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Growth and adhesion to HT-29 cells inhibition of Gram-negatives by *Bifidobacterium longum* BB536 e *Lactobacillus rhamnosus* HN001 alone and in combination

R. INTURRI, A. STIVALA, P. M. FURNERI, G. BLANDINO

Biomedical and Biotechnological Sciences, University of Catania, Catania, Italy

Abstract. – OBJECTIVE: The aim of this study was to test the inhibitory effect of supernatants of broth cultures of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001, both individually and in combination, against Gram-negative strains (uropathogens, enteropathogens and a reference strain). Moreover, *in vitro* protection of *B. longum* BB536 and *L. rhamnosus* HN001, both individually and in combination, against pathogen adhesion to HT-29 cell line, was investigated.

MATERIALS AND METHODS: The inhibitory activity was performed by the agar diffusion test and *in vitro* antagonistic activity against pathogen adhesion to human epithelial intestinal HT-29 cells was performed using standardized culture techniques.

RESULTS: The study showed that *B. longum* BB536 and *L. rhamnosus* HN001, individually and in combination have inhibitory activity against the majority of the Gram negative strains tested. Furthermore, the results showed that both probiotic strains have a good capacity to inhibit pathogenic adhesion to HT-29 cells. Moreover, the ability of *B. longum* BB536 and *L. rhamnosus* HN001 to inhibit pathogenic adhesion increased when they were used in combination.

DISCUSSION: The combination of *B. longum* BB536 and *L. rhamnosus* HN001 showed inhibitory activity against Gram-negatives and an improved ability to reduce their adhesion properties and to compete with them.

CONCLUSIONS: The simultaneous presence of the two-probiotic strains could promote competitive mechanisms able to reduce the adhesion properties of pathogen strains and have an important ecological role within the highly competitive environment of the human gut.

Key Words

B. longum BB536, *L. rhamnosus* HN001, Pathogens, Inhibitory activity, Intestinal adhesion antagonism, Antagonistic activity of *B. longum* BB536, *L. rhamnosus* HN001 alone and in combination against Gram-negatives.

Introduction

The potential antagonistic or symbiotic effects that exist among probiotic strains used in combination have not been well investigated. A relevant feature of a probiotic strain is the ability to inhibit the adhesion and the intestinal colonization of enteropathogenic strains[1-5]. Probiotic strains can antagonize enteropathogenic strains in the intestine with several mechanisms: competing for adhesion sites or nutritional sources, modulating the immune response, decreasing the luminal pH by producing lactic acid, acetic acid and specific protein compounds such as bacteriocins[1,6-13]. In particular, inhibition of adhesion of pathogens to intestinal epithelial cells by probiotic bacteria is a property closely correlated with the strain[12,14]. It has been shown that probiotic strains, belonging to the *Lactobacillus* and *Bifidobacterium* genus, can play a protective role against enteropathogenic infections[14-17].

The literature provides data about some microbiological characteristics and effects on human health of the *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001, currently present in combination in a food supplement marketed in Italy[17-23].

The aim of this study was to test the ability of *B. longum* BB536 and *L. rhamnosus* HN001, alone or in combination, to inhibit *in vitro* Gram-negative pathogens and to counteract their adhesion to HT-29 cells.

Materials and Methods

Bacterial Strains and Culture Conditions

The probiotic strains tested in this study were individual lyophilised powders of *Bifi-*

Corresponding Author: Giovanna Blandino, MD; e-mail: blandino@unict.it

dobacterium longum BB536 and *Lactobacillus rhamnosus* HN001, provided by Alfa Wassermann S.p.A., (Bologna, Italy). These strains are present in combination in the food supplement ZirCombi (total charge of 5×10^9/sachet) formulated by Giellepi S.p.A. Health Science (Milan, Italy).

Bifidobacterium longum BB536 and *Lactobacillus rhamnosus* HN001 were individually cultivated in de Man Rogosa & Sharpe (MRS, Oxoid, Milan, Italy) broth or agar with 0.25% cysteine (MRSc) and incubated for 24-48 h at 37°C under anaerobic and aerobic conditions respectively. To isolate *Bifidobacterium longum* BB536, when the probiotic strains were co-cultivated, Bifidobacterium Selective Medium agar (BSM, Oxoid, Rodano, MI, Italy) and an incubation of 48 h at 37°C under anaerobic conditions were used[24].

Gram-negative strains tested in the study were: *Escherichia coli* EC4219, *Salmonella enteritidis* SEN6 and *Salmonella typhi* STN12 (enteropathogens) and *Escherichia coli* EC3960 (uropathogen) from the collection of the Bacteriological Laboratory of the Department of Biomedical and Biotechnological Sciences, University of Catania, Italy. For *in vitro* inhibitory activity assay *Escherichia coli* ATCC 25922 was also tested. Gram-negative strains were cultivated in Brain-Heart-Infusion broth or agar (BHI, Oxoid, Milan, Italy) and incubated for 24 h at 37°C under aerobic conditions.

In Vitro Inhibitory Activity

The inhibitory activity of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001 alone or in combination, against Gram-negative strains was performed modifying the agar diffusion test described by Jara et al[25]. Each Gram-negative strain was spread (1.0×10^9 CFU/mL) onto the surface of Muller Hinton (MH, Oxoid, Milan, Italy) agar plates. A culture of 72-96 h of each probiotic strain or their combination, grown in MRSc broth, was centrifuged (5000 rpm for 15 min) and each supernatant was filtered through a 0.22 µm filter (Millex-GP Syringe Filter Unit, Millipore, Billerica, MA, USA). Then the supernatants were deposited in 6 mm wells previously set up in plates where Gram-negatives were spread. The inhibitor effect was detected by a growth-free inhibition zone around the wells containing the supernatant tested, after 24 h of incubation. The results were expressed as the mean of three individual experiments with duplicate samples.

In Vitro Antagonism of B. longum BB536 and L. Rhamnosus HN001 Against Adhesion to the HT-29 Cell Line by Gram-Negative Isolates

The *in vitro* antagonism of *Bifidobacterium longum* BB536 and *Lactobacillus rhamnosus* HN001, both individually and in combination, against the adhesion of *Escherichia coli* EC3960, *Escherichia coli* EC4219, *Salmonella enteritidis* SEN6 and *Salmonella typhi* STN12 to the human intestinal cell lines HT-29 was performed modifying the method described by Serafini et al[14].

When *B. longum* BB536 and *L. rhamnosus* HN001 were tested in combination, a 1:1 ratio was used to highlight the characteristics of the individual strains. For the tests, HT-29 cells, provided by the American Type Culture Collection (ATCC), were prepared as described by Inturri et al[26] and incubated to reach confluence (about 1.0×10^7 CFU/mL)[14]. Each probiotic strain, their combination, and the pathogens, grown on solid media under their typical growth conditions, were washed twice (8000 rpm for 8 min) with PBS buffer (Sigma-Aldrich, St. Louis, MO, USA) and resuspended in RPMI medium (total bacterial count about 1.0×10^8 CFU/mL). The bacterial suspension was added to the HT-29 cells at a ratio of 10:1 and the plates were incubated for 2 h at 37°C. After incubation, the HT-29 monolayer was washed to remove unadhered bacteria and then was treated with a 0.25% trypsin-EDTA solution. The bacterial count was performed after plating on suitable agar media, and using routine growth conditions. The results were expressed as a relative percentage of adhesion (CFU adhered bacteria/CFU added bacteria × 100). To determine inhibition of pathogen adhesion, the individual probiotic strain or their combination was incubated for 1 h on HT-29 cells; after the addition of the pathogenic strain (about 1.0×10^9 CFU/mL) the incubation was continued for 1 h. The same procedure for pathogen inhibition was performed to assess pathogen displacement, except that the individual probiotic strain or the combination was added after 1 h of pathogen incubation. To determine competition for adhesion on HT-29 cells, the individual probiotic strain or the combination was incubated together with the pathogen for 2 h. The adhesion ratio was calculated as a percentage of adhesion cells (probiotic or pathogenic strain) added in combination divided by the percentage of adhered cells (probiotic or pathogenic strain) added alone[14].

The data represent the mean of three independent experiments with duplicate samples.

Results

In Vitro Inhibitory Activity

Table I. shows the inhibition values of the fresh cells free supernatant (CFS) of the broth cultures of *B. longum* BB536 and *L. rhamnosus* HN001 alone and in combination against tested Gram-negatives.

The supernatants of *B. longum* BB536 (CFS_{BB536}), of *L. rhamnosus* HN001 (CFS_{HN001}) and *B. longum* BB536/*L. rhamnosus* HN001 combination ($CFS_{BB536+HN001}$) showed inhibitory activity against the Gram-negatives tested. In particular, good inhibitory activity (++) was observed for CFS_{BB536} and $CFS_{BB536+HN001}$ against *E. coli* ATCC 35218, *E. coli* EC3960 and *S. typhi* STN12. The results also showed no antagonism effect between the probiotic strains tested.

In Vitro Antagonism of B. Longum BB536 and L. Rhamnosus HN001 Against Adhesion to the HT-29 Cell Line of Gram-negative isolates

B. longum BB536 showed an inhibitory effect against adhesion of *E. coli* EC3960 and *E. coli* EC4219 to HT-29 cells. When *B. longum* BB536 was simultaneously incubated with these pathogenic strains showed competition only against *E. coli* EC3960. *B. longum* BB536 did not show the ability to displace *E. coli* EC3960 and *E. coli* EC4219 already adhered to HT-29 cells (Figure 1A). Moreover, *B. longum* BB536 tested against *S. enteritidis* SEN6 and *S. typhi* STN12 was able to inhibit, displace and compete with both pathogens (Figure 1B).

L. rhamnosus HN001 showed an inhibitory effect against *E. coli* EC3960, *E. coli* EC4219, *S. enteritidis* SEN6 and *S. typhi* STN12 and also the ability to compete with them (Figure 2 A-B) for adhesion to HT-29. Instead, *L. rhamnosus* HN001 did not show the ability to displace all the tested pathogens (Figure 2 A-B).

Compared to the individual strains, the two-probiotic strains in combination showed a greater inhibitory effect against *E. coli* EC3960, *E. coli* EC4219, *S. enteritidis* SEN6 and *S. typhi* STN12 (Figure 3 A-B) and also a better ability to compete with them for adhesion to HT-29 (Figure 3 A-B).

The probiotic strains in combination were able to displace *E. coli* EC3960 and *S. enteritidis* SEN6.

Discussion

Strains of *Lactobacillus* spp. and *Bifidobacterium* spp. have been shown to play a protective role against enteropathogenic infections by several mechanisms[5, 14-16, 22, 25-32]. Adhesion to intestinal epithelial cells is certainly an important factor for *Lactobacillus* spp. and *Bifidobacterium* spp. strains to prevent and/or to reduce adhesion of enteropathogenic strains[27-29,33]. However, antagonistic effects could exist between the probiotic strains used in combination. Some studies have shown that strains of *Lactobacillus rhamnosus* and *Bifidobacterium longum* can reduce cytotoxic effects, adhesion and invasion of enteropathogenic bacteria with a potential consequent reduction in the severity of some intestinal infections[11,29,30,33]. Serafini et al[14] showed that *B. bifidum* PRL2010 can contrast adhesion to intestinal epithelial cells of enteropathogenic strains by mechanisms of inhibition, competition, and displacement. A previous study[26] on *B. longum* BB536 and *L. rhamnosus* HN001 showed a higher survival rate under gastrointestinal conditions and ameliorated adhesion properties for each strain when they are tested in combination. The study also showed no *in vitro* antagonistic effect between the two strains in combination[26]. The results presented in this study show the abili-

Table I. Inhibition of tested supernatants against the Gram-negative strains.

Strains	Supernatants of probiotic strains		
	CFS_{BB536}	CFS_{HN001}	$CFS_{BB536+HN001}$
E. coli ATCC 35218	++	+	++
E. coli EC3960	++	+	++
E. coli EC4219	+	+	+
S. enteritidis SEN6	+	+	+
S. typhi STN12	++	+	++

Notes: +++: ≥ 20 mm; ++: 19-15 mm; +: 14-10 mm, -: <10 mm; CFS_{BB536}: *B. longum* BB536 supernatant; CFS_{HN001}: *L. rhamnosus* HN001 supernatant; $CFS_{BB536+HN001}$: *B. longum* BB536 + *L. rhamnosus* HN001 supernatant.

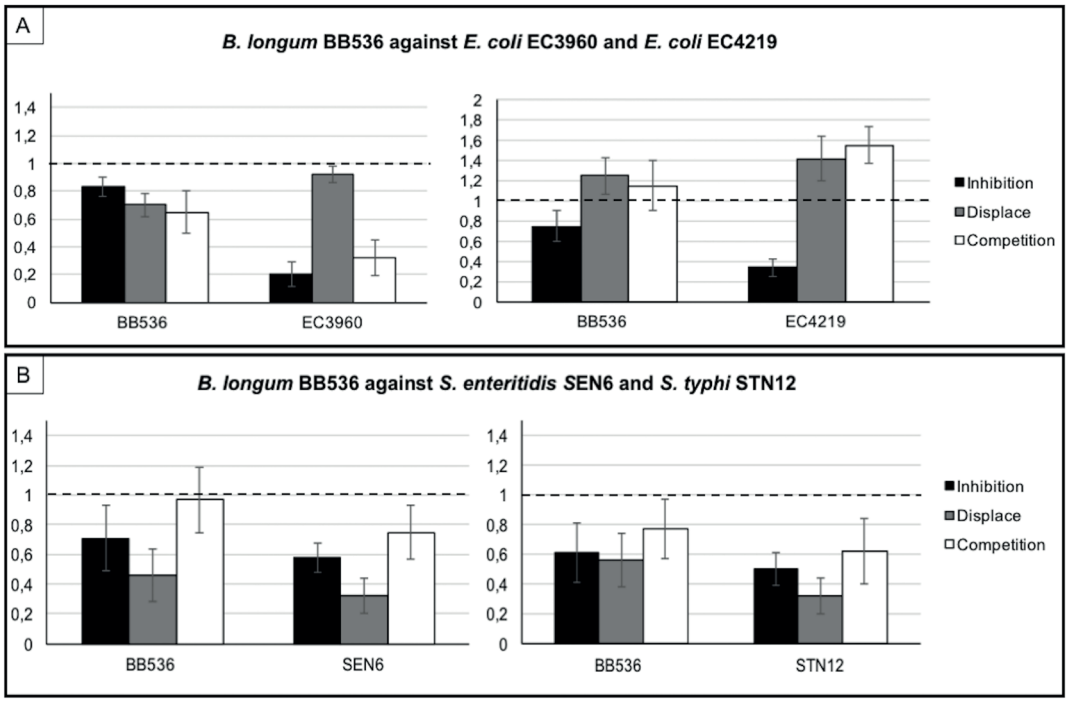

Figure 1. Ability of *B. longum* BB536 to inhibit (black bar), to displace (grey bar) or to compete (white bar) for adhesion to the HT-29 intestinal epithelial cells of: the pathogenic strains *Escherichia coli* EC3960 and *Escherichia coli* EC4219 (**A**) and the pathogenic strains *Salmonella enteritidis* SEN6 and *Salmonella typhi* STN12 (**B**). The dotted line (ratio = 1) represents the adhesion value to HT-29 cells by each strain added individually. The vertical lines indicate the standard deviation.

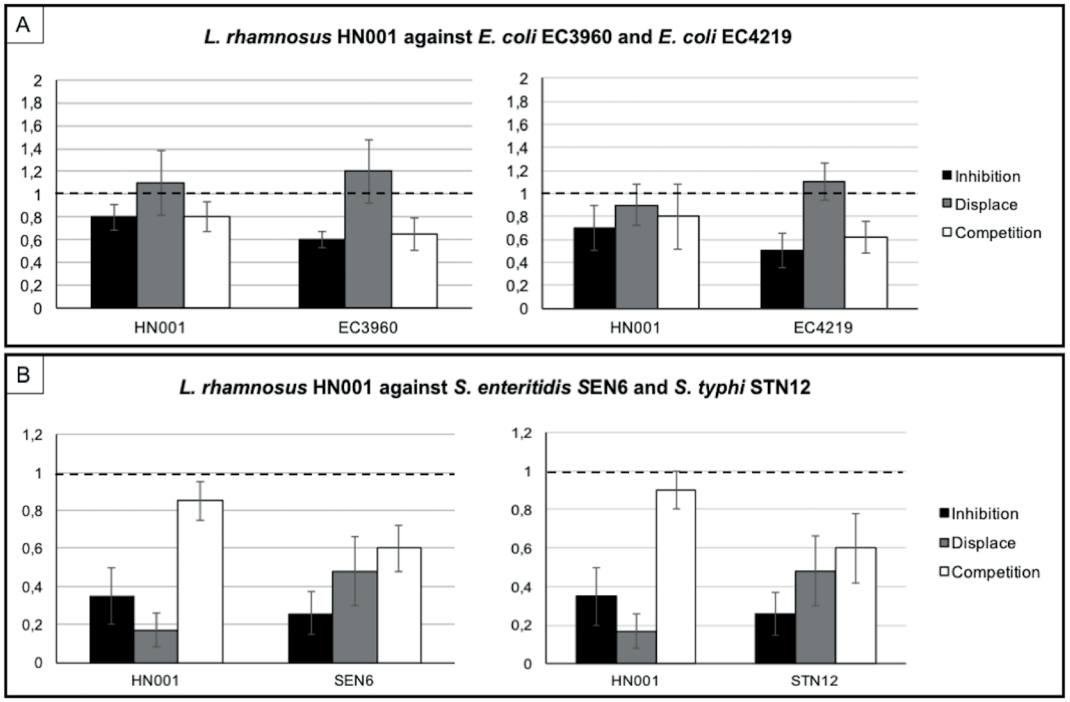

Figure 2. Ability of *L. rhamnosus* HN001 to inhibit (black bar), to displace (grey bar) or to compete (white bar) for adhesion to the HT-29 intestinal epithelial cells of: the pathogenic strains *Escherichia coli* EC3960 and *Escherichia coli* EC4219 (**A**) and the pathogenic strains *Salmonella enteritidis* SEN6 and *Salmonella typhi* STN12 (**B**). The dotted line (ratio = 1) represents the adhesion value to HT-29 cells by each strain added individually. The vertical lines indicate the standard deviation.

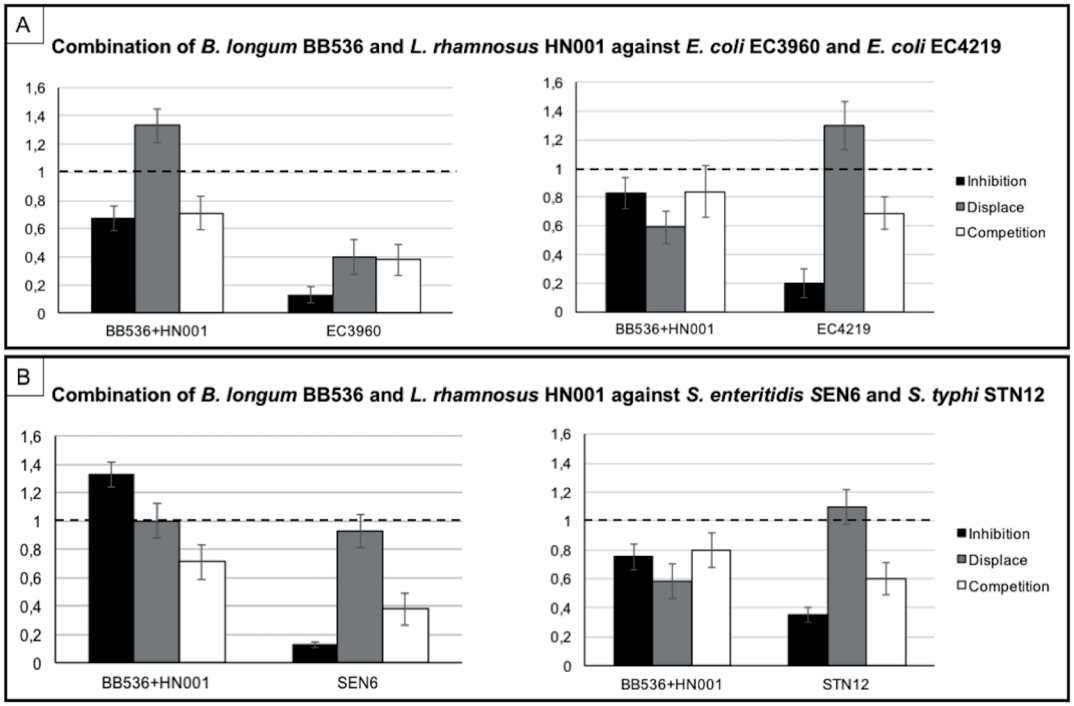

Figure 3. Ability of the probiotic strains *B. longum* BB536 and *L. rhamnosus* HN001 in combination to inhibit (black bar), to displace (grey bar) or to compete (white bar) for adhesion to the HT-29 intestinal epithelial cells of: the pathogenic strains *Escherichia coli* EC3960 and *Escherichia coli* EC4219 (**A**) and the pathogenic strains *Salmonella enteritidis* SEN6 and *Salmonella typhi* STN12 (**B**). The dotted line (ratio = 1) represents the adhesion value to HT-29 cells by each strain added individually. The vertical lines indicate the standard deviation.

ty of *B. longum* BB536 and *L. rhamnosus* HN001 to inhibit Gram-negative pathogens.

Moreover, the probiotic strains tested in combination confirmed the inhibition values of the strain with higher inhibitory activity. This *in vitro* inhibitory activity could be due to the production of protein or non-protein inhibitory substances, such as lactic acid for *Lactobacillus* and acetic acid for *Bifidobacterium*[31]. Our study showed that the probiotic strains *B. longum* BB536 and *L. rhamnosus* HN001 have a good ability to prevent/reduce adhesion of Gram-negative pathogens to HT-29. In particular, the ability of probiotic strains to inhibit the adhesion of pathogens could be due to their strong initial adhesion to the cells. Under the experimental conditions, there is evidence for improved antagonism effects (inhibition, competition and, at least extend, displacement) by the two-probiotic strains in combination against the adhesion of Gram-negative pathogens to HT-29 cells.

Conclusions

The good adhesive behavior shown by *B. longum* BB536 and *L. rhamnosus* HN001 alone or in combination may reflect a potential ecological role within the highly competitive environment of the human gut. However, the simultaneous presence of the probiotic strains tested in this study could promote *in vivo* competitive mechanisms able to reduce the adhesion ability of pathogen strains.

Many factors, such as the cellular differentiation phase of the intestinal cells used and the formation of tight junctions at the time of infection, as well as the production of various types of exopolysaccharides can influence the adhesion of probiotic strains to intestinal cells and the pathogen displacement but the molecular mechanisms should be investigated[12,14,15,27-29,34,35]. Therefore, further studies are necessary to understand better the mechanisms involved in the antagonism of *B. longum* BB536 and *L. rhamnosus* HN001 against Gram-negative pathogens and in particular when these probiotic strains are tested in combination.

Acknowledgments section

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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