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

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1 **Abstract**
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4 28 To evaluate functions of *Lactobacillus* strains, isolated from fermented food, in restoration of
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7 29 ampicillin-induced disruption based on mucosal barrier, gut microbial community and
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10 30 metabolome analyses, three *Lactobacillus* strains, *L. plantarum* CGMCC12436 (LacP), *L.*
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12 31 *casei* CGMCC 12435 (LacC) and *L. rhamnosus* strain GG (LacG) were individually
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15 32 administered to ampicillin-pretreated mice. All three strains significantly restored
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18 33 concentrations of endotoxin and diamine oxidase to control levels. **Linear discriminate**
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21 34 **analysis based on 16S rRNA sequencing of faecal bacteria revealed that the restoration of**
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23 35 **microbial communities by *Lactobacillus* strains was more effective than natural restoration.**
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26 36 Correlation analysis between microbiota and metabolites indicated that, the higher level of
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29 37 acetate in LacC group was positively correlated with increased relative abundance of
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32 38 *Citrobacter*, *Bifidobacterium* and S24-7. **Furthermore, LacC down-regulated the expression of**
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34 39 **NF- κ B p65 and modulated the ampicillin-induced inflammatory responses.** The LacC strain
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37 40 could particularly attenuate ampicillin-induced disruption by optimisation of microbial taxa
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40 41 and enhancement of acetate and butyrate production.
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45 43 **Keywords:** gut dysbiosis; microbial ecology; metabolites; immunity; functional food
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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

1 67 (Arpaia & Rudensky, 2014). Some bacterial fermentation products, particularly SCFA, were
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4 68 considered as key signs of colonic health, but the specific relationship between the microbial
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7 69 community and metabolites under the status of antibiotic-induced dysbiosis is poorly
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10 70 understood. The intestinal immune response should be balanced between the tolerance for
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12 71 unexpected immune molecules and pathogen-induced inflammation in the host cells and
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15 72 commensal bacteria, with the balance developed by mucus production and antimicrobial
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18 73 peptides to establish a barrier between host tissue and the microbes (Johansson, Larsson, &
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21 74 Hansson, 2011; Vaishnava et al., 2011). Toll-like receptors (TLRs) are a group of pattern
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23 75 recognition receptors, that play a crucial role in mucosal immune response (de Kivit, Tobin,
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26 76 Forsyth, Keshavarzian, & Landay, 2014) and can recognise microbe-associated molecular
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29 77 patterns (MAMPs). As one of these MAMPs, lipopolysaccharide (LPS) can be increased
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32 78 through antibiotic use and cause an innate immune response via TLR4 in intestinal epithelial
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35 79 cells (Collado-Romero, Arce, Ramirez-Boo, Carvajal, & Garrido, 2010). It is well accepted
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37 80 that LPS initiates a signalling pathway through TLR4 to activate NF- κ B, and leads to
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40 81 inflammation and removal of infection by pro-inflammatory cytokines including TNF- α and
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43 82 IL-1 β (Doyle & O'Neill, 2006). One strain of *Lactobacillus* has been proven to down-regulate
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46 83 the expression of pro-inflammatory cytokines in a TLR4-dependent NF- κ B signal pathway
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48 84 (Shimazu et al., 2012).

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51 85 A number of *Lactobacillus* strains, tested as cocktails or individual strains, were shown to
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54 86 alleviate gut-related disorders or metabolic diseases such as obesity, diabetes and
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57 87 non-alcoholic fatty liver disease in mice (Aronsson et al., 2010; Simon et al., 2015; Wang et
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59 88 al., 2015; Xu, Wan, Fang, Lu, & Cai, 2012; Yoo et al., 2013). Importantly, different probiotic

1 89 strains were proven to have remarkably different abilities to modulate gut metabolism and
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4 90 immune response (Fåk & Bäckhed, 2012; Million et al., 2012; Yin, Yu, Fu, Liu, & Lu, 2010).
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7 91 It remains unclear whether specific *Lactobacillus* strains can be administered to regulate the
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10 92 alteration of the gut microbiota and subsequently promote the production of beneficial
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12 93 metabolites. Moreover, it has been reported that a *Lactobacillus* mixture played a beneficial
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15 94 role in the immune response in mice through balancing anti- or pro-inflammatory cytokines
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18 95 (Taranu, Marin, Pistol, Motiu, & Pelinescu, 2015).
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21 96 In the present study, a model of dysbiosis was constructed by exposing healthy adult mice
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23 97 to a therapeutic-dose of ampicillin, leading to perturbed gut microbiota. The
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26 98 ampicillin-induced dysbiosis was confirmed by determining the caecal index, endotoxin
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29 99 levels and the expression of tight-junction proteins. The different *Lactobacillus* species (*L.*
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32 100 *plantarum*, *L. casei* and *L. rhamnosus*) were compared to investigate their impact on the
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35 101 restoration of microbiota in an ampicillin-induced dysbiotic state. The metabolic composition
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37 102 of faecal water was measured using ¹H NMR, and the relationship between the microbial
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40 103 community and SCFA was correlated to characterise the function of *Lactobacillus* strains
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43 104 tested. Our findings provide a novel insight that the administration of different *Lactobacillus*
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46 105 strains after ampicillin-induced dysbiosis exhibited distinct effects in modulation of the
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48 106 microbial community, metabolites and the immune system.
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52 53 108 **2 Materials and Methods**

54 55 56 109 2.1 Culturing of bacteria

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59 110 *L. plantarum* CGMCC12436 and *L. casei* CGMCC12435 were isolated from a traditional
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1 111 fermented cream from Inner Mongolia, China, and the *L. rhamnosus* strain GG (LGG) (ATCC
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4 112 533103) was purchased from ATCC. All strains were held in long-term storage (-80°C in 30%
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7 113 sterile glycerol) in the Culture Collection of the Food Microbiology (CCFM) of Jiangnan
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10 114 University. These three strains were cultured in de Man Rogosa and Sharpe (MRS) broth at
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12 115 37°C overnight. The bacterial cultures were centrifuged and the pellets were resuspended in
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15 116 0.9% saline solution to give a final concentration of $\sim 1 \times 10^9$ CFU per 0.2 mL respectively.

18 117 2.2 Experimental animals and ethics statement

20 118 The experiments were carried out with four-week-old male C57BL/6 mice obtained from
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23 119 Slack Experimental Animal Co., LTD (Shanghai, China). Mice were caged in groups of two
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26 120 or three. Throughout the experiments, distilled water and standard laboratory chow were
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29 121 provided *ad libitum*. Light conditions (12 h light/dark cycle), temperature (21°C) and air
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32 122 humidity were tightly controlled. The experimental procedures and numbers of animals used
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35 123 were approved by the Ethics Committee of Jiangnan University in China (JN No.
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37 124 20160608-20160831/47). The experiments were designed in order that both the number of
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40 125 animals used and their suffering were minimised.

43 126 2.3 Experimental groups, timelines and treatment

45 127 Mice were allocated to one of six groups (Table 1) and acclimatised for 1 week.
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48 128 Ampicillin (Sigma, USA) was dissolved in 0.9% saline solution (500 mg kg⁻¹), and mice were
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51 129 treated via oral gavage with ampicillin for 2 weeks, except for the control group (Con,
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54 130 n=20-24) which were treated with 0.9% saline solution for 2 weeks. After 2 weeks, the group
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57 131 of dysbiotic mice (Amp) and half the group of control mice were sacrificed, whilst the
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59 132 remaining control mice received saline via oral gavage for another 4 weeks; the natural
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1 133 restoration group (NaR) pre-treated with ampicillin received saline via oral gavage for 4
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4 134 weeks; dysbiotic mice were treated via oral gavage with *L. rhamnosus* GG (LacG group), *L.*
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7 135 *plantarum* CGMCC12436 (LacP group), or *L. casei* CGMCC12435 (LacC group) respectively,
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10 136 for 4 weeks.

11 137 2.4 Quantification of gut permeability in the serum

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15 138 The concentrations of endotoxin (ET) and diamine oxidase (DAO) were determined in
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18 139 serum samples using Enzyme-Linked Immunosorbent Assay (ELISA) kits (SenBeiJia
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21 140 Biological Technology Co. Ltd., Nanjing, China). Gut permeability was also measured using
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23 141 4000 Da fluorescent dextran-FITC (DX-4000-FITC) (Sigma-Aldrich, USA) as described in
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26 142 the supplementary methods.

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32 144 Total genomic DNA was extracted from thawed faecal samples with the FastDNA Spin
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34 145 Kit for Soil (MP Biomedical, USA) according to the manufacturer's instructions.
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37 146 Subsequently, the 16S rRNA gene was amplified by PCR with a forward (5'- CCT AYG GGR
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40 147 BGC ASC AG -3') and reverse (5'- GGA CTA CNN GGG TAT CTA AT -3') barcoded primer
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43 148 set, targeting the V3-V4 region. PCR products were gel-purified and the amplicon DNA
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45 149 concentration was determined, the libraries were prepared using the TruSeq DNA LT Sample
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48 150 Preparation Kit (Illumina, USA) and sequenced on the Illumina MiSeq platform (500 cycles
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51 151 paired-end). The detailed methods of QIIME and Linear Discriminate Analysis (LDA) with
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54 152 Effect Size (LEfSe) analyses are described in the supplementary methods.

55 56 153 2.6 Faecal metabolomic analysis

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59 154 Metabolites in the faecal samples of mice were analysed by ¹H NMR analysis at
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1 155 Quadram Institute Bioscience (United Kingdom). Faecal water was prepared by mixing ~100
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4 156 mg of frozen faecal samples with 12 times the volume of a phosphate buffer that consisted of
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7 157 1.9 mM Na₂HPO₄, 8.1 mM NaH₂PO₄, 150 mM NaCl, and 1 mM TSP (sodium
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10 158 3-(trimethylsilyl)-propionate-d₄) in D₂O (deuterium oxide). After homogenising thoroughly
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12 159 with a pellet pestle motor (Kimble Kontes, USA), samples were centrifuged at 14,000 g for 10
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15 160 min at 4°C. High resolution ¹H NMR spectra were recorded using a Bruker AV 600
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18 161 spectrometer (Bruker, Rheinstetten, Germany). The spectra were analysed as previously
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21 162 described and further plotting is listed in the supplementary methods (Le Gall et al., 2011).

23 163 2.7 Correlation between taxa abundance and metabolites

26 164 To evaluate the relationship between the most abundant taxa and observed metabolites, a
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29 165 correlation test was performed, and associated *p*-values were adjusted for multiple testing in R.
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32 166 The *physeq* package was used to obtain taxa abundance and meta data information, the
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35 167 *ggplot2* package was used to plot figures, “Pearson” was selected as the method to
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37 168 characterise correlation coefficient, and “adjust meta variables (panel on the correlation plot)”
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40 169 was selected to adjust *p*-values for multiple comparisons using Benjamin and Hochberg.

43 170 2.8 ELISA analysis of inflammatory cytokines in the colon

45 171 Colon tissues (100 mg) were homogenised in 900 µL PBS using a Scientz-50 tissue mill
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48 172 (Lanzhi, Ningbo, China), centrifuged at 13,000 g for 10 min at 4°C, and the supernatants were
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51 173 transferred into sterile tubes. The levels of secretory immunoglobulin A (sIgA), nuclear factor
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54 174 kappa-light-chain-enhancer of activated B cells (NF-κB), monocyte chemotactic protein 1
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56 175 (MCP-1), interferon-γ (IFN-γ), regenerating islet derived protein 3 gamma (Reg3γ) and
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59 176 interleukin 1β (IL-1β) were determined using respective ELISA kits following the

1 177 manufacturer's protocols (Nanjing SenBeiJia Biological Technology Co. Ltd. China).

4 178 2.9 Immunofluorescence

7 179 Colon tissue sections from different groups of mice were fixed by 4% paraformaldehyde
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10 180 in 0.1 M phosphate buffer. The tissues were excised, post fixed for 3 h in the perfusion
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12 181 fixative, cryoprotected for 72 h in 30% sucrose in 0.1 M phosphate buffer. Transverse sections
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15 182 (20 μ m) were cut and the slides were incubated with primary NF- κ B p65 rabbit polyclonal
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18 183 antibody (Thermo Fisher Scientific, USA) (1:200 dilution) for 2 h at 37°C. Following
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21 184 incubation, sections were washed in PBS and incubated with secondary goat anti-rabbit
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23 185 antibody (1:100 in PBS) (Jackson Immuno Research, USA) for 1 h in a dark room. The
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26 186 sections were incubated with 4,6-Diamidino-2-phenylindole dihydrochlorid (DAPI) (Sigma
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29 187 Aldrich), washed twice, and visualised under a Leica fluorescence microscope. All
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32 188 micrographs were taken with identical exposure times and focused on the centre of each well.

34 189 2.10 Statistical analysis

37 190 Data were represented as mean \pm standard error of the mean (SEM). The gut permeability
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40 191 data were analysed using one-way ANOVA, followed by Dunnett's multiple comparisons test
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43 192 in GraphPad Prism 5. The mRNA expression of tight-junction proteins, metabolites and levels
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45 193 of cytokines were calculated using one-way ANOVA followed by Tukey's multiple
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48 194 comparisons test in GraphPad Prism 5. $P < 0.05$ was considered statistically significant.

51 195 2.11 Data deposition

54 196 The raw sequence data have been deposited in the NCBI Sequence Read Archive
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56 197 (Accession no. SRP146081 and BioProject Accession no. PRJNA471394).

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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 ($p <$

1 221 0.01, $p < 0.0001$ and $p < 0.001$ respectively). LacC treatment enhanced the expression of
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4 222 ZO-1 and Occludin to the control level in the ileum which was more effective than LacG ($p <$
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7 223 0.05 and $p < 0.001$ respectively), while LacP promoted the expression of Claudin-1 in the
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10 224 ileum to the control level. These data demonstrated that, with regards to the expression of
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12 225 tight-junction proteins, the disruption by ampicillin and the enhancement by *Lactobacillus*
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15 226 strains mainly occurred in the ileum, and the LacC strain showed a promotion of ZO-1 and
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18 227 Occludin while the LacP strain positively affected Claudin-1 levels.

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21 228 3.3 Ampicillin-induced colonic microbiome disruption can be largely restored by
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23 229 *Lactobacillus* administration

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26 230 Microbial species richness was indicated by the inverse of the classical Simpson diversity
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29 231 (Invsimpson Index) as shown in Fig. 3A, which was calculated to eliminate the sampling
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32 232 effects of the Shannon Index. The bacterial diversity was greatly reduced by ampicillin
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35 233 treatment ($p < 0.001$), and three groups of the administration of single *Lactobacillus* strain
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37 234 enhanced the level of alpha-diversity to that of the Con group, which was higher than that
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40 235 observed in the NaR group ($p < 0.001$). Principal coordinate analysis (PCoA) based on
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43 236 Phyloseq's Weighted Unifrac showed that ampicillin-treated mice had a considerably altered
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46 237 (clustering by distance) microbial community compared to that of the Con mice (Fig. 3B and
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48 238 C). The profiles of the microbial composition of the Con group and *Lactobacillus*-restored
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51 239 groups were clustered more closely to each other than that of naturally-restored mice (Fig. 3B
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54 240 and C), indicating that tested *Lactobacillus* strains could restore the antibiotic-treated
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57 241 microbiota structure towards the normal profile.

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59 242 Ampicillin treatment led to an increase in *Proteobacteria* and a severe depletion of
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1 243 *Bacteroidetes* and *Verrucomicrobia* at the phylum level (Fig. 3D). Natural restoration did not
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4 244 lead to the recovery of *Bacteroidetes*, while LacC, LacG and LacP groups had an increase in
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7 245 the abundance of *Bacteroidetes* towards, or above (LacC), that of the Con group. Although the
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10 246 microbiota was not completely restored, the abundance of *Proteobacteria* was improved to
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12 247 the control level by LacC and LacP strains, but not by the LacG strain.

15 248 We further examined compositional changes of the microbiome at the family or genus
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18 249 level using high-throughput amplicon sequencing. Following antibiotic cessation, taxa
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21 250 including *Enterobacteriaceae*, *Klebsiella* and *Enterococcus* were dramatically increased in
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23 251 Amp mice compared to the Con mice, whereas *Akkermansia*, *Lachnospiraceae* and *Dorea*
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26 252 were absent in the Amp group (Fig. S1). The cluster of the NaR grouped closer to the Amp
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29 253 group rather than the Con group, indicating that natural restoration for four weeks after
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32 254 ampicillin disruption was not effective in recovering the microbial community to the normal
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34 255 level (Fig. S2). An evaluation of relative abundance (Fig. S4) indicated that *Coprobacillus*,
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37 256 *S24-7* and *Eubacterium* were enhanced in the LacC, LacG and LacP groups after
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40 257 ampicillin-induced depletion, whilst there was no observed restoration in the NaR group.
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43 258 Meanwhile, the relative abundance of *Klebsiella* and *Enterococcus* was reduced by LacC,
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45 259 LacG and LacP strains after their increase due to ampicillin exposure. These data indicated
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48 260 that each of the three *Lactobacillus* strains altered the community structure of the gut
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51 261 microbiota in a manner different from the ampicillin-induced dysbiotic state towards that of
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54 262 the Con group. Furthermore, the LEfSe analysis revealed that the number of significantly
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56 263 altered taxa was lower in LacC (15) than NaR (25) (Fig. 4A and B). The significantly altered
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59 264 taxa after administration of LacG or LacP was 15 and 13 respectively (Fig. S3), demonstrating

1 265 that restoration of the microbiota by *Lactobacillus* strains enabled a stronger shift towards the
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4 266 initial state than observed by natural restoration.
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7 267 3.4 The LacC strain restored the faecal metabolome following alteration by
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10 268 ampicillin-induced dysbiosis

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12 269 The impact of ampicillin-induced microbial community alterations on the faecal
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15 270 metabolite profiles was evaluated by ¹H NMR spectroscopy, which revealed that ampicillin
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18 271 exposure altered the faecal metabolome composition as can be seen by the clear separation
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21 272 between the Amp group and the Con group (Fig. 5A). The LacC group clustered closer to the
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24 273 Con group than the LacG or LacP groups, with NaR positioned between the Con and Amp
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26 274 groups, indicating that LacC was more effective in restoration of the faecal metabolome than
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29 275 natural recovery or LacG and LacP strains. Some metabolites, such as amino acids and
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32 276 carbohydrates produced by the colonic microbiota, were found to have decreased in the faecal
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35 277 samples of ampicillin-treated mice (Fig. S5). In particular, the SCFA reflected by acetate,
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37 278 propionate and butyrate were significantly decreased or diminished ($p < 0.0001$) by the
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40 279 antibiotic use (Fig. 5B).

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43 280 The administration of *Lactobacillus* strains could partly restore the SCFA profile,
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45 281 however, strain-dependent differences were observed. Acetate, propionate and butyrate levels
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48 282 were found to be significantly enhanced in the LacC group compared to the Amp group ($p <$
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51 283 0.0001 , Fig. 5B), increasing towards the same levels as seen in the Con group. The LacG
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54 284 group increased the levels of acetate and propionate to levels higher than the NaR group, but
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57 285 had less effect in the recovery of butyrate levels. However, SCFA levels were not significantly
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59 286 altered in the LacP group. In addition, the increases in lactate levels observed following
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1 287 ampicillin administration were significantly reduced by all three *Lactobacillus* groups and the
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4 288 NaR group ($p < 0.0001$, Fig. 5B). Taken together, these results showed that the LacC strain
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7 289 mitigated the decrease of SCFA in ampicillin-treated mice and was more effective in the
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10 290 restoring the whole metabolic profile.

11 12 291 3.5 The relationship between altered faecal metabolome and changes in the microbial 13 14 15 292 community

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18 293 To assess microbiota-metabolome associations, the functional correlation between
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21 294 alterations in microbial taxa and metabolites was assessed using Pearson's correlation
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23 295 coefficient method, based on 60 bacterial taxa and 8 acids that potentially contributed to the
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26 296 observed differences between the Amp and Con groups (Fig. 5C). Acetate, butyrate and
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29 297 succinate were highly correlated with alterations in proportions of bacterial taxa following
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32 298 ampicillin use. The decrease in acetate within the Amp group was positively correlated with
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35 299 the decreased relative abundance of *Staphylococcus*, *Streptophyta* and *Planococcaceae* ($p <$
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37 300 0.01). The decreased level of butyrate was positively correlated with changes in the relative
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40 301 abundance of *Rikenellaceae*, *Helicobacter*, *Lactobacillus*, *Lactobacillaceae*, and
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43 302 *Epulopiscium* whilst was negatively correlated with *Morganella*, *Enterobacter* and
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45 303 *Enterobacteriaceae* in the Amp group. In particular, the enhanced level of acetate in the LacC
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48 304 group was positively correlated with an increase in the relative abundance of *Citrobacter*,
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51 305 *Bifidobacterium*, *Eubacterium*, *S24-7*, *Rikenellaceae* and *Clostridiaceae* ($p < 0.05$, Fig. 5D),
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54 306 and was negatively correlated with members of *Ruminococcus*, *Ruminococcaceae*, *Dorea*,
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56 307 *Coprococcus*, *Bilophila*, *Lachnospiraceae* and *Desulfovibrionaceae* ($p < 0.05$, Fig. 5D).
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59 308 However, no significant correlations were observed between microbial taxa and butyrate in
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1 309 either the LacC group or LacP (Fig. 5D, E).

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4 310 3.6 *Lactobacillus* strains modulated NF- κ B signalling and colonic inflammation caused by
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7 311 ampicillin-induced dysbiosis

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9 312 The serum endotoxin in ampicillin-treated mice was significantly increased ($p < 0.0001$,
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11
12 313 Fig. 1C) compared to the Con mice, indicating that LPS-stimulated TLR4 and NF- κ B
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15 314 expression might be induced following ampicillin exposure. NF- κ B levels determined by
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18 315 ELISA (Fig. 6B) were significantly increased ($p < 0.0001$) after ampicillin treatment, and
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21 316 reduced to control levels by the administration of LacC or LacP. Consistent with the ELISA
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24 317 results of NF- κ B, ampicillin treatment increased the level of NF- κ B p65 (Fig. 6A), and all the
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27 318 tested *Lactobacillus* strains could partly reduce the level of p65 compared to that achieved by
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30 319 natural restoration. Also, due to the positive feedback activation of the NF- κ B signal pathway,
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33 320 the level of IL-1 β was significantly increased by ampicillin treatment ($p < 0.0001$), and
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36 321 reduced by LacC to the level of the Con group (Fig. 6B). Therefore, the LacC strain seemed
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39 322 to protect against ampicillin-induced inflammatory responses through the regulation of NF- κ B
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42 323 expression in the colon.

43 324 To investigate whether disturbance of the microbiota induced alterations in mouse
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46 325 intestinal immune homeostasis, the local expression levels of several inflammatory mediators
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49 326 were measured in the colon. In addition, the effects of different *Lactobacillus* strains against
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52 327 inflammatory mediator expression were also evaluated. Levels of secretory IgA (sIgA) were
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55 328 significantly increased in antibiotic-induced mice ($p < 0.0001$) whilst LacG and LacC reduced
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58 329 the levels of sIgA to that of the Con group. In ampicillin-treated mice, the expression of
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61 330 Reg3 γ was increased in the colon, and decreased in the LacG group compared to the NaR

1 331 group ($p < 0.0001$, Fig. 6B).

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4 332 The inflammatory cytokines including IFN- γ and MCP-1 were also examined to evaluate
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7 333 colonic inflammation. Levels of IFN- γ and MCP-1 were considerably increased in the Amp
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10 334 group compared to the Con group ($p < 0.0001$). IFN- γ was modulated towards the level
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12 335 observed in the Con group by the LacC group but none of the *Lactobacillus* groups could
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15 336 reverse MCP-1 to the same level as the Con group. Taken together, inflammatory mediators
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18 337 were activated in the colon following treatment with ampicillin, and the LacC strain was more
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21 338 effective in modulating the levels of sIgA, Reg3 γ and IFN- γ whilst LacG administration
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23 339 reduced the level of Reg3 γ .

24 25 26 340 27 28 341 **4 Discussion**

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31 342 By generating ampicillin-induced dysbiosis in the gut microbiota and related metabolome,
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34 343 we could analyse the chronic effects of ampicillin on the host and investigate restoration
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37 344 strategies (Scott et al., 2016). Previous studies applied mouse models to explore the
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40 345 relationship between antibiotic treatment and subsequent changes in host physiology and gut
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43 346 microbiota composition (Aguilera et al., 2015; Bech-Nielsen et al., 2012; Mahana et al., 2016;
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45 347 van Opstal et al., 2016). Although some informative alterations can be measured by
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48 348 examining the microbiota or the metabolome alone, correlation analyses were employed in
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51 349 this study to assess the relationship between the microbiota and metabolome and provide
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54 350 functional information. Furthermore, by comparing the effects of the three *Lactobacillus*
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56 351 strains tested here, the present study revealed substantial evidence of associations between
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59 352 microbial taxa and metabolites in the ampicillin-induced and *Lactobacillus*-restored mice.

1 353 The Amp mice exhibited a decreased microbial diversity. A reduction in microbial
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4 354 diversity is associated with multiple gastrointestinal diseases (Guarner, 2015; Le Chatelier et
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7 355 al., 2013). Several microbial taxa including *Enterobacteriaceae*, *Clostridia*, *Erwinia*, and
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10 356 *Klebsiella* were found to be enhanced in the NaR mice (after ampicillin treatment). Among
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12 357 these changed taxa, the high frequency of *Klebsiella* and *Enterobacteriaceae* has been
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15 358 observed in patients with gastroenteritis and irritable bowel syndrome (Ganji et al., 2016). In
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18 359 the Amp mice, the increased relative abundance of *Enterobacteriaceae* and decreased
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21 360 *Lachnospiraceae* were strongly associated with gut inflammation. The reduction of *Klebsiella*
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23 361 and *Enterococcus* and the enhancement of *Coprobacillus*, *Bacteroidales* and *Eubacterium* in
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25
26 362 all three *Lactobacillus* treatment groups suggested that *Lactobacillus* administration
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29 363 contributed to the promotion of a stable gut microbial community. In particular, the relative
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32 364 abundance of *S24-7* (family) was enhanced in the LacC group and these butyrate-producing
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34 365 bacteria are beneficial to intestinal epithelial cell health (Villanueva-Millan, Perez-Matute, &
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37 366 Oteo, 2015). The decrease of the SCFA was not only associated with perturbation of the
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40 367 microbial communities but also related to the integrity of mucosal barrier in the Amp group.
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43 368 SCFA are reported to be associated with maintenance of intestinal barrier function; acetate
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45 369 was shown to be crucial in the inhibition of enteropathogens (Fukuda et al., 2011), and
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48 370 butyrate production could lead to increased mucin production and promotion of tight-junction
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51 371 integrity (Jung, Park, Jeon, & Han, 2015). The majority of gut butyrate-producers including
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54 372 *Faecalibacterium*, *Eubacterium*, and *Roseburia* utilise pathway in which butyryl-CoA is
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56 373 converted to butyrate (Louis, Young, Holtrop, & Flint, 2010). In this study, following an
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59 374 initial decrease due to ampicillin exposure, the abundance of *Eubacterium* improved in each
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1 375 of the *Lactobacillus* administration groups, which may explain the increase of butyrate
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4 376 observed in these groups.
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7 377 The correlation between microbial communities and faecal metabolites provided crucial
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10 378 evidence on the function of bacteria, with alterations at the microbiota-level leading to a
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12 379 changed metabolome, which could potentially influence gut disease (Claesson et al., 2012;
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15 380 Tremaroli & Bäckhed, 2012). We observed a positive correlation between the relative
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18 381 abundance of *S24-7* (belong to *Bacteroidales*) and levels of faecal acetate and butyrate. It was
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20
21 382 previously reported that consumption of common bean and chickpea reduced
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23 383 colitis-associated inflammation, whilst promoting the levels of SCFA and *S24-7* (Power et al.,
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25
26 384 2016). After the administration of the LacC strain, acetate levels were significantly enhanced,
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29 385 and this increase was positively correlated with the relative abundance of *Citrobacter*,
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32 386 *Bifidobacterium*, *Eubacterium* and *Rikenellaceae* in this study. Among these acetate-related
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35 387 gut bacteria, the selective increase of *Bifidobacterium* has been shown to protect against
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37 388 enteropathogenic infection through the enhancement of acetate (Fukuda et al., 2011); in an
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40 389 elderly health study, genera including *Eubacterium* were associated with long-stay subjects
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43 390 and acetate production, as well as gene counts for acetate-producing enzymes, were
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45 391 significantly higher in long-stay subjects (Claesson et al., 2012). Interestingly, the increased
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48 392 abundance of *Rikenellaceae*, which was associated with the increase of acetate in the LacC
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51 393 group, had previously been associated with potentially beneficial effects on gut health. The
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54 394 decrease in *Rikenellaceae* observed in the Amp and NaR groups had recently been observed
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56 395 in the intestinal dysbiosis of spondyloarthritis (Lin et al., 2014). These analyses of the
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59 396 functional relationship between microbial taxa and SCFA further specified the possible
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1 397 mechanism of functional restoration of LacC strain in the metabolome and microbiome.
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4 398 Exposure to ampicillin increases levels of endotoxin, activation of the NF- κ B pathway
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7 399 and upregulation of the pro-inflammatory cytokines, which are in accordance with the
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10 400 observations during LPS exposure (Lawrence, 2009). The SCFA-driven inhibition of histone
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12 401 deacetylases (HDACs) tends to improve an anti-inflammatory cell phenotype that is critical
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15 402 for maintaining immune homeostasis. A number of studies identified the inactivation of
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18 403 NF- κ B and downregulation of pro-inflammatory cytokines by SCFA (Usami et al., 2008;
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21 404 Vinolo et al., 2011). Also, LPS-induced expression of pro-inflammatory cytokines was
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23 405 attenuated by *Lactobacillus jensenii* through the down-regulation of the TLR4-dependent
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26 406 NF- κ B pathway and the mitogen-activated protein kinase (MAPK) in a porcine intestinal
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29 407 epithelial cell line (Shimazu et al., 2012). In this study, *L. casei* (LacC), *L. plantarum* (LacP)
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32 408 and *L. rhamnosus* GG (LacG) were administrated to ampicillin-treated mice and the levels of
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35 409 NF- κ B and IL-1 β were found to be reduced in the colon by LacC. Collectively, these results
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37 410 confirm that LacC treatment could modulate the host immune responses through the
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40 411 TLR4-dependent NF- κ B pathway in ampicillin-induced mice, although as yet it is unclear
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43 412 whether this was through the action of SCFA or a beneficial modulation of the microbiome
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45 413 structure.
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51 415 **5 Conclusions**

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54 416 We demonstrated that the three strains of different *Lactobacillus* species are able to
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56 417 individually restore antibiotic-induced alterations of the microbiome and the metabolic profile
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59 418 in mice. We found significant differential changes in colonic microbial taxa and clades by
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1 419 LEfSe analysis through the comparison of three *Lactobacillus*-restored groups with a natural
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4 420 restoration group. Correlation analysis of associations between the microbiota and
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7 421 metabolome indicated that LacC strain can promote specific bacterial taxa and SCFA to
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10 422 attenuate ampicillin-induced dysbiosis, suggesting strain-specific effects on functionally
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12 423 relevant gut disease. Furthermore, we confirmed that LacC reduced inflammatory activity by
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15 424 regulating the NF- κ B pathway and pro-inflammatory cytokines in ampicillin-induced
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18 425 dysbiotic mice. These strain-specific *Lactobacillus* treatments offer the potential to mediate
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21 426 antibiotic-associated gastrointestinal disturbances and diseases, although clinical trials would
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23 427 be necessary to confirm their potential beneficial effects in humans.
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26 428

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47
48 436 control in Jiangsu Province.
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50 51 437

54 438 **Conflict of Interest**

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56 439 All authors declared no conflict of interest.
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1 **Figure Legends**

2 **Fig. 1. Effects of *Lactobacillus* strains administration after antibiotic disturbance on gut**

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4 **injury.** A. Level of DX-4000-FITC measured by ELISA in serum samples from different mice
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611 **injury.** A. Level of DX-4000-FITC measured by ELISA in serum samples from different mice
612 groups. B. Caecal indexes weighted and calculated through caecum tissues from different
613 groups. C. Endotoxin and D. DAO concentrations measured by ELISA in serum samples from
614 all mice groups.

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

626 **Fig. 2. The alleviation of intestinal barrier disruption by *Lactobacillus* treatment.**

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

1 631 with the mean of the Con group, ns means no significant difference, * $p < 0.05$, ** $p < 0.01$,

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4 632 *** $p < 0.001$. Values are represented as mean \pm SEM of 5-8 mice per group.

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7 633 **Fig. 3. The restorative effect of *Lactobacillus* strains following ampicillin-induced**
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10 634 **dysbiosis via microbial diversity and bacterial abundance measurements.**

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12 635 A. Microbial α -diversity of faecal samples indicated by Shannon Index, Simpson Index and
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15 636 Invsimpson Index. The richness of each sample was estimated in a phyloseq data object, and
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18 637 one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine
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21 638 statistical significance, with the p value obtained by comparing the mean of each group with
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23 639 the mean of the Con group, ns means no significant difference, * $p < 0.05$, ** $p < 0.01$, *** $p <$
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29 641 B. Principal coordinates analysis (PCoA) and C. clustering of distance based on Phyloseq's
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32 642 Weighted Unifrac to present differences in microbial community structure between samples
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34 643 from Amp (●), Con (●), LacC (●), LacG (●), LacP (●) and NaR (●) groups. The first
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37 644 principal component (PC1) and second principal component (PC2) explained 51.6% and 16.2%
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40 645 of the variance in the Weighted UniFrac metrics, respectively. Each point represents the faecal
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43 646 microbiome of a single sample.

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45 647 D. The abundance of bacterial phyla in faecal samples from Amp, Con, LacC, LacG, LacP
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47
48 648 and NaR mice groups. The rectangles representing *Bacteroidetes*, *Firmicutes*, *Proteobacteria*,
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50
51 649 *Tenericutes* and *Verrucomicrobia* were stacked in order and the aggregate height of the
52
53
54 650 stacked bar reflects the quantitative information.

55
56 651 **Fig. 4. Comparison of differential microbial communities from mice groups using LEfSe.**

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59 652 A. Circular cladogram of biologically and statistically consistent differences in faecal
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1 653 microbial clades between NaR and Con groups. In the panel, each circle's diameter was
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4 654 proportional to the taxon's abundance, green = taxon significantly enriched in NaR, red =
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7 655 taxon significantly enriched in Con and yellow = non-significant. The cladogram
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10 656 simultaneously highlights specific genera/families and high-level trends.

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12 657 B. Histogram of LDA scores computed for taxa that have differential abundance in NaR and
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15 658 Con groups of mice. The magnitude of the LEfSe scores represents the degree of difference in
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18 659 relative abundance between features in the NaR and Con groups.

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21 660 C. Circular cladogram of biologically and statistically consistent differences in faecal
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23 661 microbial clades between LacC and Con groups. Green = taxon significantly enriched in LacC,
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26 662 red = taxon significantly enriched in Con and yellow = non-significant. The cladogram
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29 663 simultaneously highlights specific genera/families and high-level trends.

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32 664 D. Histogram of LDA scores computed for taxa that have differential abundance in LacC and
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35 665 Con groups of mice. The magnitude of the LEfSe scores represents the degree of consistent
36
37 666 difference in relative abundance between features in the LacC and Con groups.

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40 667 **Fig. 5. The restoration effect of selected *Lactobacillus* strains after ampicillin-induced**
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43 668 **dysbiosis based on metabolomic analyses and the correlation between taxa abundance**
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45 669 **and metabolite alterations.**

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47
48 670 A. Principal component plot analysis of faecal metabolites altered in the Amp (●), Con (●),
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51 671 LacC (●), LacG (●), LacP (●) and NaR (●) groups. The first principal component (PC1)
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54 672 and second principal component (PC2) explained 36.8% and 17.6% of the variance
55
56 673 respectively. Each point represents the faecal metabolites in a single sample.

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58
59 674 B. Distribution of intensities for the selected four acids based on the metabolomic analysis.

1 675 Each point represents the faecal metabolites in a single sample, and the mean and SEM were
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4 676 indicated by horizontal lines. One-way ANOVA followed by Tukey's multiple comparisons
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7 677 test was used to determine statistical significance, and the p value was obtained by comparing
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10 678 the mean of each group with the mean of the Con group, ns means no significant difference,
11
12 679 $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$.

15 680 C. Correlation analysis of microbial taxa and acids, as quantified using NMR intensity,
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18 681 between the Con and Amp groups. Top 60 microbial taxa and metabolites in the correlation
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20
21 682 matrix were filtered prior to the Pearson's correlation coefficient method being applied.
22
23 683 Significant microbiota-metabolite correlations were determined based on adjusted p -values
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25
26 684 for multiple comparisons using Benjamin and Hochberg, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
27
28
29 685 Positive correlations between taxa and acids were presented in red, and negative correlations
30
31
32 686 were presented in blue.

34 687 Correlation plot of top 27 taxa associated with acetate or butyrate in the Con and LacC groups
35
36
37 688 (D), or Con and LacP groups (E). Pearson's correlation coefficient method was applied and
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39
40 689 significant microbiota-metabolite correlations were determined based on adjusted p -values for
41
42
43 690 multiple comparisons, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. Positive correlations between taxa
44
45
46 691 and acids were presented in red, and negative correlations were presented in blue.

48 692 **Fig. 6. Effects of ampicillin and subsequent administration of *Lactobacillus* strains on**
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50
51 693 **immune and host-bacterial interaction markers.**

54 694 A. Immunofluorescence analysis of NF- κ B p65 in the colons of mice from Con, Amp, NaR,
55
56 695 LacG, LacC and LacP groups. The expression of p65 (green light) and DAPI (blue light) was
57
58
59 696 observed under the same exposure times (Representative images, $n = 4$ /group).

1 697 B. ELISA analysis of levels of sIgA, NF- κ B, Reg-3 γ , IFN- γ , MCP-1 and IL-10 in the colons
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4 698 of mice from Con, Amp, NaR, LacG, LacC and LacP groups. One-way ANOVA followed by
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7 699 Tukey's multiple comparisons test was used to determine statistical significance, and the p
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10 700 value was obtained by comparing the mean of each group with the mean of the Con group, ns
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12 701 means no significant difference, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Values
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15 702 are represented as mean \pm SEM of 5-8 mice per group.
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1 703 **Table 1. Animal experimental design**

Groups	Antibiotic treatment (2 weeks)	<i>Lactobacillus</i> 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
<i>L. rhamnosus</i> GG (LacG)	Ampicillin (500 mg kg ⁻¹)	LacG 10 ⁹ CFU in 0.9% saline
<i>L. casei</i> CGMCC 12435 (LacC)	Ampicillin (500 mg kg ⁻¹)	LacC 10 ⁹ CFU in 0.9% saline
<i>L. plantarum</i> CGMCC12436 (LacP)	Ampicillin (500 mg kg ⁻¹)	LacP 10 ⁹ CFU in 0.9% saline

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Figure 1
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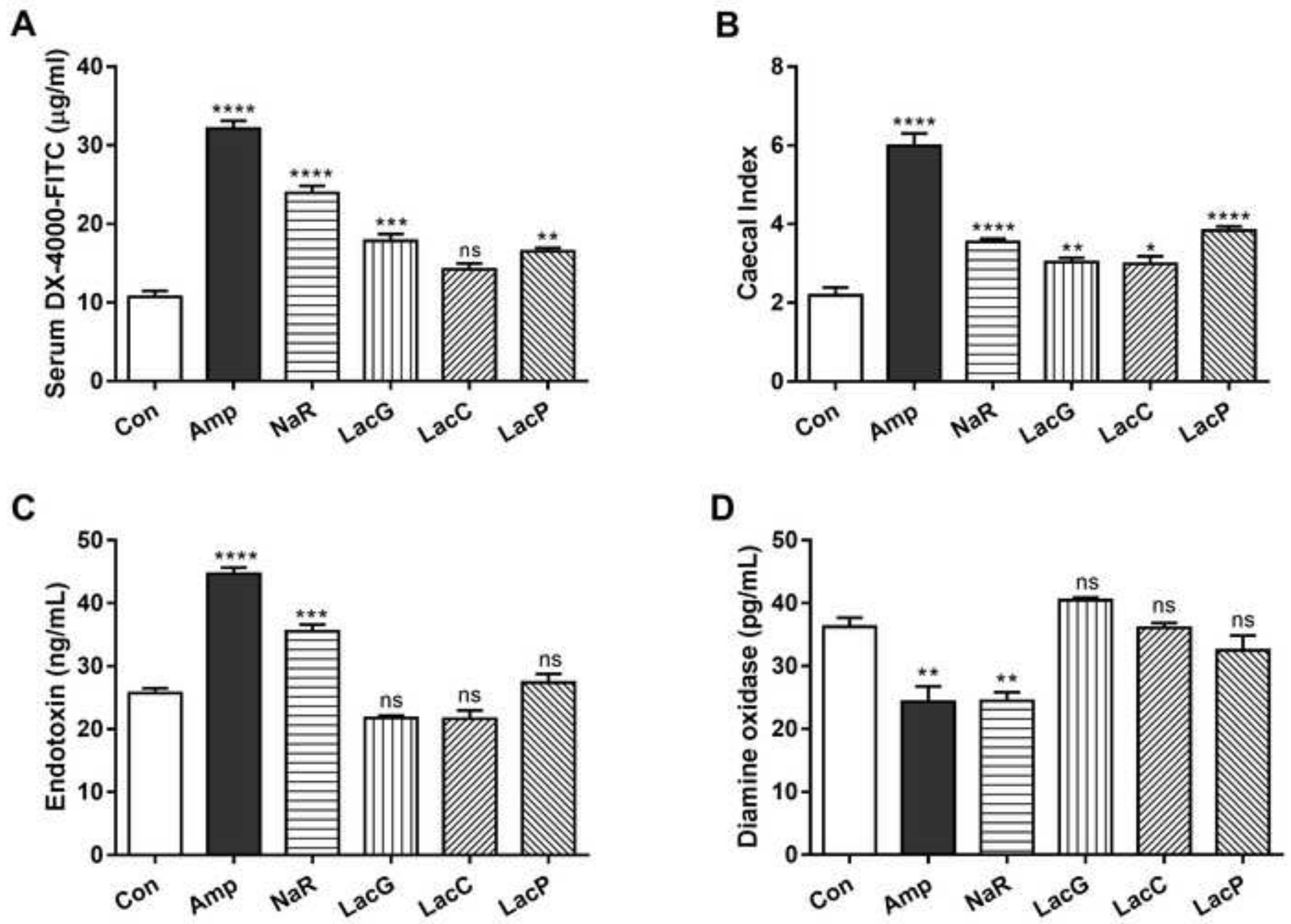


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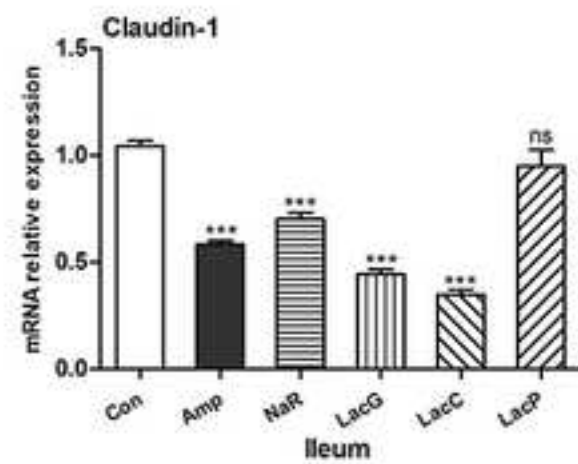
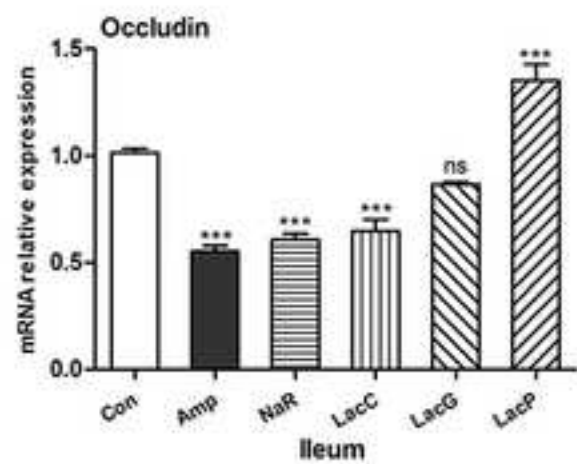
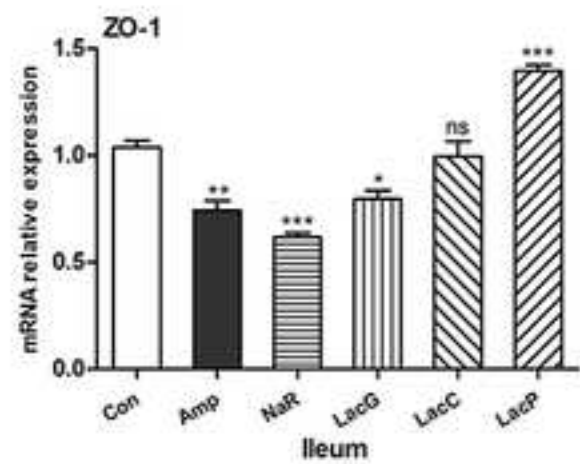
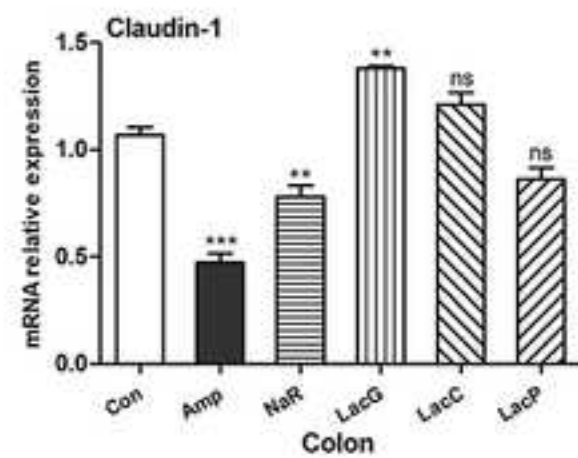
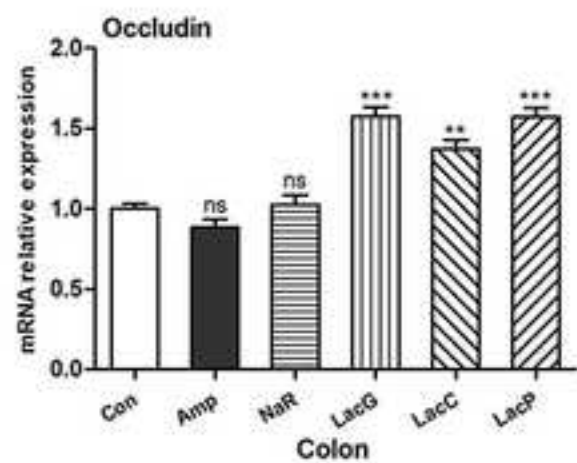
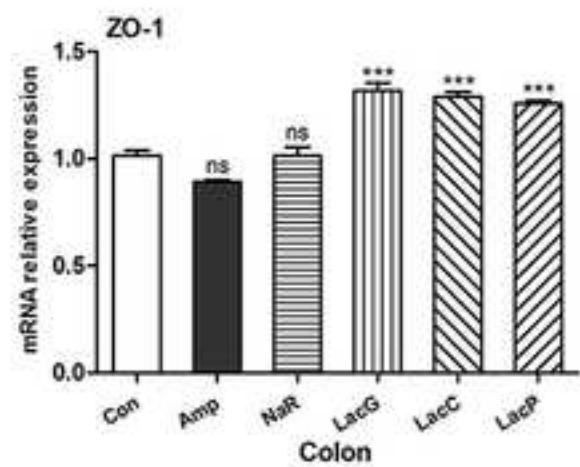


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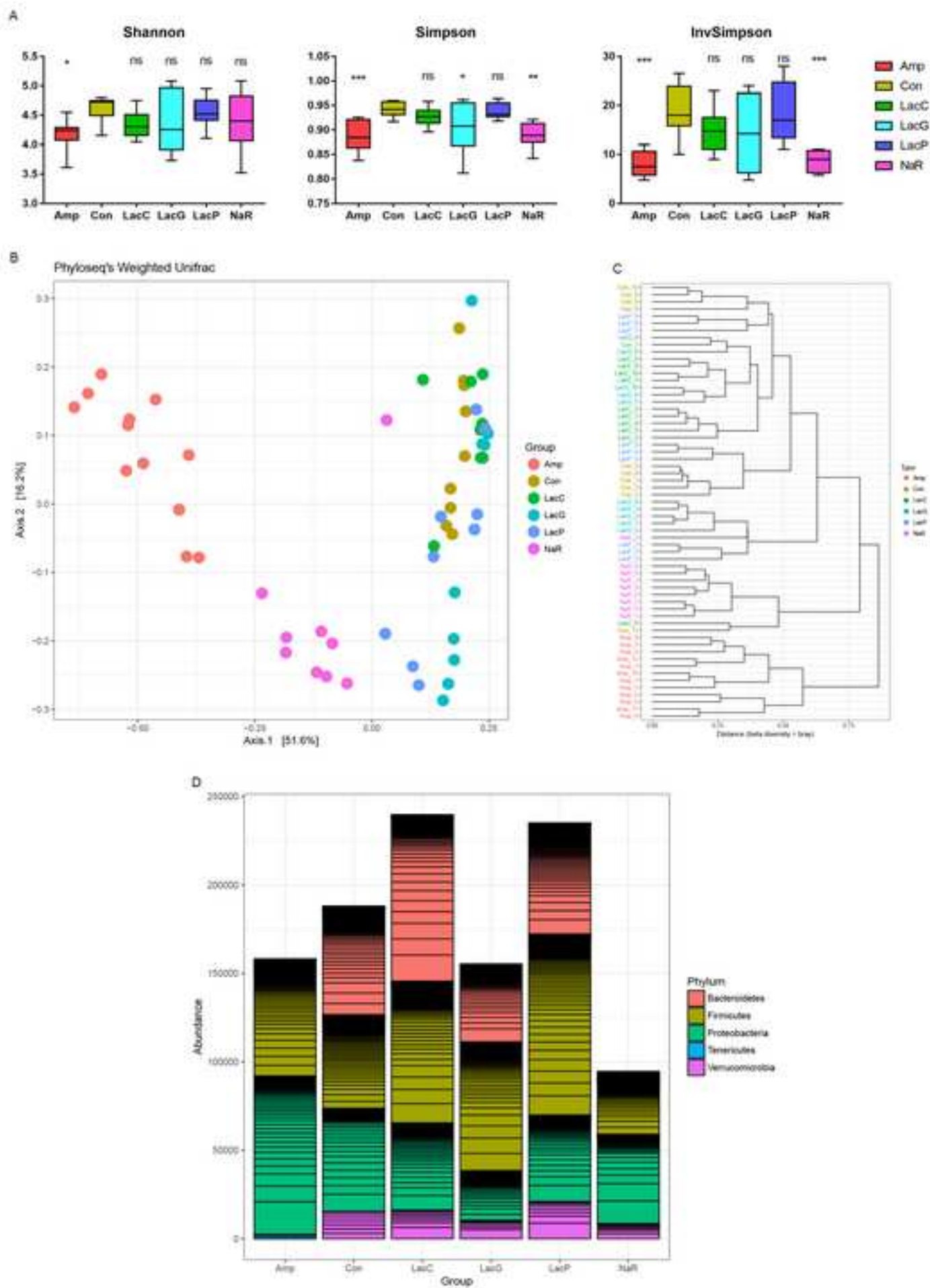


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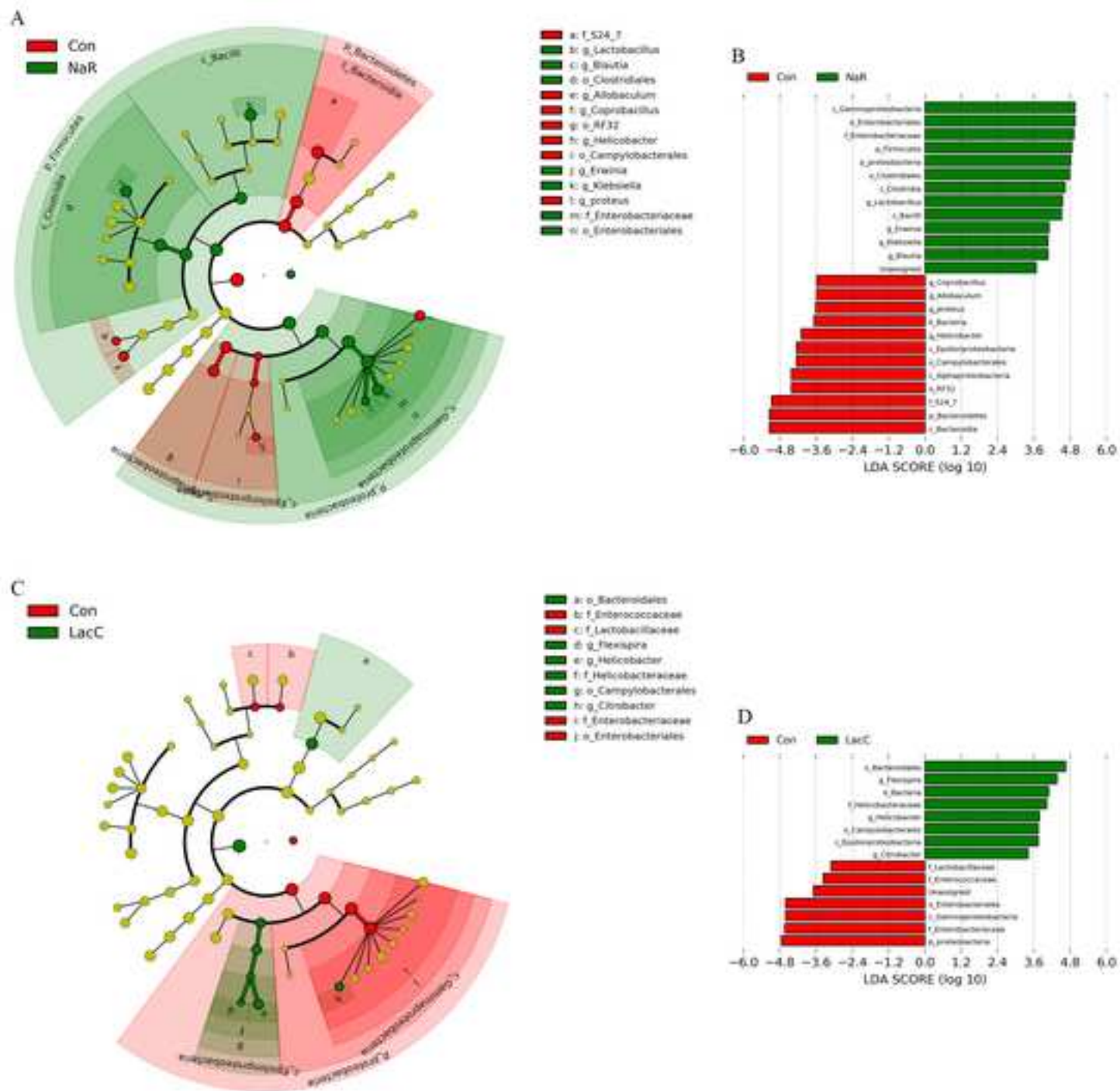


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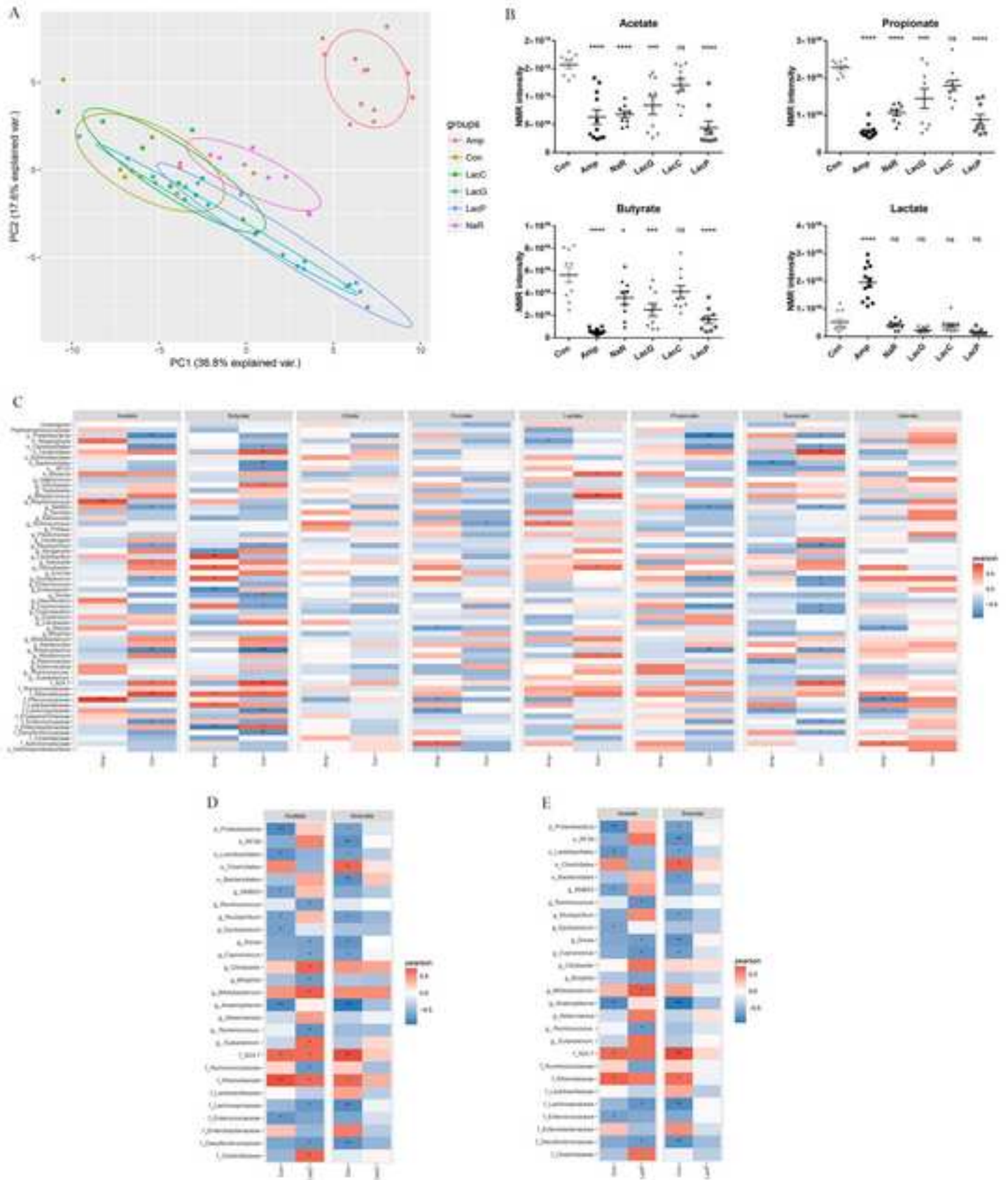
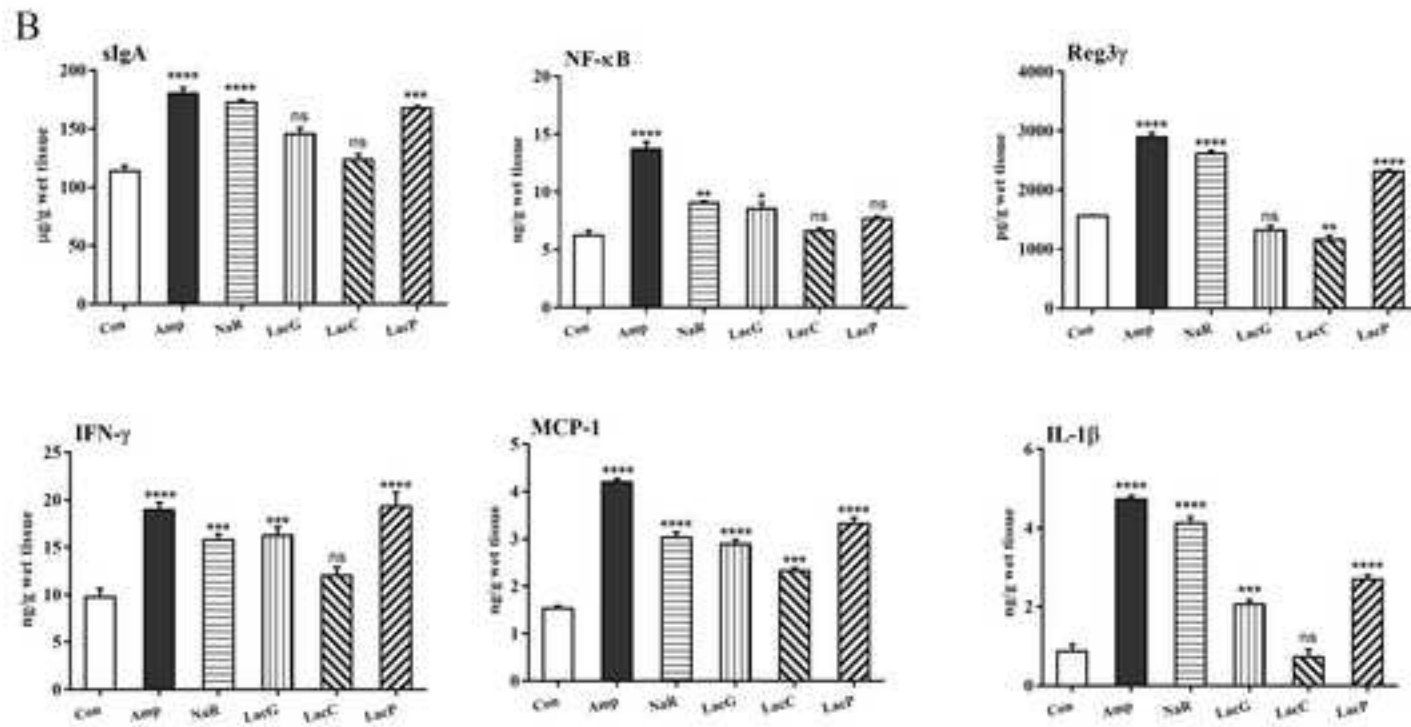
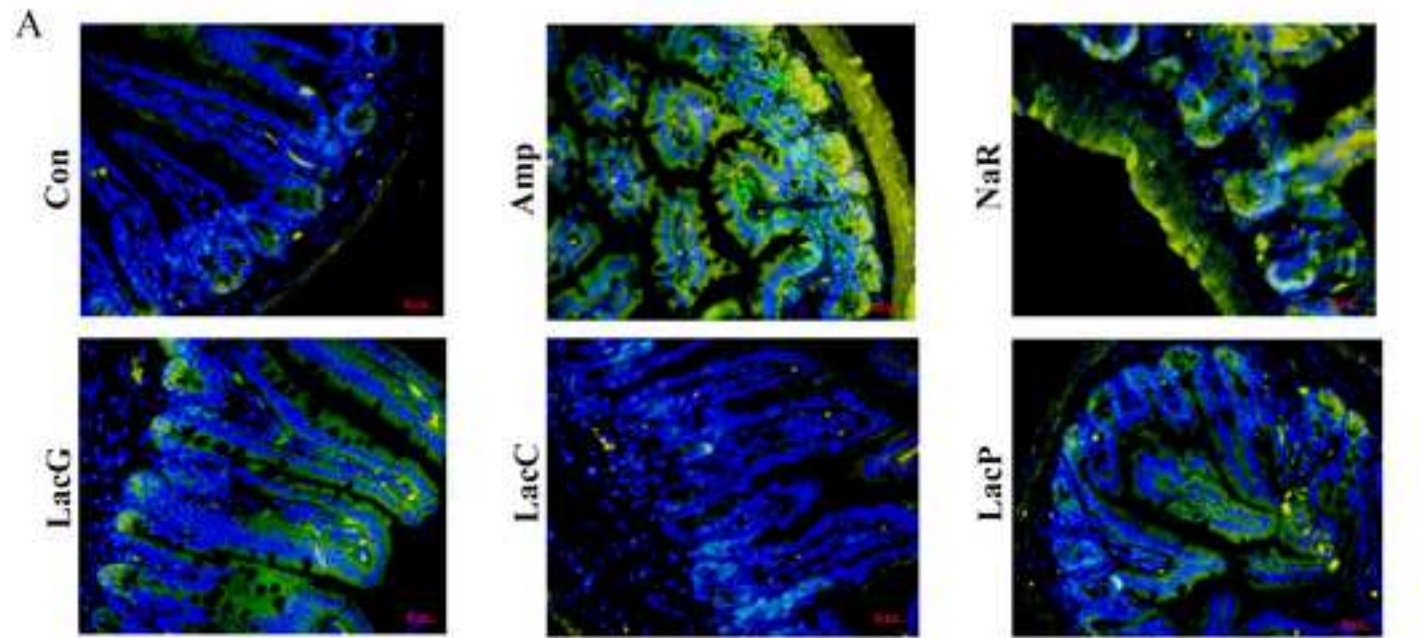
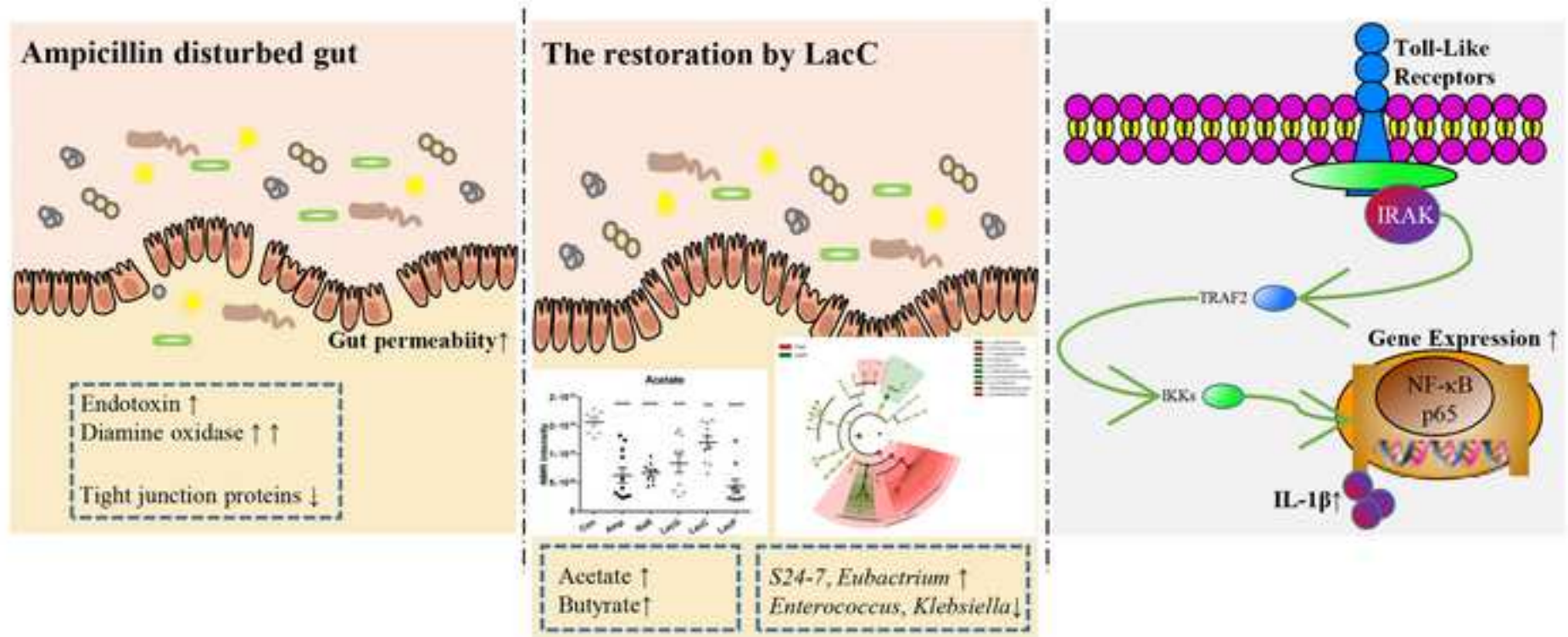


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***Conflict of Interest**

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