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# Glycomacropeptide: Biological Activities and Uses

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## Abstract

Glycomacropeptide (GMP) is a milk-derived bioactive peptide that comprises 15–20% of proteins present in whey, being the third most abundant. It is released from  $\kappa$ -casein by enzymatic digestion, either physiologically or in industry during cheese making process. GMP has many biological activities that are of particular interest for the manufacture of novel functional foods. Specifically, health promoting activities of this whey peptide are related to: antimicrobial, anticariogenic, gastric acid inhibitory, cholecystokinin releasing, prebiotic, and immune modulatory. GMP is also a peptide with promising use in food industry, due to its nutritional value and its emulsifying, foaming, and gelling properties. Besides, GMP has received much attention due to its use as an indicator of milk adulteration with cheese whey. This chapter summarizes the current knowledge about biological activities of GMP, going in-depth in immune regulatory properties, exposes the potential uses of GMP in industry, and finally reviews different methods used to detect GMP as adulteration index with cheese whey.

**Keywords:** glycomacropeptide, bioactive peptide, health promoting activities, whey component, milk adulteration

## 1. Introduction

As already mentioned in other chapters, milk whey is a liquid by-product generated after obtaining cottage cheese or curd (proteins coagulated by acid and heat), also known as cheese whey, that for many years has been considered a waste product, and sent to bodies of water, soil, and sewage systems. However, currently it is used due to its multiple nutritional and functional properties [1].

In Mexico, the production of whey in 2016 was estimated at 1,010,000 tons, 47% of which was discharged to soil, drains, and bodies of water. Despite the fact that multiple uses have been found to cheese whey, this has become a serious environmental problem [2]. This by-product is composed of water, lactose, proteins, peptides, fat, and mineral salts [3]. One of the peptides of interest is glycomacropeptide (GMP), which is obtained after the coagulation of milk  $\kappa$ -casein during cheese production and represents 15–20% (w/w) of the total proteins contained in milk whey [4].

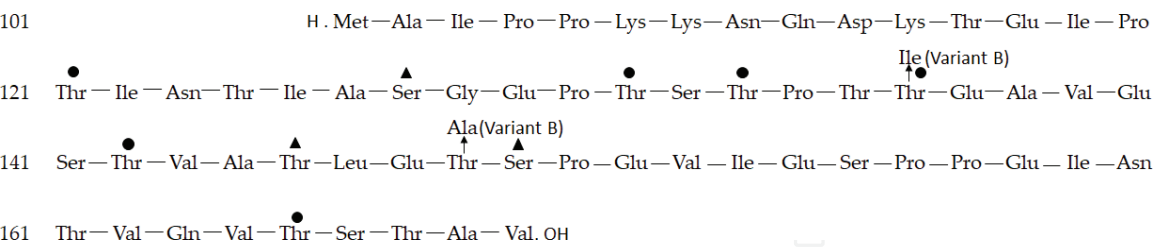
GMP is the C-terminal fragment released by the proteolytic action of the endopeptidase chymosin (renin) on  $\kappa$ -casein during the initial stages of cheese making, or by the action of pepsin during the gastric digestion.  $\kappa$ -casein is hydrolyzed at phenylalanine<sup>105</sup>-methionine<sup>106</sup> bond, forming two very different

polypeptides. One is called para-κ-casein (residues 1–105), and it is slightly cationic at pH 6.6, hydrophobic and poorly soluble, which remains in cheese curd; and the other is GMP (residues 106–169), that is strongly polar so diffuses into the aqueous phase, being eliminated during the draining with the cheese whey (as reviewed in [5]).

## 2. Chemical properties and molecular structure of GMP

GMP has 64 amino acid residues, with an isoelectric point (pI) between 4 and 5. Fifty percent of GMP is deglycosylated and is known as caseinomacropепptide (CMP) [5]. However, milk GMP can present different types of carbohydrates, such as: sialic acid, galactosyl, and N-acetylgalactosamine, which generate different glycosylated forms of the molecule. GMP is rich in amino acids such as proline, glutamine, serine, and threonine, but deficient in tryptophan, tyrosine, phenylalanine, and cysteine. The absence of aromatic amino acids in its primary structure causes that GMP does not present absorption at the wavelength of 280 nm. However, GMP can be detected at wavelengths between 205 and 226 nm and absorption differences between 210 and 280 nm are used for the characterization of GMP (as reviewed in [5]). The composition of GMP can be variable and depends on the source of serum and the fractionation technology used in its isolation [3] (**Figure 1**).

As reviewed by Neelima [7], the three-dimensional structure of GMP cannot be evaluated due to its crystallization which is not possible, so it can only be seen from a purely theoretical approach. GMP is a peptide that does not possess defined secondary and tertiary structure. However, three-dimensional structure of GMP has been predicted by means of protein modeling and shows that a large part of the peptide has a strong negative charge, whereas there are three small domains with a positive charge at the N-terminal end. At pH 7.0, its mean value of the hydropathy is  $-0.322$ , and GMP is more hydrophilic than hydrophobic. The hydropathy value decreases when glycosylation of GMP increases, due to the greater amount of sialic acid residues.



**Figure 1.** Primary structure of bovine GMP variant A and B, where • indicates its three phosphorylation sites and ▲ the most important glycosylation sites. Modified from Thomä-Worringer et al. [6].

## 3. Biological activities of GMP

The use of GMP is growing, since it is a bioactive peptide with unique nutritional and nutraceutical properties. Many biological activities of GMP have been reported, highlighting antimicrobial, anticariogenic, gastric acid inhibitory, cholecystokinin (CCK) releasing, prebiotic, and immune modulatory. Of particular interest is GMP's capacity to modulate the immune response, due to its potential use in treatment or prevention of different immunopathologies.

One of the first antimicrobial effects observed in GMP was due to its ability to bind cholera toxin and *Escherichia coli* enterotoxins. Chinese hamster ovary (CHO)-K1 cells undergo morphological changes in presence of cholera toxin and GMP at 20, 100, and 1000 µg/mL was able to suppress this morphological change by more than 70%. Treatment of GMP with proteinases lowered this activity, but removal of sialic acid abolished it [8]. Curiously, as GMP doses increased the inhibitory effect decreased. Authors demonstrated that sialic acid is mediating this inhibitory activity, so it could be inferred that GMP at high doses has less sialic acid available probably as consequence of its aggregation into polymers. Likewise, GMP has showed inhibitory activity on CHO-K1 cells morphological change induced by *E. coli* thermolabile enterotoxins [9]. Later, the binding ability of GMP to intestinal pathogenic bacteria was evidenced, mainly to enterohemorrhagic *E. coli* (EHEC O157). This activity decreased when the peptide was desialylated and peroxidated, showing that sialic acid is essential to GMP attachment. Besides, GMP prevented in a dose-dependent manner the adhesion of EHEC O157 to Caco-2 cells and when it was conjugated with xylooligosaccharide or carboxymethyl dextran, the release of IL-8 by Caco-2 was also suppressed [10]. The same inhibitory effect on pathogenic bacteria adhesion to Caco-2 cells was corroborated with 3[H] thymidine-labeled enteropathogenic *E. coli* (EPEC) *Salmonella typhimurium* or *Shigella flexneri* [11]. Besides, it has been reported that GMP generates dose-dependent inhibition of enterotoxigenic *E. coli* (ETEC) K88 adhesion to ileal mucosa *in vitro* [12]. Finally, a recent report showed that GMP also prevents the attachment of several strains of EHEC and EPEC to Caco-2 and HT-29 mammalian cells [13]. In this study, it was demonstrated that GMP, beyond inhibiting bacterial adhesion, is able to maintain the structural integrity of tight junctions on Caco-2 monolayers, thus delaying the paracellular translocations of EPEC. However, it is important to clarify that authors did not measure the expression level of proteins associated with these junctions.

There are several *in vivo* assays that show a probable protective effect of GMP against pathogenic bacteria. GMP has been reported to neutralize in 80% the rate of incidence of diarrhea induced by cholera enterotoxins LT-1 in mice, and abolished that induced by LT-2 [9]. On the other hand, the addition of GMP into diets of piglets diminished the bacterial count in mucosal scraping and abated the attachment of ETEC K88 to the intestine, mainly on the ileal mucosa where the receptor for *E. coli* is located [12]. Moreover, a diet supplemented with 1% GMP protected weaning piglets from damage caused by *E. coli* infection, and prevented the reduction of growth, morphological damage, and the increase in intestinal permeability associated to infection [14].

In association with this antimicrobial effect, an anticariogenic activity to GMP has been demonstrated. Firstly, *in vitro* assays using different products that contain GMP as an active principle showed that it could inhibit the adhesion of bacteria inducing dental plaque and carious in oral cavity to surface plastic, such as *Streptococcus mutans*, *S. sanguis*, and *Actinomyces viscosus* [15]. Later assays corroborated these results, as the incorporation of GMP with or without caseinophosphopeptide to salivary films modified the adhesion capacity of *S. sobrinus* and *S. mutans* to bovine enamel discs [16]. This antimicrobial effect was linked to a remineralization activity of GMP, as demonstrated later through an experimental protocol in human that showed that GMP alone or combined with xylitol promotes more remineralization than commercial fluoride toothpaste [17]. The anticariogenic and remineralization activity of GMP have been claimed in several patents [15, 17].

Several studies have related GMP with the inhibition of gastric secretion. First ones were mostly developed using dogs by a group of Russian researchers. The first evidence that GMP inhibits gastric secretion was showed by Shlygin and co-workers [18] using gastrin to evoke it. Subsequent works demonstrated similar effect using



different gastric secretion stimulants [19]. Some years later, it was proposed that this inhibitory effect was caused by a GMP fragment rather than the whole molecule [20, 21]. Later, injecting dogs with a protein fraction obtained from the gastric content of unweaned rats, it was observed an inhibition in dog gastric secretion to a food stimulus [22]. This inhibitory action was similar to that induced by GMP in dogs. GMP was also demonstrated to inhibit gastric motility after its intravenous injection in dogs [23]. All these experiments point out that at physiological conditions GMP may be playing a crucial role in the preservation of active milk proteins in newborn animal during natural breast feeding. In addition to dogs, other experimental models such as rats, pigs, and calves and also isolated organs were used to demonstrate that GMP induces gastric secretion inhibition in association with a decrease in blood of some regulatory digestive hormones, as gastrin and CCK (as reviewed in [24]). However, variations in used gastric stimuli, GMP dose, and origin, via of administration and experimental approach may be the cause of the differences in the reported intensity to this GMP activity.

Related with the effect of this bioactive peptide on digestive hormones, GMP has also been associated with appetite control. Several *in situ* studies with Wistar rats have suggested that glycosylated forms of GMP A variant could regulate food intake through CCK secretion, a hormone involved in satiety, as GMP stimulate its release [25, 26]. Nevertheless, in studies in human, GMP had no effect in the regulation of food intake over a short-term period [27], neither in the loss of body weight after 12 months of sustained consumption [28]. Likewise, GMP with different degree of glycosylation does not modify the concentration of CCK in human plasma [29]. As other authors have pointed out [27], these inconsistencies related to GMP's effect on CCK release are probably due to dose changes in both animal models and human trials.

For many years, the prebiotic properties of GMP have been discussed. The first evidence that GMP might possess prebiotic activity arose with the bifidobacterial growth promoting effect of human's colostrums and milk by *in vitro* assays [30]. In an attempt to discern the component of the milk responsible for this activity, an important role of N-acetyl-glucosamine (GlcNAc) and oligosaccharides with GlcNAc [31] was pointed out. Subsequent investigations were quite contradictory, until the prebiotic activity of GMP was demonstrated for the first time in 1984 [32]. This work showed that human GMP is a promoter of bifidobacterial growth, although the effect was lost when it was hydrolyzed, as well as when bovine GMP was used, showing the importance of the GMP peptide chain to function as a prebiotic. Eight years later, it was reasserted that peptide fraction was decisive for the prebiotic effects on bifidus [33]; while other researchers leaned by sialic acid as the inductor of the effect [34]. Following this line, the supplementation of milk with 2% GMP increased the *in vitro* growth of *Bifidobacterium lactis* [35]. Besides, the addition of 2 mg/mL of cow GMP to the growth medium, promoted the growth of *Lactobacillus rhamnosus* and *Bifidobacterium thermophilum*, and apparently glycosylation was not an essential factor to carry out this function [36]. On the other hand, using an artificial colon model to simulate colonic fermentation, GMP was shown to modulate the gut microbiota of elderly subjects by promoting the growth of several health-relevant taxa, like *Coprococcus* and *Dorea*, both related to resistance to pathobiont colonization [37]. Finally, a recent *in vitro* research has shown that GMP promotes the growth of *Bifidobacterium longum* ssp. *infantis* in a dose-dependent manner and modulates its genes expression. Again, they found that the effect was lost with periodate GMP, suggesting that its activity is due to the oligosaccharides present in the molecule [38].

In the last years, several research groups have demonstrated that oral treatment with GMP modifies *in vivo* the microbiota in gut using different experimental approaches. First works were developed in mice, and showed that after 15 days of

GMP treatment, there was a significant increase in *Lactobacillus* and *Bifidobacteria* in fecal samples, at the same time the number of *Enterobacteriaceae* and coliforms decreased [39]. Feeding mice for 8 weeks with a GMP-enriched diet reduced *Desulfovibrio* bacteria in normal and phenylketonuric mice; a bacteria that is associated with the pathogenesis of inflammatory bowel disease (IBD). Likewise, normal mice increased Firmicutes and phenylketonuric ones Bacteroidetes; specifically, the genus *Allobaculum* (associated with body weight loss [40]) and *Bacteroidales*, respectively [41]. In this case, the prebiotic property of GMP was associated with an increase in short chain fatty acid production, mainly acetate and propionate, that may have an important role in the regulation of immune response. So, although the prebiotic activity of GMP has been demonstrated, more research is needed to clarify whether the peptidic or carbohydrate fraction or both are involved in this bioactivity.

### 3.1 The immunomodulatory properties of GMP

GMP has been shown to modulate the immune response in a number of different ways. First, we summarize literature reports about regulatory activity of GMP on immune cells demonstrated by *in vitro* assays, and later, we will focus on those studies in which the regulatory effect of GMP on immune response was analyzed in animal models.

In relation to the immunomodulatory effects of GMP on immune cells, different *in vitro* approaches have corroborated the inhibitory action of GMP on splenocyte proliferative response to mitogens, and have shown that both sialic acid residues and polypeptide portions of GMP are essential in this inhibitory effect. In 1992, it was shown that the GMP fraction obtained from  $\kappa$ -casein inhibited proliferation of mouse splenocytes induced by *Salmonella typhimurium* lipopolysaccharide (LPS) [42]. Furthermore, GMP displayed an inhibitory activity on the proliferative response induced by concanavalin A (Con A) and phytohemagglutinin (PHA) [42, 43]. Initially, it was found that sialic acid was the key in this inhibitory activity, as it was lost after digestion with neuraminidase [44]. However, the inhibitory effect of GMP was increased after digestion with trypsin and pronase, which suggests that the peptidic chain is also involved in this immunomodulatory activity. In this regard, the same working group showed that the inhibitory effect on PHA-induced proliferative response is higher when the number of sialic acid residues is increased and that on LPS-induced proliferation is highest with a GMP fraction containing two sialic acid residues [45]. Both inhibitory effects decreased significantly after neuraminidase digestion. They also suggested that phosphate group at serine-149 plays a role in GMP binding to the mitogen receptor, as they observed a reduced inhibitory activity after GMP chymotrypsin digestion. Regarding to the associated mechanism to GMP inhibitory effect on splenocyte proliferation, it was shown that GMP stimulates the synthesis of a soluble inhibitory component, an interleukin (IL-1) receptor antagonist or IL-1ra [46, 47]. Moreover, GMP was able to bind to mouse CD4<sup>+</sup> helper T cells and to suppress the expression of the IL-2 receptor on the cell membrane, inhibiting the PHA-induced proliferation of mouse splenocytes [48]. Subsequently, the inhibitory action of GMP on LPS-induced cellular proliferation was confirmed in mouse splenocytes, although they did not report any effect on PHA- or Con A-stimulated cells [49]. But later, controversy about the effect of GMP on the *in vitro* proliferation of spleen cells was generated, as it was reported that GMP increases the proliferation response of lymphocytes stimulated by Con A [50]. In this study, an increase in Foxp-3 and IL-10 expression was also demonstrated. Besides, authors showed an inhibition on secretion of IFN- $\gamma$  and TNF- $\alpha$  and on STAT4 activation when cells were stimulated by Con A in presence of GMP. The same team studied the action of GMP on monocyte cell line THP-1 and they found that GMP increases the secretion

of TNF, IL-1 $\beta$ , and IL-8 by THP-1 cells, and this effect is mediated via MAP kinase and NF- $\kappa$ B pathways [51].

On the other hand, GMP is also able to downregulate dendritic cell response to LPS by inducing a slight but significant decrease in the production of IL-6, IL-1 $\beta$ , and TNF- $\alpha$ , but without changing the production of IL-12 and IL-10 [49]. Strikingly, the regulatory effect of GMP on neutrophils is the opposite, as it improves proliferation and phagocytic activity of the human macrophage like cells U937 [52]. However, the observation that both polypeptide and carbohydrate portions are essential for GMP biological effects is reinforced in this study, as peptides of pepsin-digested GMP and sialic acid-rich GMP fractions significantly enhanced cell proliferation and phagocytic activities stimulated by non-digested or asialo-GMP on U937 cell. Also, an upregulatory effect of GMP on production of IgA by LPS-stimulated splenocytes has been reported, being correlated with an increase in the population of IgA positive cells [53].

There are several studies that analyze the immunomodulatory activity of GMP on immune response when it is orally administered to experimental animals. In the context of splenocytes response to mitogens, two *in vivo* studies were carried out to analyze the possible immunomodulatory activity of GMP. First one was developed in 1998 and demonstrated that mice fed with a GMP-supplemented diet show an enhanced proliferative response of spleen cells to Con A, without generating significant changes in the response to LPS or PHA [54]. Later in 2012, it was showed that oral intake of GMP by rats reduces the proliferative response of splenocytes induced by Con A [55]. In both *in vivo* studies, animals were antigen-immunized because antibody response was also measured. All together *in vitro* plus *in vivo* studies, point out the inhibitory effect of GMP on splenocyte proliferation to mitogens. The opposite response reported by one *in vitro* [50] and *in vivo* [54] assay was quite possibly due to concentration-dependent effects or assay-used conditions.

The effect of orally administered GMP on humoral immunity has also been studied. Mice fed with GMP have shown suppressed levels of specific IgG to dietary and injected antigens, with no change in IgM, IgA, and IgE antibody response [54]. In this regard, a recent study showed that oral administration of GMP to mice resulted in a greater number of IgA positive plasma cells in the intestinal lamina propria [56]. All these results [54, 56] plus *in vitro* ones [53] about Igs production fit together, suggesting an immuno-suppressing activity of GMP on systemic humoral response, but an immuno-stimulating activity on humoral mucosal immunity.

Martínez-Augustin and co-workers [57, 58] have studied the immunomodulatory action of GMP in experimental models of intestinal inflammation. They have demonstrated that GMP administered orally to rats exerts an anti-inflammatory effect in ileitis and colitis induced with trinitrobenzenesulfonic acid (TNBS); said anti-inflammatory effect shows a degree of efficacy similar to that of sulfasalazine, a drug widely used in the treatment of inflammatory bowel disease. GMP was shown to protect rats from TNBS-induced colonic and ileal inflammatory damage, by reducing the damage score and the extent of necrosis, and also by diminishing the increased alkaline phosphatase colonic activity and inducible oxide nitric synthase expression. IL-1 $\beta$  and IL-1ra messenger RNA levels were significantly decreased in colon as a consequence of GMP administration; and myeloperoxidase activity and levels of IL-1 $\beta$  and IL-17 were decreased in ileum. Initially, the authors assumed that the action mechanism of GMP was not related to anti-oxidative activity or to regulatory cell induction, as glutathione or TGF- $\beta$  levels in colon and Foxp-3 in ileum were not affected [57, 58]. However, when GMP was orally administered to rats, an increase on Foxp3 expression in spleen cells was observed, although secretion of cytokines by *ex vivo* Con A-stimulated splenocytes did not change [50]. Putting together these results with the regulatory activity of



GMP on monocytes (THP-1) and splenocytes cytokine response obtained by the same working group and previously mentioned in this review [50, 51], authors concluded that the intestinal anti-inflammatory action of GMP is likely to be mediated by the direct modulation of monocyte or splenocyte activity, especially by hampering the activation of Th1 cells while favoring the differentiation of Treg cells [50].

In recent years, a Mexican laboratory led by Salinas [55, 59–61] has focused on the study of the immunomodulatory activity of GMP in experimental allergy models. They found that oral administration of GMP to rats before and during sensitization with allergen significantly reduces the level of allergen-specific IgE in serum, and also decreases the proliferative response and the production of IL-13 by splenocytes stimulated by the allergen [55]. Treatment of animals with GMP also protected them from systemic anaphylaxis as GMP administration increased survival rates and lessened signs of severity of anaphylactic shock. Moreover, GMP reduced the intensity of urticarial inflammatory reaction when sensitized animals were intradermally challenged with the allergen [55]. With these results, it was demonstrated the immunomodulatory properties of GMP on allergic sensitization and its beneficial effect on clinical signs associated to early-phase allergic reaction. Then, they investigated whether GMP may impact on late-phase and chronic inflammatory allergic reactions, using two experimental models that after repetitive exposure to allergens displayed local recruitment and activation of immune cells with persistent production of inflammatory mediators in affected tissues, together with substantial changes in the extracellular matrix and alterations in structural cells [62]. Specifically, they used experimental models of asthma and atopic dermatitis prophylactically administered with GMP, that is to say, prior to and during pathology establishment. As expected, GMP intake resulted in reduction of IgE titers in serum. In addition to this, in asthma model, GMP substantially decreased blood eosinophilia and suppressed the recruitment of inflammatory cells to the bronchoalveolar compartment. GMP also inhibited eosinophils infiltration, goblet cells hyperplasia, and collagen deposit in lung tissue [59]. Equivalent results were obtained in allergen-induced atopic dermatitis model, where GMP reduced the intensity of cutaneous inflammatory process and edema, abolished pruritus, and reduced eosinophils recruitment and mast cells hyperplasia in dermis [60]. In both models, expression of IL-5 and IL-13 was markedly inhibited in lung and skin, while expression of IL-10 was increased. Their research then turned to the mechanism by which GMP modulates the allergic response. They demonstrated that GMP administration increases the amount of *Lactobacillus* and *Bifidobacterium* present in gut of allergen-sensitized animals after 3 days of oral treatment, and that of *Bacteroides* after 17 days. Interestingly, this intestinal microbiota is associated with protection in allergy. GMP intake also increased the production of TGF- $\beta$  by splenocytes of sensitized animals in response to allergen and impacted mast cell function, inhibiting their activation and also the release of histamine in response to allergens. No change in tissue mast cell number was found [61]. These results obtained in experimental allergy models again show a double way by means GMP exerts its control on inflammation; on one hand, through a direct modulation of immune cells activity involved in the process, and on other side by potentiating a regulatory microenvironment against the Th2-inflammatory one. More studies are needed to understand which immune cell receptors recognize GMP and which intracellular signals activate or inhibit.

Finally, there are few studies that analyze the role of GMP on cancer. In a rat model of pharmacological-induced colorectal cancer, oral administration of 100 mg/kg of GMP decreased the number of aberrant crypt foci although no effect was observed at doses of 10 and 50 mg/kg. On the other hand, there was no change in methylation and expression level of p16 and MUC2, two tumor suppressor genes [63]. Additionally,



through an *in vitro* assay GMP was showed to inhibit the expression of p65 NF- $\kappa$ B in human colorectal tumor HT-29 cells activated with LPS, key element in colorectal cancer induced by inflammatory bowel diseases [64].

Although more studies are needed in relation to some biological activities, current results propose GMP as a good candidate to be used as a functional ingredient in food industry.

## 4. Potential uses of GMP

Today, one of the objectives of the food industry is the development of novel food products with beneficial properties for health. For its different health benefits, GMP can be used in therapeutic and dietary foods, or as a functional ingredient in various special products, like oral care products.

### 4.1 Therapeutic and nutritional applications

It is crucial to demonstrate that GMP is hypoallergenic to be used in food compositions. In this regard, Takahashi and collaborators patented a food composition that contained GMP and a mixture of free amino acids (leucine, lysine, methionine, cysteine, phenylalanine, tyrosine, tryptophan, arginine, histidine, and glycine) [65]. The composition presented good taste, good absorption and digestion properties, and a high nutritional value. They demonstrated that this composition was hypoallergenic, as after repeated injections of the GMP composition together with an adjuvant used to induce experimental allergy in mice, no antibody against GMP was detected in serum by Ouchterlony. Although this method is not very accurate, GMP hypoallergenicity was later corroborated by Milkelsen and collaborators using ELISA test to show absence of specific antibodies in mice after being sensitized both systemically or orally with GMP [66].

Due to the particular amino acid composition of GMP, devoid of aromatic amino acids (phenylalanine, tryptophan, and tyrosine), it can be used for special diets of people suffering from phenylketonuria (PKU), being an adequate choice as a source of proteins [67]. On the other hand, GMP has low amount of methionine but high amount of branched chain amino acids (valine and isoleucine), which makes this peptide an excellent candidate to be used for the control of liver diseases, as this type of amino acids are good as caloric sources [68]. There is a patent to use of GMP to improve female's health [69]. The inventors claim that administration of a composition comprising GMP can improve the health of the females. They used murine models fed with GMP composition and showed that females decreased final fat mass and percent body fat, when comparing with females that received a diet based on caseins or free amino acids as source of proteins. In relation to bone characteristics, femur length was larger in GMP administered mice, although only females showed less femoral weakness and greater bone mineral content and density as compared to those fed with amino acids or casein diets, respectively.

### 4.2 Dietary supplementation

As previously mentioned, research results suggest that GMP has an effect on the feeling of fullness but this does not translate into a lower food intake [27, 28]. For an application in food intake regulation and in potentially body weight management, more work is required. Understanding dose, timing, and delivery mode, including food form and composition, in relation to the pattern of release of CCK, is needed for the use of GMP as appetite suppressant [70].

### 4.3 Food additive

GMP has physicochemical properties that make it attractive for use as an additive in food products. According to studies on the functional properties of GMP, it can act as an emulsifier, foaming, and gelling agent.

GMP as an emulsifier presents stability to pH variations, which is attractive for foods that undergo pH changes during their process, such as the case of fermented milk products [71]. The best emulsifying capacity was obtained at alkaline pH. However, it has been observed that emulsions with GMP as emulsifying agent are not stable during storage when they have received thermal treatment [72]. Besides, GMP modified covalently with disaccharides or fatty acids can present an improved function and even increase its biological activity [73, 74]. Therefore, in order to modify the emulsification activity of GMP, this peptide has been conjugated with other molecules such as lactose [73] and fatty acids [74]. The conjugation of GMP with lactose was carried out through the reaction of Maillard, and this conjugate showed a better emulsifying capacity without significantly reducing the solubility of GMP [73].

Currently, foams have many industrial uses of great importance in the production of beer, soaps, whipped cream, shaving cream, aerosols, etc. The formation of a foam requires the participation of a surfactant capable of diffusing to the air/water interface to lower the surface tension. GMP complies with this property, although the foams formed with GMP are stronger or more stable when combined with other foaming proteins [75, 76]. In order to improve the foam properties of the proteins, synergistic mixtures of biopolymers and pH variations have been made that can modify their charge and, consequently, their foam ability. In relation to this, by combining sodium caseinate with GMP, synergistic interactions take place between these molecules on foaming and on stability at pH 5.5 [77]. Non-glycosylated GMP has better foaming properties than glycosylated GMP [78]. This is due to the glycosidic structures favor a combination of hydrophilic and electrostatic effects, which prevents an orderly adsorption of the glycosylated GMP molecules at the air/water interface; whereas, non-glycosylated GMP forms a very stable network at the interface.

On the other hand, gels are semi-solid systems that consist of a network of solids (three-dimensional network of polymers) with an inside trapped-liquid. They are of great importance in food and pharmaceutical industry as many gelled products are manufactured throughout the world (gummies, gelatins, jelly jams, bakery fillings, and therapeutic or cleaning agents). Generally, gelling agents are proteins and polysaccharides. Gelling properties of GMP has been studied and it is known that its gelation depends on pH and temperature, reporting that even aqueous solutions with low GMP amounts can be gelled at pH below 4 [79]. Besides, GMP can potentiate gelling capacity of other substances. Thus, by fermenting goat milk to which GMP was added, a more ordered and structured gel was obtained, in addition to obtaining a better elasticity in it, as compared to that obtained when whey protein concentrate was added [80]. The influence of GMP on the gelation made by gelatin has also been studied and when these two compounds are mixed, lower concentration of both substances are needed to get a gel as compared with the ones needed when they are used separately [81]. This synergistic effect in gelation is very important in the food industry for the preparation of desserts and foods based on gels.

### 4.4 Oral care products

Dental caries is one of the chronic diseases that most often affect humans. Due to the anticariogenic and remineralization properties demonstrated to GMP and

previously reviewed in biological activities section, nowadays GMP is being incorporated to some oral care products [15–17].

## 5. GMP as an indicator of milk adulteration with cheese whey

One of the problems presented by the dairy industry is the adulteration of milk with whey cheese, which is very cheap and not detected by sensorial tests. Cheese whey does not cause harm to health, however, it affects milk-derived products manufacturers financially and can affect the consumers nutritionally, so the addition of cheese whey is considered a fraud. Due to GMP present in cheese whey, the detection of this peptide may indicate the addition of cheese whey to milk. Some of the methods that detect GMP as an indicator of the presence of cheese whey are described below.

### 5.1 Electrophoretic methods

*Sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE)*: This method was standardized to analyze pasteurized milk and milk powder. Samples has to be previously treated with 24% trichloroacetic acid (TCA) to eliminate interfering k-casein and later with 50% TCA to precipitate GMP, which is resuspended in Tris-buffer 0.05 M HCl, 1 mM EDTA, pH 7.2 [82]. Analyzing under these conditions cheese whey and milk added with cheese whey, a protein fraction of 20.8 kDa is identified corresponding to GMP that allowed to detect adulteration with whey up to 1%. This protein fraction is not detected in samples of acid whey or in raw milk. This technique had been previously used to analyze milk drinks that were distributed in schools as part of a program of the Brazilian government [83]. However, the method showed sensitivity to detect 5% of added whey, probably because they did not treat the sample with TCA. In later works, it was able to detect 40 and 20 µg of GMP in samples analyzed by electrophoresis in SDS-PAGE and in cellulose acetate strips, respectively, due to the use of thiobarbituric acid and malachite green dye reactions instead of Coomassie blue as developing agents [84].

*Capillary electrophoresis (CE)*: A variant of electrophoresis is CE, a technique that has the advantage of allowing a rapid detection of GMP. This method has been used to identify GMP as an indicator of the presence of cheese whey in buttermilk powder and skim milk. An advantage of this method is that it is usually reproducible, repeatable and sensitive; however, the interpretation of the results is difficult [85].

### 5.2 Chromatographic method

High performance liquid chromatography (HPLC) has been widely used to identify GMP as indicative of milk adulteration with cheese whey. In order to carry out the analysis, it is necessary to pre-treat the samples with TCA to precipitate proteins that can interfere (k-casein) and to concentrate GMP [86]. Similarly, a rapid and sensitive HPLC method on a gel permeation column was developed to detect GMP to follow the hydrolysis of k-casein by chymosin in milk [87]. The only pretreatment given to samples was addition of TCA (final concentration 8%) to precipitate the interfering caseins and whey proteins. This method was widely used by several researchers to analyze different samples, such as skimmed milk powder [88]. Cation-exchange chromatography has also been used to detect GMP, previously removing caseins from whey samples by precipitation with HCl at pH 4.6, neutralizing with TCA at 2–8% and analyzing supernatants [89]. On the other hand, a Reversed-Phase HPLC (RP-HPLC) method was developed and validated to separate and quantify GMP and was demonstrated to be precise, sensitive, and reliable [90].



The determinations were performed in the linear range of 15–200 µg/mL and the detection limit was 2 µg/mL. The method was applied to the analysis of rennet and acid whey, whey protein concentrates produced by the dairy industry, and also for the detection of rennet whey in powdered milks.

The European Commission uses two methods to detect the presence of cheese whey in milk: a gel permeation chromatography and subsequently a RP-HPLC as a confirmatory test [91]. However, it has been shown that the sensitivity of this method is affected by the presence of acidified rennet whey, which makes it difficult to detect the addition of whey [92]. Besides, the HPLC methodology used to analyze compounds like GMP in dairy products usually includes extractions with solvents, sample's preparation require a lot of time and reactivities, the equipment is very sophisticated and demands trained personal.

### 5.3 Spectroscopy methods

Spectroscopy has also been used to detect GMP. The medium infrared spectroscopy (MIR) was used to analyze milk powder in order to detect GMP as adulteration parameter. Although this method is fast, it is not widely used because derived spectra are not very easy to interpret, in addition to its high cost [93]. On the other hand, by liquid chromatography/electrospray coupled to mass spectrometry, milk products were analyzed and it was able to quantify GMP from concentration of 10 pmol, although the method was not used to detect milk adulteration [94].

### 5.4 Immunochemical methods

Immunoassays are analytical methods of great application in the food area, and have the advantages that they are quick, sensitive, and that the sample to be analyzed requires little or no treatment. Several immunochemical methods have been developed in order to identify and quantify GMP in milk. Firstly, it is necessary to produce antibodies against GMP and later, these antibodies can be used for the development of the different immunochemical methods that detect it. Some of these assays are described below:

*Enzyme-linked immunosorbent assay (ELISA):* It is an immunoassay widely used to analyze foods. It has the advantage that is simple, sensitive, and fast, in addition to being inexpensive. Two main ELISA assays has been developed to detect GMP in milk samples. An inhibition ELISA method was performed to detect bovine rennet whey solids in skim milk powders that presented a detection limit of 0.1% (w/w) and used enzyme-labeled monoclonal antibodies against bovine k-casein [95]. On the other hand, Chávez and co-workers [96] developed a sandwich ELISA using polyclonal antibodies against GMP, that showed a limit of detection of 0.047% (w/w).

*Western blot assay:* As ELISA, this technique is an immunoassay designed to detect proteins in complex samples and also has great specificity. Using the same polyclonal antibodies against GMP previously mentioned, Chávez and co-workers developed a western blot system to detect GMP [97]. When analyzing cheese whey, this antibody recognized three protein fractions of 20.1, 14, and 45 kDa. The detection limit of the test was 0.5% (v/v) to liquid cheese whey and 0.001% (w/w) to whey powder.

*Immunochromatographic lateral-flow assay:* The development of immunochromatographic systems for quality control is a relatively new field of research and has been applied to milk. There are commercial immunochromatographic sticks which contain monoclonal antibodies specific to GMP labeled with colloidal particles that present a limit detection of 4% (v/v) of milk whey. Using these immunosticks, it has been possible to identify GMP in different samples of commercial milks [98].



Besides, it has been developed an immunochromatographic lateral-flow test that used two specific anti-bovine  $\kappa$ -casein monoclonal antibodies, with a detection limit of 15 ng/ml of GMP and 1% (v/v) of cheese whey [99].

In summary, different techniques and methods have been developed and used to detect GMP as an index of adulteration of milk with cheese whey. Some of them can also be used to quantify GMP in food products. The aim of this area of research is to achieve one that bring together being cheap, fast, easy to develop, and to interpret the results, with high sensitivity and a limited sample processing. These characteristics will allow people to use them at the time and place of milk reception.

## 6. Conclusions

GMP possesses several nutritional and health promoting properties. Among them, it exerts important modulatory effects on the immune system that are beneficial in a number of different inflammatory conditions. GMP immune response mechanism of action might be mediated by increasing healthy intestinal microbiota, by inhibiting splenocyte proliferation, by promoting both local and systemic regulatory environment, and also by directly modulating immune cell functions. More research is needed to support these findings, as we cannot exclude a possible effect of products derived from GMP digestion on *in vivo* immunomodulatory activity. Besides, GMP is a peptide of promising industrial potential. It has a unique heat stability and solubility under acidic conditions that may suggest several uses in food. Studies on the functional properties of GMP may indicate new possible uses as a food additive. On the other hand, several methods have been developed and applied to detect GMP as an indicator of milk adulteration with cheese whey; however, none of them has been established as an international official method. Rapid, reliable, and inexpensive tests to detect GMP should be worked out to readily detect those cases of adulteration.

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## Conflict of interest

Authors declare that there is no conflict of interest between the authors of the chapter entitled: "Glycomacropeptide: Biological activities and uses."

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