

Effects of hydrolysed casein, intact casein and intact whey protein on energy expenditure and appetite regulation: a randomised, controlled, cross-over study

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Abstract

Casein and whey differ in amino acid composition and in the rate of absorption; however, the absorption rate of casein can be increased to mimic that of whey by exogenous hydrolysis. The objective of the present study was to compare the effects of hydrolysed casein (HC), intact casein (IC) and intact whey (IW) on energy expenditure (EE) and appetite regulation, and thereby to investigate the influence of amino acid composition and the rate of absorption. In the present randomised cross-over study, twenty-four overweight and moderately obese young men and women consumed three isoenergetic dietary treatments that varied in protein source. The study was conducted in a respiration chamber, where EE, substrate oxidation and subjective appetite were measured over 24 h at three independent visits. Moreover, blood and urine samples were collected from the participants. The results showed no differences in 24 h and postprandial EE or appetite regulation. However, lipid oxidation, estimated from the respiratory quotient (RQ), was found to be higher after consumption of IW than after consumption of HC during daytime ($P=0.014$) as well as during the time after the breakfast meal ($P=0.008$) when the food was provided. Likewise, NEFA concentrations were found to be higher after consumption of IW than after consumption of HC and IC ($P<0.01$). However, there was no overall difference in the concentration of insulin or glucagon-like peptide 1. In conclusion, dietary treatments when served as high-protein mixed meals induced similar effects on EE and appetite regulation, except for lipid oxidation, where RQ values suggest that it is higher after consumption of IW than after consumption of HC.

Key words: Dairy proteins: Energy expenditure: Substrate oxidation: Appetite

Evidence supports that isoenergetic meals with a high proportion of dietary energy from protein are more satiating^(1,2) and stimulate diet-induced thermogenesis (DIT)⁽³⁾ to a greater extent than energy intake from carbohydrate (CHO) and fat; consequently, intake of high-protein meals may lead to weight loss or reduce body-weight gain over time^(4–6). Proteins have unique characteristics related to their content of amino acids, structure and rate of absorption and digestion. Therefore, it is speculated that different proteins have diverse metabolic effects^(7,8), and some studies have suggested that they differ in their effects on appetite^(3,8–10) and energy expenditure (EE)^(3,11).

In many Western countries, dairy products are a major source of dietary protein, and two recent meta-analyses have shown that intake of dairy products combined with energy restriction, but not combined with *ad libitum* diets, may favour weight loss^(12,13). Casein and whey are the major proteins found in dairy products. They differ in the way they are digested and absorbed. Whey is absorbed rapidly and induces a high transient rise in the levels of plasma amino acids, whereas casein coagulates in the acidic environment in the stomach, delaying gastric emptying with a subsequent slower and prolonged rise in the levels of plasma amino acids⁽¹⁴⁾. Moreover, whey and casein differ in their amino

Abbreviations: CHO, carbohydrate; DIT, diet-induced thermogenesis; E%, percentage of energy; EE, energy expenditure; ER, energy requirements; GLP-1, glucagon-like peptide 1; HC, hydrolysed casein; IC, intact casein; IW, intact whey; RQ, respiratory quotient.

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acid composition, and some studies have found that whey is more satiating than casein^(9,15), but the results have been inconsistent^(3,8,16). Likewise, there is no clear evidence that one dairy protein is superior to the other when their effects are examined on EE^(3,8,16,17).

The absorption rate of proteins can be manipulated through exogenous hydrolysis, and the absorption rate of hydrolysed casein has been found to be more rapid than that of intact casein and to approach the absorption rate of whey^(18,19). Hydrolysis of proteins, especially casein, may consequently affect metabolic parameters. A recent study in mice has demonstrated that consumption of hydrolysed casein reduced body weight and fat mass, partly through increased β -oxidation in white adipose tissue, compared with consumption of intact casein⁽²⁰⁾. Moreover, it has been observed that hydrolysed casein tend to induce a lower respiratory quotient (RQ) than intact casein⁽²⁰⁾. To our knowledge, the satiating effects of casein and its hydrolysate on appetite and EE have not been previously compared in human subjects, while few studies have investigated the satiating effects of whey and its hydrolysate. From these studies, it has been concluded that hydrolysed and intact whey induce similar effects on appetite^(21,22), which is supported by similar absorption rates found by Calbet & Holst⁽¹⁸⁾. However, this finding is not consistent, as Morifuji *et al.*⁽²³⁾ found that hydrolysed whey is absorbed more rapidly than intact whey. Therefore, whether whey, casein and its hydrolysate have different effects on appetite regulation and EE, as well as the potential mechanisms underlying such possible differences remain to be elucidated.

The objective of the present study was to compare the effects of hydrolysed casein, intact casein and intact whey on EE and appetite regulation, and thereby to investigate the influence of amino acid composition and the rate of absorption. On the basis of the expected differences in absorption rates, we hypothesised that whey and hydrolysed casein would be more satiating and have a greater effect on EE shortly after protein consumption, whereas intact casein would be more satiating and have a greater thermogenic effect several hours after protein consumption. Moreover, we hypothesised that whey would have a more satiating effect than hydrolysed casein due to its higher protein quality. Finally, we expected that consumption of hydrolysed casein would result in greater lipid oxidation than consumption of intact casein, as observed previously in mice⁽²⁰⁾.

Subjects and methods

Subjects

A total of thirty-six overweight to moderately obese (BMI 27–35 kg/m²) men and women aged 22–40 years were recruited for the study by advertisements at local educational institutions and through web pages from October 2012 to July 2013. All subjects participated in a medical screening, and thirty-three subjects were selected for the study based on the following criteria: healthy; no food allergies; non-vegetarians; non-smokers; no drug abuse; low-to-moderate

alcohol consumption; physically active <10 h/week; no use of medication (except for contraceptives); no blood donation 3 months before the study; no participation in other clinical studies 4 weeks before the study; Hb level >8 mmol/l; fasting blood glucose concentration <6.1 mmol/l; non-pregnant and non-lactating women. Moreover, all subjects had a stable weight at baseline (\pm 3 kg within the previous 3 months), and were instructed not to change their dietary patterns and activity levels throughout the study period. Of the total subjects, seven dropped out between the screening period and the initiation of the study as they could not fit the experimental visits into their schedules, and two were excluded at their first visit due to illness. Finally, twenty-four subjects completed the study. The baseline characteristics of the study participants are presented in Table 1.

The present study was conducted according to the guidelines laid down in the Declaration of Helsinki II, and all procedures involving human subjects were approved by the Danish National Committee on Health Research Ethics. Written informed consent was obtained from all subjects after verbal and written information about the study procedures. The study was registered at ClinicalTrials.gov as NCT01677273.

Experimental design

The present study was a randomised, double-blind, three-way cross-over trial. All men were tested with at least 2 weeks between the visits, whereas women had to be in the same phase of their menstrual cycle at each visit. The subjects received the following three dietary treatments in a random and balanced order: (1) hydrolysed casein (HC, Hylvital Casein CMA 500; Friesland Campina Domo) with a degree of hydrolysis of 37%; (2) intact casein (IC, Miprodan[®] 30; Arla Foods Amba); (3) intact whey (IW, Lacprodan[®] SP-9225 Instant; Arla Foods Ingredients).

Each intervention visit lasted 40 h, during which the subjects spent 25 h in a respiration chamber (Fig. 1). The subjects were asked to consume a standardised meal (17% of energy (E%) from protein, 50 E% from CHO and 33 E% from fat) at home

Table 1. Characteristics of the study subjects at baseline (Mean values and standard deviations, n 24)

Characteristics	Mean	SD
Sex (n)		
Male	19	
Female	5	
Age (years)	29	6
Body weight (kg)	95.6	10.1
Height (cm)	178.0	8.5
BMI (kg/m ²)	30.1	2.3
Fat-free mass (kg)*	63.8	10.1
Fat mass (kg)*	31.7	8.7
Fat mass (%)*	33.1	8.6
Systolic blood pressure (mmHg)	126	11
Diastolic blood pressure (mmHg)	79	9
Fasting plasma glucose (mmol/l)	5.1	0.4

* Body composition was determined by dual-energy X-ray absorptiometry.

between the proteins. On day 1, the subjects were served three meals at 09.30, 14.00 and 19.30 hours (Fig. 1), contributing to 20, 40 and 40% of ER, respectively. For breakfast, the subjects were served a protein shake (26E% from protein, 50E% from CHO and 24E% from fat), but for lunch (18E% from protein, 57E% from CHO and 25E% from fat) and dinner (17E% from protein, 58E% from CHO and 25E% from fat), the subjects were provided with additional standardised foods to meet their ER. The subjects were not allowed to drink water *ad libitum*, but 200 ml of water were served with all meals and, in addition, three times during day 1. On day 2, the subjects were served a protein shake for breakfast (identical to day 1) and an *ad libitum* lunch meal (15E% from protein, 55E% from CHO and 30E% from fat) at 12.30 hours, during which period the subjects were asked to eat until they felt comfortably satiated.

Energy expenditure and substrate oxidation

EE was calculated from respiratory gas exchange measured in two identical respiration chambers⁽²⁵⁾. The respiration chambers are rooms measuring 14.7 m³ equipped with a bed, desks, chairs, telephone, television, computer and toilet facilities. The room temperature was kept constant at 22.5°C. Gas exchange was calculated from measured airflow and concentrations of O₂ and CO₂ at the outlet of the chamber as well as in the fresh air going in. Urine was quantitatively collected for 24 h in containers with HCl, and protein oxidation was calculated from the amount of urinary N multiplied by 6.25.

Measurements of 24 h total EE, RQ and substrate oxidation were made starting from 09.30 hours on day 1 until 09.30 hours on day 2, and presented as postprandial effects after the breakfast meal on day 1 (09.30–14.00 hours) during daytime (09.30–01.00 hours), during night-time (01.00–06.00 hours) and over the total 24 h period. BMR was assessed from 08.00 to 09.00 hours on day 2, and sleeping metabolic rate was determined from 01.00 to 06.00 hours on day 2. The subjects were told to avoid physical activity, but spontaneous physical activity was measured by a microwave radar based on the Doppler principle (Sisor Mini-Radar; Static Input System). Substrate oxidation was calculated from 24 h urinary N, O₂ consumption and CO₂ production. CHO and lipid oxidation were calculated according to the method described by Elia & Livesey⁽²⁶⁾.

Appetite assessment and energy intake

Visual analogue scales were used to assess subjective appetite sensations as well as palatability of meals. The scales were based on a series of questions presented individually on sheets in a visual analogue scale booklet. They consisted of a 100 mm horizontal line with words anchored at each end, expressing the most positive and the most negative ratings of hunger, satiety, fullness and prospective food consumption, and desire to eat something fatty, salty, sweet or savoury, and, finally, the palatability of the test meals. On day 1, visual analogue scales were filled out every 30 min as well as after each meal (09.40 hours (10 min), 14.15 hours (285 min) and 19.40

hours (610 min)) from 09.30 to 23.00 hours. On day 2, they were filled out every 30 min and after each meal (09.40 hours and after the *ad libitum* meal) from 09.30 to 12.30 hours.

Energy intake was assessed from an *ad libitum* lunch meal served at 12.30 hours on day 2. The meal consisted of spaghetti Bolognese (15E% from protein, 55E% from CHO and 30E% from fat). The subjects were instructed to eat until they felt comfortably satiated. The time spent was self-determined, but registered for the analysis of data.

Biochemical measures

Blood plasma or serum samples were analysed to measure the concentrations of insulin, glucose, total glucagon-like peptide-1 (GLP-1) and NEFA.

Blood samples were drawn into 2 ml sodium fluoride–oxalate tubes and 2 ml serum clot activator tubes (Vacuette; Hettich) for analyses of glucose and insulin concentrations, respectively. For analysis of GLP-1 concentration, blood samples were collected into 6 ml EDTA tubes, to which 300 µl dipeptidyl peptidase IV inhibitor were added. For analysis of NEFA concentration, blood samples were collected into 4 ml EDTA tubes. The glucose, GLP-1 and NEFA samples were immediately centrifuged for 10 min at 4°C at 2500 rpm, pipetted into 2 ml cryotubes and stored at –80°C until further analysis. Before centrifugation, insulin samples were allowed to coagulate for 20–30 min. Serum insulin concentration was quantified by a solid-phase, two-site chemiluminescent immunometric assay (Siemens Healthcare Diagnostics) on an Immulite 1000 analyser. Concentrations of glucose and NEFA were analysed by ABX Pentra 400 (Horiba ABX) and total GLP-1 concentration was analysed by RIA (antiserum no. 89390)⁽²⁷⁾.

Finally, 24 h urinary N was determined by the Dumas combustion method, using a VarioMax CN analyser (Elementar).

Statistical analyses

Power calculations were based on previous studies examining protein-induced changes in EE^(11,28,29). With a sensitivity of 0.80 and a significance level of 0.05, power calculation indicated a sample size of twenty-four when the minimal detectable difference was set to 0.22 with a within-subject standard deviation of 0.25.

Data are presented as means with their standard errors, unless otherwise specified.

Repeated-measures analyses were conducted using linear mixed models to examine the differences between proteins in relation to EE, RQ, substrate oxidation, biochemical measures and subjective appetite. All analyses included adjustment for age, sex and order of intervention, and subjects were included as random effects. Moreover, in the analysis of EE, RQ and substrate oxidation, respiration chambers were additionally included as random effects, and analyses were also controlled for spontaneous physical activity. A two-way ANOVA was used to examine the differences (means with their standard errors) in BMR and *ad libitum* energy intake between the dietary treatments. Furthermore, associations between insulin and glucose, insulin and GLP-1, insulin and

lipid oxidation and NEFA and lipid oxidation were explored using Pearson's correlation coefficients.

Repeated-measures analyses were also used to analyse the data on EE, RQ, substrate oxidation, biochemical measures and subjective appetite in the postprandial period after the breakfast meal on day 1 (09.30–14.00 hours). Moreover, data on EE, RQ and substrate oxidation were analysed during daytime (09.30–01.00 hours), during night-time (01.00–06.00 hours) and over the total 24 h period. BMR values were analysed from 08.00 to 09.00 hours on day 2, and data on subjective appetite were additionally analysed during daytime (09.30–23.00 hours) and after the breakfast meal on day 2 (09.30–12.45 hours).

Finally, peak magnitude and time to peak were analysed to determine the levels of glucose, insulin and GLP-1, and nadir magnitude and time to nadir were analysed to determine the levels of glucose and NEFA.

All statistical models were checked for normality and variance homogeneity, and all data that were non-normally distributed were transformed. Moreover, all multiple pairwise comparisons were Bonferroni-adjusted.

Statistical analyses were performed using the STATA software, version 11.2 (StataCorp LP, 2011), and statistical significance was set at $P < 0.05$.

Results

Energy expenditure

The results revealed that 24 h EE did not differ from 24 h energy intake (10 396 (SEM 255) kJ/24 h) and between the dietary treatments (Table 3), which indicates that the subjects remained in energy balance during all the visits. Likewise, there was no difference between the dietary treatments in relation to either the postprandial EE after consumption of the breakfast meal on day 1, daytime EE, sleeping metabolic rate, or BMR after adjustment for spontaneous physical activity (Table 3).

Respiratory quotient and substrate oxidation

Total protein oxidation in relation to total EE over 24 h was similar between the dietary treatments (HC 22.2 (SEM 0.7)%, IC 21.5 (SEM 0.8)% and IW 22.1 (SEM 0.7)%).

There was no effect observed for the time \times treatment interaction on the RQ at either time interval studied (after breakfast: $P = 0.42$; daytime: $P = 0.69$; 24 h: $P = 0.58$; night-time: $P = 0.22$); however, an effect of treatment was observed during daytime ($P = 0.048$) and after the breakfast meal ($P = 0.029$) on day 1. The mean postprandial RQ value was 0.02 units higher after consumption of HC than after consumption of IW ($P = 0.036$) during daytime (Fig. 2(a)), as well as during the time after the breakfast meal (0.02 units, $P = 0.018$) on day 1 (Fig. 2(b)), when the food was provided, and these results remained significant after adjustment for 24 h energy balance. There was no difference in RQ between the dietary treatments during night-time and when calculated over the total 24 h period.

Accordingly, there was no effect observed for the time \times treatment interaction on either lipid (after breakfast: $P = 0.50$; daytime: $P = 0.72$; 24 h: $P = 0.53$; night-time: $P = 0.16$) or CHO oxidation (after breakfast: $P = 0.46$; daytime: $P = 0.70$; 24 h: $P = 0.53$; night-time: $P = 0.16$) at all time intervals studied. However, an effect of treatment was observed on lipid oxidation during daytime and after the breakfast meal on day 1 (after breakfast: $P = 0.008$; daytime: $P = 0.014$). Postprandial lipid oxidation was 13% higher after ingestion of IW than after ingestion of HC during daytime ($P = 0.036$) and 15% higher after ingestion of the breakfast meal on day 1 ($P = 0.015$). There was no difference between the HC and IC treatments or between the IW and IC treatments (Fig. 3). Accordingly, an effect of treatment was observed on CHO oxidation after the breakfast meal on day 1 ($P = 0.041$). It was 18% lower after ingestion of IW than after ingestion of HC, but did not differ between the HC and IC treatments or between the IW and IC treatments (Fig. 3). There was no difference in substrate oxidation during night-time and during the 24 h period between the dietary treatments.

Biochemical measures

There were no significant differences in baseline values for serum insulin and plasma glucose, NEFA and GLP-1 concentrations. Likewise, no effects of the time \times treatment interaction were observed for postprandial serum insulin, plasma glucose or NEFA concentrations (Fig. 4). However, the overall postprandial plasma glucose concentration was higher

Table 3. Energy expenditure
(Mean values with their standard errors)

	HC		IC		IW		<i>P</i>
	Mean	SEM	Mean	SEM	Mean	SEM	
24 h EE (kJ/24 h)*	10 410	252	10 474	271	10 445	266	NS
EE after breakfast on day 1 (kJ/4.5 h)*	2208	55	2224	58	2230	56	NS
Daytime EE (kJ/15.5 h)*	7649	197	7674	205	7693	205	NS
SMR (kJ/6 h)*	1642	39	1669	45	1635	40	NS
BMR (kJ/h)†	382	10	378	11	381	10	NS
SPA (%/24 h)*	14.4	1.3	14.7	1.3	14.4	1.1	NS

HC, hydrolysed casein; IC, intact casein; IW, intact whey; EE, energy expenditure; SMR, sleeping metabolic rate; SPA, spontaneous physical activity.

* Data were analysed by a repeated-measures analysis using a mixed linear model.

† BMR was analysed by a two-way ANOVA ($n = 24$).

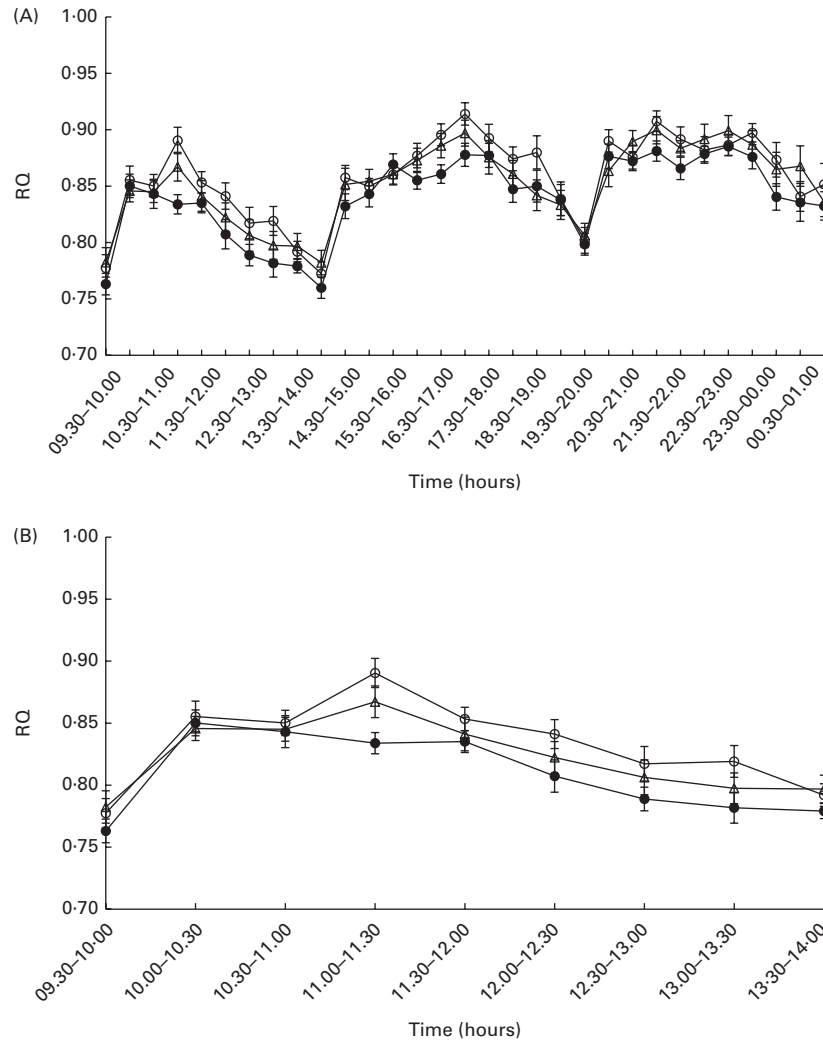


Fig. 2. Postprandial respiratory quotient (RQ) values during daytime (A) and after the breakfast meal (B) on day 1 (n 24). Values are means, with their standard errors represented by vertical bars. Repeated-measures analyses showed no effect for the time \times treatment interactions (after breakfast: $P=0.42$; daytime: $P=0.69$), but the RQ value was higher for hydrolysed casein (\circ) than for intact whey (\bullet) during daytime ($P=0.036$), as well as during the time after the breakfast meal on day 1 ($P=0.018$). \blacktriangle , Intact casein.

after ingestion of IC than after ingestion of HC ($P=0.033$), with no difference being observed between the HC and IW treatments and between the IW and IC treatments. Moreover, the difference in plasma glucose concentration was not explained by the difference in serum insulin concentration, which was similar after ingestion of all the three dietary treatments. Accordingly, there were no significant differences observed between the dietary treatments in relation to peak magnitude and time to peak for glucose and insulin concentrations, as well as no significant differences between the dietary treatments in relation to nadir magnitude and time to nadir were observed for glucose concentration. On the contrary, nadir magnitude for NEFA concentration was lower after consumption of HC than after consumption of IW ($P<0.001$) and IC ($P=0.003$), with no significant difference in time to nadir. Furthermore, the mean postprandial concentration of NEFA was higher after consumption of IW than after consumption

of HC ($P=0.003$) and IC ($P<0.0001$), with no difference being observed between the HC and IC treatments (Fig. 4). No overall effect of treatments was observed for GLP-1 concentration, but a time \times treatment interaction effect was found ($P=0.012$). At 15 min, GLP-1 concentration was higher after ingestion of IC than after ingestion of HC and IW ($P<0.05$) with no difference being observed between the HC and IW treatments. GLP-1 concentration was higher after consumption of HC than after consumption of IW at 60 and 90 min ($P=0.042$ and $P=0.015$, respectively) and higher after consumption of HC than after consumption of IC at 90 min ($P=0.012$). It did not differ between the IW and IC treatments (Fig. 4), and there were no significant differences observed between the dietary treatments in relation to peak magnitude and time to peak for GLP-1 concentration.

Serum insulin concentration was correlated with plasma GLP-1 (r 0.3042, $P<0.0001$) and glucose (r 0.3519, $P<0.0001$) concentrations. Moreover, NEFA and insulin concentrations

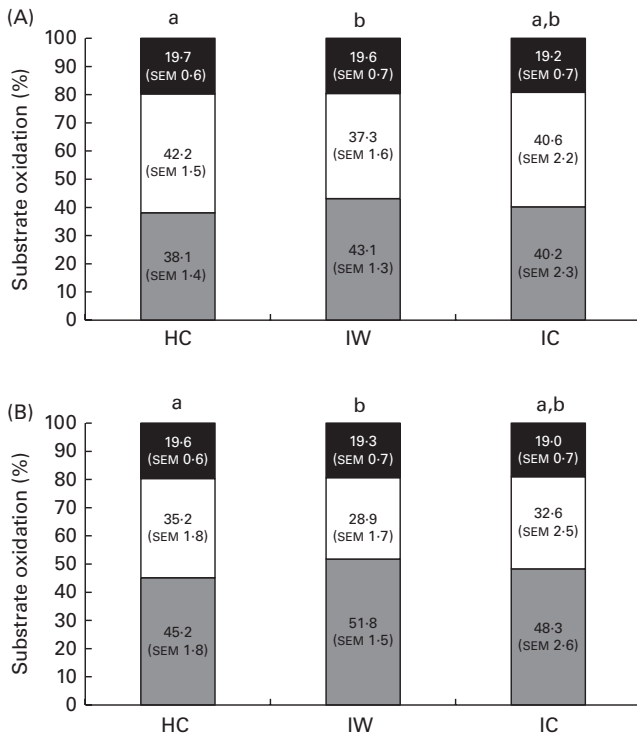


Fig. 3. Substrate oxidation during daytime (A) and after the breakfast meal (B) on day 1 (*n* 24). There was no difference in protein oxidation between the dietary proteins. Repeated-measures analyses showed that intact whey (IW) stimulated larger lipid oxidation and smaller carbohydrate oxidation than hydrolysed casein (HC) during daytime as well as during the time after the breakfast meal on day 1. There was no difference between the HC and intact casein (IC) treatments or between the IW and IC treatments. Values are means, with their standard errors. ^{a,b}Mean values with unlike letters were significantly different (*P*<0.05). ■, Lipid; □, carbohydrate; ■, protein.

were positively ($r=0.4894$, $P<0.0001$) and negatively ($r=-0.1813$, $P=0.0002$) correlated, respectively, with lipid oxidation.

Appetite

There were no effects observed for the time × treatment interaction and for treatment on satiety, hunger, fullness and prospective food consumption during daytime, as well as during the time after the breakfast meal on day 1 or day 2. Thirst was also not affected differently by the three dietary treatments at any time intervals studied. However, IC elicited a lower desire to eat savoury foods than IW during daytime ($P=0.006$), and tended to elicit a lower desire to eat savoury foods after ingestion of the breakfast meal on day 1 ($P=0.075$). There was no difference observed between the HC and IC treatments or between the HC and IW treatments, as well as between any of the treatments after the breakfast meal on day 2. IC also elicited a lower desire to eat sweet foods than HC after ingestion of the breakfast meal on day 1 ($P=0.015$) and day 2 ($P=0.021$). Moreover, IW tended to induce a lower desire to eat sweet foods than HC after ingestion of the breakfast meal on day 2 ($P=0.087$). However, there was no difference observed between the dietary treatments during daytime. Likewise, there was no difference observed

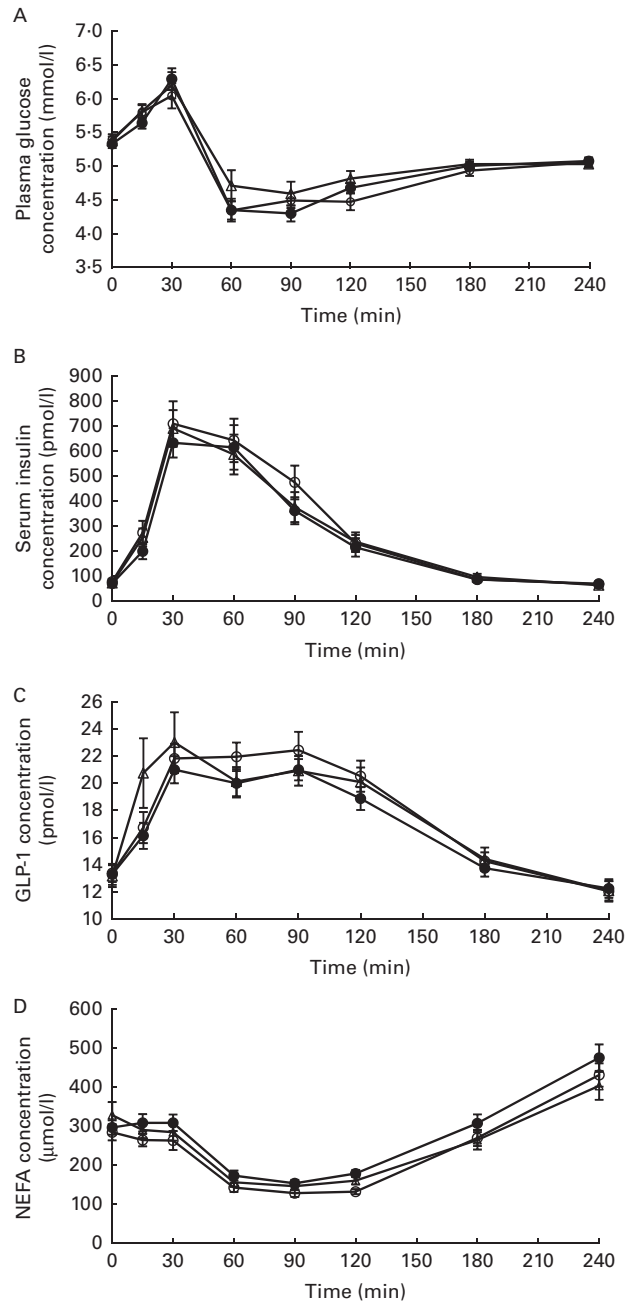


Fig. 4. Response of (A) glucose, (B) insulin, (C) glucagon-like peptide 1 (GLP-1) and (D) NEFA concentrations after ingestion of the breakfast meal on day 1 (0–240 min; *n* 24). Glucose concentrations were higher after ingestion of intact casein (IC, △) than after ingestion of hydrolysed casein (HC (○); $P=0.033$); however, there was no difference between the HC and intact whey (IW, ●) treatments or between the IW and IC treatments. No difference was observed in insulin concentrations between the dietary treatments. For GLP-1, there was a significant effect of the time × treatment interaction. At 15 min, GLP-1 concentration was higher after ingestion of IC than after ingestion of HC and IW ($P<0.05$), with no difference being observed between the HC and IW treatments. However, at 60 and 90 min, GLP-1 concentration was higher after ingestion of HC than after ingestion of IW and IC ($P<0.05$ and $P<0.01$, respectively), but did not differ between the IW and IC treatments. There were no significant differences observed at any other time points. The concentration of NEFA was higher after ingestion of IW than after ingestion of HC ($P=0.003$) and IC ($P<0.0001$), with no difference being observed between the HC and IC treatments.

between the dietary treatments in relation to the desire to eat salty or fatty foods at any of the time intervals studied.

Moreover, there was no difference in energy intake observed between the dietary treatments after the *ad libitum* lunch meal on day 2 (HC: 3571 (SEM 218) kJ; IW: 3780 (SEM 216) kJ; IC: 3667 (SEM 180) kJ), and palatability assessed after the *ad libitum* meal did not depend on the dietary treatments.

The palatability of the test meals was rated lower after consumption of HC than after consumption of IW and IC ($P < 0.01$), and an effect of the time \times treatment interaction was found for feelings of comfort ($P = 0.038$). After breakfast, the subjects felt less comfortable after consumption of HC than after consumption of IW at 10, 30 and 60 min ($P < 0.01$), and tended to feel less comfortable at 150 min ($P = 0.075$). Moreover, immediately after the breakfast meal on day 1 (10 min), the subjects felt less comfortable after consumption of HC than after consumption of IC ($P = 0.003$). There was no difference in feelings of comfort observed between the IW and IC treatments during daytime, as well as during the time after the breakfast meal on day 1; however, the subjects tended to feel less comfortable after consumption of HC than after consumption of IW ($P = 0.093$).

Discussion

The present study showed that whey, casein and hydrolysed casein induced similar effects on appetite regulation and on 24 h total and postprandial EE after ingestion of high-protein mixed meals. However, we showed that the RQ value was lower after consumption of whey than after consumption of hydrolysed casein, also after adjustment for energy balance. This might indicate higher lipid oxidation after ingestion of whey than after ingestion of hydrolysed casein, which was supported by the response of NEFA concentration.

Proteins are more satiating than CHO and fat^(2,3,30), and Belza *et al.*⁽¹⁾ recently showed that proteins stimulate satiety in a dose-dependent manner. However, little is known about the effects of different protein sources. Proteins differ in their amino acid composition, which may influence their effects on satiety. Casein and whey are both complete proteins, but whey has a higher content of branch-chained amino acids and contains the components glycomacropeptide and α -lactoglobulin, which have previously been found to stimulate satiety^(31–33). Despite this finding, there is no clear evidence that whey is more satiating than casein or vice versa⁽⁸⁾. In the present study, no difference was observed between the whey, casein and hydrolysed casein treatments, which is supported by several human studies^(16,32,34). However, the results have been inconsistent. Hall *et al.*⁽⁹⁾ found that whey stimulates satiety compared with casein during 180 min following ingestion. Accordingly, they observed a higher plasma concentration of the appetite-regulating hormones cholecystokinin, GLP-1 and glucose-dependent insulinotropic polypeptide after consumption of whey. This finding is partly supported by Veldhorst *et al.*⁽¹⁵⁾, who demonstrated a decreased feeling of hunger after consumption of whey compared with casein at a protein dose of 10 E%. However, they observed no difference at a dose of 25 E%. They

suggested that the inconsistent results may be explained by the difference in protein concentration, as the concentration of certain amino acids may need to be above a particular threshold to promote a relatively stronger hunger suppression or greater satiety. Their results suggested that certain proteins will reach these threshold concentrations at lower doses than other proteins. At high-protein loads, it may not be possible to distinguish between different complete proteins as the concentration of all amino acids rapidly increases above this threshold. In the present study, the percentage of energy from proteins varied from 17 to 26 E%, suggesting that the protein load may be too high to detect a difference between the proteins. However, other factors may play a role. For example, Acheson *et al.*⁽³⁾ found that casein is more satiating than whey at a protein content of 50 E%. They continued their measurements for 330 min, which indicated that timing of measurements may also play an important role. In support, Boirie *et al.*⁽¹⁴⁾ showed that plasma amino acid concentrations are higher after consumption of whey than after consumption of casein at 100 min and higher after consumption of casein than after consumption of whey at 300 min. This suggests that the effects of casein may not be fully developed when appetite measures are obtained shortly (180 min) after protein consumption as in the study by Hall *et al.*⁽⁹⁾, and that the concentration of amino acids after consumption of whey may have returned to baseline levels when the measurements are continued for 330 min. Accordingly, we expected that whey and hydrolysed casein would have a greater satiating effect than casein shortly after protein consumption, and that casein would be more satiating several hours after consumption of proteins. Moreover, we expected that gastric emptying and the rate of absorption would influence the effect on EE in a similar way.

We observed no differences between the dietary proteins in relation to postprandial or 24 h total EE. In support of this finding, Lorenzen *et al.*⁽¹⁶⁾ also did not find a difference in DIT between whey and casein; however, the results have been inconsistent^(3,17). Previously it has been shown that when whey and casein are served in mixed meals, it may mask the differences in kinetics, mostly due to a slower absorption rate of whey⁽³⁵⁾. Therefore, this may explain the reason why we observed no differences in appetite regulation and EE between the dietary proteins. Moreover, it can be speculated that the differences in kinetics may be masked by other proteins present in the shakes/meals. However, they only make up a very small amount of total protein (2.7 E% of 26 E% in the shakes) and were also similar between the dietary treatments, why it is unlikely to affect the present results. However, other factors may play important roles such as those suggested by Acheson *et al.*⁽³⁾ who found that consumption of whey induced a larger increase in DIT and cumulative EE at 330 min than consumption of casein, although the proteins were served as shakes that additionally contained fat and CHO. The authors suggested that the higher DIT observed following consumption of whey may be explained by the beneficial effect on protein synthesis. Boirie *et al.*⁽¹⁴⁾ showed that protein synthesis was 2-fold more rapid, measured at 40–140 min, after consumption of

wey than after consumption of casein. In contrast to the present study and the study by Lorenzen *et al.*⁽¹⁶⁾ where casein was served as a caseinate, in the study by Acheson *et al.*⁽³⁾, casein was served as micellar casein. Caseinates are absorbed more rapidly than the micellar form, which may explain why the difference in absorption rates between proteins used in the present study may be very small.

Furthermore, comparison of studies may be hampered by the use of different methodologies, timings of measurements, energy contents and proportions of protein in the meals served. The majority of studies investigating the effects of protein on EE have measured the thermogenic effect after one single meal using ventilated hood systems, whereas the present study used respiration chambers. It can be speculated that the 'dead space' of the chambers (14.7 m³) may be too large to allow the detection of small and transient changes in EE. However, respiration chambers were also used in the study by Acheson *et al.*⁽³⁾, but these chambers may have very different response times. Moreover, as DIT is only 10% of the total EE, it can be speculated that a rather large meal is necessary to induce a detectable increase in postprandial EE. The meal size in the study by Acheson *et al.*⁽³⁾ was similar to the breakfast meal in the present study (20% of ER), but the protein content was much greater (50 E%).

To our knowledge, no human studies have compared the effects of casein and its hydrolysate on EE or appetite regulation; however, a recent study in mice has shown that intact casein and hydrolysed casein induce similar effects on EE⁽²⁰⁾. The authors also examined the effect on the RQ and showed that hydrolysed casein tends to induce a lower RQ than intact casein during light periods (07.30–19.00 hours), indicating that the absorption rate may play an important role. In the present study, no difference in RQ was found between hydrolysed casein and intact casein; however, we found that hydrolysed casein induced a slightly higher mean RQ value (0.86 (SEM 0.01) *v.* 0.84 (SEM 0.01), respectively) than whey during daytime when food was provided, and these results remained significant after adjustment for energy balance. A higher RQ value suggests higher CHO and lower lipid oxidation, but could also indicate higher amino acid oxidation, which would drive the RQ value to 0.8⁽³⁶⁾. A limitation of the present study was that there was no continuous measurement of protein oxidation. Only the data on the average 24 h protein oxidation were obtained from 24 h N excretion. However, data on the RQ and substrate oxidation were analysed over rather long postprandial time intervals (≥ 4.5 h post-meal consumption), and based on previous findings^(14,18), we expected that amino acids, from all the three dietary proteins, would be absorbed within this time period.

Furthermore, data on the RQ and substrate oxidation were supported by the response of plasma NEFA concentrations. NEFA concentrations were positively correlated with lipid oxidation and have previously been associated with a lower RQ value⁽³⁷⁾ and lower glucose oxidation⁽³⁸⁾. In accordance with a lower RQ value after consumption of whey, the response of NEFA concentration was increased compared with hydrolysed casein. Furthermore, insulin is known to suppress lipolysis⁽³⁹⁾. Therefore, it can be speculated that insulin may

have a modulating effect on lipolysis and consequently lipid oxidation. In the present study, insulin was weakly correlated with lipid oxidation estimated from the RQ; however, we observed no differences in postprandial response of insulin concentration between the dietary proteins. Data on the differences in the insulinotropic effects of whey and casein in healthy human subjects are sparse; however, some studies have suggested a beneficial effect of whey^(40,41). Moreover, it has been previously demonstrated that the response of insulin concentration is associated with the availability of plasma amino acids^(23,42), with rapidly absorbed proteins inducing a greater response of insulin concentration than slowly absorbed proteins. Accordingly, Deglaire *et al.*⁽⁴³⁾ showed a higher insulin response after ingestion of hydrolysed casein than after ingestion of casein, especially during the initial postprandial hours when plasma amino acids were higher.

In contrast, Akhavan *et al.*⁽²¹⁾ investigating the effects of intact and hydrolysed whey on glucose and insulin concentrations found that intact whey stimulates a lower postprandial glucose concentration than hydrolysed whey, despite similar insulin concentrations. This indicates that intact proteins regulate blood glucose levels in a non-insulinotropic manner. A higher blood glucose concentration following consumption of hydrolysed whey may stimulate a higher RQ value, which was not measured. This also suggests that gastric emptying may affect the magnitude and timing of postprandial blood glucose and insulin increases and that intact whey is emptied more slowly than hydrolysed whey because whey, but not hydrolysed whey, stimulates the secretion of gut peptides that are involved in glycaemic control and gastric emptying. This is though not a consistent finding. Calbet & Holst⁽¹⁸⁾ showed that whey and casein, as well as their hydrolysates, stimulate the secretion of incretins. Likewise, we showed that at some time points, GLP-1 concentrations were higher after consumption of hydrolysed casein than after consumption of intact casein and intact whey. In contrast, there was no overall difference in GLP-1 concentration between the dietary proteins, which is in accordance with the data on insulin and appetite regulation. Data from previous studies investigating the effect of different protein sources are inconsistent^(9,18,44). For example, Hall *et al.*⁽⁹⁾ observed an increase in GLP-1 concentration after consumption of whey than after consumption of casein in accordance with a greater satiating effect, whereas Calbet & Holst⁽¹⁸⁾ showed no difference in GLP-1 response between whey, casein and their hydrolysates. Finally, we observed a higher mean glucose concentration after consumption of casein than after consumption of hydrolysed casein, although there was no difference in insulin concentration. This may, however, be a matter of chance finding due to multiple testing.

The strength of the present study was the randomised cross-over design, where the subjects were kept under controlled settings for 40 h, including the measurements. Moreover, proteins were served in mixed meals, which reflect real-life situations, and measurements were continued for a total 24 h period. However, the study has also some obvious limitations. As mentioned previously, protein oxidation was not assessed as a function of time, and fat and CHO oxidation were not independently tested but estimated from the RQ values.

Moreover, gastric emptying and differences in absorption rates were not measured. Finally, as shown in Fig. 4, a blood sample taken at 45 min would have been advantageous as all biochemical measures were likely to reach peak or nadir magnitude between 30 and 60 min. Therefore, the lack of the blood sample taken at this time period limits the analyses of peak and nadir magnitudes.

Conclusion

In conclusion, data from the present study suggest that repeated meals with whey, hydrolysed casein and casein induce similar effects on appetite regulation and postprandial and total EE over 24 h, which may be explained by similar absorption rates when proteins are served as high-protein mixed meals. However, we found that whey resulted in a slightly lower RQ value than hydrolysed casein, indicating a potentially higher lipid oxidation and lower CHO oxidation. Future studies are needed to investigate whether the effects on substrate oxidation can be verified and whether they persist over time, and thereby can contribute to a beneficial effect on body weight. Moreover, future studies should focus on the effect of the relative amount of protein in the meals served, and the effect of serving protein in mixed meals *v.* the effect of serving proteins alone, as this may influence gastric emptying and the rate of absorption.

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