RESEARCH LETTER

Specific probiotic strains and their combinations counteract adhesion of *Enterobacter sakazakii* to intestinal mucus

Maria Carmen Collado[1], Erika Isolauri[2] & Seppo Salminen[1]

[1]Functional Foods Forum, University of Turku, Turku, Finland; and [2]Department of Paediatrics, University of Turku, Turku, Finland

Correspondence: Maria Carmen Collado, Functional Foods Forum, University of Turku, Itäinen Pitkäkatu, 4A 5th floor, FI-20014 Turku, Finland. Tel.:+358 2 333 6822; fax: +358 2 333 6862; e-mail: marcol@utu.fi

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Abstract

Enterobacter sakazakii is an opportunistic pathogen and an occasional contaminant in powdered infant formula. Interaction between specific probiotics and *E. sakazakii* may reduce the risk of infection. The aim of this study was to characterize *in vitro* the ability of probiotics (alone and in combinations) to inhibit, compete with and displace the adhesion of *E. sakazakii* to immobilized human mucus and to assess their capacity to aggregate with pathogen. Specific probiotic strains have proved to aggregate *E. sakazakii* cells and, through competitive exclusion, inhibition and displacement of the adhered pathogen, were able to inhibit *E. sakazakii* action on intestinal mucus. The ability to inhibit and to displace adhered pathogen depended on both the probiotic and the pathogen, suggesting that several complementary mechanisms are involved in the processes. We suggest that the selection of specific probiotic strains and their combinations may be a useful means of counteracting *E. sakazakii* contamination in infant formula and thus to reduce the risk of emerging infection. This approach may also allow the development of new probiotic combinations to counteract the risks associated with other pathogens by improving the intestinal barrier against pathogens.

Introduction

Enterobacter sakazakii is an opportunistic pathogen and an occasional contaminant in powdered infant formula (Lai, 2001; van Acker *et al.*, 2001; Drudy *et al.*, 2006). The reservoir for *E. sakazakii* is unknown, but a number of studies have established that infant food made of powdered infant formula products may be a source and vehicle of infection (Lai, 2001; van Acker *et al.*, 2001; Drudy *et al.*, 2006). Recent reports have suggested the presence of *E. sakazakii* in powdered infant formulas at frequencies ranging from 0% to 22%, but the microorganism is usually present at levels below 1 CFU per 100 g of dry powder (Muytjens *et al.*, 1988; Nazarowec-White & Farber, 1997a, b; FAO/WHO, 2004, 2006). Currently, the European Food Safety Authority (EFSA) recommends the introduction of a performance objective for powdered infant formula and follow-up formula (EFSA, 2004). Implementation of this objective is designed to eliminate *Salmonella* and *E. sakazakii* from infant milk powders. Because *E. sakazakii* is not capable of surviving pasteurization processes, it is possible that the contamination occurs during subsequent processing of infant formula (Nazarowec-White & Farber, 1997a, b). It is presumed that most cases involve outgrowth of *E. sakazakii* after rehydration and preparation of the formula. Thus, inappropriate preparation and maintenance of reconstituted infant formula, above all extended storage at suitable growth temperatures, appear to be prerequisites for infection.

Some studies have demonstrated a considerable diversity in acid resistance among *E. sakazakii* isolates and there is no apparent relationship between the acid resistance of individual strains and their thermal resistance (Edelson-Mammel *et al.*, 2006). Thus, *E. sakazakii* may be adapted to survive the light acidity of the neonatal stomach and could cause severe infection in a newborn with an undeveloped intestinal microbiota. This, together with the fact that the infant formula matrix may protect the pathogen during gastrointestinal tract (GIT) transit and maintain its viability for potential infection, increases the importance of developing methods to reduce *E. sakazakii* contamination in formula and subsequent infection of infants.

GIT colonization begins immediately after birth and is influenced by the mother's microbiota, the mode of delivery,

the infant's diet and hygiene levels (Fuller, 1991; Gueimonde *et al.*, 2007). Breast milk has been shown to be a source of commensal bacteria, which enhance gut microbiota and mucosal barrier development (Harmsen *et al.*, 2000; Gueimonde *et al.*, 2007). The intestinal microbiota plays an important role in the health of the host due to involvement in nutritional, immunologic and physiological functions (Hooper & Gordon, 2001), and deviations have been reported to be related to disturbances to the intestinal microbiota (Kalliomäki *et al.*, 2001), which may influence later health. Thus, when breastfeeding is not possible, the inclusion of probiotics in infant formula has been suggested as one way to aid promotion of the microbiota on the intestinal mucosa to improve the resistance to gastrointestinal pathogens. Specific probiotics in infant formula focus on aiding healthy gut microbiota development and may constitute a new model of preventing pathogen action through competitive exclusion and aggregation with pathogens (Collado *et al.*, 2005, 2006, 2007, 2008). Thus, the inclusion of probiotics in powdered infant formula may enhance their resemblance to breast milk.

The aim of this study was to assess the ability of specific probiotic strains to adhere to the human intestinal mucus and their capacity to aggregate, to inhibit, to compete with and displace *E. sakazakii*-type strain (ATCC 29544) (Farmer *et al.*, 1980) adhering to immobilized human intestinal mucus (Ouwehand *et al.*, 2002; Collado *et al.*, 2005; Vesterlund *et al.*, 2005).

Materials and methods

Bacterial strains and culture conditions

Probiotics were obtained and selected from the culture collection of Nestle S.A. (Vevey, Switzerland) and included the following strains: *Streptococcus thermophilus* NCC 2496, *Lactobacillus rhamnosus* NCC 4007, *Lactobacillus paracasei* NCC 2461, *Bifidobacterium longum* NCC 3001 and *Bifidobacterium lactis* NCC 2818.

Recent studies have established that the majority of *E. sakazakii* isolates from different environments (e.g. infant formula, faeces) are grouped into the same cluster as the *E. sakazakii* ATCC 29544-type strain (Iversen *et al.*, 2006, 2007). Thus, we selected *E. sakazakii* ATCC 29544 as a model strain for the study.

All probiotic strains and also, an *E. sakazakii* strain were grown in Gifu anaerobic medium (GAM Nissui Pharmaceutical, Tokyo, Japan) and metabolically labelled by addition of tritiated thymidine (5-^{3}H-thymidine 120 Ci mM^{-1}; Amersham Biosciences, UK). After overnight incubation at 37 °C under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2; Concept 400 anaerobic chamber, Ruskinn Technology, Leeds, UK), radiolabelled bacteria were harvested and washed with phosphate-buffered saline buffer (130 mM sodium chloride, 10 mM sodium phosphate, pH 7.2). Absorbance ($A_{600\,nm}$) was adjusted to 0.25 ± 0.05 to standardize the bacterial concentration (10^8 cells mL^{-1}). Probiotic combinations were prepared by mixing equal amounts of each probiotic strain.

Human mucus was dissolved (0.5 mg protein mL^{-1}) in HEPES–Hanks buffer (HH; 10 mM HEPES, pH 7.4) and the solution was immobilized onto wells of polystyrene microtitre plates (Maxisorp, Nunc, Denmark) by an overnight incubation at 4 °C as described previously (Collado *et al.*, 2005; Gueimonde *et al.*, 2006).

Adhesion assays to human mucus

Human intestinal mucus was isolated from the healthy part of resected colonic tissue as described earlier (Ouwehand *et al.*, 2002). Before use, protein concentration was determined using bovine serum albumin (Sigma, St Louis, MO) as the standard (Collado *et al.*, 2005). Human mucus was dissolved (0.5 mg protein mL^{-1}) in HH (10 mM HEPES, pH 7.4) and 100 μL of the solution was immobilized into polystyrene microtitre plate wells (Maxisorp, Nunc, Denmark) by overnight incubation at 4 °C. Adhesion to the human intestinal mucus was characterized as described previously (Collado *et al.*, 2005). An aliquot (100 μL) of standardized suspension was added to the wells and incubated for 1 h at 37 °C. Subsequently, the wells were twice washed with HH to remove unattached bacteria. Adhering bacteria were released and lysed with 1% (w/v) sodium dodecyl sulphate in 0.1 mol L^{-1} NaOH by incubation at 65 °C for 1 h. The contents of the wells were transferred to microfuge tubes containing scintillation liquid (OptiPhase 'HiSafe 3'; Wallac, Turku, Finland) and radioactivity was measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucus. Adhesion was determined in four independent experiments, and each assay was performed in quadruplicate to calculate the intra-assay variation.

Inhibition of pathogen adhesion to intestinal mucus

To test the ability of the probiotic strains to inhibit the adhesion of *E. sakazakii*, unlabelled probiotic bacteria were added to the wells and incubated for 1 h at 37 °C. Thereafter, unattached cells were removed by washing twice with HH buffer and radiolabelled *E. sakazakii* (10^8 cells mL^{-1}) were added to the wells and incubated at 37 °C for 1 h. The wells were then washed and bound bacteria were recovered after lysis as described above. Radioactivity was measured by liquid scintillation. The percentage of inhibition

was calculated as the difference between the adhesion of the pathogen in the absence and presence of probiotic strains.

Displacement of pathogen adhered to intestinal mucus

The ability of the probiotic strains to displace previously adhered *E. sakazakii* was assessed following the methodology described elsewhere (Collado *et al.*, 2005). Radiolabelled *E. sakazakii* was added to the wells, incubated for 1 h at 37 °C and after washing and removal unbound cells, non-radiolabelled probiotics (100 μL, 10^8 cells mL^{-1}) were added to the wells and incubated for 1 h at 37 °C. The wells were then washed again, and bound bacteria released and lysed as described above and radioactivity was measured. Displacement of pathogens was calculated as the difference between the adhesion of pathogens before and after the addition of the probiotic strains.

Competence between pathogen and probiotic strains to adhere to intestinal mucus

For the competition test, equal quantities of bacterial suspension of probiotic and radiolabelled *E. sakazakii* were mixed and then added to the intestinal mucus and incubated as an adhesion protocol. Then, the cells of the pathogen bound to the mucus were removed and the adhesion was calculated as described above. Pathogen adhesion was determined in four independent experiments and each assay was performed in quadruplicate to calculate intra-assay variation.

Bacterial aggregation analysis

The coaggregation test was performed as described elsewhere (Collado *et al.*, 2008). Briefly, equal volumes of a standardized cell concentration ($A_{600\,nm} = 0.25 \pm 0.05$, 10^8 cells mL^{-1}) of probiotic strains and their combinations and also the *E. sakazakii* strain were mixed and incubated at room temperature without agitation. The absorbances of the mixtures (probiotic and *E. sakazakii*) described above were monitored during 4 h of incubation. Absorbance was determined for the mixture and for the bacterial suspensions alone. The standard deviations derived from the coaggregation values of three independent experiments did not exceed 10% of the mean value. Coaggregation (%) was calculated according to the equation $[(A_{pat+probio})(A_{mix})/(A_{pat+probio})] \times 100$, where $A_{pat+probio}$ represents the $A_{600\,nm}$ of the mixed bacterial suspensions at time point 0 min and A_{mix} represents the $A_{600\,nm}$ of the mixed bacterial suspension at different times.

Statistical analysis

Statistical analysis was performed using the SPSS 11.0 software (SPSS Inc, Chicago, IL). Data were subjected to one-way ANOVA and, where appropriate, the Student–Newman–Keuls (S–N–K) test was used for comparison of means.

Results and discussion

The newborn intestinal tract is colonized by bacteria derived from the mother and the immediate environment. This colonization is reinforced by breastfeeding due to the bacteria and oligosaccharides present in breast milk but when breastfeeding is not possible, the use of infant formula is needed.

The inclusion of probiotics in infant formula focuses on aiding healthy gut microbiota development and may constitute an alternative to support the development of intestinal microbiota of a newborn and to promote the intestinal health and immune development when breastfeeding is not possible. The selection of specific probiotic strains and their combinations may be useful to counteract pathogen contamination in infant formula and thus to reduce the risk of emerging infection. At the same time, specific probiotics may be useful for healthy intestinal colonization and barrier formation to fight pathogen adhesion through competitive exclusion and also by aggregation abilities with pathogens (Collado *et al.*, 2005, 2006, 2007, 2008; Gueimonde *et al.*, 2006). Thus, the inclusion of specific probiotics in powdered infant formula may improve their resemblance to breast milk, and specific probiotic strains or combinations have been reported to prevent invasion of specific pathogens. We suggest that the interaction between probiotic bacteria and the pathogen *E. sakazakii* ATCC 29544 may be of

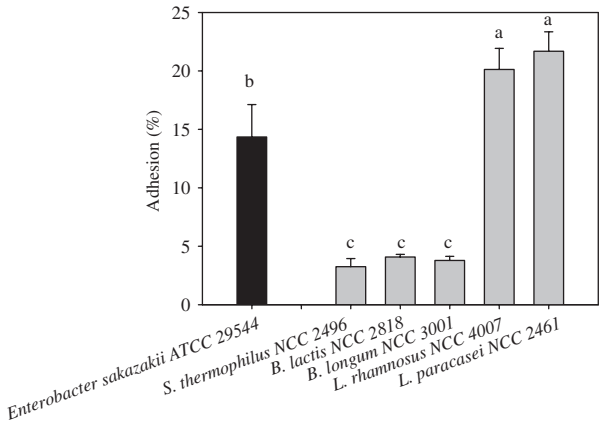

Fig. 1. Adhesion of *Enterobacter sakazakii* and probiotic strains to intestinal mucus. Results ($n = 4$) are expressed as the percentage (mean and SD) of radioactivity recovered from immobilized mucus compared with radioactivity added to mucus. Different superscripts differ significantly ($P < 0.05$) in ANOVA test.

importance for the development of infant feeding procedures that reduce the risk of *E. sakazakii* infection.

When all the studied probiotic strains were assessed alone, they adhered to the intestinal mucus model in a strain-specific manner (Fig. 1). *Lactobacillus* adhesion levels were similar to those reported for *L. rhamnosus* GG (Ouwehand *et al*., 2002; Collado *et al*., 2006, 2007), whereas the adhesion of *Bifidobacterium* strains was slightly lower than reported for commercial probiotic strains *B. lactis* Bb12 or *B. longum* 46 (Ouwehand *et al*., 2002; Collado *et al*., 2006, 2007; Gueimonde *et al*., 2006). *Enterobacter sakazakii* evinced a marked ability to adhere to intestinal mucus ($>$ 14%), indicating that it has the capacity to bind to intestinal epithelia (Mange *et al*., 2006). *Enterobacter sakazakii* adhesion was modified in the presence of probiotic strains and their combinations, in some cases being reduced more than 40% (Table 1). Results showed differences among the mechanisms tested (inhibition, displacement and competition) using all probiotic strains alone and also in combination. Competitive exclusion is the main mechanism to reduce the adhesion of pathogens as *E. sakazakii* ATCC 29544 in our study as also reported in previous studies (Collado *et al*., 2005, 2006, 2007; Gueimonde *et al*., 2006).

The degree of adhesion of probiotic strains was not proportional to the degree of *E. sakazakii* inhibition, competition and displacement. The most markedly adhesive strain, *L. paracasei* (21.7%), to intestinal mucus was not the most effective in inhibiting *E. sakazakii* adhesion (Table 1) and other probiotic strains with lower adhesion to intestinal mucus showed similar abilities to inhibit *E. sakazakii* ATCC 29544 (Table 1). Thus, the ability to inhibit the adhesion of *E. sakazakii* ATCC 29544 strain appears to depend both on the specific probiotic and the pathogen, indicating a high specificity in the mechanism of inhibition. These results, in

Table 1. Modulation of *Enterobacter sakazakii* adhesion to intestinal mucus by probiotic strains and their combination

	Inhibition of *E. sakazakii* adhesion (%) *($n = 4$)		
Probiotic combinations	Inhibition	Displacement	Competition
ST = *S. thermophilus* NCC 2496	33.2 (4.2)^{a}	19.8 (3.3)^{a}	49.9 (8.0)^{a}
BLO = *B. longum* NCC 3001	21.3 (3.2)^{b}	18.6 (4.0)^{a}	39.2 (6.2)^{a,b}
BLA = *B. lactis* NCC 2818	28.0 (4.3)^{a}	13.0 (5.0)^{a,b}	40.5 (4.2)^{a,b}
LRHA = *L. rhamnosus* NCC 4007	27.7 (4.5)^{a,b}	16.9 (5.2)^{a,b}	30.8 (5.5)^{b}
LPA = *L. paracasei* NCC 2461	26.2 (3.6)^{a,b}	15.7 (5.0)^{a,b}	33.5 (2.6)^{b}
ST-BLO	21.2 (3.7)^{b}	18.9 (4.2)^{a,b}	36.1 (5.3)^{a}
ST-BLA	32.5 (5.0)^{a}	25.4 (5.0)^{a}	40.4 (6.0)^{a}
ST-LRHA	30.6 (3.2)^{a}	20.8 (2.2)^{a,b}	32.3 (4.8)^{a}
ST-LPA	26.2 (3.0)^{a,b}	15.8 (4.0)^{b}	31.6 (3.2)^{a,b}
BLO-BLA	20.1 (2.5)^{b,c}	11.2 (6.0)^{b,c}	28.4 (5.0)^{a,b}
BLO-LRHA	19.3 (4.0)^{b,c}	13.8 (3.2)^{b,c}	26.5 (2.6)^{b}
BLO-LPA	20.6 (4.5)^{b}	17.0 (2.6)^{b}	31.5 (6.1)^{a,b}
BLA-LRHA	22.9 (5.1)^{a,b}	17.5 (5.2)^{a,b}	27.7 (5.5)^{a,b}
BLA-LPA	27.4 (3.8)^{a,b}	18.6 (3.2)^{a,b}	30.0 (4.2)^{a,b}
LRHA-LPA	33.0 (6.0)^{a}	22.5 (4.5)^{a}	22.8 (3.0)^{b}
ST-BLO-BLA	29.1 (3.0)^{a}	23.3 (6.0)^{a}	41.5 (5.0)^{a}
ST-BLO-LRHA	26.8 (3.2)^{a,b}	17.7 (3.2)^{b,c}	31.5 (4.6)^{b,c}
ST-BLO-LPA	29.2 (3.3)^{a}	16.5 (4.0)^{b,c}	30.7 (5.2)^{b,c}
ST-BLA-LRHA	29.5 (4.0)^{a}	18.5 (6.1)^{b}	32.4 (4.8)^{b,c}
ST-BLA-LPA	26.3 (5.2)^{a,b}	21.5 (5.5)^{a,b}	35.9 (4.4)^{b}
ST-LRHA-LPA	23.9 (2.0)^{b}	20.6 (2.4)^{b}	33.6 (4.6)^{b,c}
BLO-BLA-LRHA	27.7 (3.0)^{a,b}	23.7 (5.2)^{a}	40.8 (5.0)^{a}
BLO-BLA-LPA	29.2 (5.6)^{a}	24.4 (3.3)^{a}	41.8 (3.6)^{a}
BLA-LRHA-LPA	31.0 (6.0)^{a}	20.2 (5.3)^{a,b}	42.2 (4.0)^{a}
BLO-LRHA-LPA	22.7 (3.3)^{b}	21.4 (5.0)^{a,b}	35.8 (4.7)^{b}
ST-BLO-BLA-LRHA	27.8 (3.0)^{b}	15.8 (5.0)^{a}	33.4 (4.0)^{b}
ST-BLO-BLA-LPA	25.4 (5.0)^{b,c}	12.7 (3.7)^{a,b}	34.7 (6.2)^{a,b}
BLO-BLA-LRHA-LPA	23.8 (3.2)^{c}	14.4 (6.0)^{a}	38.1 (5.3)^{a}
ST-BLO-LRHA-LPA	24.4 (4.0)^{b,c}	11.5 (4.0)^{a,b}	35.0 (2.8)^{a,b}
ST-BLA-LRHA-LPA	37.1 (3.8)^{a}	11.6 (3.6)^{a,b}	30.4 (3.8)^{b,c}
ST-BLO-BLA-LRHA-LPA	25.9 (5.0)	18.2 (4.2)	42.3 (4.2)

*Control was taken as 0% of pathogen adhesion.

^{a,b,c}Different superscripts differ significantly ($P < 0.05$). Statistical analyses were made comparing within different blocks corresponding to the probiotic combinations (strain alone, 2, 3, 4 and 5 strain combination).

Results are shown as mean and SD compared with adhesion of *E. sakazakii* alone (taken as 0%) without the presence of probiotic strains.

agreement with previous reports (Collado *et al.*, 2005, 2006, 2007; Gueimonde *et al.*, 2006), indicate that the inhibition is not directly related to the adhesion ability of the strains. The ability to interact and to inhibit the adhesion of *E. sakazakii* or to displace the bacterium appears to be specific and probiotic strain-dependent. The displacement profiles of the probiotic strains and their combinations were different from those observed for the inhibition of *E. sakazakii* ATCC 29544, and the overall adhesion of the probiotic strains was not associated with the ability to inhibit or displace pathogens (Bibiloni *et al.*, 1999; Ouwehand *et al.*, 2002; Collado *et al.*, 2005, 2006).

Competitive exclusion was the most effective mechanism to inhibit pathogen adhesion (Table 1). Competitive exclusion profiles among the probiotic strains and their combinations and the pathogen were different from those observed for the inhibition and displacement of *E. sakazakii* ATCC 29544 and in agreement with earlier findings (Lee *et al.*, 2003; Collado *et al.*, 2005, 2006, 2007). Our results clearly demonstrate that the degree of adhesion of probiotic strains is not proportional to the degree of pathogen inhibition, competition and displacement. Thus, in adhesion to the mucus, other factors such as coaggregation with the pathogen could be involved. Bacterial aggregation between microorganisms from different species and strains (coaggregation) is of importance in specific ecological niches, especially in the human gut, where many probiotics are active (Jankovic *et al.*, 2003). The adhesion and coaggregation abilities of *Lactobacillus* species may promote formation of the mucosal barrier to prevent pathogen colonization (Reid *et al.*, 1988; Boris *et al.*, 1997; Schachtsiek *et al.*, 2004). Our results suggest that especially coaggregation between *E. sakazakii* and the probiotic strains could be involved in the reduction of pathogen adhesion to mucus, and this property could be used for screening specific probiotics for infant formula use. We analysed coaggregation abilities among the probiotic strains (data not shown) and also properties of coaggregation with *E. sakazakii* (Table 2). In general, the most markedly adhesive strains showed the highest percentages of coaggregation; for example, *L. paracasei* and *L. rhamnosus* showed the highest percentages for adhesion, 21.7% and 20.2%, respectively. Their combination showed one of the highest percentages of coaggregation after 4 h of incubation (20.8% coaggregation). All combinations with these lactobacilli evinced a higher capacity for aggregation than the other combinations. Moreover, the probiotic strains with strongly coaggregative properties with *E. sakazakii* were *L. paracasei* and *L. rhamnosus* (19.7% and 18.2%, respectively), and also all combinations with these lactobacilli showed a propensity for high aggregation properties with the pathogen (Table 2).

Our results demonstrate that specific probiotic strains can influence *E. sakazakii* adhesion to mucus at different pathogen concentrations ranging from 10^7–10^8 cells mL^{-1} to 10^4–10^5 cells mL^{-1} (Fig. 2). As little information is available on cell–dose interaction and infection (Nazarowec-White & Farber, 1997a, b) and as different concentrations of *E. sakazakii* cells could already cause severe infection, the phenomenon described may be of practical importance. Owing to the detection limit ($< 10^3$ cells mL^{-1}), the method used in this study could not be used to test the lower concentrations (< 10–10^2 cells mL^{-1}) reported in some formula products. However, our results suggest that at lower pathogen concentrations (10^3–10^4 cells mL^{-1}), specific probiotics are more efficient in inhibiting *E. sakazakii* adhesion to mucus (Fig. 2), because the presence of probiotic strains can reduce a higher percentage of adhered pathogen. Thus, specific probiotics may significantly influence *E. sakazakii*

Table 2. *Enterobacter sakazakii* coaggregation percentages with different probiotic strains and their combinations

	% Coaggregation with *E. sakazakii* at 37 °C [a,*] ($n = 4$)	
Probiotic combination	T. 2 h	T. 4 h
ST = *S. thermophilus* NCC 2496	8.7 (1.1)^{a,b}	14.6 (0.5)^{b}
BLO = *B. longum* NCC 3001	8.5 (0.7)^{a,b}	15.9 (1.2)^{b}
BLA = *B. lactis* NCC 2818	8.2 (1.0)^{a,b}	15.6 (0.6)^{b}
LRHA = *L. rhamnosus* NCC 4007	10.2 (1.1)^{a}	19.7 (1.6)^{a}
LPA = *L. paracasei* NCC 2461	10.8 (2.5)^{a}	18.2 (3.6)^{a}
ST-BLO	7.4 (1.1)^{b,c}	13.8 (1.1)^{c}
ST-BLA	7.8 (1.8)^{b,c}	16.8 (2.9)^{b}
ST-LRHA	9.8 (0.8)^{b}	18.4 (1.0)^{a,b}
ST-LPA	9.9 (1.6)^{b}	18.6 (0.7)^{a,b}
BLO-BLA	7.7 (1.2)^{b,c}	15.1 (0.8)^{b,c}
BLO-LRHA	11.2 (0.8)^{a}	18.1 (0.8)^{a,b}
BLO-LPA	8.8 (1.3)^{b}	16.7 (1.7)^{b}
BLA-LRHA	8.7 (1.8)^{b}	17.8 (1.4)^{a,b}
BLA-LPA	7.8 (2.4)^{b,c}	17.4 (0.5)^{a,b}
LRHA-LPA	11.1 (2.5)^{a}	20.8 (2.0)^{a}
ST-BLO-BLA	7.6 (2.0)^{b}	12.9 (0.8)^{c}
ST-BLO-LRHA	9.2 (3.5)^{a}	18.3 (0.8)^{b,c}
ST-BLO-LPA	8.1 (2.0)^{a,b}	16.6 (1.7)^{b,c}
ST-BLA-LRHA	9.4 (3.8)^{a}	18.3 (0.4)^{b,c}
ST-BLA-LPA	10.2 (1.8)^{a}	17.8 (0.9)^{b,c}
ST-LRHA-LPA	9.3 (4.0)^{a}	19.0 (1.2)^{b}
BLO-BLA-LRHA	8.7 (2.2)^{a,b}	16.3 (0.6)^{b,c}
BLO-BLA-LPA	8.8 (2.2)^{a,b}	17.0 (1.0)^{b,c}
BLA-LRHA-LPA	10.2 (3.1)^{a}	19.6 (1.3)^{b}
BLO-LRHA-LPA	10.6 (2.8)^{a}	21.5 (0.2)^{a}
ST-BLO-BLA-LRHA	8.1 (2.4)	17.6 (0.3)^{b}
ST-BLO-BLA-LPA	8.1 (2.6)	18.0 (0.8)^{v}
BLO-BLA-LRHA-LPA	9.8 (2.0)	20.7 (1.0)^{a}
ST-BLO-LRHA-LPA	9.5 (2.0)	20.4 (0.6)^{a}
ST-BLA-LRHA-LPA	10.1 (2.0)	19.3 (0.2)^{a}
ST-BLO-BLA-LRHA-LPA	7.7 (2.4)	16.7 (0.9)

*Control was taken as 0% of pathogen adhesion.

[a,b,c]Different superscripts differ significantly ($P < 0.05$). Statistical analyses were made comparing within different blocks corresponding to the probiotic combinations (strain alone, 2, 3, 4 and 5 strain combination). Results are expressed as mean and SD.

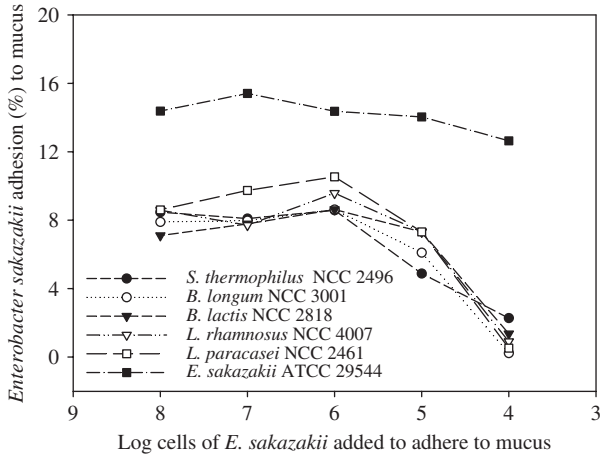

Fig. 2. *Enterobacter sakazakii* ATCC 29544 adhesion (%) at different concentrations (ranged from 8 to 4 Log cells per millilitres added to intestinal mucus) in a competitive exclusion test in absence and presence of probiotic strains. □, *Enterobacter sakazakii* alone; ●, *E. sakazakii* in presence of *Streptococcus thermophilus* NCC 2496; ○, *E. sakazakii* in presence of *Bifidobacterium longum* NCC 3001; ▼, *E. sakazakii* in presence of *Bifidobacterium lactis* NCC 2818; △, *E. sakazakii* in presence of *Lactobacillus rhamnosus* NCC 4007; ■, *E. sakazakii* in presence of *Lactobacillus paracasei* NCC 2461.

adhesion and their inclusion in infant formula may offer a new means of providing protection against infection. The results described may result in new strategies and treatment modalities and further research on this topic is warranted, especially on very low *E. sakazakii* concentrations.

Our findings are among the first to demonstrate probiotic and *E. sakazakii* interactions. We suggest that the selection of specific probiotic strains and their combinations may be a useful means of counteracting *E. sakazakii* contamination in infant formula and thus of reducing the risk of emerging infection. This approach may also allow the development of new probiotic combinations to counteract the risks associated with other pathogens. At the same time, specific probiotics may be useful for healthy intestinal colonization and barrier formation to fight pathogen adhesion. The use of safe probiotic bacteria thus offers new options in infant formula development for improving formula safety and promoting infant health.

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References

Bibiloni R, Perez PF & de Antoni GL (1999) Will a high adhering capacity in a probiotic strain guarantee exclusion of pathogens from intestinal epithelia? *Anaerobe* **5**: 519–524.

Boris S, Suarez JE & Barbes C (1997) Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *J Appl Microbiol* **83**: 413–420.

Collado MC, Gueimonde M, Hernández M, Sanz Y & Salminen S (2005) Adhesion of selected *Bifidobacterium* strains to human intestinal mucus and its role in enteropathogen exclusion. *J Food Prot* **68**: 2672–2678.

Collado MC, Jalonen L, Meriluoto J & Salminen S (2006) Protection mechanism of probiotic combination against human pathogens: *in vitro* adhesion to human intestinal mucus. *Asia Pac J Clin Nutr* **15**: 570–575.

Collado MC, Meriluoto J & Salminen S (2007) *In vitro* analysis of probiotic strains combinations to inhibit pathogen adhesion to human intestinal mucus. *Food Res Int* **40**: 629–636.

Collado MC, Meriluoto J & Salminen S (2008) Interactions between pathogens and lactic acid bacteria: aggregation and coaggregation abilities. *Eur Food Res Technol* **226**: 1065–1073.

Drudy D, Mullane NR, Quinn T, Wall PG & Fanning S (2006) *Enterobacter sakazakii*: an emerging pathogen in powdered infant formula. *Clin Infect Dis* **42**: 996–1002.

Edelson-Mammel S, Porteous MK & Buchanan RL (2006) Acid resistance of twelve strains of *Enterobacter sakazakii*, and the impact of habituating the cells to an acidic environment. *J Food Sci* **71**: M201–M207.

EFSA (2004) European Food Safety Authority. Opinion commission related to the microbiological risks in infant formulae and follow-on formulae. *EFSA J* **13**: 1–34.

FAO/WHO (2004) Food and Agriculture Organization/World Health Organization. *Enterobacter sakazakii* and other microorganisms in powdered infant formula: meeting report, MRA series 6. World Health Organization. Geneva, Switzerland.

FAO/WHO (2006) Food and Agriculture Organization/World Health Organization. *Enterobacter sakazakii* and *Salmonella* in powdered infant formula: meeting report. Food and Agriculture Organization. Rome, Italy.

Farmer JJ, Asbury MA, Hickmann FW & Brenner DJ (1980) *Enterobacter sakazakii*: a new species of "*Enterobacteriaceae*" isolated from clinical specimens. *Int J Syst Bacteriol* **30**: 569–584.

Fuller R (1991) Factors affecting the composition of the intestinal microflora of the human infant. *Nutritional Needs of the 6–12 Month Infant* (Heird WC, ed), pp. 121–130. Raven Press, New York.

Gueimonde M, Jalonen L, He F, Hiramatsu M & Salminen S (2006) Adhesion and competitive inhibition and displacement of human enteropathogens by selected lactobacilli. *Food Res Int* **39**: 467–471.

Gueimonde M, Laitinen K, Salminen S & Isolauri E (2007) Breast milk: a source of *Bifidobacteria* for infant gut development and maturation? *Neonatology* **92**: 64–66.

Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG & Welling GW (2000) Analysis of intestinal flora development in breast-fed and formula-fed infants by

using molecular identification and detection methods. *J Pediatr Gastroenterol Nutr* **30**: 61–67.

Hooper LV & Gordon JI (2001) Commensal host-bacterial relationships in the gut. *Science* **292**: 1115–1118.

Iversen C, Waddington M, Farmer JJ & Forsythe SJ (2006) The biochemical differentiation of *Enterobacter sakazakii* genotypes. *BMC Microbiol* **26**: 94.

Iversen C, Lehner A, Mullane N, Bidlas E, Cleenwerck I, Marugg J, Fanning S, Stephan R & Joosten H (2007) The taxonomy of *Enterobacter sakazakii*: proposal of a new genus *Cronobacter* gen. nov. and descriptions of *Cronobacter sakazakii* comb. nov. *Cronobacter sakazakii* subsp. *sakazakii*, comb. nov., *Cronobacter sakazakii* subsp. *malonaticus* subsp. nov., *Cronobacter turicensis* sp. nov., *Cronobacter muytjensii* sp. nov., *Cronobacter dublinensis* sp. nov. and *Cronobacter genomospecies*. *BMC Evol Biol* **7**: 64.

Jankovic I, Ventura M, Meylan V, Rouvet M, Elli M & Zink R (2003) Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. *J Bacteriol* **185**: 3288–3296.

Kalliomäki M, Salminen S, Arvilommi H, Kero P, Koskinen P & Isolauri E (2001) Probiotics in primary prevention of atopic disease: a randomised placebo-controlled trial. *Lancet* **357**: 1076–1079.

Lai KK (2001) *Enterobacter sakazakii* infections among neonates, infants, children, and adults. Case reports and a review of the literature. *Medicine* **80**: 113–122.

Lee Y-K, Puong KY, Ouwehand AC & Salminen S (2003) Displacement of bacterial pathogens from mucus and Caco-2 cell surface by lactobacilli. *J Med Microbiol* **52**: 925–930.

Mange JP, Stephan R, Borel N, Wild P, Kim KS, Pospischil A & Lehner A (2006) Adhesive properties of *Enterobacter sakazakii* to human epithelial and brain microvascular endothelial cells. *BMC Microbiol* **6**: 58.

Muytjens HL, Roelofs-Willemse H & Jaspar GH (1988) Quality of powdered substitutes for breast milk with regard to members of the family *Enterobacteriaceae*. *J Clin Microbiol* **26**: 743–746.

Nazarowec-White M & Farber JM (1997a) Incidence, survival and growth of *Enterobacter sakazakii* in infant formula. *J Food Prot* **60**: 226–230.

Nazarowec-White M & Farber JM (1997b) Thermal resistance of *Enterobacter sakazakii* in reconstituted dried-infant formula. *Lett Appl Microbiol* **24**: 9–13.

Ouwehand AC, Salminen S, Tölkkö S, Roberts P, Ovaska J & Salminen E (2002) Resected human colonic tissue: new model for characterizing adhesion of lactic acid bacteria. *Clin Diag Lab Immunol* **9**: 184–186.

Reid G, McGroarty JS, Angotti R & Cook RL (1988) *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. *Can J Microbiol* **34**: 344–351.

Schachtsiek M, Hammes WP & Hertel C (2004) Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein Cpf mediating coaggregation with and aggregation among pathogens. *Appl Environ Microbiol* **70**: 7078–7085.

van Acker J, de Smet F, Muyldermans G, Bougatef A, Naessens A & Lauwers S (2001) Outbreak of necrotizing enterocolitis associated with *Enterobacter sakazakii* in powdered milk formula. *J Clin Microbiol* **39**: 293–297.

Vesterlund S, Paltta J, Karp M & Ouwehand AC (2005) Measurement of bacterial adhesion – *in vitro* evaluation of different methods. *J Microbiol Methods* **60**: 225–233.