Biofunctional milk protein ingredients: Caseinophosphopeptides in perspective

A.B. Nongonierma & R. J. FitzGerald*

Department of Life Sciences and Food for Health Ireland (FHI),
University of Limerick, Limerick, Ireland.

Please cite as:

*Corresponding author: dick.fitzgerald@ul.ie

Tel: +353 (0) 61 202598

Fax: + 353 (0) 61 331490
Abstract

A significant amount of information is available linking consumption of milk proteins with the beneficial modulation of key outcomes associated with diet related conditions such as cardiovascular disease, obesity and type two diabetes. While intact milk proteins per se may have some direct health promoting effects, the functional food potential of dairy proteins is mainly attributed to the peptides formed following hydrolysis. Evidence/emerging evidence for the potential role of dairy peptides as hypotensive, mineral binding, immunomodulatory, anticancer, opioid agonist and antagonist, antimicrobial, gut health enhancing, hypo-cholesterolemic, insulinotropic and neuromodulatory agents now exists. Some information outlining the biofunctional potential of caseinophosphopeptides (CPPs) is presented herein.
Introduction

The terms “functional foods” and “nutraceuticals” are relatively broad and need to be carefully defined. Their meaning may be distinguished on the basis that they describe foods/food ingredients which on the one hand have potential disease preventative versus disease curative roles, i.e., functional foods and nutraceuticals, respectively. In both cases, functional foods and nutraceuticals are foods/food ingredients that contain biofunctional components which may beneficially modulate physiological functions. Furthermore, in order for these components to have an effect in vivo, the bioactive components must be bioavailable, i.e., they must reach the target site/organ in sufficient amounts to have a physiological effect. The real distinction between the two is that functional foods are ultimately foods whereas nutraceuticals, having potential disease curative functions, could be considered as drug-like agents. There are also instances where the above two terms may be confused with technofunctional foods/ingredients. This term relates to foods or ingredients which confer specific technofunctional properties such as emulsification, foaming, gelation, water-binding, aggregation properties, etc., to foods.

There is a growing interest in biofunctional foods/food ingredients given their potential in the prevention of chronic diseases. From a consumer perspective, biofunctional foods are associated with the general concept of ‘wellness’ and lifestyle choice where an individual may choose to consume specific foods/food ingredients based on their ability to modulate controllable risk factors associated with various chronic diseases. These include diseases/conditions such as cardiovascular disease, diabetes and obesity. Biofunctional foods are therefore a growing global market and as a consequence represent a real opportunity for diversification and innovation in the food sector.

It is well documented that intact milk proteins possess many potential bioactivities. These include the antibacterial activity associated with the whey protein lactoferrin. Milk proteins, in addition,
contain an extensive range of bioactive peptide sequences which are encrypted within their primary structures (FitzGerald and Meisel, 2003). As a consequence, different milk protein-derived peptides are being marketed as biofunctional foods and ingredients which may beneficially modulate the physiological responses of different systems within the human body. Nevertheless, some conflicting evidence exists in relation to the efficacy of these biofunctional peptides. This article presents some of the scientific information with respect to caseinophosphopeptides (CPPs), casein derived phosphorylated peptides, as a means to ascertaining those parameters which may dictate the overall bioactivity of milk protein-derived biofunctional peptides. It is proposed that this exercise may provide some useful insights for the development of peptides as biofunctional ingredients for the reduction of chronic disease risk.

1 Physiological targets of milk bioactive peptides

Beyond their basic nutritional role in the provision of nitrogen and essential amino acids, milk proteins and peptides have the potential to beneficially modify a range of different physiological systems within the human body. Milk protein-derived bioactive peptides may, for example, modulate control systems involved in the cardiovascular, nervous, gastrointestinal and immune systems. The general mode of action is that these peptides ultimately alter specific interactions involving cellular receptors or enzymes which lead to the induction of a physiological response. Therefore, the targeted utilisation of these peptides can beneficially modulate physiological systems within the human body. A diverse range of potential physiological targets have been identified, mostly using in vitro assays to date, for peptide sequences which are encrypted within the primary structures of the milk proteins. These include peptides having immunomodulatory, opioid, mineral binding/bone formation, hypotensive, antithrombic, anticancer, etc, properties (Clare and Swaisgood, 2000; FitzGerald and Meisel, 2003; Korhonen and Pilhanto, 2006).

Milk bioactive peptides have significant potential to modulate various human health conditions
via their application as health promoting and disease risk reducing agents. Furthermore, these peptides have possible synergistic effects in human health maintenance. The application of bioactive peptides to modulate various conditions associated with the metabolic syndrome is one such example. The so-called metabolic syndrome is manifested in conditions associated with dyslipidemia, impaired glucose metabolism, central obesity and hypertension. It is interesting to note that for each of these conditions, one or more milk-derived peptides may exist which could, individually or indeed in synergy, beneficially modulate these conditions (Figure 1). Looking at cardiovascular disease, for example, it has been shown (at least in vitro and in some instances in vivo) that different milk protein-derived peptides display angiotensin converting enzyme (ACE) inhibitory, antithrombotic, anti-inflammatory, mineral binding and anti-oxidant activities. All these activities have the potential to reduce the incidence of controllable risk factors associated with cardiovascular disease. In addition to conditions associated with the metabolism syndrome, Figure 1 shows that opioid peptides and CPPs may play a role in the reduction of stress and poor bone health, respectively.

Since these bioactive peptides are encrypted within the primary structures of the different milk proteins they must first be released in order to become active. A number of different release approaches exist as follows:

- **in vitro** hydrolysis following a targeted enzymatic route using proteolytic activities from mammalian, microbial and plant sources or via the utilisation of physical and/or chemical processes such as ultrasonic, microwave and chemical treatments.

- hydrolysis by bacterial proteolytic/peptidolytic activities during the manufacture of fermented dairy products. Such hydrolysis may also occur **in vivo** following the action of intestinal microflora naturally found in the human body.
- *in vivo* digestion via the combined action of gastric and pancreatic hydrolases during gastrointestinal transit following oral ingestion. Subsequently, epithelial and serum peptidases may mediate further degradation assuming transport across the intestinal mucosa and transfer into the serum.

**Figure 1.** Summary of milk peptides that may beneficially modulate lifestyle diseases and conditions. (ACE: angiotensin converting enzyme; CPPs: Caseinophosphopeptides)

The translation of *in vitro/ex vivo* observations in relation to a particular bioactive peptide to ingredients/products beneficial to human health represents a major scientific challenge. Detailed analysis of the scientific information in connection with the application of CPPs as mineral bioavailability enhancement agents provides some critical insights into the above.
2 CPPs

A range of CPP ingredients and CPP containing products are currently available in the marketplace (Table 1). These CPPs originate from the four caseins found in bovine milk.

Table 1. Some commercial ingredients and products containing caseinophosphopeptides (CPPs).

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Manufacturer</th>
<th>Format</th>
</tr>
</thead>
<tbody>
<tr>
<td>CE90CPP</td>
<td>DMV, International</td>
<td>ingredient</td>
</tr>
<tr>
<td>Lacprodan D1-2021</td>
<td>Arla Foods</td>
<td>ingredient</td>
</tr>
<tr>
<td>Peptigen 110</td>
<td>MD Foods</td>
<td>ingredient</td>
</tr>
<tr>
<td>Capolac</td>
<td>Arla Foods</td>
<td>ingredient</td>
</tr>
<tr>
<td>CPPB and CPPC</td>
<td>Armor Proteins</td>
<td>ingredient</td>
</tr>
<tr>
<td>CPP-I, II &amp; III</td>
<td>Meiji Seika</td>
<td>ingredient</td>
</tr>
<tr>
<td>Recaldent</td>
<td>Cadbury Adams</td>
<td>ingredient</td>
</tr>
<tr>
<td>Products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tekkotsu Inryou</td>
<td>Suntory</td>
<td>soft drink</td>
</tr>
<tr>
<td>Kotsu Calcium</td>
<td>Asahi</td>
<td>soft drink</td>
</tr>
<tr>
<td>MI Paste &amp; MI Paste Plus</td>
<td>GC, America</td>
<td>tooth paste</td>
</tr>
</tbody>
</table>

CPPs are encrypted within the primary sequences of caseins and may be released following the approaches already described. They correspond to peptide fragments which are rich in clusters of phosphorylated seryl (and occasionally threonine) residues. They are reported to improve mineral bioavailability by acting as a mineral carrier and/or by enhancing mineral solubility. The caseins are phosphorylated during milk biosynthesis via the activity of specific kinases present in the mammary gland (FitzGerald, 1998). The number of serine/threonine phosphate groups may be influenced by genetic polymorphism of the proteins. Therefore, different levels of phosphorylation may be observed in the individual caseins as follows:

- \( \alpha_{s1}\)-casein: 8-9 phosphate groups
- α₅₂-casein: 10-13 phosphate groups
- β-casein: 5 phosphate groups
- and κ-casein: 1-2 phosphate groups.

The two most studied CPPs originate from αₛ₁- and β-casein. Both of these CPPs, i.e., αₛ₁-casein f(59-79)5P and β-casein f(1-25)4P, contain a specific sequence known as the “acidic motif”. This motif consists of 3 serine phosphate groups followed by two glutamic acid residues (Figure 2). At neutral pH, the “acidic motif” is a highly charged region and this has been linked with the ability of CPPs to bind minerals (Ca²⁺, Zn²⁺, Fe²⁺, Mn²⁺, etc.). CPPs have been reported to improve dietary bioavailability of those bivalent cations and as a consequence CPPs may play a major role in modulating mineral uptake and bone formation. CPPs have also been associated with anticariogenic effects in addition to promoting remineralisation of dental enamel (Reynolds, 1997). Apart from their mineral binding properties, CPPs are also reported to have immunomodulatory and antioxidant properties.

<table>
<thead>
<tr>
<th>αₛ₁-casein f(59-79)5P</th>
<th>β-casein f(1-25)4P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gln-Met-Glu-Ala-Glu-Ser(P)-Ile-Ser(P)-Ser(P)-Glu-Glu-Ile-Val-Pro-Asn-Ser(P)-Val-Glu-Gln-Lys</td>
<td>Arg-Glu-Leu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Asp(P)-Leu-Ser(P)-Ser(P)-Ser(P)-Glu-Glu-Ser-Ile-Thr-Arg</td>
</tr>
</tbody>
</table>

Figure 2. Peptide sequence of tryptic caseinophosphopeptides (CPPs) from αₛ₁-casein f(59-79)5P and β-casein f(1-25)4P highlighting the “acidic motif” (fragment underlined in the sequence).
Several studies performed in mammals including humans, pigs and rats have demonstrated the *in vivo* formation of CPPs following ingestion of milk and dairy products (Table 2). CPPs have been found in the distal small intestine of rat, and in the stomach, duodenum and distal ileum of human subjects (Hartmann and Meisel, 2007) following the consumption of dairy ingredients and products.

**Table 2.** Examples of studies reporting the formation of caseinophosphopeptides (CPPs) *in vivo* following the ingestion of milk and dairy products (adapted from FitzGerald (1998))

<table>
<thead>
<tr>
<th>System</th>
<th>Diet</th>
<th>CN origin</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rat</strong> (small intestine)</td>
<td>CN</td>
<td>nd</td>
<td>Naito <em>et al.</em> (1972)</td>
</tr>
<tr>
<td><strong>Minipig</strong> (jejunal fluid)</td>
<td>CN</td>
<td>αs1-CN</td>
<td>Meisel and Frister (1988)</td>
</tr>
<tr>
<td><strong>Human</strong> (stomach and duodenum)</td>
<td>milk/yoghurt</td>
<td>αs1/β-CN</td>
<td>Chabance <em>et al.</em> (1998)</td>
</tr>
<tr>
<td><strong>Human</strong> (ileostomy fluid)</td>
<td>milk/CPPs</td>
<td>nd</td>
<td>Meisel <em>et al.</em> (2003)</td>
</tr>
</tbody>
</table>

(CN: casein; nd: not determined)

Despite the fact that CPPs are present in the gastrointestinal system following dairy protein or CPPs ingestion, conflicting evidence exists in the literature regarding the relationship between Ca$^{2+}$ absorption and CPP consumption. Some studies have demonstrated a positive role for CPPs on Ca$^{2+}$ absorption while others have shown no effect (Table 3).
Table 3. Summary of the action of caseinophosphopeptides (CPPs) on Ca$^{2+}$ bioavailability (+: increase in the Ca$^{2+}$ bioavailability; no effect: no improvement in the Ca$^{2+}$ bioavailability). Adapted from FitzGerald (1998) and Meisel et al. (2003).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Study type</th>
<th>Method</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>rachitic children</td>
<td>in situ</td>
<td>Ca$^{2+}$ balance</td>
<td>+</td>
</tr>
<tr>
<td>rat</td>
<td>in situ</td>
<td>Ca$^{2+}$ balance</td>
<td>+</td>
</tr>
<tr>
<td>rat</td>
<td>in situ</td>
<td>Ca$^{2+}$, tube ligation</td>
<td>+</td>
</tr>
<tr>
<td>chicken</td>
<td>in situ</td>
<td>Ca$^{2+}$, tube ligation</td>
<td>+</td>
</tr>
<tr>
<td>rat</td>
<td>in vivo</td>
<td>bone measurement</td>
<td>no effect</td>
</tr>
<tr>
<td>pig</td>
<td>in vivo</td>
<td>Ca$^{2+}$ solubilisation</td>
<td>+</td>
</tr>
<tr>
<td>rat</td>
<td>in vivo</td>
<td>Ca$^{2+}$ balance</td>
<td>no effect</td>
</tr>
<tr>
<td>pig</td>
<td>in vivo</td>
<td>bone mineralisation</td>
<td>no effect</td>
</tr>
<tr>
<td>human</td>
<td>in vivo</td>
<td>absorption</td>
<td>+</td>
</tr>
<tr>
<td>rat embryonic bone</td>
<td>in vitro</td>
<td>bone deposition</td>
<td>+</td>
</tr>
<tr>
<td>human (post-menopausal)</td>
<td>in vivo</td>
<td>absorption</td>
<td>+</td>
</tr>
<tr>
<td>human</td>
<td>in vivo</td>
<td>absorption</td>
<td>+</td>
</tr>
</tbody>
</table>

3 Parameters influencing CPP modulated mineral bioavailability

The different outcomes from studies carried out on Ca$^{2+}$ bioavailability in the presence of CPPs may find their origin in several parameters. The conflicting bioavailability results may be due to differences in the methodology utilised for mineral quantification, the presence of chelating agents such as phytates, the food matrix, CPP preparation, CPP dose or CPP:mineral ratio. Aspects of the last four parameters will be discussed in the following sections.

3.1 Food matrix effects on mineral bioavailability

Since CPPs are being used as ingredients or additives for their mineral binding properties they are generally formulated in a food matrix. However, different studies have shown that components within the food matrix may play a significant role in the bioavailability of dietary minerals. For instance, the presence of inorganic phosphate (Pi) may lead to a decreased absorption of dietary...
Ca\(^{2+}\) due to the formation of insoluble calcium phosphate complexes. However, on inclusion of CPPs, the bioavailability of dietary Ca\(^{2+}\) may be maintained in the presence of phosphate. In a rat ileum model the absorption of Ca\(^{2+}\) by (Pi) decreased by 40% at a Ca:Pi = 1:1 and was decreased by 60% at a Ca:Pi = 1:2 in the presence of CPPs. However, in the absence of CPPs Ca\(^{2+}\) absorption decreased by 90% at a Ca:Pi = 1:1 and was decreased by 97% at a Ca:Pi = 1:2 (values compared to the absorption of Ca\(^{2+}\) from CaCl\(_2\) in the absence of Pi, Erba et al. (2001)). This specific example clearly demonstrates that the absorption of Ca\(^{2+}\) from a medium containing Pi can be significantly maintained in the presence of CPPs.

CPPs have been detected in the intestinal fluid of humans with an ileostomy. Higher levels were detected after ingestion of milk as compared to none or very low CPP levels detectable following CPPs ingestion (Meisel et al., 2003). The results from this study therefore indicate that milk has a protective effect against CPP degradation in the gastrointestinal tract. Surprisingly, direct ingestion of CPPs resulted in their almost total degradation and as a consequence they were only detected at very low levels in the ileum fluid. Food matrix therefore influences the stability of bioactive peptides in this case the stability of CPPs.

In other studies, fruit beverage appears to have the ability to protect CPPs against degradation by gastrointestinal enzymes. A fruit beverage (a mixture of 7.2% grape concentrate, 4.2% orange concentrate, 24.5% apricot puree, 5.1% sugar, 0.35% pectin, 0.054% L-ascorbic acid and 58.7% osmosis water) was supplemented with either milk or CPPs and it was subsequently subjected to simulated gastro intestinal digestion (SGID) using a combination of gastric and pancreatic enzyme activities (García-Nebot et al., 2009). Subsequent to SGID, 10 different CPPs, with one presenting the “acidic cluster”, were identified in the fruit beverage sample supplemented with milk. However, when CPPs were added to the fruit beverage, 16 different CPPs were identified following SGID with more than one peptide containing the acidic cluster sequence (Figure 3).
These results indicate that fruit beverages may be good vehicles/matrices for the delivery of CPPs.

**Figure 3.** Release/retention of caseinophosphopeptides (CPPs) following simulated gastrointestinal digestion (SGID) of fruit beverage supplemented with either milk or CPPs. Adapted from García-Nebot et al. (2009)

Clear evidence therefore exists on differences in the relative stability of CPPs during *in vivo* and *in vitro* digestion. These findings highlight the need to assess/validate both the stability and the bioactivity of biofunctional peptides within the food delivery matrix. As already mentioned, there may be a need for the application of specific reformulation and/or protective methodologies (such as encapsulation) if the biofunctional peptide is not stable in a given food matrix.

**3.2 Influence of CPP preparation**

The nature of the CPP preparation is another important factor that may affect its ability to modulate mineral bioavailability. Differences in the mineral uptake promoting potential of $\alpha_{s1}$-casein f(59-79)5P and $\beta$-casein f(1-25)4P have been reported. For example, a higher number of HT29 epithelial cells responsive to $\text{Ca}^{2+}$ uptake was found with $\beta$-casein f(1-25)4P than with $\alpha_{s1}$-
casein f(59-79)5P (Ferrarretto et al., 2003). This result may be linked to differences in the binding affinity for Ca\(^{2+}\) by these two particular fragments. It was shown that β-casein f(1-25)4P had a lower Ca\(^{2+}\) binding affinity and higher stoichiometry (Ca\(^{2+}\):peptide, 0.85 mM\(^{-1}\) and 4 Ca\(^{2+}\):peptide, respectively) than α\(_{s1}\)-casein f(59-79)5P which had a higher binding affinity and lower stoichiometry (0.63 mM\(^{-1}\) and 1 Ca\(^{2+}\):peptide, respectively) (Meisel and Olieman, 1998). Similarly, iron uptake by Caco-2 intestinal cell cultures and rat duodenal loop has been shown to depend on the CPP preparation, being about 2 fold higher with β-casein f(1-25)4P than with α\(_{s1}\)-casein f(59-79)5P (Kibangou et al., 2005).

In addition to the milk protein source of the CPP, peptide sequence may also play an important role on mineral bioavailability modulation. It has been demonstrated that Ca\(^{2+}\) uptake by intestinal cells depends on amino-acid residues upstream and downstream from the “acidic motif”. The central role of the phosphorylated acidic domain and of the N-terminal region of β-casein f(1-25)4P have been clearly demonstrated (Ferrarretto et al., 2003). Removal of phosphorus residues or removal of the N terminal region yielding β-casein f(17-25)3P both resulted in a dramatic decrease in Ca\(^{2+}\) uptake by HT29 cells. This result indicates that further degradation of the CPPs once released *in vivo* following gastrointestinal digestion may significantly affect Ca\(^{2+}\) uptake by intestinal cells and may even result in a complete loss of Ca\(^{2+}\) uptake. Similarly, these results indicate during the generation of CPPs *in vitro*, that optimum conditions (hydrolysis or fermentation) must be employed in order to release peptide fragments which retain biofunctionality.

### 3.3 Influence of CPP dose and CPP:mineral ratio

CPP dose and CPP:mineral ratio have also been demonstrated to affect Ca\(^{2+}\) uptake by intestinal cells. At constant extracellular Ca\(^{2+}\), a dose-response relationship was reported between the concentration of β-casein f(1-25)4P (from 50 to 200 μmol L\(^{-1}\)) and Ca\(^{2+}\) uptake by HT29 cells.
However, no similar dose-response relationship was observed for αs1-casein f(59-79)5P (Gravaghi et al., 2007). Additional to CPP dose, the concentration of extracellular Ca\(^{2+}\) also affects Ca\(^{2+}\) uptake by HT29 cells, with maximal biological activity being observed at 4 mmol L\(^{-1}\) extracellular Ca\(^{2+}\). A further increase in extracellular Ca\(^{2+}\) concentration (6 mmol L\(^{-1}\)) resulted in a decrease in the Ca\(^{2+}\) uptake by the HT29 cells. In this specific example, optimum conditions for Ca\(^{2+}\) uptake by HT29 cells were 4 mmol L\(^{-1}\) extracellular Ca\(^{2+}\) and 200 μmol L\(^{-1}\) β-casein f(1-25)4P.

The extracellular Ca\(^{2+}\) concentration was also shown to affect the formation of CPP aggregates. Both β-casein f(1-25)4P and αs1-casein f(59-79)5P formed aggregates in the presence of Ca\(^{2+}\) having similar hydrodynamic radii (60 nm). However, the main difference originated from the relative concentration of β-casein f(1-25)4P aggregates (100%) as compared to that of αs1-casein f(59-79)5P (4.5%) in the presence of 4 mmol L\(^{-1}\) extracellular Ca\(^{2+}\). Ca\(^{2+}\) is required for the formation of these aggregates as its binding to CPPs results in lower electrostatic repulsion and/or higher intermolecular bonds enabling the formation of supramolecular structures. The lesser extent of aggregate formation with αs1-casein f(59-79)5P was postulated to be due to higher electrostatic repulsions as this fragment possesses more negatively charged phosphorylated groups compared to β-casein f(1-25)4P. The aggregation and Ca\(^{2+}\) uptake behaviour of CPPs were superimposable, suggesting that the bioactive form of β-casein f(1-25)4P may be aggregated (Gravaghi et al., 2007).

The above represent just some of the parameters which contribute to the efficacy of CPPs. It is worth noticing that other factors exist and that the ultimate physiological effect observed may be a cumulative response to multiple, even opposing, effects.

4 Some conclusions relative to peptide biofunctionality
The different aspects addressed herein relative to the biofunctionality of CPPs may be extended to other bioactive peptides originating from milk and alternative biological materials. Some of the main lessons learnt to date during the study of bioactive peptides may be summarised as follows:

- numerous factors affect the potential of peptides to act as biofunctional agents as illustrated with the specific example of CPPs. The conflicting reports relative to the ability of CPPs to beneficially modulate mineral bioavailability may be related to the methodology employed, food matrix, peptide structure, dose utilised and potency differences between preparations, etc.

- while bioactivity may be demonstrated in vitro it is important to appreciate that peptides are sensitive biological materials which can be degraded or altered during different steps in food processing and then later on during food digestion and/or during transport in the blood stream. These different steps must be accounted for when addressing the bioactivity of milk peptides as minor changes in peptide sequence may result in a complete loss in their bioactivity.

- the mechanisms of action governing many bioactivities are still poorly understood and a greater understanding of same may lead to a more targeted utilisation of bioactive peptides within functional/formulated food systems. In addition, knowledge of the mechanism(s) of action is a key element in discovery of novel bioactive peptide sequences.

- the fact that peptides may display multifunctional bioactivities needs to be considered as some peptides may have several actions within the body. This multifunctionality may lead to synergetic effects.

- finally, regulatory requirements are important as these will ultimately dictate success in the development of new bioactive peptides or new applications of these ingredients for the
marketplace. Not alone must bioactive peptides be effective, they must be safe for consumption and abide by the regulations in place for the specific application (food matrix) in the target market associated with a given geographical location. These regulatory aspects are addressed in the regulation on nutrition and health claims adopted in December 2006 by the EU to ensure that health claims on foods were supported by scientific evidence. The regulation verified by EFSA includes different aspects such as “general function” in article 13.1, “new function” health claims in article 13.5 and “claims regarding disease risk reduction and child development or health” in article 14.

In summary, bioactive peptides represent complex additives/ingredients that must be studied in the context of their final utilisation in order to confirm their potential to modulate specific functions within the human body.

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