Casein glycomacropeptide is well tolerated in healthy adults and changes neither high-sensitive C-reactive protein, gut microbiota nor faecal butyrate

a restricted randomised trial

Wernlund, Pernille G; Hvas, Christian L; Dahlerup, Jens F; Bahl, Martin Iain; Licht, Tine Rask; B Knudsen, Knud E; Agnholt, Jørgen S

Published in:
The British Journal of Nutrition

Link to article, DOI:
10.1017/S0007114520003736

Publication date:
2021

Document Version
Peer reviewed version

TITLE PAGE

Casein glycomacropeptide is well tolerated in healthy adults and changes neither high-sensitive C-reactive protein, gut microbiota nor faecal butyrate: a restricted randomised trial

Authors

Pernille G Wernlund,[1,2] Christian L Hvas,[1] Jens F Dahlerup,[1] Martin I Bahl,[3] Tine R Licht,[3] Knud E B Knudsen,[4] and Jørgen S Agnholt.[1]

Author Affiliations

[1]*Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark.*

[2]*Department of Clinical Medicine, Aarhus University, Aarhus, Denmark.*

[3]*National Food Institute, Technical University of Denmark, Denmark.*

[4]*Department of Animal Science, Aarhus University, Tjele, Denmark.*

Corresponding author

Pernille Glahn Wernlund, MD, Aarhus University Hospital, Department of Hepatology and Gastroenterology, Palle Juul-Jensens Boulevard 99, DK-8200 Aarhus N, Denmark. Tel +4578453820, Email pernillewernlund@clin.au.dk

This peer-reviewed article has been accepted for publication but not yet copyedited or typeset, and so may be subject to change during the production process. The article is considered published and may be cited using its DOI
10.1017/S0007114520003736
The British Journal of Nutrition is published by Cambridge University Press on behalf of The Nutrition Society

Short running title

Glycomacropeptide for healthy adults

Keywords

Glycomacropeptide, milk protein, whey protein, healthy adults, bioactive peptides, anti-inflammatory, immunomodulation.

Abbreviations

ANCOM, analysis of composition of microbiomes; ASV, amplicon sequence variant; BMI, body mass index; CGMP, casein glycomacropeptide; CRP, C-reactive protein; hsCRP, high-sensitive C-reactive protein; PCoA, principal coordinates analysis; PKU, phenylketonuria; REDCap, Research Electronic Data Capture; SD, standard deviation.

Word counts: abstract, 227 words; main text, excluding references and legends, 5847 words.

ABSTRACT

Casein glycomacropeptide (CGMP) is a bioactive milk-derived peptide with potential anti-inflammatory effects. Animal studies suggest that CGMP may work by altering gut microbiota composition and enhancing butyrate production. Its effects on intestinal homeostasis, microbiota and metabolites in humans are unknown. The aim of the present study was to assess both the intestinal and systemic immunomodulatory effects of orally ingested CGMP. We hypothesised that a daily oral CGMP intake would reduce high-sensitive C-reactive protein in healthy adults. In a single-centre limited but randomised, double-blinded, reference-controlled study, we compared the effects of a four-week intervention of either 25 grams of oral powder-based chocolate-flavoured CGMP or a reference drink. We included twenty-four healthy adults who all completed the study. CGMP had no systemic or intestinal immunomodulatory effects compared with a reference drink, either with regard to high-sensitive C-reactive protein, or faecal calprotectin level, faecal microbiota composition or faecal short-chain fatty acid content. CGMP ingestion did not affect satiety or body weight, and it caused no severe adverse events. The palatability of CGMP was acceptable and adherence was high. CGMP did not induce or change gastrointestinal symptoms. In conclusion, we found no immunomodulatory effects of CGMP in healthy adults. In a minor group of healthy adults, oral ingestion of 25 grams of CGMP during four weeks was safe, well tolerated, had acceptable palatability, and was without any effects on body weight.

INTRODUCTION

Casein glycomacropeptide (CGMP), a milk-derived protein, has been recognized as a bioactive peptide with immunomodulatory properties.[1] Bioactive peptides are defined as peptide sequences with a beneficial effect on body functions beyond their known nutritional value.[2] This effect may stem from direct impacts on the gastrointestinal tract via receptors and cell signalling in the gut or may, less likely, arise from absorption of the peptides into the systemic circulation.[3] Certain milk-derived peptides exert multifunctional properties such as anti-thrombotic, anti-microbial, anti-oxidant, opiate, and immunomodulatory effects.[3-6] Directly applied, they may modify the gut microbiota.[7,8]

Extensive investigation of the gut microbiome during the past decade has paved the way for a deeper understanding of the interaction between commensal bacteria in the colon and human health.[9,10] Specific patterns of gut microbiota composition have been associated with the development and clinical course of several diseases, such as diabetes, obesity, inflammatory bowel disease, and rheumatic arthritis.[11] Even though a concise definition of a healthy microbiota composition does not yet exist, there is some consensus as to which phyla are considered beneficial for intestinal homeostasis and which are not.[12,13]A proxy for a healthy gut microbiota is the relative amount of the short-chain fatty acid butyrate – the common understanding being, the more butyrate the better.[10,14,15]

Different diets are associated with different microbiota compositions.[16,17] Dietary changes may therefore be a feasible way to manipulate intestinal microbiota to achieve health effects. In children with Crohn's disease, a polymeric diet improves disease activity equally to corticosteroid treatment,[17] and plant-based meals may increase beta cell function in type 2 diabetes.[18]

Bovine CGMP is a small peptide weighing approximately 7 kDa and containing 64 amino acid residues. It is enzymatically cleaved from kappa-caseins in milk during cheese production.[1] CGMP lacks both sulphur-containing and aromatic amino acids. However, commercial CGMP preparations comprise a small residual amount of aromatic amino acids, including phenylalanine. Due to its special amino acid composition, it has been suggested as a potential nutritional supplement for patients with phenylketonuria (PKU) under close monitoring of blood phenylalanine.[19,20]

A rodent and two human cell studies found that CGMP may potentially decrease enteric infections by reducing the cell adherence of cholera toxin, *Salmonella* Typhimurium, *Shigella flexneri*, and both enterohaemorrhagic and enteropathogenic *Escherichia coli*.[21-24] Thereby, CGMP may limit bacterial invasion. In other cell-based *in vitro* studies and rodent models of colitis, CGMP has prebiotic and anti-

inflammatory effects.[25-31] In piglets, CGMP has caused the number of lactobacilli as well as the relative amount of butyrate to increase.[32] A human study in healthy term infants suggested that CGMP and alpha lactalbumin, also a bioactive milk-derived peptide, may cause the intestinal microbiota to evolve more similarly to that of breast-fed infants than would a standard infant formula.[33] Another infant trial found that CGMP and alpha lactalbumin promotes the maturation of the adaptive immune system and a delayed involvement of the innate immune system.[34] In human *ex vivo* settings, CGMP may exert anti-bacterial and anti-cariogenic effects by reducing counts of *Streptococcus mutans* in dental plaque samples from healthy children.[35] *In vitro* studies reached similar conclusions.[36,37]

Besides the above mentioned human trials, CGMP has been investigated in – unsuccessful – attempts to induce satiety.[38,39] One clinical study assessed the potential of using orally ingested CGMP as an anti-inflammatory agent and found that the addition of CGMP to maintenance treatment in patients with clinically active distal ulcerative colitis had clinical effects comparable to those achieved by increasing the usual first choice of medical treatment, mesalazine, (in doses between 1600-3200 mg) to maximum dose (4800 mg per day).[40] Little is known about the clinical effect of CGMP on intestinal homeostasis, systemic inflammation, and gastrointestinal symptoms.

The aim of the present study was to investigate the immunomodulatory effects of orally ingested CGMP in healthy adults. We hypothesised that oral intake of CGMP would decrease intestinal and systemic inflammation compared with the intake of a reference drink.

SUBJECTS AND METHODS

Study design

This was a single-centre randomised, double-blinded, reference-controlled study, conducted in healthy adults. The study interventions were oral intake of powder-based chocolate-flavoured CGMP or a reference-drink during four weeks. A crossover design was deselected because it would compromise blinding due to the different amounts of powder in the CGMP and reference sachets. The duration of the study was decided based on careful considerations. A pilot study conducted in patients with ulcerative colitis, observed anti-inflammatory clinical effects of CGMP after four weeks.[40] Studies of dietary-induced CRP changes reported CRP decreases after two to three weeks.[41,42] Regarding the impact of dietary intervention on microbiota composition, studies found changes after five days to four

weeks of intervention.[15,43,44] Consequently, we considered a four week intervention period most optimal.

Study subjects

We included twenty-four healthy Caucasians aged between 18 and 60 years. They were assessed at the Department of Hepatology and Gastroenterology, Aarhus University Hospital, Denmark from June 2016 to June 2017.

Inclusion criteria were body mass index (BMI) of 18-25 kg/m^2 and absence of lactose intolerance, milk protein allergy, and chronic disease (ulcerative colitis, Crohn's disease, coeliac disease, rheumatoid arthritis, autoimmune arthritis, psoriasis, diabetes, or multiple sclerosis). We excluded subjects with prior resection of the intestine (apart from the appendix) and those who had been admitted to hospital, had been taking antibiotics, had experienced diarrhoea, or had had bloody stools three months prior to inclusion. We also excluded pregnant and nursing women as well as subjects who did not understand or speak Danish. The participants answered a health status questionnaire prior to inclusion.

The twenty-four subjects were randomised 1:1 to either CGMP or a reference-drink. The randomisation list was produced on www.randomization.com and attained by a third party, the Hospital Pharmacy Aarhus, Aarhus University Hospital. Treatment was blinded for both study participants and investigators.

Study interventions

Study powders were pre-packed in daily portions. Participants dissolved the powder in approximately 250 ml of water, shook it to homogenise, and stored it in the refrigerator for 15 minutes to optimise its taste. The CGMP used was Lacprodan® CGMP-20 provided by Arla Foods Ingredients Group P/S (Viby J, Denmark) produced with chocolate flavour. Arla Foods Ingredients also produced the reference powder. Table 1 shows the ingredients of the study interventions. A daily portion of CGMP powder comprised 25 g of 95% pure CGMP. CGMP is enzymatically released from kappa-casein and consists of the 64 amino acids in the carboxy-terminus. Kappa-casein has several genetic variants but in bovine CGMP mainly variant A and B are present. These two variants differ by two amino acids. CGMP is rich in proline, glutamine, serine, isoleucine and threonine, but deficient in the aromatic

amino acids, arginine, cysteine, and histidine. Post-translationally, the peptide is both glycosylated and phosphorylated. Glycosylation involves the sugars, sialic acid, galactosyl, and N-acetylgalactosamine, which are present as mono-, di-, tri-, or tetrasaccharides. Due to the different modifications CGMP is quite heterogeneous.[1,45] The reference-drink consisted of 15 g of skimmed milk powder and flavourings. Similarity of taste and texture was optimised to secure participant blinding. Due to the significant difference in protein and energy amount, and overall weight of the daily CGMP and reference intervention (Table 1), the participants received the intervention sachets from unblinded study personnel to secure investigator blinding.

Outcome measures

The primary outcome was a decrease in hsCRP in the CGMP group compared to the reference group. Secondary outcomes comprised a shift in microbiota composition towards higher alpha-diversity and a higher proportion of butyrate-producing organisms in the CGMP group. We also anticipated CGMP to reduce any present intestinal symptoms. Outcome measures were assessed after 4 weeks.

Data collection and recording of symptoms

Study data were collected and managed using the Research Electronic Data Capture (REDCap) tools hosted at Aarhus University (www.redcap.au.dk). REDCap is a secure, web-based application designed to support data capture for research studies.[46] Participants filled out questionnaires online on medicine use, smoking status, alcohol consumption, physical activity, depression, intestinal symptoms, and blinding of interventions. The investigators also filled out questionnaires on blinding at all post-randomisation visits.

Each week, participants received a link by email and filled out an online questionnaire about their daily intake of the study drink. They were asked whether their daily study drink intake was 0, 25, 50, 75, or 100%. The palatability of the study products was assessed at the end of the intervention period, and participants were asked to rate the taste on a scale from awful (0) to excellent (100). Dietary habits were screened on three consecutive days before the intervention started and on three consecutive days during the last week of the study period by use of a dietary assessment questionnaire. The ingredients in the study products are included in the analysis of dietary intake. Participants' height was measured at baseline. Their weight was assessed at both baseline and after 4 weeks using the same equipment and

standardised according to clothes and no footwear in order to minimize inaccuracies. Participants fasted overnight and were weighed the following morning. Adverse events were evaluated after 4 weeks or if the subjects contacted the investigators because of study drinks side effects.

Blood samples

Venous blood samples were drawn and analysed for hsCRP, leukocyte count, and albumin at baseline and after 4 weeks. Plasma samples were cryopreserved for later analysis. High-sensitive CRP analysis was done on an ADVIA Chemistry XPT System (Siemens, Erlangen, Germany) and ranged from 0•2 to 200 mg/l. The Chemistry XPT labels the samples with the lowest possible outcome as "below 0•2 mg/l." Those samples were truncated to 0•1 mg/l in order to be able to run relevant statistics. A value below 3 mg/l is considered as normal.

Plasma cytokines

Plasma cytokines were analysed using a BD Cytometric Bead Array (BD Biosciences, Franklin Lakes, NJ, USA) and a MACSQuant Analyser 10 (Miltenyi Biotec, Bergisch Gladbach, Germany). We used the Human Inflammatory Cytokines Kit (cat.no. 551811) to examine the cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α. Plasma samples were prepared and analysed according to the manufacturer's instruction. In order to lower the detection level to 2•5 pg/ml, we conducted three additional dilutions to the standard curve. The rationale for investigating these specific cytokines were, that they are part of inflammatory processes in general, and especially IL-1β, IL-6, and TNF-α are also linked to low grade inflammation[47] and hence could be of interest in assumable healthy adults.

Faecal samples

Data on faecal samples, 24-hours faeces wet-weight, and faecal consistency were obtained at baseline and after 4 weeks. Faecal consistency was assessed by the subjects themselves using the Bristol stool scale.[48] To obtain the 24-hours faeces wet-weight, the subjects were provided with a faecal collection device and asked to weigh their faeces throughout 24 hours using an extradited weight. After the weighing procedure, three containers were filled and immediately stored at -20°C. Within 48 hours, they were moved to the study lab. Without thawing, they were divided into smaller containers appropriate for analysis and stored at -80°C.

Faecal calprotectin

Faecal calprotectin was analysed using a second-generation EliA Calprotectin 2 test (Thermo Fischer, Waltham, MA, USA) with a range from 4 to 6,000 mg/kg faeces. This analysis is part of the clinical routine analysis performed at the Department of Clinical Biochemistry, Aarhus University Hospital, Denmark. Values below 50 mg/kg faeces are considered normal.

Microbiota - Extraction of DNA and amplicon library preparation

Faecal samples were stored at -80^{o}C until DNA extraction. Community DNA was extracted by using the MoBio PowerLyzer® Power Soil® DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA) according to the manufacturer's recommendations with approximately 100 mg material per sample. DNA concentrations were measured fluorometrically with the Qubit dsDNA HS kit (Life Technologies, Carlsbad, CA, USA). The bacterial community composition was determined by amplification and sequencing of the V3-region of the 16S ribosomal RNA gene using the Ion Torrent PGM platform (Life Technologies) as previously describe.[49] Briefly, the V3-region of the 16S rRNA gene was amplified using a universal forward primer (PBU 5'-A-adapter-TCAG-barcode-CCTACGGGAGGCAGCAG-3') with a unique 10–12-base-pairs barcode for each bacterial community (IonXpress barcode as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5'-trP1-adapter-ATTACCGCGGCTGCTGG-3'). Polymerase chain reaction products were purified using the MAGBIO HigPrep™ PCR-96-well protocol according to the manufacturer's recommendations. DNA concentrations were determined with the Qubit HS assay (ThermoFisher Scientific, Carlsbad, CA, USA). Amplicon libraries were constructed by mixing equal amounts of PCR products from each original community. Sequencing was performed on an Ion Personal Genome Machine® (PGM™, ThermoFisher Scientific) using Ion PGM Hi-Q kit, 200bp sequencing and Ion 318™ Chip.

Microbiota - Bioinformatics

Sequence data in FASTQ format were initially processed in a CLC Genomic Workbench (version 8.5, Qiagen, Aarhus, Denmark) in order to de-multiplex and remove sequencing primers, retaining reads only if both forward and reverse primers were correctly identified with 100% homology as previously described.[49] Next, The DADA2 version1.12.1 pipeline[50] incorporated in RStudio[51] was used to

generate an amplicon sequence variant (ASV) table with taxonomy assigned against the RDP database (rdp_train_set_16). The MaxEE parameter was set to 2 and all samples were pooled for sample inference. Further downstream processing was performed in QIIME2.[52] The ASV table was filtered to include only ASVs classified as bacteria. We excluded the Cyanobacteria/Chloroplast group as well as ASVs with a total abundance less than 20 across all samples and samples with less than 7730 reads in total (3 samples). This yielded total 650 ASVs in 45 samples with a median read depth of 11,679 (range 7,735-47,898). A rooted phylogenetic tree was generated with the function qiime phylogeny align-to-tree-mafft-fasttree after which the qiime diversity core-metrics-phylogenetic pipeline was run to assess alpha-diversity (Shannon index and number of observed ASVs), beta-diversity (principal coordinates analysis (PCoA) plots based on weighted and un-weighted UniFrac distances) as well as relative abundance distributions at different taxonomic levels. Sampling depth was set at 7,730 reads. Differential abundance testing at the ASV level was performed with analysis of composition of microbiomes (ANCOM) analysis implemented in QIIME2.[53]

Short-chain fatty acids

Concentrations of faecal SCFA and other acids including lactic acid were determined by gas-liquid chromatography (HP-6890 Series, Hewlett Packard Enterprise, Palo Alto, CA, USA).[54] The total SCFA concentration was calculated as the sum of the formic acid, acetate, propionate, isobutyrate, butyrate, isovaleric, and valeric acid concentrations. The branched-chain fatty acid concentration was calculated as the sum of the isobutyrate and isovaleric acid concentrations. We calculated the amount of 24-hour acid excretion by multiplying the 24-hour faeces weight and the acid concentration. In the statistical analysis, we use the amount of different SCFA.

Ethics statement

This study was conducted according to the guidelines in the Declaration of Helsinki and all procedures involving human subjects were approved by the Central Denmark Region Committee on Health Research Ethics (journal no. 1-10-72-369-15, 2 March 2016). All the participants gave written informed consent to participation. The study was registered at www.clinicaltrials.gov with study identifier NCT02832700.

Statistics

The number of participants was estimated based on a presumption of finding a mean high-sensitive C-reactive protein (hsCRP) of approximately 2•8 mg/l in this group of healthy adults who were not biochemically screened prior to inclusion.[55] Other studies found standard deviation (SD) of 1•6-1•8 in healthy cohorts.[56] We regarded a variation in hsCRP of 2•3 mg/l as the minimal clinically important difference. In order to achieve a power of 80% (type 2 error of 0•2) and a type 1 error below 0•05, it was calculated that a total of 20 participants (ten participants in each group) was needed[57] Consequently, we planned to include twenty-four individuals to have a small margin in case of up to 15% drop-outs.

Descriptive statistics are expressed as medians and range. Non-paired data were compared with the two-tailed unpaired t-test. If data did not show a Gaussian distribution, they were log-transformed to obtain this. If this was not achievable, the non-parametric Wilcoxon ranksum test was used. In the case of paired samples, model validation, by inspection of Bland-Altman plots and probability plots of the residuals, was performed before using the two-tailed paired t-test. (This does not apply for the microbiota analysis.) If criteria were not met, the Wilcoxon signed-rank test was applied. Dichotomous data were analysed with Fischer's exact test. We considered a two-tailed p-value below 0•05 as significant. STATA/IC 14.2 (StataCorp, College Station, Texas, USA) and GraphPad Prism 8.3.0 (Graph Pad Software, Inc., San Diego, California, USA) were used to perform the statistical analysis. The illustrations are made in Graph Pad Prism 8.3.0 (Graph Pad Software, Inc., San Diego, California, USA).

RESULTS

Study population

Baseline characteristics for the study participants are summarized in Table 2.

We screened twenty-eight individuals and included twenty-four healthy adults. The flow chart of the study process is shown in Figure 1. Due to difficulties finding lean male participants, we decided to include males with a BMI up to 30 kg/m^2. Subsequently, we recruited four male participants with a BMI between 25•5 and 29•0 kg/m^2, all of whom by chance were randomised to the reference group. The mean BMI was statistically significantly lower in the CGMP group than in the reference group.

The median age of the participants was 35 years in the CGMP group and 36 years in the reference group (P=0•40). The age span ranged from 30 to 51 years in the CGMP group, and from 24 to 59 years in the reference group.

All participants received the interventions and completed the study. The primary and secondary outcomes were analysed according to the originally assigned intervention. One participant in each intervention group had a hsCRP above 3 mg/l at baseline (9•8 mg/l in the CGMP group and 4•1 mg/l in the reference group). All other biochemical values were within normal values for healthy adults, except for one participant in the reference group who had a faecal calprotectin of 211 mg/kg faeces at baseline, probably due to a recent upper airway infection. Plasma concentrations of the measured cytokines were all below detection limit at baseline.

The mean daily intake of protein and energy per kg body weight was higher in the CGMP group at baseline than in the reference group (Table 3). Intakes of fibres, cereals, and yoghurt were not statistically significantly different between the groups at baseline (Table 4). None of the participants were vegans, vegetarians, or using probiotic supplements besides the intake of yoghurt at any time during the study period. The composition of the gut microbiota did not differ between the groups at baseline. We found no differences in Shannon indices between the CGMP group (5•4 (95% CI: 5•0, 5•8) and the reference group (5.3 (95% CI: 4•9, 5•7) at baseline (P=0•69). The mean number of ASVs did not differ between the CGMP group (191 (95% CI: 154, 229)) and the reference group (185 (95% CI: 155, 215)) (P=0•77) at baseline.

Local and systemic inflammation markers

In the CGMP group, we found a median hsCRP of 0•7 (range: 0•1, 8•6) mg/l at the end of the study period. In the reference group, the median was 0•4 (range: 0•1, 3•1) mg/l at study end (P=0•82). HsCRP did not change from baseline to the end of the study period in either the CGMP (P=0•27) or the reference group (P=0•93) (Table 5). The median leukocyte count was 5 (range: 3, 10) $\cdot 10^9$/l in the CGMP and 5 (range: 3, 7) $\cdot 10^9$/l in the reference group. No difference was found between the groups (P=0•98). We found no changes from baseline to week 4 in either the CGMP (P=0•65) or the reference group (P=0•25). The median faecal calprotectin was 29 (range: 6, 84) mg/kg faeces in the CGMP group and 29 (range: 3, 47) mg/kg faeces in the reference group (P=0•48) at study end. No

differences were found between the groups concerning albumin (P=0•77) at study end. We found no changes from baseline to week 4 within either faecal calprotectin or albumin (data not shown).

Plasma cytokines

Cryopreserved plasma samples were available from all participants at baseline and week 4. Plasma concentrations of the cytokines, IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α were all below the detection limit both at baseline and in the end of the study period.

Microbiota

The alpha diversity expressed as mean Shannon index at week 4 in the CGMP group (5•4 (95% CI: 5•1, 5•8) was not different from that of the reference group (5•2 (95% CI: 4•8, 5•6) (P =0•36, unpaired t-test). Neither within the CGMP group (P=0•82, paired t-test) nor within the reference group (P=0•64, paired t-test) did we find any temporal change in Shannon index from baseline to week 4. Similarly, the bacterial richness expressed as mean number of observed ASVs was not different at week 4 in the CGMP group (202 (95% CI: 169, 234)) and the reference group (185 (95% CI: 153, 217)) (P=0•42, unpaired t-test), and no change over time was observed either in the CGMP group (P=0•11, paired t-test) or in the reference group (P=0•98, paired t-test). The beta-diversity was visualized by PCoA plots based on weighted (Figure 2) and un-weighted UniFrac distances. Analysis of similarities (ANOSIM) based on both the weighted and un-weighted UniFrac distance matrices showed no significant difference between the treatment groups at baseline and week 4, nor temporal changes within the groups from baseline to week 4 (P>0•25 in all comparisons). Analysis of composition of microbiomes (ANCOM) at the ASV level showed no significant differences between the CGMP and reference groups at week 4 or at baseline.

Short-chain fatty acids

We found no differences in either butyrate or total SCFA between the groups at baseline or at study end and no changes within the two intervention groups during the study (Figure 3A). Although we observed a statistically significant drop in faecal valerate in the CGMP group during the study period, there was no difference between valerate in the two study groups at week 4 (Figure 3B). Within both the CGMP and the reference group, isobutyrate (Figure 3C) and branched-chain fatty acids (data not shown) did

fall from baseline to study end, but there were no differences between the groups. We found no differences in the other acids, either between or within the groups (data not shown).

Clinical changes

The participants in the CGMP group significantly increased their daily intake of protein during the study (P=0•005) (Table 3, Figure 4A). At the end of the study period, the daily mean intake of protein per kg body weight was higher in the CGMP group than in the reference group (P=0•003). In the CGMP group, the intake of the intervention added 25•2 g (95% CI: 12•9, 37•6) to the mean daily total protein dietary intake, independent of body weight. In the reference group, the addition was 5•2 g (95% CI: -5•5, 15•9). The mean daily intake of energy per kg body weight did not differ between the CGMP and the reference group at the end of the study period (Table 3). No changes in energy intake occurred during the study (Table 3, Figure 4B). This indicates that CGMP consumption led to reduced consumption of other foods. The median fibre, cereals, and yoghurt intake did not differ between the CGMP and the reference group at study end and no temporal changes occurred between baseline and week 4 in the CGMP or the reference group (Table 4). Despite the increased intake of protein in the CGMP group, we found no changes in the participants' body weight during the study (Figure 4C). We found no difference in weight between the CGMP group (67•3 (95% CI: 61•0, 73•6 kg) and the reference group (76•7 (95% CI: 65•9, 87•4 kg) (P=0•10) at the end of the intervention period.

Intestinal symptoms such as abdominal pain, rumbling, nausea, passage of gas, and bloating were recorded both before and during the intervention. We found no differences in either group between before and at the end of the intervention period or between groups at the end of the intervention period (data not shown). Concerning defecation urge, mucus in stools and incomplete emptying, we found no differences between the groups (Figure 5). One person in the CGMP group reported occasional blood in the stools. We did not suspect that to be related to intake of CGMP. Furthermore, the participant had a normal faecal calprotectin at study end, making it unlikely that there was blood in the stools at that time.

We screened the participants' level of physical activity and found them to be moderately active.[58] Evaluated by Becks Depression Score,[59] none of the participants had depressive symptoms. There was no difference between the groups concerning physical activity and depression, and no changes occurred during the study (data not shown).

Perception and adherence to study products

At the end of the study, we assessed the participants' perception of the study drink. In the CGMP group, palatability was found to be acceptable (54 (range: 7, 93) median score), while the reference drink was found significantly more palatable (78 (range 48, 100) median score) (Figure 6) (P=0•02). Daily adherence was documented once a week. Adherence data were available for 87% of the days in the CGMP group and for 88% in the reference group. Adherence was 97% in both the CGMP group (97 (95% CI: 92, 100) %) and the reference group (97 (95% CI: 82, 100) %) (P=0•59).

The blinding of the participants was investigated using Fischer's exact test. In the CGMP group, three participants (25%) thought they received CGMP, nine (75%) that they received the reference drink. In the reference group, six participants (55%) thought they received CGMP, five (45%) that they received the reference drink. This difference was not statistically significant (P=0•21), indicating a successful blinding. Two participants from the CGMP group reported mild side effects during the intervention period. One experienced more belching of gas than usual throughout the 4 weeks; the other felt epigastric discomfort shortly after consuming the drink during the whole study period. No adverse or severe adverse events were reported.

DISCUSSION

This is the first study to assess the potential immunomodulatory effects of orally ingested CGMP in healthy adults. A daily intake of 25 g of CGMP during four weeks caused no weight changes and was found to be safe and well tolerated. We demonstrated no decrease in systemic inflammation markers evaluated by blood hsCRP and leukocyte count. We analysed the faecal calprotectin levels, the faecal microbiota, and SCFA, and we observed no local gastrointestinal immunomodulatory effects. We conclude that in healthy participants, CGMP neither diminished gastrointestinal symptoms, e.g. incomplete emptying, nor had any severe side effects.

These clinical results are in line with those of previous studies reporting that CGMP did not change satiety or body weight compared with skimmed milk powder or whey proteins.[38,40,60,61] In the CGMP group, we observed a significant increase in protein intake due to the intervention. Since it was not accompanied by a reduction in overall energy intake, we do not consider it to represent a satiety inducing effect but simply a replacement of one or more food items with CGMP. Conclusively, a daily intake of 25 g of CGMP shows no effect on satiety or body weight. Despite the increased daily protein intake in

the CGMP group, we found no effects on the microbiota composition. A study in athletes found a negative effect of protein supplementation on microbiota composition,[62] The supplement consisted of whey isolate and beef hydrolysate, which suggest that the source of protein may play an important role. Future studies are needed to elucidate this area since the source of protein may play an important role in altering gut flora.

No previous study has investigated the anti-inflammatory effects of CGMP in healthy adults. In a prior study in patients with distal ulcerative colitis, CGMP had potential anti-inflammatory effects in the colon.[40] However, the healthy adults included in the present study had no signs of local or systemic inflammation, and the intervention did not change the levels of hsCRP.

The findings of the present study are partly in agreement with those of an earlier in vitro study investigating the prebiotic potential of CGMP in an artificial colon model of elderly persons.[63] Regarding the SCFA production, no changes were found in either of the two studies.

The choice of reference intervention may have affected our results if the reference drink possesses either anti- or pro-inflammatory effects. Clinical studies of proteins' effect on gastrointestinal inflammatory parameters are sparse. Since protein gut fermentation generates potentially harmful metabolites such as ammonia, phenols and hydrogen sulphide,[64] we deliberately avoided a protein dense fraction for comparison. To secure blinding, we needed to achieve a texture not easily distinguishable from that of protein, which is difficult with a saccharide-based drink. Furthermore, we anticipated that a saccharide like maltodextrin would induce intestinal side effects such as bacterial overgrowth[65] and thereby confound our findings. In an attempt to pick the lesser of two evils, we chose a drink of skimmed milk powder with only a small amount of protein, but enough to ensure a protein-like texture. We chose not to make the reference drink isoenergetic in order to avoid too many disaccharides and the relatively high glycaemic index that these disaccharides possess.

Our study has important limitations. According to our records of dietary intake, the fibres, cereals, and yoghurt intake did not differ between the two groups but the CGMP group did have a slightly higher average intake of protein and energy at baseline compared to the reference group. The protein intake continued to differ throughout the study period. This difference may very well affect our results and conceal any bowel protective effects of CGMP, since the CGMP group, due to a higher intake of potentially damaging protein, may have had a worse starting point than the reference group, even though we were not able to objectify it. Our study lacks control of the participants' everyday diet

during the study period. Even though we screened the participants' dietary intake both before and at the end of the intervention period, we cannot be certain that the reported diet reflects the actual intake, which changes with weekdays, holidays, etc. These obstacles may have been avoided or their impact minimised, if the diet for the participants was supplied during the study period and the precise amount taken by each participant was measured. Furthermore, the small sample size might have led us to overlook important differences of clinical interest. A larger number of participants or alternatively a cross over study design may have revealed differences in microbiota composition between groups. A cross over design may as well have abated the impact of individual every day diets.

Selection bias may have affected our results if the higher mean BMI in the reference group at baseline reflects a higher level of systemic inflammation as seen in obese individuals.[66] Two persons in the reference group with a BMI of 27.5 and 29.0 kg/m^2, respectively, mainly drive the difference in mean BMI. Because none of our participants were obese, defined as having a BMI at or above 30 kg/m^2, we do not expect that obesity-related inflammation has affected our results.[67] On the other hand, the CGMP group had a higher mean intake of both protein and energy at baseline, which may make them more prone to be inflamed than the reference group. Importantly, none of the inflammation markers were increased in any of the groups.

Even though the age medians are not statistically significantly different between the groups, the participants cover a relatively wide age span (30 to 51 years in the CGMP group and 24 to 59 years in the reference group). This may affect the representativeness of our results, especially with regard to the microbiota composition analysis, since differences in BMI and age are associated with differences in alpha-diversity and other microbiota compositional parameters.[68-71] Potentially, the small sample size in the present study in addition to the BMI differences and vast age span may have blurred the results and caused us to miss CGMP induced differences in microbiota composition.

Some protein sources, such as red and processed meat, may be associated with an increased risk of developing disease, e.g. colorectal cancer and coronary heart disease.[72,73] Safe protein sources that may be recommended to the public to enhance health are therefore needed. CGMP may be such an alternative. Patients with PKU depend on a specific combination of amino acids; at the same time, they seek products with a higher palatability than can be obtained with amino acid-based nutrition. In this regard, CGMP is a sensible option because of its amino acid composition and palatability, though it has to be applied under surveillance of blood phenylalanine.[20,74,75] In the future, a safe, palatable protein

with anti-inflammatory potential might be of benefit in patients with various degrees of intestinal inflammation, e.g. patients with metabolic syndrome or inflammatory bowel disease. Recently, CGMP has been found to exert anti-oxidative and anti-inflammatory effects in a cell model mimicking the oxidative stress, and low-grade inflammation that are characteristics of metabolic syndrome.[76]

In conclusion, the present study supports earlier findings that CGMP is safe and well tolerated and has an acceptable palatability and no effects on satiety and body weight. We found neither immunomodulatory effects nor effects on markers of intestinal immune homeostasis in healthy subjects with no signs of inflammation. Thus, CGMP may be ingested without severe gastrointestinal side effects. As a consequence, we perceive these findings to be useful in relation to healthy adults and to the management of some diseases, for instance PKU, and to the investigation of effects in patients with active inflammation. The results of the present study do not exclude that CGMP may have anti-inflammatory effects in healthy adults but this needs further investigation. Due to the rather small sample size in the present study, more *in vivo* studies of CGMP are warranted to assure a valid evaluation of its potential.

ACKNOWLEDGEMENTS

The authors thank Mette Mejlby Hansen in the research lab of the Department of Hepatology and Gastroenterology, Aarhus University Hospital, Aarhus, Denmark for tireless support, and Marlene Danner Dalgaard and Neslihan Bicen at the DTU Multi-Assay Core facility for performing the 16S rRNA sequencing.

FINANCIAL SUPPORT

Funding was provided by grants from the Novo Nordisk Foundation [NNF16OC002314], the Danish Colitis Crohn Foundation [no grant number]. and Arla Foods Ingredients [no grant number]. Arla Foods Ingredients provided the study test products. Arla Foods Ingredients had no role in the design, analysis, or writing of this article.

CONFLICT OF INTEREST

Arla Foods Ingredients financed two thirds of PGW's PhD-salary. Arla Foods Ingredients had no influence on the study design; collection, analysis, and interpretation of data, writing of the report or on the decision to submit the report for publication; The other authors declare no conflicts of interest.

AUTHORSHIP

JFD, JSA, CLH, and PGW designed and conducted the research, wrote the manuscript, and had primary responsibility for its final contents. MIB, TRL, and KEBK provided essentials. All authors analysed the data or performed statistical analyses and read and approved the final manuscript.

REFERENCES

1. Brody EP (2000) Biological activities of bovine glycomacropeptide. *Br J Nutr* **84 Suppl 1**, S39-46.
2. Majumder K, Mine Y, Wu J (2016) The potential of food protein-derived anti-inflammatory peptides against various chronic inflammatory diseases. *J Sci Food Agric* **96**, 2303-2311.
3. Möller NP, Scholz-Ahrens KE, Roos N *et al.* (2008) Bioactive peptides and proteins from foods: indication for health effects. *Eur J Nutr* **47**, 171-182.
4. Hill DR, Newburg DS (2015) Clinical applications of bioactive milk components. *Nutr Rev* **73**, 463-476.
5. Mohanty DP, Mohapatra S, Misra S *et al.* (2016) Milk derived bioactive peptides and their impact on human health - A review. *Saudi J Biol Sci* **23**, 577-583.
6. Fiat AM, Migliore-Samour D, Jollès P *et al.* (1993) Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *J Dairy Sci* **76**, 301-310.
7. Arumugam M, Raes J, Pelletier E *et al.* (2011) Enterotypes of the human gut microbiome. *Nature* **473**, 174-180.
8. Qin J, Li R, Raes J *et al.* (2010) A human gut microbial gene catalogue established by metagenomic sequencing. *Nature* **464**, 59-65.
9. Rehman A, Rausch P, Wang J *et al.* (2016) Geographical patterns of the standing and active human gut microbiome in health and IBD. *Gut* **65**, 238-248.
10. Singh RK, Chang H-W, Yan D *et al.* (2017) Influence of diet on the gut microbiome and implications for human health. *J Transl Med* **15**, 73-73.
11. Bäckhed F, Fraser CM, Ringel Y *et al.* (2012) Defining a healthy human gut microbiome: current concepts, future directions, and clinical applications. *Cell Host Microbe* **12**, 611-622.
12. Wong JMW, de Souza R, Kendall CWC *et al.* (2006) Colonic health: fermentation and short chain fatty acids. *J Clin Gastroenterol* **40**, 235-243.
13. Liu L, Li L, Min J *et al.* (2012) Butyrate interferes with the differentiation and function of human monocyte-derived dendritic cells. *Cell Immunol* **277**, 66-73.

14. Partula V, Mondot S, Torres MJ *et al.* (2019) Associations between usual diet and gut microbiota composition: results from the Milieu Interieur cross-sectional study. *Am J Clin Nutr* **109**, 1472-1483.
15. David LA, Maurice CF, Carmody RN *et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559-563.
16. Berni Canani R, Terrin G, Borrelli O *et al.* (2006) Short- and long-term therapeutic efficacy of nutritional therapy and corticosteroids in paediatric Crohn's disease. *Dig Liver Dis* **38**, 381-387.
17. Borrelli O, Cordischi L, Cirulli M *et al.* (2006) Polymeric diet alone versus corticosteroids in the treatment of active pediatric Crohn's disease: a randomized controlled open-label trial. *Clin Gastroenterol Hepatol* **4**, 744-753.
18. Kahleova H, Tura A, Klementova M *et al.* (2019) A plant-based meal stimulates incretin and insulin secretion more than an energy- and macronutrient-matched standard meal in type 2 diabetes: A randomized crossover study. *Nutrients* **11**, 486.
19. National Institutes of Health Consensus Development P (2001) National Institutes of Health Consensus Development Conference Statement: phenylketonuria: screening and management, October 16-18, 2000. *Pediatrics* **108**, 972-982.
20. Daly A, Evans S, Chahal S *et al.* (2019) Glycomacropeptide: long-term use and impact on blood phenylalanine, growth and nutritional status in children with PKU. *Orphanet J Rare Dis* **14**, 44.
21. Kawasaki Y, Isoda H, Tanimoto M *et al.* (1992) Inhibition by lactoferrin and kappa-casein glycomacropeptide of binding of Cholera toxin to its receptor. *Biosci Biotechnol Biochem* **56**, 195-198.
22. Nakajima K, Tamura N, Kobayashi-Hattori K *et al.* (2005) Prevention of intestinal infection by glycomacropeptide. *Biosci Biotechnol Biochem* **69**, 2294-2301.
23. Bruck WM, Kelleher SL, Gibson GR *et al.* (2006) The effects of alpha-lactalbumin and glycomacropeptide on the association of CaCo-2 cells by enteropathogenic Escherichia coli, Salmonella typhimurium and Shigella flexneri. *FEMS Microbiol Lett* **259**, 158-162.
24. Feeney S, Ryan JT, Kilcoyne M *et al.* (2017) Glycomacropeptide reduces intestinal epithelial cell barrier dysfunction and adhesion of entero-hemorrhagic and entero-pathogenic escherichia coli in vitro. *Foods* **6**.

25. Sawin EA, De Wolfe TJ, Aktas B *et al.* (2015) Glycomacropeptide is a prebiotic that reduces Desulfovibrio bacteria, increases cecal short-chain fatty acids, and is anti-inflammatory in mice. *Am J Physiol Gastrointest Liver Physiol* **309**, G590-601.
26. Requena P, Daddaoua A, Martinez-Plata E *et al.* (2008) Bovine glycomacropeptide ameliorates experimental rat ileitis by mechanisms involving downregulation of interleukin 17. *Br J Pharmacol* **154**, 825-832.
27. Daddaoua A, Puerta V, Zarzuelo A *et al.* (2005) Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. *J Nutr* **135**, 1164-1170.
28. Ortega-Gonzalez M, Capitan-Canadas F, Requena P *et al.* (2014) Validation of bovine glycomacropeptide as an intestinal anti-inflammatory nutraceutical in the lymphocyte-transfer model of colitis. *Br J Nutr* **111**, 1202-1212.
29. Cheng X, Gao D, Chen B *et al.* (2015) Endotoxin-Binding Peptides Derived from Casein Glycomacropeptide Inhibit Lipopolysaccharide-Stimulated Inflammatory Responses via Blockade of NF-κB activation in macrophages. *Nutrients* **7**, 3119-3137.
30. Lalor R, O'Neill S (2019) Bovine kappa-casein fragment induces hypo-responsive M2-like macrophage phenotype. *Nutrients* **11**, 19.
31. O'Riordan N, O'Callaghan J, Buttò LF *et al.* (2018) Bovine glycomacropeptide promotes the growth of Bifidobacterium longum ssp. infantis and modulates its gene expression. *J Dairy Sci* **101**, 6730-6741.
32. Gustavo Hermes R, Molist F, Francisco Pérez J *et al.* (2013) Casein glycomacropeptide in the diet may reduce Escherichia coli attachment to the intestinal mucosa and increase the intestinal lactobacilli of early weaned piglets after an enterotoxigenic E. coli K88 challenge. *Br J Nutr* **109**, 1001-1012.
33. Bruck WM, Redgrave M, Tuohy KM *et al.* (2006) Effects of bovine alpha-lactalbumin and casein glycomacropeptide-enriched infant formulae on faecal microbiota in healthy term infants. *J Pediatr Gastroenterol Nutr* **43**, 673-679.
34. Andersson Y, Hammarstrom ML, Lonnerdal B *et al.* (2009) Formula feeding skews immune cell composition toward adaptive immunity compared to breastfeeding. *J Immunol* **183**, 4322-4328.
35. Masoud K, Farg M, El-Batawy O *et al.* (2019) Inhibitory effect of alpha lactalbumin and casienglycomacropeptide on Mutans streptococci count in dental plaque. *Int J of Adv Res* **7**, 58-67.

36. Schüpbach P, Neeser JR, Golliard M *et al.* (1996) Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. *J Dent Res* **75**, 1779-1788.
37. Elgamily H, Safwat E, Soliman Z *et al.* (2019) Antibacterial and remineralization efficacy of casein phosphopeptide, glycomacropeptide nanocomplex, and probiotics in experimentaltoothpastes: An in vitro comparative study. *Eur J Dent* **13**, 391-398.
38. Keogh JB, Clifton P (2008) The effect of meal replacements high in glycomacropeptide on weight loss and markers of cardiovascular disease risk. *Am J Clin Nutr* **87**, 1602-1605.
39. Keogh JB, Woonton BW, Taylor CM *et al.* (2010) Effect of glycomacropeptide fractions on cholecystokinin and food intake. *Br J Nutr* **104**, 286-290.
40. Hvas CL, Dige A, Bendix M *et al.* (2016) Casein glycomacropeptide for active distal ulcerative colitis: a randomized pilot study. *Eur J Clin Invest* **46**, 555-563.
41. Jenkins DJA, Kendall CWC, Marchie A *et al.* (2003) Effects of a dietary portfolio of cholesterol-lowering foods vs lovastatin on serum lipids and C-reactive protein. *JAMA* **290**, 502-510.
42. King DE, Egan BM, Woolson RF *et al.* (2007) Effect of a high-fiber diet vs a fiber-supplemented diet on C-reactive protein level. *Arch Intern Med* **167**, 502-506.
43. O'Keefe SJ, Li JV, Lahti L *et al.* (2015) Fat, fibre and cancer risk in African Americans and rural Africans. *Nat Commun* **6**, 6342.
44. Bonder MJ, Tigchelaar EF, Cai X *et al.* (2016) The influence of a short-term gluten-free diet on the human gut microbiome. *Genome Med* **8**, 45.
45. Cordova-Davalos LE, Jimenez M, Salinas E (2019) Glycomacropeptide bioactivity and health: A review highlighting action mechanisms and signaling pathways. *Nutrients* **11**.
46. Harris PA, Taylor R, Thielke R *et al.* (2009) Research electronic data capture (REDCap)--a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* **42**, 377-381.
47. Minihane AM, Vinoy S, Russell WR *et al.* (2015) Low-grade inflammation, diet composition and health: current research evidence and its translation. *Br J Nutr* **114**, 999-1012.
48. Lewis SJ, Heaton KW (1997) Stool form scale as a useful guide to intestinal transit time. *Scand J Gastroenterol* **32**, 920-924.

49. Nielsen LN, Roager HM, Casas ME *et al.* (2018) Glyphosate has limited short-term effects on commensal bacterial community composition in the gut environment due to sufficient aromatic amino acid levels. *Environ Pollut* **233**, 364-376.
50. Callahan BJ, McMurdie PJ, Rosen MJ *et al.* (2016) DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods* **13**, 581-583.
51. (assessed 2016) RStudio: Integrated Development for R . [Online]. https://rstudio.com/; http://dx.doi.org/10.1007/978-81-322-2340-5
52. Bolyen E (2018) QIIME 2: Reproducible, interactive, scalable and extensible microbiome data science. *PeerJ Prepr* **9-10**.
53. Mandal S, Van Treuren W, White RA *et al.* (2015) Analysis of composition of microbiomes: a novel method for studying microbial composition. *Microb Ecol Health Dis* **26**, 27663.
54. Jensen MT, Cox RP, Jensen BB (1995) Microbial production of skatole in the hind gut of pigs given different diets and its relation to skatole deposition in backfat. *Animal Science* **61**, 293-304.
55. Ma Y, Griffith JA, Chasan-Taber L *et al.* (2006) Association between dietary fiber and serum C-reactive protein. *Am J Clin Nutr* **83**, 760-766.
56. Ockene IS, Matthews CE, Rifai N *et al.* (2001) Variability and classification accuracy of serial high-sensitivity C-reactive protein measurements in healthy adults. *Clin Chem* **47**, 444-450.
57. Julious SA (2004) Sample sizes for clinical trials with normal data. *Stat Med* **23**, 1921-1986.
58. Craig CL, Marshall AL, Sjöström M *et al.* (2003) International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc* **35**, 1381-1395.
59. Beck AT, Ward CH, Mendelson M *et al.* (1961) An inventory for measuring depression. *Arch Gen Psychiatry* **4**, 561-571.
60. Poppitt SD, Strik CM, McArdle BH *et al.* (2013) Evidence of enhanced serum amino acid profile but not appetite suppression by dietary glycomacropeptide (GMP): a comparison of dairy whey proteins. *J Am Coll Nutr* **32**, 177-186.
61. Chungchunlam SM, Henare SJ, Ganesh S *et al.* (2014) Effect of whey protein and glycomacropeptide on measures of satiety in normal-weight adult women. *Appetite* **78**, 172-178.
62. Moreno-Pérez D, Bressa C, Bailén M *et al.* (2018) Effect of a Protein Supplement on the Gut Microbiota of Endurance Athletes: A Randomized, Controlled, Double-Blind Pilot Study. *Nutrients* **10**.

63. Ntemiri A, Chonchúir FN, O'Callaghan TF *et al.* (2017) Glycomacropeptide sustains microbiota diversity and promotes specific taxa in an artificial colon model of elderly gut microbiota. *J Agric Food Chem* **65**, 1836-1846.
64. Yao CK, Muir JG, Gibson PR (2016) Review article: insights into colonic protein fermentation, its modulation and potential health implications. *Aliment Pharmacol Ther* **43**, 181-196.
65. Nickerson KP, Chanin R, McDonald C (2015) Deregulation of intestinal anti-microbial defense by the dietary additive, maltodextrin. *Gut Microbes* **6**, 78-83.
66. Yudkin JS (2003) Adipose tissue, insulin action and vascular disease: inflammatory signals. *Int J Obes Relat Metab Disord* **27 Suppl 3**, S25-S28.
67. *NHLBI Obesity Education Initiative Expert Panel on the Identification, Evaluation, and Treatment of Obesity in Adults (US). Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults: The Evidence Report. Bethesda (MD): National Heart, Lung, and Blood Institute; 1998 Sep. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2003/.*
68. Odamaki T, Kato K, Sugahara H *et al.* (2016) Age-related changes in gut microbiota composition from newborn to centenarian: a cross-sectional study. *BMC Microbiol* **16**, 90.
69. Salazar N, Arboleya S, Fernández-Navarro T *et al.* (2019) Age-Associated Changes in Gut Microbiota and Dietary Components Related with the Immune System in Adulthood and Old Age: A Cross-Sectional Study. *Nutrients* **11**, 1765.
70. Yun Y, Kim H-N, Kim SE *et al.* (2017) Comparative analysis of gut microbiota associated with body mass index in a large Korean cohort. *BMC Microbiol* **17**, 151.
71. Ley RE, Turnbaugh PJ, Klein S *et al.* (2006) Microbial ecology: human gut microbes associated with obesity. *Nature* **444**, 1022-1023.
72. Chao A, Thun MJ, Connell CJ *et al.* (2005) Meat consumption and risk of colorectal cancer. *JAMA* **293**, 172-182.
73. Bernstein AM, Sun Q, Hu FB *et al.* (2010) Major dietary protein sources and risk of coronary heart disease in women. *Circulation* **122**, 876-883.
74. van Calcar SC, Ney DM (2012) Food products made with glycomacropeptide, a low-phenylalanine whey protein, provide a new alternative to amino Acid-based medical foods for nutrition management of phenylketonuria. *J Acad Nutr Diet* **112**, 1201-1210.
75. Lim K, van Calcar SC, Nelson KL *et al.* (2007) Acceptable low-phenylalanine foods and beverages can be made with glycomacropeptide from cheese whey for individuals with PKU. *Mol Genet Metab* **92**, 176-178.
76. Foisy-Sauvé M, Ahmarani L, Delvin E *et al.* (2020) Glycomacropeptide Prevents Iron/Ascorbate-Induced Oxidative Stress, Inflammation and Insulin Sensitivity with an Impact on Lipoprotein Production in Intestinal Caco-2/15 Cells. *Nutrients* **12**.

TABLES

Table 1. Study products ingredients.

	Ingredients per daily portion	
	CGMP	Reference
Energy, kJ	627	244
Fat, g	0•6	0•6
Carbohydrate, g	9•2	10•6
Protein, g	27	3
Lacprodan CGMP-20, %	68•4	0
Sugar, sucrose, white, %	11•4	36•0
Cocoa, %	4•6	14•4
Skimmed milk powder, %	14•0	44•3
Flavour, vanilla sugar, %	1•7	5•4
Sum, %	100	100

CGMP, casein glycomacropeptide.

Table 2. Baseline characteristics. Values are medians with range. Non-parametric statistics were used.

	CGMP		Reference		
	Median [a]	Range	Median [a]	Range	P
n	13		11		
Female, n (percent)	7 (54%)		5 (45%)		0•69
Age, years	35	30, 51	36	24, 59	0•40
Age groups, n					
20-30 years	1		2		
31-40 years	10		6		
41-50 years	1		0		
51-60 years	1		3		
Weight, kg	66	52, 82	79	54, 101	0•13
BMI, kg/m^2	21	20, 24	24	20, 29	0•04*
Alcohol, units/week[b]	6	3, 15	7•5	1•5, 21	0•62
Smoker, current or past, n	1		0		0•36
hsCRP, mg/l	0•5	0•1, 9•8	0•5	0•1, 4•1	0•75
Leukocytes, $\cdot 10^9/l$	5	3, 7	6	3, 8	0•42
Albumin, g/l	42	37, 46	41	37, 50	0•23
Faecal calprotectin, mg/kg	29	7, 29	29	5, 211	0•19

CGMP, casein glycomacropeptide; hsCRP, high-sensitive C-reactive protein.

[a] Unless in case of numbers (n). [b] One unit equals 8 g of alcohol. * Median value significantly different from the reference group.

Table 3. Daily intake per kg body weight. Data were analysed using parametric statistics.

	CGMP		Reference		P
	Mean	95% CI	Mean	95% CI	
Baseline					
Protein, g/kg	1•2	1•0, 1•4	0•9	0•7, 1•1	0•008*
Energy, kJ/kg	124	103, 145	93	73, 113	0•03*
Week 4					
Protein, g/kg	1•5	1•3, 1•7	1•0	0•7, 1•2	0•003*
Energy, kJ/kg	126	102, 152	111	79, 143	0•24
Temporal change from baseline to week 4					
Protein, P		0•005*		0•14	
Energy, P		0•70		0•10	

CGMP, casein glycomacropeptide. *Marks significant P-values, i.e. P < 0•05.

Table 4. Daily intake of fibres, cereals, and yoghurt. Data were analysed using non-parametric statistics.

	CGMP		Reference		P
	Median	Range	Median	Range	
Baseline					
Fibres, g	20	12, 31	16	7, 28	0•05
Cereals, g	23	0, 60	26	0, 107	0•88
Yoghurt, dl	0•3	0, 2	0•4	0, 2	0•87
Week 4					
Fibres, g	16	8, 38	16	7, 35	0•85
Cereals, g	23	0, 97	13	0, 105	0•92
Yoghurt, dl	0•0	0, 2	0•8	0, 2	0•08
Temporal change from baseline to week 4					
Fibres, P		0•42		0•84	
Cereals, P		0•53		0•84	
Yoghurt, P		0•22		0•18	

CGMP, casein glycomacropeptide.

Table 5. Primary outcome. Median values with range. Data were analysed using non-parametric statistics.

	hsCRP baseline mg/l		hsCRP week 4 mg/l	
	Median	Range	Median	Range
CGMP	0•5	0•1, 9•8	0•7	0•1, 8•6
Reference	0•5	0•1, 4•1	0•4	0•1, 3•1

CGMP, casein glycomacropeptide; hsCRP, high-sensitive C-reactive protein.

FIGURE LEGENDS

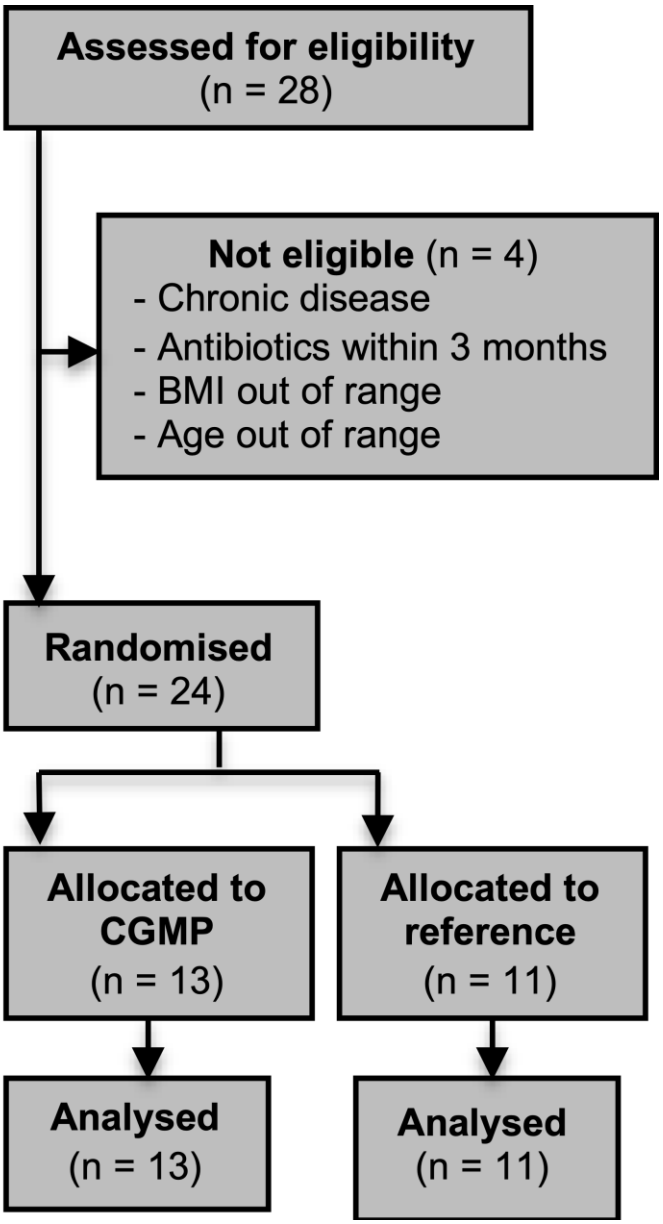

Figure 1. CONSORT study flow diagram. CONSORT, Consolidated Standards of Reporting Trials. CGMP, casein glycomacropeptide.

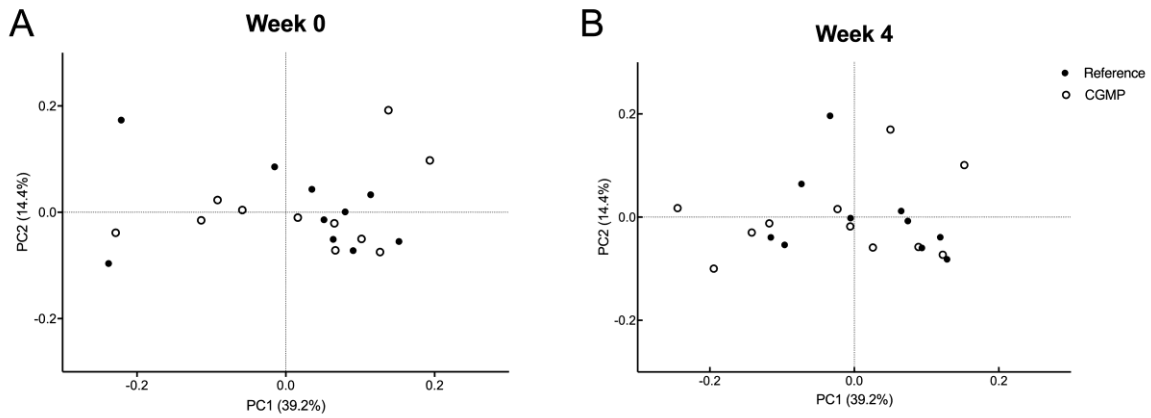

Figure 2. Intestinal microbiota composition. (A) Baseline (Week 0). (B) Week 4. PCoA plot of weighted UniFrac distances. The variation explained by the included principle coordinates is indicated on the respective axis. CGMP, casein glycomacropeptide. ○, CGMP group; ●, reference group.

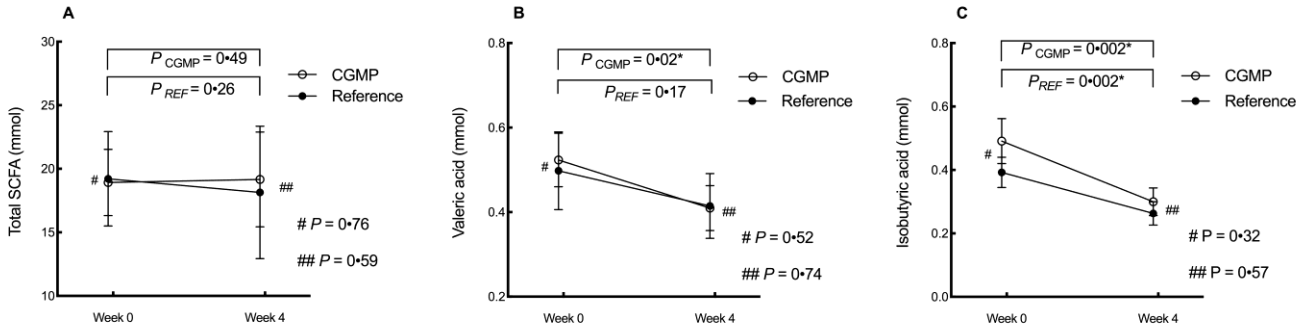

Figure 3. Short-Chain Fatty Acids 24-hour faecal production. (A) Group means of the total amount of SCFA. (B) Group means of valeric acid. (C) Group means of isobutyric acid. Error bars show the standard error of the mean. CGMP, casein glycomacropeptide; REF, reference. P_{CGMP} and P_{REF} mark the p-values from paired t-test on differences between baseline mean and mean at week 4 within the CGMP or the reference group, respectively. *Significant difference. #, ## Unpaired t-test applied for comparison of means at baseline and week 4. ○, CGMP group; ●, reference group.

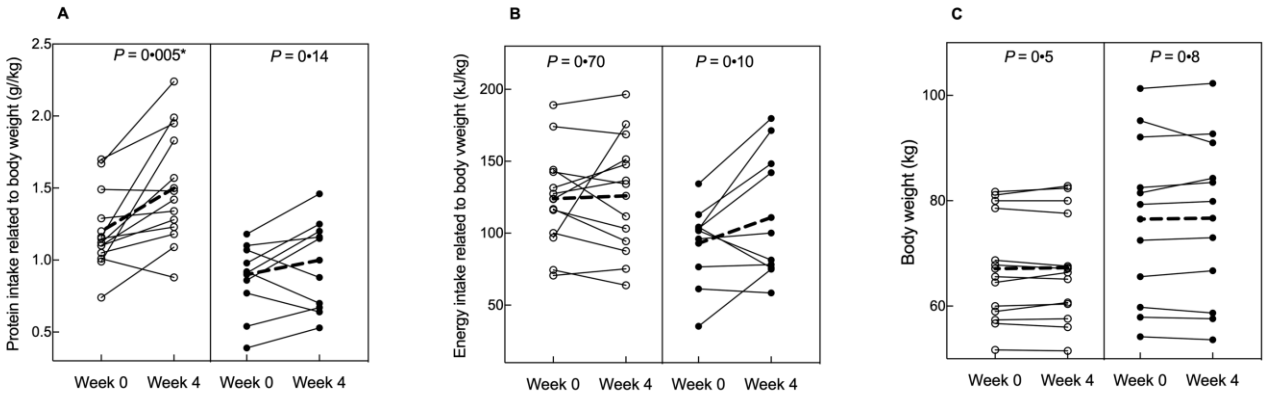

Figure 4. Protein and energy intake and body weight. Each solid line shows the individual change from baseline (week 0) to after 4 weeks. The dashed line shows the mean change in the group from baseline to after week 4. (A) Daily protein intake shown in g protein per kg body weight. The mean change in the CGMP group is 0•3 (95% CI 0•1, 0•5) g/kg and 0•1 (95% CI -0•1, 0•2) g/kg in the reference group. (B) Daily energy intake shown in kilojoule per kg body weight. The mean change in the CGMP group is 3 (95% CI: -14, 20) kJ/kg and in the reference group it is 18 (95% CI -4, 41) kJ/kg. (C) Weight of the study participants. The mean change in the CGMP group is 0•2 (95% CI -0•5, 0•8) kg and in the reference groups it is 0•1 (95% CI -1•1, 1•3) kg. CGMP, casein glycomacropeptide. The P-values refer to a paired t-test. CGMP, casein glycomacropeptide; SD, standard deviation. *Significant difference. ○, CGMP group; ●, reference group.

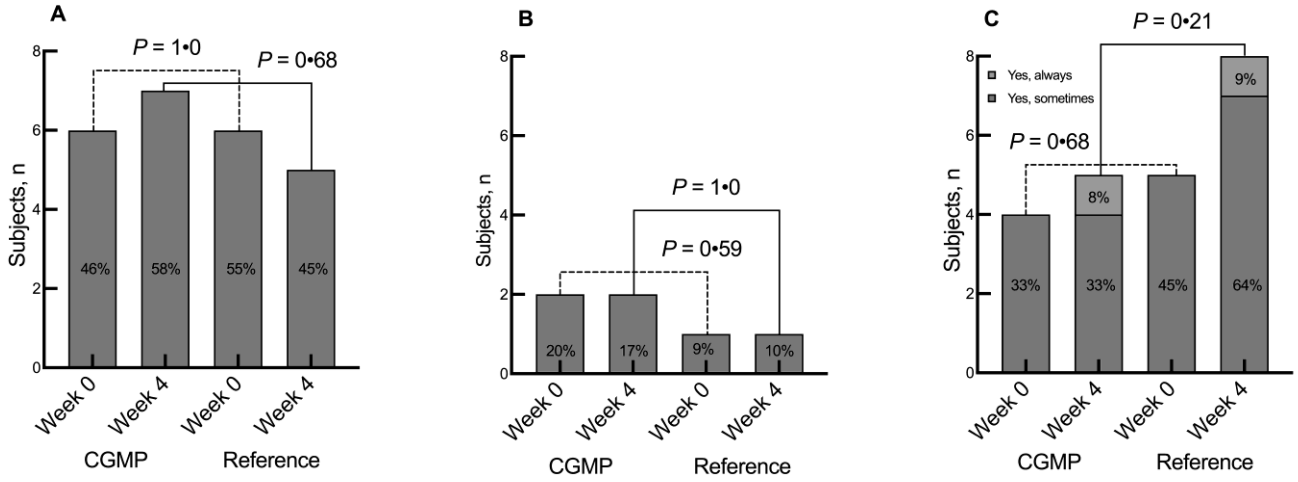

Figure 5. Intestinal symptoms. (A) Defecation urge. (B) Mucus in stools. (C) Incomplete emptying. The bars show the number of participants who answered "yes, sometimes" (dark grey) or "yes, always" (light grey) when asked if they experienced the symptom in question. The percentages show the number of participants with that specific symptom in relation to the number of answers. CGMP, casein glycomacropeptide.

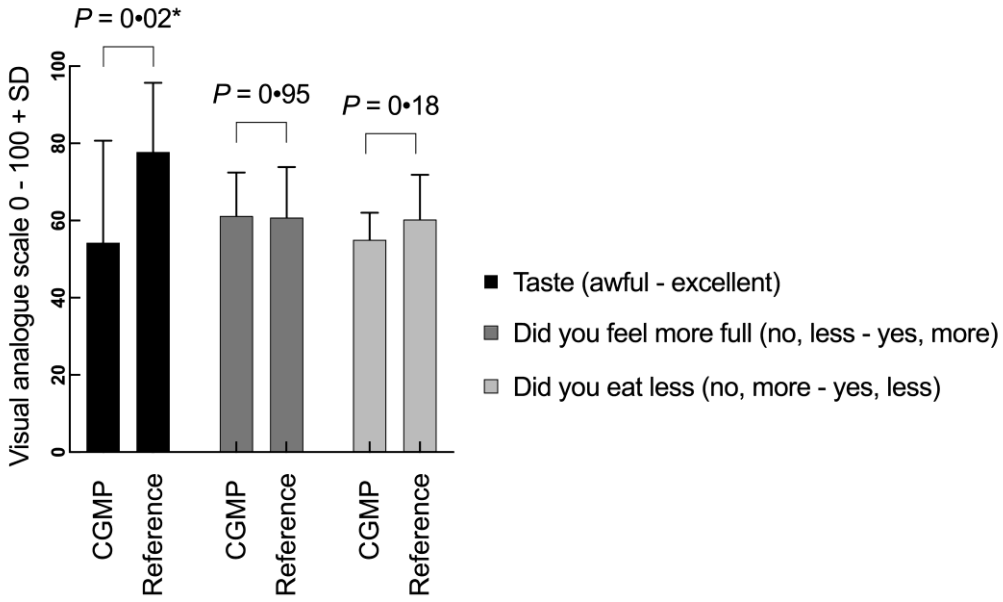

Figure 6. Palatability of the study interventions. Data compared with an unpaired t-test. X-axis show means. Error bars show SD. CGMP, casein glycomacropeptide; SD, standard deviation. *Significant difference.