Better Efficacy of *Lactobacillus casei* in Combination with *Bifidobacterium bifidum* or *Saccharomyces boulardii* in Recovery of Inflammatory Markers of Colitis in Rat

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ABSTRACT

Alteration of intestinal microflora has an important role in Inflammatory Bowel Disease (IBD) and probiotics by balancing microflora and modulating inflammatory cytokines seem effective in management of IBD. In the present study, two combinations of probiotics *Lactobacillus casei* (*L. casei*) plus *Bifidobacterium bifidum* (*B. bifidum*) or plus *Saccharomyces boulardii* (*S. boulardii*) were tested for their potential to prevent or treat experimental colitis in rats. Nine groups of animals including sham (normal group), control (vehicle-treated), dexamethasone as standard, treatment groups (oral administration of *L. casei* and mixture of *L. casei*-*B. bifidum* and *L. casei*-*S. boulardii* after induction of colitis) and prevention groups (oral administrations of *L. casei* and mixture of *L. casei*-*B. bifidum* and *L. casei*-*S. boulardii* before induction of colitis) were used. Tumor necrosis factor-α (TNF-α), myeloperoxidase (MPO) and lipid peroxidation (LPO) were determined in all groups. In treatment groups, histological scores and TNF-α, MPO and LPO levels attenuated significantly but colonic lesions in the prevention groups did not recover. The results revealed that, combination of *L. casei*-*B. bifidum* and *L. casei*-*S. boulardii* alleviated inflammatory parameters in colitis but these combinations were ineffective to prevent colitis. In addition, we found that mixture of probiotics is significantly more effective than *L. casei* group in improving barrier function of epithelium in experimental colitis model.

Key words: *Lactobacillus casei*, *Bifidobacterium bifidum*, *Saccharomyces boulardii*, ulcerative colitis, tumor necrosis factor alpha (TNF-α), myeloperoxidase (MPO)

INTRODUCTION

Ulcerative Colitis (UC) and Crohn’s Disease (CD) are two forms of Inflammatory Bowel Disease (IBD) that have no exact cure yet, although some drugs are used such as aminosalicylates, corticosteroids and immunosuppressives. With the notion that intestinal microflora has important role in IBD, probiotics have been trialed and found effective in management of IBD (Elahi *et al.*, 2008; Rahimi *et al.*, 2008a, b; Salari *et al.*, 2012; Nikfar *et al.*, 2010) and irritable bowel syndrome (Nikfar *et al.*, 2008).
Probiotics are defined as live microorganisms that modulate intestinal microbial in favor of Gastrointestinal (GI) health. These agents grow in foods such as yogurt, fermented milk and whey cultures where they produce beneficial substances with improving effect on human health (Ghasemi-Niri et al., 2011).

Several kinds of probiotics have been used in human such as Lactobacilli, Bifidobacterium, Streptococcus, Enterococcus, nonpathogenic Escherichia coli and Saccharomyces boulardii (S. boulardii) species (Geossens et al., 2003). In particular, Lactobacillus and Bifidobacterium strains and S. boulardii were effective on functional immune system and inhibition of oxidative stress process in human colon cells (Ghasemi-Niri et al., 2011; Lee et al., 2009; Imaoka et al., 2008).

Previous studies indicated that Lactobacillus species indirectly stimulate anti-inflammatory cytokines and reduce secretion of pro-inflammatory cytokines such as tumor necrosis factor (TNF-α). Lactobacillus casei (L. casei) reduces myeloperoxidase (MPO) activity and lipid peroxidation (LPO). S. boulardii is thermophilic nonpathogenic yeast that is used against antibiotic-associated diarrhea and colitis in human (Czerucka and Rampal, 2002). In previous reports, the clinical efficacy of S. boulardii in IBD has been demonstrated in the GI tract. It inhibits the inflammatory responses (Buts and Bernasconi, 2005) via inhibiting the activation of nuclear factor-kappa B (NF-κB). Furthermore, S. boulardii reduced intestinal inflammation by decreasing mucosal expression of proinflammatory cytokine genes in a Trinitrobenzene Sulphonic Acid (TNBS)-induced colitis model in rat (Lee et al., 2009).

Bifidobacterium was beneficial in control of intestinal inflammation via enhancement of blood cells IL-10 and reduction of IL-8 in IBD patients (Reddy et al., 2007).

In TNBS-induced colitis, the infiltration of immune cells into the lumen starts injury through attack of free radicals.

In the present study, we aimed to evaluate the efficacy of two combination of probiotics including L. casei with B. bifidum (LB) and L. casei with S. boulardii (LS) in whey culture in comparison to L. casei in the recovery of intestinal mucosa barrier after TNBS-induced colitis in rats besides their possible preventive effect against colitis.

MATERIALS AND METHODS
Chemicals: TNBS from Sigma-Aldrich Chemie (GmbH Munich, Germany), thiobarbituric acid (TBA), trichloroacetic acid (TCA), n-butanol, Hexadecyl Trimethyl Ammonium Bromide (HETAB), hydrochloric acid diamine tetra acetic acid (EDTA), O-dianisidine hydrochloride, acetic acid, sodium acetate, Coomassie reagent, bovine serum albumin (BSA), sodium sulphate, phosphoric acid, H_2O_2, potassium dihydrogen phosphate, sodium carbonate, Na-K-tartrate, cupric sulphate from Merek Chemical Co. (Tehran, Iran) and rat-specific Tumor Necrosis Factor-α (TNF-α) kits from (Bender Med System GmbH, Austria), whey powder from Pooyan-Milk Co. (Tehran, Iran), Lactobacillus casei, Bifidobacterium bifidum and Saccharomyces boulardii were used in this study.

Preparation of probiotics: Whey was reconstituted (2-4% w/v) with water to prepare liquid whey having lactose concentration of (70-72% w/v). Whey contained protein (10-12% w/v) and ash (8.8-5% w/v) and was supplemented with yeast extract (<50 w/v) and lipid (2% w/v). The whey culture (10% w/v) was sterilized at 121°C for 20 min. After sterilization we had three vials, the first one was inoculated with 10⁷ CFU/100 mL of L. casei and the second one with 10⁷ CFU/100 mL of L. casei and 10⁷ CFU/100 mL of B. bifidum and the third one was inoculated with 10⁷ CFU/100 mL of L. casei and 10⁷ CFU/100 mL of S. boulardii. They were stored at 37°C for 48 h.
under stationary conditions. Eventually whey culture contained 10^8 CFU/100 mL of *L. casei*, 10^8 CFU/100 mL of *B. bifidum* and 10^8 CFU/100 mL of *S. boulardii*.

**Animals:** Male Wistar rats (220-250 g) were maintained under standard conditions of temperature (23±1°C), relative humidity (55±10%) and 12/12 h light/dark cycle and fed with a standard pellet diet and water ad libitum. They were housed individually in standard polypropylene cages. All ethical themes of studies on animals were considered carefully and the experimental protocol was approved by the local institute ethical committee with number TUMS-14076.

**Experimental design:** Nine groups of male rats containing six in each group were used in study. Colitis was induced by rectal administration of TNBS. One group of animals received normal saline instead of TNBS (sham). Other eight groups that received TNBS were: control (no treatment), Dexta that treated with dexamethasone (as standard) at 1 mg kg⁻¹, three treatment groups that treated with whey (2 mL day⁻¹): *L. casei, L. casei-B. bifidum* (LB) and *L. casei-S. boulardii* (LS) for 12 days after induction of colitis and three prevention groups which received 12 days whey (2 mL day⁻¹): *L. casei, L. casei-B. bifidum* (LB) and *L. casei-S. boulardii* (LS) before induction of colitis. Whey and dexamethasone were dissolved in water and administered to rats orally by gavage.

**Induction of colitis:** For induction of colitis, 36-h-fasted rats were anesthetized with administration of 50 mg kg⁻¹ pentobarbital sodium intraperitoneally and were positioned on their right side. Then 0.3 mL of a mixture containing six volumes of 5% TNBS plus 4 volume of 99% ethanol was instilled through anus by use of a rubber cannula (8 cm long). After instillation of TNBS, the rats were maintained in a supine Trendelenburg position to reduce leakage of TNBS through anus.

**Sample preparation:** Treatments duration was 10 days. On the 11th day, animals were sacrificed by an overdose of ether inhalation. Abdomen was immediately opened and the colon was removed. Then colon was cut in pieces and cleaned with saline and examined for macroscopic changes and scored as described later. Then samples were divided into two pieces, one piece for histopathology assessment (maintained in 10 mL formalin 10%) and the other for measuring biomarkers. The first one was weighed and maintained in -20°C for 24 h. The latter, the colonic samples were homogenized in 10 volume ice cold potassium phosphate buffer (50 mM, pH 7.4), then sonicated and centrifuged for 30 min at 3500 xg. The supernatants were transformed into several microtubes for biochemical assays and all were kept at -80°C until analyses.

**Macroscopic and microscopic recognition of colonic damage:** The severity of colonic tissue damage was measured using colon macroscopic scoring as follows: Normal appearance with no damage = score 0; Localized hyperemia without ulceration = score 1; Linear ulceration without significant inflammation = score 2; Linear ulceration with inflammation at one site = score 3; Two or more sites of ulceration and extending more than 1 cm along the length of colon = score 4; If damage extended more than 2 cm along the length of colon and the score enhanced by 1 for each increased cm of involvement = score between 5-8.

For microscopic examination, formalin-fixed colon samples were embedded in paraffin and stained with hematoxylin and eosin. Then results were expressed by microscopic scoring of colonic damage as follows (Ghasemi-Niri et al., 2011):
Score 0: No damage
Score 1: Focal epithelial edema and necrosis
Score 2: Disperse swelling and necrosis of the villi.
Score 3: Necrosis with neutrophil infiltration in submucosal
Score 4: If tissue had wide spread necrosis with massive neutrophil infiltration and hemorrhage.

Determination of TNF-α: TNF-α was measured using enzyme linked immunosorbent assay (ELISA) kit. The primary wave length was 450 nm and the reference wavelength was 620 nm (Abdolghaffari et al., 2010).

Myeloperoxidase activity in colonic mucosa: In a separate experiment, the MPO activity was determined by 3.2 mL of 50 mM phosphate buffer containing 0.167 mg mL⁻¹ O-dianisidine hydrochloride and 0.0005% H₂O₂ that was combined with 0.1 mL of supernant. The absorbance of the reaction mixture was measured at 450 nm for 3 min. MPO activity was expressed as units per gram of total protein. Details were noted previously (Ghazanfari et al., 2006).

Lipid peroxidation: Thiobarbituric Acid-reactive Substances (TBARS) were measured in colon tissue, using 1,1,3,3-tetraethoxypropane as a standard and from a standard curve of TBA adduct formation that produce a complex with absorbance at 532 nm. Results were described as μg mg⁻¹ protein. Details were noted previously by Ghazanfari et al. (2006).

Total protein of colon tissue: The concentration of protein in the colon homogenate was measured by the Bradford method using BSA as the standard. Results were reported as mg mL⁻¹ of the homogenized tissue.

Statistical analysis: All data are expressed as mean±SEM. Differences between groups were analyzed by ANOVA and Newman-Keuls test. The p<0.05 was considered statistically significant.

RESULTS
Macroscopic and microscopic evaluation of the colonic damage: Results of colonic damage are shown in Table 1 and Fig. 1. Control group had severe ulceration, dilatation, necrosis, massive infiltration of inflammatory cells in the mucosa and sub-mucosa, crypt abscesses, severe edema and

<table>
<thead>
<tr>
<th>Groups</th>
<th>Macroscopic score</th>
<th>Microscopic score</th>
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<tbody>
<tr>
<td></td>
<td>Mean±SEM Median Min-Max</td>
<td>Mean±SEM Median Min-Max</td>
</tr>
<tr>
<td>Sham</td>
<td>0.00±0.00 0.0 0-0.0</td>
<td>0.00±0.00 0 0-0.0</td>
</tr>
<tr>
<td>Control</td>
<td>6.68±0.66* 6.0 5.0-7.0</td>
<td>3.80±0.40* 4 2.0-4.0</td>
</tr>
<tr>
<td>DEXA</td>
<td>1.80±0.34 2.0 0.0-3.0</td>
<td>2.50±0.40 2 1.0-4.0</td>
</tr>
<tr>
<td>L. casei (treatment)</td>
<td>2.57±0.25 2.0 2.0-4.0</td>
<td>1.71±0.28 2 1.0-4.0</td>
</tr>
<tr>
<td>LB (treatment)</td>
<td>2.70±0.60 2.0 2.0-4.0</td>
<td>1.90±0.30 2 1.0-4.0</td>
</tr>
<tr>
<td>LS (treatment)</td>
<td>3.25±1.29* 2.0 2.0-7.0</td>
<td>1.40±0.29* 2 1.0-3.0</td>
</tr>
<tr>
<td>L. casei (prevention)</td>
<td>5.00±0.866* 5.5 2.0-7.0</td>
<td>3.30±0.35** 3 2.0-4.0</td>
</tr>
<tr>
<td>LB (prevention)</td>
<td>6.00±0.86* 5.5 2.0-7.0</td>
<td>3.30±0.31** 3 2.0-4.0</td>
</tr>
<tr>
<td>LS (prevention)</td>
<td>5.30±0.87* 5.5 2.0-7.0</td>
<td>3.30±0.31** 3 2.0-4.0</td>
</tr>
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a,b,c: Significantly different from sham, control, dexta and L. casei group at p<0.05, respectively.
Fig. 1(a-d): Photomicrograph of colon trans-mural histology in experimental groups, Ulcerative colitis was produced after TNBS administration, characterized by complete degeneration and necrosis of epithelium and crypt abscesses, severe edema, hemorrhages and congestion (a) and massive infiltration of inflammatory cells in the mucosa and sub-mucosa (a, b). As shown in pictures (c) and (d), treatment with LB and LS did not reduce the morphological alteration associated with TNBS administration so that multifocal areas of epithelial necrosis and severe inflammatory cell infiltration are seen.

Hemorrhages induced by TNBS, in comparison to Sham group which had regular mucosal layer without any damage (p<0.001). Dexamethasone improved macroscopic scores in colitis rats in comparison to controls (p<0.01). Treatment groups with LB significantly showed mild inflammatory cell infiltration and improved mucosal and epithelium layer (p<0.01) when compared to control group. LB and LS were more effective in improving mucosal layer when compared with L. casei group. The prevention groups of LB and LS showed significant difference with Sham group (p<0.01). Histological examination of control group showed multifocal degenerative changes in the lining epithelium and areas of necrosis, extensive mucosal and sub-mucosal damage with congested blood vessels, severe edema and hemorrhages along with extensive infiltration of inflammatory cells and increase in macrophage and lymphocyte levels in ulcer region and submucosa whereas in Sham group, features of colons were within normal limits (p<0.01). In Dexa group, no ulcer, necrosis and inflammation in mucosa and submucosa was demonstrated. Treated groups showed no necrosis in crypts and deceased polymorphonuclear leucocytes (PMN). In addition, ulcer improvement and reduction in macrophage and lymphocyte levels in mucosa and submucosa region was observed. However, in prevention groups there was an increase in amount of macrophage, lymphocyte, inflammatory cells, ulcer and necrosis in mucosa.

**Colonic TNF-α level**: TNF-α was higher in controls when compared to Sham group (p<0.05). In Dexa group, TNF-α noticeably decreased when compared to controls (p<0.05). In treatment groups, administration of *L. casei* (p<0.05), LB (p<0.05) and LS (p<0.05) significantly reduced TNF-α in
Fig. 2: Changes in TNF-α contents in studied groups, a, b, c, dSignificantly different from sham, control, dexta and L. casei group at p<0.05, respectively.

Fig. 3: Changes in myeloperoxidase (MPO) activity in studied groups, a, b, c, dSignificantly different from sham, control, dexta and L. casei group at p<0.05, respectively.

comparison to control. Also more reduction in TNF-α was observed in LB and LS groups in comparison to L. casei group (p<0.05). TNF-α was significantly different in prevention groups of L. casei, LS, LB when compared with Sham and Dexta groups (p<0.05) (Fig. 2).

Colonic MPO activity: Colonic MPO activity in control group was significantly higher than that of sham group (p<0.05). MPO activity was significantly lower in Dexta group in comparison to control group (p<0.05). Treatment with L. casei, LB and LS (p<0.05) decreased MPO activity significantly when compared with control group, also LB and LS was different in comparison to L. casei (p<0.05). MPO activity was significantly higher in L. casei, LB treatment groups when compared to Dexta group (p<0.05) (Fig. 3). However, in prevention groups L. casei, LB and LS, the MPO activity was higher than that of Dexta and Sham groups (p<0.05).

Colonic lipid peroxidation level: TBARS as a marker of lipid peroxidation noticeably increased in controls in comparison to Sham group (p<0.05). TBARS was significantly lower in Dexta group than that of controls (p<0.05). A significant decrease in TBARS was shown in treatment groups L. casei, LB and LS in comparison to controls (p<0.05). TBARS was lower in LB and LS treatment groups than that of L. casei group (p<0.05). In L. casei, LB and LS prevention groups, TBARS was not significantly lower in comparison to Sham and Dexta groups (p<0.05) (Fig. 4).
Fig. 4: Changes in TBARS contents in studied groups. * b, c dSignificantly different from sham, control, dexe and L. casei group at p<0.05, respectively

DISCUSSION

Results of the present study demonstrated the beneficial effects of LB and LS in an experimental model of ulcerative colitis induced by TNBS. Combination of LB and LS significantly improved macroscopic and histological scores of colitis and reduced biochemical markers of inflammation as one of the potent pathological factors in IBD development, such as inflammatory cytokines, cellular lipid peroxidation, neutrophils and enhanced the antioxidant power of colonic tissue. We also observed that combinations of LB and LS improved mucosal layer significantly more than single L. casei group.

In IBD, pro-inflammatory cytokines such as TNF-α, are widely secreted during inflammation from monocytes and macrophages. In addition, T cells, B cells, NK cells and mast cells are increased. Therefore, a successful treatment should reduce these factors to show its therapeutic effect in IBD (Rezaie et al., 2007).

In previous studies, L. casei has positively affected IBD via decreasing TNF-α level (Tien et al., 2006). In addition, the effect of S. boulardii on human colon cells and reducing inflammation in TNBS-induced colitis model of rats were demonstrated. S. boulardii also increases expression of peroxisome proliferator-activated receptor-gamma (PPAR-γ) and inhibits secretion of IL-8 secretion most probably through modulating TNF-α. Furthermore, S. boulardii inhibits expression of pro-inflammatory cytokine genes in the colonic cells. S. boulardii is also effective against infectious pathogens in the GI tract. It degrades toxin A by producing serine protease that inhibits binding of pathogens to the intestinal membrane (Ghasemi-Niri et al., 2011; Lee et al., 2009; Castagliuolo et al., 1996). Colonic epithelium contains the highest amount of PPAR-γ that is known as a modulator of cellular metabolism (Pajas et al., 1997), adipocyte differentiation (Rosen and Spiegelman, 2001), macrophage lipid transport (Chawla et al., 2001) and finally regulating process of inflammation. S. boulardii increases PPAR-γ gene transcription which is down regulated by inflammatory cytokines Kelly et al. (2004).

In the other hand, Bifidobacterium strains influence production of anti-inflammatory cytokines such as IL-10 in UC patients. IL-10 reduces TNF-α by affecting macrophages (Imaoka et al., 2008).

In conclusion, present results reveal the potential beneficial effect of combination of LB and LS in recovering inflammation and involved elements as confirmed by biochemical and histopathological examinations. In this study, LB and LS when used as treatment were more effective than prevention.

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