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A mixture of *Lactobacillus* species isolated from traditional fermented foods promote recovery from antibiotic-induced intestinal disruption in mice

Running headline: JUP-Y4 promotes gut function

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Abstract

Aims: This study evaluated the antibiotic-induced changes in microbial ecology, intestinal dysbiosis and low-grade inflammation; and the combined effect of four different *Lactobacillus* species on recovery of microbiota composition and improvement of gut barrier function in mice.

Methods and Results: Administration of the antibiotic ampicillin for 2 weeks decreased microbial community diversity, induced caecum tumefaction and increased gut permeability in mice. Application of a probiotic cocktail of four *Lactobacillus* species (JUP-Y4) modulated the microbiota community structure and promoted the abundance of potentially

beneficial bacteria such as *Akkermansia*. Ampicillin administration led to a decline in *Bacteroidetes* from 46.6±3.91% to 0.264±0.0362%; the addition of JUP-Y4 restored this to 41.4±2.87%. This probiotic supplementation was more effective than natural restoration, where the levels of *Bacteroidetes* were only restored to 29.3%±2.07%. Interestingly, JUP-Y4 treatment was more effective in the restoration of microbiota in faecal samples than in caecal samples. JUP-Y4 also significantly reduced the levels of D-lactate and endotoxin (lipopolysaccharide, LPS) in the serum of mice, and increased the expression of tight-junction proteins while reducing the production of inflammatory cytokines (TNF-α, IL-6, MCP-1, IFN-γ and IL-1β) in the ileum and the colon of antibiotic-treated mice.

Conclusions: JUP-Y4 not only promoted recovery from antibiotic-induced gut dysbiosis, but also enhanced the function of the gut barrier, reduced inflammation, and lowered levels of circulating endotoxin in mice.

Significance and Impact of Study: Consumption of a mixture of *Lactobacillus* species may encourage faster recovery from antibiotic-induced gut dysbiosis and gut microbiota-related immune disturbance.

Keywords: antibiotics; *Lactobacillus*; intestinal microbiota; immunology; diseases

Introduction

Antibiotics have been used as medical therapies for almost 100 years, with considerable benefits to healthcare and improved survival rates of patients with previously intractable microbial infections. The medical use of antibiotics, especially broad-spectrum ones such as

ampicillin, has increased drastically worldwide. Epidemiological studies show a relationship between the overuse of antibiotic and an increased risk of dysbiosis and inflammatory diseases (Cho *et al*. 2012; Canova *et al*. 2014; Goulet 2015). Furthermore, recent animal studies have confirmed that antibiotic exposure can induce gut microbiome alterations that ultimately affect host metabolic and inflammatory responses associated with inflammatory bowel disease (IBD), irritable bowel syndrome (IBS), necrotizing enterocolitis, and chronic diarrhoea (Fouhy *et al*. 2012; Cox *et al*. 2014; Nobel *et al*. 2015), as well as extra-intestinal disorders such as type 2 diabetes, food allergy, asthma and obesity (Swidsinski *et al*. 2008b; Tannock 2008; De *et al*. 2010).

Several animal studies and human clinical trials have demonstrated the protective effects of dietary supplements comprised of particular strains of probiotic *Lactobacillus*, against gut diseases including antibiotic-associated diarrhoea (AAD) and *Clostridium difficile* infection (CDI) (Mileti *et al*. 2009; Gao *et al*. 2010; Gareau *et al*. 2010; Kale-Pradhan *et al*. 2010; Zhu *et al*. 2011). For example, *L. casei* strain ATCC334, inhibited the progression of colon cancer by mediating apoptosis through the c-Jun *N*-terminal kinase pathway (Konishi *et al*. 2016). Consumption of *L. plantarum* was reported to alleviate the side effects of antibiotic therapy, including the risk of nausea and developing loose or watery stools (Lonnermark *et al*. 2010). Furthermore, administration of *L. rhamnosus* and *L. helveticus* prevented microbial dysbiosis in an IBD mouse model (Emge *et al*. 2016). However, to date there have been few studies on the protective effects achieved using a combination of different lactic acid bacteria against antibiotic-induced alterations in intestinal microbiota and inflammation; this is despite the fact that the benefits of using mixtures of probiotics against other conditions, have been well documented (Jernberg *et al*. 2007; Buffie *et al*. 2012; Brown *et al*. 2017).

It is increasingly evident that many fermented foods contain live bacteria some of which are genetically similar to strains used as probiotics (Marco *et al*. 2017), and there is evidence

that *Lactobacillus* strains isolated from fermented foods can provide health benefits (Rousseaux *et al*. 2007). The aim of this study was to evaluate the effectiveness of a mixture of four *Lactobacillus* species on restoration of gut microbiota and regulation of immunity in mice following treatment with antibiotic.

Material and Methods

Culturing of bacteria

The strains of *Lactobacillus* species used in this experiment were isolated from Chinese traditional fermented foods and included: *L. plantarum* CCFM2602 and *L. casei* CCFM2710 isolated from a yogurt-like dairy product from Inner Mongolia; *L. rhamnosus* CCFM492 obtained from pickled Chinese cabbage; and *L. helveticus* CCFM671 isolated from fermented camels' milk from Xinjiang. All strains were held in long-term storage (at -80°C in 30% sterile glycerol) in the Culture Collection of the Food Microbiology (CCFM) of Jiangnan University. Prior to use the four strains described above were cultured in de Man Rogosa and Sharpe (MRS) broth at 37°C overnight without shaking. The bacterial cultures were centrifuged and the pellets re-suspended in 0.9% saline solution and mixed in equal volumes (the concentration of each strain was adjusted to 0.5×10^9 CFU per 0.2 mL using the plate counting method), to give a final concentration of 2×10^9 CFU per 0.2 mL. The mixture of the four *Lactobacillus* strains was designated as Jiangnan University Probiotic-Ying 4 strains (JUP-Y4).

Animal experiments

The experimental protocol was approved by the Ethics Committee of Jiangnan University, Wuxi, China (JN No. 201501671), and every effort was made to minimise animal suffering. Four-week-old male C57BL/6J mice were purchased from Shanghai Slack Experimental Animal Co., Ltd. (China) and maintained in a designated pathogen-free facility.

After a one-week acclimatisation period, the mice were randomly allocated into two groups (Table 1): the Control group (Cont; n=6) which were fed a regular diet (*ad libitum*) throughout the experimental period and a second group of mice (n=18) that all received ampicillin (500 mg kg^{-1}) via oral gavage twice-daily for 14 days. After this modelling period, the mice in the second group were randomly allocated into three sub-groups (n=6 per group) as follows: the Ampicillin group (Amp) which was sacrificed (at 14 d); the Natural Restoration (NatR) group which received 0.9% saline solution (0.2 mL) via oral gavage for 4 weeks in addition to regular diet; and the Probiotics group (JUP-Y4) which received 2×10^9 CFU (0.2 mL per dose) of JUP-Y4 preparation (in saline) via oral gavage for 4 weeks in addition to regular diet.

Throughout the experiment, the weight of each mouse was recorded twice weekly, and faecal samples were collected after 7, 14 and 28 days. Blood (0.5-1.5 mL) from each mouse was collected into anticoagulant tubes after sacrifice. At the end of the experiment, the remaining mice were sacrificed (both NatR and JUP-Y4 groups, at 14 d+4 weeks) and tissues, including the colon, caecum (and its contents) and ileum, were excised and frozen immediately in liquid nitrogen before further analysis. To collect the faeces, the mice were placed in a sterile room and wiped lightly using 75% alcohol. Then fresh faecal samples were collected and transferred into sterile precooled tubes, and then stored at -80 °C.

DNA extraction and 16S rRNA gene sequencing

Total genomic DNA was extracted from the faecal samples and also the contents of the caecum using the FastDNA Spin Kit for Soil (MP Biomedical, USA) according to the manufacturer's instructions. The V4 region of the bacterial 16S rRNA gene was amplified from each sample with a forward (5'-AYT GGG YDT AAA GNG-3') and reverse (5'-TAC NVG GGT ATC TAA TCC-3') primer set and using the conditions described by Zhao *et al*. (2013). After purification, the PCR products were quantified using a Quant-iT PicoGreen dsDNA Assay Kit (Life Technologies, USA). Libraries were prepared using the TruSeq DNA LT Sample Preparation Kit (Illumina, USA) and analysed on a MiSeq sequencer platform with the MiSeq Reagent Kit (500 cycles-PE, Illumina).

Bioinformatics analysis

Following removal of sequencing primers and barcode trimming, the sets of sequences that shared identity (>97%) were defined as operational taxonomic units (OTUs). These OTUs were assigned using the Ribosomal Database Project (RDP) Naive Bayes classifier (Wang *et al*. 2007). Representative sequences from each cluster identified were aligned using the PyNAST aligner to the green genes core set in QIIME (Caporaso *et al*. 2010), which was also used to calculate alpha and beta diversity and rarefaction curves. The Shannon diversity index was estimated to evaluate the ecological diversity of the microbiota in each sample. Similarity between the microbial communities was determined using a UniFrac analysis (Lozupone and Knight 2005).

Enzyme-linked immunosorbent assays (ELISAs) of blood and tissues

Blood samples were each centrifuged at 3,000 *g* for 10 min. The sera were each mixed with limulus lysate reagent to evaluate endotoxin (lipopolysaccharide, LPS) levels (P D Cani 2009), and an enzymatic assay kit was used to determine D-lactate concentrations (Chen *et al*. 2012). Ceca, ilea and colon tissue samples were each homogenised with a Scientz-50 tissue mill (Lanzhi, Ningbo, China). The resulting homogenates were centrifuged twice at 13,000 *g* for 10 min and the supernatants were transferred to fresh tubes. The levels of tumour necrosis factor alpha (TNF-α), monocyte chemotactic protein 1 (MCP-1), interleukin-6 (IL-6) and interferon-γ (IFN-γ) were determined using specific ELISA kits following the manufacturer's instructions (Nanjing SenBeiJia Biological Technology Co. Ltd. China).

Real-time RT-PCR

RNA was extracted from colon and ileal tissue samples using TRIzol reagent (Ambion, USA) and reverse transcribed into cDNA using a Reverse Transcriptase kit following the manufacturer's instructions (TaKaRa, Japan). Gene expression was determined on a real-time quantitative PCR system (CFX Connect; Bio-Rad), using appropriate primers and iTaq Universal SYBR green Supermix (Bio-Rad, USA). Primer sets utilised for the determination of the gene expression of TNF-α were (forward 5-TAG CCA GGA GGG AGA ACA GA-3 and reverse 5-TTT TCT GGA GGG AGA TGT GG-3), IL-1β (forward 5-TTG AAG AAG AGC CCA TCC TC-3 and reverse 5-CAG CTC ATA TGG GTC CGA C-3), MCP-1 (forward 5-TCA CTG AAG CCA GCT CTC TCT-3 and reverse 5-GTG GGG CGT TAA CTG CAT-3), IFN-γ (forward 5-CGG TGA GAA GAT GTT CCA TGC CAC-3 and reverse 5-TCT CCT TCA GGA CAA TGT CAA ACA-3). Relative quantification of the target gene

was analysed using normalisation with the β-actin gene (forward 5-GGCTGTATTCCCCTCCATCG-3 and reverse 5-CCAGTTGGTAACAATGCCATGT-3) and calculated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Statistical analysis

A one-way analysis of variance (ANOVA) and a Tukey's *post hoc* test were performed, and 95% confidence intervals were used. The analyses were performed using SPSS software (version 16.0; SPSS Inc., USA). Comparisons between different groups were achieved using the two-tailed student's *t* test (**P* < 0.05 and ***P* < 0.01; GraphPad Prism version 5.00). Differences were considered significant at a *P* value < 0.05. Data are presented as means ± standard deviation (SD).

Results

Impact of a mixture of *Lactobacillus* strains on antibiotic-induced intestinal physiology

Compared with the Cont group, antibiotic treatment (Amp group) significantly increased the caecal weight, as indicated by the caecum index (caecum weight/mouse weight) (*P*<0.01, Figure 1). Caecal tumidity was still severe in NatR group where the caecum index was also significantly higher than the Cont group (*P*<0.01). However, in the JUP-Y4 treatment group, the caecum index was reduced to the same level as that in the control group. Gut permeability was indicated by the levels of endotoxin and D-lactate in the serum, which increased in the antibiotic group but decreased at a faster rate following JUP-Y4 treatment compared with the NatR group. (Figure 2). The expression of intestinal epithelial tight-junction proteins,

including ZO-1, occludin and claudin-1, in the ileum and caecum segments was inhibited after antibiotic exposure but restored by the use of JUP-Y4 (Figure 3).

JUP-Y4 restores diversity of the gut microbiota in mice with antibiotic-induced dysbiosis

The calculated Shannon diversity index value (Figure 4A) indicated that antibiotic exposure significantly reduced gut microbial diversity in the Amp mice group. The final diversity index values in the NatR group (4.52±0.20) were lower than both that of the Cont group (5.98±0.38) and the JUP-Y4 group, the latter of which had the highest diversity value of 6.56±0.26.

The abundance matrix obtained from faecal samples was subjected to an unweighted UniFrac PCoA, which clearly separated the Amp group from the Cont group. The JUP-Y4 and NatR groups were distributed in between the other two groups (Figure 4B). Clustering of the group means, which was based on the Mahalanobis distances derived from PCoA scores, confirmed a statistically significant separation between the microbiota from the Cont, Amp, NatR and JUP-Y4 groups with the position of the JUP-Y4 group being much closer to the Cont group than the NatR group (Figure 4C). In summary, treatment with the probiotic, JUP-Y4, shifted the overall structure of the antibiotic-disrupted gut microbiota toward that of the Cont group.

Changes in the key phylotypes of gut microbiota are reversed by JUP-Y4 treatment after antibiotic exposure

Gut microbiota analysis at the phylum level

After antibiotic exposure, the gut microbiota in both the faeces and caecum were largely dominated by *Proteobacteria* (Figure 5). In faeces, the abundances of *Firmicutes* and *Bacteroidetes* were restored following treatment with JUP-Y4 to values more similar to those in the Cont group than in the NatR group (Figure 5A). In caecal samples, the phylum-level structure of the gut microbiota was even more disordered in the NatR group, where *Firmicutes* accounted for 61.5 ± 2.6% compared with 26.8 ± 5.7% in the Cont group (Figure 5B). The treatment with JUP-Y4 helped to decrease the relative levels of *Firmicutes* to 42.2 ± 5.3% which was still higher than the Cont group, reflecting the contribution of the added *Lactobacillus* species to the overall abundance of *Firmicutes*.

Gut microbiota analysis at the genus level

At the genus level, antibiotic treatment significantly increased the abundances of *Staphylococcus*, *Acinetobacter*, *Azorhizophilus*, *Enterococcus* and *Pseudomonas* relative to the Cont group in faeces, whereas the abundances of other genera decreased (Figure 6). In faecal samples, the JUP-Y4 treatment enriched the populations of *Akkermansia*, *Alistipes* and *Porphyromonadaceae* while reduced the abundances of *Desulfovibrionales*, *Anaerotruncus*, *Sporobacter*, *Robinsoniella*, *Oscillibacter*, *Dorea*, *Ruminococcus*, *Clostridia* and *Helicobacter* compared with the NatR group. Accordingly, the levels of these genera in the JUP-Y4 group were closer to the levels in the Cont group than levels in the NatR group. A cluster analysis in a heat map (Figure 6) indicated that treatment with JUP-Y4 restored the

gut microbiota structure and abundance to levels similar to those in the Cont group, whereas the NatR group struggled to reach the same levels following antibiotic treatment.

Results from caecal samples were consistent with results from faecal samples; the abundances of *Acinetobacter*, *Burkholderia*, *Pseudomonas*, *Betaproteobacteria*, *Proteobacteria* and *Enterococcaceae* increased in response to antibiotic use, whereas other genera decreased (Figure 7). However, in the case of the caecal samples, restoration of the microbiota following JUP-Y4 treatment was not different to that in the NatR mice cohort.

Consumption of a mixture of *Lactobacillus* species (JUP-Y4) improves immune function in the intestines of antibiotic-exposed mice

Antibiotic administration influenced intestinal immune cell homeostasis, the protein levels of TNF-α, IL-6, MCP-1 and IFN-γ were significantly higher in the colon and ileal tissues of antibiotic-exposed mice compared with the Cont treatment (Figure 8). Notably, levels of these inflammatory cytokines were reduced by varying degrees in the JUP-Y4 treatment compared with the NatR group.

The expression of specific cytokines at the mRNA level was also determined. The transcript levels of TNF-α, IL-1β, MCP-1 and IFN-γ increased significantly in the colon and ilea of antibiotic-treated mice compared with the Cont group (Figure S1). However, expression of these transcripts was reduced more following treatment with JUP-Y4, than in the NatR group.

Discussion

In this study, we used a mouse model of antibiotic exposure that exhibited significant alterations in intestinal bacterial communities and immune cell homeostasis. We observed that treatment with a cocktail of *Lactobacillus* species (JUP-Y4; containing *L. plantarum, L. casei, L. rhamnosus* and *L. helveticus*) derived from fermented foods restored the gut microbiota community structure, enhanced microbial diversity and reduced the intestinal inflammation caused by antibiotic administration.

It is noteworthy that there was a difference in the gut microbiota from faecal and caecal samples in the present study, as indicated by the more effective restoration of gut microbiota by JUP-Y4 in the former than the latter. Previous studies have also shown differences between the composition of caecal and faecal microbial communities in mice (Pang *et al*. 2012; Gu *et al*. 2013). A report by Tanca *et al.* (2017) indicated that taxon-specific functions, oxalate degradation and glutamate metabolism varied between microbiota communities from caecal and faecal samples. The beneficial modulatory effects of treatment with a cocktail of *Lactobacillus* species following antibiotic exposure were greater in microbiota from faecal samples than microbiota from caecal samples, implying that the probiotic cocktail was more effective in the colon than in the caecum.

A single dose of antibiotic is sufficient to induce gut microbiota dysbiosis (Knoop *et al*. 2016). In susceptible hosts, these antibiotic treatments could trigger intestinal dysbiosis and systemic bacterial translocation (Sartor and Balfour 2008; Shaw *et al*. 2010; Shaw *et al*. 2011). A previous study also showed that commensal bacteria affected the expression of host genes associated with mucosal barrier function, xenobiotic metabolism and nutrient absorption (Hooper *et al*. 2001). These antibiotic-induced subclinical intestinal abnormalities favour the expansion of opportunistic microbes, enhance inflammatory responses and

ultimately lead to the development of chronic diseases, although in this study we did not study the antibiotic treatment in the context of any specific disease. Serum D-lactate and endotoxin levels were measured as markers of gut permeability (Bischoff *et al*. 2014). Several pathologies including gastrointestinal diseases and metabolic syndrome have been linked to alteration of intestinal barrier function and permeability of the gut epithelium, (Sun *et al*. 2001; Camilleri *et al*. 2012). In our study, we have demonstrated that the antibiotic administration to mice increased the levels of serum D-lactate and endotoxin.

The mechanisms accounting for the protective effect of JUP-Y4 against intestinal dysbiosis may be attributed to three aspects: modulation of the gut microbiota community structure; promotion of beneficial bacteria; and activation of the host-adaptive immune system. The composition of the intestinal microbiome fluctuated for two weeks in response to the administration of oral antibiotics. At both phylum and genus level, the structure and abundance of gut microbiota could be restored by the use of our cocktail JUP-Y4. The JUP-Y4 probiotic treatment promoted beneficial bacteria, and these oligosaccharides and short-chain fatty acids producing bacteria can stimulate microbial interactions, the hosts immune responses, and metabolic signalling (Derrien *et al*. 2011).

In the present study, antibiotic-induced changes in the gut microbiota were better restored by treatment with the JUP-Y4 cocktail than via natural recovery. We observed that it was difficult to reduce the abundance of some opportunistic pathogenic bacteria, including *Desulfovibrionales*, *Clostridia* and *Helicobacter* during natural recovery. However, probiotic treatment decreased the abundance of *Desulfovibrionales*, which are associated with Crohn's disease (Chiodini *et al*. 2015) and human infections such as appendicitis and cholecystitis (Swidsinski *et al*. 2008a; Kaakoush *et al*. 2012). In addition, the abundances of some beneficial bacteria, such as *Akkermansia* species, increased to higher levels following probiotic treatment than during natural recovery. *Akkermansia* species are thought to be

biomarkers for intestinal health (Swidsinski *et al*. 2011) and their abundance is inversely correlated with the severity of Crohn's disease, ulcerative colitis (Png *et al*. 2010), appendicitis, obesity (Santacruz *et al*. 2010) and paediatric autism (Wang *et al*. 2011). Moreover, the microorganisms isolated from fermented food could alter the composition and function of the intestinal microbiota, although the significance of these changes and their importance to probiotic efficacy is under debate (van Hylckama Vlieg *et al*. 2011; Kato-Kataoka *et al*. 2016). In this study, we have showed the JUP-Y4 is able to modulate the microbiota more effectively after antibiotic-induced disturbance.

Antibiotic-exposed mice are known to produce higher levels of pro-inflammatory cytokines, including TNF-α, IL-6, MCP-1 and IFN-γ in intestinal tissues than the untreated mice (Suzaki *et al*. 1999; Knoop *et al*. 2016). In this study, we also detected increases in the levels of these four cytokines following ampicillin treatment. The application of JUP-Y4 decreased intestinal inflammation in antibiotic-treated mice with associated reductions in levels of all four cytokines when quantified either at the protein level or by transcription analysis. In another study a strain of *L. paracasei* was also able to downregulate the expression of the IL-6 and TNF-α in neomycin-administered mice which suffered from Crohn's disease (Verdú 2006). Furthermore, some probiotics have been shown to activate anti-inflammatory mechanisms and regulate mucosal immune responses (Gareau *et al*. 2010). For example, *L. casei* DN-114001 attenuated pathogenic activation of the pro-inflammatory signal transduction cascade by inhibiting the NF-κB signal transduction pathway (Tien *et al*. 2006). In other hand, the tight junction integrity could strengthen the intestinal barrier, thus limiting the ability of antigens to enter the body and prevent adverse immune responses. *L. plantarum* MB452 up-regulated the expression of four tight junction proteins (occludin, ZO-1, ZO-2 and cingulin) and some proteasome-associated genes in Caco-2 cells, which indicated protection of intestinal barrier function by this probiotic (Anderson *et al*. 2010). *L. plantarum*

CCFM8610 (Zhai *et al*. 2016) reversed disruption of tight junctions, alleviated inflammation and decreased intestinal permeability in mice with chronic cadmium exposure. Similarly, *L. rhamnosus* GG ameliorated chronic alcoholic liver disease by positively modulating intestinal tight junctions via miR122a regulation (Zhao *et al*. 2015). In such antibiotic-induced mice, mucosal inflammation and various aspects of immune function have been influenced either directly or indirectly by the gut microbial signal. The mixture of *Lactobacillus* species in JUP-Y4 reported in this study improved tight junction integrity of the intestinal epithelial cells.

In summary, antibiotic-induced changes in the development or composition of the gut microbiota and intestinal barrier integrity can disturb the relationship between the microbiota and the host immune system, eventually leading to altered immune responses, which may underlie multiple host inflammatory disorders. The ability of JUP-Y4 to modulate antibiotic-induced gut dysbiosis is reflected in the restoration of the gut microbiota and intestinal barrier, suggesting the potential for this probiotic mixture to restore gut homeostasis much faster than by natural restoration after the cessation of antibiotic treatment. Although the translation of these outcomes to clinical practice needs further clinical research, probiotic interventions intended to selectively promote bacteria could become an important dietary strategy to enhance recovery from dysbiosis and related immune disturbances associated with antibiotic use.

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Conflict of interest

No conflict of interest declared.

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Table

Table 1 Animal experimental protocol

Group	Acclimatisation for 7 days	Antibiotic model assay for 14 days	*Lactobacillus* therapy assay for 28 days
Control (Cont)	Regular diet	0.9% saline solution	0.9% saline solution
Ampicillin (Amp)	Regular diet	Ampicillin (500 mg kg^{-1})	Sacrificed
Natural Restoration (NatR)	Regular diet	Ampicillin (500 mg kg^{-1})	0.9% saline solution
Mixture of Probiotics (JUP-Y4)	Regular diet	Ampicillin (500 mg kg^{-1})	*Lactobacillus* species

A cocktail of *Lactobacillus* species was administered to mice via gavage in 0.9% saline solution. Equal volumes of 0.9% saline solution (0.2 mL) were administered to the non-treatment group (Cont).

Figure Legends

Figure 1 Administration of a mixture of *Lactobacillus* species (JUP-Y4) attenuates antibiotic-induced caecum tumefaction.

The caecum index was calculated as: caecum weight (g)/mouse weight (g). Control (Cont) indicates the Non-treatment group, Ampicillin (Amp) indicates oral gavage with ampicillin (500 mg kg^{-1}) for 2 weeks, Natural restoration (NatR) indicates oral gavage with 0.9% saline solution for 4 weeks after ampicillin gavage, and JUP-Y4 indicates oral gavage with a *Lactobacillus* cocktail (*L. plantarum* CCFM2602, *L. casei* CCFM2710, *L. rhamnosus* CCFM492 and *L. helveticus* CCFM671) for 4 weeks after gavage with ampicillin. Data are

shown as means ± standard deviations. Two-tailed student's *t* test determined statistical significance, $^{**}P < 0.01$ and ns means no significant difference. n = 6 mice per group

Figure 2 Administration of a mixture of *Lactobacillus* species (JUP-Y4) mitigates antibiotic-induced gut barrier dysfunction.

Levels of D-lactate (A) and endotoxin (B) in the serum of mice. All group names correspond to those described in the legend of Figure 1. Data are shown as means ± standard deviations. The values of groups with different letters differed significantly in an ANOVA, followed by Tukey's *post hoc* test ($P < 0.05$). n = 6 mice per group

Figure 3 Effects of administration of a mixture of *Lactobacillus* species (JUP-Y4) on the expression of mRNAs corresponding to the tight-junction proteins ZO-1 (A), occludin (B) and claudin-1 (C) in ileal and caecal tissues of mice.

Gene expression levels are expressed as values relative to the control group. All group names correspond to those described in the legend of Figure 1, ( ) Control group (Cont), ( ) Ampicillin group (Amp), ( ) Natural Restoration group (NatR), ( ) Mixed Probiotics group (JUP-Y4). Data are shown as means ± standard deviations. The values of groups with different letters differed significantly in an ANOVA, followed by Tukey's *post hoc* test ($P < 0.05$)

Figure 4 Administration of a mixture of *Lactobacillus* species (JUP-Y4) alters the diversity and structure of antibiotic-disturbed gut microbiota.

(A) Shannon index of microbiota diversity from faecal samples of mice from different groups (α-diversity), * $P<0.05$, compared with control, ** $P<0.01$, compared with control, using Independent Samples *t* test. (B) Unweighted UniFrac PCoA plot, characterised by an abundance of diverse bacteria in samples. The first principal component (PC1) and second

principal component (PC2) respectively explained 28.27% and 10.92% of the variance in the unweighted UniFrac metrics. Each point represents the faecal microbiota from a single mouse. (■) Control group (Cont), (●) Ampicillin group (Amp), (▼) Natural Restoration group (NatR), (▲) Mixed Probiotics group (JUP-Y4). (C) Clustering of the group means based on the Mahalanobis distances derived from the PCA. All of group names correspond to those described in the legend of Figure 1. n = 6 mice per group

Figure 5 Relative abundances of the main phyla after antibiotic exposure and restoration following administration of a mixture of *Lactobacillus* species (JUP-Y4).

The abundances of microbial phyla in faecal samples (A) and caecum content samples from different groups (B). Data are expressed as means ± standard deviations. n = 6 mice per group. All of group names correspond to those described in the legend of Figure 1, the different colours of legends represent different phyla, (▬) *Actinobacteria*, (▬) *Bacteroidetes*, (▬) *Deferribacteres*, (▬) *Firmicutes*, (▬) *Other*, (▬) *Proteobacteria*, (▬) *TM7*, (▬) *Verrucomicrobia*

Figure 6 Heatmap analysis of the abundances of 40 microbial taxa in faecal samples from mice that were administered a mixture of *Lactobacillus* species (JUP-Y4) after antibiotic exposure.

The abundance of a taxon is expressed by colour as indicated. All of group names correspond to those described in the legend of Figure 1

Figure 7 Heatmap analysis of the abundances of 40 taxa in caecal content samples from mice that were administered a mixture of *Lactobacillus* species (JUP-Y4) after antibiotic exposure.

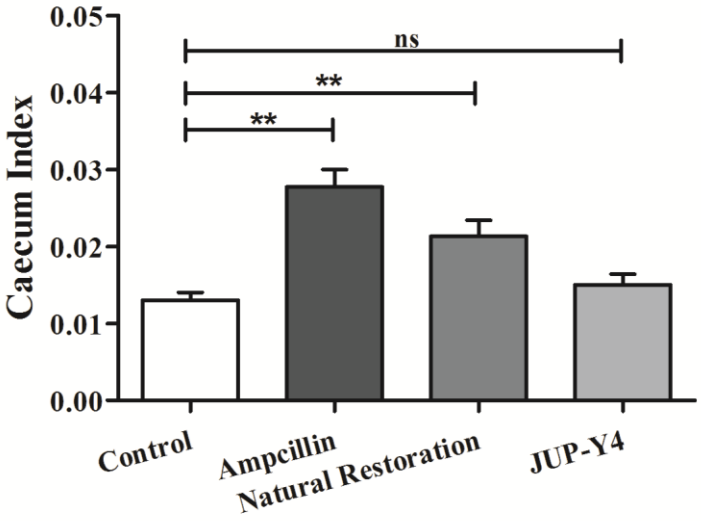
The abundance of a taxon is expressed by colour as indicated. All of group names correspond to those described in the legend of Figure 1

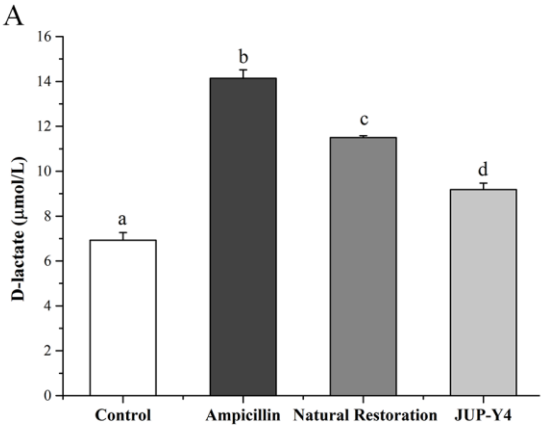
Figure 8 Production of cytokines in the antibiotic-induced ileum, caecum and colon of mice that were administered a mixture of *Lactobacillus* species (JUP-Y4).

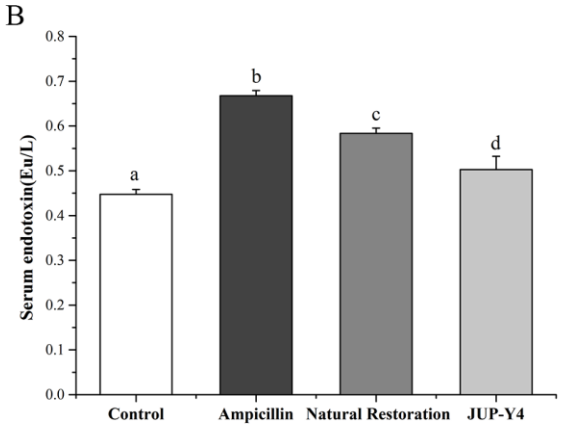
The levels of cytokines measured were TNF-α (A), IL-6 (B), MCP-1 (C) and IFN-γ (D). All of group names correspond to those described in the legend of Figure 1, (□) Control group, (■ Ampicillin group, (▒ Natural Restoration group, (▒ JUP-Y4 treatment group. Data are shown as means ± standard deviations. The values of groups with different letters differed significantly in an ANOVA, followed by Tukey's *post hoc* test (P <0.05). n = 6 mice per group

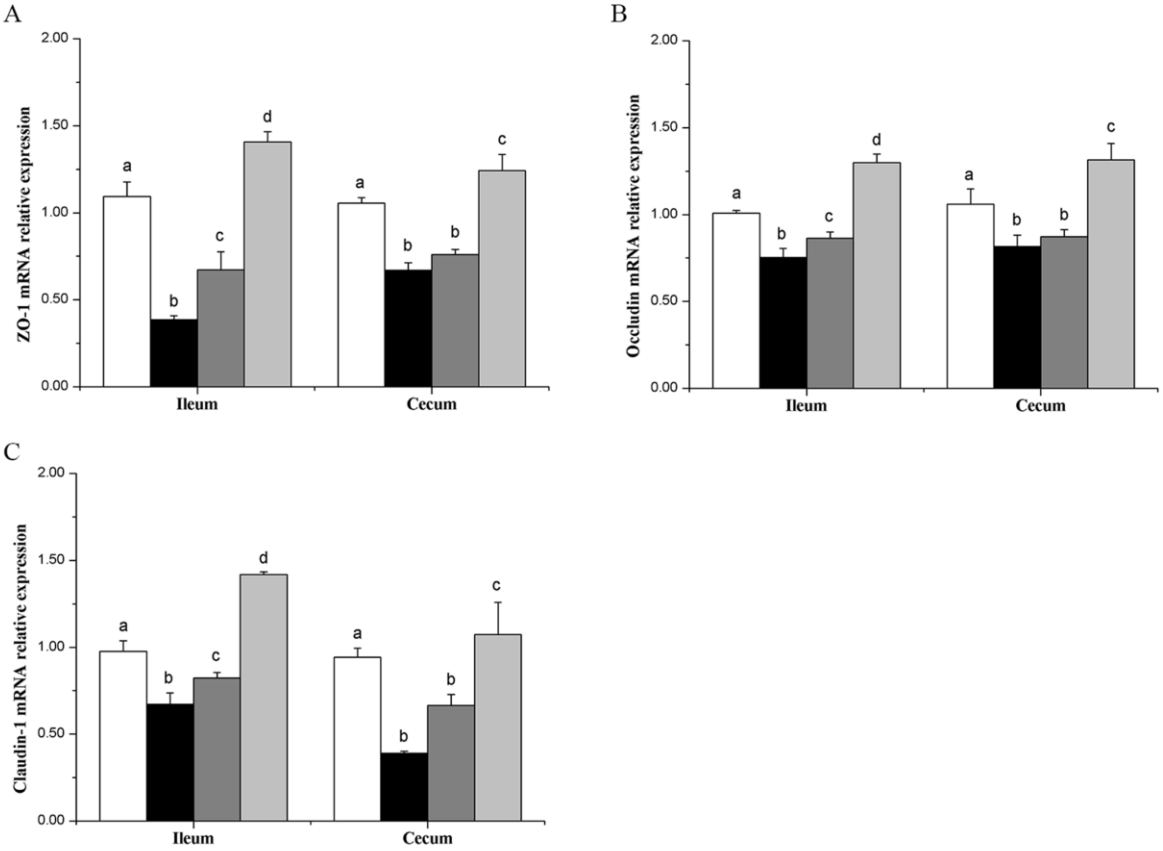
Figure S1 Effects of a mixture of *Lactobacillus* species on the expression of genes encoding inflammatory factors in the ilea and colons of ampicillin-exposed mice.

Levels of mRNA of TNF-α (A), IL-1β (B), MCP-1 (C) and IFN-γ (D) in the intestines of mice. Gene expression levels are expressed as values relative to the control group. All of the group names correspond to those described in the legend of Figure 1, (□) Control group (Cont), (■ Ampicillin group (Amp), (▒ Natural Restoration group (NatR), (▒ Mixed Probiotics group (JUP-Y4). Data are shown as means ± standard deviations. The values of groups with different letters differed significantly in an ANOVA, followed by Tukey's post hoc test (P < 0.05). n = 6 mice per group









A

Group	Shannon Diversity Index
Control	5.98 ± 0.38
Ampicillin	1.01**± 0.30
Natural Restoration	4.52** ± 0.20
JUP-Y4	6.56* ± 0.26

* P < 0.05, ** P<0.01