The ciprofloxacin resistance of *Lactobacillus* species isolated from probiotic food products in the Philippines is due to mutations in *gyrB* and *parC* genes

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The consumption of probiotic products in the Philippines is prevalent, with more than 2.7 million bottles of a single product consumed per day. Probiotics are microorganisms that are administered for their beneficial activity on animal and human health. On one hand, there are some concerns on probiotics as potential conduits of lateral antibiotic resistance gene transfer to gut pathogens. On the other hand, probiotics are also being tapped as potential cell-based therapeutics against pathogens to augment the antimicrobial activity of antibiotics. Therefore, it is of interest to the general public for widely consumed probiotics to be assessed for their susceptibility to antibiotics that are medically important in the country. In this study, the antibiotic susceptibilities of probiotic bacteria that were isolated from commercial food products in the Philippines were assessed. Three *Lactobacillus* species — *L. paracasei*, *L. casei,* and *L. delbrueckii* subsp. *bulgaricus*, were isolated from probiotic food products. A modified agar well diffusion method and a minimum inhibitory concentration (MIC) microdilution assay were utilized to test these isolates. Our results demonstrated that the isolated species of *Lactobacillus* bacteria are more susceptible to amoxicillin and doxycycline than the reference bacteria, *Escherichia coli* (ATCC 25922). These findings suggest that they likely do not harbor transferrable resistance genes to these antibiotics. However, we also found that the isolates are recalcitrant to ciprofloxacin, which is likely due to mutations in their *gyrB* and *parC*. These are chromosomal genes that encode for the target enzyme of ciprofloxacin. Therefore, with their ciprofloxacin resistance genes being chromosomal in nature, the odds of lateral transfer of ciprofloxacin resistance genes to gut pathogens is less compared to plasmid-encoded resistance genes. We believe that routine antibiotic resistance profiling should be conducted on probiotic microorganisms that are widely consumed as food products or feed supplement in agricultural industries. This will provide valuable information to reduce risks and maximize the benefits of probiotics.

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INTRODUCTION

The consumption of probiotic products containing the beneficial *Lactobacillus* species is very popular in the Philippines. *Lactobacillus* is a common inhabitant of the gastrointestinal (GI) tract and is considered as one of the most common species of probiotic bacteria. Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO 2001, Hill et al. 2014). They are preferably from human origins and possess a "generally recognized as safe" (GRAS) status (Rönkä et al. 2003, Kumar et al. 2015). Probiotic bacteria are also found in fermented milk products, vegetables, fruits, fish, meats, sausages, rice, cassava, sugar cane, coconut, soya, and others (Sanni et al. 2002, Banaay et al. 2013). The intake of these probiotic food products can expand the richness of good bacteria in the gut leading to a possible augmentation of host protection against pathogenic species.

Researches have documented several mechanisms by which probiotics protect the gut from invading microorganisms (Vanderbergh 1993). Most probiotics reduce gut pH by producing lactic, acetic, succinic, propionic, and hydrochloric acids to inhibit pathogen growth (van der Wielen et al. 2002, Murry et al. 2004, Makras et al. 2006). Some probiotic species prevent pathogens from attaching to gut epithelial cells, thus preventing colonization and infection (Hudault et al. 1997, Lee et al. 2003, Eom et al. 2015). Others produce soluble factors that are inhibitory to pathogens (Michetti et al. 1999, Lorca et al. 2001, Mukai et al. 2002, Gong et al. 2010). Probiotics can also modulate the host's immune response to pathogens (Servin 2004, Salva et al. 2010, Castillo et al. 2011).

Like any other bacteria, probiotics are affected by their habitat. Probiotics colonize a complex microenvironment in the gut that is constantly altered by food (De Filippo et al. 2010, Maslowski and Mackay 2011) or antibiotics (Dethlefsen et al. 2008, Perez-Cobas et al. 2013) ingested by the host. The diet of the host can define the dominant species of the gut, as well as the diversity and relative abundance of its microbial flora (Walker et al. 2011). Antibiotics, on the other hand, are strong effectors that can modify the gut microenvironment. Antibiotics can act as a double-edged sword, targeting specific pathogens while inhibiting the growth of other commensals of the gut (Jernberg et al. 2007, Jakobsson et al. 2010). By suppressing both types of bacteria, antibiotics can influence the interplay of ecologic and metabolic processes between commensals and pathogens or the host (Willing et al. 2011). This can lead to a higher chance of perturbing normal microbial populations in the gut (Perez-Cobas et al. 2013), replacing the dominant species in the gut (De La Cochetiere et al. 2005), fluctuating the gut's taxonomic richness and evenness (Dethlefsen et al. 2008), and developing a condition called gut dysbiosis (Myers 2004).

Clinical practices have devised a system of coupling antibiotic therapy with probiotics to counteract dysbiosis (Reviewed in Ciorba, 2012). The supplementation of probiotics helps commensals, which are the normal dominant species of the gut, control the number of pathogenic bacteria. Thus, a healthy equilibrium of microflora is maintained (Fooks and Gibson, 2002). However, it is important to consider that probiotics have innate resistance to some antibiotics. Furthermore, for bacteria to be considered as probiotic supplement to antibiotic treatment, they should have reduced susceptibility to antibiotics (Salminen et al. 1998, Ljungh and Wadstrom 2006). These characteristics will permit probiotics to withstand the effects of antibiotics while executing their beneficial effects to the host (Tong et al. 2007). It should be noted also that probiotics and other commensals share a niche with pathogenic bacteria in the GI tract, making it an ideal microenvironment for lateral transfers of resistance genes among microorganisms (Sommer et al. 2009, Imperial and Ibana 2016,). Therefore, it is important to assess the susceptibility of *Lactobacillus* bacteria in Philippine food products to determine their relative risk for harboring antibiotic resistance genes. Antibiotics to which *Lactobacillus* bacteria are highly susceptible would suggest low probability of carrying antibiotic resistance genes. However, this may reduce their efficiency when co-administered with antibiotics. Should antibiotic resistance be observed in probiotics, the mechanism of resistance must be elucidated to assess the risk of lateral transfer of resistance genes to other microorganisms in the gut (Imperial and Ibana 2016). Thus, in this study, we isolated and characterized probiotic bacteria from Philippine food products, investigated their susceptibility to commonly used antibiotics, and further examined the possible mechanism of their observed antibiotic resistance.

MATERIALS AND METHODS

Isolation and identification of probiotic microorganisms from commercial probiotic food products

Six fermented probiotic food products were purchased from three major supermarkets in Quezon City, Philippines. The products were designated as products A, B, C, D, E, and F. One hundred microliter (100 µL) aliquot of each product was enriched in DeMan-Rogosa-Sharpe (MRS; Himedia, Mumbai, India) broth and then subcultured on MRS agar plates at 35°C under aerobic conditions. A single colony from each MRS agar plate was picked and propagated further in MRS agar plates for purification and then to MRS broth. Aliquots of stocks of the isolates were placed in cryogenic tubes and stored at -80°C in 1:1 of bacterial culture to 80 % glycerol ratio (Zayed and Roos 2004).

To characterize and identify the isolated bacteria from each food product, the isolates were Gram stained, subjected to catalase test, and their genomic DNA was extracted following a standard boiling method. In brief, a loopful of each isolate was transferred into microfuge tubes containing phosphate buffered saline (PBS) and washed by mixing the culture for ten seconds using a vortex at maximum speed. The bacterial suspension was centrifuged at 10,000 × g for 15 minutes. PBS was aspirated, leaving the bacterial pellet in the tube. One milliliter (mL) of TE buffer was added to the pellet, mixed, and incubated in a dry bath at 100°C for 30 minutes. The supernatant containing the DNA was aspirated, transferred to new tubes, and stored at -20°C until use.

The identification of the isolates was performed through the amplification of their 16S rRNA gene using primer pair 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 520R (5'- ACC GCG GCT GCT GGC-3') (Hayashi et al. 2005). The PCR condition consisted of an initial denaturation temperature of 95°C for 5 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 51°C for 45 seconds, and extension at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes. The PCR product of ≈500 bp was visualized using 1.5% agarose gel stained with SYBR™ Safe DNA gel stain (Invitrogen, ThermoFisher Scientific, Carlsbad, CA) and UV trans-illuminator. The amplicons of the three isolates were submitted to the Philippine Genome Center for sequence analysis of the amplified 16S rRNA gene. Sequence matching of the PCR products was then performed using the BLASTN application of the National Center for Biotechnology Information (NCBI). The maximum identity score and query coverage of more than 95% or greater were utilized in identifying the isolates.

Measurement of the growth of isolated probiotic bacteria in microaerophilic and aerobic growth conditions

A 24-hour culture of each probiotic isolate was diluted to adjust the cell concentration to approximately 1.0×10^6 CFU/mL. Following the preparation of the determined baseline cell concentration, 250 µL of each culture was dispensed into wells of a 96-well plate. Replicates of 9 wells for each isolate were prepared together with a blank control of sterile MRS broth. The plate was placed in a Multiskan GO UV/Vis microplate spectrophotometer (Thermo Scientific, MA, USA), programmed to incubate at 35°C and to shake the plate prior to an absorbance reading every hour for 28 hours. This condition allowed the cells to grow at the bottom of the well under microaerophilic conditions. Another set of plate was prepared and placed in the spectrophotometer, programmed to 35°C incubation, with regular shaking every 5 minutes to induce aerobic growth. The absorbance was measured every hour for 34 hours. The measurements in both growth conditions were plotted in a line graph to show the growth stages of the isolates.

Determination of antibiotic susceptibility of isolated microorganisms from probiotic products

To determine the antibiotic susceptibility of the isolated bacteria, a modified agar well diffusion method was used. The pour plate technique was performed to allow visible growth and inhibition. The test antibiotics were amoxicillin, ciprofloxacin and doxycycline. Antibiotic stocks were prepared at a concentration of 100 mg/mL and stored at -20°C for no more than 4 months. Each aliquot was used promptly, and unused portions were discarded. The concentrations of the antibiotics used were based on Clinical and Laboratory Standards Institute or CLSI (2014) recommendation of 10 µg amoxicillin, 5 µg ciprofloxacin, and 30 µg doxycycline. The isolated bacteria were mixed with warm MRS agar, plated, and allowed to solidify. Six (6) millimeter holes were bored and carefully dispensed with the antibiotics. Mueller-Hinton Agar (MHA) plates inoculated with *Escherichia coli* ATCC 25922 were also used to serve as reference. The plates were incubated at 37°C for 18–24 hours, followed by the measurement of zones of inhibition (ZI). The Kirby-Bauer technique was also performed to validate the results of the modified method.

To further assess the antibiotic susceptibility of the isolated probiotic bacteria, Minimal Inhibitory Concentration (MIC) was determined following established broth microdilution technique. The antibiotics were diluted in MRS broth, and serial two-fold dilutions of amoxicillin, ciprofloxacin, and doxycycline at 50 to 1.56 µg/mL concentrations were prepared on 96-well culture plates. The reference and each isolated probiotic bacterium from food products were prepared by inoculating bacterial colonies from a 24-hour culture agar plates in culture broth to achieve a turbidity of 1.0 McFarland standard. Subsequently, bacterial suspension was added to the plates with antibiotics at densities equivalent to 1.5×10^8 and 1.5×10^5 CFU/mL.

The plates were then incubated for 24 hours at 37°C. Optical density readings at 600 nm were taken. The MIC was recorded as the lowest concentration that gave an OD reading of 50% (MIC-50) and 20% (MIC-80) of the value for no antibiotic control (100% growth).

Analysis of the quinolone resistance determining region (QRDR) of the isolates

Two of the quinolone resistance-determining genes of the isolates were analyzed for amino acid substitutions. The *gyrB* and *parC* genes of probiotic bacterial isolates were amplified using the *gyrB* primer pair (F: 5'-CTGCCGGGCAAACTGGCAGA -3' and R: 5'-TCGACGTCCGCATCGG TCAT -3') that targets a region of the gene encoding for lysine 447 and serine 464 (Cattoir et al. 2006) and the *parC* primer pair (F: 5'- AAACCTGTTCAGCG CGCATT -3' and R: 5'- GTGGTGCC GTTAAGCAAA -3') that amplifies the gene encoding for serine 80 and glutamic acid 84 (Vila et al. 1996). The PCR condition for *gyrB* amplification consists of initial denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, 40°C for 30 seconds, 72°C for 30 seconds, and a final extension at 72°C for 5 minutes. For the amplification of the *parC* gene, PCR was programmed with an initial denaturation at 94°C for 3 minutes, 30 cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 5 minutes. The expected amplified DNA fragment sizes are ≈300 bp for *gyrB* and ≈400 bp for *parC*. The amplified gene fragments were sent to the Philippine Genome Center for sequence analysis. Resultant sequences were aligned with QRDR sequences of *gyrB* and *parC* genes from GenBank and then translated to peptide sequences using the BioEdit program (Hall 1999). Amino acids 447 and 464 in the QRDR of *gyrB* and amino acids 80 and 84 in the QRDR of *parC* genes found in the probiotic isolates were compared to GenBank peptides of other *Lactobacillus s*pp. and quinolone-susceptible strains of *E coli.*

RESULTS

Characteristics and identities of the probiotic bacterial isolates

Colonies that formed on the MRS agar plate inoculated with aliquots from the commercial probiotic food products exhibited similar colonial characteristics per plate. Only four products showed colony growth on the MRS agar. Products A and B produced small colonies with the following characteristics: pinpoint to pinhead size, glistening white, domed-shaped, circular, and regular margins (Figure 1). Products D and E grew colonies that have similar characteristics seen from plates inoculated with products A and B. No growth on MRS agar plates was observed in products C and F. Gram staining and catalase testing indicated that all four isolated bacteria were Gram-positive bacilli and catalase-negative bacteria.

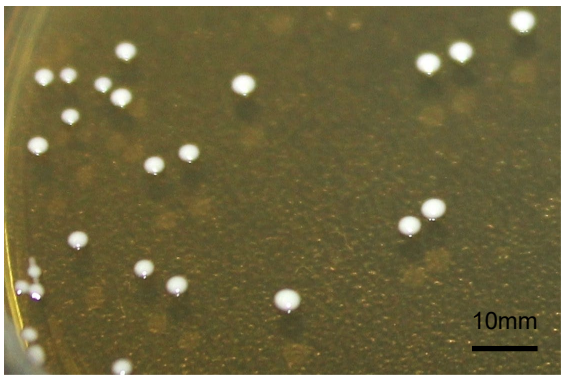

Figure 1: Representative colonial morphology of the isolated lactic acid bacteria (LAB) on MRS agar incubated at 35°C for 24 to 48 hours under aerobic condition. Each LAB isolate exhibit pinpoint to pinhead colonies that are glistening white, domed-shaped, circular with regular margins.

Sequence matching analyses of amplified 16S rRNA gene products, performed using the BLASTN application of NCBI, revealed the identities of the isolates. Query coverage and identity score of more than 95% or greater were observed in analyses of gene sequences. BLASTN results showed that isolates were all lactic acid bacteria from the genus *Lactobacillus* (Table 1). The matching identities of the isolates from products A, B, and D are *L. paracasei*, *L. casei*, and *L. delbrueckii* subsp. *bulgaricus,* respectively. These isolates were selected and used in the succeeding experiments. Reference code IRL14-01 was assigned for the *L. paracasei* isolate, IRL14-02, for the *L. casei* isolate, and IRL14-03 for the *L. delbrueckii* subsp. *bulgaricus* for future reference. The isolate from product E, which is also *L. casei,* was not pursued in succeeding experiments. No live bacteria were isolated from products C and F.

Growth of the isolates in microaerophilic and aerobic conditions

Using a UV-Vis spectrophotometer, the growth curve of isolates in microaerophilic and aerobic conditions were assessed by measuring the optical densities of cultures. Measurements every hour were plotted in Figure 2. In microaerophilic conditions (Figure 2.A), the three isolates were in a lag phase—a period of

Table 1: Species identification using 16S rRNA gene sequence analyses of isolated lactic acid bacteria (LAB) showed high sequence homology with *Lactobacillus* species[1]

Product	Query Coverage	Identity Score	Identity
A	97%	97%	*Lactobacillus paracasei*
B	98%	98%	*Lactobacillus casei*
D	98%	96%	*Lactobacillus delbrueckii* subsp. *bulgaricus*
E	97%	99%	*Lactobacillus casei*

[1]BLAST-N results of species identification by 16S rRNA gene sequence analyses showed that LAB isolates from product A matches *L. paracasei*, product B matches *L. casei*, product D matches *L. delbrueckii* subsp. *bulgaricus*, and product E matches *L. casei*.

adaptation during the first two hours of incubation. Signs of exponential growth were observed on the third and fourth hours of incubation, which continued until the 16th hour for *L. casei* and the 21st hour for both *L. delbrueckii* subsp. *bulgaricus* and *L. paracasei.* A steady stationary growth was observed beyond these periods until the 28th hour of incubation.

In contrast, the aerobic growth of isolates (Figure 2.B) showed slow and steady growth with lesser concentration of cells. The isolates were in lag phase for five to six hours. Cell doubling of all isolates followed, but growth was slow and gradual with a gentle steep on the 16th hour of incubation. Further exponential growth was noted until 34 hours of incubation. No evidence of stationary growth was obtained until the end of the experiment (34hrs).

When the growth turbidity absorbances of isolates at 600nm were compared between the two conditions, the aerobic culture absorbance value is less than 0.30 while in microaerophilic condition, the growth absorbances—0.66 to 0.96, were 2× to 3× higher than in the aerobic culture.

From the measurements of optical densities per hour of incubation, the exponential growth rate and doubling time were computed. In microaerophilic culture, *L. delbrueckii* subsp. *bulgaricus* and *L. paracasei* had an exponential growth rate of 0.3623 and 0.3845, respectively, while *L. casei* showed the highest rate of exponential growth at 0.4712. All isolates had a doubling time of less than two hours (Table 2). Cultivation of isolates in aerobic conditions had a different effect on their growth. There was a reduction in their exponential growth rate, and their doubling time was extended. The rates of exponential growth of *L. casei*, *L. delbrueckii* subsp. *bulgaricus*, and *L. paracasei* were 0.1338, 0.1372, and 0.1809, respectively. Their doubling time increased from less than four hours to five hours (Table 2). The highest rate of exponential growth and the shortest doubling time were exhibited by *L. paracasei* under microaerophilic culture condition.

The results of the experiment suggest that the isolates favor microaerophilic condition for growth. The cell density of the isolates increased up to 3x in culture with minimal concentration of oxygen compared in culture with atmospheric oxygen.

Antibiotic susceptibility of the probiotic isolates

The assessment of susceptibility to antibiotics of the isolated *Lactobacillus* spp. from different Philippine commercial food

Table 2: Exponential growth rate and doubling time of isolated lactic acid bacteria in microaerophilic and aerobic conditions at 35°C for 28 to 34 hours.

Bacteria	Microaerophilic		Aerobic	
	Exponential Growth Rate	Doubling Time (h)	Exponential Growth Rate	Doubling Time (h)
Lactobacillus delbrueckii subsp. *bulgaricus*	0.3623	1.6970	0.1372	5.0506
Lactobacillus casei	0.4712	1.4710	0.1338	5.1784
Lactobacillus paracasei	0.3845	1.8024	0.1809	3.8312

products by agar well diffusion assay is summarized in Table 3. The reference bacterium, *E. coli* ATCC 25922, is susceptible to all three antibiotics. Its ZI were within the range as prescribed by CLSI. Interestingly, based on the size of ZI, all *Lactobacillus* isolates were relatively more susceptible to amoxicillin and doxycycline. However, they were more recalcitrant to ciprofloxacin than *E. coli*. The isolates treated with amoxicillin and doxycycline had ZI of approximately 24 to 28 millimeters (mm). These are significantly wider in diameter than that of *E. coli*, which ranges from 18 to 19 mm ($p \leq 0.05$). When ciprofloxacin was used against the isolates, their observed measurements were 11 to 13 mm, which were significantly less than the ZI of *E. coli*, which was 32.33±1.63 mm ($p \leq 0.05$).

Table 3: Growth inhibition zones of antibiotics against the isolated lactic acid bacteria[1].

Bacteria	Zone of Inhibition (mm)		
	Amoxicillin 20µg/ml	Ciprofloxacin 5µg/ml	Doxycycline 30µg/ml
Lactobacillus delbrueckii subsp. *bulgaricus*	$^{S}28.00\pm1.07^{a}$	$^{R}13.33\pm0.82^{b}$	$^{S}28.33\pm1.03^{a}$
Lactobacillus casei	$^{S}28.17\pm1.11^{a}$	$^{R}13.00\pm1.10^{bc}$	$^{S}27.83\pm0.75^{a}$
Lactobacillus paracasei	$^{S}24.00\pm1.63^{b}$	$^{R}11.42\pm0.80^{c}$	$^{S}28.17\pm0.98^{a}$
Escherichia coli	$^{S}19.25\pm0.63^{c}$	$^{S}32.33\pm1.63^{a}$	$^{S}18.83\pm1.72^{b}$

*[1] Results of the agar well diffusion assay indicate the susceptibility of the isolates to amoxicillin and doxycycline and their resistance to ciprofloxacin. Significant difference among the isolates within antibiotic treatment (n=6) was analyzed using One-Way ANOVA. *$p \leq 0.05$. Right hand superscripts indicate statistical difference; different letters within each antibiotic treatment are significantly different at 95% confidence level. LEGEND: ^{R} : Resistant; ^{S} : Susceptible; ^{I} : Intermediate.

Photomicrographs of the growth inhibition of the four test bacteria by different antibiotics are shown in Figure 2.

The modified agar well diffusion data were corroborated by outcomes of MIC determination by broth microdilution. The quantification of the minimum concentration of antibiotics to inhibit 80% of cells of the test bacteria confirmed the susceptibility profile of *Lactobacillus* isolates to amoxicillin, ciprofloxacin, and doxycycline as compared to that of *E. coli* (Figure 3). Quality control ranges set by CLSI for *E. coli* were carried out in this assay. At a concentration of less than 1 µg, amoxicillin could easily kill the *Lactobacillus* isolates, while *E. coli* was inhibited by approximately 5 µg of the antibiotic. When these isolates were exposed to doxycycline, their cells were also killed by less than 1 µg of the antibiotic. *E. coli*, on the other hand, was inhibited by at least 1.2 µg of doxycycline. As demonstrated previously, the *Lactobacillus* isolates were found less susceptible to ciprofloxacin, as compared with the reference bacterium. This assay supported this observation by revealing a

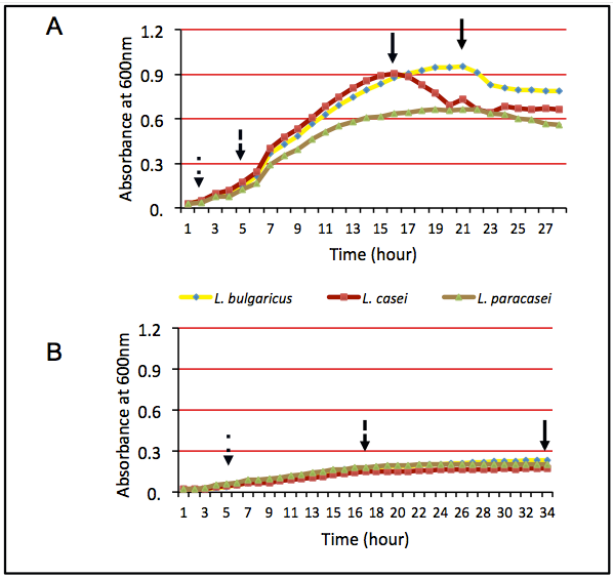

Figure 2: Growth curves of the three isolated lactic acid bacteria in microaerophilic and aerobic conditions based on broth culture turbidity measurements using spectrophotometry indicate faster growth rates in microaerophilic condition. The isolates were cultured in MRS broth under microaerophilic (A) and aerobic (B) growth conditions until the stationary phase was reached. Different stages of growth are marked—lag phase (dotted-line arrow), logarithmic phase (broken-line arrow) and stationary phase (straight-line arrow).

Figure 3: Zones of inhibition of Lactobacillus isolates and the reference bacterium, E. coli ATCC 25922 by amoxicillin, ciprofloxacin and doxycycline. The isolates are susceptible to amoxicillin and doxycycline but were resistant to ciprofloxacin.

higher concentration of ciprofloxacin needed to kill the *Lactobacillus* isolates. While *E. coli* was inhibited by as low as 0.01 µg of the antibiotic, the *Lactobacillus* isolates were controlled only by 2.5 µg of ciprofloxacin or higher. Statistical analyses also showed significant difference in the concentration needed to inhibit the isolates, as compared with *E. coli.* The MIC-80 profile of the isolates at cell concentration of 1.5×10^8 CFU/mL was also similar to the results generated from MIC-80 at 1.5×10^5 CFU/mL, MIC-50 at 1.5×10^8 CFU/mL, and 1.5×10^5 CFU/mL (data not shown).

Quinolone resistance-determining region (QRDR) of the isolates

The decreased susceptibility of isolates to the quinolone drug ciprofloxacin may be attributed to mutations in their quinolone resistance-determining genes. To test this hypothesis, specific amino acids in the QRDR of their *gyrB* and *parC* genes were analyzed. Studies have demonstrated the roles of amino acids 447 and 464 in *gyrB* and of amino acids 80 and 84 in *parC* in quinolone resistance. In wild type bacteria, susceptibility to quinolone antibiotics is attributed to lysine 447 and serine 464 (*gyrB*) and to serine 80 and glutamic acid 84 (*parC*). Any substitution of these amino acids may confer resistance to quinolone drugs (Vila et al. 1996, Guillemin et al. 1998, Bansal and Tandon 2011, Cattoir et al. 2006). In this experiment, substitutions were noted in all four amino acids (Table 4). Lysine 447 in *gyrB* of all three isolates was substituted to aspartic acid, while serine 464 was substituted into alanine and leucine by *L. delbrueckii* subsp. *bulgaricus* and *L. casei,* respectively, but was retained by *L. paracasei*. The two amino acids in the *parC* gene were also substituted by all isolates. Serine 80 was changed into aspartic acid, and glutamic acid 84 into alanine. *GyrB* and *parC* gene sequences of *L. delbrueckii* subsp. *bulgaricus*, *L. casei*, and *L. paracasei* from GenBank were also compared in the amino acid substitution analysis. All of the *Lactobacillus* species have substitutions in *gyrB*, but only strains of *L. delbrueckii* subsp. *bulgaricus* have substitution in the *parC* gene. Glutamic acid 84 was substituted to glycine. Gene sequences of *gyrB* and *parC* from strains of *E. coli* were also taken from the nucleotide database for comparison. These two strains were found to be susceptible to ciprofloxacin, and mutations in the specific codons were not observed.

DISCUSSION

Probiotics confer benefits to the host (FAO/WHO 2001, Hill et al. 2014); they help maintain a healthy equilibrium of gut microflora, especially during antibiotic therapy (Fooks and Gibson 2002). On one hand, probiotics, if susceptible to the co-administered antibiotic, may not be useful to the host. Therefore, it is important to consider that probiotics have innate resistance to antibiotics. In this study, we evaluated lactic acid bacteria from commercial probiotic food products in the Philippines. The genotypic identity of each isolate was determined and their growth characteristics in two different culture conditions were examined. Their susceptibility to three common antibiotics was assessed as well as their potential mechanism of antibiotic resistance.

Probiotic food products were sampled to isolate lactic acid bacteria. The isolates were identified as three species of *Lactobacillus* namely, *L. delbrueckii* subsp. *bulgaricus, L. casei* and *L. paracasei.* Probiotics, such as *Lactobacillus* species, have general beneficial activities, but they use different mechanisms to confer benefits to the host. Most probiotics have species-specific or strain-specific benefits (Grimoud et al. 2010). Furthermore, the same species of *Lactobacillus* but isolated from different samples may have varying biological activities

Table 4: Amino acid substitutions in the *gyrB* and *parC* quinolone resistance-determining region (QRDR) of *E. coli* and *Lactobacillus* species[1].

Microorganisms from GenBank	Substitution of QRDR in			
	gyrB		*parC*	
	Lys447	Ser464	Ser80	Glu84
Escherichia coli str. K-12	-	-	-	-
Escherichia coli O157:H7	-	-	-	-
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 11842	Arg	Asn	-	Gly
Lactobacillus delbrueckii subsp. *bulgaricus* ND02	Arg	Asn	-	Gly
Lactobacillus delbrueckii subsp. *bulgaricus* 2038	Arg	Asn	-	Gly
Lactobacillus casei ATCC 393	Arg	Asn	-	-
Lactobacillus casei 12A	Arg	Asn	-	-
Lactobacillus casei str. Zhang	Arg	Asn	-	-
Lactobacillus paracasei ATCC 334	Arg	Asn	-	-
Lactobacillus paracasei JCM 8130	Arg	Asn	-	-
Lactobacillus paracasei 8700:2	Arg	Asn	-	-
Isolates from Probiotic Products[1]				
Lactobacillus delbrueckii subsp. *bulgaricus* isolate	Asp	Ala	Asp	Ala
Lactobacillus casei isolate	Asp	Leu	Asp	Ala
Lactobacillus paracasei isolate	Asp	-	Asp	Ala

[1]The isolates have substitutions in amino acids Lys447 and Ser464 in *gyrB* and in amino acids Ser80 and Glu84 in *parC,* which may confer resistance to ciprofloxacin.

(Georgieva et al. 2015). The isolates, which are three distinct species, may also work differently in their host. Therefore, the *Lactobacillus* isolates in this study were characterized. Their susceptibility to common antibiotics was assessed to determine their potential as synergist of antibiotics for treatment of gut infection.

To understand the growth characteristics of each isolate, their growth rate and doubling time were determined. The *Lactobacillus* isolates can grow in both aerobic and microaerophilic conditions, more optimally in the latter. Many have reported that these species of *Lactobacillus* favor an anaerobic condition for growth (Yuki et al. 1999, Ljungh et al. 2002, Tharmaraj and Shah 2003). Therefore, these isolates may survive and grow in an anaerobic environment such as the human gut. The results also suggest their ability to adapt in a new environment at a relatively short time of 2 to 3 hours. Their doubling time of less than 2 hours indicates an advantage in competing for food and suitable niche in the gut. Among the isolates, *L. paracasei* has the highest rate of exponential growth and shortest doubling time. If these isolates have rapid growth rate, especially *L. paracasei,* they may create a barrier for the gut which could protect it from invading pathogens, maintaining gut health and homeostasis (Gill 2003, Fang et al. 2010, Yeung et al. 2013).

Consumption of fermented products enriches the gut with probiotic bacteria. But the survival and growth of probiotics may be affected by food or drugs that the host consumes (De Filippo et al. 2010, Maslowski and Mackay 2011). If the host is undergoing antibiotic therapy, the growth of these probiotic bacteria could be affected (Dethlefsen et al. 2008, Perez-Cobas et al. 2013). The isolates are susceptible to amoxicillin and doxycycline and recalcitrant to ciprofloxacin. If consumed during amoxicillin/doxycycline therapy, the *Lactobacillus* isolates may be killed. Therefore, it is helpful to profile the antibiotic susceptibility of probiotics to aid in the selection of species/strains of probiotics for co-administration with a specific antibiotic. The CLSI (2014) had established the protocol for antibiotic susceptibility testing of common pathogens. It may be useful if such protocol will be adapted for probiotics. With this, the efficiency of co-administering probiotics with antibiotics is improved by not using probiotics, which are susceptible to the antibiotic of choice for therapy.

Although the results have suggested the recalcitrance to ciprofloxacin of our isolates, it is important to note that antibiotic resistance is a trait useful for species of probiotic bacteria (Salminen et al. 1998, Ljungh and Wadstrom 2006). That trait may protect them from the antibiotic during treatment. The ability to withstand the killing effect of ciprofloxacin provides our isolates an advantage over gut pathogens such as

Salmonella (Truusalu et al. 2008). Strains of *L. delbrueckii* subsp. *bulgaricus, L. casei* and *L. paracasei* have been found to control gut pathogens such as *Salmonella* and confer other benefits to the host (Simone et al. 1988, Hudault et al. 1997, Jankowska et al. 2008). Co-administration of these probiotic bacteria with ciprofloxacin for treatment of *Salmonella* infection may present a synergistic effect in eliminating the pathogen (Tong et al. 2007, Truusalu et al. 2008). Therapy would come not only from the action of antibiotics but also from the antimicrobial and immunomodulatory activities of the co-administered probiotic.

It is also important to know that probiotic bacteria once consumed by the host may share a niche with pathogens in the gut, their presence in the microenvironment may trigger different interactions to happen—between probiotics and other commensals or between probiotics and pathogens. If they have reduced susceptibility to antibiotics, similar to the recalcitrance to ciprofloxacin of our isolates, there is a possibility that probiotics sharing a microenvironment with the gut microbiota, including pathogens, can increase the risk of lateral transfers of resistance genes (Sommer et al. 2009).

Interestingly, our study revealed that the reduced susceptibility of the isolates to ciprofloxacin is attributed to a mechanism encoded in their chromosome—the variation in the QRDR of their *gyrB* and *parC* genes. Ciprofloxacin, a fluoroquinolone, exerts antibacterial activity by inhibiting DNA synthesis through interaction with DNA gyrase and topoisomerase IV (Hooper 1999). These enzymes are encoded by *gyrB* and *parC* genes, respectively. However, bacteria counteract this mechanism through point mutations in the quinolone-resistance determining regions of these genes. Specific amino acids that have been reported to play important roles in quinolone drug susceptibility are lysine 447 and serine 464 in *gyrB* and serine 80 and glutamic acid in *parC* genes (Vila et al. 1996, Guillemin et al. 1998, Cattoir et al. 2006, Bansal and Tandon 2011). To our knowledge, our study is the first to report in the Philippines of *gyrB* and *parC* amino acid substitutions in *Lactobacillus* spp. that were isolated from commercial food products. The results of our experiment corroborated with other studies that tested *Lactobacillus* spp. to ciprofloxacin. Previous studies proposed that these species have innate or natural resistance to this antibiotic (Katla et al. 2001, Danielsen and Wind 2003, Kirtzalidou et al. 2011). It has been suggested that the intrinsic ciprofloxacin resistance trait do not pose significant risk for consumers with respect to transfer to other gut-associated bacteria (Drago et al. 2011, Lee et al. 2011, Guo et al. 2017). Several studies have verified that the chromosomally encoded ciprofloxacin resistance is unlikely to be transferred from one bacterium to another. An example is the lateral transfer of *gyrA*- and *parC*-related ciprofloxacin resistance from *L. brevis* KB290 to the gut bacteria—*Enterococcus faecalis* (Fukao et al. 2009). Another is the transfer of *gyrA* gene mutation from ten ciprofloxacin-resistant strains of *Lactobacillus* to either *E. faecalis* or *Lactococcus lactis* MG1614 (Guo et al. 2017). Therefore, if the ciprofloxacin resistance of our isolates is found in their chromosome, although possible, our isolates do not pose much threat in transferring ciprofloxacin resistance to other bacteria such as gut pathogens.

Our current study is limited to three types of antibiotics. We recommend further studies on the susceptibilities of our probiotic isolates to other classes of antibiotics. Probiotics may be profiled for their antibiotic susceptibilities to aid in selecting probiotic strains for co-administration with antibiotics. It is also essential to evaluate whether their antibiotic resistance is not transferred easily to other bacteria. These efforts could mitigate the potential of probiotics as conduits of transferable antibiotic resistance genes, and maximize their health benefits.

CONCLUSION

In sum, our findings provide an experimental evidence that probiotics for co-administration with antibiotics must be properly selected. Antibiotic susceptibility profiling of probiotics may aid in securing their survival in the gut during antibiotic therapy. This will support their potential synergistic activity with antibiotics in eliminating pathogens as well as in providing health benefits to the host. Furthermore, because the ciprofloxacin resistance of our *Lactobacillus* isolates is encoded in their chromosome, the lateral transfer of antibiotic resistance to other bacteria is less likely compared to plasmid-encoded resistance genes. It is also worth considering to evaluate novel and commercially available probiotics for the risks of transferring antibiotic resistance genes to other bacteria if used in combination with antibiotics. Together, these results provided important insights on the potential impacts of probiotics and commonly used antibiotics to gut microbial ecology and health.

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CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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