Isolation and Identification of Acetic Acid Bacteria from Philippine Fermented Rice Cake Batters by 16S rRNA Gene Sequence Analysis

Audrey Mae Villamin Orillaza¹, Honey Bhabes R Iñigo¹, *Baby Richard Ragudo Navarro¹ (1. Institute of Food Science and Technology, College of Agriculture and Food Science, University of the Philippines Los Baños (Philippines))

Keywords: Acetic acid bacteria, fermented rice cake, 16S rRNA sequence analysis, phylogenetics

As part of our work to study the microflora of Philippine traditional fermented foods, batters from fermented rice cakes, or puto in the vernacular, from different parts of the Philippines were sampled and used for the isolation, screening and purification of acetic acid bacteria (AAB) by culture-based methods. Pure AAB isolates were then identified by DNA-based methods [i.e., cetyl trimethylammonium bromide (CTAB) DNA extraction, polymerase chain reaction (PCR), and 16S rRNA gene sequence analysis], DNA base composition determination, phenotypic characterization, and phylogenetic analysis. Six isolates were obtained from three types of rice cake batter: puto Calasiao, puto Lanson, and puto Boac batters. The AAB isolates were identified to belong to the genera Acetobacter at 94-99% homology with DNA base compositions ranging from 54.40-55.74 mol% GC content. The isolates were Gram-negative, catalase-positive rods that oxidize ethanol to acetic acid and grow in mannitol agar and in most sugars. None of them were cellulose producer or motile. 02CPPu1-2 produced a water-soluble brown pigment in glucose-yeast extract-peptone (GYP) medium and 24BMTa2-3 yielded γ-pyrones from D-glucose. From the phylogenetic tree deduced from the 16S rRNA gene sequence analysis results, the isolates clearly formed an independent clade distinct from the type strains of other genera of acetic acid bacteria. The puto Lanson and puto Boac batter isolates were closely related to A. pasteurianus and A. lovaniensis, respectively. On the other hand, the puto Calasiao isolates were associated with none of the type species of AAB. Overall, our data suggest that the fermented rice cake batter isolates comprise a possibly new species of acetic acid bacteria under the genus Acetobacter. This is very interesting considering that all the isolates were sourced from batters of only traditionally fermented rice cakes. DNA-DNA hybridization and detailed phenotypic characterization are recommended to verify this new species possibility, which may be linked the difference in geographical location, raw material and processing technique employed in traditional rice cake making in the Philippines.
Isolation and Identification of Acetic Acid Bacteria Isolates from Philippine Fermented Rice Cake Batters by 16S rRNA Gene Sequence Analysis

Audrey Mae V. Orillaza,* Honey Bhabes R. Iñigo, and Baby Richard R. Navarro

Institute of Food Science and Technology, College of Agriculture and Food Science, University of the Philippines Los Baños, Philippines

*Corresponding author: avorillaza@up.edu.ph

ABSTRACT

As part of our work to study the microflora of Philippine traditional fermented foods, batters from fermented rice cakes, or puto in the vernacular, from different parts of the Philippines were sampled and used for the isolation, screening and purification of acetic acid bacteria (AAB) by culture-based methods. Pure AAB isolates were then identified by DNA-based methods [i.e., cetyl trimethylammonium bromide (CTAB) DNA extraction, polymerase chain reaction (PCR), and 16S rRNA gene sequence analysis], DNA base composition determination, phenotypic characterization, and phylogenetic analysis. Six isolates were obtained from three types of rice cake batter: puto Calasiao, puto Lanson, and puto Boac batters. The AAB isolates were identified to belong to the genera Acetobacter at 94-99% homology with DNA base compositions ranging from 54.40-55.74 mol% GC content. The isolates were Gram-negative, catalase-positive rods that oxidize ethanol to acetic acid and grow in mannitol agar and in most sugars. None of them were cellulose producer or motile. 02CPPu1-2 produced a water-soluble brown pigment in glucose-yeast extract-peptone (GYP) medium and 24BMTa2-3 yielded γ-pyrones from D-glucose. From the phylogenetic tree deduced from the 16S rRNA gene sequence analysis results, the isolates clearly formed an independent clade distinct from the type strains of other genera of acetic acid bacteria. The puto Lanson and puto Boac batter isolates were closely related to A. pasteurianus and A. lovaniensis, respectively. On the other hand, the puto Calasiao isolates were associated with none of the type species of AAB. Overall, our data suggest that the fermented rice cake batter isolates comprise a possibly newspecies of acetic acid bacteria under the genus Acetobacter. This is very interesting considering that all the isolates were sourced from batters of only traditionally fermented rice cakes. DNA-DNA hybridization and detailed phenotypic characterization are recommended to verify this new species possibility, which may be linked the difference in geographical location, raw material and processing technique employed in traditional rice cake making in the Philippines.

Keywords: Acetic acid bacteria, fermented rice cake, puto, 16S rRNA sequence analysis, CTAB method, DNA extraction, phylogenetics

1. INTRODUCTION

The Philippines, a tropical country in Southeast Asia, is recognized as one of the centers of microbial diversity. This is in part due to the various traditional methods of food preservation in the country. An archipelago of 7107 islands, it has different food preservation methods that vary among islands, and hence a wide array of fermented foods (Sanchez, 2008) consumed throughout the archipelago, which are part and parcel of our culture (Banaay et al., 2013). However, it is common knowledge that the market of traditional Philippine fermented foods has always remained local, for instance, domestic for bagoong, patis, alamang, and suka, regional for some such as buro and tuba, and even provincial for some such as etag, pindang, bahalina, and native longanisa (i.e., longanisang Vigan and Lucban). In fact, a few of our traditional fermented foods such as sabeng and tengba never reach the market, their production only linked to festivities that celebrate the richness or sanctity of our indigenous cultures.
This is unfortunate considering that the top 5 most consumed food items in the US are all fermented foods, namely, beer, bread, cheese, wine and fermented meat in descending order. Moreover, with the trend in globalization, a few Asian fermented food products have already seeped through the changing Western culinary landscape, with soy sauce now considered the most used condiment not only in Asia but also in the world, while a few such as kimchi and fish sauce are slowly finding their niche in international cuisines. Thus said, there is clearly an explosive market that awaits other fermented foods in the future with the increasing population and intensifying food insecurity across the globe. It is therefore vital that active up-to-date research on traditional Philippine fermented foods be carefully carried out with the purpose of making them at par, in terms of quality and product image and design, with fermented foods of other countries that have reached global commercialization. Note that, in recent years, traditional fermented foods have become increasingly relevant not only because of their guaranteed safety, high nutritional quality or unique sensory profile, but also because of their potential huge market contribution, as proved by the global probiotic market projected to reach USD 46.6 billion by 2020, with Europe as the largest and Asia-Pacific region as the fastest-growing markets (Elegado et al., 2016).

Currently, there is a dearth of solid statistics concerning our traditional fermented foods, most likely linked to the limited market and consumer research on them. Nonetheless, it is unequivocal that our traditional fermented foods can stand up to those of other countries in terms of flavor, nutritional value and health benefits. It is only the lack of consistent quality and use of non-standardized and unhygienic manufacturing processes that have relegated our food products to their local status and inferior image. In addition, since most of the traditional food fermentation industries in the Philippines are rural, seasonal, labor-intensive, informal, and capital-deficient, their supply remains much too limited to establish a huge market such that their market and ultimately their consumption remain confined only to places where they are produced. Also, most producers of our traditional fermented foods are local farmers, fisherfolks, and housewives who are poor and capital-deficient, which logically dictates the choice of the least expensive methods of production (even if these methods are non-GMP-compliant and non-HACCP-certified) as well as understandably highlight the need for easy money turn-over (vending “unripe” products), which often result in compromised product qualities and products that do not reach their full bloom. Lastly, there is extremely limited scientific and technological knowledge about our traditional fermented food products, particularly about their microbial and biochemical aspects because of the lack of research institutes passionate for, dedicated to, and fully equipped for research and development of our traditional fermented food products. (Only large private food industries are technologically equipped for food science research, most of which however neither prioritize nor sense the importance of our traditional food products.) This is in large contrast to the comprehensive research knowledge on wine and cheese in France, on balsamic vinegar in Italy, on soy sauce in Japan, and on kimchi in Korea, which perhaps explain why these fermented food products, unlike ours, command global respect, as these products are continuously being researched and polished to perfection. These might as well be the reasons behind the meager research on Philippine traditional fermented food products, as well as behind the insignificant contribution of the fermentation food industry to our gross domestic products compared with that of the agricultural industry. But the crux of the matter is this: without research on our fermented foods, there will be no improvement in them; without improvement, there will be no increase in their market share; without an increase in market share, there will be no research attention on them. Therefore, it is crucial that extensive research on Philippine fermented foods be carefully performed if such food products are to infiltrate the global food market. This could be started through the use of genotypic methods side by side with biomolecular analyses in conducting an in-depth accurate analysis of the fermenting microflora (including unculturable microorganisms) of these fermented foods.

Thus, as part of our comprehensive research on Philippine fermented foods, in this study, we isolated, screened, purified and identified acetic acid bacteria (AAB) from batters of traditionally fermented rice cakes by culture-based methods and molecular methods. We focused on acetic acid bacteria since in previous works, lactic acid bacteria (e.g., Leuconostoc mesenteroides, Streptococcus faecalis, and Lactobacillus plantarum) and yeasts (e.g. Saccharomyces cerevisiae) have already been isolated from fermented rice cake batters; these microorganisms are expected as they are commonly associated with cereal fermentation (Kelly, Asmundson, Harrison, & Huang, 1995; Sanchez, 1999; Tamang et al., 2016; Uchimura, Garcia, & Flores, 1984). We consider it interesting to determine the proliferation of...
other fermentative microorganisms such as acetic acid bacteria (AAB) in rice cake fermentation. Our objectives were to isolate, identify and characterize AAB from different Philippine traditional fermented rice cakes.

2. MATERIALS AND METHODS

2.1 Sampling
Fermenting batters from local fermented rice cakes were obtained on site or purchased from local producers and processed for AAB isolation by inoculating a loopful of each batter onto glucose-yeast extract-peptone (GYP) slants within 12 h of collection and incubating it at room temperature. The slants were kept in an ice box once growth had been observed.

2.2 Isolation, screening, purification, and storage
In the laboratory, 5 mL of sterile physiological saline solution (PSS) was added to each GYP slant with growth, and the cell culture was suspended by aseptically scraping it using a wire loop and then vortexing the mixture. Appropriate dilutions of the suspension were then spread- plated on GYP agar plates with CaCO₃ and then incubated at 30°C for 18-48 h. Colonies that formed a zone of clearing on the GYP agar plates were then individually transferred onto GYP slants and incubated as described above. The cultures were again suspended in PSS, and appropriate dilutions of the suspension were then streaked on GYP agar plates with CaCO₃ as acid production indicator. The plates were then incubated at 30°C for 18-48 h. After incubation, colonies with a zone of clearing were picked up and again transferred onto GYP slants. Resuspension and replating were repeated several times until visual and microscopic examinations of colonies and cells of each isolate showed homogeneous morphological characteristics. The pure isolates were then subjected to Gram staining by the Hucker method (Hucker & Conn, 1923) and to catalase test using the method of MacFaddin (2000). Only pure isolates from GYP agar that were Gram-negative and catalase-positive were presumed to be AAB and stored in glycerol solution.

2.3. DNA extraction
DNA was extracted from each AAB isolate using a modified cetyl trimethylammonium bromide (CTAB) DNA extraction protocol (Wilson, 1987). 5 mL of 24-h GYP broth culture at 30°C was centrifuged at 12,000 rpm for 45 s at room temperature. The cell pellet obtained was suspended in 200 µL of Tris-EDTA (TE) buffer (pH 8), to which 25 µL of 10% sodium dodecyl sulfate (SDS) and 5 µL of 25 mg mL⁻¹ proteinase K were added. The mixture was then incubated with gentle shaking at 37°C for 1 h. The resulting viscous lysate was added with 45 µL of 5 M sodium chloride (NaCl) and 40 µL of CTAB solution (10% CTAB in 0.7 M NaCl), and then with an equal volume of chloroform:isoamyl alcohol (24:1); this was left to stand for 10 min, centrifuged at 12,000 rpm for 10 min at room temperature, added with an equal volume of cold isopropanol, and mixed gently. The resulting mixture was centrifuged at 8,000 rpm for 5 min at 4°C, and the supernatant was decanted to obtain a DNA pellet, which was then washed with 1 mL of 70% ethanol by centrifugation at 12,000 rpm for 5 min at 4°C. The supernatant was discarded, and the remaining precipitate was air-dried for 5-10 min and redissolved in 100 µL of TE buffer. This DNA solution was subjected to spectrophotometry and agarose gel electrophoresis to confirm its purity and quality, respectively.

2.4. Polymerase chain reaction (PCR) amplification
PCR amplification was done based on the optimized protocol of Dalmacio et al. (2011), in which the V1-V8 region of the 16S rRNA gene was amplified using universal primers: 8F (5' AGAGTTTGATCCTGGCTCAG 3') and 1492R (5' GGTTACCTTGTTACGACTT 3'). The PCR reaction mixture (1x TE buffer, 0.5 U Taq polymerase, 0.3 µM each of the bacterial 8f and 1492r primers, 1.5 mM MgCl₂, 0.2 mM dNTP, ≥ 30 ng/µL template DNA, and nanopure water) was subjected to an optimized amplification program as follows: initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, and elongation at 72°C for 1.75 min, and final elongation at 72°C for 5 min. The PCR products were subjected to electrophoresis on 1.0% (w/v) agarose gel with 0.5x Tris-acetate EDTA (TAE) buffer and visualized using ethidium bromide for confirmation of the desired length of 1.5 kb.

2.5. 16S rRNA gene sequencing
The amplified DNAs of the AAB isolates were sent to First Base Laboratories in Malaysia for 16S rRNA gene sequencing using the same primers mentioned above and determined of their identity
and % homology to type strains of different species of acetic acid bacteria using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

### 2.6. Determination of DNA base composition
DNA base composition expressed as mol% GC content was determined using an online GC calculator (http://www.endmemo.com/bio/gc.php).

### 2.7. Phenotypic characterization
Cell form was determined by growing AAB isolates on GYP agar. Unless otherwise stated, the isolates were incubated at 30°C for 18-48 h. The oxidation of ethanol to acetic acid as indicated by a zone of clearing after 2-3 days of incubation, and catalase production as indicated by evolution of gas were tested in GYP agar with CaCO₃. Motility was also determined by growing the isolates in soft GYP agar stabs. The formation of cellulose and a water-soluble brown pigment was examined by visual observation in GYP broth and agar cultures, respectively. The production of dark brown γ-pyrones from D-glucose and D-fructose was determined by adding FeCl₃ to 11-day broth cultures. Growth in mannitol agar and various sugars (i.e., D-glucose, D-fructose, D-xyllose, D-sucrose, D-galactose, D-sorbitol, D-maltose, and D-starch) in broth cultures was also determined.

### 2.8. Phylogenetic analysis
DNA sequences of the AAB isolates and type species of the 14 valid AAB genera (i.e., *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Ameiyamæa*, *Tanticharoenia*, *Asaia*, *Swaminathania*, *Kozakia*, *Neoasaia*, *Granulibacter*, *Acidimonas*, *Komagataeibacter*, *Saccharibacter*, and *Neokomagataea*) (Mamlouk & Gullo, 2013) were subjected to multiple sequence alignment using CLUSTAL W and the neighbor-joining method (Saitou & Nei, 1987) with 1000 bootstrapping replicates (Felsenstein, 1985) to construct the phylogenetic tree (Nei & Kumar, 2000) using Mega X software (Kumar, Stecher, Li, Knyaz, & Tamura 2018).

### 3. RESULTS AND DISCUSSION

#### 3.1. Sampling and isolation, screening and purification of AAB isolates

Samples of batter from four types of local fermented rice cakes (i.e., *puto Calasiao* from Calasiao, Pangasinan; *puto Lumban* from Lumban, Laguna; *puto Lanson* from Irosin, Sorsogon; and *puto Boac* from Boac, Marinduque) procured from their towns of production were used in AAB isolation. Initially, seven isolates with acid production ability in GYP agar were isolated. This number was whittled down to six aerobic, acid-producing, Gram-negative, ellipsoidal to rod-shaped isolates after preliminary characterization and purification. The six isolates were sourced from *puto Calasiao*, *puto Lanson*, and *puto Boac* batters; no isolates were obtained from the *puto Lumban* batter.

The predominant microorganisms in fermented rice cakes include LAB and yeasts such as *Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Lactobacillus delbrueckii*, *Lactobacillus fermenti*, *Lactobacillus lactis*, *Pediococcus cerevisiae*, *Geotrichum candidum*, *Torulopsis holmii*, *Torulopsis candida* and *Trichospora pibulans*, which have been isolated from *idli*, *dosa* and *dhokla*, varieties of steamed blend of rice and black gram (*Phaseolus mungo*) in India (Blandino et al., 2003), as well as *Lactobacillus casei*, *Lactobacillus brevis*, *Leuconostoc mesenteroides* and *Saccharomyces cerevisiae*, which are found in *jeung-pyun*, a sponge-like bread in Korea (Park et al., 2017). However, AAB have been shown to be in symbiotic relationship with LAB and yeasts in *jiaozi*, a traditional steamed bread in China. Li et al. (2016) have identified *Acetobacter tropicalis* (22.8%), together with *Saccharomyces cerevisiae* (42.9%), *Pedocioccus pentosaceus* (38.6%), *Wicherhamomyces anomalus* (27.0%), *Lactobacillus plantarum* (24.3%), *Saccharomycopsis fibuliger* (22.2%), *Torulaspora delbrueckii* (7.9%), *Enterococcus durans* (5.7%), *Bacillus cereus* (2.9%), and *Enterococcus faecium* (1.4%) in *jiaozi* by combined culture-based method and PCR-DGGE analysis. One possible reason for AAB seemingly being the minor microflora in fermented rice cakes is their late proliferation in the fermenting batter, growing only after yeasts and LAB have already taken hold during fermentation. Thus, their growth is hindered by the predominance of these earlier colonizers of the fermenting batter, such that they only exist in very small numbers. At such small numbers, they are not generally isolated by traditional culture-based methods using common growth media, being classified as ‘VBNC’.

Another possible reason for the low AAB load in *puto* batter is linked to the inherent nature of AAB. AAB generally thrive in the natural environment (e.g., soil, herbs, fruit, and flowers) and a wide variety of fermentation substrates (Crotti et al., 2010) that are good sources of simple sugars, not
starch.

### 3.2. Molecular identification of AAB isolates

Through the alignment of their DNA nucleotide sequences to sequences in the BLAST database, the six potential AAB isolates were all confirmed to share 94-99% nucleotide sequence homologies to known species of acetic acid bacteria belonging to the genus *Acetobacter*. The four isolates of *puto* Calasiao showed >94% homologies with the following AAB species indicated: 02CPPu1-2 with *Acetobacter orientalis* (at 94% homology), 02CPPu2-1 with *Acetobacter persici* (at 95%) and both 02CPPu2-2 and 02CPPu3-1 with *Acetobacter malorum* (at 98 and 99%, respectively). The isolates from *puto* Lanson (12ISPu1-1) and *putong* Boac, on the other hand, were found to have 97 and 99% homologies with *Acetobacter pasteurianus* and *Acetobacter lovaniensis*, respectively.

The DNA base contents of the six AAB isolates ranged from 54.40 to 55.74 mol% GC content (Table 1), which fit the DNA base content range of the genus *Acetobacter*.

**Table 1.** Phenotypic characteristics and DNA base composition of AAB isolates from local fermented cake batter.

<table>
<thead>
<tr>
<th>Test</th>
<th>Isolate Code</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>02CPPu1-2</td>
</tr>
<tr>
<td>A. Cell form</td>
<td>short rods</td>
</tr>
<tr>
<td>B. Oxidation of ethanol to acetic acid</td>
<td>+</td>
</tr>
<tr>
<td>C. Catalase test</td>
<td>+</td>
</tr>
<tr>
<td>D. Cellulose production</td>
<td>-</td>
</tr>
<tr>
<td>E. Formation of brown soluble pigment</td>
<td>+</td>
</tr>
<tr>
<td>F. Motility test</td>
<td>-</td>
</tr>
<tr>
<td>G. Τ-pyrones from sugars</td>
<td>-</td>
</tr>
<tr>
<td>H. Growth in MYPY</td>
<td>+</td>
</tr>
<tr>
<td>I. Growth in sugars</td>
<td>+</td>
</tr>
<tr>
<td>J. G+C content (mol%)</td>
<td>55.03</td>
</tr>
</tbody>
</table>

Positive, +; negative, -, weak, (+)

In this study, the AAB isolates from the batters of three of the rice cakes sampled, namely, *puto* Calasiao, *puto* Lanson, and *putong* Boac, were found to belong to the genus *Acetobacter*, differently from those of the *puto* Lumban batter. According to Raspor and Goranovic (2008), *Acetobacter* strains
prefer alcohol-enriched environments, which explain the presence of *Acetobacter* in the fermented rice cake batter samples. Note that all the batter samples were obtained in the late fermentation stage prior to steaming or baking, and hence fermentation by yeasts and/or lactic acid bacteria is almost complete, making the conditions in the fermenting batter supportive of AAB growth, that is, rich in alcohol as a result of the alcoholic fermentation by yeasts and with a slightly acidic pH of approximately 5.0 as a result the addition of lye, sugar and other flavoring ingredients, which bumped up the low batter pH of approximately 3.5 caused by lactic acid production by LAB. Furthermore, *Acetobacter* species have an optimum growth pH range of 5-6.5 (although they can grow even as low as pH 3-4) at 28-30 °C (Mamlouk & Gullo, 2013), the very same conditions present in local fermented rice cake batter.

### 3.3. Phylogenetic relationship among AAB isolates

From the phylogenetic tree (Fig. 1) constructed based on the alignment of 905 bp 16S rRNA gene sequences, all the AAB isolates from the local fermented rice cake batter samples distinctly clustered with the type strains of all known *Acetobacter* species, separate from the other type species of other AAB genera. Moreover, our isolates formed an independent clade together with *A. pasteurianus* and *A. lovaniensis*. 12ISPu1-1 and 24BMTa2-3 from *puto* Lanson and *puto* Boac batters corroborated their BLAST database homologies to *A. pasteurianus* and *A. lovaniensis*, respectively. On the other hand, all four isolates from *puto* Calasiao batter interestingly formed a tight-knit clade not associated with the AAB species to which they had high % homologies based on BLAST alignment results.

![Phylogenetic tree of AAB isolates](image)

*Fig. 1. Phylogenetic relationships of AAB isolates from 16S rRNA gene sequence clustering. The tree was made based on an alignment of 905 bp of 16S rRNA gene sequences and constructed by neighbor-joining method. Numbers indicate the bootstrap percentage values derived from 1000 replications. Sequences used in this study are represented in sample codes. Lactobacillus fermentum strain 1 (GenBank accession number FJ462686.1) is used as an outgroup.*

Interestingly, despite the differences in preparation method, ingredients and geographic location of *puto* Calasiao, *puto* Lanson, and *puto* Boac, all the AAB species isolated from all three fermented rice cakes were of the same genus, *Acetobacter*. This suggests that similar microorganisms are at work in the fermentation of our local fermented rice cakes, regardless of type. This is evident in the deduced phylogenetic tree of the six isolates together with the type strains of all valid *Acetobacter* species and the type species of the other 13 valid AAB genera. Despite the homology data obtained from the nucleotide sequence alignment with the BLAST database suggesting the wide variety of species (i.e., *A. malorum*, *A. persici*, *A. tropicalis*, *A. pasteurianus*, and *A. lovaniensis*) responsible in rice cake
fermentation, results of the phylogenetic analysis indicate otherwise. All our six isolates formed a highly distinct clade, with the four isolates from 
*puto* Calasiao forming a clade that is entirely separate from all known valid *Acetobacter* species. Therefore, this strongly suggests that the four aforementioned isolates constitute a new species in the genus *Acetobacter*. Thus, it is important that DNA-DNA hybridization of the *puto* Calasiao isolates with all valid *Acetobacter* species as well as quinone analysis be conducted to confirm this possibility. If confirmed, it will be highly interesting to study why a highly specific AAB microflora is associated with Philippine rice cake fermentation. This could likely lead to hitherto unknown fermentation mechanisms by AAB that utilizes starch as substrate.

### 3.4. Phenotypic characteristics relevant to the acetification by AAB species

As shown in Table 1, all the AAB isolates are Gram-negative, catalase-positive rods. They oxidize ethanol to acetic acid. They also grow in mannitol agar and in most of the sugar sources, particularly starch. None of them are cellulose producer or motile. 02CPPu1-2 produces a brown water-soluble pigment in GYP medium and 24BMTa2-3 yields \( \gamma \)-pyrones from D-glucose.

Majority of the phenotypic characteristics of the isolates were reflective of the species indicated in the BLAST homology search. Their growth in mannitol agar confirmed their identity as *Acetobacter* utilizing mannitol as an energy source. 02CPPu1-2 was noted to produce a brown water-soluble pigment, similarly to a few *Acetobacter* species such as *A. polyoxogenes* isolated from vinegar broth (Entani et al., 1985) and *A. aurantius* now under genus *Frateuria* isolated from golden-rayed lily (*Lilium auratum* Lindl.) (Swings et al., 1980), as well as to *Gluconacetobacter liquefaciens* (Navarro and Komagata, 1997). 12ISPu1-1 was observed to ferment all representative sugars in the study but its homologous species *A. pasteurianus* prefers only glucose, mannitol and ethanol as carbon sources (Konig et al., 2009). 24BMTa2-3 produced \( \gamma \) pyrones from D-glucose. It was also the only isolate that did not ferment D-sucrose, exactly the same as its homologous species *A. lovaniensis* (Konig et al. 2009), unequivocally confirming its identity. Furthermore, note that all the isolates fermented D-starch, the major component of rice-based products. This characteristic is not typical of *Acetobacter* species (Sievers & Swings, 2015), a possible indication of the unique fermentation mechanism conducted by these rice cake batter isolates. More importantly, this ability to grow on starch provides a strong support to the possibility not just of a new species but perhaps also of a new genus.

AAB isolates are generally associated with dough acidification, which favors LAB growth, as well as with the production of enzymes and exopolysaccharides (such as levan) resulting in the hydrolysis of biochemical compounds and in the formation of the structural network of bread in the absence of gluten proteins in rice flour, respectively (Korakli et al., 2001; Tieking et al., 2003). However, the exact role of our isolates in fermented rice cake fermentation remains to be elucidated, what with the yet to be confirmed identity of the *puto* Calasiao. Further analyses (e.g., DNA-DNA hybridization, as mentioned earlier, and detailed phenotypic characterization). Likewise, microbial succession analysis using PCR-DGGE must be conducted to determine the fermentation mechanism wherein the VBNC state of AAB can also be resolved.

### 4. CONCLUSION

From this study, the similarity in the fermenting microflora isolated from batters of various Philippine fermented rice cakes in Luzon was highlighted. Although a wide variety of AAB species were identified by BLAST search analysis, namely, *A. malorum*, *A. orientalis*, *A. persici*, *A. pasteurianus* and *A. lovaniensis* from fermented rice cake batters from Pangasinan, Sorsogon, and Marinduque, results of phylogenetic analysis, indicated otherwise. The deduced phylogenetic tree showed that the isolates from the *puto* Calasiao batter formed a tight-knit clade completely separate from all known species of *Acetobacter* and the other 13 genera of AAB. This points to a hitherto undiscovered group of starch-fermenting *Acetobacter* strains that may perhaps constitute a new *Acetobacter* species, at least, if not an altogether novel genus in the family Acetobacteraceae.

**ACKNOWLEDGMENT**
We thank the UP OVPAA Balik Scientist Program for the financial support, and the UPLB Institute of Animal Science for granting us access to the equipment and facility used in the study.

REFERENCES


