Lactobacillus plantarum-mediated regulation of dietary aluminum induces changes in the human gut microbiota: An *in vitro* colonic fermentation study

Running title: Probiotic regulating Al induced human gut dysbiosis

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Abstract

The gut microbiota has been identified as a target of toxic metals and a potentially crucial mediator of the bioavailability and toxicity of these metals. In this study, we show that aluminium (Al) exposure, even at low-dose, affected the growth of representative strains from the human intestine via pure culture experiments. *In vitro*, *Lactobacillus plantarum* CCFM639 could bind Al on its cell surface as shown by electron microscopy and energy dispersive X-ray analysis. The potential of *L. plantarum* CCFM639 to reverse changes in human intestine microbiota induced by low-dose dietary Al exposure, was investigated using an *in vitro* colonic fermentation model. Batch fermenters were inoculated with fresh stool samples from healthy adult donors and supplemented with 86 mg/L Al and/or 10^9 CFU of *L. plantarum* CCFM639. Al exposure significantly increased the relative abundances of Bacteroidetes (*Prevotella*), Proteobacteria (*Escherichia*), Actinobacteria (*Collinsella*), Euryarchaeota (*Methanobrevibacter*), Verrucomicrobiaceae; and decreased Firmicutes (*Streptococcus*, *Roseburia*, *Ruminococcus*, *Dialister*, *Coprobacillus*). Some changes were reversed by the inclusion of *L. plantarum* CCFM639. Alterations in gut microbiota induced by Al and *L. plantarum* CCFM639 inevitably led to changes in metabolite levels. The short-chain fatty acids (SCFAs) contents were reduced after Al exposure but *L. plantarum* CCFM639 could elevate their levels. SCFAs had positive correlations with beneficial bacteria, such as *Dialister, Streptococcus, Roseburia*, and negative correlations with *Erwinia, Escherichia, Serratia*. Therefore, dietary Al exposure altered the composition and structure of the human gut microbiota and this was partially mitigated by *L. plantarum* CCFM639. This probiotic supplementation is potentially a promising and safe approach to alleviate the harmful effects of dietary Al exposure.

Keywords: aluminum toxicity; gut microbiota; lactic acid bacteria; probiotic; short chain fatty acids; 16S rRNA sequencing

1. Introduction

The human gastrointestinal (GI) tract harbors a complex and dynamic population of microorganisms, including bacteria, archaea, fungi, protists, and viruses, among which bacteria are the major inhabitants [1]. The number of microorganisms in the human GI is approximately 10^{13}–10^{14} that, together, have 300 times more genes than the human genome. The gut microbiota should, therefore, be considered as a virtual super-organ [2]. The gut microbiota plays important roles in human physiology and metabolism, including extraction of indigestible nutrients from food, synthesis of vitamins, maintenance of intestinal homeostasis, and modulation of the immune system [3]. The microbiota is dynamic and susceptible to changes in the host gut environment induced by exposure to exogenous substances, such as dietary factors, toxic metals, or probiotics [4,5]. In turn, the gut microbiome can affect host physiology by producing metabolites or transforming compounds in the gastrointestinal tract [2]. In the gut, short-chain fatty acids (SCFAs) are produced, mainly by anaerobic bacteria, from dietary components. These important metabolites have immunomodulatory and anti-inflammatory functions mediated via regulation of T cell homeostasis in the gut [6].

Aluminum (Al) is the most abundant metal on earth. This metal is lightweight, strong, non-corroding and easily processed. For these reasons, it is widely used in daily life and by many industries: construction, aircraft, food production. Increases in Al contamination of the environment and accumulation in the food chain have led inevitably to continuous increases in human oral Al exposure. In several European countries, the daily Al exposure levels from food and water were reported to range from 0.2 - 1.5 mg/kg body weight (bw) per week in the general population [7]. The European Food Safety Authority (EFSA) has established a health-based guidance value of 1 mg/kg bw per week. Approximately 40% of ingested Al accumulates in the gut, which has been underestimated as a target organ for Al exposure [8]. Several studies have recently identified the gut microbiota as the first protective barrier against toxic metals, including Al. Accordingly, the gut microbiota may be a crucial mediator of the bioavailability of these metals [9,10]. For example, the gut microbiota may interact with metals via active absorption or passive binding [11]. Moreover, the gut microbiota and its metabolites, including SCFAs, can influence the transfer of metals into the body indirectly, which could affect intestinal barrier integrity [12]. Toxic metals may induce changes in the gut microbiota that lead to, or exacerbate, the toxicities associated with these metals.

Probiotics are defined as live microorganisms that, when administered in adequate amounts,

confer a health benefit on the host [13] and are widely used in industrial probiotic foods and beverages [14]. They are known to have beneficial effects on the GI tract and nervous system [15,16], as well as having various other functions, such as reduction of lactose intolerance, modulation of immune system, alleviation of food allergy, decrease of blood pressure and prevention of osteoporosis [17,18]. It's worth mentioning that probiotics can reduce hazardous substances, such as toxic metal and acrylamide, in food products and the host [19-21]. Many probiotic strains are isolated from the gastrointestinal tract itself. Administration of probiotics can lead to changes in the levels of specific microbial species and SCFAs in the gut. For example, supplementation with *Lactobacillus salivarius* Ls-33 altered the relative abundance of *Clostridium* spp. in the feces of obese juveniles [22]. Ingestion of fermented milk containing *Bifidobacterium animalis* can reduce the abundance of some pathogenic bacteria and potentiate the production of SCFAs [23].

Studies in mice and fish reported that high-dose of Al exposure (up to ~200 mg/kg bw) affected the intestinal microbiota [10,24], but little has been reported about the effects of dietary Al exposure on the human gut microbiota at doses relevant to real-life exposure. *L. plantarum* CCFM639, a probiotic supplement, has been used previously to regulate changes in the intestinal microbiota induced by exposure to high doses of Al in an animal model [24]. It remains unclear whether administration of CCFM639 could reverse changes to the human intestinal microbiota induced by low-dose dietary Al exposure. *In vitro* gut fermentation models are powerful tools for investigating the impact of dietary components on the human gut microbiota [25]. These models can simulate the physiological environment of the gut, including pH, temperature, and anaerobic conditions [26]. Although not a complete substitute for human studies with *in vivo* models, *in vitro* analysis can be an accurate systematic approach to analyzing different parameters and end points in colonic fermentation [26]. Batch cultures constitute the simplest forms of *in vitro* models and have been used to investigate efficacy of probiotics and prebiotics [27-29]. In this study, we investigated the effects of dietary Al exposure and supplementation with the probiotic, *L. plantarum* CCFM639, on gut microbial composition and levels of SCFAs in healthy adults using an *in vitro* batch culture model.

2. Materials and Methods

2.1 Probiotic preparation

L. plantarum CCFM639 (CGMCC 9664) was obtained from the Culture Collection of the Food

Microbiology department (CCFM), Jiangnan University (Wuxi, China) and cultured in De Man Rogosa Sharpe (MRS) broth at 37 ºC for 18 h with 2% inoculation. After three generations of activation, 20 mL of the fermentation broth was inoculated into 1L MRS for 18 h at 37 ºC. The cell pellets were collected by centrifugation at 8000×g and 4ºC for 5 min and then washed three times with sterile saline solution (NS; 0.85%) for the following experiments.

2.2 Al exposure studies with pure bacterial cultures

2.2.1 Pure bacterial cultures.

In vitro experiments using pure cultures were done on representative strains to determine whether they were affected by Al. The following strains were evaluated and obtained from the in-house culture collection of the Quadram Institute Bioscience: *L. plantarum* CCFM639 (CGMCC 9664), *Lactobacillus rhamnosus* GG (ATCC 53103), *Escherichia coli* 1BO4 (isolated from human feces), *Salmonella typhimurium* ATCC SL1344, *Bifidobacterium longum* B78 (isolated from human feces), *Clostridium perfringens* NCTC3310 and *Bacteroides thetaiotaomicron* VP1-5482 (ATCC 29148). The two *Lactobacillus* strains were cultured in MRS broth and the *E. coli* and *S. typhimurium* were cultured in LB (Luria-Bertani) broth. All other strains were cultured in BHI (Brain Heart Infusion) broth. The strains were inoculated at a dose of 2% (v/v), approximately 10^6 CFU, in 100-well honeycomb plates supplemented with different concentrations of $AlCl_3 \cdot 6H_2O$ (43, and 86 mg/L Al ions) and incubated for 24 or 48 h at 37ºC under anaerobic conditions (85% N_2, 5% O_2, 10% CO_2, in a MACS-MG-1000 controlled atmosphere cabinet, Don Whitley Scientific, UK). Absence of Al (0 mg/L) was used as the control group for each strain. Optical density (OD_{600}) values were measured automatically every hour by a Magellan Microplate Reader (Tecan Life Sciences, Mannedorf, Switzerland).

2.2.2 Electron microscopy and energy dispersive X-ray analysis.

The Al binding assay [30] and samples for transmission electron microscopy (TEM) and scanning electron microscopy (SEM) were prepared as described previously [20]. After the Al binding assay, bacterial cells were harvested by centrifugation at 8000×g for 20 min, washed with phosphate-buffered saline solution (PBS) (pH 7.2), and resuspended in PBS. 25% glutaraldehyde was added to the bacterial suspension and the cells were left to fix for 1.5 h, then centrifuged and washed with sodium cacodylate buffer (0.05 M). The cell pellets were mixed at a 1:1 ration with molten 2% agarose, and then chopped into small pieces (about 1 mm^3). These samples were left overnight in a 2.5% glutaraldehyde/0.05 M

142 sodium cacodylate buffer (pH 7.2) and then transferred to a Leica EM TP tissue processor (Leica
143 Microsystems UK Ltd., Milton Keynes, UK). The samples were infiltrated with a resin (London Resin
144 Ltd., London, UK) and ethanol mixture, and the tissue blocks of the samples were placed into gelatine
145 capsules containing fresh resin and polymerized overnight at 60 ºC. The sections were cut using
146 Reichert-Jung Ultracut E ultramicrotome, and examined and imaged using a FEI Tecnai G2 20 Twin
147 TEM at 200 kV. The samples for SEM observation were prepared as following, after the Al binding
148 assay, the bacterial cells were harvested and fixed with glutaraldehyde (2.5% v/v) for 4 h. The cells
149 were then washed with PBS three times and dehydrated with graded alcohols. An identical volume of
150 isoamyl acetate was used to displace the graded alcohols. The bacterial samples were lyophilized and a
151 Hitachi S-3400N SEM was used to observe the cellular morphology. The TEM and SEM were
152 equipped with energy dispersive X-ray (EDX) microanalysis systems and the elemental composition of
153 the selected areas observed using the electron microscope was analyzed.
154
155 2.3 *In vitro* colonic fermentation models
156 Fecal samples were collected from healthy volunteers with no diagnosed gastrointestinal diseases
157 and no history of probiotic or antibiotic usage within the previous 4-week period. The study was
158 approved by the Institute of Food Research (now Quadram Institute Bioscience) Human Research
159 Governance committee (IFR01/2015). Informed consent was obtained from all participating volunteers.
160 The trial was registered at http://www.clinicaltrials.gov (NCT02653001). Fresh fecal samples were
161 collected every morning between 6:00 and 10:00 and processed within 1 hour. Specifically, 15 g of
162 feces from central area were suspended in sterile deoxygenated phosphate-buffered saline PBS (pH 7.0)
163 at a ratio of 1:10 and homogenized using a Stomacher 400 circulator (Seward Ltd., Worthing, West
164 Sussex, UK) at 230 rpm for 45 s [31]. The processed fecal samples were then used for *in vitro* colonic
165 fermentation (Fig.1).
166 To each vessel (300 mL), 15 mL of the processed fecal sample and 135 mL of sterile basal growth
167 medium (BGM) were added. Composition of BGM media included peptone water 2 g/L, yeast extract 2
168 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4.7H_2O$ 0.01 g/L, $CaCl_2.6H_2O$ 0.01 g/L,
169 $NaHCO_3$ 2 g/L, Tween 80 2 mL, glucose 10 g/L, vitamin K1 10 μL, cysteine HCl 0.5 g/L, bile salts 0.5
170 g/L (pH 7.0) [31]. The experimental groups were designated as follows: (A) control (no addition), (B)
171 LP group (10^9 CFU of *L. plantarum* CCFM639 added), (C) Al group (86 mg/L of Al ion added) and (D)

Al + LP group (10^9 CFU of *L. plantarum* CCFM639 and 86 mg/L of Al added). There were three replicate vessels per experimental group. The vessels were maintained at 37ºC by a circulating water jacket and supplied with nitrogen to maintain an anaerobic environment. The pH value was maintained at 6.8 using Fermac 260 pH control units (Electrolab Biotech Ltd., Tewkesbury, Gloucestershire, UK). 15 mL aliquots were collected from each vessel for further analysis after 0, 8, and 24 h. Three four-vessel experiments were conducted in total using feces from 3 different donors.

At each sampling time, the total number of bacteria and the number of *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Lactobacillus*, and *Enterobacter* spp. in each vessel were estimated by colony counts using Wilkins Chalgren, Bacteroides, Beerens, Clostridia, MRS, and McConkey selective agar plates, respectively (Oxoid, Basingstoke, Hampshire, UK). The two *Lactobacillus* strains were incubated at 37ºC for 24 h in aerobic condition, while other strains were cultured in anaerobic conditions at the same temperature for 24 h or 48 h

2.4 Evaluation of the microbiota

At each sampling time 12 mL of fermentation broth from each vessel was separated by centrifugation at 13,000×*g* and 4ºC for 10 min. The supernatant was removed and the pellets resuspended in 1 mL of PBS. This step was repeated three times, after which time DNA was extracted from each pellet using the Fast DNA Spin Kit for Soil (Qbiogene, Carlsbad, California, USA) according to the manufacturer's instructions. The bacterial 16S rRNA gene was amplified using the forward primer 515F (5′-barcode-GTG CCA GCM GCC GCG G-3′) and the reverse primer 907R (5′-CCG TCA ATT CMT TTR AGT TT-3′) [32]. Amplicons were added, 8-base barcoded, and an Illumina MiSeq sequencer used for sequencing. The original data files were analyzed using the QIIME platform (version 1.17). UPARSE was used for cluster analysis of operational taxonomic units (OTUs), and UCHIME was used for identification and removal of chimeric sequences. OTU strain types were identified using the Ribosomal Database Project (RDP) Naive Bayes classifier.

2.5 Quantification of SCFAs

At each sampling time (0, 8, and 24 h) a 1-mL aliquot of fermentation broth was collected from each vessel and centrifuged at 4000×g and 4ºC for 15 min. Subsequently, 900 µL of supernatant was added to 100 µL of NMR buffer (100 ml D_2O containing 0.26 g NaH_2PO_4, 1.41 g K_2HPO_4, 0.1% NaN_3,

and 1 mM deuterated trimethyl silylpropionate (TSP) as a reference compound) before ^{1}H NMR spectroscopic analysis of the SCFAs, as described in a previous study [31]. The metabolites were quantified using the software Chenomx® NMR Suite 7.0TM. It is a specialised software that directly quantifies compounds from signals in the 1H NMR spectra. It relies on the principle that the NMR signal area of a compound is directly proportional to its concentration. We use an internal reference called TSP that we add to the buffer solution (1 mM TSP) to quantify all the compounds detected in Chenomx. This obviates the need for a calibration curve or further calculations, as the software provides the data directly.

2.6 Data analysis

One-way analysis of variance and nonparametric tests were used to analyze the results with three repetitions. Data were expressed as means ± standard deviations (SD). The analyses were performed using Origin 8.6 software (Originlab, USA). *P* values of < 0.05 indicated a statistically significant difference. Alpha diversity and beta diversity of the gut microbiota were analyzed based on the levels of OTUs. Moreover, a linear discriminate analysis effect size (LEfSe) was applied to determine the significance of differences in the relative abundances of gut microbiota between the control and the Al only groups and between the Al and Al + LP groups, using the Galaxy website (http://huttenhower.sph.harvard.edu/galaxy?tdsourcetag=s_pctim_aiomsg) [33]. A parametric Kruskal-Wallis test and linear differential analysis (LDA) were used to identify significant differences and estimate their effect sizes. The results of LDA classification can be graphically visualized by projecting the classes (preferably three or more) into the space of canonical variates, or discriminant functions [33]. Results were considered significant at an adjusted P value of less than 0.05 and an LDA score of at least 2.0. The association between the gut microbiota and SCFAs was explored via the Pearson's correlation coefficient (two-tailed), one of the most common used correlation coefficient, which was plotted as a heat map using the R package "corrplot".

3. Results

3.1 Effects of Al exposure on pure cultures

The Al ion concentrations of 43 and 86 mg/L were selected according to the total dietary Al exposure as assessed in several European countries [7]; these two Al exposure concentrations had little or no effect on the growth of *L. plantarum*, *L. rhamnosus, Ba. thetaiotaomicron*, *E. coli*, and *S.*

typhimurium (Figs. 2A, B, D-F). The growth of *Bi*. *longum* was significantly reduced at an Al concentration of 43 mg/L ($P < 0.05$); at 86 mg/L the lag and logarithmic phases were greatly extended and cultures did not reach the stationary period, even after 48 h (Fig. 2C). Growth of *C. perfringens* in the absence of Al entered the logarithmic phase quickly, and the stationary phase was achieved within 10 h. The trend in the growth curve in cultures exposed to 43 mg/L of Al ion was similar to that of control cultures, but the OD_{600} level in the stationary phase was significantly increased in the presence of Al ($P < 0.05$). Interestingly, at an Al concentration of 86 mg/L, the lag phase was extended to approximately 10 h, followed by a short logarithmic phase and a stable period with the highest OD_{600} reading (Fig. 2G). These results indicate that *Bi. longum* and *C. perfringens* were sensitive to Al, whereas *L. plantarum, Ba*. *thetaiotaomicron*, *E. coli*, and *S. typhimurium* exhibited good tolerance to Al.

We examined the morphology of CCFM639 using electron micrographs after Al binding (Fig. 3). SEM revealed obvious Al deposits on the cell surface, with no morphological changes after Al binding (Fig. 3B). TEM revealed that Al was deposited on cell surfaces, but that it did not enter the cells (Fig. 3D). EDX did not detect Al in the control cells (Fig. 3E) but revealed a distinct Al peak in the Al-treated samples (Fig. 3F), thus demonstrating the association of Al with the cells.

3.2 Effects of Al and *L. plantarum* CCFM639 on the gut microbiota in an *in vitro* colon model

3.2.1 Growth of specific groups of bacteria

The analyses of pure cultures revealed that an Al concentration of 86 mg/L had greater effects on the representative strains than the lower concentration. Accordingly, 86 mg/L Al was selected for the *in vitro* colonic fermentation experiments. In the fermentation study, no significant differences were observed among the four vessels with respect to the total number of bacteria and the abundance of *Lactobacillus* spp. ($P > 0.05$) (Table 1). However, compared with the control, the number of *Bacteroides* increased significantly within 24 h in the Al-only treatment ($P < 0.05$), while co-inoculation of Al and *L. plantarum* CCFM639 (Al+ LP) prevented this increase. *Bifidobacterium* spp. were highly sensitive to treatment of both Al only and Al + LP; compared with the control, the abundance of *Bifidobacterium* spp. was significantly reduced at 8 and 24 h with both of these treatments, but the reduction was not as great when Al was added together with *L. plantarum* CCFM639 at 8 h ($P < 0.05$). Notably, *L. plantarum* CCFM639 administration resulted in an increased

abundance of *Bifidobacterium* species at 8 h in the absence of Al ($P < 0.05$). For *Clostridium* spp., Al only significantly promoted growth at both 8 and 24 h, whereas co-treatment with *L. plantarum* CCFM639 significantly reversed these trends ($P < 0.05$), giving counts which were more similar to the untreated controls. Moreover, compared with the control, *L. plantarum* CCFM639 significantly reduced the numbers of *Clostridia* at 24 h in the absence of Al exposure. The levels of *Enterobacteriaceae* were also significantly increased after Al exposure at 24 h, whereas co-inoculation with *L. plantarum* CCFM639 reduced this population so that it was not significantly different to the control ($P < 0.05$). Overall, *L. plantarum* CCFM639 has a mitigating effect on the Al-induced imbalance in the microbiota, particularly with regards to *Bifidobacterium* and *Clostridia*.

3.2.2 Intestinal microbiota diversity and composition

Shannon indices revealed no significant differences between the four treatment groups at both the 8 h and 24 h time points (Fig. 4A). However, the Principal coordinates analysis (PcoA) plot (Fig. 4B) revealed clear distinctions between the control, Al, and Al + LP groups indicating significant differential clustering of the microbiota composition at 24 h; PC1 and PC2 explained 38.2% and 17.3% of the variance, respectively.

We further examined compositional changes in the gut microbiota at the phylum, class, order, family and/or genus levels using high-throughput amplicon sequencing (Fig. 4C, 4D). After 24 h, the relative abundances of the five predominant bacterial phyla - Firmicutes, Actinobacteria, Euryarchaeota, Bacteroidetes, and Proteobacteria - were 56.10%, 35.70%, 4.10%, 3.12%, and 0.92%, respectively, in the control group (Fig. 4C). Al exposure enhanced the abundances of Proteobacteria (24 h, 6.90%) and Bacteroidetes (at 8 h only, 12.96%) and reduced the abundance of Firmicutes (24 h, 46.67%), whereas these changes were not as large in the Al + LP group. Interestingly, the LP group exhibited an increase in the abundance of Actinobacteria (24 h, 40.84%) and a decrease in the abundance of Bacteroidetes (24 h, 1.48%), compared with the control.

At the family and genus levels, various taxa were significantly decreased in the Al only group compared with the control ($P < 0.05$); these included Streptococcaceae, *Streptococcus*, Lactobacillaceae, *Roseburia*, *Dialister*, *Coprobacillus*, and *Ruminococcus*. In contrast significant increases were observed in Enterobacteriaceae, *Escherichia*, *Erwinia*, *Serratia*, Coriobacteriaceae, *Collinsella*, *Actinomyces*, Odoribacteraceae, Porphyromonadaceae, Rikenellaceae, Barnesiellaceae,

Prevotellaceae, *Prevotella*, Clostridiaceae, Verrucomicrobiaceae, Methanobacteriaceae, *Methanobacteria*, and *Methanobrevibacter* (Fig. 5; Table S1). Compared to the Al group, significant increases in the relative abundances of Lactobacillaceae, *Lactobacillus*, *Pediococcus*, *Weissella* and Bacillaceae, and decreases in the abundances of Odoribacteraceae, Porphyromonadaceae, *Parabacteroides*, Erysipelotrichaceae, Lachnospiraceae, Bacteroidales, Rikenellaceae, Coriobacteriaceae, *Collinsella*, *Eggerthella*, and Verrucomicrobiaceae were observed in the Al + LP group (Fig. 5; Table S1).

3.2.3 Significant changes in the composition of intestinal microbiota

At the genus level, the abundances of *Bifidobacterium*, *Lactobacillus*, *Pediococcus*, *Streptococcus*, *Dialister*, *Roseburia*, and *Ruminococcus* spp. were significantly reduced in the Al only group, compared with the control, whereas the abundances of *Clostridium*, *Escherichia*, and *Erwinia* spp. were significantly increased (Fig. 6, $P < 0.05$). In the Al + LP group, *L. plantarum* CCFM639 administration significantly increased the abundances of *Lactobacillus* and *Pediococcus* ($P < 0.05$), to a greater extent than in the LP group. Moreover, *L. plantarum* CCFM639 treatment caused a large decrease in the abundance of *Streptococcus*, both alone and in the presence of Al ($P < 0.05$).

3.2.4 Impact of Al and CCFM639 on SCFA levels

Al exposure and *L. plantarum* CCFM639 treatments had little effect on levels of SCFA after 8 h, except for a decrease in acetate in all three treatment groups compared to the control (Fig. 7). After 24 h, however, the levels of acetate, butyrate, and propionate all decreased significantly in the Al-only group, whereas co-treatment with *L. plantarum* CCFM639 significantly alleviated the decrease of butyrate and propionate ($P < 0.05$). Interestingly, butyrate levels were also increased significantly by treatment with *L. plantarum* CCFM639 only at 24 h ($P < 0.05$).

3.2.5 Correlations of the gut microbiota with SCFAs

Pearson correlation analysis indicated negative correlations between levels of the three SCFAs and *Actinomyces*, Bacteroidetes, Bacteroidia, Bacteroidales, Enterobacteriaceae, Erysipelotrichales, *Erwinia*, *Escherichia*, Gammaproteobacteria, Odoribacteraceae, Parabacteroides, Porphyromonadaceae, *Prevotella*, Prevotellaceae, Proteobacteria, *Serratia*, Verrucomicrobia and Verrucomicrobiaceae

(Pearson rank correlation coefficient r: -0.50 to -0.95, Fig. 8). Positive correlations between the SCFAs levels and *Coprobacillus*, *Dialister*, *Roseburia*, *Ruminococcus*, *Streptococcus* and Streptococcaceae were also observed ($P < 0.05$; r: 0.49 to 0.99). Moreover, for Bacilli, Lactobacillales, Lactobacillaceae, *Lactobacillus* and *Pediococcus*, there were positive correlations with butyrate and propionate levels, but negative correlations with acetate level. However, *Collinsella* and Erysipelotrichceae showed positive correlations with acetate and butyrate levels. Interestingly, negative correlations between Bacilli, Lactobacillales, Lactobacillaceae, *Lactobacillus*, *Pediococcus*, Veillonellaceae, *Veillonella* and Bacteroidetes, Bacteroidia, Bacteroidales, Erysipelotrichaceae, Odoribacteraceae were also observed ($P < 0.05$).

4. Discussion

Previous studies have focused largely on Al toxicity to the liver, kidney, and brain in mice [34,21,30], but there have been few studies on the effects of dietary Al on the human gut microbiota at doses relevant to real-life exposure. However, the gut microbiota plays important roles in human physiology and metabolism, and Al-induced changes in these bacteria may be an important mechanism for Al toxicity. Thus, it is necessary to explore the effects of dietary Al on the human gut microbiota. Al has toxic effects on microorganisms, mainly via competition with Fe and Mg, and by binding to DNA, ATP enzymes, or enzyme substrates [35]. Analyses of pure cultures showed that the effects of Al on microorganisms were largely strain-dependent. A previous study also found an approximately 10-fold difference in Al resistance abilities among strains [30]. Strains of *L. rhamnosus*, *L. plantarum*, *E. coli*, *S. typhimurium*, and *Ba. thetaiotaomicron* were relatively resistant to Al at physiologically-relevant levels (86 mg/L). In contrast, *Bi. longum* was sensitive to Al, and its growth was significantly reduced. In contrast, Al promoted the growth of *C. perfringens*, possibly because Al can form a complex with superoxide to catalyze the reduction of Fe^{3+} to Fe^{2+} and promote the use of Fe [36]. Fe is a critically-important nutrient for *Clostridium* spp., which are strict anaerobes. These results were consistent with the results from intestinal microbiota analyses under *in vitro* colonic fermentation conditions. According to the results of our *in vitro* pure cultures and previous studies, we hypothesized that the ingestion of a low dose of Al would inevitably affect the gut microbiota [24,10]. The probiotic *L. plantarum* CCFM639 had an excellent Al-binding ability in our previous study [30]. Here, EM observation and EDX analysis confirmed that Al was bound to the surfaces of *L. plantarum* CCFM639

cells, thus reducing the Al concentration in the fermentation broth and reducing the toxic effects of this metal on the gut microbiota.

Batch cultures constitute the simplest forms of *in vitro* models used to study the human gut microbiota. These are usually composed of a single bioreactor vessel with basal media, incubated under constant physiological temperature (37 ^{o}C), pH (6.8), and anoxic atmosphere (N_2) with short period of incubation (usually 24-48 h) [37]. The reasons for short incubation time are nutrient depletion and accumulation of inhibitory bacterial metabolites, leading to a rapid progression to the stationary phase [38]. *In vitro* colonic models do not always provide accurate models of what occurs *in vivo*, as they lack an epithelial mucosa, host immunological interactions, and neuroendocrine system functionality [39]. However, they enable changes in the microbiota to be monitored, in terms of numbers and metabolism, attributable to the addition of exogenous substance, or disease state that is to be assessed. The single vessel batch culture in this study are a quick, simple, and cost-effective means of studying the gut microbiome [37]. They have been widely used in investigating the effects of probiotics, prebiotics or other food ingredients on the composition and metabolism of the human gut microbiota [29,40].

Alpha diversity analysis revealed no significant differences among the control, LP, Al and Al + LP groups in overall richness of gut microbiota. Possibly, a low-dose dietary Al exposure or ingestion of a single probiotic may not have a significant effect on the overall richness but may lead to changes in the relative abundances of specific families or genera [41]. Our results indicated an increase in abundances of the genera *Escherichia*, *Erwinia*, *Serratia*, *Collinsella*, *Prevotella*, *Clostridium*, *Methanobacteria*, and *Methanobrevibacter* and decreases in abundances of the genera *Streptococcus*, *Roseburia*, *Ruminococcus*, *Dialister*, and *Coprobacillus* in Al group. Similar changes were also observed in mice exposed to other toxic metals, such as Cd, Pb, and Cr [42]. Some changes in microbial abundance induced by Al exposure, namely in the phyla Bacteroidetes, Actinobacteria, and Verrucomicrobia and the family Lactobacillaceae, were restored to control levels by the addition of *L. plantarum* CCFM639 in the Al + LP treatment. Wu et al. reported that the probiotic *L. plantarum* TW1-1 could also modify Cr-induced changes in the structure of the gut microbiota through a process called ‘gut remediation’ [42].

In humans, *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Ruminococcus* spp. are the predominant genera of anaerobic bacteria in the gut microbiota, followed by facultative anaerobes such

as *Escherichia*, *Enterobacter*, *Enterococcus*, and *Lactobacillus*. An increase in the abundance of Proteobacteria is associated with disrupted anaerobiosis, an indicator of gut dysbiosis [43]. Al exposure dramatically increased the relative abundances of organisms in the phylum Proteobacteria, including the class Gammaproteobacteria, family Enterobacteriaceae, and genera *Escherichia*, *Erwinia*, and *Serratia*. Proteobacterial blooms have been observed in humans with low-level or severe intestinal inflammation, including those with inflammatory bowel disease (IBD), necrotizing enterocolitis, or irritable bowel syndrome [44]. High levels of the class Gammaproteobacteria have been observed in pregnant women with IBD and their newborns [45]. The abundance of Enterobacteriaceae, which has a relatively higher oxygen tolerance, is low in the gut. However, gut inflammation is particularly conducive to proliferation of Enterobacteriaceae [46]. *L. plantarum* CCFM639 administration partially counteracted the Al-induced increase in Enterobacteriaceae (Table 1), which may be due to *L. plantarum* CCFM639 secreting SCFAs that affect the growth of Enterobactericeae [47]. Wei et al., found that proliferation of the genus *Serratia* was related to downregulation of gut immune responses in fungus-infected mosquitoes [48]. *Serratia marcescens*, an opportunistic pathogen, was significantly more abundant in patients with Crohn's disease (CD) compared with healthy individuals [49]. The abundance of *Erwinia* spp. increased in patients with systemic sclerosis, an autoimmune gastrointestinal disease associated with high morbidity and mortality [50]. Another study demonstrated that Al had pro-inflammatory effects in both animals and humans [51].

The relative abundances of Porphyromonadaceae and Odoribacteraceae (Phylum Bacteroidetes) and of Coriobacteriia, Coriobacteriales, Coriobacteriaceae, and Collinsella (Phylum Actinomycetales) were significantly more abundant in the Al-only treatment compared with the control, but became less abundant after *L. plantarum* CCFM639 supplementation in the Al + LP treatment. The genus *Odoribacter* has been identified in the inflammatory processes associated with IBD, CD, ulcerative colitis, and colon cancer [52]. *Collinsella* spp. correlated strongly with the production of the proinflammatory cytokine IL-17A and chemokines [53]. In addition, an increased abundance of *Collinsella* may reduce tight junction protein expression and increase gut permeability, thus increasing transfer of toxic metals through the gut barrier. Furthermore, the families Bacteroidaceae, Prevotellaceae, and Rikenellaceae, and the genus *Prevotella*, were also in greater abundance in the Al-only group, compared with the control. Bacteroidaceae spp. are known to promote secretion of IL-17 by Th17 cells, thus triggering inflammatory responses [54]. An increase in the abundance of

Bacteroides may increase the secretion of the proinflammatory cytokines IL-6 and IL-23 [55]. The abundances of other members of the phylum Actinomycetales, namely Actinomycetales, Actinomycetaceae, and *Actinomyces*, were also in greater abundance in the Al-only group, compared with the control, while the abundance of *Eggerthella* was lower in the Al + LP group compared with Al only group. *Eggerthella* has been positively associated with the frailty index in elderly people [56]. Also, Wang et al. reported that a higher abundance of *Eggerthella* spp., which may be pathogenic, was related to abnormalities in glutamate and bile acid metabolism in the guts of autistic children [57]. Thus, *L. plantarum* CCFM639 may alleviate Al toxicity by regulating the abundances of Bacteroidetes and Actinomycetales spp.

Al exposure and *L. plantarum* CCFM639 administration had dramatic effects on the relative abundances of Firmicutes spp. Al exposure decreased the relative abundance of the phylum Bacilli, compared with the control, including the orders Lactobacillales, families Streptococcaceae and Lactobacillaceae, and the genus *Streptococcus,* many of which are considered beneficial. In contrast, *L. plantarum* CCFM639 treatment led to increases in Bacilli, including the orders Lactobacillales and Bacillales; family Lactobacillaceae; and genera *Lactobacillus*, *Pediococcus,* and *Weissella*. Abundances of the genera *Roseburia* and *Ruminococcus* were lower in the Al group compared with the control. *Roseburia* spp. produce SCFAs, particularly butyrate. A decrease in the abundance of *Roseburia* has often been associated with reduced production of SCFAs and negative effects on the gut microbiota [58]. Decreased relative abundances of *Veillonella* and *Streptococcus* have also been observed in the gut microbiota of patients with autism spectrum disorder, and *Streptococcus* has been negatively associated with inflammation [59]. The subclass Erysipelotrichia has been correlated positively with the levels of alpha tumor necrosis factors (TNF-α) and inflammation [60]. Therefore, Al exposure led to increases in the relative abundances of some harmful bacteria and decreases in the relative abundances of beneficial bacteria, whereas *L. plantarum* CCFM639 administration had the opposite effects and, when added together with Al, was often able to mitigate its effects.

Changes in gut microbiota composition induced by Al and *L. plantarum* CCFM639 led to changes in metabolite levels. We identified positive correlations between beneficial bacteria, such as *Dialister*, *Streptococcus*, *Roseburia*, and levels of SCFAs; and negative correlations between *Erwinia*, *Escherichia*, Prevotellaceae, *Serratia* and levels of SCFAs. These fatty acids exert anti-inflammatory functions on various gut immune cells [6], thus decreases in SCFA-producing species may induce a

shift to an inflammation-promoting microbiota. Various bacteria in the Phylum Firmicutes, including *Coprococcus*, *Roseburia*, *Ruminococcus* and *Dialister*, produce butyrate. Accordingly, a decrease in Firmicutes spp. decreases the production of butyrate [61]. Al exposure led to reductions in these SCFA-producing strains, which corresponded to reductions in SCFAs production. Consistent with our hypothesis, the levels of the three main SCFAs decreased significantly in the Al-only group, whereas co-*L. plantarum* CCFM639 administration significantly elevated the levels of butyrate and propionate. Therefore, *L. plantarum* CCFM639 counteracted the Al-induced changes in human gut microbiota possibly due to its Al binding ability and metabolites [24]. Initial Al sequestration of *L. plantarum* CCFM639 could lead to a decrease of Al level in intestine, thereby counteracting the Al-induced changes in gut microbiota. Moreover, some metabolites of CCFM639, such as SCFAs, may increase beneficial bacteria and decrease harmful bacteria, thus altering the composition of the gut microbiota and therefore the total bacterial metabolite profile.

5. Conclusions

In conclusion, daily dietary Al exposure affects the diversity and community structure of the human gut microbiota, leading to increases in the relative abundances of harmful bacterial species such as *Escherichia*, *Erwinia*, *Serratia* and Odoribacteraceae, and decreases in the abundances of beneficial bacterial species such as *Streptococcus*, Lactobacillales, and Veillonellaceae. The levels of SCFAs were reduced after Al exposure. However, inclusion of the probiotic *L. plantarum* CCFM639, which binds Al, mitigated some of the negative changes described above. *L. plantarum* CCFM639 may alleviate Al toxicity by regulating gut microbiota and the levels of SCFAs. This probiotic supplement is potentially a promising and safe approach to the alleviation of the harmful effects of daily dietary Al exposure.

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Competing interests

The authors declare that they have no competing interests.

Author Contributions

Conceptualization, Qixiao Zhai, Wei Chen and Arjan Narbad; Data curation, Leilei Yu, Hui Duan and Shi Cen; Funding acquisition, Fengwei Tian, Qixiao Zhai, Wei Chen and Arjan Narbad; Investigation, Leilei Yu and Qixiao Zhai; Methodology, Leilei Yu, Melinda Mayer and Gwénaëlle Le Gall.; Project administration, Lee Kellingray, Melinda Mayer and Arjan Narbad; Supervision, Fengwei Tian, Jianxin Zhao, Hao Zhang; Writing - original draft, Leilei Yu and Hui Duan; Writing - review & editing, Leilei Yu, Hui Duan, Melinda Mayer, and Arjan Narbad.

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670 **Table 1.** Effects of Al and *L. plantarum* CCFM639 on the predominant microbiota in a colonic fermentation model

Predominant microbiota	Fold of control, ±SD							
	8 h				24 h			
	Control	LP	Al	Al + LP	Control	LP	Al	Al + LP
Total anaerobes	1.00 ^{a}	1.07±0.10 ^{a}	0.88±0.09 ^{a}	1.07±0.23 ^{a}	1.00 ^{A}	0.99±0.18 ^{A}	0.97±0.17 ^{A}	1.04±0.13 ^{A}
Bacteroides	1.00 ^{a}	1.04±0.09 ^{a}	1.16±0.23 ^{a}	1.05±0.10 ^{a}	1.00^{A}	0.99±0.11^{A}	1.39±0.19^{B}	1.03±0.16^{A}
Bifidobacterium	1.00^{a}	1.14±0.06^{b}	0.20±0.04^{c}	0.52±0.07^{d}	1.00^{A}	0.98±0.10^{A}	0.31±0.05^{B}	0.46±0.07^{B}
Clostridium	1.00^{a}	0.94±0.17^{a}	4.94±0.66^{b}	1.09±0.16^{a}	1.00A	0.62±0.07^{B}	3.14±0.24^{C}	0.95±0.11^{A}
Lactobacillus	1.00 ^{a}	0.99±0.07 ^{a}	0.91±0.10 ^{a}	1.01±0.06^{a}	1.00 ^{A}	0.94±0.04 ^{A}	0.97±0.02 ^{A}	0.98±0.14 ^{A}
Enterobacteriaceae	1.00 ^{a}	1.05±0.13 ^{a}	1.23±0.10 ^{a}	0.99±0.23 ^{a}	1.00^{A}	0.92±0.05^{A}	1.47±0.15^{B}	1.15±0.12^{A}

671 Different superscript letters a-d and A-D indicate statistically significant differences among the four groups after 8 and 24 h, respectively ($P < 0.05$).
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Figure Legends

Figure 1. The flow chart of the *in vitro* colonic fermentation models.
A: The illustration of colonic fermentation model. B: The flow diagramme indicating sampling points and analysis. Co., control; LP, *L.plantarum*; Al, Aluminium

Figure 2. Effects of Al exposure on the growth of representative strains in pure *in vitro* culture.

The asterisks indicate a statistically significant difference relative to the control group ($P < 0.05$).

Figure 3. Scanning electron microscopy (SEM), transmission electron microscopy (TEM), and energy dispersive X-ray (EDX) analyses of *L. plantarum* CCFM639 before and after Al binding.
A, C, and E depict the SEM, TEM, and EDX results of the untreated biomass. B, D, and F depict the SEM, TEM, and EDX results of biomass after Al binding. Scale bar = 100 nm.

Figure 4. Effects of Al and *L. plantarum* CCFM639 supplementation on gut microbiota diversity and relative abundance.
A: Shannon index analysis of microbial alpha diversity. B: Principal coordinates analysis (PCoA) of differences in the microbial community structures among the four groups. C: Relative abundances of the gut microbiota at the phylum level after 8 and 24 h, respectively. D: Relative abundances of the gut microbiota at the class, family and genus levels after 8 and 24 h, respectively.

Figure 5. Comparison of different compositions of gut microbiota after Al exposure and *L. plantarum* CCFM639 supplementation based on a LefSe analysis.
A and C: Circular cladograms of statistically significant differences in the gut microbiota between the control and Al group at 8 and 24 h, respectively. In the panel, the diameters of the circles exhibit positive correlations with the relative abundances. Green, red, and yellow circles indicate microbial species that are significantly enriched in the control or Al group or are not significantly affected, respectively. p, phylum; c, class; o, order; f, family; g, genus. a and c: Histograms of LDA scores for statistically significant differences between the control group (green bars) and Al group (red bars) at 8 and 24 h, respectively. B and D: Circular cladograms comparing the Al group and Al + LP group at 8 and 24 h, respectively. b and d: Histograms for the Al group and Al + LP group at 8 and 24 h, respectively.

Figure 6. Effects of Al and *L. plantarum* CCFM639 treatment on the relative abundances of specific gut bacteria.

Different letters indicate statistically significant changes among the four groups ($P < 0.05$).

Figure 7. Effects of Al exposure and *L. plantarum* CCFM639 on short-chain fatty acids based on metabolomic analysis.
Letters (a-b and A-D) indicate statistically significant changes between the four groups at 8 and 24 h, respectively ($P < 0.05$).

Figure 8. Correlation between abundances in the gut microbiota and changes in SCFAs.
The colors and values indicate the distribution of Pearson's correlation coefficients. Significant negative and positive correlations are represented by red and blue circles, respectively ($P < 0.05$). The intensity of the color represents the strength of the correlation.