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Antimicrobial susceptibility profiles of genera *Bifidobacterium*, *Enterococcus*, and *Lactobacillus* probiotic strains

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Abstract

Antimicrobial-associated diarrhea (AAD) is a well-known side effect of antimicrobial therapy. Probiotics are sometimes used to prevent AAD, but their effectiveness remains unclear. To clarify the appropriate combination of antimicrobial agents and probiotic strains to prevent AAD, the minimum inhibitory concentrations (MICs) of oral antimicrobial agents for different probiotic strains were determined by the broth microdilution method. Fifteen probiotic strains and 10 antimicrobial agents were included in this study. Two of the three enterococci were associated with high MICs (>64 μg/ml) for β-lactams. Fosfomycin had a high MIC (>512 μg/ml) for lactobacilli. However, most bifidobacteria, enterococci, and lactobacilli were associated with low MICs (1-4 μg/ml) for new quinolones. Probiotic strains associated with high MIC values were considered viable in the intestinal tract. This study suggests that appropriate antimicrobial agents and the selection of probiotic strains are important for the prevention of AAD. In the future, it will be necessary to investigate the effects of probiotics on preventing AAD in clinical cases.

Introduction

Antimicrobial-associated diarrhea (AAD) occurs in approximately 20% of patients treated with antimicrobial agents[1)] and is the most common adverse effect of antimicrobial therapy. One cause of AAD is *Clostridioides difficile*-associated diarrhea (CDAD) caused by this pathogen. CDAD accounts for 15-25% of AAD cases[2)] and is thought to be caused by disruption of the intestinal microbiota, which in turn occurs due to antimicrobial administration, resulting in the abnormal growth of *C. difficile* and toxin production[2)]. Risk factors for CDAD include antimicrobial use, older age, and prolonged hospitalization[3)], and antimicrobial use is a particularly important risk factor. The impact of CDAD on the health care system is significant and associated with longer hospital stays, higher healthcare costs, and increased mortality[4)]. Therefore, the prevention of CDAD is important in clinical practice.

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host"[5)]. However, whether there is a difference in health effects between live and dead bacteria remain uncertain, and at this point, it is thought that live bacteria should be taken. Recently, several studies have reported that probiotics reduce the incidence of AAD and CDAD[6-9)]. In contrast, some reports have found no evidence regarding the effectiveness of probiotics in pre-

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venting AAD and CDAD[10], leading to controversy among medical professionals. One of the factors contributing to the differences in study results could be the wide variety of antimicrobials and probiotics administered. We hypothesized that probiotics could prevent AAD and CD-associated enteritis when the appropriate probiotic is selected according to the antimicrobial administered. In the present study, to clarify the appropriate combination of antimicrobial agents and probiotics, the minimum inhibitory concentrations (MICs) of oral antimicrobial agents against each probiotic strain used in clinical practice were determined by the broth microdilution method.

Materials and methods

Bacterial strains

We selected probiotics that are commonly used in clinical practice for acute gastroenteritis and those that are contained in commercially available foods. Bacterial strains were provided by the manufacturers of the probiotics. The genera tested were *Bifidobacterium*, *Enterococcus*, and *Lactobacillus*. Bifidobacteria treatments included *Bifidobacterium longum* LBR (Kowa Pharmaceuticals, Tokyo, Japan), *Bifidobacterium infantis* SMR (Wakamoto Pharmaceutical, Tokyo, Japan), *B. longum* plus *B. infantis* (Kowa Pharmaceuticals), *Bifidobacterium bifidum* G9-1 (Biofermin Pharmaceuticals, Kobe, Japan), *B. longum* (Morinaga Milk Industry, Tokyo, Japan), *Bifidobacterium breve* (Morinaga Milk Industry), *Bifidobacterium animalis* subsp. *animalis* (Morinaga Milk Industry), *B. animalis* subsp. *lactis* (Morinaga Milk Industry), *Bifidobacterium pseudolongum* (Morinaga Milk Industry) ; for enterococci, *Enterococcus faecalis* 129 BIO 3B (Biofermin pharmaceuticals), *E. faecalis* 129 BIO 3B-R (Biofermin pharmaceuticals), and *E. faecalis* PCR (Wakamoto Pharmaceutical) were used. The lactobacilli used comprised *Lactobacillus acidophilus* 4AR (Wakamoto Pharmaceutical), *Lactobacillus rhamnosus* (Morinaga Milk Industry), and *Lactobacillus casei* (Morinaga Milk Industry).

Antibiotics

Penicillins, cephems, fluoroquinolones, fosfomycin (FOM), clindamycin (CLDM), tetracyclines, macrolides, and glycopeptides, which are commonly used in clinical practice were tested. The penicillins used were amoxicillin (AMPC) and amoxicillin/clavulanic acid (ACV), the cephems used were cefaclor (CCL) and cefcapene (CFPN), the fluoroquinolone used was levofloxacin (LVFX), the tetracycline used was minocycline (MINO), the macrolide used was azithromycin (AZM), and the glycopeptide used was vancomycin (VCM).

Determination of MICs

The MICs (μg/ml) of the antibiotics were determined by the broth microdilution method in accordance with the "M07-A8. Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically ; Approved Standard, Eighth Edition," by the Clinical and Laboratory Standards Institute (CLSI) for enterococci, and "M11-A7. Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria ; Approved Standard, Seventh Edition," by CLSI for anaerobic bacteria. Each strain was inoculated onto BL agar medium. AnaeroPack-Anaero (Mitsubishi Gas Chemical, Tokyo, Japan) and BL agar media were placed in an AnaeroPack Rectangular Jar (Mitsubishi Gas Chemical) and incubated at 37°C for 48 hours. After culturing, the test bacteria were removed and suspended in saline to a McFarland standard of 2, then 0.025 mL of the suspended bacterial solution was added to *Brucella* broth and mixed evenly to make an inoculum. Next, 0.1 mL of the inoculum solution was dispensed into each well of the frozen plate (original plate : custom order, Eiken Chemical, Tokyo, Japan). AnaeroPack-Anaero and frozen plates were placed in an AnaeroPack Rectangular Jar and incubated at 37°C for 48 hours, and then, susceptibility was determined.

Results

Susceptibility of bifidobacteria to β-lactam antibiotics

Table 1 shows the susceptibility of bifidobacteria to β-lactam antibiotics. Bifidobacteria were associated with low MICs (0.06-2 μg/ml) for AMPC and ACV. The MICs of CCL tended to be higher than those of AMPC and ACV ; further, a wide range of MICs (2 to >128 μg/ml) was observed for CCL. Two of the nine *Bifidobacterium* species (*B. longum* LBR and *B. longum* SMR) were associated with high MICs (>128 μg/ml) for CCL. *B. infantis* SMR and *B. breve* were associated with MICs of 16 μg/ml for CFPN. For other *Bifidobacterium* species, MICs were low (≤0.25-4 μg/ml).

Susceptibility of bifidobacteria to other antimicrobial agents

Table 1 shows the susceptibility of bifidobacteria to other antimicrobial agents. The MIC (4 μg/ml) of LVFX was low for most *Bifidobacterium* species. *B. animalis* subsp. *lactis* and *B. breve* were associated with MICs of 8 and 64 μg/ml, respectively, for LVFX. Further, for *B. infantis* SMR and *B. animalis* subsp. *Animalis*, FOM MICs were 256 μg/ml. Other *Bifidobacterium* species were associated with low MICs (32-128 μg/ml) for FOM. For one of the nine *Bifidobacterium* species (*B. longum* LBR) a high CLDM MIC (128 μg/ml) was observed. Other *Bifidobacterium* species were associated with low MICs (≤0.12-2 μg/ml). For one of the nine species, *Bifidobacterium* (*B. animalis* subsp. *lactis*), an MIC of 8 μg/ml for MINO was noted. Meanwhile, other *Bifidobacterium* species were associated with low

Table 1 Susceptibility of bifidobacteria to antimicrobial agents using the broth microdilution method

	MICs (μg/ml)									
species	AMPC	ACV	CCL	CFPN	LVFX	FOM	CLDM	MINO	AZM	VCM
Bifidobacterium longum LBR	1	1	>128	4	4	64	128	0.5	>16	0.5
Bifidobacterium infantis SMR	2	2	>128	16	4	256	≦0.12	0.5	≦0.5	0.5
Bifidobacterium longum+infantis	1	1	16	2	4	128	≦0.12	0.5	≦0.5	0.5
Bifidobacterium bifidum G9-1	0.06	0.06	32	1	4	64	≦0.12	0.5	≦0.5	2
Bifidobacterium longum	0.25	0.25	2	1	4	128	≦0.12	0.5	≦0.5	0.5
Bifidobacterium breve	0.25	0.25	32	16	64	64	≦0.12	0.5	≦0.5	0.5
Bifidobacterium animalis subsp. *animalis*	0.06	0.06	4	≦0.25	4	256	2	0.5	≦0.5	0.5
Bifidobacterium animalis subsp. *lactis*	0.25	0.25	4	0.5	8	64	≦0.12	8	≦0.5	1
Bifidobacterium pseudolongum	0.12	0.12	4	≦0.25	4	32	≦0.12	0.5	≦0.5	0.5

AMPC, amoxicillin ; ACV, amoxicillin/clavulanate ; CCL, cefaclor ; CFPN, cefcapene ; LVFX, levofloxacin ; FOM, fosfomycin ; CLDM, clindamycin ; MINO, minocycline ; AZM, azithromycin ; VCM, vancomycin

Table 2 Susceptibility of enterococci to antimicrobial agents using the broth microdilution method

	MICs (μg/ml)									
species	AMPC	ACV	CCL	CFPN	LVFX	FOM	CLDM	MINO	AZM	VCM
Enterococcus faecalis 129 BIO 3B	0.5	0.5	128	256	4	32	128	≦0.12	>16	2
Enterococcus faecalis 129 BIO 3B-R	>64	>64	>128	>512	2	16	128	2	>16	2
Enterococcus faecalis PCR	>64	>64	>128	>512	2	16	128	≦0.12	>16	1

AMPC, amoxicillin ; ACV, amoxicillin/clavulanate ; CCL, cefaclor ; CFPN, cefcapene ; LVFX, levofloxacin ; FOM, fosfomycin ; CLDM, clindamycin ; MINO, minocycline ; AZM, azithromycin ; VCM, vancomycin

MICs (0.5 μg/ml). For one of the nine species of *Bifidobacterium* (*B. longum* LBR), MICs (>16 μg/ml) above the measurement sensitivity were noted for AZM. In contrast, this agent showed high activity, with MICs ≤ 0.5 μg/ml, for other *Bifidobacterium* species. *Bifidobacterium* species were associated with low MICs (0.5-2 μg/ml) for VCM.

Susceptibility of enterococci to for *β*-lactam antibiotics

Table 2 shows the susceptibility of enterococci to β-lactam antibiotics. Beta-lactams showed a wide range of MICs (0.5 to >512 μg/ml) against enterococci. *E. faecalis* 129 BIO3B showed good susceptibility to AMPC and ACV with MICs of 0.5 μg/ml, whereas *E. faecalis* 129 BIO3B-R and *E. faecalis* PCR were associated with high MICs (>64 μg/ml) for AMPC and ACV. The MICs (128 to >512 μg/ml) of CCL and CFPN were high for all three enterococci.

Susceptibility of enterococci to other antimicrobial agents

All *Enterococcus* species showed good susceptibility to LVFX, with MICs ranging from 2 to 4 μg/ml. Enterococci were associated with low MICs (16-32 μg/ml) for FOM. Further, for enterococci, a high CLDM MIC (128 μg/ml) was observed. Enterococci were also associated with low MICs (≤0.12-2 μg/ml) for MINO and VCM. Finally, enterococci were associated with MICs (>16 μg/ml) above the measurement sensitivity for AZM.

Susceptibility of lactobacilli to *β*-lactam antibiotics

Table 3 shows the susceptibility of lactobacilli to β-lactam antibiotics. Here, lactobacilli were associated with low MICs (0.12-1 μg/ml) for AMPC and ACV. For lactobacilli, a high MIC (>128 μg/ml) for CCL and a low MIC (2-4 μg/ml) for CFPN were noted.

Susceptibility of lactobacilli to other antimicrobial agents

Table 3 shows the susceptibility of lactobacilli to other antimicrobial agents. One of the three species, *L. acidophilus* 4AR, was associated with an MIC of 16 μg/ml for LVFX. For other *Lactobacillus* species, low MICs (1-2 μg/ml) for LVFX were noted. Lactobacilli were associated with high MICs (>512 μg/ml) for FOM, and *L. acidophilus* 4AR was associated with MICs of 8, 32, and >16 μg/ml for CLDM, MINO, and AZM, respectively, whereas *L. rhamnosus* and *L. casei* were associated with lower MICs for CLDM, MINO, and AZM (2, 0.5, and ≤ 0.5 μg/ml, respectively). For *L. acidophilus* 4AR, a low MIC (1 μg/ml) for VCM was noted. For *L. rhamnosus* and *L. casei*, VCM MICs >2 μg/ml, above the measured sensitivity, were observed.

Table 3 Susceptibility of lactobacilli to antimicrobial agents using the broth microdilution method

	MICs (μg/ml)									
species	AMPC	ACV	CCL	CFPN	LVFX	FOM	CLDM	MINO	AZM	VCM
Lactobacillus acidophilus 4AR	0.12	0.12	>128	2	16	>512	8	32	>16	1
Lactobacillus rhamnosus	1	1	>128	4	2	>512	2	0.5	≦0.5	>2
Lactobacillus casei	1	1	>128	2	1	>512	2	0.5	≦0.5	>2

AMPC, amoxicillin ; ACV, amoxicillin/clavulanate ; CCL, cefaclor ; CFPN, cefcapene ; LVFX, levofloxacin ; FOM, fosfomycin ; CLDM, clindamycin ; MINO, minocycline ; AZM, azithromycin ; VCM, vancomycin

Discussion

This study revealed the optimal combination of antimicrobial agents and probiotic strains to prevent AAD. The probiotic strains commonly used in Japan show good susceptibility to various oral antimicrobial agents. Therefore, when using probiotics to prevent AAD, it is necessary to consider the sensitivity of the antimicrobial agent and the bacteria used as probiotic and to select an appropriate probiotic.

E. faecalis 129 BIO 3B-R and *E. faecalis* PCR were associated with high MICs for AMPC, ACV, CCL, and CFPN, indicating that they might be effective when β-lactam antibiotics are used. For *E. faecalis* 129 BIO 3B, high MICs for CCL and CFPN were noted, indicating that this strain could be effective when cephem antibiotics are used. *B. longum* LBR, *B. infantis* SMR, *L. acidophilus* 4AR, *L. rhamnosus*, and *L. casei* were associated with high MICs for CCL and low MICs for CFPN, indicating that they might be effective when using a narrow spectrum cephem. FOM had a high MIC against lactobacilli, indicating that these strains might be effective when FOM is used. *B. longum* LBR and enterococci were associated with high MICs for CLDM, indicating that they could be effective with CLDM use. However, the MICs of LVFX for bifidobacteria, enterococci, and lactobacilli were low, except for some bacteria (*B. breve* and *L. acidophilus* 4AR), suggesting that these strains are likely to be killed in the intestinal tract. In addition, bifidobacteria, enterococci, and lactobacilli were associated with low MICs for MINO, except for some bacteria (*B. animalis* subsp. *Lactis* and *L. acidophilus* 4AR), suggesting that they were likely to be killed in the intestinal tract.

Since it is challenging to accurately measure the concentration of antimicrobial agents in the intestinal tract, it is difficult to estimate the extent to which probiotic strains survive during antimicrobial administration. According to previous reports, fecal concentrations of AMPC, ACV, CEX, FOM, LVFX, FOM, AZM, and VCM are <1.20 μg/g, <0.3 μg/g, 3-6 μg/g, 197-393 μg/g, 17.9-65.2 μg/g, 197-393 μg/g, 23.5-2116 μg/g, and 242-2570 μg/g, respectively[11-17]. Breakpoints for probiotic strains are not specified by the CLSI or the Japanese Society for Chemotherapy, but EUCAST specifies breakpoints for enterococci and gram-positive anaerobes (including bifidobacteria and lactobacilli). The breakpoint of AMPC and ACV for gram-positive anaerobes is 8 μg/ml, whereas that for enterococci is 8 μg/ml ; the breakpoint of LVFX for enterococci for is 4 μg/ml, the breakpoint of CLDM for gram-positive anaerobes is 4 μg/ml, the breakpoint of VCM for gram-positive anaerobes is 2 μg/ml, and that of enterococci is 4 μg/ml[18]. In addition, cut-off values for bifidobacteria, enterococci, and lactobacilli have been proposed by FEDAP. The cut-off value of CLDM for bifidobacteria is 1 mg/L, and the cut-off values of tetracycline for bifidobacteria, enterococci, and lactobacilli are 8 mg/l, 4 mg/l, and 4-32 mg/l, respectively[19]. Based on these reports, we thought that probiotics could be used in combination when MICs were significantly higher than 8 μg/ml for AMPC and ACV, 6 μg/ml for CCL and CFPN, 65.2 μg/ml for LVFX, 393 μg/ml for FOM, 4 μg/ml for CLDM, 32 μg/ml for MINO, 2116 μg/ml for AZM, and 2570 μg/ml for VCM. *E. faecalis* 129 BIO 3B-R and *E. faecalis* PCR were viable when AMPC, ACV, CCL, CFPN, and CLDM were used, *E. faecalis* 129 BIO 3B was viable when CCL, CFPN, and CLDM were used, *B. longum* LBR was viable when CCL and CLDM were used, *B. infantis* SMR was considered sufficiently viable in the intestine when CCL was used, and lactobacilli were considered sufficiently viable in the intestine when CCL and FOM were used. In contrast, most of the probiotic strains were considered likely to die in the intestine when LVFX or MINO are used, but there were variations in intestinal concentrations and cut-off values. *B. breve* and *L. acidophilus* 4AR were considered likely to survive in the intestine when LVFX is used, whereas *B. animalis* subsp. *Lactis* and *L. acidophilus* 4AR were considered to have the potential to survive in the intestine when MINO is used. There were several strains of bacteria associated with MICs >16 μg/ml for AZM and >2 μg/ml for VCM, which are values above the measurement sensitivity. However, the intestinal concentrations of AZM and VCM when administered orally were extremely high, although they varied as described previously herein, and the

strains that could survive in the intestine were not identified.

Studies on the relationship between AAD and the use of antimicrobial agents have reported that antimicrobial agents with a broad antimicrobial spectrum, such as third-generation cephems, fourth-generation cephems, and carbapenems, are more likely to cause AAD[20]. Penicillins, cephalosporins, clindamycin, and fluoroquinolones have also been identified as risk factors for CDAD[21]. Oral antimicrobial agents account for 90% of antimicrobial agents used based on sales volumes in Japan, and the use of broad-spectrum antimicrobial agents such as third-generation cephalosporins, macrolides, and fluoroquinolones is high at 76%[22], suggesting a high risk of developing AAD and CDAD. In this experiment, the highest MICs for penicillin and cephem antibiotics were observed with *E. faecalis* 129 BIO 3B-R and *E. faecalis* PCR, which were much higher than the expected concentrations of antibiotics in stool. Therefore, the combination of *E. faecalis* 129 BIO 3B-R and *E. faecalis* PCR with penicillin and cephem antibiotics might prevent the development of AAD and CDAD. *B. breve* and *L. acidophilus* 4AR were associated with the highest MICs for fluoroquinolones, but these were lower than the expected concentrations of antimicrobial agents in the stool, and thus, these strains might be killed. However, the concomitant use of probiotics with higher MICs could prevent the development of AAD and CDAD. In this study, macrolide antimicrobials and strains associated with potential benefits based on their use were not identified.

This study had three limitations. The first was related to whether probiotic strains that are resistant to certain antimicrobials are safe for the host to which they are administered. Probiotics are used for their infection-fighting effects, such as preventing the onset of AAD. However, since probiotics are live bacteria, the bacteria used could cause bacteremia. Land et al. reported a case of bacteremia caused by *L. rhamnosus* in a patient who received the probiotic strain *L. rhamnosus* GG[23]. Bertelli et al. also reported a case of bacteremia caused by *B. longum*[24]. Most bacteremia caused by probiotics reported to date have occurred in immunocompromised individuals or those with diseases of the gastrointestinal tract. Although probiotics are unlikely to cause bacteremia in healthy individuals, the possibility of infection should be considered in cases with a high risk of infection. The second is the propagation of resistant genes. In clinical practice, the increase in resistant strains owing to horizontal plasmid spread is a problem, and it is important to prevent the spread of resistance to enteric bacteria. *E. faecalis* BIO 3B-R and *E. faecalis* PCR, which showed resistance to β-lactam antibiotics in our study, were obtained by selecting resistant strains from susceptible strains via in vitro culture with increasing doses of antibiotics. Yamashita et al. reported that the resistance genes of artificially resistant *E. faecalis* BIO-4R strains were not transmitted to recipient bacteria and the strain did not contain cyclic DNA, indicating that the resistance in *E. faecalis* BIO-4R strains was not a result of plasmid transmission. We believe that resistance genes are not transmitted via plasmids[25]. However, the mechanism of drug resistance is still unknown, and we need to continue monitoring the spread of this resistance. The third question is whether resistance transmission from intestinal bacteria to probiotic strains will occur. Klarin et al. examined the antimicrobial susceptibility of *Lactobacillus plantarum* 299v re-cultured from the stool of patients treated with antimicrobial agents in combination with *L. plantarum* 299v and found neither evidence of susceptibility to the acquisition of genetic material encoding antimicrobial resistance nor any reduction in antimicrobial susceptibility. They also reported that there was no evidence of decreased susceptibility[26].

Conclusion

This study revealed the optimal combination of antimicrobial agents and probiotics to prevent AAD. The use of probiotics containing *E. faecalis* BIO 3B-R and *E. faecalis* PCR when using β-lactams and probiotics containing *L. acidophilus* 4AR, *L. rhamnosus*, and *L. casei* when using FOM might prevent AAD. In the future, it is necessary to investigate the effectiveness of probiotics in preventing AAD in clinical cases.

Conflict of interest

None to declare

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プロバイオティクス用菌株の抗菌薬感受性

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【要旨】 抗菌薬関連下痢症は抗菌薬治療中のよく知られた副作用である。抗菌薬関連下痢症を予防するためにプロバイオティクスを使用することがあるが、その効果ははっきりしていない。抗菌薬関連下痢症を予防するための抗菌薬とプロバイオティクス用菌株の適切な組み合わせを明らかにするため、プロバイオティクス各菌株に対する経口抗菌薬の最小発育阻止濃度（MIC）を微量液体希釈法によって測定した。15 種類のプロバイオティクス用菌株と 10 種類の抗菌薬を対象とした。3 種中 2 種の Enterococci はβラクタム系抗菌薬に対して高い MIC（>64 μg/ml）を有していた。Lactbacilli はフォスフォマイシンに対して高い MIC（>512 μg/ml）を有していた。一方で、殆どの Bifidobacteria、Enterococci、Lactobacilli はニューキノロン系抗菌薬の MIC（1〜4 μg/ml）が低かった。高い MIC 値を示したプロバイオティクス用菌株は腸管内でも生存可能と考えられた。本研究では、抗菌薬関連下痢症の予防のためには適切な抗菌薬とプロバイオティクス用菌株の選択が重要であることが示唆された。今後は臨床例におけるプロバイオティクスの抗菌薬関連下痢症予防効果を検討していく必要がある。

〈キーワード〉 プロバイオティクス、抗菌薬感受性、抗菌薬関連下痢症