Original Article
Food Science and Technology

ISSN 0101-2061 (Print)
ISSN 1678-457X (Online)

DOI: https://doi.org/10.1590/fst.20322

In vitro and in vivo evaluation of probiotic as immunomodulatory and anti-*Campylobacter* agent

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Abstract

Human campylobacteriosis can be transmitted from animals to human either directly or through contaminated food. So, food safety plays a critical role in preventing the prevalence of food-borne diseases. Probiotics microorganisms are considered one of the most promising new strategies in controlling gastrointestinal tract (GIT) infection. In this study, two probiotic strains *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20080 and *Bifidobacterium bifidum* were investigated as anti-*Campylobacter* agent both in vitro and in vivo. Where they showed elevated antibacterial activity when used as mixed culture with a significant downregulation in the mice intestinal gene expression of IL-1β mRNA and TNF-α mRNA induced by *Campylobacter jejuni*. Besides, the histopathological investigation of mice intestinal tissue showed great morphological changes in the infected groups with *C. jejuni* compared with Probiotics treated group that showed healthy intestinal sections with well-defined and enhanced villi. Generally, our findings demonstrate that probiotics have a beneficial and significant effect in the reduction and treatment of destructive effect of *C. jejuni* on mice gut.

Keyword: probiotic; pro-inflammatory genes; *Campylobacter jejuni*; mice.

Practical Application: Protective effect of probiotic against infection of *C. jejuni* induced inflammation in gut of female BALB/c mice.

1 Introduction

Food safety is the key role in the reduction of incidence of food-borne diseases, since pathogenic bacteria may transmitted from animals to humans either directly or through contaminated food (Santini et al., 2010). *Campylobacter* spp. are widely distributed microorganisms, that live throughout the gastrointestinal tract (GIT) as commensals in many animal species, causing human food- borne diarrheal disease worldwide (Bratz et al., 2015) . There are more than 30 species and subspecies in this large and diverse category of Gram-negative bacteria. Since, it mainly transmitted from animal species like poultry where infection is asymptomatic to human in many developed countries (Śmiałek et al., 2021). Human campylobacteriosis had recorded noticeably increase in many country that over the number of *Salmonella* infection which considered as major public health concern (European Food Safety Authority, 2010). Mostly of campylobacteriosis come after handling and consumption of contaminated poultry meat so removal of *Campylobacter* in the poultry reservoir is a critical stage in controlling foodborne bacterial infection (Saint-Cyr et al., 2016). Human illness could be attributed to different species of *Campylobacter* such as C. *jejuni, C. upsaliensis.,, C. lari*, and *C. coli* but the most virulent species is *C. jejuni* which responsible for majority of zoonotic infections (Hugas et al., 2009). *Campylobacter* infection may cause variable symptoms such as abdominal pain, fever and bloody diarrhea (Jin et al., 2021). Furthermore, diarrhetic disease is about 10 times higher from *Campylobacter* infection than from other infection such as *Escherichia coli* O157: H, *Salmonella* species or *Shigella* species (Zilbauer et al., 2008).

In order to reduce the transmission of the pathogen through different processing stage not only good hygiene but also other approach should be applied to minimize the presence of *Campylobacter* in the farm (Gibbens et al., 2001). There are different possible ways may be used to reduce contamination of *Campylobacter* including reduction in environmental exposure or increasing host resistance or recently using antimicrobial alternatives that reduce colonized *Campylobacter* in the chicken (Saint-Cyr et al., 2016). For many years, treatment of *Campylobacter* infection involved antibiotics that become less effective over the time and may lead to the occurrence of antibiotic-resistant bacterial strains (Ipe et al., 2020). Wo´zniak and Wieliczko recorded an increase of enrofloxacin-resistant *Campylobacter* strains isolated percentage that from poultry in Poland from 52.1% in 1994 to 93.6% in 2008 (Woźniak & Wieliczko, 2011). Ongoing with this results tetracyclines resistance strains also observed by these authors. As well, at the dawn of the twenty-first century

Received 13 Feb., 2022
Accepted 22 Mar., 2022
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a multi-resistant strains of *Campylobacter* were emergence (Woźniak & Wieliczko, 2011). Accordingly, it becomes a crucial demand to search for other biological alternatives.

The prevention of *Campylobacter* spp. at the farm level could be achieved by different approach including vaccines, bacteriophage and feed additives which varying from medium-chain fatty acids (MCFA), compounds derived from plants, organic acids, bacteriocins probiotics and prebiotics (Micciche et al., 2019). In previous study comparison had made between 12 feed additives used as anti-*Campylobacter*, only three of them remain effective including different species of probiotic (Guyard-Nicodème et al., 2016).

Probiotics microorganisms could be yeast or bacteria that consist of one or mixed organisms mainly non-pathogenic, non-toxic and have a positive influence on the host's health when oral administration method take place (Vuong et al., 2016). It often referred to as beneficial bacteria which are commonly utilized included in species of *Lactobacillus, Bifidobacterium, Streptococcus, Escherichia coli, Saccharomyces,* and *Bacillus* (Helmy et al., 2017; Thibodeau et al., 2017; Massacci et al., 2019).

Probiotic was recorded for their anti-*Campylobacter* ability in numerous research paper (Śmiałek et al., 2021). Since it exerts their antimicrobial effects via different mechanisms including competing for nutrients and mucosal adhesion sites with pathogenic microorganisms (Papadimitriou et al., 2015), modulation of the immune system (Quinteiro-Filho et al., 2015) or by production of secondary metabolites such as volatile fatty acids and bacteriocins (Taha-Abdelaziz et al., 2019).

Hence in the current research, we explored the protective effect of probiotics on campylobacteriosis-induced inflammation in mice. In which we *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20080 and *Bifidobacterium bifidum* because they're already in a lot of commercial products

2 Materials and methods

2.1 In vitro studies

Bacterial strain and culture condition

Two strains of probiotic were used in this research, *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20080 and *Bifidobacterium bifidum* that provided by ATCC (Manassas, VA, USA). Both bacterial strains were cultivated on deMan Rogosa Sharpe medium (MRS, BTL, Łódź, Poland) at 37 °C for 48 h in 5% CO2. One *Campylobacter jejuni* ATCC 33291, was used as main pathogen to test the inhibitory effect of the probiotic strains.

Detection of antibacterial activity of probiotic strains – well diffusion method

The inhibitory effect of the two probiotic strains were tested by well-diffusion agar assay with slight modification according to (Santini et al., 2010). 24 h cultures of *Lactobacillus delbrueckii* and *Bifidobacterium bifidum* on MRS medium with an optical density OD of 0.5 measured at 600 nm (OD 600 = 0.5, in 0.9% NaCl). *Campylobacter jejuni* sub-cultured on Nutrient agar medium (Oxoid) plates and grown for 24 h at 37 °C. Using a McFarland reader a direct colony suspension method applied, McFarland standard (1.5×10^8 CFU/mL) in the saline tube was prepared, *Campylobacter jejuni* was spread on the Nutrient Agar (1.5% agar, Oxoid) plates using sterile swap. Three Wells each of 5 mm in diameter were made on each agar plate using a sterile metal cylinder then filled with 50 µl for each probiotic bacteria separately then with mixed culture of both probiotic strains. Then, the plates were incubated at 37 °C for 24 h and the inhibition zone was determined in terms of a millimeter. These tests were performed in triplicate.

2.2 In vivo studies

Starter cultures

Two probiotic strains *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20080 and *Bifidobacterium bifidum* have been provided by ATCC (Manassas, VA, USA). In order to get active and viable bacterial strains, the two probiotics were inoculated separately in de Man, Rogosa, Sharpe broth media (MRS) (Oxoid CM0359; Thermo Fisher Scientific, UK) then incubated for 24 h at 30 °C. The bacterial culture subjected to centrifugation for 10 min at 4000 rpm then the pellets were washed in phosphate buffer saline (PBS) twice. After all, the cells were resuspended in PBS and mixed with a ratio of 108 : 108 cfu/g just before oral mice feeding by gavage tubes.

Experimental design

The rats were given tap water and a well-balanced food ad libitum. All experiments were carried out in line with Directive 95/701/EEC of the European Community. The animal care protocols were in accordance with the ninth edition of the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and the Institutional Animal Ethics Committee for Laboratory Animal Care at Helwan University's Zoology Department, Faculty of Science, gave their approval (Approval number: HU2021/Z/MFE0521-01).

In order to conduct the in vivo experiment, female BALB/c mice (n = 28; 8 weeks old) were taken from VACSERA (Giza, Egypt). The mice were tested with 10^9 colony forming units (CFU) *Campylobacter jejuni* strain 81-176 using 0.3 mL sterile phosphate buffered saline (PBS) by gavage as described earlier (Bereswill et al., 2011).

Mice were placed into two groups four groups (n = 7). Group I: represented the normal (non-infected) control. Group II: infected untreated positive control group mice. Group III: (pretreatment groups) were administered 100 µL of probiotics solution by oral gavage two weeks before infection. Groups IV: infected mice with *C. jejuni* orally/day and treated at the same time with 100 µL of probiotics solution by oral gavage.

Two weeks post infection, Mice were massacred., and their intestine tissues were promptly removed. For histological investigation, intestinal tissue samples were preserved in 10% formalin at 80 °C until intestinal slices were processed., the samples were frozen at 80 °C without formalin before processing for molecular analysis.

Real Time PCR

An RNeasy Plus Mini kit (Qiagen,Valencia, CA, USA) was used to extract total RNA from the intestinal tissue samples. RevertAid H Minus Reverse Transcriptase was used to reverse transcribe RNA (Fermentas, Thermo Fisher Scientific Inc.,Waltham, MA, USA). The Applied Biosystems 7500 Instrument was used to perform real-time PCR experiments. With the help of power SYBR Green, (Life Technologies, Carlsbad, CA, USA) the relative gene expression was assessed & Pfaffi's comparative threshold cycle approach (2001). Jena generated the PCR primers for the TNF- and IL-1 genes.

Bioscience GmbH (Jena, Germany). The Primer-Blast program from NCBI was used to create primers. GAPDH was used to standardize the mRNA levels in each sample.

The primer sets used were as follows:

Name sense (50—30) antisense (50—30)

IL-1β GACTTCACCATGGAACCCGT GGAGACTGCCCATTCTCGAC

TNF-α AGAACTCAGCGAGGACACCAA GCTTGGTGGTTTGCTACGAC

GAPDH GCATCTTCTTGTGCAGTGCC GATGGTGATGGGTTTCCCGT

Histopathological investigation

Immediately after scarification, samples of the intestine out of each group were extracted and fixed in 10% neutral formalin for 24 h before being processed into paraffin blocks for light microscopy. Slices of 4-5 µm were taken from the ready blocks and subjected to stained with eosin and hematoxylin. To assess the pathological alterations, the prepared samples were examined under a Nikon microscope.

Analytical statistics

One-way analysis of variance (ANOVA) was used to examine data for multiple variable comparisons. For all statistical analyses in this study P-values are two-sided and $P < 0.05$ was significant. The results were presented as means ± standard error. Duncan's test was applied as a post hoc test to compare significance across groups, according to the statistical package software SPSS ver. 22. (Chicago, IL).

3 Results

3.1 In vitro studies

Antibacterial activity of probiotic strains – well diffusion method

A mixed culture of *Lactobacillus delbrueckii* and *Bifidobacterium bifidum* showed elevated inhibitory effect when applied to plates seeded with *Campylobacter jejuni* (5 ± 1 mm inhibition zone) in comparison to using each probiotic strain separately (2 ± 1 mm inhibition zone) as showed in (Figure 1)

3.2 In vivo studies

During inflammation caused by infection with *C. jejuni*., the nucleus has an important role in immunity related by pro-inflammatory genes that encodes IL-1β and TNF-α. The results exhibited a significant upregulation in the expression of IL-1βmRNA and TNF-α mRNA in the *C. jejuni*-infected groups (Figure 2), while the Probiotics-treated groups showed a significant downregulation in gene expression level compared to the control group.

Histopathological patterns showed in the gut (Figure 3): the results of examination showed that normal structure in the control non-infected group, while showed that changes

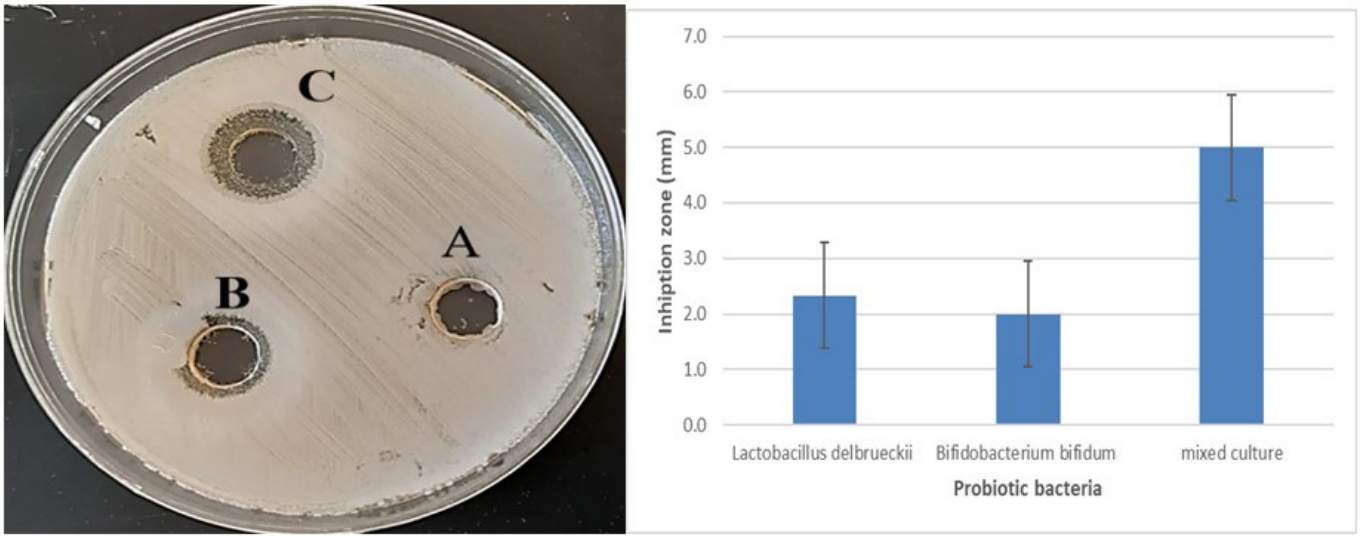

Figure 1. Well-diffusion agar assay, showing different inhibition zone of *Campylobacter jejuni* by probiotic strain. (A) *Bifidobacterium bifidum*, (B) *Lactobacillus delbrueckii*, and (C) mixed culture of both probiotic strains. Data presented are mean values from three replicates.

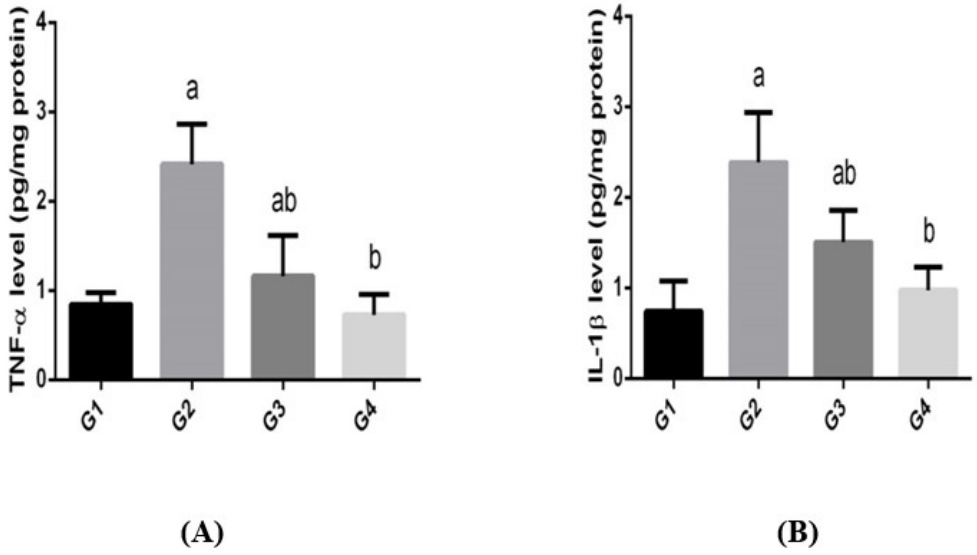

Figure 2. (A and B) The effect of probiotics on intestinal gene expression of *TNF-α* mRNA and IL-1β mRNA induced by *C. jejuni*. a: $p < 0.05$, significant change compared to negative group (G 1); b: $p < 0.05$, significant change compared to infected group (G 2), pretreatment groups were administered 100 µL of probiotics solution by oral gavage two weeks before infection (G3) and Infected mice with *C. jejuni* orally/day and treated at the same time with 100 µL of probiotics solution by oral gavage (G4). Values are means ± SEM (n = 7).

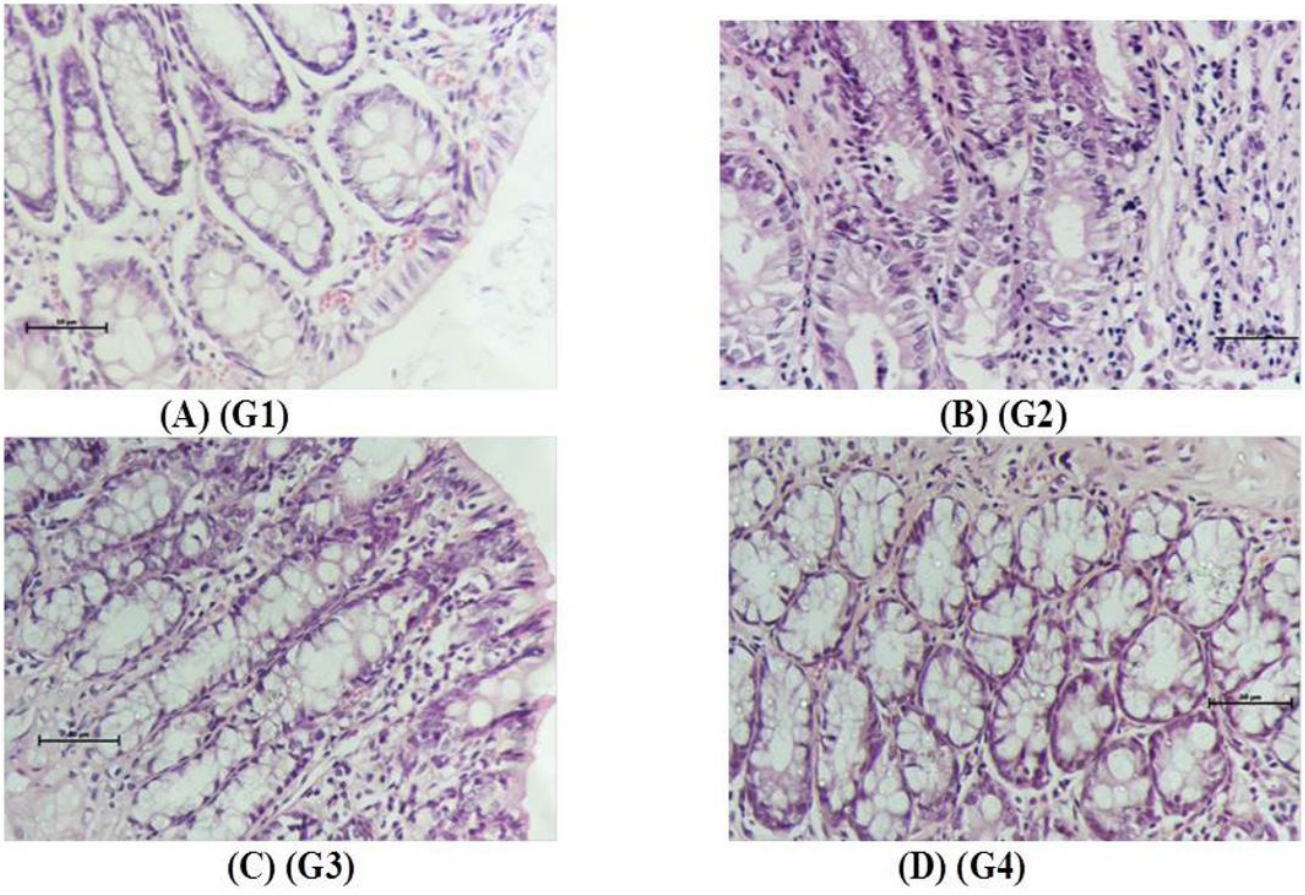

Figure 3. Photomicrograph of intestinal tissue stained with H&E stain (100×) showing (A) normal structure in the mucosa layer, submucosa, muscularis and lamina propria for control group (G1). (B) Infected group presenting a degeneration of the lamina propria and destructed villus (G2). (C) The protected group, given probiotics and after 1 week infected with *C. jejuni* and showing improved villus (G3). (D) Infected group and treated at the same time with probiotics and showing a little degeneration of lamina propria (G4).

in morphological structure of infected groups compared with treated groups.

The infected group exhibited degeneration of the lamina propria, destructed villi, and the nuclei of the columnar cells were fused and lost together to form membranous-like shape, whereas the infected group treated with Probiotics showed normal intestinal sections with enhanced villi and well-defined.

4 Discussion

For many enteric bacterial infections, the small intestine is considered a significant site of infection in both humans and animals (Garner et al., 2009). *Campylobacter* is one of the most common causes of bacterial gastroenteritis that has been recorded and produced gastrointestinal inflammatory problems that extended for a long time and may occur having a post-infection duration of weeks to months all over the worldwide (Genger et al., 2020). Pathogens are primarily spread through the food chain when undercooked meat is consumed or via contaminated surface (Backert et al., 2017).

However, *C. jejuni* induced immune cell responses in the large intestine that are proinflammatory by signaling dependent of lipooligosaccharide (LOS) from *C. jejuni* (Janssen et al., 2008; Nachamkin et al., 2008; Lane et al., 2010; Hermans et al., 2012; Taha-Abdelaziz et al., 2019)

Probiotics are a broad category of symbiotic microorganisms isolated from the intestine. When ingested, it improves one's health by modulating the gut flora. Bacteria that are widely used include *Bifidobacteria* and *Lactobacilli.* The obtained results showed elevated inhibitory effect when mixed culture of *Lactobacilli* and *Bifidobacteria* spp. against *C. jejuni* since these probiotic strains make a symbiotic association with the host body and they invade the mucus membrane located on the intestinal epithelial cells and by releasing bactericidal chemicals (e.g. bacteriocins [biological toxins of proteins formed by bacteria to prevent the growing of closely related pathogenic bacterial strain(s)], antibiotics, hydrogen peroxide, and free fatty acids) they prevent the growth and adhesion of pathogenic microorganisms (Gagnon et al., 2011; Aliakbarpour et al., 2012; Papadimitriou et al., 2015). Additionally, they elevated aggregation and adhesion capability enables their establishment in the GIT and the elimination of pathogenic bacteria (Deng et al., 2020). In agreement with our finding, the repressing activity of six *Lactobacilli* spp. against *C. jejuni* in vitro from both the *Lactobacilli* spp. cell cultured and the neutralized cell-free supernatant inhibited *C. jejuni* growth with formation of clear inhibition zones on Mueller-Hinton (MH) agar. Furthermore, other investigation mentioned that exposure of *C. jejuni* to most of *Lactobacilli* spp. undergo downregulation of genes responsible for invasion and motility (Mortada et al., 2020). Therefore, it was growing interest to reduce the harmful effect of different food born pathogen with studying potential of probiotics as possible antibacterial agents with low biological hazards (European Food Safety Authority, 2015).

Furthermore, other study demonstrates that different probiotic bacterial strains have anti-inflammatory ability by downregulation of lipopolysaccharide (LPS) production that initiate pro-inflammatory pathways. As an example, different strains of *Lactobacillus reuteri* were found to reduce proinflammatory cytokine release, including IL-6 and TNF-α, in necrotizing enterocolitis (NEC)-affected neonatal rats, that was enhanced with probiotics TLR-4 inhibition signaling through the pathway of nuclear factor-kappa B (NF-kB) (Brisbin et al., 2008; Hemarajata & Versalovic, 2013; Thomrongsuwannakij et al., 2016).

Several in vitro and in vivo studies have demonstrated the effectiveness of probiotics in the prevention and treatment of enteric illnesses. Probiotic microorganisms such as *Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacill casei, Lactobacillus gasseri,* and *Bifdobacterium lactis*, for example, they have already been found to prevent growth, metabolism and attachment of gastrointestinal pathogens including *C. jejuni, Shigella, Salmonella, Vibrio cholerae* and enterotoxigenic *Escherichia coli* (Perdigon et al., 1995; Silva et al., 2012).

ROS formation play a key role in triggering the inflammatory response and their toxicity multiplies during several normal processes in tissues and cells, which is symptomatic of the pathogenesis of various diseases, including *C. jejuni*. (Lee et al., 2012; Heimesaat et al., 2019).

Nuclear factor-κB has critical implications of immunity, since it triggers the expression of pro-inflammatory genes encoding COX-2, iNOS, IL-1β, TNF-α, and IL-6. That is stimulated by ubiquitination, phosphorylation, and subsequent proteolytic degradation of the IκB protein by activated IκBkinase (IKK). The released NF-κB translocate and bind to the nucleus as a transcription factor to κB motifs promoters of the target genes, resulting in its transcription. Abnormal NF-κB activity is associated with several inflammatory disorders, mostly anti-inflammatory drugs that stop expression of inflammatory cytokine by preventing the NF-κB pathway. Therefore, an NF-κB suppressor has clinical prospective in inhibition of inflammatory disorders (Gagnon et al., 2011; Aliakbarpour et al., 2012; Papadimitriou et al., 2015).

The outcome of the histological analysis revealed that the infected group with *C. jejuni* had morphological alterations. In which, intestinal cells were severely damaged and disintegrated as a result of the treatment., that were in agreement with numerous authors (Lane et al., 2010; Hermans et al., 2012; Taha-Abdelaziz et al., 2019).

Pro-inflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) are released during inflammation that is crucial for host-defense responses to infection and injury. Inflammation has a vital aspect in the protection of the host., that includes a variety of responses to external stimuli such as pathogen infection, chemical exposure or bacterial endotoxin poisoning (Lane et al., 2010; Taha-Abdelaziz et al., 2019).

In our study, we evaluated the effect of probiotics, as anti-inflammatory prevented by signaling shift of proinflammatory gene expression. On the other hand, there was a substantial upregulation in the IL-1β and TNF-α gene expression tiers resulted from *C. jejuni* infection in comparison to the control group, these results in agreement with other studies have suggested that the phytochemical in probiotics lead to significant decrease in the level of gene expression, that is capable of lowering the levels of inflammatory proteins (Taha-Abdelaziz et al., 2019).

5 Conclusions

In conclusion, treatment with probiotics against *C. jejuni* infection was significantly effective in preventing the development of gut toxicity through improving the histological features of the gut and by down regulating proinflammatory cytokines genes.

Conflicts of interest

The authors declare no conflicts of interest.

Availability of data

The data used to support the findings of this study are included within the article.

Author contributions

M.F.E. and S.M.K. contributed to study design. W.A.A. and H.M.Y. contributed to data acquisition. M.A.A., H.A., H.S., and A.F.A. organized the database, performed the statistical analysis. All authors revised, improved, read, and approved the final manuscript. All authors have read and agreed to the published version of the manuscript.

Acknowledgements

Princess Nourah bint Abdulrahman University Researchers Supporting Project number (PNURSP2022R214), Princess Nourah bint Abdulrahman University, Riyadh, Saudi Arabia and also was supported by Researchers Supporting Project number (RSP-2021/97), King Saud University, Riyadh, Saudi Arabia.

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