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Development of a synbiotic product for newborns and infants

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ABSTRACT

The capability of ten commercial fibers of selectively stimulating the growth of four *Bifidobacterium* strains were studied with the purpose of developing a synbiotic product for infants. Two galacto-oligosaccharides (GOS), one fructo-oligosaccharide (sc-FOS), four inulins with different polymerization degree (DP), a gluco-oligosaccharide, an arabinogalactan and a hydrolyzed guar gum were used (10 gl^{-1}). The prebiotic score was calculated comparing the capability of the fibers of stimulating the growth of bifidobacteria compared to infant pathogens. GOS, sc-FOS, low DP inulin (oligofructose) and the gluco-oligosaccharide could stimulate growth. However, the fibers showing the highest prebiotic score were oligofructose (Orafti®HIS), sc-FOS (Actilight®950P) and the GOS Vivinal®. Lyophilized strain survival in simulated gastro-intestinal conditions was also assayed to define suitable ways of administration. Survival in gastric juice at pH 2.5 was poor, whereas it was higher at pH 4, a value closer to newborn pH. Microencapsulation in a lipid matrix ensured strain survival also at pH 2.5. Survival to 1 gl^{-1} bile salts was acceptable. The results allowed to conclude that *Bifidobacterium breve* B632 strain, in a lyophilized or microencapsulated form, has the potential for use in synbiotic products targeted to infants coupled to a mixture of GOS and FOS or oligofructose.

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1. Introduction

Probiotics and prebiotics constitute a central growing market for the food/pharmaceutical industry and this has driven a lot of research aimed at understanding their activity (Kumar et al., 2014). The children associated market is of great relevance, because infants are very susceptible to diseases and non-chemotherapeutic treatments are particularly looked forward for this target group (Mugambi, Young, & Blaauw, 2014). Moreover, the baby food industry is particularly interested in probiotics and prebiotics with the aim of improving the quality of formula milks and post-weaning milks. The health status of the gut in infants is extremely important for the well-being of the whole organism in the successive stages of life (Bischoff, 2011). The stimulation of beneficial bacteria by prebiotic fibers in the infant gut is essential because these microbes, mainly belonging to the *Bifidobacterium* and *Lactobacillus* genera, play useful roles in prevention of diseases (Di Gioia, Aloisio, Mazzola, & Biavati, 2014; Turroni et al., 2012). In this context, the development of synbiotic formulas, i.e. mixtures of prebiotics and probiotics, in which the prebiotic fiber sustains the growth of the probiotic microorganism(s) supplied or of other beneficial host bacteria (Slavin, 2013), has a central role in infant nutrition. On the contrary, the growth of potential pathogenic or harmful microbes should not be enhanced by the metabolization of the fiber (Huebner, Wehling, & Hutkins, 2007). It is also important to underline that oligosaccharides may have additional beneficial effects for the host, because they can bind dangerous toxins and prevent adhesion of pathogens to the gut epithelium thus negatively affecting pathogen interaction with the host (Hickey, 2012; Newburg, 2000).

A number of works have developed and used a quantitative measure of the prebiotic efficacy of selected fibers with the use of a score, referred to as prebiotic score (Huebner et al., 2007; Marotti et al., 2012), which compares the extent to which a fiber supports selective growth of beneficial bacteria with respect to growth on glucose (i.e. an easily metabolized substrate) and to the growth of potential pathogenic bacteria. Potential pathogens to be used in this evaluation have to be selected considering the target for the prebiotic fiber under study.

The ability of bifidobacteria to utilize a large variety of oligosaccharides makes them able to adapt and compete in an environment with changing nutritional conditions such as the infant gut. A recent work has allowed the identification of three *Bifidobacterium breve* and one *Bifidobacterium longum* subsp. *longum*

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strains as potential probiotic strains for the treatment of enteric disorders in newborns such as infantile colics or as preventive agents for infantile diarrhea of bacterial origin (Aloisio et al., 2012). These strains possess strong antimicrobial activity against coliforms and other pathogenic bacteria, do not possess transmissible antibiotic resistance traits and are not cytotoxic for the gut epithelium. In addition, the capability of one of these strains, namely *B. breve* B632, of reducing the amount of gas forming coliforms has also been shown in an *in vitro* slurry model system simulating the intestinal microbiota of a 2-month-old colicky infant (Simone et al., 2014).

In this work, several commercial fibers, including fibers usually employed in the human diet but also less commonly used plant derived oligosaccharides, have been assayed for their capability of selectively stimulating the growth of the previously selected *Bifidobacterium* strains with the aim of developing a synbiotic product for infants. Moreover, the survival of the same strains in simulated gastro-intestinal conditions (stomach acidic pH and bile salts) has been checked in order to define suitable ways of administering them to newborns and infants with the aim of planning a validation clinical trial.

2. Materials and methods

2.1. Strains and culture conditions

Four *Bifidobacterium* strains (*B. breve* B632, B2274, B7840 and *B. longum* B1975), previously selected (Aloisio et al., 2012) as probiotics for the treatments of enteric disorders in newborns, were used. *Bifidobacterium* strains were grown on Tryptone, Phytone, Yeast extract (TPY) broth (tryptone, 10 gl^{-1}, soy peptone, 5 gl^{-1}, glucose, 10 gl^{-1}, yeast extract, 2.5 gl^{-1}, K_2HPO_4, 1.5 gl^{-1}, $MgCl_2 \cdot 6H_2O$, 0.5 gl^{-1}, cystein-HCl, 0.5 gl^{-1}, Tween 80, 0.5 gl^{-1}, pH 6.5). The medium was modified (m-TPY) to perform the growth experiment with potential prebiotic fibers: it did not contain the carbon source (glucose), which was provided by the fiber, and had a halved amount of tryptone, peptone and yeast extract. m-TPY pH was the same of the original medium.

Escherichia coli ATCC25645, two gas-forming coliforms isolated from colicky infants feces, i.e. *Klebsiella pneumonia* GC23a and *Enterobacter cloacae* GC6a (Savino et al., 2011), and *Clostridium difficile* M216, isolated from hospitalized patients (unpublished results), were used as potential pathogen strains. *E. coli*, *K. pneumonia* and *E. cloacae* strains were grown on M9 medium (Howard-Flanders & Theriot, 1966) with 10 gl^{-1} glucose (pH 6.9), whereas Reinforced *Clostridium* Medium (RCM, Merck, Germany, pH 6.8) was used for *C. difficile*. Modified M9 medium (m-M9) for prebiotic activity tests did not contain glucose. The modified RCM broth (m-RCM) was prepared with half of the concentration of peptone and yeast extract and no glucose. Modified media pH was the same of the original media. Inoculated cultures (20 ml l^{-1} inoculum) were incubated at 37 °C for 24–48 h under anaerobic conditions.

2.2. Evaluation of the prebiotic activity of the fibers

2.2.1. Commercial fibers used in the study

Two galacto-oligosaccharides (GOS), one fructo-oligosaccharide (FOS), four inulins having a different degree of polymerization (DP), a gluco-oligosaccharide, an arabinogalactan and a partially hydrolyzed guar gum were used. Available information on the fibers used, including composition, DP, origin, the commercial name as well as the provider, are listed in Table 1.

2.2.2. Prebiotic activity assays and prebiotic score

Prebiotic activity of the assayed fibers was evaluated according to a modification of Marotti et al. (2012) by evaluating the capability of the fiber of stimulating bifidobacteria with respect to potentially pathogenic strains. Fibers were added to autoclaved media. After fiber addition, in order to favor solubilization, the media were heated at 100 °C. Bifidobacteria growth was determined in m-TPY with 10 gl^{-1} of each fiber. As pathogenic strains, a 1:1:1 mixture of *E. coli* ATCC25645, *K. pneumoniae* GC23a and *E. cloacae* GC6a (referred to as enteric mixture) and a culture of *C. difficile* M216 were used. The enteric mixture was prepared by growing each strain separately on m-M9 with 10 gl^{-1} glucose and then mixing the cultures in a 1:1:1 ratio. The mixture was inoculated (20 ml l^{-1}) in m-M9 with 10 gl^{-1} prebiotic fiber. A culture of *C. difficile* (A_{620} 0.6) was prepared in m-RCM broth with 10 gl^{-1} glucose and inoculated (20 ml l^{-1}) in m-RCM broth supplemented with 10 gl^{-1} prebiotic fiber. Strains were also grown on the modified media with no added carbon source and with 10 gl^{-1} glucose (negative and positive controls, respectively). The assay was performed in 96 well plates, which were inoculated and incubated anaerobically at 37 °C. Bacterial growth was determined by measuring A_{620} after 0, 6, 24, 30 and 48 h of incubation in a microwell plate reader (Multiskan, Thermo Electron, Finland). Each assay was replicated three times. The growth curves for *Bifidobacterium* strains, for the enteric mixture and for *C. difficile* grown in the presence of tested prebiotic fibers were generated by plotting the average A_{620} versus incubation time. The prebiotic score (PS) was calculated as follows (Marotti et al., 2012):

PS = {(A_{620} of *Bifidobacterium* strain on the fiber at 24 h−A_{620} nm of *Bifidobacterium* strain on the fiber at 0 h)/(A_{620} of *Bifidobacterium* strain on glucose at 24 h−A_{620} of *Bifidobacterium* strain on glucose at 0 h)}−{(A_{620} nm of enteric mixture or *C. difficile* strain on the fiber at 24 h−A_{620} of enteric mixture on the fiber at 0 h)/($A_{620}$of enteric mixture on glucose at 24 h−A_{620} of enteric mixture on glucose at 0 h)}.

Factorial ANOVA was applied to analyze prebiotic scores using the Statistica Software (ver. 8.0, StatSoft, Tulsa, OK, USA). Values of $P < 0.05$ were considered to be significant.

2.3. Evaluation of strain survival under simulated intestinal conditions

Survival of *Bifidobacterium* strains was checked in human gastric juice, kindly provided in a lyophilized form by Probiotical SpA, Novara, Italy, and suspended in sterile water to obtain its original volume. Gastric juice was used at its pH (2.5) and at pH increased to 4 with NaOH 0.1 mol $l^{-1}$to simulate the newborn gastric pH (Kageyama, 2002) and was filter sterilized. In order to simulate duodenal conditions, 1 gl^{-1}porcine bile salts (Oxgal, Sigma−Aldrich) at pH 7 were used. To perform the survival assays, 0.1 g of lyophilized *Bifidobacterium* strains (nominal concentration 10^9 CFU g^{-1}) were suspended in 0.9 ml of gastric or bile juice solutions in sterile glass tubes and mixed. The cell suspension was incubated anaerobically at 37 °C. Cells suspended in PBS at pH 7.0 served as controls. Furthermore the survival to gastric juice at pH 2.5 of *B. breve* B632 strain in a microencapsulated form (provided by Probiotical S.p.A.) was assayed. Microencapsulation was performed in a vertical fluid bed drier Glatt GPCG2 LabSystem. 60 g of lyophilized strain (3.2×10^9 CFU g^{-1}) were mixed with 90 g of polyglycerol esters of saturated fatty acids. Final concentration of microencapsulated cells was 10^9 CFU g^{-1}.

Enumeration was performed after sampling 100 μl immediately after mixing the free cells with gastric juice or bile solution (time 0) and at pre-established times (30 and 60 min for simulated gastric conditions and 60 and 120 min for bile salts). The sampled amount

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