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Determination of nonprotein tryptophan in yoghurts by selective fluorescence and HPLC

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The concentrations of total nonprotein (protein-bound and free) and free tryptophan were determined in various kinds of market yoghurts prepared from raw, skimmed or partially skimmed cow's milk, with or without fruit, coffee or cereals. The mean values of total nonprotein tryptophan in plain yoghurt made from whole or skimmed milk were 3·45 ± 0·46 and 3·25 ± 0·38 mg/kg, respectively, that is, five to six times higher than in cow's milk. Instead, the free fraction of tryptophan was 2·76 ± 0·50 and 2·44 ± 0·36 mg/kg, respectively (seven to eight times higher than in cow's milk). In yoghurts with fruit. the mean values were quite similar to those of plain yoghurt, although they varied within each type of yoghurt. The highest contents of both fractions of nonprotein tryptophan were found in yoghurts with coffee or cereal, due to the contribution of these kinds of food. The increase in free tryptophan in yoghurt improves its nutrition value, as more tryptophan is made available for the brain synthesis of the neurotransmitter serotonin, of which tryptophan is the precursor.

INTRODUCTION

Milk plays an important role in human nutrition, contributing 20–30% of proteins introduced in diet in developed countries (Hambraeus, 1982). However, most milk is consumed as processed products, one of which is yoghurt.

Yoghurt is a fermented milk product in which *Lactobacillus bulgaricus* and *Streptococcus thermophilus*, normally the essential microbial species, together improve the nutritional value of fermented milks. In fact, they not only contain the same components as the milk, but also some products of transformation by these microorganisms (Blanc, 1984). Due to the proteolytic activity of *L. bulgaricus* (So, 1984), milk proteins are partly digested, thus favouring absorption of amino acids (Blanc, 1984).

Some authors have observed a marked increase in free amino acid contents (Bossi *et al.*, 1982; Vass *et al.*, 1983; Khamagaeva *et al.*, 1985), in particular lysine, threonine, valine, leucine, tyrosine and phenylalanine (Bossi *et al.*, 1982) in fermented milk with respect to raw milk. Significant amounts of free D-amino acids have also been detected in yoghurt, attributed to the action of microorganisms (Palla *et al.*, 1989; Brueckner & Hausch, 1990).

The amino acid pattern and percentage of free amino acids change from sample to sample in fermented milk products, probably on account of the different strains of microorganisms employed (Palla *et al.*, 1989). Khamagaeva *et al.* (1985) observed that the levels of total free amino acids in sour milk fermented with *L. bulgaricus* (26·33 mg%) were higher than in milk fermented with *Bifidobacteria* (17·75 mg%), but the latter contained more essential amino acids (40 vs 17% of the total).

Amino acids are generally assumed to occur in foods as constituents of proteins. They may also be present in foods in free form, although in very small amounts (Ling *et al.*, 1961; Johnson, 1974; George & Lebenthal, 1981), but free tryptophan values are not reported in the literature.

Recently (Allegri *et al.*, 1993), we have begun to investigate the occurrence of nonprotein tryptophan (protein-bound and free) in processed foods, in particular those which undergo heat treatment such as ultra-high temperature (UHT) milk, and in products obtained by fermentation processes (yoghurt and cheese). Also, for these foods, there are no data in the literature regarding free tryptophan contents.

In this paper we demonstrate that nonprotein tryptophan is also present in yoghurt made from cow's milk and that its concentrations are significantly higher than in raw milk.

Various kinds of yoghurt are now marketed. We therefore analysed yoghurts prepared with raw, skimmed or partially skimmed milk, with or without fruit, coffee and cereals.

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MATERIALS AND METHODS

Materials

The following kinds of market yoghurts made from cow's milk, all containing *L. bulgaricus* and *S. thermophilus*, were analysed (10 samples of each flavour from each manufacturer. When not reported, the amounts of fruit, cereals and coffee are not specified by the manufacturer):

(a) Ala Zignago (Ala S.p.A., Fossalta di Portogruaro, Venice): — made from whole milk, with or without apricot 7%, coffee 4%, prune 8·4% + wheat bran 0·7%;
— made from skimmed milk.
(b) Biospega (Spega S.r.l., Monticello Conte Otto, Vicenza):
— made from whole milk, with or without cereals + coconut;
— made from skimmed milk
(c) Galbani (Galbani S.p.A., Melzo, Casale Cremasco, Cremona):
— made from whole milk;
— made from skimmed milk.
(d) Danone (Gervais Danone S.p.A.. Casale Cremasco, Cremona):
— made from whole milk, with or without citrus fruits, peach, strawberry, berry fruits, banana, apricot, cereals;
— made from skimmed milk, with or without pineapple, strawberry, berry fruits.
(e) Il Compatto — Torre in Pietra (Torre in Pietra S.p.A., Torrinpietra, Rome):
— made from whole milk, with or without strawberry, berry fruits, banana, cereals.
(f) Parmalat (Parmalat S.p.A.. Collecchio, Parma):
— made from whole milk, with or without banana, strawberry, pear + fig, apricot, berry fruits, cereals.
(g) Torvis (Torvis S.R.L., Torviscosa, Udine):
— made from skimmed milk.
(h) Yogo Di' (Alvi Cooperativa latte Alto Vicentino s.c.a r.l., Bassano del Grappa, Vicenza):
— made from whole milk, with or without citrus fruits, pineapple, strawberry, blueberry, other berry fruits, banana, apricot, coffee, malt;
— made from skimmed milk, with or without apricot.
(i) Yomo (Yomo S.p.A., Milan):
— made from whole milk, with or without pineapple, citrus fruits (orange and lemon), strawberry, raspberry, blueberry, cherry, banana, pear, apricot, cereals, coffee;
— made from skimmed milk, with or without strawberry and berry fruits.

Besides yoghurts, 10 samples of UHT cow's milk from commercial sources were analysed.

Preparation of samples and analytical procedure

The yoghurt samples were diluted 1:4 with distilled water. Aliquots were used to measure pH, ranging from 3·7 to 4·0. An aliquot was then centrifuged for 10 min at 3000 rpm.

The supernatant (10 μl) was used to determine total nonprotein tryptophan (protein-bound and free). The free form of tryptophan was obtained by ultrafiltering 4 ml of the supernatant through an ultrafiltration cell (Amicon Model 12, Amicon, Oosterhout, The Netherlands) with an XM-50 (Diaflo) membrane (Amicon). Only the first 0·4 ml of filtrate was collected (Eccleston 1973), 10 μl of which was used to determine free tryptophan.

Total nonprotein (protein-bound and free) and free tryptophan were measured by a combined high-performance liquid chromatography (HPLC) fluorescence system, using the stop-flow technique according to the methods of Bettero *et al.* (1984) and Costa *et al.* (1987).

Apparatus

A Perkin-Elmer series 3B liquid chromatograph (Norwalk, CT, USA) equipped with an LS-4 fluorimetric detector connected by an ASV-1 automatic switching valve to an ISS-100 intelligent sampling system, and a Perkin-Elmer 3600 luminescence spectrophotometer connected to a Perkin-Elmer Sigma 15 data system were employed. Chromatographic separation was achieved at room temperature by an RP-8 (5 μm) Merck column equipped with a precolumn packed with Lichroprep RP-8 (20–40 μm). The mobile phase consisted of acetonitrile–aqueous phosphate (0·004 M, pH 3·5) in isocratic elution (15:85, v/v). The flow rate was 1 ml/min. Excitation and emission wavelengths were 285 and 345 nm respectively.

A 10 μl sample of milk or yoghurt solution was injected directly by an automatic sampling device, after filtration through a 0·45 μm millipore filter (Millipore, Molsheim, France).

RESULTS

The contents of total nonprotein (protein-bound and free) and free tryptophan (mg/kg) in cow's milk and various kinds of plain yoghurt derived from cow's milk are given in Table 1. The solutions of these yoghurts, obtained from whole and skimmed milk and prepared as in the experimental section, showed a mean value of pH 3·9. As Table 1 shows, the tryptophan concentrations in various samples reveal significant differences ($P < 0·001$), as compared with ordinary milk samples, both for total nonprotein (protein-bound and free) and free tryptophan. The free fraction varied among the yoghurts, ranging from 64% in the Galbani sample to 96% in the Biospega sample, as a percentage of free plus bound tryptophan. The values of nonprotein tryptophan in yoghurts made from whole and skimmed milk also showed some fluctuations. In plain yoghurt made from whole or skimmed milk, the mean values of total nonprotein tryptophan were 3·45 ± 0·46 and 3·25 ± 0·38 mg/kg, respectively, that is, five to six times higher than in cow's milk (0·59 ± 0·05 and 0·60 ± 0·06

Table 1. Contents (mean values ± S.D.) of total nonprotein (protein-bound and free) and free tryptophan (mg/kg) in plain yoghurt made from whole or skimmed cow's milk

Yoghurt	Tryptophan (mg/kg)			
	Whole milk		Skimmed milk	
	Total	Free	Total	Free
Cow's milk (UHT long life)	0·59 ± 0·05	0·35 ± 0·08	0·60 ± 0·06	0·36 ± 0·10
Ala Zignago	3·89 ± 0·36	2·85 ± 0·37	3·81 ± 0·53	2·89 ± 0·35
Biospega	3·44 ± 0·53	3·32 ± 0·64	3·04 ± 0·41	2·92 ± 0·37
Danone	3·19 ± 0·53	2·69 ± 0·49	3·06 ± 0·69	2·28 ± 0·64
Galbani	3·03 ± 0·55	1·91 ± 0·42	3·25 ± 0·24	2·10 ± 0·10
Parmalat[a]	2·88 ± 0·49	2·28 ± 0·28		
Torre in Pietra	4·65 ± 0·55	4·13 ± 0·66		
Torvis			3·85 ± 0·20	2·39 ± 0·55
Yogo Di	3·52 ± 0·17	2·73 ± 0·58	3·15 ± 0·15	2·72 ± 0·09
Yomo	3·00 ± 0·45	2·20 ± 0·48	2·62 ± 0·40	1·79 ± 0·37
Mean ± S.D.	3·45 ± 0·46[b]	2·76 ± 0·50[b]	3·25 ± 0·38[b]	2·44 ± 0·36[b]

[a]Kyr: yoghurt and milk fermented with *Lactobacillus acidophilus* and *Bifidobacterium*.
[b]Statistically significant values with respect to cow's milk at the $P < 0.001$ level (Student's *t*-test).

mg/kg). The free form of the amino acid was seven to eight times higher (2·76 ± 0·50 and 2·44 ± 0·36 mg/kg) than in cow's milk (0·35 ± 0·08 and 0·36 ± 0·10 mg/kg).

Tables 2 and 3 show the contents of total nonprotein tryptophan and free form (mg/kg) in various fruit-flavoured yoghurts obtained from whole and skimmed milk, respectively. The pH values of these samples (dilution 1:4) ranged from 3·7 (raspberry) to 3·9 (berry fruits). The mean values of total nonprotein and free tryptophan varied, depending on the kind or amount of fruit, and were generally similar to those of plain yoghurt. However, the values fluctuated within each type of yoghurt. The percentage of free tryptophan ranged from about 60 to 90%, as percentages of free plus bound tryptophan.

Total nonprotein and free tryptophan concentrations of the various kinds of yogurts with cereals or coffee are given in Table 4. There was little variation in the pH values for these yoghurts (pH 3·8–4·0). The values of both fractions of nonprotein tryptophan are clearly much higher than those of plain yoghurt, although large variations in tryptophan contents appear from this table, depending on the different contribution of this kind of food.

DISCUSSION

According to Miller & Kandler (1964, 1967), yoghurt containing *L. bulgaricus* and *S. thermophilus* and ready for consumption has a content of free amino acid (300–500 mg/litre) higher than that of milk. These authors observed that the most intensive proteolysis occurred during the lag phase of growth of the microorganisms. Miller & Kandler (1967) also found that the content of free amino acids was influenced to some extent by the predominating species of organisms present in the raw milk flora. In a yoghurt product kept at 4°C for 5 days, they observed that there was a striking increase in the concentration of all amino acids; the proline concentration above all was up to 500 μmol/litre (Miller & Kandler, 1966).

In a study on the amino acids of yoghurt, Rasic *et al.* (1971) reported that glutamic acid and proline were increased in the highest quantity in yoghurt, whereas methionine was present in the lowest quantity. Among the essential amino acids, valine, lysine and leucine were the most representative. Breslaw & Kleis (1973) found free amino acid contents in finished yoghurt products considerably higher than those obtained by Miller & Kandler (1964), i.e. 956 and 995 mg/litre.

The results of this study indicate that the quantities of nonprotein and free tryptophan also increase during yoghurt manufacture, from the yoghurt milk to the final product, the levels of both fractions being markedly higher than in the original milk. We did not usually find great differences in the concentrations of free and total nonprotein tryptophan in various fruit-flavoured yoghurts, their values being very similar to those of plain yoghurt. However, some foods such as coffee or cereals added to yoghurt considerably increased the content of non-protein tryptophan. For example, we found that various samples of ordinary lyophilized coffee contain from 30 to 100 mg/kg of non-protein tryptophan (data not published).

The variation in tryptophan levels within each kind of yoghurt is an indication of how proteolysis influences the free amino acid content in these foods. In fact, the digestibility of proteins in yoghurts varies according to the activity of the microorganisms present in them and to the period elapsing between the date of production and that of testing.

The increase in free tryptophan in yoghurt improves the nutrition value, as it makes more tryptophan avail-

Table 2. Concentrations (mean values ± S.D.) of total nonprotein (protein-bound and free) and free tryptophan (mg/kg) in fruit-flavoured yoghurts made from whole cow's milk

Yoghurt	Tryptophan (mg/kg)																					
	Citrus fruits		Peach		Pineapple		Strawberry		Raspberry		Blueberry		Cherry		Berry fruits		Banana		Pear		Apricot	
	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free	Total	Free
Ala Zignago																					2·44 ±0·48	2·16 ±0·25
Danone	3·11 ±0·39	2·19 ±0·66	3·23 ±0·59	2·70 ±0·66			3·75 ±0·48	2·81 ±0·06							3·09 ±0·30	2·61 ±0·48	3·10 ±0·50	1·95 ±0·06			3·50 ±0·62	3·30 ±0·54
Parmalat[a]							5·50 ±0·72	4·20[d] ±0·65							5·19 ±0·33	3·28 ±0·56	2·65 ±0·09	1·78 ±0·12			2·04 ±0·16	1·71 ±0·26
Torre in Pietra							2·30 ±0·28	2·28 ±0·20							3·44 ±0·52	2·37 ±0·54	3·27 ±0·27	2·55 ±0·14				
Yogo Di	2·92 ±0·48	1·99 ±0·17			2·53 ±0·44	2·12 ±0·17	3·37 ±0·31	2·04 ±0·23			2·35 ±0·23	2·04 ±0·08			2·05 ±0·19	1·75 ±0·09	4·00 ±0·28	2·66 ±0·13			2·81 ±0·53	2·17 ±0·30
Yomo	3·30 ±0·52	2·94 ±0·59			3·96 ±0·24	3·21 ±0·07	4·35 ±0·28	3·51 ±0·50	2·94 ±0·64	2·01 ±0·96	2·68 ±0·07	1·77 ±0·28	3·91 ±1·25	3·11 ±0·54			4·55 ±0·91	3·31 ±0·90	2·81 ±0·44	2·28 ±0·28	3·69 ±0·63	3·27 ±0·44
Mean ±SD	3·11 ±0·47	2·37[b] ±0·48	3·23 ±0·59	2·70[b] ±0·66	3·24 ±0·34	2·66[b] ±0·12	3·85 ±0·42	2·97[b] ±0·33	2·94 ±0·64	2·01[b] ±0·96	2·52 ±0·15	1·90[c] ±0·18	3·91 ±1·25	3·11[b] ±0·54	3·44 ±0·34	2·50[b] ±0·42	3·51 ±0·41	2·45[b] ±0·27	2·81[d] ±0·44	2·28 ±0·28	2·90 ±0·49	2·52[b] ±0·36

[a]Kyr: yoghurt and milk fermented with *L. acidophilus* and *Bifidobacterium*.
[b]Values not significantly different from those of plain yoghurt.
[c]Statistically significant values with respect to those of plain yoghurt at the $P < 0.001$ level (Student's *t*-test).
[d]Statistically significant values with respect to those of plain yoghurt at the $P < 0.005$ level (Student's *t*-test)

Table 3. Concentrations (mean values ± S.D.) of total nonprotein (protein-bound and free) and free tryptophan (mg/kg) in fruit-flavoured yoghurts made from skimmed cow's milk

Yoghurt	Tryptophan (mg/kg)							
	Pineapple		Strawberry		Berry fruits		Pear	
	Total	Free	Total	Free	Total	Free	Total	Free
Danone	3·50 ±0·66	2·73 ±0·37	2·84 ±0·84	2·46 ±0·53	3·29 ±0·84	2·35 ±0·92		
Yogo Di							2·77 ±0·42	2·14 ±0·10
Yomo			4·46 ±0·41	3·25 ±0·08	3·04 ±0·52	2·79 ±0·63		
Mean ± S.D.	3·50 ±0·66	2·73 ±0·37	3·65 ±0·63	2·85 ±0·31	3·16 ±0·68	2·57 ±0·78	2·77 ±0·42	2·14[a] ±0·10

[a]Statistically significant values with respect to those of plain yoghurt at the $P < 0.005$ level (Student's t-test).

Table 4. Concentrations of total nonprotein (protein-bound and free) and free tryptophan (mg/kg) in yoghurts with cereals or coffee (mean values ± S.D.)

Yoghurt	Tryptophan (mg/kg)					
	Cereals		Coffee		Malt	
	Total	Free	Total	Free	Total	Free
Ala Zignago	10·03 ±2·30	6·25 ±2·24	7·36 ±1·65	5·28 ±0·73		
Biospega	6·80 ±1·85	5·00 ±1·48				
Parmalat	8·52 ±1·56	6·88 ±1·12				
Torre in Pietra	6·80 ±1·15	3·80 ±0·88				
Yogo Di	5·48 ±0·37	4·00 ±1·34				
Yomo	9·20 ±2·65	6·02 ±0·53	10·49 ±2·57	8·60 ±1·61	8·91 ±1·68	6·00 ±1·60

able to the brain (Tagliamonte *et al.*, 1973). In fact, it is known that tryptophan is the only amino acid bound 80–90% to plasma albumin (McMenamy & Oncley, 1958). The small free fraction, the only one capable of crossing the blood–brain barrier, has great functional importance, since its availability appears to be an important factor in the brain synthesis of the neurotransmitter serotonin, of which tryptophan is the precursor (Fernstrom & Wurtman, 1971; Knott & Curzon, 1972; Tagliamonte *et al.*, 1973; Gessa & Tagliamonte, 1974).

In a comparative study of the plasma amino acids of rats fed on milk and yoghurt, Wong *et al.* (1984) observed that yoghurt-fed rats showed higher levels of plasma amino acids, indicating improved digestibility and/or amino acid absorption, which may contribute to the growth-stimulating effect of yoghurt. Vass *et al.* (1983) in feeding experiments also found that rats showed higher weight gain from fermented milk diets than from nonfermented milk diets.

In conclusion, yoghurt improves the nutritional value of milk, as the protein digestibility increases during fermentation, due to the proteolysis characteristic of microorganisms. Of the various kinds of market yoghurts, some variations in proteolysis occur, considering the different contents in free amino acids as reported in the literature. This also happens for nonprotein tryptophan.

The chemical changes which take place during yoghurt manufacture are, therefore, of great interest, as the nutritional value of yoghurt also depends on them.

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