GMR Genetics and Molecular Research
Online Journal
ISSN:1676-5680

Determining the role of a probiotic in the restoration of intestinal microbial balance by molecular and cultural techniques

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Genet. Mol. Res. 14 (1): 1526-1537 (2015)
Received April 29, 2014
Accepted November 12, 2014
Published February 20, 2015
DOI http://dx.doi.org/10.4238/2015.February.20.8

ABSTRACT. The human intestine has a vast variety of microorganisms, and their balance is dependent on several factors. Antibiotics affect microfloral balance and allow naturally opportunistic organisms to multiply. Azithromycin is the most widely used macrolide antibiotic, active against a wide number of pathogens including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. It is currently used in the treatment of cystic fibrosis patients. The use of probiotics has advantages in gastrointestinal conditions, including infectious diarrhea and imbalance due to antibiotic use. In this research, the effect of azithromycin on the intestinal microbiota of Sprague Dawley rats and the role of *Lactobacillus acidophilus* in the restoration of the balance by employing molecular and cultural techniques was investigated. PCR with universal primers targeting the V3 region of the 16S rRNA gene followed by DGGE was used to characterize the overall intestinal microbiota composition. Cultivable fecal bacteria count using microbiological media and semi-quantitative PCR with group-specific primers were also utilized to analyze the effects of antibiotic and probiotic on microflora. We found that the total amount of 16S rRNA gene and fecal aerobic bacterial count was reduced following

azithromycin administration along with elimination of non-pathogenic *Escherichia coli,* but it was restored by the use of the probiotic. The results from PCR with group-specific primers showed that *Bacteroides* sp was present in the control and probiotic groups, but it was nearly eliminated in the antibiotic group. Moreover, semi-quantitative PCR revealed that the numbers of Enterobacteriaceae were nearly the same in the probiotic group and decreased in the antibiotic group, while *Bifidobacterium* was significantly increased in the probiotic group and decreased in the antibiotic group ($P < 0.05$) as compared with that in the control group. Azithromycin-induced dysbiosis can result in prolonged deleterious effects on the host. The present study revealed that the use of lactic acid bacteria particularly *L. acidophilus* helped to restore intestinal microfloral balance.

Key words: Intestinal microflora; Azithromycin; Polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE); *Lactobacillus acidophilus*

INTRODUCTION

In the past decade there has been an increased interest in the study of intestinal microbial balance. The intestinal microbiota plays important roles such as maturation of the immune system (Mazmanian et al., 2005) and intestinal response to epithelial cell injury (Rakoff-Nahoum et al., 2004). The human intestine has vast a variety of microorganisms (Gill et al., 2006), and their balance is dependent on several factors such as gastric acidity, gut motility, bile salts, colonic pH, and competition between microorganisms for nutrients and intestinal binding sites (Marshall, 1999). Their disturbance may cause a variety of diseases or abnormal physiological states.

Drug administration may cause disturbance of the gut microbiota (Clayton et al., 2006). It is believed that antibiotics can affect the intestinal microfloral balance (Sullivan et al., 2001) and allow naturally opportunistic organisms to grow and multiply. Antibiotic therapy produce some side effects in the host, including disturbance of the metabolism and absorption of vitamins, alteration of susceptibility to infections (Levy, 2000), and overgrowth of yeast and/or *Clostridium difficile* (Sullivan et al., 2001).

Azithromycin (deoxo-azamethyl-homo-erythromycin, AZM) is the most widely used synthetic analogue having antimicrobiological properties (Mazzei et al., 1993). It is active against a wide number of pathogens including *Streptococci*, *Staphylococcus aureus*, *Propionibacterium acnes, Listeria monocytogenes*, *Haemophilus influenzae*, *Neisseria gonorrhoeae, Mycobacterium avium*, and *Chlamydia trachomatis*. AZM belongs to the macrolide antibiotics and is currently widely used in the treatment of cystic fibrosis patients (Jaffe et al., 1998). It is currently recommended as chronic therapy for cystic fibrosis patients infected with *Pseudomonas aeruginosa* (Southern et al., 2004; Flume et al., 2007). Long-term administration of AZM helps to reduce or stabilize clinical symptoms of airway inflammation in patients chronically infected with *P. aeruginosa* (Equi et al., 2002; Wolter et al., 2002; Saiman et al., 2003). Some side effects are associated with this macrolide therapy. One study has reported a significant increase in mild adverse effects (wheezing, diarrhea and nausea) in patients receiv-

ing azithromycin (Saiman et al., 2003).

The use of probiotics has advantages in gastrointestinal conditions, including infectious diarrhea and imbalance due to antibiotic use (Sullivan and Nord, 2005). Probiotics are live microorganisms, which when administered in certain numbers exert health benefits in the host. They reduce the incidence of common infectious diseases (Weizman et al., 2005) and produce antipathogenic effects on extraintestinal sites (Peral et al., 2009), such as the vaginal tract (Martinez et al., 2009) and stomach (Park et al., 2007). They play a role in keeping the gut microbial ecosystem stable by restoring normal microflora (Erickson and Hubbard, 2000; Isolauri et al., 2001; Macfarlane and Cummings, 2002) and also take part in the removal of carcinogens, decreasing cholesterol and enhancing the availability of nutrients (Parvez et al., 2006). Currently *Lactobacillus* and *Bifidobacterium* species are attracting great interest as health supplements from both consumers and researchers because of increased awareness of their beneficial roles in health and nutrition (Stanton et al., 2001).

Recent advancements in molecular analysis of bacterial species have provided new tools to discover a highly diverse intestinal ecosystem (Tannock, 2001). Polymerase chain reaction together with denaturing gradient gel electrophoresis (PCR-DGGE) is one of the powerful techniques used for the exploration of the vast variety of microorganisms (Muyzer et al., 1993), and it is used to study their structure and evolution from the gastrointestinal tract (Zoetendal et al., 2002). This technique is useful for analyzing the bacterial diversity profile in different disease conditions (Liu et al., 2010). DGGE is applied to identify sequence variations in a number of genes (Muyzer et al., 1993) and 50% of the sequence variants can be detected in DNA fragments up to 500 bp (Myers et al., 1985). By the attachment of a GC clamp to one end of the DNA fragment, this percentage can be increased to nearly 100% (Sheffield et al., 1989).

This study aimed to demonstrate the effect of AZM on the intestinal microbial ecosystem and the role of *Lactobacillus acidophilus* in intestinal microbial balance by employing molecular and cultural techniques, since there is a correlation between molecular and cultural measurements when individual species are measured. Deviation is due to picking up DNA from non-viable cells by molecular techniques.

MATERIAL AND METHODS

Animals and treatments

Twenty-four male Sprague Dawley rats (weighing 200 ± 2 g) of SPF grade were supplied by the Animal Lab Center of Dalian Medical University. All rats were housed in a standard facility, allowed unrestricted access to water and food and distributed into three groups: control, antibiotic-administered and antibiotic followed by probiotic. AZM was given at 200 mg/kg to the antibiotic and probiotic groups for 7 days. After antibiotic administration, *L. acidophilus* 878 (1 x 10^9 CFU/day) was administered to probiotic group for 7 days. *L. acidophilus* 878 was provided by China Medical Culture Collection (CMCC). Drug and probiotic were given by gavage (0.2 mL). Average body weight of all groups was also calculated to check the effects of antibiotic and probiotic. Luria-Bertani (LB), MacConkey's, eosine methylene blue (EMB), de Mann Rogosa Sharpe (MRS), and sorbitol MacConkey's (SMAC) agar were used for microbial culture techniques.

DNA isolation

Fecal samples were collected from each rat in each group after 7 days following antibiotic course and at the end of the probiotic administration period and stored at -80°C until analysis, and total bacterial DNA was extracted by using E.Z.N.A. Stool DNA kit (OMEGA, USA), according to the manufacturer protocol. DNA integrity was determined visually after electrophoresis on a 1% agarose gel containing ethidium bromide. DNA concentration was measured spectrophotometrically using BioPhotometer plus (NanoVue, USA). Extracted DNA samples were stored at -20°C.

PCR amplification

Primers targeting the V3 region of bacterial 16S rRNA (TaKaRa Biotechnology Co., Ltd., Japan) were used for PCR amplification as shown in Table 1. An automated thermocycler (Thermo USA) was used to perform PCR amplification using a 50-µL reaction mixture containing 2 µL template genomic DNA, 5 µL 10X *Ex*Taq buffer (Mg^{2+} plus), 8 µL dNTP mixture, 5 µL 1% BSA, 1 µL 10 pmol of each primer, 0.5 µL 1.25 U *ExTaq* polymerase (TaKaRa), and sterile Milli-Q water to volume. PCR program was as follows: 94°C for 5 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s; and finally, 72°C for 7 min. The PCR products were evaluated by 2% agarose gel electrophoresis containing ethidium bromide at 100 V for 45 min.

Table 1. Primers targeting V3 region of bacterial 16S rRNA.

F-primer	5'-GC clamp- CCTACGGGAGGCAGCAG-3'
R-primer	5'-ATT ACC GCG GCT GCT GG-3'
GC clamp	CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G

DGGE

DGGE was performed on D-Code™ Universal Mutation Detection System (Bio-Rad, Hercules, CA, USA). Briefly, PCR products were loaded on 8% polyacrylamide gel containing 35-55% gradient of urea and formamide. Electrophoresis was performed, first for 10 min at 200 V, and subsequently for 16 h at 70 V in a 1X TAE buffer at a constant temperature of 60°C. The gels were stained with 0.5 µg/mL ethidium bromide for 60 min, washed with deionized water, and viewed with a Gel Documentation System (Bio-Rad).

DGGE analysis

DGGE profiles were analyzed by the Phoretix 1D software (Phoretix, Newcastle upon Tyne, USA). The similarity score and clustering were determined using the unweighted pair group method with arithmetic averages (UPGMA). The analysis was performed by the SPSS software version 11.5. The Shannon-Weaver diversity index (H') was computed to determine the intestinal microbial diversity by the formula:

$$\text{Shannon-Weaver Index (H')} = -\sum (Pi)(\ln Pi)$$
$$\text{Evenness (E)} = H' / \ln S \qquad \text{(Equation 1)}$$

where *Pi* = proportion of bands/species in the sample and *S* = number of bands.

Sequence analysis

Selected bands were excised from the gel with a sterile scalpel, washed with deionized water and incubated in 35 µL TE buffer at 4°C overnight. The extracted gel mix was heated at 90°C for 10 min, and 4 µL of the solution served as the template for PCR re-amplifying by using the same set of primers and programs described previously but without GC clamp. PCR products were electrophoresed on a 2% agarose gel, purified and then cloned into the PMD19-T Easy vector (TaKaRa), transformed into competent *Escherichia coli* Nova blue cells, and screened for positive plasmid insertions according to the manufacturer protocol. Plasmid DNA was extracted from positive clones, amplified by PCR and sent for sequencing (TaKaRa).

Semi-quantitative PCR

The genus *Bacteroides*, Enterobacteriaceae and *Bifidobacterium* were semi-quantified by using group-specific primers as shown in Table 2. An automated thermocycler (Thermo USA) was used to perform semi-quantitative PCR. Each 25-µL reaction mixture contained 1 µL template, 2.5 µL 10X *Ex*Taq buffer, 4 µL dNTP mixture, 2.5 µL 1%BSA, 1 µL of each primer, and 2.5 U *ExTaq* polymerase (TaKaRa). For Enterobacteriaceae, the amplification program was set as 95°C for 1 min, then 40 cycles of 95°C for 5 s, 55°C for 30 s, and 72°C for 30 s, and finally 72°C for 7 min. For *Bifidobacterium*, the program was set as 95°C for 1 min, then 40 cycles of 95°C for 10 s, 55°C for 15 s, and 72°C for 50 s, and finally 72°C for 7 min. The PCR products were evaluated by 2% agarose gel electrophoresis and semi-quantified by using Gel Pro Analyzer 4.0. PCR with group-specific primers targeting *Bacteroides* sp was also performed to check the presence of *Bacteroides* sp in all three groups as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 20 s, 55°C for 20 s, and 72°C for 30 s. All samples were assayed simultaneously in three parallel reactions.

Table 2. Group specific primers targeting 16S rRNA used in semi-quantitative PCR.

Genus	Primer	Sequence (5'-3')
Enterobacteriaceae	Ecol-F	CATTGACGTTACCCGCAGAAGAAGC
	Ecol-R	CTCTACGAGACTCAAGCTTGC
Bifidobacterium	Bifid-F	CTCCTGGAAACGGGTGG
	Bifid-R	GGTGTTCTTCCCGATATCTACA
Bacteroides	Bact-F	GGTTCTGAGAGGAGGTCCC
	Uni-R	GCTGCCTCCCGTAGGAGT

Bacterial culture of feces and identification of predominant bacteria

Standard plate count was performed for the cultivation of aerobic, enteric, coliform, and lactic acid bacteria from fresh fecal samples. Briefly, 1 g fecal samples from each group was serially diluted and seeded onto LB agar (for aerobes), MacConkey's agar (for Enterobacteriaceae) and MRS agar (for lactic acid bacteria). All plates were incubated for 24-48 h at 37°C. Isolated colonies from MacConkey's agar were streaked onto EMB and SMAC agar for the identification of bacteria. All samples were assayed in duplicate.

RESULTS

DGGE analysis

The intestinal microbial diversity from control, antibiotic and probiotic groups was analyzed to compare the H' of the bands from the DGGE profile. Number of bands significantly decreased ($P < 0.05$) in the antibiotic group as compared to control and probiotic groups as indicated in Table 3.

For sequencing in the DGGE, clear and selected bands were cut from the gel based on quantity analysis. Bands in the same position but in different lanes were excised and sequenced to confirm that they had the same identity. As shown in Figure 1, the control and probiotic groups shared bands (Bands A) at the same position, while there was nearly no band at the corresponding place from the antibiotic group, revealing microbial disturbances. Unfortunately, we could not determine the sequence of selected bands. So we decided to go for PCR using group-specific primers.

A dendrogram was constructed from DGGE profiles using the Phoretix 1D software. Figure 2 illustrates that two clusters were formed. Cluster 1 contained the control and probiotic groups, while in cluster 2, there were two subclusters: subcluster 1 related to the antibiotic group and subcluster 2 related to the probiotic group.

Table 3. Microbial diversity index analysis (means ± SD).

Parameters	Normal group	Antibiotic group	Probiotic group
Number of bands	7.0 ± 2.0	3.50 ± 0.57*	5.50 ± 2.3
Shannon-Weaver index (H')	1.75 ± 0.59	1.07 ± 0.16	1.46 ± 0.74
Evenness (E)	0.89 ± 0.18	0.86 ± 0.07	0.85 ± 0.21

*P value < 0.05 (*t*-test) considered statistically significant.

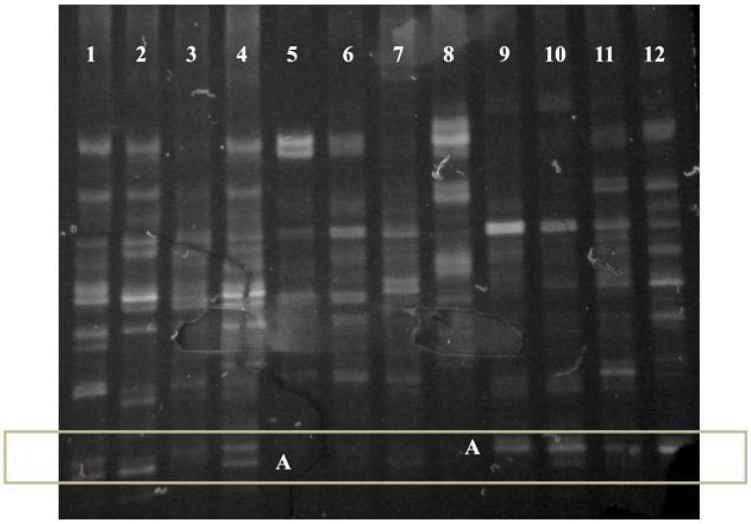

Figure 1. Denaturing gradient gel electrophoresis. *Lanes 1-4* = control group, *lanes 5-8* = antibiotic group, *lanes 9-12* = probiotic group.

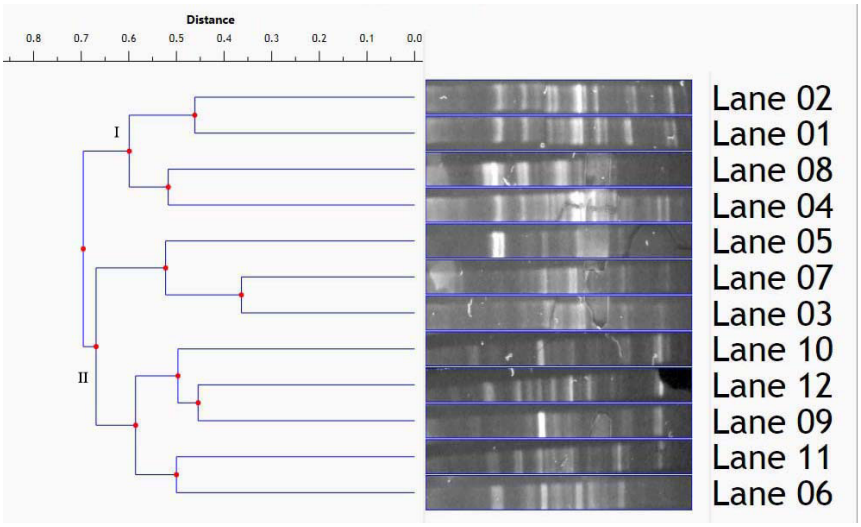

Figure 2. Dendogram of DGGE profiles analyzed by UPGMA. *Lanes 1-4* = control group, *lanes 5-8* = antibiotic group, *lanes 9-12* = probiotic group.

Semi-quantitative PCR

Enterobacteriaceae, *Bifidobacterium* and *Bacteroides* are the most important bacterial groups in the intestine, and they were identified by PCR with group-specific primers (Figures 3-5). Enterobacteriaceae were nearly the same in the probiotic group and decreased in the antibiotic group, while *Bifidobacterium* was significantly increased in the probiotic group and decreased in the antibiotic group ($P < 0.05$) as compared with the control group (Table 4). *Bifidobacterium* to Enterobacteriaceae (B/E) ratio is a sign of microbial colonization resistance in the gut. This ratio was lower in the antibiotic group than that in the control and probiotic groups. As shown in Figure 4, the control and probiotic groups indicated the presence of *Bacteroides* sp in the fecal samples, while AZM produced a deleterious effect on this group of bacteria.

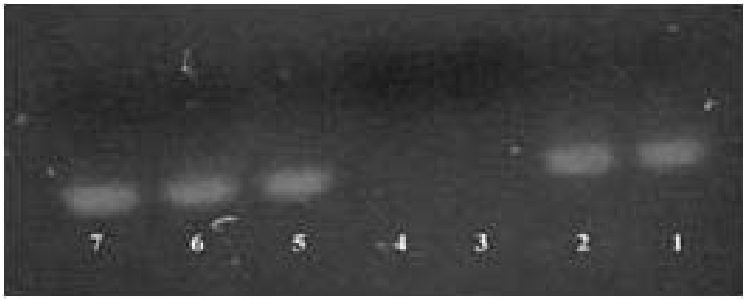

Figure 3. PCR using *Bacteroides* group specific primers. *Lanes 1-2* = control group, *lanes 3-4* = antibiotic group, *lanes 5-7* = probiotic group.

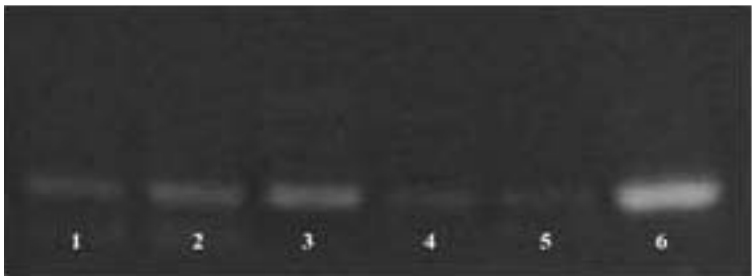

Figure 4. Semi-quantitative PCR using *Bifidobacterium* group specific primers**.** *Lanes 1-3* = probiotic group, *lanes 4-5* = antibiotic group, *lane 6* = control group.

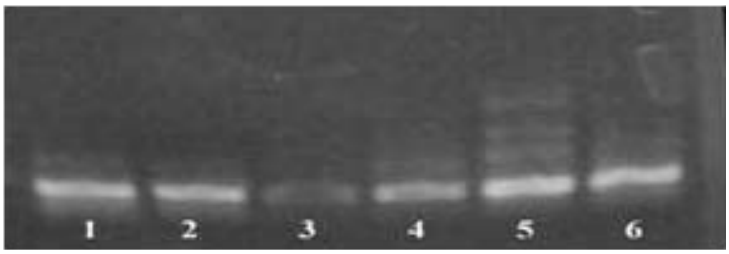

Figure 5. Semi-quantitative PCR using Enterobacteriaceae group specific primers**.** *Lanes 1-2* = probiotic group, *lanes 3-4* = antibiotic group, *lanes 5-6* = control group.

Table 4. Semi-quantitative PCR analysis of bacterial population (means ± SD).

Bacterial genus	Control group	Antibiotic group	Probiotic group
Enterobacteriaceae	6.39 ± 0.65	3.55 ± 0.65	5.50 ± 0.06*
Bifidobacterium	5.79 ± 0.17	1.73 ± 0.03*	2.97 ± 0.03*

*P value < 0.05 (*t*-test) considered statistically significant.

Bacterial culture of feces and identification of predominant bacteria

Figure 6 illustrates the average body weight of rats, and Figure 7 shows the log CFU/g values for fecal aerobic, enteric, coliform, and lactic acid bacterial counts in all three groups. AZM therapy reduced the number of aerobic, enteric, and particularly lactose-fermenting bacteria, as well as body weight, indicating a detrimental impact on the host microbial ecosystem. While probiotic therapy restored intestinal balance and increased the counts of aerobic, enteric and lactic acid bacteria. *E. coli* was dominant in the control and probiotic groups but not in the AZM-administered group according to bacterial culture of fecal samples onto MacConkey's and EMB agar. It produced lactose-fermenting pink and greenish metallic sheen colonies on respective agar plates. The API 20E identification scheme was also utilized to confirm isolated bacteria. Sorbitol-fermenting *E. coli* colonies on SMAC agar confirmed the presence of non-pathogenic strain in the control and probiotic groups. Lactic acid bacterial count was higher in

the probiotic group as compared with that in the control and antibiotic groups indicating the beneficial role of the probiotic in the gut.

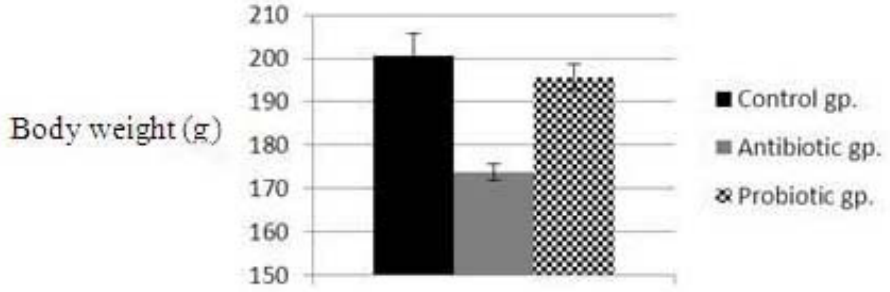

Figure 6. Average body weight of rats during study.

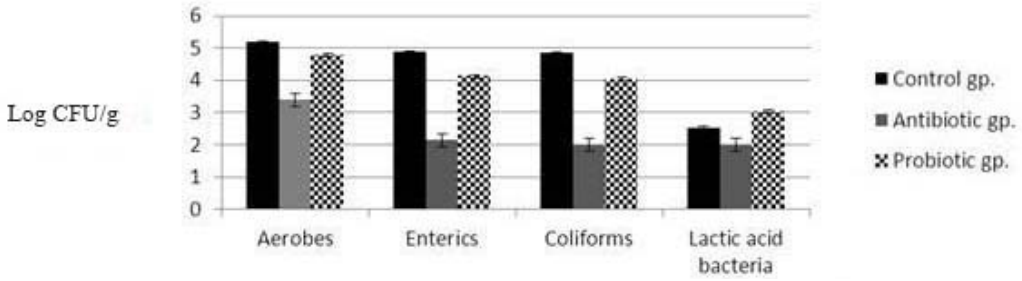

Figure 7. Bacterial culture of feces using different bacteriological media.

DISCUSSION

Culturing of microorganism is the gold standard technique in the field of microbiology, but it fails to represent the complete information of intestinal microflora which comprises many non-cultivable bacteria. In contrast, molecular methods based on the direct analysis of environmental DNA without any culture step have been developed to study microbial communities. Among these methods, PCR-DGGE and PCR-temporal temperature gradient gel electrophoresis (Muyzer et al., 1993) have been widely used for profiling environmental or food-associated microbial ecosystems. Bands excised from the gels and sequenced to get taxonomic identity is the most important feature of DGGE. In the current study, we assessed the potential of *L. acidophilus* as it is widely used in the preparation of probiotic-supplemented food products. Bile acid tolerance makes this bacterium a better candidate to establish its colony in the gut. This bacterium inhibits the colonization of pathogens by increased IgA production (Perdigon et al., 1995). *L. acidophilus* CMCC 878 is an authorized representative strain of this species.

The qualitative composition of intestinal microbiota from each group was analyzed by DGGE with universal primers. DGGE profiles showed the typical characteristics of general bacteria in the intestine. Each band derived possibly from one phylogenetically distinct community; hence, an estimation of species number could be based on the total number of the bands in the profile (Hu et al., 2007). Number of bands and intestinal microbial diversity

of the AZM-administered group were lower than that of the control and probiotic groups. A previous study reported that the administration of antibiotics may cause disturbance of the gut microbiota (Clayton et al., 2006). This allows natural opportunists to grow and increase their population. AZM is widely used for the treatment of bacterial lung infections in patients with cystic fibrosis. The current study demonstrated the possible side effects of this antibiotic on the gut microbial ecosystem.

According to PCR with group-specific primers, *Bacteroides* sp was identified in the control and probiotic groups, but AZM administration nearly eliminated *Bacteroides* sp, indicating antibiotic-induced intestinal imbalance and the restoration of microbial balance by probiotic use. *Bacteroides* sp improves human health by improving the efficiency of nutrition (Bäckhed et al., 2004), increasing the vascularization of the gut mucosa (Stappenbeck et al., 2002), and maintaining the intestinal microbial balance (Hooper et al., 2001; Sears, 2005). A previous study has shown that probiotics improve the ecology of intestinal microbiota (Barrow, 1992). Probiotics have been used to provide health benefits to the host by enhancing the growth of beneficial intestinal bacteria (Rastall et al., 2005). Decreased *Bacteroides* sp with antibiotic administration is associated with side effects in the intestinal tract. The result is in agreement with those obtained in a previous study that indicated a decrease in *Bacteroides* sp in the intestine of macrolide antibiotic-treated mice (Li et al., 2013).

PCR with group-specific primers is considered to be a precise and sensitive method for accurate quantification of individual species. In this study, semi-quantitative PCR also revealed the deleterious effects of AZM on gut ecology and restoration of this balance by probiotic therapy. Enterobacteriaceae were nearly the same in the probiotic-fed group and reduced in antibiotic-administered group as compared with the control group, while AZM significantly decreased and probiotic significantly increased *Bifidobacterium* as compared with the control group. Emmelot and Van der Waaij in 1980 found that oral doses of neomycin and polymyxin B eliminated Enterobacteriaceae in conventional mice. Enterobacteriaceae are the group of Gram-negative enteric bacteria consisting of normal, pathogenic and opportunistic organisms while the *Bifidobacterium* group is considered probiotics and provide protection against different gut infections. They also restore the normal intestinal flora during antibiotic therapy.

To ascertain that the antibiotic induced intestinal dysbiosis, fecal aerobic bacterial count was evaluated, which was lower in the antibiotic group than that in the control and probiotic groups. Lactose-fermenting bacterial colonies on MacConkey's agar were nearly the same in the control and probiotic groups. Predominant bacteria were isolated and identified as a non-pathogenic strain of *E. coli*. But this bacterial strain was absent in the AZM-administered group. *E. coli* is a normal gut inhabitant and provides different benefits to the host, including synthesis of vitamin K. Some strains of *E. coli* (such as *E. coli* O157:H7) are pathogenic, unable to ferment sorbitol and form colorless colonies on SMAC agar.

CONCLUSION

Minimal disruption in the intestinal microbiota by AZM can result in prolonged injurious effects on the ability of the host to resist infections. The present study revealed that the use of lactic acid bacteria, particularly *L. acidophilus*, helps to restore intestinal microbial balance. *L. acidophilus* as a probiotic could be the best candidate to treat gut infections because of their additional ability to tolerate bile acids. Additional studies are necessary to determine the potential of these bacteria to restrict the growth and colonization of opportunistic and

pathogenic bacteria.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

Research supported by the National Basic Research Program of China with a “973” project (#2007CB513006).

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