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Saccharomyces boulardii ameliorates clarithromycin- and methotrexate-induced intestinal and hepatic injury in rats

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Abstract

Saccharomyces boulardii is a probiotic used for the prevention of antibiotic-associated diarrhoea. We aimed to investigate whether *S. boulardii* could alter the effects of clarithromycin (CLA) and methotrexate (MTX) on oro-caecal intestinal transit and oxidative damage in rats. Rats were divided into two groups receiving a single dose of MTX (20 mg/kg) or CLA (20 mg/kg per d) for 1 week. Groups were treated with either saline or *S. boulardii* (500 mg/kg) twice per d throughout the experiment. The control group was administered only saline. Following decapitation, intestinal transit and inflammation markers of glutathione (GSH), malondialdehyde and myeloperoxidase were measured in intestinal and hepatic tissues. CLA and MTX increased intestinal transit, while *S. boulardii* treatment slowed down CLA-facilitated transit back to control level. Both MTX and CLA increased lipid peroxidation while depleting the antioxidant GSH content in the hepatic and ileal tissues. Conversely, lipid peroxidation was depressed and GSH levels were increased in the ileal and hepatic tissues of *S. boulardii*-treated rats. Increased ileal neutrophil infiltration due to MTX and CLA treatments was also reduced by *S. boulardii* treatment. Histological analysis supported that *S. boulardii* protected intestinal tissues against the inflammatory effects of both agents. These findings suggest that *S. boulardii* ameliorates intestinal injury and the accompanying hepatic inflammation by supporting the antioxidant state of the tissues and by inhibiting the recruitment of neutrophils. Moreover, a preventive effect on MTX-induced toxicity is a novel finding of *S. boulardii*, proposing it as an adjunct to chemotherapy regimens.

Key words: ***Saccharomyces boulardii*: Clarithromycin: Methotrexate: Intestinal toxicity: Hepatic toxicity**

Probiotics are viable micro-organisms that confer health benefits to the host when administered in adequate amounts. They have been used to treat several acute infectious and chronic intestinal diseases[1]. *Saccharomyces boulardii* is a probiotic yeast that has been shown to be effective in the prevention of antibiotic-associated diarrhoea (AAD)[2]. It exerts trophic effects, both in the mucosa and the endoluminal fluid of the small intestine, which appears to be mediated by the endoluminal release of polyamines[3]. As secretion of IgA and polymeric Ig receptors into the lumen of the small intestine impairs the attachment of micro-organisms and external antigens to intestinal epithelial cells, the proliferation of pathogens in the gut lumen is thus prevented[4]. In a mouse model of inflammatory bowel disease, *S. boulardii* treatment was shown to inhibit inflammatory bowel disease by suppressing $CD4^+$ T-cell number and interferon-γ production within the colon, suggesting that both humoral and cellular immune defences are involved in the beneficial effects of *S. boulardii* on AAD[1]. Recent data support the efficacy of *S. boulardii* in gastrointestinal inflammatory conditions, including bacterial infections and inflammatory bowel disease, through modulation of host pro-inflammatory responses by controlling inflammation at different levels, such as the NF-κB and the mitogen-activated protein kinase pathways[5,6]. However, the effects of probiotics on oro-caecal motility or

Abbreviations: AAD, antibiotic-associated diarrhoea; CLA, clarithromycin; GSH, glutathione; MDA, malondialdehyde; MPO, myeloperoxidase; MTX, methotrexate.

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gastrointestinal antioxidant capacity in inflammatory models have not been described before.

AAD is caused by antibiotics used to treat infections by enteric pathogens via the direct effects on the intestinal mucosa or due to the metabolic consequences of reduced concentrations of faecal flora[7]. However, neither mechanism is clearly established as the cause of AAD. Although the frequency of AAD varies among antibacterial agents, diarrhoea occurs in 7·5% of patients treated with clarithromycin (CLA)[8]. CLA, a semi-synthetic macrolide antibiotic that inhibits bacterial protein synthesis, is clinically active against Gram-positive and Gram-negative cocci, Gram-negative bacilli (primarily *Haemophilus influenzae*, *Legionella* species, *Moraxella catarrhalis*, *Campylobacter jejuni*, *Bordatella pertussis* and *Helicobacter pylori*)[9]. It undergoes extensive hepatic metabolism, mainly by hydroxylation, and there is a substantial first-pass effect. Unchanged CLA and its metabolites are eliminated in the faeces and urine[9]. Despite the extensive use of CLA in the treatment of skin, soft tissue, upper and lower respiratory tract infections, sexually transmitted *Chlamydia trachomatis* infection and as a component of anti-*H. pylori* regimens, AAD and hepatotoxicity frequently limit its use[10].

Methotrexate (MTX), a folic acid antagonist, is widely used as a cytotoxic chemotherapeutic agent for leukaemia and other malignancies. MTX inhibits dihydrofolic acid reductase, and thus interferes with DNA synthesis, repair and cellular replication[11]. As the cytotoxic effect of MTX is not selective for cancer cells, normal tissues that have a high rate of proliferation, including the haematopoietic cells of the bone marrow and the actively dividing cells of the gut mucosa, are more susceptible to toxicity. Additionally, two rat studies have shown increased intestinal transit with parenteral MTX[12,13], though the relevant data are lacking in human subjects. Thus, the efficacy of the agent is often limited by severe hepatic and intestinal mucosal damage.

The purpose of the present study was to elucidate the putative protective effects of *S. boulardii* on oxidative damage induced by two different mechanisms, namely, metabolic alteration of faecal flora by CLA and direct cellular toxicity by MTX. We also aimed to study the impact of the yeast therapy on AAD-induced and MTX-induced diarrhoea.

Materials and methods

Animals

Adult male Sprague–Dawley rats (250–320 g) supplied by the Marmara University Animal Centre (DEHAMER) were housed individually in a light- and temperature-controlled room on a 12 h light–12 h dark cycle, where the temperature (22 ± 2°C) and relative humidity (65–70%) were kept constant. The animals were fed a standard pellet laboratory chow, and food was withdrawn overnight before emptying experiments, but free access to water was allowed. Experiments were approved by the Marmara University Animal Care and Use Committee.

Administration of drugs

Rats either received a single intraperitoneal injection of MTX (20 mg/kg; Onco-Tain, Faulding Pharmaceutics Plc) or they were administered daily with CLA (20 mg/kg per d; Deva İlaç Sanayii) for 7 d by an orogastric catheter under brief diethyl ether anaesthesia. Following the single MTX injection or accompanying the CLA administration, rats were also treated with either saline or *S. boulardii* (Reflor®, Biocodex; 500 mg/kg twice per d) by an orogastric catheter for seven consecutive days. *S. boulardii* is available as a lyophilised preparation in Turkey, which is stable for over 1 year at room temperature when protected from moisture and maintains high viability counts over prolonged periods[6]. The viability analysis of the probiotic showed $2{\cdot}5 \times 10^9$ living cells per 250 mg sachet, as provided by the manufacturer. The dose selection of the test drugs was made according to the previous *in vivo* rat studies[13–16]. In control rats, following a single dose of saline injection, saline was administered orally for 7 d. Groups consisted of seven to nine rats.

Measurement of intestinal transit

All rats were fasted overnight and intestinal transit studies were performed by giving 1 ml of a mixture of Arabic gum (gum Arabic from Acacia tree, Sigma Chemical) and activated charcoal through an intraduodenal catheter on the 8th day of the experiment at 08.00 hours[17]. After 20 min, rats were killed by decapitation, the abdomen was opened and ligatures were made around the pylorus and ileocaecal valve. The small intestine was dissected and freed from its mesentery, with its continuity retained. The intestine was then measured by laying it longitudinally. To avoid movement of intraluminal contents, the intestine was not stretched. The total length of the small bowel and the length of small bowel filled with the black meal were recorded. Intestinal transit index (%) was expressed as the fraction of the total length of the small bowel filled with the black material.

Measurement of tissue myeloperoxidase activity

Tissue-associated myeloperoxidase (MPO) activity is frequently utilised to estimate tissue neutrophil accumulation in inflamed tissues. The method of assay of MPO activity in the present study was similar to that previously described by others[18]. The ileum and liver tissue samples (0·2–0·3 g) were homogenised in ten volumes of ice-cold potassium phosphate buffer (50 mM-K_2HPO_4, pH 6·0) containing hexadecyltrimethylammonium bromide (0·5%, w/v). The homogenate was centrifuged at 41 400 ***g*** for 10 min at 4°C, and the supernatant was discarded. The pellet was then rehomogenised with an equivalent volume of 50 mM-K_2HPO_4 containing 0·5% (w/v) hexadecyltrimethylammonium bromide and 10 mM-EDTA (Sigma). MPO activity was assessed by measuring the H_2O_2-dependent oxidation of *o*-dianizidine.2HCl. One unit of enzyme activity was defined as the amount of MPO present per g of tissue weight that caused a change in absorbance of 1·0/min at 460 nm and 37°C.

British Journal of Nutrition

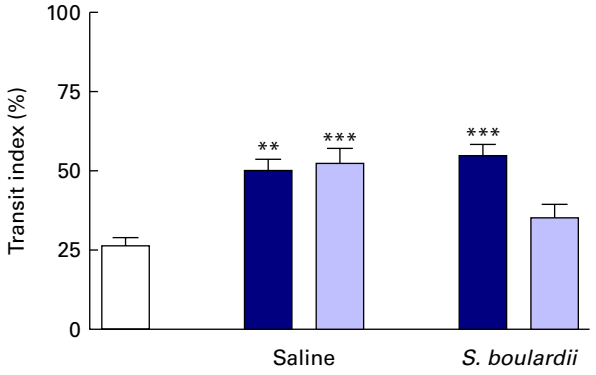

Fig. 1. Intestinal transit index (%) in orally *Saccharomyces boulardii*- or saline-treated rats that received either methotrexate (■) injection or daily clarithromycin (▨) by oral administration. Values are means, with standard errors represented by vertical bars. Mean values were significantly different compared with the saline-treated control (□) group: ** $P<0{\cdot}01$, *** $P<0{\cdot}0001$. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

Determination of lipid peroxidation (malondialdehyde) and glutathione levels

Ileal and hepatic tissue samples were homogenised in a 10 ml volume of ice-cold 10 % trichloroacetic acid, in an Ultra Turrax tissue homogeniser. Homogenised tissue samples were centrifuged at 3000 rpm for 15 min at 4°C. The supernatant was removed and recentrifuged at 15 000 rpm for 8 min. Glutathione (GSH) measurements were performed using a modification of the Ellman procedure[19]. Lipid peroxidation was quantified by measuring the formation of thiobarbituric acid-reactive substances, as described previously[20]. Lipid peroxide levels were expressed in nmol malondialdehyde (MDA) per g tissue.

Histopathological preparation and analysis

For the light microscopic investigations, tissue specimens from the ileum were fixed with 10 % formaldehyde and processed routinely for embedding in paraffin. Approximately, 5 μm-thick tissue sections were stained with haematoxylin and eosin and examined under an Olympus BX51 photomicroscope. Microscopic scoring was done by an experienced histologist, who was unaware of the treatments. Scores were given as 0, none; 1, mild; 2, moderate; 3, severe for each criterion, using the semi-quantitative scale as follows: (1) degeneration of surface and crypt epithelium; (2) degeneration of villus structure; (3) inflammatory cell infiltration. The microscopic score of the ileum was calculated as the sum of the scores given to each criterion, and at least five microscopic areas were examined to score each specimen. The maximum score that could be given was '9'[21].

Statistical analysis

All data are expressed as means with their standard errors. Each group consisted of seven to nine rats. Groups of data were compared with an ANOVA followed by Tukey's multiple comparison tests, and for pair-wise comparisons, Student's *t* test and Mann–Whitney *U* test were used. Results were considered significant when *P* was less than 0·05. Calculations were made using GraphPad Prism 3.0 (GraphPad Software).

Results

At the end of the experiment, all the rats in the groups had survived and no significant differences were observed among the weights of the animals.

Effect of Saccharomyces boulardii *on intestinal transit*

MTX and CLA significantly facilitated intestinal transit by nearly 2-fold in the saline-treated groups ($P<0{\cdot}01$ and $<0{\cdot}001$; Fig. 1). *S. boulardii* administration had no significant effect on increased intestinal transit due to MTX treatment. Nevertheless, *S. boulardii* depressed CLA-induced rapid intestinal transit, but was not statistically different from the transit index of the control group ($P>0{\cdot}05$).

Ileal and hepatic malondialdehyde and glutathione levels

MDA levels determined in the ileum and liver tissues were found to be significantly higher in both the saline-treated MTX and CLA groups, as compared with those in the tissues of the control group ($P<0{\cdot}01$; Fig. 2(a) and (b)). In the ileum, *S. boulardii* administration significantly decreased the MTX- or the CLA-associated lipid peroxidation ($P<0{\cdot}01$). In the liver, *S. boulardii* was not effective on MTX-associated MDA elevation, but it depressed hepatic MDA of the CLA-treated group significantly ($P<0{\cdot}01$).

The levels of the major cellular antioxidant GSH in both tissues were significantly decreased in the saline-treated MTX and CLA groups ($P<0{\cdot}01$–$0{\cdot}001$; Fig. 3(a) and (b)). Treatment with *S. boulardii* reduced the reductions in GSH

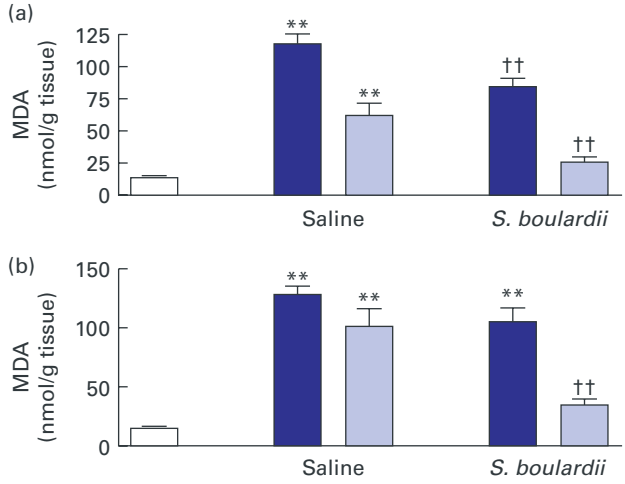

Fig. 2. Malondialdehyde (MDA) levels in the (a) ileum and (b) liver of the orally *Saccharomyces boulardii*- or saline-treated rats that received either methotrexate (■) injection or daily clarithromycin (▨) by oral administration. Values are means, with standard errors represented by vertical bars. ** Mean value was significantly different compared with the saline-treated control (□) group ($P<0{\cdot}01$). †† Mean value was significantly different compared with the respective saline-treated group ($P<0{\cdot}01$). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

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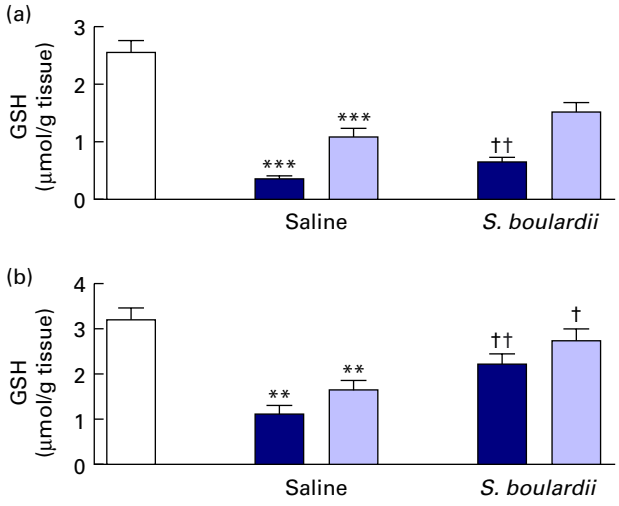

Fig. 3. Glutathione (GSH) levels in the (a) ileum and (b) liver of the orally *Saccharomyces boulardii*- or saline-treated rats that received either methotrexate (■) injection or daily clarithromycin (□) by oral administration. Values are means, with standard errors represented by vertical bars. Mean values were significantly different compared with the saline-treated control (□) group: ** $P<0·01$; *** $P<0·001$. Mean values were significantly different compared with the respective saline-treated group: † $P<0·05$; †† $P<0·01$. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

content in the hepatic and ileal tissues of both the MTX- and the CLA-treated groups ($P<0·05$–$0·01$).

Tissue myeloperoxidase activity

MPO activities in the ileal tissues were increased in the saline-treated MTX and CLA groups ($P<0·001$; Fig. 4(a)), indicating recruitment of neutrophils to the inflamed ileum. *S. boulardii* administration reduced both the MTX- and CLA-induced elevation in MPO activity in the ileal tissue ($P<0·001$ and $P<0·01$). The MTX-induced increase in the hepatic MPO activity ($P<0·001$, Fig. 4(b)) was depressed in the *S. boulardii*-treated group ($P>0·05$). However, CLA treatment with or without *S. boulardii* had no impact on hepatic MPO activity.

Histopathological results

The light microscopic findings of the small-bowel mucosa were entirely normal in the control group. By contrast, histological analysis revealed the presence of MTX-induced damage in the ileum (Fig. 5). In the saline-treated MTX group, the ileal mucosa showed mild degeneration of the surface epithelium, flattened villi and severe inflammatory cell infiltration. In the MTX group treated with the probiotic, the ileum showed mild degeneration in the surface epithelium and mild inflammation. Severe degeneration in the surface epithelium and villi, and severe inflammation in the saline-treated CLA group were replaced with mild degeneration in the surface epithelium and moderate inflammation in the *S. boulardii*-treated CLA group. Histopathological scores of the ileum showed that *S. boulardii* treatment reduced the degeneration of the surface and crypt epithelium, the villus structure and depressed inflammatory cell infiltration of both the MTX and CLA-treated groups ($P<0·05$, Fig. 6).

Discussion

The present findings revealed that CLA and MTX increased intestinal transit significantly, as compared with the control rats, while *S. boulardii* treatment slowed down CLA-facilitated transit back to control level. The results showed that both MTX and CLA increased lipid peroxidation of the ileal and hepatic tissues, along with depletion of the antioxidant GSH content in both tissues. Moreover, antioxidant GSH levels were increased in the ileal and hepatic tissues of *S. boulardii*-treated rats, and lipid peroxidation in these tissues was depressed. Accordingly, increased ileal neutrophil infiltration due to the pro-inflammatory MTX and CLA treatments was also reduced by *S. boulardii* treatment. In addition, histological analysis supported that *S. boulardii* significantly protected the intestinal tissues against the inflammatory effects of both agents. These findings suggest that *S. boulardii* ameliorates intestinal injury and the accompanying hepatic inflammation by supporting the antioxidant state of the tissues and inhibiting recruitment of pro-oxidant neutrophils to the tissues.

The macrolide group antibiotic CLA has a stimulatory effect on gut motility because it has a fourteen-membered ring like erythromycin, which stimulates gastroduodenal motility through the activation of motilin receptors[22,23]. Although having no direct action on motilin receptors, the ability of *S. boulardii* to counteract the disturbed motility induced by CLA could be explained by the beneficial effects of *S. boulardii* on altered gut flora. Within the intestinal lumen, *S. boulardii* may restore motility by suppressing the inflammatory state, as demonstrated in the present study. Since it is metabolised by cytochrome P450 isoenzymes[24], asymptomatic

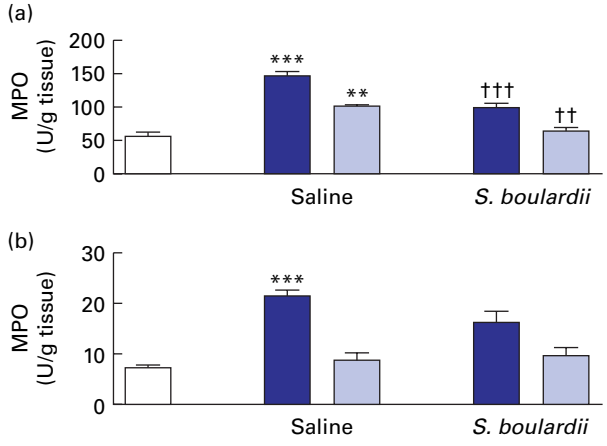

Fig. 4. Myeloperoxidase (MPO) activity in the (a) ileum and (b) liver of the orally *Saccharomyces boulardii*- or saline-treated rats that received either methotrexate (■) injection or daily clarithromycin (□) by oral administration. Values are means, with standard errors represented by vertical bars. Mean values were significantly different compared with the saline-treated control (□) group: ** $P<0·01$; *** $P<0·001$. Mean values were significantly different compared with the respective saline-treated group: †† $P<0·01$; ††† $P<0·001$. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

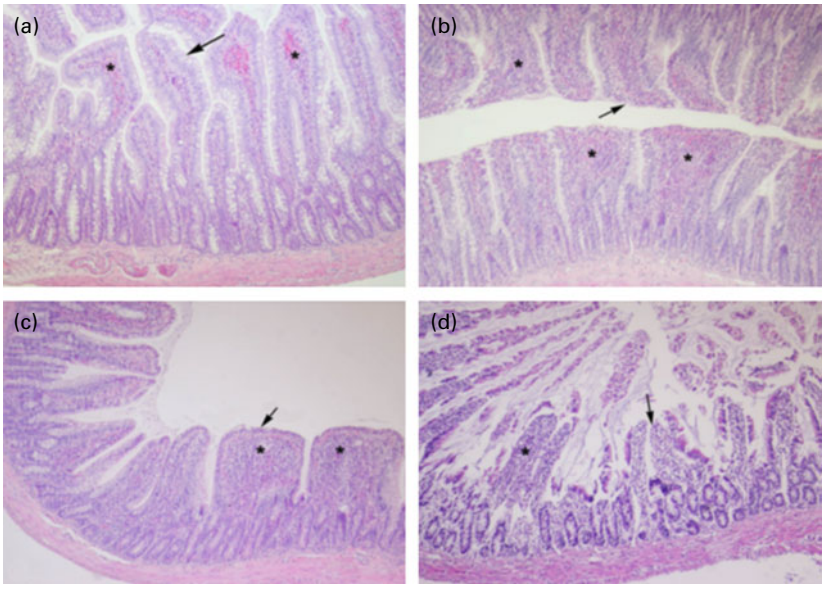

Fig. 5. Micrographs of ileal tissues. (a) *Saccharomyces boulardii*-treated methotrexate (MTX) group: mild degeneration in the surface epithelium (→), mild inflammatory cell infiltration (*). (b) Saline-treated MTX group: mild degeneration in the surface epithelium (→), severe inflammatory cell infiltration (*). (c) *S. boulardii*-treated clarithromycin (CLA) group: mild degeneration in the surface epithelium (→), moderate inflammatory cell infiltration (*). (d) Saline-treated CLA group: severe degeneration in the surface epithelium (→), severe inflammatory cell infiltration (*). Haematoxylin and eosin staining, original magnification ×100. (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

elevation of transaminases and cholestatic liver injury are well-known in patients treated with this antibiotic[10]. An earlier study in rats has suggested that hepatocellular injury induced by another macrolide antibiotic, troleandomycin, involved the local depletion of GSH via toxic metabolites formed in cytochrome P 450[25]. According to a meta-analysis by Johnston *et al.*[26], a dose of 5–40 billion colony-forming units/d of *S. boulardii* had the most promise of decreasing AAD. In accordance with this report, the present findings demonstrate that CLA and MTX increased the intestinal transit, and the drug-induced ileal and hepatic oxidative injury was accompanied by a depletion of hepatic and intestinal GSH stores. Furthermore, *S. boulardii* (approximately in a dose of 2·5 billion colony-forming units/d) effectively reversed all these alterations and normalised the intestinal motility. Apart from its beneficial effects on the prevention of AAD in human subjects, including regimens containing CLA[27], *S. boulardii* has not been investigated before for its anti-inflammatory effect on antibiotic-induced gut inflammation. The present results demonstrate that in two different models of drug-induced oxidative injury of the liver and the intestine, *S. boulardii* showed anti-inflammatory and antioxidant actions. Regarding the beneficial effects of probiotics in experimental colitis, *Lactobacillus salivarius* treatment resulted in decreased MPO activity and increased GSH content in the colon together with reductions in TNF-α and leukotriene B4 levels[28]. Various studies investigated the mechanism to explain the anti-inflammatory effects of *S. boulardii*. *In vivo* and *in vitro* extracellular signal-regulated kinases 1/2 mitogen-activated protein kinase activity and IL-8 production were shown to decrease with *S. boulardii* treatment in *Clostridium difficile* toxin A-induced enteritis[29]. The anti-inflammatory action of the yeast was further intensified by increasing the barrier integrity of *Shigella*-infected tissues, thus preventing the migration of pro-inflammatory factors[30]. Additionally, recent data showed that the yeast produces a low-molecular weight soluble factor that blocks NF-κB activation and NF-κB-mediated *IL-8* gene expression in intestinal epithelial cells and monocytes[31].

For many chemotherapeutic agents, including MTX, chemotherapy-induced gut toxicity remains a major dose-limiting side effect, which may limit the efficacy of chemotherapy, affect overall malnutrition, aggravate cancer cachexia and may even contribute to worsened prognosis[32]. Patients undergoing chemotherapy experience symptoms of nausea, vomiting, cramping, diarrhoea, abdominal pain and, in its

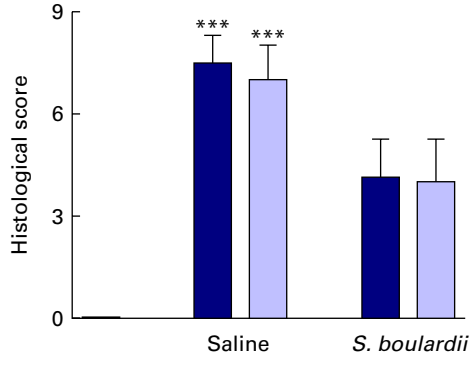

Fig. 6. Histological scores of ileal damage of *Saccharomyces boulardii*- or saline-treated rats that received either methotrexate (■) injection or daily clarithromycin (□) by oral administration. Values are means, with standard errors represented by vertical bars. *** Mean value was significantly different compared with the saline-treated control (□ group ($P<0·001$). † Mean value was significantly different compared with the respective saline-treated. Mean vaule group ($P<0·05$). (A colour version of this figure can be found online at http://www.journals.cambridge.org/bjn).

most severe form, necrosis and perforation of the bowel[33]. Despite several symptomatic treatment options in present practice, there is still an obvious requirement to develop new agents to protect the bowel during cancer chemotherapy. *S. boulardii* was shown to significantly suppress expression of pro-inflammatory cytokine genes[34] and reduced diarrhoea, weight loss, leucocyte migration and mucosal inflammation in chemotherapy-induced mucosal damage[35]. Consequently, mucositis shown in various models as well as MTX mucositis in the present study were alleviated by *S. boulardii;* therefore, further evaluation is warranted for the use of the yeast in chemotherapy-induced intestinal injury in human subjects.

Probiotics have been demonstrated to support intestinal epithelial barrier function by preventing the adherence of pathogens to the mucosal surface[36] and by enhancing phagocytosis and non-specific immune response[37]. Previously, *Streptococcus thermophilus* was shown to diminish the severity of small-intestinal mucositis in MTX-treated rats, as monitored by the ^{13}C-sucrose breath test, where increased tissue MPO activity was also depressed by the probiotic[38]. We found increased MPO activity in both the ileal and hepatic tissues of the rats treated with MTX, while *S. boulardii* treatment decreased the MPO levels in the ileum and liver, suggesting decreased neutrophil infiltration to the inflamed tissues. Supporting our findings, an earlier study has shown reduced transepithelial migration of polymorphonuclear leukocytes in the presence of the yeast[30]. Regarding the hepatic effects of *S. boulardii* on the MTX-treated group, the increased GSH levels, as compared with the saline-treated MTX group, have not been described before. This finding points to the antioxidant effects of *S. boulardii* on the liver.

The anti-inflammatory effects of *S. boulardii*, as shown in the present study, constitute a growing interest in the nutrition arena. Trials in a wide range of animal models are currently being conducted to identify which component of *S. boulardii* works to reduce the inflammatory status. Whether the live yeast cell *per se* or the wall structure of the killed yeast is responsible for the beneficial effects is a matter of debate. The study by Generoso *et al.*[39] showed similar effects with viable or heat-killed *S. boulardii* on preventing bacterial translocation and increasing the anti-inflammatory cytokine, IL-10, levels in rats having intestinal injury. By contrast, Zanello *et al.*[40] reported that viable and killed *S. cerevisiae,* but not viable *S. boulardii,* was effective in protecting against intestinal inflammation induced by enterotoxigenic *Escherichia coli*. Even more conflicting were the results by Jawhara *et al.*[41] stating that the β-glucan fraction of any yeast (*S. cerevisiae*, *S. boulardii* or even *Candida albicans*) has protective effects against intestinal inflammation in mice.

CLA is an antibiotic that might cause altered gut flora more than MTX. Altered gut microbiota is thought to cause chronic liver injury via bacterial translocation through the permeable tight junctions of the gut into the portal bloodstream. Since the data from other animal models have shown that translocation from the gut to the liver axis is reduced with *S. boulardii* treatment[42,43], this is likely to be a mechanism for the decreased inflammation with the probiotic treatment. Probiotic administration appears to decrease both the hepatic and ileal MDA levels in the CLA-treated group. However, the mechanism of liver damage is totally different in the MTX group, which is apparent in the failure of the probiotic to depress hepatic MDA levels, suggesting that the probiotic may not be fully effective against drug-related hepatotoxicity.

In conclusion, our findings suggest that *S. boulardii* effectively suppressed the oxidative damage in rats due to MTX- or CLA-induced inflammation in the ileum and liver. These results are confirmed by the alleviated histological scores in the gut when *S. boulardii* was added to the treatment with a pro-inflammatory antibiotic or chemotherapeutic agent. The present results suggest that antioxidant protection is another mechanism through which *S. boulardii* is beneficial in the prevention of AAD. Moreover, the effects of that probiotic on MTX-induced toxicity is a novel finding proposing *S. boulardii* as an adjunct to clinical regimens in an attempt to prevent the intestinal and, in part, hepatic side effects.

Acknowledgements

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