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Sphingoid bases in infant formulas

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Abstract

Sphingolipids are a group of lipids present in all eukaryotic cells. They consist of a long-chain sphingoid base that is amide-bound to a fatty acid and a polar group on C-1 sphingosine. Sphingosine is the most widespread base in mammals. The goal of this study is to determine the concentrations of free and total sphingosine and sphinganine in infant formulas and human milk. Following the extraction of sphingolipids, base and acidic hydrolysis was preformed. Sphinganine and sphingosine were determined by means of high-performance liquid chromatography. The results of this research illustrate the differences between the concentrations of sphingoid bases in infant formulas and human milk. On the basis of the obtained results, it can be concluded that despite all efforts made to produce infant formulas as similar to human milk as possible, in terms of their structure and the amount of their constituents, there are differences that could be biologically significant and thus need to be further researched.

Keywords: Sphingosine; Sphinganine; Infant formulas; Human milk; HPLC

1. Introduction

Sphingolipids are a group of lipids present in all eukaryotic cells (Vesper et al., 1999). They consist of a long-chain sphingoid base that is amide-bound to a fatty acid producing ceramide. A C-1 hydroxyl group of ceramide is substituted with various polar constituents to give complex sphingolipids (Merrill et al., 1997). Sphingoid bases are long-chain amino alcohols that differ in alkyl chain lengths (from C_{14} to C_{22}) and branching, in the presence or absence of a hydroxyl group at the C-4 position or elsewhere, the 4,5-*trans*-double bond, double bond(s) at other positions and methyl group(s) on the alkyl side chain or on the amino group (Vesper et al., 1999). The most widespread long-chain bases in mammals are D-*erythro*-sphingosine and DL-sphinganine (Fig. 1). Sphingolipids have been implicated in cell growth and differentiation, oncogenesis, and cell to cell contact (Olivera & Spiegel, 1992; Hakomori, 1990) for a long time. They act as cell surface receptors for some viruses and bacteria; they can modulate the functions of certain growth factors, and they may be effectors of protein kinases, ion transporters, and other membrane proteins. However, it has become evident that the lipid backbones of sphingolipids are highly bioactive compounds which may play important roles in cell regulation. These include ceramide, sphingosine, sphingosine-1-phosphate, sphingosine phosphorylcoline and di- and trimethylsphingosine. Sphingolipid metabolites appear to serve as second messengers for growth factors, cytokines, and other agonists, and when elevated abnormally, they could lead to disease (Spiegel & Merrill, 1996). Thus, these bioactive compounds play an important role in normal cell functioning as well as in disease development.

The concentration of free sphingoid bases in cells is low. Generally, the literature offers little data on sphingoid base content in human bodily fluids (Castegnaro et al., 1998; Ribar, Mesarić, & Sedić, 2003; Solfrizzo, Avantaggiato, & Visconti, 1997; Van der Westhuizen, Brown, Marasas, Swanevelder, & Shephard, 1999) as well as in food. Sphingolipids are constituents of most foods, but their amounts are relatively small, and there is no evidence that dietary sphingolipids are required for either growth or survival.

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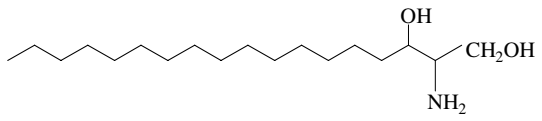
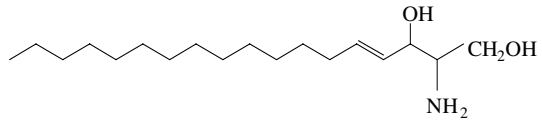


D-*erythro*-sphinganine



D-*erythro*-sphingosine

Fig. 1. The structures of sphingosine and sphinganine.

Milk contains different sphingolipids. The major sphingolipid of human milk is sphingomyelin. Although there is no evidence that dietary sphingolipids are essential for growth and development, the digestion products derived from them in the intestine, i.e. ceramide and sphingosine, have been proven to have a profound impact on the developmental fate of many cell types. This is due to the fact that they act as intracellular messengers, involved in cell cycle regulation and apoptosis induction.

In the current study, we compare for the first time the concentrations of sphingoid bases in human milk and commercially available infant formulas.

2. Materials and methods

2.1. Materials

Fourteen commercially available infant formulas: Bebimil 0, Bebimil 1, Bebimil 2, Hipp pre, Hipp 1, Hipp 2, Laktovit plus, Aptamil 1, Aptamil 2, Aptamil 3 (Agrana, Austria), Humana 1, Humana 2 (Humana GmbH, Germany), Milumil 1, Milumil 2 (Milupa GmbH & Co KG, Germany) were prepared according to the instructions of their producers. All infant formulas were based on cow's milk. Human milk samples were obtained from 20 healthy mothers who had delivered full-term infants (samples being obtained from 3 to 42 days postpartum). Milk samples were collected at the Department of Gynaecology and Obstetrics, Zagreb University Hospital Center (Zagreb, Croatia). All milk samples were frozen and stored immediately at −20°C until analysis.

2.2. Chemicals

Standards of sphingoid bases (C18 D-sphingosine and C18-DL-*erythro*-dihydrosphingosine), ortho-phthalaldehyde (OPA), and 2-mercaptoethanol were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany); $CHCl_3$ (pro analysi), CH_3OH (HPLC gradient grade) and CH_3OH (pro analysi) were purchased from Riedel de Hähn AG (Seelze, Germany); NH_4OH, KOH and HCl were purchased from Kemika (Zagreb, Croatia).

2.3. Isolation of sphingosine and sphinganine

Sphingolipids were extracted from infant formulas and human milk, then subjected to acid (Yoo, Norred, & Riley, 1996) and base hydrolysis according to the method of Riley et al. (Riley, Wang, & Merrill, 1994) with minor modifications (volumes of alkaline water and chloroform were changed; chloroform phase did not dry over Na_2SO_4 tightly packed in Pasteur pipette drying tube).

2.4. Extraction procedures

A mixture containing 2.25 ml $CHCl_3$: CH_3OH = 1:2 (v/v) and 0.150 ml of 2 N NH_4OH solution was added to 0.5 ml of milk sample (or to100 mg of infant formulas), and the content was thoroughly mixed on a vortex. Then the mixture was incubated at 37 °C for 1 h in a closed screw tube and cooled down to room temperature; then, 1.5 ml $CHCl_3$ and 3 ml alkaline water (0.1 ml 2 N NH_4OH + 250 ml distilled deionised water, pH 8.0–10.0, prepared daily) were added and centrifuged for 20 min at 3000 rpm for phase separation.

Following the centrifugation, the upper aqueous phase was discarded, and 3 ml alkaline water was added to the lower chloroform phase; the content was mixed and centrifuged again. Rinsing of the chloroform phase with alkaline water was repeated once, after which the aqueous phase was removed, and the chloroform phase was evaporated to dryness. Thus prepared, the sample could be stored overnight in nitrogen at +4 °C or subjected to base or acid hydrolysis.

2.5. Base hydrolysis

The objective of base hydrolysis is the splitting of acylglycerolipids (to obtain free sphinganine and sphingosine) and hydrolysis of lysosphingolipids (free sphingoid bases modified on the sphinganine or sphingosine C1 atom hydroxyl group) in order to release free sphinganine and sphingosine. The phospholipids (phosphatidylethanol amine and phosphatidyl serine) that may react with orthophtaldialdehyde (OPA) reagent for sphingoid base derivation are successfully eliminated by the base treatment (Merrill et al., 1988). This procedure does not release sphingoid bases from complex sphingolipids.

Base hydrolysis was performed by the modified method described by Riley et al (Riley et al., 1994). A dry extract was dissolved in 1 ml of 0.1 M methanolic KOH: $CHCl_3$ = 4:1 (v/v) mixture and placed for 1 min in an ultrasonic bath, after which it was incubated at 37 °C for 1 h. The mixture was cooled down, and 1 ml $CHCl_3$ and 1 ml alkaline water were added, gently mixed and centrifuged for 20 min at 3000 rpm. The upper aqueous phase was then discarded, and the lower chloroform phase was rinsed with 2 ml alkaline water and centrifuged again for 10 min at 3000 rpm. The upper aqueous phase was then again removed, and the chloroform phase was evaporated to dryness and stored at −20 °C until analysis.

2.6. Acid hydrolysis

The objective of acid hydrolysis is to obtain total sphinganine and sphingosine released from complex sphingolipids. Acid treatment leads to hydrolysis of amide-bound fatty acid and any group esterified on the sphingoid base C-1 atom. Acid hydrolysis was performed by the method described by Yoo et al. (Yoo et al., 1996). To dry extract, 0.5 ml aqueous methanolic HCl (1 N HCl, prepared immediately before use) was added, and the tube was capped by a Teflon cap and left to stay for approximately 15 h at 68 °C. Upon cooling down to room temperature, 0.5 ml saturated methanol KOH (30 g KOH was dissolved in 100 ml CH_3OH and filtered through wrinkled filter paper), 0.5 ml alkaline water, 0.1 ml of 2 N NH_4OH solution and 0.6 ml $CHCl_3$ were added, and the mixture was stirred and centrifuged for 20 min at 3000 rpm. Then, the aqueous phase was discarded and the chloroform phase was rinsed 3 times with 0.9 ml alkaline water, evaporated to dryness, and stored in nitrogen at −20 °C until analysis.

The initial milk volumes for the base and acid hydrolysis were 0.5 ml and 10 μl, respectively. The initial infant formula masses for the base and acid hydrolysis were 100 mg and 10 mg, respectively.

2.7. Chromatographic analysis

2.7.1. Instruments

High performance liquid chromatography (HPLC) was performed using the following Perkin–Elmer equipment: isocratic pump with one piston, fluorescence detector, interface, column oven, autosampler and software for chromatography (Turbochrom 4.1.2). Analytical column (Radial-Pak™ cartridge, Nova-Pak™ C_{18}, 10 cm × 0.8 cm, 4 μm), column module and holder with precolumn filter (Guard-Pak assembly, Nova-Pak C_{18}, 4 μm) were purchased from Waters Corporation. Device for HPLC solvent filtration, centrifuge, microcentrifuge, vortex, water bath, univapo 100 H evaporator with Unicryo MC 21 cooling unit, ultrasonic bath and thermostat were also used.

2.7.2. Chromatographic conditions

The sphingoid bases were analyzed with high performance liquid chromatography (HPLC). The ortho-phthalaldehyde reagent (OPA) reacting with sphingoid base via its amino group was used for sphingoid base derivation before their column application. OPA reagent was prepared by means of dissolving 5 mg reagent and 5 μl 2-mercaptoethanol in 0.1 ml ethanol, making up to 10 ml with boric buffer (pH adjusted to 10.5 by 1 M KOH). Thus prepared reagent remains stable for 7 days if stored in the dark at +4 °C.

2.7.3. Standard mixture

A standard mixture is prepared by adding 3 μl each of 10 μM C18 D-sphingosine and C18 DL-*erythro*-dihydrosphingosine. Then 194 μl of mobile phase and 100 μl OPA reagent are added. The mixture is stirred and left for 20–30 min at room temperature. The mixture is then filtered and injected.

2.7.4. Sample preparation

A sample was prepared for HPLC by adding 250 μl mobile phase to the dry extract (obtained by base or acid hydrolysis), mixing for 1 min on a vortex, and adding 50 μl OPA reagent. The mixture was then mixed for 30 s on a vortex, filtered by centrifugation through a filter of 0.45-μm pore size (for 1 min), and left for 1 h at room temperature before injection. The comparison of the sample chromatogram with the standard mixture chromatogram was used in order to identify particular sphingoid bases in the sample.

2.7.5. High performance liquid chromatography conditions

A mixture of CH_3OH: H_2O = 9:1 (v/v), filtered through nitrocellulose filter paper of 0.45-μm pore size and degassed by the passage of helium for 3–5 min, was used as a mobile phase. The flow rate was 2 ml/min. Analyses were performed at emission wavelength of 440 nm and excitation wavelength of 334 nm. The injected sample volume was 50 μl.

2.8. Statistical analysis

Statistical analysis of the results was performed using T-test (Ivanković et al., 1991).We used STATISTICA for Windows, Version 5.0, StatSoft, Inc., Tulsa, OK, USA.

3. Results and discussion

Sphingolipids as a constituent of eukaryotic cell membranes do not have only a structural function. They are also vital for the regulation of cell growth, their survival as well as for their death (Spiegel & Merrill, 1996). Although they are not essential constituents of food, recent research emphasizes the role of their decomposition products in the preservation of health. Therefore, it is necessary to bring a certain amount of sphingolipids into an organism through food. On the basis of data from well known literature (Pan & Izumi, 1999; Rueda, Puente, Hueso, Maldonado, & Gil, 1995; Takamizawa, Iwamori, Mutai, & Nagai, 1986), it can be concluded that there is a difference in the amount and structure of sphingolipids between human milk and infant formulas. Milk is a complex liquid that consists mainly of water, carbohydrates, fats, proteins, mineral substances and vitamins. It contains 3.8–3.9 g of fat/100 ml, which is present in the form of globules emulsified in the water phase of milk (Koletzko et al., 2001). Human milk fat is the major source of energy for the breast fed infant, contributing some 40–55% of the total energy intake. The lipids present in milk are triglycerides (98%), phospholipids (0.7%), cholesterol (0.5%), as well as other complexes like sphingomyelin, gangliosides and neutral glycosylceramides (Jensen, 1996; Jensen et al., 1995). The

major sphingolipid of human milk is sphingomyelin. C_{18}-sphingosine contributes 60% of the sphingoid bases of human milk sphingomyelin (Bouhours & Bouhours, 1981). Human milk sphingomyelin differs from cow's milk sphingomyelin in its sphingoid base composition. Relative proportions of sphinganine vs sphingosine bases are similar, but cow milk sphingomyelin is more heterogenous and contains a higher proportion of C_{16}-sphingosine and branched chain bases (Morrison, 1969). Some studies on fatty acid synthesis in brain of neonatal mouse suggest that a part of the long chain fatty acids required for normal myelin formation may have a nutritional origin (Bourre, Paternau-Jouas, Daudu, & Baumann, 1977). If such a requirement exists in human infancy, milk sphingolipids are the best candidate to fulfill it. Thus, it can be concluded that sphingolipids are important constituents of both human milk and infant formulas.

As there is no literature on the concentration of sphingoid bases in infant formulas, the goal of this study was to determine the concentrations of free and total sphingosine and sphinganine in infant formulas and to explore weather there is a difference in relation to human milk. After the extraction of sphingolipids, they were subjected to base and acid hydrolysis, after which the concentrations of free and total sphinganine and sphingosine were determined by means of high performance liquid chromatography (HPLC). Sphingoid bases were identified by comparing their retention time with the standard retention time under the same conditions (Fig. 2). Analysis was performed using 14 samples of infant formulas and 20 samples of human milk provided by healthy women. The results of this study show that the concentrations of free sphingosine in all samples of infant formulas (Fig. 3), ($P = 0.001$) were significantly lower than in human milk. The comparison of medium-concentration values of free sphingosine in particular samples of infant formulas shows that they were lower in all samples than in human milk. Statistically speaking, the concentrations of free sphinganine in infant formulas were also significantly lower than the ones in human milk (Fig. 4), ($P < 0.001$). The comparison of medium-concentration values of free sphinganine in particular samples of infant formulas to human milk shows that they were lower in 13 out of 14 examined samples (Fig. 4). The concentrations of total sphingosine in infant formulas were also significantly lower ($P < 0.001$), (Fig. 5). Comparing medium-concentration values of total sphingosine in particular samples of infant formulas with human milk shows that they were significantly lower in all samples of infant formulas (Fig. 5). The comparison of total sphinganine in particular kinds of infant formulas and human milk showed that the concentrations were lower in all nine kinds of formulas (Fig. 6). However, in this variable, there is no statistically significant difference between all samples of infant formulas and human milk ($P = 0.09$). Because sphingomyelin is the major sphingolipid of milk, it could be assumed that it is the main source of milk sphingoid bases.

The development of new technology has shown that human milk contains numerous nutrients, enzymes, hormones and growth factors, many of which undergo certain modifications in the process of lactation. However, the knowledge about these substances in infant formulas is still quite limited (Goldman & Garza, 1987). It is well-known that human milk completely fulfills the needs of an infant. It contains a whole set of biochemical, metabolical, nutritious, and immunological components. Human milk acts as an infant's first vaccination, significantly lowering the risks of disease. It is also well-known that mortality caused by infective diseases is somewhat higher for infants who were fed with infant formulas (Jason, Nieburg, & Marks, 1984; Laegried, Otnaess, & Fuglesang, 1986). Furthermore, infants fed with human milk are less prone to infections of gastrointestinal tract in comparison with infants fed with

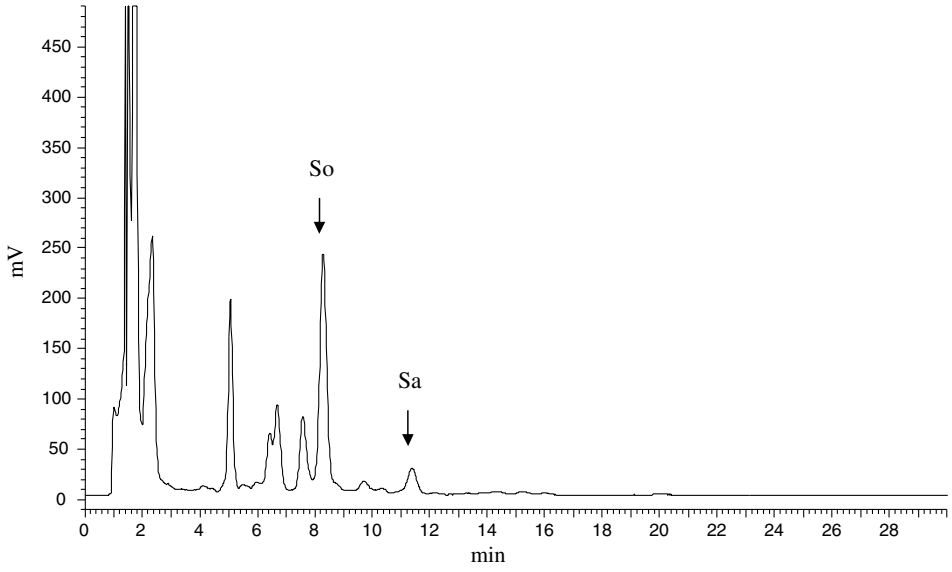


Fig. 2. HPLC chromatogram of total sphinganine and sphingosine in infant formulas.

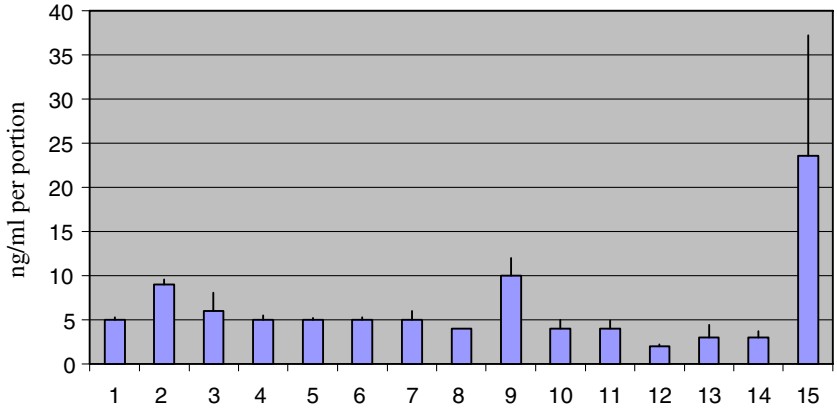


Fig. 3. The concentrations of free sphingosine in infant formulas ($\bar{x} \pm$ SD). "ng/ml per portion" mean: ng/ml of prepared infant formulas (which is mixture of infant formulas powder and water). 1 = bebimil 1; 2 = bebimil 2; 3 = humana 1; 4 = humana 2; 5 = hipp 1; 6 = hipp 2, 7 = laktovit plus, 8 = hipp pre, 9 = bebimil 0, 10 = milumil 1, 11 = milumil 2; 12 = aptamil 1; 13 = aptamil 2; 14 = aptamil 3; 15 = human milk.

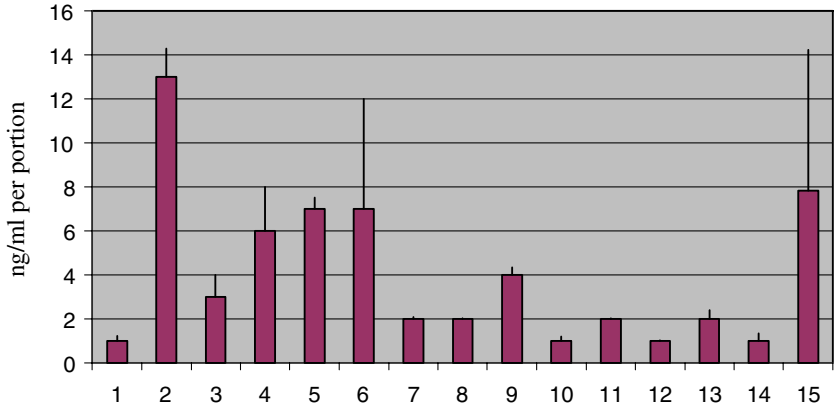


Fig. 4. The concentrations of free sphinganine in infant formulas ($\bar{x} \pm$ SD). "ng/ml per portion" mean: ng/ml of prepared infant formulas (which is mixture of infant formulas powder and water). 1 = bebimil 1; 2 = bebimil 2; 3 = humana 1; 4 = humana 2; 5 = hipp 1; 6 = hipp 2, 7 = laktovit plus, 8 = hipp pre, 9 = bebimil 0, 10 = milumil 1, 11 = milumil 2; 12 = aptamil 1; 13 = aptamil 2; 14 = aptamil 3; 15 = human milk.

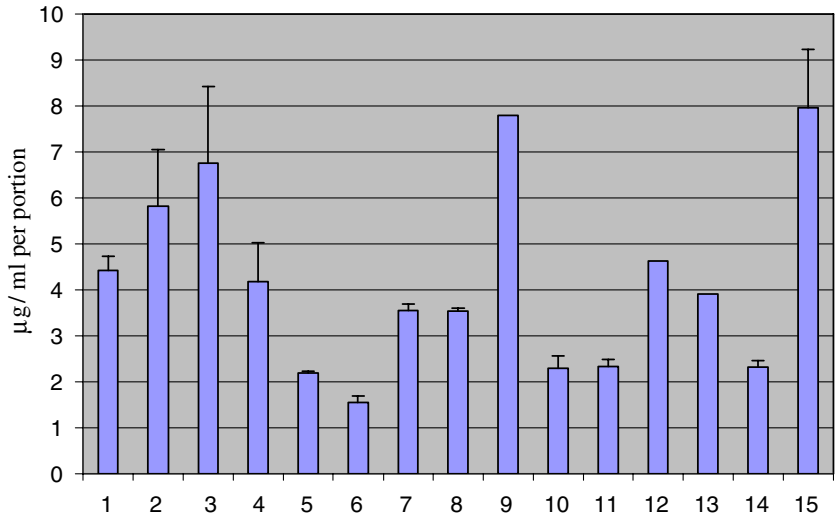


Fig. 5. The concentrations of total sphingosine in infant formulas ($\bar{x} \pm$ SD). "µg/ml per portion" mean: µg/ml of prepared infant formulas (which is mixture of infant formulas powder and water). 1 = bebimil 1; 2 = bebimil 2; 3 = humana 1; 4 = humana 2; 5 = hipp 1; 6 = hipp 2, 7 = laktovit plus, 8 = hipp pre, 9 = bebimil 0, 10 = milumil 1, 11 = milumil 2; 12 = aptamil 1; 13 = aptamil 2; 14 = aptamil 3; 15 = human milk.

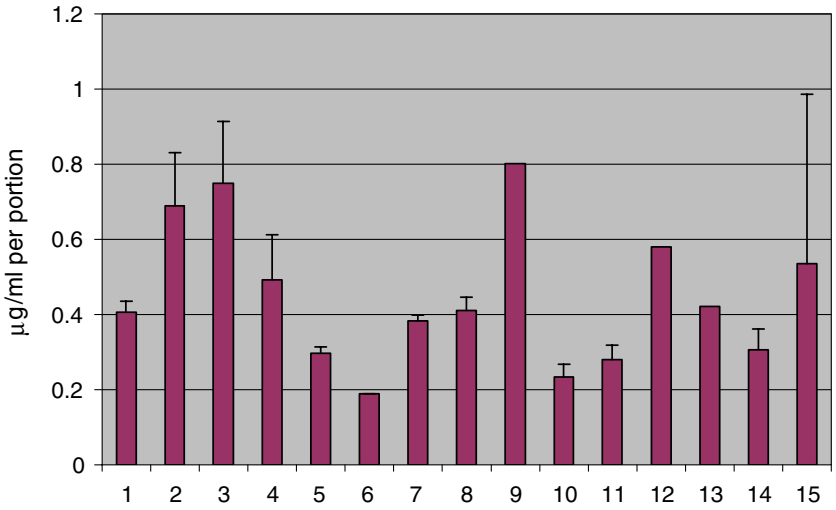


Fig. 6. The concentrations of total sphinganine in infant formulas ($\bar{x} \pm$ SD). "µg/ml per portion" mean: µg/ml of prepared infant formulas (which is mixture of infant formulas powder and water). 1 = bebimil 1; 2 = bebimil 2; 3 = humana 1; 4 = humana 2; 5 = hipp 1; 6 = hipp 2, 7 = laktovit plus, 8 = hipp pre, 9 = bebimil 0, 10 = milumil 1, 11 = milumil 2; 12 = aptamil 1; 13 = aptamil 2; 14 = aptamil 3; 15 = human milk.

cow's milk or artificial infant formulas (Kovar, Serdula, Marks, & Fraser, 1984). Some substances that are considered to be favourable for the protective function of human milk against bacteria caused diseases are present in significantly lower amounts in cow's milk (Welsh & May, 1979). Human milk also aids the decrease in occurrences of respiratory infections and allergies. Children who were breastfeed for a long period of time have lower rates of adolescent diabetes and children's lymphomas. Colostrum contains less fat, and more protein (mostly antibodies IgA that provide an infant with part of his or her passive immunity), proving that breastfeeding decreases the risk of food allergies which can be a consequence of early contact with protein, especially that from a cow. Human milk and infant formulas are significantly different in terms of their nutritious values, amounts of enzymes, growth factors and immunities.

The fact that the concentrations of sphingoid bases are higher in human milk than in infant formulas enhances the importance of breast feeding in order to provide sufficient amounts of sphingoid bases for an infant organism. Significant differences between particular kinds of infant formulas in terms of the concentrations of sphingoid bases that were proven by this study could be the consequence of seasonal, nutritious, and lactation differences of the cow's milk that was used for the production of the different formulas. High variability within human milk samples could be explained by nutritious or lactation times differences.

There is a lot of research highlighting the function of sphingolipids for the preservation of health. The metabolism of sphingolipids in immunological cells creates other lipid messengers (ceramide, sphingosine-phosphate, ceramide-1-phosphate, sfingosine-1-phosphate) which are components of signal ways (Baumruker & Prieschl, 2002) and which control main phases of immunological cell development, differentiation, activation and proliferation. Numerous experiments on animals have shown that sphingomyelin and other sphingolipids can inhibit the development of spontaneous and chemically induced intestinal tumours (Dillehay, Webb, Schmelz, & Merrill, 1994; Schmelz et al., 1996; Schmelz et al., 2001). It is well-known that ceramides have a proapopotic and anti-proliferation effect, while sphingosine-1-phosphate has the opposite effect (Spiegel & Milstien, 2000). One of the most striking responses of carcinoma cells to chemotherapy is the accumulation of endogenous ceramide (Cabot, Giuliano, Han, & Liu, 1999; Charles et al., 2001; Lucci, Han, Liu, Giuliano, & Cabot, 1999; Maurer, Metelitsa, Seeger, Cabot, & Reynolds, 1999). Radiotherapy also causes the accumulation of endogenous ceramide and apoptosis induction (Haimovitz-Friedman et al., 1994). The addition of glycosphingolipids from milk and C_{16} ceramide directly to animal food results in the suppression of colon neoplasia (Schmelz, Sullards, Dillehay, & Merrill, 2000), while C_8 ceramide significantly reduces focal cerebral ischemia in SH (spontaneously hypertensive) rats (Furuja, Ginis, Takeda, Chen, & Hallenbeck, 2001). Research on the effects of sphingolipids could detect a mechanism by means of which sphingolipids from food reduce colon carcinogenesis. It could imply that the digestion of complex sphingolipids creates bioactive sphingoid bases that have an important function in signal transduction and cell regulation, either through the inhibition of cell growth, the induction of cell differentiation, or the stimulation of apoptosis (Davis, Flaws, Young, Collins, & Colburn, 2000; Merrill et al., 1997).

Based on the obtained results, it can be concluded that despite all efforts made in order to produce infant formulas as similar to human milk as possible, there are still differences that could be biologically important, influencing brain growth, the risk of food allergies, and non-immuno-

globuline effects against bacterial toxins. All of the above-mentioned highlights the importance of breast-feeding for an infant organism as it provides sufficient amounts of sphingolipids which have a whole set of biochemical functions.

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