High Concentrated Probiotics Improve Inflammatory Bowel Diseases Better than Commercial Concentration of Probiotics

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ABSTRACT

The incidence of inflammatory bowel diseases has increased during recent decades in Korea as well as Asian countries. Probiotics have been clinically administered to improve intestinal inflammation in inflammatory bowel disease (IBD). In this study, we identified that higher concentrations of probiotics called "Amanlac" probiotics protected intestinal tissues with the regulation of cytokine production and the improvement of intestinal injury of mice with dextran sodium sulfate (DSS)-induced colitis much better than commercial probiotics. "Amanlac" probiotics significantly ameliorated gross and pathological scores of colitis caused by DSS in a concentration-dependent manner based on the following mechanisms; inflammatory markers such as IL-1β, TNF α and COX-2, as well as MMPs and ICAM1 were significantly lower in colon tissues of probiotics-treated mice following DSS treatment compared with DSS-treated control mice, but the overall efficacy of "Amanlac" probiotics was significantly improved than conventional concentration of probiotics. In conclusion, the administration of higher concentrated probiotics helps to successfully maintain intestinal homeostasis, while also improving intestinal inflammation.

Key words: "Amanlac" probiotics, inflammatory bowel diseases, dextran sodium sulfate

INTRODUCTION

Crohn's disease and ulcerative colitis, collectively referred to as inflammatory bowel diseases (IBD), are chronic, immune-mediated conditions that develop in genetically susceptible individuals due to an abnormal, defective recognition of a variety of microbial antigens by different elements of the innate immune system, resulting in a complex aggravated inflammatory response. This involves the participation of a wide range of 'immune' and 'non-immune' cells, which ultimately results in intestinal damage and the patient's symptoms like diarrhea and hematochezia[(1)].

Probiotics are defined as live microbial food supplements that when ingested, they can survive gastrointestinal tract and exert positive influence on host health. However, the underlying mode of action of probiotics is complex and not yet fully elucidated in spite of acknowledged efficacy. Many mechanisms have been inferred to explain probiotic actions such as antagonism against intestinal pathogens, enhancement of mucosal barrier activity, or modulation of host's immune functions[(2,3)].

Toxic bowels and overall declining health can be caused by the loss of good intestinal flora, after which the overgrowth of harmful intestinal bacteria and parasites, whereas well-functioning bowels and small intestine contain a balance of healthy and harmful microorganisms, which keep each other in check and the digestive system balanced and healthy. This intestinal flora, as it is called, helps break down food, protect us from toxins and antigens, and aid us in absorbing essential nutrients. A dysbiotic microbiota is an ecological disorder of the bacterial community and the concept is often associated with the pathogenesis of IBD[(4,5)]. However, the precise mechanisms of action are not fully understood. Though higher concentration of probiotics can provide the better effects in this condition of dysbiosis, but higher acidity by lactic acid bacilli culture limits this anticipation. The recent advancement in culturing technology enables us to provide higher concentration of probiotics, *e.g.*, more than 10^{12} cfu/g without affecting lowered culture media pH. In the current study we hypothesized that highly concentrated amounts of probiotics, "Amanlac" probiotics from 10^9 to 10^{12} cfu/g, can yield better efficacy than commercially available concentration of probiotics, 10^9 cfu/g.

MATERIALS AND METHODS

I. *Probiotics*

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Various concentrations of lactic acid bacteria mixture for this study were obtained from amBio Co., Ltd (Busan, Korea). They were prepared as trial formula of Amanlac® Probiotics and their formulation of lactic acid bacteria is shown in Table 1. Amanlac® Probiotics high dosage (HD), middle dosage (MD), and low dosage (LD) were made of lactic acid bacteria manufactured by membrane cell-recycling reactor (MCR) process of amBio. Co. Ltd. The Products by MCR process have high density of viable cells and low concentration of lactic acid. The commercial probiotics made of lactic acid bacteria were purchased from Korean market.

Table 1. Added lactic acid bacteria in tested samples

Samples	Lactic acid bacteria (cfu/g)			
	L. plantarum	*B. lactis*	*S. thermophilus*	Total
Amanlac® Probiotics HD	6.50E+11	1.38E+11	6.80E−10	8.56E−11
Amanlac® Probiotics MD	4.23E+10	8.97E+09	4.42E−09	5.56E−10
Amanlac® Probiotics LD	1.06E+10	2.24E+08	1.11E−08	1.39E−09
Control	1.06E+10	2.24E+08	1.11E−08	1.39E−09

Table 2. Primer sequence for PCR

Gene (NCBI accession No.)	Forward	Reverse
COX-2 (NM_011198.3)	GAAATGGCTGCAGAGTTGAA	TCATCTAGTCTGGAGTGGGA
iNOS (NM_010927.3)	TTTTCCCAGGCAACCAGACG	GTAGCGGGGCTTCAGAATGG
ICAM-1 (NM_010493.2)	TGTGCTTTGAGAACTGTGGC	GGTTCTGTCCAACTTCTCAG
MMP-2 (NM_008610.2)	GAG TAA GGG GAT CGC CGT GCA	AAG AGG TTG CAA CTC TCC TTG G
MMP-3 (NM_010809.1)	ACA GAG CTG TGG GAA GTC AAT G	TTT GGC GAA CCT GGG AAG GTA
MMP-7 (NM_010810.4)	TGG CCT GCC CAT GAC TGG AA	TGG GTG GCA GCA AAC AGG AA
MMP-9 (NM_013599.2)	AGC AGT CTC TAC GGC CGG CTT	TCC GCT TCG GGT CCG TAC ACG
MMP-11 (NM_008606.2)	TCA CTG AGG TGC ACG AGG GA	ATG GTG GAA ACC GCG TCG AA
MMP-13 (NM_008607.2)	ATG ATC TTT AAA GAC AGA TTC TTC TGG	TGG GAT AAC CTT CCA GAA TGT CAT AA
GAPDH (NM_008084.2)	GGTGCTGAGTATGTCGTGGA	TTCAGCTCTGGGATGACCTT

II. *Animal Model of DSS-induced Colitis*

A total of sixty C57BL/6 mice were divided into 6 groups, 10 mice per group, respectively; a vehicle control group (normal group), 2.5% dextran sodium sulfate (DSS, molecular weight ¼ 36,000 - 50,000; MP Biomedicals) in tap water ingestion for 1 week (DSS group). "Amanlac probiotics" (high concentrated lactobacilli cultured in special membrane than broth) were pretreated 2 weeks before DSS treatment. "Amanlac" probiotics were resuspended in PBS before administration by gavage to each animal. Animals received 10^9 cfu/g [DSS + low dose of "Amanlac" probiotics (LD)], 10^{11} cfu/g [DSS + medium dose of "Amanlac" probiotics (MD)], 10^{12} cfu/g [DSS + high dose of "Amanlac" probiotics (HD)] daily in 200μL PBS for 3 weeks, whereas vehicle control received only PBS. Last group was pretreated with commercial probiotics (DSS + COM), which was *lactobacillis* in concentration of 10^9 cfu/g. Clinical phenotypes such as hematochezia and rectal prolapse were investigated and charted daily. There were no mortalities observed in all groups. After 7 days of DSS ingestion, all mice were killed and colons were removed, and rinsed with PBS. The lengths of colon were measured, and isolated tissues were subjected to extraction of mRNA.

III. *ELISA Assay*

Immunoreactive IL-1β and TNF-α were measured in serum using enzyme linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D systems. MN. USA).

IV. *RNA Isolation and RT-PCR*

Total RNA was isolated from mouse tissues using Trizol (Gene All. Seoul. Korea). Reverse transcription-PCR (RT-PCR) was performed according to the manufacturer's instruction using a RT-PCR kit (Bioneer, Daejeon, Korea). The primers used were as Table 2. PCR was performed with a 40 μL reaction volume. The amplification cycle (denaturation step at 94°C for one minute, an annealing step at 60°C for one minute, and an extension step at 72°C for 30 seconds) was repeated 30 times and followed by a final extension for 10 minutes at 72°C.

V. *Statistical Analysis*

The data are presented as means ± SD. The ANOVA test or the Student t-test for unpaired results was used to evaluate differences between more than 3 groups or between 2 groups, respectively. Differences were considered to be significant for values of $p < 0.05$.

RESULTS AND DISCUSSION

I. *Effects of Different Concentrations of Probiotics on the Severity of DSS-induced Colitis in C57BL/6 Mice*

Administration of "Amanlac" probiotics revealed that this bacterium did not affect the expected increase in body weight, the length of colon or cause any other signs of a disease, including rectal bleeding and diarrhea (data not shown), implying that this bacterium does not disturb the physiological balance in mouse intestine.

Next, we investigated whether "Amanlac" probiotics provide an improved protective effect against colonic inflammation. Three different concentrations of "Amanlac" probiotics increased the lengths of colon of the mice treated with DSS as well as commercial probiotics (Figure 1A). Whereas mice provided with DSS alone had marked weight loss due to colonic inflammation compared

with the vehicle-treated mice, mice fed with DSS and "Amanlac" probiotics were resistant to weight loss (data not shown). To determine the severity of colitis, clinical parameters of colitis such as rectal bleeding and diarrhea were assessed (Figure 1B). "Amanlac" probiotics significantly ameliorated clinical signs of colitis caused by DSS in a concentration-dependent manner. Shortening of the colon length (one of the macroscopic signs of colitis representing the severity of colitis) was less evident in mice treated with DSS + "Amanlac" probiotics than mice treated with DSS alone. These data suggest that "Amanlac" probiotics ameliorate the development and progress of DSS-induced colitis and high concentration of "Amanlac" probiotics yield higher efficacy than lower concentration of "Amanlac" probiotics and commercial probiotics.Then, we further examined whether "Amanlac" probiotics have a better protective effect on histological damages in colonic mucosa of colitic mice. The hematoxylin and eosin-stained colonic tissues from mice treated with Amanlac probiotics and DSS showed histologically intact colonic mucosa and substantially reduced leukocyte infiltration in the colon, whereas mice treated with DSS alone revealed highly inflamed colonic mucosa, represented by the severe erosion and the massive leukocyte infiltration (Figure 2A). Evaluating several histopathological parameters of colonic inflammation (ulceration, neutrophil infiltration, and edema), colonic tissue sections obtained from DSS + "Amanlac" probiotics-treated mice, especially medium dose of "Amanlac" probiotics showed statistically significant improvement (Figure 2B). These data indicate that "Amanlac" probiotics reduced mucosal damages in DSS-induced colitis much better than commercially available probiotics.

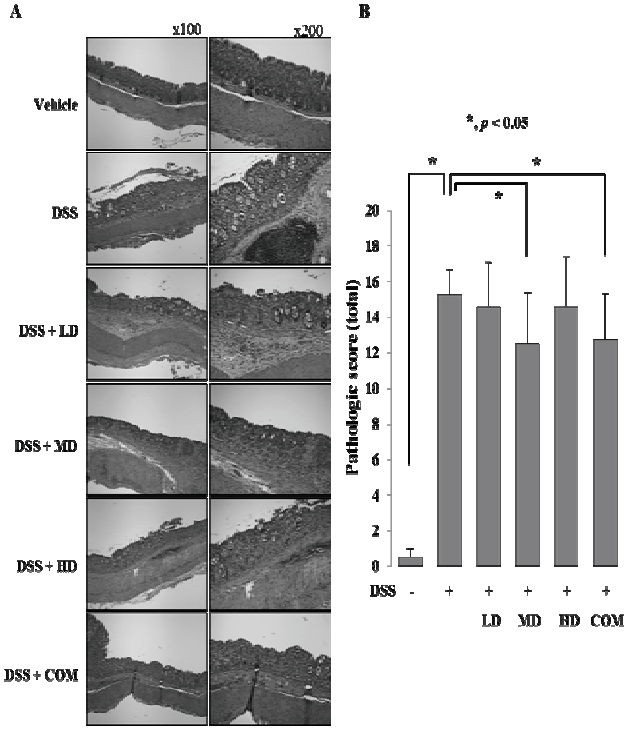

Figure 2. Effects of Amanlac probiotics on H & E staining (A) and total pathologic scores (B). LD: low dose of "Amanlac" probiotics (1×10^9 cfu/g), MD: medium dose of "Amanlac" probiotics (1×10^{10} cfu/g), HD: high dose of "Amanlac" probiotics (1×10^{11} cfu/g), COM: commercially available probiotics (1×10^9 cfu/g).

II. *Effects of Probiotics on the Expressions of Inflammatory Cytokines and Enzymes of DSS-induced Colitis in Mice*

The etiology of IBD remains unknown, by which it was nominated as idiopathic bowel disease, but the characteristic disproportionate inflammatory response in the gut may develop through various mechanisms at the cellular and subcellular level. Tumor necrosis factor (TNF)-α is one crucial mediator of this abnormal immune response, and in recent years, biological therapies targeting TNF-α have significantly improved the management of IBD refractory to conventional therapies. In addition,

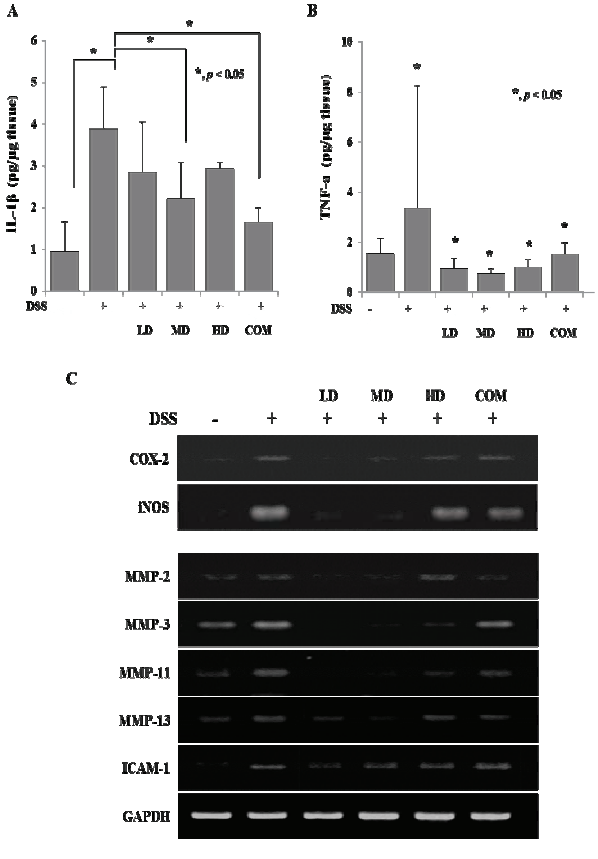

Figure 3. Effects of "Amanlac" probiotics on the levels of IL-1β (A), TNF-α (B) and the mRNA expressions of inflammatory enzymes (C) in DSS-induced colitis. LD: low dose of "Amanlac" probiotics (1×10^9 cfu/g), MD: medium dose of "Amanlac" probiotics (1×10^{10} cfu/g), HD: high dose of "Amanlac" probiotics (1×10^{11} cfu/g), COM: commercially available probiotics (1×10^9 cfu/g).

interleukin (IL)-1β, produced by activated macrophages, has been known to an important mediator of the inflammatory response, and is involved in a variety of cellular activities, including cell proliferation and differentiation[6]. IL-1β and TNF-α were significantly lower in colon tissues (Figure 3A and B) of probiotics-treated mice following DSS treatment compared with DSS-treated control mice.

Up-regulation of IL-1β induces the expression of cyclooxygenase-2 (COX-2) to contribute to inflammatory pain hypersensitivity[7]. Therefore, we tested the effects of "Amanlac" probiotics on representative inflammatory enzymes. As expected, the mRNA expression of COX-2 and iNOS were significantly inhibited in the colon tissues from the mice treated with "Amanlac" probiotics (Figure 3C).

III. *Effects of Probiotics on the Intestinal Epithelial Cell Migration of DSS-induced Colitis in Mice*

DSS has been reported to provide direct toxic damage to intestinal epithelial cells leading to loss of epithelial barrier integrity and consequently acute colitis[8]. Therefore, we examined whether "Amanlac" probiotics affects the extracellular matrix proteins in DSS-induced colitis and found that DSS treatment induced the expressions of matrix metalloproteinases (MMPs) as evident in the colonic mucosa of DSS-treated mice (Figure 3C). Pretreatment of "Amanlac" probiotics inhibited the expressions of MMPs and intercellular adhesion molecule (ICAM)-1. These data indicate that "Amanlac" probiotics could reduce intestinal mucosal damages through attenuating protease activation in DSS-induced colitis.

CONCLUSIONS

We demonstrated that higher dose of probiotics improved severity of DSS-induced colitis and inhibited the levels of inflammatory markers in the colon tissues much better than commercially available concentration of probiotics. Therefore, it is appealing to design probiotic-based preventive diet in order to either maintain GI tract homeostasis or prevent development of chronic inflammation. Here in, we show that the administration of higher concentrated probiotics called "Amanlac" probiotics developed with the advancement of culture technology helps to successfully maintain intestinal homeostasis, while also curing intestinal inflammation in IBD.

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