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Original Research Article

Impact of Dietary Cucurbits Induce Expression of Probiotics mapA-gene and Its Implication in the Adhesion to Human GI cells-INT407

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Abstract

Probiotic Lactobacilli co-exist as adhered entities with epithelial surface of human gut to impart barrier function. These dominant inhabitants must be supported by certain dietary intervention to maintain enteric homeostasis. In the present study extracts from three cucurbit fruits Lagenaria siceraria, Luffa cylindrica and Cucurbita maxima were prepared and investigated for their effects on three strains of probiotic Lactobacilli (L. rhmnosus, L. plantarum and L. acidophilus). We found extracts to support the adhesion properties of Lactobacilli showed significantly to the human intestinal cells-INT407. Expression of mucus adhesion promoting gene (mapA) of Lactobacilli was targeted for transcriptional analysis and found to be up-regulated in presence of mucin and extracts as well. These studies suggest that cucurbit extract mixed with probiotics in certain proportion might be used as prophylactic and therapeutic agents.

Abbreviations: GI- Gastrointestinal, INT407- a human intestinal cell line 407.

Keywords: Adhesion, Cell line, Cucurbits, Gut, Probiotics.

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Introduction

Millions of symbiotic bacteria including probiotics inhabit the human gut and have been implicated to confer health benefit by several mechanisms. Probiotic *Lactobacilli* adhere the epithelial surface to modulate barrier function of the gut [1,2]. These bacteria have capacity to suppress colitogenic and oncogenic bacterial activities in the gut [3]. Diet influences activities of gut microflora. Bad dietary habit alters the bacterial composition resulting into opportunistic pathogens to be dominant in GI tract. Altered compositions of gut microflora with declined probiotics have been reported in diseased persons [4]. Because it stimulates the host animals' physiological and immunological systems, bacterial adherence to the GI tract is thought to play a significant role in this health-promoting impact. Therefore, mucus, tissue samples, cell lines, and extracellular matrix components have all been used to study the adherence of bacteria to the GI tract or the colonisation of the GI tract by these organisms [5,6]. Many animals' gastrointestinal systems include the bacteria Lactobacillus, which is thought to benefit the host in a number of ways [7]. Numerous investigations have demonstrated that giving these probiotic bacteria orally alters the host animals' immune systems [8]. Studies examining the molecular mechanisms behind Lactobacilli's adherence to the host GI tract have yielded conflicting results, despite the evidence of colonisation of the GI tract following probiotic administration of Lactobacilli or its usage in oral administration.

Crypt stem cells of intestinal *villi* are well protected through adhered probiotics and a non cellular layer of mucus to inhibit aberrant colonization of pathogenic bacteria. Colonization of oncogenic bacteria like *Salmonella* and *Helicobacter* are capable of inducing inflammation and tumorigenic transformation of Crypt stem cells and other cells of *villi*. Therefore, the epithelial surface must be protected against pathogenic and oncogenic stressors. Gut modulating dietary phytochemicals has been implicated to protect the gut directly and through activities of probiotic action indirectly. Although its biological and ecological roles in bacterial adherence have been clarified, this protein is thought to be an adhesion factor. A mucus adhesion promoting protein (MapA) from Lactobacilli allowed us to demonstrate the bacteria's capacity to bind mucus [9]. The cell-surface protein MapA has a theoretical pI of 9.7 and a molecular weight of 26 kDa. Strong electrolyte solutions can be used to extract this protein. Furthermore, a mouse colonisation experiment conducted in vivo revealed that MapA is one of Lactobacilli's adhesion factors [10]. At the amino acid level, MapA are thought to have a high degree of similarity (94%). Since no prior study has employed intestinal cells to examine the receptor-like molecules involved in bacterial adherence, MapA may have affinity for molecules in the human GI tract.

Dietary cucurbits have been found as gastro-protective and their therapeutic implications have also been addressed in scientific literature of traditional medicine but lacking the experimental validation. Therefore, we attempt to reveal a significant role of cucurbit fruits in host microbe interaction of probiotic *Lactobacilli* to gastrointestinal epithelial cells in cell culture system [11].In this study three strains of *Lactobacilli* (*L. rhmnosus, L. plantarum* and *L. acidophilus*) were taken to evaluate the effect of cucurbit extracts on adhesion of these organisms to intestinal INT407 cells [11,12]. In order to prevent experimental colitis and inhibit cytokine-induced epithelial cell death, *L. rhamnosus* GG activates the epidermal growth factor receptor (EGFR) pathway [13]. Furthermore, this probiotic bacterium attenuates enterohemorrhagic *Escherichia coli* O157:H7 [14]. *L. plantarum* is capable of modulating the expression of GI epithelial tight junction proteins [15]. *L. acidophilus* enhances the secretion of IgA titer significantly for *Salmonella enterica subsp. enterica serovar Typhi* [16,17]. Since ancient times, cucurbits have been used for therapeutic purposes. It has recently been determined that cucurbits are the richest source of secondary metabolites with therapeutic benefits and vital nutritional additions [12,18,19]. These include ribosome-inactivating proteins, alkaloids, flavonoids, cucurbitacins, and various other bioactive substances with pharmacological and nutritional significance. Lagenaria siceraria (Ls), Luffa cylindrica (Lc) and Cucurbita maxima (Cm) are some of the most important fruits which have been consumed as vegetables throughout the world. In the present study, we investigated the adhesion of probiotics to human intestinal cells-INT407 cells as an *in vitro* of the intestinal surface. In particular, we sought to elucidate the correlation between the adhesions of probiotics to INT407 and their potential effect on human health. We also investigated the enhancement of gut homeostasis through the transitory effect of probiotics on the synergistic effects of probiotics to cucurbits extract promote the inhibition of pathogen binding to host and their ability to trigger signalling pathways.

Materials and Methods

Chemicals and reagents: Culture media, antibiotics, and supplements for mammalian and bacterial cell culture were purchased from HiMedia (Mumbai, India). Cell-culture flasks and microtiter plates were procured from Nunc (USA). General laboratory reagents were obtained from Sigma (Germany) and Merck (Mumbai, India). Taq DNA polymerase, Taq buffer, dNTPs, Proteinase K, and DNA ladders were procured from GeNei (Bangalore, India). Genomic DNA isolation kits were obtained from Taurus Scientific (USA). The Total Protein Extraction Kit was purchased from G-Biosciences (USA). Trizol reagent for RNA extraction was obtained from Merck (Mumbai, India), and the cDNA synthesis kit was purchased from Bio-Rad (USA).

Probiotic strains and culture conditions: Three standard Lactobacillus strains were procured from the Microbial Type Culture Collection (MTCC) as showed (Table 1), Institute of Microbial Technology, Chandigarh, India. All strains were cultured and maintained in MRS broth and agar under standard micro-aerophilic conditions. Routine subculture was performed every 48 hours to maintain viability and purity.

Table 1: Probiotic *Lactobacilli* used in the studies

Strains	**MTCC Code**	**Growth medium**	**Temperature (^{0}C)**	**pH**
L. rhamnosus	**1408**	**MRS**	**30-37**	**6.5±0.02**
L. plantarum	1407	MRS	30-37	6.5±0.02
L. acidophilus	447	MRS	30-37	6.5±0.02

Biochemical and molecular identification of probiotic strains: Each MTCC strain was subjected to standard biochemical characterization according to Bergey's Manua [20]. Gram staining, catalase, oxidase, motility, and carbohydrate fermentation profiles were recorded to confirm phenotypic identity. Molecular confirmation was performed by amplifying the 16S rRNA gene (Table 2) following the method of Rani A et al. (2008) [21]. Genomic DNA was extracted using the MO BIO UltraClean kit (MO BIO Laboratories, USA). PCR was carried out in a 50 µl reaction mixture containing 1× PCR buffer, 2 mM $MgCl_2$, 200 µM of each dNTP, 2.5 U Taq polymerase, 2 µM of each primer, and 50 ng DNA template. Cycling conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, annealing at 53–58°C for 90 s, and extension at 72°C for 2.5 min, with a final extension at 72°C for 10 min. PCR products were visualized on a 1.5% agarose gel to confirm genus-specific amplicon size [21].

Table 2: Primersoptimization used for transcriptional expression of genes used in this study

16srRNA F-5'TGCCTAATACATGCAAGTCGA 3'
R-5'GTTTGGGCCGTGTCTCAGT 3'
MapA
F-5'TGGATTCTGCTTGAGGTAAG 3'
R-5' GACTAGTAATAACGCGACCG 3'
β-actin -F-5' ACGGGTCACCCACACTGTGC 3'
R-5' CTAGAAGCATTTGCGGTGGACGATG 3'

Preparation of cucurbit extracts: Fresh fruits of *Lagenaria siceraria*, *Luffa cylindrica* and *Cucurbita maxima* were collected from a local market in Jamia Nagar, New Delhi, and authenticated. Moisture content was determined using a standard moisture meter (A&D Company Ltd., Tokyo, Japan). Samples were homogenized in 80% ethanol using a Polytron homogenizer (IKA T18, Japan) and a Waring blender. The homogenate was filtered and centrifuged at 1600×g for 10 minutes. The supernatant was filtered again and centrifuged once more at 1600×g. Solvent was removed by rotary evaporation followed by lyophilization to yield powdered extracts. Extracts were stored in vacuum desiccators until use [22].

Phytochemical analysis: Phytochemical profiling included total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity.

- **TPC** was measured using the Folin–Ciocalteu reagent [23] and expressed as mg gallic acid equivalents (GAE)/g extract.
- **TFC** was estimated by the aluminium chloride method [24] and expressed as mg quercetin equivalents (QE)/g extract.
- **Antioxidant activity** was evaluated using the DPPH radical-scavenging assay following [25].

Cell line and culture conditions: The human intestinal epithelial cell line *INT407* was obtained from the National Centre for Cell Sciences (NCCS), Pune, India. Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 mg/l streptomycin. Cultures were maintained at 37°C in a humidified incubator with 5% CO_2 and sub-cultured as required.

Adhesion assay (qualitative): Adhesion of *L. acidophilus*, *L. plantarum* and *L. rhamnosus* was examined by seeding *INT407* cells at 1×10^6 cells/well in 12-well plates and allowing them to reach 70% confluence. Freshly grown probiotic cultures (10^9 CFU/ml) were added and incubated for 2 hours at 37°C in 5% CO_2 [26]. After incubation, wells were washed with PBS and stained with Gram's crystal violet to visualize bacterial adherence under an inverted microscope.

Quantitative adhesion assay: Quantitative adhesion was measured using the CFU-recovery method described by Tuomola EM et al. (1998) [26]. *INT407* cells (1×10^6/well) were incubated with mid-log Lactobacillus cultures (10^9 CFU/ml) for 2 hours. After washing, cells were lysed with 0.1% Triton X-100 for 10 minutes. Lysates were serially diluted, plated on MRS agar, and incubated at 37°C for 24 hours. Adhesion (%) was calculated as:

(adhered CFU ÷ initial CFU) × 100

Semi-quantitative RT-PCR for *mapA* expression: Probiotic strains treated with and without cucurbit extracts were grown for 24 hours at 37°C. Total RNA was isolated using the Trizol method, and cDNA was synthesized using the Bio-Rad kit. Semi-quantitative PCR was performed with primers specific for *mapA* and β-actin according [27]. Amplification conditions consisted of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s for 35 cycles. β-actin served as an internal control.

Protein isolation and SDS-PAGE: Bacterial cultures grown for 24 hours in MRS broth were harvested at 16,000×g for 2 minutes. Cell pellets were resuspended in Laemmli sample buffer, boiled for 5 minutes, and processed using the Total Protein Extraction Kit (G-Biosciences, USA). Proteins were resolved on 12% SDS-PAGE gels prepared with standard 5% stacking and 12% resolving compositions [28]. Gels were stained with Coomassie Brilliant Blue and destained with methanol–acetic acid solution.

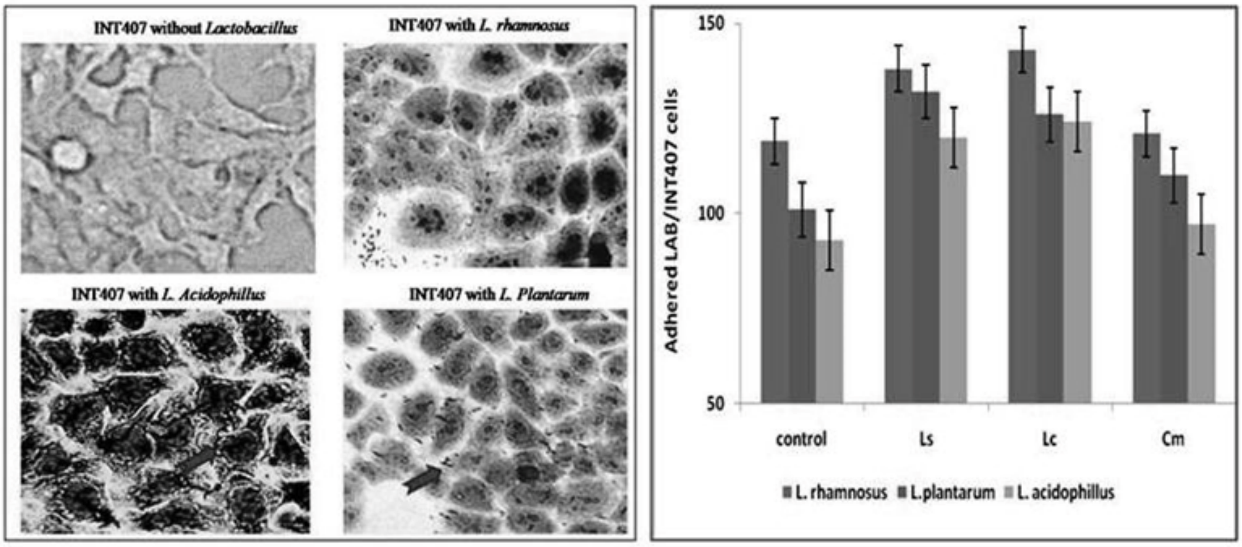
Cytotoxicity assay (MTT): Cytotoxicity of cucurbit extracts toward *INT407* cells was evaluated using the MTT assay [29]. Cells were exposed to extract concentrations ranging from 25–400 µg/ml for 24 hours. MTT solution (5 mg/ml) was added for 4 hours, and formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm to determine viability.

Pathogen exclusion assay: Pathogen exclusion was evaluated using the competitive adhesion method described by Adlerberth et al. [30]. *INT407* cells were preincubated with extract-treated or untreated Lactobacilli for 2 hours. After washing, *Escherichia coli* or *Salmonella enterica* (10^8 CFU/ml) were added and incubated for 1 hour [31]. Cells were lysed with 0.1% Triton X-100, and released pathogens were plated on selective agar. Inhibition was calculated relative to pathogen-only controls [32].

Statistical analysis (expanded): All experiments were performed in triplicate unless stated otherwise, and each independent experiment contained three technical replicates. Data are expressed as mean ± SEM to reflect both central tendency and variability. Normality of the data distribution was assessed before applying statistical tests. Comparisons between control and treated groups were performed using a two-tailed Student's *t*-test, and differences were considered statistically significant at $p< 0.05$. For experiments involving multiple treatment groups, such as extract-treated probiotics or concentration-dependent cytotoxicity assays, data were additionally checked for consistency across replicates. Graphical analysis and statistical calculations were carried out using Microsoft Excel and GraphPad Prism (version 9.0). Inhibition percentages, adhesion fold-changes, and densitometry values were calculated and plotted using the same software. All statistical interpretations were based on the average of independent experimental outcomes rather than individual plate readings to avoid pseudo-replication.

Results

Adhesion of probiotic Lactobacilli to *INT407:* Adhesion to host epithelial cells is a key functional attribute of probiotics, and all three Lactobacilli tested (*L. rhamnosus*, *L. plantarum* and *L. acidophilus*) demonstrated the ability to attach to INT407 cells. Microscopy showed clear binding of the bacteria to the epithelial surface (Figure 1). Lactobacilli express a range of surface-associated proteins that support adhesion, including members of the S-layer family (Figure 2). One such protein, mucus adhesion-promoting protein (MapA), is widely recognized for its role in epithelial interaction. In this study, the **mapA** gene was successfully amplified from all three Lactobacillus strains, and its protein product was detected, confirming that each strain expresses*MapA* under the tested conditions.



(A) (B)

Figure 1: (A) and (B) showed the adhession of *Lactobacilli* to INT407

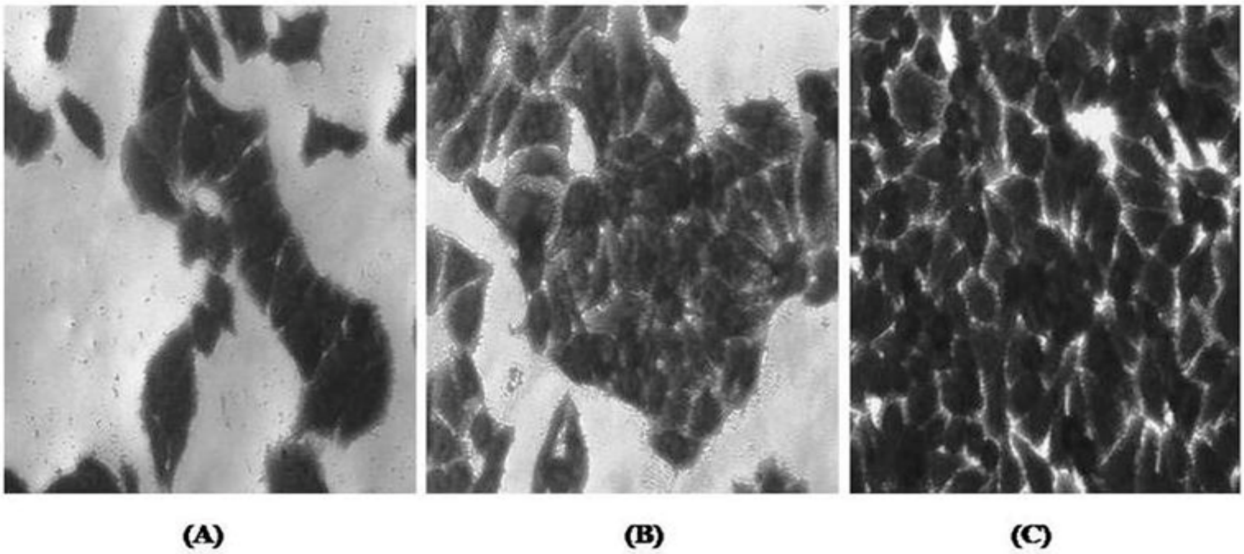


Figure 2: (A), (B) and (C) showed the adhered Crystal violet stained Lactobacilli to INT-407 cells. Bacterial surface layer proteins of various types have been reported to be involved to adhere on epithelial cell surface layer of the gut. One example is mucus adhesion promoting protien (MapA)

Expressional analysis of *MapA*gene: The expression of the *mapA* gene was evaluated in the presence and absence of 5% mucin and cucurbit extracts. Both treatments modulated *mapA* expression compared with untreated controls (Figure 3). The increase in expression in response to mucin exposure is consistent with the known role of mucin as a cue for probiotics to enhance their adhesion machinery [27]. Extract-treated cultures showed a similar trend, suggesting that cucurbit phytochemicals may influence the regulation of adhesion-related genes.

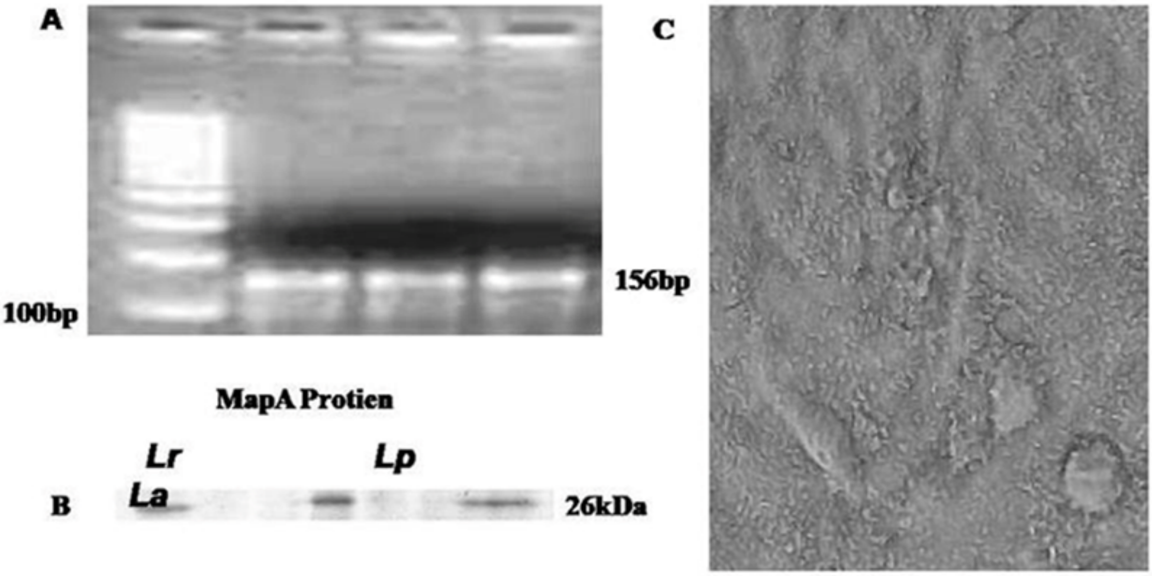


Figure 3: Showing adhesion of lactic acid bacteria (LAB) to INT407 cells. Mucus adhesion promoting protein (MapA) facilitates adhesion of Lactobacilli to epithelial lining of the gut. A: PCR product of mapA gene; B: CBR stained MapAprotiens; C: INT-407 with adhered Lactobacilli.

Adhesion of Lactobacilli to mucin-coated surfaces: Mucin is the natural protective barrier lining the intestinal epithelium. When *INT407* plates were coated with mucin, both mucin-treated and extract-treated Lactobacilli adhered strongly to the surface, whereas negative controls showed little to no attachment (Figure 4). The enhanced adhesion on mucin reflects the biological relevance of *MapA*-mediated interaction and further supports the expression results described above.

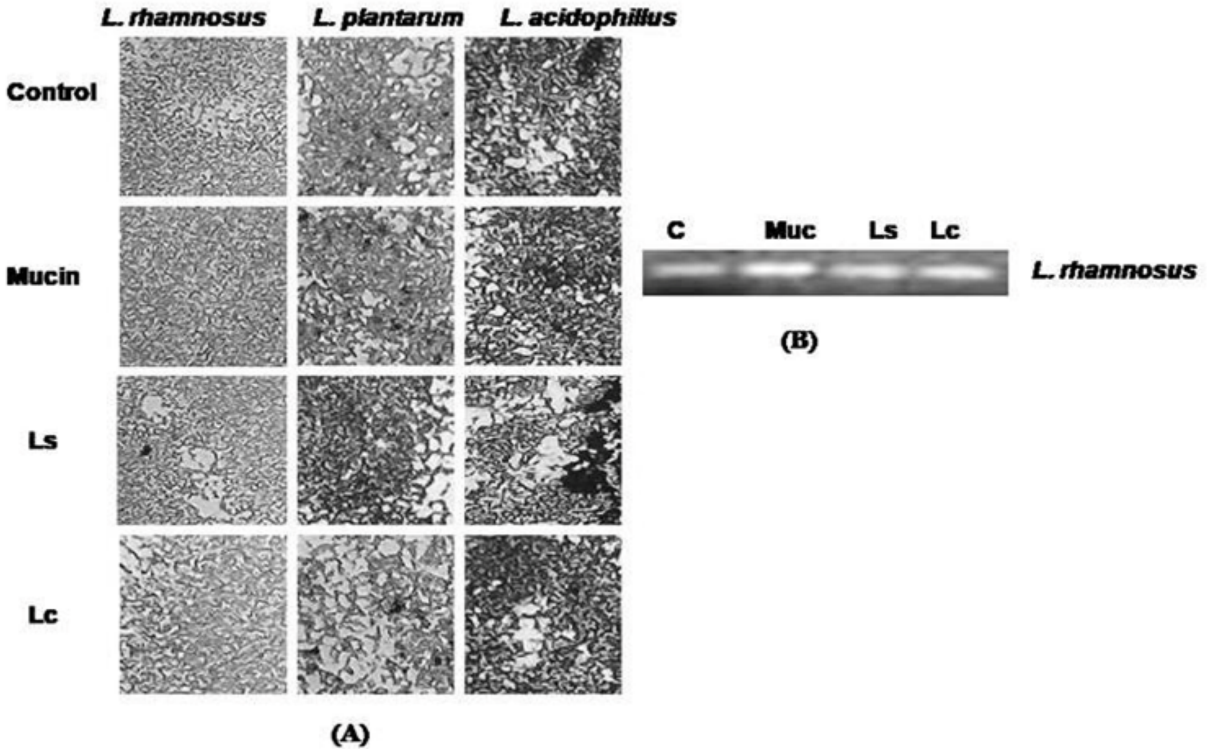


Figure 4: Showing adhesive potential of *Lactobacilli* grownin the presence and absence of mucin (5%) and extracts. Fresh culture of *Lactobacilli* grown at 37^{0}C for 20h were incubated at 4^{0}C for 24h to allow adherence onto the surface of mucin coated 96 well round bottomed microtiter plate. Gram staining was performed and photographed by inverted microscope. **(A):***Lactobacilli;* **(B)**: mRNA expression of MapA gene in mucin and extract treated *Lactobacilli* with the normal control. C: Control, Muc:Mucin, Ls: *Lagenaria siceraria*, Lc: *Luffa cylindrica*.

Quantitative adhesion of probiotic Lactobacilli: Quantitative CFU-based adhesion analysis showed that all three Lactobacilli had measurable baseline adhesion to INT407 cells, ranging between 8–14% of the initial inoculum, which aligns with reported epithelial adherence characteristics [30]. Treatment with cucurbit extracts led to a significant increase in adhesion. *Luffa cylindrica* produced the strongest effect, elevating adhesion to 18–29%, equivalent to a 1.8–2.1-fold increase over untreated controls. Such enhancement is consistent with earlier evidence showing that plant-derived phytochemicals can strengthen probiotic–host interactions [33]. Among the strains, *L. rhamnosus* showed the greatest responsiveness, possibly due to its flexible surface protein architecture, a feature also described previously [26]. Overall, extract treatment improved adhesion in a strain-dependent manner ($p< 0.05$), supporting the hypothesis that cucurbit bioactives modulate Lactobacillus adhesion behaviour.

Biochemical characterization: Biochemical profiling confirmed that all three strains were Gram-positive, catalase-negative, oxidase-negative, and non-motile rods, fully consistent with the classical descriptions in Bergey's Manual [20]. PCR analysis of the 16S rRNA gene produced a clear single band of the expected size (~1500 bp), confirming the identity of each MTCC strain [21]. This agreement between biochemical and molecular results indicates that the cultures were pure and accurately maintained.

Phytochemical composition of extracts: All cucurbit extracts contained detectable levels of phenolic and flavonoid compounds. *Luffa cylindrica* showed the highest phenolic content (20–24 mg GAE/g extract), consistent with prior phytochemical evaluations of cucurbit species [22]. Flavonoid levels followed a similar pattern. Antioxidant activity, assessed through the DPPH assay, reached up to 60% radical scavenging by 200 µg/ml in *L. cylindrica* comparable to earlier report of antioxidant-rich cucurbit extracts [25]. These phytochemical profiles align closely with the functional outcomes, supporting the idea that higher phenolic and flavonoid levels may enhance probiotic adhesion and metabolic activity [23],

Cytotoxicity on INT407 cells: Cytotoxicity testing using the MTT assay showed that cell viability remained above 90% at extract concentrations up to 200 µg/ml. These findings agree with earlier reports that cucurbit phytochemicals exhibit low toxicity toward mammalian cells [22]. The MTT method is sensitive for detecting metabolic impairment [29], yet no reduction in viability or adverse morphological changes were observed. This confirms that the improved adhesion recorded in treated groups reflects true biological modulation and not damage-induced artifacts.

Pathogen exclusion by extract-treated Lactobacilli: All extracts improved the ability of Lactobacilli to prevent pathogen binding. Without extracts, probiotics reduced *E. coli* and *Salmonella* adhesion by **20–25%**, consistent with established probiotic–pathogen competition mechanisms [32]. When Lactobacilli were pre-grown with *Luffa cylindrica* extract, pathogen exclusion increased markedly:

- **48% reduction** in *E. coli* adhesion
- **42% reduction** in *Salmonella* adhesion

This enhanced performance is likely due to stronger probiotic adhesion and more effective receptor occupation, as suggested in earlier studies [31]. The observed improvements support previous findings that bioactive-enhanced probiotics provide stronger epithelial protection against enteric pathogens [34].

Discussion

The present study examined how dietary cucurbit extracts influence the adhesion, gene expression and functional activity of three probiotic *Lactobacillus* strains. Adhesion to intestinal epithelial surfaces is a central determinant of probiotic efficacy, as it enhances competitive exclusion, immune modulation and barrier reinforcement [30]. Our quantitative adhesion assays confirmed that all Lactobacilli tested adhered naturally to INT407 cells, which agrees with established behaviour of these probiotic species. More importantly, treatment with cucurbit extracts produced a marked increase in adhesion, with *Luffa cylindrica* showing the strongest enhancement. Similar enhancement of probiotic adhesion by plant-derived compounds has been documented previously [33], suggesting that cucurbit phytochemicals may act as signalling molecules influencing cell surface interactions. The observed increase in adhesion was strongly correlated with the up-regulation of the *mapA* gene, a key mucin-binding adhesin in Lactobacilli. Previous studies have shown that expression of adhesion proteins such as *MapA* can be modulated by environmental cues, including mucin exposure and dietary components [27]. Our findings support this concept and further indicate that cucurbit phytochemicals may enhance probiotic adherence through transcriptional activation of adhesion-associated genes. The improved adhesion response of *L. rhamnosus* also aligns with earlier reports highlighting its robust and adaptable surface protein machinery [26]. Phytochemical profiling revealed that *Luffa cylindrica* contained higher levels of phenolic and flavonoids, consistent with earlier studies reporting strong bioactivity in this species [22]. Phenolic and flavonoids are known to act as antioxidants and signalling modulators and can influence microbial gene expression and stress responses [23]. This may explain why extracts rich in these compounds produced greater enhancement of adhesion and *mapA* expression.

Cytotoxicity testing confirmed that cucurbit extracts were non-toxic at all working concentrations, consistent with their known safety in nutritional studies [22]. This demonstrates that the increased adhesion was not due to epithelial cell damage, which can artificially expose binding sites. Rather, the effect represents a genuine interaction between probiotic cells and cucurbit-derived metabolites. The functional relevance of this enhanced adhesion was demonstrated in pathogen exclusion assays. Extract-treated probiotics significantly reduced adherence of *E. coli* and *Salmonella*, with the strongest effect seen for *Luffa cylindrica*. Probiotic-mediated pathogen inhibition has been attributed to competitive binding, steric hindrance and secretion of antimicrobial molecules [32]. Our findings expand on this framework by showing that plant extracts can further strengthen this function, likely by increasing the density and strength of probiotic attachment to epithelial surfaces. This is consistent with previous work demonstrating that enhanced probiotic adhesion improves resistance against enteric pathogens [34]. Taken together, the data indicate that cucurbit extracts enhance probiotic adhesion, up-regulate the *mapA* gene and strengthen pathogen exclusion. The phytochemical richness of cucurbits-especially phenolic and flavonoids-may be responsible for modulating bacterial physiology in a manner that improves host–microbe interactions. These findings provide experimental validation for the traditional use of cucurbit vegetables in digestive health and support their potential utility as synergistic components in probiotic formulations.

Further work is needed to isolate the specific phytochemicals responsible for this activity and to map the full regulatory network underlying adhesion-related gene expression. Studies using purified compounds, transcriptome profiling and in vivo gut colonization models would help clarify the molecular mechanisms involved. Nonetheless, the present study establishes a strong foundation for incorporating cucurbit-derived metabolites into probiotic or functional food applications aimed at improving gastrointestinal health.

Conclusion

This study demonstrates that extracts from dietary cucurbits significantly enhance the functional properties of probiotic *Lactobacillus* strains. All three extracts improved bacterial adhesion to INT407 intestinal epithelial cells, with *Luffa cylindrica* showing the strongest effect. This enhanced adhesion was accompanied by increased expression of the *mapA* adhesion gene, indicating that cucurbit phytochemicals influence bacterial surface physiology at the molecular level. Because adhesion is a key determinant of probiotic persistence and competitive behavior in the gut, these findings highlight a meaningful interaction between dietary plant metabolites and beneficial gut bacteria. The phytochemical analyses revealed that cucurbit extracts contain bioactive compounds such as phenolic and flavonoids, which may act as signalling molecules that modulate probiotic gene expression and adhesion behavior. Importantly, the extracts were non-toxic to INT407 cells at all working concentrations, confirming that the observed effects were not due to epithelial cell injury.

Functionally, extract-treated probiotics showed enhanced ability to prevent the adhesion of pathogenic *E. coli* and *Salmonella*. This improvement in competitive exclusion reinforces the biological relevance of stronger probiotic adhesion and suggests that cucurbit-rich diets or formulations may support microbial defense mechanisms in the gut. Overall, the results indicate that cucurbit extracts can be used to augment probiotic performance by strengthening adhesion, enhancing gene expression, and improving pathogen exclusion. These findings provide a scientific basis for exploring cucurbits as supportive components in functional foods, symbiotic formulations or dietary strategies aimed at promoting gastrointestinal health. Further studies involving purified phytochemicals, mechanistic analyses and in vivo models will help define their full therapeutic potential.

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Conflict of Interest: The authors do not have any conflict of interest.

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