Probiotics from Philippine dairy products are bactericidal for pathogens with transferable multiple drug resistance

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Probiotic bacteria isolated from locally available commercial dairy products, namely, Nestle Chamyto Probiotic Drink, Yakult Probiotic Drink, Nestle Cereal Infant Cereal, and Nido 3+ Powdered Milk were established to have antimicrobial activities using the agar spot assay. Tester strains used were extended-spectrum β-lactamase (ESBL)-producing strains of Enterobacter aerogenes, Klebsiella pneumoniae, Proteus mirabilis, and Escherichia coli, metallo-β-lactamase (MBL)-producing strain of Pseudomonas aeruginosa, and three strains of methicillin resistant Staphylococcus aureus (MRSA). The probiotics inhibited the growth of all the tested multiple drug resistant strains. Further tests showed that the isolate from Chamyto displayed bactericidal activities on all the test strains. Yakult and Nestle Cereal probiotics were bactericidal for all strains except for ESBL-producing E. coli. Nido 3+ probiotic isolates were bactericidal for all strains except for ESBL-producing K. pneumoniae. The probiotic isolates were identified phenotypically and through 16S rDNA analysis to be Lactobacillus casei or Lactobacillus paracasei. The in vitro bactericidal activities of the isolated probiotics show great potential for further research in treatment and prophylaxis of infections from multiple drug resistant bacteria.

KEYWORDS
Probiotics, Lactobacillus, antibacterial, multiple drug resistant bacteria, extended-spectrum β-lactamase-producing Enterobacteriaceae, metallo-β-lactamase-producing Pseudomonas aeruginosa, methicillin resistant Staphylococcus aureus, ESBL, MRSA, MBL

INTRODUCTION
The rapid emergence of drug resistant strains of different microbial pathogens, especially those with multiple resistances, is a major health problem because of their high occurrence worldwide (Rouveix 2007). In the Philippines, the ready access of broad spectrum antibiotics to the general public who is mostly unaware of the proper antimicrobial usage, or on the issue of drug resistance poses a hindrance to the control of these strains.
Among the drug resistant bacterial pathogens that are often implicated in various life-threatening diseases are the extended-spectrum β-lactamase (ESBL)-producing strains of Enterobacteriaceae such as Enterobacter aerogenes, Klebsiella pneumoniae, Proteus mirabilis, and Escherichia coli; metallo-β-lactamase (MBL)-producing Pseudomonas aeruginosa and methicillin resistant Staphylococcus aureus (MRSA). ESBL producers are recalcitrant to treatment with all penicillins, cephalosporins and monobactams (CLSI 2010), while MBL producers are resistant to almost all broad spectrum β-lactams including carbapenems (Fujimura et al. 2007). MRSA is clinically resistant to all β-lactams (CLSI 2010). This complete resistance to β-lactams caused the switch to the use of glycopeptide antibiotic, specifically vancomycin. However, strains possessing high-level resistance, intermediate resistance, and tolerance to vancomycin have already been reported (Fridkin et al. 2003, Chang et al. 2003; Fridkin 2001; May et al. 1998). In addition, because the resistance genes of these three groups are found in plasmids (for ESBL and MBL) or in gene cassettes located in the chromosome (for MBL and MRSA), they are often also resistant to other classes of antimicrobials such as aminoglycosides and quinolones (Takashi 2007; Cabrera and Rodriguez 2009; Zhang et al. 2009). For the same reason, the genes coding for these are transferable to other bacteria, converting susceptible strains to resistance.

Probiotics are live microbial food supplements which improve the microbial balance in a host animal, thus providing beneficial health effects (Fuller 1989). Probiotics have likewise been found to have immunostimulatory properties (Trois et al. 2008), promoting the non-specific (Herich & Levkut 2002) and specific immune responses (Alvarez et al. 1998). In addition, they have been shown to competitively exclude other gut pathogens, preventing the adhesion of these pathogens to intestinal and urogenital epithelia (Chan et al. 1985). Probiotics have been reported to produce antimicrobial substances, which include organic acids such as lactic acid and acetic acid, diacetyl, hydrogen peroxide (produced by Lactobacillus upon transfer to an aerobic environment from an anaerobic one), and bacteriocins and microcins (Silva et al. 1987). Other metabolites of probiotic bacteria which can contribute to their effects include biosurfactants (Rodrigues et al. 2004), adhesion inhibitors (Meurman 2005), and co-aggregation molecules (Ekmeckci et al. 2009; Gupta and Garg 2009).

While there have been numerous studies on the antibacterial activities of different genera of probiotics (Lactobacillus, in particular), the significance and potential of probiotics in the prevention or reduction of emergent drug resistant strains and transmission of resistant genes has only been given minimal attention as of the moment. The increasing emergence of drug-resistant strains of pathogenic bacteria has become a great concern, as this may pose a problem for the control of nosocomial as well as community-acquired bacterial infections. Hence, this study aimed to assess the antimicrobial activities of probiotics from locally available dairy products on multiple drug resistant strains of bacterial pathogens. In addition, the identities of the isolated probiotics were determined using phenotypic characterization and 16S rRNA gene (or 16S rDNA) sequencing to ensure that these were the isolates from the dairy products, and that the antimicrobial activities were in fact from the probiotic isolates of interest.

MATERIALS AND METHODS

Test Microorganisms

The multiple drug resistant (MDR) bacterial strains and probiotic strains that were tested upon are listed in Tables 1 and 2, respectively. The MDR strains were obtained from the culture collection of the Microbiology Laboratory of De La Salle University Manila. Strains Enterobacter aerogenes MISC 253...
ESBL-CTX M3 and Proteus mirabilis MISC 182 ESBL CTX M2 were kind gifts from Dr. James H. Jorgensen of the University of Texas Health Center, San Antonio, Texas. The purity, identities and antimicrobial susceptibility patterns of all the tester strains were verified after they were revived from the -80°C glycerol stock, before they were used in the study. The antimicrobial susceptibility patterns were confirmed using the disc diffusion method (CLSI, 2010). All the tester strains showed the resistance phenotypes to the different antimicrobials reflected in Table 1.

The probiotics were isolated from locally available commercial milk products.

Isolation and Phenotypic Identification of Probiotics from Dairy

The dairy samples were serially diluted from 10⁻¹ to 10⁻³ and spread-plated in the amount of 100 µl onto Elliker (Lactic) Agar (Elliker et al. 1956). The plates were incubated in a candle jar (5% CO₂) for 48-72 hours. Because the indicated probiotic Bifidus BL in Cerelac is reported to be Bifidobacterium lactis (Fisher 2007), the plates for these were anaerobically incubated in a jar with pyrogallol and potassium hydroxide. The colonies on the plates were observed for their morphology. White or cream-colored punctiform colonies that were convex and had entire margins were selected from the plates for spore staining and Gram staining to check their microscopic morphology.

Non-sporeforming Gram positive bacilli in long or short chains were characterized using the following assays: catalase production, gelatin hydrolysis, sulfide and indole production, motility, and lactose fermentation. Catalase-negative, non-motile isolates, which did not hydrolyse gelatin, produce sulfide or indole, and which produced acid from lactose were furthered identified using 16srDNA sequencing.

Genotypic Identification using 16S rDNA

Chromosomal DNA was extracted using InstaGene Matrix™ (Bio-Rad Laboratories, USA) according to manufacturer’s instructions. The colonies were washed twice with 500 µl of TBE buffer. InstaGene Matrix resin in a volume of 100 µl was added, and then mixed in a thermomixer at 56°C for 15-30 min. After vortexing at high speed for 10 s, the mixture was boiled for 8 min, mixed again for 10 s and centrifuged for 3 min at 14,000 x g. The supernatant served as the source of DNA template for the polymerase chain reaction (PCR).

Universal primers were used to amplify the 1.5 kbp of 16S rDNA through PCR. The sequence for the forward primer is: 5'-CAGGCCCTAACACATGCAAGTC-3', while the sequence for the 16S reverse primer is 5'-GGGCGGWGTGTACAAGGC-3' (Kawai et al. 2002). The PCR mix was comprised of the following: 1X PCR buffer, 1.5 mM MgCl₂, 2mM of the dNTPs, 0.5 µM 16S forward primer, 0.5 µM 16S reverse primer, and 0.05 U/µL Taq DNA polymerase, and 1 µl of DNA extract. For the negative control, sterile deionized distilled water was utilized.

PCR was carried out in a PTC-100 Peltier Thermal Cycler (Bio-Rad Laboratories, USA) using the following conditions: 10 min at 94°C, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and final extension at 72°C for 5 min. The amplicons were viewed using a UV transilluminator after electrophoresis in 1.6% agar and staining with ethidium bromide. The amplicons were sent to Macrogen Inc., Seoul, Korea for purification and sequencing. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) Analysis in the...
Testing for Antibacterial Activity of the Probiotics

The agar spot method was utilized for determining the antibacterial activity of the isolated probiotics. The probiotics were grown in Elliker broth (Elliker et al. 1956) at 37˚C for 48-72 hr and standardized to the turbidity of MacFarland 1.0 (3.0 x 10^8 CFU/mL cell density). Two µl of the probiotic isolates were spotted aseptically onto the Elliker agar medium, which was found to support good growth of all the four probiotic strains. The plates were then incubated in 5% CO₂ for 48-72 hours.

On the other hand, cultures of the MDR test bacteria were prepared in nutrient broth and incubated at 37˚C for 16-18 hr. They were then transferred to fresh nutrient broth and incubated at 37˚C for 4 hr. The turbidity of the bacteria was then standardized to that of MacFarland 0.5 (1.5 x 10^8 CFU/mL cell density). One ml of each of the MDR test strains was then inoculated into a flask with 100 ml molten nutrient agar. Approximately 7 ml of the agar were poured as an overlay on the plate with the grown probiotic, prepared as described above. This was allowed to solidify, before being incubated aerobically at 37˚C for 16-18 hr. The diameters of the clear zones were measured to the nearest mm. The zones of inhibition were then swabbed, and tubes of nutrient broth were inoculated. After incubation at 37˚C for 24 hr, the tubes were checked for turbidity. A clear tube indicated bactericidal activity of the probiotic, while a turbid tube indicated bacteriostasis (Chuayana et al. 2003). Each assay was done in triplicate.

RESULTS AND DISCUSSION

Identification of the Isolated Probiotics

The identities of the isolated bacteria were confirmed using phenotypic characterization and 16S rDNA sequencing to ascertain the reliability of the results of the antimicrobial assays to be those of the probiotics from the dairy products. All isolates from the dairy products were found to be nonmotile, Gram positive non-sporeforming bacilli in chains (Fig. 1), negative for catalase, hydrogen sulfide and indole production, and gelatin hydrolysis. Lactose was fermented with acid production but no gas. These are characteristics of lactobacilli.

BLAST analyses of the 16S rDNA amplicons from the four probiotic isolates identified the probiotics as Lactobacillus casei or Lactobacillus paracasei with 100% homology and E value of 0.0, the same identities as those indicated on the product literature of Yakult, Chamyto and Nido 3+ (Table 2). Lactobacillus PROTECTUS (of Nido 3+) and Lactobacillus FORTIS (of Chamyto) are reported to be L. paracasei (Linantud et al. 2009). Although the Nido 3+ product claims to also contain a bifidus culture, it was not successfully isolated. Lastly, the isolate from Nestle Cerelac (advertised as Bifidus BL) was identified as L. casei or L. paracasei. The identity as such was further verified through the product specialists of Cerelac (personal communication, January 2011). It is noted that the 16S rDNA analyses were not able to differentiate between L. casei and L. paracasei. This limitation in the ability of the method to distinguish species has been observed in many studies, and other methods such as microarray-based technologies with 16S rDNA and other housekeeping genes have been used in such cases (Janda and Abbott 2007).
Antimicrobial Activity of the Isolated Probiotics

All of the isolated probiotics from the four dairy products inhibited the growth of all the MDR bacterial strains as shown by zones of inhibition with mean diameters that ranged from 10.67mm to 38mm (Table 3, Fig. 2, 3). The in vitro inhibition of the multiple drug resistant strains by the probiotic strains may have been due to the secretion of organic acids, primarily lactic acid, thereby decreasing the pH of the medium to one that is unsuitable for growth (Marianelli et al. 2010). Other possibilities include the secretion of other antimicrobial compounds such as bacteriocins, low molecular weight peptides, and possibly hydrogen peroxide (Gupta and Garg 2009). Fayolle-Messaoudi et al. (2005) noted that there may be a synergistic effect of the lactic acid secreted by Lactobacillus strains as well as the non-lactic acid molecules on Gram-negative bacteria. The lactic acid is said to increase the permeability of the outer membrane of the gram negative pathogens, making them more susceptible to the activity of antimicrobial molecules.

It is especially worth noting that most of the antimicrobial activities exhibited by the probiotics were proven to be bactericidal in nature on further subculture in nutrient broth, with the exception of the isolates from Yakult and Cerelac against E. coli ESBL 168, and the isolate from Nido 3+ against K. pneumoniae SHV-12 (Table 3). An earlier investigation by Chuayana et al. (2003) showed that the bacteria they isolated from Yakult (identified as L. casei strain Shirota in the product literature) was bacteriostatic against methicillin-susceptible S. aureus and antimicrobial susceptible P. aeruginosa. Since probiotics exert strain-specific effects against other bacteria, the indicator strains they tested may have been less susceptible. Linantud et al. (2009) also investigated the antimicrobial activity of bacteria from Yakult and Chamyto for E. coli and other Gram negative bacilli. However, they found that only the probiotics from Yakult displayed any

### Table 1. Bacterial strains tested for response to probiotics from local dairy products.

<table>
<thead>
<tr>
<th>Strain Name</th>
<th>Resistance Phenotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRSA NBP-2</td>
<td>Resistant to all β-lactams and erythromycin</td>
<td>DLSU Manila culture collection</td>
</tr>
<tr>
<td>MRSA NBP-9</td>
<td>Resistant to all β-lactams</td>
<td>DLSU Manila culture collection</td>
</tr>
<tr>
<td>MRSA NBP-12</td>
<td>Resistant to all β-lactams</td>
<td>DLSU Manila culture collection</td>
</tr>
<tr>
<td>Klebsiella pneumoniae 67 ESBL SHV-12</td>
<td>AZT, CAS, CTX, CRO, CFX, TE, NN, SXT</td>
<td>Cabrera &amp; Rodriguez (2009)</td>
</tr>
<tr>
<td>Escherichia coli 168 ESBL SHV-12</td>
<td>AZT, CAZ, CTX, CRO, CFX, CXT, TE, AK, CIP, NN</td>
<td>Cabrera &amp; Rodriguez (2009)</td>
</tr>
<tr>
<td>Enterobacter aerogenes MISC 253 ESBL-CTX M3</td>
<td>Resistant to all penicillins, cephalosporins, AZT, TE, AK, CIP, NN, SXT</td>
<td>Lewis et al. (2007)</td>
</tr>
<tr>
<td>Proteus mirabilis MISC 182 ESBL-CTX M2</td>
<td>Resistant to all penicillins, cephalosporins and AZT</td>
<td>Lewis et al. (2007)</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa PA 97 MBL</td>
<td>AK, GM, CIP, CRO, NET, IMI, SXT, AZT, CAP, CTX</td>
<td>Balhon et al. (2006)</td>
</tr>
</tbody>
</table>

AK, amikacin, AZT, aztreonam, CAZ, ceftazidime, CFX, cefuroxime, CIP, ciprofloxacin, CRO, co-trimoxazole, CTX, cefotaxime, CXT, cefotaxin, DLSU, Do La Salle University, GM, gentamicin, IMI, imipenem, MRSA, methicillin resistant Staphylococcus aureus, NET, nitrofurantoin, NNN, tobramycin, SXT, sulfamethoxazole-trimethoprim, TE, tetracycline.

### Table 2. Probiotic strains in dairy products and tested against multiple drug resistant bacterial strains

<table>
<thead>
<tr>
<th>Strains Reported in Product Literature</th>
<th>Source</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus casei Shirotai 610</td>
<td>Yakult Probiotic Drink</td>
<td>Yakult Philippines Inc.</td>
</tr>
<tr>
<td>Lactobacillus Fortis 6071</td>
<td>Chamyto Probiotic Drink</td>
<td>Nestle Philippines Inc.</td>
</tr>
<tr>
<td>Lactobacillus Protetex 6072</td>
<td>Nido 3+ Powdered Milk</td>
<td>Nestle Philippines Inc.</td>
</tr>
<tr>
<td>Bifidus culture</td>
<td>Nido 3+ Powdered Milk</td>
<td>Nestle Philippines Inc.</td>
</tr>
<tr>
<td>Bifidus BL</td>
<td>Nestle Cereal Infant Cereal</td>
<td>Nestle Philippines Inc.</td>
</tr>
</tbody>
</table>
inhibitory activity, and only against *Vibrio cholerae*. To test the probiotics, they utilized a blank disk method where they immersed sterile blank disks in the dairy products for 5 minutes, then placed these onto the surface of Mueller Hinton plates previously streaked with the enteric bacteria. It is highly likely that the reason the inhibitory activity was scant, if present, was because the probiotics were not first cultured in appropriate media. It is only then that the organic acids and other antimicrobial compounds would be optimally secreted as the source of inhibitory activity. Secondly, lactobacilli grow poorly on Mueller Hinton (Ocaña et al. 2006). Robust growth is always recommended if one wants to test the probiotic, so that they may have an optimal release of metabolites.

Based on the results of the present study, the *in vitro* capacity of the probiotics isolated from the commercial milk products to inhibit the growth of and exert bactericidal effects on multiple drug resistant bacteria shows promise for future *in vivo* studies. *In vivo* studies are a necessary step in evaluating the potential efficacy of probiotics for use in the prophylaxis or treatment of infections caused by microbial pathogens. The MDR strains tested in the study were obtained from clinical specimens, and therefore are medically important as these strains are resistant to the actions of all β-lactams (MRSA) (CLSI 2010), to all penicillins, cephalosporins and monobactams (ESBL-producers) (CLSI 2010), and to almost all broad spectrum β-lactams including carbapenems (MBL producers) (Fujimura et al. 2007). In addition, strains have been found that are resistant to other classes of antimicrobials such as fluoroquinolones and aminoglycosides (Takashi 2007; Cabrera and Rodriguez 2009; Zhang et al. 2009). Aside from the multiple resistances, the genes coding for these are found in transferable plasmids or gene cassettes, and thus can convert susceptible bacterial strains to resistance.

This leaves behind very few options for physicians, so one cannot rely on antibiotics alone, as the pathogens will continue to develop resistance to newer drugs that will be developed. Probiotic supplements can help to provide an intervention due to the bactericidal effects of select strains, without worsening the problems caused by overuse of antibiotics. While each antibiotic has a specific mode of action (such as inhibiting transpeptidation), bacteriocins isolated from probiotic strains have several modes of action, reducing the probability of a resistance development (Sleator 2010). The results also support past studies which have demonstrated the benefits that consumption of probiotics can provide for our health.

**CONCLUSION**

It is inevitable for bacteria to continue developing resistance to antibiotics, not only because of their high rates of mutation and transferability of drug resistance genes, but also because antibiotics pose a selective pressure against these bacteria, prompting drug-resistant strains to outcompete the susceptible ones (Pray 2008). The rise of multi-drug resistant strains has therefore led researchers to look for alternative therapies such as probiotics that would decrease our reliance on antibiotic use.

The results of this *in vitro* study clearly demonstrated that probiotics have strong potential in this regard because of their capacity to kill the multiple drug resistant bacteria. The results also suggest that the consumption of products containing probiotics can help to protect an individual from developing infection caused by such bacteria. Thus, probiotics can be used...
as a helpful tool for addressing the worldwide issue of antibiotic resistance.

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CONFLICTS OF INTEREST

We certify that there are no conflicts of interest related to the present study, nor in the preparation or submission of the manuscript.

CONTRIBUTION OF THE INDIVIDUAL AUTHORS

Mary Catherine A. Mercado: conduct of the entire study, preparation of manuscript.

Esperanza C. Cabrera: provision of the research problem and design, supervision of the conduct of the study, preparation of manuscript.

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