

African Journal of Biotechnology Vol. 10(12), pp. 2302-2307, 21 March, 2011

Available online at <http://www.academicjournals.org/AJB>

DOI: 10.5897/AJB10.580

ISSN 1684-5315 © 2011 Academic Journals

## Full Length Research Paper

# Infant formula as a substrate for bifidogenesis: An *in vitro* investigation

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Accepted 20 January, 2011

Six commercially available infant formulas (IF) without added prebiotic, were investigated as substrates for the growth of bifidobacteria isolated from the feces of 3 day old babies. This investigation tracked, during 48 h, the microbiological and biochemical changes in prepared infant formula meals, caused by the action of bifidobacteria, isolated from babies' feces. Pancreatin digestion preceded inoculation of the bifidobacteria biomass. The reference substrate was mature breast milk. This investigation showed important differences in bifidogenic effect between individual infant formulas and mature breast milk. In relation to dry biomass from the mature breast milk, dry biomass obtained in individual infant formulas ranged from 71.4% (IF-1) to 34.6% (IF-6). The results showed that, with the similar declared nutritive and biological characteristics, there are important differences between individual infant formulas. The results obtained showed that, an *in vitro* bifidogenic index test which covers tracking of microbiological and biochemical changes should be developed as a useful tool in determining the suitability of infant formulas and it should be one of the first steps before clinical investigations.

**Key words:** Infant formula, bifidobacteria, prebiotics, bifidogenic effect.

## INTRODUCTION

At birth, the sterile infant's gut becomes inoculated with bacteria from the mother and from the environment. Within a few days, the type of milk feeding becomes one of the most important factors for the further development of the intestinal microbiota (Satokari et al., 2002). Many studies have shown differences in the fecal microbiota between breast-fed and formula-fed infants. In general, the microbiota of breast-fed infants mainly contains bifidobacteria, while the microbiota of formula-fed infants is more diverse, with bifidobacteria often being the predominant species, but also containing substantial

quantities of *Bacteroides*, *Enterobacteriaceae* and *Clostridium* species (Harmsen et al., 2000; Leahy et al., 2005; Penders et al., 2006; Solis et al., 2010).

Human breast milk is the best food for a newborn baby (Pronczuk et al., 2004; WHO, 1981). In addition, it protects against infections (Pettigrew et al., 2003) and atopy (Gdalevich et al., 2001). Mothers who are not able to nurse their babies, often for different reasons, feed them with infant formulas "substitutes for breast milk" (Hamosh, 1999; The Commission of the European Communities, 2006). The complex composition of breast milk is impossible to fully attain in infant formulas, especially that related to the biologically active components (immunoprotective and other non-immunogenous protective substances, growth factors, hormones and enzymes) (Alles et al., 2004; Agostoni and Domellof, 2005). In addition, breast milk contains over one hundred different oligosaccharide structures that together with the other milk components, are the major source of prebiotic effect (Guidel-Urbano and Goni, 2001; Euler et al., 2005).

Non-digestible oligosaccharides in human milk contri-

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**Abbreviations:** BI, Bifidogenic index; BML, bifidobacteria medium with lactulose; DBM, dry biomass; IF, infant formula; MBM, mature breast milk; MRAL, mole ratio of acetic and lactic acid; TNB, total number of bifidobacteria; TOA, total organic acids.

bute to the bifidogenic microbiota of breast-fed infants. These oligosaccharides are prebiotics, they are not digested by human gastrointestinal enzymes, but enter the colon intact, where they serve as fermentable substrates for the colonic microbiota (Gibson and Roberfroid, 1995), preferably bifidobacteria (Roberfroid, 2000).

The bifidogenic effect (Gibson and Roberfroid, 1995) is the growth stimulation of bifidogenic bacteria, which are probiotics, in the intestinal tract by the effect of food ingredients, prebiotics for these bacteria. (Schrezenmeir and De Vrese, 2001; Simmering and Blaut, 2001; WHO, 2003; Manning and Gibson, 2004). The bifidogenic effect is in addition to its usual properties of nutritive and biological value, one of the most important biological indicators of the resemblance of infant formulas to breast milk. For this reason, it is necessary to know and also control this property.

Manufacturers are trying to produce infant formula that closely resembles the mothers' breast milk. Although, the composition of all infant formulas, without added prebiotics, is relatively similar, it can be assumed that, despite that, there are still differences in their effect on the development of the intestinal flora of the babies, primarily on the growth of bifidobacteria. Therefore, this study measured differences in microbiological and biochemical parameters resulting from the growth of bifidobacteria in different infant formulas, in comparison to their growth in mature breast milk.

## MATERIALS AND METHODS

### Chemicals

All chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany), Sigma (Stenheim, Germany) and Torlak (Belgrade, Serbia).

### Substrates

Six commercially available infant formulas, without added prebiotic, (designations IF-1 to IF-6) were prepared according to the instructions of their producers. All infant formulas were based on cow's milk. It was included only the "starting milks" or "first milks"; formulated to meet the needs of healthy full-term infants, from 0 to 4 months of age. Table 1 indicates the general composition of each formula described in the information provided on each packet.

Bifidobacteria medium with lactulose (BML) broth (Nebra and Blanch, 1999) was a control substrate. Mature breast milk (MBM) was the reference substrate and it comprised of equal volumes of 20 breast milks obtained from 20 healthy mothers who had delivered full-term infants (samples being obtained from 3 to 10 days postpartum). Milk samples were collected in the Clinic for gynecology and obstetrics, University Clinical Centre, Belgrade, Serbia. All breast milk samples were frozen and stored at -20°C until analysis.

### Material for the isolation of bifidobacteria and preparation of inoculum

Material for the isolation of the accumulated mixed culture of

*Bifidobacterium* spp. was 1 g mixture of the 30 feces of healthy, vaginally and interm born babies, who were exclusively nursed and did not consume antibiotics. The feces were sampled three days after birth, directly upon excretion. All samples were frozen and stored at -20°C until preparation of the inoculum.

Bifidobacteria were isolated on a solid BML. The inoculum was prepared from the third 48 h subculture on a BML agar slant, into 10 ml liquid BML which was then incubated for 48 h at 37°C in an anaerostat (produced by "Heraeus") under an atmosphere of carbon dioxide (CO<sub>2</sub>). The total volume required was prepared by mixing several individual inoculums in order to obtain a large enough biomass for all the required seeding.

### Identification of the *in vitro* bifidobacterial isolates

Isolated colonies growing on BML were identified as members of the genus *Bifidobacterium* using rapid kits (RAPID ID 32A<sup>®</sup>; bioMérieux, Marcy-l'Etoile, France). These test kits are based on evaluation of a range of enzymes and allow classification to the genus level within 4 h.

### Pancreatic digestion – digestion simulation (Despopoulos and Silbernagl, 2003)

Digestion was carried out in wide-necked Erlenmeyer flasks of total volume 300 ml. Substrates (100 ml volumes) were added individually to Erlenmeyer flasks, together with 50,000 USNF units (General Committee of Revision, 1989) of amylase and protease and 4,000 USNF units of lipase (pancreatin powder; A. Constantino & Co., Italy).

Pancreatic digestion was performed by immersion in a water bath at 37°C for 2 h, with occasional shaking. Digestion was halted by sterilization (121°C for 20 min), allowing thermal deactivation of the enzymes. For one series of experiments, two Erlenmeyer flasks with the same substrates were prepared, one of which was used after inoculation for the determination of the parameters at the beginning of the experiment.

### *In vitro* bifidogenesis

Sterile "digested" substrates were inoculated with 1.0 ml of inoculum from the same accumulated mixed culture of bifidobacteria. Bifidogenesis lasted for 48 h at 37°C in anaerostat with CO<sub>2</sub>.

### Microbiological and analytical methods

#### Description of a native microscopic preparation (Gojic-Cvijovic and Vrvic, 2003)

A native preparation was obtained from the substrate and observed with a light microscope operating in the bright field ("Laboval", Zeiss, Germany) and observed using bright field microscopy.

#### Determination of the total number of bifidobacteria (TNB) (Collins et al., 2004)

Suitable dilutions of substrates and pure cultures of bifidobacteria were prepared in physiological saline and spread-plated onto BML agar. BML agar plates were incubated for 48 h at 37°C in a CO<sub>2</sub> atmosphere and plates with isolated bifidobacteria colonies counted to obtain TNB (expressed in colony-forming units per ml (cfu/ml)).

**Table 1.** Composition of infant milk-based formulas according to the information provided on labels.

Formula	Lipid <sup>a</sup>	Protein <sup>a</sup>	Carbohydrate <sup>a</sup>	Main ingredients <sup>b</sup>
IF 1	23.1	12.8	58.3	Skimmed milk, lactose, starch, palm olein, demineralized whey milk, colza, coconut and sunflower oils, soybean lecithin
IF 2	24.0	12.5	58.6	Demineralized whey milk, skimmed milk, palm olein, lactose, colza, palm and corn oils, soybean lecithin
IF 3	24.4	11.7	58.6	Demineralized whey milk, milk powder (partially demineralized), vegetable oils (palm, colza, sunflower), lactose
IF 4	26.0	9.5	58.0	Skimmed milk, vegetable oils (palm, coconut, sunflower, soybean), LC-PUFA (arachidonic and docosahexaenoic acid), lactose, milk proteins, soybean lecithin
IF 5	25.9	10.9	58.3	Skimmed milk, demineralized whey powder, vegetable oils, corn starch, LCP oils (vegetable, fish)
IF 6	26.0	10.7	58.3	Lactose, vegetable oil, skimmed milk, corn starch, serum proteins, egg phospholipids

<sup>a</sup>Expressed as g/100g of powder; <sup>b</sup> Ingredients are listed in the order in which they appear on the label. Beside this main ingredients, all infant formulas had also micronutrients (mineral components, vitamins and other microcomponents such as taurine).

#### Determination of the dry biomass (DBM) (Gojic-Cvijovic and Vrvic, 2003)

The dry biomass was determined gravimetrically from 10 ml culture-inoculated substrate, by centrifugation at 4,500 rpm for 10 min, triple water rinsing and measured using a hygrometer type "Shimazu"-moisture balance MOC-120H. The results are expressed in g/100 ml.

#### Determination of pH

The pH was measured using a pH-meter type PHM 26, with a combined glass electrode type GK 2401C ("Radiometer").

#### Determination of the total organic acids (TOA)

Total organic acids were determined by automatic potentiometric titration (Titrator type TT 2, "Tacussel", France, combined glass electrode type GK 2401C, "Radiometer") of 50 ml samples with a standard 0.1 M NaOH solution. The results were calculated as lactic acid and are expressed in g/100 ml.

#### Determination of the mole ratio of acetic and lactic acid (MRAL)

The molar ratio of acetic and lactic acid was determined by enzymatic tests in accordance with the producer's instructions («RANDOX», Crumlin, UK) (Manual Acetic and Lactic acid, 1999), modified for these substrates (Filtration through a 0.2 µm pore size membrane and without alkaline hydrolysis).

#### Statistical analysis

All statistical analysis of microbial counts, microbiological and biochemical parameters were calculated by statistical program SPSS, version 11.5. One-way ANOVA test was used to compare the observed parameters of all eight substrates, while for intergroup comparisons, the Bonferroni test was used. Values of  $p < 0.05$  were considered significant. Five replicates of each of the eight substrates were prepared and the parameters for each replicate

(Microscopy, TNB, DBM, pH, TOA and MRAL) were measured individually. Microscopy, TNB, DBM, pH and TOA were measured at the start and end of the study, while MRAL was measured only at the end after bifidogenesis.

## RESULTS

An *in vitro* artificial model as simulation of gastrointestinal transit was used to study bifidogenic effect of six commercial infant formulas, without adding prebiotic (IF1 and IF6). This model consisted of incubation chambers (Erlenmeyer flask), each containing an infant formula (IF1 and IF6) and also comparable chambers containing mature breast milk (MBM) and selective medium with lactulose broth (BML). All substrates were mixed with digestive enzymes using incubation techniques for solubilisation of nutrients and were subsequently inoculated and incubated with bifidobacteria.

At the beginning and end of the investigation, the microbiological (description of the native microscopic preparation, the total number of bifidobacteria and their dry biomass) and biochemical indicators (pH, total bacterial generated organic acids and at the end of the experiment mole ratio of the obtained acetic and lactic acids) were determined. These indicators were a reflection of the changes of the substrates caused exclusively by the action of bifidobacteria and the stimulation of their multiplication under the effects of infant formula as prebiotics.

Before addition of inoculums, the measured pH was lowest in MBM 6.55, while other samples had relatively similar initial pH in a range from 6.65 IF-5, over 6.70 IF-6, 6.73 IF-3, 6.78 IF-1 and highest pH was measured in samples IF-2 6.82 and IF-4 6.89, which was close to the initial pH of the control substrate (BML) 6.80.

The mole ratio of the produced acetic and lactic acids (3:2) is a characteristic of the genus *Bifidobacterium*

**Table 2.** Microbiological indicators of the bifidogenic effect at the end of the experiment (given as mean  $\pm$  SD).

Substrate	TNBx10 <sup>7</sup> (cfu/ml)	DBM (g/100 ml)
IF 1	1.34 $\pm$ 0.164	0.227 $\pm$ 0.009
IF 2	1.60 $\pm$ 0.265	0.162 $\pm$ 0.010
IF 3	1.02 $\pm$ 0.053	0.164 $\pm$ 0.008
IF 4	1.08 $\pm$ 0.278	0.143 $\pm$ 0.011
IF 5	2.00 $\pm$ 0.583	0.113 $\pm$ 0.004
IF 6	0.55 $\pm$ 0.374	0.109 $\pm$ 0.004
BML	0.30 $\pm$ 0.035	0.095 $\pm$ 0.001
MBM	0.80 $\pm$ 0.132	0.318 $\pm$ 0.006

IF, Infant formula; BML, bifidobacteria medium with lactulose; MBM, mature breast milk; TNB, total number of bifidobacteria; DBM- dry biomass.

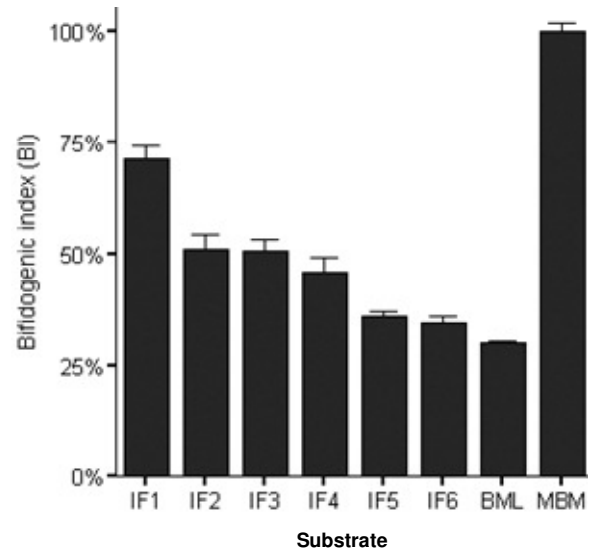
(without generation of CO<sub>2</sub>) (De Vries and Stouthamer, 1968), which was, besides the RAPID ID 32A<sup>®</sup> identification system for anaerobes, used as an additional indicator that the changes really were the consequence of the bifidogenic effect of the substrate. The microbiological indicators at the end of the experiment are given in Table 2.

The values at the beginning of the study were identical, with that in the native preparation, the bacterial cells were extremely small, of irregular characteristically elongated shape, in all substrates the TNB was 6.14 x 10<sup>5</sup> cfu/ml and dry biomass 0.005 g/100 ml.

The total number of bifidobacteria for all substrates at the end of the experiment was practically the same (10<sup>7</sup> cfu/ml). Also, there was no statistically significant difference in TNB between infant formulas and reference substrate (Table 2). There was no direct correlation between the total number and the dry biomass of the bifidobacteria, which is a clear consequence of their ability to undergo polymorphism. As expected, the most suitable substrate for the production of biomass was mature breast milk. However, important differences in the dry biomass were found between the different infant formulas. For that reason, the bifidogenic index (BI) was calculated. The BI (developed in the current study) is the ratio of bifidobacteria dry biomass obtained in the same series of the experiment at the end of the test with infant formula, breast milk multiplied by hundred, that is expressed in percentages, as a discriminatory criterion for the estimation of the bifidogenic effects. The values obtained in this study are shown in Figure 1.

In the current study, the BIs obtained from commercial infant formulas were significantly different ( $p < 0.05$ ) to the BI of MBM. In relation to the dry biomass from mature breast milk, the dry biomass obtained in the individual infant formulas was within the limits from 71.4 (IF-1) to 34.6% (IF-6). Changes in the biochemical indicators are given in Table 3.

The bacterially generated TOA at the beginning of the experiment were 0.015 g/100 ml in all substrates. At the



**Figure 1.** Estimation of the bifidogenic effects. Bifidogenic index (BI). IF, Infant formula; BML, bifidobacteria medium with lactulose; MBM, mature breast milk.

end of the process, as the result of the *in vitro* bifidogenesis, the pH in all substrates was 3.5 and 3.6 (Between 3.47 for IF-5 and 3.62 for IF- 4, with a mean value of 3.55), which corresponds to lactic buffer with a high buffer capacity, since the pH is approximately the pK<sub>a</sub> of lactic acid (pK<sub>a</sub> = 3.86) (Lurie, 1978). The concentration of the bacterially generated total organic acids in all infant formulas was higher in relation to the reference substrate and the differences in relation to mature breast milk were from 5.8 (IF-2 and IF-3) to 21.4% (IF-6). Although, bacterially generated TOA increased noticeably in all samples, the difference between the samples and the reference substrate (MBM) was not statistically significant ( $p > 0.05$ ).

The mole ratio of acetic and lactic acids in all the investigated substrates was very close to 3:2, which is a physiological-biochemical characteristic of the genus *Bifidobacterium*. This result is an additional confirmation that, the isolated accumulated mixed culture consists only of bifidobacteria species and that the changes of the substrate were only the consequence of bacterial action, that is, the result of bifidogenesis.

## DISCUSSION

Since all the replicates were performed simultaneously with the same inoculum and mature breast milk, possible mistakes were avoided due to the absence of standard mature breast milk and standard inoculum. In order to obtain an "average" inoculum and breast milk substrate, all replicates were prepared with inoculum, which was obtained from 1 g of feces, which was comprised of a

**Table 3.** Changes in the biochemical indicators (given as mean  $\pm$  SD).

Substrate	pH <sub>0</sub>	pH <sub>48</sub>	TOA (g/100 ml)	MRAL
IF 1	5.58 $\pm$ 0.036	3.61 $\pm$ 0.015	0.179 $\pm$ 0.007	3.46 : 1.90
IF 2	5.68 $\pm$ 0.011	3.54 $\pm$ 0.021	0.162 $\pm$ 0.013	3.22 : 1.90
IF 3	5.64 $\pm$ 0.011	3.59 $\pm$ 0.015	0.163 $\pm$ 0.002	3.06 : 1.75
IF 4	5.65 $\pm$ 0.011	3.62 $\pm$ 0.020	0.171 $\pm$ 0.003	2.98 : 1.88
IF 5	5.50 $\pm$ 0.026	3.47 $\pm$ 0.010	0.171 $\pm$ 0.003	3.10 : 1.94
IF 6	5.65 $\pm$ 0.025	3.54 $\pm$ 0.015	0.186 $\pm$ 0.001	3.10 : 1.83
BML	5.71 $\pm$ 0.030	3.75 $\pm$ 0.020	0.149 $\pm$ 0.006	3.12 : 1.95
MBM	5.77 $\pm$ 0.017	3.49 $\pm$ 0.020	0.154 $\pm$ 0.001	3.11 : 1.80

<sup>0</sup> pH at the beginning of the experiment; <sup>48</sup> pH at the end of the investigation; IF, infant formula; BML, bifidobacteria medium with lactulose; MBM, mature breast milk; TOA, total organic acids; MRAL, mole ratio of acetic and lactic acid.

mixture of feces collected from 30 babies. In addition, the mature breast milk substrate was a mixture of 20 breast milk samples obtained from 20 healthy mothers.

Comparing the differences in the values of microbiological and biochemical indicators of the bifidogenic effect, it can be seen that, the differences in the microbiological indicators were more distinct. For this reason, we proposed and used the BI. Using the BI calculation developed in the current study, the differences in the bifidogenic effect between individual infant formulas were clearly seen. With this *in vitro* investigation of infant formula, the bifidogenic effect can be easily monitored and objectively (all the conditions were controlled) determined and the quality of the infant formula evaluated in relation to this parameter.

Since the concentration of the bacterially generated organic acids in all infant formulas was higher in relation to the reference and control substrate, this is very significant considering previous work in which it was stated that production of TOA with a lower pH may have an impact on the growth of other groups of bacteria, yeasts and fungi, since these are inhibited by weak acids in combination with a low pH (Adams and Hall, 1988; Moon, 1983; McWilliam Leitch and Stewart, 2002). Additionally, it has been reported that bifidobacteria and other lactic acid bacteria, secrete compounds bacteriocins, which directly influence the growth of other colonic bacteria, including pathogens (Gibson and Wang, 1994).

Based on the results obtained during investigation of six commercial infant formulas, it can be concluded that the specimen IF-1 is the best one; the products, IF-2 to IF-4, have the same or similar bifidogenic effect and would belong to a medium category product, while the last two infant formulas would be in the lowest class. Since all the investigated products have similar declared nutritive and biological value, it is obvious that, their particular formulation plays a very important role, thus affecting their influence on growth of bifidobacteria or some contain substances which inhibit the activity of bifidobacteria.

Based on all the *in vitro* obtained and discussed results, concerning this investigation developed for the estimation

of the bifidogenic effect of infant formula in relation to mature breast milk as a reference substrate, the following conclusion can be made: The determined microbiological (description of the native microscopic preparation, total number of bifidobacteria and their dry biomass) and biochemical indicators (pH, bacterially generated total organic acids and the mole ratio of the obtained acetic and lactic acids) are suitable for tracking the *in vitro* bifidogenesis process. However, the dry biomass of bifidobacteria at the end of the investigation was the best indicator according to which it was possible to compare infant formulas in relation to the bifidogenic effect, as one of the most important parameters of their biological value and general quality.

Despite the fact that the dry biomass is a distinct indicator of the bifidogenic effect of infant formula, it is necessary to monitor both the microbiological and biochemical indicators in order to unambiguously establish whether the obtained changes in substrates are exclusively the consequence of bifidobacteria activity.

The objectivity of the current investigation lies in the fact that, all the conditions during the experiment were controlled and the bifidogenic index, which is proposed here as a discriminatory criterion for the evaluation of the bifidogenic effect on the basis of the values obtained for six tested commercial infant formulas, clearly categorized the tested products.

Further investigation should be directed to standardization of mature breast milk (in order to gain an average sample) and also to standardization of inoculum which would enable further evaluation of the BI test for measuring bifidogenic effect. Evolving an *in vitro* BI test would enable a reliable method for the examination of the biological value of infant formulas and it should produce quantitative discrimination in terms of food quality, which should be one of the most important factors in selection or development of infant formulas.

*In vitro* tests have not been described in the literature yet, but clinical tests are performed since they are more reliable. However, they are also time-consuming, expensive and connected to a series of problems, firstly with the number of babies on which they are conducted and

their individual differences (Puccio et al., 2007; Ziegler et al., 2007).

Further operations on the *in vitro* bifidogenic effect of infant formulas should be continued by investigating as many products which can be found on the market as possible, in order that they could also be discussed in relation to this property concerning the quality and utilization of infant formulas and the obtained results compared with clinical (*in vivo*) tests.

The final aim should be the standardization of the procedure for testing the bifidogenic effect and the introduction of this indicator into the criteria related to the scientific-based quality of infant formulas, that is, in a way that the bifidogenic effect is institutionalized.

## ACKNOWLEDGEMENTS

This work was partially financed by the Ministry of Science and Technological Development of the Republic of Serbia (Grants No. 142018B) and the authors wish to acknowledge their assistance.

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