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A LONG WAY FROM THE BIFIDOGENIC EFFECT TO ANTIOXIDATIVE AND PHARMACOLOGIC PROPERTIES OF BREAST MILK AND INFANT FORMULAS

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Bifidogenic effect and bifidogenic index

Our examination of infant formulas bifidogenic effect-development stimulation of bifidogenic bacteria-probiotics in the intestinal tract by the effect of food ingredients which are bifidogenic factors-prebiotics for these bacteria, (Heine, 1989, Lilly and Stillwell, 1965, Gibson and Roberfroid, 1995, Schrezenmeir and de Vrese, 2001, Reid, 2008) started twenty five years ago with an *in vitro* investigation (Vučetić *et al.*, 1989). The cooperation in these research were established with “IMPAZ” (Industry of dairy products in Zaječar, Serbia), now “Impamil” Ltd., Belgrade-Zemun (up to day only producer of infant formulas in Serbia) and Institute of neonatology, University pediatrics clinic and Center for the welfare of infants, children and adolescent, all in Belgrade.

Comparative study of mature breast milk, adapted milk-food for babies first 12 months, adapted milk-food for babies first 12 months with 5% (m/m) consume sugar-saccharose and reference one substrate have shown that tested substances, as bifidogenic factors (Dubey and Mistry, 1996), do not show more significant differences in respect to the determined biochemical parameters, which could have physiological effects (Vrvic *et al.*, 2000). In relation to bifidobacteria biomass as a probiotic, the breast milk is the best substrate through the baby’s food is quite acceptable, particularly with an addition of saccharose where biomass is slightly less. *In vitro* and in clinical study we have shown that inulin and fructooligosaccharides (FOS), as a prebiotic additive in infant formula, influence on bifidogenesis in infants in a manner comparable to the effect of mature breast milk (Sokić *et al.*, 2008, Lugonja *et al.*, 2010). We also 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.

Comparing the value differences for certain microbiological and biochemical indicators of bifidogenic effect, it is noticed that the differences in microbiological indicators are more distinct, that’s why we introduced **bifidogenic index** (BI), as a discriminatory criterion for estimation of the bifidogenous effects, which represents the ratio of bifidobacteria dry biomass, obtained in the same series of the experiment at the end of the test, with infant formula and breast milk multiplied by 100, respectively expressed in percentages (Martinov *et al.*, 2011). Based on the results obtained during investigation of six commercial infant formulas, it was concluded that the one specimen is the best one; the three products, 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. Our results confirmed that the bifidogenic index of infant formulas is proportional with concentration of lactulose which is one of well-known prebiotic (Jakovljevic *et al.*, 2008).

In the next study we determined digestibility, anti-radical activity and the effects on bifidobacterial growth of (1→3),(1→6)-β-D-glucans isolated by alkaline/acid extraction from *Saccharomyces. cerevisiae* (baker's yeast) in relation to the level of glucan purity (Laugier *et al.*, 2011). Our findings demonstrate that baker's yeast β-D-glucan is not degraded by human enzymes, and hence the structure and molecular mass of the glucan are preserved in digestive system, which is crucial for glucan's biological activity. In relation to its digestibility and bifidogenic efficiency, our results show this baker's yeast β-D-glucans should qualify as an indigestible supplement, suitable for use as a functional prebiotic food ingredient of infant formula and other products.

Antioxidative and pharmacologic properties of breast milk and infant formulas

As human mature breast milk is an “gold standard” (Newton, 2004, Walker, 2010) for baby's nutrition we continue to examine ways to achieve similar quality for infant formulas based on bovine milk.

Our study in last two years was to compare mature breast milk with standard infant formulas by examining their antioxidative properties and effects on non-vascular smooth muscle contraction.

In general, breast milk there are higher antioxidative activity compared with the infant formulas as shown in Table 1.

Table 1
Activities of antioxidant defense system in breast milk and different infant formula
(adapted from Lugonja *et al.*, 2013a)

Sample	Antioxidant components			Total proteins, [g/L]
	Total SOD, [U/g protein]	-SH, [μmol/L]	GSH-Px, [nmol NADPH · min ⁻¹ · g ⁻¹ protein]	
1	5.00 ± 2.3 ^a	35.6 ± 2.4 ^a	9.72 ± 2.16 ^a	12.02 ± 0.31 ^a
2	1.56 ± 0.56 ^b	23.3 ± 3.1 ^b	1.58 ± 0.25 ^b	15.57 ± 0.21 ^b
3	0.96 ± 0.13 ^b	12.4 ± 1.2 ^b	4.10 ± 0.16 ^b	17.93 ± 0.35 ^b
4	1.63 ± 0.35 ^b	25.1 ± 1.6 ^b	8.03 ± 0.21 ^a	19.46 ± 0.15 ^b
5	0.96 ± 0.23 ^b	35.2 ± 2.6 ^a	4.32 ± 0.15 ^b	21.60 ± 0.42 ^b
6	1.01 ± 0.21 ^b	15.52 ± 1.2 ^b	6.83 ± 0.81 ^a	19.45 ± 0.35 ^b
7	0.72 ± 0.15 ^b	12.5 ± 1.3 ^b	3.11 ± 0.65 ^b	16.51 ± 0.13 ^b
8	1.32 ± 0.42 ^b	14.3 ± 1.1 ^b	2.51 ± 0.42 ^b	17.35 ± 1.6 ^b

1-Mature human breast milk; 2-Mil 1 (Impamil, Serbia)-Infant formula (IF) for 0-6 months-old infants; 3-Mil 2 (Impamil, Serbia)-IF for 6-12 months-old infants; 4-Mil 3 (Impamil, Serbia)-IF for infants after 12 months; 5-Mil Pre (Impamil, Serbia)-IF for premature infants; 6-Mil FL (Impamil, Serbia)-IF free of lactose; 7-Bebelac (Danone, Holland)-IF for 0-6 months-old infants and 8-Aptamil (Milupa, Germany)-IF for 0-6 months-old infants.

SOD-Superoxide dismutase; -SH-free sulfhydryl group; GSH-Px-Glutathione peroxidase and NADPH-Reduced nicotinamide adenine dinucleotide phosphate.

The results are expressed as mean ±SD. Different superscript letters (^a and ^b) indicate a statistical significance between samples. SOD activity was significantly different at P < 0.005. The content of the -SH groups differed significantly at P < 0.05. GSH-Px activity differed significantly at P < 0.01.

The presence of total proteins differed significantly at P < 0.05.

Statistically significant differences in total SOD were observed between the breast milk (sample 1) and all infant formulas (samples 2–8, $P < 0.005$). GSH-Px was statistically different in samples 2, 3, 5, 7, and 8 compared with breast milk (sample 1, $P < 0.05$; Table 2). The content of -SH groups was statistically different in all infant formulas, except for sample 5 (Mil Pre, which contains a two-fold higher level of proteins) compared with sample 1 ($P < 0.0$).

The EPR (electron paramagnetic resonance) spectra obtained in the Fenton (Fenton, 1894) system (Fig. 1) showed that the production of hydroxyl radicals was

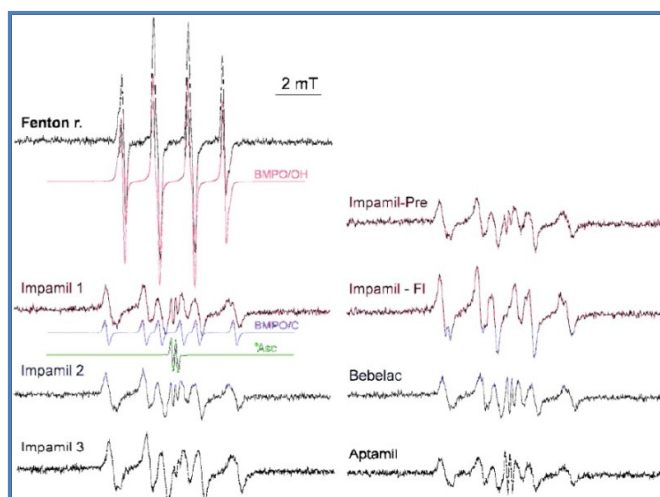


Fig. 1. (A) Fenton reaction in phosphate buffered saline (pH 7.4), Fe^{2+} (0.6 mmol/L), and H_2O_2 (3 mmol/L). Gray curves represent the spectral simulation of the 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-N-oxide (BMPO) adduct with the $\cdot\text{OH}$ radical. (B) Fenton reaction in sample 1. Gray curve represents the spectral simulation of the BMPO adduct with a carbon-centered radical. The signal intensity of the BMPO adduct with carbon-centered radical was 81 ± 10 . (C) Fenton reaction in sample 1 without a spin trap. Lines characteristic of ascorbyl radical are present (connected with the signal of the ascorbyl radical in B). (D) Fenton reaction in sample 2, signal intensity 108 ± 11 . (E) Fenton reaction in sample 3, signal intensity 111 ± 7 . (F) Fenton reaction in sample 4, signal intensity 171 ± 10 . (G) Fenton reaction in sample 5, signal intensity 107 ± 16 . (H) Fenton reaction in sample 6, signal intensity 145 ± 7 . (I) Fenton reaction in sample 7, signal intensity 94 ± 13 . (J) Fenton reaction in sample 8, signal intensity 80 ± 20 (Lugonja *et al.*, 2013a).

affected in breast milk and the infant formulas. In all systems, the hydroxyl radical reacted with biomolecules (proteins and ascorbate) to produce carbon-centered and ascorbyl radicals (Fig. 1). The signal of an ascorbyl radical emerged in the breast milk exposed to the Fenton system (Fig. 1B). The radical was stable and was detected by EPR without a spin trap (Fig. 1C). The level of these reactive byproducts was lowest in the breast milk. In samples 2 to 6, the level of carbon-centered radical was significantly higher than in breast milk (up to two-fold). In samples 7 and 8, the level of the carbon centered radical was similar to that in breast milk, but the level of the ascorbyl radical was clearly higher (Lugonja *et al.*, 2013a).

The aim of our newest study is to investigate the significance of breast milk and infant formula in the prevention of oxidative stress, by electrochemical determination of the total antioxidant potential, demonstrating the relationship between the antioxidant capacity of milk and postnatal age. Human breast milk, commercial UHT milk, and infant formulas supplemented with prebiotics were used. The potentiometric measurement indicates that

human breast milk has the highest redox potential, while the commercial UHT milk has very low potential. Infant formulas also have high potential. The main advantage of electrochemical methods used to assess the total antioxidant activity of milk was that they directly monitored the electron donating ability of the compounds and could be used for the quantitative analysis of the total antioxidants of different types of milk (Lugonja *et al.*, 2013b).

Our results at Fig. 2 showed that breast milk induces the relaxation of non-vascular smooth muscle (isolated rat uteri obtained from virgin female Wistar rats), whereas no such effect was observed with the infant formulas (Lugonja *et al.*, 2013a).

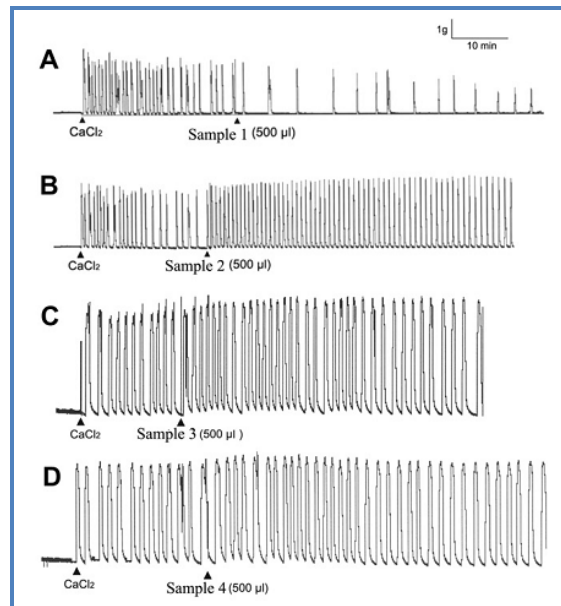


Fig.2. The effects of 500 μL of breast milk (sample 1) and different infant formulas on Ca^{2+} induced uterine contractions. Representative records from each examined group are presented ($n=5$). (A) Sample 1, (B) sample 2, (C) sample 3, (D) sample 4 (adapted from Lugonja *et al.*, 2013a).

Those pharmacologic effects may be due to the higher level of SOD in human milk, which is known to induce relaxing effects, in addition to its anti-inflammatory and other properties. Pertinent to the observed effects, human milk has been shown to have an antihypertensive activity. Milk casein-derived angiotensin-converting enzyme, inhibitory tripeptides: isoleucine-proline-proline (Ile-Pro-Pro) and valine-proline-proline (Val-Pro-Pro), which are present in breast milk, have been shown to exert antihypertensive effects in human subjects and to attenuate the development of hypertension in experimental models. It should be stressed that these tripeptides and SOD can preserve their structure and function in the human gastrointestinal system. Therefore, they should be capable of increasing infant digestion through their effects on the smooth muscles in the intestinal walls (Tsopmo and Friel, 2007, Ehlers *et al.* 2011).

Concluding Remarks

Human milk provides better antioxidant protection to baby's and exert direct pharmacologic effects compared with infant formulas because of the presence of specific components found only in human mature breast milk. We are confident that our results indicate that further development of infant formulas to closely resemble to mother milk health effects in baby's should be application of specific oligosaccharides (such as β -D-glucans from baker's yeast) as compensation for specific human oligosaccharides-prebiotics and further increasing of infant formulas bifidogenic and antioxidative capabilities and relaxation effects on gastrointestinal tract and on the mood of babies.

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Izvod

Dugačak put od bifidogenog efekta do antioksidativnih i farmakoloških osobina majčinog mleka i infant formula

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Dojenje-hranjenje novorodjenčeta majčinim mlekom, pruža prednosti za uspešan i zdrav početak života u odnosu na onu, koja su hranjena infant formulama. Uzrok je, naravno, u razlikama u sastavu humanog i kravljeg mleka koje je, skoro dominantno, osnova za proizvodnju hrane za bebe.

Naši radovi na ispitivanju i razvoju hrane za odojčad i malu decu započela su pre četvrt veka, u saradnji sa Industrijom mlečnih proizvoda-„IMPAZ“ iz Zaječara, čiji je naslednik sada „Impamil“ doo iz Beograda-Zemuna, onda i sada jedini domaći proizvođač ovih strateški važnih dijetetskih proizvoda.

Infant formule slične po osnovnim i propisanim nutritivnim i biološkim osobinama razlikuju se u kvalitetu po nekim od zakonski neobaveznih svojstava, koja su od značaja za pravilan razvoj i napredovanje bebe. Zato je i naš cilj da učinimo osobine i efekte infant formula sto bližim majčinom mleku. Put ne može biti dodatak originalnih komponenti majčinog mleka, već nadoknaditi nedostajuće drugim, koje mogu pozitivno uticati na zdravlje dece. Zato su naši prvi radovi bili usmereni ka bifidogenom efektu i definisanju bifidogenog indeksa kao jednih od ključnih kriterijuma za infant formule vrhunskog kvaliteta, što je dokazano nizom studija i naučnih radova u prestižnim časopisima. Posebna pažnja posvećuje se razvoju novih prirodnih prebiotika.

Antioksidativne osobine su u fokusu interesovanja ne samo u ishrani beba, već uopšte za vitalan život čoveka, pa smo u svetlu tih trendova dizajnirali i izučavali infant formule u poredjenju sa majčinim mlekom, kao „zlatnim standardom“. Majčino mleko sadrži moćan antioksidativni sistem, ali i infant formule, u zavisnosti od namene i proizvođača imaju sposobnost da uklanjaju reaktivne vrste slobodnih radikala i na taj način imaju protektivnu ulogu za organizam bebe.

Poznato je relaksantno dejstvo majčinog mleka na digestivni trakt bebe. Ovo farmakološko svojstvo, manjeg intenziteta, potvrđeno je u laboratorijskim ispitivanjima i za infant formule.

Naši rezultati pokazuju da opšta strategija razvoja infant formula, koje blisko odgovaraju osobinama majčinog mleka treba da se bazira na biomimikriji: sastava majčinog mleka u pogledu sadržaja antioksidanasa male molekulske mase i primeni specifičnih prebiotičkih polisaharida, kao što su glukani ćelijskog zida pekarskog kvasca, kao potencijalna zamena za oligosaharide humanog mleka. Svi naši dosadašnji i tekući radovi otvaraju nove perspektive i pravce za budući razvoj sastava infant formula najvišeg kvaliteta.