



Plasma glucagon-like peptide-1 responses to ingestion of protein with increasing doses of milk minerals rich in calcium

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(Submitted 19 March 2021 – Final revision received 9 July 2021 – Accepted 4 August 2021 – First published online 9 August 2021)

Abstract

A high dose of whey protein hydrolysate fed with milk minerals rich in calcium (Capolac[®]) results in enhanced glucagon-like peptide-1 (GLP-1) concentrations in lean individuals; however, the effect of different calcium doses ingested alongside protein is unknown. The present study assessed the dose response of calcium fed alongside 25 g whey protein hydrolysate on GLP-1 concentrations in individuals with overweight/obesity. Eighteen adults (mean ± SD: 8M/10F, 34 ± 18 years, 28.2 ± 2.9 kgm⁻²) completed four trials in a randomised, double-blind, cross-over design. Participants consumed test solutions consisting of 25 g whey protein hydrolysate (CON), supplemented with 3179 mg (LOW), 6363 mg (MED) or 9547 mg (HIGH) Capolac[®] on different occasions, separated by at least 48 h. The calcium content of test solutions equated to 65, 892, 1719 and 2547 mg, respectively. Arterialised-venous blood was sampled over 180 min to determine plasma concentrations of GLP-1_{TOTAL}, GLP-1_{7–36amide}, insulin, glucose, NEFA, and serum concentrations of calcium and albumin. *Ad libitum* energy intake was measured at 180 min. Time-averaged incremental AUC (iAUC) for GLP-1_{TOTAL} (pmol·l⁻¹·min⁻¹) did not differ between CON (23 ± 4), LOW (25 ± 6), MED (24 ± 5) and HIGH (24 ± 6). Energy intake (kcal) did not differ between CON (940 ± 387), LOW (884 ± 345), MED (920 ± 334) and HIGH (973 ± 390). Co-ingestion of whey protein hydrolysate with Capolac[®] does not potentiate GLP-1 release in comparison with whey protein hydrolysate alone. The study was registered at clinical trials (NCT03819972).

Key words: Calcium: Protein: GLP-1: Appetite: Energy intake

Glucagon-like peptide-1 (GLP-1) is a peptide hormone produced by intestinal L-cells⁽¹⁾. Its release stimulates glucose-dependent insulin secretion⁽²⁾, delays gastric emptying⁽³⁾ and reduces food intake⁽⁴⁾. These actions, among others, act to preserve metabolic health such that GLP-1 agonism has been targeted for obesity and type 2 diabetes (T2D) management⁽⁵⁾. Strategies including bariatric surgery, administration of GLP-1 agonists and dipeptidyl peptidase-IV inhibitors (DPP4) rapidly inactivates GLP-1 upon binding) have been effective at increasing/preserving GLP-1 action and promoting weight loss and greater glycaemic control^(5–8). However, it is also important to understand the regulation of endogenous GLP-1 through nutrition, whereby enhancement of gut hormones through specific targeted nutrition can be implemented for wide-scale use.

Nutrition potently regulates enteroendocrine cell action and subsequent peptide hormone release through nutrient sensing and absorption⁽⁹⁾. Recent evidence has implicated the calcium-sensing receptor (CaSR), which is expressed on intestinal L-cells, with facilitating gut hormone release across *in vitro*^(10,11),

ex vivo^(12,13) and *in vivo* rodent models⁽¹⁴⁾. CaSR is responsive to extracellular calcium and amino acids/peptides at physiological fasting and postprandial concentrations^(15,16).

The administration of calcium and hydrolysed protein to stimulate GLP-1 secretion was first investigated using an *ex vivo* rodent model⁽¹²⁾. Rat small intestine was perfused with a KHB buffer in the presence or absence of 10 mM phenylalanine. Calcium was then introduced with increasing dose from 0.1 to 10 mM. GLP-1 concentrations were augmented with increasing calcium dose only in the presence of phenylalanine⁽¹²⁾. More recent work in lean humans is consistent with these observations, whereby plasma GLP-1_{TOTAL} iAUC was 25 % greater over 2 h following acute ingestion of 50 g whey protein with Capolac[®] compared with the ingestion of 50 g whey protein alone⁽¹⁷⁾. Furthermore, peak GLP-1_{TOTAL} concentrations measured following the ingestion of whey protein and Capolac[®] were some of the highest recorded in humans following nutrient ingestion (91 ± 20 pmol·l⁻¹).

The previous study selected a large dose of protein as part of a proof of principle design. However, it remains unclear whether

Abbreviations: CaSR, Ca-sensing receptor; GLP-1, glucagon-like peptide-1; NEFA, non-esterified fatty acids; T2D, type 2 diabetes.

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this effect persists in an overweight/obese population and when feeding a practically relevant dose of protein more reflective of that ingested during a standard meal, i.e. 25 g. For instance, total protein content of breakfast, lunch and dinner for adults is approximately 15 g, 23 g and 35 g, respectively⁽¹⁸⁾. Moreover, the dose of calcium required alongside protein to effectively stimulate the relevant gut hormone response is yet to be established. Accordingly, the aim of this study was to assess the dose–response of calcium fed alongside a practically relevant dose of protein on GLP-1 release in individuals with overweight/obesity. It was hypothesised that GLP-1 concentrations would rise with increasing dose of Ca.

Methods

Experimental design

Eighteen overweight men and women (Table 1) were recruited to participate in a double-blinded randomised crossover study with four trial arms. Inclusion criteria included age 18–65 years, BMI 25.0–34.9 kg·m⁻², no history of metabolic disease and free from allergies or alterations to calcium or milk proteins. Following written consent, participants were randomly assigned to a counterbalanced trial sequence by a researcher who was not involved in data collection. The study protocol was approved by the University of Bath Research Ethics Approval Committee for Health (REACH REF: EP 17/18 250). All procedures were carried out in accordance with the declaration of Helsinki. The study was registered at <https://clinicaltrials.gov/> (NCT03819972).

Pre-Trial standardisation

Participants recorded their habitual diet for 24 h prior to trial 1, then replicated this diet for 24 h prior to each subsequent trial. They also refrained from caffeine, alcohol and any vigorous physical activity for 24 h prior to each trial day. Participants were provided with a standardised meal (Tesco spinach and ricotta cannelloni 440 g; 463 kcal, 56.3 g carbohydrate, 10.1 g sugars, 25.0 g fat, 23.8 g protein) which was consumed no later than 22.00 the evening before a trial day. Women were tested at different phases of their menstrual cycle as this was considered most representative of real life. The washout period between trials was between 2 and 7 d.

Table 1. Participant characteristics (Mean values and standard deviations)

	Total		Males		Females	
	Mean	SD	Mean	SD	Mean	SD
Sample size (n)	18		8		10	
Age (years)	34	18	28	14	38	20
Body mass (kg)	81.2	12.5	89.0	12.3	74.9	8.8
BMI (kg·m ⁻²)	28.2	2.9	29.1	3.4	27.4	2.4
Fasting glucose (mmol·l ⁻¹)	5.5	0.5	5.5	0.5	5.5	0.7
Fasting insulin (pmol·l ⁻¹)	41	20	43	20	40	21

Trial days

Participants arrived at the laboratory between 08.00 and 10.00 h following a 10–14 h overnight fast (standardised within participants). Water consumption was permitted *ad libitum*, with volume recorded on the first trial, and replicated on the subsequent trials. Height was measured using a stadiometer (Seca Ltd., Birmingham, UK), with participants barefoot in the Frankfurt plane. Body mass was measured using digital scales (Tanita, Amsterdam, The Netherlands) with participants barefoot and wearing light clothing.

Arterialised-venous blood samples were obtained by retrograde cannulation of a pre-heated dorsal hand vein or, if unsuccessful, an antecubital arm vein (standardised within participants). After a baseline blood sample and visual appetite scale, test solutions were consumed within a 5-min window (trial commenced upon the first mouthful of the test solution). The time taken to ingest the test solution on trial 1 was recorded and replicated for each subsequent trial. Following ingestion, palatability and blinding validation scales were completed. Blood samples were taken at 15, 30, 45, 60, 90, 120 and 180 min post-ingestion. Appetite visual analogue scales were obtained at baseline and every hour throughout the postprandial period. At the end of this period, participants ingested an *ad libitum* lunch (Tesco Hearty Food Co cheese and tomato pasta 400 g; nutritional info per 100 g, 118 kcal, 19.9 g carbohydrate, 15.9 g sugars, 6.8 g fat, 14.3 g protein) until they were comfortably full and did not want to consume any more. This was followed by another visual analogue scale and palatability scale. Upon the completion of the final trial participants completed a restrained eating questionnaire. The protocol is outlined in Fig. 1.

Test solutions

There were four test solutions consumed in a randomised, counterbalanced order over four trials: (1) whey protein hydrolysate [29.8 g dry powder providing 25 g protein and 15 mg calcium; CON; Lacprodan® DI-3091; (Arla Foods Ingredients, Viby J, Denmark)]; (2) Whey protein hydrolysate plus milk minerals rich in calcium, Capolac® [Arla Foods Ingredients, Viby J, Denmark (3179 mg to provide 842 mg calcium; LOW)]; (3) Whey protein hydrolysate plus Capolac® (6363 mg to provide 1669 mg calcium; MED); and (4) Whey protein hydrolysate plus Capolac® (9547 mg to provide 2497 mg calcium; HIGH). Calcium concentrations for each condition increased linearly up to the maximal tolerable dose in HIGH. The calcium content in the local tap water was 99.58 mg·l⁻¹⁽¹⁹⁾, and day-to-day variation is < 15 mg which is unlikely to alter any responses measured⁽¹⁷⁾. A tap water contribution of 50 mg calcium per 500 ml drink is included in the composition given in Table 2. Test solutions were prepared by two researchers not involved with data collection by dissolving both the protein and Capolac® together in 500 ml water. The composition of test meals is given in Table 2.

Blood sampling and analysis

A 10-ml blood sample was collected for sampling of arterialised venous blood at each time point. This was dispensed into

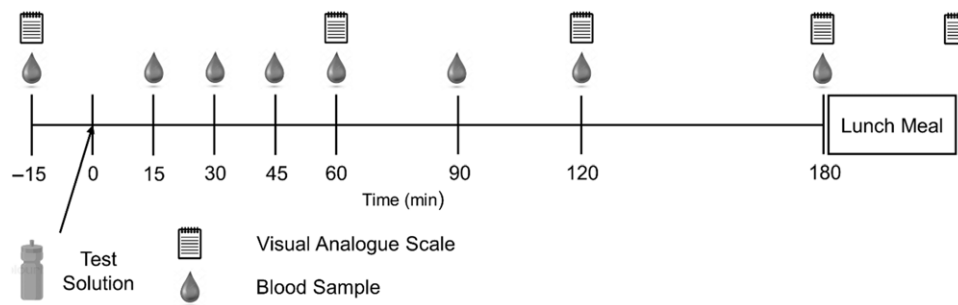


Fig. 1. Schematic of trial days.

Table 2. Nutritional composition of the test meal for each condition

Ingredient	Condition			
	CON*	LOW†	MED‡	HIGH§
Energy (kJ)	438	444	449	455
Energy (kcal)	105	106	107	109
Water (ml)	500	500	500	500
Sucralose (mg)	80	80	80	80
Calcium (mg)	65	892	1719	2547
Phosphorus (mg)	60	410	760	1110
Magnesium (mg)	15	37	60	82
Protein (g)	25	25	25	25
Carbohydrate (g)	0.6	0.9	1.1	1.4
Fat (g)	< 0.1	< 0.1	< 0.1	< 0.1

* CON, whey protein hydrolysate.

† LOW, whey protein hydrolysate with a low dose of Capolac®.

‡ MED, whey protein hydrolysate with a medium dose of Capolac®.

§ HIGH, whey protein hydrolysate with a high dose of Capolac®.

|| Reported calcium value includes the estimated tap water content.

(1) 5-ml EDTA collection tubes (Sarstedt, Nümbrecht, Germany); (2) 3-ml untreated blood collection tubes (Sarstedt, Nümbrecht, Germany) and (3) 2-ml p800 tubes (BD, New Jersey, USA) containing a cocktail of enzyme inhibitors. Plasma samples (EDTA and p800 tubes) underwent immediate centrifugation, whereas untreated blood collection tubes were allowed to clot at room temperature for 15 min before being centrifuged to extract blood serum. All samples were centrifuged at 4000 g for 10 min at 4°C. Plasma and serum were then collected and frozen immediately on dry ice. Samples were stored at -80°C until further analysis.

Plasma GLP-1_{TOTAL}, GLP-1_{7-36amide} and insulin were measured using commercially available enzyme-linked immunosorbent assays (ELISA, GLP-1, Merck Millipore, Watford, UK; Insulin, Mercodia, Uppsala, Sweden). The antibodies in the GLP-1_{TOTAL} assay are specific to both GLP-1_{7-36amide} and GLP-1_{9-36amide}, and therefore the GLP-1_{TOTAL} assay is indicative of total secretion whereas the GLP-1_{7-36amide} assay is indicative of hormone action⁽²⁰⁾. All samples for comparison between treatments within each participant were included on the same plate, and the respective intra-plate CV for GLP-1_{TOTAL}, GLP-1_{7-36amide} and insulin were 6.1, 6.1 and 7.4%, respectively. Plasma glucose and non-esterified fatty acids (NEFA) and serum calcium and albumin were analysed in singular using a spectrophotometric analyser (Randox, Daytona, Randox Laboratories Ltd.).

Subjective appetite ratings

Subjective ratings of appetite were assessed using a previously validated 100-mm visual analogue scale⁽²¹⁾. The four questions from this scale: 'How hungry do you feel?', 'How full do you feel?', 'How satisfied do you feel?' and 'How much do you think you can eat?' can be used and then converted into a composite appetite score. This score combines hunger, fullness, satisfaction and prospective consumption using the following equation⁽²²⁾.

$$\text{Appetite score} = (\text{hunger} + \text{prospective food consumption} + (100 - \text{fullness}) + (100 - \text{satisfaction}))/4.$$

Statistical analysis

The primary outcome was the iAUC for plasma GLP-1_{TOTAL}. Plasma GLP-1_{7-36amide}, insulin, glucose and NEFA, and serum calcium and albumin were secondary outcomes, alongside visual analogue scales for appetite and *ad libitum* energy intake. The sample size calculation was based on our previous data⁽¹⁷⁾ in which the mean difference for postprandial plasma GLP-1_{TOTAL} between the co-ingestion of whey protein and Capolac® *v.* whey protein alone was $9.1 \pm 6.9 \text{ pmol}\cdot\text{l}^{-1}\cdot 120 \text{ min}^{-1}$ (mean \pm SD). Using this effect size ($d = 1.32$), approximately eighteen participants (sixteen participants for blood data) provide a 90% probability (85% for blood data) of detecting such an effect with an α -level of 0.05 using a one-way, repeated-measures ANOVA with two tails. Data are presented as means \pm SD in text, and means \pm 95% normalised confidence intervals (nCI) in figures, corrected for between-participant variation, such that the magnitude of the nCI directly infers the contrast between paired means at each time point rather than variance of individual values around the mean⁽²³⁾. The iAUC or total AUC (tAUC) was calculated for all variables (other than *ad libitum* energy intake) using the Time Series Response Analyser⁽²⁴⁾. One-way ANOVA were employed to assess differences between non-time dependent variables (i.e. iAUC, *ad libitum* energy intake), whereas differences between time-dependent variables were analysed using a two-way repeated-measures ANOVA (condition-time). ANOVA are considered robust to violations to normality assumptions⁽²⁵⁾. Where data points were missing, mixed effects models were employed. Where an interaction effect was determined, *post hoc* comparisons were adjusted for multiple comparisons using the stepwise Holm-Bonferroni correction. Simple linear regression was used to determine

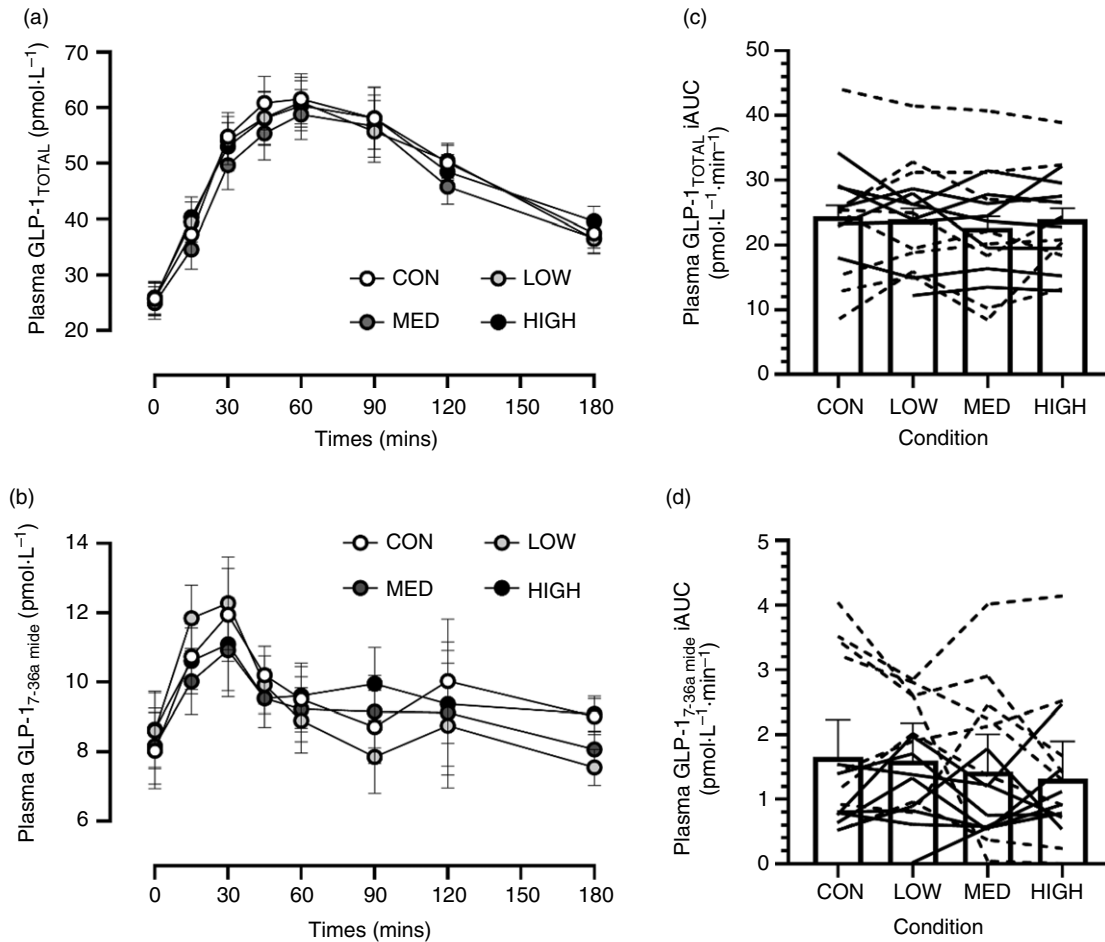


Fig. 2. Plasma GLP-1_{TOTAL} (a), GLP-1_{7-36amide} concentrations (b) and time-averaged iAUC values for plasma GLP-1_{TOTAL} (c) and GLP-1_{7-36amide} (d) following ingestion of protein hydrolysate (CON) and protein hydrolysate and a low (LOW), medium (MED) and high (HIGH) dose of Capolac®. Solid and dashed lines represent individual data for males and females, respectively. Data are means ± 95% nCI, n 16, CON = 15, LOW = 15, MED = 16, HIGH = 16. GLP-1 glucagon-like peptide-1. Significance was set at $P \leq 0.05$.

whether BMI and HOMA-IR could predict GLP-1 concentrations. A two-tailed P value of ≤ 0.05 was deemed statistically significant. Results were analysed using Microsoft Excel version 16.0 and GraphPad Prism version 8.4.3.

Results

Plasma GLP-1

For postprandial change in plasma GLP-1_{TOTAL} and GLP-1_{7-36amide} concentrations, there was no main effect of trial ($P = 0.24$ and $P = 0.34$, respectively) or trial x time interaction (Fig. 2(a)–(b); $P = 0.22$ and $P = 0.43$, respectively). There was also no significant differences in plasma GLP-1_{TOTAL} time-averaged iAUC (Fig. 2(c); $P = 0.57$, CON (24 ± 9 pmol·l⁻¹·min⁻¹); LOW (24 ± 8 pmol·l⁻¹·min⁻¹); MED (23 ± 8 pmol·l⁻¹·min⁻¹); HIGH (24 ± 7 pmol·l⁻¹·min⁻¹)) and GLP-1_{7-36amide} time-averaged iAUC between conditions (Fig. 2(d); $P = 0.59$, CON (1.7 ± 1.2 pmol·l⁻¹·min⁻¹); LOW (1.6 ± 0.9 pmol·l⁻¹·min⁻¹); MED (1.4 ± 1.1 pmol·l⁻¹·min⁻¹); HIGH (1.3 ± 1.0 pmol·l⁻¹·min⁻¹)). GLP-1_{TOTAL} time-averaged iAUC did not differ between males and females ($P = 0.90$); however, GLP-1_{7-36amide} time-averaged iAUC was greater for females compared with males

($P = 0.02$). There was, however, no difference between conditions for males or females, respectively, for both GLP-1_{TOTAL} time-averaged iAUC ($P = 0.53$, $P = 0.74$) and GLP-1_{7-36amide} time-averaged iAUC ($P = 0.32$, $P = 0.39$). Peak plasma GLP-1_{TOTAL} concentrations were 64 ± 19 pmol·l⁻¹, 62 ± 17 pmol·l⁻¹, 61 ± 14 pmol·l⁻¹ and 62 ± 17 pmol·l⁻¹ for CON, LOW, MED and HIGH conditions, respectively, and were not statistically different ($P = 0.75$). Similarly, peak plasma GLP-1_{7-36amide} concentrations did not differ between conditions ($P = 0.30$). There was also no main effect of trial ($P > 0.99$) or trial x time interaction for GLP-1_{7-36amide}:GLP-1_{TOTAL} ratio ($P = 0.99$). Change in postprandial GLP-1 iAUC (HIGH minus CON) was not significantly correlated with either BMI (Fig. 3(a), $r^2 = 0.05$, $P = 0.42$) or Homeostatic Model Assessment of Insulin Resistance (HOMA-IR; Fig. 3(b), $r^2 = 0.08$, $P = 0.30$), nor was peak GLP-1_{TOTAL} significantly correlated with BMI (Fig. 3(a), $r^2 = 0.09$, $P = 0.27$) or HOMA-IR (Fig. 3(a), $r^2 = 0.02$, $P = 0.63$).

Plasma insulin, glucose and non-esterified fatty acids

Following the ingestion of test drinks, there was no effect of trial or trial x time interaction for plasma insulin (Fig. 4(a), $P = 0.92$ and $P = 0.67$, respectively), plasma glucose (Fig. 4(b), $P = 0.92$

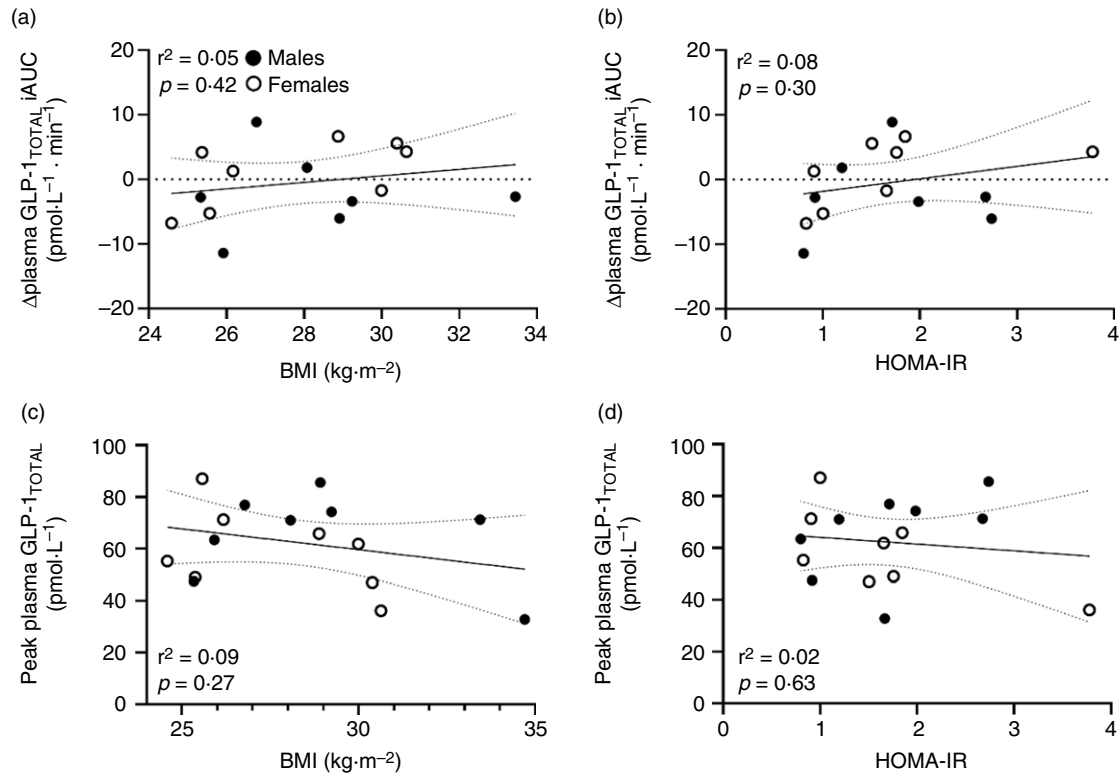


Fig. 3. Simple linear regression for plasma GLP-1_{TOTAL} iAUC (change from HIGH minus CON) and BMI (a) and HOMA-IR (b) and for peak plasma GLP-1 and BMI (c) and HOMA-IR (d). Filled circles and open circles represent male and females, respectively. $n = 16$. GLP-1 glucagon-like peptide-1. Significance was set at $P \leq 0.05$.

and $P = 0.41$, respectively) or plasma NEFA (Fig. 4(c), $P = 0.35$ and $P = 0.11$, respectively). The subsequent time-averaged iAUCs for plasma insulin ($P = 0.83$, CON (85 ± 61 pmol·l⁻¹·min⁻¹); LOW (82 ± 96 pmol·l⁻¹·min⁻¹); MED (86 ± 90 pmol·l⁻¹·min⁻¹); HIGH (83 ± 84 pmol·l⁻¹·min⁻¹)) and plasma glucose ($P = 0.79$, CON (0.10 ± 0.25 mmol·l⁻¹·min⁻¹); LOW (0.08 ± 0.22 mmol·l⁻¹·min⁻¹); MED (0.06 ± 0.13 mmol·l⁻¹·min⁻¹); HIGH (0.05 ± 0.08 mmol·l⁻¹·min⁻¹)), and the time-averaged tAUC for plasma NEFA ($P = 0.57$, CON (0.24 ± 0.10 mmol·l⁻¹·min⁻¹); LOW (0.24 ± 0.12 mmol·l⁻¹·min⁻¹); MED (0.24 ± 0.09 mmol·l⁻¹·min⁻¹); HIGH (0.27 ± 0.08 mmol·l⁻¹·min⁻¹)) did not differ between conditions.

Serum calcium and albumin

There was no effect of trial or trial x time interaction for either serum calcium (Fig. 5(a), $P = 0.70$ and $P = 0.45$, respectively) or albumin (Fig. 5(b), $P = 0.92$ and $P = 0.41$, respectively). There was also no differences observed in the time-averaged iAUC for serum calcium ($P = 0.37$, CON (0.10 ± 0.14 mmol·l⁻¹·min⁻¹); LOW (0.10 ± 0.14 mmol·l⁻¹·min⁻¹); MED (0.05 ± 0.10 mmol·l⁻¹·min⁻¹); HIGH (0.16 ± 0.26 mmol·l⁻¹·min⁻¹)) or serum albumin ($P = 0.31$, CON (1.8 ± 2.6 g·l⁻¹·min⁻¹); LOW (1.7 ± 2.8 g·l⁻¹·min⁻¹); MED (0.9 ± 2.0 g·l⁻¹·min⁻¹); HIGH (3.1 ± 5.2 g·l⁻¹·min⁻¹)) between conditions.

Ad libitum lunch and visual analogue scales

There was no difference in *ad libitum* lunch energy intake between conditions (Fig. 6(a); $P = 0.35$, CON (940 ± 387 kcal); LOW (884 ± 345 kcal); MED (920 ± 334 kcal); HIGH (973 ± 390

kcal)). Equally, there were no effects for trial or trial x time interaction for ratings of perceived fullness, satisfaction, hunger, prospective consumption or overall appetite score (Fig. 6(c), all $P > 0.05$). There was a significant difference in postprandial appetite iAUC between conditions (Fig. 6(b), $P = 0.04$), although differences did not remain following *post hoc* stepwise Holm-Bonferroni corrections.

Standardisation and blinding

The test drinks were well tolerated by all participants and were correctly identified on only 26% of occasions, with a third of participants unable to identify a single drink correctly. There was no difference in the time taken to ingest the test drink between conditions ($P = 0.07$; 260 ± 80 s, 257 ± 91 s, 264 ± 83 s and 288 ± 72 s, for CON, LOW, MED and HIGH, respectively). Data were checked for order effects and the only parameters that exhibited any evidence of systematic variance over trial were plasma glucose and serum albumin, for which there was a main trial effect of trial sequence (both $P = 0.03$), but without any sequence x trial interaction.

Discussion

The present study reveals that, contrary to our hypothesis based on previous data, the postprandial plasma GLP-1 response is not accentuated by the addition of Capolac® to ingested whey protein hydrolysate (25 g), regardless of the calcium dose across a range of 65–2547 mg. Neither plasma concentrations of insulin,



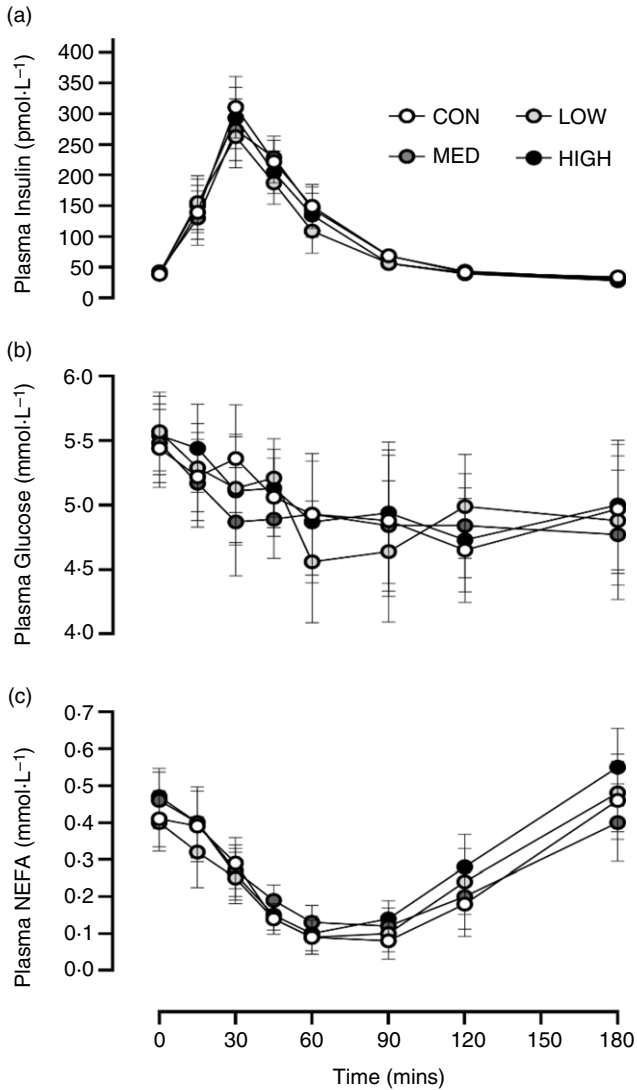


Fig. 4. Plasma insulin (a), glucose (b) and NEFA concentrations (c) following ingestion of protein hydrolysate (CON), and protein hydrolysate and a low (LOW), medium (MED) and high (HIGH) dose of Capolac®. Data are means \pm 95% nCI, *n* 16. NEFA, non-esterified fatty acids. Significance was set at $P \leq 0.05$.

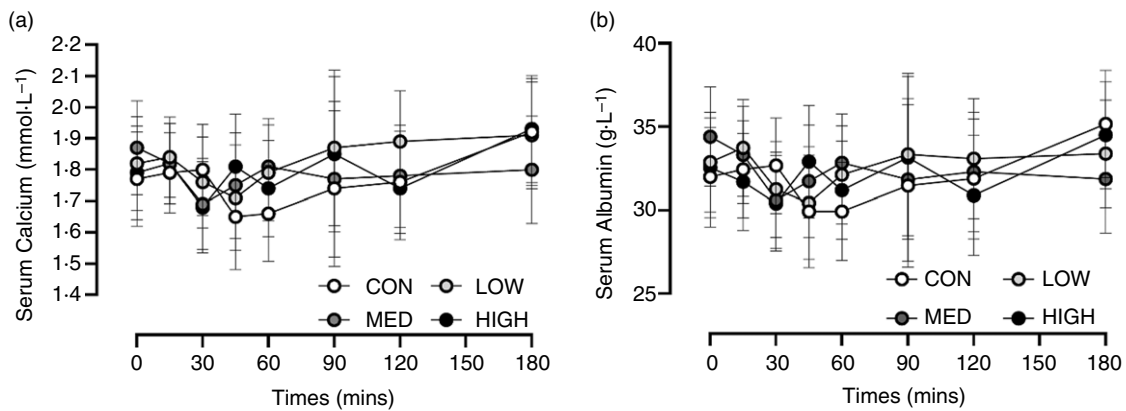


Fig. 5. Serum calcium (a) and albumin concentrations (b) following ingestion of protein hydrolysate (CON) and protein hydrolysate and a low (LOW), medium (MED) and high (HIGH) dose of Capolac®. Data are means \pm 95% nCI, *n* 16. Significance was set at $P \leq 0.05$.

glucose and NEFA nor serum concentrations of calcium and albumin differed meaningfully between conditions. There is also no effect of calcium dose on *ad libitum* energy intake from the lunch meal or overall subjective ratings of appetite.

The absence of any difference in plasma GLP-1_{TOTAL} or GLP-1_{7-36amide} concentrations between conditions in the present study is interesting considering these findings contradict previous work where supplementing meals with calcium enhances GLP-1 concentrations in comparison with meals without supplementation⁽²⁶⁻²⁸⁾. Furthermore, the combination of calcium and protein/amino acids has been shown to potently stimulate GLP-1 release through co-ingestion, *in vivo*⁽¹⁷⁾, and administration, *ex vivo*⁽¹²⁾. The contrasting results of the present study compared with previous research may be explained by either the protein dose of the test drinks or the population sampled in the study.

In our previous work, a large dose of whey protein hydrolysate was selected in a proof of concept study design⁽¹⁷⁾. Interestingly, plasma GLP-1_{TOTAL} time-averaged iAUC in the HIGH condition of the present study (25 g whey protein hydrolysate) was almost half the concentration ($24 \pm 6 \text{ pmol}\cdot\text{l}^{-1}\cdot 180 \text{ min}^{-1}$) of that recorded following the consumption of 50 g whey protein hydrolysate (double the dose of the present study) and $\sim 1000 \text{ mg}$ Capolac® ($45 \pm 6 \text{ pmol}\cdot\text{l}^{-1}\cdot 120 \text{ min}^{-1}$)⁽¹⁷⁾. Equally, the protein-calcium synergy reported *ex vivo*, used isolated loops of rat small intestine where phenylalanine was perfused at a very large dose equating to $10 \text{ mmol}\cdot\text{l}^{-1}$ ⁽¹²⁾. Following the ingestion of a protein-rich meal (50 g bovine serum albumin) in humans, ileal concentrations of phenylalanine as free and peptide amino acids reach (mean \pm SEM) 0.36 ± 0.08 and $1.65 \pm 0.33 \text{ mmol}\cdot\text{l}^{-1}$, respectively⁽²⁹⁾, which means enhanced stimulation of GLP-1, mediated by protein and calcium, has only been reported at high protein concentrations. Within the intestinal epithelium, L-cells capable of releasing GLP-1 increase in density along the length of the intestinal epithelium, with the greatest number located in the colon⁽⁹⁾. This may suggest that a larger protein dose is required to reach more distal sections of the intestine for calcium to influence GLP-1 release. Additionally, while at low doses, calcium is

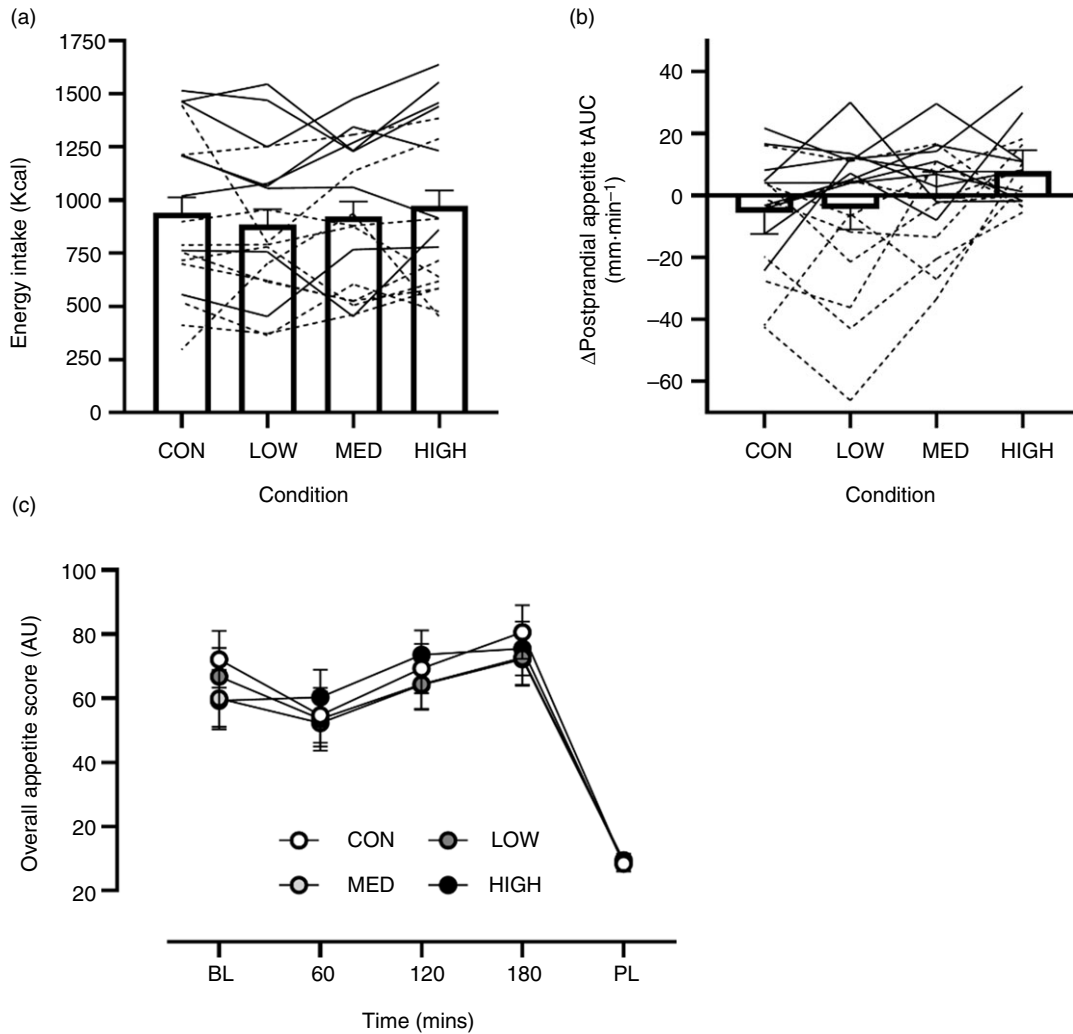


Fig. 6. *Ad libitum* energy intake (a), time-averaged postprandial tAUC values for appetite (b), and overall appetite score (c) following ingestion of protein hydrolysate (CON) and protein hydrolysate and a low (LOW), medium (MED) and high (HIGH) dose of Capolac®. Solid and dashed lines represent individual data for males and females, respectively. PL, post lunch. Data are means \pm 95% nCI, n 18, significance was set at $P \leq 0.05$.

primarily absorbed in the duodenum and jejunum via saturable transcellular processes, at normal to high doses, the non-saturable paracellular diffusive pathway occurs throughout the intestine but dominates more distally, in the jejunum and ileum⁽³⁰⁻³²⁾. Considering the ingestion of large calcium doses in the present study, the limiting factor for protein and calcium-mediated GLP-1 release could be the amount of protein fed. Protein and calcium-induced GLP-1 release is thought to act via the synergistic stimulation of the CaSR^(12,16). The CaSR is able to sense amino acids in the presence of extracellular calcium concentrations of 0.5–10 mM⁽¹⁵⁾. Furthermore, while being directly activated by calcium, occupancy of the L-amino acid-binding site enhances the sensitivity of CaSR to calcium^(16,33), meaning that protein and calcium may work through allosteric activation of the receptor to further enhance GLP-1 concentrations. CaSR is also thought to be expressed on the basolateral membrane meaning protein absorption is paramount for GLP-1 release⁽¹³⁾. Therefore, insufficient protein absorption and binding may be limiting to CaSR-mediated GLP-1 release.

Another potential explanation as to why the addition of calcium did not exert the expected effect on GLP-1 could relate to the population studied. In the present study, individuals who were classified as overweight/obese were recruited to determine whether protein- and calcium-mediated GLP-1 release, seen in lean participants⁽¹⁷⁾, was preserved. GLP-1 release in adults with overweight and obesity has been shown to be impaired in comparison with lean controls^(34,35), which could suggest a disruption in the signalling pathways resulting in GLP-1 release. However, these findings are inconsistent and reduced β -cell function, rather than a reduction in GLP-1 release, is often cited as a driver of the impaired incretin effect often observed in obese and diabetic populations^(36,37). Moreover, in the present study, there was no correlation between BMI or HOMA-IR and GLP-1_{TOTAL} iAUC, or between BMI or HOMA-IR and peak plasma GLP-1_{TOTAL}, suggesting that body composition and insulin sensitivity were not associated with GLP-1 concentrations, at least in the present study. Importantly, if population differences in GLP-1 release do exist and are mediated by factors such as

BMI and insulin sensitivity, this impairment may not be linear with increasing BMI/insulin resistance but instead occur at distinct thresholds, that could not be captured in the population sampled in this study.

Despite no differences in postprandial plasma GLP-1 concentrations between conditions, the average peak GLP-1_{TOTAL} concentration for the HIGH condition (62 ± 17 pmol·l⁻¹) was greater than those reported following the ingestion of 30 g whey protein isolate (120 kcal) in healthy older men (n 8) and women (n 8) (~ 40 pmol·l⁻¹)⁽³⁸⁾ and 45 g whey protein isolate (311 kcal) in nine obese young women (~ 50 pmol·l⁻¹)⁽³⁹⁾. Whilst inter-study comparisons should be made with caution the higher plasma peak GLP-1 concentrations in the present study are unlikely due to the assay employed as it is been shown to have good precision and specificity for GLP-1_{TOTAL}⁽⁴⁰⁾. Furthermore, fasting concentrations of GLP-1_{TOTAL} (26 ± 17 pmol·l⁻¹) are similar to those reported in the aforementioned studies. Greater plasma GLP-1 concentrations observed in the present study could be at least partly due to the form of whey protein ingested, whereby hydrolysed whey protein is likely to increase free amino acid exposure to the intestine⁽⁴¹⁾.

Serum calcium was not different between conditions in the present study despite such large differences in dose consumed. Calcium balance results mainly from the net effects of intestinal absorption and renal, intestinal and parathyroid gland excretion into the dominant calcium pool, bone^(31,42). PTH and vitamin D are two key calcium-regulating hormones, which integrate to increase bone resorption or intestinal absorption at high and low calcium concentrations, respectively^(42,43). These mechanisms maintain calcium concentrations within a narrow physiological range of ~ 2.5 mM (10%)^(31,42). The content of other minerals such as phosphorus and magnesium in each test solution differed marginally (Table 2); however, this is not likely to influence gut hormone concentrations. Previously, GLP-1_{TOTAL} iAUC was not different following ingestion of calcium citrate (< 1 mg phosphorus, < 1 mg magnesium) *v.* milk minerals rich in calcium (551 mg phosphorus, 26 mg magnesium)⁽¹⁷⁾.

In conclusion, the co-ingestion of a practically relevant dose of whey protein with a high dose of Capolac® did not potentiate GLP-1 release in comparison with whey protein hydrolysate alone, in people with overweight/obesity. Furthermore, *ad libitum* energy intake and subjective ratings of appetite did not differ between conditions. Future research should investigate whether there is a protein dose threshold for protein- and calcium-mediated GLP-1 release and whether this effect is specific to metabolically healthy populations or also present in clinical populations with metabolic diseases.

Acknowledgements

The authors thank the volunteers for their time and effort in participating in this study. This work was funded by Arla Foods Ingredients and the University of Bath.

J. D. W., J. T. G., L. H. B. and U. R. M. designed the research, J. D. W. conducted the research, H. A. S. and A. H. prepared the test meals, J. D. W. and H. A. S. analysed the data, J. D. W. performed

the statistical analysis, J. D. W. primarily wrote the paper and all authors read and approved the final version of the manuscript.

J. T. G. is a named investigator on research grants funded by BBSRC, MRC, British Heart Foundation, Lucozade Ribena Suntory, Arla Foods Ingredients, Kennisentrum Suiker and Voeding and has received paid consultancy for PepsiCo, Lucozade Ribena Suntory and SVGC. J. A. B. is a named investigator on research grants funded by BBSRC, MRC, British Heart Foundation, Rare Disease Foundation, EU Hydration Institute, GlaxoSmithKline, Nestlé, Lucozade Ribena Suntory, ARLA Foods and Kennis Centrum Suiker; has completed paid consultancy for PepsiCo, Kellogg's and SVGC; receives an annual stipend as Editor-in Chief of International Journal of Sport Nutrition & Exercise Metabolism; and receives an annual honorarium as a member of the academic advisory board for the International Olympic Committee Diploma in Sports Nutrition.

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