In Vitro Validation Studies For Adhesion Factor and Adhesion Efficiency of Probiotic Bacillus Licheniformis MCC 2514 and Bifidobacterium Breve NCIM 5671 on HT-29 Cell Lines

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Abstract

Probiotic bacterial adhesion to the epithelial cell is a composite process and *in vivo* adhesion studies can be strengthened with the improved *in vitro* models for preliminary screening of potentially adherent strains. With this rationale, the study aimed is the first report to demonstrate the colonizing efficiency of probiotic *Bacillus licheniformis* MCC 2514 in comparison to *Bifidobacterium breve* NCIM 5671on HT-29 cell line. *B. licheniformis* (54.28 ± 0.99%) and *Bif. breve* (70.23 ± 0.85%) adhered in a higher percentage on fibronectin and mucin, respectively. However, the adhesion was higher for *B. licheniformis* when compared to *Bif. breve*. In adhesion score, *B. licheniformis* obtained about 138.85 ± 12.32, whereas *Bif. breve* got the score of 43.05 ± 9.12. The same trend continued in the adhesion percentage study, where *B. licheniformis adhered* 75.5 ± 5.2%, higher than *Bif. breve* adhered 32.66 ± 3.2%. In invasion assay, both the bacteria significantly decreased the colonization of the pathogen *Kocuria rhizophila* ATCC 9341 about 97.32 ± 0.81% in the competitive assay, 97.87 ± 0.73% in exclusion assay and 82.19 ± 2.51% in displacement assay. The cytotoxicity effects of the test bacterial strains against HT-29 cell line through MTT assay determined no viability loss in the treated cells. Therefore, the data obtained from the *in vitro* studies showed that both *B. licheniformis* and *Bif. breve* had shown significantly good invasion on pathogen and adhesion capacity on HT-29 cell line.

1. Introduction

Probiotics are 'live micro-organisms which when administered in adequate amounts confer a health benefit on the host' (Hill et al. 2014). Bacterial adherence to the host tissue and colonization has been the prominent step for bacterial infection (Ayala et al. 2017). The capability to adhere to the mucosal surface can be a reasonable advantage for thriving in humans' gastrointestinal tract (Bernet et al. 1994). In many probiotic bacterial strains, effective colonization can also be evaluated to adhere to the epithelium cell where the mucosal surface has been suggested to be an important trait for activity (Duary et al. 2011). Studies have suggested a wide range of adhesion factors responsible for the adhesion of bacteria to the human gut system. The major common bacterial adhesion factor is fibronectin-binding protein (Romberger 1997), mucus binding protein (Johansson et al. 2008), extracellular appendages such as flagella, pili and fimbriae (Erdem et al. 2007), sortase dependent proteins (Marraffini et al. 2006), lactobacilli surface layers proteins (S-layers) (Palva 2005), multifunctional *Lactobacillus* mucus adhesion protein (Tassell and Miller 2011), lectin-like mucus adhesins (Juge 2012), Numerous human enteric pathogens attach to the human histo blood group antigens (HBGAs) demonstrated on the gut mucosa, such as *Helicobacter pylori*, *Norwalk virus* and *Campylobacter jejuni* (Lindell et al. 2008; Magalhães et al. 2010). The probiotic micro-organisms, which are used as starter cultures in different food products, adhere to intestinal epithelium cells and play a crucial part in the host immunomodulation process(Neish 2009; Swain et al. 2014).

Even after being beneficial bacteria, *Bacillus* spp. never gained much importance because of some toxic and pathogenic family members such as *B. cereus*, *B. weihenstephanensis*, *B. anthracis*, and *B. thuringiensis* (Elshaghabee et al., 2017). Most of the studies on *Bacillus* concentrated only on industrial

usage and very few on food products where the exposure to probiotic aspects of *Bacillus* are not well studied. Understanding the probiotic attribute of the *Bacillus licheniformis* would lead to new opportunities in the field of *Bacillus* utilization as probiotic bacteria. A combination of facultative anaerobic *Bacillus licheniformis* and anaerobic *Bifidobacterium breve* can be the better competitor for the gut system's pathogen. Bifidobacteria inhabits in the gut system in the very early stages of life, making a permanent member of the GIT and bacilli a non-member. The introduction of these two bacteria, which colonize in different parts of the colon, can affect the colonizing factor of the pathogen.

B. licheniformis MCC 2514 is a native isolate obtained from goat milk, which has exhibited major probiotic attributes such as the ability to survive under gastric conditions like low pH, pepsin; intestinal conditions such as high pH; in the presence of trypsin and pancreatin. It also exhibited antioxidant, antimicrobial, cell hydrophobicity and auto-aggregation property (Shobharani and Halami 2014). *Bif. Breve* is a native isolate obtained from an infant fecal sample that has exhibited safety and functional attributes such as phytase activity, milk fermentation ability, antioxidant activity and antimicrobial activity (Achi and Halami 2019). In the gastrointestinal tract, the epithelium is being protected using many mechanisms from pathogen bacteria. One of the main mechanisms in reducing pathogenic infection is competition of micro-organism for adhesion sites with antimicrobial components produced by probiotic bacteria or deploying probiotic bacteria itself against pathogen bacteria(Ouwehand and Vesterlund 2003; Baccigalupi et al. 2005). This study was carried out to understand the binding and adhesion efficiency of very less studied *Bacillus* spp. as a co-culture with well-studied bifidobacteria under *in vitro* condition also in addition comparative evaluation of the *Bacillus licheniformis* and *Bifidobacterium breve* as single culture and as a co-culture against the foodborne pathogen *Kocuria rhizophila*, factors affecting adhesion, bacterial adhesion, inhibition studies and cytotoxicity study was assessed.

2. Materials And Methods

2.1 Materials

McCoy's medium, phosphate buffer saline, trypsin-EDTA solution, T25 cell culture plates, T75 cell culture plates, de Man, Rogosa and Sharpe (MRS) agar, brain heart infusion broth, trypton-yeast broth, fetal bovine serum (Himedia, Mumbai, India), 5 mg streptomycin and 5000 units penicillin per ml, DMSO, L-cystine HCl, Giemsa stain, mucin and fibronectin (Sigma, Bangalore, India) 6 well plates (Corning, India), 12 well plates (Eppendroff, India), were procured for experimental studies until otherwise mentioned.

2.2 Bacterial strains and culture conditions

Bacillus licheniformis MCC 2514 isolated from goat milk (Shobharani and Halami 2014), a fecal isolate of *Bifidobacterium breve* NCIM 5671 (Achi and Halami 2019) and *Kocuria rhizophila* ATCC 9341 were used in the study. Potential probiotic cultures *Bacillus licheniformis* and *Bifidobacterium breve* are native laboratory isolates used to screen the adherence potential against cancerous epithelium HT-29 cell lines, which was procured from National Centre for Cell Sciences (NCCS), Pune, India. *B. licheniformis* were

cultured in optimized trypton-yeast media, *Bif. breve* was grown in MRS medium with 0.05% L-cystine HCl and *K. rhizophila* was cultured in BHI (Brain Heart Infusion) medium. *B. licheniformis* and *K. rhizophila* were grown at 37° C for about 18 h in a shaker incubator at 150 rpm and *Bif. breve* was incubated on CO_2 incubator at 37°C for 24 h. In the entire study, *B. licheniformis* and *Bif. breve* was used as an individual culture and as co-cultures.

2.3 Adhesion to mucin and fibronectin

The ability of *B. licheniformis* and *Bif. breve* to bind fibronectin and mucin was evaluated as described by Roos and Jonsson (Roos and Jonsson 2002; Archer and Halami 2015). Briefly, the 96 well microtiter plates were coated with fibronectin (50 µg/mL) and 150 µl/well of mucin (100 µg/ml) dissolved in 50 mM Na_2CO_3 buffer with a pH of 9.7. The 96 well plates were incubated overnight at 4° C with minimum shaking and later blocked with phosphate-buffered saline (PBS) of 0.2 mL supplemented with 1 % Tween20 for one h and it was thoroughly washed with PBST (0.05 % Tween 20 with PBS). Overnight grown bacterial cultures were washed using PBST, diluted to the OD600 of 1.0 in the same buffer and 150 µL was added to the wells and incubated at 37°C for one h. All the wells were thoroughly washed with PBST and cell adherence was witnessed under the inverted microscope. Besides, to remove the adhered bacteria, 0.2 mL of Triton X-100 (0.5 %) was used and incubated at 37°C for two h. From every well, 100 µL of cell suspension was diluted and plated in respective medium and CFU count was observed.

2.4 Maintenance of HT-29 Cell lines

The HT-29 Epithelium cells were grown in McCoy's medium with 10% (v/v) of heat-inactivated (56°C, 30 mins) fetal bovine serum, 5 mg streptomycin and 5000 U penicillin /ml in 25 cm^2 culture flask at 37°C in 5 % CO_2. The incubated cells were fed with fresh medium every alternate day. After cells reaching confluency around 80%, the cells were collected by incubating adhered cells with 0.25% Trypsin-EDTA solution at 37°C. The cells were centrifuged (1200 rpm, 2 mins at room condition) and the cells were re-suspended in McCoy's medium. Before incubating, the cells were counted using a hemocytometer (Rohem, India) and introduced into respective assay plates. For six-well plates, 3 x 10^5 cells/well, for 12 well plates 7.5 x 10^4 cells / well and T25 flask 1 x 10^6 cells / well were added as described earlier by Gagnon et al. 2013.

2.5 Binding efficiency on HT-29 cell line

Adhesion assay on HT-29 was carried out with the previously described procedure with slight changes (Duary et al., 2011). Adhesion of the *B. licheniformis and Bif. breve* cultures to the HT-29 cell line were measured. The cell suspension with 3 x 10^5 cells added in 3 ml McCoy's medium was transferred to each well into six-well adherent culture plates. The medium was replaced every alternate day. When these cells reached a confluency of 80%, the medium was replaced every day repeatedly for about 15–20 days. The exhausted medium was decanted three h prior to the adhesion assay and cells were introduced with McCoy's medium without antibiotics and incubated at 37° C for three h in 5% CO_2. After the incubation period, cells were thoroughly washed with 3 ml phosphate-buffered saline (PBS) twice. The *B.*

licheniformis and *Bif. breve* (at 1 x 10^8 CFU/ml) cells were added to 1.0 ml McCoy's medium (without antibiotics and FBS) and introduced into different wells. These treated plates were incubated at 37°C for four h in 5% CO_2. The monolayers were washed five times thoroughly using sterile PBS.

2.6 Adhesion score, adhesion percentage and SEM analysis

By following the procedure described in Sect. 2.5, adhesion study experiments were carried out, 3 ml of methanol was introduced into each well containing the cells and incubated for 10 min at room condition. Methanol was aspirated completely and cells were stained using 1:20 diluted Giemsa for 90 min at ambient condition. The wells which were fixed are washed using pure ethanol to take out the unwanted stain. The air-dried plates were examined under 100x magnification (BX 5, Olympus, Japan). The bacteria were counted in 20 random microscopic fields and were labeled into different groups according to their counts, such as strongly adhesive (> 100 bacteria/field), adhesive (41−100 bacteria/field) and non-adhesive (≤ 40 bacteria/field). Continuing the adhesion assay procedure, the cell monolayer from the treated plate was separated using trypsinization. A 0.25% trypsin-EDTA solution was introduced into each well of six-well plates and incubated for 15 min at room condition. The detached cells were gently homogenized to make a uniform suspension. The cells were then plated on Tryptone-yeast agar for *B. licheniformis* and plated on MRS agar for *Bif. breve* using serial dilution. After the incubation at 37°C for 24−48 h, colonies were counted (B_1 CFU/ml). Bacterial cells initially added to each well of six-well plates were also counted (B_0 CFU/ml).

The adhesion percentage was calculated using the below formula:

% adhesion= (B_1 / B_0) * 100

For scanning electron Microscopy observation, the HT-29 cells were grown and maintained on glass coverslips until the monolayer is achieved. Later cells were fixed using 2.5% glutaraldehyde solution and incubated at 4°C for overnight. The ascending gradation of alcohol dehydration (30%, 50%, 70%, 90%, 95% and 100%) was carried on coverslips and finally, air-dried coverslips were subjected to gold plating and observed under SEM (LEO 435 VP, Carl Zeiss, Cambridge, UK) (Manjulata et al. 2018).

2.7 Inhibition of *Kocuria rhizophila* from colonizing HT-29 cell line

Inhibition of foodborne pathogen *Kocuria rhizophila* by *B. licheniformis* and *Bif. breve* from colonizing on HT-29 cell line was measured by the following assays as described before with slight modifications (Kumar et al. 2011).

For competition assay, both *B. licheniformis* and *Bif. breve* (1 x 10^8 CFU/ml) and *K. rhizophila* (1 x 10^8 CFU/ml) added in one ml Mc Coy's medium (without antibiotics and FBS) and suspended into HT-29 cells and incubated at 37°C for 90 min in 5% CO_2 condition. After the incubation time, non-adhered bacterial cells were detached by washing the wells thoroughly with PBS trice and adhered bacterial cells were

obtained by treating with 0.25% trypsin at 37°C for 10 min. The obtained bacterial cells were plated in respective medium (Optimized tryptone-yeast medium for *B. lichenifomis*, MRS agar for *Bif. breve* and BHI agar for *K. rhizophila*) for their culturing. The total bacterial count was denoted in log CFU/ml. Control or un-treated wells were maintained for both *B. lichenifomis* and *Bif. breve* and *K. rhizophila*, and they were standard for all of the assays. For exclusion assay, *B. lichenifomis* and *Bif. breve* (1×10^8 CFU/ml) cells were introduced to the HT-29 cell line and incubated at 37°C for 90 min in 5% CO_2. Weakly attached cells were removed by thoroughly washing with PBS. After washing, *K. rhizophila* (1×10^8 CFU/ml) cells were added to the HT-29 cells, which are already colonized by *B. lichenifomis* and *Bif. Breve* was allowed for incubation at 37° C. At the end of the incubation, weakly attached cells were detached using PBS wash thoroughly, and bacterial cells adhered to were obtained using trypsinization and CFU/ml. For displacement assay, initially *K. rhizophila* in the concentration of 1×10^8 CFU/ml cells were introduced on HT-29 cells and incubated at 37° C for 90 min in 5% CO_2.. Weakly attached cells were detached by washing thrice with PBS wash. After the wash, *B. lichenifomis* and *Bif. breve* (1×10^8 CFU/ml) were added to HT-29 cells which are already adhered with *K. rhizophila*. The treatment which is mentioned in the exclusion assay was followed. All three assays were carried out in triplicates, and results were interpreted statistically. SEM analysis was determined with glutaraldehyde and alcohol dehydration in ascending gradation (Manjulata et al., 2018).

2.8 Cytotoxicity studies using MTT assay and confocal microscope

To study the cytotoxic effects of bacteria on the epithelium cell line, MTT assay and confocal microscopy were performed (Xi et al. 2009). The *B. licheniformis* and *Bif. breve* cultures to the HT-29 cell line were measured. The HT-29 cell suspension with 4×10^4 cells /ml cell concentration prepared in 0.1 ml complete McCoy's medium and it was transferred to the individual well of 96-well culture plates. These plates were incubated at 37°C for 48 h in 5% CO_2 for better adhesion and growth. After the incubation, the 96 wells were thoroughly washed using PBS and cells were fed with McCoy's medium lacking antibiotics of 1×10^8 CFU/ml of both *B. licheniformis and Bif. breve* and were incubated at 37°C for four h. Later, the incubated cells were washed 3 to 5 times thoroughly using PBS to remove bacteria from the wells and a 10 µl of MTT reagent was added to the cell containing fresh Mc Coy's medium and incubated at 37°C for four h in 5% CO_2. The medium was aspirated from the well and 100 µl of DMSO reagent was added and incubated at 37°C for 30 mins. Reading taken at 570 nm and the percentage of the viability was calculated using the below equation

% Viability = Q_1/Q_0 x 100. ($Q_0 =$ control reading, Q_1 = treated reading)

For confirmation of cytotoxicity on HT 29 cell line, confocal microscopy for the *B. licheniformis* and *Bif. breve* cultures were performed. The HT-29 cell with the concentration of 3×10^5 cells was prepared in 3 ml McCoy's medium and it was added to every individual well of 12-well culture plates. It was incubated at 37°C for 48 h for better adhesion and growth. After the incubation, wells were thoroughly washed using

sterile PBS and cells were fed with McCoy's medium lacking antibiotics with the bacterial concentration of 1 x 10^8 of both *B. licheniformis* and *Bif. breve* and incubated at 37°C for four h. The incubated cells were then washed with sterile PBS several times to remove the bacterial cells and treated with 0.5 ml of absolute chilled alcohol and incubated for two h at -20°C. After incubation, the cells were suspended in 0.5 ml of PBS to avoid drying of the cell until the microscopy observation. Fluorescence dyes such as Acridine orange and ethidium bromide (EtBr) dye were added 5 mins before the observation under the confocal microscope (LSM 700, Carl Zeiss, Germany).

2.9 Statistical analysis

All the data was subjected to statistical analysis was performed using one-way ANOVA using the Graph Pad Prism 7. All data are denoted as mean ± standard deviation (SD). In all the experiments, significance was set at $P < 0.05$.

3. Results

3.1 Binding efficiency of *B. licheniformis* and *B. breve* to mucin and fibronectin

The adhesion factors play a critical role in the adhesion process in bacteria on epithelium cells in GIT. The adhesion efficiency in *B. licheniformis* and *Bif. breve* using mucin and fibronectin was analyzed in this experiment. On adhesion to mucin, both the bacteria have shown better adhesion of 70.23 ± 0.85 % for *Bif. breve* and 65.01 ± 0.11% for *B. licheniformis.* In adhesion to fibronectin, B. licheniformis had a higher adhesion percentage of 54.28 ± 0.99 compared to *Bif. breve* of 39.66 ± 0.74%, indicating both the bacteria had better adhesion on both mucin and fibronectin. The all values are the mean ± SD (*n = 3*).

3.2 Adhesion of probiotic bacteria on HT-29 cells line using score and percentage method

Adhesion efficiency can be calculated on the number of bacteria adhered to GIT. The numbers were estimated using the score and percentage method. Considering scoring method for adhesion analysis, both *B. licheniformis* and *Bif. breve* adhered to HT-29 cell lines through different points. In this evaluation, *B. licheniformis* adhesion score with 138.85 ± 12.32 adhere significantly strong when compared to *Bif. breve* with a score of 43.05 ± 9.12. Inoculation of *B. licheniformis* and *Bif. breve* as a co-culture did not show much deviation. *B. licheniformis* has the same adhesion score 135.43 ± 8.49, significantly higher adhesion on the comparison with *Bif. breve* 34.04 ± 8.81 (Fig. 1). The microscopic observation gives an identity proof for the adhesion of bacteria in Fig. 1.

Adhesion percentage was calculated by plating on MRS agar for *Bif. breve* and TY agar for *B. licheniformis* (Table 1). Adhesion of *B. licheniformis* was significantly high of 75.5 ± 5.2% in comparison to the adhesion of *Bif. breve* 32.6 ± 3.2% which was moderate. It was observed that under co-culturing of the test bacteria, the percentage of *Bif. breve* was decreased to 24.5 ± 1.1% and *B. licheniformis* was 75.5

± 2.6% with significant changes. The results indicated that *B. licheniformis* had better adhesion when compared to *Bif. breve* (Fig. 1). The adhesion efficiency of test bacterial results was documented using a scanning electron microscope for better understanding. Adhesion of the bacteria in combination adhered together can be observed in the same field circled in Fig. 1.

Table 1
Adhesion percentage of *B. licheniformis* and *Bif. Breve* in CFU/ml.

Group	CFU/ml
B. licheniformis Control	$8.1 \pm 0.2 \times 10^7$
B. licheniformis After adhesion	$6.1 \pm 0.4 \times 10^7$
B. licheniformis in combination After adhesion	$6.1 \pm 0.2 \times 10^7$
Bif. breve Control	$7.8 \pm 0.0 \times 10^7$
Bif. breve After adhesion	$2.5 \pm 2.6 \times 10^7$
Bif. breve in combination After adhesion	$1.9 \pm 0.0 \times 10^7$
The value presented is the mean ± SD (n = 4) of adhesion of *B. licheniformis & Bif. breve* on HT-29 cell lines	

3.3 Invasion assays of probiotic bacteria on *K. rhizophila*

The inhibition efficiency of probiotic bacteria against pathogen *K. rhizophila* was performed using three assays. The control log CFU/ml recorded was 7.62 ± 0.021 for *B. licheniformis*, 7.50 ± 0.036 for *Bif. breve* and 7.73 ± 0.20 for *K. rhizophila.* The *K. rhizophila* was treated with both test cultures individually as well as a co-culture (Tables 2 & 3). Untreated control remained constant for all three experimental assays. The results of the assay were documented as a scanning electron microscopic observation (Fig. 2).

Table 2

Invasion assay against *K. rhizophila* using probiotic *B. licheniformis* and *Bif. Breve*

	K. rhizophila	Initial log CFU/ml of *K. rhizophila*	Final log CFU/ml of *K. rhizophila*	Reduction of Adhesion in % *K. rhizophila*
Competition assay	Against *B. licheniformis*	7.73 ± 0.20	7.02 ± 0.02	89.44 ± 2.02
	Against *Bif. breve*		7.16 ± 0.08	75.16 ± 4.58
	Against *B. licheniformis & Bif. breve*		6.20 ± 0.17	97.32 ± 0.81
Exclusion assay	Against *B. licheniformis*	7.73 ± 0.20	6.93 ± 0.16	85.58 ± 3.77
	Against *Bif. breve*		7.160 ± 0.10	76.13 ± 3.20
	Against *B. licheniformis & Bif. breve*		6.10 ± 0.17	97.87 ± 0.73
Displacement assay	Against *B. licheniformis*	7.73 ± 0.20	7.2 ± 0.06	71.33 ± 5.18
	Against *Bif. breve*		7.25 ± 0.09	70.61 ± 5.07
	Against *B. licheniformis & Bif. breve*		7.04 ± 0.03	82.19 ± 2.51
The value presented is the mean ± SD (n = 3) of adhesion of *K. rhizophila* on HT-cell lines				

<div align="center">Table 3

Invasion assay against *K. rhizophila* using probiotic *B. licheniformis* and *Bif. Breve* (plating CFU/ml)</div>

	K. rhizophila	Initial CFU/ml of *K. rhizophila*	Final CFU/ml of *K. rhizophila*
Competition assay	Against *B. licheniformis*	$6.23 \pm 0.41 \times 10^7$	$1.06 \pm 0.04 \times 10^7$
	Against *Bif. breve*		$1.56 \pm 0.38 \times 10^7$
	Against *B. licheniformis & Bif. breve*		$1.66 \pm 0.47 \times 10^6$
Exclusion assay	Against *B. licheniformis*	$6.23 \pm 0.41 \times 10^7$	$9 \pm 0.29 \times 10^6$
	Against *Bif. breve*		$1.50 \pm 0.29 \times 10^7$
	Against *B. licheniformis & Bif. breve*		$1.33 \pm 0.47 \times 10^6$
Displacement assay	Against *B. licheniformis*	$6.23 \pm 0.41 \times 10^7$	$1.76 \pm 0.20 \times 10^7$
	Against *Bif. breve*		$1.83 \pm 0.33 \times 10^7$
	Against *B. licheniformis & Bif. breve*		$1.10 \pm 0.08 \times 10^7$
The value presented is the mean ± SD (n = 3) of adhesion of *K. rhizophila* against probiotic cultures on HT-29 cell lines			

Invasion assay using simultaneous colonization (Competition assay), the count of *K. rhizophila* when treated with *B. licheniformis* the count of *K. rhizophila* was decreased to 7.02 ± 0.02 log CFU/ml ($10.56 \pm 2.02\%$) and with *Bif. breve* the count of *K. rhizophila* decreased to 7.16 ± 0.08 CFU/ml ($24.84 \pm 4.58\%$). When both *B. licheniformis* and *Bif. Breve* used as co-culture, the pathogen reduction was shown to be 6.20 ± 0.17 CFU/ml ($2.68 \pm 0.81\%$), which indicates that test culture has decreased the count of pathogen significantly ($p < 0.05$). On exclusion assay wherein the cells of *K. rhizophila* are treated with *B. licheniformis*, the count of *K. rhizophila* was decreased to 6.93 ± 0.16 log CFU/ml ($14.42 \pm 3.77\%$) and when treated with *Bif. breve* it decreased to 7.16 ± 0.10 CFU/ml ($23.87 \pm 3.20\%$). Co-culturing of both *B. licheniformis* and *Bif. Breve*, it reduced to 6.10 ± 0.17 CFU/ml ($2.13 \pm 0.73\%$), indicating that the test culture has decreased the pathogen significantly ($p < 0.05$).

During the post-colonization of probiotics over *K. rhizophila (displacement assay)*, *K. rhizophila* was treated with *B. licheniformis* and the count of *K. rhizophila* was decreased to 7.2 ± 0.06 log CFU/ml

(28.67 ± 5.18%) when treated with *Bif. breve* decreased to 7.25 ± 0.09 CFU/ml (29.39 ± 5.07%). In the co-culturing of both *B. licheniformis* and *Bif. Breve*, it reduced to 7.04 ± 0.03 CFU/ml (17.81 ± 2.51%). The results indicated that test culture had decreased the pathogen significantly (p < 0.05) (Fig. 2). The invasion of probiotic bacteria on pathogen can be seen where the cell count of the *K. rhizophila* is very low when treated with *B. licheniformis* and *Bif. breve*. Co-culturing of bacteria incubated together can be seen in the highlighted circle in Fig. 2. Overall, the simultaneous and pre inoculation of probiotic bacteria has inhibited the pathogen.

3.4 Viability count of HT-29 on the treatment of probiotic bacteria

The viability of the epithelium cells (HT-29) on the treatment of *B. licheniformis* and *Bif. breve* was checked using MTT assay. Viability count did not show much deviation on the treatment either with *B. licheniformis* or *Bif. breve* alone or in combination. Viability count did not show much deviation with the treatment of test bacteria as a co-culture compared to the treatment of bacteria as an individual culture. At 1×10^8 cell concentration, the Percentage of viability was found as 93.53 ± 0.53%, 94.78 ± 2.27% and 98.80 ± 0.97% on the treatment of *B. licheniformis*, *Bif. breve* and in combination, respectively. The viability of the cell line was documented using confocal microscopic observation Fig. 3.

4. Discussion

In vivo animal models are the chosen approach for proving safety and probiotic bacterial efficiency towards effective colonization in the gut. Traditional safety valuation methods depending on toxicological screening in animals might not be valid in the circumstance of foods in many cases (Fao et al. 2002). No particular guiding principle is present for evaluating probiotics, but studies such as a repeated administration of chronic toxicity and acute toxicity assessment have been suggested (Ishibashi and Yamazaki 2001). Some *in vitro* studies have been carried out for estimating various characters of potential probiotic bacteria. The bile content and tolerance of the low pH in the upper part of the gut system are the first line of challenge and colonizing in the lower part of the intestine is the second and most important aspect. Adhesion to the intestine is the well-preferred mechanism observed in probiotic bacteria for their establishment and demonstrates probiotic effect (Lee and Salminen 1995).

Concerning microbiota, the mucus has a dual role where it provides a primary adhesion site and matrix where bacteria can multiply and increase. It also protects from undesired contact with pathogenic bacteria (Van Klinken et al. 1995). Few strains of *Lactobacillus fermentum* have shown good adhesion to mucin and fibronectin (Archer and Halami 2015). Certain strains of *B. cereus* have shown great adhesion on mucin and fibronectin (Sánchez et al. 2009) and *B. licheniformis* had shown higher mucin binding capacity in the study conducted earlier. An earlier study has shown greater adhesion of spores to hydrophilic and hydrophobic surfaces than vegetative cells (Shobharani and Halami 2014). On similar lines to the existing reports, the present study on both *B. licheniformis and Bif. breve* has shown a greater binding capacity to mucus membrane. *Bif. breve* adhered in higher percentage in comparison to *B.*

licheniformis. In the fibronectin study, *B. licheniformis* has shown higher adhesion percentage when compared to *Bif. breve*. Depending on the hydrophobicity characteristic, the *Bacillus* spores can also adhere to a different kind of surface, such as stainless steel (Peng et al. 2001). *B. licheniformis* has exhibited good cell hydrophobicity toward xylene, toluene, and hexadecane under *in vitro* conditions stimulated with solvents (Shobharani and Halami 2014).

In this investigation, the adhesion capacity of the probiotic bacteria was evaluated using adhesion score and adhesion percentage. On proportional valuation based on adhesion score, *B. licheniformis* showed significantly strong adhesion compared to *Bif. breve*. Both the bacteria were incubated together for compatibility and to check the probiotic effect in combination. No much deviation was found concerning *B. licheniformis* and a slight decrease was observed in the case of *Bif. breve*. In another study reported earlier, maximum adherence was found by *Lactobacillus acidophilus* LA 1 strain of 124 ± 13 (Bernet et al. 1994) and by *Lactobacillus paraplantarum* 128 (Manjulata et al. 2018), which gives a similar result to that of *B. licheniformis*. Other studies with *Lactobacillus* sp. have displayed a lesser adhesion score of 38–55 (Jacobsen et al. 1999) compared to *B. licheniformis* but higher than *Bif. breve*. The adhesion score of *Bif. breve* aligned with earlier studies reported with an adhesion score of 30 ± 2 on HT-29 cell line (Bernet et al. 1993) and *Bif. longum* has been reported with a little higher adhesion score (Del Re et al. 2000). The spore-producing *B. licheniformis* had significantly higher adhesion when compared to *Bif. breve*. When both the cultures were inoculated in combination together, the result was a similar but slight decrease in *Bif. breve* cell count was noticed. Other studies suggest *that B. tequilensis has shown* less adhesion percentage on human colon carcinoma epithelial cells such as HCT-116 (Rani et al. 2016) and *B. subtilis* on HT29-16E, Caco-2 and HEp-2 cell line. *Bif. bifidum* and *Bif. longum* have demonstrated higher adhesion percentage on Caco-2 cell line in previous studies (Achi and Halami 2019).

Adhesion of pathogens to the epithelium is an important step as it permits the discharge of enzymes and toxins, starting necrotic processes directly to the target cell and assisting the invasion (Bernet et al. 1994). By deploying the probiotic bacteria and the production of antimicrobial component and other organic compounds from these bacteria, using microbial pathogens, the decrease in pathogenic contaminations through competition for adhesion sites can cause greater damage for pathogen colonization (Kumar et al. 2011). The inhibitory study was carried out to understand the efficiency of *B. licheniformis* and *Bif. breve* against colonizing of foodborne pathogen *K. rhizophila*. In invasion assay, the adhesion of *K. rhizophila* was out numbered on the introduction of probiotic *B. licheniformis* and *Bif. Breve*. In all the three assay's, the reduction of *K. rhizophila* was in the range of 70–97%. Scanning electron microscope analysis confirmed the above-mentioned effect. In similar studies, *Lactobacillus plantarum* has outnumbered the pathogen *Vibrio parahaemolyticus* in all assay types (Kumar et al. 2011). In another study, adhesion of *Salmonella enterica* was decreased *by Lactobacillus paracasei* by fourfold in competitive and in exclusion experiment about seven-fold (Bernet et al. 1994)

Previous studies showed the *B. subtilis* and *Bif. indicus* seemed to show no indication of any toxicity(Hong et al. 2008). Azimirad et al. (2017) has demonstrated the non-toxicity of *B. coagulans* and *B. subtilis* on HT29 cell lines(Azimirad et al. 2017). No cytotoxicity effect was found by using

exopolysaccharides produced *Bif. longum* on HT-29 (Inturri et al. 2017). In this study, our data suggested that both the bacteria, i.e., *B. licheniformis* and *Bif. breve* did not show any cytotoxic effect and no viability of HT-29 cell was affected when used as lone culture and combination. HT- cell line29 is the most commonly used human intestinal cell line displaying the physiological and morphological characteristics of normal human enterocytes, which has been used to explain the mechanism mediating bacterial adhesion (Kerneis et al. 1991; Bernet et al. 1994). The obtained results can be correlated to the human gut environment. The *in vitro* data can be used as a reference for the *in vivo* studies. The adhesion and invasion efficiency displayed by the *B. licheniformis* and *Bif. breve* on HT-29 cells can be expected during the *in vivo* studies. However, evaluating the results through in vivo or clinical trials will enhance the utilization of these bacterial strains in combination with food product development, nutraceutical or any other human beneficial usage.

5. Conclusion

The potential probiotic *B. licheniformis* has shown very good adhesion capacity on the gastrointestinal tract compared to well-studied *Bif. breve* which was indicated by the HT-29 cell line. The adhesion factor such as fibronectin-binding protein in *B. licheniformis* and mucin binding protein in *Bif. breve* was dominated and demonstrated the most influenced factor which is affecting the adhesion. *B. licheniformis* had displayed higher adhesion potential when compared to *Bif. breve*. Both the bacteria have shown good invasion ability against the *K. rhizophila.* During simultaneous colonization and pre-colonization, *K. rhizophila* has reduced drastically and in post-colonization, *K. rhizophila* has shown better colonization compared to the other two assays. Both the bacteria did not show any toxicity on the HT-29 cell line, which proved that both are efficient probiotic bacteria regarding colonization in the gut system. The spore-forming bacteria always have an advantage in adhesion when compared to the non-spore bacteria. Hence, the *B. licheniformis* has shown greater adhesion efficiency when compare to *Bif. breve*. Overall it is concluded that both under *in vitro* conditions *B. licheniformis* and *Bif. breve* has shown better adhesion and invasion ability and a better contender to colonize and gastrointestinal tract. The outcome of *In vitro* studies will be additional validation before performing the *in vivo* studies of probiotic and probiotic food formulation.

Declarations

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Conflict of interest

The authors have no conflict of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable

Consent for publication

Not applicable

Authors' contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Rohith HS. Rohith HS wrote the first draft of the manuscript and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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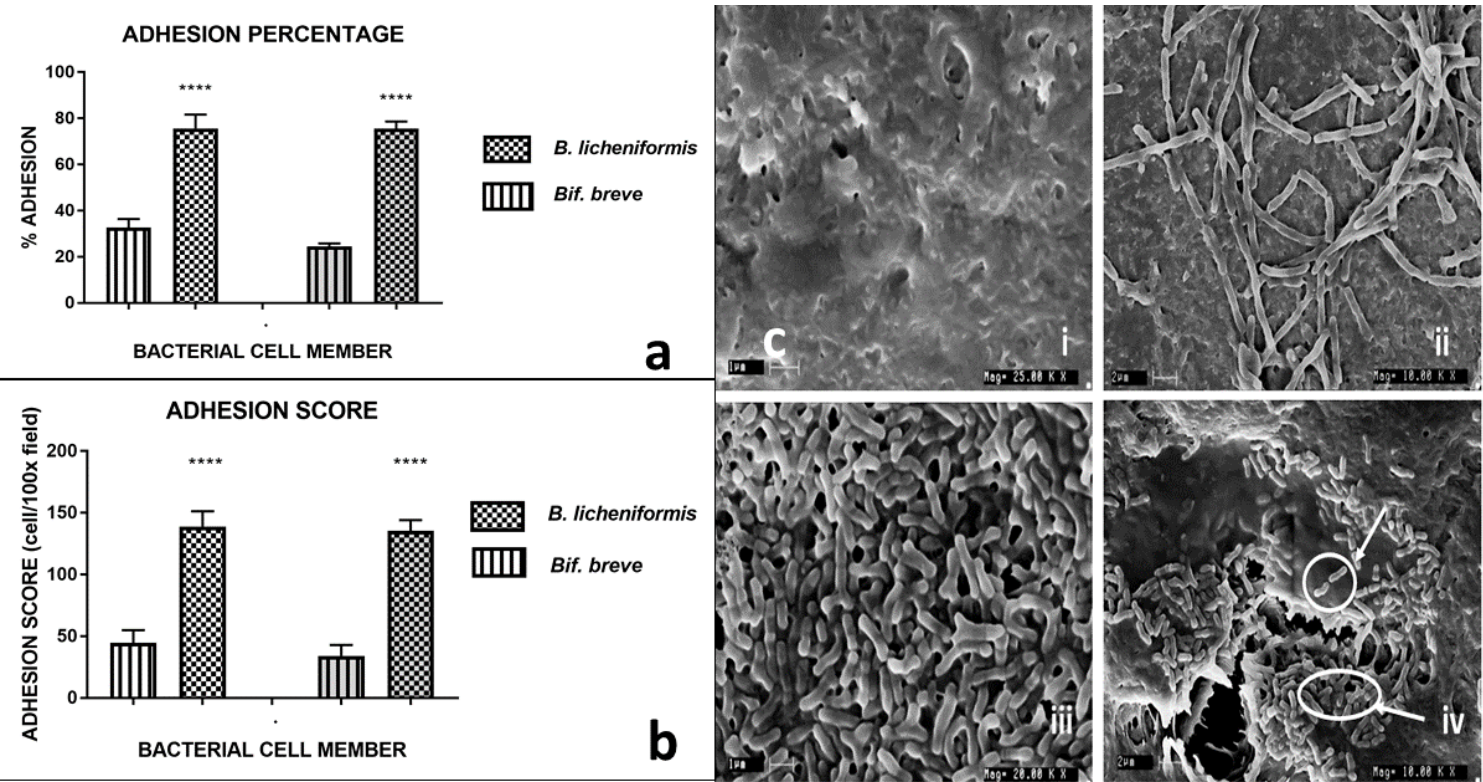
Figures

Figure 1

Binding efficiency of probiotic bacteria on HT-29 cell lines. a: Adhesion score of B. licheniformis and Bif. breve on HT-29 cell line. The values presented are the mean ±SD (n = 20). P-value <0.05 (**** = <0.0001), b: Adhesion percentage of B. licheniformis and Bif. breve on HT-29 cell line. The values presented are the mean ±SD (n = 3). P-value <0.05 (****= <0.0001), c: Scanning electron microscopic study of bacterial adhesion on HT-29 cell line (i: untreated cell, ii: B. licheniformis, iii: Bif. breve, iv: B. licheniformis and Bif. breve {upper circle indicates B. licheniformis and the lower circle indicates Bif. breve}). Magnification = a, c: 20.00 K X; b, d : 10.00 K X
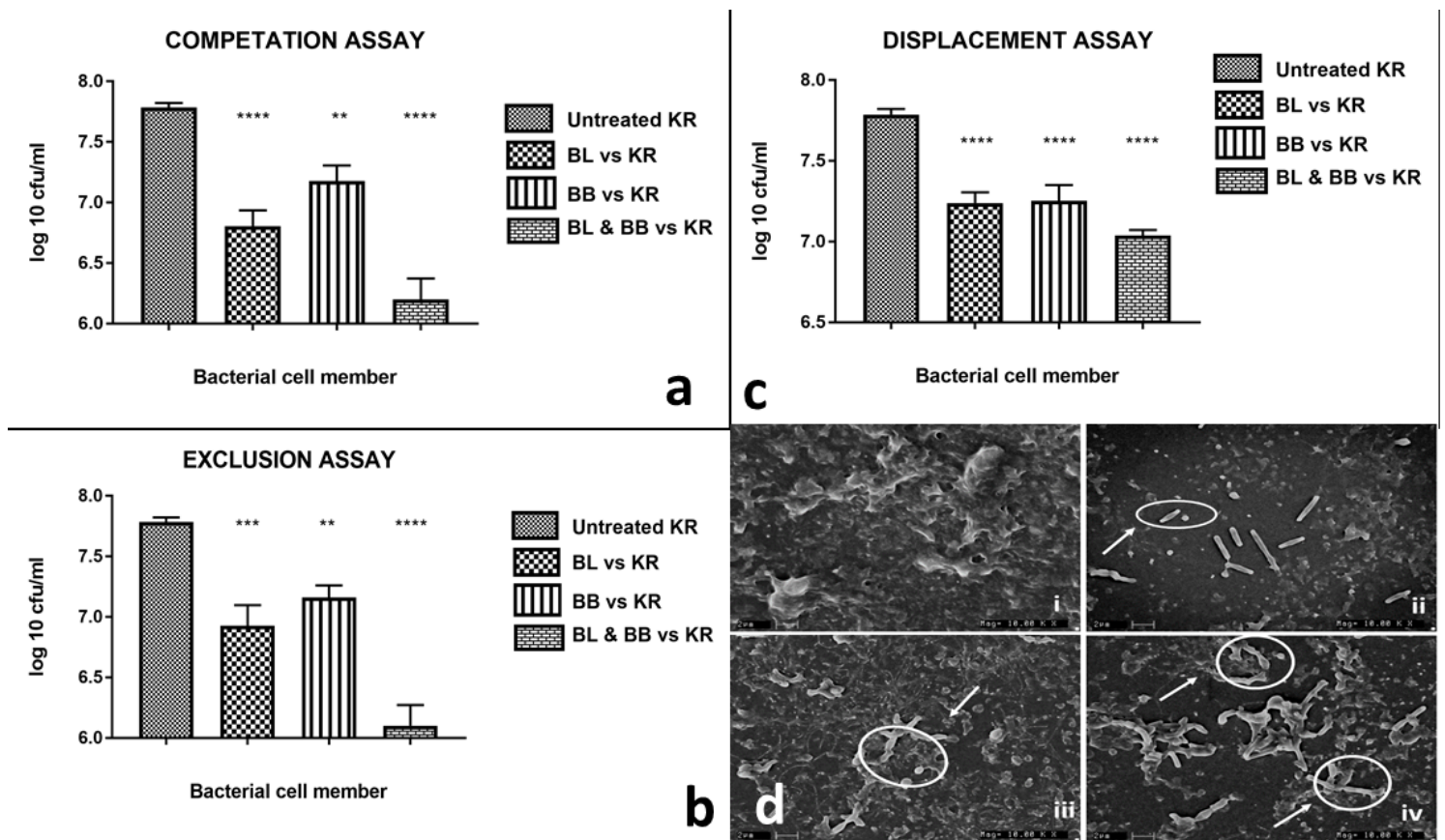
Figure 2

Invasion assay against K. rhizophila a: Competition assay, b: Exclusion assay, c: Displacement assay, d: Scanning Microscopic analysis of invasion assay on HT-29 cell line (i: untreated cell line, ii: B. licheniformis against K. rhizophila, iii: Bif. breve against K. rhizophila, iv: B. licheniformis and Bif. breve against K. rhizophila) encircled in image d(ii) indicates the combination of B. licheniformis and K. rhizophila, encircled in image d(iii) indicates the combination of Bif. breve and K. rhizophila, d(iv) indicate the combination of B. licheniformis, Bif. breve and K. rhizophila. Abbr. ML: K. rhizophila, BL: B. licheniformis, BB: Bif. breve). The values presented are the mean ±SD (n = 3). P-value = <0.05. (****= <0.0001, ***=0.0001, **=0.0012). Magnification: 10.00 K X
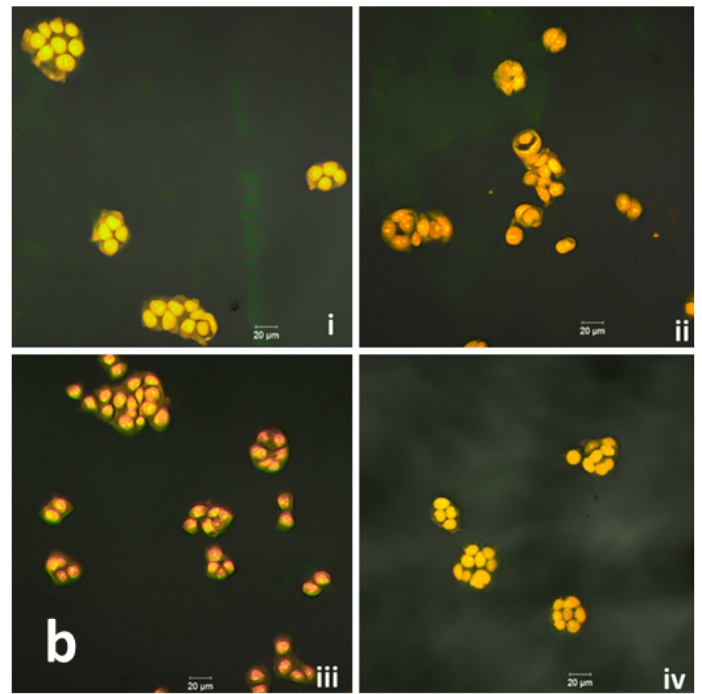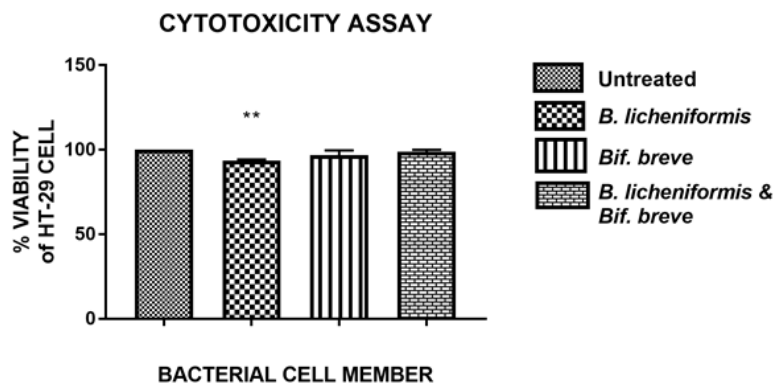
Figure 3

a: Cytotoxicity assay on HT-29 using MTT method using B. licheniformis and B. breve. The values presented are the mean ± SD (n = 3) P-value = <0.05. b: Confocal microscopic observation for the HT-29 cell viability (i: untreated, ii: B. licheniformis, iii: Bif. breve, iv: B. licheniformis and Bif. breve) Magnification: 10μM.