Research Article

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Role of Probiotics Administration on Laboratory Mice and Their Effects on The Blood Physiological and Immunological Parameters

Fatin Mohammad Askander, Haider Rasheed Alrafas, Hanaa Khaleel Ibraheim.

Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq.

Corresponding Author Email Address: haider.alrafas@uobasrah.edu.iq
ORCID ID: https://orcid.org/0000-0002-5564-8969
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Abstract

This study looked at what happens to the innate immune system when lab mice are regularly given probiotics (*Lacticaseibacillus rhamnosus* and *Bacillus claus*). It looked at the total number of lymphoid cells in a mesenteric lymph node, mucus production, physiological parameters, and blood biochemical levels. It also looked at how these things might affect the results of experiments. This study utilized sixty female mice. All animals were placed under the same conditions and treated daily for seven days with a combination of probiotics, including *Lacticaseibacillus rhamnosus* and *Bacillus clause* (100 ul oral gavage). Blood samples were collected to evaluate biochemical and hematological parameters such as (Hb, PCV, RBCs, WBCs, ALT, AST enzymes, and urea). In contrast, colon tissue and monistic lymph nodes were collected, and cell counting was performed to evaluate the innate lymphoid cell level through the experimental period.There were no notable significant variations in the Hb and RBC readings values. However, the WBC count in peripheral blood and local lymph nodes of animals increased significantly after 7 days of treatment. Also, a significant change in mucus secretion in colon mucosa is associated with probiotic administration. In determining the biochemical parameters of blood, including Creatinine, Urea, AST, and ALT. These results notably showed that there were no significant changes in these parameters in all experimental layouts.The regular treatment of probiotics is one of the most important elements that may affect laboratory mice's general immune conditions, especially the WBCs and innate immune responses. This is represented by increasing mucosa and submucosa colon tissue thickness and immune cell infiltration and increasing the level of innate lymphoid cells type three in the colon mucosa, which both represent the essential elements of innate immunity of the digestive tract.

Key word: Probiotic, Innate Lymphoid Cells 3, Immunohistochemistry.

Introduction

Probiotics are defined as living microbes that, when provided in sufficient quantity, confer a benefit to the host's health (1). This definition was accepted by the Agriculture Organization of the United Nations and the World Health Organization (FAO/WHO). Probiotics have been used to change the gut microbiota of the digestive tract to improve the integrity and function of the intestinal barrier and preserve an equilibrium of immunological response in managing the illness (2). Probiotics containing Bacillus and Lactobacillus are advantageous because they can produce endospores that can withstand environmental stresses, including heat, bile secretions, and an acidic pH in the stomach. These characteristics help *Lacticaseibacillus rhamnosus* and *Bacillus clause* probiotics endure the unusual environment of the digestive tract. They could easily pass to the tracts in intact condition, where they can benefit the body. *Lacticaseibacillus rhamnosus* and *Bacillus clausii* are the safest types of bacteria to use on both adults and children who have problems with their gut bacteria. Studies have shown that *Lacticaseibacillus rhamnosus* and *Bacillus clausus* lower the amount of rotavirus and adenovirus that kids with acute diarrhea excrete and the number of times they go to the bathroom (3, 4). At the core of the innate immune system lies the immune system's defense against external pathogens. The innate immune system of the gut detects pathogen patterns rapidly and broadly. Intestinal epithelial cells, macrophages, monocytes, neutrophils, eosinophils, basophils, dendritic cells (DCs), and natural killer cells (NKs) are all part of this defense system. In contrast, the body's adaptive immune system, which is present throughout, offers long-lasting and extremely selective immunity by identifying particular infections (5). DCs and intestinal macrophages release pro-inflammatory cytokines, antimicrobial peptides, and pathogen patterns identified when they detect Pathogen-associated molecular patterns (PAMPs) of bacteria. As a result, signal pathways such as NF-kB become active and create more IL-1 (6, 7). The most current group of cells involved in innate immune response has been identified as the innate lymphoid cell (ILC) family. These cells are derived from common lymphoid progenitors (CLPs). As a result of pathogenic tissue injury, ILCs regulate both adaptive and innate immune cells and secrete signaling chemicals that contribute to immunity. ILCs are predominantly tissue-resident cells sporadically detected in the bloodstream (8). They are present in non-lymphoid and Lymphoid tissue (immune-related). They are crucial for preserving mucosal immunity and homeostasis and are particularly common at mucosal surfaces. They can be differentiated from other immune cells by their typical lymphoid shape, absence of modified antigen receptors on T and B cells (due to their lack of the RAG gene), and phenotypic markers frequently observed on myeloid or dendritic cells (9). The communication between intestinal ILCs and immune cells, commensal microbiota, and epithelial cells occurs continuously. While Rorγt + ILCs are necessary for the early stages of immunity in response to the digestive system infection,

they also maintain epithelial integrity while maintaining homeostasis. The immune response against the gut pathogen *Citrobacter rodentium* depends on IL-22. This cytokine directly affects intestinal epithelial cells and increases the synthesis of antimicrobial proteins RegIIIγ and RegIIIβ, as well as antimicrobial peptides such as Cathelicdin and β-defensins (10). DC and CX3CR1+ mononuclear cells in the gut are important producers of IL23 or IL1β after they detect bacterial antigens. IL22, GM-CSF, and IL17 are effector cytokines produced by ILC3 as a response to these Cytokines. Th17, Th22, and ILC3 release the cytokine IL22 essential for maintaining homeostasis (11).

Materials and Methods

The experimental work was conducted at the College of Veterinary Medicine, University of Basrah. The study used a total of sixty female mice, aged between seven and ten weeks and weighing between twenty and twenty-five grams. The mice were housed at 22°C temperature and 50-60% humidity. A regular 12-hour cycle of darkness and light was maintained (lights off at 7 p.m., lights on at 7 a.m.). All experimental animals had standard dietary rations and water (12). Two groups of five each were formed. The Naïve group was given 100 ul Distal water oral gavage. The Probiotics group was treated daily for seven days with a probiotic combination including *Lacticaseibacillus rhamnosus* and Bacillus clause (100 ul oral gavage).

At the endpoint of the experiment, the retro-orbital plexus was where the blood samples were collected using a sterilized method. (13). Hematological parameters were measured in EDTA-treated tubes, and serum was measured in a sterile labeled contender tube (Clot Activator with Gel). To confirm all blood values established before each analysis using control blood, an automated veterinary hematology counter (CBC) was carried out using the pocH-100iV DiffLAM (Sysmex® - Roche), and an external quality evaluation procedure was put in place. The VITROS® 350 Chemistry System, an automated spectrophotometer, was used to analyze the blood serum for clinical biochemistry parameters. The biochemical kits and calibration controls were used as mentioned in the manufacturer's instructions. Lab Test Diagnosis was the source of all biochemical kits.

Serial sections of colon tissue fixed in Paraffin resin were stained with (H&E) hematoxylin and eosin staining to aid in distinguishing between various cell and tissue types and to reveal crucial details regarding the pattern, shape, and structure of cells in colon tissue samples (13) . The histology score was calculated by multiplying the percentage of each of the three histologic features listed below by the percentage of the region of involvement (14). The staining of immunohistochemistry The mouse colon tissue was treated with antibodies against inducible ILC3 in certain regions. Paraffin-embedded slices were immunohistochemically examined using the avidin-biotin indirect immunoperoxidase technique (15). Follow the directions provided by Vector Laboratories, Burlingame, CA, the Vectastain Elite ABC kit maker.

GraphPad Prism® 8 (San Diego, CA) was used to conduct the statistics (20). Three duplicates of the trials were used. Student T-test was used for statistical comparisons, followed by the Mann-Whitney test to check the differences between non-parametric groups. The significance of the differences was detected using the $P<0.05$ threshold.

Results

Probiotic-treated mice's complete blood counts showed no appreciable changes in normal levels of erythrocyte indices, such as hematocrit (HCT), hemoglobin concentration (HGB), red cell distribution width (RDW-CV), mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red blood cell (RBC), mean corpuscular hemoglobin (MCHC), and red cell distribution width (RDW-SD) (Fig1). When comparing the PLT counts of probiotic-treated mice to those of naive mice, no discernible alterations were found in the number of platelets (PLT), platelet dimensions width (PDW), mean platelet volume (MPV), as shown in (Fig 2). The automated leukocyte count showed that the lab mice that were given probiotics had significantly higher numbers of immune cells, such as lymphocytes (LY), monocytes (MON), and neutrophils/segmented (NEU/SEG). Other WBC types did not change, as shown in (Fig. 3). When comparing probiotic-treated lab mice to naive mice, the biochemistry for blood serum assays showed discernible changes in aspartate transaminase (AST), alanine transaminase (ALT), urea (UR), alkaline phosphatase (AP), total protein (TP), globulin (GLB), cholesterol (CHL), albumin (ALB), and triglyceride (TG). An automated spectrophotometer processed all sera. Data show means ± standard Error Mean (SEM), (Fig 4). The histopathological scoring for colonic tissue undergone with H&E staining showed no significant changes in the general histological stricture in the colonic tissue. However, the section showed increased immune cell infiltration in the colonic lamina propria without notable histopathological changes (Fig 5). The immunohistochemical evaluation for colon tissue demonstrated a significant increase in the number of innate lymphoid cells type three ILC3 in the colon lamina propria of mice that had probiotics treatment compared to naive animals (Fig 6).

Discussion

The physical and biochemical blood examinations confirm the general effect of *Lacticaseibacillus rhamnosus* and *Bacillus clausii* as probiotics on lab mice. The mice parameters, including ALT, AST, urea, and creatinine levels, are, on average, higher in all experimental animals. The reason for this is that it takes longer to achieve the elevation of those enzymes than the experiment was intended to (1,16), however there is no evidence that probiotic treatments improve liver and kidney function (16,17)

A gross pathological and histopathological examination was performed to confirm the research hypothesis. The histopathological examination showed no significant changes in general colon histology structure, with a slight improvement in immune cell activity recorded in the colon tissue section that was processed with H&E after the probiotics

treatment. In general, Probiotics help restore the natural hemostasis of the microbiota in the Gastrointestinal tract (including the stomach, intestines, and colon) In case of illness or treatment that disrupts it. There is some clear evidence that probiotics may be helpful in gut mucosa illness cases, including ulcerative colitis (18,19).

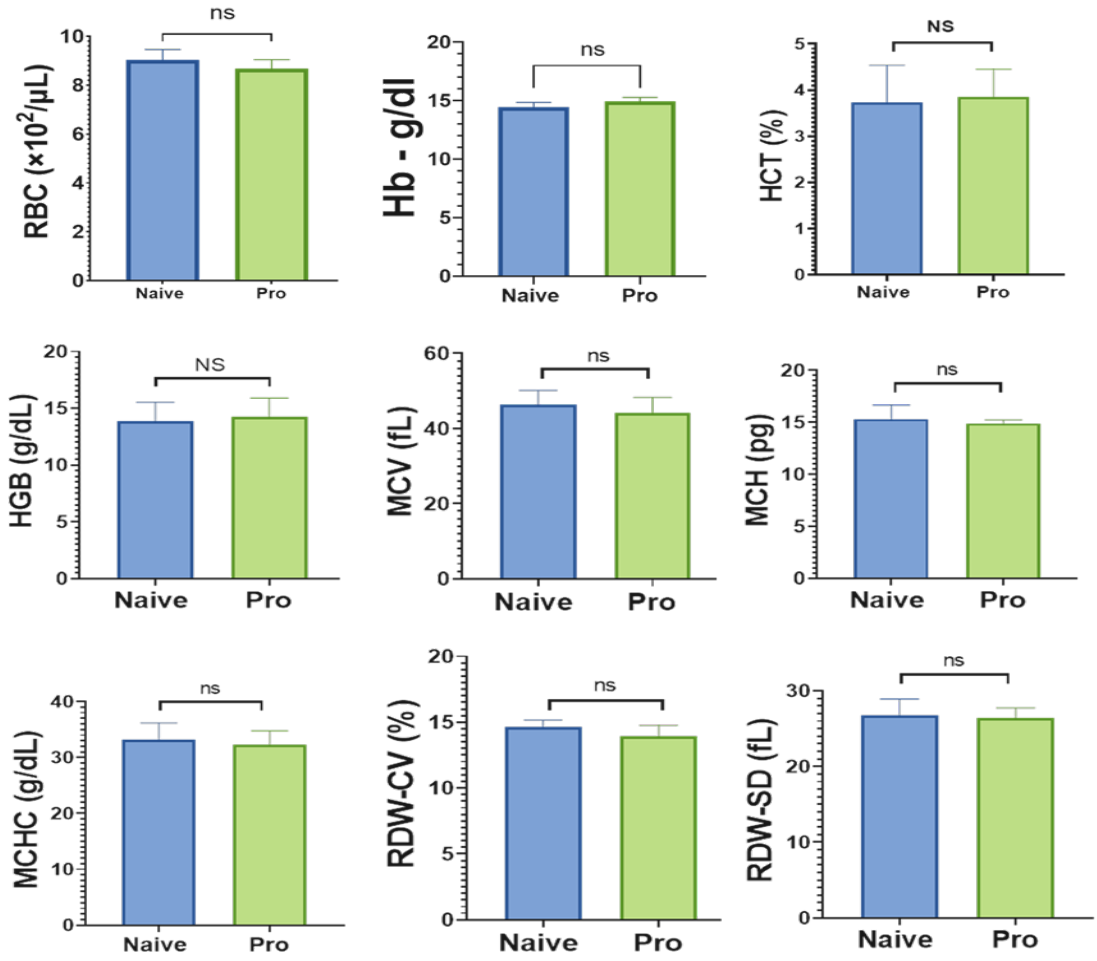

Fig (1): Probiotic administration changes normal erythrocyte indices levels: A) RBC, red blood cell ; **B)** HCT, hematocrit; **C)** HGB, hemoglobin concentration; **D)** MCV, mean corpuscular volume; **E)** MCH, mean corpuscular hemoglobin; **F)** MCHC, mean corpuscular hemoglobin concentration; **G)** RDW-CV, red cell distribution width - coefficient of variation; **H)** RDW-SD, red blood cells dimension width - standard deviation. **CBCs** were performed in an automated veterinary hematology counter pocH-100iV Diff™ (Sysmex® - Roche). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using Student t test In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

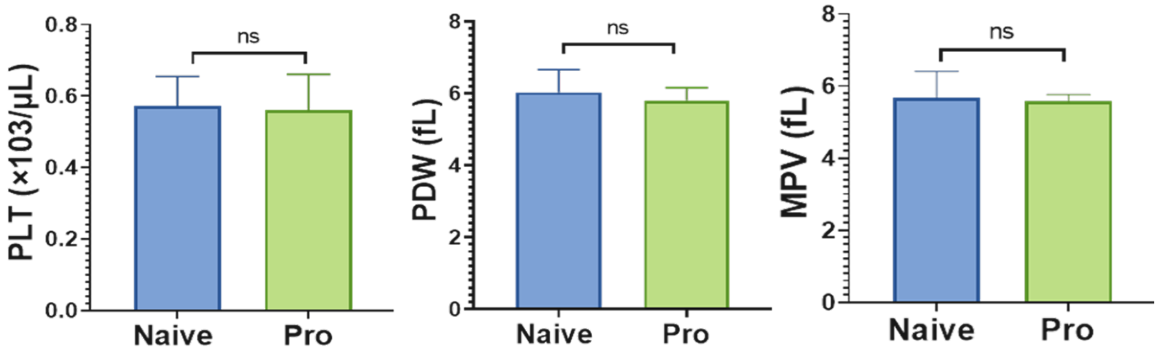

Fig (2): Probiotics administration changes normal platelets indices levels: A) PLT, number of platelets; **B)**PDW, platelet dimensions width.; **C)** MPV mean platelet volume; . **CBCs** were performed in an automated veterinary hematology counter pocH-100iV Diff™ (Sysmex® - Roche). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using student t test. In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

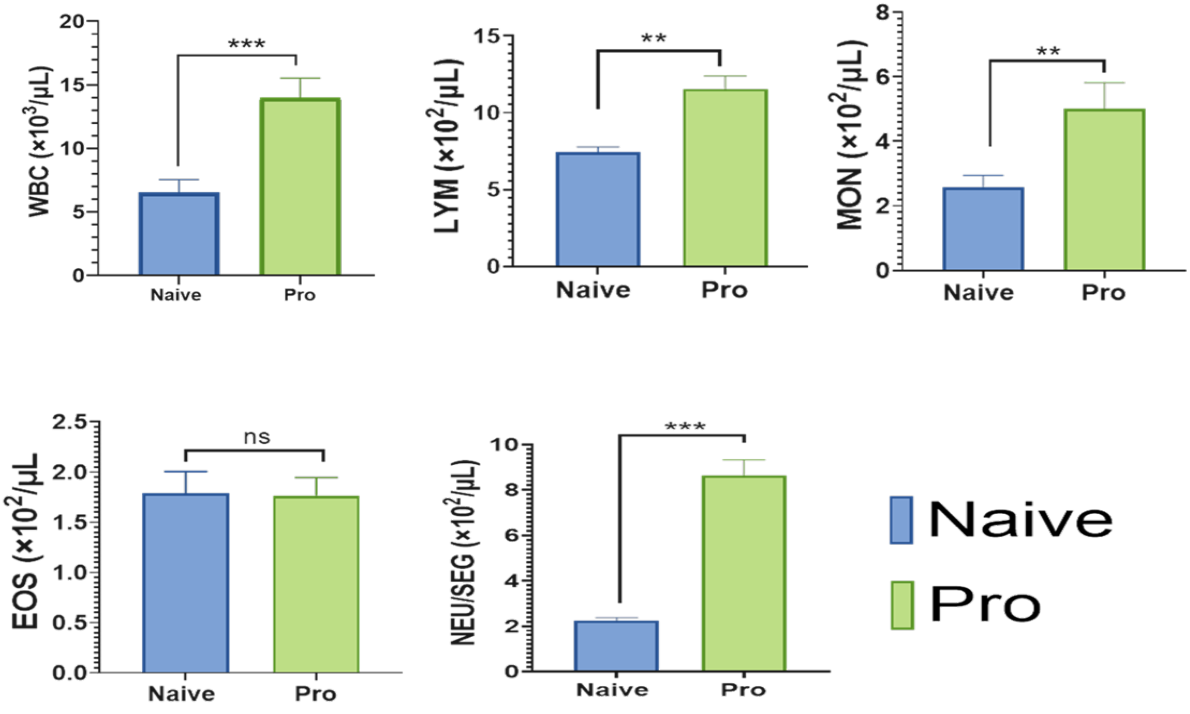

Fig (3): Probiotics administration changes normal leukocyte indices levels: A) WBC, number of white blood cells ; **B)** LYM, lymphocytes; **C)** EOS, eosinophils; **D)** MON, monocytes; **E)** NEU/SEG, neutrophils/Segmented. **CBCs** were performed in an automated veterinary hematology counter pocH-100iV Diff™ (Sysmex® - Roche). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using student t test. In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

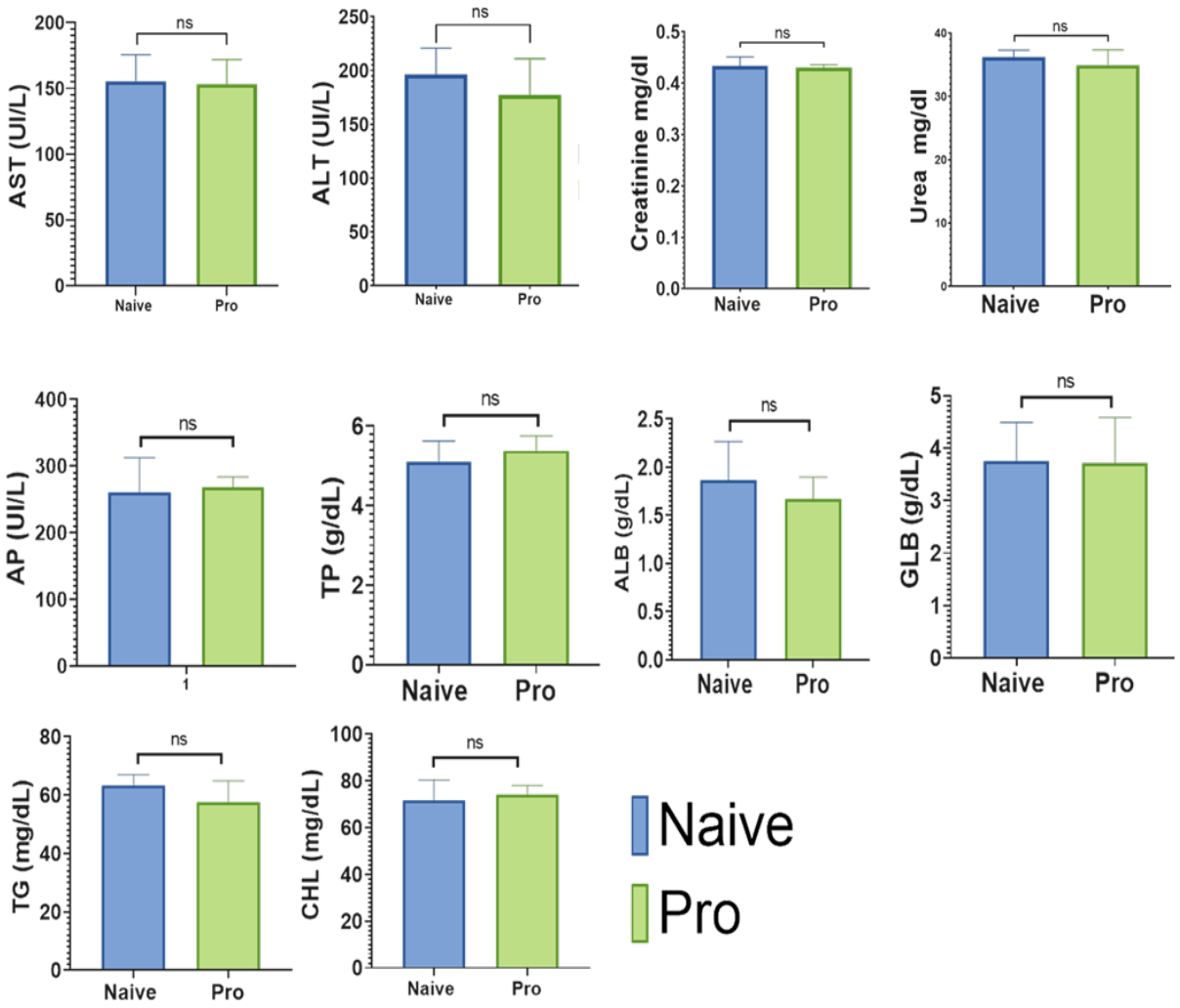

Fig (4): Probiotics Administration changes serum biochemical indices levels: **A**) AST, aspartate transaminase; **B)** ALT, alanine transaminase; **C)** UR, urea; **D)** AP, alkaline phosphatase; **E)** TP, total protein; **F)** GLB, globulin; **G)** CHL, cholesterol; **H)** ALB, albumin; **I)** TG, triglycerides…Sera were processed by(automated spectrophotometer). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using student t test. In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

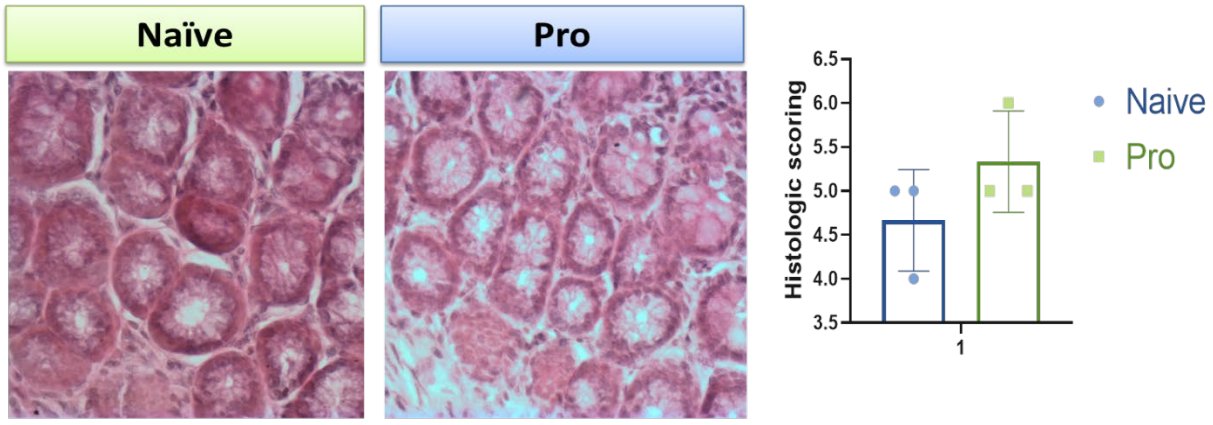

Fig (5) Probiotics Administration changes the histopathological in colon mucosal tissue (H&E) 40 X . A) Naïve control group B) Probiotic treatment group C) Histopathological The statistic score was calculated by multiplying the percentage of each of the histologic section listed below by the percentage of the region of involvement (Mosli et al., 2017). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using student t test. In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

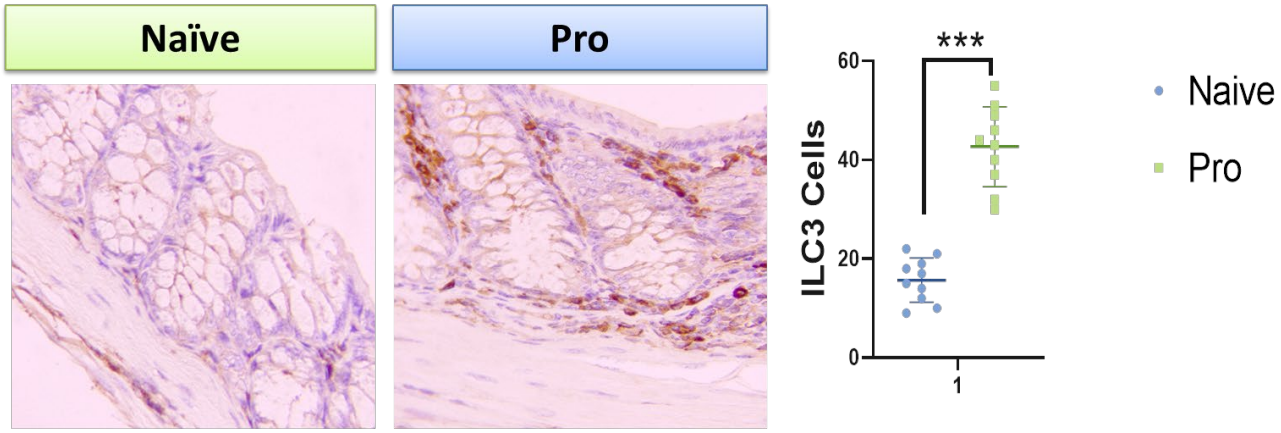

Fig (6) Probiotics Administration changes the level of Type 3 innate lymphoid cells (ILC3)in colon mucosal tissue. A) Naïve control group B) Probiotic treatment group C) ILC3 Cells The statistic score was calculated by multiplying the percentage of each of the histologic section listed below by the percentage of the region of involvement (de Boer et al 2020). Data show means ± standard Error Mean (SEM), calculated with GraphPad Prism version 8.01. Significance (P-value: * < 0.05, ** < 0.01, *** < 0.005, **** < 0.001) was determined by using student t test. In all data presented in the figure, 5 mice were used in each group. The data presented are representative of at least 3 independent experiments.

The particular reason that *Lacticaseibacillus rhamnosus* and *Bacillus clausii* are used as probiotics and have a therapeutic effect on the Gastrointestinal tract is the ability to survive and thrive through the digestive system while adhering to the intestinal epithelial cells. Compared to related strains, this strain was shown to be an excellent mucus-adhering Bacillus and *Lactobacillus* strain (20). Additionally, the scientific paper reported that it can form biofilms, which enhance the protection and elevation of the cytoskeleton integrity and strengthen integrity to prevent different pathogens' colonization (21). The strong adhesive capacity and efficacy of LGG against digestive system pathogens in vivo and in vitro in humans have been documented (22). The study by (23) confirms that *L. rhamnosus* GG adheres very well to the mucosa of the intestines. Immunohistochemistry was performed on the colon section to determine the types of immune cells enrolled after being treated with probiotics. The experimental results showed a significant increase in the ILC3 associated with probiotic administration in treated mice. However, there is strong evidence that ILC3 plays a double-edged role in gastrointestinal mucosal immunity, especially in inflammatory bowel disease (24). In the gastrointestinal system, ILC3 plays an important role in immune responses. They maintain intestinal homeostasis by protecting the mucosa from infections by various pathogens. Epithelial cells produce antimicrobial peptides (AMPs) due to the secretion of IL22, IL-17, and GM-CSF, causing them to kill pathogens (23,24). Through the expression of an MHC-II molecule, T cells respond to commensal bacteria, support intestinal dendritic cells (DC) tolerance function, and regulate epithelial glycosylation (24 ,25).

Conclusion

The regular treatment of probiotics *Lacticaseibacillus rhamnosus* and *Bacillus clausii* is one of the most important elements that may affect the general immune conditions of lab mice, especially the WBCs and the innate immune responses represented by Innate Lymphoid cells type 3(ILC3). This is represented by increasing mucosa and submucosa colon tissue thickness and immune cell infiltration and increasing the level of innate lymphoid cells type three in the colon mucosa, which both represent the essential elements of innate immunity of the digestive tract.

Conflicts of interest

The authors declare that there is no conflict of interest.

Ethical Clearance

This work is approved by The Research Ethical Committee.

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دور إعطاء البروبيوتيك على الفئران المعملية وتأثيراته على المعايير الفسيولوجية والمناعية للدم

فاتن محمد اسكندر، حيدر رشيد الرفاس، هناء خليل إبراهيم

قسم الأحياء المجهرية، كلية الطب البيطري، جامعة البصرة، البصرة، العراق.

الخلاصة

في هذه الدراسة، تم إجراء الفحص لمعرفة تاثير الإعطاء المنتظم للبروبيوتيك *Lacticaseibacillus rhamnosus* و *Bacillus clause* على الفئران المختبرية و تاثيرها على الاستجابة المناعية الفطرية، بما في ذلك العدد الإجمالي للخلايا الليمفاوية على العقدة الليمفاوية المساريقية، وإفراز المخاط، والمعايير الفسيولوجية، ومستويات الكيمياء الحيوية في الدم، وكيف تؤثر هذه العوامل على النتيجة المحتملة للتجارب. تم استخدام ستين فأرة أنثى في هذه الدراسة. تم وضع جميع الحيوانات تحت نفس الظروف وعولجت يوميًا لمدة سبعة أيام بمزيج من البروبيوتيك بما في ذلك *Lacticaseibacillus rhamnosus* و *Bacillus clause* 100 ul عن طريق الف وتم جمع عينات الدم لتقييم المعايير الكيميائية الحيوية والدموية متظمنة Hb ، PCV، RBCs، WBCs، ALT، AST، واليوريا، بينما تم إجراء عد الخلايا من أنسجة القولون والعقدة الليمفاوية الأحادية حيث تم جمعها لتقييم مستوى الخلايا الليمفاوية الفطرية خلال الفترة التجريبية.وقد تبين عدم وجود فروق كبيرة ملحوظة في قيم قراءات الهيموجلوبين وكريات الدم الحمراء، إلا أن عدد كريات الدم البيضاء في الدم المحيطي والعقد الليمفاوية الموضعية للحيوانات زاد بشكل ملحوظ بعد 7 أيام من العلاج. كما يوجد تغير كبير في إفراز المخاط في الغشاء المخاطي للقولون مرتبط بإعطاء البروبيوتيك. وفي تحديد المعايير الكيميائية الحيوية للدم بما في ذلك الكرياتينين واليوريا و ALTوAST، أظهرت هذه النتائج بشكل ملحوظ عدم وجود تغييرات كبيرة في هذه المعايير في جميع المخططات التجريبية. يعتبر العلاج المنتظم بالبروبيوتيك أحد أهم العناصر التي قد تؤثر على الحالة المناعية العامة للفئران المعملية وخاصة كريات الدم البيضاء والاستجابات المناعية الفطرية. ويتمثل ذلك في زيادة سمك أنسجة القولون المخاطية وتحت المخاطية وتسلل الخلايا المناعية وزيادة مستوى الخلايا الليمفاوية الفطرية من النوع الثالث في الغشاء المخاطي للقولون، وكلاهما يمثلان العناصر الأساسية للمناعة الفطرية للجهاز الهضمي.

الكلمة المفتاحية: البروبيوتيك، الخلايا الليمفاوية الفطرية النوع الثالث، المناعة النسيجية.