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Randomized control trials

Intraileal casein infusion increases plasma concentrations of amino acids in humans: A randomized cross over trial

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SUMMARY

Background: Activation of the ileal brake by casein induces satiety signals and reduces energy intake. However, adverse effects of intraileal casein administration have not been studied before. These adverse effects may include impaired amino acid digestion, absorption and immune activation.
Objective: To investigate the effects of intraileal infusion of native casein on plasma amino acid appearance, immune activation and gastrointestinal (GI) symptoms.
Design: A randomized single-blind cross over study was performed in 13 healthy subjects (6 male; mean age 26 ± 2.9 years; mean body mass index 22.8 ± 0.4 kg/m^{-2}), who were intubated with a naso-ileal feeding catheter. Thirty minutes after intake of a standardized breakfast, participants received an ileal infusion, containing either control (C) consisting of saline, a low-dose (17.2 kcal) casein (LP) or a high-dose (51.7 kcal) of casein (HP) over a period of 90 min. Blood samples were collected for analysis of amino acids (AAs), C-reactive protein (CRP), pro-inflammatory cytokines and oxylipins at regular intervals. Furthermore, GI symptom questionnaires were collected before, during and after ileal infusion.
Results: None of the subjects reported any GI symptoms before, during or after ileal infusion of C, LP and HP. Plasma concentrations of all AAs analyzed were significantly increased after infusion of HP as compared to C ($p < 0.001$), and most AAs were increased after infusion of LP ($p < 0.001$). In total, 12.49 ± 1.73 and 3.18 ± 0.87 g AAs were found in plasma after intraileal infusion of HP and LP, corresponding to 93 ± 13% (HP) and 72 ± 20% (LP) of AAs infused as casein, respectively. Ileal casein infusion did not affect plasma concentrations of CRP, IL-6, IL-8, IL-1β and TNF-α. Infusion of HP resulted in a decreased concentration of 11,12-dihydroxyeicosatrienoic acid whereas none of the other oxylipins analyzed were affected.
Conclusions: A single intraileal infusion of native casein results in a concentration and time dependent increase of AAs in plasma, suggesting an effective digestion and absorption of AAs present in casein. Also, ileal infusion did not result in immune activation nor in GI symptoms.
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1. Introduction

The increasing prevalence of overweight causes increased concerns for health and health care costs worldwide. There is a clear need for therapeutic and preventive strategies, as current strategies have not proven to be successful on the long term.

One potential strategy to reduce energy intake is activation of the so-called ileal brake. The ileal brake is a feedback mechanism

Abbreviations: AAs, amino acids; AUC, area under the curve; ANOVA, mixed analysis of variance; C, control; CRP, C-reactive protein; CV, coefficient of variation; GI, gastrointestinal; GLP-1, glucagon-like peptide 1; PYY, peptide YY; HP, high protein; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-8, interleukin-8; LLOQ, lower limit of quantification; LOD, limit of detection; LP, low protein; RYGP, Roux-en-Y gastric bypass; SEM, standard error of the mean; TNF-α, tumor necrosis factor-α.

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that slows or 'brakes' the process of proximal gastrointestinal digestion and absorption, and food intake. The ileal brake is activated when energy-containing macronutrients reach the ileum. Ileal brake activation induces enhanced satiety signals, satiation and reduction of energy intake [1,2]. Recently, we have shown that not only ileal appearance of lipid but also of the other macronutrients, carbohydrate (sucrose) and protein (casein), results in increased satiation and a reduction of food intake [3]. This reduction in food intake underlines the relevance of the ileal brake as potential food based strategy in the prevention or treatment of overweight and obesity. Despite its potential, it should be investigated whether undigested native protein infused into the ileum does not result in adverse effects such as protein malabsorption or immune activation.

Intraileal infusion bypasses the proximal parts of the gastrointestinal (GI) tract as is also achieved by bariatric surgery. One of the most frequently applied bariatric procedures worldwide is the Roux-en-Y gastric bypass (RYGB). RYGB effectively results in weight loss and improvement of type II diabetes [4]. Although the mechanism is not completely understood yet, the proposed mechanism by which RYGB may exert its beneficial effects is enhanced stimulation of enteroendocrine cells in the distal small intestine by undigested nutrients, resulting in the release of the L-cell products glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) [5]. It has been suggested that weight loss after RYGB may be attributable to limited food intake which can be partly explained by gastric restriction and malabsorption. However, evidence has increased that the positive health effects induced by RYGB may be due to altered GI physiology [4] and not due to gastric restriction and malabsorption. RYGB results in rapid emptying of gastric content with prolonged small intestinal transit times [6] and only a small proportion of the reduction in energy intake after RYGB is due to malabsorption [7]. Malabsorption may result in an increased risk of developing nutritional deficiencies, such as protein deficiency [8]. Under physiological conditions, gastric acid activates the conversion of pepsinogen into the proteolytic enzyme pepsin which denatures orally ingested protein. Protein is further digested by trypsin in the small intestine. In response to orally ingested protein, amino acids in the form of peptides or free amino acids are found in the intraluminal content of the jejunum and ileum [9].

Recently it was found by Bojsen-Møller et al. (2015) that RYGB accelerates caseinate digestion and amino acid absorption, and it was suggested that protein digestion is not impaired after RYGB [10]. However, it remains to be investigated whether native casein, directly infused into the ileum and thus bypassing the proximal parts of the small intestine results in malabsorption. If this is the case, protein may be available for colonic fermentation resulting in potential harmful effects for the host's health [11]. Such effect could manifest itself by immune activation [12]. This might be shown by the production and release of pro-inflammatory cytokines such as interleukin-1β (IL-1β), IL-6, IL-8 and tumor necrosis factor-α (TNF-α) [12], or other pro-inflammatory mediators such as C-reactive protein (CRP) and eicosanoids, a subset of oxylipins. The release of these lipid mediators can be triggered by a variety of stimuli. They have been associated to be involved in pain and fever and are known to be potent regulators of the host immune response [13,14].

The aim of this study was to investigate the effects of intraileal native protein infusion on digestion, AA absorption and immune activation. This was done in a study evaluating the effects of intraileal infusion of native protein on food intake previously performed by our group [3]. It was hypothesized that ileal infusion of native casein results in plasma appearance of AAs in casein and does not result in acute adverse effects such as incomplete digestion of casein protein or inflammation. This was investigated by analyzing plasma AAs at several time points after intraileal infusion of both a low-dose and high-dose of casein in a randomized single-blind cross over study as reported by van Avesaat et al. (2015) [3]. Inflammatory mediators such as pro-inflammatory cytokines and oxylipins were analyzed to investigate acute adverse inflammatory effects.

2. Material and methods

2.1. Study design

In the present study plasma samples from a previously published single blind randomized placebo controlled cross over study were analyzed to investigate the effects of intraileal protein infusion on plasma profiles of AAs and inflammation markers [3]. Thirteen subjects (6 male, 7 female, age 26 ± 2.9 years, BMI of 22.8 ± 0.4 kg m^{-2}) completed the study.

The effects on AAs and inflammation markers of the following interventions were investigated 1) saline infusion (control (C)), 2) low dose protein infusion (5 g casein (LP), 17.2 kcal) and 3) high dose protein infusion (15 g casein (HP), 51.7 kcal). Each treatment was infused into the ileum over a 90-min period, on separate test days. Order of interventions were randomly assigned to each participant and based on a randomization protocol (via randomizer.org) defined prior to the start of the study.

Casein (energy density: 3.45 kcal/g, Dutch Protein & Services, Tiel, The Netherlands) was used as protein source. The AA composition of casein is shown in Table 1 (Eurofins Food Testing Netherlands B.V., Heerenveen, The Netherlands). Casein was dissolved in a total volume of 180 ml tap water and infused into the ileum a rate of 2 ml/min (0.6 kcal/min; total infusion time 90 min).

2.2. Experimental design

Each subject participated in all three test days. On all test days an intravenous catheter was placed in a forearm vein for blood collection. At 8.30 AM, a basal blood sample was obtained. Subsequently, a standardized breakfast meal consisting of a sandwich

Table 1
Amino acid composition of native casein expressed as % (w/w).

Amino acid	% (w/w)
Glutamic acid[a]	18.60
Proline	9.21
Leucine	8.09
Lysine	6.95
Aspartic acid[a]	6.11
Valine	5.55
Serine	4.89
Tyrosine	4.57
Phenylalanine	4.35
Isoleucine	4.33
Threonine	3.68
Arginine	3.08
Alanine	2.62
Histidine	2.40
Methionine	2.22
Glycine	1.56
Tryptophan	1.15
Cysteine	0.39
Total	89.75

[a] The amino acid composition of casein was analyzed by acid protein hydrolysis followed by amino acid analysis using ninhydrin according to ISO 13903:2005. This analytical method is unable to separate aspartic acid from asparagine and glutamic acid from glutamine. Tryptophan and cysteine were analyzed according the EU152/2008 (F) and ISO 13903:2005 protocol.

and an egg (sunny side up, 210 kcal) was consumed. Ileal substrate infusion was performed from $t = 30$ to $t = 120$ min after breakfast ingestion. Participants were blinded to the infusion treatment. One hour after ending the infusion ($t = 180$ min), the volunteer received a standardized *ad libitum* lunch meal (sandwiches with egg salad (energy density: 2.2 kcal/g)). At $t = 240$ the last blood sample was taken and the test day was finished.

2.3. Questionnaires

GI symptoms were evaluated using a questionnaire addressing complaints such as headache, nausea, stomach-ache, diarrhea, and other symptoms. Symptoms were graded on a 4-point scale with the grade 0 representing 'not present' to 4 'strongly present'. Subjects were asked to mark how they felt at the moment before ($t = 15$ and $t = 30$), during ($t = 60$ and $t = 90$) and after the infusion stopped ($t = 120$ and $t = 240$).

2.4. Sample collection

Venous blood samples were drawn 15 min before breakfast ($t = -15$ min), and at 30, 45, 60, 90, 120, 150, 180, 210 and 240 min after breakfast, respectively. Blood was collected in ice-chilled EDTA coated tubes (Becton & Dickinson, Franklin Lakes, NJ, USA). Immediately after blood collection, tubes were centrifuged at a rate of 3000 revolutions per minute at 4 °C for 10 min. Plasma was transferred into aliquots and stored on dry ice for the rest of the test day. At the end of the test day, samples were stored at −80 °C until further analysis of AAs, cytokines and oxylipins.

2.5. Plasma amino acid profiles

Plasma AA profiles were analyzed including the AAs present in casein (Table 1, except cysteine), as well as other AAs and amines, i.e. 1-methylhistidine, 3-methylhistidine, asymmetric dimethylarginine, citrulline, DL-3-amino isobutylene, glycyl-glycine, homoarginine, hydroxyl-lysine, amino adipic acid, α-amino-n-butyric acid, kynurenine, L-4-hydroxy-proline, L-glutamic acid, homoserine, trimethylamine, phosphor-ethanolamine, putrescine, symmetrical dimethylarginine, sarcosine, serotonin, taurine, gamma-aminobutyrine, gamma-L-glutamyl amine, glycine, ethanol, glutamine, o-acetyl-L-serine. AAs and amines were analyzed using an LC–MS method as described previously [16].

2.6. Plasma profiles of pro-inflammatory cytokines and C-reactive protein

Plasma profiles of CRP, TNF-α, IL-1β, IL-6, IL-8 were analyzed using an in-house developed and validated multiplex immunoassay based on Luminex technology (xMAP, Luminex, Austin TX USA). The assay was performed as described previously [17,18]. Aspecific heterophilic immunoglobulins were pre-absorbed from all samples with heteroblock (Omega biologicals Bozeman MT, USA). Acquisition was performed with the Biorad FlexMAP3D (Biorad laboratories, Hercules USA) in combination with xPONENT software version 4.2 (Luminex). Data were analyzed by 5-parametric curve fitting using Bio-Plex Manager software, version 6.1.1 (Biorad).

For TNF-α the range was 1.2–5000 pg/ml and the limit of detection (LOD) for TNF-α was 0.7 pg/ml, with an inter-assay coefficient of variation (CV) of 3.0 ± 1.8% and an intra-assay CV of 7.6 ± 0.6%. For IL-1β the range of the assay was 1.2–5000 pg/ml and the LOD was 0.4 pg/ml with an intra-assay CV of 3.4 ± 2.9% and an inter-assay of 3.5 ± 2.5%. For IL-6 the range of the assay was 2.4–10,000 pg/ml and the LOD for IL-6 was 0.9 pg/ml, with an intra-assay CV of 4.4 ± 2.7% and an inter-assay CV of 11.0 ± 4.6%. For IL-8 the range of the assay was 2.4–10000 pg/ml and the LOD was 1.3 pg/ml with an intra-assay CV of 5.8 ± 3.6% and inter-assay CV of 13.7 ± 6.8%. For CRP the range was 12.2–50,000 pg/ml and the LOD was 11.7 pg/ml whereas the upper limit of detection was 42,691.6 pg/ml. The samples were diluted to be within the range of the assay.

Not all cytokine concentrations were above the lower limit of quantification (LLOQ) of the assay. For TNF-α, 63% of the samples was below the LLOQ (1.2 pg/ml). For IL-1β, 25% of the samples was below the LLOQ (2.2 pg/ml). For IL-6, 11% of the samples was below LLOQ (2.8 pg/ml). For IL-8, 46% of the samples was below LLOQ

Table 2
Overview of oxylipins analyzed assigned to their precursor fatty acids.

	Arachidonic acid	Dihomo-γ-linoleic acid	Docosahexa-enoic acid	Eicosapenta-enoic acid	Linoleic acid
Prostanoids/thromboids	TXB2 PGF2α PGE2 13,14-Dihydro-PGF2α	PGF1α			
Diols	14,15-DiHETrE 11,12-DiHETrE 8,9-DiHETrE 5,6-DiHETrE		19,20-DiHDPA	17,18-DiHETE 14,15-DiHETE	12,13-DiHOME 9,10-DiHOME
Epoxides					12(13)-EpOME 9,10-EpOME
Alcohols	12-HETE 5-HETE 15-HETE 20-HHETE 11-HETE		17-HDoHE 14-HDoHE 10-HDoHE	12-HEPE 5-HEPE	9-HODE 13-HODE
Ketones					13-KODE 9-KODE
Triols					9,12,13-TriHOME 9,10,13-TriHOME

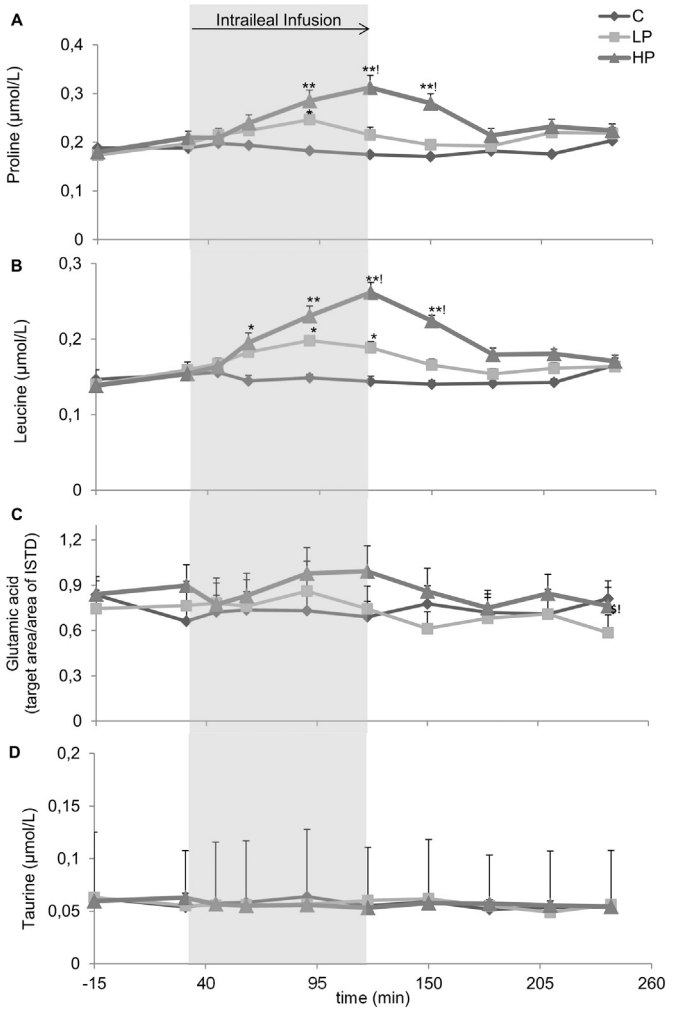

Fig. 1. Plasma profiles of proline (A), leucine (B), glutamic acid (C) and taurine (D) after intraileal infusion (infusion time t = 30–120 min) of control (C), low-dose casein protein (LP) or high-dose casein (HP) (n = 13). An interaction effect (treatment*time) for proline (A) and leucine (B) was observed. Proline and leucine concentrations increased after HP infusion ($p < 0.0001$) and LP infusion ($p < 0.0001$). Glutamic acid (C) increased after HP infusion ($^{\$!}$treatment effect $p < 0.005$) as compared with both placebo and LP infusion (this panel represents ratios of the peak area's/internal standard peak areas). Taurine concentrations (D) were not affected by HP or LP infusion. $^{*}p < 0.05$ compared with placebo at the same time point, $^{**}p < 0.0001$ compared with placebo at the same time point, $^{!}p < 0.001$ compared with LP at the same time point. Note: in Supplemental data Table 1 the results of all amino acids are presented.

(4.5 pg/ml). Since these samples were below the detection range of the assay, they were excluded from the data analysis.

2.7. Plasma profiles of oxylipins

The oxylipin platform (Table 2) covers classical and non-classical eicosanoids from different polyunsaturated fatty acids, including ω-6 and ω-3 PUFAs such as linoleic acid, arachidonic acid and dihomo-γ-linoleic acid (all ω-6), eicosapentaenoic acid and docosahexaenoic acid (both ω-3). The samples were analyzed by liquid chromatography tandem mass spectrometry as described previously [19].

2.8. Data analysis

Intervention effects on plasma AA concentrations, pro-inflammatory cytokines and oxylipins were analyzed within participants with a mixed model analysis of variance (ANOVA) including the fixed factors treatment (infusion of C, infusion of LP and infusion of HP), time and the interaction between treatment and time. Because of the crossover design, intervention effects within subjects were compared by including the random factor subject.

All statistical analyses were performed using the SAS statistical software package (SAS version 9; SAS institute, Cary, NC, USA). Data were visually checked on normality and on constant variance of residuals by plots of residuals vs. corresponding predicted values. All data met the criteria for ANOVA assumptions. If an intervention effect occurred post hoc comparisons were made using Tukey–Kramer adjustment to correct for multiple testing. Data are presented as the mean ± SEM (unless specified otherwise) and considered significant at $p < 0.05$.

Since infused amounts of native casein (5 g LP vs. 15 g HP) were known, the amount of AAs infused as casein was calculated based on the AA composition of the native casein infused (Table 1). To calculate the absolute amount of AA in gram, plasma AA concentrations (µmol/L) were corrected for the total blood volume (female 65 ml/kg, male 75 ml/kg [20]). To estimate the increase in AAs due to LP and HP infusion, the total area under the curves (AUCs) during the infusion period (t = 30 until t = 180) were calculated by applying the trapezoid method. The AUC after C treatment was subtracted from AUCs after LP and HP infusion per individual to correct for control AA concentrations. In case the AUC was negative after placebo correction, the value was excluded from further analysis (n = 3 for LP and n = 1 for HP).

3. Results

3.1. Questionnaires

Subjects did not report any feelings of nausea, intestinal cramps, diarrhea, headache, heartburn, belching or other parameters of impaired wellbeing before, during or after infusion of C, LP or HP.

3.2. Amino acid plasma concentrations

Baseline AA concentrations were not different between treatments. Overall, a treatment and time interaction effect was found ($p < 0.001$); AAs present in native casein increased after LP and HP over time as compared to C ($p < 0.001$), whereas amines or metabolites not present in casein such as taurine did not (Fig. 1 and Supplemental data Tables 1 and 2). Post-hoc analysis showed that both LP and HP resulted in increased plasma concentrations over time as compared to C of 15 out of 19 analyzed AAs in casein, namely alanine, arginine, asparagine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine and valine ($p < 0.001$). Aspartic acid was only increased after HP ($p < 0.0001$). Concentrations of glutamic acid could not be assessed due to high plasma glutamic acid concentrations. Therefore these results are shown in relative peak areas as compared to an internal standard of glutamic acid concentration (Fig. 1C). Glutamic acid was increased after HP and LP as compared to C ($p < 0.005$). Plasma concentrations of glutamine and glycine did not change after LP and HP infusion.

HP resulted in a further increase of the following AAs as compared to LP; aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, tyrosine, tryptophan and valine ($p < 0.001$). After infusion of LP and HP the AAs present in native casein increased when the infusion started (t = 30), and peak plasma concentrations were reached at the time the infusion stopped (t = 120) (Fig. 1A, B). All other amines and AAs not present

in casein, such as taurine (Fig. 1D), did not show a change in plasma concentrations after LP, HP or C.

In Table 3 the mean AUCs (g) are shown per AA for LP and HP. Also the total amount of AAs found in plasma after intraileal infusion of LP and HP was calculated as a percentage of amount of AAs infused as casein (4.44 g for LP and 13.38 g for HP). In total, 3.18 ± 0.87 g and 12.49 ± 1.73 g AAs were found in plasma after intraileal infusion of LP and HP, respectively. These numbers correspond to 72 ± 20% (LP) and 93 ± 13% (HP) of the amount of AAs infused as casein.

3.3. Cytokines and CRP

Plasma concentrations of IL-6, IL-8, IL-1β, TNF-α and CRP were not affected by either LP, HP or C (Fig. 2). Plasma concentrations of the cytokines analyzed did not change over time and no interaction effect between time and protein infusion was found.

3.4. Oxylipins

Only an effect of infusion ($p < 0.001$), time ($p < 0.001$) and interaction between infusion and time was found for 11,12-DiHETrE ($p < 0.05$) (Fig. 3). Concentrations of 11,12-DiHETrE decreased after HP ($p < 0.001$) and LP ($p < 0.01$) as compared to C. Plasma concentrations of 11,12-DiHETrE did not differ between LP as compared to HP.

An infusion and time effect ($p < 0.05$) was found for 5,6-DiHETrE and 19,20-DiHDPA. These oxylipins decreased after HP as compared to C, and LP ($p < 0.05$). However, no interaction between time and treatment was found.

None of the other oxylipins presented in Table 2 were affected by ileal infusion of either LP, HP or C.

4. Discussion

To our knowledge this is the first study showing that intraileal infusion of native casein results in AA plasma recoveries of approximately 72–93%. These results suggest that ileal infusion of native protein casein at 5 and 15 g may result in near complete digestion and absorption of its AAs. Pro-inflammatory mediators or GI symptoms did not increase in response to intraileal protein infusion, indicating that intraileal casein infusion does not induce adverse effects in healthy subjects.

The present study aimed to investigate adverse effects of intraileal protein infusion. This was done by 1) estimating AA digestion and absorption after LP and HP reducing the possibility of casein malabsorption and 2) evaluating immune activation after ileal protein infusion.

For casein specific AAs, plasma recoveries of 72 ± 20% and 93 ± 13% were estimated after LP and HP. It cannot be excluded that part of the increased AA concentrations after LP and HP result from endogenous protein breakdown. However the following arguments suggest that the increase in casein specific AAs was due to intraileal casein infusion; 1) data of the present study show reproducible and dose-dependent AA concentration patterns after infusion of both LP and HP for all AAs present in casein. In contrast, plasma concentrations of amines (e.g. taurine) not present in casein did not change after infusion of both LP and HP; 2) Starting ileal protein infusion resulted in an immediate increase in plasma AA concentrations, whereas these concentrations decreased immediately after ending the infusion; 3) Effects on AA plasma concentrations were studied within subjects making direct comparisons between undigested casein and saline possible.

In this study we showed that pro-inflammatory markers were not affected by protein infusion, nor were GI symptoms. Subjects did not report any feelings of nausea, intestinal cramps, diarrhea, headache, heartburn, belching or other overall GI symptoms before, during or after C, LP or HP. The results on subjective feelings of wellbeing during intestinal protein infusion are in line previous studies. In studies of Geraerts (2011) and Ryan et al. (2013) it was shown that intraduodenally infused protein did not result in any GI complaints [21,22].

Recently Neurath et al. proposed that factors in the diet such as dietary protein may contribute to an impaired intestinal barrier

Table 3
AAs in plasma AUCs (30–180 min) after C, LP and HP.

Amino acid	LP ($n = 10$)					HP ($n = 12$)			
	Infused AA (as casein)	AUC plasma AA			Infused AA (as casein)	AUC plasma AA			
	Mean (g)	Mean (g)	SEM	p-value*	Mean (g)	Mean (g)	SEM	p-value*	p-value#
Alanine	0.13	0.44	0.18	ns	0.39	0.88	0.35	<0.05	ns
Arginine	0.15	0.56	0.12	<0.005	0.46	0.72	0.15	<0.0001	ns
Aspartic acid	0.31	0.03	0.01	ns	0.92	0.09	0.04	<0.05	ns
Asparagine		0.06	0.05	ns		0.14	0.05	ns	ns
Glutamic acid	0.90	−0.49	0.90	ns	2.79	0.31	0.89	ns	ns
Glutamine				ns				ns	ns
Glycine	0.08	0.18	0.12	ns	0.23	0.26	0.10	ns	ns
Histidine	0.12	0.31	0.12	<0.05	0.36	0.40	0.10	0.0034	ns
Isoleucine	0.22	0.23	0.08	ns	0.65	0.81	0.12	<0.0001	<0.0001
Leucine	0.40	0.38	0.13	ns	1.21	1.52	0.30	<0.0001	<0.0001
Lysine	0.35	0.47	0.29	ns	1.04	2.35	0.59	0.0003	<0.005
Methionine	0.11	0.04	0.03	ns	0.33	0.28	0.04	<0.0001	<0.0001
Phenylalanine	0.22	0.17	0.07	ns	0.65	0.28	0.06	<0.005	ns
Proline	0.46	0.26	0.18	ns	1.38	0.26	0.18	<0.005	<0.005
Serine	0.24	0.12	0.12	ns	0.73	1.11	0.25	<0.05	ns
Threonine	0.18	0.13	0.09	ns	0.55	0.55	0.14	0.0005	<0.05
Tryptophan	0.06	0.09	0.03	ns	0.17	0.33	0.07	<0.0001	<0.05
Tyrosine	0.23	0.08	0.06	ns	0.69	0.73	0.15	<0.0001	<0.0001
Valine	0.28	0.44	0.23	ns	0.83	1.45	0.26	<0.0001	<0.005
Total	4.44	3.18	0.87	ns	13.38	12.49	1.73	<0.0001	<0.0001

*p-value LP or HP as compared to C, #p-value as compared LP, ns; not significant.

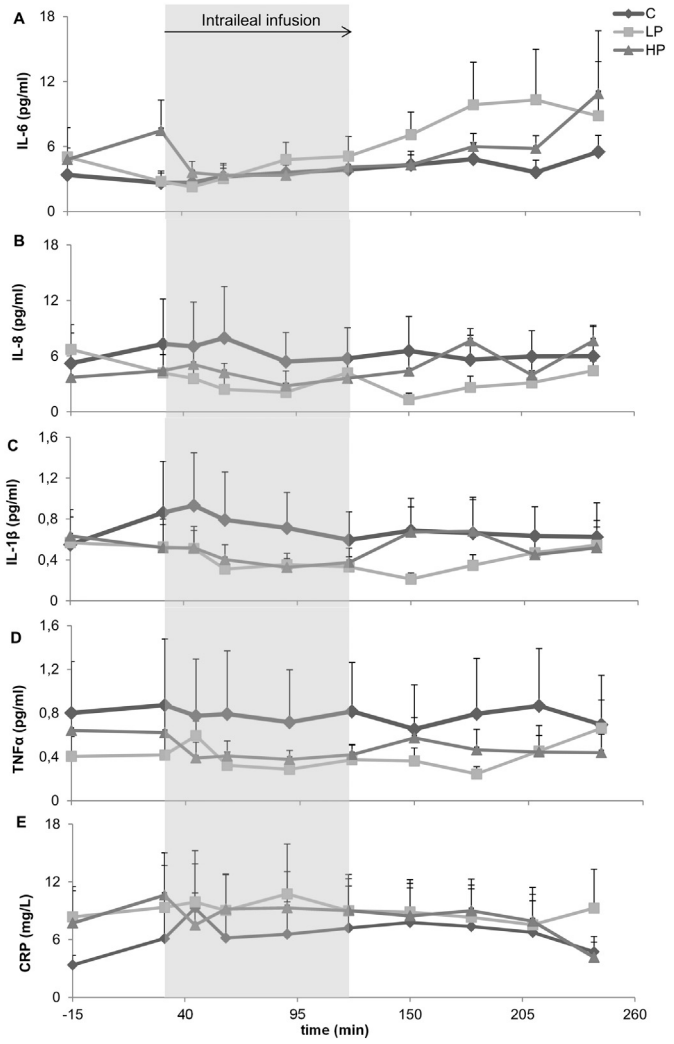

Fig. 2. Plasma concentrations of IL-6 (A), IL-8 (B), IL-1β (C), TNF-α (D) and CRP after intraileal infusion (infusion time $t = 30–120$ min) with control (C), low-dose casein (LP) or high-dose casein (HP). None of the treatments affected plasma concentrations of the cytokines analyzed. For IL-6, 2 subjects plasma concentrations were below the LLOQ ($n = 11$). For IL-8, 8 subjects plasma concentrations were below the LLOQ ($n = 5$). For IL-1β, 3 subjects had plasma concentrations below the LLOQ ($n = 10$) and for TNF-α 8 subjects had plasma concentrations below the LLOQ ($n = 5$).

function, resulting in the release of pro-inflammatory cytokines [12]. Another possible trigger for pro-inflammatory responses when protein is not efficiently digested may be protein fermentation in the colon [11]. Therefore, it was reasonable to postulate that ileal protein infusion could induce an inflammatory response. However, in our study none of the pro-inflammatory cytokines evaluated were affected by ileal infusion of 5 or 15 g casein. Furthermore, almost the total amount of AAs infused as casein was found in blood plasma suggesting that casein was already digested before entering the colon. Additionally, none of the oxylipins analyzed were induced by protein infusion, except for 11,12-DiHETrE, which plasma concentrations decreased after infusion of HP. Recently, increased plasma concentrations of 11,12-DiHETrE have been found in patients with nonalcoholic steatohepatitis (NASH), making it a candidate biomarker for the non-invasive detection of NASH [23]. However, in our study the decrease of 11,12-DiHETrE was rather small and the exact function of this oxylipin is still unknown. Therefore we believe this effect is has little, if any, biological significance.

Several proteolytic enzymes, as well as the expression of peptide- and amino acid-transporters (e.g. peptide transporter 1 and B^0AT1) are known to be present in the ileum [24–27]. These enzymes and transporters may be involved in the digestion and absorption of casein in the ileum. It was hypothesized that intraileal infusion of native casein results in efficient casein digestion and absorption of casein specific AAs. It was found that LP and HP resulted in recoveries of 72 ± 20% and 93 ± 13% for casein specific AAs, suggesting that these AAs were indeed efficiently digested and absorbed. To our knowledge, this is the first study investigating the effects of undigested proteins in the human ileum on its digestibility. Previous studies evaluated postprandial kinetics and digestibility of oral protein intake in healthy volunteers [9,28–30], as well as casein digestibility after RYGB surgery [10]. Our estimated AA recoveries after ileal protein infusion are in line with AA recoveries reported by Mahé et al. In that study orally ingested [^{15}N] casein was recovered in the jejunum mainly in the form of peptides and subsequently efficiently absorbed in the upper part of the small intestine [28]. Luminal jejunal recovery of orally ingested casein was 82.6 ± 9.5%. In another study by Gaudichon et al. (2002) ingestion of a meal containing milk protein resulted in calculated true digestibility percentages of AAs between 90 and 100% [30]. Although the recovery percentages in these studies are in the same range as our findings on AA recoveries, direct comparisons are difficult. Both studies investigated the ingestion of oral protein loads containing more protein as compared to the amounts of protein infused in the present study e.g. 30 g milk protein [30] and 368 mmol casein [28] as compared to 5 or 15 g of casein in the present study. Although the AA recovery of 15 g of ileal casein infusion was approximately 80%, more studies are needed to confirm efficient protein digestion and subsequent AA absorption after intraileal delivery of higher amounts of protein.

This study has limitations that need to be considered. First, the present study design was not optimal to draw conclusions on amino acid digestion and absorption. Since we did not used labeled casein protein we cannot prove that the increased AAs concentrations were due to digestion and absorption of ileal infused casein. Also we cannot exclude a possible interaction effect between breakfast ingestion and casein infusion. Therefore, we can only conclude that it is highly likely that the increased amino acid plasma concentrations originated from casein digestion. Secondly, data of the present study only concern acute responses. The effects of repeatedly targeting the ileum with protein on adverse effects remain to be investigated. However based on available literature of patients undergoing RYGB it seems unlikely that repeatedly targeting the ileum results in adverse effects or protein malabsorption. A recent report showed that ingestion of caseinate by RYGB patients

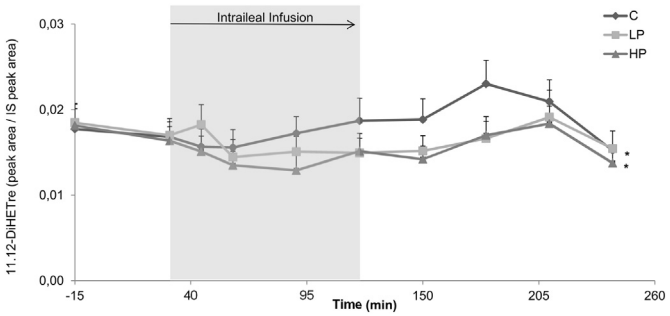

Fig. 3. Results of 11,12-DiHETre after intraileal infusion (infusion time $t = 30–120$ min) with control (C), low-dose casein (LP) or high-dose casein (HP) ($n = 13$). An effect for time ($p < 0.001$), infusion ($p < 0.001$) and interaction between time and infusion ($p < 0.001$) was found for 11.12-DiHETre. *$p < 0.05$ both LP and HP resulted in decreased 11,12-DiHETre.

results in efficient and even accelerated protein digestion and absorption as compared to protein ingestion before RYGB [10]. Furthermore, it has been proposed that malabsorption contributes only a small proportion to the reduction in net energy absorption which is observed after RYGB [4,7]. These data suggest that it is not very likely that repeatedly targeting the ileum with native casein results in adverse effects in healthy subjects. Thirdly, the present study was performed with healthy subjects with a normal BMI and may not give the same results in other groups, such as subjects with comorbidities or overweight individuals. Fourthly, we only measured a selection of inflammation parameters and therefore it is possible that effects could have been missed.

Our group previously showed that intraileal infusion of casein, sucrose and safflower oil results in increased satiation and decreased food intake [3]. A reduction in food intake by ileal protein infusion indicates that the ileal brake may be a target in the prevention of overweight and obesity. The data of the present study show that ileal casein infusion results in high plasma recoveries of casein specific AA and does not induce acute adverse effects. These data provide evidence that targeting the ileal brake may not only be a potent but also a safe target for weight management.

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Conflict of interest

The authors have declared that no competing interests exist.

Author contribution

The authors' responsibilities were as follows: FT, HH and AM: designed the research; MvA and DR: conducted the research; DR: analyzed data and performed the statistical analyses; DR, MvA, FT, HH, AM and RW: contributed to interpretation of the results; DR and HH: wrote the manuscript; HH: had primary responsibility for the final content of the manuscript. All authors approved the final version of the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.clnu.2016.01.012.

References

[1] Maljaars PW, Peters HP, Mela DJ, Masclee AA. Ileal brake: a sensible food target for appetite control. A review. Physiol Behav 2008;95:271–81.
[2] Shin HS, Ingram JR, McGill AT, Poppitt SD. Lipids, CHOs, proteins: can all macronutrients put a 'brake' on eating? Physiol Behav 2013;120C:114–23.
[3] van Avesaat M, Troost FJ, Ripken D, Hendriks HF, Masclee AA. Ileal brake activation: macronutrient-specific effects on eating behavior? Int J Obes (Lond) 2015;39:235–43.
[4] Manning S, Pucci A, Batterham RL. Roux-en-Y gastric bypass: effects on feeding behavior and underlying mechanisms. J Clin Invest 2015;125:939–48.
[5] Holst JJ. Enteroendocrine secretion of gut hormones in diabetes, obesity and after bariatric surgery. Curr Opin Pharmacol 2013;13:983–8.
[6] Dirksen C, Damgaard M, Bojsen-Møller K, Jørgensen N, Kielgast U, Jacobsen S, et al. Fast pouch emptying, delayed small intestinal transit, and exaggerated gut hormone responses after Roux-en-Y gastric bypass. Neurogastroenterol Motil 2013;25:346-e255.
[7] Odstrcil EA, Martinez JG, Santa Ana CA, Xue B, Schneider RE, Steffer KJ, et al. The contribution of malabsorption to the reduction in net energy absorption after long-limb Roux-en-Y gastric bypass. Am J Clin Nutr 2010;92:704–13.
[8] Sawaya RA, Jaffe J, Friedenberg L, Friedenberg FK. Vitamin, mineral, and drug absorption following bariatric surgery. Curr Drug Metab 2012;13:1345–55.
[9] Adibi SA, Mercer DW. Protein digestion in human intestine as reflected in luminal, mucosal, and plasma amino acid concentrations after meals. J Clin Invest 1973;52:1586–94.
[10] Bojsen-Moller KN, Jacobsen SH, Dirksen C, Jorgensen NB, Reitelseder S, Jensen JB, et al. Accelerated protein digestion and amino acid absorption after Roux-en-Y gastric bypass. Am J Clin Nutr 2015;102(3):600–7.
[11] Windey K, De Preter V, Verbeke K. Relevance of protein fermentation to gut health. Mol Nutr Food Res 2012;56:184–96.
[12] Neurath MF. Cytokines in inflammatory bowel disease. Nat Rev Immunol 2014;14:329–42.
[13] Serhan CN, Savill J. Resolution of inflammation: the beginning programs the end. Nat Immunol 2005;6:1191–7.
[14] Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 2001;294:1871–5.
[16] Noga MJ, Dane A, Shi S, Attali A, van Aken H, Suidgeest E, et al. Metabolomics of cerebrospinal fluid reveals changes in the central nervous system metabolism in a rat model of multiple sclerosis. Metabolomics 2012;8:253–63.
[17] de Jager W, Hoppenreijs EP, Wulffraat NM, Wedderburn LR, Kuis W, Prakken BJ. Blood and synovial fluid cytokine signatures in patients with juvenile idiopathic arthritis: a cross-sectional study. Ann Rheum Dis 2007;66:589–98.
[18] Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–64.
[19] Strassburg K, Huijbrechts AM, Kortekaas KA, Lindeman JH, Pedersen TL, Dane A, et al. Quantitative profiling of oxylipins through comprehensive LC–MS/MS analysis: application in cardiac surgery. Anal Bioanal Chem 2012;404:1413–26.
[20] Nadler SB, Hidalgo JH, Bloch T. Prediction of blood volume in normal human adults. Surgery 1962;51:224–32.
[21] Geraedts MC, Troost FJ, Munsters MJ, Stegen JH, de Ridder RJ, Conchillo JM, et al. Intraduodenal administration of intact pea protein effectively reduces food intake in both lean and obese male subjects. PLoS One 2011;6:e24878.
[22] Ryan AT, Luscombe-Marsh ND, Saies AA, Little TJ, Standfield S, Horowitz M, et al. Effects of intraduodenal lipid and protein on gut motility and hormone release, glycemia, appetite, and energy intake in lean men. Am J Clin Nutr 2013;98:300–11.
[23] Loomba R, Quehenberger O, Armando A, Dennis EA. Polyunsaturated fatty acid metabolites as novel lipidomic biomarkers for noninvasive diagnosis of nonalcoholic steatohepatitis. J Lipid Res 2015;56:185–92.
[24] Borgstrom B, Dahlqvist A, Lundh G, Sjovall J. Studies of intestinal digestion and absorption in the human. J Clin Invest 1957;36:1521–36.
[25] Silk D, Grimble G, Rees R. Protein digestion and amino acid and peptide absorption. Proc Nutr Soc 1985;44:63–72.
[26] van der Wielen N, van Avesaat M, de Wit NJ, Vogels JT, Troost F, Masclee A, et al. Cross-species comparison of genes related to nutrient sensing mechanisms expressed along the intestine. PLoS One 2014;9:e107531.
[27] Terada T, Shimada Y, Pan X, Kishimoto K, Sakurai T, Doi R, et al. Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract. Biochem Pharmacol 2005;70:1756–63.
[28] Mahe S, Roos N, Benamouzig R, Davin L, Luengo C, Gagnon L, et al. Gastrojejunal kinetics and the digestion of [15N]beta-lactoglobulin and casein in humans: the influence of the nature and quantity of the protein. Am J Clin Nutr 1996;63:546–52.
[29] Lacroix M, Bos C, Leonil J, Airinei G, Luengo C, Dare S, et al. Compared with casein or total milk protein, digestion of milk soluble proteins is too rapid to sustain the anabolic postprandial amino acid requirement. Am J Clin Nutr 2006;84:1070–9.
[30] Gaudichon C, Bos C, Morens C, Petzke KJ, Mariotti F, Everwand J, et al. Ileal losses of nitrogen and amino acids in humans and their importance to the assessment of amino acid requirements. Gastroenterology 2002;123:50–9.