Title: Glycomacropeptide is a prebiotic that reduces Desulfovibrio bacteria, increases cecal short chain fatty acids and is anti-inflammatory in mice

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Running head: GMP is a Prebiotic with Beneficial Effects on Microbiota

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ABSTRACT –

Background: Glycomacropeptide (GMP) is a 64-amino acid (AA) glycoprophosphate with application to the nutritional management of phenylketonuria (PKU), obesity and inflammatory bowel disease (IBD). GMP is a putative prebiotic based on extensive glycosylation with sialic acid, galactose and galactosamine.

Objective: To determine the prebiotic properties of GMP by characterizing cecal and fecal microbiota populations, short chain fatty acids (SCFA) and immune responses.

Methods: Weanling PKU (Pahenu2) and wild type (WT) C57Bl/6 mice were fed isoenergetic AA, GMP or casein diets for 8 weeks. The cecal content and feces were collected for microbial DNA extraction to perform 16S microbiota analysis by Ion Torrent PGM sequencing. SCFA were determined by GC, plasma cytokines using a Bio-Plex Pro assay, and splenocyte T cell populations using flow cytometry.

Results: Changes in cecal and fecal microbiota are primarily diet dependent. The GMP diet resulted in a reduction from 30-35% to 7% in Proteobacteria, genera Desulfovibrio, in both WT and PKU mice with genotype-dependent changes in Bacteroidetes or Firmicutes. Cecal concentrations of the SCFA acetate, propionate and butyrate were increased with GMP. The percentage of stimulated spleen cells producing interferon-gamma (IFN-γ) was significantly reduced in mice fed GMP compared to casein. Plasma concentrations of IFN-γ, TNF-α, IL-1β and IL-2 were reduced in mice fed GMP.

Conclusions: GMP is a prebiotic based on reduction in Desulfovibrio, increased SCFA and lower indices of inflammation compared with casein and AA diets in mice. Functional foods made with GMP may be beneficial in the management of PKU, obesity and IBD.
Glycomacropeptide (GMP) or caseinomacropeptide is a bioactive 64-amino acid glycophosphopeptide derived from κ-casein in bovine milk that is used in the formulation of novel functional foods. GMP is produced during the manufacture of cheese, when rennet (chymosin) cleaves the C-terminal of κ-casein between Phe 105 – Met 106 releasing GMP into the cheese whey and precipitating para-κ-casein to form cheese, Figure 1A [61]. GMP is released in the adult human GI tract by pepsin mediated hydrolysis after milk ingestion [5]. GMP comprises 20-25% of the proteins in whey protein isolate and whey protein concentrate that are produced from cheese whey. Use of GMP as a food ingredient requires isolation of GMP from cheese whey using commercial techniques primarily based on chromatography or ultrafiltration.

GMP encompasses a heterogeneous group of peptides due to genetic variance and post-translational modifications including phosphorylation and glycosylation. The amino acid (AA) profile of GMP is unique. Pure GMP contains no aromatic AA (phe, tyr and trp), as well as no his, cys, and arg, and concentrations of iso and thr that are 2- to 3-fold greater, respectively, than those found in typical dietary proteins [14]. GMP is an acidic, highly polar peptide (isoelectric point below 4) that is hydrophilic and heat stable, with good functional properties for food formulation [30]. The theoretical mass of GMP is between 7-11 KDa. The primary structures of bovine GMP variants A and B, the two major genetic variants of GMP, are shown in Figure 1B. GMP contains three primary phosphorylation sites located on ser residues. Glycosylation of GMP at thr residues via
OH linkages occurs with five different mucin-type carbohydrate chains containing: N-acetylgalactosamine (sialic acid) [64], galactose, or N-acetylgalactosamine [61]).

Approximately 75% of glycosylated GMP molecules include trisaccharide and tetrasaccharide chains as shown in Figure 1B. GMP is a putative prebiotic based on this high degree of glycosylation.

GMP demonstrates a number of biological activities relevant to GI physiology and metabolic disease due to its unique composition as summarized in reviews [3,10,61]. GMP has been shown to bind and inactivate toxins of Escherichia coli and Vibrio cholerae [28] and inhibit the adhesion of cariogenic bacteria [1]. This protection from pathogens has been linked with glycosylation of GMP, especially sialic acid [39], as many pathogens and enterotoxins adhere to cells by recognizing carbohydrate receptors. GMP enhances zinc absorption in rhesus monkeys linked with its negative charge [29]. Conflicting studies suggests that GMP promotes satiety in humans [42,63] with limited evidence that stimulation of cholecystokinin by GMP may mediate reduced food intake [2]. GMP exerts immunomodulating activities as reflected in increased cytokine production in human monocytes [45] and decreased stimulation of T helper 1 lymphocytes in rat splenocytes [47]. GMP administered by oral gavage shows significant anti-inflammatory effects in rat models of colitis and ileitis, resulting in normalization of spleen mass and reduced expression of IL-1β in ileum and colon [8,31,38,46], as we have observed in PKU mice [57]. GMP may provide a beneficial source of protein in functional foods for the management of inflammatory bowel disease (IBD).
GMP provides a physiological source of low-phe protein for humans with the genetic condition phenylketonuria (PKU), in part, because it is the only known dietary protein that contains a mere trace of phe and can be made into a variety of palatable medical foods for the essential management of PKU [35,62]. A medical food, as defined by the US Food and Drug Administration (FDA), is a “food which is formulated to be consumed or administered enterally under the supervision of a physician and which is intended for the specific dietary management of a disease or condition for which distinctive nutritional requirements, based on recognized scientific principles, are established by medical evaluation” [18]. Our murine studies, which characterize the nutritional and neurochemical properties of GMP, confirm the nutritional adequacy of GMP supplemented with limiting AA and illustrate anti-inflammatory effects, reduced body fat mass, and positive effects on bone size and strength with chronic ingestion of this peptide [54,57,58]. A unifying feature and potential mechanism for the reported beneficial activities of GMP is its putative role as a prebiotic that promotes a beneficial gut microbiota and modulates immune function.

There is no definitive evidence of the prebiotic effects of dietary GMP on the intestinal microbiome and associated immune responses [6,10]. The extensive glycosylation and sialic acid content of GMP suggests it is a potent prebiotic with application to functional foods for the management of PKU, obesity, IBD and bone health. Our objective was to determine the prebiotic properties of GMP by characterizing cecal and fecal microbiota populations and cecal concentrations of short chain fatty acids (SCFA) from PKU and wild type mice fed diets containing GMP as the primary protein source. Plasma cytokines and splenocyte T cell populations were
assessed to characterize the anti-inflammatory effects of GMP associated with alterations in the microbiota. Our findings indicate that GMP is a prebiotic based on modulation of the GI microbiota in association with increased cecal concentrations of SCFA and beneficial immune effects.

**METHODS**

**Animals and Experimental Design**

The University of Wisconsin-Madison Institutional Animal Care and Committee approved the facilities and protocols used in this study. Experimental animals were produced from a breeding colony of PKU mice by breeding C57BL/6J mice heterozygous for the \( \text{Pah}^{enu2} \) mutation to yield homozygous PKU mice and WT control mice [25,32]. Experimental mice were genotyped for the presence of the \( \text{Pah}^{enu2} \) mutation [57]. A series of three experiments was performed. Experiment 1 utilized a 2x2x3 factorial design to examine the effects of genotype (PKU and WT), sex (male and female), and diet (high-phe casein, low-phe AA, and low-phe GMP) as well as their interactions on growth, spleen mass, and mass of jejunum and colon. Experimental animals were fed from weaning (3 weeks of age) through young adulthood, 20 to 22 weeks of age. Experiment 2 was used to investigate the prebiotic properties of GMP by examining gut microbiota populations, SCFA concentrations, and plasma cytokine concentrations in WT mice fed either the casein or GMP diet and PKU mice fed either the AA or GMP diet. Mice were fed the experimental diet from weaning through 8-9 weeks of age. Experiment 3 further investigated the anti-inflammatory properties of GMP using flow cytometry to characterize spleen and blood cells in both WT and PKU
mice fed either the casein or GMP diets. These mice were fed the experimental diets from weaning through 20 to 22 weeks of age.

At weaning, mice were randomized to one of the three diets, separated by sex, and housed with littermates in shoe-box cages. The facility was maintained at 22°C on a 12-12-h light-dark cycle with mice being fed ad-libitum and having free access to water. All the diets were isoenergetic and formulated based on the AIN-93G diet composition [44] with the protein source being the only source of variation across diets (Harlan Teklad, Madison, WI; TD.09667 – TD.09669, TD.120645) [57]. The protein in the casein diet was provided by 20% (wt/wt) casein plus 0.3% L-cystine, the AA diet included 17.5% free AAs which provided higher luminal osmolality [51], and the GMP diet had 20% GMP (LACPORDEN CGMP-10, Arla Foods Ingredients, Viby J, Denmark) plus 1.5 times the NRC requirement for 5 limiting AA in order to provide a complete protein source. The cGMP-10 from Arla Foods Ingredients contains 6-7% sialic acid. The AA profile of the diets was previously reported [57]. The content of the sulfur AA, methionine and cysteine is similar across the 3 diets (8-9 g/kg diet). However, 3-3.5 g/kg of cystine was added to the casein and AA diets whereas no cystine, only met, was added to the GMP diet.

For experiment 1, the small intestine and colon were dissected, weighed, and then the jejunum separated from the rest of the small intestine. A 4 cm segment was removed 6 cm from the proximal end of the jejunum and weighed. Mucosa was scraped from this segment, dried, and then weighed. At the end of experiment 2, mice were placed into individual metabolic chambers (Tecniplast, West Chester, PA) for 3 days and feces were collected every 24 hours. Blood was collected by cardiac puncture.
into syringes containing a final concentration of 2.7 mmol/l EDTA, and plasma was
isolated by centrifugation at 4°C. The cecum was dissected and cecal contents
collected into microfuge tubes and frozen at -70°C. Spleens were dissected, weighed
and used immediately for flow cytometric analysis.

Gut Microbiota Analysis

DNA Extraction

The cecum digesta was homogenized in 1.5ml PBS and total DNA from 200 μL
of the homogenate was isolated using the QIAamp DNA Stool Mini Kit (Quiagen
Sciences, MD) with modifications to the manufacturer's instructions. These
modifications included an initial mechanical cell disruption step by inclusions of 0.1 mm
glass beads (Sigma-Aldrich) followed by exposure to six 1 min beating at maximum
speed in a Mini-beadbeater-96 (Biospec Products, INC., Bartlesville, OK) with intervals
of 2 min on ice. Subsequently, a heat treatment step was performed for 5 min at 95°C.
The DNA was further purified by phenol:chloroform:isoamyl alcohol (25:24:1, pH 8)
extraction, phase separation using Phase Lock Gels (5 PRIME) and ethanol
precipitation using pellet paint co-precipitant (EMD Millipore). DNA was quantified by
Qubit® 2.0 Fluorometer (Invitrogen).

Ion Torrent PGM Sequencing and Microbiota Analysis

Partial 16S rRNA sequences were determined on a 318 v2 chip using the
IonTorrent Personal Genome Machine System at University of Wisconsin-Madison,
Biotechnology Center. Briefly, the V1-V2 region was amplified using forward primers
that contained a sample-specific bar-code with an Ion A adapter and a key sequence,
while the associated reverse primer contained a truncated P1 (trP1) adapter. The
sequence of these primers were: forward (8FM – 5’ – CCA TCT CAT CCC TGC GTG  
TCT CGg ACT CAG BBB BBB BBB BAG AGT TTG ATC MTG GCT CAG – 3’) with 
the Ion A adapter in italics, the key sequence in italics and underlined, the 13 bp bar 
code designated as Bs, and the 16S primer sequence in capital letters; reverse (357R – 
5’ – CCT CTC TAT GGG CAG TCG GTG ATC TGC TGC CTY CCG TA- 3’) with the 
trP1 adapter in italics and the 16S primer sequence in capital letters. All PCR reactions 
were quality-controlled for amplicon saturation by gel electrophoresis. The amplicons 
from all reactions were pooled in equal amounts and purified using AxyPrep Mag PCR 
beads (Corning, Inc.). The resulting products were quantified using PicoGreen 
(Invitrogen) and Qubit fluorometer (Invitrogen) before sequencing. The data processing 
pipeline removed low-quality reads that: 1) did not completely match the PCR primer 
and barcode; 2) were shorter than 300 bp or longer than 400 bp in length; or 3) had an 
average quality score <22. Data analysis was performed primarily in QIIME 1.8 
framework [4]. Operational Taxonomic Units (OTUs) were chosen with QIIME picking 
OTU workflow based upon sequence similarity. Taxonomic identities were assigned 

Cecal Short Chain Fatty Acids Analysis

1 mL of cecal contents were added to a microfuge tube containing 20 μL of 50% 
H₂SO₄. Sample was centrifuged for 3 min and 600 μL of supernatant transferred to a 
new microfuge tube. 120 μL of 25% metaphosphoric acid was added and allowed to 
stand for 30 min. Samples were centrifuged and supernatant transferred to a GC vial. 
Concentrations of individual SCFA were measured on a Perkin Elmer Autosystem GC 
using a 4% Carbowax 20 M on 80/120 mesh Carbopack B-DA, 1.8 m × 2 mm column
(Supelco Inc.). Nitrogen was used as a carrier gas, and the injector temperature was 170°C and the detector temperature were 175°C. The column temperature was maintained at 115°C for 45 min. Nitrogen flow was maintained at 25 mL/min.

Identification of SCFA peaks were based on a purchased external standard from Sigma Chemical Co. (St. Louis, MO) [19,60].

**Plasma Cytokines**

Cytokine assays were performed by using a Bio-Plex™ Mouse Cytokine 23-Plex Panel (1 × 96-well) (BioRad, USA) according to the manufacturer’s instructions. Plasma samples were diluted 1:4 with mouse sample diluent. The desired number of wells were pre-wet with 100 μL of Bio-Plex assay buffer. The multiplex bead working solution was vortexed for 15–20 s and 50 μL were pipetted into each well. The buffer was then immediately removed using Bio-Plex Pro Magnetic Wash Station™. Wells were washed twice by adding 100 μL of wash buffer. 50 μL of diluted standard or prepared sample were added to each well and the plate incubated for 30 min. Thereafter, the plate was washed 3 times with 100 μL of Bio-Plex wash buffer. 25 μL of vortexed Bio-Plex Detection Antibody working solution was added to each well, incubated for 30 min, and then washed 3 times with Bio-Plex wash buffer. 50 μL of vortexed 1x streptavidin-PE was added to each well, incubated for 30 min. and then washed 3 times with Bio-Plex wash buffer. Beads were resuspended with 125 μL assay buffer, incubated for 30 s and then immediately read on the Bio-Plex 200 System [BioRad, USA]. Cytokine concentrations were calculated from the standard curve using Bio-Plex manager software. Samples were run in duplicate.
Flow Cytometry

Spleens were dissociated by mechanical disruption and digested with collagenase/dispase (20 ug/ml, Roche, Indianapolis, IN) and DNAse I (300 ug/ml, Roche) for 30 min at 37°C and passed through a 70 um cell strainer (BD Biosciences, San Jose, CA). Heparinized blood was collected via cardiac puncture and RBCs were removed by dextran sedimentation. Remaining RBCs were lysed with ammonium chloride. Cells were stained at 4°C in PBS with Live/Dead Violet Fixable Stain kit (Invitrogen, Carlsbad, CA), washed, then stained in PBS with 0.5 mM EDTA, 0.2% BSA, 0.09% azide, and 2% normal rat serum (Jackson ImmunoResearch, West Grove, PA). Anti-mouse CD3-eFluor450 (eBioscience, San Diego, CA), CD4-Alexa Fluor 700, CD8-APC-eFluor780 (eBioscience, San Diego, CA), CD44-PE, CD62L-PE-Cy7, IFNγ-PerCPCy5.5 (eBioscience, San Diego, CA), Granzyme B-FITC (eBioscience, San Diego, CA), and Perforin-APC antibodies were purchased from BD Biosciences except as indicated. Isolated spleen cells were also stimulated with PMA and ionomycin. Anti-Rat/Hamster CompBeads (BD Biosciences) were used to set compensation. Data were collected on an LSRII cytometer (BD Biosciences) and analyzed with FlowJo 7.6.1 (TreeStar, Ashland, OR).

Statistical Analysis

Data were analyzed by three-way ANOVA using generalized linear model (GLM) to identify the main treatment effects of genotype, sex, diet, as well as their two and three way interactions. Differences between the treatment groups were detected using a protected Fisher's Least Significant Difference (LSD) test (SAS Institute, 2007, Cary, NC). Data transformations were performed where appropriate to fit assumptions of
normality and equal variance prior to statistical analysis. Data are presented as mean ± SE. P-values < 0.05 are considered significant. Where there was no significant interaction, data were pooled into treatment groups by their respective main effects. Data for SCFA concentration and plasma cytokine concentration were analyzed by one-way ANOVA. For gut microbiota analysis data are presented as mean ± SE. The statistical difference between treatments was tested by Monte-Carlo in package ade4 [13] of R 2.14.0 [43] as described by de Carcer et al [9]. The dominant genera that were promoted or inhibited were determined by correspondence analysis in package ade4 of R 2.14.0 as described by de Carcer et al [9]. An unweighted UniFrac analyses on tables of OTU counts was performed with beta_diversity_through_plots.py script of QIIME. The resulting distances were summarized in the PCoA biplot.

RESULTS

Experiment 1: Body and organ mass

Body Weight and Spleen Mass

The casein control diet provided higher phe content than the AA and GMP diets. Thus, it was not surprising that WT mice fed the casein diet showed significantly greater final body weight compared to WT or PKU mice fed the AA or GMP diets (WT casein 29 ± 1 vs. WT AA 23 ± 1, WT GMP 23 ± 1, PKU casein 23 ± 1, PKU AA 23 ± 1, PKU GMP 22 ± 1; g final body mass). Food intake was not significantly different in WT mice fed the casein, AA and GMP diets. PKU mice fed the GMP diet showed similar food intake to WT mice but lower food intake compared with PKU mice fed the casein or AA diets consistent with greater metabolic efficiency due to ingestion of GMP [57]. Spleen mass expressed relative to body weight showed significant treatment effects for both
genotype and diet without interaction. PKU mice had significantly larger spleens compared to WT mice, Figure 2A. Regardless of genotype, mice fed the GMP diet had significantly smaller spleens compared with mice fed either the casein or AA diet, Figure 2B.

Gut Mass

The mass of the jejunum showed significant treatment effects for both genotype and diet without interaction. PKU mice demonstrated significantly greater intact jejunum wet mass and jejunum mucosa dry mass compared with WT mice that was independent of diet, Figure 3A and 3C. Regardless of genotype, PKU and WT mice fed the GMP diet showed significantly lower intact jejunum wet mass and jejunum mucosa dry mass compared with mice fed the casein or AA diets, Figures 3B and 3D. Consistent with greater intestinal absorptive area, we previously reported significantly greater energy expenditure and food intake in PKU mice compared with WT mice [57]. However, the increases relative to WT mice were of the greatest magnitude in PKU mice fed the casein and AA diets compared with the GMP diet, e.g., a 13-15% increase in oxygen consumption over 48 hr with the casein and AA diets compared to a 3% increase with GMP diet. These data suggest that because PKU mice expend more energy and need to eat more diet to grow at a rate similar to WT mice, their small intestine adapts to a larger mass to facilitate nutrient absorption, and the response is attenuated in mice fed the more physiologic GMP diet [57].

Mice fed the GMP diet showed a significantly lower colon mass compared to mice fed the casein and AA diets (24.5±0.6 casein; 25.3±0.8 AA, 22.5±0.5 GMP; mg/cm intact wet colon; p=0.006, GMP vs casein and AA diets, n=45). The implication of the
lower colon wet mass in mice fed the GMP diet is unclear. Interestingly, mean fecal mass was increased by 11% to 33% with consumption of the GMP diet compared to the casein and AA diets, respectively (GMP=0.43±0.02, casein=0.37±0.02, and AA=0.33±0.03; g feces/day; p<0.05 for GMP vs AA; n=12). The greater fecal mass induced by ingestion of GMP, despite similar food intake compared to casein in both Experiment 1 and Experiment 2, is consistent with the putative prebiotic effects of GMP as investigated in Experiment 2.

Experiment 2: Gut Microbiota, Short Chain Fatty Acids, & Plasma Cytokines

Microbiota Populations at the Phyla Level

Intestinal microbiota populations were characterized in both the cecum and feces and are presented as percent of the population. A total of 1,792,910 filtered reads were obtained from the 54 samples; the number of reads varied from 16,268 to 61,124 with an average of 33,202 reads per sample. In cecal contents from both WT and PKU mice fed the GMP diet there was a significant reduction from 30-35% to 8-10% in the Proteobacteria phylum compared to WT mice fed the casein diet and PKU mice fed the AA diet, p = 0.002 and p = 0.001 respectively, Figure 4A. PKU mice fed the GMP diet had a significant increase in Bacteroidetes from 23.3% to 55.8% compared to mice fed the AA diet. In contrast to PKU mice, WT mice fed the GMP diet had a significant increase in Firmicutes from 29.6% to 56.7% compared to WT mice fed the casein diet. In the feces Bacteroidetes was the primary phylum. PKU mice fed the GMP diet had a significant increase in Bacteroidetes from 74.4% to 86.7% compared to PKU mice fed the AA diet, Figure 4B.
*Microbiota Populations at the Genera level*

Pairwise comparisons of the treatment groups demonstrates that WT mice fed the casein diet have a significantly different gut microbiome population than WT mice fed the GMP diet in both the cecum and feces. PKU mice fed the AA diet have a significantly different gut microbiome population than PKU mice fed the GMP diet in both the cecum and feces. In the cecal contents of WT mice fed the GMP diet there was a significant reduction of the *Desulfovibrio* genera compared to WT mice fed the casein diet, from 29.5% to 7.3%, \( p = 0.001 \), Figure 5A. Similar to WT mice, PKU mice fed the GMP diet had a significant reduction in *Desulfovibrio* from 34.6% to 6.9%, compared to PKU mice fed the AA diet, \( p = 0.001 \). WT mice fed the GMP diet saw a significant increase in *Allobaculum*, 4.7% to 28.2%, compared to WT mice fed the casein diet, \( p = 0.012 \). PKU mice fed the GMP diet had a significant increase in *Bacteroidales; f__S24-7;g__* compared to PKU mice fed the AA diet, from 17.7% to 52.0%, \( p = 0.001 \). In the feces, *Desulfovibrio* is reduced in both WT and PKU mice fed GMP compared to WT mice fed the casein diet and PKU mice fed the AA diet, \( p = 0.017 \) and \( p = 0.021 \) respectively, Figure 5B.

The first two dimensions of principal coordinate analysis (PCoA) plot, Figure 6, depicts unweighted unifrac distances between microbial communities. Each point represents the microbial community of the cecum sample from one mouse and illustrates a diet effect by the clustering of the communities.

*Short Chain Fatty Acid Concentrations*

Consistent with fermentation of the carbohydrate residues in GMP, both WT and PKU mice fed the GMP diet demonstrated greater cecal concentrations of acetate,
propionate, and butyrate compared to mice fed the casein and AA diets, Figure 7. WT mice fed the GMP diet showed significantly greater cecal concentrations of acetate and propionate and greater butyrate concentrations (p=0.027, p=0.022, and p=0.062, respectively) compared to WT mice fed the casein diet. PKU mice fed the GMP diet showed significantly greater cecal concentrations of butyrate (p=0.04) and greater cecal concentrations of acetate and propionate (p=0.058-0.086) compared to PKU mice fed the AA diet. The sum of acetate, butyrate and propionate concentrations in cecum was highest in the PKU GMP group (9.2±2.5, PKU GMP; 5.6±0.79 WT GMP; 4.4±0.81 PKU AA; and 2.8±0.64 WT casein; mmol; p<0.075 for PKU GMP vs PKU AA and p<0.029 for WT GMP vs WT casein). Isobutyrate, 2-methylbutyrate, isovalerate, and valerate concentrations were not different between the groups, data not shown.

**Plasma Cytokine Concentrations**

Inflammatory cytokine concentrations were measured in plasma of WT mice fed the casein or GMP diets and PKU mice fed the AA or GMP diets. Plasma concentrations of IFN-γ and TNF-α were significantly reduced in WT mice fed the GMP diet compared to WT mice fed the casein diet, p = 0.01, Figure 8A and 8B. Similarly, WT mice fed the GMP diet had significantly lower plasma concentrations of IL-1α, IL-1β, IL-2, and IL-10 compared to WT mice fed the casein diet, Figure 8C-8F. Although there were trends for lower plasma concentrations in IFN-γ and IL-2 in PKU mice fed the GMP diet compared to the AA diet, this did not reach significance.
Experiment 3: Flow Cytometry

Flow Cytometric Analysis of Splenocytes and Blood Cells

To investigate the potential anti-inflammatory properties of GMP, splenic T cells were characterized by flow cytometry. Splenocytes were stimulated with PMA and ionomycin and both WT and PKU mice fed the casein diet had significantly elevated CD8⁺ CD62L⁻CD44hi effector cytotoxic T cells compared to mice fed the GMP diet, p < 0.0001, Figure 9A. Stimulated splenocytes from mice fed the casein diet also had significantly greater production of CD8⁺ IFN-γ compared to mice fed the GMP diet, p = 0.0043, Figure 9B. Splenocytes were also analyzed for expression of CD4⁺ IFN-γ, but there were no significant differences between groups, data not shown.

Flow cytometry was used to analyze anti-inflammatory effects of GMP in the blood. The results seen in the spleen were replicated as production of CD8⁺ IFN-γ was significantly greater in mice fed the casein diet compared to mice fed the GMP diet, p < 0.0001. Similar to spleen, there were no significant differences in production of CD4⁺ IFN-γ between the groups, data not shown.

Discussion

There is considerable evidence that dietary prebiotics modulate the GI microbiota with therapeutic applications to conditions including irritable bowel syndrome, IBD, obesity and bone health [22,48,49]. The International Scientific Association for Probiotics and Prebiotics defines a dietary prebiotic as “a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the GI microbiota, thus conferring benefit(s) upon host health” [49]. Most prebiotics are poorly digested carbohydrates. The dairy protein GMP is considered a putative prebiotic based
on its high degree of glycosylation that includes mucin-like oligosaccharide chains that contain sialic acid, galactose and galactosamine [61]. However, there is no definitive evidence that GMP is a prebiotic [10]. We demonstrate for the first time that chronic ingestion of GMP in both WT and PKU mice acts as a prebiotic based on specific modulation of the GI microbiota (reduced *Desulfovibrio*) that is beneficial based on increased cecal concentrations of SCFA and lower indices of inflammation.

Irrespective of genotype and compared to isoenergetic diets containing either casein or AAs as the primary protein source, GMP induced greater fecal output with similar food intake and a dramatic reduction (from 30-35% to 7%) in the Proteobacteria phylum, *Desulfovibrio* genus in cecum. Parallel changes were noted in feces. This was offset by genotype-dependent increases in the Bacteroidetes or Firmicutes phyla. The *Desulfovibrio* genus includes sulfate metabolizing bacteria that reduce sulfites and sulfates obtained from the diet and sulfated mucopolysaccharides found in mucin leading to the generation of hydrogen sulfide, a cytotoxic compound [21]. Compelling data in human subjects demonstrate that humans with ulcerative colitis have an increase in prevalence of *Desulfovibrio* [53]. Of note, 5-aminosalicylic acid inhibits sulfate reduction and reduces fecal hydrogen sulfide levels suggesting that its efficacy in treating colitis may reside in its ability to reduce the prevalence of sulfate reducing bacteria, e.g., act like an antibiotic [40]. Moreover, dextran sulfate sodium is an established experimental rodent model of chronic colitis based on the fermentation of sulfated substrates by metronidazole-sensitive fecal bacteria to yield toxic hydrogen sulfide products that induce colonic inflammation and ulceration [37]. Lastly, studies with 3 models of IBD, dextran sulfate, trinitrobenzenesulfonic acid, and lymphocyte-transfer
all demonstrate that gavage of a small amount of GMP (15 mg/day in combination with a stock diet versus over 200 mg dietary GMP/day ingested in a semi-purified diet as used in the current study) reduces disease severity and shows anti-inflammatory effects. This is the first study to establish an association between ingestion of GMP and alterations in the GI microbiota associated with the pathogenesis of IBD. Taken together, these data suggest that GMP may exert its anti-inflammatory effects in preclinical models of IBD, in part, by reducing the prevalence of sulfate reducing bacteria, e.g. *Desulfovibrio*.

The mechanisms underlying GMP’s ability to specifically reduce *Desulfovibrio* in the cecum and feces likely reflect the dual contributions of dietary sulfur [17] and endogenous sulfur from intestinal mucins [21]. The total sulfur content of the GMP, casein and AA diets provided by met and cysteine or cystine (reduced dimer of cysteine) is similar, 8-9 g/kg diet, and exceeds the minimum requirement (5 g/kg diet) [34]. The GMP diet is supplemented with met whereas the casein and AA diets are supplemented with cystine (3-3.5 g cystine/kg diet) to provide for nutritional adequacy [44]. Supplemental cystine included in the casein and AA diets, and often used in human nutritional products, may provide a substrate that directly promotes the growth of *Desulfovibrio*. However, the similar total sulfur content of the three diets suggests indirect effects of GMP to reduce the prevalence of *Desulfovibrio, possibly by altering mucin synthesis*. Sulfate reducing bacteria metabolize sulfated mucopolysaccharides found in mucin [21] and mucin composition is altered by the source of dietary protein, although the effects of GMP on mucin composition has not been reported [7,15,16,59]. Indeed, high intake of sulfur compounds from high protein foods rich in methionine and
cysteine is associated with relapse in patients with ulcerative colitis [27]. Additional studies will be required to understand the complex interactions between dietary intake of GMP and cystine, the composition of intestinal mucins and bile acids [12], and sulfate reducing bacteria.

The ability of GMP to increase cecal levels of the SCFA, acetate, butyrate and propionate, suggests improved intestinal barrier function [20] and the potential for reduced systemic inflammation [23]. Moreover, Desulfovibrio metabolize SCFA which may account for reduced SCFA in mice fed casein or AA diets who show elevated levels of Desulfovibrio compared to GMP [33,41,50]. Carbohydrate availability and a lower intestinal pH induced by increased levels of SCFA reduces the ability of intestinal microbiota to metabolize the aromatic AAs phe, tyrosine and tryptophan to phenolic and indolic compounds which have been associated with a variety of disease states in humans and animals [56]. This may be relevant to emerging chronic complications of PKU treated with an AA diet including chronic kidney disease [26] and osteopenia [24]. In contrast, the prebiotic effects of GMP associated with increased cecal SCFA levels reflect a healthy microbiota and intestinal anti-inflammatory properties.

Although the relationship between elevated levels of Desulfovibrio and IBD may be coincidental [53], Desulfovibrio clearly flourish in an inflammatory environment [21]. The intestinal anti-inflammatory activity of GMP has been reported in rodent models of IBD where the GMP is administered by gavage pre-or post-induction of IBD, rather than being ingested in a diet as studied herein. These acute studies associate GMP’s ability to reduce the severity of IBD with its ability to boost innate immunity but block T cell-
driven adaptive immunity [31,38]. Consistent with these studies, both WT and PKU mice fed the GMP diet had reduced levels of cytotoxic T cells, marked by CD44^{hi}CD62L^{lo} antibodies compared to WT and PKU mice fed the casein diet. After stimulation with PMA and ionomycin the CD8^{+} cytotoxic T cells produced less IFN-γ, part of the adaptive immune response [55] in mice fed the GMP diet relative to mice fed the casein diet. This finding is supportive of the conclusion drawn by Ortega G et. al [38] that GMP’s mechanism to reduce severity of IBD is by blocking T cell-driven adaptive immunity. Reduction of plasma concentrations of T_{H1} associated cytokines, IFN-γ and IL-2 [52], in mice fed the GMP diet is consistent with the data produced by López-Posadas et. al [31] suggesting that GMP may act through these mechanisms. Concentrations of plasma IL-1β were also reduced in mice fed the GMP diet, similar to the suggested mechanism by Daddaoua A et al [8] as to how GMP functions to reduce symptoms of hapten-induced colitis in rats. As our cytokine concentrations are in plasma and consistent with the results seen in specific tissues, this suggests that ingestion of GMP has systemic anti-inflammatory effects. Reduced plasma cytokine levels may be relevant to the positive effects of GMP on bone strength [36,58]. In summary, chronic ingestion of GMP, like gavage of GMP in murine models of IBD, shows systemic and intestinal anti-inflammatory effects.

In conclusion, we demonstrate for the first time that GMP meets the criteria for a prebiotic, e.g., a specific change in the GI microbiota with beneficial effects on the host. GMP specifically reduces Desulfovibrio (associated with the pathogenesis of IBD and generation of hydrogen sulfide a cytotoxic compound), increases SCFAs (associated with enhanced intestinal barrier function and reduced pH) and induces anti-inflammatory
effects which previous reports have linked with attenuation of inflammation and intestinal damage in preclinical models of IBD [10]. Palatable functional and medical foods can be made with GMP and such foods may be a beneficial in the management of PKU, obesity and IBD. Additional research is needed to elucidate the complex interactions between GMP, the GI microbiota, intestinal mucin composition and immune function.

Acknowledgements:
We would like to thank Sandy Bertics in the Department of Dairy Science for her assistance in analyzing SCFA concentrations. We would also like to thank Lori Neal and Laura Knoll for their assistance in sharing flow cytometry methodology and undergraduate student Jennifer Mallon for help in maintenance of the mouse colony.

Grants:
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Disclosure:
Denise M. Ney is a co-inventor on U.S. Patent 8,604,168 B2, "Glycomacropeptide Medical Foods for Nutritional Management of Phenylketonuria and other Metabolic Disorders," which is held by the Wisconsin Alumni Research Foundation and licensed to Cambrooke Therapeutics, LLC. Peggy Steele, a member of Dr. Steele’s family, is employed by DuPont Inc., a supplier of bacterial cultures to the food industry.
References:


43. RDevelopmentCoreTeam. R: A language and environment for statistical computing. 2011.


46. Requena P, Daddaoua A, Martínez-Plata E, González M, Zarzuelo A, Suárez MD, de Medina FS, Martínez-Augustin O. Bovine glycomacropeptide ameliorates


Figure 1. A: Glycomacropeptide (GMP) or caseinomacropeptide is a bioactive peptide released from one of the casein milk proteins (κ-casein) during cheesemaking. Rennet (chymosin) cleaves κ-casein between phe 105 and met 106 releasing GMP, a glycosylated peptide into the whey. GMP constitutes 20-25% of nitrogen in most whey products. GMP is a unique peptide lacking aromatic acids and thus has been isolated from whey for use in medical foods needed for the management of phenylketonuria. It is an acidic, highly polar peptide (isoelectric point below 4.0) that is hydrophilic and heat stable, with a theoretical molecular mass between 7-11 kDa.

B: Bovine GMP represents a heterogeneous group of 64 amino acid peptides due to genetic variance (variants A and B) and post-translational modification, including phosphorylation at serine residues and O-glycosylation at threonine residues. The primary structure of bovine variant A is shown; the 2 sites corresponding to mutational differences in the B variant are indicated. Glycosylated forms of GMP include five different mucin-type carbohydrate chains containing N-acetylneuraminic acid (sialic acid), N-acetylgalactosamine, or galactose. Approximately 75% of glycosylated GMP molecules include trisaccharide and tetrasaccharide chains as shown.

Figure 2. Relative spleen mass in wild-type (WT) and phenylketonuria (PKU) mice fed casein, amino acid (AA) or glycomacropeptide (GMP) diets from weaning through 18 ± 3 weeks of age. Values shown are means + SE (n=45-70) for significant main effects of genotype (A) and diet (B); there was no significant interaction of genotype and diet. Relative spleen mass was significantly greater in PKU compared with WT mice and lower with ingestion of the GMP diet compared to the casein and AA diets.
Figure 3. Intact jejunal wet mass (A, B) and jejunal mucosa dry mass (C, D) in WT and PKU mice fed casein, AA or GMP diets from weaning through 21 weeks of age. Values shown are means + SE (n=45-70) for significant main effects of genotype and diet; there was no significant interaction of genotype and diet. Jejunal mass was significantly greater in PKU compared with WT mice and significantly lower with ingestion of the GMP diet compared to the casein and AA diets.

Figure 4. WT and PKU mice fed the GMP diet have altered bacterial populations from WT mice fed the casein diet and PKU mice fed the AA diet. Relative bacterial phyla abundance was calculated compared to total bacteria in both the cecum (A) and feces (B). In cecal contents from both WT and PKU mice fed the GMP diet there was a significant reduction in the Proteobacteria phylum compared to WT mice fed the casein diet and PKU mice fed the AA diet, p = 0.002 and p = 0.001 respectively. In the feces PKU mice fed the GMP diet had a significant increase in Bacteroidetes relative to PKU mice fed the AA diet, p = 0.047

Figure 5. Relative bacterial genera abundance was calculated compared to total bacteria in both the cecum (A) and feces (B) in WT and PKU mice fed either the casein, AA, or GMP diets. In the cecal contents of WT mice fed the GMP diet there was a significant reduction in Desulfovibrio compared to WT mice fed the casein diet, p = 0.001. PKU mice fed the GMP diet had a significant reduction in Desulfovibrio compared to PKU mice fed the AA diet, p = 0.001.
Figure 6. Cecal bacterial communities clustered using principal coordinates analysis (PCoA) of the unweighted UniFrac distance. Each point corresponds to a cecal sample from an individual mouse colored according to genotype and diet. PC1 and PC2 are plotted on x and y axes with percentage of variation explained in the parentheses. All 4 groups are circled to look at group effects of diet (A) and GMP diet separated from the casein and AA diets (B).

Figure 7. Cecal concentrations of the short chain fatty acids (SCFA) acetate (A), propionate (B) and butyrate (C) in WT mice fed casein and GMP diets and in PKU mice fed AA and GMP diets from weaning through 8-9 weeks of age. Values are means + SE, n=6-8.

Figure 8. Plasma cytokine concentrations for: interferon-gamma (IFN-γ, A), tumor necrosis factor-alpha (TNF-α, B), interleukin 1-α (IL-1α, C), interleukin 1 β (IL-1β, D), interleukin-2 (IL-2, E), and interleukin-10 (IL-10, F) in PKU mice fed AA and GMP diets and in WT mice fed casein and GMP diets from weaning through 8-9 weeks of age. Values are means + SE, n=6-8.

Figure 9. Spleen cells were stimulated with PMA and ionomycin and were collected from both WT and PKU mice fed either the casein or GMP diet. (A) Cells were gated on CD8 and plots are representative of mice fed either the Casein or GMP diets. Histogram shows mean ± SE percentage of CD8⁺ CD44^hi^CD62L^lo^ cells. (B) Percentage of CD8⁺ cells producing IFN-γ detected by intracellular cytokine staining.
Glycomacropeptide (GMP) is derived from K-casein in bovine milk. Chymosin added for cheese manufacture.

Whey Protein - GMP comprises 20-25% of whey proteins.

Calcium Phosphate

Casein Micelle

Glycomacropeptide – 64 amino acid chain

Mucin – type carbohydrate chains

- Sialic acid
- N-Acetyl-Galactosamine
- Galactose

Glycosylation sites
Figure 2

A

Relative Spleen Mass (g / 100g Body Weight)

0.21
0.24
0.27
0.30
0.33
0.36
0.39
0.42
0.45

WT PKU

B

Relative Spleen Mass (g / 100g Body Weight)

0.21
0.24
0.27
0.30
0.33
0.36
0.39
0.42
0.45

Casein AA GMP

p < 0.0001

p < 0.0001
Figure 3

**A**

Jejunum Mass (mg/cm)

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**B**

Jejunum Mass (mg/cm)

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**C**

Jejunum Mucosa Dry Mass (mg/cm)

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**D**

Jejunum Mucosa Dry Mass (mg/cm)

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Figure 5

A

Casein

WT

GMP

PKU

AA

B

Casein

WT

GMP

PKU

AA

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Figure 6.

A.

B.
Figure 7

A

Butyrate (mM)

0.0 0.2 0.4 0.6 0.8 1.0 1.2 1.4 1.6

AA GMP

Casein GMP

WT PKU

p = 0.0619

p = 0.0439

B

Propionate (mM)

0.0 0.4 0.8 1.2 1.4

AA GMP

Casein GMP

WT PKU

p = 0.0219

p = 0.0587

C

Acetate (mM)

0 1 2 3 4 5 6 7 8 9 10

AA GMP

Casein GMP

WT PKU

p = 0.0268

p = 0.0219

p = 0.0861

p = 0.0587

p = 0.0619

p = 0.0439
Figure 8

A. IFN-γ (pg/ml) with p = 0.0110
B. TNF-α (pg/ml) with p = 0.0105
C. IL-1α (pg/ml) with p = 0.0153
D. IL-1β (pg/ml) with p = 0.0431
E. IL-2 (pg/ml) with p = 0.0481
F. IL-10 (pg/ml) with p = 0.0329
Figure 9

A.

Casein GMP

CD8+ CD44hi/CD62Llo cells (%)

p < 0.0001

CASEIN GMP

CD8+ IFN-γ cells (%)

p = 0.0015

B.