Hypocholesterolaemic effect of *Bifidobacterium animalis* subsp. *lactis* (Bb12) and trypsin casein hydrolysate

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**Abstract**

Hypercholesterolaemia is one of the most important risk factors in the development of cardiovascular disease. In this study, we report the in vitro potential of *Bifidobacterium animalis* subsp. *lactis* (Bb12) cultures and bovine casein hydrolysates formed by trypsin and Bb12 culture to reduce cholesterol levels. Cholesterol levels in vitro were reduced by up to 48% after incubation with Bb12 and up to 87% after incubation with trypsin hydrolysates, whereas unhydrolysed bovine casein did not affect cholesterol levels. Individual peptide fractions, obtained from size-exclusion chromatography, from casein hydrolysates formed by trypsin after a 48 h hydrolysis, reduced cholesterol levels by 2.7–50%. The molecular masses of these fractions, containing hypocholesterolaemic peptides, were below 1200 Da, as determined by LC-MS.

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1. Introduction

Coronary heart disease (CHD) is one of the major causes of death in the industrialised countries (Pereira & Gibson, 2002). An elevated serum cholesterol level is implicated as an important risk factor for CHD and other atherosclerotic diseases, and a reduction of 1% of serum cholesterol has been reported to reduce the risk of death in the industrialised countries (Pereira & Gibson, 2002). One of the major preventative strategies for treatment of the disease is to manage blood cholesterol and triglyceride levels, through diet and drug therapies, most notably the use of statins (El-Gawad, El-Sayed, Hafez, El-Zein, & Saleh, 2005; Pereira & Gibson, 2002). Other approaches to cholesterol reduction include the use of phytoestrogens, soy protein, soluble fibres, probiotic bacteria and prebiotic compounds (Taylor & Williams, 1998).

*Bifidobacterium animalis* subsp. *lactis* (Bb12) is one of the most commonly used probiotic microorganisms; it is a gram-positive bacterium found in the large intestine of most mammals. A number of health benefits have been reported for different *Bifidobacterium* species, including the promotion of gastrointestinal health (*Bifidobacterium longum*) (Marteau, de Vrese, Cellier, & Schrezenmeir, 2001) and enhancement of immunity (*Bifidobacterium lactis* HNO19) (Gill, Rutherford, Cross, & Gopal, 2001). In vivo studies of the hypocholesterolaemic effect of fermented milk products have been demonstrated in human subjects (Lewis & Burmeister, 2005) and animals (Kawase, Hashimoto, Hosoda, Morita, & Hosono, 2000; Larson, Donovan, & Potter, 1996). However, the mechanism of cholesterol reduction is still unclear. A number of hypotheses have been proposed to explain the hypocholesterolaemic effects of probiotic bacteria and the bioactive peptides produced by their proteolytic activity. Probiotic bacteria ferment indigestible carbohydrates to produce short chain fatty acids in the intestine, which can influence hepatic cholesterol synthesis and/or the redistribution of cholesterol from the plasma to the liver. Probiotic bacteria may also interfere with cholesterol absorption from the intestine by deconjugating bile salts, preventing reabsorption or by directly assimilating cholesterol (Hosono & Tono-o, 1995; Liong & Shah, 2005; Pereira & Gibson, 2002; Tahri, Crociani, Ballongue, & Schneider, 1995). Hypcholesterolaemic peptides have been described from several proteins (Li & Papadopoulos, 1998) and result in cholesterol reduction by binding to cholesterol by electrostatic and hydrophobic interactions (Seelig & Seelig, 1996), or by reducing the micellar solubility of cholesterol and inhibiting cholesterol absorption (Nagaoka et al., 2001).

A cholesterol recognition/interaction consensus sequence (CRAC) has been identified (ATVLNYYWRDNS) with the cholesterol interacting with arginine, tyrosine, valine and leucine amino acid side-chains (Li, Yao, Degenhardt, Teper, & Papadopoulos, 2001). The reduction in cholesterol level has been hypothesised to be due to the direct interaction of arginine and tyrosine with cholesterol, forming cholesterol–apo-protein complexes (Li et al., 2001) and has been reported to be dependent on the content of...
arginine in the protein (Klimov, Kozhevnikova, Klueva, & Belova, 1992). 

The objectives of the study were to determine the in vitro cholesterol-reducing effects of *B. animalis* subsp. *lactis* (Bb12) and crude trypsin casein hydrolysates. Additionally, the crude trypsin hydrolysate was fractionated, by molecular weight, and in vitro cholesterol-reducing effects determined.

2. Materials and methods

2.1. Materials

UHT skimmed milk was purchased locally and all chemicals (MRS broth, sodium thioglycolate, oxgall, cholesterol, KOH, n-hexane, o-phthalaldehyde reagent, β-cyclodextrin solution, ethanol, sulphuric acid, Sephadex G-25, trypsin enzyme (EC.3.4.21.4, bovine pancreas, type III) and soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Company (UK). Acetonitrile, HPLC grade water and trifluoroacetic acid (TFA) were purchased from Fisher Scientific and Acros organics and the bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (USA). Freeze-dried *Bifidobacterium animalis* subsp. *lactis* (Bb12) was a gift from Chr. Hansen Company (UK).

2.2. Culture preparation

Culture medium was prepared according to the method described by Mishra and Prasad (2005), using freshly prepared MRS broth supplemented with 0.2% sodium thioglycolate as an antioxidant and 0.3% oxgall. The medium was sterilized at 121°C for 15 min, and cholesterol was added at a concentration of 1.5 mg ml⁻¹ and mixed thoroughly. An aliquot of 9 ml of the media containing cholesterol was inoculated with 1 ml of active *Bifidobacterium animalis* subsp. *lactis* (Bb12) culture (0.5 g freeze-dried Bb12 in 250 ml of MRS broth; incubated aerobically at 37°C for 24 h). Control experiments were carried out with 10 ml of media. All of the samples were incubated aerobically at 37°C for up to 48 h. After incubation, the samples were cooled to 4°C and 48 h. After incubation, the samples were cooled to 4°C. Control experiments were run in triplicate and analysed for standard deviation using Minitab 15 (Minitab Ltd, Coventry, UK). Statistical software. Two sample differences were determined using a two sample t-test. Multiple differences were determined using ANOVA with Tukey’s pairwise analysis. All data was assumed to be normally distributed and results were deemed significant when p < 0.05 throughout.

2.3. Preparation of bovine casein hydrolysates

Casein was prepared from UHT skimmed milk by precipitation at pH 4.6 using 4 M HCl, followed by centrifugation at 13,000 rpm for 3 min. The whey was discarded and the casein precipitate was washed three times with distilled water to remove any residual traces of whey proteins. The precipitate was then re-dissolved in 1 M NaOH and neutralised to pH 7.5, using 4 M HCl. Trypsin hydrolysates were prepared by adding 15 mg of trypsin (10,000 units mg⁻¹) to 15 ml of crude casein solution (11 mg ml⁻¹) and incubating at 37°C for up to 48 h. After incubation, trypsin inhibitor (16 mg) was added to the casein solution (10,000 units mg⁻¹) (Steiner & Fratalli, 1969). Protein concentrations were determined using the BCA method.

Bb12 hydrolysates were prepared by adding 30 mg of freeze-dried Bb12 to 15 ml casein solution (11 mg ml⁻¹) and incubating aerobically at 37°C for up to 48 h. Unhydrolysed casein was used as the control.

2.4. Cholesterol determination

Cholesterol determination was carried out as described by Rudel and Morris (1973) and modified by Mishra and Prasad (2005). Absorbances were read at 550 nm and the percentage cholesterol reduction was calculated using the formula,

\[ \% \ \text{Cholesterol reduction} = \frac{C - S}{C} \times 100 \]  

where C is the average cholesterol reading for the control samples and S is the average cholesterol reading of the spent broth for Bb12 culture or the casein hydrolysate formed by trypsin or Bb12 culture. The experiments were run in triplicate and analysed for standard deviation using Minitab 15 (Minitab Ltd, Coventry, UK) statistical software. Two sample differences were determined using a two sample t-test. Multiple differences were determined using ANOVA with Tukey’s pairwise analysis. All data was assumed to be normally distributed and results were deemed significant when p < 0.05 throughout.

2.5. Effects of casein hydrolysates from trypsin or Bb12 culture on cholesterol

100 µl of unhydrolysed casein (control) and trypsin or Bb12 hydrolysates (samples), at concentrations of 11 mg ml⁻¹, were added to a cholesterol solution containing 1.5 mg cholesterol dissolved in 85 µl β-cyclodextrin solution. Triplicate samples were incubated for 1.5 h at 40°C. The reduction in cholesterol level was calculated using Eq. (1).

2.6. Separation of crude casein hydrolysate on Sephadex G25

Peptides from the casein hydrolysate were separated by size-exclusion chromatography on a Sephadex G25 column (60 × 2.5 cm) at room temperature and eluted under isocratic conditions with 20 mM Tris–HCl buffer, pH 7.0, containing 4.5 M urea and 0.35 M NaCl. An aliquot of 4 ml of trypsin hydrolysate, at a concentration of 3 mg ml⁻¹, was loaded onto the column and eluted at a flow rate of 16 ml h⁻¹ and 4 ml fractions were collected and then pooled into five fractions, (F1–F5) Fig. 1.

![Fig. 1. Size-exclusion chromatography profile (280 nm) of crude casein hydrolysate obtained after 48 h of hydrolysis with trypsin. Elution with 20 mM Tris–HCl buffer, pH 7.0, containing 4.5 M urea and 0.35 M NaCl under isocratic conditions. The collected fractions are marked by numbers above the peak.](image-url)
2.7. Reverse-phase high-performance liquid chromatography (RP-HPLC) separation of peptides

Fractions F1–F4 and casein samples hydrolysed with trypsin or Bb12 for 48 h were fractionated by RP-HPLC on a C18 column (250 × 7.75 mm, 300 Å, ACE, Hichrom Ltd., UK). The peptides were eluted with 100% buffer A for 3 min and a linear gradient of 100% buffer A to 60% buffer B over 70 min (A: 95% (v/v) water, 5% (v/v) acetonitrile, 0.1% TFA (v/v)); B 85% (v/v) acetonitrile, 15% (v/v) water, 0.1% TFA (v/v)). Peptides were eluted at a flow rate of 1 ml min⁻¹ and monitored at 220 nm.

2.8. The effects of casein hydrolysate fractions from trypsin on cholesterol

All samples and controls were adjusted to the same protein concentration of 0.18 mg ml⁻¹, by diluting them with gel permeation buffer. Aliquots of 1 ml of unhydrolysed casein (control) and each fraction (F2–F4) were added to the cholesterol solution (1.5 mg cholesterol dissolved in 85 μl β-cyclodextrin solution) and the level of cholesterol reduction was measured as described earlier.

2.9. Determination of degree of hydrolysis (%DH)

The ortho-phthalaldehyde (OPA) method was used to determine the degree of hydrolysis of casein hydrolysates formed by Bb12 and trypsin, as described by Church, Swaisgood, Porter, and Catignani (1983) with modifications by Donkor, Henriksson, Vasiljevic, and Shah (2005). Fifty microlitres of unhydrolysed casein or casein hydrolysed by trypsin or Bb12 were added to 3 ml of OPA reagent containing 2-mercaptoethanol, mixed briefly and left to stand at room temperature (21 °C) for 10 min. The absorbance of the solution was measured at 340 nm. The degree of hydrolysis for each sample was determined according to the following equation:

\[
\text{% degree of hydrolysis} = \frac{(S - C)}{D} \times 100
\]

where C is the unhydrolysed casein reading (control), S is the sample reading of the casein hydrolysate formed by Bb-12 or trypsin (samples), and D the difference between the sample reading after 48 h (taken as 100% degree of hydrolysis) and the unhydrolysed casein reading taken as 0%.

2.10. Separation and molecular mass identification of peptide fractions using LC-MS

Fractions F2–F4 were analysed on an Agilent 1200 LC-MSD and operated by Agilent Technologies ChemStation software for LC/MSD, using a C18 column (2.1 × 100 mm, 1.8 μm) at 35 °C using a flow rate of 0.4 ml min⁻¹ and monitored by a diode-array detector from 214–280 nm. The peptides were separated using a linear gradient of 98% A and 2% B to 58% A and 42% B over 40 min (A: water, 0.1% (v/v) trifluoroacetic acid and B: acetonitrile, 0.1% (v/v) TFA). The mass spectrometer was operated in the positive mode with a nebuliser pressure of 50 psi, using a drying gas flow of 11 l/min⁻¹ and a drying temperature of 350 °C. Mass spectra were recorded over the mass/charge range of 100–1200 m/z. The spectral data were processed and presented as peptide masses for each eluted peak.

3. Results and discussion

3.1. Effect of Bb-12 cultures on cholesterol

The direct effect of Bifidobacterium animalis subsp. lactis (Bb12) cultures, on cholesterol levels in the presence of bile salts in vitro, showed reductions of approx. 25% and 48% after 24 h and 48 h incubations, respectively (Fig. 2). These results showed a significant difference in cholesterol reduction between the 24 h and 48 h incubations. These results are in general agreement with those of Tahri et al. (1995) who studied three strains of Bifidobacteria, B. longum, B. Breve and B. animalis, grown in trypticase–peptone–yeast extract (TPY) medium containing cholesterol for 24 h which resulted in cholesterol reductions of 50%, 60% and 39%, respectively. Similar observations were reported for some Lactobacillus casei strains inoculated in MRS broth containing cholesterol, resulting in a 44% reduction in cholesterol level when incubated for up to 48 h (Mishra & Prasad, 2005). In another study, Lactobacillus reuteri and Lactobacillus acidophilus were reported to absorb cholesterol from MRS broth medium, resulting in cholesterol reductions of up to 60% and 50%, respectively (Bottazzi, Zacconi, Gonzaga, & Paladino, 1986).

Bb12 culture was also able to grow aerobically in MRS broth medium supplemented with 0.3% oxgall (as bile salts) and demonstrated cholesterol-reducing ability. The Bb12 culture was oxygen-tolerant, in agreement with reports of Meile et al. (1997) and Bouluc, Raymond, Fustier, Champagne, and Vuillemond (2006). The mechanism(s) responsible for this cholesterol reduction have been attributed to assimilation and absorption of cholesterol into the bacterium and binding to cell wall peptidoglycans and by incorporation into cellular membranes during bacterial growth (Hosono & Tono-oka, 1995; Liong & Shah, 2005; Tahri et al., 1995).

3.2. Effect of crude casein hydrolysates formed by trypsin or Bb12 on cholesterol

Unhydrolysed crude casein was shown to have no cholesterol-reducing activity. However, increasing levels of hydrolysis were correlated with increasing cholesterol reduction in vitro (Table 1). The Table also shows that, for a given level of hydrolysis, the peptides produced by Bb12 were more effective in reducing cholesterol levels than those produced by trypsin, a level of hydrolysis of casein by Bb12 of 51.2%, resulting in an 87.5% reduction in cholesterol, higher than that produced by trypsin.
after 48 h of hydrolysis. There were significant differences in the reduction of cholesterol by peptides produced by Bb12 and trypsin in relation to % hydrolysis, the peptides produced by Bb12 being more effective than those produced by trypsin in reducing cholesterol levels. Trypsin has a narrow specificity and cleaves peptide bonds preferentially on the carboxylic side of the amino acid residues (lysine and arginine) (Antal et al., 2001; Pedroccho et al., 2004). The extracellular proteases of Bifidobacterium spp. are poorly characterised; however, aminopeptidase activities (general aminopeptidases, proline iminopeptidase, X-prolyl dipeptidase) were found to be higher for B. animalis spp. lactis when grown in a milk-based medium than in MRS broth (Janer, Arigoni, Lee, Peláez, & Requena, 2005). An endopeptidase has been sequenced and cloned, the predominant peptide bond cleavage being on the amino side of phenylalanine residues, with a post-proline secondary cleavage site (Janer et al., 2005). It would be expected, therefore, that Bb12 could produce a mixture of extracellular proteases, with a range of specificities to digest proteins, when Bb12 was in a nutrient-rich environment. The recent elucidation of the genome sequence of B. animalis spp. lactis ADD01 should enable identification of proteolytic enzymes and other proteins associated with other probiotic functions (Kim et al., 2009).

The hypocholesterolemic effect of the crude casein hydrolysates formed by trypsin has been postulated to be due to the interaction of lysine and arginine residues with cholesterol. The content of arginine and lysine residues in casein is approx 12.3 mol% (Belitz, 1992; Li et al., 2001). Similar results were reported for yogurt containing B. animalis spp. lactis (El-Gawad et al., 2005). Similar results were reported by Yoshikawa et al. (2000) where β-lactoglobulin peptides were able to reduce serum cholesterol when orally administered to mice. In another study, peptides from soy proteins, with low molecular weights, were shown to have cholesterol-lowering properties (Lovati et al., 2000), whereas, peptides derived from soybean protein, having high molecular weights of 1000–10,000, have also been found to lower cholesterol level (Sugano et al., 1990). Small peptides were superior (in reducing cholesterol) to large peptides, due to their easier absorption into the intestine without further hydrolysis by digestive enzymes (Meisel & Bockelmann, 1999).

The casein hydrolysate, formed by trypsin after 48 h of incubation, showed a higher hypocholesterolemic effect than did the individual fractions obtained after size-exclusion chromatography containing hypocholesterolemic peptides from crude casein hydrolysate formed by trypsin after the maximum hydrolysis time of 48 h. This might be due to the synergistic effect of these peptides together in the whole crude casein. Such effect, although different from cholesterol reduction, would resemble other observations which have been reported by Clare and Swaisgood (2000) where total antibacterial effect in milk was higher than that of the sum of the individual contributions of immunoglobulin and non-immunoglobulin defence proteins. This suggests that the recovery of hypocholesterolemic peptides from whole crude casein hydrolysate, for use as a food supplement, would not be as effective in reducing cholesterol as consumption of the whole crude hydrolysate.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time (h)</th>
<th>% Hydrolysis</th>
<th>% Cholesterol reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bb12</td>
<td>24</td>
<td>37.8</td>
<td>51.5 (16.9)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>51.2</td>
<td>87.5 (8.7)</td>
</tr>
<tr>
<td>Trypsin</td>
<td>24</td>
<td>94.2</td>
<td>78.5 (13.7)</td>
</tr>
<tr>
<td></td>
<td>48</td>
<td>100</td>
<td>81.7 (6.2)</td>
</tr>
</tbody>
</table>

3.3. Effects of individual casein fractions from size-exclusion chromatography on cholesterol

The casein hydrolysate formed by trypsin, after 48 h of incubation, was first fractionated according to molecular weight by size-exclusion chromatography on Sephadex G25, (Fig. 1). The cholesterol-reducing activity of each of the fractions F1–F5 was determined taking into account their perspective concentration. Fractions F1 and F5 were found to have no effect on cholesterol reduction whilst fractions F2, F3 and F4 showed reductions in cholesterol levels of 50%, 37% and 3%, respectively (Fig. 3). There were significant differences in the mean responses to cholesterol reduction for the fractions F2, F3 and F4 (one way ANOVA); further analysis, using Tukey’s pairwise method, supports the observation that F4 is where the significant difference arose. RP-HPLC of fraction 2 is shown in Fig 4 and was compared with those from fractions 3 and 4. The molecular weights of the peptides in fractions F2, F3 and F4, were determined by LC-MS, and were all found to be below 1200 Da. Using an average residue molecular weight of 119 Da (calculated for casein), the largest peptide would consist of six amino acid residues. These results are in general agreement with the results of Proulx, Ward, Gauthier, and Roy (1994) where most of the peptides formed by trypsin (89%) were less than 2000 Da, with 11% being over 2000 Da.

Similar results were reported by Yoshikawa et al. (2000) where β-lactoglobulin peptides were able to reduce serum cholesterol when orally administered to mice. In another study, peptides from soy proteins, with low molecular weights, were shown to have cholesterol-lowering properties (Lovati et al., 2000), whereas, peptides derived from soybean protein, having high molecular weights of 1000–10,000, have also been found to lower cholesterol level (Sugano et al., 1990). Small peptides were superior (in reducing cholesterol) to large peptides, due to their easier absorption into the intestine without further hydrolysis by digestive enzymes (Meisel & Bockelmann, 1999).

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![Fig. 3. Cholesterol reduction of elution fractions from size-exclusion. Error bars indicate the standard deviation.](image-url)
Bb12 has been shown to grow in milk and hydrolyse casein to release peptides that reduce cholesterol levels in vitro. However, in food applications, as a bacterium, it requires optimum conditions and time to grow to produce enzymes that are released into the medium to generate beneficial peptides. This process takes longer than does the direct addition of a purified enzyme, such as trypsin for economic addition to a food, unless the Bb12-generated peptides can be shown to have an enhanced effect over those generated by trypsin. Addition of trypsin-derived casein peptides would be more viable. However, there may be a synergistic effect on the addition of Bb12 cultures and the peptides derived from them in cholesterol reduction, as the mechanisms by which they reduce cholesterol levels are different. These studies have shown cholesterol reduction in vitro; further studies should be carried out on the peptides produced by the action of Bb12 on bovine casein to characterise the effects, in vivo, of adding milk cultured with Bb12 (containing both Bb12 and the peptides) and trypsin-derived peptides on cholesterol levels. Bb12 and its casein-derived peptides may have a greater cholesterol reducing effect than those of the peptides alone.

4. Conclusion

The current in vitro study of cholesterol reduction showed that unhydrolysed crude casein had no effect, whereas trypsin or Bb12 crude casein hydrolysates and Bb12 culture in MRS broth significantly reduced cholesterol levels (24–87%).

Bb12 has been shown to grow in milk and hydrolyse casein to release peptides that reduce cholesterol levels in vitro. However, in food applications, as a bacterium, it requires optimum conditions and time to grow to produce enzymes that are released into the medium to generate beneficial peptides. This process takes longer than does the direct addition of a purified enzyme, such as trypsin for economic addition to a food, unless the Bb12-generated peptides can be shown to have an enhanced effect over those generated by trypsin. Addition of trypsin-derived casein peptides would be more viable. However, there may be a synergistic effect on the addition of Bb12 cultures and the peptides derived from them in cholesterol reduction, as the mechanisms by which they reduce cholesterol levels are different. These studies have shown cholesterol reduction in vitro; further studies should be carried out on the peptides produced by the action of Bb12 on bovine casein to characterise the effects, in vivo, of adding milk cultured with Bb12 (containing both Bb12 and the peptides) and trypsin-derived peptides on cholesterol levels. Bb12 and its casein-derived peptides may have a greater cholesterol reducing effect than those of the peptides alone.

References


Fig. 4. Profile of fraction 2 (crude casein hydrolysed by trypsin for 48 h) from size-exclusion, separated by RP-HPLC column.


