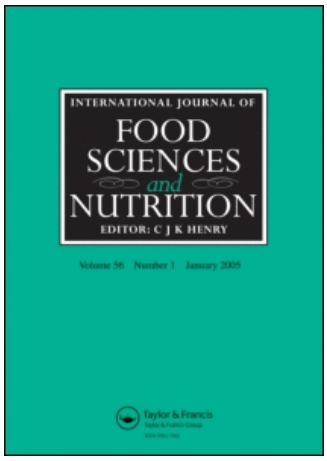

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Composition of the non-protein nitrogen fraction of goat whole milk powder and goat milk-based infant and follow-on formulae

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Abstract
The non-protein nitrogen fraction of goat whole milk powder and of infant and follow-on formulae made from goat milk was characterized and compared with cow milk powder and formulae. Goat milk infant formula contained 10% non-protein nitrogen, expressed as a proportion of total nitrogen, compared with 7.1% for cow milk formula. Goat follow-on formula contained 9.3% and cow 7.4% non-protein nitrogen. Urea, at 30%, was quantitatively the most abundant component of the non-protein nitrogen fraction of goat milk and formulae, followed by free amino acids at 7%. Taurine, glycine and glutamic acid were the most abundant free amino acids in goat milk powders. Goat milk infant formula contained 4 mg/100 ml total nucleotide monophosphates, all derived from the goat milk itself. Goat milk has a very different profile of the non-protein nitrogen fraction to cow milk, with several constituents such as nucleotides at concentrations approaching those in human breast milk.

Keywords: *Infant formula, follow-on formula, non-protein nitrogen, goat milk, nucleotides*

Introduction

The total nitrogen component of milk consists of protein and non-protein nitrogen. Non-protein nitrogen accounts for 18–30% of the total nitrogen content of human milk, consisting of urea, free amino acids, nucleotides, creatinine and other nitrogen-containing moieties (Carlson 1985; Donovan & Lonnerdal 1989; Agostoni et al. 2000). These components have a variety of functions in the neonate. For instance, urea is converted to amino acids that are utilized for protein synthesis (Heine et al. 1986; Jackson 1994). Free amino acids are similarly utilized or used to support intestinal functions (Fuller & Reeds 1998; Duggan et al. 2002). Several studies suggest nucleotides and polyamines in milk are important for development of immune response in infants (Peulen et al. 1998; Duchen & Thorell 1999; Dandrifosse et al. 2000; Hawkes et al. 2006). Thus, increasing evidence indicates non-protein nitrogen components, although present at low concentrations, can have a profound impact on

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the development of metabolic, immunological and physiological processes of the infant.

In circumstances where a mother is not able to feed her infant breast milk, a substitute for human milk is necessary to satisfy the nutritional requirements of infants. Infant formula is for use by infants during the first 4–6 months of life and follow-on formula is used after 4 months, usually until 12 months of age. Infant formula must provide all the nutrients sufficient for adequate growth of healthy newborn infants when supplied as the sole source of nutriment, whereas follow-on formulae are typically used as the principal liquid element in a progressively diversified diet.

Traditionally, cow milk has been used as a low-cost ingredient for manufacture of infant formula and follow-on formula, because of its widespread availability. Other milk sources such as goat milk may also be used as a source of nitrogen for these formulations. Goat milk is widely consumed in some European countries, the Middle East and Bangladesh (Haenlein 2004). Goat whole milk powders are manufactured in several European countries, the USA, Australia and New Zealand, and goat milk infant formula has been in use in Australia, New Zealand, Taiwan, Korea, Russia and China. In many countries, goat milk infant and follow-on formulae comprise approximately 5% of infant formula sales.

Goat milk has been studied as a substitute for cow milk by several authors and found to be highly nutritious, benefiting growth and skeletal mineralization in children (Mack 1953; Hachelaf et al. 1993; Razafindrakoto et al. 1993). Several animal-based studies have also indicated enhanced protein, mineral and fat utilization from goat milk compared with cow milk (Murry et al. 1999; Lopez-Aliaga et al. 2000, 2003; Barrionuevo et al. 2002, 2003; Alferez et al. 2003; Campos et al. 2003). Despite these, there are few studies that have addressed the composition and nutritive value of goat milk formulae. The amino acid and mineral digestibility of goat milk infant formula was studied in piglets and shown to be similar to cow milk infant formula (Rutherfurd et al. 2006a,b). The growth rates of healthy newborn infants on goat infant formula were also equivalent to cow infant formula or breast milk (Grant et al. 2005).

Formulae based on cow milk contain 5–17% non-protein nitrogen of varying composition depending on the ingredients used to manufacture the formula (Donovan & Lonnerdal 1989; Agostoni et al. 2000; Ferreira 2003). Goat and cow milk have different levels of a range of constituents, including taurine, nucleotides, polyamines, which are all components of the non-protein nitrogen fraction of milk. Thus it is to be expected that the non-protein nitrogen component of a formula made from goat milk would be different to that of a formula made from cow milk. In this study we have characterized the non-protein nitrogen fraction of whole milk powder and infant and follow-on formulae made from New Zealand goat milk and compared these with cow milk and cow milk-based formulae.

Materials and methods

Samples

Four whole milk powders, three infant formulae and three follow-on formulae based on goat milk were analyzed for total and non-protein nitrogen and non-protein nitrogen constituents. The whole milk powders were made at four different times of the year to determine the influence of stage of lactation on non-protein nitrogen

composition. In addition, two whole milk powders, four infant and four follow-on formulae based on cow milk were also analyzed. All whole milk powders and formulae were obtained from the Dairy Goat Co-operative (N.Z.) Ltd (Hamilton, New Zealand). Infant and follow-on formulae were established formulae with a 80:20 casein:whey ratio and are available internationally. These were made from a base of milk solids with added vegetable oils, lactose, vitamins and minerals and formulated to comply with the CODEX standard 72 on Infant formula (CODEX Alimentarius 1981). All milk was of New Zealand origin.

Whole milk powders or formulae were reconstituted to 13% w/v prior to analysis.

Biochemical analyses

Total nitrogen was determined on a LECO analyzer by the Dumas method (ISO 2002). Non-protein nitrogen was determined using the Kjeldahl method (International Dairy Federation 1993) after precipitiation of the milk proteins with 12% trichloroacetic acid.

Nucleotides were measured by reverse-phase high-performance liquid chromatography (HPLC), based on the method described by Sugawara et al. (1995). Reconsituted milk powders were first extracted with perchloric acid as described by Gil & Sanchez-Medina (1981). Reversed-phase separations were carried out at 25°C using a 250 mm × 3.2 mm i.d. Phenomenex Prodigy 5 μm ODS3 100A column (Phenomenex NZ Ltd, Aukland NZ). The peaks associated with individual nucleosides and monophosphate nucleotides were integrated. Individual purified monophosphate nucleotides and nucleosides were treated identically to the milk powder samples and used as reference material to calculate the concentrations in the samples.

Recovery of individual nucleosides added to goat whole milk powder ranged from 82 to 96% and 89 to 103% for nucleotide monophosphates (Table I). In both instances, moieties containing guanine had the lowest recoveries. This suggests that these compounds may be slightly underestimated.

Polyamines were measured by reverse-phase HPLC following extraction with perchloric acid and derivatization with dansyl chloride. 1,6-Diaminohexane (1.0 μg) was added as an internal standard to milk powders (5 g) and mixed with 25 ml of 0.4 M perchloric acid. Aliquots (1 ml) of the supernatant were removed after centrifugation of the slurry for 10 min at 3,000 × g. Derivatization was achieved by

Table I. Recovery of individual nucleotide monophosphates and nucleosides added to goat whole milk powder and measured by reverse-phase HPLC following perchloric acid extraction.

Compound	Recovery (%)
Cytidine	95.6
Cytidine monophosphate	98.5
Adenosine	96.3
Adenosine monophosphate	102.7
Guanosine	82.1
Guanosine monophosphate	89.2
Uridine	90.3
Uridine monophosphate	98.1
Inosine	90.5
Inosinic acid	103.2

addition of 200 μl of 2 M NaOH, 300 μl saturated sodium bicarbonate solution and 2 ml dansyl chloride in acetone (optimum pH 8–10) and incubation at 40°C for 40 min. Following this, 250 μl concentrated ammonia was added and incubated for a further 30 min at 40°C. After cooling to room temperature, hexane (6 ml) was added and the mixture centrifuged at 1,000 $\times g$ for 10 min. Approximately 4–5 ml clear upper layer was transferred to a glass vial and evaporated to dryness at 45°C under nitrogen. The polyamines were reconstituted with 0.5 ml acetonitrile. Reversed-phase separations were carried out at 25°C using a 250 mm × 3.2 mm i.d. Phenomenex Prodigy 5 μm ODS3 100A column with fluorescent detection of polyamines. Reference standards of individual purified polyamines, made up in 0.4 M perchloric acid, were used to calculate the concentrations in the samples.

The analysis of sialic acid in milk powders was based on the method described by Wang et al. (2001). Only protein-bound sialic acid was measured as this is the main form of sialic acid in ruminant milk (Martin et al. 2001). Protein was precipitated from reconstituted milk powder using 10% trichloroacetic acid and then hydrolyzed at 80°C using sulphuric acid. The sialic acid released was determined by a modification of the thiobarbituric colorimetric method, described by Tram et al. (1997). The absorbance was read at 549 nm. Concentrations of sialic acid were determined by extrapolation of a standard curve generated using sialic acid standards in a concentration range of 31–500 μg/ml.

Carnitine was analyzed using an enzymatic method described by Woollard et al. (1997).

Urea was measured enzymatically on a Hitachi 717 Autoanalyzer (Tokyo, Japan). Creatinine was measured by the Jaffe method (Masson et al. 1981) on a Hitachi 717 Autoanalyzer.

Free amino acids were measured by the Pharmacia LKB-Alpha Plus Amino Acid Analyser (Cambridge, UK). Milk samples were deproteinized by ultrafiltration using 5000 NMWL membrane (Millipore, Milford, MA, USA). The amino acids were separated by ion-exchange chromatography and detected following reaction with ninhydrin. Cysteine and methionine were measured by ion-exchange HPLC (Millipore Corporation, Waters, Chromatography Division, Milford, MA, USA) with post-column *o*-phthaldehyde derivatization and fluorescence detection.

Results

Non-protein nitrogen content

The concentration of total nitrogen in reconstituted goat whole milk powder and infant and follow-on formulae are 524, 222 and 306 mg/100 ml, respectively, compared with cow milk powder or formulae at 530, 219 and 316 mg/100 ml, respectively. The non-protein nitrogen content of these powders were also similar – 42, 22 and 27 mg/100 ml for the goat powders, compared with 38, 17 and 22 mg/100 ml for the cow powders. The non-protein nitrogen, expressed as a proportion of total nitrogen, of powders from goat milk were between 9 and 40% higher than those made from cow milk (Figure 1).

Non-protein nitrogen composition

The composition of the non-protein fraction of goat milk powder and goat milk formulae is presented in Table II. Nucleosides or nucleotides were not measured in

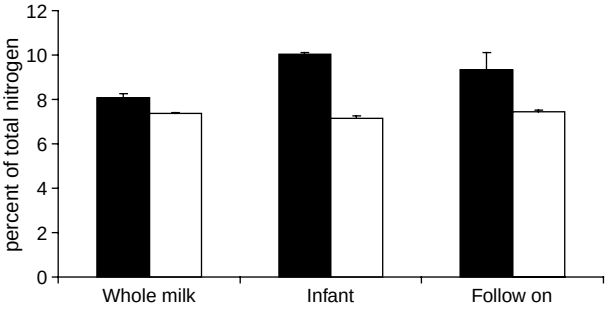

Figure 1. Non-protein nitrogen content, expressed as a percentage of total nitrogen, of goat (■) or cow (□) whole milk powder, infant and follow-on formulae. Data presented as the mean (± SD).

the cow milk powders because of low levels in cow milk (Johke 1974; Gil & Sanchez-Medina 1981). Urea was quantitatively the major component in both goat and cow milk powders. The next most abundant source of nitrogen in the goat milk powders were the free amino acids. In contrast, there were much lower concentrations of free amino acids in cow whole milk powders, representing only 2% of the total non-protein nitrogen content. For infant and follow-on formulae made from cow milk, free amino acids represented the next most abundant source of nitrogen, due to the addition of further taurine.

Free amino acids

The individual amino acids comprising the free amino acid pool of goat milk, infant and follow-on formulae are presented in Table III. The three most abundant amino acids are taurine, glutamic acid and glycine. Several amino acids that were detected

Table II. Composition of the non-protein nitrogen fraction of whole milk powders or infant and follow-on formulae made from goat or cow milk.

	Goat			Cow		
	Whole milk powder	Infant formula	Follow-on formula	Whole milk powder	Infant formula	Follow-on formula
Total nucleotide monophosphates[a]	10.1	4.0	5.7	ND	ND	ND
Polyamines	0.06	0.016	0.037	0.041	0.012	0.016
Free amino acids	21.3	9.7	12.4	5.9	9.2	9.9
Urea	28	11	14	22	7	14
Creatinine	1.4	1.0	1.0	1.8	1.0	1.0
Carnitine	2.1	1.6	1.6	2.1	1.7[b]	1.8[b]
Sialic acid	10.5	4.8	5.0	19.9	6.8	11.1

The concentrations of individual components were determined in reconstituted powders and their nitrogen content calculated. All concentrations are in mg/100 ml reconstituted powder or formulae. ND, not determined. [a]The nucleosides were converted to monophosphate equivalents and summed with the nucleotide monophosphates to obtain total nucleotide monophosphate levels. [b]Contains added carnitine.

Table III. Free amino acids in whole milk powder, infant and follow-on formulae made from goat or cow milk.

	Goat			Cow		
	Whole milk powder	Infant formula	Follow-on formula	Whole milk powder	Infant formula	Follow-on formula
Alanine	0.7	0.2	ND	0.6	0.2	ND
Arginine	0.8	ND	ND	0.8	ND	ND
Glutamic acid	2.1	0.9	1.3	2.8	2.5	2.1
Glutamine	1.4	0.4	ND	ND	ND	ND
Glycine	2.6	0.6	1.2	0.7	0.2	0.2
Histidine	0.4	0.4	0.2	0.4	0.5	0.6
Isoleucine	0.2	ND	ND	ND	ND	ND
Leucine	0.2	ND	ND	ND	ND	ND
Lysine	1.0	0.4	0.8	0.7	0.4	0.6
Serine	1.8	0.2	0.2	0.3	ND	0.1
Tyrosine	0.2	ND	ND	0.2	ND	ND
Valine	0.7	ND	ND	ND	ND	ND
Taurine	9.8	71.6[a]	85.9[a]	0.5	56.0[a]	63.8[a]

Individual amino acids were measured by HPLC. All concentrations are in mg/100 ml reconstituted powder or formulae. ND, not detected. [a]Includes some taurine added during process.

in the whole milk powder were below the lower detection limit of the assay in either the infant or follow-on formula.

Nucleotides

Individual nucleosides and nucleotide monophosphates in whole milk powders and infant and follow-on formulae made from goat milk are presented in Table IV. Both nucleosides and nucleotide monophosphates are present in significant amounts in the goat milk powders. The most abundant species are uridine and adenosine monophosphate. The total nucleotide monophosphate levels, calculated by converting the nucleosides to monophosphate equivalents and adding to the nucleotide monophosphates, are 10.1, 4.0 and 5.7 mg/100 ml for whole milk powder, infant formula and follow-on formula, respectively.

Table IV. Individual nucleosides and nucleotide monophosphates in whole milk, infant and follow-on formula made from goat milk.

	Nucleosides[a] (mg/100 ml)			Nucleotide monophosphates (mg/100 ml)			
	Uridine	Guanosine	Inosine	Cytidine monophosphate	Uridine monophosphate	Adenosine monophosphate	Guanosine monophosphate
Whole milk	2.5	0.2	2.2	0.7	1.0	0.1	1.6
Infant formula	1.0	0.1	0.6	0.2	0.8	0.1	0.5
Follow-on	1.4	0.1	1.0	0.3	1.1	0.1	0.7

Nucleosides and nucleotides were measured by reverse phase HPLC following perchloric acid extraction of the milk powders. All concentrations are in mg/100 ml reconstituted powder or formulae. [a]Cytosine, adenosine and inosine monophosphate were not detected in any samples.

Nucleosides or nucleotides were not measured in the cow milk powders because of low levels.

Stage of lactation

The effect on stage of lactation or season on goat milk was evaluated by analyzing four separate powders manufactured at different times of the season (Table V). There was a general trend for an increase in non-protein nitrogen with stage of lactation, but most components varied erratically between 1 and 8 months.

Discussion

Whole milk powders made from goat milk from New Zealand have an average 8.1% of total nitrogen as non-protein nitrogen, in keeping with levels reported in unprocessed goat milk (Mehaia & Al-Kanhal 1992; Hadjipanayiotou 1995; Tripaldi et al. 1998; García-Ruiz et al. 2000). Our results further show that both infant formula and follow-on formula made from goat milk contains 10 and 9% of total nitrogen as non-protein nitrogen, respectively.

The main component of the non-protein nitrogen fraction was urea. Approximately 30% of the nitrogen in the non-protein nitrogen fraction of goat infant formula is urea, compared with only 18% for the casein-dominant cow infant formula analyzed in this study. This compares with around 50% for human milk and 37% for casein-dominant formula based on cow milk (Donnovan & Lonnerdal 1989). However, the urea content of different infant formula varies significantly depending on the source of the ingredients and the manufacturing processes used (Donnovan & Lonnerdal, 1989). It is thought that some urea nitrogen is available as a source of amino nitrogen for protein synthesis (Heine et al. 1986; Jackson 1994), but the relative importance of urea to infant nutrition is not known (Fuller & Reeds 1998).

The other main components of the non-protein nitrogen content of goat milk are the free amino acids. The four most abundant amino acids in goat milk powders were taurine, glycine, glutamic acid and glutamine, confirming reports of Mehaia & Al-Kanhal (1992) and Tripaldi et al. (1998) for fresh goat milk. This is very similar to the pattern in human milk, for which the three most abundant free amino acids are listed as glutamic acid, taurine and alanine (Donnovan & Lonnerdal 1989; Mehaia &

Table V. Effect of stage of lactation (months after kidding) on non-protein nitrogen content and composition in goat whole milk powders.

	1–2 months	3–4 months	5–6 months	7–8 months
Non-protein nitrogen	41.7	41.2	43.6	43.8
Total nucleotide monophosphates	10.6	10.5	9.1	10.3
Polyamines	0.05	0.06	0.06	0.07
Taurine	8.7	9.6	10.3	10.5
Free amino acids	12.9	9.4	15.2	8.7
Urea	29.4	28.9	25.7	28.1
Creatinine	1.4	1.4	1.4	1.3
Carnitine	2.1	2.1	2.1	2.1
Sialic acid	9.3	10.1	12.2	10.5

All concentrations in mg/100 ml reconstituted powder.

Al-Kanhal, 1992; Agostoni et al. 2000; Ferreira 2003; Chuang et al. 2005) with either serine (Chuang et al. 2005), glycine (Donnovan & Lonnerdal 1989) or glutamine (Mehaia & Al-Kanhal 1992; Agostoni et al. 2000) listed as the fourth. When expressed in proportion to the different protein contents, the concentration of taurine in goat milk is approximately 60% of the level in human milk, whereas in cow milk it is only 3%. Infant and follow-on formulae made from both cow and goat milk are further supplemented with taurine, so the taurine levels in these represent indigenous and added taurine.

Goats in New Zealand are predominantly pasture fed, and kid in the same month of the year. As a result, at any time of the year milk composition is affected by stage of lactation and quality of pasture feed, which varies across the season (Auldist et al. 1998). These factors can also affect proteolytic enzyme activity that could impact on non-protein nitrogen (Nicholas et al. 2002). In this study, however, samples taken from four different times of the season only had a minor impact on the non-protein nitrogen composition of goat milk.

Increasing evidence suggests that, although present at low concentrations, individual components of the non-protein nitrogen fraction of milk have a profound impact on the development of metabolic, immune and physiological processes of the infant. For instance, several trials have demonstrated that nucleotides, when added to cow-based formula, induce higher antibody response to immunization (Pickering et al. 1998; Schaller et al. 2004; Hawkes et al. 2006), immune cell development (Buck et al. 2004) and reduce the incidence of diarrhea (Brunser et al. 1994; Yau et al. 2003) in infants. Unlike cow milk, goat milk contains a complex array of nucleotides and nucleosides (Johke 1974; Gil & Sanchez-Medina 1981), which are also carried over into infant and follow-on formulae manufactured from goat milk. Cow-based infant formula, without nucleotide supplementation, contains 1 mg/100 ml nucleotide monophosphates (Pickering et al. 1998; Yau et al. 2003; Ferreira, 2003; Hawkes et al. 2006) and is almost exclusively cytosine monophosphate (Sugawara et al. 1995; Ferreira 2003). In contrast, the total nucleotide content of infant formula made from goat milk, when converted to monophosphate equivalents, averaged 4 mg/100 ml, which is in the same range as studies showing a beneficial effect of cow infant formula supplemented with nucleotide monophosphates (Brunser et al. 1994; Hawkes et al. 2006). For comparison, the total level of monophosphate equivalents in human milk ranges from 2.4 to 4.2 mg/100 ml (Johke 1974; Gil & Sanchez-Medina 1981, 1982; Sugawara et al. 1995). The calculation used to yield the total level of monophosphate equivalents in goat milk powders or human milk do not take into account the nucleosides that may be released from RNA or DNA, which contribute to the total potentially available nucleoside content of human milk (Leach et al. 1995).

Polyamines are another component of the non-protein nitrogen fraction that may have functional benefit for the infant. For instance, polyamines are important for optimal growth and maintenance of gastrointestinal cells and maturation of enzyme function (Bardocz et al. 1995, Capano et al. 1998; Greco et al. 2001). High dietary intake of polyamine has been implicated in reducing the incidence of food allergy in infants (Peulen et al. 1998; Duchen & Thorell 1999, Dandrifosse et al. 2000). The present study found that the concentrations of polyamines in goat milk powders and infant and follow-on formulae are slightly higher than cow milk or cow-based formulae, which confirms earlier data for raw milk (Ploszaj et al. 1997). However, the levels present in goat or cow infant formula are much lower than levels found in

human milk (Romain et al. 1992) and represent only a minor component of the non-protein nitrogen fraction of these formulae.

In conclusion, the non-protein nitrogen fraction of goat milk powder and infant and follow-on formulae manufactured from goat milk is 8–10% of total nitrogen. The profile of the non-protein nitrogen fraction of goat milk is very different to that of cow milk, with several constituents such as nucleotides at concentrations approaching those in human breast milk. No additional nucleotides are required to adjust nucleotide levels of infant formula when made from goat milk.

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