Immune modulation effects and safety of Lactobacillus casei variety rhamnosus in a chemotherapy-induced intestinal mucositis mouse model

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Research article

Keywords: intestinal mucositis, chemotherapy, probiotics, immune modulation, enterocytes, intestinal stem cells

Posted Date: March 30th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-19846/v1

Abstract

Background Intestinal mucositis remained one of the most deleterious side effects in cancer patients undergoing chemotherapy. 5-Fluorouracil (5-FU) treatment was reported to affect the abundance of gut microbiota. In this study, we hypothesize that the probiotics could preserve gut ecology, ameliorate inflammation and protect epithelium by maintaining the tight junction integrity via immune modulations of enterocytes and intestinal stem cells. Our aim is to characterize these changes and to investigate the immune modulation effects and safety of probiotic via a 5-FU-induced intestinal mucositis mouse model.

Methods 5-FU-injected BALB/c mice were used. They were either orally administrated saline or probiotic suspension of Lactobacillus casei variety rhamnosus (Lcr35). Diarrhea score, serum pro-nflammatory cytokines, intestinal histology and T-cells subtypes were assessed. Immunostaining analysis for intestinal stem cells CD44 and Ki67 proliferation were processed. Samples of blood and internal organs were investigated for bacteria translocation.

Results Diarrhea was attenuated significantly after oral Lcr35 administration. Serum pro-inflammatory cytokines were significantly increased in 5-FU group and were reversed by Lcr35. There was a tremendous rise of CD3+/CD8+ count in the 5-FU group. The CD8+ count was reversed in the 5-FU+Lcr35 group. 5-FU caused a significant decrease of CD3+CD4+/CD3+CD8+ ratio and was reversed by Lcr35. 5-FU significantly stimulated the expression of CD44 stem cells and was restored by Lcr35. We also found 5-FU could increase the number of Ki67 proliferative cells. No bacterial translocation was found in this study.

Conclusions Our results showed 5-FU caused intestinal inflammation via Th1 and Th17 responses. 5-FU could stimulate stem cells and proliferation cells in a mouse model. We demonstrated chemotherapy could decrease immune competence. Probiotics were shown to modulate immune response. This is the first study to analyze the immune modulation effects and safety of Lactobacillus strains on enterocytes and intestinal stem cells in a 5-FU-induced mucositis mouse model. The model therefore seems well suited to study the effects of different probiotics on chemotherapy-induced mucositis, prior to performing clinical human studies.

Background

Mucositis is a common and clinically significant side effect of chemotherapy that can affect any portion of the gastrointestinal tract. The incidence of chemotherapy-induced mucositis has been reported as 50–80% of patients treated with high-dose chemotherapy [1, 2, 3] Intestinal mucositis can cause treatment delays, interruptions of anticancer drugs and increased complication rates. [1] In 2014, the Multinational Association of Supportive Care in Cancer/International Society of Oral Oncology published an updated clinical practice guideline for mucositis and was considered as the keystone of prevention and treatment of mucositis. [4] However, managements of intestinal mucositis remain mostly symptomatic at present. [5]

In recent years, probiotics had been demonstrated therapeutic effects in clinical diseases such as inflammatory bowel disease and chemotherapy-induced mucositis. Because commensal bacteria play pivotal roles in both the innate and adaptive immune systems of the host, intestinal dysbiosis is considered part of the reasons in the pathophysiology of chemotherapy-induced mucositis. [6, 7] Therefore, normalization of intestinal homeostasis could be an appropriate strategy to improve the status of patients receiving chemotherapy. In recent years, the use of probiotics to alleviate damage to intestinal mucosa has been supported by clinical consensus. [8] Besides, we previously discovered that various Lactobacillus strains could relieved the intestinal barrier damages induced by Salmonella lipopolysaccharide. [9] We also demonstrated Lactobacillus strain and mixture of Lactobacillus and Bifidobacterium strains could attenuate inflammation and protect epithelium by maintaining the tight junction integrity and reduce the severity of 5-FU-induced intestinal mucositis in a mouse model. [10] Much progress has been made in recent years in terms of understanding of the pathological and signaling alterations occurring in the gut subsequence to chemotherapy treatment. [11] Recently we also successfully demonstrated that gut microbiota of mice undergoing chemotherapy exhibited a distinct disruption in bacterial composition. Probiotic did modulate the abundance and diversity of gut microbiota. [12]

In this study, we hypothesize that the probiotics could preserve gut ecology, ameliorate inflammation and protect epithelium by maintaining the tight junction integrity via immune modulations of enterocytes and intestinal stem cells. Our aim is to characterize these changes and to investigate the immune modulation effects and safety of probiotic via a 5-FU-induced intestinal mucositis mouse model.

Methods

5-FU treatment

5-FU (Fluorouracil-TEVA®, Netherland) was injected intraperitoneally (IP) at a single dose of 30 mg/kg/day at the first day to cause intestinal mucositis and diarrhea as described in our previous study. [10] IP saline was injected for alternative in control group. Body weight changes and diarrhea score were recorded and assessed daily and the results were compared. We used Bowen's score system to assess diarrhea severity. [13] Severity was classified into four grades according to the stool consistency.

Probiotic preparation

Lactobacillus casei variety *rhamnosus* (Lcr35, Antibiophilus®, France) (1×10^7cfu) was used in this experiment. Probiotic was diluted in sterile saline and administered by oral gavages as described in our previous research. [10] The mice received 100 µL of saline or suspension containing $1x10^7$ CFU of the probiotic daily for 5 days. This probiotic strain was chosen because it is widely used clinically in chronic gastrointestinal disorders in our country and shown promising results in maintaining tight junction integrity in our previous study. [9]

Animal trial

Male Balb/c mice were used in our experiments. They were obtained from Taiwan's National Laboratory Animal Center under a 12h light/dark cycle with a temperature of 22±1℃ and a humidity of 55±10%. All mice were given *ad libitum* access to autoclaved food (Laboratory autoclavable rodent diet 5010) and water. The mice were at the age of about 6 weeks with weight 24±3gm and were randomly assigned as four groups (n=4-5). The mice were injected saline or 5-FU IP at the first day. Mice in each control group and experimental group were then orally administrated saline or probiotic suspension of Lcr35 daily. Body weight was measured daily. On day 5 post-treatment, mice were submitted to euthanasia for blood sampling. Mice were treated by inhaled anesthesia by using 2-5% isoflurane for 3 mins. The anesthetized mice were confirmed by pressed toe for no reflex action. Then the mice were treated by cardiac puncture. After the maximal volume of blood was collected, the mice were treated for cervical dislocation to assure death. The whole **euthanasia/sacrifice method** followed the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition. It addresses the welfare concerns of those who fear that the collection of tissues (in particular, animal blood by intracardiac puncture) from live animals in the immediate postslaughter period creates undue suffering. Although the heart may continue to beat (which is necessary for the successful collection of fetal blood), in the absence of breathing there is little likelihood of return to a state of consciousness.

Ethics Statement

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of MacKay Memorial Hospital (MMH-A-S-105-26). IACUC has been accredited, approved and authorized by government office, Agriculture and Food Agency Council of Agriculture, Executive Yuan, Taiwan. All methods were performed in accordance with the relevant guidelines and regulations in this animal study.

Cytokines and flow cytometry analysis

Blood samples were collected and centrifuged from the heart after sacrifice. The serum was analyzed by the Bio-Plex Pro^{TM} Mouse Cytokine multi-Plex Panel kit (Bio-Rad Laboratories. Inc. United States). Targets of cytokines included IL-1β, IL-4, IL-6, IL-17A, IFN-γ, MCP-1 and TNF-α. The results were expressed as pg/ml.

To evaluate subtypes of T-cells, the peripheral blood monocyte cells were collected from whole blood by using BD FACS™ Lysing Solution. Then the blood cells were calculated by complete blood cell count (HEMAVET®). A total of 3X105 cells were washed with PBS and re-suspended in 1 ml PBS. The suspension (50 ml) was incubated with anti-CD3-PE, CD4–FITC, CD8-PerCP-Cy5.5 (BD Pharmingen™, CA) for 30 min at 4°C, and then washed with cold PBS. After incubation, cells were post-fixed and permeated with 300 µl of Cell Fix 1× (BD Cytofix and Cytoperm) and kept at 4°C in the dark. The cells were re-suspended in 1 ml PBS. Then, the cells were stained by IL-4-PE and IL-17A-PE for 30 min at 4°C, and then washed with cold PBS. Data were recorded using a BD FACSCalibur and analyzed using the BD CellQuest Pro software (both Becton Dickinson, NJ, United States). The results were multiplied by percentage of T-cell subtypes and quantity of leukocytes.

Histological analysis for villus height, crypt depth and goblet cells

Jejunum specimens with 2-cm ring each were collected after sacrifice and were processed and fixed in 10% buffered neutral formalin. Sections were routinely haematoxylin-eosin stained for tissue morphology. Periodic acid-Schiff and Alcian blue (PAS+AB) stained for goblet cells were expressed as the number of goblet cells per villus-crypt. Specimens were viewed under a TissueFAXS automatic scanning system, captured by a digital camera and analyzed by HistoQuest software (TissueGnostics, Vienna, Austria). Immunostaining analysis for CD44 and Ki67 were processed and assessed.

Safety of probiotic

Blood samples were collected and cultured for possible bacteria. Specimens from liver, spleen and mesenteric lymph-nodes were homogenized and seeded on MRS, BHI and BIM-25 agar plate for bacteria investigation. Cultured bacteria from plate colony were identified by the genomic sequence.

Statistical analysis

Parametric data were presented as mean with standard deviation. Statistical significance was analyzed by one-way ANOVA. Data were analyzed with IBM SPSS software (version 21.0; SPSS Institute, Chicago, USA). Values of $p \leq 0.05$ were considered statistically significant.

Results

Effects of Lcr35 on body weight change and diarrhea score of mice with intestinal mucositis induced by 5-FU

All mice tolerated the experiments well and no animal exhibited signs of adverse effects. No cachexia or mortality were found. The mice were weighted and compared daily. The average body weight increased both in the saline and Lcr35 groups (100.76±0.27% and 101.31±0.76%, respectively), though there was no significant difference between the 2 groups. (Figure 1) In contrast, body weight in the 5-FU group decreased considerably. Body weight was sharply decreased from 2nd day in mice exposed to 5-FU when compared to the body weight in saline groups. Furthermore, in 5-FU injected mice, the decrease in BW was significantly less severe following Lcr35 administrations comparing to those without probiotic administration (91.41±1.57% vs 87.53±0.63%, p=0.009).

Diarrhea score of the mice were recorded and compared too. There was no diarrhea noted both in the saline group and Lcr35 group. On the contrary, remarkable diarrhea developed in the two 5-FU groups 24 hours later but diarrhea was relieved after Lcr35 administration (Figure 2). Improved diarrhea score in 5-FU+Lcr35 group (2.00±0.00) was found when compared to 5-FU group (2.75±0.14, p=0.001) 5 days later.

Effect of Lcr35 on pro-inflammatory cytokines production

Effect of *Lcr35* on pro-inflammatory cytokines production assays was shown in Figure 3. Serum levels of IL-1β (a), IL-4 (b), IL-6 (c), IL-17A (d), (MCP-1 (e), TNF-α (f) and IFN-γ (g) were evaluated. Values were represented in mean ± SEM. Serum levels of these pro-inflammatory cytokines were significantly increased in 5-FU group when compared to saline group. This suggested a severe pattern of intestinal mucositis in mice. On the contrary, administration of the probiotic obviously reduced the expression levels of IL-1β, IL-6, MCP-1, TNF-α and IFN-γ (p=0.0001, p=0.004, p=0.003, p=0.003, p=0.04, p=0.001 and p=0.0001, respectively) when compared to 5-FU group (Figures 3a to 3g).

Flow cytometry of T-cell subtypes

Effect of Lcr35 administration on T-cell subtypes was assessed and shown in Figure 4. We found there was a tremendous rise of $CD3^+/CD8^+$ lymphocyte count in the 5-FU group (1.45±0.10 K/μl) when compared to the saline group (0.21±0.01 K/μl). (Figure 4a) The CD8 T lymphocytes in the 5-FU+Lcr35 group (0.46±0.04 K/μl) was significantly lower than 5-FU group (p=0.0001). Besides, there was a significant increase of $CD3^+/CD4^+$ T lymphocyte count in the 5FU group when compared to the saline group. (Figure 4b) The $CD3^+/CD4^+$ T lymphocytes in the 5-FU+Lcr35 group (2.25±0.08 K/μl) was lower than 5-FU group ((2.50±0.15 K/μl) though no significant difference was found (P=0.194). The $CD4^+$/IL4 T lymphocytes in the 5-FU+Lcr35 group (0.96±0.15 K/μl) was significantly higher than 5-FU group (0.18±0.04 K/μp=0.001) (Figure 4c). Similarly, there was a tremendous rise of $CD4^+$/IL17A lymphocyte count in the 5FU group when compared to the saline group. The $CD4^+$/IL17A T lymphocytes in the 5-FU+Lcr35 group (0.16±0.02 K/μl) was significantly higher than 5-FU group (0.08±0.02 K/μl, p=0.004) (Figure 4d).

Intestinal stem cells (CD44 stem cell and Ki67 proliferation)

Intestinal stem cells were represented by CD44 markers and Ki67 proliferation cells (Figure 5). An increase in CD44 expression of intestinal stem cells and Ki67 proliferation were found in immunolabelled jejunal specimens from mice after 5-FU challenge. 5-FU significantly stimulated the expression of CD44 and was restored by administration of Lcr35, though not to the S+S or S+Lcr35 levels. 5-FU could increase the numbers of Ki67 proliferative cells, but there were no significant differences between 5-FU+S and S+S groups and 5-FU+S and 5-FU+Lcr35 groups, respectively.

Effect of Lcr35 on histological changes in the intestinal mucosa

Effects of Lcr35 on histological changes and stem cells in the intestinal mucosa from mice exposed to 5-FU were shown in Figure 6. Morphology was shown by hematoxylin and eosin staining. Goblet cells were found in PAS+AB stained sections. 5-FU caused substantial changes in the intestinal mucosal layer including flattened epithelial layer, shortened villi and lamina propria with inflammatory cells infiltration. The crypts looked small and narrow. No mitoses were found.

The probiotic effects on the villus height in the jejunum were assessed. The villus height of Lcr35 group (477.5 ± 6.7μM) was significantly higher than saline control group (441.9±11.9 μM, p=0.012) (Figure 7a).

However, 5-FU significantly decreased villus height (332.2±7.8 μM) and this effect was restored by Lcr35 resulting a significant lengthened jejunal villi (400.4±6.4 μM, p=0.002) compared with 5-FU group though it did not reach the original height level as in the control saline group.

Similarly, 5-FU significantly lengthened crypt depth of the intestine compared with the saline group (149.0±6.8 μM vs 70.5±2.5 μM, p<0.001) (Figure 7b). However, the crypt depth was significantly improved by Lcr35 treatment (123.8±6.9 μM vs 149.0±6.8 μM, p=0.004). Changes in villus height to crypt depth ratio were similar to that found in villus height. 5-FU markedly decreased the ratio in jejunal sections (2.19±0.16 vs 6.28±0.19, p<0.001) (Figure 7c) but this effect was significantly restored by Lcr35 (3.17±0.31 vs 2.19±0.16, p=0.012) treatment.

Jejunum goblet cells after staining with PAS+AB were also counted in jejunal villus and crypt. Similar to the previous findings on villus height, we found the saline group and Lcr35 group had the highest number of goblet cells (Figure 7). However, the jejunum showed a significant decrease in total goblet cell numbers after 5-FU administration (goblet cells per villus: 15.57±0.87 vs 3.63±0.19, Fig. 7d; goblet cells per crypt: 7.65±0.54 vs 1.62±0.19, Fig. 7e). This effect was relieved by Lcr35 administration with an increase of goblet cell numbers compared with 5-FU groups though without significant differences (goblet cells per villus: 5.94±1.17 vs 3.63±0.19, p=0.162, Fig, 7d; goblet cells per crypt: 2.13±0.07 vs 1.62±0.19, p=0.52, Fig. 7e).

Safety and translocation

Regarding the safety of probiotic administration, cultured bacteria were identified by the genomic sequence. We did identify 2 bacterial strains (*E coli* str. *K-12*; *E coli* O157:H7 str. *Sakai*; *E coli* UMN026) in mesentery lymph node in the saline group. Two bacterial strains (*Enterococcus dispar* ATCC 51266 genomic scaffold; *Enterococcus faecalis*; *Enterococcus casseliflavus* EC20) were identified in the 5-FU group. However, no bacterial translocation was found in the samples of blood, liver and spleen tissues (Table 1).

Discussion

Intestinal mucositis is a frequently encountered adverse effects in cancer patients undergoing chemotherapy and currently there are no effective preventive and control measures. [1, 4, 5] 5-FU treatment was reported to affect the abundance of gut microbiota. In recent years, probiotics had been demonstrated therapeutic effects in chemotherapy-induced mucositis. However, the results are inconsistent. [13, 14] We previously demonstrated various *Lactobacillus* strains had shown beneficial effects on the mucosal barrier of intestines and could enhance tight junction integrity. [9] In this study, we hypothesize that the probiotics could preserve gut ecology, ameliorate inflammation and protect epithelium by maintaining the tight junction integrity via immune modulations of enterocytes and intestinal stem cells. Our aim is to characterize these changes and to investigate the immune modulation effects and safety of probiotic via a 5-FU-induced intestinal mucositis mouse model.

Weight loss and diarrhea score

In our mouse model study, body weight in the 5-FU group decreased considerably by day 3 after 5-FU administration. The weight of the 5-FU + Lcr35 decreased with less intensity in relation to that of the 5-FU group. On the contrary, we found that in those mice in the probiotic group, their degree in body weight loss was significantly lesser than those in the 5-FU and saline groups. Our results were similar to the findings of other studies in the literatures. [1, 15] In our experiment, no diarrhea was noted in the saline and Lcr35 groups. However, marked diarrhea developed in the two 5-FU groups 24 hours later. We demonstrated diarrhea scores improved significantly after oral Lcr35 administrations. Previous studies reported that more than one third of the oncology patients undergoing chemotherapy experienced severe intestinal mucositis. [16] Benson et al reviewed that chemotherapeutic protocol containing 5-FU has been demonstrated with a higher risk for chemotherapy-induced diarrhea. [17]

Cytokines analyses

In our study, we showed those mice in 5-FU+saline groups had significantly higher levels of pro-inflammatory cytokines. This suggested a severe pattern of intestinal mucositis in mice. However, the levels of these cytokines were significantly reversed after administration of probiotic in the 5FU+Lcr35 group. We demonstrated that the protective effects of Lcr35 on 5-FU-induced mucositis was probably by triggering Th1 immune response via down-regulations of the cytokines IFN-γ and TNF-α. In an earlier study, Justino et al reported that *Saccharomyces boulardii* lowered pro-inflammatory cytokine levels (TNF-α, IL-1β, and CXCL-1) in the rat jejunum and ileum induced by 5-FU. [18] The mechanism of *Saccharomyces boulardii*'s protective effect might be similar to the mechanism of Lcr35's action in our study. Up to date the exact mechanism of chemotherapy-induced intestinal mucositis remains unclear. Previous studies had suggested that it involved a five-stage process. [19-21] Soares et al suggested possible pathophysiology of mucositis development including the generation of reactive oxygen species and the up-regulation of pro-inflammatory cytokines causing further mucosal injury eliciting further tissue damage. [22] Few studies have assessed the effects of *Lactobacillus acidophilus* on inflammation. One of these studies found lower levels of leukocyte migration in animals treated with *Lactobacillus acidophilus* in a model of intestinal mucositis induced by irinotecan. [23] Several studies have reported reduced inflammatory effects using other probiotic species. [18, 24]

Flow cytometry

We found there was a tremendous rise of $CD3^+/CD8^+$ lymphocyte count in the 5FU group when compared to the saline groups. However, it was reversed after probiotic administration. The CD8 T lymphocytes of 5-FU+Lcr35 group was significantly lower than 5-FU group. Besides, there was a significant increase of $CD3^+/CD4^+$ lymphocyte count in the 5FU group when compared to the saline groups. We suggested the protective effect of Lcr35 on 5-FU-induced mucositis was by down-regulations of the lymphocytes $CD3^+/CD8^+$ and $CD8^+$/ IFN-γ cells in 5-FU+Lcr35 group. The Lcr35 could also activate the T helper cells by stimulating the $CD4^+/IL4^+$ cell maturation.

Similarly, there was a tremendous rise of $CD4^+$/IL17A lymphocyte count in the 5FU group when compared to the saline group. Interestingly, the level of $CD4^+$ T lymphocytes further increased after probiotic administration. The amount of $CD4^+$/IL17A lymphocyte count in the 5-FU+Lcr35 group was significantly higher than 5-FU group. Th17 immune response was demonstrated in $CD4^+$/IL-$17A^+$ lymphocytes activation in 5-FU+Lcr35 group. Roles of $CD4^+$/IL17A lymphocytes on intestinal immunity and the pathophysiology of chemotherapy-induced mucositis have been investigated recently. [25] Edelblum et al recently found that $CD4^+$ T cells, and in particular Th17 cells, were necessary to limit acute *Salmonella typhimurium* invasion in CA-MLCK mice. Studies in germ free CA-MLCK mice showed that commensal bacteria are required for both $CD4^+$ T-cell expansion and early protection against bacterial invasion. [26]

Intestinal stem cells and crypt proliferation

For further exploring the mechanism of probiotics, we also looked at the intestinal stem cells and crypt proliferation in this study. Intestinal stem cells represented by CD44 markers and crypt proliferation with Ki67 expression were shown by IHC methods. Marked CD44 expression of intestinal stem cells and Ki67 proliferation were found in immunolabelled jejunal specimens from mice after 5-FU challenge and with Lcr35 administration. In our study, 5-FU significantly stimulated the expression of CD44 and was restored by administration of Lcr35, though not to the S+S or S+Lcr35 levels. 5-FU could increase the numbers of Ki67 positive cells, but there were no significant differences between 5-FU+S and S+S groups and 5-FU+S and 5-FU+Lcr35 groups, respectively. The actual role of probiotic on stem cells proliferation remains unclear and requires further investigation.

Athiyyah et al investigated the probiotic effect of *Lactobacillus plantarum* IS-10506 in activating and regenerating leucine-rich repeat-containing G-protein-coupled receptor (Lgr) 5- and B lymphoma Moloney murine leukaemia virus insertion region (Bmi)1-expressing intestinal stem cells in rodents following *Escherichia coli* serotype O55:B5 lipopolysaccharide exposure. [27] Their results demonstrated that the probiotic *Lactobacillus plantarum* IS-10506 activated intestinal stem cells to counter inflammation and might be useful for maintaining intestinal health, especially when used as a prophylactic agent.

Histological analysis on villus height, crypt depth and goblet cells

In our mice model, the 5-FU + Lcr35 group experienced a significant improvement of histopathological changes, as shown by photomicrographs. Previous studies on the effects of chemotherapy-induced mucositis on villus height and crept depths were not consistent. [28, 29] This inconsistency might be due to differences in the choices of probiotic strains or regimens. Stringer et al demonstrated 5-FU could influence the mucin dynamics and might interrupt intestinal barrier function. [30] They showed a marked decrease in goblet cell number following 5-FU administration. In this study, we also demonstrated a marked decrease in goblet cell number in mice with 5-FU-induced mucositis and Lcr35 administration with or without 5-FU injection could both increase goblet cell numbers.

Safety and translocation

Probiotics are defined as living bacteria that can confer health benefits to the host. However, potential side-effects including sepsis development, presence of virulence factors and translocation of live bacteria into local tissues are possible. [31, 32] In the present study, we did identify 2 bacterial strains (*E coli* str. K-12; *E coli* O157:H7 str. *Sakai*; *E coli* UMN026) in mesentery lymph node in the saline group. Two bacterial strains (*Enterococcus dispar* ATCC 51266 genomic scaffold; *Enterococcus faecalis*; *Enterococcus casseliflavus* EC20) were identified in the 5-FU group. However, no bacterial translocation was found in the samples of blood, liver and spleen tissues (Suppl Table 1). Risk of systemic infection with Lcr35 administration in this mice model was not likely.

Pathophysiology of chemotherapy-induced mucositis and roles of probiotics

The pathophysiology of chemotherapy-induced mucositis is complex and most likely involves multiple different processes. [33, 34] In 2004, Sonis published the famous five-phase model theory to explain the pathophysiology of mucositis. [19] Over the past decade, this model has been built upon, with advances in our understanding in regard to cell kinetics, epithelial junctions, inflammation, the microbiome and the innate immune system. [35]

Studies have shown that chemotherapy increase intestinal permeability, induce the generation of reactive oxygen species and pro-inflammatory cytokines, and modulate gut microbiota. [19, 34] Our study showed Lcr35 could reduce levels of proinflammatory cytokines in the intestine in 5-FU–treated mice. Proinflammatory cytokines such as TNF-α and IL-6 contributed to the severity and maintenance of injury in intestinal mucositis [36] and IL-4 was found to participate as a proinflammatory cytokine in a model of 5-FU–induced intestinal damage. [37] Thus, the reduction of these cytokines suggested that the probiotic had strong anti-inflammatory activity.

We previously demonstrated *Lactobacillus* were associated with the maintenance of the tight junction integrity. [9] However, beneficial effects of probiotics on chemotherapy-induced mucositis were not consistent in the literature. [38, 39] In the current study, we determined the effect of probiotic treatment on the expressions of pro-inflammatory cytokines. We further explored the effects of probiotic on stem cells, T cells and cell proliferation. Our results showed convincing protective effect and safety of probiotics on the chemotherapy induced mucositis. Recently we successfully demonstrated that probiotic did modulate the abundance and diversity of gut microbiota of mice undergoing chemotherapy [12] Previous studies in the literature seldom determined the effect of probiotics treatment on the expressions of pro-inflammatory cytokines. Furthermore, the safety of probiotics administrations was rarely investigated.

Limitations

There are several limitations in this study. One limitation is that the small sample size of mice models used in this experiment. Besides, the mice used in this study were indeed normal mice without malignancy, we confessed the model could not mimic or represent the actual situation happened in the clinical patients receiving chemotherapy. The duration of the experiment should be extended in future studies to evaluate the long-term influence of probiotics on microbiota modifications, rather than only the

acute changes. Nevertheless, the greatest challenge for animal model is the difficulty in translating results obtained from current model to the wide range of human patient groups, with varying ages, cancer diagnoses, and to treatments covering a wide range of drugs and doses of chemotherapy.

Conclusions

Our results showed that 5-FU causes intestinal inflammation via Th1 and Th17 responses. 5-FU could stimulate stem cells and proliferation cells in a mouse model. Oral administration of probiotic Lcr35 can ameliorate chemotherapy-induced intestinal mucositis. This is the first study to analyze the immune modulation effects and safety of Lactobacillus casei variety rhamnosus on enterocytes and intestinal stem cells in a 5-FU-induced mucositis mouse model. The model therefore seems well suited to study the effects of different probiotics on chemotherapy-induced mucositis, prior to performing clinical human studies.

Abbreviations

5-FU
5-Fluorouracil
Lcr35
Lactobacillus casei variety rhamnosus
IP
intraperitoneally

Declarations

Ethics approval and consent to participate

Animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of MacKay Memorial Hospital (MMH-A-S-105-26). IACUC has been accredited, approved and authorized by government office, Agriculture and Food Agency Council of Agriculture, Executive Yuan, Taiwan. All methods were performed in accordance with the relevant guidelines and regulations in this animal study.

Consent to publish

We confirm here that all authors have contributed to and agreed on the content of the manuscript, and the respective roles of each author. We confirm that the manuscript has not been published previously, in any language, in whole or in part, and is not currently under consideration elsewhere.

Availability of data and materials

All data generated or analysed during this study are included in this published article Further information are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by research grants from the Taipei MacKay Memorial Hospital (MMH-104-84 and MMH-105-60). We declared that the funding body did not involve or interfere in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' Contributions

Conceived and designed the experiments: CYY WTC JSCC HCL.

Performed the experiments: CYY MLC JSCC.

Analyzed the data: CYY WTC CBJ SWC MLC.

Contributed reagents/materials/analysis tools: CYY WTC CBJ SWC MLC JSCC.

Wrote and approved the paper: CYY WTC CBJ SWC MLC JSCC HCL.

All authors have read and approved the manuscript.

Acknowledgements

The authors thank the Taiwan Mouse Clinic for technical support in the animal experiments.

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Tables

Table 1. Translocation of probiotic to mesentery lymph node, spleen, liver and blood of 5-FU treated mice fed with or without Lcr35 were assessed. Cultured bacterial were from plate colony and identified by the genomic sequence.

a: *E coli* str. K-12; *E coli* O157:H7 str. *Sakai*; *E coli* UMN026 were identified

b: *Enterococcus dispar* ATCC 51266 genomic scaffold; *Enterococcus faecalis*; *Enterococcus casseliflavus* EC20 were identified

	Mesentery lymph node	Spleen	Liver	Blood
S+S	2/4[a]	0/4	0/4	0/4
S+Lcr35	0/5	0/5	0/5	0/5
5-FU+S	2/4[b]	0/4	0/4	0/4
5-FU+Lcr35	0/5	0/5	0/5	0/5

Plate colony culture (bacteria positive/mice number)

Supplemental Information Note

The Supplemental Table mentioned on page 20 was omitted by the authors in this version of the paper.

Figures

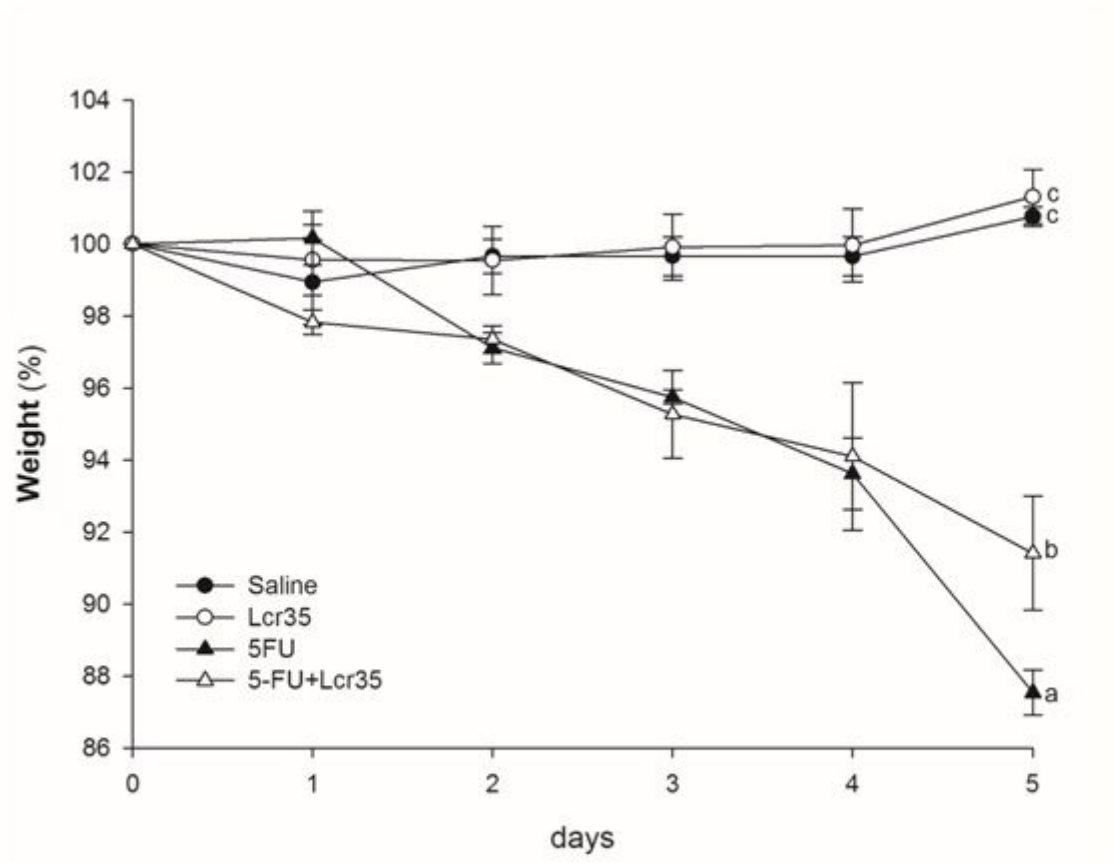

Figure 1

Daily body weight change in percentage of saline or 5-FU-injected mice with/without probiotic Lcr35 administration. The mice were weighted daily and the results of all groups were compared with those in 5-FU-saline groups for 5 days. In the control groups, the mice were injected saline and administrated with saline or Lcr35. In the experimental groups, the mice were injected 5-FU and administrated with or without Lcr35. Data of starting bodyweight are expressed 100% from day 0. Body weight percentage was sharply decreased from 2nd day in mice exposed to 5-FU. The weight percentage of 5-FU+Lcr35 group (91.41±1.57%) was significantly decreased compare to 5-FU group (87.53±0.63%) (P=0.009). Statistical analysis was performed by one-way ANOVA.

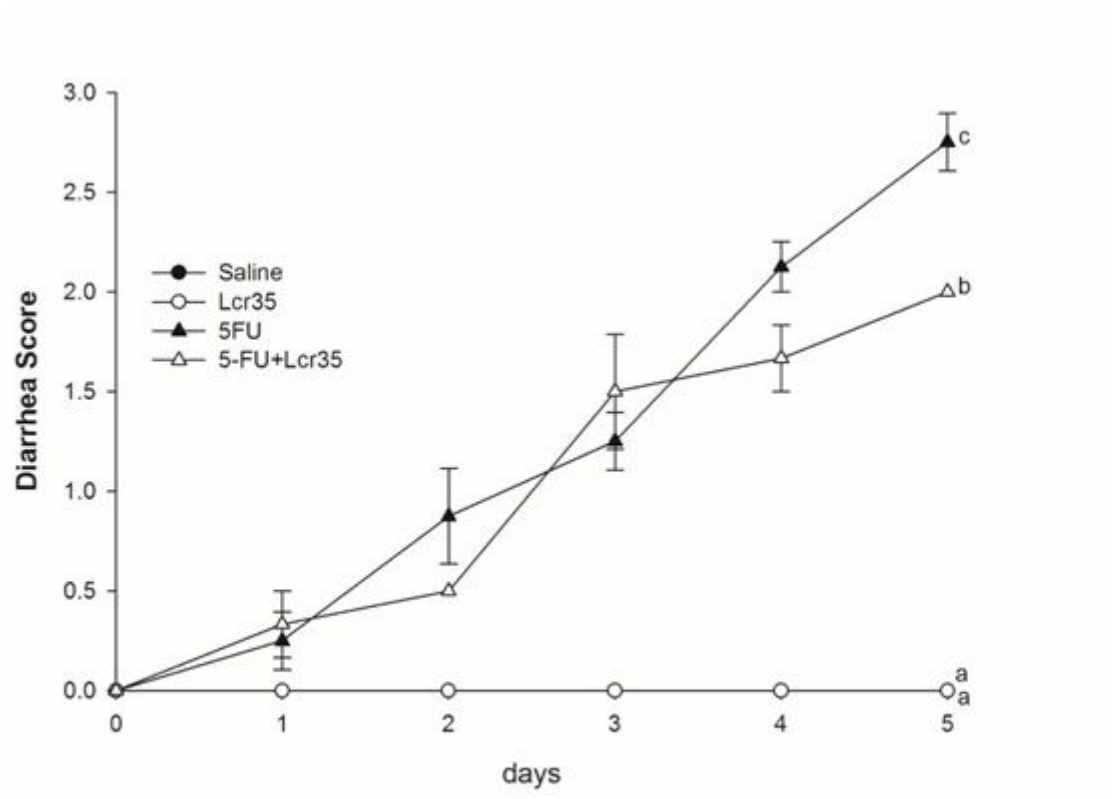

Figure 2

Diarrhea score after administrating probiotic Lcr35 with/without 5-FU treatment. The mice were recorded daily and the results of all groups were compared with those in 5-FU + saline group for 5 days. In the control groups, the mice injected saline and administrated with saline or Lcr35. In the experimental groups, the mice injected 5-FU and administrated with or without Lcr35. Diarrhea score was increased from 1st day after the mice was exposed to 5-FU. The diarrhea score of 5-FU+Lcr35 group (2.00±0.00) was significantly decreased when compared to 5-FU group (2.75±0.14) (P=0.001). The severity of diarrhea was attenuated in those mice treated with probiotics in the 5-FU groups. Statistical analysis was performed by one-way ANOVA.

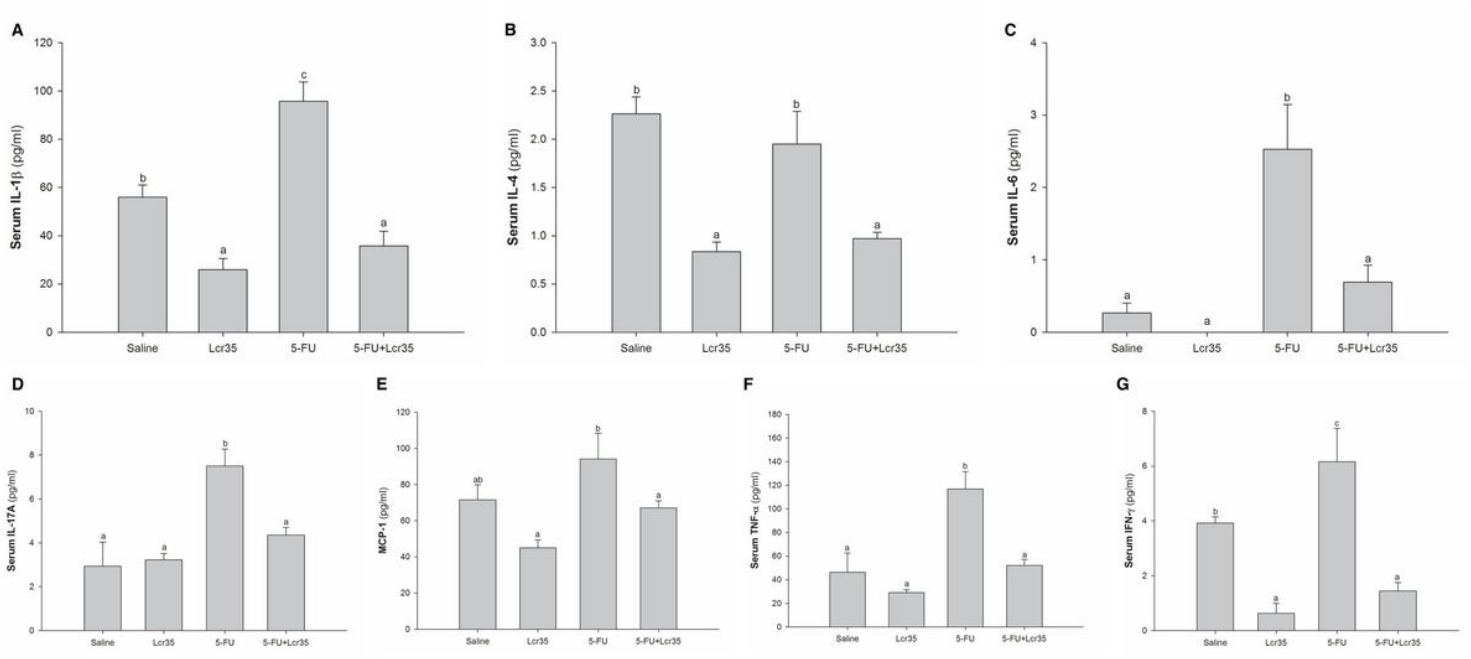

Figure 3

Up-regulations of IL-1β, IL-4, IL-6, IL-17A, MCP-1, TNF-α and IFN-γ, in mucositis mice were followed after injection with 5-FU. Mucositis mice were fed with or without probiotic. Gene expressions of IL-6, IL-1β and TNF-α were determined by Q-PCR. (a) Serum IL-1β level of 5-FU+Lcr35 group (35.8±6.1 ng/ml) was significantly lower than 5-FU group (95.8±8.0 ng/ml) (P=0.0001). (b) Serum IL-4 level of 5-FU+Lcr35 group (0.97±0.06 ng/ml) was significantly lower than 5-FU group (1.95±0.34 ng/ml) (P=0.004). (c) Serum IL-6 level of 5-FU+Lcr35 group (0.69±0.23 ng/ml) was significantly lower than 5-FU group (2.53±0.62 ng/ml) (P=0.003). (d) Serum IL-17A level of 5-FU+Lcr35 group (4.35±0.35 ng/ml) was significantly lower than 5-FU group (7.50±0.78 ng/ml) (P=0.003). (e) Serum MCP-1 level of 5-FU+Lcr35 group (67.1±3.8 ng/ml) was significantly lower than 5-FU group (94.1±14.2 ng/ml) (P=0.04). (f) Serum TNF-α level of 5-FU+Lcr35 group (52.2±4.9 ng/ml) was significantly lower than 5-FU group (116.9 ±14.7 ng/ml) (P=0.001). (g) Serum IFN-γ level of 5-FU+Lcr35 group (1.45±0.31 ng/ml) was significantly lower than 5-FU group (6.16 ±1.21 ng/ml) (P=0.0001). Statistical analyses were performed by one-way ANOVA.

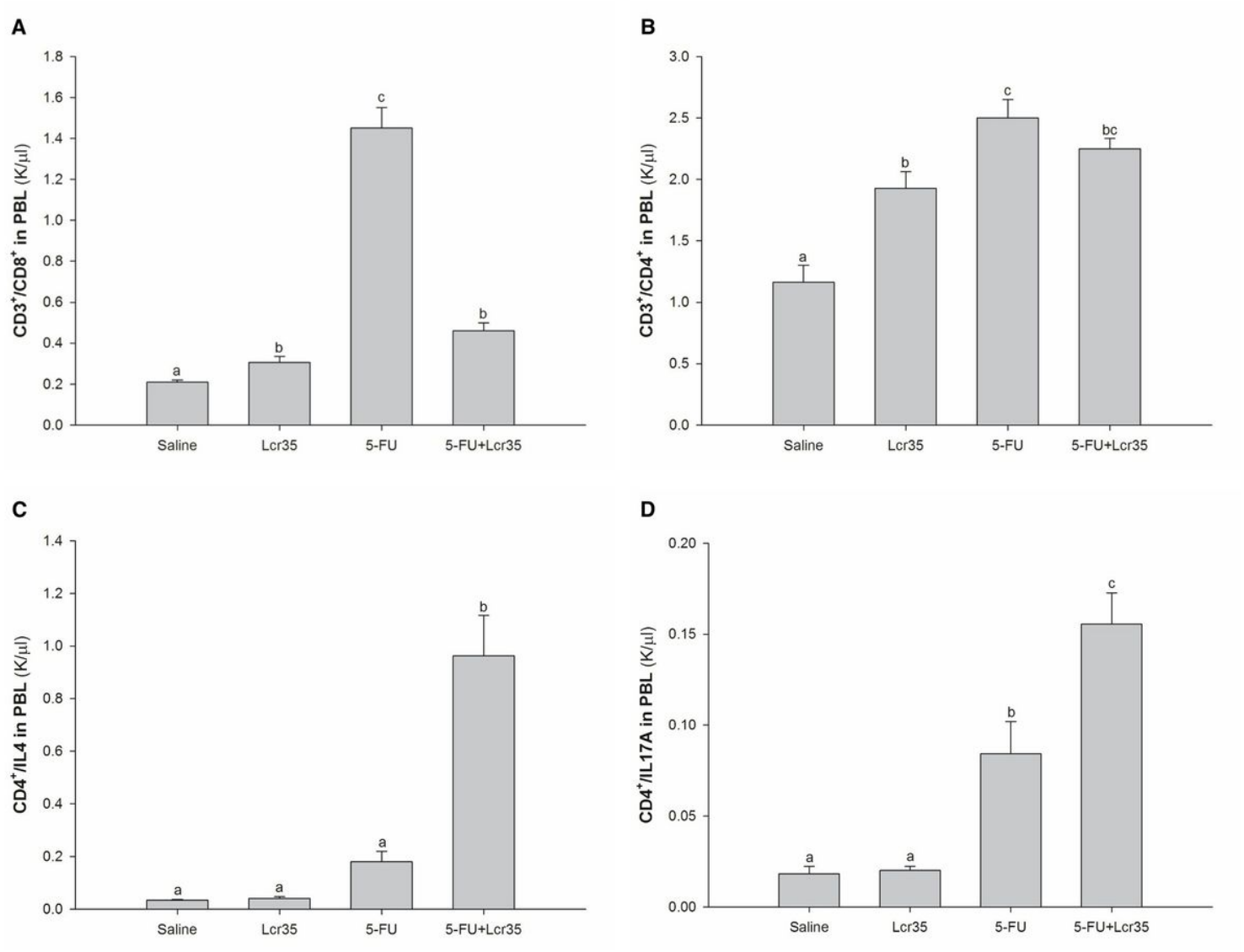

Figure 4

Effect of Lcr35 administration on 5-FU-induced mucositis on T lymphocyte count by flow cytometry analysis. (a) A tremendous rise of CD3+/CD8+ lymphocyte count in the 5FU group (1.45±0.10 K/µl) when compared to the saline group (0.21±0.01 K/µl). CD8 T lymphocytes of 5-FU+Lcr35 group (0.46±0.04K/µl) was significantly lower than 5-FU group (P=0.0001). (b) A significant increase of CD3+/CD4+ T lymphocyte count in the 5FU group (2.50±0.15 K/µl) when compared to the saline group (1.16±0.14 K/µl). CD3+/CD4+ T lymphocytes of 5-FU+Lcr35 group (2.25±0.08 K/µl) was lower than 5-FU group though no significant difference was found (P=0.194). (c) The CD4+/IL4 T lymphocytes of 5-FU+Lcr35 group (0.96±0.15 K/µl) was significantly higher than 5-FU group (0.18 ±0.04 K/µl) (P=0.001). (d) A tremendous rise of CD4+/IL17A lymphocyte count in the 5FU group when compared to the saline group. The CD4+/IL17A T lymphocytes of 5-FU+Lcr35 group (0.16±0.02 K/µl) was significantly higher than 5-FU group (0.08± 0.02 K/µl) (P=0.004). Statistical analysis was performed by one-way ANOVA.

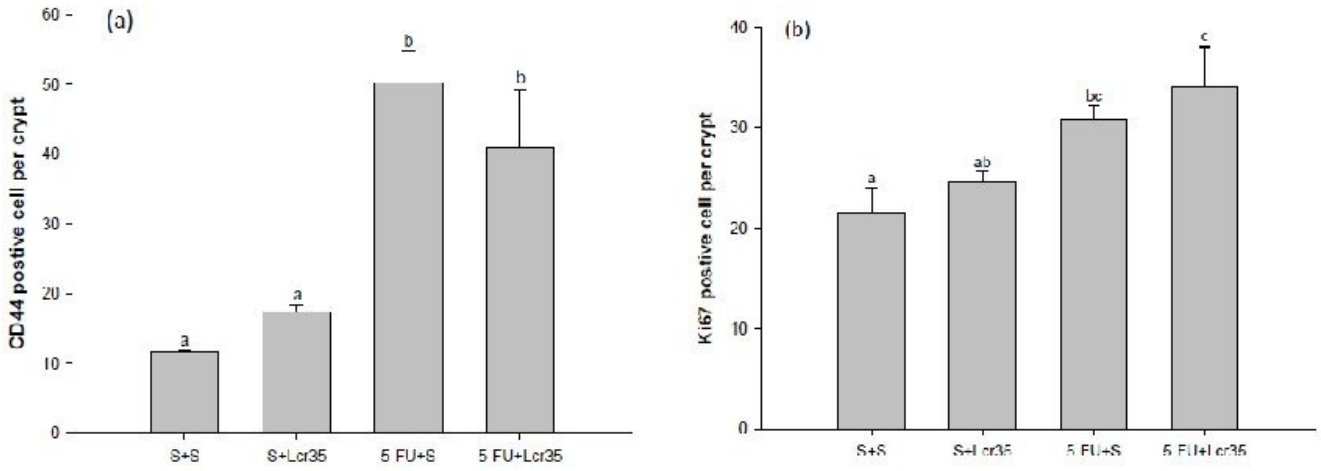

Figure 5

Effects of Lcr35 on CD44 positive stem cells and Ki67 proliferative cells in the intestinal mucosa from mice exposed to 5-FU. CD44 positive (a) and Ki67(b) cells after staining were counted in per crypt. 5-FU significantly stimulated the expression of CD44 and was restored by administration of Lcr35, though not to the S+S or S+Lcr35 levels. 5-FU could increase the numbers of Ki67 positive cells, but there were no significant differences between 5-FU+S and S+S groups and 5-FU+S and 5-FU+Lcr35 groups, respectively. Values were represented as mean ± SEM and were analyzed using one-way ANOVA.

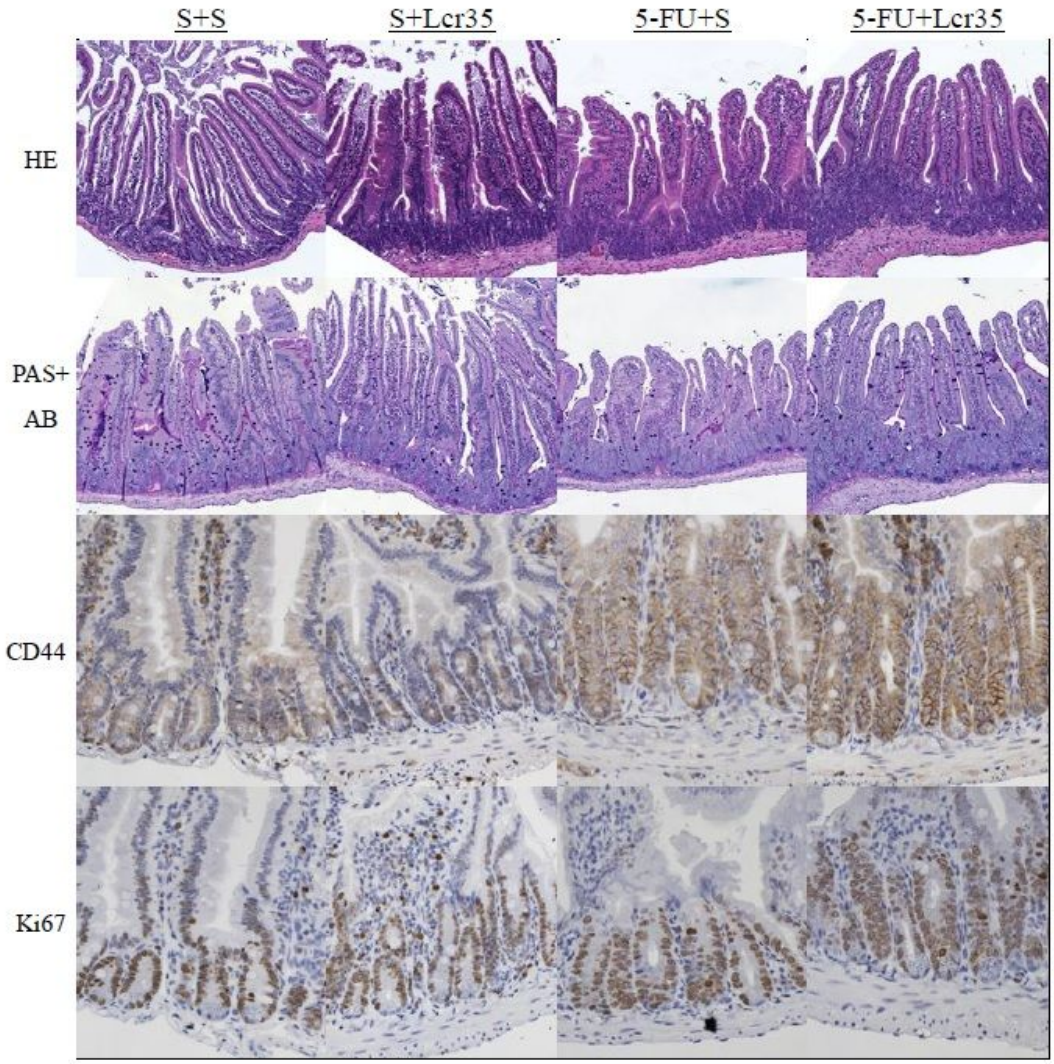

Figure 6

Representative histology of jejunum showing villus height and crypt depth with haematoxylin and eosin stain in mice on day 5 challenged with 5-FU. Microscopical findings of intestinal mucosa from mice exposed to 5-FU-induced mucositis at the jejunum. Goblet cells were found in PAS+AB stained sections. Intestinal stem cells CD44 markers and proliferation of crypt (Ki67 expression) were shown by IHC methods. CD44 analysis of intestinal stem cells and Ki67 immunolabeling of jejunum from mice were assessed after 5-FU challenged with Lcr35 administration.

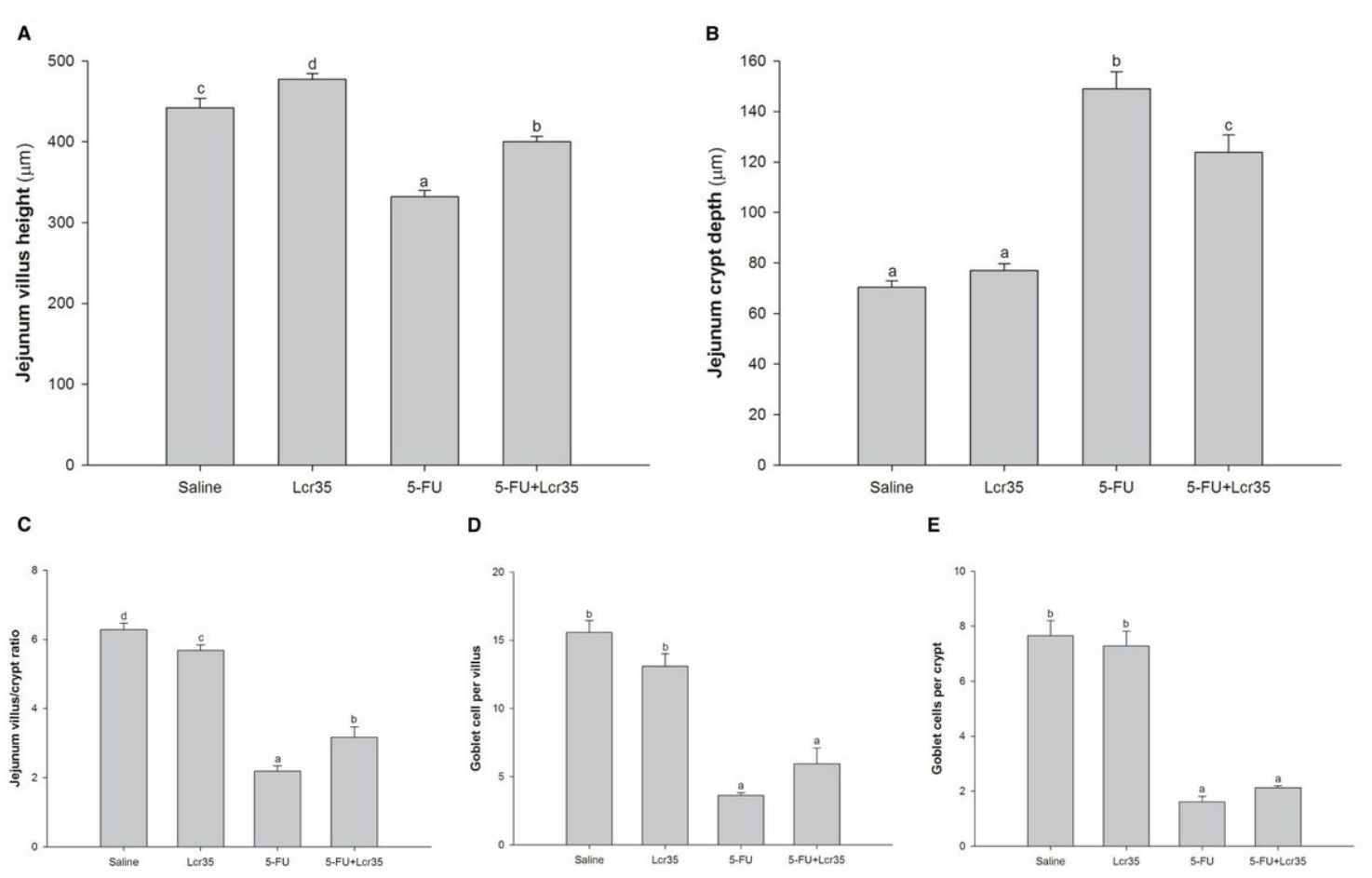

Figure 7

Effects of Lcr35 on villus height, crypt depth and goblet cells in the intestinal mucosa from mice exposed to 5-FU. (a) The villus height of Lcr35 group (477.5±6.7 μM) was significantly higher than saline group (441.9±11.9 μM) (P=0.012). The villus height of 5-FU+Lcr35 group (400.4±6.4 μM) was significantly higher than 5-FU group (322.2±7.8 μM) (P=0.002). (b) The crypt depth of 5-FU+Lcr35 group (123.8±6.9 μM) was significantly shorter than 5-FU group (149.0±6.8 μM) (P=0.004). (c) The villus/crypt ratio of 5-FU+Lcr35 group (3.17±0.31) was significantly higher than 5-FU group (2.19±0.16) (P=0.012). (d) The goblet cells per villus of 5-FU+Lcr35 group (5.94±1.17) was higher than 5-FU group (3.63±0.19) (P=0.16) but without obvious significant difference. (e) The goblet cells per crypt of 5-FU+Lcr35 group (2.13±0.07) was higher than 5-FU group (1.62±0.19) (P=0.52) but without significant difference. Statistical analysis was performed by one-way ANOVA.

Supplementary Files

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