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Effect of α-linolenic acid (ALA) on proliferation of probiotics and its adhesion to colonic epithelial cells

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Abstract

The effects of α-linolenic acid (ALA) on the proliferation and adhesion of probiotics would be investigated in the present study. Effects of ALA on intestinal flora were studied by animal fecal anaerobic fermentation system *in vitro*, which were analyzed by high-throughput sequencing. Results showed that treatment with ALA could promote the proliferation of probiotics *Lactobacillus* and *Bifidobacterium*, and inhibit the growth of harmful bacteria *Enterococcus* and *E. coli*. ALA restored the abnormal intestinal flora caused by high-fat diet, which was beneficial to the improvement of intestinal flora structure. In addition, adhesive characteristics of probiotics to epithelial colon cells NCM460 were detected by plate counting and Gram staining, which indicated that ALA promoted adhesion of probiotics to colonic cells. In conclusion, ALA could promote the proliferation and adhesion of intestinal probiotics, which provides a basis for ALA to exert the healthy activities of intestinal probiotics.

Keywords: α-linolenic acid (ALA); high-fat diet; probiotics; proliferation; adhesion.

Practical Application: As a functional substance promoting the health of the intestinal micro-ecosystem.

1 Introduction

α-linolenic acid (ALA), as the sole dietary source of plant-derived ω-3 polyunsaturated fatty acids, has been extensively studied (Paschos et al., 2007; Salem Junior & Eggersdorfer, 2015). Studies have shown that ALA has a strong effect on reducing risks of chronic diseases such as atherosclerosis, thrombosis and hyperlipidemia (Rodriguez-Leyva et al., 2010; Zhao et al., 2004; Winnik et al., 2011). Recently, dietary supplementation with ALA has been shown to improve colonic inflammation of experimental colitis rats (Shimizu et al., 2007; Wen et al., 2019).

As to the intestinal micro-ecology, formation of the stable and diverse ecosystem of microorganisms is closely related to intestinal health, including metabolic syndrome, colon cancer, obesity and diabetes (Tremaroli & Bäckhed., 2012; Delzenne et al., 2011; Korem et al., 2015; Erdman & Poutahidis., 2015), and the dysbiosis of gut microbes was closely associated with obesity (Cotillard et al., 2013). In recent years, studies have shown that probiotics play a beneficial role in intestinal health by inhibiting growth of harmful bacteria and enhancing host intestinal mucosal barrier (Liu et al., 2016). *Bifidobacterium bifidum* and *Lactobacillus acidophilus* have been proved to be the important probiotics, and adhesion of probiotics is a necessary prerequisite for probiotics to play their physiological functions (Zhang, 2019; Bai et al., 2012; Kotzamanidis et al., 2010).

However, effects of ALA on intestinal micro-ecology of animals on high-fat-diet, and adhesion ability of probiotics has not yet been reported. Thus, effects of ALA on intestinal flora of rats on high-fat-diet would be investigated in this study exploiting the *in vitro* animal fecal anaerobic culture system, as well as the effect of ALA on adhesion of probiotics with colonic epithelial cells NCM460. The present study will provide experimental evidence for candidates of ALA as a functional food ingredient to improve the intestinal health.

2 Materials and methods

2.1 Materials

ALA standard (purity 98%) was purchased from Harvey Biotechnology Co., Ltd Co., LTD (Beijing). Mediums (LBS, BS, EMB, MRS or Enterococcus agar medium) were purchased from Bowei Biotechnology Co., Ltd (Shanghai). NCM460 cells were purchased from Guangzhou Biotechnology Co., Ltd. Bacterial (*Lactophilus acidophilus* (AS1.2686) or *Bifidobacterium bifidum* (CDMCCL-1.324)) was obtained from Guangzhou Microbial Strain Collection Center. Cell culture medium and trypsin were purchased from Thermo Fisher Scientific (Beijing) Co., Ltd.

2.2 In vitro animal fecal anaerobic fermentation system experiment

Twenty SD rats on normal diet or high-fat diet were supplied by Shanghai Slack Laboratory Animal Co., Ltd. Fecal specimens were collected, which were dissolved with sterile normal saline and filtered by vortex. The concentration of fecal bacteria was adjusted to 10^{-1}.

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According to methods described in our previous studies (Wang et al., 2021), fecal bacteria, BHI broth, and ALA samples (1:7:2) were mixed and cultured for 48 h at 37 °C in the anaerobic glove box, with the gas of N_2, CO_2 and H_2 (85: 10: 5). The number of bacteria in the fermentation broth with selective medium agar was determined by colony counting method. *E. coli* or *Enterococcus* was cultured in the incubator for 24-36h. *Lactobacillus* or *Bifidobacterium* was cultured under anaerobic conditions for 36-48h.

2.3 Analysis of gut microbiota of animal fecal anaerobic fermentation system

Following the above *in vitro* fermentation experiment, total DNA of bacteria were extracted by TIA Namp Stool DNA Kit (Tiangen Biotech Co. Ltd., Beijing) and were stored at -20 °C. 16S rRNA sequencing of the samples was performed by Shanghai Gensky Biotechnology Co., Ltd.

2.4 Effects of ALA on probiotics adhesion with NCM460 cells

The probiotics (*Bifidobacterium bifidum* or *Lactobacillus acidophilus*) were cultured and activated in the nutrient medium for 48 h in an anaerobic work station. Then the probiotics were collected by centrifuge and washed with PBS for three times. The concentration of probiotics was diluted to 1×10^8 CFU/mL by DMEM medium.

ALA (5, 25, 50 μg/mL) and NCM460 cells were mixed and cultured in an incubator at 37 °C, 5% CO_2 atmosphere for 24 h. Then the supernatant was discarded and washed twice with PBS. DMEM culture medium (without antibodies) and the probiotic suspension were added to each well and were cultured for 1-2 h. The adhesive number and adhesive rate of probiotics were calculated according to methods described in our previous studies (Wang et al., 2021). Adhesive number (CFU/cell) = Number of adherent colonies in culture plate/ Number of cells in culture. Adhesive rate (%) = $(N_1/N_0) \times 100\%$, Where N_1 represents number of post-adhesion colonies, and N_0 represents the number pre-adhesion.

2.5 Statistical analysis

The data were represented as mean ± s.d and analyzed by one-way of variance (ANOVA). Tukey test via the SPSS software to compared. GraphPad Prism was used for figures production.

3 Results and discussion

3.1 Effect of ALA on intestinal flora of animal fecal anaerobic fermentation system

Effects of ALA on population of four kinds of bacterial strain in rat fecal anaerobic fermentation system were explored, which were harmful bacteria (*E. coli* and *Enterococcus*) and probiotics (*Lactobacillus* and *Bifidobacte*). As shown in Table 1, compared with normal-diet control, a significant increase in the number of *E. coli* and *Enterococcus* in high-fat-diet group, and the number of *Lactobacillus* markedly decreased. Treatment with ALA improved population of probiotics (*Bifidobacteria* or *Lactobacillus*), and decreased the population of harmful bacteria *(E. coli and Enterococcus*) significantly. Therefore, treatment with ALA has a positive effect on the proliferation of probiotics.

3.2 Effect of ALA on gut microbiota of rat fecal anaerobic fermentation system

Microbiota diversity analysis

Alpha diversity and richness indexes (*Chao-1*, *ACE*, *Shannon* and *Simpson*) were used to evaluate diversity of intestinal bacteria of rat feces fermented *in vitro* (Table 2), which showed that

Table 1. Effect of ALA on population of intestinal bacteria of rat fecal fermentation *in vitro*.

Groups	Dose (μg/mL)	*Bifidobacterium*	*Lactobacillus*	*Enterococcus*	*E. coli*
Normal-diet	0	10.980 ± 0.038	10.782 ± 0.086	8.563 ± 0.199	10.613 ± 0.083
ALA	5	11.084 ± 0.150	11.031 ± 0.116*	8.535 ± 0.161	10.402 ± 0.029*
	25	11.437 ± 0.287*	11.193 ± 0.159*	7.943 ± 0.295*	10.812 ± 0.031
	50	11.241 ± 0.226*	11.138 ± 0.293*	8.594 ± 0.153	10.522 ± 0.157
High-fat-diet	0	10.965 ± 0.031	10.554 ± 0.012*	9.224 ± 0.161*	11.561 ± 0.407*
ALA	5	11.022 ± 0.100	10.703 ± 0.151	8.678 ± 0.260#	10.546 ± 0.043#
	25	11.228 ± 0.056#*	11.100 ± 0.260#	8.896 ± 0.122#	11.046 ± 0.426
	50	11.024 ± 0.029	10.852 ± 0.009#	8.373 ± 0.105#	10.556 ± 0.026#

Compared with normal-diet control group, *P < 0.05; Compared with High-fat-diet control group. #P < 0.05.

Table 2. Effect of ALA on Alpha diversity of gut microbiota.

Groups	Dose (μg/ mL)	Chao1	ACE	Shannon	Simpson
Normal-diet	0	95.20 ± 24.04	88.14 ± 20.71	2.17 ± 0.06	0.16 ± 0.01
ALA	5	70.74 ± 6.84	77.09 ± 5.91	2.24 ± 0.01	0.15 ± 0.01
High-fat-diet	0	85.60 ± 11.74	85.49 ± 10.62	2.11 ± 0.09	0.21 ± 0.02*
ALA	5	89.17 ± 16.10	92.01 ± 14.98	2.07 ± 0.08	0.22 ± 0.01**

Compared with Normal-diet control group, *P < 0.05; **P < 0.01.

treatment with ALA significantly influenced Simpson values and the diversity. Results of Figure 1 showed that high-fat diet or intervention of ALA affected the structure of intestinal flora. What's more, compared with high-fat-diet control group, ALA treatment significantly increased the relative abundance of bacteria (*Lachnospiraceae* or *Porphyromonadaceae*) (Figure 1 D), which indicated that intervention of ALA would be beneficial to the improvement of intestinal flora structure.

Intestinal Microbial composition analyzed at the level of phylum and genus

As shown in Figure 2, the intestinal microorganisms were mainly composed of *Bacteroidetes*, *Proteobacteria* and *Firmicutes* analyzed at the phylum level. Compared with normal-diet control group, a higher abundance of *Proteobacteria* was observed in high-fat-diet control group. Compared with the high-fat-diet group, *Firmicutes* showed a relatively lower abundance, while *Bacteroidetes* showed a relatively higher abundance in ALA treatment group. The ratio of *Firmicutes/Bacteroidetes (F/B)* is an important indicator of evaluating the structure of intestinal flora (Turnbaugh et al., 2009), and studies have shown that intestinal flora *F/B* ratio of obese mice was lower than that of normal mice (Wang et al., 2015; Armougom et al., 2009).

Analyzed at the level of Genus, intestinal microbiota was mainly composed of *Escherichia/Shigella*, *Bacteroides*, *Clostridium_XIVa*, *Parabacteroides*, *Enterococcus*, *Phascolarctobacterium*, *Proteus*, *Flavonifractor*, *Lactobacillus*, *Anaerostipes* and *Parasutterella* (Figure 3). Compared with normal-diet control group, the

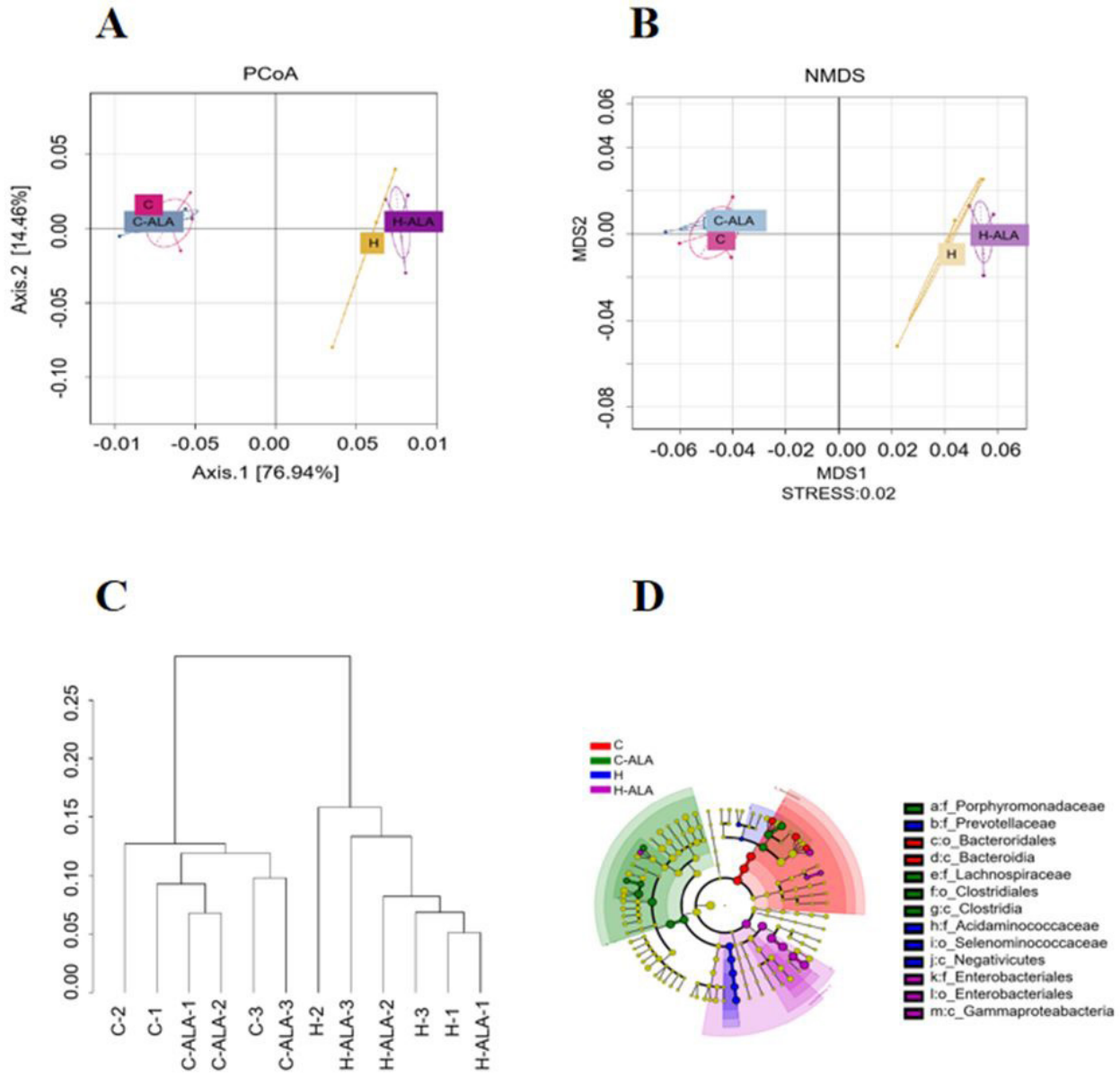

Figure 1. Effects of ALA on the diversity of gut microbiota. (A) Weighted UniFrac distances PcoA; (B) NMDS non-metric multidimensional scaling analysis; (C) Cluster analysis. (D) LEfSe Analysis.

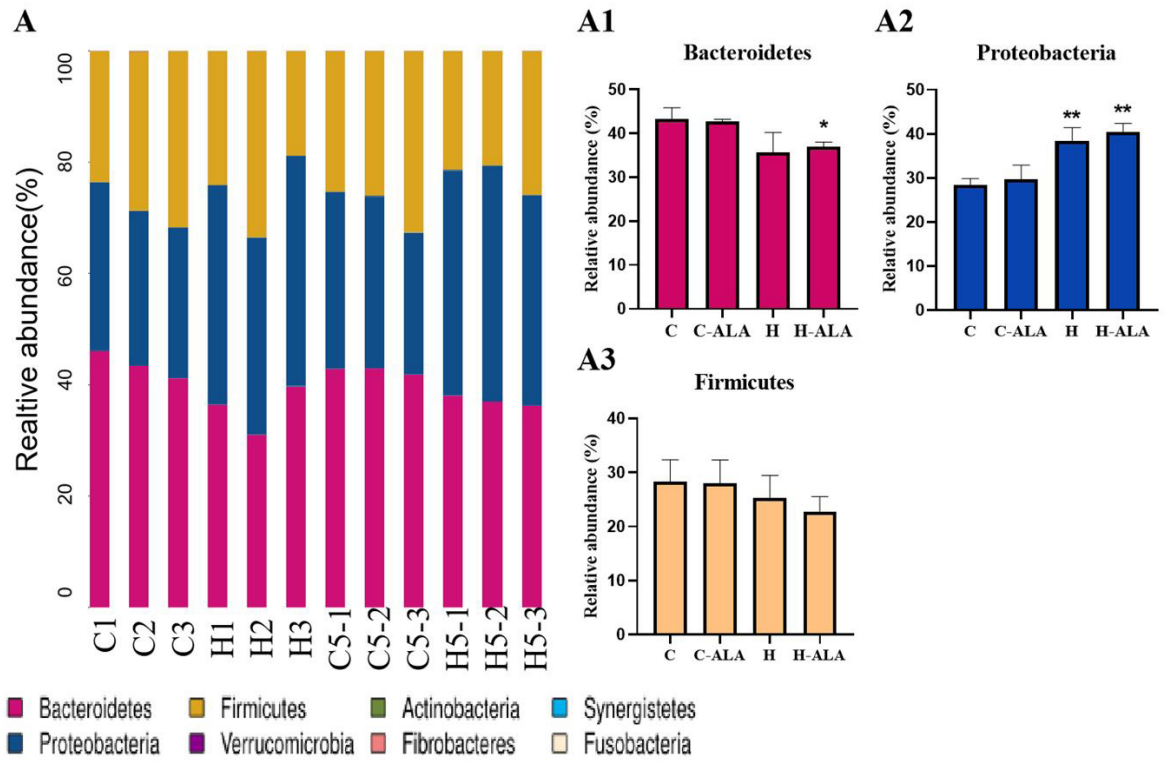

Figure 2. Effects of ALA on composition of gut microbiota analyzed at the phylum level. (A) Stacked histogram of microbiological composition. The relative abundance of *Bacteroidetes* (A1), *Proteobacteria* (A2), and *Firmicutes* (A3). C: Normal-diet Control group; C-ALA: Normal-diet Control + ALA (5 μg/mL) treatment group; H: High-fat-diet control group; H-ALA: High-fat-diet + ALA (5 μg/mL) treatment group. *$p < 0.05$, **$p < 0.01$, compared with control group.

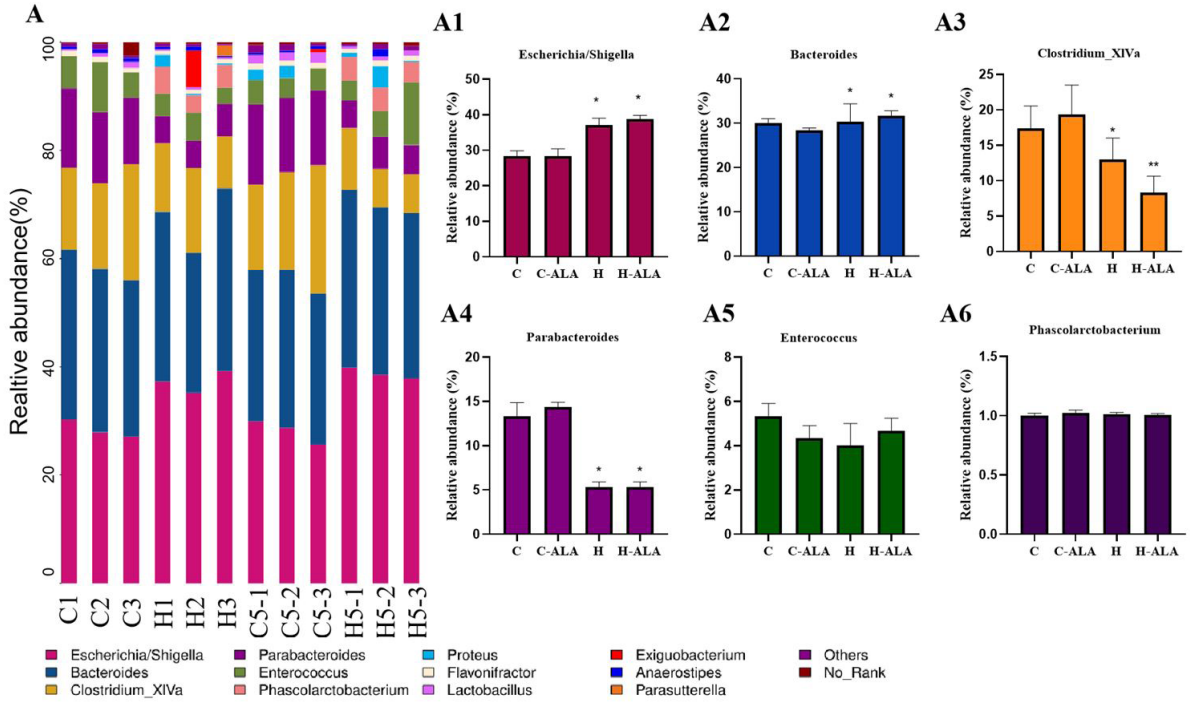

Figure 3. Effects of ALA on composition of gut microbiota analyzed at the genus level. (A) Stacked histogram of microbiological composition. The relative abundance of *Escherichia / Shigella* (A1); *Bacteroides* (A2); *Clostridium_XIVa* (A3); *Parabacteroides* (A4); *Enterococcus* (A5); *Phascolarctobacterium* (A6). *$p < 0.05$, **$p < 0.01$, compared with the control group.

relative abundance of *Enterococcus* apparently decreased as of ALA intervention. Compared with high-fat-diet control group, treatment with ALA increased the relative abundance of *Lactobacillus* and *Phascolarctobacterium*, and decreased the relative abundance of *Clostridium_XIVa*.

Above all, the shifts of relative abundance of microbiota were induced by ALA treatment, promoting the proliferation of intestinal probiotics while inhibiting the growth of harmful intestinal bacteria, and leading to the improvement of intestinal flora structure.

3.3 Effect of ALA on adhesive ability of probiotics with colonic cells NCM460

Adhesion of probiotics with colonic epithelial cells is an important step in colonization (Xu et al., 2019). As shown in Figure 4 and Figure 5, the adhesion of probiotics to colonic cells NCM460 was observed by gram staining. Probiotics (*Lactobacillus acidophilus* or *Bifidobacterium bifidum*) adhered to the colonic epithelium around the cells and appeared as rods. Compared with normal-diet control, treatment with ALA markedly increased the adhesive activity of *Lactobacillus acidophilus* or *Bifidobacterium bifidum* in a dose-dependent manner ($P < 0.01$), which were consistent with the results of the adhesive number or adhesive rate in Table 3 and Table 4. Therefore, treatment

Table 3. Effects of ALA on adhesive ability of *Lactobacillus acidophilus* with NCM460 cells.

Dose of ALA (μg/mL)	Adhesive number / (CFU/cell)	Adhesive rate (%)
0	3.627 ± 0.960	0.91
5	3.787 ± 0.380	0.95
25	4.020 ± 1.047	1.01
50	6.473 ± 0.405**	1.62

Compared with Normal control group; **P < 0.01.

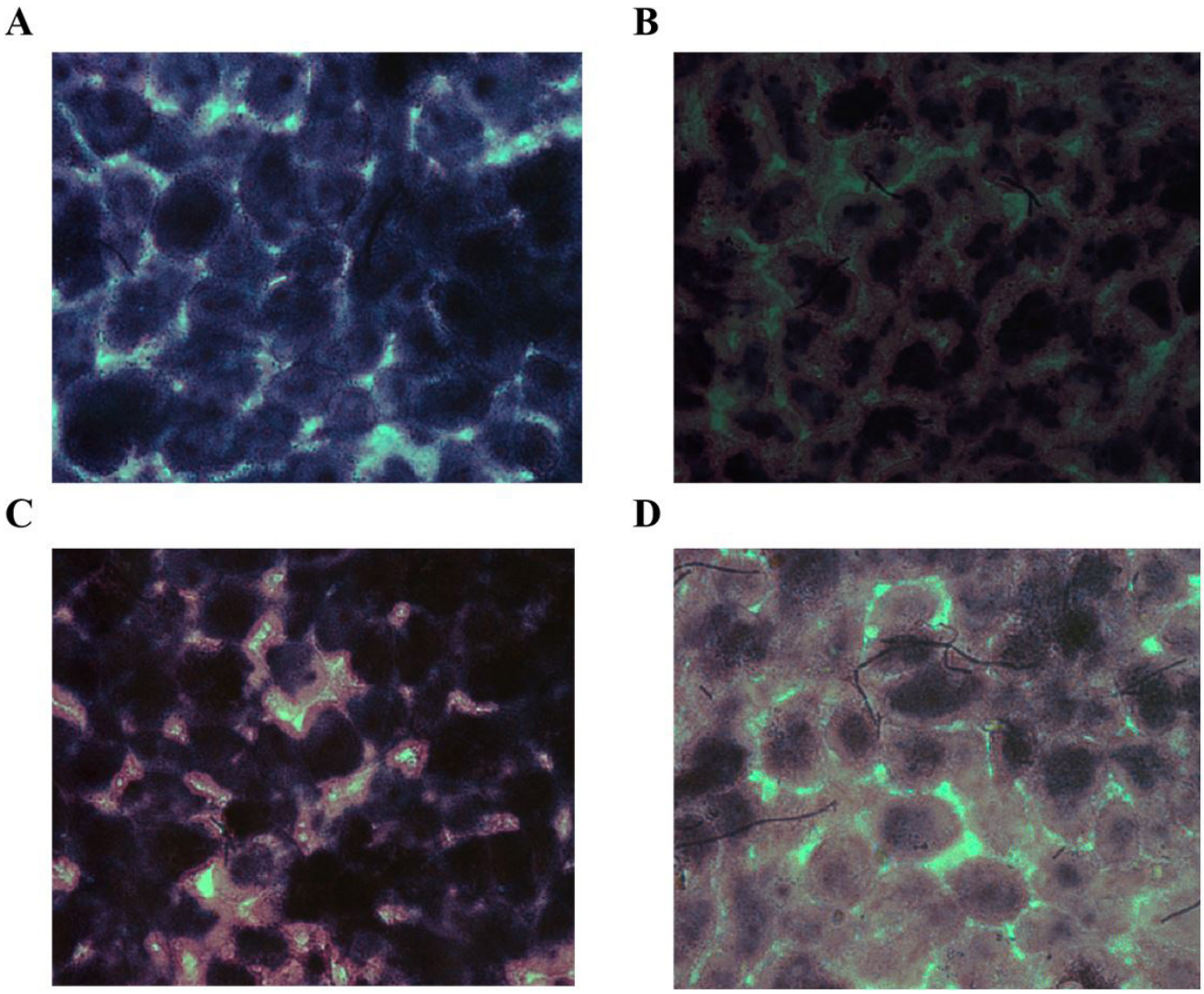

Figure 4. Effects of ALA on the adhesive ability of *Lactobacillus acidophilus* with NCM460 cells (Gram staining, 1000×). (A) Normal control group; (B) ALA (5 μg/mL) group; (C) ALA (25 μg/mL) group; (D) ALA (50 μg/mL) group.

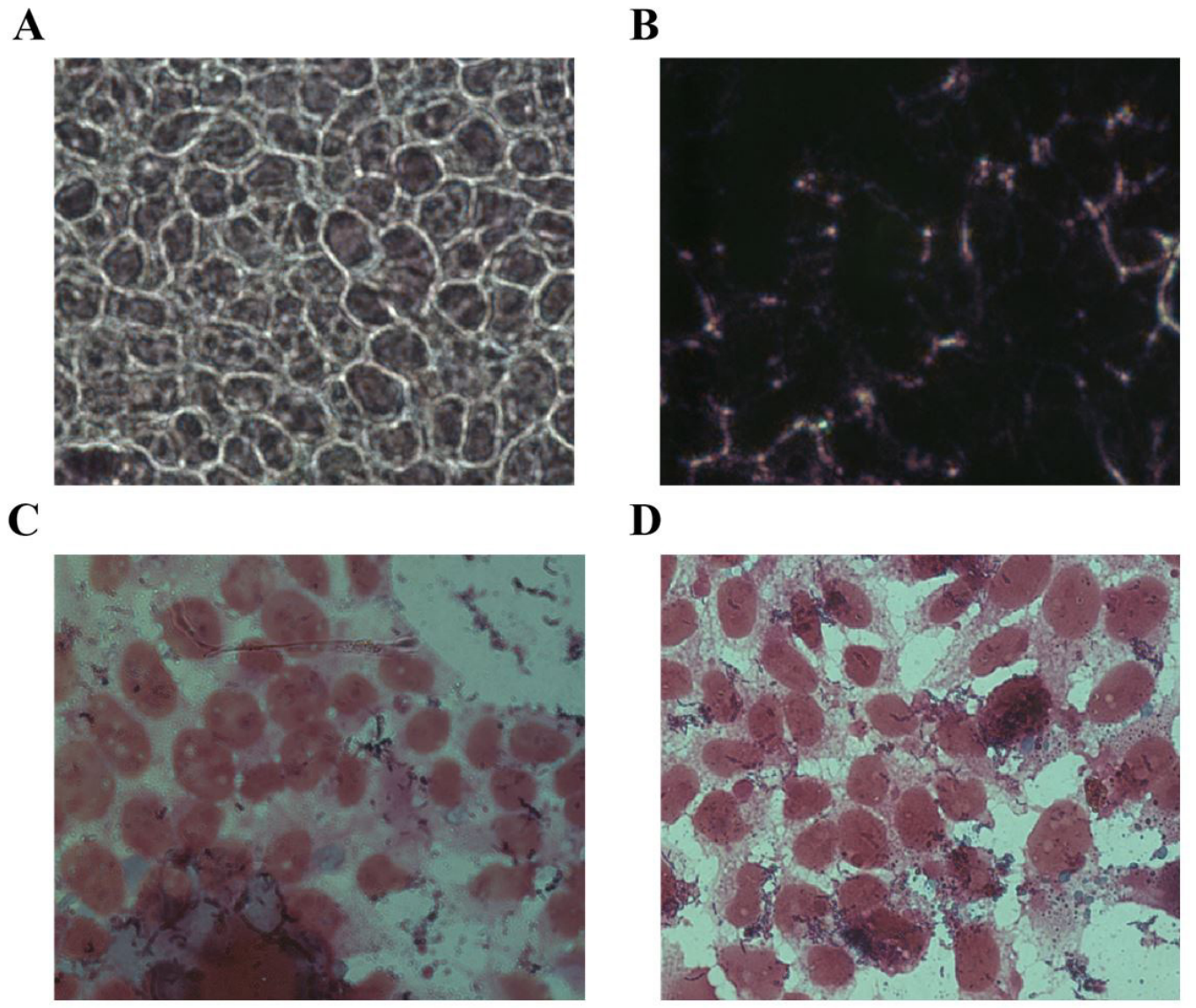

Figure 5. Effects of ALA on adhesive ability of *Bifidobacterium bifidum* to NCM460 cells (Gram staining, 1000×). (A) Normal control group; (B) ALA (5 μg/mL) group; (C) ALA (25 μg/mL) group; (D) ALA (50 μg/mL) group.

Table 4. Effect of ALA on adhesive ability of *Bifidobacterium bifidum* with NCM460 cells.

Dose of ALA (μg/mL)	Adhesive number / (CFU/cell)	Adhesive rate (%)
0	2.425 ± 0.318	0.61
5	3.460 ± 2.065	0.87
25	5.160 ± 0.085**	1.29
50	5.470 ± 0.806**	1.37

Compared with Normal control group; **P < 0.01.

with ALA could promote the adhesion of probiotics to colonic epithelial cells NCM460 cells, which indicated that ALA could promote the healthy function of probiotics.

4 Conclusion

Effects of α-linolenic acid (ALA) on proliferation and adhesion of intestinal probiotics were investigated in the present study. Results showed that ALA promoted proliferation of probiotics *Lactobacillus acidophilus*, and inhibited the population of *E.coli* at a certain concentration range markedly. According to the analysis of microbial composition, treatment with ALA increased the relative abundance of *Lactobacillus* or *Phascolarctobacterium*, while reduced the relative abundance of *Clostridium_XIVa* and *Enterococcus*, which indicated that ALA was beneficial to improve the intestinal flora structure. Moreover, ALA promoted adhesion of probiotics with colonic epithelial cells NCM460 dose-dependently, which made probiotics play the healthy function more effectively. This study provides the experimental basis for

the future research on the beneficial effect of ALA on intestinal micro-ecology.

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