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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

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]

[1]*Department of Hepatology and Gastroenterology, Aarhus University Hospital, 8200 Aarhus, Denmark*
[2]*Department of Clinical Medicine, Aarhus University, 8200 Aarhus, Denmark*
[3]*Research Group for Gut, Microbes and Health, National Food Institute, Technical University of Denmark, 2800 Kgs. Lyngby, Denmark*
[4]*Department of Animal Science, Aarhus University, 8830 Tjele, Denmark*

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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 homoeostasis, 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 daily oral CGMP intake would reduce high-sensitive C-reactive protein (hsCRP) in healthy adults. In a single-centre limited but randomised, double-blinded, reference-controlled study, we compared the effects of a 4-week intervention of either 25 g 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, with regard to either hsCRP or faecal calprotectin level, faecal microbiota composition or faecal SCFA 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 g of CGMP during 4 weeks was safe, well tolerated, had acceptable palatability and was without any effects on body weight.

Key words: Glycomacropeptide: Milk protein: Whey protein: Healthy adults: Bioactive peptides: Anti-inflammatory effects: Immunomodulation

Casein glycomacropeptide (CGMP), a milk-derived protein, has been recognised 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, antioxidant, 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 homoeostasis and which are not[12,13]. A proxy for a healthy gut microbiota is the relative amount of the SCFA 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 β-cell function in type 2 diabetes[18].

Abbreviations: ASV, amplicon sequence variant; CGMP, casein glycomacropeptide; CRP, C-reactive protein; hsCRP, high-sensitive C-reactive protein.

* **Corresponding author:** Pernille G. Wernlund, email pernillewernlund@clin.au.dk

Bovine CGMP is a small peptide weighing approximately 7 kDa and containing sixty-four amino acid residues. It is enzymatically cleaved from κ-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 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 α-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 α-lactalbumin promote 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 and 3200 mg) to maximum dose (4800 mg/d)[40]. Little is known about the clinical effect of CGMP on intestinal homoeostasis, 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 4 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 4 weeks[40]. Studies of dietary-induced C-reactive protein (CRP) changes reported CRP decreases after 2–3 weeks[41,42]. Regarding the impact of dietary intervention on microbiota composition, studies found changes after 5 d to 4 weeks of intervention[15,43,44]. Consequently, we considered a 4-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 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 bloody stools 3 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 min to optimise its taste. The CGMP used was Lacprodan® CGMP-20 provided by Arla Foods Ingredients Group P/S (Viby J) 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 the CGMP powder comprised 25 g of 95 % pure CGMP. CGMP is enzymatically released from κ-casein and consists of the sixty-four amino acids in the carboxy-terminus. κ-Casein has several genetic variants, but in bovine CGMP, mainly variants 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

Table 1. Study product ingredients (Percentages)

	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.

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 high-sensitive CRP (hsCRP) in the CGMP group compared with the reference group. Secondary outcomes comprised a shift in microbiota composition towards higher α-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 tools hosted at Aarhus University (www.redcap.au.dk). Research Electronic Data Capture 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 minimise 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, leucocyte count and albumin at baseline and after 4 weeks. Plasma samples were cryopreserved for later analysis. hsCRP analysis was done on an ADVIA Chemistry XPT System (Siemens) 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) and a MACSQuant Analyser 10 (Miltenyi Biotec). We used the Human Inflammatory Cytokines Kit (catalogue 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 was 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-h 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-h faeces wet weight, the subjects were provided with a faecal collection device and asked to weigh their faeces throughout 24 h using an extradited weight. After the weighing procedure, three containers were filled and immediately stored at –20°C. Within 48 h, they were moved to the study laboratory. 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 Fisher) with a range from 4 to 6000 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°C until DNA extraction. Community DNA was extracted by using the MoBio PowerLyzer® Power Soil® DNA Isolation Kit (MoBio Laboratories) 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). 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 described[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-bp barcode for each bacterial community (IonXpress barcode as suggested by the supplier, Life Technologies) and a universal reverse primer (PBR 5′-trP1-adapter-ATTACCG CGGCTGCTGG-3′). PCR products were purified using the MAGBIO HigPrep™ PCR-ninety-six-well protocol according to the manufacturer's recommendations. DNA concentrations were determined with the Qubit HS assay (Thermo Fisher Scientific). 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™, Thermo Fisher Scientific) using Ion PGM Hi-Q kit, 200 bp sequencing and Ion 318™ Chip.

Microbiota: bioinformatics

Sequence data in FASTQ format were initially processed in a CLC Genomic Workbench (version 8.5; Qiagen) 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 version 1.12.1 pipeline[50] incorporated in RStudio[51] was used to generate an amplicon sequence variant (ASV) table with taxonomy assigned against the Ribosomal Database Project (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 ASV classified as bacteria. We excluded the Cyanobacteria/Chloroplast group as well as ASV with a total abundance <20 across all samples and samples with <7730 reads in total (three samples). This yielded total 650 ASV in forty-five samples with a median read depth of 11 679 (range 7735–47 898). 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 α-diversity (Shannon index and number of observed ASV), β-diversity (principal coordinates analysis plots based on weighted and unweighted UniFrac distances) as well as relative abundance distributions at different taxonomic levels. Sampling depth was set at 7730 reads. Differential abundance testing at the ASV level was performed with analysis of composition of microbiomes analysis implemented in QIIME2[53].

SCFA

Concentrations of faecal SCFA and other acids including lactic acid were determined by GLC (HP-6890 Series, Hewlett Packard Enterprise)[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-h acid excretion by multiplying the 24-h faeces weight and the acid concentration. In the statistical analysis, we use the amount of different SCFA.

Ethics statement

The present 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 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 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 twenty 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 % dropouts.

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 rank sum 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 Fisher's exact test. We considered a two-tailed *P* value below 0·05 as significant. STATA/IC 14.2 (StataCorp) and GraphPad Prism 8.3.0 (Graph Pad Software, Inc.) were used to perform the statistical analysis. The illustrations are made in Graph Pad Prism 8.3.0 (Graph Pad Software, Inc.).

Results

Study population

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

We screened twenty-eight individuals and included twenty-four healthy adults. The flow chart of the study process is shown in Fig. 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.

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Table 2. Baseline characteristics*
(Median values and ranges; numbers and percentages)

	CGMP		Reference		
	Median	Range	Median	Range	*P*
n	13		11		
Female					
n	7		5		0·69
%	54		45		
Age (years)	35	30–51	36	24–59	0·40
Age groups (*n*) (years)					
20–30	1		2		
31–40	10		6		
41–50	1		0		
51–60	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/weeks)‡	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
Leucocytes (× 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.
* Non-parametric statistics were used.
† Median value significantly different from the reference group.
‡ One unit equals 8 g of alcohol.

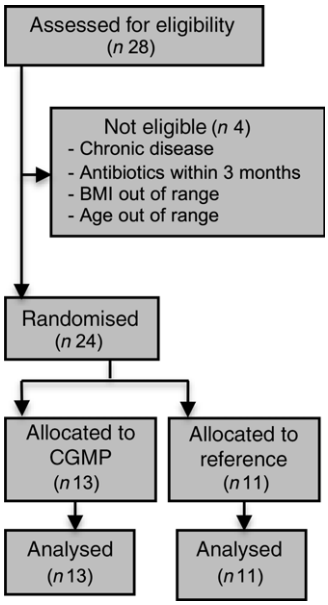

Fig. 1. Consolidated Standards of Reporting Trials (CONSORT) study flow diagram. CGMP, casein glycomacropeptide.

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 an 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.

Table 3. Daily intake per kg body weight†
(Mean values and 95 % confidence intervals)

	CGMP		Reference		
	Mean	95 % CI	Mean	95 % CI	*P*
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.
* $P < 0·05$.
† Data were analysed using parametric statistics.

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 yogurt were not statistically significantly different between the groups at baseline (Table 4). None of the participants was vegans, vegetarians or using probiotic supplements besides the intake of yogurt 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 ASV 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.

Table 4. Daily intake of fibres, cereals and yogurt*
(Median values and ranges)

	CGMP		Reference		
	Median	Range	Median	Range	*P*
Baseline					
Fibres (g)	20	12–31	16	7–28	0·05
Cereals (g)	23	0–60	26	0–107	0·88
Yogurt (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
Yogurt (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		
Yogurt (*P*)	0·22		0·18		

CGMP, casein glycomacropeptide.
* Data were analysed using non-parametric statistics.

Table 5. Primary outcome*
(Median values and ranges)

	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

hsCRP, high-sensitive C-reactive protein; CGMP, casein glycomacropeptide.
* Data were analysed using non-parametric statistics.

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 leucocyte count was 5 (range 3–10) $\times 10^9$/l in the CGMP and 5 (range 3–7) $\times 10^9$/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 α-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 ASV 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 β-diversity was visualised by principal coordinates analysis plots based on weighted (Fig. 2) and un-weighted UniFrac distances. Analysis of similarities 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 at the ASV level showed no significant differences between the CGMP and reference groups at week 4 or at baseline.

SCFA

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 (Fig. 3(a)). 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 (Fig. 3(b)). Within both the CGMP and the reference group, isobutyrate (Fig. 3(c)) 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, Fig. 4(a)). 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, Fig. 4(b)). This indicates that CGMP consumption led to reduced consumption of other foods. The median fibre, cereals and yogurt 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 (Fig. 4(c)). We found no difference in weight between the CGMP group (67·3 (95 % CI 61·0, 73·6) kg)

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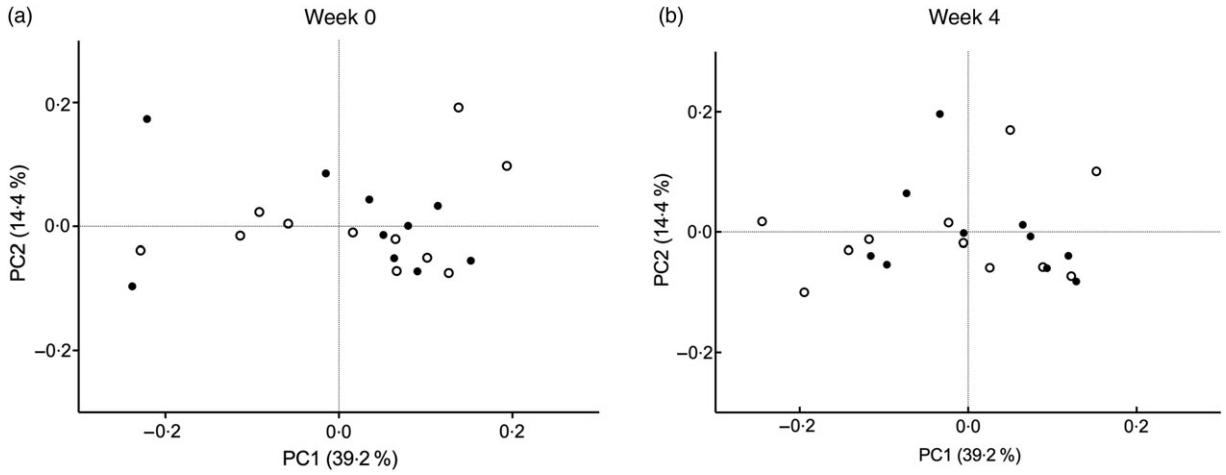

Fig. 2. Intestinal microbiota composition. (a) Baseline (week 0). (b) Week 4. Principal coordinates (PC) analysis plot of weighted UniFrac distances. The variation explained by the included principal coordinates is indicated on the respective axes. ○, Casein glycomacropeptide group; ●, reference group.

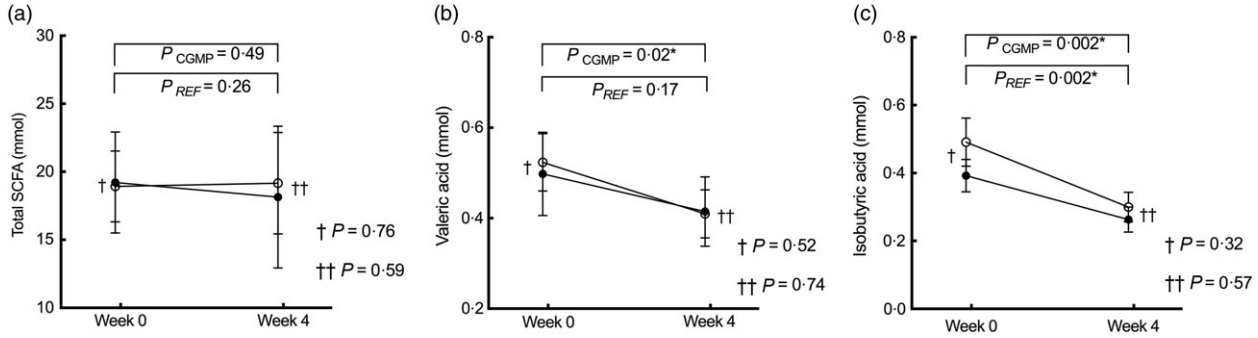

Fig. 3. SCFA 24-h faecal production. (a) Group means of the total amount of SCFA. (b) Group means of valeric acid. (c) Group means of isobutyric acid. Error bars show the standard errors of the mean. CGMP, casein glycomacropeptide; REF, reference. P_{CGMP} and P_{REF} mark the *P* values from paired *t* test on differences between baseline mean and mean at week 4 within the CGMP or the REF group, respectively. * Significant difference. †, †† Unpaired *t* test applied for comparison of means at baseline and week 4. –○–, CGMP group; –●–, REF group.

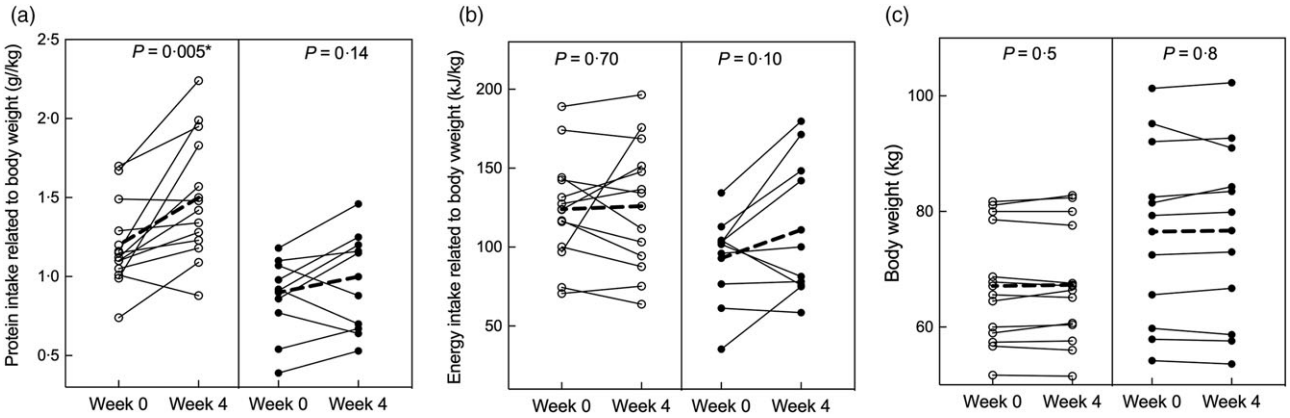

Fig. 4. Protein and energy intake and body weight. Each solid line shows the individual change from baseline (week 0) to after 4 weeks. The dashed line shows the mean change in the group from baseline to after week 4. (a) Daily protein intake shown in g protein per kg body weight. The mean change in the casein glycomacropeptide (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 kJ 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 group, it is 0·1 (95 % CI –1·1, 1·3) kg. *P* values refer to a paired *t* test. * Significant difference. ○, CGMP group; ●, reference group.

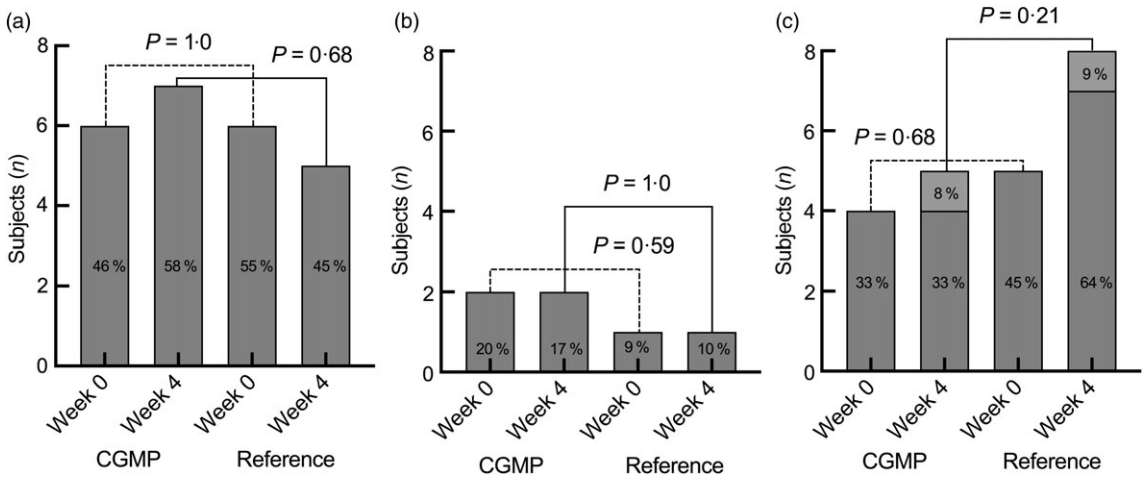

Fig. 5. Intestinal symptoms. (a) Defecation urge. (b) Mucus in stools. (c) Incomplete emptying. The bars show the number of participants who answered 'yes, sometimes' (dark grey) or 'yes, always' (light grey) when asked if they experienced the symptom in question. The percentages show the number of participants with that specific symptom in relation to the number of answers. CGMP, casein glycomacropeptide. ■, Yes, always; ■, yes, sometimes.

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 and 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 (Fig. 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 Beck's 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).

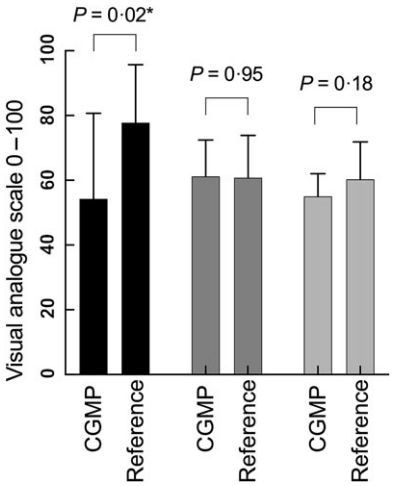

Fig. 6. Palatability of the study interventions. Data compared with an unpaired *t* test. The X-axis shows means. Error bars show standard deviations. CGMP, casein glycomacropeptide. * Significant difference. ■, Taste (awful–excellent); ■, did you feel more full (no, less–yes, more); ■, did you eat less (no, more–yes, less).

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) (Fig. 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 Fisher's exact test. In the CGMP group, three participants (25 %) thought they received CGMP and nine (75 %) that they received the reference drink. In the reference group, six participants (55 %) thought they received CGMP and 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 4 weeks caused no weight changes and was found to be safe and well tolerated. We demonstrated no

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decrease in systemic inflammation markers evaluated by blood hsCRP and leucocyte 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, for example, 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 yogurt 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 with 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 crossover study design may have revealed differences in microbiota composition between groups. A crossover 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 was 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 was 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 (30–51 years in the CGMP group and 24–59 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 α-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, for example, colorectal cancer and CHD[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 phenylketonuria 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, for example, patients with the

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 the 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 homoeostasis 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, phenylketonuria, 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.

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J. F. D., J. S. A., C. L. H. and P. G. W. designed and conducted the research, wrote the manuscript and had primary responsibility for its final contents. M. I. B., T. R. L. and K. E. B. K. provided essentials. All authors analysed the data or performed statistical analyses and read and approved the final manuscript.

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