Potential prebiotic properties of whey protein and glycomacropeptide in gut microbiome

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Abstract

Proteins in whey have prebiotic and antimicrobial properties. Whey protein comprises numerous bioactive proteins and peptides, including glycomacropeptide (GMP), a hydrophilic casein peptide that separates with the whey fraction during cheese making. Glycomacropeptide has traditionally been used as a protein source for individuals with phenylketonuria and also has prebiotic (supporting the growth of *Bifidobacterium* and lactic acid bacteria) and antimicrobial activities. Glycomacropeptide supplementation may help positively modulate the gut microbiome, help treat dysbiosis-related gastrointestinal disorders and improve overall health in consumers.

Keywords: whey protein, glycomacropeptide (GMP), prebiotic effect, gut microbiome.

Introduction

Whey is a byproduct of the cheese manufacturing process. It is the liquid portion that is drained away after curd formation. Bovine milk is the most common source of whey due to its predominance in the dairy industry. The extracted product referred to as whey protein is a mixture of numerous proteins including β-lactoglobulin (~65%), α-lactalbumin (~25%), bovine serum albumin (~8%), lactoferrin (~1%), lactoperoxidase (0.25-0.5%) and immunoglobulins (<1%) (Boscaini et al., 2020).

Bovine whey protein is used in a large array of products, including supplements to promote muscle synthesis and infant formula. Whey protein helps increase muscle synthesis which can help with muscle and exercise recovery in athletes (Daniel et al., 2017; Moore, 2019), and decrease the risk of age-related sarcopenia (Liao et al., 2019; Yang et al., 2012) (Figure 1). Bovine whey protein is added to most infant formulas to mimic human milk. Beyond serving as a source of amino acids, the proteins in whey can exert additional functions, including prebiotic, antimicrobial (Kareb and Aïder, 2019), and enhancing gastrointestinal health (Li et al., 2018). For example, many whey proteins, including lactoferrin, lactoperoxidase and immunoglobulins have direct or indirect antimicrobial activity (Bielecka et al., 2022). Perhaps due to these bioactivities, whey protein supplementation can alter the gut microbiome (at least in animal models) (Boscaini et al., 2020; Nilaweera et al., 2017) and thereby impact metabolism (Boscaini et al., 2020) (Figure 1).

Glycomacropeptide (GMP) is a hydrophilic peptide cleaved from κ-casein during cheesemaking and makes up about 20% by mass of commercial whey protein (Neelima et al., 2013). About half of the GMP in whey is the unglycosylated form known as caseinomacropeptide, whereas the other 50% consists of 14 different glycovariants (Robitaille,

2013). Glycomacropeptide has several functions, including prebiotic (it promotes the growth of *Bifidobacterium* and lactic acid bacteria (Córdova-Dávalos et al., 2019) (Figure 1), antimicrobial and immunomodulatory (Laura et al., 2019).

Impact of whey on the gut microbiome

Whey protein supplementation can impact the gut microbiome. In an infant fecal culture system with 3 feeding conditions; 1) bovine milk, 2) formula with α-lactalbumin, and 3) formula with GMP, both formulas supplemented with α-lactalbumin and GMP both supported *Bifidobacterium* as the predominant organism and decreased *Bacteroides, Clostridium* and *Escherichia coli* (Brück et al., 2003). In the same study, all groups reduced enteropathogenic *E. coli* and *Salmonella* Typhimurium after initial inoculation of these pathogens into the fecal culture system (Brück et al., 2003).

Whey protein feeding has also been shown to alter the microbiome in animal models. Nilaweera et al. found that whey protein isolate (WPI) supplementation reduced the susceptibility of mice to sucrose-induced microbial changes to a larger degree than those supplemented with casein (Nilaweera et al., 2017). The study indicated that whey protein reduced the abundance of Firmicutes and Actinobacteria, which is associated with enhanced metabolic health and reduced inflammation, and increased Bacteroidetes, a phylum that can break down complex carbohydrates and produce beneficial short-chain fatty acids (SCFAs). In the same study, whey protein feeding increased the families Unclassified_*Sutterellaceae*, *Sutterellaceae, Anaeroplasmanaceae*, Unclassified_*Porphyromonadaceae* and *Porphyromonadaceae*, and decreased *Streptococcaceae* and *Enterobacteriaceae*, resulting in the increase of potential healthy gut microbiomes (Nilaweera et al., 2017). Similarly, mice with

autoimmune prostatitis fed glycated whey had decreased Firmicutes and an increased *Porphyromondaceae*, among other families and genera (Chen et al., 2020). In mice fed a high-fat diet (HFD), whey protein increased *Lactobacillaceae* and *Clostridiaceae* at the family level and *Desulfovibrio* and *Mucisprillum* at the genus level (McAllan et al., 2014). Boscaini et al. found that 5-week-old mice fed whey (compared with casein) on a HFD had increased *Streptococcaceae* and *Lactococcus* at the family and genus levels, increased *Lactococcus lactis* and *Bacteroides vulgatus* at the species level and increased abundance of *Lactobacillus murinis* (Boscaini et al., 2020). Sprong et al. found that casein supplemented with either whey protein or threonine and cysteine both increased *Lactobacillus* and *Bifidobacteria*, decreased markers of inflammation and increased mucin secretion in rats with dextran sulfate sodium-induced colitis (Sprong et al., 2010). Chen et al. found that non-obese diabetic mice fed whey protein-derived early glycation products for 6 months had increased *Allobaculum, Anaerostipes, Bacteroides, Parabacteroides* and *Prevotella* and decreased *Adlercreutzia* and *Roseburia* at the genus level (Chen et al., 2020). In this study, some of the changes in the microbiome correlated with immune markers measured, which suggested that the microbial changes may have contributed to the observed anti-inflammatory effects (Chen et al., 2020). Specifically, total splenocytes were negatively correlated with *Bacteroides* (*uniformis* and *acidifaciens*), *Parabacteroides*, *Prevotella*, and *Anaerostipes*; splenic M1 macrophages were negatively correlated with *Bacteroides* (*uniformis* and *acidifaciens*) and *Parabacteroides*; and splenic CD4+ T-cells were negatively correlated with *Bacteroides acidifaciens* (Chen et al., 2020).

Nielsen et al. found that piglets given WPC with high α-lactalbumin tended to have higher alpha-diversity and a higher abundance of *Clostridiaceae, Enterobacteriaceae, Streptococcus* and *Streptomyces* than those supplemented with WPC with low α-lactalbumin

(Nielsen et al., 2020). However, the microbial composition resulting from dietary interventions did not show evidence of correlation with physiological changes or functional, or performance aspects of preterm pigs (Nielsen et al., 2020).

Though dietary whey protein modulates the gut microbiome in fecal cultures, murine models and piglet models, the few human studies have not been able to replicate these results. This lack of findings in humans may be due to the large degree of variation in human gut microbiomes, behaviors, environmental factors and genetics. Two studies on primarily overweight or obese adult humans found that whey protein did not significantly alter the gut microbiome (Cronin et al., 2018; Reimer et al., 2017). However, one study on endurance athletes fed between whey isolate and beef hydrolysate for 10 weeks indicated an altered gut microbiome with increased *Bacteroidetes* and decreased health-related taxa, suggesting a potential negative impact of long-term protein supplementation that requires further research (Moreno-Pérez et al., 2018). A summary of recent studies related to whey protein diets is listed in Table 1.

Impact of GMP on the gut microbiome

Glycomacropeptide can promote the growth of beneficial organisms and inhibit the adhesion of pathogens to intestinal cells (Córdova-Dávalos et al., 2019). Córdova-Dávalos et al. provided an extensive review of GMP's ability to prevent the adhesion of pathogens (e.g., *S.* Typhimurium and enterohemorrhagic *E. coli* 0157) and toxins (e.g., cholera toxin, *E. coli* enterotoxin) to intestinal cells (Córdova-Dávalos et al., 2019). Glycomacropeptide also demonstrates an enhancement in the growth of some specific probiotic organisms. For example, bovine and caprine GMP both improved the growth of *Lactobacillus rhamnosus* RW-9595-M

and *Bifidobacterium thermophilum* RBL67 in a dose-dependent manner and to a greater degree than bovine β-lactoglobulin (Robitaille, 2012).

The mechanism by which GMP exhibits prebiotic activity is unclear. Though studies on the antimicrobial impact of GMP tend to implicate the glycosylation structures in its bioactivity (Feeney et al., 2017), studies examining growth-promoting ability are less concordant. Some studies suggest the prebiotic effect stems from the glycan moieties of GMP. For example, periodate oxidation to remove the glycans from GMP significantly reduced its bifidogenic effect, which was interpreted as evidence that its prebiotic activity is linked to glycosylation (O'Riordan et al., 2018). This study also found that GMP induces the expression of some glycogenes, but repressed others, perhaps representing a glycan-structure specific response (O'Riordan et al., 2018).

Further, sialyl glycopeptide concentrate (SGC) created through digestion and ultrafiltration of GMP-containing whey protein concentrate (G-WPC) outperformed G-WPC as the sole carbon source for the growth of certain *Bifidobacteria* (Fukudome et al., 2021). Although the glycan component of GMP is often hypothesized to be the basis for prebiotic activity, the peptide portion may be involved as well, as periodate-treated GMP did possess a small growth-promoting effect (O'Riordan et al., 2018). Similarly, Tian et al. found that GMP's *Bifidobacteria* growth-promoting effect did not directly depend on sialic acid content and may be related to its high glutamine, leucine, and alanine contents, despite the poor proteolytic activity of most *Bifidobacteria* (Tian et al., 2014). Glycomacropeptide hydrolysate produced with papain (GHP) had a stronger growth-promoting effect on *Bifidobacterium animalis subsp. lactis* (Bb12) than intact GMP or GMP hydrolyzed by trypsin (GHT), although GHP had the lowest sialic acid content of the three (Tian et al., 2014). Robitaille found that glycosylated, unglycosylated, and

mixed GMP treatments equally promoted the growth of lactic acid bacteria, indicating that the glycosylation state is not necessarily a factor in prebiotic activity, even though neither *Bifidobacteria* strain studied was proteolytic (Robitaille, 2012). Robitaille suggested that GMP allows improved growth of *Bifidobacteria* and *Lactobacillus* in acidic media during fermentation by triggering metabolic adaptations (Robitaille, 2012). Regardless of the mechanism, GMP is effective in encouraging the growth of probiotic organisms *in vitro* and could be used in the production of probiotics or as a functional ingredient to promote the growth of probiotic cultures in fermented dairy products and influence beneficial organisms in the gut (O'Riordan et al., 2018; Robitaille, 2013; Tian et al., 2014).

Like whey protein, GMP can impact the gut microbiome. In an *in vitro* human fecal culture system, GMP addition supported stable *Bifidobacterium* presence and decreased Bacteroides, *Clostridium* and *E. coli* (Brück et al., 2003). Chen et al. demonstrated that feeding GMP to mice promoted the growth of *Lactobacillus* and *Bifidobacteria* while decreasing *Enterobacteriaceae* and coliforms (Chen et al., 2012).

In piglets, compare with control diet, diet supplemented with 1.5% casein GMP resulted in positive changes to the gut microbiome (increased *Lactobacillus* and decreased *Enterobacteria*) (Hermes et al., 2012). Supplementation of sows with a combination of GOS and casein glycomacropeptide (GOS+GMP) during late gestation through farrowing induced changes to the intestinal microbiome in both the sow and their offspring, when comparing to control treatment based on nutrient requirements from National Research Council (Wu et al., 2020). At the phylum level, Fusobacteria became more prevalent in GOS+GMP-fed sows, whereas their offspring had an increase in Synergistetes and a decrease in Patescibacteria. At the genus level, GOS+GMP-fed sows had higher *Prevotella*, *Fusobacterium*, and *unclassified_f_Prevotellaceae*,

and their offspring had higher *norank_f_Ruminococcaceae*, *Christensenellaceae_R-7_group*, *Ruminococcaceae_UCG-005*, and *Ruminococcaceae_UCG-010* (Wu et al., 2020). Beyond these changes in the microbiome, this supplementation improved the number of live and healthy piglets, total litter weight, and average birth weight of live piglets (Wu et al., 2020). These findings align with previous work demonstrating that maternal diet impacts the health and microbial composition of the intestinal tract of offspring (Kashtanova et al., 2016). The study design did not allow identification of any effect due to GMP alone.

Effects of GMP on the microbiome in humans

Though GMP has growth-promoting effects on probiotic organisms *in vitro* and in animal models, these results are not consistently replicable in humans. Wernlund et al. found that GMP supplementation in healthy human subjects had no effects on Shannon or observed diversity and no changes in microbiome composition or fecal short-chain fatty acids (Wernlund et al., 2020). Moreover, that study showed no effect of GMP on gastrointestinal symptoms. Likewise, the study found no effect of GMP on high-sensitivity C-reactive protein, fecal calprotectin, indicating little systemic immunomodulatory impacts (Wernlund et al., 2020). A possible explanation for the observed lack of change in humans is the increased variability in human genetics, environments, behaviors and microbiomes compared to laboratory mice.

A recent crossover study (Hansen et al., 2023) in which obese women consumed twice or thrice daily GMP supplements (15 g GMP + 10 g whey protein/dose) found that compared to baselines, the high dose GMP resulted in lower overall alpha-diversity and the low dose resulted in lower relative abundance of the genus *Streptococcus.* The observed changes in the gut microbiome were unlikely to be the cause of the observed increases in satiety and higher area

under the curves of the glucoregulatory/satiety hormone amylin and the C-peptide of insulin and lower glucagon in a blood test after a GMP meal tolerance test compared with a baseline soy meal tolerance test. In this study, GMP supplementation did not affect weight, markers of systemic inflammation or plasma short-chain fatty acids (Hansen et al., 2023).

GMP effects on dysbiosis

Glycomacropeptide may be able to improve dysbiosis induced by old age or metabolic syndromes. In the elderly, a loss of microbial diversity may be a contributing factor in suboptimal health (Ntemiri et al., 2017). In an artificial colon model of elderly gut microbiota, the addition of GMP increased microbial diversity and increased the growth of the beneficial gut bacteria *Coprococcus* and *Clostridium* cluster XIVb (Ntemiri et al., 2017). In fecal cultures from free-living subjects GMP supplementation increased *Roseburia* and tended to increase *Dorea*, whereas in fecal culture from subjects residing in long-stay facilities *Pseudoflavonifactor* increased (Ntemiri et al., 2017). Type 2 diabetes is also associated with a loss of microbial diversity and changes to the microbial composition (Yuan et al., 2020). Mice with type 2 diabetes had increased *Helicobacteraceae* and *Lachnospiraceae* and decreased *Bacteridales_S24-7_group* (Yuan et al., 2020). Feeding glycomacropeptide hydrolysates (GHP) to these diabetic mice recovered lost microbial diversity, reduced *Helicobacteraceae*, and increased *Ruminococcaceae* and *Bacteroidales_S24-7_group*, the ratio of Bacteroidetes:Firmicutes, and *Ruminisclostridium*, *Blautia*, and *Allobaculum* (Yuan et al., 2020). Similarly, feeding non-hydrolyzed GMP increased the abundance of *Allobaculum* in wild-type mice and the abundance of *Bacteroidales;f_S24-7;g_* in mice with phenylketonuria and reduced

Desulfovibrio in both wild-type and phenylketonuria mice, which is associated with inflammatory bowel disease (IBD) (Sawin et al., 2015).

Impacts of GMP-induced microbial shifts on physiology

The changes in the gut microbiome induced by GMP can have physiological impacts (Table 2). For example, in a study by Yuan et al., supplementing glycomacropeptide hydrolysates to mice with high fat diet and streptozotocin-induced type 2 diabetes induced antidiabetic effects that correlated with changes in the microbiome (Yuan et al., 2020). Similarly, microbial changes in piglets whose mothers were supplemented with a combination of GOS and GMP were positively correlated with mRNA transcript levels for claudin-1, claudin-2, occludin, mucin-4, and mucin-13 (Wu et al., 2020).

Future Perspective

Though many studies have examined the effects of GMP in animal models (e.g., neonatal piglets (Wu et al., 2020), rhesus monkeys (Kelleher et al., 2003), and mice (Nilaweera et al., 2017)), studies in humans are scarce. Moreover, we know little about how changes in the microbiome induced by GMP affect physiology. Future research is needed to examine the effects of whey protein and GMP on the microbiome in humans and determine their clinical impacts. Moreover, more studies examining the long-term effect of whey protein and GMP supplementation on gut health and immune function are needed (Pena et al., 2018). Further research can guide the use of GMP and whey protein as therapeutics.

Conclusions

Both whey protein and GMP have a range of bioactivities, including prebiotic action and antimicrobial actions, and may enhance human health beyond provision of their amino acids. In animal models, whey protein supplementation frequently results in increased gut microbiota diversity, increased growth of beneficial microbial species and decreased markers of inflammation. GMP's capacity to promote the growth of beneficial organisms makes it a potential prebiotic dietary supplement. More research is needed to determine the extent to which whey protein and GMP affect the microbiome in humans and the extent to which any such changes affect overall physiology.

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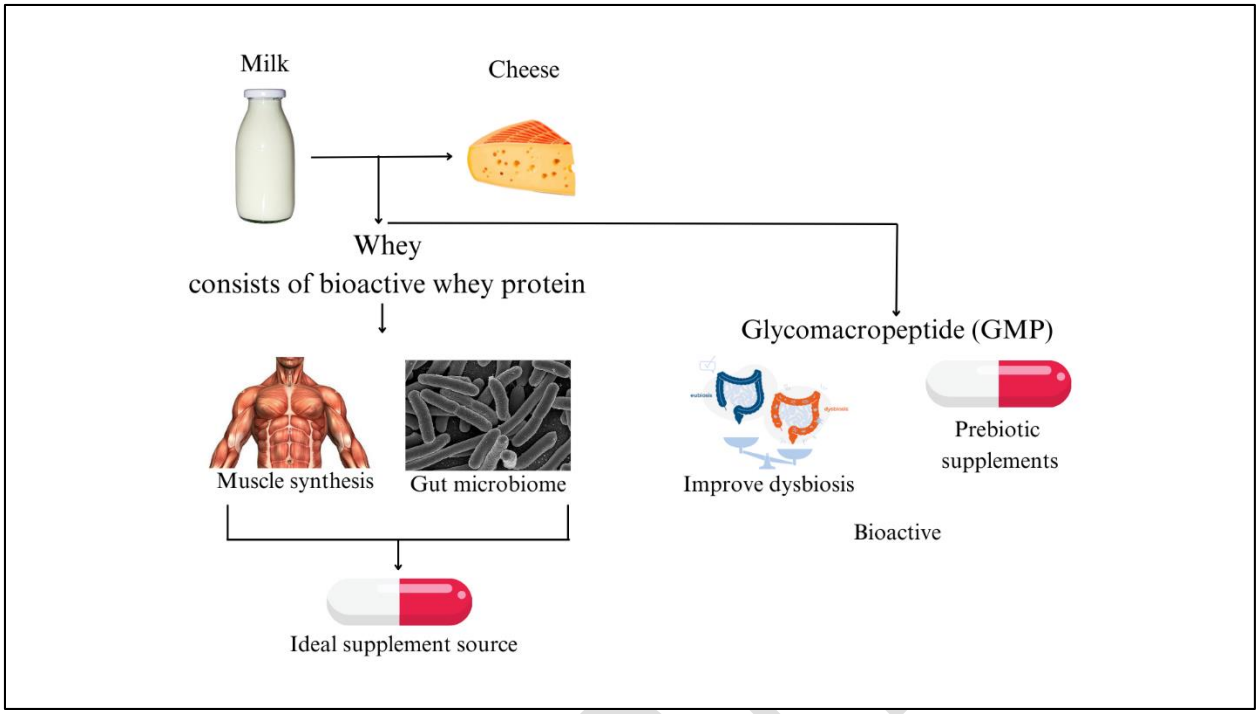

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405 **Figure 1:** Overall relations between whey and GMP with their primary functions.

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407 **Table 1:** A summary of the recent studies examining the effect of different whey protein diets on gut microbiome diversity

Area	Criteria of study	Outcome: changes in gut microbiome (whey only)		Reference
		Increase	Decrease	
Animal studies	Mice fed whey protein isolate (WPI) for 17 weeks	*Bacteroidetes*	Firmicutes and Actinobacteria	Nilaweera et al., 2017
	Non-obese diabetic mice fed glycated whey proteins for 6 months	*Porphyromondaceae*	Firmicutes	Chen et al., 2020
	Mice on a high-fat diet (HFD) fed for 21 weeks	*Lactobacillaceae*	*Clostridiaceae/Clostridium*	McAllan et al., 2014
	Mice on a HFD fed WPI versus casein for 5 weeks	*Lactobacillus murinus*	*HsL* and *Lpl* expression	Boscaini et al., 2020
	Rats with 3% dextran sulfate sodium-induced colitis fed cheese whey protein for 14 days	*Lactobacilli* and *Bifidobacteria*	N/A	Sprong et al., 2010
	Preterm piglets fed α-lactalbumin-enriched whey protein concentrate (WPC) versus regular WPC for 19 days	*Clostridiaceae*, *Enterobacteriaceae*, and *Lachnospiraceae*	None noted	Nielsen et al., 2020
	Pre-pubertal male rats under 60 days of food restriction and refeeding with casein- or whey-based diet	*Burkholderiales* (phylum Proteobacteria), *Bacilli* (phylum Firmicutes), and *Lactibacillaceae*	*Erysipelotrichales*, *Cytophagales*, and *Flavobacteriales*	Masarwi et al., 2018
	Mice fed high-fat-whey protein concentrate and high-fat whey-protein hydrolysate for 9 weeks	Bacteroidetes phylum (*B. rodentium, B. acidifaciens* and *B. stercoris*)	*Firmicutes*	Monteiro et al., 2016
	Rats with and without tumors fed a whey-based diet containing medium-chain triglycerides	*Muribaculaceae* and *Peptostreptococcaeceae*	*Ruminococcaceae*	Wardill et al., 2023
Human study	Endurance athletes fed whey isolate or beef hydrolysate for 10 weeks	Bacteroidetes phylum	*Citrobacter*, *Klebsiella*, *Coprococcus*, *Roseburia*, and *Blautia* genera	Moreno-Pérez et al., 2018
	With or without whey protein supplement on adult engaged in aerobic and resistance training for 8 weeks	*Lactococcus* phage, β-diversity of gut virome	None noted	Cronin et al., 2018
	Adults with overweight/obesity assigned snack bar with whey protein for 12 weeks	None	None	Reimer et al., 2017

In vitro study	In vitro infant fecal culture exposed to whey protein hydrolysate enriched in essential amino acids	*Lactobacillus acidophilus*, *Proteobacteria*, *Streptococcus*, and *Bacteroides*	Actinobacteriota:Bacteroidota (ratio)	Feng et al., 2022

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409 **Table 2:** A summary of glycomacropeptide (GMP) effects on gut microbiome diversity

Area/Criteria of Study	Specific effect	Reference
General population health	Decrease *Streptococcus* abundance in the gut	Hansen et al., 2023
	Enhance indicators of satiety and glycemic control	
	Positive relationship of GMP + GOS feeding with increased mRNA transcript levels for claudin-1, claudin-2, occludin, mucin-4, and mucin-13	Wu et al., 2020
	Changes in the microbiome were positively correlated with antidiabetic effects after feeding glycomacropeptide hydrolysates	Yuan et al., 2020
	Maternal GMP + GOS supplementation improved litter characteristics in piglet (number of live and healthy piglets, total litter weight, and average birth weight of live piglets)	Wu et al., 2020
	Maternal GMP + GOS supplementation increased immunoglobulins (IgA, IGF-1, IgG, IgM) in neonatal piglets	
Microbiome - Dysbiosis	Increased *Lactobacillus*, stable *Bifidobacterium*, decreased *Bacteroides*, *Clostridium*, and *E. coli* in human fecal cultures	Brück et al., 2003
	Improved microbial diversity in an artificial colon model for elderly people: increased growth of *Coprococcus*, *Clostridium cluster XIVb*, *Roseburia*; decreased *Dorea*	Ntemiri et al., 2017
	Reduced *Helicobacteraceae*; increased *Ruminococcaceae, Bacteroidales_S24-7_group* in mice with type-2 diabetes	Yuan et al., 2020

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