Investigation on whey proteins profile of commercially available milk-based probiotics health drinks using fast protein liquid chromatography (FPLC)

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Abstract

Purpose – The main aim of this study is to investigate the whey protein profiles of different commercially available fermented milk drinks that might have been influenced by the growth of probiotics bacteria that have been added according to the claims made by the manufacturer.

Design/methodology/approach – The growth and the subsequent effect of probiotics on whey proteins were investigated through the peptide profiles of the hydrolysed whey protein. The profiles of whey proteins in skimmed milk and the four other probiotic fermented milk drinks were obtained by using the FPLC technique. Changes in whey proteins profiles in fermented milks were evaluated by comparing them with those of unfermented skimmed milk (control). The four samples were those of Yakult, Actimel, Muller and Tesco probiotic cranberry drinks.

Findings – This work has shown that all samples demonstrated a degree of protein hydrolysis. The high level of hydrolysis in “Yakult” and “Actimel” drink samples might have been due to the nature of the process, the length of time of fermentation or the high level of proteolytic activities of the micro-organisms used. When compared with casein, it seems that whey proteins are more resistant to hydrolysis. The results also indicated that only traces of α-lactalbumin were left in the whey sample from “Yakult” drink. There were noticeable reductions in the other three samples. Orotic acid, on the other hand, showed a decrease in their concentration in all whey protein samples when compared with the skimmed milk sample, except for the “Actimel” sample, which showed a noticeable increase.

Originality/value – This work has shown that there were distinct differences between the control sample (skimmed milk) and the four commercially available probiotic milk-based fermented health drinks when a direct comparison was carried out between these samples.

Keywords Drinks, Proteins, Milk, Bacteria, Health foods

Paper type Research paper

Introduction

Milk is a complex mixture of carbohydrates, fats, proteins, vitamins and minerals. The protein content is 3.3-3.6 per cent, of which casein is the major component (80 per cent). Whey protein, as the soluble fraction in milk, comprises 0.6 per cent, of which 0.3 per cent is β-lactoglobulin (A and B), and α-lactalbumin. Other proteins present are represented by serum proteins including BSA, immunoglobulins, lactoperoxidase and lactoferrin. For a long time, milk proteins were considered to provide only nutrition components such as nitrogen and essential amino acids for young mammals (Hambræus, 1992) but in the last decades, several studies have indicated that milk proteins have important nutritional values and health benefits.
Cultured milk products such as yoghurt have been used for a very long time and for many reasons, one of which was for its perceived health benefits mainly associated to the presence of lactic acid bacteria (LAB) that could retard the growth of other harmful bacteria. In the last few decades the term “probiotic” has been widely used for products specifically produced to contain these beneficial bacteria. The word probiotics is derived from Greek, which means “pro life”. The recent definition of probiotics has been given by Salminen et al. (1998) as being “a live microbial feed supplement that is beneficial to health” which was accepted by the working party of the European scientists.

The main health benefits of regular consumption of probiotic containing products include the improvement of the intestinal microbial balance (Bergogne-Berezin, 2000; Larsen et al., 2003); alleviating the symptoms of lactose intolerance through the production of lactase (Pettoello et al., 1989; Marteau et al., 2001); strengthening the immune system (Jolles et al., 1981; Migliore-Samour et al., 1989; Fiat et al., 1993; California Dairy Research Foundation, 2002); reducing the risk of colon cancer in human studies (Rafter, 2002) and protection against breast cancer (Van’t Veer et al., 1991); reducing some forms of food allergies (Pelto et al., 1996); lowering the blood cholesterol levels (Taylor and Williams, 1998; Ouwehand et al., 1999); may suppress the blood pressure of hypertensive individuals (Takano, 1998), playing a key role in the prevention and treatment of diarrhoea (Saavedra et al., 1994; Scarpignato and Rampal, 1995; Hilton et al., 1997; Rolfe, 2000), inhibiting the growth of some pathogenic bacteria (Sušković et al., 1997; Yusof et al., 2000; Ibrahim et al., 2003; Gagnon et al., 2004).

However, the properties of milk constituents, especially proteins have been of interest to many researchers for their use in a wide range of high quality traditional dairy products, as well as for the development of the “new generation” products. Nowadays, dairy products are becoming one of the main products on the chilled cabinet of all retailers. Internationally, Japan is the highest consumer of functional foods, which includes probiotics, and there are more than 50 different types of milk products that contain probiotic organisms marketed there. In Europe, products containing probiotics are becoming very popular but their use is mainly associated with dairy products especially cultured milk drink and yoghurt (Hilliam, 2000).

So far, there is no set of standards in the food legislation to regulate the claim made on certain food products for being probiotic. For example, the claim for the presence of the probiotic Lactobacillus rhamnosus strain GG in cheese has not been substantiated (Coeuret, 2003; Coeuret et al., 2004). Furthermore, in another study it was found that the declared probiotic strain on the label could not be detected from products such as those of Actimel (Danone, France) and Yakult (the Netherlands), instead, is was found to be Lactobacillus paracasei ssp. paracasei (Temmerman et al., 2002).

The main aim of this study was to investigate the whey protein profiles of different commercially available fermented milk products that might have been influenced by the growth of probiotics bacteria that have been added according to the claims made by the manufacturers.

Studying the level of whey protein hydrolysis during fermentation and the change in their profile, could suggest that the presence of the bacteria have produced some peptides which could have a significant importance, especially those having a biological activities such as lowering the cholesterol level and suppressing the blood pressure of hypertensive individuals, as mentioned above. The microbial analysis of
these samples was not carried out at this stage of the study, pending further investigation into their growth and survival under controlled environment.

**Materials and methods**

Pasteurised skimmed milk and four different milk-based fermented health drinks (Danone’s Actimel 0 per cent fat, Muller’s Vitality Strawberry, Tesco’s Probiotics Cranberry and Yakult’s Light) were purchased locally and were analysed the following day after being stored overnight at 4°C. The declared bacterial cultures of the samples were different, containing one or a mixture of two probiotics (see Table I). No microbiological examination was conducted on these samples. All chemicals, tris and NaCl, were purchased from Fisher and Sigma chemicals Company (UK). Sterile membrane filters were purchased from Whatman (UK). Individual whey protein standards were purchased from Sigma (UK). BCA protein assay kit was purchased from PIERCE (USA) and the Mono Q HR 5/5 anion-exchanger column was that of Pharmacia (UK).

**Methods**

The growth and the subsequent effect of probiotics on whey proteins were investigated through the peptide profiles of the hydrolysed whey protein. The profiles of whey proteins in skimmed milk and the four other probiotic fermented milk drinks were obtained by using the FPLC technique as described by Andrews et al. (1985). Changes in whey proteins profiles in fermented milks were evaluated by comparing them with those of unfermented skimmed milk (control). All experiments were carried out in triplicates unless otherwise stated.

All buffers, represent in the mobile phase were prepared on the same day of the experiments, filtered through 0.2 μm membrane filters to ensure high purity for the mobile phase, and deaerated under vacuum to avoid air bubbles formation within the detector cell. Buffer A, contained 20mM of Tris-HCl at pH 7.0, while eluting buffer B was similar to buffer A but containing 0.4mM NaCl. Linear gradient elution, with buffers A and B, was used to fractionate the whey proteins.

**Protein samples preparation**

Whey protein samples from skimmed milk and the four different types of probiotic drinks were prepared after acidification of the milk with 1M HCl to pH 4.6 followed by centrifugation at 13,000 rpm for 8 mins. The whey samples were decanted and then

<table>
<thead>
<tr>
<th>Samples</th>
<th>Protein (g/100 g)</th>
<th>Carbohydrates (g/100 g)</th>
<th>Fat</th>
<th>pH</th>
<th>Declared organisms on label</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skimmed milk</td>
<td>3.3</td>
<td>5.0</td>
<td>0.1</td>
<td>6.6</td>
<td>Lactobacillus casei Imunitass</td>
</tr>
<tr>
<td>Actimel (Danone)</td>
<td>2.8</td>
<td>11.7</td>
<td>0.0</td>
<td>4.3</td>
<td>Lactobacillus casei DN-114001</td>
</tr>
<tr>
<td>Yakult Light</td>
<td>1.3</td>
<td>12.2</td>
<td>0.04</td>
<td>4.0</td>
<td>Lactobacillus casei Shiroti</td>
</tr>
<tr>
<td>Muller Vitality strawberry</td>
<td>2.6</td>
<td>13.8</td>
<td>1.4</td>
<td>4.3</td>
<td>Bifidobacterium sp. and Lactobacillus acidophilus</td>
</tr>
<tr>
<td>Tesco probiotic cranberry</td>
<td>2.0</td>
<td>12.2</td>
<td>0.9</td>
<td>4.1</td>
<td>Lactobacillus acidophilus La-5</td>
</tr>
</tbody>
</table>

**Table I.**

Chemical composition of skimmed milk and four commercially available fermented probiotic milk drinks.
diluted with buffer A to get a protein concentration of 10 mg/mL. All samples were filtered through a 0.2 \( \mu \)m filters fitted into a syringe to be ready for injection to the FPLC.

Protein determination

All protein samples were measured using the BCA Protein Assay Kit, which is a colorimetric method based on the reaction of bicinchoninic acid with protein. This method combines the well-known reduction of \( \text{Cu}^{+2} \) to \( \text{Cu}^{+1} \) by protein in an alkaline medium (the biuret reaction) with the highly sensitive colorimetric detection of the cuprous cation (\( \text{Cu}^{+1} \)) using a unique reagent containing bicinchoninic acid.

Fast performance liquid chromatograph (FPLC)

Several methods have been used for the separation and fractionation of proteins such as casein and whey proteins. These methods include:

- size exclusion/gel filtration (Andrews et al., 1985; Matar et al., 1996), high-performance liquid chromatography (HPLC) using ion-exchange materials (Andrews et al., 1985; Egito et al., 2002; Tanabe et al., 2003), reversed-phase HPLC (Matar et al., 1996; Cornelia et al., 2002; Tazuzin et al., 2003; McCann et al., 2005; El-Zahar et al., 2005) and hydrophobic interactions (Ferreira et al., 2000; Ferreira et al., 2001);

- electrophoresis using polyacrylamide gels (PAGE) with or without urea (Mayer and Hörtner, 1992; Carretero et al., 1994; Egito et al., 2002; Veloso et al., 2002) or sodium dodecyl sulphate (SDS-PAGE) (Matar et al., 1996; Jin and Park, 1996; Tanabe et al., 2003) isoelectric focusing (IEF) (Kim and Jimenez-Flores, 1994), and capillary electrophoresis that shows extremely good resolution (Molina et al., 2000; Cartoni et al., 1999; Miralles et al., 2000); and

- immunological methods (Levieux and Venien, 1994; Haza et al., 1996).

FPLC technique was used in this work using Mono Q column HR 5/5 which is an anion exchange column, packed with particle size of 10\( \mu \)m, represents the stationary phase. A 500 \( \mu \)L of filtered sample, dissolved in buffer A, was injected into the column through the injection port at room temperature and the sample was automatically pushed through the column by high pressure pump using Dionex Gradient pump model GP 40 at a pressure of 29 bar (430 psi). The column was operated at a flow rate of 1 mL/min for optimum, fast and efficient separation. The mobile phase was based on a two buffer systems; buffer A, initially held at 100 per cent for 3 mins followed by buffer B to give the desired gradient. After 23 min, buffer B was run at 100 per cent for further 3 min followed by a 100 per cent of buffer A for 4 min, ready for the next injection. Whey protein fractions were monitored by Dionex model AD 20 Absorbance Detector at 280 nm. The results of the chromatographic separation for the individual whey protein fractions were displayed on a chart recorder connected to the detector.

Results and discussion

The study of the individual factions of casein and whey proteins and their hydrolysates from milk, cheese and other dairy products has been of major interest to many scientists (Molina et al., 2000; Veloso et al., 2002; Tanabe et al., 2003).
The overall results of whey protein hydrolysis in probiotic drinks showed that probiotic microorganisms have some effect on whey proteins by hydrolysing it into smaller peptides and amino acids during the fermentation process.

The changes in whey protein profile
Whey proteins are generally more sensitive to heat treatment, unaffected by changes in pH and are relatively more resistant to proteinases compared to caseins (Chen and Ledford, 1971; Yamauchi and Kaminogawa, 1972; Andrews, 1983). The effect of probiotics on the profile of whey protein fractions in probiotic health drinks was studied and compared with the profile of whey protein fractions of skimmed milk to investigate whether the fermentation process had any effect on the proteins. The individual whey protein fractions were identified after running a standard solution containing \( \alpha \)-lactalbumin, \( \beta \)-lactoglobulin and bovine serum albumin (BSA). Orotic acid, a vitamin like substance that is normally associated with fermentation and the growth of lactic acid bacteria was also added to this standard. All the samples were prepared and adjusted to a protein concentration of 10 mg/ml and 500 \( \mu \)L of the sample was injected into the FPLC.

For \( \alpha \)-lactalbumin fraction, the results showed that there was only traces of this fraction left in “Yakult” drink that contained \( L. \) casei Shirota (Figure 1) and there was a noticeable reduction in the other three samples (Figure 2, Figure 3 and Figure 4) when compared with the sample taken from skimmed milk (Figure 5). Such reduction might be due to the level of denaturation of this fraction during the heat treatment of the milk prior to fermentation or due to its utilisation by the individual microorganisms involved in the fermentation process especially if prebiotic such as inulin was added into the milk, as was the case with “Actimel” sample.

Researchers have shown that \( \alpha \)-lactalbumin fraction is more sensitive to hydrolysis with pepsin (El-Zahar et al., 2005) and was then completely hydrolysed into small peptides within 30 mins (Reddy et al., 1988; Schmidt and Poll, 1991).

![Figure 1. The FPLC separation of Yakult drink whey proteins on the Mono Q column](image)
There was no indication of any presence of β-lactoglobulin A and B in all the four drink samples (Figures 1-4). Such a complete absence of these fractions could be due to the same reasons mentioned above for the α-lactalbumin fraction.

The level of heat treatment, time and temperature, which was used for the preparation of all the probiotic drink samples, is not known. However, it is expected that the milk has been subjected to a heat treatment ranging from 80-90°C up to 105°C for 5-20 min which is well above the normal pasteurisation treatment. Information provided by one of the manufacturers has also indicated that UHT treatment has been used. Such high thermal treatment could cause varying degrees of denaturation of the two main whey proteins fractions, α-lactalbumin, β-lactoglobulin (Griffin et al., 1993; Qi et al., 1997) as well as the formation of insoluble complexes with α-casein Hugunin (1999), especially the interaction
between β-lactoglobulin and κ-casein, via the disulphide bond (Park and Lund, 1984; Sawyer, 1969) and with lactose to a certain degree. This study showed that any reduction of whey protein concentrations could not be associated solely to a specific factor/s. Although some variations in whey protein profiles between the control milk sample (used in the experiment) and of the original skimmed milk (used in the preparation of the drink samples) might exist, it was considered that such differences were too small to affect the overall whey protein profiles. Several factors remained unknown regarding the extent of the heat treatment used for processing the milk prior to the fermentation of the drink samples and the stage of the addition of the probiotic microorganisms into the milk and the length of time to
get the desired bacterial population of about $10^7$/ml. All these factors could have some effect on the overall level of whey protein hydrolysis and its profile.

Orotic acid, which is not a whey protein but is normally associated with fermented milk, is usually eluted as an early fraction. There was a slight decrease in its concentration in the three samples, Figures 1, 3 and 4, when compared with the control sample, Figure 5. However, some increase in its concentration was noticeable only in “Actimel”, Figure 2. Researchers have indicated that a reduction of up to 55 per cent of orotic acid has been reported during fermentation with probiotic and normal yogurt cultures and that the level of reduction varied between the cultures (Fernandez-Garcia and McGregor, 1994; La Torre et al., 2003; Østlie et al., 2003). Orotic acid thought to be an intermediate agent in the synthesis of nucleotides as well as its influence on the growth of starter cultures (Fernandez-Garcia and McGregor, 1994) and for its effect in lowering the cholesterol level in human population (Buonopane et al., 1992) as well as in rats (Rao et al., 1981).

**Conclusion**

The current research work has shown that there were distinct differences between the control sample, skimmed milk, and the four commercially available probiotic milk-based fermented health drinks when a direct comparison was carried out between these samples.

Although all the samples showed a degree of protein hydrolysis, its high level in “Yakult” and “Actimel” drinks might have been due to the nature of the process, the length of time of fermentation or the high level of proteolytic activities of the microorganisms used. The effect of heat treatment i.e. the time and temperature used during processing could have caused denaturation of the whey proteins which could also accelerate the level of hydrolysis. In the absence of any information from the manufacturers regarding the real processing procedure, which is quite understandable, such variations in the level of protein hydrolysis is inconclusive. Further investigation under controlled conditions and using pure cultures could give a better understanding of the protein hydrolysis.

**References**


