Title page

Therapeutic Potential of Probiotics to counter *Campylobacter* Infections: An *In vitro* and *In vivo* Evaluation in Mice

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Abstract

Probiotics, including Lactic Acid Bacteria (LAB), have gained considerable attention due to their potential health benefits for humans and animal livestock. This research aimed to isolate probiotic bacteria of human origin and explore their therapeutic potential against *Campylobacter* strains, which are recognized as the leading bacterial cause of human gastroenteritis worldwide. A collection of 230 LAB isolates from human stools were screened for the ability of cell free supernatants to inhibit the growth of three strains of *C. jejuni* and a *C. coli*. Co-culture and agar-well diffusion assays identified seven isolates with inhibitory activity against *Campylobacter* ssp., which were characterized as probiotic strains by their tolerance to pH 2 and bile salts, and then identified as *Enterococcus* spp. using 16S rRNA gene sequencing:: *E. faecalis* NM231, *E. faecium* NM234, *E. faecium* NM233, *E. faecium* NM113, *E. durans* NM232, *E. faecalis* NM235, and *E. faecium* NM236. In vivo evaluation was undertaken by the oral administration of selected probiotics to groups of mice prior to challenge with *C. jejuni*. Histopathological examinations showed challenge groups receiving either mixed probiotics or *E. faecium* NM234 were protected against *C. jejuni*-induced jejunal, colonic, ovarian and uterine tissue damage. Immunohistochemical detection of the challenged mice revealed the presence of *C. jejuni* antigen in jejunal and colonic sections but was absent in mice receiving either mixed probiotics or *E. faecium* NM234. This study introduces *E. faecium* NM234 as a promising probiotic against *Campylobacter*.

Keywords: Probiotics, *Campylobacter*, foodborne, immunohistochemistry, zoonotic diseases.

1. Introduction

Probiotics have emerged as a compelling area of scientific research due to their significant contributions to human and animal health. These beneficial microorganisms, found in various niches, play a pivotal role in countering pathogenic threats and promoting well-being. The gastrointestinal tract (GIT) and salivary glands serve as natural reservoirs for several probiotic organisms, underlining their potential to bolster our defenses against infections arising from foodborne pathogens (Terai et al., 2015; Pruthviraj et al., 2023). Among the plethora of probiotic candidates, the *Enterococcus* genus constitutes vital members of the Lactic Acid Bacteria (LAB) group that have garnered attention for their

therapeutic properties (Franz et al., 2011; Krawczyk et al., 2021). Various enterococcal species; including *E. faecalis, E. faecium, E. lactis, E. hirae* (Adnan et al., 2017), and *E. durans* (Li et al., 2018) have been utilized as probiotics in both human and animal applications. Notably, enterococci possess the ability to thrive in challenging environments including high salt content, and temperature fluctuations, which together with the ability to synthesize robust proteolytic and lipolytic enzymes, render them of increasing importance to the food industry (Haghshenas et al., 2017).

Enterococci are gram-positive, catalase-negative, non-spore-forming bacteria commonly found in various fermented foods (Nami et al., 2017). *E. faecalis* and *E. faecium*, the most prevalent species among enterococci, have demonstrated a range of therapeutic properties, including their ability to combat pathogenic organisms and contribute to the reduction of cholesterol levels (Chandel et al., 2019). Moreover, enterococci have shown anti-carcinogenic effects by inducing apoptosis and downregulating cell proliferation genes in cancer cell lines (Nami et al., 2022). Enterococci have also been shown to activate human immune cells in vitro by releasing interleukin 12, a key factor in the generation of T helper 1 responses (Mansour et al., 2014). The mechanisms through which enterococci act include: the secretion of antimicrobial substances, competitive exclusion of pathogenic bacteria for nutritional sources and adhesion sites, functional enhancement of the intestinal barrier, and immunomodulation (Iqbal et al., 2021).

According to the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC), *Campylobacter* are recognized as the leading bacterial cause of human gastroenteritis worldwide, ranking among the top four global contributors to diarrheal diseases (Kirk et al., 2015). While *Campylobacter* infections are typically mild, they can pose a significant threat to vulnerable populations, including young children, the elderly, and individuals with weakened immune systems. *Campylobacter* species exhibit distinctive spiral, "S"-shaped, or curved rod-like structures. They are ubiquitously present in most warm-blooded animals. The species most commonly associated with human diseases are *Campylobacter jejuni* and *C. coli* (Kaakoush et al., 2015). *Campylobacter* transmission can occur through the consumption of or direct contact with raw or undercooked poultry, seafood, and meat that has been contaminated with the bacterium (Whiley et al., 2013).

C. jejuni is a gram-negative zoonotic pathogen capable of causing mild to severe disease in humans and colonizing the intestinal tracts of birds and other animals asymptomatically (Burnham and Hendrixson 2018; Tegtmeyer et al., 2021). Over the past decade, *C. jejuni* has emerged as a major cause of gastroenteritis worldwide (Kaakoush et al., 2015).

Campylobacter infections in sheep can result in abortion and economic losses in infected herds. In the USA, for instance, ovine abortion attributed to *C. jejuni* has increased significantly since the 1980s, to become a leading cause of ovine abortion (Kreuder et al., 2019). The term "Campylobacter" refers to its primary morphological characteristic as a curved rod but is frequently observed with spiral morphology and can adopt various morphological forms under adverse environmental conditions, including a helical filamented rod and/or coccoid forms. These morphological changes significantly impact its biological and pathogenic properties, altering its transmission, colonization, and interactions with its host (Frirdich and Gaynor, 2013; Frirdich et al., 2019). The cytoplasmic membrane of *C. jejuni* is surrounded by peptidoglycan (PG), forming a rigid layer that maintains its shape and protects the cell from lysis, playing a vital role in its macromolecular structure (Vollmer et al., 2008).

C. jejuni possesses a group of proteases and peptidases that are essential in cellular processes. These enzymes are involved in protein quality control, protein transport across inner and outer membranes into the periplasm, and interactions with the host cell, including the cleavage of crucial host cell factors such as epithelial cell junction proteins, contributing to intestinal tissue damage (Linz et al., 2023).

In light of these attributes, this research endeavors to further explore the therapeutic potential of probiotic isolates. Through a series of experimental investigations, we aimed to assess the ability of LAB isolates to counteract the pathogenic bacterium *Campylobacter* sp. and evaluate their effectiveness in protecting against infections in various anatomical tissues in a mouse model, including the jejunum, colon, and reproductive system. In addition, we sought to elucidate the mechanisms through these probiotics contribute to the inhibition of *C. jejuni* and their potential as agents in bolstering defense mechanisms against foodborne pathogens.

2. Material and Methods

2.1.Microorganisms and growth conditions

LAB isolates were cultivated in de Man, Rogosa & Sharpe (MRS) broth and agar (Laboratories Conda S.A., Madrid, Spain) at 37°C under anaerobic conditions using an anaerobic jar and AnaeroGen (Oxoid Basingstoke, UK). The *Campylobacter* strains *C. jejuni* OR1, *C. coli* OR12 (El-Shibiny et al., 2005), *C jejuni* 81116 (Pearson et al., 2007), and *C. jejuni* 12661 (Sacher et al., 2018) were cultured in Mueller Hinton (MH) broth and agar as well as *Campylobacter* Blood-Free Selective Medium (CCDA) (Oxoid GmbH, Wesel,

Germany) under microaerobic conditions at 35°C for 48 hours, following the protocol established by Jeong et al. (2014).

2.2.Isolation of LAB from human fecal samples

Fecal samples, each weighing 1 gram, were collected from a diverse group of healthy individuals under the age of 40. These samples were suspended in 5 ml of phosphate-buffered saline (PBS) with a pH of 7.0 and subsequently stored at -40°C for preservation. To obtain viable LAB cultures, serial dilutions of each fecal sample, extending up to 10^{-7}, were used to inoculate MRS broth supplemented with 0.05% w/v L-cysteine (Loba Chemie, Mumbai, India) and then anaerobically incubated at 37°C within an anaerobic jar supplemented with AnaeroGen (Oxoid Basingstoke, UK) for 48 h. The cultures were plated onto MRS agar (Laboratories Conda S.A., Madrid, Spain) containing 0.05% w/v L-cysteine. Following 48 hours of incubation, individual colonies were selected from the agar plates. These colonies underwent Gram staining and a catalase test for initial characterization. Subsequently, the selected LAB isolates were preserved in 40% glycerol at -40°C, in accordance with the protocol established by Rubio et al. (2014).

2.3.Screening of fecal isolates for Campylobacter spp. inhibition by their cell-free supernatants

To assess the inhibitory potential of the fecal isolates against *Campylobacter* spp., cell free supernatants were prepared from the LAB isolates as well as from *Campylobacter* strains. Primarily, the bacterial isolates and strains were cultured overnight in broth; the cells were harvested and suspended in PBS until they reached an optical density (O.D.) of 0.22 at a wavelength of 600 nm. Afterward, this suspension was inoculated into fresh broth followed by incubation at 37°C for 48 h. The culture experienced centrifugation at 4000 rpm for 10 minutes and the supernatants were filtered through 0.22-μm membrane filters (Minisart polyethersulfone, Sartorius, Göttingen, Germany) and neutralized to pH of 7.0 (Mansour et al., 2018). A 400 μL aliquot of the *Campylobacter* suspension from each strain was added to 10 mL broth with 2 mL of each isolate supernatant individually or 2 mL MRS broth at pH 7.0 and pH 3.9 for controls with and without pH neutralization, respectively. These were incubated at 37 °C for 0 and 48 h, and the growth was measured at 600 nm on each time interval against a blank (5.5 mL MH / 1 mL MRS). The O.D600 in the control samples after 48 h of incubation was defined as 100% growth. Differences in O.D600 measurements at 0 and 48 h of incubation of the test samples were calculated as percentages of the value

obtained from the control. The isolates which exhibited growth inhibition percentage as 50% or more were picked. Each test was performed in triplicate.

2.4.Co-culture of isolates with Campylobacter spp.

The co-culture protocol as described by Folkerts et al. (2010) was used to evaluate the inhibitory potential of selected isolates against *Campylobacter* strains, whereby a fresh culture of the selected isolate was diluted 10-fold with MRS broth and 600 µL from each were placed in combination with 200 µL of *Campylobacter* culture (diluted 1:100). The co-cultures were incubated anaerobically at 37 °C for 48 h. Serial dilutions were spread on MRS (anaerobic) and CCDA agar plates (microaerobic), to determine the total count on each medium.

2.5.Agar well diffusion assay

The potential of the selected isolates to inhibit *Campylobacter* growth were assessed using the agar well diffusion assay method as described by Jeong et al. (2014). In this assay, 100 µL of inoculum containing 10^8 colony-forming units (CFU)/ml of each *Campylobacter* bacterium were evenly spread on Mueller Hinton Agar (MHA) plates. Subsequently, wells were created in each inoculated plate and filled with 30 µL of neutralized cell-free supernatant (pH 7) obtained from the isolates following 24 hours of growth in MRS broth. As a negative control, MRS broth alone was employed. All MHA plates were then incubated at 37ºC for 24 hours and subsequently assessed for the presence of inhibition zones exceeding 1 mm in diameter. Each test was conducted in triplicate to ensure the reliability of the results.

2.6.In vitro assessment of the competitive inhibition of adhesion of C. jejuni OR1 by probiotic isolates

To evaluate the potential of probiotic strains to competitively inhibit the adhesion of *C. jejuni* OR1 in an in vitro setting, HT29 cells, serving as a representative system that mimics the intestinal epithelium, were employed as per the methodology outlined by Candela et al. (2008). Briefly, probiotic strains and *C. jejuni* OR1 were independently cultured in liquid media overnight at 37 °C. The bacterial cell concentration was adjusted to 10^8 colony-forming units (CFU) per mL. Then, HT29 cells were seeded on glass coverslips within 24-well plates and incubated at 37 °C with 5% carbon dioxide for 24 hours to establish a monolayer. Each well received 1 mL of either a probiotic strain or saline solution (as a control), followed by a 2-hour incubation period. Post-incubation, the probiotic strain or

saline solution was aspirated, and the HT29 cells were washed with PBS. Subsequently, 1 mL of *C. jejuni* OR1 was introduced into each well, and the cells were incubated for an additional 2 hours. Following this incubation, *C. jejuni* OR1 was removed, and the HT29 cells were washed with PBS and fixed with methanol. The cells were then stained with Giemsa stain. Observation and enumeration of *C. jejuni* OR1 cells that adhered to the HT29 cells were performed.

2.7. Determination of the probiotic characteristics

Acid tolerance assay

To assess acid tolerance, cells from overnight cultures were subjected to a controlled acidic environment. Specifically, the cells were incubated at 37°C in 10 mL of pH 2.0 PBS for duration of 3 hours. Following this acid challenge, the cells were promptly collected and resuspended in PBS adjusted to pH 7.0 to neutralize the acidic conditions. Subsequently, serial dilutions (10-fold) of the cell suspensions were prepared and plated onto MRS agar plates. These plates were then incubated at 37°C for a period of 48 hours to facilitate colony growth. The viable count was determined based on the number of colonies formed on the MRS plates. To quantify the survival rate, the results were compared to the initial bacterial count before exposure to the acidic environment, as outlined by Kusada et al. (2021).

Bile Salt Tolerance

To assess bile salt tolerance, we followed the method outlined by Gilliland et al. (1975). The assay involved subjecting bacterial cells to varying concentrations of oxgall bile (Sigma-Aldrich, St. Louis, MO, USA) in MRS broths for duration of 4 hours. Specifically, three concentrations of oxgall bile were used: 0.3%, 0.7%, and 2% w/v. Following the incubation period, the treated bacterial cells were plated on MRS agar plates and then incubated at 37°C for 24 hours to facilitate colony formation. Viable counts were performed at three time points: 0, 3, and 24 hours after incubation. Survival rates were calculated by comparing the viable counts obtained at these time points to those of a control group that did not undergo exposure to oxgall bile. This assay was conducted in triplicate to ensure the reliability of the results.

Susceptibility to antibiotics

The susceptibility of the isolates to antibiotics was determined using the disc diffusion method as outlined by Patel et al. (2011). Nine different antibiotics, namely ampicillin,

amoxicillin, chloramphenicol, kanamycin, tetracycline, ciprofloxacin, gentamycin, vancomycin, and erythromycin, were tested. The antibiotic discs were obtained from bioMérieux (Lyon, France). The diameters of the inhibition zones around the antibiotic discs were measured in millimeters (mm) and compared to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI). Based on these comparisons, the isolates were categorized as resistant, intermediate, or susceptible to each antibiotic.

Assessment of hemolysin activity

The assessment of hemolysin activity, which is the ability of some bacteria to lyse red blood cells and cause hemolysis, was conducted on Columbia blood agar (Oxoid) supplemented with 5% v/v human blood. The plates were incubated at 37ºC for 48 hours and the bacteria were observed for the presence of clearance zones around the colonies, which indicate the degree of hemolysis (Bazireh et al., 2020). Thus, green zones around the colonies were estimated as α-hemolytic, clear zones around the colonies were estimated as ß-hemolytic, and no-zones around the colonies were interpreted as ϒ-hemolytic.

2.8.Genomic DNA extraction and PCR

Genomic DNA extraction was performed using the AxyPrep bacterial genomic DNA miniprep kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Primers; FD1 '5CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3' and RD1 '5CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3' (Weisburg et al., 1991) were used for amplification the 16S rRNA gene. The primers were obtained from Bioserve Company (Cairo, Egypt). PCR was conducted using a T100 thermal cycler system (Bio Rad, Hercules, CA, USA) with PCR Master Mix from Fermentas (Life Sciences, Vilnius, Lithuania). PCR amplifications were analyzed by electrophoresis. The purification of the PCR products was carried out using a QIAquick PCR purification kit (Qiagen, Hilden, Germany).

2.9.DNA sequencing and analysis

Sequencing was performed utilizing the dideoxy chain termination method as described by Sanger et al. (1977). A comprehensive analysis was then undertaken to unravel sequence similarities and gain insights into genetic relationships. The BLAST search tool, available through the National Centre of Biotechnology Information (NCBI) (Altschul et al.,

1990) was employed to explore sequence homologies and make comparisons against a diverse genetic database. Subsequently, the obtained sequences were aligned using the CLUSTALW interface, and a phylogenetic tree was constructed using the MEGA7.0 software tool, accessible at http://www.megasoftware.net/.

2.10. Nucleotide sequence accession numbers

The amplified sequences of the 16S rRNA gene have been deposited in the GenBank database under accession numbers: OQ860807 *E. faecalis*-NM231, OQ860810 *E. faecium*-NM234, OQ860809 *E. faecium*-NM233, KC878684.1 *E. faecium*-NM113, OQ860808 *E. durans*-NM232, OQ860811 *E. faecalis*-NM235 and OQ860812 *E. faecium*-NM236.

2.11. In vivo evaluation of the probiotic isolates

Experimental animals

A total of 42 female Swiss albino mice, 12 weeks of age, were obtained from the National Research Centre (NRC), Egypt and housed at the NRC's animal facility. The mice were carefully maintained under controlled environmental conditions, including a temperature range of 18-20°C, 20% humidity, and a 12-hour light/12-hour dark cycle. All animal experiments adhered to the ethical guidelines of the NRC (Egypt) and received approval from its ethical committee. At the conclusion of the one-month experiment, the mice were humanely euthanized via decapitation under anesthesia, administered by a trained individual using thiopental sodium (50 mg/kg), as previously demonstrated (Atef et al., 2018; Marquardt et al., 2018) following the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition.

Administration of probiotic isolates and challenge with C. jejuni OR1

For the administration of probiotic isolates and the subsequent challenge with *C. jejuni* OR1, the 42 mice were divided into seven groups (n=6), each receiving specific treatments as detailed in Table 1. A consistent dose of 10^9 colony-forming units (CFU) in 200 µL of PBS was orally administered for both probiotic and *C. jejuni* OR1. The groups included: 1) Control (A), which received only 200 µL of PBS buffer; 2) Control (B), serving as the positive control group for probiotics, received a mixture of seven probiotic strains *(E. faecalis* NM231, *E. faecium*-NM234, *E. faecium*-NM233, *E. faecium*-NM113, *E. durans*-NM232, *E. faecalis*-NM235, *E. faecium*-NM236) without any challenge; 3) Mice treated with the same mixture of seven probiotic strains and challenged with *C. jejuni* OR1 at the specified dose; 4) Mice

treated with the probiotic strain *E. faecium*-NM234, while 5) another group received *E. faecalis*-NM235, 6) a separate group was treated with E. *faecium*-NM236. All three groups 4, 5 and 6 were subsequently challenged with *C. jejuni* OR1at the specified dose; and 7) a challenge control group which solely received *C. jejuni* OR1 orally at the specified dose.

Table 1

Experimental mice groups with treatments including probiotic and *C. jejuni* OR1 challenge

Group	Administration	Challenge
1- Negative control (a)	PBS buffer	NA
2- Probiotic control (b)	Mix Probiotics (NM231, NM234, NM233, NM113, NM232, NM235, NM236)	NA
3-	Mix Probiotics (NM231, NM234, NM233, NM113, NM232, NM235, NM236)	*C. jejuni* OR1
4-	Probiotic strain NM234	*C. jejuni* OR1
5-	Probiotic Strain NM235	*C. jejuni* OR1
6-	Probiotic Strain NM236	*C. jejuni* OR1
7- Challenge control	PBS buffer	*C. jejuni* OR1

NA: not applicable.

Histopathology protocol

Following the humane sacrifice of the experimental mice, a postmortem-examination was conducted, and tissue specimens were collected from the jejunum, colon, ovaries, and uterus. These tissue specimens were initially fixed in 10% neutral buffered formalin (NBF) overnight and subsequently transferred to 70% ethanol. This step was undertaken to preserve the epitopic structural features of the *C. jejuni* antigenic protein, safeguarding it from the effects of formalin fixation. Standard tissue processing procedures, including dehydration, embedding in paraffin wax, and sectioning at 3-5 µm thickness, were meticulously carried out. The resulting paraffinized tissue blocks were prepared for subsequent histopathological and immunohistochemical examinations. Tissue slides were stained with hematoxylin and

eosin (H & E) for histopathological analysis, according to the method described by Suvarna et al. (2012).

Immunohistochemistry protocol (IHC)

The previously prepared paraffinized tissue blocks obtained from the jejunum, colon, ovary, and uterine tissue specimens, were sectioned at a thickness of 3 µm and mounted on positively charged slides. The antigen retrieval process for the *C. jejuni* antigen was initiated by immersing the slides in a 10% sodium citrate buffer solution, followed by autoclaving at 20-25 PSI pressure (Pounds per Square Inch) for a duration of 5 minutes. Subsequently, the tissue slides were incubated with a rat anti-*C. jejuni* IgG3 monoclonal antibody, which was preserved in a solution containing 0.02% sodium azide and diluted in PBS buffer with 4% bovine serum albumin (BSA) and 0.05% Tween at pH 7.4. The antibody was used at a dilution rate of 1:10, as recommended by the manufacturer. The specific antibody employed in this experiment was procured from Abcam Co, UK, with the serial number ab8063. For the immunohistochemical staining process, the CRFTM anti-polyvalent HRP Polymer (DAB) detection kit was utilized, and it was imported from Scy Tek Laboratories, USA. This kit is characterized by species specificity against mice, rats, and rabbits. The experimental design included negative, positive, and challenge control groups. After the immunohistochemical procedure, the tissue slides were counterstained with hematoxylin and subsequently examined under a light microscope. Positive findings were indicated by varying degrees of coloration, ranging from light brown to golden brown or deep brown, which corresponded to the antigenic intensity observed in the examined tissues, as compared to the three control groups slides (Kim et al., 2016).

3. Results

3.1.Isolation of lactic acid bacteria from stool samples

The isolation of colonies from the stool samples has been processed as described in the methods section, the gram positive and catalase negative colonies were picked and inspected for their tolerance to pH 2.0, bile salts up to 3%, and resilience to pancreatic enzymes. Thus, we identified 230 isolates to screen against the *Campylobacter* strains.

3.2.Screening the isolates against Campylobacter strains in vitro

The supernatants of the 230 isolates were subjected to initial screening against four *Campylobacter* strains: *C. jejuni*-OR1, *C. coli*-OR12, *C. jejuni*-81116, and *C. jejuni*-12661. Among these isolates, 15 were identified that displayed more than 50% inhibition with neutralized and non-neutralized supernatants, as determined by the quantification of *Campylobacter* viable counts.

3.3.Co-culture of the selected isolates with Campylobacter spp.

When the co-culture method was applied using the selected fifteen isolates, the results revealed the growth reduction of the four *Campylobacter* strains (Table 2). Viable counts were determined after the incubation for 48 h at 37 °C of the co-cultures on MRS agar anaerobically and CCDA agar microaerobically to support the growth of LAB and *Campylobacter* respectively. The results revealed obvious reduction in *Campylobacter* strain growth, where the *Campylobacter* strains showed counts of 1.7–3.3 Log 10 CFU/mL from the co-cultures compared to their growth alone recorded at 7.00 Log 10 CFU/mL. The viable count of fifteen LAB isolates did not change upon the co-culture with *Campylobacter* strains compared to their controls.

3.4.Agar well diffusion

To further confirm their inhibitory capacity, the 15 isolates were subsequently subjected to agar well diffusion test (Table 3) against all four strains of *Campylobacter* spp. This assay allowed for the detection of inhibition zones, providing additional evidence of their inhibitory potential. Consequently, seven isolates exhibiting substantial inhibitory effects were selected for further investigation.

Table 2

Log 10 CFU/mL enumerated on MRS and CCDA agar plates after co-culture with the fifteen isolates with *Campylobacter* strains

Isolate Code no.	Log 10 CFU/ml on agar plates after co-culture							
	***C. jejuni* OR1**		***C. coli* OR12**		***C. jejuni* 81116**		***C. jejuni* 12661**	
	MRS	**CCDA**	**MRS**	**CCDA**	**MRS**	**CCDA**	**MRS**	**CCDA**
1	9.30	2.08	9.18	2.08	9.18	2.08	9.18	2.30
10	9.18	3.30	9.18	2.08	9.18	2.08	9.18	3.00

11	9.00	3.25	9.18	2.08	9.18	2.08	9.18	2.30
23	9.30	2.00	9.11	1.03	9.18	2.00	9.18	1.84
24	9.08	1.70	9.18	2.08	9.18	2.08	9.18	2.08
26	8.30	3.08	9.18	2.08	9.18	2.08	9.18	2.08
32	9.30	1.84	9.18	2.08	9.18	2.08	9.18	2.08
34	9.30	1.95	9.18	2.08	9.18	2.08	9.18	2.08
38	8.00	2.60	9.18	2.08	9.18	2.08	9.18	2.84
40	8.18	2.30	9.18	2.08	9.18	2.08	9.18	2.95
52	9.18	2.08	9.18	2.08	9.18	2.08	9.18	2.15
53	9.18	2.08	9.18	2.08	9.18	2.08	9.18	2.15
67	9.08	2.00	9.18	2.08	9.18	2.08	9.11	2.11
84	9.00	2.08	9.00	2.08	9.00	2.08	8.00	3.04
91	9.00	2.00	9.00	2.08	9.00	2.00	8.30	3.00

Control *C. jejuni* OR1 in axenic culture produced a viable count of 7.00 Log 10 CFU/mL.

Table 3

Agar diffusion diameter of inhibition zone for the probiotic isolates against *Campylobacter* strains

Species determination & name	**Isolate Code no.**	**Inhibition zone diameter (mm)**			
		***C. jejuni* OR1**	***C. coli* OR12**	***C. jejuni* 81116**	***C. jejuni* 12661**
***E. faecium* NM113**	**32 (1)**	**25±1**	**10±1**	**8±1**	**13±1**
***E. faecalis* NM231**	**1 (2)**	**21±0**	**13±1**	**6±1**	**12±1**
***E. durans* NM232**	**34 (3)**	**24±0**	**10±1**	**10±1**	**12±1**
***E. faecium* NM233**	**24 (4)**	**23±1**	**8.5±1**	**10±1**	**10±0**
***E. faecium* NM234**	**23 (5)**	**28±1**	**12±1**	**12±1**	**9±1**
***E. faecalis* NM235**	**67 (6)**	**23±1**	**8±1**	**11±1**	**10±0**
***E. faecium* NM236**	**91(7)**	**23±1**	**12±0**	**6±1**	**12±1**
	38 (8)	21±1	14±1	10±1	15±1
	52 (9)	20±1	14±1	17±1	10±0
	10 (10)	20±1	12±0	10±1	8.2±1
	26 (11)	21±0	0	10±1	13±0
	53 (12)	20±0	8±0	5±1	3±0
	40 (13)	21±1	0	5±1	19±1
	84 (14)	20±1	11±1	10±1	0
	11 (15)	20±0	0	5±1	10±1

In the absence of the probiotic the campylobacters show no inhibition zone.

Values represent averages ± standard deviations of triplicate experiments.

3.5.Adhesion assays against C. jejuni-OR1

We investigated the *in vitro* capacity of the seven candidate probiotic isolates (1, 23, 24, 32, 34, 67, and 91) to competitively inhibit the adhesion of *C. jejuni*-OR1 to HT29 colorectal cells in culture. Figure 1 illustrates the ability of these seven isolates to hinder the adhesion of *C. jejuni*-OR1 to HT29 cells. Notably, isolate number 23 exhibited the highest inhibition rate, while isolate number 34 displayed the lowest inhibition rate among isolates tested.

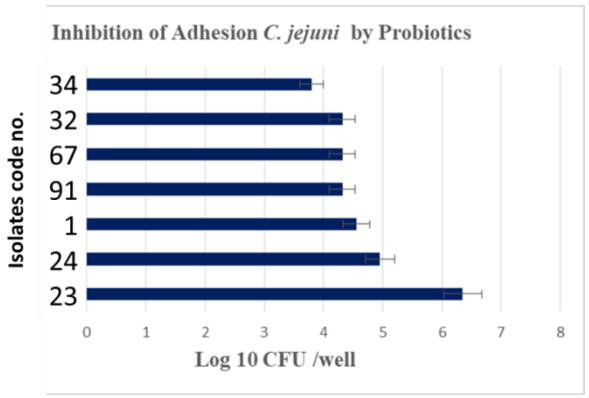

Fig. 1. Inhibition of *C. jejuni*-OR1 adhesion to HT29 cells by selected probiotic isolates

3.6.Evaluation of probiotic characteristics

The seven isolates underwent a comprehensive evaluation of their probiotic characteristics, encompassing tolerance to acid and bile salts, antibiotic resistance, and hemolytic activity. In terms of acid tolerance, the seven isolates exhibited a robust to survive under low pH conditions, with survival rates ranging from 88.5% to 95.5% when exposed to pH 2.0 for 3 h (Table 4). The isolates displayed noteworthy resilience to bile salts over 3 h incubation with survival rates ranging from 98.5% to 80.5% at concentrations of 0.3% to 1% (Table 4). Furthermore, the susceptibility of the isolates to ten antibiotics, as outlined in the Methods section, was assessed using the agar diffusion method. The results presented in Table (5) reveal that all seven isolates demonstrated different patterns of susceptibility, but all

were resistant to chloramphenicol and susceptible to tetracycline. Screening on blood agar plates confirmed that none of the seven isolates displayed any hemolytic activity.

Table 4 Acid tolerance and bile salt resistance of selected isolates.

		Acid Tolerance Survival (%)	**Bile Salt Resistance (Survival %)**	
Species determination	Isolate	**pH 2.0 (3 h)**	**Bile Salt (0.3%)**	**Bile Salt (1%)**
***E. faecium* NM113**	1	95.5 ± 2.0	95.5 ± 1.0	80.5 ± 1.3
***E. faecalis* NM231**	23	90.5 ± 1.0	98.5 ± 1.0	85.2 ± 1.0
***E. durans* NM232**	24	90.0 ± 1.4	96.4 ± 2.0	83.5 ± 1.2
***E. faecium* NM233**	32	88.5 ± 1.5	95.5 ± 2.0	81.5 ± 1.5
***E. faecium* NM234**	34	91.2 ± 2.0	97.5 ± 1.5	84.7 ± 2.0
***E. faecalis* NM235**	67	90.5 ± 2.0	97.5 ± 1.0	85.5 ± 1.2
***E. faecium* NM236**	91	89.5 ± 1.0	98.4 ± 1.0	86.5 ± 1.0

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Table 5 Antibiotic susceptibility of selected isolates.

		Diameters of inhibition zone (mm) and susceptibility								
Species determination	**Isolate**	**Van 30 µg**	**Ery 15 µg**	**Gen 10 µg**	**Kan 30 µg**	**Amx 30 µg**	**Tet 30 µg**	**Amp 10 µg**	**Cm 30 µg**	**Cip 15 µg**
***E. faecium* NM113**	**1**	**I**	**S**	**S**	**I**	**S**	**S**	**I**	**R**	**S**
***E. faecalis* NM231**	**23**	**I**	**I**	**S**	**S**	**S**	**S**	**R**	**R**	**S**
***E. durans* NM232**	**24**	**I**	**R**	**I**	**S**	**I**	**S**	**R**	**R**	**S**
***E. faecium* NM233**	**32**	**R**	**S**	**I**	**S**	**I**	**S**	**R**	**R**	**I**
***E. faecium* NM234**	**34**	**S**	**I**	**S**	**S**	**S**	**S**	**R**	**R**	**I**
***E. faecalis* NM235**	**67**	**I**	**I**	**R**	**S**	**I**	**S**	**I**	**R**	**S**
***E. faecium* NM236**	**91**	**R**	**I**	**S**	**S**	**I**	**S**	**I**	**R**	**I**

Diameters of inhibition zones were measured, and the results expressed in terms of resistant (R), intermediate (I) and susceptible (S) according to cut off levels proposed by the NCCLS. Data represents three independent experiments. Van, Vancomycin; Ery, erythromycin; Te, tetracycline; Amp, ampicillin; Amx, Amoxicillin; Cip, ciprofloxacin; Gen, Gentamycin; Cm, chloramphenicol; Kan, Kanamycin.

3.7.Molecular identification

The seven isolates that exhibited competitive inhibition against *Campylobacter* strains underwent molecular identification through the amplification and sequencing of their 16S rRNA genes. The sequencing analysis confirmed their classification within the *Enterococcus* genus. Subsequently, partial sequences of their 16S rRNA genes were submitted to GenBank, and each isolate was assigned a unique accession number. The isolates were identified and designated as follows: *E. faecalis*-NM231 (OQ860807), *E. faecium*-NM234 (OQ860810), *E.*

faecium-NM233 (OQ860809), *E. faecium*-NM113 (KC878684.1), *E. durans*-NM232 (OQ860808), *E. faecalis*-NM235 (OQ860811), *E. faecium*-NM236 (OQ860812).

3.8.Histopathological observations

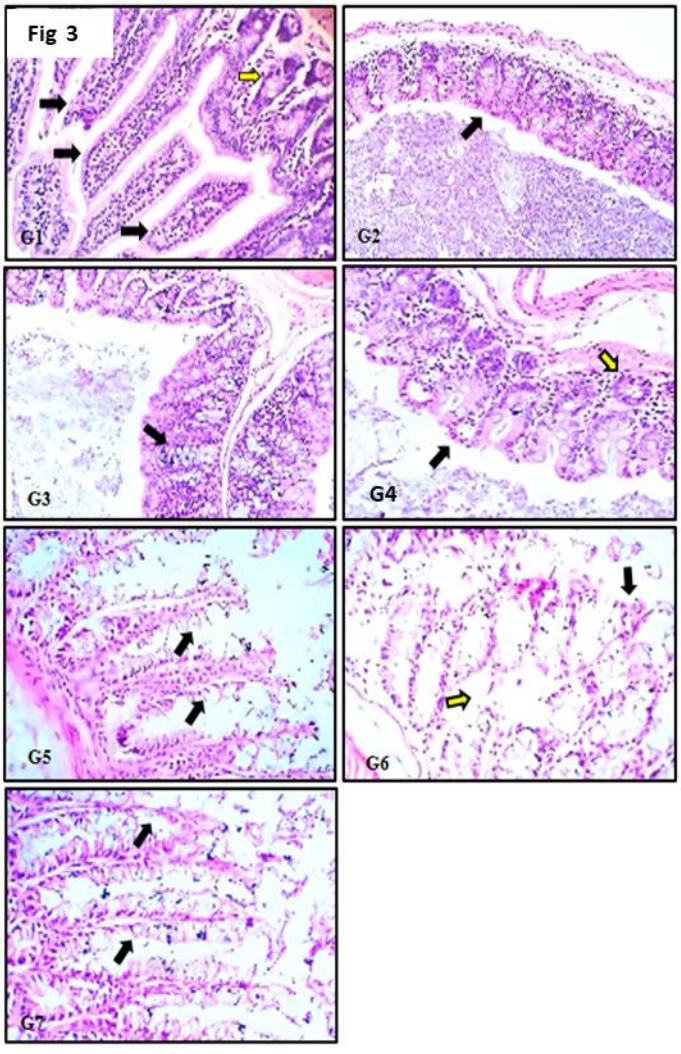
Jejunum and Colon

Histopathological examination revealed the maintenance of tissue integrity in group (2). This group was treated exclusively with the seven selected probiotic strains: *E. faecalis*-NM231, *E. faecium*-NM234, *E. faecium*-NM233, *E. faecium*-NM113, *E. durans*-NM232, *E. faecalis*-NM235, and *E. faecium*-NM236, without any challenge from *C. jejuni*. Group (3), which received the seven *Enterococcus* probiotic strains but was also challenged with *C. jejuni*, showed similar normal tissue histology. Group (4), treated with a combination of *E. faecium*-NM234 and *C. jejuni*, exhibited histological features similar to those of the negative control mice (group 1) and differed from the *C. jejuni*-treated mice (challenge control-group 7), which displayed significant histological abnormalities. These groups exhibited intact and healthy jejunum with normal crypts of Lieberkühn. In the colon, they displayed normal villi with an intact lining epithelium and active goblet cells as in Figure (3) [G1, G2, G3, and G4].

Conversely, the groups treated with *E. faecalis*-NM235 and *E. faecium*-NM236 probiotic strains (G5 and G6, respectively) showed no protection against *C. jejuni* infection. This was evident from the severe desquamation in the lining epithelium of the intestinal villi in the jejunum, coupled with severe degeneration and necrosis of the crypts of Lieberkühn as in Figure 3 (G5 and G6). These observations mirrored those seen in the *C. jejuni*-treated mice (challenge control-group 7), which exhibited severe necrosis and exfoliation of the intestinal villi lining epithelium (Figure 3 - G7).

Fig 2: Histopathological findings in the jejunum and colon tissues

G1 (negative control group (a)): jejunum of PBS-treated mice showed complete normal archticture and tissue details of jejunal tissue, as intact healthy intestinal villi (black arrow) and normal crypts of lieberkuhn (yellow arrow) X 200. **G2 (positive control group (b)):** Colon of treated mice with the mix of the selected seven probiotic strains without *C. jejuni* challenge exhibited normal intestinal villi with intact lining epithelium and active goblet cells (arrow) X 100. **G3:** Colon of the of treated mice with the mix of the selected seven probiotic strains and challenged with *C. jejuni* showed normal intestinal villi with intact lining epithelium and active goblet cells (arrow) X 100. **G4:** Colon of the treated mice with *E. faecium* NM234 and challenged with *C. jejuni* displayed healthy, active intestinal villi associated with the intact lining epithelium and active goblet cells (black arrow) and normal crypts of lieberkuhn (yellow arrow) X 200. **G5:** Jejunum of the mice treated with *E. faecium* NM235 and challenged with *C. jejuni* showed complete desquamation for the lining epithelium of the intestinal villi leaving the intestinal villi tissue core alone (black arrows) X 400. **G6:** Jejunum of the treated mice *E. faecium* NM236 probiotic strains and challenged with *C. jejuni* also showed severe exfoliation for the lining epithelium of the intestinal villi (black arrow) accompanied with severe degeneration and necrosis of the crypts of lieberkuhn (yellow arrow) X 200. **G7 (challenge control group):** The treated mice with *C. jejuni* demonstrated severe necrosis and exfoliation of the entire lining epithelium of most of the intestinal villi (arrows) X 400.



Ovary and Uterus

The histopathological examination of ovarian and uterine tissues revealed a consistent tissue protection pattern against *C. jejuni* infection in mice groups subjected to treatment with the selected seven probiotic strains, both with and without *C. jejuni* challenge. This protective effect was also evident in the groups treated with the *E. faecium*-NM234 probiotic strain (G2, G3, and G4) when compared to the negative control mice group (G1).

In these groups, the ovarian tissue displayed a structurally normal appearance with multiple follicular stages and corpora lutea. The uterine tissue exhibited well-developed endometrial folding and uterine glands, reflecting normal histological features as shown in Figure 4 (G1, G2, G3, and G4). Conversely, the mice groups treated with *E. faecalis*-NM235 and *E. faecium*-NM236 probiotic strains (G5 and G6) did not exhibit protective effects on ovarian and uterine tissues. These groups displayed histopathological alterations, including moderate degeneration and desquamation in ovarian follicle granulosa cells. The uterus exhibited signs of suppurative endometritis, characterized by a moderate diffuse infiltration of inflammatory cells, primarily polymorphic nuclear neutrophils, in the uterine lumen, along with exfoliations in the endometrial lining epithelium as shown in Figure 5 (G5 and G6). These histopathological changes closely resembled those observed in the group solely treated with *C. jejuni*, serving as the challenge control (G7), which exhibited ovarian degenerative changes and uterine suppurative endometritis as in Figure 5 (G7).

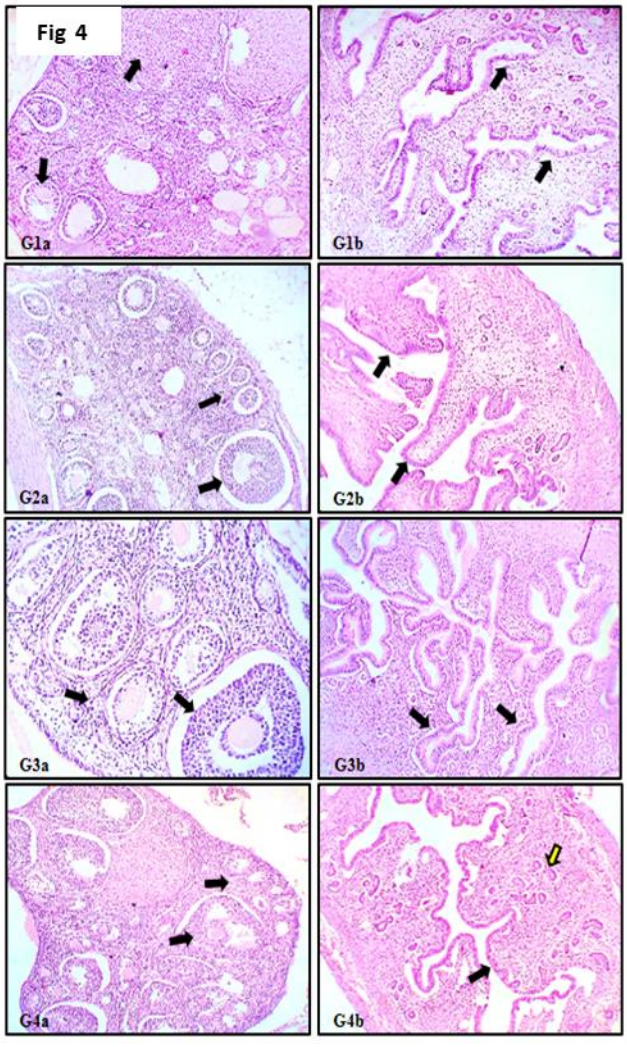


Fig 3: Histopathological findings in ovary and uterus (groups 1, 2, 3, and 4)

G1a & b: The control mice group showed that; the ovarian tissue appeared normal, active with different developmental stages of the ovarian follicles and corpora lutea (arrows in fig. G1a) X 100**.** The uterus displayed normal and well-developed

endometrial folding and active uterine glands (arrows in fig. G1b) X 100. **G2 a & b:** The selected seven probiotic strains-treated mice without challenge showed normal, active ovary in the follicular stage with different developmental stages of the ovarian follicles adjacent to each other (arrows in fig. G2a) X 100. The uterus displayed physiologically active endometrial layer with the development of primary and secondary endometrial folding (arrows in fig. G2 b) X 100. **G3 a & b:** The selected seven probiotic strains-treated mice challenged with *C. jejuni* challenge exhibited active ovarian tissue in the follicular stage with the presence of multiple mature ovarian follicles and secondary follicles adhering to each other (arrows in fig. G3a) X 100. The uterus showed a well-developed active endometrium with the presence of a primary and secondary endometrial folding and narrow uterine lumen (arrows in fig. G3b) X 100. **G4a & b:** The *E. faecium* NM234 probiotic-treated mice showed normal, active ovarian tissue with overcrowded follicles in different stages (arrows in fig. G4a). Uterus showed the development and folding of endometrium (black arrow in fig. G4a) with active and evenly distributed uterine glands (yellow arrow in fig G4b) X 100.

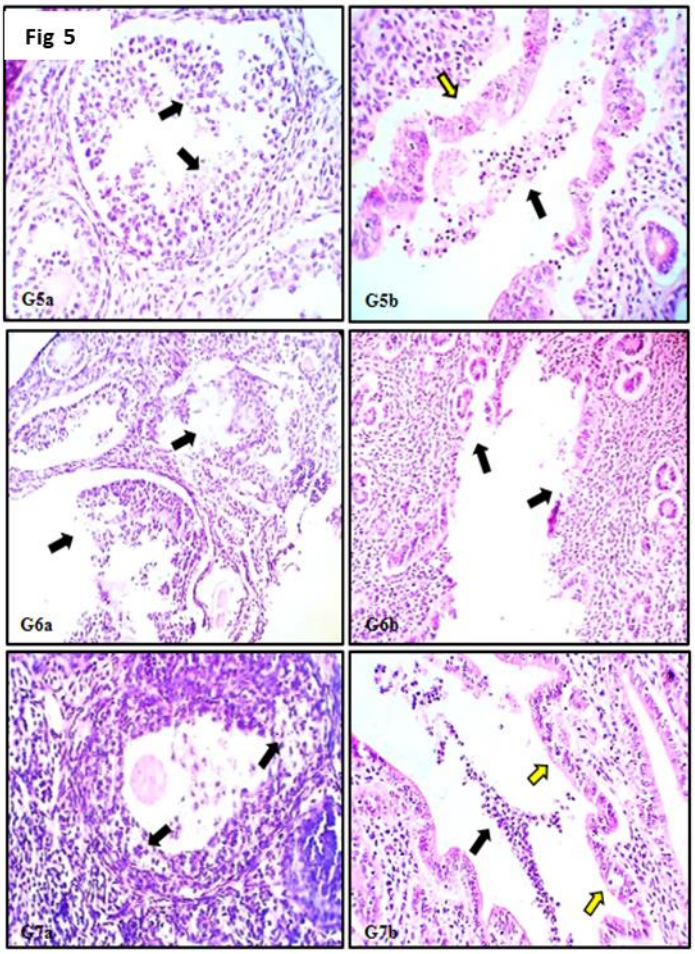


Fig 5: Histopathological findings in ovarian and uterine tissues (groups G5, G6, and G7)

G5a & b: The mice treated with *E. faecalis*-NM235 showed that; moderate desquamation and degeneration of the granulosa cells of the ovarian follicles (arrows in fig. G5a) X 200**.** The uterus exhibited severe suppurative endometritis which displayed diffuse infiltration of the polymorphic nuclear neutrophils in the lumen of the uterus (black arrow in fig. G5b), associated with exfoliations in the endometrial lining epithelium (yellow arrow) X 200. **G6a**

& b: The mice group treated with *E. faecium*-NM236 revealed that; severe degeneration and necrosis of several ovarian follicles (arrows in fig. G6a) X 100. The uterus illustrated severe ulcerative endometritis characterized by multifocal desquamations in the lining epithelium of the endometrium (arrows in fig. G6b) X 100. Ovary of the mice treated with *C. jejuni* displayed moderate degeneration and desquamation of the granulosa cells in the antral follicles (arrows) X 200. **G7a & b:** The *C. jejuni*-treated mice (challenge control group) displayed moderate degeneration and desquamation of the granulosa cells in the antral follicles of the ovarian tissue (arrows in fig. G7a) X 200. The uterus illustrated a case of suppurative endometritis which characterized by intra-luminal aggregation of the inflammatory cells in the uterine lumen mainly macrophages and neutrophils (black arrow in fig. G7b). Also moderate degeneration in the lining epithelium of the endometrium accompanied with nuclear pyknosis (yellow arrows) X 200.

3.9.Immunohistochemical detection of C. jejuni antigen.

Intestinal immunohistochemical findings (IHC).

In groups (2, 3, and 4), the probiotic protection was evidenced by the absence of immunoreactivity in both the jejunum and colon. This absence indicated that the *C. jejuni* antigen was not detected within the lining epithelium of the intestinal villi, Peyer's patches, or crypts of Lieberkühn. These findings were matching to the negative control mice group (G 1), as illustrated in Figure 6 (G1, G2, G3, and G4).

Conversely, mice groups treated with *E. faecalis*-NM235 and *E. faecium*-NM236 probiotic strains (G5 and G6) exhibited a strong positive golden brown immunoreaction. This reaction indicated the presence and localization of the *C. jejuni* antigen within the secretory and absorptive cells of the intestinal villi, as well as in the Peyer's patches as shown in Figure (6) (G5 and G6). These findings closely resembled the observations in the *C. jejuni*-treated mice, which served as the challenge control group (G7). In this control group, a strong positive golden-brown immunoreaction was evident in the infiltrated inflammatory cells among the basal crypts of Lieberkühn in the colon as shown in Figure 6 (G7).

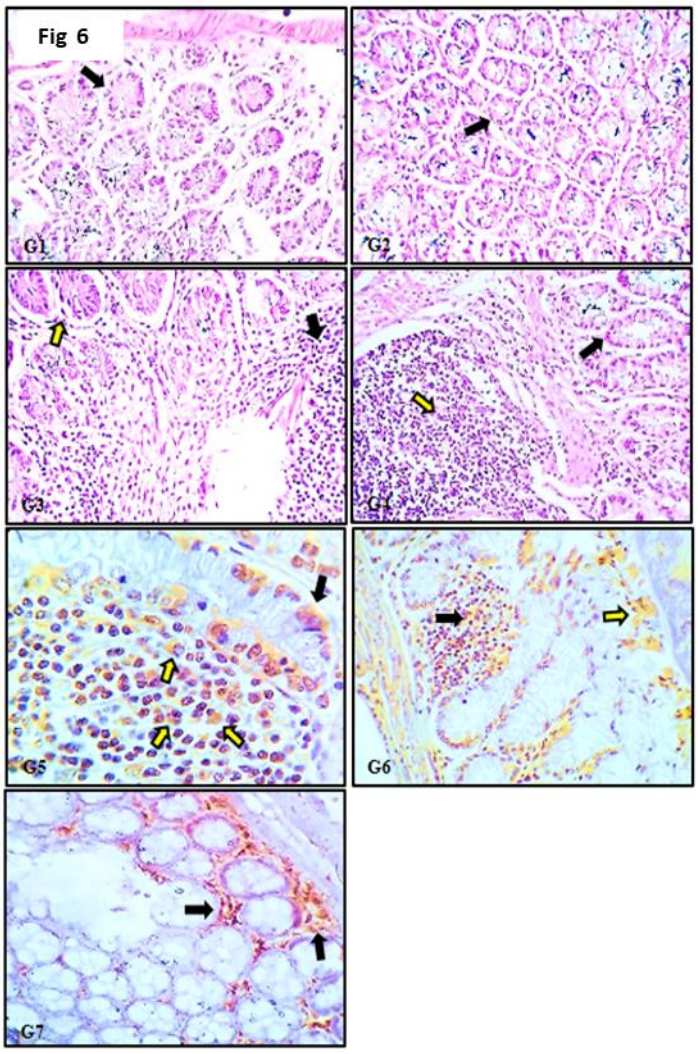


Fig. 6. Immunohistochemical findings for the detection of the *C. jejuni* antigen in jejunum and colon tissue.

G1 (negative control group): Jejunum of the PBS-treated rats showed no immunoreaction against the *C. jejuni* antigen in a histologically normal crypts of Lieberkühn (arrow) X 400. **G2:** Colon of the selected seven probiotic strains-treated mice-without *C. jejuni* challenge exhibited negative immunoreaction and absence of the *C. jejuni* antigen (to confirm the freeness of tissue from *C. jejuni* antigen) in the crypts of Lieberkühn (arrow) X 200. **G3:** Jejunum of the selected seven probiotic strains-treated mice-with *C. jejuni* challenge showed negative immunoreaction due to absence of *C. jejuni* antigen in peyer's patches (black arrow) and crypts of Lieberkühn (yellow arrow) X 400. **G4:** Jejunum of the *E. faecium*-NM234 probiotic strain-treated mice displayed negative immunoreaction in cells of both intestinal villi (black arrow) and lymphatic nodules (yellow arrow) X 400. **G5:** Jejunum of the *E. faecalis* NM235 strain probiotic strain-treated mice showed strong positive golden brown immunoreaction against the localized *C. jejuni* antigen in the immune cells of the intestinal lymphatic nodules (yellow arrows) and also found in the intestinal secretory cells (black arrow) X 1000. **G6:** Jejunum of *E. faecium*-NM236 probiotic strains-treated mice illustrated also strong positive golden brown immunoreaction against the localized *C. jejuni* antigen in cells of the intestinal lymphatic nodules (black arrow) and also found in the absorptive cells of the intestinal villi (yellow arrow) X 200. **G7 (challenge control group):** The *C. jejuni*-treated mice illustrated strong positive golden brown immunoreaction in the infiltrated inflammatory cells among several crypts of lieberkuhn in the basal line of jejunum (arrows) X 400.

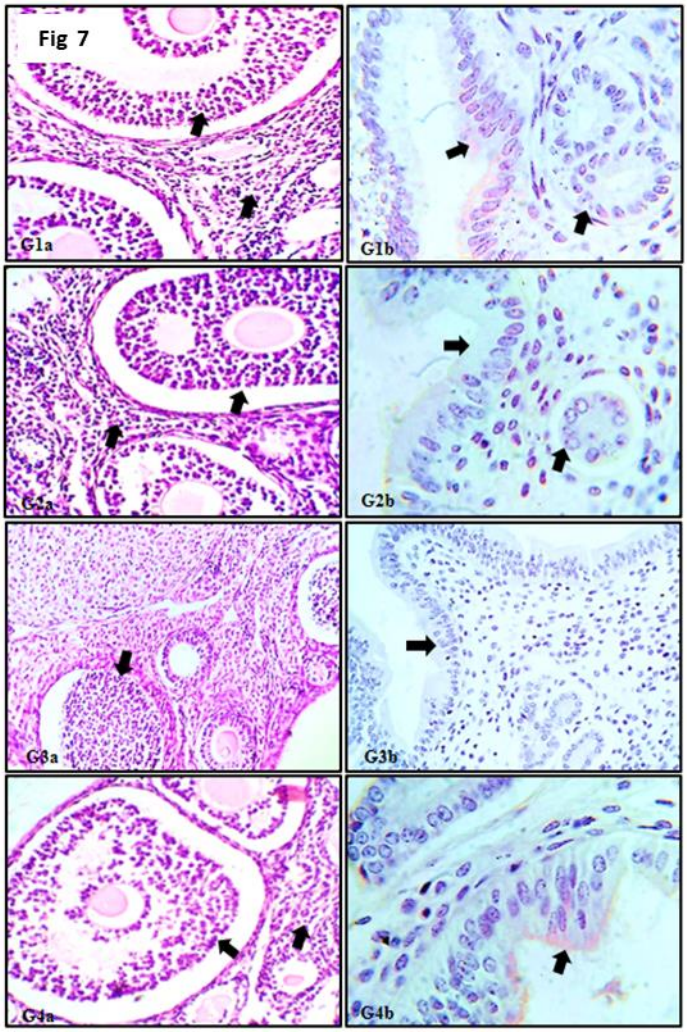
Ovarian and Uterine Immunohistochemical Findings

In the ovaries and uteri of groups (G2, G3, and G4), the immunoreactive findings were negative for the *C. jejuni* antigen. This absence of immunoreactivity was observed in both mature follicles and ovarian interstitial stromal cells, as well as in the endometrial epithelium and uterine glands. These findings were consistent with those of the negative control group (G1) as shown in Figure 7 (G1, G2, G3, and G4).

On the other side; both of the *E. faecalis*-NM235 and *E. faecium*-NM236 probiotic strains-treated mice groups (G5 and G6) respectively exhibited no protection for the reproductive system where, the ovary showed moderate to strong positive intra-cytoplasmic golden brown immunoreaction against the *C. jejuni* antigen, which was detected in the infiltrated macrophages inside the ruptured mature follicles. Moreover, uterus detected a strong positive immunoreaction against the *C. jejuni* antigen in the intra-luminal infiltrated neutrophils and in the endometrial epithelium as in Figure 6 (G5 and G6). These findings were compared with the findings in the *C. jejuni*-treated mice in the challenge control group (G7) which displayed a strong positive intra-cytoplasmic golden-brown immunoreaction in the infiltrated macrophages near to the mature ovarian follicle and also noticed intra-cytoplasmic in the infiltrated neutrophils in the lamina propria of the endometrium as in Figure 6 (G7). Conversely, in *E. faecalis*-NM235 and *E. faecium*-NM236 probiotic strain-treated mice groups (G5 and G6), protection against *C. jejuni* infection in the reproductive system was not observed. The ovaries exhibited moderate to strong positive intracytoplasmic golden-brown immunoreactivity against the *C. jejuni* antigen, primarily within infiltrated macrophages inside ruptured mature follicles. Furthermore, the uteri displayed strong positive immunoreactivity against the *C. jejuni* antigen in intra-luminal infiltrated neutrophils and the endometrial epithelium as shown in Figure 8 (G5 - G6). These findings were comparable to those observed in the *C. jejuni*-treated mice, serving as challenge control group (G7). In this control group, strong positive intracytoplasmic golden-brown immunoreactivity was noted in infiltrated macrophages near mature ovarian follicles, as well as within infiltrated neutrophils in the lamina propria of the endometrium as shown in Figure 8 (G7).

Fig. 7. Immunohistochemical findings for the detection of the *C. jejuni* antigen in ovarian and uterine tissues (groups G1, G2, G3, and G4).

G1 a & b: The PBS-treated mice showed that; Negative immunoreaction against the *C. jejuni* antigen in both mature follicles and the interstitial stromal cells of ovary (arrows in fig. G1a) X 400. Uterus also illustrated negative findings for the *C. jejuni* antigen in the endometrial epithelium and uterine glands (arrows in fig. G1b) X 1000. **G2 a & b:** The selected seven probiotic strains-treated mice without *C. jejuni* challenge showed negative immunoreactive findings against the *C. jejuni* antigen (to confirm the absence of the *C. jejuni* antigen in the sectioned tissues) in the ovarian mature follicles (arrows in fig. G2a) X 400, in the endometrial epithelium and uterine glands (arrows in fig. G2b) X 1000. **G3 a & b:** The selected seven probiotic strains-treated mice-with *C. jejuni* challenge showed negative immunoreactive findings against the *C. jejuni* antigen in the ovarian mature follicles (arrow in fig. G3a X 200), in the endometrial epithelium and uterine glands (arrow in fig. G3b X 400). **G4a & b:** The *E. faecium* NM234 probiotic strain-treated mice displayed negative immunoreaction in cells of the ovarian tissue (arrows in fig. G4a) X 400 and in the endometrium (arrow in fig. G4b) X 1000.



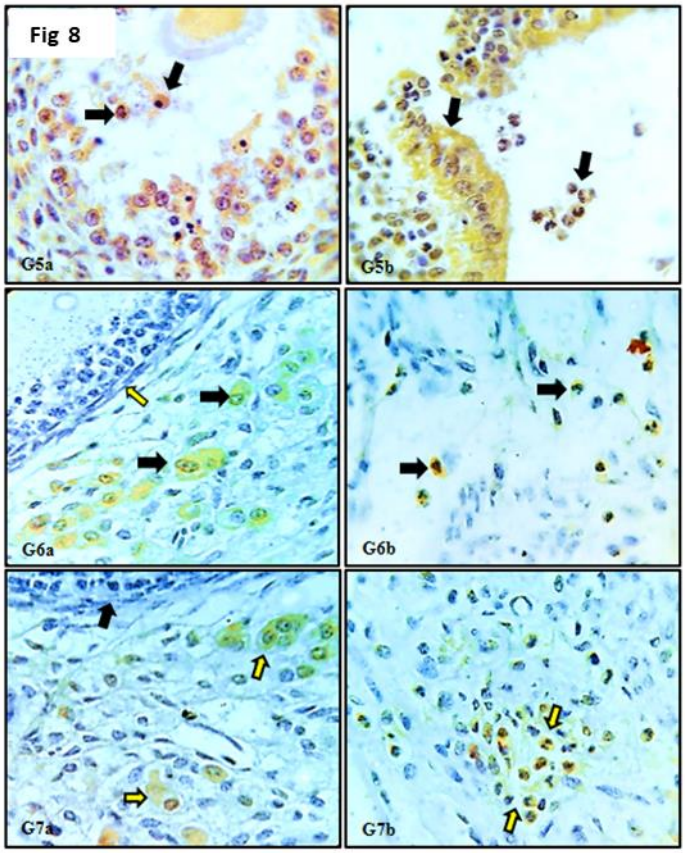


Fig. 8. Immunohistochemical findings for the detection of the *C. jejuni* antigen in ovarian and uterine tissues (groups G5, G6, and G7).

G5 a & b: *E. faecalis* NM235 probiotic strains-treated mice showed that; moderate positive intra-cytoplasmic golden-brown immunoreaction against the *C. jejuni* antigen in the infiltrated macrophages inside the ruptured mature follicles (arrows in fig. G5a) X 1000. Uterus also illustrated strong positive immunoreaction against the *C. jejuni* antigen in the endometrial epithelium and in the intra-luminal infiltrated neutrophils (arrows in fig. G5b) X 1000. **G6 a & b:** The *E. faecium* NM236 probiotic strain-treated mice revealed that; The ovary showed strong positive intra-cytoplasmic golden-brown immunoreaction against the *C. jejuni* antigen in the infiltrated macrophages (yellow arrows in fig. G6a) around the mature ovarian follicle (black arrow in fig. G6b) X 1000. Uterus also showed the same findings in the infiltrated neutrophils in the endometrial submucosa (arrows in fig. 40) X 1000. **G7 a & b:** The *C. jejuni*-treated mice gave a strong positive intra-cytoplasmic golden-brown immunoreaction in the infiltrated macrophages (yellow arrows in fig. G7a) adjacent to the mature ovarian follicle (black arrow in fig. G7a) X 1000. A positive immunoreaction was also noted intra-cytoplasmic in the infiltrated neutrophils in the lamina propria of the endometrium (arrows in fig. G7b) X 1000.

4. Discussion

Campylobacter remains a significant cause of foodborne infection in developed (EFSA, 2022) and developing countries (Paintsil et al., 2023). Symptoms of *Campylobacter* infection include diarrhea, cramping, abdominal pain, and fever within two to five days after exposure to the organism (Connerton and Connerton, 2017). Furthermore, it has been implicated in reproductive losses and abortions in various animal species, making it a concerning pathogen. The primary mode of infection transmission is through the gastrointestinal tract, where it colonizes humans and various animal species, serving as a reservoir for infection through fecal shedding (Lashley et al. 2019). Biosecurity measures aimed to reduce the exposure and transmission of campylobacters on farm and during post-harvest processing have had a limited impact in developed countries but are not feasible for small holders and subsistence farmers. Sustainable and affordable intervention strategies are sorely needed.

Probiotics represent a valuable natural strategy to limit the impact of intestinal pathogens, our study aimed to isolate probiotic bacteria from human gut microbiome able to compete the *C. jejuni* and *C. coli* as the major species responsible for human infection (Śmiałek et al., 2021). We screened 230 isolates against *Campylobacter* spp. from which fifteen isolates passed the initial evaluation by exhibiting inhibition over 50% in neutralized supernatants. We reduced these to seven candidate probiotic strains on the basis of their ability to out-compete four strains of *Campylobacter* spp. when tested by co-culture, produced inhibition in agar well diffusion and succeeded to inhibit *C. jejuni*-OR1 adhesion to HT29 cells in vitro.

The seven isolates were identified on the basis of the 16S rRNA gene sequences as *E. faecalis* NM231, *E. faecium* NM234, *E. faecium* NM233, *E. faecium* NM113, *E. durans* NM232, *E. faecalis* NM235, *E. faecium* NM236. To achieve their health benefits, probiotic strains must be able to survive and maintain their activity as they pass through the human body. They face the acidic gastric juice in the stomach expressing low pH (2-3) (Derrien and van Hylckama Vlieg, 2015) then they expose to the bile salt which cause disruption to the membrane of the sensitive microorganisms while they pass through the stomach loop of the small intestine (Ilango et al., 2016). Therefore, the seven strains were evaluated for their

ability to act as probiotics and demonstrated their tolerance to low pH (2.00) and bile salts up to 1%. They expressed high tolerance and stability in these harsh conditions and their survival ranged from (80.5 – 98.5%).

For evaluation of their safety, the seven isolates were tested for their susceptibility to nine antibiotics where they showed susceptibility or intermediate status to most of the antibiotic tested but could be differentiated from each other. All of them showed resistance to chloramphenicol while two isolates showed resistance to vancomycin, and one isolate resistance to ampicillin. However, the other studies have shown that the resistance of *Enterococcus* spp. to be wide-ranging according to the source, species and strain (Yerlikaya and Akbulut 2019). The assessment of hemolytic activity is strongly recommended for evaluation the safety of the probiotic strains, even if they have GRAS (Generally recognized as safe) status. None of the seven isolates showed α- hemolytic or β-hemolytic activity. The absence of hemolysin is indicative that they are non-virulent strains (Wang et al., 2020).

It is established that the action of probiotics can be strain-specific, however the reduction of adhesion and inhibitory action of *Enterococcus* spp. against *Campylobacter* strains could be the result of releasing of antimicrobial compounds such as hydrogen peroxide, organic acids, bacteriocins known as "Enterocins" or other bacteriostatic substances which suppress the pathogens (Achemchem et al., 2012; Ait Meddour et al., 2015; Elmoslih et al., 2017).

When the strains administrated to mice groups, the histopathological findings in our study revealed a protective performance against the *C. jejuni* infection for two groups of mice containing either a mix of the seven prebiotic strains (G3) orthe strain *E. faecium* NM234 alone (G4). In contrast to the infection control these group (G7) did not show evidence of pathological changes in the jejunum, colon, ovary, and uterus. While groups (G5) and (G6) which contain single strains of *E. faecalis* NM235 and *E. faecium*-NM236 respectively demonstrated significant differences in terms of tissue protection exhibited severe degenerative and necrotic changes in these tissues, including supportive endometritis which were similar to the challenge control group (G7), where only *C. jejuni* was administered. Immunohistochemical examination confirmed the histological results as it revealed no detection of the *C. jejuni* antigen in jejunum, colon, ovary and uterus in groups (G5) and (G6) that confirmed no protection against invasion and colonization for the *C. jejuni* for these two probiotic strains in contrast to probiotic strains in the other groups (G3) and (G4).

The mechanisms underlying *C. jejuni* pathogenesis involve several stages, including intestinal cell adhesion, colonization of the digestive lining epithelium, targeted cell invasion, and toxin production. Additionally, *C. jejuni* can secrete a heat-labile exotoxin known as cytolethal distending toxin (CDT), which induces degenerative and necrotic changes in eukaryotic cells (Pedersen et al., 2018). Our findings in groups G5 and G6, particularly the occurrence of suppurative endometritis and the immunohistochemical detection of the *C. jejuni* antigen within the examined tissues, align with previous research conducted by (Sanad et al., 2014). These reports highlight the similarity in the pathogenesis of *C. jejuni* infections in different species, including humans and animals. This pathogenesis encompasses gastroenteritis, placentitis, endometritis, and fetal infection, ultimately culminating in late-stage pregnancy abortions. In essence, across various pregnant species, including humans, cattle, and sheep, *C. jejuni* infection is consistently associated with abortion. This is primarily attributed to the development of necrotizing suppurative placentitis and endometritis, which coincide with an upregulation of host genes responsible for pro-inflammatory cytokines. Consequently, these processes lead to suppurative inflammation and cellular necrosis. Additionally, Kreuder et al. (2019) have reported on the reproductive impact of *C. jejuni*, highlighting its capacity to trigger abortion storms and impose significant economic losses on infected ovine flocks.

Furthermore, the presence of *C. jejuni* in the intestinal, ovarian, and uterine tissues in groups G5 and G6, as confirmed by immunohistochemical analysis, may be attributed to the bacterium's repertoire of proteases and peptidases. These enzymes play critical roles in various cellular processes of *C. jejuni*, including protein quality control and transport across membranes,

However, from a histopathological perspective, these enzymes are unequivocally classified as virulence factors. They play a pivotal role in promoting cellular invasion and the cleavage of crucial host cell factors, including epithelial cell junction proteins. Ultimately, these contribute to the observed intestinal tissue damage (El-Shibiny et al., 2005).

In conclusion, our study provides valuable insights into the protective potential of specific probiotic strains directed against *C. jejuni* infection in various tissues, specially strain *E. faecium* NM234. While some probiotic strains demonstrated effectiveness in preventing tissue damage and invasion by *C. jejuni*, others were less successful in providing protection. These findings emphasize the complexity of host-pathogen interactions and the need for further research to elucidate the specific mechanisms underlying probiotic-mediated protection against *C. jejuni* infections. Understanding these mechanisms can inform the

development of more effective probiotic-based interventions to mitigate the impact of *C. jejuni* in both humans and animals, particularly in cases involving reproductive complications and abortion. Finally, we propose the probiotic bacterium *E. faecium* NM234 as a candidate against *Campylobacter* infection for pharmaceutical and animal feed preparations. As a result, additional studies will be conducted on the seven strains, including whole genome sequencing and transcriptome profiling, to guarantee their safety and genetic function before moving on to clinical and feed formulations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Ethical Approval

This study was approved according to Ethics of Medical Research Committee of National Research Centre, Al Buhouth st. Dokki- Cairo Egypt (No 7497082023).

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