The divergent restoration effects of *Lactobacillus* strains in antibiotic-induced dysbiosis

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Abstract

To evaluate functions of *Lactobacillus* strains, isolated from fermented food, in restoration of ampicillin-induced disruption based on mucosal barrier, gut microbial community and metabolome analyses, three *Lactobacillus* strains, *L. plantarum* CGMCC12436 (LacP), *L. casei* CGMCC 12435 (LacC) and *L. rhamnosus* strain GG (LacG) were individually administered to ampicillin-pretreated mice. All three strains significantly restored concentrations of endotoxin and diamine oxidase to control levels. Linear discriminate analysis based on 16S rRNA sequencing of faecal bacteria revealed that the restoration of microbial communities by *Lactobacillus* strains was more effective than natural restoration. Correlation analysis between microbiota and metabolites indicated that, the higher level of acetate in LacC group was positively correlated with increased relative abundance of *Citrobacter*, *Bifidobacterium* and *S24-7*. Furthermore, LacC down-regulated the expression of NF-κB p65 and modulated the ampicillin-induced inflammatory responses. The LacC strain could particularly attenuate ampicillin-induced disruption by optimisation of microbial taxa and enhancement of acetate and butyrate production.

Keywords: gut dysbiosis; microbial ecology; metabolites; immunity; functional food

1 Introduction

The intestinal distal gastrointestinal tract is colonised by trillions of microbes, and this extensive microbial community comprising approximately 10^{12} CFU in the colon, influences gastrointestinal physiology, metabolism, immunity and susceptibility to disease infection (Lozupone, Stombaugh, Gordon, Jansson, & Knight, 2012). The disruption of the "balanced" microbiota, referred to as dysbiosis, can drive functional and inflammatory changes in animals and humans (Petersen & Round, 2014). In the last decade, a large number of studies have announced significant alterations in the structure of microbial communities in patients and mice models of dysbiosis-related diseases such as inflammatory bowel diseases, diabetes, obesity, asthma and autism (Becattini, Taur, & Pamer, 2016; Karlsson et al., 2013). Alterations to the intestinal microbiota during a critical developmental period also had lasting metabolic consequences (Abrahamsson et al., 2014; Parracho, Bingham, Gibson, & McCartney, 2005). Antibiotic-induced dysbiosis was linked to changes in colonic microbial ecology; ampicillin has been shown to decrease the number of *Bifidobacteria*, increase *Candida*, and reduce the production of short-chain fatty acids (SCFA) (Hawrelak & Myers, 2004; Mangin, Suau, Gotteland, Brunser, & Pochart, 2010). It was suggested that, due to the inability to differentiate between commensals and pathogens, antibiotics perturbed the microbiota structure and the evolutionary relationship between the immune system and the host symbionts (Aguilera, Cerda-Cuellar, & Martinez, 2015; Buffie et al., 2012; Cho et al., 2012).

Antibiotic use could induce dysregulation of metabolic activities conducted by colonic microbiota (Lee & Hase, 2014). Microbial metabolites are capable of manipulating the metabolic integrity of intestinal epithelial cells and causing intestinal immune responses

(Arpaia & Rudensky, 2014). Some bacterial fermentation products, particularly SCFA, were considered as key signs of colonic health, but the specific relationship between the microbial community and metabolites under the status of antibiotic-induced dysbiosis is poorly understood. The intestinal immune response should be balanced between the tolerance for unexpected immune molecules and pathogen-induced inflammation in the host cells and commensal bacteria, with the balance developed by mucus production and antimicrobial peptides to establish a barrier between host tissue and the microbes (Johansson, Larsson, & Hansson, 2011; Vaishnava et al., 2011). Toll-like receptors (TLRs) are a group of pattern recognition receptors, that play a crucial role in mucosal immune response (de Kivit, Tobin, Forsyth, Keshavarzian, & Landay, 2014) and can recognise microbe-associated molecular patterns (MAMPs). As one of these MAMPs, lipopolysaccharide (LPS) can be increased through antibiotic use and cause an innate immune response via TLR4 in intestinal epithelial cells (Collado-Romero, Arce, Ramirez-Boo, Carvajal, & Garrido, 2010). It is well accepted that LPS initiates a signalling pathway through TLR4 to activate NF-κB, and leads to inflammation and removal of infection by pro-inflammatory cytokines including TNF-α and IL-1β (Doyle & O'Neill, 2006). One strain of *Lactobacillus* has been proven to down-regulate the expression of pro-inflammatory cytokines in a TLR4-dependent NF-κB signal pathway (Shimazu et al., 2012).

A number of *Lactobacillus* strains, tested as cocktails or individual strains, were shown to alleviate gut-related disorders or metabolic diseases such as obesity, diabetes and non-alcoholic fatty liver disease in mice (Aronsson et al., 2010; Simon et al., 2015; Wang et al., 2015; Xu, Wan, Fang, Lu, & Cai, 2012; Yoo et al., 2013). Importantly, different probiotic

strains were proven to have remarkably different abilities to modulate gut metabolism and immune response (Fåk & Bäckhed, 2012; Million et al., 2012; Yin, Yu, Fu, Liu, & Lu, 2010). It remains unclear whether specific *Lactobacillus* strains can be administered to regulate the alteration of the gut microbiota and subsequently promote the production of beneficial metabolites. Moreover, it has been reported that a *Lactobacillus* mixture played a beneficial role in the immune response in mice through balancing anti- or pro-inflammatory cytokines (Taranu, Marin, Pistol, Motiu, & Pelinescu, 2015).

In the present study, a model of dysbiosis was constructed by exposing healthy adult mice to a therapeutic-dose of ampicillin, leading to perturbed gut microbiota. The ampicillin-induced dysbiosis was confirmed by determining the caecal index, endotoxin levels and the expression of tight-junction proteins. The different *Lactobacillus* species (*L. plantarum*, *L. casei* and *L. rhamnosus*) were compared to investigate their impact on the restoration of microbiota in an ampicillin-induced dysbiotic state. The metabolic composition of faecal water was measured using ^{1}H NMR, and the relationship between the microbial community and SCFA was correlated to characterise the function of *Lactobacillus* strains tested. Our findings provide a novel insight that the administration of different *Lactobacillus* strains after ampicillin-induced dysbiosis exhibited distinct effects in modulation of the microbial community, metabolites and the immune system.

2 Materials and Methods

2.1 Culturing of bacteria

L. plantarum CGMCC12436 and *L. casei* CGMCC12435 were isolated from a traditional

fermented cream from Inner Mongolia, China, and the *L. rhamnosus* strain GG (LGG) (ATCC 533103) was purchased from ATCC. All strains were held in long-term storage (-80°C in 30% sterile glycerol) in the Culture Collection of the Food Microbiology (CCFM) of Jiangnan University. These three strains were cultured in de Man Rogosa and Sharpe (MRS) broth at 37°C overnight. The bacterial cultures were centrifuged and the pellets were resuspended in 0.9% saline solution to give a final concentration of ~1×10^9 CFU per 0.2 mL respectively.

2.2 Experimental animals and ethics statement

The experiments were carried out with four-week-old male C57BL/6 mice obtained from Slack Experimental Animal Co., LTD (Shanghai, China). Mice were caged in groups of two or three. Throughout the experiments, distilled water and standard laboratory chow were provided *ad libitum*. Light conditions (12 h light/dark cycle), temperature (21°C) and air humidity were tightly controlled. The experimental procedures and numbers of animals used were approved by the Ethics Committee of Jiangnan University in China (JN No. 20160608-20160831/47). The experiments were designed in order that both the number of animals used and their suffering were minimised.

2.3 Experimental groups, timelines and treatment

Mice were allocated to one of six groups (Table 1) and acclimatised for 1 week. Ampicillin (Sigma, USA) was dissolved in 0.9% saline solution (500 mg kg^{-1}), and mice were treated via oral gavage with ampicillin for 2 weeks, except for the control group (Con, n=20-24) which were treated with 0.9% saline solution for 2 weeks. After 2 weeks, the group of dysbiotic mice (Amp) and half the group of control mice were sacrificed, whilst the remaining control mice received saline via oral gavage for another 4 weeks; the natural

restoration group (NaR) pre-treated with ampicillin received saline via oral gavage for 4 weeks; dysbiotic mice were treated via oral gavage with *L. rhamnosus* GG (LacG group), *L. plantarum* CGMCC12436 (LacP group), or *L. casei* CGMCC12435 (LacC group) respectively, for 4 weeks.

2.4 Quantification of gut permeability in the serum

The concentrations of endotoxin (ET) and diamine oxidase (DAO) were determined in serum samples using Enzyme-Linked Immunosorbent Assay (ELISA) kits (SenBeiJia Biological Technology Co. Ltd., Nanjing, China). Gut permeability was also measured using 4000 Da fluorescent dextran–FITC (DX-4000–FITC) (Sigma-Aldrich, USA) as described in the supplementary methods.

2.5 Microbiome analysis

Total genomic DNA was extracted from thawed faecal samples with the FastDNA Spin Kit for Soil (MP Biomedical, USA) according to the manufacturer's instructions. Subsequently, the 16S rRNA gene was amplified by PCR with a forward (5'- CCT AYG GGR BGC ASC AG -3') and reverse (5'- GGA CTA CNN GGG TAT CTA AT -3') barcoded primer set, targeting the V3-V4 region. PCR products were gel-purified and the amplicon DNA concentration was determined, the libraries were prepared using the TruSeq DNA LT Sample Preparation Kit (Illumina, USA) and sequenced on the Illumina MiSeq platform (500 cycles paired-end). The detailed methods of QIIME and Linear Discriminate Analysis (LDA) with Effect Size (LEfSe) analyses are described in the supplementary methods.

2.6 Faecal metabolomic analysis

Metabolites in the faecal samples of mice were analysed by ^{1}H NMR analysis at

Quadram Institute Bioscience (United Kingdom). Faecal water was prepared by mixing ~100 mg of frozen faecal samples with 12 times the volume of a phosphate buffer that consisted of 1.9 mM Na_2HPO_4, 8.1 mM NaH_2PO_4, 150 mM NaCl, and 1 mM TSP (sodium 3-(trimethylsilyl)-propionate-d_4)) in D_2O (deuterium oxide). After homogenising thoroughly with a pellet pestle motor (Kimble Kontes, USA), samples were centrifuged at 14,000 *g* for 10 min at 4℃. High resolution ^1H NMR spectra were recorded using a Bruker AV 600 spectrometer (Bruker, Rheinstetten, Germany). The spectra were analysed as previously described and further plotting is listed in the supplementary methods (Le Gall et al., 2011).

2.7 Correlation between taxa abundance and metabolites

To evaluate the relationship between the most abundant taxa and observed metabolites, a correlation test was performed, and associated *p*-values were adjusted for multiple testing in R. The *physeq* package was used to obtain taxa abundance and meta data information, the *ggplot2* package was used to plot figures, "Pearson" was selected as the method to characterise correlation coefficient, and "adjust meta variables (panel on the correlation plot)" was selected to adjust *p*-values for multiple comparisons using Benjamin and Hochberg.

2.8 ELISA analysis of inflammatory cytokines in the colon

Colon tissues (100 mg) were homogenised in 900 μL PBS using a Scientz-50 tissue mill (Lanzhi, Ningbo, China), centrifuged at 13,000 *g* for 10 min at 4℃, and the supernatants were transferred into sterile tubes. The levels of secretory immunoglobulin A (sIgA), nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), monocyte chemotactic protein 1 (MCP-1), interferon-γ (IFN-γ), regenerating islet derived protein 3 gamma (Reg3γ) and interleukin 1β（IL-1β） were determined using respective ELISA kits following the

manufacturer's protocols (Nanjing SenBeiJia Biological Technology Co. Ltd. China).

2.9 Immunofluorescence

Colon tissue sections from different groups of mice were fixed by 4% paraformaldehyde in 0.1 M phosphate buffer. The tissues were excised, post fixed for 3 h in the perfusion fixative, cryoprotected for 72 h in 30% sucrose in 0.1 M phosphate buffer. Transverse sections (20 μm) were cut and the slides were incubated with primary NF-κB p65 rabbit polyclonal antibody (Thermo Fisher Scientific, USA) (1:200 dilution) for 2 h at 37°C. Following incubation, sections were washed in PBS and incubated with secondary goat anti-rabbit antibody (1:100 in PBS) (Jackson Immuno Research, USA) for 1 h in a dark room. The sections were incubated with 4,6-Diamidino-2-phenylindole dihydrochlorid (DAPI) (Sigma Aldrich), washed twice, and visualised under a Leica fluorescence microscope. All micrographs were taken with identical exposure times and focused on the centre of each well.

2.10 Statistical analysis

Data were represented as mean ± standard error of the mean (SEM). The gut permeability data were analysed using one-way ANOVA, followed by Dunnett's multiple comparisons test in GraphPad Prism 5. The mRNA expression of tight-junction proteins, metabolites and levels of cytokines were calculated using one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism 5. $P < 0.05$ was considered statistically significant.

2.11 Data deposition

The raw sequence data have been deposited in the NCBI Sequence Read Archive (Accession no. SRP146081 and BioProject Accession no. PRJNA471394).

3 Results

3.1 Effects of *Lactobacillus* strains on intestinal injury in antibiotic-induced dysbiotic mice

Gut permeability and the caecal index were investigated as indicators of alteration to intestinal integrity. The Amp mice showed significantly increased levels of DX-4000-FITC in serum and caecal index, which indicated an increase in gut permeability and an enlargement of the caecum after antibiotic use compared to the Con group ($p < 0.0001$, Fig. 1A and B). The endotoxin concentration was increased ($p < 0.0001$, Fig. 1C) and the enzyme activity of serum DAO was decreased in the Amp group compared to the Con group ($p < 0.01$, Fig. 1D), demonstrating the damage of mucosal integrity after antibiotic exposure. After four-weeks restoration, no difference was observed among these four intestinal integrity biomarkers in the natural restoration group (NaR) compared to controls. Treatment with LacC markedly reduced the level of DX-4000-FITC and caecal index, and proved to be more effective than LacG and LacP (Fig. 1A and B). In addition, all groups of *Lactobacillus* treatment modified the levels of endotoxin and DAO towards the control level (Fig. 1C and D).

3.2 Effects of *Lactobacillus* strains on intestinal barrier disruption in antibiotic-induced dysbiotic mice

To evaluate potential effects of *Lactobacillus* treatment on paracellular communications in the intestines, we measured mRNA expression of the tight-junction proteins ZO-1, Occludin and Claudin-1 in the colon and ileum (Fig. 2). In the colon, the expression of ZO-1 and Occludin were not statistically affected by ampicillin-induced dysbiosis, however, expression of Claudin-1 was significantly decreased ($p < 0.001$) after antibiotic use. In the ileum, levels of all tight-junction proteins were remarkably reduced by ampicillin use ($p <$

0.01, $p < 0.0001$ and $p < 0.001$ respectively). LacC treatment enhanced the expression of ZO-1 and Occludin to the control level in the ileum which was more effective than LacG ($p < 0.05$ and $p < 0.001$ respectively), while LacP promoted the expression of Claudin-1 in the ileum to the control level. These data demonstrated that, with regards to the expression of tight-junction proteins, the disruption by ampicillin and the enhancement by *Lactobacillus* strains mainly occurred in the ileum, and the LacC strain showed a promotion of ZO-1 and Occludin while the LacP strain positively affected Claudin-1 levels.

3.3 Ampicillin-induced colonic microbiome disruption can be largely restored by *Lactobacillus* administration

Microbial species richness was indicated by the inverse of the classical Simpson diversity (Invsimpson Index) as shown in Fig. 3A, which was calculated to eliminate the sampling effects of the Shannon Index. The bacterial diversity was greatly reduced by ampicillin treatment ($p < 0.001$), and three groups of the administration of single *Lactobacillus* strain enhanced the level of alpha-diversity to that of the Con group, which was higher than that observed in the NaR group ($p < 0.001$). Principal coordinate analysis (PCoA) based on Phyloseq's Weighted Unifrac showed that ampicillin-treated mice had a considerably altered (clustering by distance) microbial community compared to that of the Con mice (Fig. 3B and C). The profiles of the microbial composition of the Con group and *Lactobacillus*-restored groups were clustered more closely to each other than that of naturally-restored mice (Fig. 3B and C), indicating that tested *Lactobacillus* strains could restore the antibiotic-treated microbiota structure towards the normal profile.

Ampicillin treatment led to an increase in *Proteobacteria* and a severe depletion of

Bacteroidetes and *Verrucomicrobia* at the phylum level (Fig. 3D). Natural restoration did not lead to the recovery of *Bacteroidetes*, while LacC, LacG and LacP groups had an increase in the abundance of *Bacteroidetes* towards, or above (LacC), that of the Con group. Although the microbiota was not completely restored, the abundance of *Proteobacteria* was improved to the control level by LacC and LacP strains, but not by the LacG strain.

We further examined compositional changes of the microbiome at the family or genus level using high-throughput amplicon sequencing. Following antibiotic cessation, taxa including *Enterobacteriaceae*, *Klebsiella* and *Enterococcus* were dramatically increased in Amp mice compared to the Con mice, whereas *Akkermansia*, *Lachnospiraceae* and *Dorea* were absent in the Amp group (Fig. S1). The cluster of the NaR grouped closer to the Amp group rather than the Con group, indicating that natural restoration for four weeks after ampicillin disruption was not effective in recovering the microbial community to the normal level (Fig. S2). An evaluation of relative abundance (Fig. S4) indicated that *Coprobacillus*, *S24-7* and *Eubacterium* were enhanced in the LacC, LacG and LacP groups after ampicillin-induced depletion, whilst there was no observed restoration in the NaR group. Meanwhile, the relative abundance of *Klebsiella* and *Enterococcus* was reduced by LacC, LacG and LacP strains after their increase due to ampicillin exposure. These data indicated that each of the three *Lactobacillus* strains altered the community structure of the gut microbiota in a manner different from the ampicillin-induced dysbiotic state towards that of the Con group. Furthermore, the LEfSe analysis revealed that the number of significantly altered taxa was lower in LacC (15) than NaR (25) (Fig. 4A and B). The significantly altered taxa after administration of LacG or LacP was 15 and 13 respectively (Fig. S3), demonstrating

that restoration of the microbiota by *Lactobacillus* strains enabled a stronger shift towards the initial state than observed by natural restoration.

3.4 The LacC strain restored the faecal metabolome following alteration by ampicillin-induced dysbiosis

The impact of ampicillin-induced microbial community alterations on the faecal metabolite profiles was evaluated by ^{1}H NMR spectroscopy, which revealed that ampicillin exposure altered the faecal metabolome composition as can be seen by the clear separation between the Amp group and the Con group (Fig. 5A). The LacC group clustered closer to the Con group than the LacG or LacP groups, with NaR positioned between the Con and Amp groups, indicating that LacC was more effective in restoration of the faecal metabolome than natural recovery or LacG and LacP strains. Some metabolites, such as amino acids and carbohydrates produced by the colonic microbiota, were found to have decreased in the faecal samples of ampicillin-treated mice (Fig. S5). In particular, the SCFA reflected by acetate, propionate and butyrate were significantly decreased or diminished ($p < 0.0001$) by the antibiotic use (Fig. 5B).

The administration of *Lactobacillus* strains could partly restore the SCFA profile, however, strain-dependent differences were observed. Acetate, propionate and butyrate levels were found to be significantly enhanced in the LacC group compared to the Amp group ($p < 0.0001$, Fig. 5B), increasing towards the same levels as seen in the Con group. The LacG group increased the levels of acetate and propionate to levels higher than the NaR group, but had less effect in the recovery of butyrate levels. However, SCFA levels were not significantly altered in the LacP group. In addition, the increases in lactate levels observed following

ampicillin administration were significantly reduced by all three *Lactobacillus* groups and the NaR group ($p < 0.0001$, Fig. 5B). Taken together, these results showed that the LacC strain mitigated the decrease of SCFA in ampicillin-treated mice and was more effective in the restoring the whole metabolic profile.

3.5 The relationship between altered faecal metabolome and changes in the microbial community

To assess microbiota-metabolome associations, the functional correlation between alterations in microbial taxa and metabolites was assessed using Pearson's correlation coefficient method, based on 60 bacterial taxa and 8 acids that potentially contributed to the observed differences between the Amp and Con groups (Fig. 5C). Acetate, butyrate and succinate were highly correlated with alterations in proportions of bacterial taxa following ampicillin use. The decrease in acetate within the Amp group was positively correlated with the decreased relative abundance of *Staphylococcus*, *Streptophyta* and *Planococcaceae* ($p < 0.01$). The decreased level of butyrate was positively correlated with changes in the relative abundance of *Rikenellaceae*, *Helicobacter*, *Lactobacillus*, *Lactobacillaceae*, and *Epulopiscium* whilst was negatively correlated with *Morganella*, *Enterobacter* and *Enterobacteriaceae* in the Amp group. In particular, the enhanced level of acetate in the LacC group was positively correlated with an increase in the relative abundance of *Citrobacter*, *Bifidobacterium*, *Eubacterium*, *S24-7*, *Rikenellaceae* and *Clostridiaceae* ($p < 0.05$, Fig. 5D), and was negatively correlated with members of *Ruminococcus*, *Ruminococcaceae*, *Dorea*, *Coprococcus*, *Bilophila*, *Lachnospiraceae* and *Desulfovibrionaceae* ($p < 0.05$, Fig. 5D). However, no significant correlations were observed between microbial taxa and butyrate in

either the LacC group or LacP (Fig. 5D, E).

3.6 *Lactobacillus* strains modulated NF-κB signalling and colonic inflammation caused by ampicillin-induced dysbiosis

The serum endotoxin in ampicillin-treated mice was significantly increased ($p < 0.0001$, Fig. 1C) compared to the Con mice, indicating that LPS-stimulated TLR4 and NF-κB expression might be induced following ampicillin exposure. NF-κB levels determined by ELISA (Fig. 6B) were significantly increased ($p < 0.0001$) after ampicillin treatment, and reduced to control levels by the administration of LacC or LacP. Consistent with the ELISA results of NF-κB, ampicillin treatment increased the level of NF-κB p65 (Fig. 6A), and all the tested *Lactobacillus* strains could partly reduce the level of p65 compared to that achieved by natural restoration. Also, due to the positive feedback activation of the NF-κB signal pathway, the level of IL-1β was significantly increased by ampicillin treatment ($p < 0.0001$), and reduced by LacC to the level of the Con group (Fig. 6B). Therefore, the LacC strain seemed to protect against ampicillin-induced inflammatory responses through the regulation of NF-κB expression in the colon.

To investigate whether disturbance of the microbiota induced alterations in mouse intestinal immune homeostasis, the local expression levels of several inflammatory mediators were measured in the colon. In addition, the effects of different *Lactobacillus* strains against inflammatory mediator expression were also evaluated. Levels of secretory IgA (sIgA) were significantly increased in antibiotic-induced mice ($p < 0.0001$) whilst LacG and LacC reduced the levels of sIgA to that of the Con group. In ampicillin-treated mice, the expression of Reg3γ was increased in the colon, and decreased in the LacG group compared to the NaR

group ($p < 0.0001$, Fig. 6B).

The inflammatory cytokines including IFN-γ and MCP-1 were also examined to evaluate colonic inflammation. Levels of IFN-γ and MCP-1 were considerably increased in the Amp group compared to the Con group ($p < 0.0001$). IFN-γ was modulated towards the level observed in the Con group by the LacC group but none of the *Lactobacillus* groups could reverse MCP-1 to the same level as the Con group. Taken together, inflammatory mediators were activated in the colon following treatment with ampicillin, and the LacC strain was more effective in modulating the levels of sIgA, Reg3γ and IFN-γ whilst LacG administration reduced the level of Reg3γ.

4 Discussion

By generating ampicillin-induced dysbiosis in the gut microbiota and related metabolome, we could analyse the chronic effects of ampicillin on the host and investigate restoration strategies (Scott et al., 2016). Previous studies applied mouse models to explore the relationship between antibiotic treatment and subsequent changes in host physiology and gut microbiota composition (Aguilera et al., 2015; Bech-Nielsen et al., 2012; Mahana et al., 2016; van Opstal et al., 2016). Although some informative alterations can be measured by examining the microbiota or the metabolome alone, correlation analyses were employed in this study to assess the relationship between the microbiota and metabolome and provide functional information. Furthermore, by comparing the effects of the three *Lactobacillus* strains tested here, the present study revealed substantial evidence of associations between microbial taxa and metabolites in the ampicillin-induced and *Lactobacillus*-restored mice.

The Amp mice exhibited a decreased microbial diversity. A reduction in microbial diversity is associated with multiple gastrointestinal diseases (Guarner, 2015; Le Chatelier et al., 2013). Several microbial taxa including *Enterobacteriaceae*, *Clostridia*, *Erwinia*, and *Klebsiella* were found to be enhanced in the NaR mice (after ampicillin treatment). Among these changed taxa, the high frequency of *Klebsiella* and *Enterobacteriaceae* has been observed in patients with gastroenteritis and irritable bowel syndrome (Ganji et al., 2016). In the Amp mice, the increased relative abundance of *Enterobacteriaceae* and decreased *Lachnospiraceae* were strongly associated with gut inflammation. The reduction of *Klebsiella* and *Enterococcus* and the enhancement of *Coprobacillus*, *Bacteroidales* and *Eubacterium* in all three *Lactobacillus* treatment groups suggested that *Lactobacillus* administration contributed to the promotion of a stable gut microbial community. In particular, the relative abundance of *S24-7* (family) was enhanced in the LacC group and these butyrate-producing bacteria are beneficial to intestinal epithelial cell health (Villanueva-Millan, Perez-Matute, & Oteo, 2015). The decrease of the SCFA was not only associated with perturbation of the microbial communities but also related to the integrity of mucosal barrier in the Amp group. SCFA are reported to be associated with maintenance of intestinal barrier function; acetate was shown to be crucial in the inhibition of enteropathogens (Fukuda et al., 2011), and butyrate production could lead to increased mucin production and promotion of tight-junction integrity (Jung, Park, Jeon, & Han, 2015). The majority of gut butyrate-producers including *Faecalibacterium*, *Eubacterium*, and *Roseburia* utilise pathway in which butyryl-CoA is converted to butyrate (Louis, Young, Holtrop, & Flint, 2010). In this study, following an initial decrease due to ampicillin exposure, the abundance of *Eubacterium* improved in each

of the *Lactobacillus* administration groups, which may explain the increase of butyrate observed in these groups.

The correlation between microbial communities and faecal metabolites provided crucial evidence on the function of bacteria, with alterations at the microbiota-level leading to a changed metabolome, which could potentially influence gut disease (Claesson et al., 2012; Tremaroli & Bäckhed, 2012). We observed a positive correlation between the relative abundance of *S24-7* (belong to *Bacteroidales*) and levels of faecal acetate and butyrate. It was previously reported that consumption of common bean and chickpea reduced colitis-associated inflammation, whilst promoting the levels of SCFA and *S24-7* (Power et al., 2016). After the administration of the LacC strain, acetate levels were significantly enhanced, and this increase was positively correlated with the relative abundance of *Citrobacter*, *Bifidobacterium*, *Eubacterium* and *Rikenellaceae* in this study. Among these acetate-related gut bacteria, the selective increase of *Bifidobacterium* has been shown to protect against enteropathogenic infection through the enhancement of acetate (Fukuda et al., 2011); in an elderly health study, genera including *Eubacterium* were associated with long-stay subjects and acetate production, as well as gene counts for acetate-producing enzymes, were significantly higher in long-stay subjects (Claesson et al., 2012). Interestingly, the increased abundance of *Rikenellaceae*, which was associated with the increase of acetate in the LacC group, had previously been associated with potentially beneficial effects on gut health. The decrease in *Rikenellaceae* observed in the Amp and NaR groups had recently been observed in the intestinal dysbiosis of spondyloarthritis (Lin et al., 2014). These analyses of the functional relationship between microbial taxa and SCFA further specified the possible

mechanism of functional restoration of LacC strain in the metabolome and microbiome.

Exposure to ampicillin increases levels of endotoxin, activation of the NF-κB pathway and upregulation of the pro-inflammatory cytokines, which are in accordance with the observations during LPS exposure (Lawrence, 2009). The SCFA-driven inhibition of histone deacetylases (HDACs) tends to improve an anti-inflammatory cell phenotype that is critical for maintaining immune homeostasis. A number of studies identified the inactivation of NF-κB and downregulation of pro-inflammatory cytokines by SCFA (Usami et al., 2008; Vinolo et al., 2011). Also, LPS-induced expression of pro-inflammatory cytokines was attenuated by *Lactobacillus jensenii* through the down-regulation of the TLR4-dependent NF-κB pathway and the mitogen-activated protein kinase (MAPK) in a porcine intestinal epithelial cell line (Shimazu et al., 2012). In this study, *L. casei* (LacC), *L. plantarum* (LacP) and *L. rhamnosus* GG (LacG) were administrated to ampicillin-treated mice and the levels of NF-κB and IL-1β were found to be reduced in the colon by LacC. Collectively, these results confirm that LacC treatment could modulate the host immune responses through the TLR4-dependent NF-κB pathway in ampicillin-induced mice, although as yet it is unclear whether this was through the action of SCFA or a beneficial modulation of the microbiome structure.

5 Conclusions

We demonstrated that the three strains of different *Lactobacillus* species are able to individually restore antibiotic-induced alterations of the microbiome and the metabolic profile in mice. We found significant differential changes in colonic microbial taxa and clades by

LEfSe analysis through the comparison of three *Lactobacillus*-restored groups with a natural restoration group. Correlation analysis of associations between the microbiota and metabolome indicated that LacC strain can promote specific bacterial taxa and SCFA to attenuate ampicillin-induced dysbiosis, suggesting strain-specific effects on functionally relevant gut disease. Furthermore, we confirmed that LacC reduced inflammatory activity by regulating the NF-κB pathway and pro-inflammatory cytokines in ampicillin-induced dysbiotic mice. These strain-specific *Lactobacillus* treatments offer the potential to mediate antibiotic-associated gastrointestinal disturbances and diseases, although clinical trials would be necessary to confirm their potential beneficial effects in humans.

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Conflict of Interest

All authors declared no conflict of interest.

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Figure Legends

Fig. 1. Effects of *Lactobacillus* strains administration after antibiotic disturbance on gut injury. A. Level of DX-4000-FITC measured by ELISA in serum samples from different mice groups. B. Caecal indexes weighted and calculated through caecum tissues from different groups. C. Endotoxin and D. DAO concentrations measured by ELISA in serum samples from all mice groups.

Mice of the Ampicillin group (Amp) were treated with ampicillin for 2 weeks, mice of the control group (Con) were treated with saline for 2 weeks or 6 weeks; the natural restoration group (NaR) pre-treated with ampicillin (2 weeks) received saline for another 4 weeks, and dysbiotic mice, through pre-treatment with ampicillin for 2 weeks were treated with either *Lactobacillus rhamnosus* GG (LacG group), *L. plantarum* CGMCC12436 (LacP group), or *L. casei* CGMCC12435 (LacC group) respectively, for 4 weeks. These group names were used throughout this research paper. One-way ANOVA followed by Dunnett's multiple comparisons test was used to determine statistical significance, and the *p* value was obtained by comparing the mean of each group with the mean of the Con group, ns means no significant difference, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. Values are represented as mean ± SEM of 5-8 mice per group.

Fig. 2. The alleviation of intestinal barrier disruption by *Lactobacillus* treatment. Real-time PCR analysis of ZO-1, Occludin and Claudin-1 mRNA expression normalised to β-actin in the colons and ileums of mice from Con, Amp, NaR, LacG, LacC and LacP groups. One-way ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance, and the *p* value was obtained by comparing the mean of each group

with the mean of the Con group, ns means no significant difference, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Values are represented as mean ± SEM of 5-8 mice per group.

Fig. 3. The restorative effect of *Lactobacillus* strains following ampicillin-induced dysbiosis via microbial diversity and bacterial abundance measurements.

A. Microbial α-diversity of faecal samples indicated by Shannon Index, Simpson Index and Invsimpson Index. The richness of each sample was estimated in a phyloseq data object, and one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine statistical significance, with the *p* value obtained by comparing the mean of each group with the mean of the Con group, ns means no significant difference, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

B. Principal coordinates analysis (PCoA) and C. clustering of distance based on Phyloseq's Weighted Unifrac to present differences in microbial community structure between samples from Amp (●), Con (●), LacC (●), LacG (●), LacP (●) and NaR (●) groups. The first principal component (PC1) and second principal component (PC2) explained 51.6% and 16.2% of the variance in the Weighted UniFrac metrics, respectively. Each point represents the faecal microbiome of a single sample.

D. The abundance of bacterial phyla in faecal samples from Amp, Con, LacC, LacG, LacP and NaR mice groups. The rectangles representing *Bacteroidetes*, *Firmicutes*, *Proteobacteria*, *Tenericutes* and *Verrucomicrobia* were stacked in order and the aggregate height of the stacked bar reflects the quantitative information.

Fig. 4. Comparison of differential microbial communities from mice groups using LEfSe.

A. Circular cladogram of biologically and statistically consistent differences in faecal

microbial clades between NaR and Con groups. In the panel, each circle's diameter was proportional to the taxon's abundance, green = taxon significantly enriched in NaR, red = taxon significantly enriched in Con and yellow = non-significant. The cladogram simultaneously highlights specific genera/families and high-level trends.

B. Histogram of LDA scores computed for taxa that have differential abundance in NaR and Con groups of mice. The magnitude of the LEfSe scores represents the degree of difference in relative abundance between features in the NaR and Con groups.

C. Circular cladogram of biologically and statistically consistent differences in faecal microbial clades between LacC and Con groups. Green = taxon significantly enriched in LacC, red = taxon significantly enriched in Con and yellow = non-significant. The cladogram simultaneously highlights specific genera/families and high-level trends.

D. Histogram of LDA scores computed for taxa that have differential abundance in LacC and Con groups of mice. The magnitude of the LEfSe scores represents the degree of consistent difference in relative abundance between features in the LacC and Con groups.

Fig. 5. The restoration effect of selected *Lactobacillus* strains after ampicillin-induced dysbiosis based on metabolomic analyses and the correlation between taxa abundance and metabolite alterations.

A. Principal component plot analysis of faecal metabolites altered in the Amp (●), Con (●), LacC (●), LacG (●), LacP (●) and NaR (●) groups. The first principal component (PC1) and second principal component (PC2) explained 36.8% and 17.6% of the variance respectively. Each point represents the faecal metabolites in a single sample.

B. Distribution of intensities for the selected four acids based on the metabolomic analysis.

Each point represents the faecal metabolites in a single sample, and the mean and SEM were indicated by horizontal lines. One-way ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance, and the *p* value was obtained by comparing the mean of each group with the mean of the Con group, ns means no significant difference, $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

C. Correlation analysis of microbial taxa and acids, as quantified using NMR intensity, between the Con and Amp groups. Top 60 microbial taxa and metabolites in the correlation matrix were filtered prior to the Pearson's correlation coefficient method being applied. Significant microbiota-metabolite correlations were determined based on adjusted *p*-values for multiple comparisons using Benjamin and Hochberg, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Positive correlations between taxa and acids were presented in red, and negative correlations were presented in blue.

Correlation plot of top 27 taxa associated with acetate or butyrate in the Con and LacC groups (D), or Con and LacP groups (E). Pearson's correlation coefficient method was applied and significant microbiota-metabolite correlations were determined based on adjusted *p*-values for multiple comparisons, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Positive correlations between taxa and acids were presented in red, and negative correlations were presented in blue.

Fig. 6. Effects of ampicillin and subsequent administration of *Lactobacillus* strains on immune and host-bacterial interaction markers.

A. Immunofluorescence analysis of NF-κB p65 in the colons of mice from Con, Amp, NaR, LacG, LacC and LacP groups. The expression of p65 (green light) and DAPI (blue light) was observed under the same exposure times (Representative images, n =4/group).

B. ELISA analysis of levels of sIgA, NF-κB, Reg-3γ, IFN-γ, MCP-1 and IL-10 in the colons of mice from Con, Amp, NaR, LacG, LacC and LacP groups. One-way ANOVA followed by Tukey's multiple comparisons test was used to determine statistical significance, and the *p* value was obtained by comparing the mean of each group with the mean of the Con group, ns means no significant difference, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Values are represented as mean ± SEM of 5-8 mice per group.

Table 1. Animal experimental design

Groups	Antibiotic treatment (2 weeks)	*Lactobacillus* therapy assay (4 weeks)
Control (Con)	0.9% saline solution	0.9% saline solution or sacrifice
Ampicillin (Amp)	Ampicillin (500 mg kg^{-1})	Sacrifice
Natural Restoration (NaR)	Ampicillin (500 mg kg^{-1})	0.9% saline solution
L. rhamnosus GG (LacG)	Ampicillin (500 mg kg^{-1})	LacG 10^9 CFU in 0.9% saline
L. casei CGMCC 12435 (LacC)	Ampicillin (500 mg kg^{-1})	LacC 10^9 CFU in 0.9% saline
L. plantarum CGMCC12436 (LacP)	Ampicillin (500 mg kg^{-1})	LacP 10^9 CFU in 0.9% saline

Figure 1

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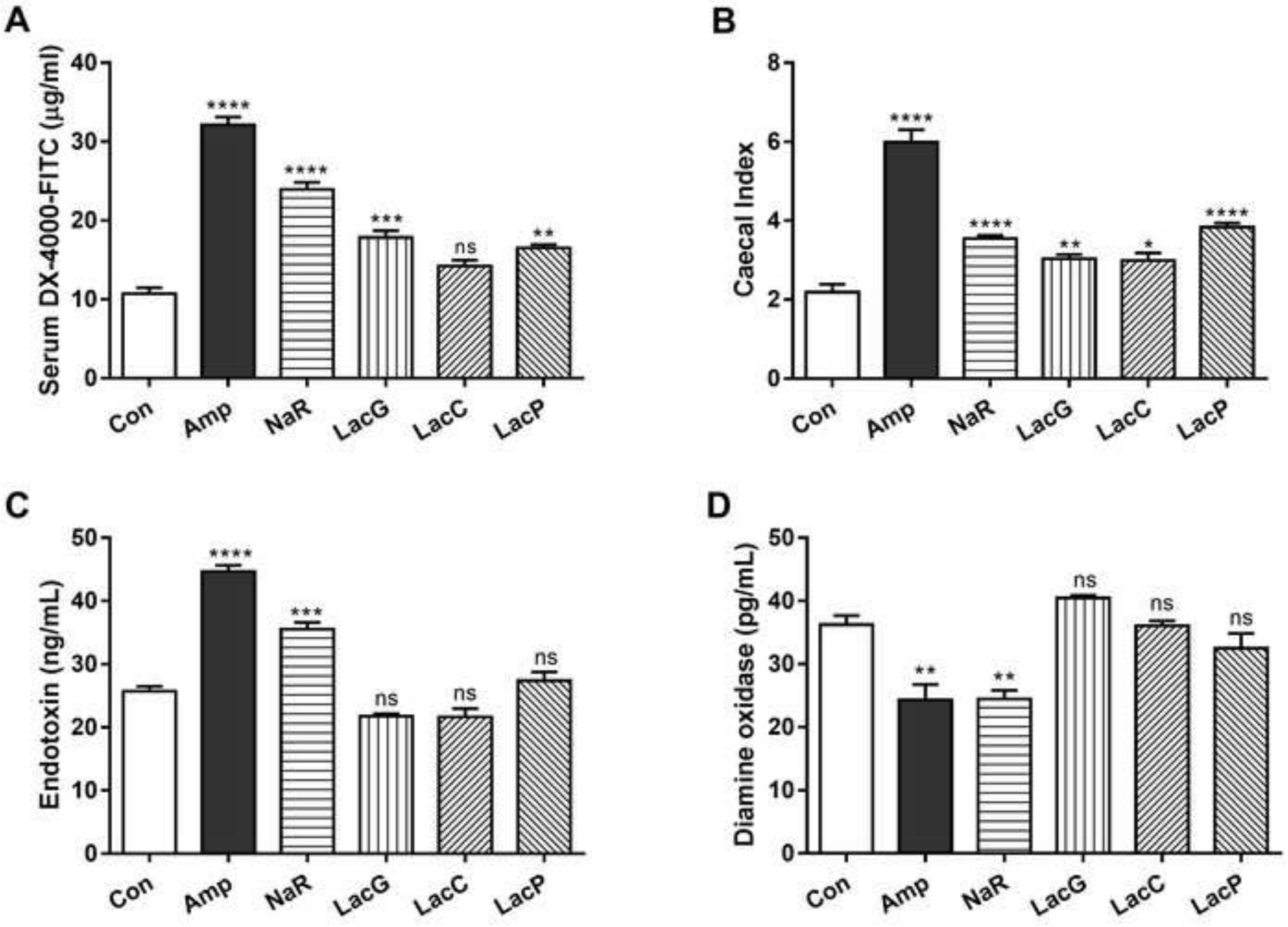

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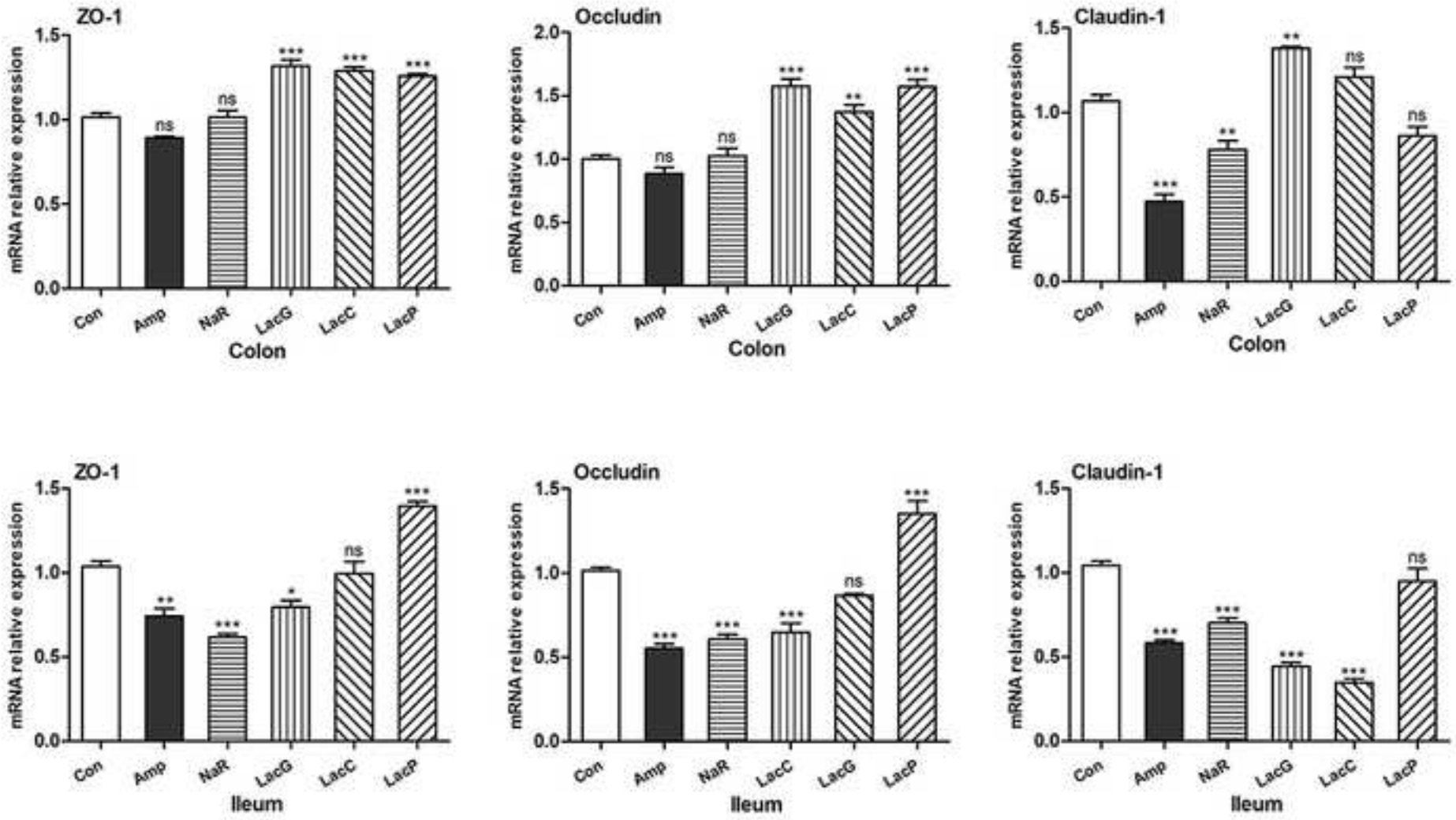

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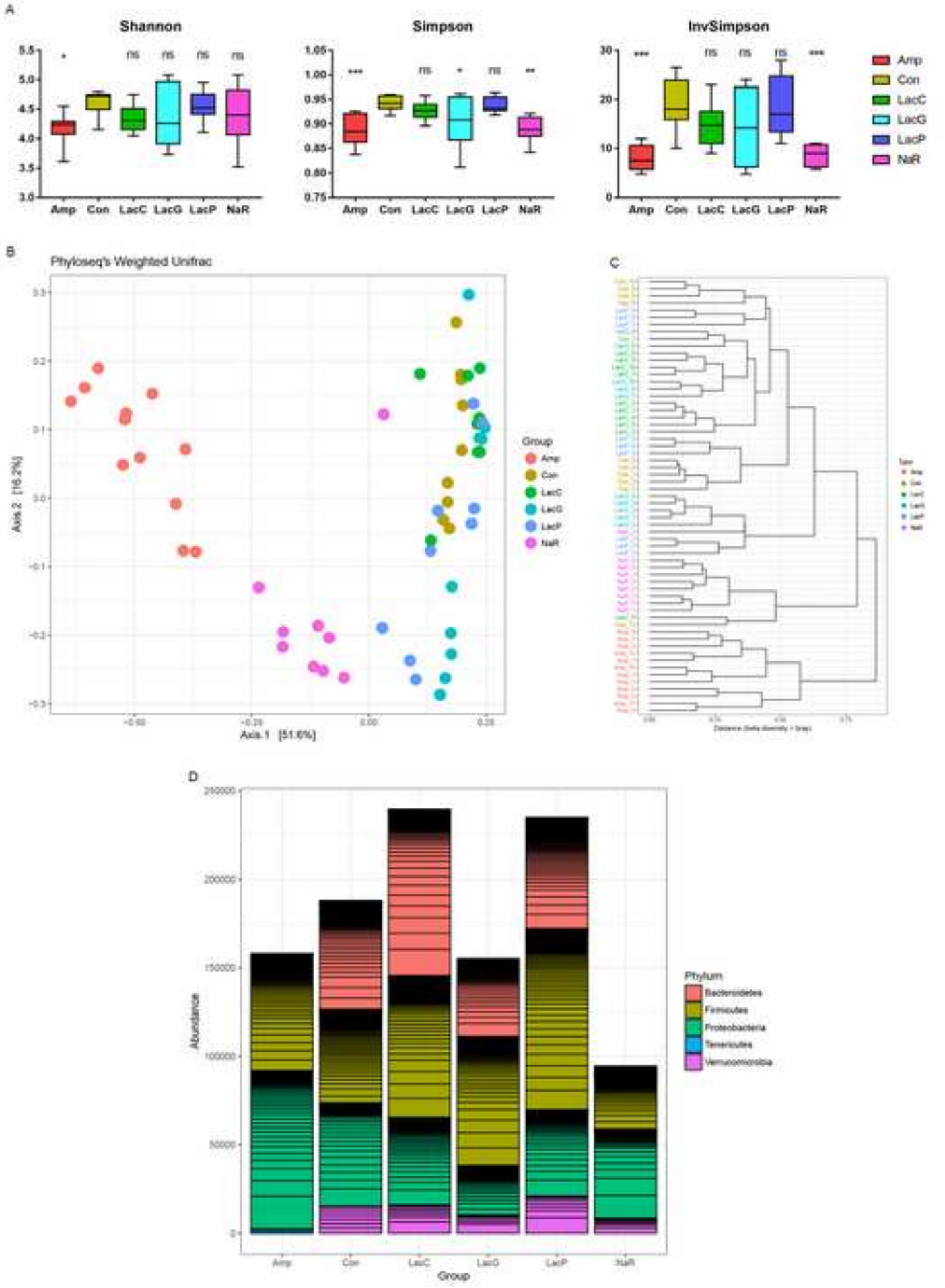

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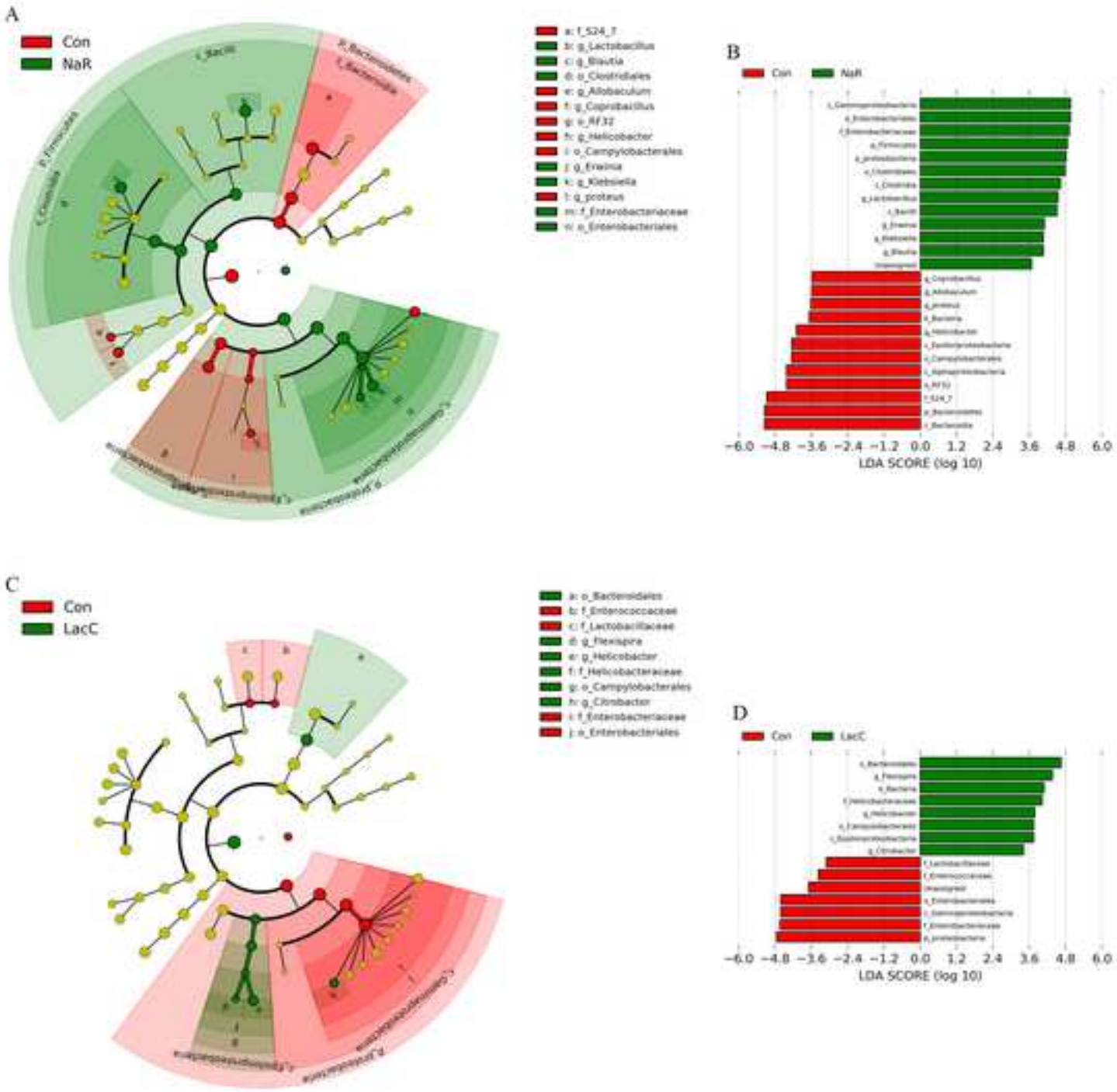

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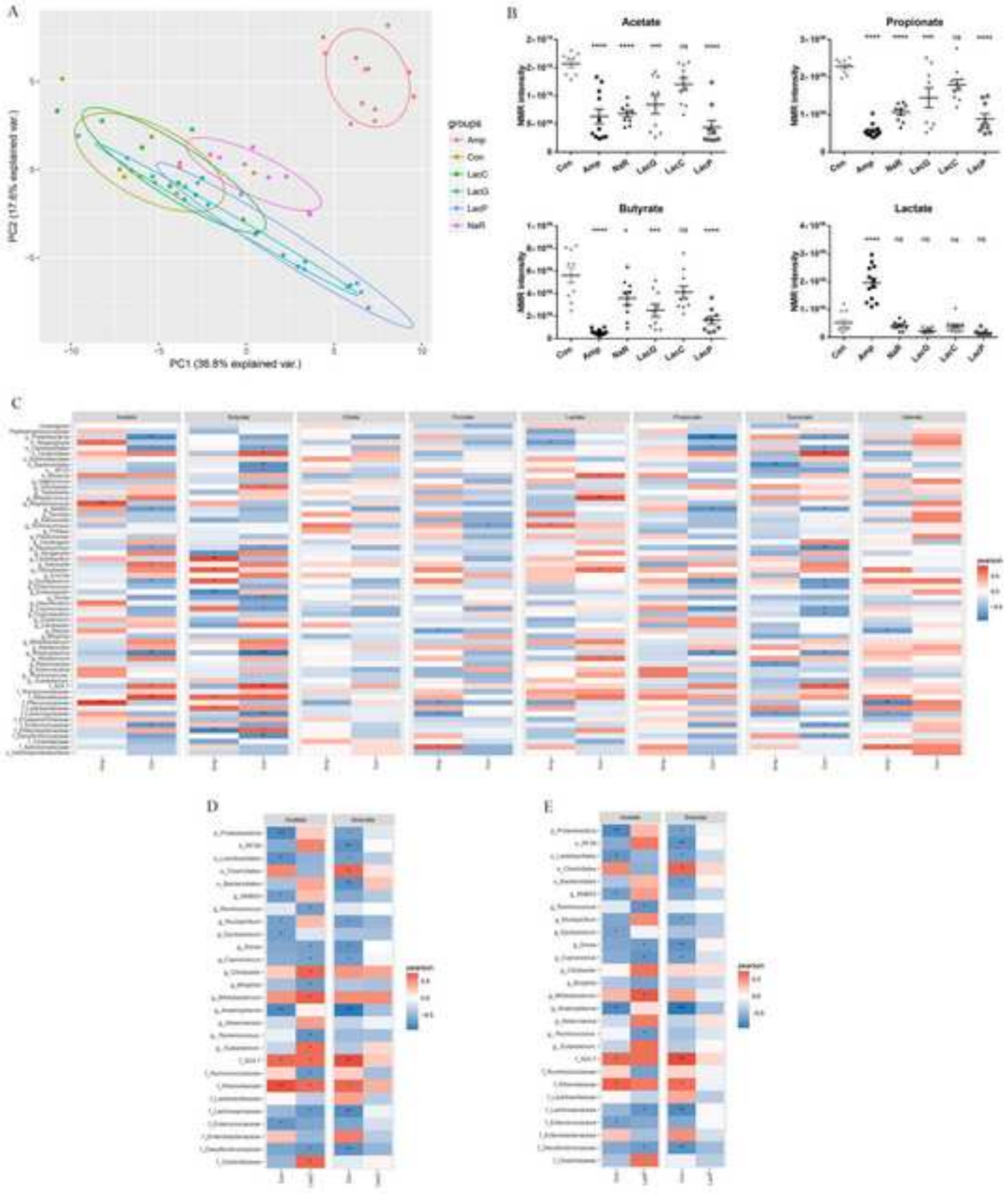

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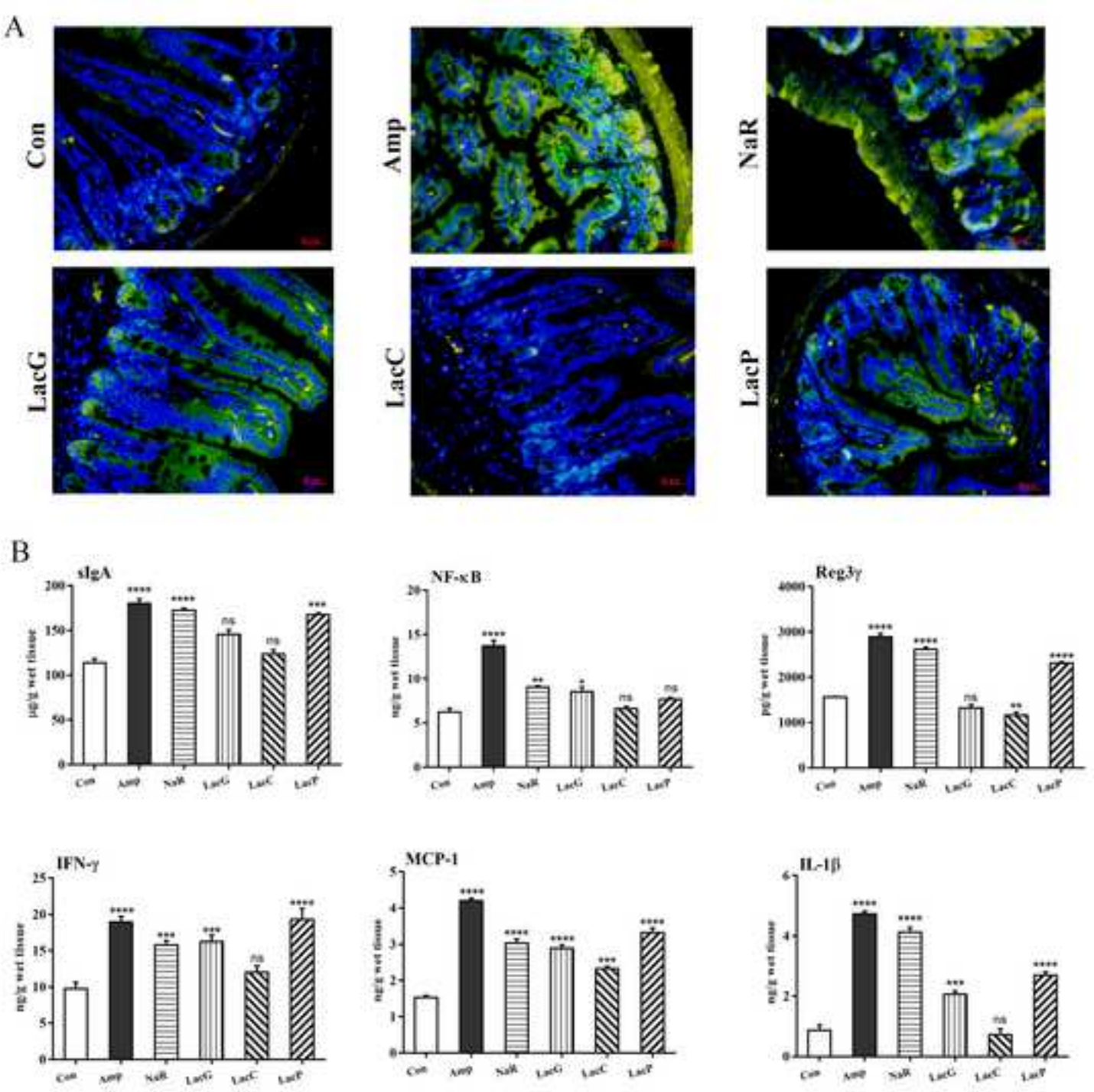
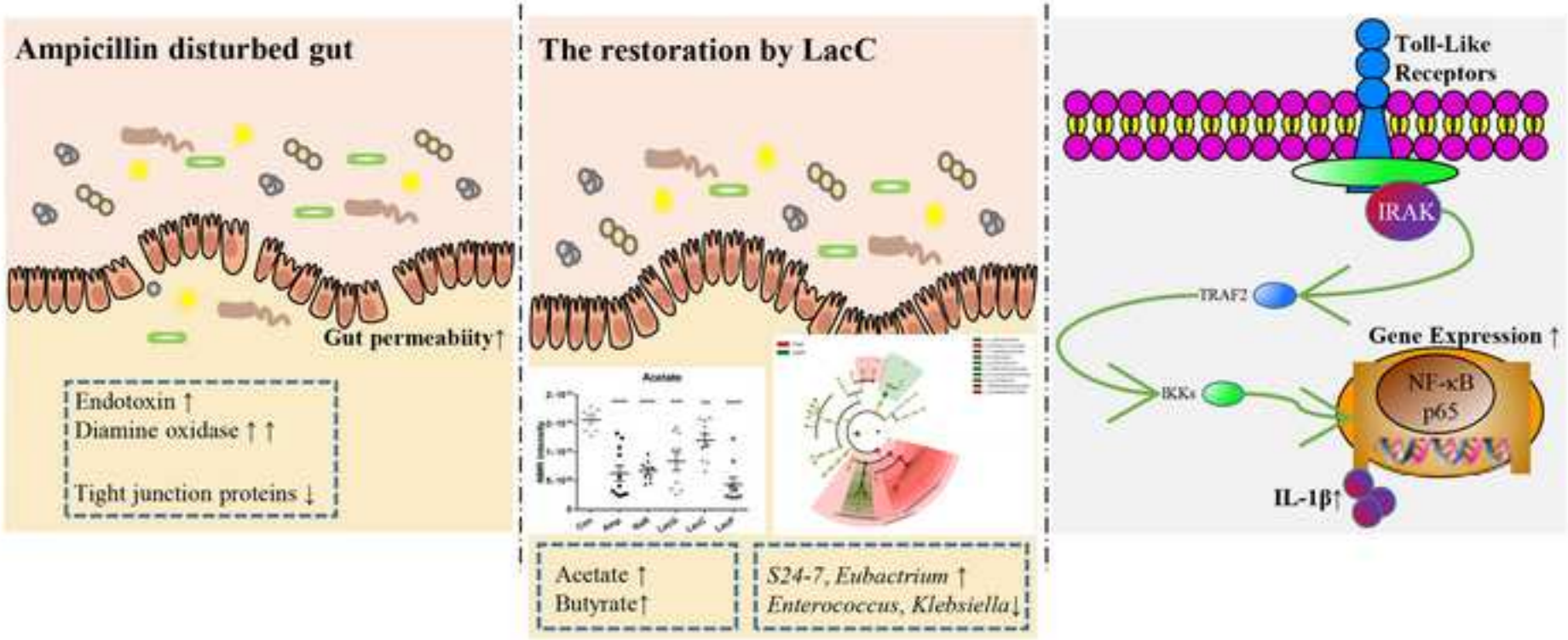


*Graphical Abstract



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***Conflict of Interest**

Declarations of interest: none.

Ethnic statement

The entire experiment was approved by the Ethics Committee of Jiangnan University in China (JN No. 20160608-20160831/47), and the procedures were carried out according to the European Community guidelines (Directive 2010/63/EU) for the care and use of experimental animals