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EFFECTS OF *LACTOBACILLUS* LB PROBIOTIC ON IMMUNE PARAMETERS AND INTESTINAL STRUCTURE OF MALE ALBINO RATS: QUALITATIVE AND QUANTITATIVE STUDY

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Abstract

Regular intake of probiotics have been shown to improve the health benefits of the host by influencing the systemic and mucosal immune systems. Probiotic sachets are characterized by the high shelf-life of the probiotic strains and have the advantage to avoid the possible effects of milk allergy on specific immune parameters. Therefore, in this study we evaluated the immunomodulatory and intestinal health effects of orally-administered *Lactobacillus* LB sachets (Lactéol® fort). Rats were supplemented with 10^9 CFU *Lactobacillus* LB for consecutive 28 days. We found that orally administered *Lactobacillus* LB induced both Th1 (TNF-α, INF-γ and IL-8) and Th2 (Il-4) cytokine production as well as immunoglobulin-A (IgA). Moreover, the histometry showed clear increase in villus heights and obvious decrease in crypt depths in animals receiving *Lactobacillus* LB. Also, the V/C ratio, goblet cell numbers, and the measures of GALT compartments were increased significantly in comparison with controls. Overall, the *Lactobacillus* LB supplementation produces immune stimulation and is possibly able to protect small intestinal mucosa via enhancing immune barrier function of the intestine and improving intestinal function. Therefore, *Lactobacillus* LB can provide effective, inexpensive, and safe remedy to prevent and/or treat functional gastrointestinal diseases.

Keywords**:** *Probiotic*; *Cytokines*; *Intestinal function*; *Immune response*; *Lactobacillus* LB

Introduction

The modulation of the immune function following probiotic consumption drew attention to the relationship between this ingested bacteria and the host immune response. Therefore, numerous studies have been conducted on the positive role of regular intake of Lactic Acid Bacteria (LAB), the most frequently used probiotics, in animals as well as humans. While the exact mechanisms are not entirely understood, it is believed that LAB has the capacity to influence both innate and adaptive immunity through exerting direct antimicrobial activity against pathogens[1], incitement of phagocytes/macrophages, and Natural Killer (NK) cells[2], stimulating the immunoglobulin A (IgA) immune response[3] or, modulating/regulating adaptive immunity into pro- and/or anti-inflammatory cytokines[4,5]. Interestingly, probiotics regulate the immune response in a very particular way, which is known as strain-specific; some boost the immune system that makes them useful in immune deficiency patients, while others regulate exaggerated immune responses linked to immunological disorders, such as Rheumatoid arthritis[6]. The most widely recognized LAB strains utilized in probiotic preparation mainly originate from *Lactobacillus* and *Bifidobacterium* genera.

Notably, cytokines play a crucial function in the immune system by binding to specific receptors on the cell membrane, stimulating one or more of the cellular cascades that lead to the elicitation, amelioration, or restraint of several cytokine-regulated genes in the nucleus[7]. They are markers for the actuation status of the body's resistance and its reactivity to external threats[8]. Hence, the immunomodulatory effects of probiotics on cytokines have received a great deal of attention. However, the precise influence of probiotics on cytokine production is a much-debated topic. For instance, Meyer et al.[8] indicated that oral consumption of yogurt containing *Lactobacillus delbrueckii ssp. bulgaricus* and *Streptococcus thermophiles* invigorate the production of pro-inflammatory and Th1 cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and interferon (IFN)-γ. On the other hand, Jang et al.[9] revealed that Th1 and Th2 cytokines were suppressed after the oral intake of *Lactobacillus rhamnosus* in the asthma mouse model.

Both luminal contents and the underlying immune cells of the lamina propria and Peyer's patches have a complicated relationship with the gut mucosal barrier. The fundamental function of the intestinal barrier is to protect the host from pathogen invasion. The intestinal morphology such as mucosal thickness, villus height, and crypts depth indicates its barrier function. Probiotic bacteria have been suggested to be beneficial by reinforcing mucosal barrier defenses via colonization, production of antimicrobial substances, and stimulation of gut-associated lymphoid tissue. The improved barrier function may then help to avoid pathogen invasion and help in antigen handling[10].

Another consistently described effect for probiotics on the intestinal immune response is their capacity to enhance secretory IgA production[11-13]. Secretory IgA plays a unique protective role against pathogens and

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toxins through a variety of non-inflammatory activities that enhance their clearance and prevent their entry into the intestinal epithelium[14].

Several systems such as pharmaceutical formulations and food-based products have been developed for the delivery of probiotics to the gastrointestinal tract. The pharmaceutical preparations are considered more efficient compared with commercial food carrier systems[15]. Lactéol® fort sachets are an example of a pharmaceutical form currently used to deliver probiotics. Such sachets are characterized by the high shelf-life of the probiotic strains used in this form and have the advantage to avoid the possible effects of milk allergy on specific immune parameters. The pharmaceutical form of the sachet consists of a combination of 10 billion lyophilized, *Lactobacillus* LB cells (*Lactobacillus fermentum* and *Lactobacillus delbrueki*).

Therefore the purpose of this study was to evaluate the immunomodulatory effect of a commercially available probiotic sachets containing *Lactobacillus* LB by detecting specific immune parameters, such as pro-inflammatory cytokines and chemokines (TNFα, IFNγ, and IL-8), anti-inflammatory cytokines (IL-4), and Immunoglobulin-A (IgA). We also investigated its effect on some parts of the digestive tract by performing histological and histometrical analysis of Gut Associated Lymphoid Tissue (GALT) in both ileum and colon, in addition to all other structural compartments.

Materials and methods

Materials

Adult male albino rats (*Rattus norvegicus*) weighing from 195 to 250 g were purchased from the breeding colony of the Ministry of Health (Helwan-Egypt). Rats were housed in plastic cages (3animals/cage) and provided water and food *ad libitum*. The experiment was performed in accordance with institutional guidelines and follow the Guide for Care and Use of Laboratory Animals. *Lactobacillus* LB was obtained as Lactéol® fort sachets from Tenth of Ramadan for Pharmaceutical Industries & Diagnostic Reagents (rameda; Under Licence of AXCAN Pharma, S.A., France). Each sachet contains *Lactobacillus* LB (*Lactobacillus delbruekii* and *Lactobacillus fermentum* 10^9 Colony-Forming Unit (CFU)).

Experimental design

After two weeks of adaptation, animals were assigned randomly to two groups (9 rats each): The control group (Con) received daily 1 ml distilled water (vehicle of Lactéol® fort) orally, *Lactobacillus* LB group (Pro) received daily (1×10^9 CFU; sachet) orally/rat for 4 weeks.

Collection of samples

At the end of the experimental period, animals were sacrificed. Blood samples were collected from the jugular vein of each rat. After 1-2 h incubation at room temperature, sera were collected followed by centrifugation at 4000 rpm for 30 min and kept at -80 for immunological measurements. Samples from the intestinal tract (ileum and colon) were removed for histological preparation.

Measurement of Pro-inflammatory cytokines and Chemokines

Proinflammatory cytokines (TNFα, IFNγ), and a chemokine (IL-8) were quantified as picograms per milliliter (pg/ml) in sera with a commercial enzyme-linked immunosorbent assay (ELISA) kits. The ELIZA detection kits (BOSTER.; Cat. No. EK0526, BOSTER; Cat. No. EK0374, and Kamiya Biomedical; Cat. No. KT-60204, respectively) were used according to the manufacturer's instructions.

Measurement of Anti-inflammatory cytokine

The cytokine IL-4 was quantified as picograms per milliliter (pg/ml) in sera with a commercial enzyme-linked immunosorbent assay (ELISA) kit. The ELIZA detection kit (BOSTER Cat. No. EK0406) was used according to the manufacturer's instructions.

Measurement of immunoglobulin-A (IgA)

Serum levels of IgA were quantified as nanograms per milliliter (ng/ml) in sera with a commercial enzyme-linked immunosorbent assay (ELISA) kit. The ELIZA detection kit (Abcam ab157735) was used according to the manufacturer's instructions.

Histopathological and Histometry studies

Sections from both ileum and colon were dehydrated through graded alcohol, embedded in paraffin, cut at 5 μm, and stained with hematoxylin and eosin (HE) using standard protocol for histological evaluation. The goblet cells were determined by staining ileum sections with the Periodic Acid Schiff reagent (PAS) technique that emphasize neutral mucin (mucopolysaccharides) in the goblet cells. All the histometrical measurements in both ileum and colon sections were performed using ImageJ software (ImageJ Java software, version 1.52 v, U. S. NIH, Bethesda, MD, USA).

For histometry, on HE-stained ileum sections the villus height, crypts depth, and the ratio of villi and crypts values (V/C ratio) were measured and calculated. The villus height was measured from the crypt-villus junction to the tip of the villus; crypt depth was measured from the base of the crypt to the crypt-villus junction[16]. For each section, a total of 10 microscopic fields on intact crypt-villus were measured. Additionally, GALT (gut-associated lymphatic tissue) in both ileum and colon sections was examined with attention paid to both the lymphatic area of individual lymphatic follicles (LF) and their different components. For this latter purpose, the lymphatic follicles were divided into their defined compartments (the cortical region, the germinal

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center, the coronal region, and the dome region) in ileum sections but the subepithelial dome region is difficult to distinguish from the corona in the colon sections according to Cesta[17]. Such compartment areas were measured (μm^2) in five LFs in randomly selected fields of each tissue sample. Furthermore, goblet cells were counted at 10 microscopic fields in the PAS-stained sections from the ileum under x250 objective.

Statistical analysis

The mean ± standard error (SEM) is used to express the data. Statistical evaluation was presented by one-way ANOVA with Tukey test using SPSS 16.0 software (SPSS Inc. Chicago, IL, USA). Differences in means were considered significant when the P-value was less than 0.05.

Results

Effect of *Lactobacillus* LB on Pro-inflammatory cytokines and Chemokine (TNF-α, IFN-γ, and IL-8)

As shown in Fig. 1.A., serum levels of TNF-α, IFN-γ, and IL-8 levels were significantly elevated ($p < 0.05$) After *Lactobacillus* LB administration in comparison with the control group.

Effect of *Lactobacillus* LB on Anti-inflammatory cytokine (IL-4)

Lactobacillus LB induced a significant increase ($p < 0.05$) in IL-4 serum concentrations as compared to those of the control group (Fig. 1.B.).

Effect of *Lactobacillus* LB on Immunoglobulin-A (IgA)

Data are shown in Fig. 1.C. revealed that oral administration of *Lactobacillus* LB administration resulted in a striking increase ($p < 0.05$) in the serum levels of IgA compared to their corresponding controls.

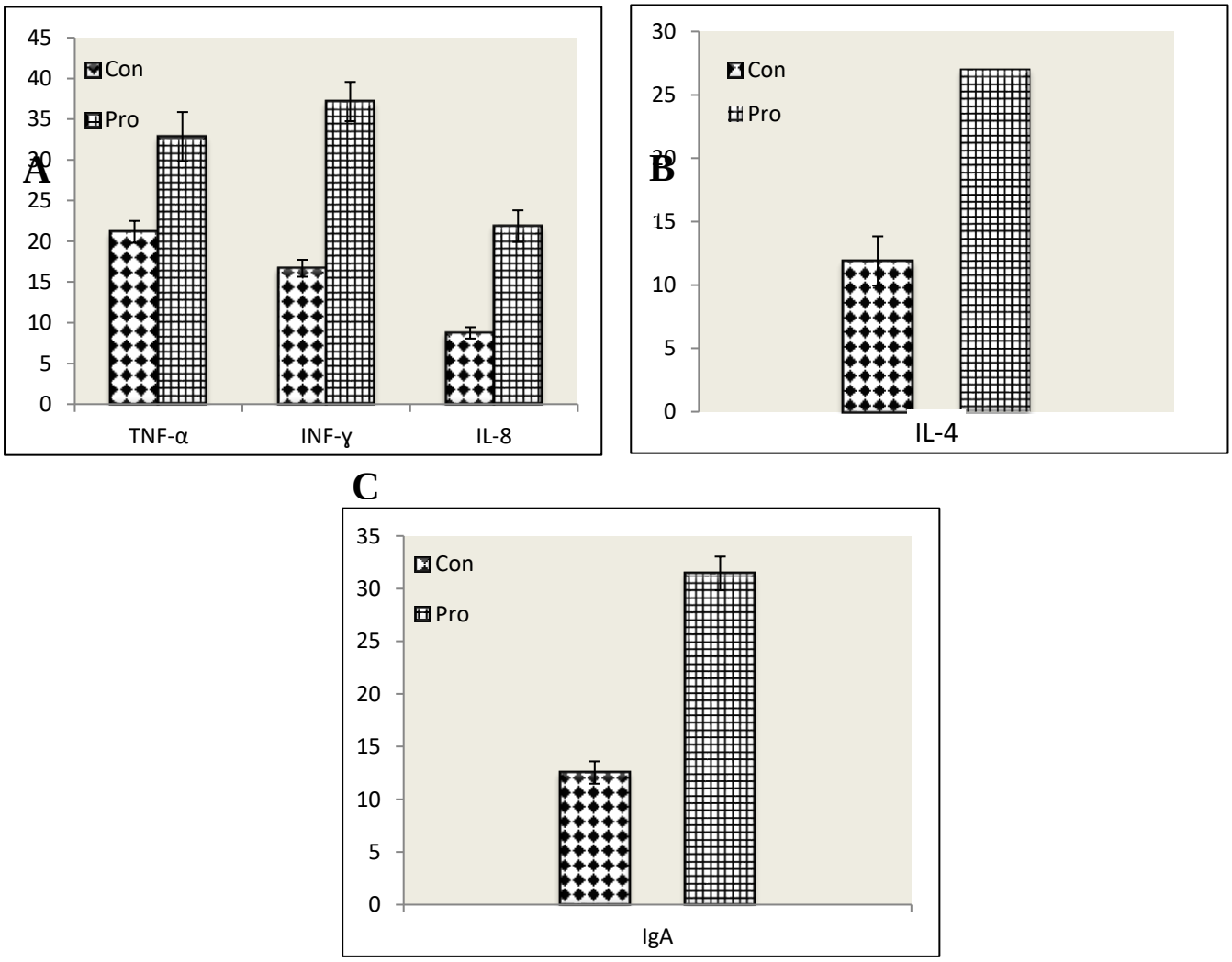

Fig. 1. A. Serum levels of TNF-α, IFN-γ, and IL-8. **B.** serum levels of IL-4. **C.** Serum levels of IgA data are expressed as means ± SEM. Con= control and Pro= *Lactobacillus LB* *= $p < 0.05$ vs. control.

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

Histology and Histometry studies

Ileum section

Investigation of tissue of control rats (Con) showed normal architecture consisting of four concentric layers: the serosa, the muscularis, the submucosa, and the mucosa. The surface of the mucosa is covered by a simple columnar epithelium, which forms enormous numbers of tall and cylindrical villi that protrude into the lumen (Fig. 2.A.). The predominant cell in the epithelium is composed of columnar with numerous mucins secreting goblet cells in between (Fig. 2.B.). The epithelium covering the villi is modified into simple tubular glands; these glands are the crypts of Lieberkühn (Fig. 2.C.). Small accumulations of lymphocytes or solitary lymphoid follicles are randomly distributed throughout the mucosa and submucosa. The most well-known representatives are Gut Associated Lymphoid Tissue (GALT) including Payer's patches (PPs) which are centrally located follicles containing a germinal center (GC) (Fig.2 A.) flanked by parafollicular or interfollicular regions (IFR). The germinal center consists of a basal zone of darkness and an apical zone of light which is superficial to the light zone with a mantle zone or a corone (C). The follicle associated epithelium (FAE) is separated from the follicle by the subepithelial dome region (SED) (Fig.2.A).

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

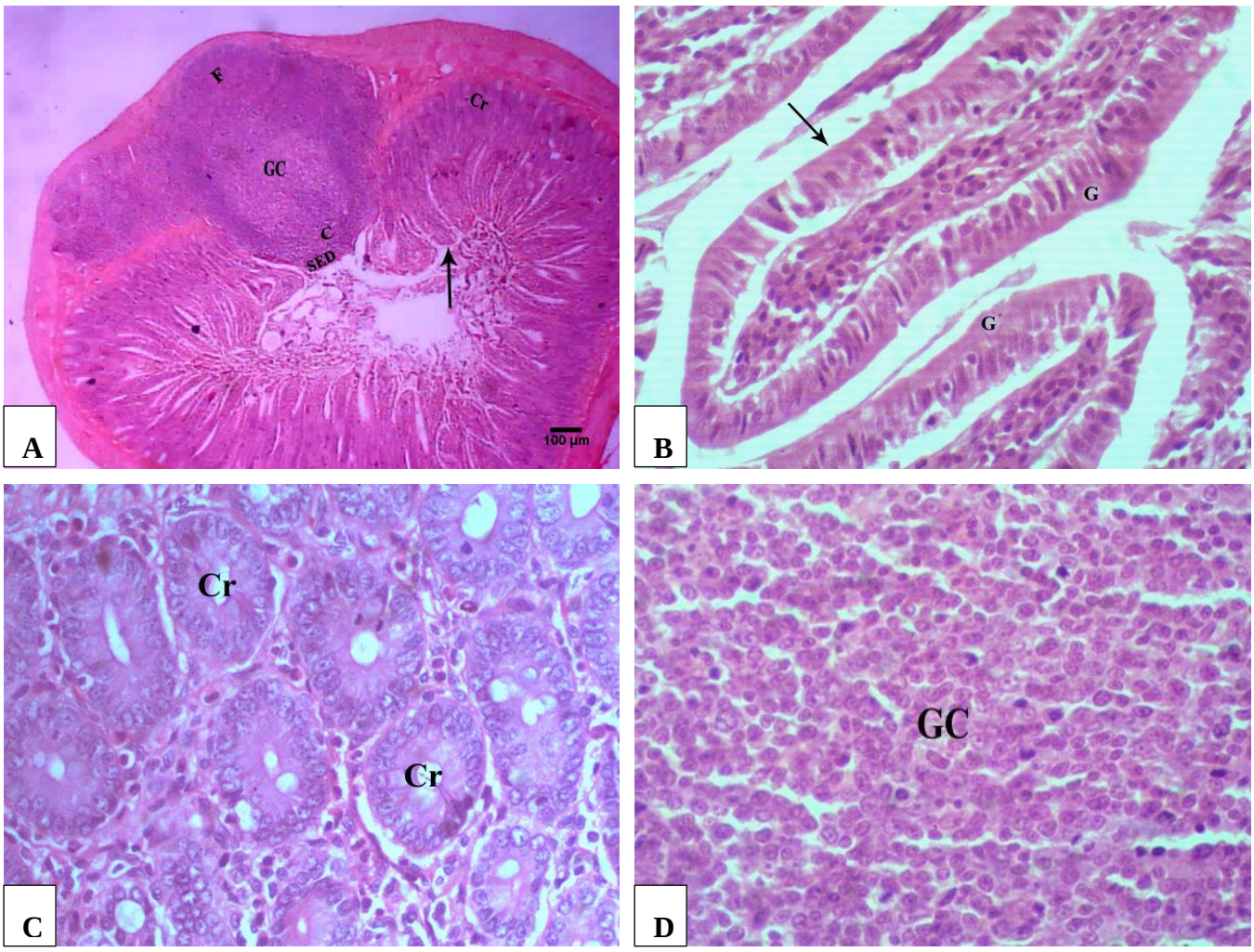

Fig 2. Histological sections of ileum A. from control adult rat showing normal morphology with the presence of, crypts of Lieberkühn (Cr) and intact villi (thin arrow) and payer's patches showing centrally located follicle (F) containing a Germinal center (GC) with corona (C) and subepithelial dome region (SED) flanked by interfollicular regions (IFR). (H&E, x 32). **B.** A magnified sector from the previous section showing finger-like villi (thin arrow) with the presence of goblet cells in between (G). (H&E, x 400). **C.** A magnified section showing normal crypts of Lieberkühn (Cr). (H&E, x 400). **D.** A magnified sector of payer's follicle revealing a normal population of lymphocyte aggregation in germinal center (GC). (H&E, x 400).

The administration of *Lactobacillus* LB was associated with increased mucosal integrity and clarity compared to the control group (Fig. 3A, B & C). The increase of phagocytic activity of macrophages was observed by detecting many tangible body macrophages in the germinal center of the PPs follicle (Fig 3D).

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

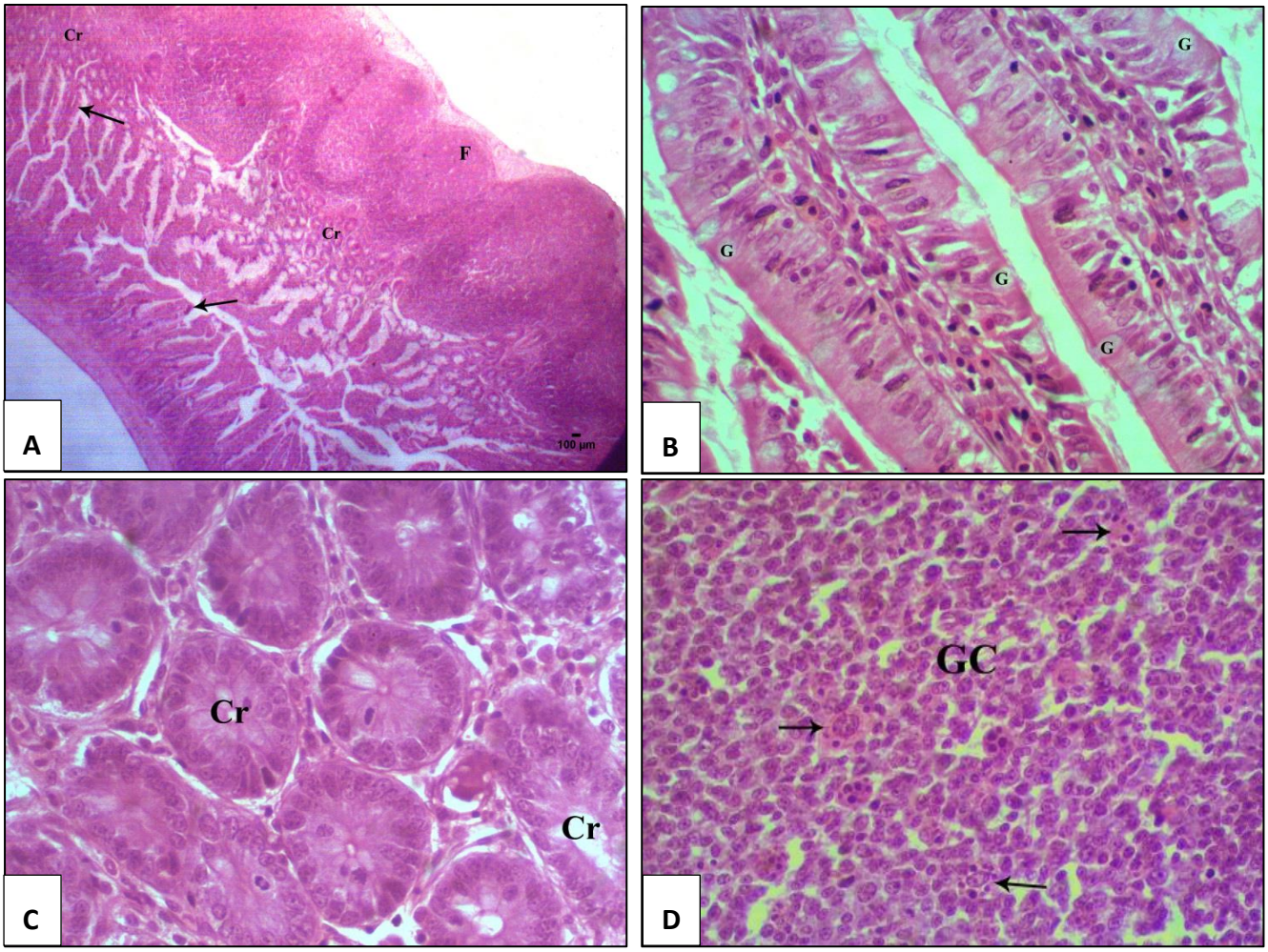

Fig. 3. Histological sections of ileum A. from orally administered *Lactobacillus* LB rat showing villi (thin arrows) and crypts of Lieberkühn (Cr). Notice the area of payer's patches with multiple lymphoid follicles (F). (H&E, x 32)**. B.** A magnified sector of villi revealing the apparent increase in the goblet cells (G) scattered in between. (H&E, x 400).. **C.** A magnified section showing better architecture of crypts of Lieberkühn (Cr). (H&E, x 400). **D.** Magnified sector of a germinal center (GC) from payer's follicle showing obvious tingible body macrophages (thin arrows). (H&E, x 400).

As shown in table (1) villi were significantly higher ($p < 0.05$) in the *Lactobacillus* LB group compared to the control one. Moreover, the number of goblet cells increased significantly after the administration of *Lactobacillus LB* comparing to those of the control group (Fig 3B). On the other hand, crypts depth showed a significant reduction versus control ($p<0.05$), therefore the villus/crypt ratio increased significantly. GALT appeared very well-organized in both control rats and rats administered *Lactobacillus* LB. Defined region measurements of Lymphoid Follicle (LF) compartments within the payer's patches were presented in Fig. 5.A. Rats administered *Lactobacillus LB* exhibited a significant increase in the total area of the lymphoid follicles and the area of the germinal centers ($p < 0.05$) compared to control animals. Nevertheless, no difference was seen in the area measurement of other defined compartments composed of the cortical region, corona region, and dome region.

Table 1. Effect of *Lactobacillus* LB on histometrical analyses related to rat ileum

	Con	Pro
Goblet cell count	166.54±13.07	222.28± 9.91*
Villi height, ileum, µm	140.51±4.21	189.61 ± 5.01 *
Crypts depth,ileum, µm	89.72±4.29	52.92 ± 3.56 *

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

V/C ratio	1.56 ± 0.98	3.58 ± 1.4*

data are expressed as means ± SEM. Con= control and Pro= *Lactobacillus* LB *= p <0.05 vs. control rats.

Colon section

The large intestine has no villi the mucosa is smooth and lined by simple columnar epithelium and numerous goblet cells. The lymphoglandular complexes (part of the GALT) in the colon sections in normal rats resemble PPs, however, they are smaller and have fewer follicles with smaller germinal centers, and here the subepithelial dome region is difficult to distinguish from the corona (Figs. 4.A&B). The observations of colon sections in rats administered *Lactobacillus* LB came in to agree with that of ileum sections. GALT nodules showed better architecture when compared to the control group (Fig. 4C). A notable increase in phagocytic activity has been observed by obvious tingible body macrophages in the germinal center (Figs. 4D).

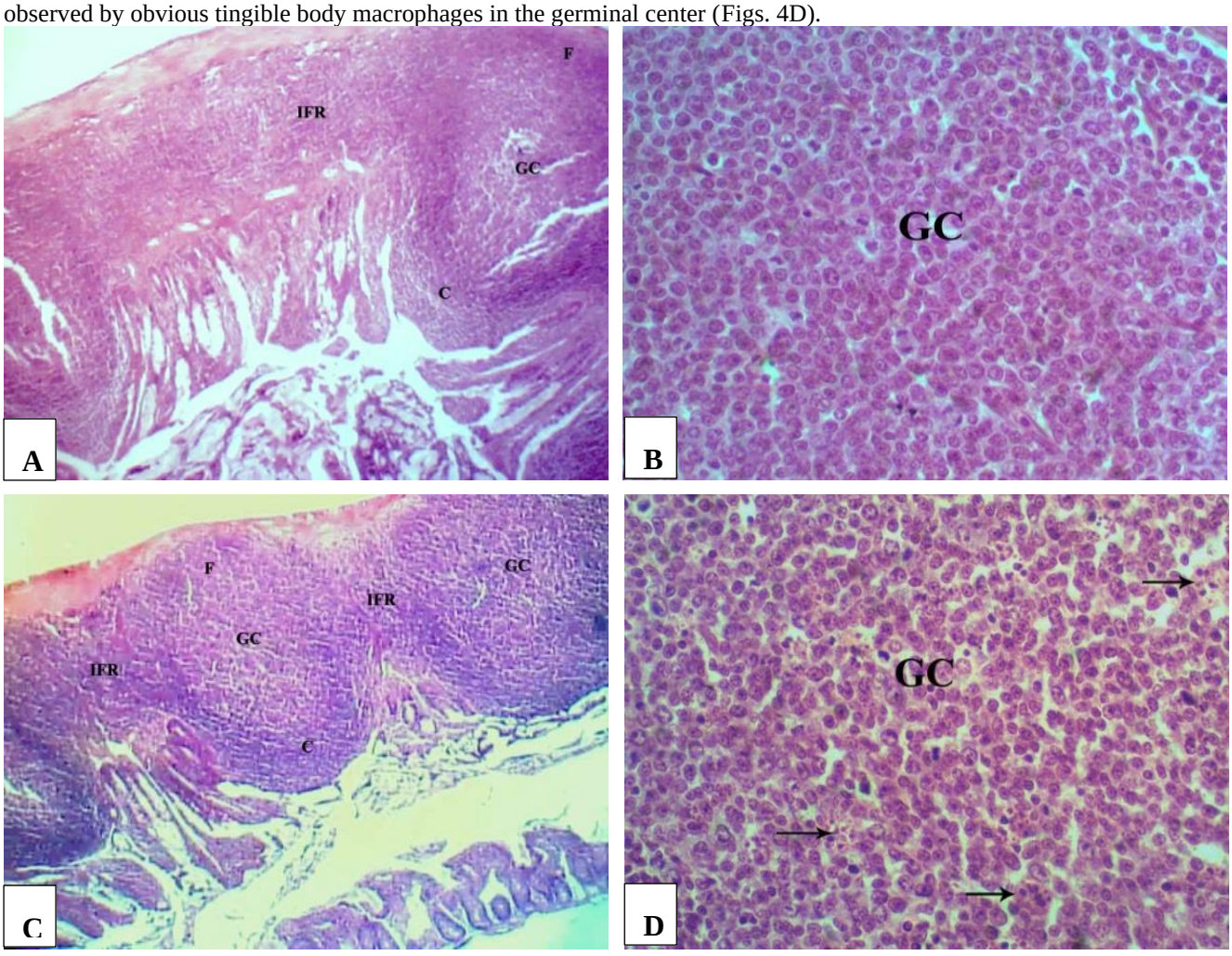

Fig. 4. Histological sections of colon A. from normal animal showing normal architecture of lymphoid follicle of the colon contains follicle (F) with a centrally located germinal center (GC). Here, the subepithelial dome region is difficult to distinguish from the corona (C). Interfollicular regions (IFR) surround the follicle (H&E, x 125). B. A magnified sector of the previous section showing crowded germinal center (GC) with lymphocytes (H&E, x 400). C. from rat administered *Lactobacillus* LB demonstrating several lymphoid follicles (H&E, x 125). D. A magnified sector of the previous section showing lymphocyte aggregation in GC with obvious tingible body macrophages (thin arrows) (H&E, x 400).

As shown in Fig. 5B the measurements of GALT were supported to those demonstrated from the payer's patches of ileum sections. The total area of the follicles and the germinal center were significantly high in animals administered *Lactobacillus* LB as compared to the control ones but no difference was seen in the other defined compartments which are the cortical region and corona region.

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

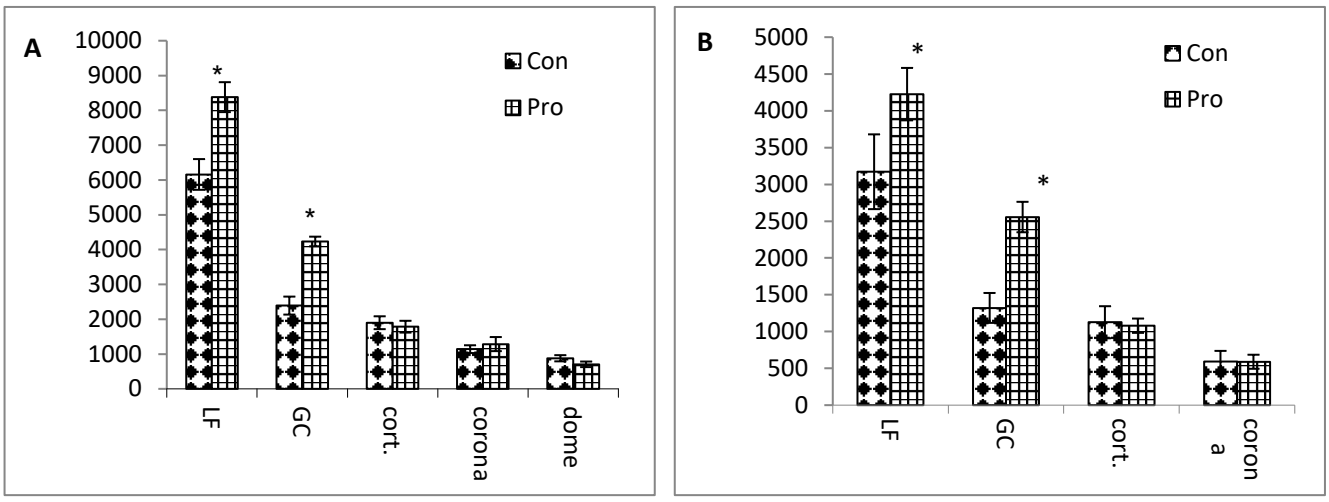

Fig. 5. Mean (± SEM) histometrical analyses of rat ileum **(A)** and colon **(B)** different areas, data are expressed as μm^2. Con= control and LB= *Lactobacillus LB* *= $p < 0.05$ vs. control rats.

Discussion

It has been established that probiotics improve the health benefits of the host by influencing the systemic and mucosal immune systems[13,18]. Previous studies revealed that lactobacilli can be used to enhance early lines of protection against invading pathogens by stimulating the immune system to release pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL-12[8,13,19]. Results presented in this study indicated that the administration of *Lactobacillus* LB (Lactéol® fort) produced immune stimulation that affected the balance of Th1/Th2, as manifested by the release of high levels of pro-inflammatory cytokines and chemokine (TNF-α, IFN-γ, and IL-8) as well as anti-inflammatory cytokine (IL-4). TNF-α cytokine is an important factor in host immune response, its stimulation by probiotic may be essential to launch the cross-talk between the immune cells related to the lamina propria and the intestinal epithelial cells[3]. It promotes phagocytosis and consequently increases macrophage activation[20]. It also plays a guiding role in the production of IL-8[21].

IFN-γ is associated with a cellular immune response[8] and is required for the maturation and proliferation of some immune at the intestine, such as dendritic cells (DCs)[3]. Supporting data obtained from trials on humans[8], animals[22,23], and *in vitro*[24,25] indicated that *Lactobacillus* strains stimulated T cell activation towards Th1 and produced high levels of IFN-γ. It has been demonstrated that these high levels of IFN-γ would occur via the effect of *Lactobacillus* on DCs maturation that increases cell population and cytotoxic ability NK cells and then promotes antiviral innate protection[13,23].

Our observations indicated that the area of germinal centers in the intestinal lymphatic follicles and the production of IgA increased significantly with the oral administration of *Lactobacillus* LB. The GALT is the first line of defense at the mucosal surface, which can be divided into inductive and effector sites. The inductive sites, mainly the Peyer's patches (PPs), consist of aggregations of lymphoid follicles where specific immune responses are presented[26]. Their surface is covered by a unique epithelium (termed follicle associated epithelium, FAE) which contains, interspersed between enterocytes, a specialized epithelial cell type, known as M cells[27]. According to Galdeano et al.[3]; Karamese et al. [5], probiotic bacteria interact with M cells and are then captured by DCs or macrophages in the lamina propria, where they increase signals to epithelial cells and/or immune cells before being transported to the mesenteric lymph nodes. De Simone et al.[28] indicated that enhancement of IFN-γ production by probiotic also contributes to antigen presentation and stimulates IgA response. Recently, it has been demonstrated that follicular helper T another distinct subset of helper T cells that are located in germinal centers play roles in enhancing germinal center formation and invigorate IgA production via interactions with germinal center B cells differentiation[29]. In support to the present explanation, oral administration of *Lactobacillus* MCC1849 induced a marked increase in the production of total IgA in the small intestine and serum by increasing the proportion and differentiating follicular helper T cells in the intestinal immune system[11].

Histometric analyses performed here showed that administration of *Lactobacillus* LB increased the height of ileal villi. This came in agreement with Di Giancamillo et al.[30]; Yang et al.[16] who revealed that villus height was longer in animals administered probiotics. According to Yang et al.[16] higher villi show more developed epithelial cells and better intestine absorption efficiency. Moreover, they demonstrated that increased villus height can be caused by the proliferation of crypt cells and reduced apoptosis. The number of crypt cells with the ability to divide represents the intestinal membrane's growth state. Changes in crypt depth reflect the rate of

ISSN: 0975-3583, 0976-2833 VOL 12, ISSUE 03, 2021

crypt cell division, which can affect digestion in the small intestine[31]. Herein, the administration of *Lactobacillus* LB significantly reduced crypt depth. These findings are consistent with Banasaz et al.[32] who revealed that a rise in the number of crypt cells was not linked with an increase in crypt depth and that crypts were shorter following mono-association with *Lactobacillus rhamnosus* GG. It was suggested that a decrease in individual cell size might be a co-phenomenon of enhanced cell proliferation. The ratio of villus height to crypt depth (V/C) indicates the condition of the small intestine. In the current study, the ratio of villus height to crypt depth (V/C) was higher in rats administered *Lactobacillus* LB than in control.

Our data demonstrated that *Lactobacillus* LB administration increased the number of goblet cells of the ileal villi to protect the mucosal barrier. Goblet cells are responsible for the secretion of the mucus layer that provides a dynamic protective barrier against potential pathogens and molecules[33] while serving as a lubricant in the intestine[34]. Previous studies demonstrated that some viable[35,36] and non-viable[37] probiotic strains have been found to induce protective responses by increasing the number of goblet cells and mucin production.

In conclusion, the findings of the current study showed that the use of *Lactobacillus* LB was associated with immune stimulation providing early lines of protection against invading pathogens and was able to protect small intestinal mucosa via enhancing gut barrier function. Therefore, the use of *Lactobacillus* LB sachets provides new insights into the protective effect of the pharmaceutical form of probiotics that may act through diverse mechanisms, which may be effective, inexpensive, and safe remedy to prevent and/or treat functional gastrointestinal diseases.

Disclosure of interests

The authors declare that there are no conflicts of interest concerning this article.

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