foods so far studied. Their antimicrobial properties have been investigated in several studies (Banaay et al., 2004; Elegado et al., 2003, 2004, 2007; Marilao et al., 2007). Although pediocins

and plantaricins show promise, their applications are limited at present because it is a well-known fact that other bacteriocins aside from nisin are not yet approved for food use. For pediocins and plantaricins, the most practical use for now would be dermatological and animal health care use. But since the bacteriocin-producing LAB are of GRAS status, those with probiotic properties such as tolerance to acidic pH (2.0 -3.0) and bile (0.3%) and adhesion properties to intestinal mucosa would be an advantage when used as adjunct inocula in fermented food products (Gervasio and Lim, 2007).

Perhaps another importance of bacteriocin-producing LAB is their effectiveness in biomedical applications. In one study, for example, partially-purified pediocin K2a2-3, through pH-mediated bacteriocin extraction method, was found cytotoxic against human colon adenocarcinoma (HT29) and human cervical carcinoma (HeLa) cells *in vitro* as determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Villarante et al., 2011). Other potential biomedical applications will be discussed in the succeeding section.

## 4. Probiotics and functional foods

An offshoot of the initial research on bacteriocins of LAB isolated from indigenous fermented foods is the emergence of probiotic research towards developing functional foods for biomedical applications. Probiotics refer to microorganisms that, when administered in adequate amounts, confers health benefits to the host. Although there are many microorganisms that can be considered as probiotics, LAB are the most common types because they produce antimicrobial compounds that inhibit other harmful microorganisms, they are able to tolerate acids and bile present in the digestive system, and they are able to adhere and establish themselves in the gut surfaces.

Many benefits have been ascribed to probiotics. For example, *Lactobacillus casei* (Shirota strain in Yakult®) have been shown effective in preventing diarrhea due to enterotoxigenic *Escherichia coli* (ETEC) and choleragenic vibrios (*V. cholerae* biotype E1 Tor and classical *V. cholerae*) using rats (Jacalne et al., 1990). This may be accounted for by its ability to kill the pathogens and inhibit further growth (Consignado et al., 1994). Because the probiotic used in the two studies mentioned is a commercial strain, current research on probiotics progressed to the search for indigenous LAB for use in the development of locally-produced functional food and investigation of their utility for biomedical applications. Metagenomic approaches to investigating LAB present in fermented foods have shown the diversity of potentially beneficial species present other than those that are readily detected by conventional culture-based methods. The development of functional food products shows potential in disease management. Research using metagenomic analysis in searching for microbial markers for use in functional foods to address certain lifestyle diseases as well as malnutrition is on the way.

### 4.1. Metagenomic and diversity studies

Traditional culture-based methods have been used for isolating LAB from fermented foods. These studies form the basis for the starter cultures used in food fermentation technologies employed for commercial production. Sanchez (2008) gives detailed information on the different

technologies and cultures used for the production of some traditional as well as developed technologies that have arisen from the culture-based studies conducted in earlier years.

In recent years culture-based approaches in LAB isolation have become more targeted for detection of bacteriocin-producers and those that have potential as probiotics. In one initiative, LAB isolates from fermented foods were screened for bacteriocin production and a PCR-based assay was used to detect specific bacteriocin-encoding genes. Acid and bile tolerance were also determined. Among all the isolates tested, *Lactobacillus fermentum* 4B1 and *Lactobacillus pentosus* 3G3 (later identified as *Pediococcus acidilactici*) have been identified as most promising for the development of new probiotic food products, hence they were chosen for subsequent biomedical application assays (Lim and Gervacio, 2007). In another study, LAB from traditionally fermented wine and vinegar from Visayas and Mindanao were isolated, identified, and tested for inhibitory activity against *Enterococcus faecium*, *Listeria innocua*, and *Staphylococcus aureus*. Five *Lactobacillus paracasei* and one *Lactobacillus brevis* showed antimicrobial properties against the tester strains (Licaros and Bautista, 2009).

With the advent of molecular techniques, the existence of non-culturable microorganisms has been acknowledged especially since the occurrence of culture-bias is already well-accepted. Culture-independent approaches, therefore, have been gaining popularity in microbial diversity studies and this includes researches on microorganisms found in fermented foods. The microbial populations in selected Philippine fermented foods were assessed through Polymerase Chain Reaction followed by Denaturing Gradient Gel Electrophoresis (PCR-DGGE) in two recent studies (Dalmacio et al., 2011; Larcia, 2010). Food samples tested include *burong mustasa* (fermented mustard), *alamang* (fermented shrimp paste), *burong isda* (fermented rice-fish mixture), *balao-balao/burong hipon* (fermented rice-shrimp mixture), *tuba* (sugar cane wine), and *sinamak* (spiced vinegar). Analysis of the 16S rRNA gene sequences revealed the presence of several LAB that have not been reported in these food products before. *Weissella cibaria*, *Lactobacillus plantarum*, *Lactobacillus pontis*, *Lactobacillus panis*, and *Lactobacilus fermentum* were detected in *burong mustasa* (Larcia, 2010). *L. panis* and *L. fermentum* were present in *alamang*; *L. pontis* and *L. plantarum* in *burong isda*; *L. panis*, *L. pontis*, and *L. fermentum* in burong hipon; and *W. cibaria*, *L. pontis*, *L. panis*, *L. fermentum* and *L. plantarum* in *burong mustasa* (Dalmacio et al., 2011).

The results of the two studies using molecular approaches in defining diversity of LAB in Philippine fermented foods show that culture-independent approaches are efficient tools for the analysis of microbial populations in fermented foods. Majority of the identified bacteria (LAB and other bacterial groups) have not been reported in culture-dependent studies. As such, the isolated bacterial 16S rRNA genes were cloned to have an initial partial 16S rRNA gene library for Philippine fermented foods (Dalmacio et al., 2011).

## 4.2. Biomedical applications

1. Anti-Obesity

Obesity is defined as an abnormal or excessive fat accumulation that presents risks to health. Probiotics can help in fighting obesity by reducing lipid absorption through its action on bile

acid metabolism, and by assimilation of cholesterol thus eliminating it from the host's system. Several studies were conducted to examine anti-obesity properties of different probiotic strains.

In one study, oral administration of *Lactobacillus paracasei* K3-4C, isolated from a locally fermented food had significant effect on lowering blood glucose levels (by 46%) and body weight (by 13%) in female BALB/c mice induced to be diabetic and obese through a 28-day high-fat diet (Parungao et al., 2006). In another study, orally administered *L. fermentum* 4B1 reduced adipose cell size, and decreased adipose tissue weight and overall body weight of mice fed with a high-fat diet for 49 days (Bautista et al., 2008). Likewise, oral administration of *P. acidilactici* 3G3 reduced body weight in diet-induced obese female Swiss mice (Parungao et al., 2009). In the last two studies described, the effects of the probiotics were determined to be comparable with the effects of the commercial anti-obesity drug Orlistat based on the parameters measured.

Recently, it has been postulated that the development of obesity may be caused by a shift in the composition of the gut microbiota towards the Firmicutes population (Ley et al., 2005). Firmicutes characterize obese versus lean/non-obese individuals together with a drop or no change in Bacteroidetes (Delzenne and Cani, 2010). Interestingly, Ley et al. (2006) found that a low fat diet had an effect to reverse the shift of Firmicutes/Bacteroidetes proportion. Because of this, dietary manipulation has been seen as a potential means of changing bacterial populations in the colonic microbiota and perhaps treating or at least preventing diseases like obesity. Although the root cause of obesity is excessive caloric intake coupled with a sedentary lifestyle (Blaut and Bischoff, 2010), Ley et al. (2005) proposed in their findings that alteration in the populations of mice gut microflora may have caused or may have been an effect of obesity. Because of this, current researches aim in using probiotics in the treatment of diseases such as obesity.

In two related studies (Arroyo and Fabiculana, 2011; Parungao et al., 2012), the effect of a functional food containing *P. acidilactici* 3G3 on microbial community changes in the gut of obese and non-obese mice was determined through PCR-DGGE. Results of these two preliminary studies showed that obese and non-obese mice had different baseline colonic microbiota. There were also indications that treatment with probiotics shifts the microbiota of obese mice towards the normal non-obese type. As these are preliminary studies, more research is warranted to elucidate the nature of the changes in gut microbiota and how it is related to obesity and the anti-obesity effects of probiotics.

2. Immuno-enhancement

A preliminary *in vitro* study to examine the immune-enhancing properties of viable and heat-killed preparations of two LAB previously isolated from traditional fermented foods (*L. fermentum* 4B1 and *P. acidilactici* 3G3) on murine peritoneal macrophage cells and spleenic T-cells showed that isolate 4B1 was able to induce NO production in murine macrophages but, like 3G3, was unable to stimulate murine T-cell proliferation (Tan et al., 2008). Furthermore, this study showed that preparations of *L. fermentum* 4B1 have the ability to induce NO production in murine macrophage cells and its effects were more potent when it was alive.

The study also showed that isolate 4B1 exhibited better immune-enhancing effect than the probiotic species found in a commercial probiotic drink. T-cell proliferation, however, was not observed in any of the treatments in this study and was attributed to the delayed stimulation in cells responding to a first-time exposure to the different probiotic strain preparations used.

3. Reduction of blood glucose levels

A study by Ngo et al. (2008) showed that oral administration of kefir, a common fermented food consumed by the elderly, significantly decreased blood glucose levels and body weight of diabetic obese male Sprague Dawley rats. The results of the study showed lower blood glucose levels (from 198.5 to 105.6 mg/dL) and clinically lower body weights (from 342.9 to 311.5 g) of the treated diabetic-obese rats than the untreated diabetic-obese control group.

4. Prevention of hypercholesterolemia

The effect of *P. acidilactici* 3G3 administration on hypercholesterolemic Swiss Albino mice was determined (Parungao et al., 2009). This strain was able to assimilate cholesterol in the *in vitro* plate assay and decrease HDL, LDL, and total cholesterol in the *in vivo* assay using mice. Strain 3G3 was also shown to adhere well to the duodenum and middle colon. Results suggest the potential of *P. acidilactici* 3G3 in preventing hypercholesterolemia.

### 4.3. Development of functional foods

The development of functional foods containing known probiotic strains stems from earlier researches on bacteriocins and isolation of potential probiotics from traditional fermented foods. The beneficial effects of probiotic-supplemented chocolate bars (Arroyo et al., 2010; Arroyo and Fabiculana, 2011), fermented mustard leaves (Calapardo et al., 2006), and coffee wine (Parungao, 2007) have been investigated. Initial studies on mango-milk and carrot juice drinks supplemented with probiotic strains have also been conducted (Bugarin et al., 2010; Elegado et al., 2005). These potential functional foods contain probiotic strains, previously isolated from traditional fermented foods such as *P. acidilactici* AA5a (Elegado et al., 2003), *L. plantarum* BS25 (Banaay et al., 2004), and *P. acidilactici* 3G3 (Lim and Gervacio, 2007). Research on functional foods is still in its infancy but this food category shows promise in disease management as well as in contributing to food security in the country. Commercial interest in probiotic food products is increasing due to the growing understanding of its health benefits. This growing industry can derive benefits from the researches conducted on this emerging food category.

## 5. Future perspectives

Aside from the research works presented earlier in this paper as well as on-going follow-up studies related to them, future goals may include research on a variety of other possible biomedical applications of LAB with potential probiotic properties. The effect of probiotics on *Helicobacter pylori* infections (that may cause peptic ulcers) may be determined. Their ability to modulate inflammatory and hypersensitivity responses as well as their effect on

irritable bowel syndrome and colitis may be investigated. Further research on possible anti-cancer properties of probiotics is warranted as follow-up studies on the work done by Villarante et al. (2011). These studies are very important as these have the potential to address some of the more serious health concerns of our society.

Much is still to be learned about the existing probiotic strains. The molecular biology and genomics of these isolates may be pursued in order to further elucidate their properties and mechanisms of action.

Determination of factors affecting probiotic viability in foods is also important as these will determine if their survival in the food, and therefore their delivery into the host, is maintained. This will constitute a quality control for functional foods.

The potential physiological effects of multiple prebiotic strains, as opposed to a single strain, are also interesting areas of research. The delivery of multiple probiotic strains may help ensure its effectiveness in an environment that contains high diversity of resident microflora. The potential benefits of synbiotics, (combination of probiotic and prebiotic) which have synergistic interaction, may also be investigated. A good combination will greatly enhance the health benefits to humans.

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