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Comparative in silico and in vivo study of the antioxidant activity of *lactoferrin*, *Geobacillus stearothermophilus*, and *Lactobacillus delbrueckii subsp. lactis* against *Rotavirus* infection in male mice

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Abstract

Rotavirus is a major cause of pediatric gastroenteritis, for which effective treatments are limited. This study investigates the antioxidant and antiviral potential of lactoferrin, *Geobacillus stearothermophilus*, and *Lactobacillus delbrueckii subsp. lactis* against *Rotavirus* infection. In this study, *Geobacillus stearothermophilus* and *Lactobacillus delbrueckii subsp. lactis* were isolated from Hammam Pharon soil and milk cheese, respectively, and identified using molecular techniques with accession numbers PP758390 and PP758383. The antioxidant effect against DPPH showed that lactoferrin exhibited the strongest scavenging ability, followed by *Geobacillus stearothermophilus* and *Lactobacillus delbrueckii subsp. lactis*. In vivo experiments involved administering lactoferrin, *Geobacillus stearothermophilus*, and *Lactobacillus delbrueckii subsp. lactis* in the drinking water of young mice for three days, followed by *Rotavirus* infection on the fourth day and sacrifice on the fifth day. The results demonstrated that lactoferrin significantly reduced the pathogenic effects of *Rotavirus*, as indicated by the normalization of inflammatory cytokines (TNF-α and IL-6) in the serum ($p \leq 0.001$). Histological examination of small intestinal sections from *Rotavirus*-infected mice revealed extensive destruction of villus structures, while mice treated with lactoferrin showed no pathological changes compared to the control group. *Geobacillus stearothermophilus*-treated mice exhibited less pathological alteration and *Lactobacillus delbrueckii subsp. lactis*-treated mice showed mild pathological changes. Additionally, molecular docking studies indicated that bacteriocin (a bacterial protein) exhibited the highest binding affinity for the Rotavirus outer membrane protein (VP6) at -261.92 kcal/mol, outperforming lactoferrin (-229.32 kcal/mol). Additionally, bacteriocin's active compounds, turimicin (-7.9 kcal/mol) and lactin (-6.5 kcal/mol), also showed strong binding to VP6, suggesting their potential as therapeutic agents against *Rotavirus*. In conclusion, this study highlights the significant antiviral potential of lactoferrin against *Rotavirus*, demonstrating its ability to mitigate pathological changes and normalize inflammatory responses in infected mice. The findings also suggest that bacteriocins, particularly those with high binding affinities to *Rotavirus* proteins, could serve as promising candidates for therapeutic interventions against *Rotavirus* infections.

Keywords *Rotavirus*, *Geobacillus stearothermophilus*, *Lactobacillus delbrueckii*, Lactoferrin, TNF-α IL-6

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Introduction

Enteric diseases, caused by microorganisms such as bacteria, viruses, and parasites, are a major global health concern, typically transmitted via contaminated water or food. These infections can also spread through person-to-person contact and significantly disrupt normal intestinal function, resulting in symptoms like nausea, vomiting, and diarrhea. Among these pathogens, *Rotavirus*, a member of the Reoviridae family, is the leading cause of pediatric gastroenteritis [1, 2] Since 1970, viruses, primarily from the four families Reoviridae (*rotavirus*), Caliciviridae (*calicivirus*), Astroviridae (*astrovirus*), and Adenoviridae (*adenovirus*), have been identified as enteric viral pathogens. These viruses cause gastroenteritis, characterized by tissue inflammation, epithelial barrier disruption, malabsorption, and diarrhea [3, 4]. *Rotavirus* is the primary cause of pediatric gastroenteritis, which leads to sudden onset of diarrhea and vomiting. In 2019, it was predicted that rotavirus was responsible for over 150,000 child deaths due to dehydration, as well as the hospitalization of millions of children under five years of age [5]. *Rotavirus* is composed of three capsid protein layers: an outer layer (VP7, VP4), a middle layer (VP6), and an inner layer (VP2). Infection in the small intestine triggers a robust cytokine response, including TNF-α and IL-6, which regulate inflammation and protect the intestinal epithelial cells [6]. *Rotavirus* infection in the small intestine causes the production of many cytokines, including TNF-α, which controls inflammation in intestinal cells, and IL-6, which safeguards epithelial cells from the adverse effects of the virus and regulates the immunological response to the virus [7]. Furthermore, immunization of children up to 5 years of age reduced nosocomial diarrhea by 75% without altering other viruses, according to [8]. Lactoferrin is a potent glycoprotein in the transferrin family that plays a vital role in host defense [9]. Numerous studies have shown that bovine lactoferrin plays a significant role in reducing the immunological responses to several viruses, such as *rotavirus* [10], hepatitis C [11], herpes simplex viruses [12], and HIV [13]. Furthermore, numerous studies have examined probiotic therapy that has specifically identified its therapeutic effect on rotavirus-induced diarrhea in children [14]. Probiotics are living microorganisms that, when consumed in adequate amounts, provide health benefits to the host and have a variety of positive effects on the body, including anti-inflammation, prevention of oxidative damage, inhibition of cancer growth, fight against viruses, and maintenance of a healthy balance of gut bacteria and immunological function [15, 16]. The most used probiotic strains for the treatment of diarrhea are *Lactobacillus*, *Bifidobacterium*, and *Saccharomyces*. However, other bacteria, such as *Enterococcus*, *Streptococcus*, and *Escherichia coli,* have also been used [17]. The administration of lactobacilli, such as *Lactobacillus rhamnosus* GG (LGG), *Lactobacillus reuteri*, *Lactobacillus acidophilus*, and *Lactobacillus gasseri*, has been demonstrated to enhance growth performance and relieve Rotavirus-induced diarrhea in pigs, calves, and mice [18]. Furthermore, [19] found that *lactobacilli* products, such as exopolysaccharide, improved *Rotavirus*-induced diarrhea and viral shedding and also prevented alteration of intestinal epithelial integrity. One of the best methods for drug discovery is computational drug design [20]. Molecular docking studies greatly expedite the identification of new drugs [21]. Therefore, the purpose of this study was to compare the possible effects of lactoferrin, *Geobacillus stearothermophilus* (*G. stearothermophilus*), and *Lactobacillus delbrueckii* subsp. *Lactis* (*L. delbrueckii* subsp. *Lactis*) on *Rotavirus* enteric infection in mice, both in vivo and in silico, using a molecular docking approach.

Material and methods

Isolation and enrichment of *Geobacillus stearothermophilus*

G. stearothermophilus was isolated from soil collected from Hammam Pharaon, Sinai, Egypt. The temperature was recorded as 55°C with a pH of 6.7 at the time of collection. Soil samples were collected and stored in aseptic plastic bags before being transferred to the laboratory under refrigerated conditions at 4°C. One gram of soil was diluted in sterile distilled water and spread on nutrient agar plates. The plates were incubated for 24 to 48 h between 55 and 70°C, as previously described by Selim et al. [22], according to previously reported by Mohapatra et al. [23], colonies were selected individually and sub-cultured on nutrient agar plates to produce pure isolates. A pure colony from this sample was designated as S1030. Gram staining, endospore staining, and catalase test revealed other properties. The isolated, purified bacteria were stored at −20 °C in 20% glycerol for additional research.

Isolation and identification of *Lactobacillus delbrueckii subsp. Lactis*

The 100-g white cheese design was carefully homogenized in a stomacher bag, stamped, and incubated for a day at 30 °C after dilution (1:10) using De Man, Rogosa, and Sharpe (MRS) broth medium (HI Media Laboratories, Thane, Maharashtra, India). After plating 0.1 mL of samples overnight on MRS agar plates, they were incubated at 37 °C in an anaerobic environment with 5% CO2. Colonies that were physically distinct and well-separated were selected and streaked onto fresh MRS agar plates for 48 h. Randomly chosen colonies exhibiting *lactobacillus*-like features were grown in MRS broth. The colony was examined for morphological and cultural characteristics

such as color and texture, and biochemical analysis as gram staining, catalase and oxidase tests [24]. The pure colony arising from this sample was designated L1030.

Bacterial genomic DNA extraction

According to a study conducted by [25], strain S1030 and strain L1030 were cultivated and maintained on nutrient agar for 24h. Bacterial genomic DNA was extracted from a fresh cell culture, and used the universal bacterial primers 27F (GAG AGT TTG ATC CTG GCT CAG) and 1492R (CTA CGG CTA CCT TGT TAC GA) were used for amplification of 16S rDNA [26]. For PCR, we used the following conditions: 50 µl reaction system, 95°C pre-denaturation for 5 min, 30 cycles of 94°C denaturation for 1 min, annealing at 60°C for 1 min, extension at 72°C for 1 min30 s and final extension for 10 m at 72°C. The amplicons were stored at 4°C until used. The samples were prepared in accordance with Macro Gen Company's instructions, using 50 ng/l of each PCR product [27]. The samples were transported to Macro Gen Company, Korea, where they were processed. Preliminary examination of sequences was performed using BLAST (http://www.ncbi.nlm/NLM.gov/BLAST), and cluster analysis was carried out using MEGA 11 software package.

In vitro assessment of the antioxidant effects of lactoferrin, *Geobacillus stearothermophilus,* and *Lactobacillus delbrueckii* subsp. *Lactis.*

Lactoferrin, a pharmaceutical product, was obtained from the Egyptian market. Bovine lactoferrin was dissolved at the dilution rate of 2 g/100 ml of distilled water [28] and *G. stearothermophilus* and *L. delbrueckii* subsp. *Lactis* at the dilution of 10 g/100 ml [29]. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free-radical scavenging assay was used to evaluate the in vitro antioxidant activity. A 0.1 mM solution of DPPH was prepared by dissolving 1.9 mg of DPPH in 100 mL of 100% methanol. The solution was then kept in the dark for 1 h. Subsequently, 1.0 mL of this solution was added to 3.0 mL of the extract solution in methanol at various concentrations. After a period of thirty minutes, absorbance was measured at 517 nm. A void was made for the addition of extract. Standard concentrations ranging from 1 to 18 µg mL^{-1} were utilized. A decrease in the absorbance of the reaction mixture resulted in an increase in free radicals scavenging. The scavenging ability was accurately defined in the following method according to [29].

$$1 - \frac{A517(sample)}{A517(blank)} \times 100$$

Animals and experimental design

Forty-eight (48) pathogen-free, juvenile male mice weighing 15-20g, and ages 4 months were obtained from the animal facility of the Egyptian Drug Authority (EDA), formerly known as the National Organization of Drug Control and Research (NADCAR). *Rotavirus* RIT4237 1500 µl with titer of 10 was purchased from VACSERA (51 Wezaret EL Zeraa Street, Agouza, Giza*).* It was inoculated on the 4th day at an inoculum dose 20 µL mouth injected per mouse to make the infection day, followed by mice scarifying on the 5th day. Bovine lactoferrin was dissolved at the dilution rate of 2 g/100 ml of distilled water [28]. Drinking water was inoculated with 20 µL 0.5 McFarland bacterial suspension per mouse every day for three days. The mice had free access to water and food. 6 animals/group and ethical Animals Committee (NODCAR/1/43/2022). All animals were kept under environmental conditions (day cycle 12 h light, 12-night, temp 20–25°C, and humidity 40%–50%). The animals were divided into six groups, each with six animals.

1. Control
2. *Rotavirus*
3. *Rotavirus*+ *L. delbrueckii subsp. Lactis*
4. *Rotavirus* +*G. stearothermophilus*
5. *Rotavirus* +*Lactoferrin*
6. *Lactoferrin*
7. *L. delbrueckii subsp. Lactis*
8. *G. tearothermophilus*

Determination of the production of TNF-α and interleukin-6

Serum was extracted from blood samples by centrifuging them for 15 min at 3000 rpm. The serum was then frozen and stored at −80 °C. TNF-α and IL-6 plasma levels were measured in triplicate using a Rat IL-6 ELISA Kit (Catalog No.: MBS355410) and Rat Tumor Necrosis Factor α ELISA Kit (Mice TNF-α) (Catalog No.: MBS2507393 96 T/48 T/24 T) that were purchased from MyBioSource (San Diego, California, USA) according to the manufacturer's guidelines.

Histological preparations

Mice were dissected at the end of the experiment; all animals were anesthetized with 1% sodium pentobarbital to obtain blood samples. Pieces from the small intestine of each mouse were removed, perfused with 0.9% saline solution, and fixed in a neutral buffered formalin solution for 24 h. The specimens were processed through an ascending alcohol series, cleared in xylene, and mounted in molten paraffin wax. Sections of 5µm thick were cut from each block, mounted on glass slides, dewaxed, and stained with hematoxylin and eosin [30]. An Olympus microscope (CX31RTSF, Olympus Corporation, Tokyo, Japan) connected to an Olympus digital camera (E330,

Olympus Corporation, China), was used to examine and photograph each slide.

Immunohistochemical staining

Proliferating cells were visualized immunohistochemically by incubating dewaxed small intestine sections with antibodies directed against proliferating cell nuclear antigens (PCNA). Rabbit polyclonal anti-PCNA primary antibody (ab152112, Abcam) was used at a dilution of (1:500) in phosphate-buffered solution. In another dewaxed section of the small intestine, interleukin-1 (IL-1) expression was detected using rabbit recombinant multiclonal anti-IL-1 primary antibody (RM1009, ab 283,818, Abcam) at a dilution of (1:500). The staining procedures followed the avidin-biotin complex technique described by [31], where rehydrated sections were washed in tree changes of phosphate-buffered solution (PBS). To reduce background staining, hydrogen peroxide was used to suppress endogenous peroxidase activity, followed by washing with two changes of tap water (5 min each). Heat-mediated antigen retrieval was performed by adding heated sodium citrate buffer with pH 6.0 in bath water for 20 min, to 95–98°C followed by cooling for 20 min at room temperature to the slides which were then allowed to cool and washed in PBS. Next, sections were incubated with the selected primary antibody at 4°C overnight, followed by washing tree times with PBS (5 min each). Secondary antibody was added to intestinal sections at room temperature (10 min), and the excess was removed by rinsing in PBS. Streptavidin-peroxidase complex was applied (10 min). In a humid chamber, a mixture of (1 drop of 3,3′-diaminobenzidine DAB and chromogen) with (2 ml DAB and substrate) was incubated with the slides for 5 min at room temperature. Finally, sections were counterstained with Harris hematoxylin, dehydrated, cleared, and mounted using Dibutyl phthalate Polystyrene Xylene (DPX). The optical density of the generated brown color was measured using six images taken from the small intestine section of each animal in each group at a magnification of 400X (Olympus microscope CX31RTSF, coupled with an Olympus digital camera E330). Image J software was manipulated to quantify the optical density of the brown pigment in each image, as described by Abd-Elhafeez et al. [31], by the following equation: Optical density = log (maximum gray intensity/mean gray intensity).

Protein–protein docking methods of lactoferrin against rotavirus outer membrane proteins

The 3D structure of the *rotavirus* outer membrane was obtained from the PDB database at resolutions of 2.8 and 2.2 Å, respectively. The specific PDB IDs for these structures was 1QHD [32]. The protein-protein docking analysis was performed using the H-Dock docking program [33] to investigate the interaction between the vp6 protein, the lactoferrin protein, and the bacteriocin protein. The H DOCK approach combines projecting the interaction terms into 3D grid-based potentials and estimating the binding energy during complex formation. The binding energy is approximated as a correlation function comprising van der Waals, electrostatics potential terms. The interaction-energy minima were determined by employing a rapid and thorough rotational docking search in conjunction with a straightforward translational scanning technique [34]. The docking operations for both cases were executed via the H DOCK web server, accessible at http://hdock.phys.hust.edu.cn/

Protein-ligand Docking Methods of bacteriocin produced by *G. stearothermophilus* and *L. delbrueckii subsp. Lactis* retrieved from database against rotavirus outer membrane proteins.

Bacteriocins are antimicrobial peptides manufactured by bacteria via ribosomes. They have a narrow or broad spectrum of biological activities [35]. Besides,some research on the bacteriocin classes revealed the chemical to be a member of bacteriocin classes with biological activity that includes antiviral and antibacterial properties [36, 37]. The RCSB Protein Data Bank (https://www.rcsb.org) was used to obtain protein structures of rotavirus outer membrane proteins, specifically VP6 with ID 1QHD as the receptor, and bacteriocin and lactoferrin proteins with IDs 2MWR and 4U9C respectively, as the ligands. During the final phase, hydrogen atoms were introduced to the target protein molecule once all water molecules had been eliminated.

Retrieval of ligands

Bacteriocin ligands (Table 1), which are chemical compounds produced by bacteria, were identified by searching the PubChem database in the structural data format (SDF) (http://www.pubchem.ncbi.nlm.nih.gov) [36, 37].

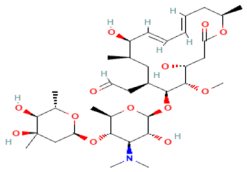
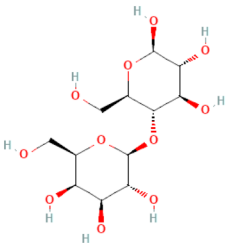
Docking analysis

The best *rotavirus* protein (VP6) and Ligand Complexes were visualized in a 2D structure using BIOVIA Discovery Studio 2021, and Molecular Docking was performed using the Auto-Dock Vina program [38].

Phylogenetic analysis

Cluster W was used to align the sequences for phylogenetic analysis. The evolutionary tree for *Geobacillus stearothermophilus* strain S1030 and *Lactobacillus delbrueckii* subsp. *Lactis* strain L1030 (Figs. 1a and b) was determined using neighbor-joining techniques by MEGA.11 software [39].

Table 1 Bacteriocin bioactive compounds obtained from database

Compound name	Chemical formula	Chemical structure
Thurincin	$C_{35}H_{59}NO_{13}$	
lactin	$C_{12}H_{22}O_{11}$	

Statistical analysis

SPSS version 20.0 was used to analyze the current data (IBM, New York, USA). The mean value ± standard error of the mean (SEM) was used to represent the measurement data. One-way ANOVA was used to assess differences between various groups, and Tukey's post hoc test was performed after the Shapiro-Wilk test was used to check for normality. Statistical significance was set at $p < 0.05$.

Results

Isolation and molecular identification of *Geobacillus stearothermophilus and Lactobacillus delbrueckii subsp. Lactis*

In the present study, the isolated thermophilic bacterial strain S1030 was identified as a spore-forming, gram-positive, rod-shaped bacillus with circular colony morphology and white-creamy coloration. Molecular analysis confirmed it as *G. stearothermophilus* strain S1030 (GenBank accession number PP758390) (https://www.ncbi.nlm.nih.gov/nuccore/PP758390). The 16S rRNA sequence analysis showed that the obtained isolate are members of the genus *Geobacillus*. The thermophilic bacterial strain S1030 was phylogenetically relevant to *G. stearothermophilus* strain S1030 (99.1%). Alternatively, the isolated lactic acid bacteria were identified as rod-shaped, or spherical, gram-positive, catalase-negative, oxidase negative and termed LAB bacteria with white to creamy-coloration after anaerobic growth in the specified culture medium. Among these, strain L1030 was identified and molecular analysis confirmed it as *L. delbrueckii* subsp. *Lactis* strain L1030 (accession number PP758383) https://ncbi.nlm.nih.gov/nuccore/PP758383.

Scavenging ability against DPPH

The experiment was conducted to study the difference in the effect of Lactoferrin, *G. stearothermophilus* and *L. delbrueckii* subsp. *Lactis* found that lactoferrin has stronger scavenging ability against DPPH than *G. stearothermophilus* and *L. delbrueckii subsp. Lactis* respectively (Table 2).

Determination of TNF-α and interlukin-6

As shown in Table 3 and Fig. 2, the serum pro-inflammatory cytokines (TNF-α and IL-6) in the *Rotavirus*- group showed a highly significant increase ($p \leq 0.001$) compared to the control group, but in the combination group (*L. delbrueckii subsp. Lactis, G. stearothermophilus*, and lactoferrin) showed a significant reduction in the level of (TNF-α and IL-6) in compared to the control group.

Lactoferrin had a remarkable and statistically significant effect on decreasing the levels of inflammatory cytokines caused by *rotavirus* treatment. These levels were then brought back to levels seen in the healthy control group ($p \leq 0.001$). However, when the group that only got *Rotavirus* was compared to groups that got *Rotavirus* along with either *L. delbrueckii* subsp. lactis or *G. stearothermophilus*, both combinations effectively lowered the virus's harmful effects. The *Rotavirus* + *L. delbrueckii* subsp. *lactis* group exhibited a remarkably substantial decrease ($p \leq 0.001$), while the *Rotavirus* + *G. stearothermophilus* group demonstrated highly significant ($p \leq 0.01$) in male mice.

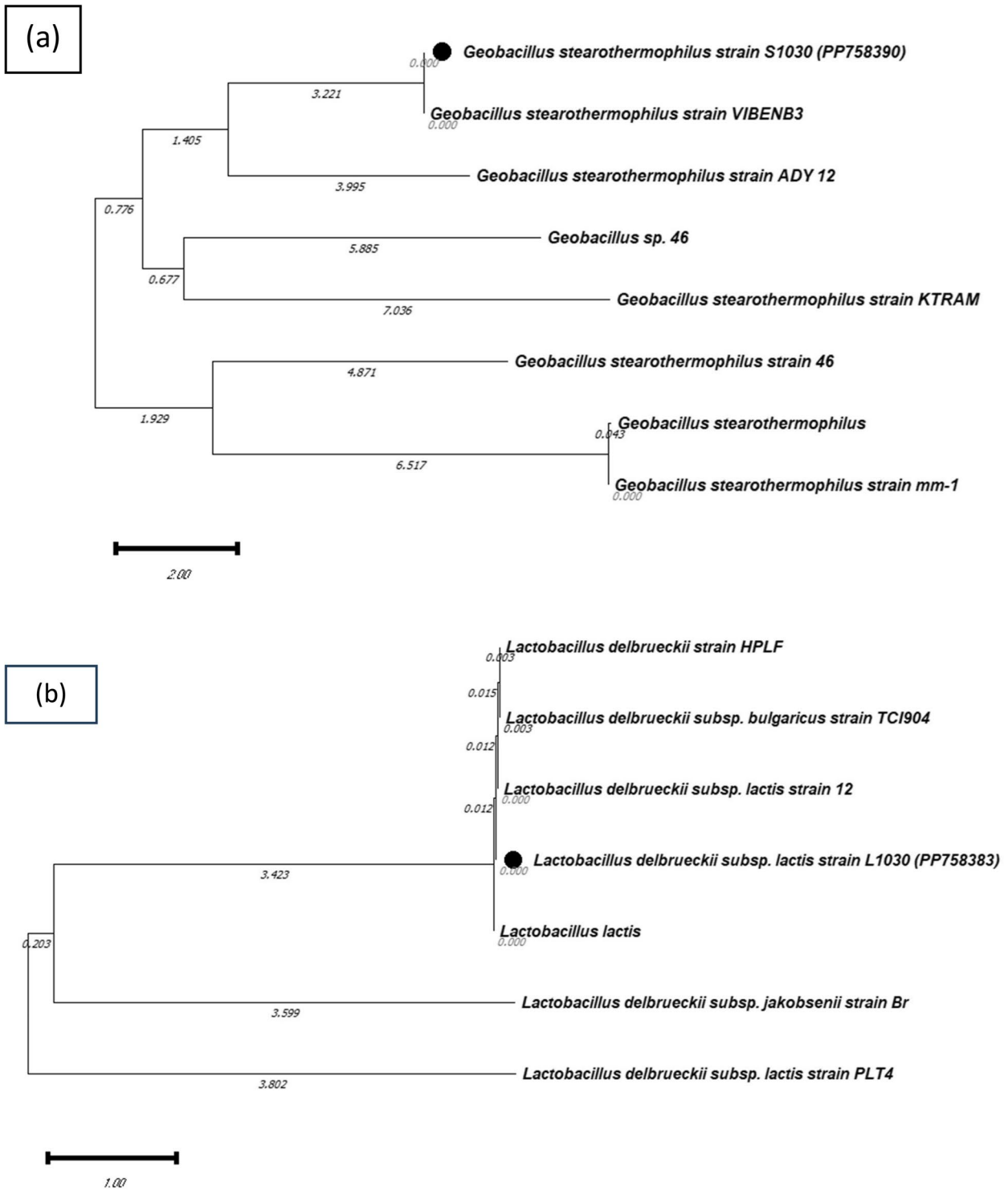


Fig. 1 Phylogenetic tree of (**a**)*G. stearothermophilus* strain S1030, (**b**) *L. delbrueckii subsp. Lactis* strain L1030 using the Neighbor-Joining method

Histological results

Light microscopical examination of control mice's small intestine sections showed no pathological changes. The mucosa of the control group appeared with normal long villi extending to the lumen and intestinal glands (crypts of Lieberkühn), which started at the bases of the villi

Table 2 Antioxidant activities of different concentrations of Lactoferrin, *G. stearothermophilus*, and *L. delbrueckii* subsp. *Lactis* against DPPH radical

Concentrations μ/ml^{-1}	Inhibition of DPPH (%) by Lactoferrin	Inhibition of DPPH (%) by *Geobacillus stearothermophilus*	Inhibition of DPPH (%) by *Lactobacillus delbrueckii subsp. Lactis*
30	55 ± 1.3	45 ± 1.1	45 ± 1.1
50	75 ± 1.8	65 ± 1.6	55 ± 1.3
70	85 ± 2.1	75 ± 1.8	65 ± 1.6

Table 3 The serum pro-inflammatory cytokines (TNF-α and IL-6) in experimental groups

Groups	TNF- α (pg/ml)	IL-6 (pg/ml)
Control	38.4	24.9
Rotavirus	172 ± 11.4^{a***}	146 ± 10.7^{a***}
Rotavirus + *L. delbrueckii subsp. Lactis*	111 ± 1.62 ^{a***b***}	91.1 ± 3.92^{a**b***}
Rotavirus + *G. stearothermophilus*	83.2 ± 5.48 ^{a**b***}	73.0 ± 5.86 ^{a**b***}
Rotavirus + Lactoferrin	56.3 ± 3.91^{b***}	47.0 ± 3.74 ^{b***}
Lactoferrin	30.17 ± 1.60	23.47 ± 1.097
L. delbrueckii subsp. Lactis	106.2 ± 7.044	85.03 ± 3.987
G. tearothermophilus	87.67 ± 4.734	72.67 ± 4.283

Data are illustrated as mean ± standard error of the mean (M ± SEM) of six animals in each group. According to the t-test: **highly significant ($p \leq 0.01$), ***Very highly significant ($p \leq 0.001$). a control group, b *Rotavirus* group

and extended to the muscularis mucosae. Each villus is formed of the central core of lamina propria surrounded by columnar absorptive epithelial cells (enterocytes) that have striated borders and goblet cells. Lamina propria is a loose connective tissue that contains lymphocytes, smooth muscle cells, lacteals, and blood vessels. In addition, some lymphocytes with round, intensely stained nuclei were distributed along different levels of the epithelial cells. Conveniently, the small intestine sections of mice were administrated with *L. delbrueckii subsp. Lactis*, *G. stearothermophilus*, or lactoferrin did not show any pathological change when compared with the control group, all examined sections were nearly normal. On the other hand, histological examination

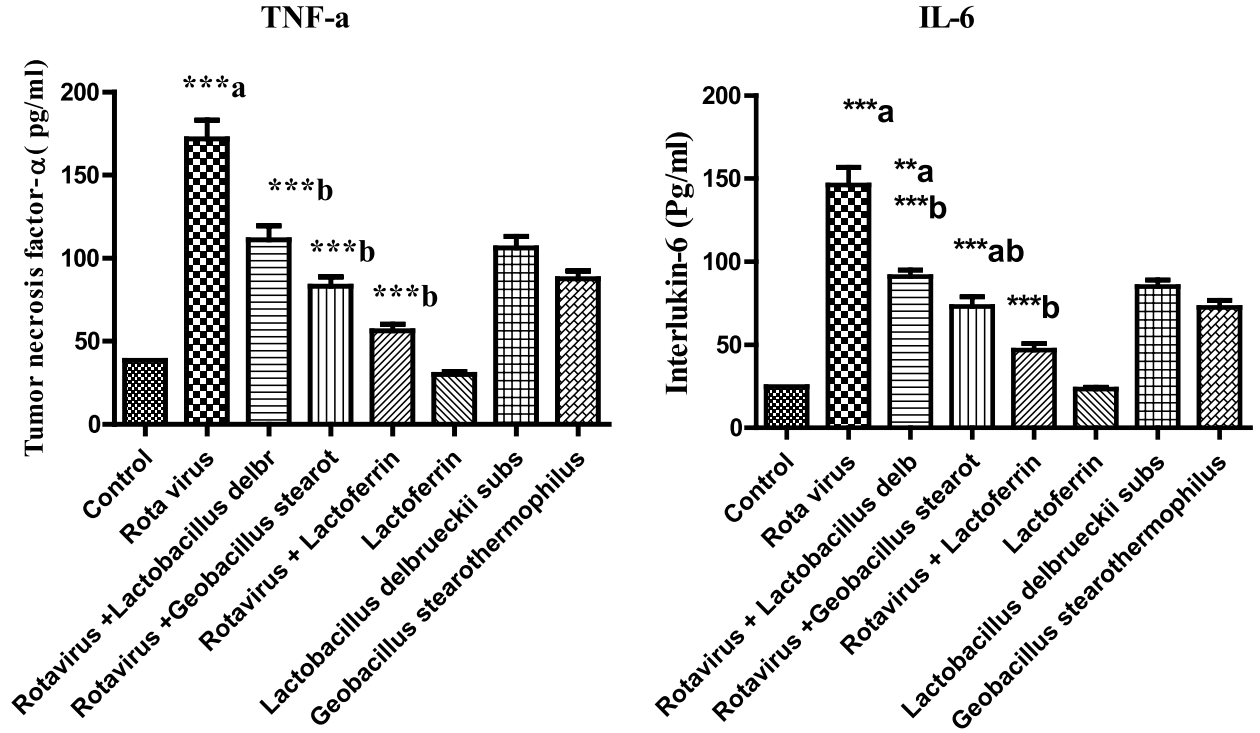


Fig. 2 The Effect of *Rotavirus* infection with *L. delbrueckii subsp. Lactis, G. stearothermophilus*, and lactoferrin protein in an enteric infection in male mice on tumor necrosis factor-α, and interleukin −6 in serum. a: control group b: *Rotavirus* group ** highly significant ($p \leq 0.01$) *** very highly significant ($p \leq 0.001$)

of small intestine sections of rotavirus-infected mice revealed massive destruction of villus structure in wide areas of intestinal mucosa in all examined animals. Villus degeneration is a common feature. In addition, some villi no longer appeared as finger-like projections in the intestinal lumen. Instead, they were short, fused, and had broad blunt apical surfaces, or swollen tips. The remaining villi showed marked widening in the subepithelial space, which may be due to edema, with a marked reduction in the thickness of lamina propria. Enterocytes were shortened and became cuboidal instead of columnar. Hyperproliferation of enterocytes, particularly at the bases of the villi, was detected in many villi, while goblet cells were reduced in number. Besides, some scattered inflammatory cells were occasionally seen in the crypts of Lieberkühn, and numerous were detected in lamina propria. Administration of *L. delbrueckii subsp. Lactis* to *Rotavirus*-infected mice did not show any histological improvement when compared with *Rotavirus*-infected group. Meanwhile, treating *Rotavirus*-infected mice with *G. stearothermophilus* showed a slight improvement in the histological structure of the small intestine as compared with the *Rotavirus*-infected group. In this group, some scattered villi appeared with nearly normal enterocytes and reduced villus subepithelial space, however many others still show degeneration and blunt apical region. On the contrary, small intestine sections of mice infected with *Rotavirus* and treated with lactoferrin revealed marked improvement in villus structure and epithelial cell integrity in all examined animals when compared with Rotavirus infected group. Most villi retained their normal appearance with well-developed enterocytes, and even damaged villi showed a marked reduction in subepithelial space. Inflammatory cells were still seen in the crypts of Lieberkühn (Fig. 3).

Immunohistochemical observations

Proliferation of small intestine epithelial cells was investigated immunohistochemically by detecting PCNA expression (Fig. 4). In experimental groups (control, *Rotavirus*, *L. delbrueckii subsp. Lactis*, *G. stearothermophilus*, and lactoferrin-treated groups, PCNA expression was observed at the crypt compartment, and it was similar between different control groups. There was no difference in the optical intensity of the brown pigment between them. Mice infected with rotavirus and those treated with either *L. delbrueckii subsp. Lactis* or *G. stearothermophilus* after *Rotavirus* infection showed a moderate increase of 0.129 ± 0.011, 0.128 ± 0.006, respectively, in the optical density of PCNA expression in the crypt epithelium when compared with the (0.118 ± 0.011) control group. On the other hand, lactoferrin administration to *Rotavirus*-infected mice reduced the brown pigment's optical density as 0.116 ± 0.010 when compared with *Rotavirus*-infected group 0.126 ± 0.007 (Fig. 5 & Table 4).

Immunohistochemical detection of IL-1 in small intestine sections of either the control group, *G. stearothermophilus* treated group, or lactoferrin treated group was similar. All examined sections showed a few numbers of

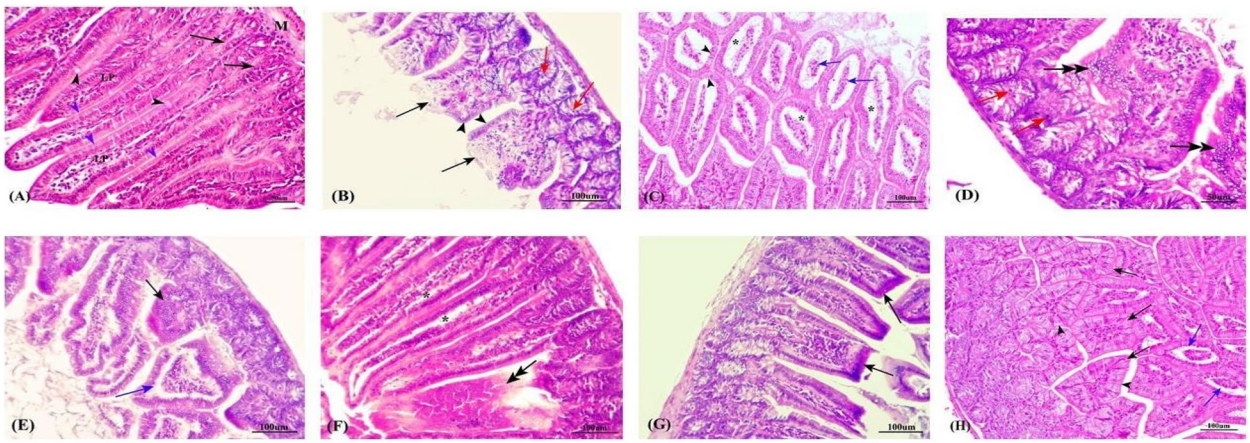


Fig. 3 Photomicrographs of sections in the small intestine of mice from different groups stained with hematoxylin and eosin, **A** control group showing intestinal villi with lamina propria (LP), enterocytes (blue arrowhead), goblet cells (black arrowhead), crypts of Lieberkühn (arrow), and muscular mucosae (M). **B**-**D** rotavirus infected group showing short villi (black arrow) with cuboidal enterocytes (arrowhead), inflammatory cells infiltrate in crypts cells (red arrow), reduced lamina propria (blue arrow), enlarged subepithelial space (star) and hyper proliferated epithelial cells (double head arrow). **E** *Rotavirus*-infected group treated with *L.delbrueckii subsp. Lactis* showing degenerated villus (black arrow) and subepithelial space (blue arrow). **F**, **G** rotavirus infected group treated with *G. stearothermophilus showing* degenerated villus (double head arrow), reduced subepithelial space (star), and villi with blunt end (arrow). **H** *Rotavirus* infected group treated with lactoferrin showed normal villi (black arrow) with columnar enterocytes (arrowhead) and sub-enterocyte space (blue arrow)

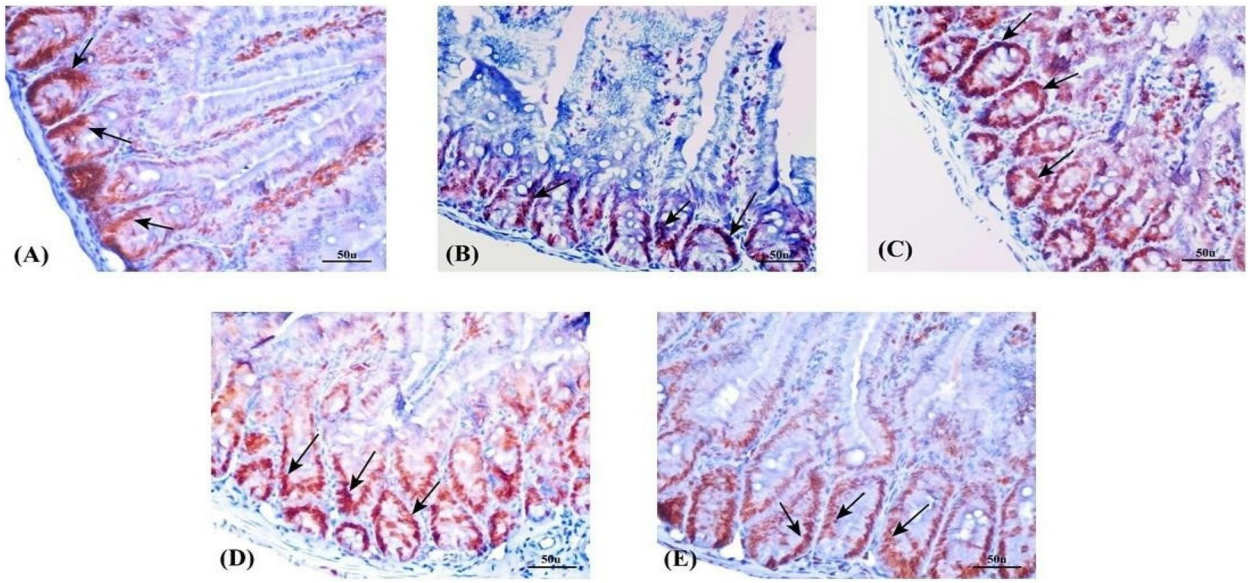


Fig. 4 Photomicrographs of sections in the small intestine of mice from different groups stained immunohistochemically for PCNA, showing positive crypt epithelial cells (arrow). **A** control group, (**B**) *Rotavirus*-infected group, (**C**) *Rotavirus*-infected group treated with *L. delbrueckii subsp. Lactis*, (**D**) *Rotavirus*-infected group treated with *G. stearothermophilus,* and (**E**) *Rotavirus*-infected group treated with lactoferrin

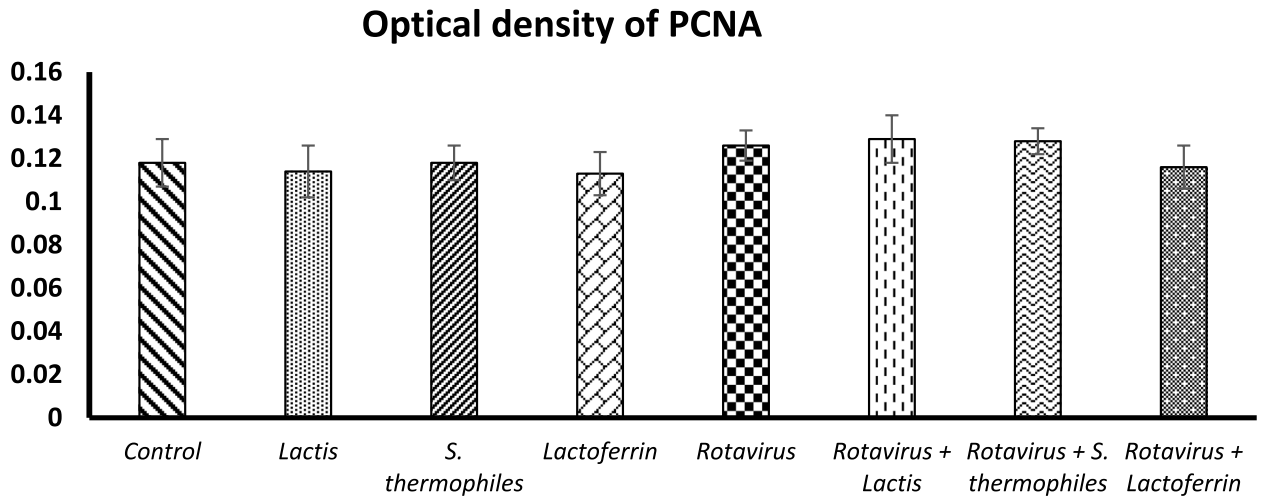


Fig. 5 Optical density of PCNA expression in different experimental groups

Table 4 Optical density of PCNA in different groups

Group	Mean ± SE
Control	0.118 ± 0.011
L. delbrueckii subsp. Lactis	0.114 ± 0.012
G. stearothermophilus	0.118 ± 0.008
Lactoferrin	0.113 ± 0.010
Rotavirus	0.126 ± 0.007
Rotavirus + L. *delbrueckii subsp. Lactis*	0.129 ± 0.011
Rotavirus + G. *stearothermophilus*	0.128 ± 0.006
Rotavirus + Lactoferrin	0.116 ± 0.010

brown pigment-positive cells distributed along lamina propria of some villi (Fig. 6). Not all villi in the examined sections were positive for IL-1, only some villi were positive. On the contrary, intestinal sections of mice infected with *Rotavirus* or *rotavirus* administrated with *L. delbrueckii* subsp. *Lactis* showed positive reactivity in lamina propria of all examined villi. There was a significant increase in the number of IL-1 positive cells in the lamina propria of each villus when compared with the control group, and the color intensity of the brown pigment of this group was darker than that of the control group.

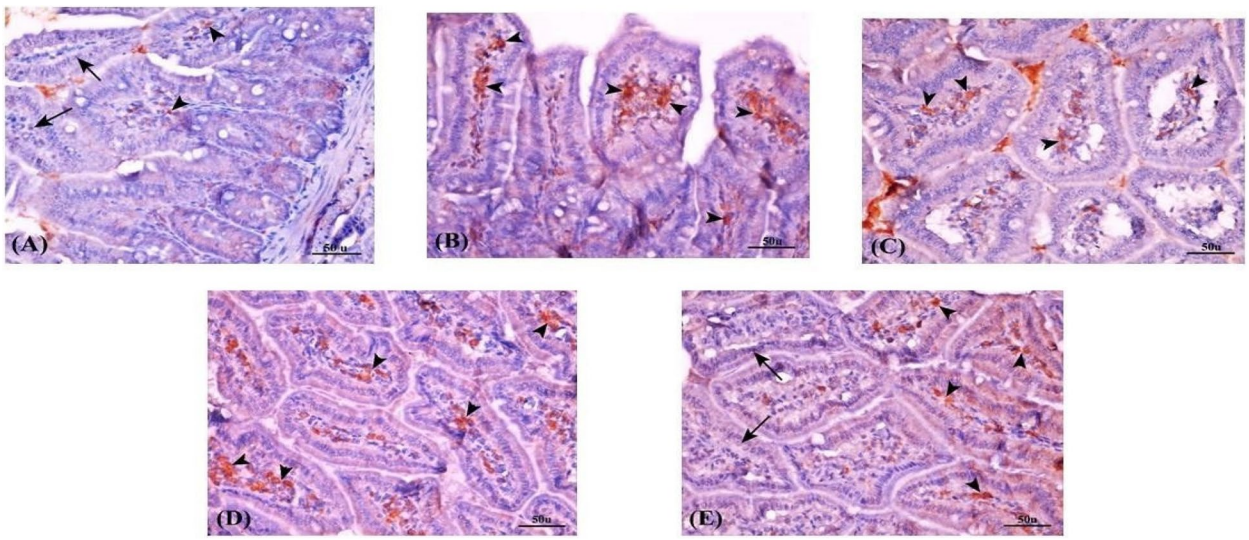


Fig. 6 Photomicrographs of sections in the small intestine of mice from different groups stained immunohistochemically for IL-1, negative villus for IL-1 (arrow), and positive cells (arrowhead). **A** control group, (**B**) rotavirus-infected group, (**C**) *Rotavirus*-infected group treated with *L. delbrueckii subsp. Lactis*, (**D**) *Rotavirus*-infected group treated with *G. stearothermophilus,* and (**E**) *Rotavirus*-infected group treated with lactoferrin

The rotavirus-infected group with *G. stearothermophilus* also showed an increased number of IL-1 positive cells in lamina propria of nearly all examined villi with obvious color intensity compared with the control group. A marked reduction in the number of positive villi for IL-1 was detected in *rotavirus* infected group treated with lactoferrin as compared with *the* infected group, however the number of IL-1 positive cells per villus –when present– was like that in the infected group (Fig. 7 & Table 5).

Molecular docking studies against rotavirus outer membrane protein (OMP)

The protein-protein docking analysis conducted between the *rotavirus* outer membrane protein VP6 (Protein Database ID: 1QHD) and the bacteriocin protein (Protein Database ID: 2MWR) yielded the maximum docking score of −261.92 kcal/mol. As shown in Fig. 8a, this result suggests that the ligand (bacteriocin) and receptor (VP6) have a substantial binding affinity. While the docking score between *rotavirus* outer membrane (VP6) against lactoferrin protein retrieved from

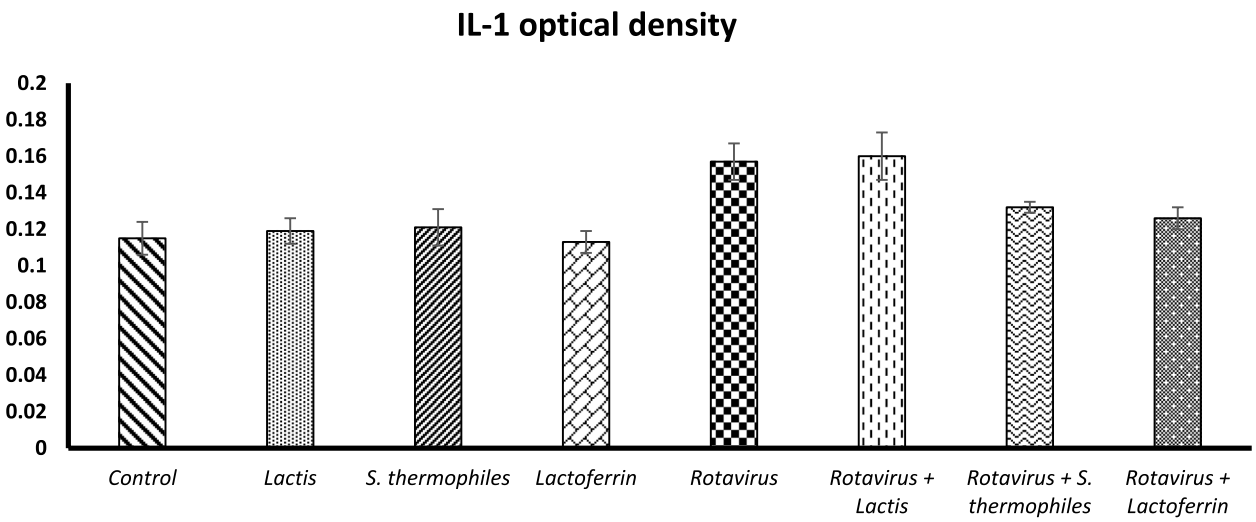


Fig. 7 Interleukin −1(IL-1) optical density in different experimental groups

Table 5 Interleukin −1(IL-1) optical density in different groups

Group	Mean ± SE
Control	0.115 ± 0.009[b]
L. delbrueckii subsp. Lactis	0.119 ± 0.007[b]
G. stearothermophilus	0.121 ± 0.010[b]
Lactoferrin	0.113 ± 0.006[b]
Rotavirus	0.157 ± 0.010[a]
Rotavirus + *L. delbrueckii subsp. Lactis*	0.160 ± 0.013[a]
Rotavirus + *G. stearothermophilus*	0.132 ± 0.003[b]
Rotavirus + Lactoferrin	0.126± 0.006[b]

[a] significant when compared with control

[b] significant when compared with rotavirus

the database with (ID: 4U9C) was -229.32 kcal/ml as Fig. (8b).

On the other hand, the binding affinity between bacteriocin ingredients retrieve from database and literatures with *rotavirus* outer membrane protein (vp6) showed high energy of -7.9 kcal/ml between turimicin and protein receptor (Fig. 9a), while the binding energy between lactin and rotavirus outer membrane protein was -6.5 kcal/ml (Fig. 9b).

Discussion

This study evaluated the potential benefits and effects of probiotic bacteria and pharmaceutical lactoferrin protein on acute intestinal diseases and their potential for treating acute diarrhea in an animal model. Probiotics (lactic acid bacterial group) were used and studied to investigate their effects on *rotavirus* enteric diseases in young mice, along with lactoferrin protein. Our study found that lactoferrin protein could improve the harmful effects of *rotavirus* on pro-inflammatory cytokines, which are signaling molecules secreted by immune cells. When administered to young male mice with enteric diseases, *rotavirus* caused a significant increase in pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) [40].

Furthermore, the current study found that oral treatment with lactoferrin after pathogenic *rotavirus* infection can scavenge the harmful effects of *rotavirus* on the enteric mucosal small intestine by suppressing the concentration of the pro-inflammatory cytokines TNF-α and IL-6 in parallel with the study conducted by Lee et al. [41]. suggested that supplementation with natural products such as lactoferrin and galacto-oligosaccharides

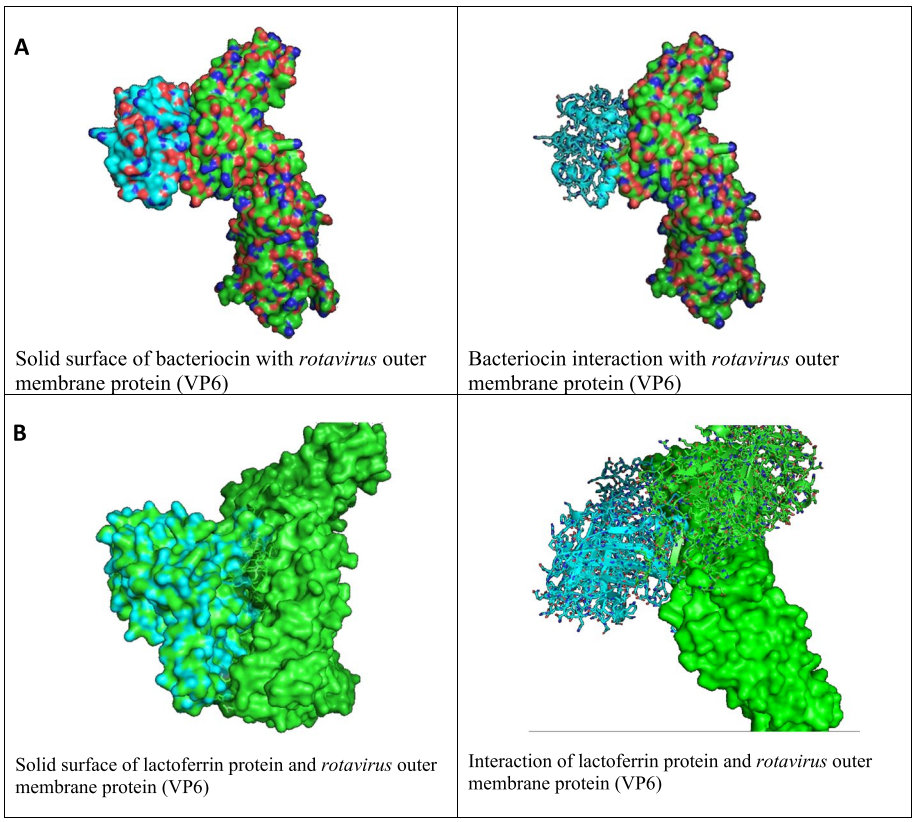


Fig. 8 Molecular docking interaction between (**a**) bacteriocin protein and *Rotavirus* (**b**) lactoferrin protein and *rotavirus* outer membrane protein (VP6)

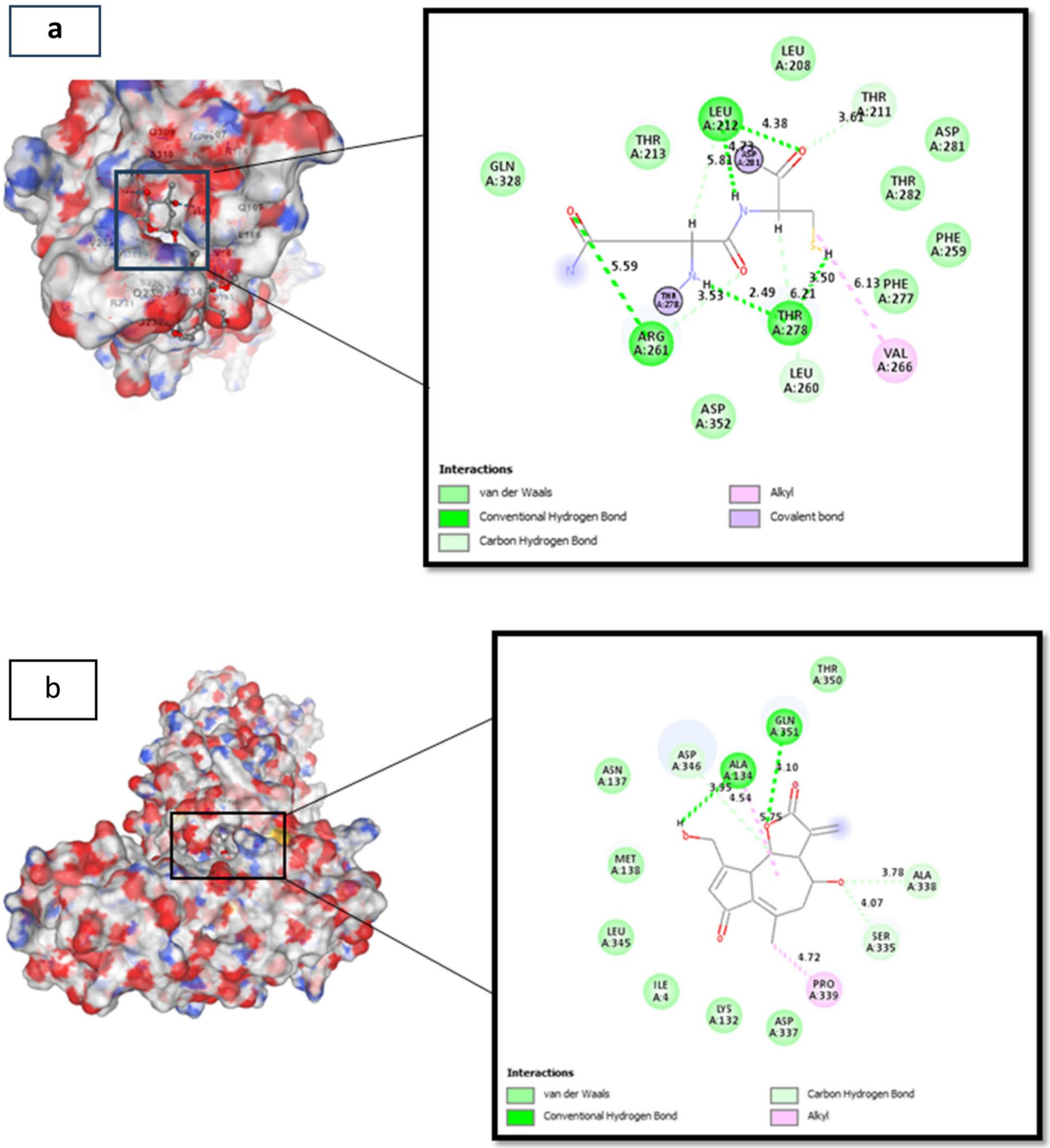


Fig. 9 Molecular docking interaction between (**a**) Turimicin -VP6 protein interaction, (**b**) Lactin – VP6 protein interaction

during incubation in young pig animals affected IgG and IgA, reduced newborn *rotavirus* infection, and enriched the pathogenic bacteria in young animals. Hence, in this investigation, lactoferrin was found to have a beneficial role in monitoring the side effects of rotavirus. In the experimental animals, milk lactoferrin has antiviral activity. It has a potential role in interacting with the virus surface and binding to virus particles to reduce the viral disorder in animals and human cells, according to Mohammadabadi and Lee [41, 42], who observed that lactoferrin and its synthetic have multiple pharmacological activities for regulating nucleic acids synthesis and can inhibit DNA duplication, antigen synthesis of rotavirus and stimulate the immune changes in vitro and in vivo

studies. The results reported in this study were similar to the survey conducted by Bellés et al. [43], which reported that treating the experimental animals with two types of bovine lactoferrin (native bovine lactoferrin and iron-saturated lactoferrin) played a major role in alleviating the decrease of TNF-α production, anti-microbial, and immune suppressants in male C57BL/6 mice treated with clindamycin. In this study, we compared the beneficial effects of three types of probiotics: lactoferrin protein, *Geobacillus stearothermophilus,* and *L. delbrueckii subsp. Lactis*, against mice with enteric viral infection, we discovered that *G. stearothermophilus* was the second probiotic to decrease the production of pro-inflammatory cytokines TNF- and IL-6, which agrees with Song et al. [44]. Additionally, recent research suggests that treatment with three different strains of *Lactoplantobacillus plantarum* strains in the development of DSS-induced colitis in C57BL/6 mice has clinical aspects of attenuating the mucosal membrane of the intestine and regulates the production of cytokines TNF-α and other anti-inflammatory cytokines [45]. Previously, the application of probiotics showed beneficial effects such as immune response [46], and disease resistance [47]. Among the probiotics used in this study, *L. delbrueckii subsp. Lactis*, *G. stearothermophilus* isolated from the cheese and thermophilic soil, respectively, have been reported as potential probiotics and an antibiotic replacement in the diet [48] This study investigated the effects of two dietary isolated probiotic supplementations besides pharmaceutical lactoferrin on growth, immune responses, and histology in mice.

Similarly, Ren et al. [49] determined that the ulcerative colitis mouse model by oral administration of dextran sulfate sodium for seven days, followed by administration of therapeutic doses of the probiotic derivatives *Lactobacillus plantarum* membrane proteins for seven days, had a significant effect on specific biomarkers. More specifically, there were significant impacts on the concentrations of cytokines (TNF-α, IFN-γ, and IL-6) in the bloodstream and biomarkers in the colon tissue (TLR4 and TGF-β). These findings indicate that the probiotic treatment had a beneficial effect on the inflammation and immunological response linked to ulcerative colitis in this experimental model. They effectively reduced the inflammatory response in mice DSS-induced colitis. Moreover, Histological observations in this study revealed many histological alterations in the small intestine sections of Rotavirus-infected mice. These alterations included degeneration, shortening, fusion, and blunting of the villi; widening of subepithelial space; reduction in goblet cells; and increase in mononuclear cells. Previous studies by Abdulazeez et al. [50]; Engevik et al. [51] reported similar histological alterations in the small intestine of mice after rotavirus infection, where villous atrophy, shortening, intervillous lymphocytic infiltrates, desquamation, and necrosis of the crypts were detected. Moderate improvement in villus structure was observed in the current experiment after administering *Geobacillus stearothermophilus* to rotavirus-infected mice. A study conducted by Rigo-Adrover et al. [52] found that milk fermented with *Geobacillus stearothermophilus* and *Bifidobacterium breve* was able to protect suckling rats from diarrhea induced by *rotavirus* infection.

One mechanism by which bacteriocins can exert their antiviral properties is via blocking host cell receptor sites and preventing viral entry [53]. The ability of the bacteriocin and lactoferrin to interfere with *rotavirus* outer membrane protein was assessed via protein–protein docking and protein-ligand docking. Therefore, this interaction could block the viral protein via a potentially different mechanism to directly inhibit viral entry. These results were similar to those of the study conducted by Won et al. [54].

Conclusion

The current study offers insights into the potential of supplementing specific probiotic strains and lactoferrin to prevent *rotavirus* infection in mice. Specifically, the study highlights the varying effectiveness of two bacterial strains. The probiotic *L. delbrueckii* subsp. *Lactis*, *G.stearothermophilus*, and lactoferrin demonstrate high efficacy in the experimental design (both in *vivo* and *silico* approaches). Furthermore, the study suggests potential mechanisms that explain the antiviral properties exhibited by *L. delbrueckii* subsp. *Lactis*, *G. stearothermophilus*, and lactoferrin. Therefore, future studies should focus on examining and testing bacteriocin proteins that contain more potent compounds for their in vivo antiviral activity.

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Authors' contributions
Conceptualization, T.I, K.G ; methodology T.I, N.E, S.T, M.S, A.H, K.D, M.A, A.E, K.G.; software, N.E, K.D, M.A, A.E, K.G; validation, T.I, N.E, S.T, M.S, A.H, K.D, M.A, A.E, K.G .; formal analysis T.I, N.E, S.T, M.S, K.G ; investigation, T.I, N.E, S.T, M.S, A.H, K.D, H.A, A.E, K.G data curation, T.I, N.E, S.T, M.S, A.H, K.D, H.A, A.E, K.G; writing—original draft preparation, T.I, N.E, S.T, M.S, A.H, K.G; writing—review and editing, T.I, N.E, S.T, M.S, A.H, K.D, H.A, A.E, K.G visualization, N.E, K.D, H.A, A.E, K.G; supervision, T.I, K.D, A.E, K.G. All authors have read and agreed to the published version of the manuscript.

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Data availability
The datasets generated and/or analyzed during the current study are available in the [NCBI Gen Bank]. The partial sequencing of Geobacillus stearothermophilus strain S1030, and Lactobacillus delbrueckii subsp. Lactis strain L1030

was deposited at NCBI Gen Bank under accession number PP758390 and PP758383 respectively.
https://ncbi.nlm.nih.gov/nuccore/PP758383.
https://www.ncbi.nlm.nih.gov/nuccore/PP758390.

Declarations

Ethics approval and consent to participate

The study was conducted in accordance with ethical standards and was approved by the [the research ethics committee for experimental and clinical studies at NODCAR]. The committee's reference number for this study is [NODCAR1/43/2022]. All participants provided informed consent before participating in the research.

Consent for publication

Not applicable

Competing interests

The authors declare no competing interests.

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