Effect of *Lactobacillus plantarum* ACTT 8014 on 5-fluorouracil induced intestinal mucositis in Wistar rats

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Abstract. Some previous studies reported that probiotics might decrease the severity of chemotherapy-induced mucositis. This study assessed the potential protective effect of *Lactobacillus plantarum* ATCC 8014 on 5-fluorouracil (5-FU) induced intestinal mucositis in the Wistar rats. The Crl:WI rats were divided into two groups of six animals (F, L) and one group of 5 animals (M). Group L received for 9 days $3.32x10^9$ CFU/ml of *Lactobacillus plantarum* orally. In the 7th day of the experiment 400 mg of 5-FU was administered intraperitoneally in groups L and F. Group M received only the vehicles. All animals were sacrificed in the 9th day. Eleven histological characteristics of mucositis were quantified from 0 (normal) to 3 (severe) for duodenum, jejunum and colon. Semiquantitative grades measured Toll-like receptor 4 (TLR4) immunopositive cells. The independent groups were analyzed using the Kruskal-Wallis test, Mann-Whitney U test, with a Bonferroni correction for alpha ($P \leq 0.016$). In the group F, treated with 5-FU, the most affected areas were the jejunum and the duodenum. The medium score of histological lesions was 27 for jejunum (minimum 25, maximum 32) and 21 for duodenum (minimum 18, maximum 29). Graded microscopic mucosal changes of the jejunum were significantly lower in group L compared with group F ($U=0$, $P=0.009$, Mann-Whitney test). The histological changes depicted on the duodenal and colonic mucosa were less severe in group L than in group F, but without reaching the statistical significance (duodenum: $U=6$, $P=0.172$, Mann-Whitney test; colon: $U=12$, $P=0.916$, Mann-Whitney test). Although the TLR4 immunoexpression was more intense in group L, no significant statistical difference was revealed at duodenum, jejunum or colonic mucosa. Significantly fewer microscopic changes were depicted in L group on the jejunum, suggesting a potential beneficial effect of *Lactobacillus plantarum* at this level in 5-FU induced mucositis.

Introduction

5-Fluorouracil (5-FU) is a potent agent against solid tumors used since 1957 for the treatment of colorectal, pancreatic and breast cancer. Its side effects include mucositis with important clinical and cancer management impact (1). The clinical term mucositis, describes the damage of mucous membranes after anticancer therapies (1,2). It affects mainly the entire gastrointestinal (GI) tract and genitourinary tract (1). The GI symptoms include nausea and vomiting, abdominal pain, distension, and diarrhoea due to direct effects of the cytotoxics on GI crypt epithelium (1).

The exact mechanisms of GI mucositis are not fully understood. The proposed pathogenetic mechanisms were initially represented by alteration in absorptive functions of cells, mucin distribution and composition, direct effects of the cytotoxic drugs resulting in considerable apoptosis at the crypt base, bacterial translocation that subsequently triggers inflammatory processes (1,3-5). An important pathogenic observation reported that germ-free mice were more resistant to chemotherapy (6), which highlighted the role of the microbiota. Recent studies documented microbiota disturbances in chemotherapy induced mucositis in animal and clinical studies (7-13). Alexander *et al* (14) reported three main clinical possibilities for the modulation of host response

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Abbreviations: 5-FU, 5-fluorouracil; GI, gastrointestinal; IL, interleukin; LPS, lipopolysaccharides; TLR, Toll-like receptor; TNF, tumor necrosis factor

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to chemotherapeutic drugs by the gut microbiota: facilitation of drug efficacy, reducing anticancer effects and mediation of toxicity.

A rat model developed by the group conducted by Logan *et al* (15), based on intraperitoneal administration of a single dose of 150 mg 5-FU/kg body weight showed clear clinical and histological signs of GI mucositis. Using this model, pathogenetic mechanisms of mucositis were revealed. Besides gut microbiota, mucins are also involved in the development of 5-FU-induced alimentary mucositis (16). The microflora found within the duodenum and jejunum mainly consists of anaerobes, *Streptococcus* spp., *Lactobacillus* spp., *Veillonelae*, *Actinomyces* spp., and a variety of fungi (17). Following 5-FU, a decrease in *Clostridium* spp., *Lactobacillus* spp., and *Streptococcus* spp., and an increase in *Escherichia* spp. could be observed in the jejunum of rats (16). In contrast to the small intestine, the colonic microflora is more diverse, consisting of over 400 different species (17) and includes large numbers of anaerobes, enterococci and Enterobacteriaeceae (17,18). In the colon, 5-FU decreased *Enterococcus* spp., *Lactobacillus* spp., and *Streptococcus* spp. In fecal samples decreasing trends were observed for *Lactobacillus* spp. and *Bacteroides* spp. and increasing trends for *E. coli*. Significantly increased trends were noted in fecal samples for *Clostridium* spp. and *Staphylococcus* spp. at 24 h (16).

The 'disturbed' gut microbiota can activate Toll-like receptors (TLRs) and subsequently can up-regulate NFκB (19) and generate the tumor necrosis factor (TNF)-α, interleukin-1β (IL-1β), and interleukin-6 (IL-6) (20). Recent studies showed that variables such as environment, metabolism, dysbiosis and genetics can modulate the mucosal TLR signaling during cancer therapy (21-23). Paradoxically, TLRs might mediate both protective and destructive responses in chemotherapy induced mucositis (21). TLR2 might accelerate host detoxification by activating the multidrug transporter ATP-binding cassette 1 (ABCB1)/MDR1 P-glycoprotein to efflux the chemotherapeutic drug. In contrast, TLR4 activation might aggravate mucosal injury by hyperresponsiveness to lipopolysaccharides (LPS) (21).

In animal models attempts to overcome these disturbances were done using probiotics (24-29), regulators of fecal metabolites (30), rifaximin (31) or Chinese medicines with inhibitory effect on cell apoptosis in the intestinal crypt (32). In humans the treatment of mucositis is based on antibiotics (33). A recent systematic review concluded that *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Bifidobacterium breve*, *Bifidobacterium infantis,* and *Saccharomyces boulardii* could be a good combination of probiotics to prevent mucositis (33).

Since not all strains of *Lactobacillus* possess the ability to confer health benefits to the host, it becomes necessary to screen and characterize numerous strains in order to obtain ideal probiotics (34).

Lactobacillus plantarum is an allochthonous lactobacilli found in the human body but does not form a stable population in the human GI tract. It is a safe, non-gas-producing lactic acid bacterium (35). Beneficial effects of *Lactobacillus plantarum* strains such as probiotic were documented on gut-heart-brain axis, in gut disorders such as inflammatory bowel diseases, in metabolic syndromes, dyslipidemia, obesity, and diabetes, and in some psychological disorders (35). A less investigated strain is *Lactobacillus plantarum* American Type Culture Collection (ATCC) 8014, with high inhibitory activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysentery, P. aeruginosa, E. coli S5 and Salmonella typhi)* (35). These properties might be used in preventing 5-FU mucositis. Only one study reported the beneficial effects of riboflavin-overproducing strain *Lactobacillus plantarum* CRL2130 on 5-FU induced mucositis (27).

This study assessed the potential protective effect of *Lactobacillus plantarum ATCC 8014* on 5-FU induced intestinal mucositis in the Wistar rats.

Materials and methods

Animals and study design. Seventeen (n=17) male Crl:WI rats, aged between 10 and 12 weeks, with a median weight of 300±30 g were used in the present study. The outbred rats, having a Charles River origin were bred and kept at the Center for Experimental Medicine from the Iuliu Hatieganu University of Medicine and Pharmacy from Cluj-Napoca. They were housed in a conventional flora animal facility at a standard temperature of 22±2˚C and a relative humidity of 55±10%, in a 12:12-h light/dark cycle (lights on, 7 a.m. to 7 p.m.) at a light intensity of 300 lx at 1 m above the floor, in open-top polycarbonate type IV-S cages (Tecniplast, Buguggiate) on autoclaved wood chip bedding (Lignocel®; J. Rettenmaier & Söhne GmBH + Co. KG). They had access to autoclaved tap water in bottles and pelleted feed (Cantacuzino Institute, Bucharest, Romania) *ad libitum*.

The experiments were based on a previous methodology used by the authors (31). The experiments, in accordance with Romanian laws, were approved by the Institutional Animal Ethics Committee. Two interventional groups of 6 animals (named L and F) and one control group (M) with 5 Wistar rats were used.

Group L received, for 9 days, 3.32×10^9 CFU/ml of *Lactobacillus plantarum* ACTT 8014 by oral gavage, the animals were allowed to eat after two hours.

In the 7th day of the experiment 400 mg of 5-FU was administered intraperitoneally in groups L and F. Group M received only the vehicles. On the 9th day all animals were sacrificed using deep ether anesthesia. The intestinal tissue samples were collected from the duodenum, jejunum and colon.

Histological assessment and TLR4 immunoexpression. The gut samples were fixed in 10% phosphate-buffered formalin (pH 7.0) for 24 h, routinely processed, embedded in paraffin wax, cut into 3-4 micrometer sections and stained with hematoxylin and eosin (H&E). We used Olympus Bx51 microscope, Olympus SP 350 digital camera for photomicrographs and Stream basic imaging software (Olympus Corporation). The degree of mucositis was assessed by a previously described method by Howarth *et al* (36). Eleven histological criteria were used for each intestinal segment (duodenum, jejunum, and colon). A semiquantitative histological assessment was obtained by rating each histological criteria from normal (0) to severe (3) by two experimental pathologists (M. Taulescu and C. Toma). They semiquantified atrophy by villous fusion and

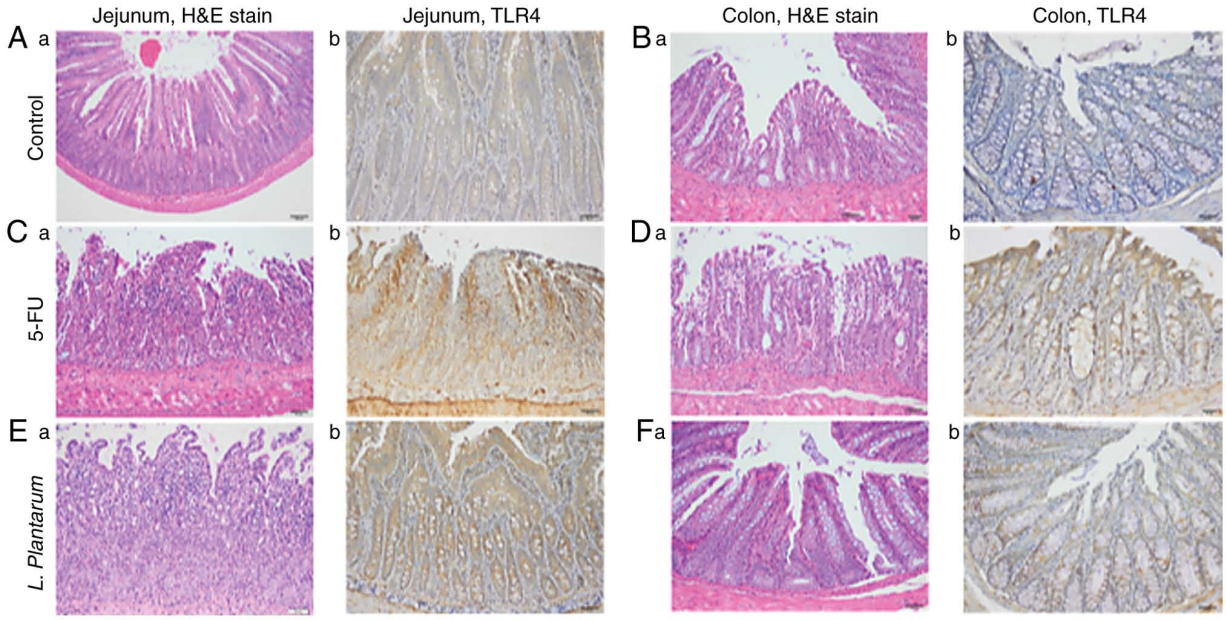

Figure 1. Microscopical findings of the intestinal mucosa from experimental Wistar rats exposed to 5-fluorouracil-induced intestinal mucositis. Control group: (Aa) jejunum and (Ba) colon, HE stain, Bar 100 μm, 50 μm; TLR4 immunoexpression in the jejunal mucosa (Ab) and colon (Bb), IHC stain, Bar 50 μm; 5-FU group: (Ca) jejunum and (Da) colon, H&E stain, Bar 50 μm; TLR4 immunoexpression in the jejunal mucosa (Cb) and colon (Db), IHC stain, Bar 50 μm, 20 μm; *L. plantarum* (ACTT 8014) group: (Ea) jejunum and (Fa) colon, H&E stain, Bar 50 μm; TLR4 immunoexpression in the jejunal mucosa (Eb) and colon (Fb), IHC stain, Bar 50 μm. 5-FU, 5-fluorouracil; H&E, hematoxylin and eosin; TLR4, Toll-like receptors 4.

stunting, disruption of brush border and surface enterocytes, reduction of goblet cells and of mitotic figures, disruption of crypt architecture and of crypt cells, formation of crypt abscesses, infiltration with polymorphonuclear cells (neutrophils and eosinophils) and lymphocytes, lymphangiectasia and congestion, thickening and edema of the submucosa and muscularis externa. Normal rat intestinal tissue was used as a baseline reference.

For immunohistochemistry, the sections were incubated at 37˚C for 12 h and were processed using automatic platform Leica BOND-MAX. The primary mouse-monoclonal antibody anti-TLR4 (ab22048, Abcam) was diluted in 1% PBS-BSA (bovine serum albumin) at 1:100. The Bond Polymer Refine Detection kit (DS9800, Novocastra) containing peroxide block, post-primary, polymer reagent, DAB chromogen and hematoxylin counterstain were used. The negative controls for each sample were prepared by replacing the primary antibody with mouse IgG1 Negative Control (Code X0931, Dako; Agilent Technologies, Inc.).

Immunopositivity for TLR4 was evaluated for mucosal epithelium, glandular epithelium, and lamina propria (37) and graded accordingly to previously described technique (38): 0 if it was the same as background, 0.5-close to background, 1-well marked positivity, 1.5 focally enhanced, 2-strong positivity, 2.5-very strong positivity.

Statistical analysis. The histological and immunohistochemistry scoring results were expressed as median IQR (interquartile range). The independent groups were analyzed using the Kruskal-Wallis test and Mann-Whitney U test. A Bonferroni correction for alpha was applied on Mann-Whitney U test, exact P-values ≤0.016 being considered statistically significant. For the statistical analysis, Soft IBM SPSS Statistics 20 was used.

Results

During the experiment, two animals from the F group presented diarrhea on the 8th day, after the 5-FU administration. No animal from group L and M had diarrhea.

In the control group, no important microscopical changes were noted at the level of the intestinal mucosa (Fig. 1Aa and Ba). The TLR4 immunopositivity was faint on epithelial surface, glandular epithelium and lamina propria at all levels of GI tract (Fig. 1Ab and Bb).

In group F, receiving only 5-FU, the most affected area was the jejunum with a medium score of 27 for jejunum (minimum 25, maximum 32). The intestinal lesions consisted of severe and diffuse villous atrophy and fusion, epithelial cell degeneration, necrosis and desquamation (erosions), multifocal crypt abscesses, edema, hemorrhages and infiltration of lamina propria with large numbers of neutrophils, lymphocytes and fewer macrophages and eosinophils (Fig. 1Ca). Low number of epithelial cell divisions was also identified at this level. Moderate degenerative and inflammatory lesions were also observed in the duodenum, with a median score of 21 (minimum 18, maximum 29) (Table I). The colonic mucosa showed mild to moderate inflammatory changes with the medium histological score of 5 (minimum 2, maximum 11) (Fig. 1Da).

In group F, moderate TLR4 staining was noted on epithelial surface, glandular epithelium and lamina propria of the jejunum (Table I and Fig. 1Ca). The intensity of TLR4 staining was also moderate on the epithelial surface, glandular epithelium and lamina propria of the duodenum (Table I). On the colon, the intensity of TLR4 staining was low on the epithelial surface, glandular epithelium and lamina propria of the colon (Table I and Fig. 1Db).

Table I. Descriptive statistics concerning microscopic degenerative lesions and TLR4 immune staining between interventional groups.

Site	Parameters	Group	Min	Median	Max	Mann-Whitney test
Duodenum	Histologic score	F	18	21	29	U=6, P=0.172
		L	13	15	23	
	TLR4 surface epithelium	F	0.5	1	2.5	U=5.5, P=0.258
		L	1	2	2,5	
	TLR4 glandular epithelium	F	0.5	1	1	U=7, P=0.439
		L	0.5	2	2	
	TLR4 lamina propria	F	0.5	1	1.5	U=9, P=0.799
		L	0.5	1	2	
Jejunum	Histologic score	F	25	27	32	U=0, P=0.009
		L	10	23	24	
	TLR4 surface epithelium	F	1	1.5	2.5	U=8, P=0.6
		L	1	2	2.5	
	TLR4 glandular epithelium	F	0.5	1.25	2	U=6, P=0.413
		L	0.5	2	2.5	
	TLR4 lamina propria	F	1	1.5	2	U=7,5, P=0.515
		L	0.5	2	2	
Colon	Histologic score	F	2	5	11	U=12, P=0.916
		L	1	6	7	
	TLR4 surface epithelium	F	0.5	0.75	1	U=7, P=0.371
		L	0.5	1	1	
	TLR4 glandular epithelium	F	0.5	0.5	0.5	U=4, P=0.074
		L	0.5	1	1	
	TLR4 lamina propria	F	0.5	0.5	0.5	U=6, P=0.273
		L	0	1	1	

TLR4, Toll-like receptors 4.

In group L (animals received *Lactobacillus plantarum* and 5-FU) the assessment of histological lesions in the jejunum mucosa depicted moderate lesions, with a medium histological score of 23 (minimum 10 and maximum 24) (Table I). The histological changes found at the level of the jejunal mucosa were represented by moderate villous atrophy and blunting, atrophy and multifocal erosions of the overlying epithelium and inflammatory infiltrates, predominated by neutrophils and lymphocytes in the lamina propria (Fig. 1Ea). The lesions in the duodenum mucosa were moderate, with a medium histological score of 15 (minimum 13 and maximum 23) (Table I). In the colonic mucosa the depicted lesions were low or absent, with a medium histological score of 6 (minimum 1 and maximum 7) (Table I and Fig. 1Fa).

In group L, the TLR4 immunoreactivity was higher on epithelial surface, glandular epithelium and lamina propria of duodenal, jejunal and colonic mucosa, compared with group F (Table I and Fig. 1 Eb and Fb).

The statistical analysis of graded microscopic degenerative lesions of the jejunum depicted significant differences between F and L groups (U=0, P=0.009, Mann-Whitney test). Graded TLR4 immunopositivity on jejunal mucosa, even more intense in L group, was not significantly different when compared with group F at the level of surface epithelium, glandular epithelium and lamina propria (Table I).

The statistical analysis of graded microscopic degenerative lesions of the duodenum did not reveal significant differences between F and L groups (U=6, P=0.172, Mann-Whitney test). Graded TLR4 immunopositivity on duodenal mucosa, although more intense in L group, was not significantly different when compared with group F at the level of surface epithelium, glandular epithelium and lamina propria (Table I).

The statistical analysis of graded microscopic degenerative lesions of the colon did not revealed significant differences between F and L groups (U=12, P=0.916, Mann-Whitney test). Graded TLR4 immunopositivity on colonic mucosa, although more intense in L group, was not significantly different when compared with group F at the level of surface epithelium, glandular epithelium and lamina propria (Table I).

Discussion

5-FU induced GI mucositis is most prominent in the small intestine (39). Our experimental model depicted severe histological lesions at jejunum in Wistar rats exposed to this chemotherapeutic agent. Moderate histological lesions were

observed in the duodenum; and mild to moderate inflammatory changes were present on colonic mucosa in animals exposed to 5-FU. How can these differences be explained as severe lesions were found in sites where the gut bacterial load is lower? The interplay between mucosal barrier, microbiota and immune system might be disrupted differently by 5-FU throughout the intestine. Several observations might explain this fact. First a higher ratio of proapoptotic to antiapoptotic genes in the small intestine than in the colon correlated with rapid changes in gene expression following chemotherapy might partially explain these differences (39,40). Also different changes in the intestinal microbiota were reported at different GI sites after 5-FU administration. As previously mentioned, following 5-FU, a decrease in *Clostridium* spp., *Lactobacillus* spp., and *Streptococus* spp., and an increase in *Echerichia* spp. were observed in the jejunum of rats (16). In the colon, 5-FU decreased *Enterococcus* spp., *Lactobacillus* spp. and *Streptococus* spp. The fecal samples showed decreasing trends in *Lactobacillus* spp. and *Bacteroides* spp., an increasing trend in *E. coli*, and significant increases in *Clostridium* spp. and *Staphylococcus* spp. at 24 h (16). Another argument is the efficacy of some probiotic to decrease the severity of the 5-FU induced mucositis.

Experimental models with probiotics reported at the beginning conflicting results as the manipulation of the small bowel microbiota was not standardized. One of the first studies reported by Smith *et al* (24) found that an association between the probiotic *Lactobacillus fermentum* BR11 and the prebiotic (fructo-oligosaccharide) did not provide additional protection for 5-FU induced mucositis, but that *Lactobacillus fermentum* BR11 has the potential to reduce inflammation of the upper small intestine. Other strains of *Lactobacillus* were found to be protective in experimental models of chemotherapy induced mucositis. *Lactobacillus acidophilus* improved the inflammatory and functional aspects of intestinal mucositis induced by 5-FU (26). Mixtures of probiotics (*Lactobacillus acidophilus* and *Bifidobacterium lactis*) or (*Lactobacillus acidophilus, Lactobacillus paracasei, Lactobacillus rhamnosus,* and *Bifidobacterium lactis*) decreased the histopathological scores in the duodenum and jejunum after mucositis, demonstrating the potential use of these probiotics as therapeutic agents against intestinal mucositis (29).

For this experiment we used also a strain of *Lactobacillus,* named *Lactobacillus plantarum*, a non-gas-producing lactic acid bacterium regarded as safe (GRAS) with Qualified Presumption of Safety (QPS) status (35). Our choice was based on previous research that reported that *Lactobacillus plantarum* was a valuable probiotic in gut disorders such as inflammatory bowel diseases, metabolic syndromes, dyslipidemia, hypercholesteremia, obesity, and diabetes, and brain health aspects involving psychological disorders (35).

We administered *Lactobacillus plantarum* 7 days prior to 5-FU administration as previous studies found that probiotics could induce mucin gene expression after a period of seven days, that exhibited protective effects in the pathogenesis of 5-FU induced mucositis (41). Probiotics might modulate the dysbiosis induced by 5-FU after 5 days (42). The animals were sacrificed after 48 h of 5-FU administration, as the histological lesions of mucositis are very well depicted at this timepoint (30).

Only one study reported the effects of *Lactobacillus plantarum* CRL2130 on 5-FU induced mucositis in mice (27). *Lactobacillus plantarum* CRL2130 is a riboflavin-overproducing strain, that showed lower macroscopic and histologic damage scores in a colitis murin model (40). In the study of Levit *et al* (27) reported significantly attenuated pathologic changes induced by 5-FU in mice such as body weight loss, diarrhea, shortening of villus height and exerted an inhibitory mechanism against oxidative stress.

We chose a less investigated strain *Lactobacillus plantarum* ATCC 8014, due to the capacity to balance the microbiota disturbed by 5-FU. This strain had high inhibitory activity against both Gram-positive (*Staphylococcus aureus*) and Gram-negative bacteria (*Shigella dysentery, P. aeruginosa, E. coli S5 and Salmonella typhi)* (34).

Our results showed significantly fewer inflammatory histological changes on jejunum, the most affected GI segment by 5-FU. We concluded that *Lactobacillus plantarum* ATCC 8014 protected the small bowel mucosa by the effects of 5-FU. Even the histological scores on duodenum and colon were lower in group receiving *Lactobacillus plantarum* ATCC 8014, the statistical significance was not achieved. Other studies are needed to detect the probiotic with high protective potential for these parts of GI tract.

As mentioned previously, the TLRs activation might mediate both protective and destructive responses in chemotherapy induced mucositis (21), data on specific effects regarding 5-FU mucositis being far less thoroughly investigated with regard to specific molecular targets when compared with irinotecan-related disease (43). TLR2 might have protective effect, by preventing intracellular accumulation of xenobiotics. We did not investigate this hypothesis for 5-FU in this experiment, this representing a limitation for our study. In contrast, we searched the immunopositivity for TLR4 on mucosal epithelium, glandular epithelium, and lamina propria of the GI tract. The activation of TLR4 might exacerbate the mucosal injury by hyperresponsiveness to LPS (21). It was assumed that *Lactobacillus plantarum* might modulate the microbiota due to its inhibitory activity against both Gram-positive and Gram-negative bacteria and subsequently in L group fewer TLR4 will be activated by LPS. In contrast, our results showed intense TLR4 immunopositivity on the whole GI tract (higher in group receiving *Lactobacillus plantarum* than in group F, but without statistical significance). The administration of this lactic acid bacteria, 7 days before 5-FU, conducted to more intense TLR4 signaling, but this activation was not subsequently followed by increased inflammatory processes, on the contrary significantly fewer degenerative and inflammatory lesions were depicted.

We might conclude from this conflicting data that more anti-inflammatory processes were involved, not related to activation of TLR4. This observation was previously reported by the Levit *et al* (40) that found *Lactobacillus plantarum* CRL2130, a riboflavin-overproducing strain showed lower macroscopic and histologic damage scores in a colitis murin model. Also Zhang *et al* (44) reported that *Lactobacillus plantarum* CQPC06 had a good protective effect against colitis in a mouse model via the IL-8 pathway. The exopolysaccharides from *Lactobacillus plantarum* N14 had anti-inflammatory capacities in porcine intestinal epithelial cells; they were able

to reduce the production of inflammatory cytokines in cells challenged with the TLR-4-agonist LPS (45,46).

The limits of our study are represented by the lack of microbiota analysis and the lack of cytokine measurement (to assess the ratio pro-inflammatory/anti-inflammatory molecules).

In conclusion, significantly fewer microscopic degenerative lesions were depicted in L group on the jejunum, suggestive of a potential beneficial effect of *Lactobacillus plantarum* ATCC 8014 at this level in 5-FU induced mucositis. As higher TLR4 immunopositivity was depicted in the L group compared with F group, in the context of significantly lower histological scores, we suppose that *Lactobacillus plantarum* ATCC 8014 might exert anti-inflammatory properties that should be better characterize in further studies.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LC, CrT, DV and MT conceived and designed the study. CrT wrote the manuscript. DMO, CB, AM, SO were responsible for the experiments. DV provided the *Lactobacillus plantarum*. CoT and MT performed the histological and immunohistochemistry analyses. LC and MT critically revised the manuscript. All the authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The experiments, in accordance with Romanian laws, were approved by the Institutional Animal Ethics Committee, Cluj-Napoca, Romania.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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