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Scanning Electron Microscopy Observation of Adhesion Properties of *Bifidobacterium longum* W11 and Chromatographic Analysis of Its Exopolysaccaride*

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Abstract

***Bifidobacterium* spp. can produce cell-bound or released exopolysaccharide (EPS) that is a beneficial trait mediating commensal-host interactions. However differences in the physico-chemical characteristics of EPS produced by different strains of *Bifidobacterium* spp. are determinant in adhesion ability and modulation of immune response. The aim of this study was to investigate the *in vitro* adhesion characteristics of *Bifidobacterium longum* W11 to intestinal epithelial cell-line HT-29, by Scanning Electron Microscopy (SEM), and chemical characteristics of its exopolysaccharide using Thin-Layer Chromatography (TLC) analysis. SEM observation showed a good adhesion of *B. longum* W11 to the HT-29 monolayer that could be increased by the production of exocellular polymers formation. TLC analysis of the purified and hydrolyzed EPS showed that the cell-surface and extracellular polysaccharide were composed mainly of fructose and glucose. Moreover, other sugars were present in smaller quantities. Information from this study on physico-chemical characteristics of EPS of *B. longum* W11 could contribute to understanding the physiology of bifidobacteria and their interaction with the host.**

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Keywords

***Bifidobacterium longum* W11, Adhesion, SEM, Exopolysaccharide, TLC**

1. Introduction

For probiotic bacteria, selected for commercial use in food and in therapeutics, the adhesion to human intestinal cells is considered a fundamental property. Adhesion enables probiotic strains to persist longer in the intestinal tract and, then, to stabilize the intestinal mucosal barrier, to provide competitive exclusion of pathogen bacteria and to better develop the metabolic and immunomodulatory activity [1]. *In vitro* evaluation tests indicated that adhesion ability and modulation of host immunity depend mainly on the strain [2]-[4]. Medina *et al.* (2007) compared the immunological properties of strains of *Bifidobacterium longum* and demonstrated that the different strains are potentially able to drive the immune response in the opposite direction *in vitro*, suggesting different immune potentials in clinical practice *in vivo* [2].

Several studies showed that some strains of *Bifidobacterium* spp. can produce cell-bound or released exopolysaccharides (EPSs) and that exopolysaccharides production is a beneficial trait mediating commensal-host interaction through immune modulation [5] [6]. However differences in the physico-chemical characteristics of EPS produced by *Bifidobacterium* spp. could be determinant for probiotic functionality and immunomodulation capability [7] [8]. Medina *et al.* (2007) studying the immunological properties of the structural cell components and the secreted molecules of the strains of *Bifidobacterium longum* did not investigate the production of exopolysaccharides [2]. The composition of EPS of *Bifidobacterium* spp. is important in understanding their interaction with the host, but the polymers have been characterized for only a few of the commercialized probiotic strains.

The strain *Bifidobacterium longum* W11 is widely employed in the formulation of most popular probiotic products and has received special attention for its probiotic effects and its long history of safe use in food supplement.

The aim of this study was to investigate the *in vitro* adhesion characteristics of *B. longum* W11 to human intestinal epithelial cell-line HT-29 by Scanning Electron Microscopy (SEM) and to characterize the chemical composition of its exopolysaccharide using chromatographic methods.

2. Materials and Methods

2.1. Bacterial Strain and Culture Conditions

Bifidobacterium longum W11, a probiotic strain commercialized by Alfa Wasserman (Italy) and produced by Probiotical (Italy), was tested, using a pack purchased in December 2013. The strain was routinely grown in MRSC broth, composed of MRS (deMan, Rogosa & Sharpe; Sigma Aldrich, Italy) plus 0.25% L-cysteine (Sigma Aldrich, Italy) at 37˚C under anaerobic conditions (80% N_2, 10% CO_2 and 10% H_2) using the AnaeroGen sachet (Oxoid, Italy) for 24 h. As standard procedure, the strain was cultivated in agar-MRSC and incubated at 37˚C in anaerobic atmosphere for 48 h.

2.2. Cell line and Culture Conditions

The human epithelial intestinal cell-line HT-29 (ATCC® HTB38™) was used in this study. The HT-29 cells were routinely grown in Dulbecco's modified Eagle's medium (DMEM, Sigma Aldrich, Italy), supplemented with 10% (v/v) heat-inactivated bovine fetal serum and 100 µg/ml of streptomycin (Sigma Aldrich, Italy), at 37˚C in a humidified atmosphere of 5% CO_2.

2.3. Observation of Adhesion on HT-29 Cells and Biopolymers Formation of *Bifidobacterium longum* W11 Using Scanning Electron Microscopy

For adhesion assays HT-29 cells (about 2×10^5 cells/ml) were seeded in 24-well tissue culture plates (Sigma Aldrich, Italy) on microscopy cover glasses in DMEM, and incubated at 37˚C in a humidified atmosphere of 5%

CO_2, until reaching 95 to 100% confluence (14 ± 1 days). Cell monolayers were washed twice with antibiotic free DMEM before bacteria cells were added. An overnight culture of *Bifidobacterium longum* W11 in MRSC-broth was centrifuged for 10 min at 3000 rpm, and the bacterial pellet was re-suspended in antibiotic free DMEM medium to a final concentration of about 1.5×10^8 CFU/ml, determined photometrically (OD_{600}) (Bio-Rad model 680). The wells with HT-29 cells and bacteria were incubated at different times (30, 60, 120 min) at 37˚C, in a humidified atmosphere of 5% CO_2.

The observation of the adhesion on HT-29 cells and biopolymers formation of *B. longum* W11 by Scanning Electron Microscopy (SEM) was done using the method described by Ali *et al*. (2009) [9]. After incubation, the HT-29 monolayer was washed three times for 5 min with Sodium Cacodylate Buffer 0.1 M, at pH 7.4, previously filtered (0.22 µm). Then, the cells were fixed with 4% (w/v) glutaraldehyde (Sigma Aldrich, Italy), in 0.1 M PBS (pH 7.2), for 12 h at 4˚C. The HT-29 monolayer, after washing three times with Sodium Cacodylate Buffer, was treated with 1% Osmium tetroxide (OsO_4) in Sodium Cacodylate Buffer 0.1 M (pH 7.4) for 1 h at 4˚C, washed again with 0.1 M Sodium Cacodylate Buffer twice for 10 min and dehydrated in a graded ethanol series (35% v/v, 50% v/v, 75% v/v, 100% v/v). Microscope cover glasses were then prepared for Critical Point Drying (CPD). The cells were dried and coated with gold. The microscope cover glasses were then examined with a Scanning Electron Microscope (SEM, Hitaki S 4000). During the experiment two wells containing only HT-29 cells were used as controls. Each assay was performed in duplicate to determine inter-assay variation.

2.4. Exopolysaccharide (EPS) Characterization

2.4.1. EPS Extraction

The exopolysaccharide (EPS) produced by *Bifidobacterium longum* W11 was extracted by the method described by Ruas-Madiedo *et al*. (2006), [10]. Cellular biomass was collected from two Petri Dishes (90 mm) with MRS agar using 2 ml of ultrapure water and a plastic L-shaped spreader. To release the polymer from the cell surface, 1 vol of 2 M NaOH was added to the cellular suspension and stirred overnight at room temperature. Afterwards the cells were removed by centrifugation (8400 g for 30 min) and EPS from the supernatant was precipitated for 3 days at 4˚C, using 2 vol. of absolute ethanol. The precipitated EPS fraction obtained after centrifugation was resuspended in ultrapure water and dialyzed (3 days at 4˚C) against the same daily-changed water using dialysis tubes (Sigma Aldrich) of 12 kDa molecular mass cutoff. The dialyzed EPS fractions were freeze-dried.

2.4.2. Quantitative Analysis of EPS

Quantitative analysis of the recovered EPS was carried out according to the method of Dubois *et al*. (1956) [11]. Briefly, two ml of EPS solution were pipetted into a colorimetric tube, and 0.05 ml of 80% phenol were added. Then, 5 ml of concentrated sulfuric acid were rapidly added. After 10 min the tubes were shaken and placed for 10 to 20 min in a water bath at 25˚C - 30˚C. Two ml of the solution were placed in a cuvette and measured by spectrophotometry (Agilent 8453). The absorbance of the characteristic yellow-orange color was measured at 490 µm for hexoses and 480 µm for pentose. The amount of EPS was determined by reference to the standard curve constructed using different concentrations (from 10 to 100 µg) of glucose solutions. All solutions are prepared in triplicate.

2.4.3. Hydrolysis of EPS

The monosaccharide composition of EPS was done modifying the method described by Yang *et al*. (2010) [12]. Briefly, the purified EPS sample (2 mg) was hydrolyzed with 1 ml of 2 M trifluoroacetic acid (TFA) at 100˚C for 1 h. Then the TFA was evaporated under a stream of nitrogen at 60˚C and the hydrolyzed EPS was solubilized in methanol.

2.4.4. Thin-Layer Chromatography (TLC) Analysis

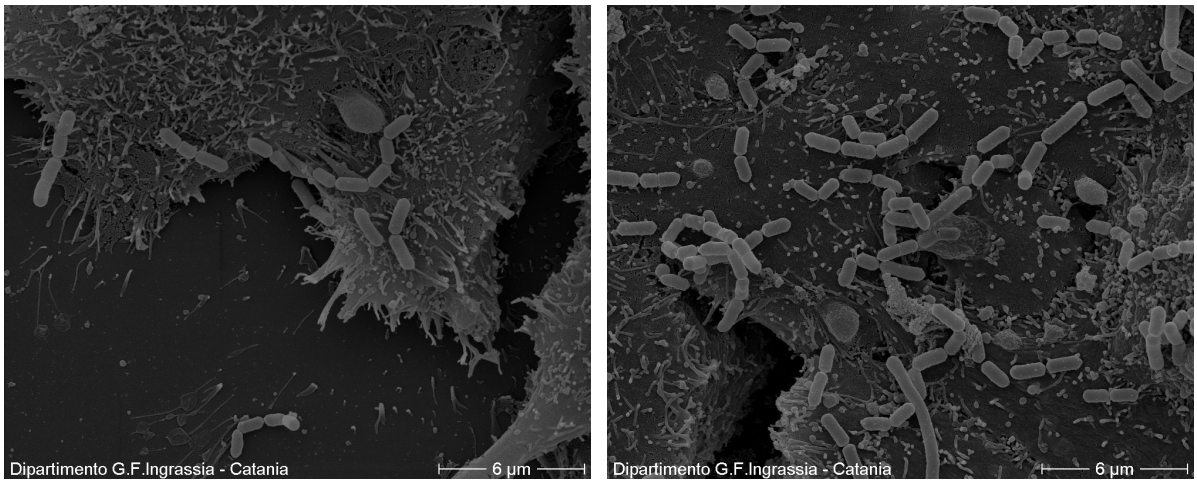
To identify the monosaccharides, the hydrolyzed EPS was analyzed by TLC using silica and cellulose as two different stationary phase supports. The silica plate was eluted using ethyl acetate-acetic acid-methanol-water/ (60:15:15:10). The spots of sugars were shown using, as chromogenic reagent, a solution of p-anisaldehyde and sulfuric acid (2:1) in acetic acid and heating the plate for 10 min at 100˚C. The cellulose plate was eluted using ethyl acetate-pyridine-water (40:20:30) (upper phase). In this case the spots of sugar were shown using, as chromogenic reagent, 0.1M p-anisidinephthalic acid in 96% ethanol and heating the plate for 10 min at 100˚C. As standard sugars, arabinose, fructose, fucose, galactose, glucose, mannose and xylose, were used.

3. Results

Scanning electron microscopy showed that adhesion of the strain to the HT-29 monolayer was already present after 30 min. Bacterial adhesivity further increased after 60 min and 120 min. **Figure 1** shows the adhesion of *Bifidobacterium longum* W11 on HT-29 monolayer at 60 min and 120 min (magnification 5000×).

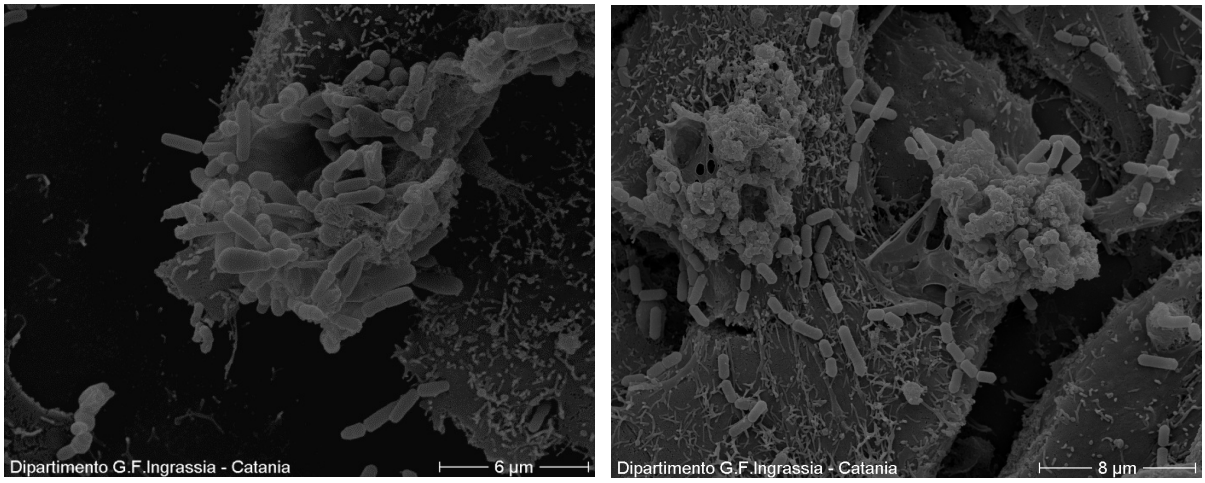
The SEM observation also showed that *B. longum* W11, adhering to HT-29 monolayer, was able to produce biopolymers. **Figure 2** shows that *B. longum* W11 and biopolymers formed a complex 3D structure, biofilm-like, at 60 min and at 120 min (magnification 5000× and 4000× respectively).

The specific methodology used for EPS extraction confirmed the production of biopolymers observed by SEM. Using spectrophotometric methods, the absorbance values showed that the recovered EPS was about 72 µg, in comparison with the standard. Using silica plates the TLC analysis of hydrolyzed and purified EPS revealed, by the retardation factor (Rf) values, the presence of fructose and glucose. These results were confirmed using cellulose plates. Using both methods, the TLC analysis showed presence of other sugars that are under examination, with different methodologies. In addition, non-carbohydrate constituents could be present.



(a) (b)

Figure 1. Monolayer of HT-29 with *Bifidobacterium longum* W11 after 60 min (a) and after 120 min (b) of incubation (magnification 5000×).



(a) (b)

Figure 2. EPS formation by *Bifidobacterium longum* W11, attached to HT29 cells, after 60 min (a) (magnification 5000×) and after 120 min (b) (magnification 4000×).

4. Discussion and Conclusion

Some of the benefits attributed to bifidobacteria have been correlated with their capacity to mediate commensal-host interaction through immune modulation and pathogen protection [6]. However, strains of some species of *Bifidobacterium* could elicit different *in vitro* responses upon interaction with human cells [7]. *Bifidobacterium longum* strains can divert immune responses *in vitro* either towards a pro-inflammatory or a regulatory profile, suggesting that different strains may have different functional roles and application in different pathological conditions [2]. Medina *et al.* (2007) [2] demonstrated that live cells of *Bifidobacterium longum* W11, strongly stimulated the production of T helper 1 cytokines (IL-2 and IFN-γ) and induced low levels of IL-10; moreover low production of IL-10 were also induced by cell-surface components. On the basis of their results the authors suggested that *B. longum* W11 could provide protection against the early stage of infection via Th_1 production. However, the authors did not characterize the structural cell components and the secreted molecules investigated for immunological properties.

Many studies have demonstrated the ability of EPS of *Bifidobacterium* spp. to mediate communication processes with the host and to trigger both innate and adaptive immune responses [1] [5]-[10] [13]-[15]. Some of the EPSs produced by *Bifidobacterium* spp. and other probiotic strains can contribute to the adhesion capacities to intestinal mucus and to the species-specific effects on immunocompetent cells [10]. The EPS consists of secreted and extracellular polymerized glycans which can be covalently linked to the bacterial surface forming a capsule, no-covalently associated with the surface or be totally secreted into the surrounding environment as slime [2] [5] [6].

The results obtained with SEM observation indicated that *B. longum* W11 was able to adhere to the HT-29 cell line and that the production of the exocellular polymers could be one of the factors contributing to its adhesion properties. Moreover, other SEM observations of *B. longum* W11 did not show a capsule layer but an EPS no-covalently associated with the surface.

Using silica and cellulose plates TLC analysis indicated by Rf value that the hydrolyzed EPS was mainly composed of fructose and glucose. The results suggested that the EPS produced by *B. longum* W11 could be a heteropolysaccharide formed by repeating units containing fructose and glucose. Further our studies are underway, using new methodologies, to better characterize the chemical composition of the EPS of *B. longum* W11.

As suggested by Hidalgo-Cantabrana *et al*. (2014) [15] studies connecting physicochemical characteristics of the EPSs of different strains of *Bifidobacterium* spp. with genetic information, could give further insight in understanding the physiology of bifidobacteria and their interaction with the host.

Our continuing research is trying to determine which of the chemical and physical characteristics of the EPS of *B. longum* W11 are responsible for the ability of the strain to interact with the host and of the immunological properties, suggested by Medina *et al*. (2007) [2].

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this article.

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