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ARTICLE

Dietary N-acylethanolamines are bioaccessible in the small intestine and modulate postprandial hormonal responses: a randomized crossover trial in subjects with ileostomy

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N-acylethanolamines (NAEs) are bioactive lipid mediators involved in the regulation of appetite, inflammation, and gut-brain signaling. This study investigated the metabolic fate of dietary NAEs following the consumption of two test meals with differing NAE content in subjects with ileostomy, and evaluated their effects on gastrointestinal hormones, glycaemia, and appetite regulation. An acute, double-blind, randomized, crossover postprandial study was conducted in ileostomy patients who consumed either a high-NAE meal (HNM) or a low-NAE meal (LNM) on two separate occasions. Ileal fluid and plasma samples were collected over an 8-hour postprandial period for analysis of NAEs and endocannabinoids (ECs). Baseline ileal microbiota composition was assessed. At the end of the 8-hour period, participants completed a buffet meal test to evaluate *ad libitum* energy intake. Dietary NAEs were significantly recovered in ileal fluids after HNM intake, with concentrations approximately 3-fold higher than after LNM, suggesting partial digestion and release from the food matrix. No significant differences in postprandial plasma NAE concentrations were observed between meals. HNM consumption led to higher postprandial levels of plasma insulin, C-peptide, and glucose-dependent insulinotropic polypeptide, despite no differences in glycemic response or subsequent *ad libitum* energy intake. Metagenomic analysis identified clusters of ileal microbial taxa associated with circulating lipid profiles, suggesting a role of the small intestinal microbiota in the metabolism of NAEs and ECs. Dietary NAEs reach the small intestine at active concentrations and may influence local signaling via GPR119, with microbiota composition influencing their release from food.

1. Introduction

The *N*-acylethanolamines (NAEs) and endocannabinoids (ECs) are a group endogenous lipid mediators playing important roles in a wide variety of biological processes such as: appetite cues, food intake, blood pressure, inflammation, glycaemia, cognition and immunity.[1] The NAEs are the congeners of the ECs and both class of compounds, along with their receptors and enzymes involved in their synthesis and breakdown, constitute the "endocannabinoid system" that is active in several tissues, including the gastrointestinal (GI) tract.[1]

The ECs consist of *N*-arachidonoylethanolamide (AEA) and 2-arachidonoylglycerol (2-AG). They may have agonist activity on cannabinoid receptors CB1 and CB2 which are located in the central nervous system (CNS) and in peripheral tissues such as in the enteric nervous system (ENS), in the liver and in the adipose tissue.[2] On the other hand, NAEs are known as "endocannabinoid-like" molecules and include oleoylethanolamine (OEA), linoleylethanolamine (LEA), palmitoyletahanolamine (PEA) and, stearoylethanolamide (SEA). AEA and NAEs are synthesized from low-abundance phospholipids, *N*-acyl-phosphatidylethanolamines (NAPE).[1,3] Differently from ECs, they can bind G-protein coupled receptor 119 (GPR119) and peroxisome proliferator-activated receptors (PPARs) located not only in the brain but also in the GI tract mucosa.[4-6] The synthesis of ECs and NAEs is closely interconnected, both among themselves and with other biological systems.[3] Emerging evidence also suggests that OEA mitigates obesity-associated dysmetabolism such as dyslipidemia in chronic diseases, by inducing the expression of genes involved in energy homeostasis, feeding behavior, and lipid metabolism regulation, including GPR119 and PPAR-α.[1,3] As a PPAR-α ligand, it stimulates fatty acid oxidation and lipolysis in white adipose tissue [7, 8] and slows gastric emptying.[9] By activating GPR119, OEA promotes the release of anorexigenic hormones such as GLP-1, thereby reducing food intake and enhancing satiety.[10]

Human studies have shown that daily supplementation with capsules containing N-oleyl-phosphatidylethanolamine (NOPE; 170 mg) and epigallocatechin-3-gallate (EGCG; 240 mg), can induce weight loss and increase feelings of fullness in overweight and obese individuals.[11, 12]

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Additionally, an 8-week supplementation with 2 × 125 mg OEA was reported to increase PPAR-α gene expression while reducing appetite, body weight, and fat mass in obese subjects.[13] However, since the supplements used in these studies contained 85% OEA along with LEA and PEA, both known to influence food intake,[14] a combined effect of the three NAEs cannot be ruled out.

Diet composition has been shown to influence fasting and postprandial plasma levels of ECs, NAPEs, and NAEs by affecting the availability of their precursors, such as fatty acids and amines.[1] For instance, consuming an oleic acid-rich breakfast increased postprandial plasma OEA levels and reduced subsequent meal energy intake in humans.[15] More recently, NAPEs, NAEs, and ECs have been identified and quantified in various foods[16] and in the intestinal fluids of fasting ileostomists.[17] Remarkably, ileal concentrations were 1.5 to 13 times higher than the EC50 values of OEA, PEA, and LEA for GPR119 activation while OEA concentrations exceeded by approximately 10-fold the levels needed to trigger a physiological response through PPAR-α.[5,10,18] Furthermore, NAEs and NAPEs were shown to be more effective in eliciting a leptogenic and anorexigenic effect from the intestinal lumen than in other tissues.[19,20]

We hypothesize that the levels of NAPEs, NAEs, and ECs in a meal may influence the concentrations of NAEs and ECs in the intestine, subsequently affecting postprandial hormonal response and appetite through interactions with receptors on the intestinal mucosa. To test this hypothesis, we conducted an acute double-blind, randomized, crossover postprandial study in a cohort of ileostomy patients who consumed, on two separate occasions, a High-NAEs meal and a Low-NAEs meal and provided ileal fluids and plasma samples over time postprandially.

2. Material and Methods

2.1 Study design

This acute double-blind randomized crossover study in a cohort of ileostomy subjects was conducted at the Human Intervention Studies Unit (HISU) at Ulster University (Coleraine, UK). The study was approved by the Office for Research Ethics Committees Northern Ireland (ORECNI) and the University of Ulster Ethical Committee in accordance with the Declaration of Helsinki. All participants signed an informed consent before the enrollment. The trial was registered at www.clinicaltrials.gov (NCT05845229). Participants (male and non-pregnant/non-lactating females) recruited to the study were aged 18–70 years, had previously undergone an ileostomy and were ≥ 2 years post-operative at time of recruitment. Participants were excluded if they were lactose intolerant, smokers, or had their surgical procedure < 2 years before. The postprandial changes in NAEs and ECs in plasma and ileal fluids were registered as the primary outcomes of the study. Secondary outcomes included postprandial changes in plasma glucose, plasma gastrointestinal hormones (i.e. insulin, GLP-1, glucose-dependent insulinotropic polypeptide (GIP), glucagon, C-peptide, ghrelin, leptin), composition of the gut microbiome and energy intake at the subsequent meal during a buffet meal test.

2.2 Dietary intervention

Fourteen participants were randomized by permuted block randomization to receive either a high-NAEs meal (HNM) or a low-NAEs meal (LNM) according to the food NAEs database.[16] The allocation ratio was 1:1. The HNM consisted of: cow's milk (150 mL), white bread (46 g), jam (10 g), cocoa powder (15 g), and whole-grain cereal (30 g). The LNM consisted of: cow's milk (150 mL), whole-grain bread (80 g), jam (10 g), butter (5 g), instant coffee powder (2 g), and dried apples (30 g). Food items were purchased from the local store Tesco shop. The nutritional composition of both breakfast are reported in the **Table 1**.

Table 1: Nutritional composition of High-NAEs meal (HNM) and Low-NAEs meal (LNM).

	HNM	LNM
Total weight (g)	251	277
Energy (kcal)	360	344
Carbohydrates (g) (% kcal)	59.2 (66)	59.2 (69)
of which sugars	20.6	33.7
Fats (g) (%)	5.4 (14)	5.4 (14)
of which saturated fats	2.5	3.0
of which monounsaturated fats	2.1	1.9
of which polyunsaturated fats	0.8	0.5
Proteins (g) (% kcal)	14.8 (16)	9.4 (11)
Dietary fibers (g) (% kcal)	7.9 (4)	10.3 (6)
NAEs (μg)[a]	124.0	9.5
of which LEA[b] (μg)	69.8	5.4
of which PEA[c] (μg)	7.0	2.7
of which OEA[d] (μg)	47.1	1.4
NAPEs[e] (mg)	57.8	12.1

[a]N-acylethanolamines;
[b]Linoylethanolamide;
[c]Palmitoylethanolamide;
[d]Oleoylethanolamide;
[e]N-acylphosphatidylethanolamides

The two breakfast meals were designed to be isocaloric (~360-344 kcal) and to provide a significantly different amount of NAEs and NAPEs. HNM provided ~13 fold more total NAEs (13 and 34 folds more LEA and OEA, respectively) and 5 fold more NAPEs than LNM (**Supplementary Table 1**). Total NAPEs were expressed as N-arachidonoylphosphatidylethanolamine equivalents according to the food NAE database[16]. Although species-specific concentrations for individual foods were not determined, all NAPEs identified included the major N-acyl chains (palmitoyl, linoleoyl, oleoyl), representing the precursors of the monitored NAEs PEA, LEA, and OEA, respectively. **Fig. 1** shows the study design. On the day before the study visit, participants followed a fixed evening meal consisting of plain chicken and boiled white rice to maintain low dietary levels of NAPEs, NAEs, and ECs.[16] In addition, after this evening meal, participants attached a new stoma bag for the overnight ileal fluids collection. Following an overnight fast, the participants visited the HISU, Ulster University (Coleraine, UK) and provided the overnight ileal fluid samples and baseline blood samples. Anthropometric measures were collected at each visit. Weight (kg) was measured using electronic SECA alpha flat scales (Seca ltd., Hamburg, Germany) and height (m) was measured using a SECA 213 portable stadiometer (Seca Ltd., Hamburg, Germany).

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Dietary behavior data were collected through the Three-Factor Eating Questionnaire (TFEQ)[21] and the power of food scale.[22] Individual psychological profiles were ascertained through the Depression, Anxiety and Stress Scale (DASS),[23] the Snaith-Hamilton Pleasure Scale,[24] and the health-related quality-of-life questionnaire (SF-12).[25] Participants also rated their baseline appetite sensations on visual analogue scale (VAS) questionnaires[26] and subsequently consumed the test meal: either a high-NAEs meal (HNM) and a low-NAEs meal (LNM). Thereafter, blood samples were collected at 2, 4, 6, 8 hours after breakfast into an EDTA-tube and, EDTA-tubes added with protease inhibitors. Ileal effluent samples were collected at 0–2, 2–4, 4–6, and 6–8 hours. At each time point, the participant removed the stoma bag and replaced it with a new one. Prior to glycaemia measurement, subjects rated their hunger, fullness and satiety by VAS questionnaires. After the final sample collection at 8 hours, participants underwent a buffet meal test. They were provided with a tray containing a variety of foods (**Supplementary Table 2**) to assess *ad libitum* energy intake. The second and third (final) clinic visit took place at least one week later, during which the same procedures were repeated for the alternate treatment. Ileal fluid samples were processed as previously described.[17]

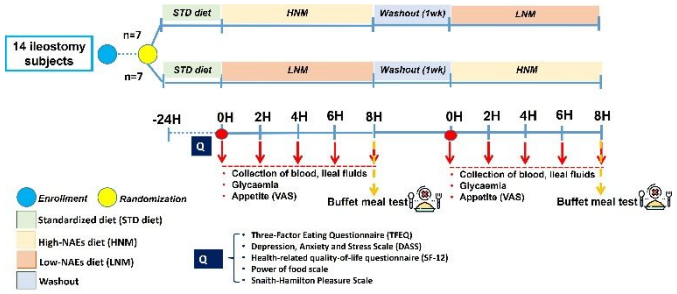

Fig. 1: Study design

2.3 Analysis of ECs and NAEs content in ileal and plasma samples

Ileal fluid and plasma samples, collected in EDTA-tubes, were processed and analyzed for ECs and NAEs as previously described.[17,27] The chromatographic separation was performed using an HPLC apparatus provided with two micropumps, Perkin-Elmer Series 200 (Norwalk, CT, USA). The compounds were separated on a Synergi Max RP 80 column (50 × 2.1 mm) (Phenomenex, USA) with a setting temperature of 30 °C and a flow rate of 0.2 mL/min. The injection volume was 10 µL. The monitored compounds were eluted by a linear gradient of H_2O and 0.2% formic acid (solvent A) and CH_3CN (solvent B). According to Tagliamonte and colleagues (2021),[27] the eluting gradient was set as follows: 50–79% B (10 min), 79–95% B (1 min), constant at 95% B (2 min) and finally returning to the initial conditions within 2 min. The acquisition was performed in positive ion mode on an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) in MRM (Multiple Reaction Monitoring). All the acquisition parameters are summarized in **Supplementary Table 3**. ECs (2-arachidonoylglycerol, 2-AG; anandamide, AEA) and NAEs (oleoylethanolamide, OEA; linoleoylethanolamide, LEA; palmitoylethanolamide, PEA; SEA, stearoylethanolamide) standards were purchased from Cayman (Cayman Chemical, Ann Arbor, MI).

2.4 Ileal gut microbiome composition

Ileal microbiome was analysed at baseline (before breakfast meal consumption) by shotgun metagenomics as previously described.[28] Briefly, microbial DNA was extracted following the SOP 07 developed by the International Human Microbiome Standard Consortium (www.microbiome-standards.org). DNA libraries were sequenced on Illumina NovaSeq platform, leading to 2x150bp, paired-end reads. One sample failed library preparation due to poor DNA quality and was removed from the analysis. Therefore, microbiome analysis was carried out on 13 subjects. Human reads were removed using the Best Match Tagger(BMtagger; https://hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf). Non-human reads were quality-filtered using PRINSEQ 0.20.4 (trimming when a base with a Phred score < 15 was found and discarding reads < 75 bp).[29] High-quality reads were imported into MetaPhlAn4[30] to obtain species-level, quantitative taxonomic profiles.

2.5 Blood glucose and gastrointestinal hormones

Glycaemia was measured at baseline and postprandial (2h, 4h, 6h, 8h) by finger pricking and using a bedside glucometer (OneTouch Sure Step; Life Scan Inc.). Accuracy of the glucometer was evaluated by using least squares linear regression analysis and was found to be 97% "clinically accurate" compared with reference (YSI2700) results. Blood samples were collected into EDTA-tubes added with protease inhibitors as previously reported.[31] Samples were centrifuged at 1800 g per 15 min at 4 °C, and the supernatants were stored at – 80 °C before analysis. The simultaneous quantification of insulin, C-peptide, ghrelin, glucose-dependent insulinotropic peptide (GIP), glucagon-like peptide 1 (GLP-1), leptin and glucagon in plasma samples, was achieved by Bio-Plex Pro immunoassay kits as previously described.[31]

2.6 Statistical analysis

The sample size was calculated by power analysis, considering the response of NAEs and ECs as endpoints. A sample size of 14 participants was calculated to identify significant changes in the response in accordance with Monteleone et al. (2012) and Gatta-Cherifi et al. (2012) with an 80% power and an $\alpha = 0.05$.[32,33] All statistical analyses were performed in R version 4.2.3. After checking for normality, variables showing a significant positive skewness, were log-transformed [ln(x + k), with k values zeroing the skewness]. Postprandial differences over time within and between the groups for normally distributed variables were evaluated with a one-way repeated measures ANOVA and Bonferroni adjustment for multiple comparisons; non-normally distributed variables were assessed with Friedman's tests and pairwise Wilcoxon's *post-hoc* tests. The postprandial incremental areas under the curve (iAUC) were calculated as previously reported[34] and differences in iAUCs between the groups were assessed by independent

sample t-test. In order to explore the different response to HNM *vs* LNM consumption of gastrointestinal hormones, a Multilevel Partial Least Squares Discriminant Analysis for cross-over design (PLS-DA, plsda function) was performed on GI hormones iAUCs (library mixOmics). Within-subject correlations among the baseline microbial compositions of the ileal fluids and the postprandial response of ileal concentrations of NAEs was performed by using rmcorr function. The resulting significant correlations were visualized as a network by using the igraph package illustrating the significant co-occurring microbial guilds and their interactions with postprandial ileal NAE concentration.

3. Results

3.1 Study participants

The participant flow is shown in **Fig. 2.** Fourteen ileostomates completed the study. One participant was unable to provide a blood sample; therefore, their data were excluded from the NAEs analysis. The general characteristics of the participants at baseline are reported in **Table 2.** Psychometric variables are reported in the **Supplementary Table 4.**

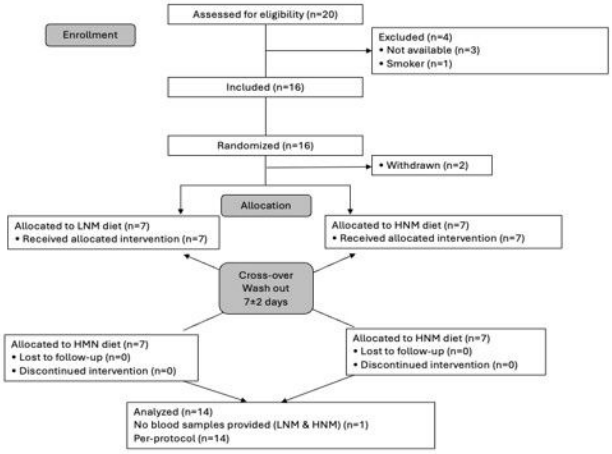

Fig. 2: CONSORT diagram of study.

Table 2: General and anthropometric characteristics of participants (n=14) at baseline.

Sex (n, F/M)	6F, 8M
Age (y)	49.5±12.6
Height (cm)	170±0.01
Weight (kg)	83.4±26.8
BMI (kg/m^2)	28.6±7.8

Data are expressed as means ± SD. n, number of participants; F, female; M, male; y, years.

3.2 Postprandial concentrations of N-acylethanolamines and Endocannabinoids in ileal fluids

No difference was found at baseline in the amount of NAEs and ECs quantified in the ileal fluids before the consumption of LNM or HNM. Among the NAEs, LEA was the most abundant with a quantity on average 50 times greater than OEA, followed by PEA and SEA. The ECs were less abundant than NAEs, with 2-AG being found at an average amount 5-fold greater than AEA (**Fig. 3**); whereas a significantly higher amount of LEA and OEA was present in ileal fluids (4-6h and 6-8h intervals) following HNM compared to LNM consumption (**Fig. 4**). Specifically, LEA concentrations were approximately 5-fold higher during the 4–6 h interval and 3-fold higher during the 6–8 h interval following HNM compared to LNM. Specifically, LEA concentrations were approximately 5-fold higher during the 4–6 h interval and 3-fold higher during the 6–8 h interval following HNM compared to LNM. Similarly, OEA concentrations were around 6-fold higher at 4–6 h and 4-fold higher at 6–8 h post-HNM intake.

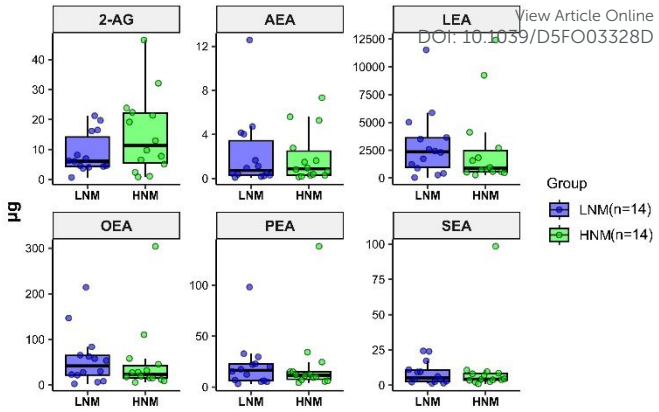

Fig. 3: Ileal fluid content of ECs and NAEs at baseline in the 13 participants before consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). 2-AG, 2-Arachidonoylglycerol; AEA, Arachidonoylthanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide.

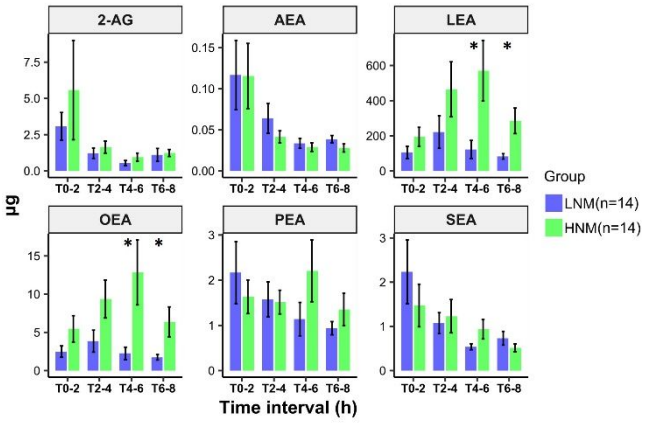

Fig. 4: Post-prandial ileal fluid content of ECs and NAEs at each collection interval post consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). Data are shown as mean ± SEM. * p–value<0.05, between-meal difference versus HNM *vs* LNM assessed by one-way repeated-measures ANOVA and Bonferroni adjustment. 2-AG, 2-Arachidonoylglycerol; AEA, Arachidonoylethanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide.

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No difference over time was shown for the other monitored compounds. Overall, the cumulative 8-hour postprandial recovery of OEA and LEA in ileal fluids was significantly higher after HNM intake, with total amounts reaching 34±6.7 µg (OEA) and 1.5±0.3 mg (LEA), compared to 10.3±1.7 µg and 0.5±0.1 mg, respectively, following LNM (**Fig. 5**).

3.3 Ileal microbiome and its association with the postprandial lipid mediators in the ileum

The composition of ileal microbiome in 13 participants at the phylum and species level is reported in **Fig. 6A and 6B**.

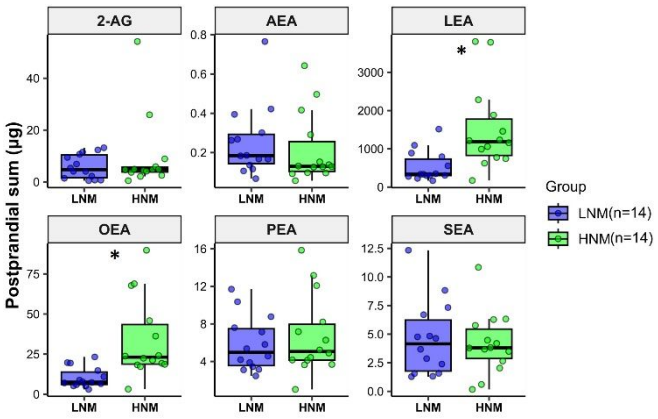

Fig. 5: Post-prandial ileal fluid sum content of ECs and NAEs upon consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). * p–value<0.05, between-meal difference versus HNM *vs* LNM assessed by independent sample t-test. 2-AG, 2-Arachidonoylglycerol; AEA, Arachidonoylthanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide.

One sample failed library preparation due to poor DNA quality and was removed from the analysis.

At phylum level, the microbiome is dominated by Bacillota (previously classified as Firmicutes; average abundance 74.9%) and Pseudomonadota (previously Proteobacteria; average abundance 20.6%), while Bacteroidota (previously Bacteroidetes) was present with an abundance >1% in only 4 subjects, with a low abundance (ranging from 0 to 8.8%). At species level, high variability among the subjects was observed (**Fig. 6B**).

For subjects where Pseudomonadota was the dominant phylum, *Escherichia coli* and *Haemophilus parainfluenzae* prevailed at species level. Within Bacillota, *Streptococcus* (*S. salivarius, S. parasanguinis, S. lutetiensis, S. gallolyticus, S. mitis*), *Romboutsia timonensis, Veillonella* spp., *Clostridium perfringens, C. disporicum* and *Ligilactobacillus salivarius* were the main species. To explore the associations between the ileal microbiome and the lipid mediators formed in the ileal fluids following the consumption of HNM and LNM, a network analysis was conducted, and results are shown in **Fig. 6C**.

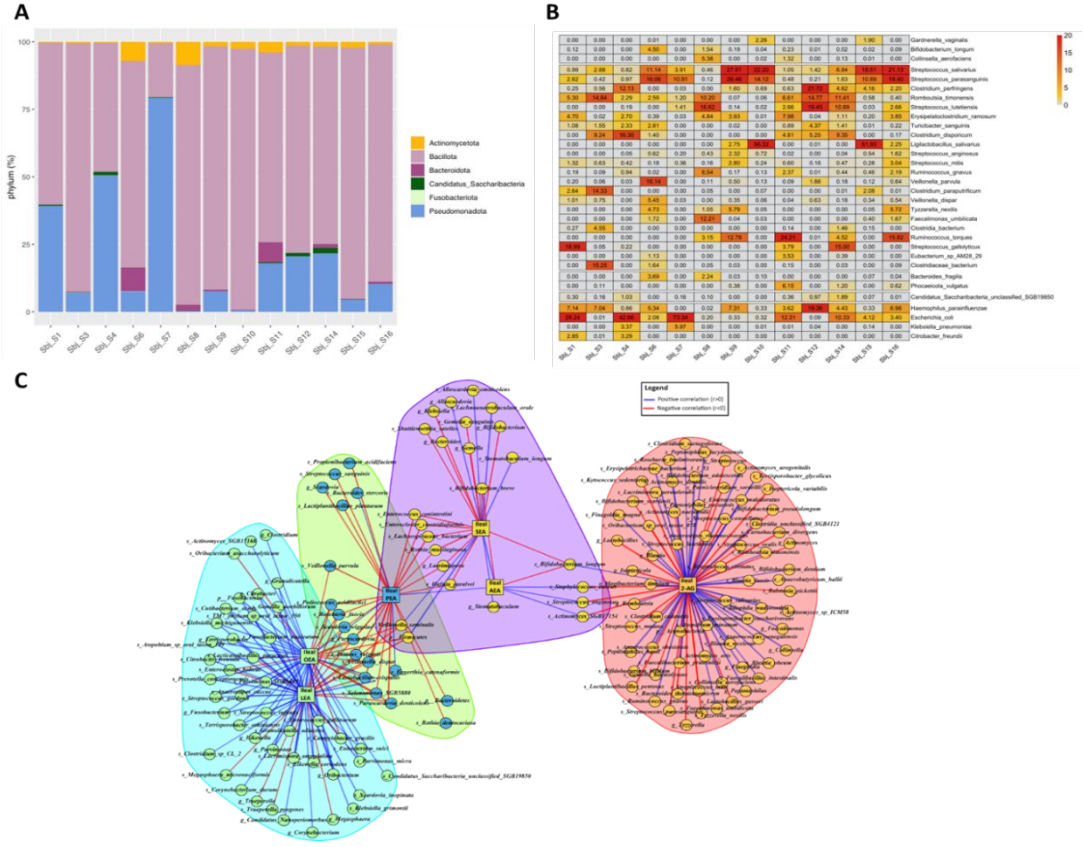

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Fig. 6: Composition of ileal microbiome in 13 participants at the phylum **(A)** and species **(B)** level; **(C):** Network illustrating significant (p-value<0.05) co-occurring microbial guilds in the intestinal lumen at baseline and their within-subject interaction with post-prandial ileal sum content of ECs and NAEs following consumption of LNM (Low-NAEs meal) and HNM (High-NAEs meal). R coefficients were calculated by performing a repeated measure correlation. Blue lines indicate positive correlations and red lines indicate negative correlations between variables correlations, < 0, respectively). Only moderate-to-strong associations (r>0.4 an r<-0.4) were included; weak correlations were excluded from the analysis.. The name of the taxon level is abbreviated as p-phylum, g-genus and s-species. 2-AG, 2-Arachidonoylglycerol; AEA, Arachidonoylthanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide.

It revealed that each lipid mediator displayed a unique interaction pattern with specific microbial taxa, highlighting their diverse roles within the intestinal ecosystem. OEA and LEA were positively correlated with a wide variety of taxa, including *Clostridium*, and *Enterococcus* species and *Fusobacteriota* phylum, while they were negatively associated with *Bacillota* phylum. Similarly, PEA, SEA and AEA exhibited overlapping clusters, sharing microbial associations such as *Bifidobacterium* and *Enterococcus* species. In contrast, 2-AG formed a separate and well-defined cluster, primarily involving *Lactobacillus* genus. A negative correlation was shown with *Lactobacillus* genus and the pro-inflammatory *R. gnavus* and a positive association with the probiotic *Bifidobacterium bifidum* and *Actinobacteriota* phylum.

3.4 Postprandial plasma *N*-acylethanolamines and Endocannabinoids

Following consumption of HNM and LNM the postprandial plasma concentrations of AEA, LEA, OEA and PEA decreased significantly at 2 hours compared to baseline (**Fig. 7**). No between-meal differences were observed for any of the monitored compounds.

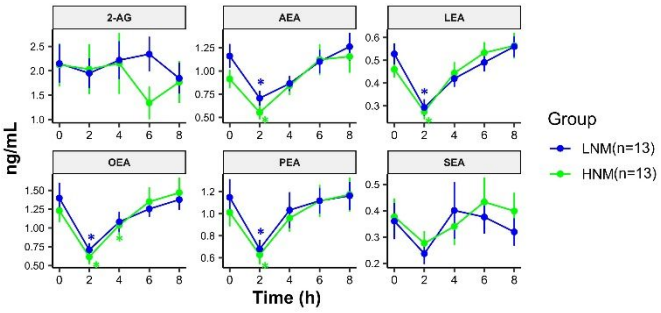

Fig. 7: Post-prandial plasma circulating ECs and NAEs upon consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). Data are shown as mean ± SEM. * p–value<0.05, within-group difference versus baseline assessed by one-way repeated-measures ANOVA and Bonferroni adjustment. 2-AG, 2-Arachidonoylglycerol; AEA, Arachidonoylthanolamide; LEA, Linoylethanolamide; OEA, Oleoylethanolamide; PEA, Palmitoylethanolamide; SEA, Stearoylethanolamide.

3.5 Gastrointestinal hormone response and glycaemia

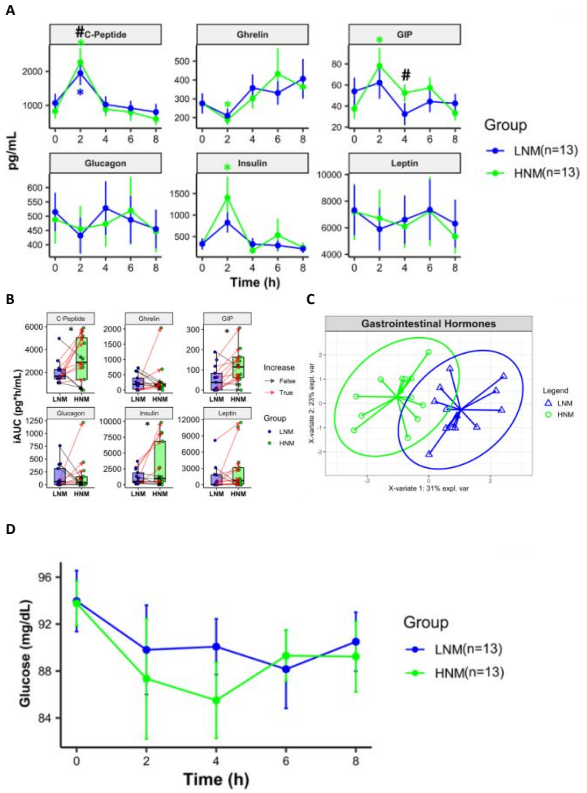

Fig. 8: A) Post-prandial concentrations of plasma gastrointestinal hormones upon consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). * p–value<0.05, within-meal difference versus HNM *vs* LNM assessed by one-way repeated-measures ANOVA and Bonferroni adjustment or non-parametric Friedman test depending on data normal distribution. GIP, Glucose-dependent insulinotropic polypeptide; B) Post-prandial incremental areas under the curve (iAUC) of the gastrointestinal hormones upon consumption of LNM (Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). * p–value<0.05, between-meal difference versus HNM *vs* LNM assessed by independent sample t-test. Red arrows indicate individual increases in iAUC, while black arrows represent individual decreases; C) The multilevel Partial Least Squares – Discriminant Analysis (PLS-DA) for cross-over design by integrating gastrointestinal hormones iAUC after HNM (High-NAEs meal, in violet) and LNM (Low-NAEs meal, in yellow). The AUC was 0.82 (p-value=0.0052) when comparing the hormones response upon HNM and LNM intake; D) Post-prandial plasma glucose upon consumption of LNM

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(Low-NAEs meal, in blue) and HNM (High-NAEs meal, in green). Data are shown as mean ± SEM.

The postprandial gastrointestinal hormones response to the LNM and HNM interventions is reported in **Fig. 8A**.
Data showed that the C-peptide and insulin response increased 2 hours after both LNM and HNM intake compared to baseline. Conversely, GIP significantly increased, while ghrelin decreased, 2 hours after HNM intake compared to baseline. The C-peptide and GIP responses were significantly higher 2 hours and 4 hours after HNM than LNM consumption, respectively. The concentration of GLP-1 was below the limit of detection in all the samples analysed. The postprandial incremental areas under the curve (iAUC) for C-peptide, GIP, and insulin were significantly higher after HNM intake compared to LNM (**Fig. 8B**), determining a distinct response between meals when analyzed using PLS-DA (**Fig. 8C**).
No between- and within-meal differences were observed for glycaemia response (**Fig. 8D**).

3.6 Energy intake at subsequent lunch

After 8 hours, participants underwent a buffet meal test to measure *ad libitum* energy intake. No significant effect was observed for either breakfast type on subsequent energy intake or the macronutrient composition of the consumed meal (**Supplementary Fig. 1)**.

4. Discussion

4.1 NAEs and ECs increase in the ileal fluids postprandially

Results of this study clearly show a causal relationship between the content of NAPEs, NAEs, and ECs in the meal and the levels of NAEs and ECs in the intestinal lumen postprandially. Indeed, baseline levels of the monitored lipids in ileal fluids were homogeneous across participants, reflecting adherence to a standardized 24-hour diet prior to each test day. In contrast, the levels of NAEs in the ileal fluids increased postprandially when the participants consumed a meal richer in NAEs and in the phosphorylated precursors NAPEs. Specifically, the amount of OEA significantly doubled and LEA tripled the basal values between 4 and 8 hours after consuming HNM. This finding confirms that food digestion in the upper GI tract releases NAEs and NAPEs from the food matrix, continuing the process initiated by mastication in the mouth.[35-37]
In a previous study, the intestinal bioaccessibility of OEA, assessed through *in vitro* digestion, was found to be higher from a liquid than a semisolid food,[38] confirming that the food matrix substantially influenced the ability of digestive enzymes to gain access to it limiting the rate and extent of the release in the lumen (for a review see[39]). To reduce the impact of physical form and structure of the two meals on the chemical digestion, in the present study the two breakfast meals were designed to include foods with a different content of NAEs and NAPEs but similar matrices and macronutrient composition. Interestingly, the ratio of cumulative OEA to LEA recovery in ileal fluids did not mirror the relative proportions of these compounds in the meals. This observation may reflect selective conversion from their respective dietary NAPE precursors or cross-regulation among NAE species within the intestinal environment.[40,41] Differences in the abundance and hydrolysis efficiency of NAPE oleoyl (18:1) versus linoleoyl (18:2) NAPE acyl chains[40], as well as luminal processes such as re-esterification or microbial metabolism, may have contributed to modulating the observed NAE profile.[41] Moreover, we observed considerable interindividual variability in postprandial NAE levels, which is likely explained by physiological differences among participants, including the lipid digestion efficiency, intestinal motility and uptake, and differences in the intestinal microbiota. Such variability is well recognized in studies of lipid-derived mediators and is consistent with the heterogeneous physiology of the small intestine.[42-44]

4.2 Ileal microbiome may influence dietary NAE metabolism

Results of the current study also suggest *in vivo* that the ileal microbiome may influence the intestinal levels of ECs and NAEs, due to microbial metabolism.
Although less densely populated than the colon, the small intestine harbors a metabolically active microbiota[43] that may contribute to the early formation and transformation of luminal NAEs from dietary precursors, including NAPEs, prior to the extensive microbial processing of dietary lipids and the subsequent formation of metabolites, including NAEs, in the colon.[41,45,46]
The ileal microbiome is a topic still underexplored in scientific literature, and due to the difficulties in sampling and ethical reasons, the knowledge is limited to subjects suffering from some intestinal diseases.[47] Compared with the colon microbiome, the small intestine is usually dominated by facultative anaerobes (e.g., Bacillota, such as *Streptococcaceae*, *Lactobacillaceae* and *Veillonellaceae*, and Pseudomonadota), due to higher oxygen concentration, while Bacteroidota abundance is usually limited. The findings of this study confirmed *Streptococcus*, *Haemophilus* and *Veillonella* as member of a core ileal microbiome, present with varying abundance in most of the subjects.[47] However, high inter-personal variability in composition was also observed. Indeed, the ileum microbiome is subject to higher fluctuations in composition and abundance compared with the colon, and dynamic changes of the microbiome are reported during fasting and fed states.[43,48] Interestingly, some of the participants of this study were characterized by a particularly high abundance of *Escherichia coli* and *Klebsiella*. The overgrowth of these taxa has been previously associated with inflammation, impaired intestinal barrier and malabsorption.[49]
Recent *in vitro* studies have demonstrated a clear role for the colonic microbiota in the formation and transformation of ECs and NAEs in the intestinal lumen. Specifically, changes in EC and NAE profiles were observed in simulated chyme undergoing fecal fermentation, with certain metabolites decreasing and others emerging over time.[45] Similarly, Roussel et al. (2024) showed that human fecal microbiota can both generate and hydrolyze specific NAEs (such as N-stearidonoylethanolamine, LEA, and PEA) from dietary polyunsaturated NAPE substrates in the Mucosal Simulator of the Human Intestinal Microbial

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Ecosystem (M-SHIME) model.[41] These findings align with the known enzymatic capacities of various intestinal microorganisms, including phospholipase, amidase, and acyltransferase activities, which can hydrolyse or remodel NAPEs and thereby influence the profile of NAEs derived from dietary precursors.

In vivo evidence further supports a microbiota-dependent contribution to luminal NAE and broader endocannabinoidome dynamics. In mice, OEA was undetectable in the cecal contents of microbiota-depleted animals but was readily produced in wild-type mice receiving an intracecal oleic acid infusion.[46] Moreover, intraperitoneal OEA administration has been shown to modulate gut microbial composition while increasing hepatic OEA levels and exerting anti-inflammatory effects,[50] suggesting bidirectional interactions between circulating NAEs and the gut microbiota mediated through host metabolic pathways.

In humans, a cross-sectional and dietary-intervention study demonstrated that the fecal endocannabinoidome profile is shaped predominantly by gut microbiota composition and dietary intakes, rather than by circulating mediator levels.[51] Reinforcing this causal link between diet, microbial ecology, and endocannabinoidome signaling, findings from an 8-week Mediterranean diet intervention showed that a personalized dietary shift elicited coordinated changes in circulating NAE profiles alongside increased abundance of key microbial taxa such as *Akkermansia muciniphila*, independent of weight loss.[27] In the present study, the analysis of the microbiome in the basal ileal fluids and the postprandial levels of ECs and NAEs in the ileal fluids, allowed the identification of clusters of microbial taxa associated with the monitored lipids, thus suggesting that also the microbial communities present in the small intestine may influence the metabolism of ECs and NAEs *in vivo*. Interestingly, OEA, PEA, LEA, and AEA formed interconnected clusters, indicating overlap in their microbial associations. For instance, *Lactobacillus* and *Steptococcus* spp. interact with several lipid mediators, predominantly showing negative associations with the postprandial formation of OEA and LEA in the ileum. Indeed, these genera, together with *Escherichia coli* (also abundant in the study participants) have been reported as involved in lipid metabolism in the small intestine.[52,53] OEA and LEA belong to the same cluster and are primarily associated with a broad range of taxa from the Fusobacteriota phylum, while negatively correlating with Bacillota. Bacillota, including certain species of *Enterococcus*, *Streptococcus*, and *Clostridium*, harbor ethanolamine utilization genes, which may facilitate the metabolism of dietary NAEs as a carbon and/or nitrogen source.[54,55] Additionally, several strains within *Lactobacillus*, *Bifidobacteria*, *Streptococcus*, *Clostridium* and *Enterococcus* are known for their capacity to metabolize ethanolamine.[54]

However, further studies integrating metagenomic functional profiling with targeted metabolomics are needed to better elucidate the microbial pathways involved in NAEs and ECs metabolism.

4.3 NAE levels in the intestine are sufficient to elicit GPR119

It is worth noting that the postprandial concentrations of NAEs in the ileal fluids were found in quantities exceeding their reported EC50 for GPR119.[5,10,18] GPR119 is a receptor for monoacylglycerols, lysophosphatidylcholine, and fatty acid ethanolamides.[56] It is expressed on enteroendocrine cells and, by sensing lipids, produces incretins, such as GLP-1 and GIP after a meal.[1,57] Therefore, results of this study suggest that OEA and LEA, delivered from the food, by activating GPR119 likely contributed to the increase in C-peptide, insulin, and particularly GIP after HNM compared to LNM.

On the other hand, GLP-1 was undetectable in the present study. In a previous study involving ileostomy subjects, the postprandial GLP-1 response was stimulated by the presence of lipids in the duodenum, occurring approximately 150 min after ingestion of a high-fat-meal (comprising 78% of energy from fats). This response was significantly lower in ileostomates compared to healthy subjects.[58] The discrepancy between GIP and GLP-1 responses is likely attributable to differences in the distribution of enteroendocrine cells. K cells, which secrete GIP, are most densely located in the duodenum, whereas L cells, responsible for GLP-1 secretion, show a gradient of increasing density along the GI tract, with the highest concentrations found in the distal ileum and colon.[59] This anatomical distribution supports the interpretation that GLP-1 release predominantly occurs in the distal gut, explaining both the diminished GLP-1 response in ileostomists and the undetectable levels observed in the present study. The lack of observable differences in glycemic responses between meals suggests that the hormonal fluctuations measured were insufficient to affect glucose regulations at the monitored time points. Moreover, the postprandial increase in GIP and the simultaneous reduction in ghrelin following HNM consumption did not appear to influence subsequent energy intake during the buffet meal. The 8-hour fasting period imposed after breakfast, prior to the buffet, may have contributed to this result, as behavioral and cognitive factors could potentially override hormonal cues.[60]

4.4 NAE levels in the bloodstream do not reflect intestinal levels

An additional finding of this study was the lack of significant differences in postprandial plasma concentrations of NAEs and ECs between the two meals, as well as the absence of correlation between ileal and plasma concentrations. These observations suggest two possible hypotheses. The first hypothesis is that dietary NAEs may not exert a direct effect on systemic circulation under the conditions of this study, possibly due to predominant intracellular physiological mechanisms (e.g., metabolic processing, receptor binding, or enzymatic degradation) that limit their release into the bloodstream.[38,39,52,61] The second hypothesis is that, absorbed NAEs are rapidly utilized by peripheral tissues, which could account for the observed postprandial decline in plasma concentrations 2 hours after breakfast. This interpretation is consistent with previous findings[1] and suggests that this rapid clearance may mask other ongoing physiological phenomena. In both scenarios, future feeding studies using isotopically labeled (e.g., ^{13}C or ^{2}H) NAEs would provide a more detailed understanding of their

metabolic fate, from ingestion through intestinal absorption to systemic distribution.

However, the lack of correlation between ileal and plasma endocannabinoidome mediators observed in our study is consistent with previous findings showing a similar disconnect between fecal or ileal tissue and circulating endocannabinoidome profiles in humans.[51,62]

This study has four main limitations. First, due to the 2-hour intervals used for glycemia monitoring, potential differences in postprandial glycemic responses between the two breakfasts, especially within the initial 2 hours after consumption, may have been missed. However, this design allowed for synchronized collection of biological samples, minimizing participant burden and improving data consistency. Second, although total carbohydrate content was comparable between the two meals, the proportion of sugars differed. However, postprandial glycemia was not significantly different following the two meals. Therefore, while we cannot entirely exclude the possibility that differences in sugar composition may have slightly influenced gastrointestinal hormone responses, any such effect is likely minimal. Third, the 8-hour interval between breakfast and buffet may have been too long to effectively evaluate the impact of breakfast composition on subsequent energy intake. After such an extended fasting period, behavioral and cognitive drivers of hunger may override hormonal or metabolic signals. Nevertheless, this extended interval enabled a more accurate assessment of the kinetics of dietary NAE bioaccessibility in ileal fluids, without interference from an additional meal. Finally, the ileostomy-based study may limit the generalizability of the findings to individuals with an intact gastrointestinal tract, although it allows direct assessment of early, diet-driven NAE bioaccessibility in the small intestine; consequently, subsequent colonic microbial transformations potentially contributing to fecal and systemic NAE pools are not captured.

5. Conclusions

In conclusion, the present study demonstrates that dietary NAEs are released into the small intestinal lumen and recovered at higher levels following a high-NAE meal, confirming their bioaccessibility under physiological conditions. However, no corresponding increase in plasma NAEs was observed, indicating that circulating levels do not reflect local intestinal concentrations. Notably, the higher ileal recovery of OEA and LEA was accompanied by increased postprandial GIP, insulin, and C-peptide responses, supporting a link between luminal NAEs and gut-derived hormonal signaling. Furthermore, baseline microbial composition was associated with variability in postprandial NAE responses, suggesting a modulatory role of the ileal microbiome. Overall, these findings highlight the importance of the small intestinal environment in shaping the local metabolism and physiological effects of dietary NAEs.

Author contributions

Paola Vitaglione: Conceptualization, Project administration, Writing - Review & Editing; **Chris R Gill:** Supervision, Resources, Writing - Review & Editing; **Silvia Tagliamonte:** Methodology, Investigation, Formal analysis, Writing – original draft, Data Curation; **Danilo Ercolini:** Writing - Review & Editing; **Francesca De Filippis:** Formal analysis, Writing - Review & Editing; **Holly R Neill:** Investigation, Data Curation; **L Kirsty Pourshahidi:** Supervision; **Brian Óg Murphy:** Investigation, Data Curation; **Koos Natalia:** Data Curation; **Brendan Curran:** Investigation, Data Curation; **Murray Nicole:** Investigation, Data Curation; **Slevin Mary:** Supervision; **Sara Dobani:** Data Curation; **Massimiliano Fontana:** Data Curation.

Conflicts of interest

There are no conflicts to declare.

Data availability

The raw sequence reads generated in this study have been deposited in the Sequence Read Archive (SRA) of the NCBI under accession number PRJNA1238284.

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Data availability

The raw sequence reads generated in this study have been deposited in the Sequence Read Archive (SRA) of the NCBI under accession number PRJNA1238284.