Probiotics with Antimicrobial Activity against Multidrug Resistant
_Pseudomonas aeruginosa_ and _Acinetobacter baumannii_

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_Pseudomonas aeruginosa_ and _Acinetobacter baumannii_ are significant opportunistic pathogens in hospitals and are resistant to most antibiotics. Multidrug-resistant _P. aeruginosa_ (MDRP A) and _A. baumannii_ (MDRAB) cause severe human nosocomial infections and are more difficult to treat than methicillin-resistant _Staphylococcus aureus_ (MRSA). Bifidobacteria are among of the most beneficial probiotics and have been widely studied for their antimicrobial activities. The present study explored the antimicrobial activity of _Bifidobacterium_ sp. isolated from healthy Koreans against MDRPA and MDRAB. The antimicrobial activity of the isolates against MDRPA and MDRAB, which are resistant to ciprofloxacin, tobramycin, gentamicin, meropenem, and ceftazidime, was determined by modified broth microdilution methods using absorbance. Among all tested bifidobacteria isolates (nine _B. adolescentis_, three _B. longum_, and two _B. pseudocatenulatum_), the culture supernatant of _B. pseudocatenulatum_ SPM1309 showed a strong growth inhibitory effect against MDRPA and MDRAB. No change in the turbidity of the mixture was observed during incubation, and its inhibitory effect occurred through bacteriostastic action. Moreover, the antibacterial activity was observed in the fraction with molecular weights <10 kDa of bifidobacteria culture supernatant, and the active fraction was heat-stable because it maintained its activity when heated at 70°C for 10 min. The results suggest that this _Bifidobacterium_ strain could have potential applications for alternative therapy in MDRPA and MDRAB infections.

Keywords: _Bifidobacterium_, antimicrobial activity, multidrug-resistant _Acinetobacter baumannii_, multidrug-resistant _Pseudomonas aeruginosa_

_Pseudomonas aeruginosa_ and _Acinetobacter baumannii_ have emerged as important nosocomial pathogens that exhibit intrinsic resistance to a large range of antibiotics and remarkable ability to develop novel mechanisms of resistance during treatment (Navon-Venezia _et al._, 2005; Falagas and Kopterides, 2006). The infections caused by these bacteria are difficult to be treated with antibiotics and are associated with a higher rate of mortality because few or no adequate therapeutic option exists (Kuo _et al._, 2004; Deplano _et al._, 2005; Falagas _et al._, 2005; Shah, 2005). According to National Nosocomial Infections Surveillance (NNIS), the prevalence of resistance in both pathogens is a significantly increasing trend (Gaynes and Edwards, 2005). Thus, novel strategies to control these strains are urgently needed.

Over the past decades, studies on beneficial health effects of probiotics or the substances they naturally produce have been actively progressing in various fields. Probiotics is defined as “good microorganisms that, administered in adequate amounts, confer health benefits on humans and that have been industrially prepared for nutritional and pharmaceutical use (Gill and Guarner, 2004; Parvez _et al._, 2006). Probiotics produce antimicrobial factors and compete with pathogens for essential nutrients, preventing excessive pathogen growth without causing...
bacterial resistance (Marteau and Shanahan, 2003). For an adequate amount of health benefits, a dose of 5 billion colony forming units (CFU) for at least 5 days (5×10⁹ CFU/day) has been recommended (Gupta and Garg, 2009). The consumption of these products is growing rapidly all over the world and probiotics are generally recognized as safe (GRAS) (Gupta and Garg, 2009). Bifidobacteria as probiotics are a major beneficial bacterial group in the healthy human gut microbiota, and have multiple health effects, including (i) improving gastrointestinal (GI) tract health; (ii) synthesizing and enhancing the bioavailability of nutrients; (iii) modulating the immune system; (iv) lowering serum cholesterol; (v) blocking and eliminating pathogens; and (vi) reducing the risk of certain cancers (Guarner and Malagelada, 2003; Iannitti and Palmieri, 2010). Numerous studies showed that bifidobacteria have antimicrobial activity against several pathogenic microorganisms. There is also increasing interest in the research of antimicrobial peptides such as bacteriocins and bacteriocin-like compounds produced by bifidobacteria (Yildirim et al., 1999; De Vuyst et al., 2004; Servin, 2004; Makras and De Vuyst, 2006; Cheikhyoussef et al., 2008; Kondepudi et al., 2012; Rodriguez et al., 2012). However, only a very small number of antimicrobial compounds produced by bifidobacteria have been purified and characterized, despite their apparent benefits to human health. Thus, there is good potential in further research in this field (Cheikhyoussef et al., 2008).

The aim of this study was to examine the antimicrobial effects of sonicated cell extract and culture supernatant of Bifidobacterium sp. isolated from human intestinal microflora against multidrug resistant P. aeruginosa (MDRPA) and A. baumannii (MDRAB) and to explore the characteristics of its antimicrobial compound(s).

**Materials and Methods**

**Preparation of Bifidobacteria samples**

For the isolation of bifidobacteria, fecal samples were collected from twenty healthy Koreans (20–30 years old). Fecal samples were diluted and seeded onto selective blood liver agar (Nissui Pharm, Japan) containing 5% sheep blood. After 48 h incubation in anaerobic conditions (90% N₂, 5% H₂, 5% CO₂) using Bactron Anaerobic Chamber (Sheldon Manufacturing Inc., USA) at 37°C, brown or reddish-brown colonies 2-3 mm in diameter were selected for further identification. A fructose-6-phosphate phosphoketolase (F6PPK) test was performed to ensure that the colonies selected were bifidobacteria (Ahn, 2005). It has been demonstrated that F6PPK activity serves as a taxonomic tool in the identification of the genus Bifidobacterium (Chevalier et al., 1990). To identify the isolated Bifidobacterium sp. at the species level, 16S rRNA sequencing was performed by Bio leaders (Daejeon, Korea). According to the 16S rRNA sequencing, the isolates contained nine strains of B. adolescentis, three strains of B. longum, and two strains of B. pseudocatenulatum (Table 1). Bifidobacterium sp. isolates were cultured at 37°C for 48 h in general anaerobic medium (GAM) broth (Nissui Pharm. Co. Ltd., Japan) under anaerobic conditions. For the preparation of bifidobacteria cell extracts, cells were harvested during the exponential growth phase by centrifugation at 4,000 rpm for 10 min, washed with PBS, and resuspended in the same buffer. These bacterial suspensions were then adjusted to a final concentration of 1.0×10⁹ CFU/ml and sonicated for 6 min (amplitude 100%). Cell extracts and culture supernatants were used after filtration (0.2 μm).

Written informed consents were obtained from all volunteers who provided samples and the protocol was approved by the Institution Review Board of Office of Research Development, Sahmyook University.

**Clinical isolates**

P. aeruginosa and A. baumannii isolated from various clinical specimens, i.e., sputum, urine, wound, blood, stool, bronchial wash fluid, and incubation tube were provided by Sahmyook Medical Center. The isolates were stored at -70°C in Nutrient broth (Difco, USA) with 20% glycerol before testing.

To select MDRPA and MDRAB, minimum inhibitory concentration (MIC) of six antibiotics [ciprofloxacin (Ildong, Korea), tobramycin (Daewoong, Korea), tigecycline (Wyeth-Ayerst, USA), gentamicin (Sigma, USA), meropenem (Yuhan, Korea), and ceftazidime (GlaxoSmithKline, UK)] against clinical isolates were measured by the agar dilution method according to the guidelines established by the Clinical Laboratory and Standards Institute (CLSI, 2012).
Assay for growth inhibition by probiotics

The antimicrobial activity of Bifidobacterium isolates against MDRPA and MDRAB was determined by modified broth microdilution methods using absorbance. MDRPA and MDRAB were cultured in Mueller-Hinton broth containing 25% (v/v) cell extract or culture supernatant of bifidobacteria prepared as described above for 24 h at 37°C. Growth inhibition was then detected by measuring absorbance of the mixtures at 625 nm using an ELISA reader (Molecular Devices, USA). As a control, MDRPA and MDRAB were cultured in Mueller-Hinton broth with the same amount of PBS or GAM broth.

Characterization of antimicrobial compound(s)

To examine the characteristics of antimicrobial compound(s), we heated the bifidobacteria sample for 10 min at 70°C or separated it by a molecular weight of 10 kDa by using Amicon® Ultra-0.5 Centrifugal Filter Devices (Millipore, USA). We then investigated the effect of heated or separated samples on growth inhibition as described above.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Significant differences were separated using Duncan’s multiple range test and commercial statistical analysis software, version 9.0 (SAS Institute, USA). All data were considered significant at P<0.05.

Results

The MICs of six antibiotics (ciprofloxacin, tobramycin, tigecycline, gentamicin, meropenem, and ceftazidime) against MDRPA and MDRAB are shown in Table 2. Most of the MDRPA exhibited high-level resistance to almost all antibiotics tested (MIC 128 μg/ml). MDRAB was likewise highly resistant to some antibiotics. Also, all MDRAB exhibited resistance to meropenem and even tigecycline (Table 2).

Among all tested bifidobacteria isolates (nine B. adolescens, three B. longum, and two B. pseudocatenulatum), the culture supernatant of B. pseudocatenulatum SPM1309 showed ~90% and ~80% stronger growth-inhibitory effect against MDRPA and MDRAB, respectively. However, the cell extract of this strain did not show such inhibition. Also, B. pseudocatenulatum SPM1309 had an inhibitory effect but same species (SPM1204) did not have such inhibitory effect on MDRPA and MDRAB. Therefore its effect was not a species-specific but a strain-specific (Figs. 1 and 2).

The turbidity of the bacterial mixture did not change during incubation for 24 h (Fig. 3). However, a colony formed when this unchanged mixture was seeded onto new agar medium, and its turbidity increased after incubation for 48 h (Fig. 3). Thus, this antibacterial activity of B. pseudocatenulatum SPM1309 was bacteriostatic in action. Moreover, the antibacterial activity was observed in the fraction with molecular weights <10 kDa of bifidobacteria culture supernatant, and active fraction was heat-stable because it maintained its activity when heated at 70°C for 10 min (Fig. 4).

Discussion

P. aeruginosa can produce disease in any part of the gastrointestinal (GI) tract from oropharynx to the rectum. The organism has been implicated in perirectal infections, pediatric diarrhea, typical gastroenteritis, and necrotizing enterocolitis. Chronic colonization of the GI mucosa is acknowledged to be an important component of P. aeruginosa diarrheal disease and systemic infections. The GI tract is an important portal of entry in Pseudomonas septicemia (Hentges et al., 1985; Marshall et al., 1993; Ohara and Itoh, 2003). Acinetobacter occasionally colonizes the gastrointestinal tract and is associated with bacteremia, and wound infections (Trottier et al., 2007).

Meropenem is a beta-lactam and belongs to the carbapenem subgroup, which is the first-line treatment of Acinetobacter infections (Bassetti et al., 2008). In the present study, all MDRAB isolates were resistant to meropenem and even tigecycline, which is a novel, broad-spectrum antibiotic that is active against MDRAB (Bassetti et al., 2008). In addition, most of the MDPA isolates exhibited high-level resistance. However, the growth of both MDRAB and MDRPA isolates were inhibited by culture supernatant of B. pseudocatenulatum SPM1309. Some studies showed that bifidobacteria have antimicrobial activity against Pseudomonas species. According to Matsumoto et al. (2007) administration of B. longum significantly decreased viable counts of P. aeruginosa in the

### Table 2. Minimum inhibitory concentration (MIC) of test antibiotics against multidrug-resistant P. aeruginosa and A. baumannii from clinical isolates

<table>
<thead>
<tr>
<th>Strains</th>
<th>CIP (μg/ml)</th>
<th>TOB (μg/ml)</th>
<th>TIG (μg/ml)</th>
<th>GEN (μg/ml)</th>
<th>MEM (μg/ml)</th>
<th>CAZ (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas aeruginosa</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPA-1</td>
<td>&gt;64</td>
<td>&gt;128</td>
<td>128</td>
<td>&lt;128</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>CPA-2</td>
<td>&gt;64</td>
<td>&gt;128</td>
<td>128</td>
<td>&lt;128</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>CPA-3</td>
<td>&gt;64</td>
<td>&gt;128</td>
<td>128</td>
<td>&lt;128</td>
<td>&gt;128</td>
<td>32</td>
</tr>
<tr>
<td>CPA-4</td>
<td>&gt;64</td>
<td>&gt;128</td>
<td>64</td>
<td>64</td>
<td>&gt;128</td>
<td>128</td>
</tr>
<tr>
<td>Acinetobacter baumannii</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAB-1</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>8</td>
<td>&gt;128</td>
<td>16</td>
<td>64</td>
</tr>
<tr>
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<td>4</td>
<td>&gt;128</td>
<td>16</td>
<td>&gt;128</td>
</tr>
<tr>
<td>CAB-3</td>
<td>&gt;128</td>
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<td>4</td>
<td>32</td>
<td>16</td>
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</tr>
<tr>
<td>CAB-4</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>4</td>
<td>&gt;128</td>
<td>16</td>
<td>&gt;128</td>
</tr>
</tbody>
</table>

CIP, ciprofloxacin; TOB, tobramycin; TIG, tigecycline; GEN, gentamicin; MEM, meropenem; CAZ, ceftazidime
Lee et al. Fig. 1. Growth inhibitory effect of culture supernatants and cell extracts of bifidobacteria against multidrug-resistant *P. aeruginosa*. MDRPA were cultured in Mueller-Hinton broth containing 25% (v/v) culture supernatant or cell extract of bifidobacteria for 24 h at 37°C. Growth inhibition was determined by measuring absorbance at 625 nm. As a control (CTL), MDRPA were cultured in Mueller-Hinton broth with the same amount of PBS or GAM broth. Data are presented as the growth rate of MDRPA. *a–h* Means with no common superscripts differ significantly (*P*<0.05).
Fig. 2. Growth inhibitory effect of culture supernatants and cell extracts of bifidobacteria against multidrug-resistant *A. baumannii*. MDRAB were cultured in Mueller-Hinton broth containing 25% (v/v) culture supernatant or cell extract of bifidobacteria for 24 h at 37°C. Growth inhibition was determined by measuring absorbance at 625 nm. As a control (CTL), MDRAB were cultured in Mueller-Hinton broth with the same amount of PBS or GAM broth. Data are presented as the growth rate of MDRPA. Means with no common superscripts differ significantly (*P* <0.05).
liver and blood. And culture of intestinal contents revealed a significantly lower viable count of *P. aeruginosa* in the jejunum of *B. longum*-treated mice. O’Riordan and Fitzgerald (1998) also reported that twelve strains of bifidobacteria exhibited a broad spectrum of antagonistic activity against both Gram-positive and Gram-negative indicators, especially *Pseudomonas* species. However, there are no studies that evaluate the effects of bifidobacteria against *Acinetobacter* species.

**Fig. 3.** The hourly change of turbidity in growth inhibitory effect of culture supernatant of *B. pseudocatenulatum* SPM1309.

Antibacterial activity of *B. pseudocatenulatum* SPM1309 was bacteriostatic in action. However, the antibacterial mechanism of *B. pseudocatenulatum* SPM1309 was not clearly elucidated. Several mechanisms have been suggested for the inhibitory action of bifidobacteria toward both gram-positive and negative pathogens, including a decrease of the local pH via the production of organic acids, the inhibitory action of undissociated organic acid molecules, competition for nutrients, competition for adhesion sites, stimulation of the host’s immunity, and production of specific antibacterial substances (Fuller, 1989; Ballongue, 1998; De Vuyst et al., 2004). We may thus speculate that *B. pseudocatenulatum* inhibited the growth of MDRPA and MDRAB by the production of specific antimicrobial compound(s).

Numerous studies report on the inhibition of a wide range of pathogenic microorganisms, by bifidobacteria, both *in vitro* and *in vivo* (Yildirim et al., 1999; De Vuyst et al., 2004; Servin, 2004; Makras and De Vuyst, 2006; Cheikhyoussef et al., 2008; Kondepudi et al., 2012; Rodriguez et al., 2012). Antimicrobial activity of bifidobacteria was first noted by Tissier (1900), who described various types of antagonistic effects of *B. bifidum* against *Escherichia coli* (Tissier, 1990). Several bifidobacteria have the ability to produce antimicrobial compounds including

**Fig. 4.** Characterization of antibacterial compound(s) from culture supernatant of *B. pseudocatenulatum* SPM1309. MDRPA (A), and MDRAB (B) were cultured in Mueller-Hinton broth containing 25% (v/v) *B. pseudocatenulatum* SPM1309 sample for 24 h at 37°C. Growth inhibition was determined by measuring absorbance at 625 nm. SPM1309, no treated culture supernatant of *B. pseudocatenulatum* SPM1309; F-1, the fraction with molecular masses lower than 10 kDa; F-2, the fraction with molecular masses higher than 10 kDa; Heated, heat treated culture supernatant of *B. pseudocatenulatum* SPM1309. *P<0.05.
Antimicrobial activity of B. pseudocatenulatum SPM1309

proteinaceous compounds called bacteriocins (Gibson and Wang, 1994; Yildirim and Johnson, 1998; Yildirim et al., 1999; Ibrahim and Salameh, 2001). Among the bacteriocins, Bifidin has been isolated from B. bifidum by a methanol-acetone extraction process and partial purification was achieved using Sephadex G-15 chromatography. Its inhibitory activity was observed after 30 h and the inhibition was maximal after 48 h. Its optimum pH was 4.8 and maximum inhibition occurred in the pH range of 4.8 to 5.5. Bifidin is heat stable as it showed no loss of activity when heated at 70℃. The purified fraction showed no loss of activity when heated at 70℃ and this fraction with molecular weights under 10 kDa, and this activity of B. pseudocatenulatum SPM1309 was observed in the fraction with molecular weights under 10 kDa, and this active fraction showed no loss of activity when heated at 70℃ for 10 min.

Bifidobacteria are used worldwide in various traditional and industrial food fermentations (Hammes and Tichaczek, 1994). Bifidobacteria dominate the indigenous microbiota of infants, but their numbers decrease in adults (Gomes and Malcata, 1999; Guarner and Malagelada, 2003). Therefore, it is important to consistently consume the beneficial bifidobacteria.

In conclusion, B. pseudocatenulatum SPM1309 had a strong growth-inhibitory effect against both MDRPA and MDRAB. The molecular masses of the antimicrobial compound(s) from B. pseudocatenulatum SPM1309 were less than 10 kDa. In addition, it had bacteriostatic action and maintained its activity when heated at 70℃ for 10 min. The results suggest that B. pseudocatenulatum SPM1309 could be a useful probiotic microorganism for the prevention or alternative therapy of MDRPA and MDRAB infections. However, further identification of the active compound(s) and more information regarding this effect in various clinical conditions are needed.

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