Milk protein digestion and the gut microbiome influence gastrointestinal discomfort after cow milk consumption in healthy subjects

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ABSTRACT

Many healthy people suffer from milk-related gastrointestinal discomfort (GID) despite not being lactose intolerant; the mechanisms underpinning such condition are unknown.

This study aimed to explore milk protein digestion and related physiological responses (primary outcome), gut microbiome and gut permeability in 19 lactose-tolerant healthy nonhabitual milk consumers [NHMCs] reporting GID after consuming cow milk compared to 20 habitual milk consumers [HMCs] without GID.

NHMCs and HMCs participated in a milk-load (250 mL) test, underwent blood sample collection at 6 time points over 6 h after milk consumption and collected urine samples and GID self-reports over 24 h. We measured the concentrations of 31 milk-derived bioactive peptides (BAPs), 20 amino acids, 4 hormones, 5 endocannabinoid system mediators, glucose and the dipeptidyl peptidase-IV (DPPIV) activity in blood and indoxyl sulfate in urine samples. Subjects also participated in a gut permeability test and delivered feces sample for gut microbiome analysis.

Results showed that, compared to HMCs, milk consumption in NHMCs, along with GID, elicited a slower and lower increase in circulating BAPs, lower responses of ghrelin, insulin, and anandamide, a higher glucose response and serum DPPIV activity. The gut permeability of the two groups was similar, while the habitual diet, which was lower in dairy products and higher in the dietary-fibre-to-protein ratio in NHMCs, possibly shaped the gut microbiome; NHMCs exhibited lower abundance of *Bifidobacteria*, higher abundance of *Prevotella* and lower abundance of protease-coding genes, which may have reduced protein digestion, as evidenced by lower urinary excretion of indoxyl sulfate.

In conclusion, the findings showed that a less efficient digestion of milk proteins, supported by a lower proteolytic capability of the gut microbiome, may explain GID in healthy people after milk consumption.

1. Introduction

More than 40% of people worldwide suffer from gastrointestinal (GI) symptoms, including abdominal pain, diarrhoea, constipation, bloating, uncomfortable fullness, nausea, and vomiting, without any underlying structural abnormalities and are diagnosed with disorders of gut-brain interactions (DGBIs) (Sperber et al., 2021). Patients with DGBIs often report increased symptoms after consuming specific foods, such as milk,

wheat, onions, garlic, chili, beans and coffee (Böhn et al., 2013; Laati-kainen et al., 2020; Black et al., 2020; Fikree and Byrne, 2021). How-ever, transient gastrointestinal discomfort (GID) after consuming cow milk occurs in the general population even in the absence of DGBIs (Pasqui et al., 2015) or lactose intolerance (Suchy et al., 2010; Laati-kainen et al., 2020; Carroccio et al., 2021), leading to a prevalence of self-perceived lactose intolerance estimated to range between 8% and 20% (Porzi et al., 2021; Nicklas et al., 2011). Due to their symptoms and self-perception of lactose intolerance, these people adopt a restrictive diet that could impact on health (Nicklas et al., 2011). Specifically, they often exclude cow milk and dairy products; this may result in suboptimal calcium intake and increase their risks of hypertension and diabetes (Misselwitz et al., 2019). However, the reasons that healthy people suffer from milk-induced GID remain obscure.

Some evidence suggests that GID is caused by the peptide β-caso-morphin-7 (BCM7), which is formed during milk digestion by β-casein and might slow GI transit by activating μ-opioid receptors (Aslam et al., 2020; Deth et al., 2015; He et al., 2017; Jianqin et al, 2015; Tulipano, 2020). Activation of μ-opioid receptors in the gut can alter gut microbial composition, impair gut barrier integrity and bile acid metabolism, and increase gastrointestinal transit time and gut inflammation (Aslam et al., 2020). However, only two randomized controlled trials focusing on BCM7 have been conducted, and their results are inconsistent (Ho et al., 2014; Jianqin et al., 2015). Additionally, many bioactive peptides (BAPs) are formed during milk digestion (for a review see Nielsen et al., 2017); these BAPs can cross the intestinal barrier (Caira et al., 2022) and modulate GI motility, digestive processes, and inflammatory responses *in vivo* (Teschemacher, 2003; Pimentel et al., 2017). The aforementioned processes are dependent on the individual enzymes and gut perme-ability. For instance, dipeptidyl peptidase-IV (DPPIV) is a brush-border enzyme that breaks down BCM7 (Tulipano, 2020) and other dietary peptides in the gut; in contrast, its soluble form (present in the blood-stream) hydrolyses endogenous peptides such as incretins (Hasan and Hocher, 2017), neuropeptides, chemokines and other chemoattractants, thus influencing metabolic, immune and inflammatory processes in the body (Trzaskalski et al., 2020). Inhibitors of DPPIV have attracted attention as therapies for many diseases, such as type 2 diabetes mellitus and immune and inflammatory diseases (Shao et al., 2020); moreover, many milk-derived BAPs inhibit DPPIV activity (Tulipano, 2020). However, the relationship between DPPIV activity in humans and milk intake is underexplored.

Mounting evidence indicates that endocannabinoids (ECs) and N-acylethanolamines (NAEs) are involved in physiological mechanisms underpinning GID (Witkamp, 2018). These lipid mediators have pleio-tropic activity and modulate several biological pathways underlying pain sensation and other phenomena, such as appetite, macronutrient metabolism, inflammation, and immunity (Witkamp, 2018). Moreover, it is widely accepted that the gut microbiome plays a crucial role in nutrient digestion, energy balance and pain regulation through bidi-rectional communication in the gut-brain axis (Rowland et al., 2018; Guo et al., 2019).

In this study, we aimed to explore milk protein digestion and related physiological responses (primary outcomes) occurring after cow milk consumption along with GID, gut permeability and the gut microbiome in healthy lactose-tolerant subjects.

2. Materials and methods

2.1. Study design and participants

The study was conducted at the University of Naples Federico II and was approved by the related Ethics Committee (Protocol number: 177/18). Each participant provided written informed consent and received no financial compensation for participation. The trial was registered at ClinicalTrials.gov (number NCT04205045).

The study design and participant flow throughout the study are shown in Fig. 1a, b. Baseline and postprandial plasma concentrations of milk-derived BAPs, GI hormones (including insulin, glucose-dependent insulinotropic peptide, ghrelin, and C-peptide), ECs, and NAEs were primary outcomes of the study. The composition of the gut microbiome; urinary excretions of lactulose, mannitol and sucralose after the gut permeability test (GPT); fasting serum DPPIV concentration; baseline and postprandial plasma concentration of amino acids (AAs) and glucose; serum DPPIV activity; postprandial GID; and urinary excretions of indoxyl sulfate, as a marker of protein digestion, were secondary outcomes.

Eligible adults (n = 101) were 18–60 years old; had a body mass index (BMI) in the range of 18.5–30 kg/m^2; were not pregnant, lactating, or taking medicines; did not have relevant organic, systemic or meta-bolic diseases; had no history of abdominal surgeries, food intolerance or alcohol abuse; and did not habitually consume probiotics, laxatives, or antibiotics. The participants were further screened based on their weekly consumption of lactose-containing cow milk (<150 mL/week or > 700 mL/week) and the absence or presence of GID after milk con-sumption as well as their results on a lactose breath test (detailed below). Participants were diagnosed with lactose intolerance and excluded from the study if the H$_2$ concentration in their breath was ≥ 20 ppm over baseline and they reported experiencing GID; participants with a positive breath test and no GID during the test (lactose mal-absorbers) as well as those with a negative breath test were included. Subjects reporting no milk-related GID and habitual milk consumption > 700 mL/week were placed in the habitual milk consumer (HMC) group, whereas those reporting milk-related GID and habitual milk consumption < 150 mL/week were placed in the nonhabitual milk consumer (NHMC) group. The frequency of self-reported GID after milk consumption in the NHMC group at recruitment is displayed in Sup-plementary Table 1.

Enrolled subjects participated in three visits to undergo anthropo-metric, lifestyle and psychological characterization, a gut permeability test and a milk-load test.

2.2. Lactose breath test

Non-invasive detection of lactose malabsorption intolerance is based on hydrogen breath test (HBT) measuring the H$_2$ concentration in the exhaled air following an oral challenge with a standard dose of lactose (Parodi et al. 2009).

Before undergoing HBT, patients were instructed to avoid probiotics, antibiotics, laxatives in the month preceding HBT, fermentable food, smoking, physical activity 24 h before and after the test. Participants consumed a standardised dinner (boiled rice and roasted chicken/fish) on the day before and the HBT was carried out in the morning – after overnight fasting by using an oral load of 20 g of lactose dissolved in 200 mL of water, in accordance with the Rome Consensus Conference approved protocol (Parodi et al., 2009) Participants who showed base-line H$_2$ value > 20 ppm were asked to repeat the test in the following days since the HBT could be no longer reliable (Misselwitz et al. 2019).

The alveolar air sample, collected every 30 min for the following 4 h after lactose administration, was obtained by having the subjects exhale, after a normal inspiration, into the Lacto FAN 2 H$_2$ Breath Test Analyser (Fisher analysen instrument GmbH. Germany) through a mouthpiece.

Each participant filled out a self-administered questionnaire for symptom assessment along with breath collection and after 6, 8, 12 and 24 h after lactose solution intake. The questionnaire included 15 items related to GI symptoms most frequently. Symptom severity was self-rated by the subjects on a 10-cm visual analogue scale (VAS) ranging from 0 (without symptoms) to 10 (maximum severity symptoms). Par-ticipants were asked to avoid milk and dairy products up to 24 h after solution ingestion, and to reintroduce milk-based products only after completing the GI symptom assessment. Compliance to the protocol was assessed by a 24-h self-recorded food diary.
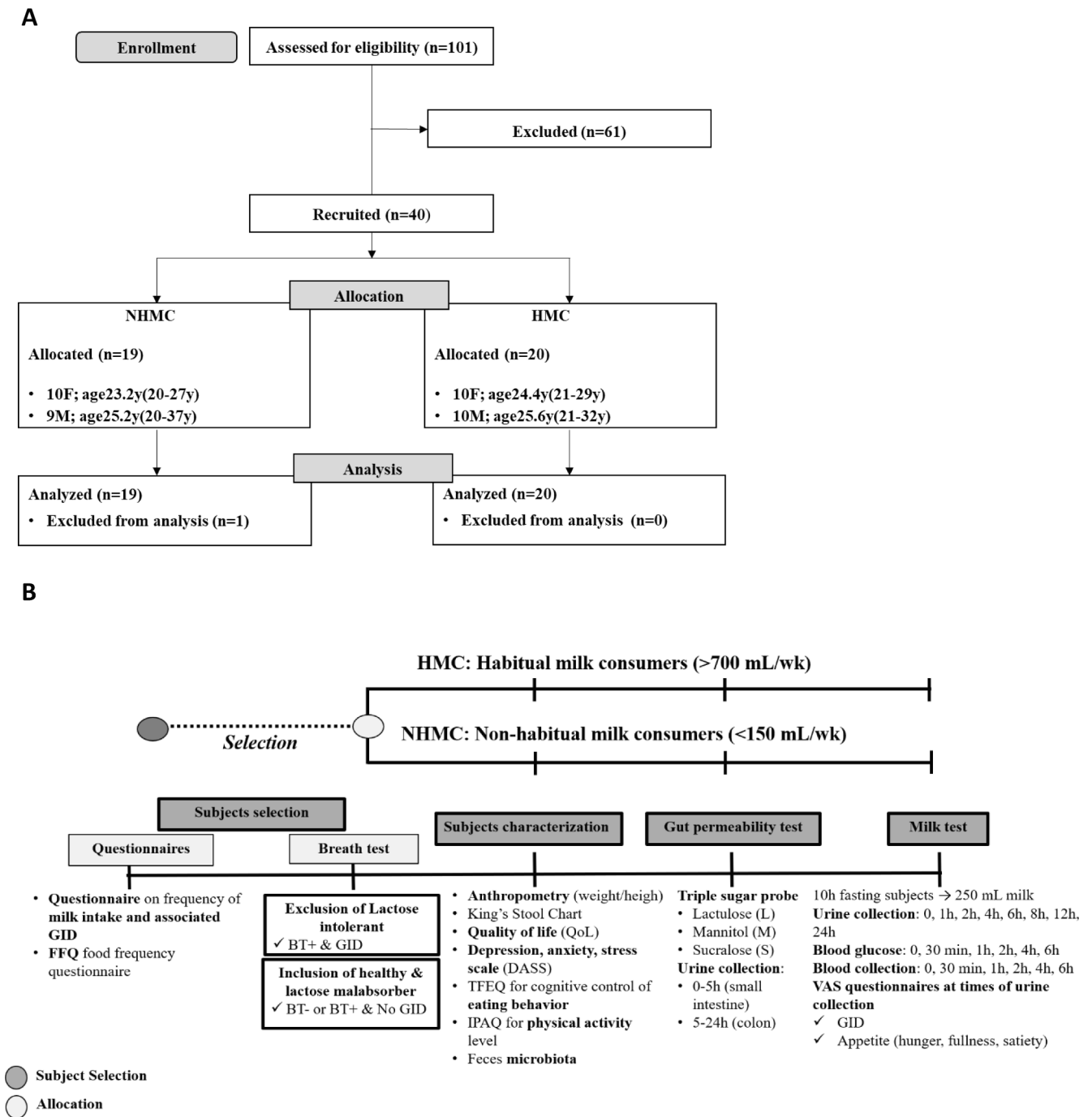
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Fig. 1. (A) Flow diagram describing the process of subject enrolment and data analysis. (B) Subjects were selected based on Questionnaires and Hydrogen Breath test (BT). Enrolled subjects, on three different occasions with one-week in between, participated in three visits to perform: (1) subject characterization; (2) gut permeability test and (3) milk-load test.

2.3. Subject characterization

Anthropometric characterization of the participants consisted of body weight and height measurements. Lifestyle characterization included a record of dietary habits, dietary behavior, physical activity and frequency and consistency of feces. Psychological characterization included assessment of individual psychological profiles (depression, anxiety and stress) and health-related quality of life. Dietary habits were recorded through a Food Frequency Questionnaire (FFQ) (Vitaglione et al., 2015), dietary behavior through the Three-Factor Eating Questionnaire (TFEQ) (Stunkard and Messick, 1985), physical activity level through the International Physical Activity Questionnaire (IPAQ) (Craig

et al., 2003) and frequency and consistency of feces through the King's Stool Chart (KSG) (Whelan et al., 2004; Whelan et al., 2008). Psychological characterization included assessment of individual psychological profile through the Depression, Anxiety and Stress Scale (DASS) (Lovibond and Lovibond, 1995) and the health-related quality-of-life questionnaire (SF-12) (Ware et al., 1996).

Before leaving the laboratory, participants were instructed on how to collect a fecal sample according to the standard operating procedure (SOP 004) of the International Human Microbiome Standards (IHMS) (https://www.microbiome-standards.org) for the gut microbiome analysis (Meslier et al., 2020).

2.4. Gut microbiome

The gut microbiome was analyzed by shotgun metagenomics. Microbial DNA extraction from fecal samples was carried out following the protocol reported by Meslier et al. (2020) according to the IHMSC SOP 07 (Meslier et al., 2020). DNA libraries were sequenced on an Illumina NovaSeq platform (Illumina, San Diego, California, USA), leading to 2x150bp, paired-end reads. Human reads were removed using the Human Sequence Removal pipeline developed within the Human Microbiome Project by using the Best Match Tagger (BMtagger; https://hmpdacc.org/hmp/doc/HumanSequenceRemoval_SOP.pdf). The resulting reads were quality-checked and filtered through Prinseq-lite v0.20.4 (with -trim_qual_right 5 and -min_len 60 parameters) (Schmieder & Edwards, 2011). Taxonomic and metabolic profiles were estimated with MetaPhlAn v3.0 and HUMAnN v3.0, respectively (Beghini et al., 2021). Genes/pathways from HUMAnN outputs were relabelled according to the KEGG database. The *diversity* function (from the R package *'vegan'*) was applied on species-level taxonomic profiles to estimate Shannon-Wiener and Simpson's alpha diversity indices. Microbial gene richness was calculated as described by Le Chatelier et al. (2013).

Reads were assembled into contigs using MEGAHIT v1.1.2 (Li et al., 2015), then genes were predicted from contigs > 1000 bp through MetaGeneMark 3.26 (Zhu et al., 2010).

DIAMOND v2.0.4 [–very_sensitive option; (Buchfink et al., 2015)] was used to align predicted genes to the MEROPS protease database (Rawlings et al., 2018). An e-value cutoff of $1e-5$ was applied, and a hit was required to display > 90% of identity over at least 50% of the query length to be kept. To obtain the gene abundance, short reads were mapped to the genes [using Bowtie2 v2.2.9, –very_sensitive_local option; (Langmead and Salzberg, 2012)] and the number of mapped reads was normalized using the RPKM method [reads per kilo-base per million mapped reads; (Mortazavi et al., 2008)].

2.5. Gut permeability test

Participants consumed a diet free of milk, dairy products and food products containing artificial sweeteners two days before the test, including a standardized lunch and dinner (boiled rice and roasted chicken/fish) on the day before the test. Then, following an overnight fast, participants drank 100 mL of a solution containing lactulose (5 g), mannitol (2 g) and sucralose (2 g) and collected 24-h urines into 2 containers (Li et al. 2016). One container collected urines excreted from baseline to 5 h (0–5 h, to assess small intestinal permeability) and the other container urines from 5 to 24 h (5–24, to evaluate colon permeability). Lactulose, Mannitol and Sucralose concentrations in urine samples were quantified by LC-MS/MS analysis.

2.5.1. Analysis of lactulose, mannitol and sucralose by LC-MS/MS

Lactulose, mannitol and sucralose concentrations in urine samples were quantified as described by Li and co-workers (2016) and Gervasoni et al. 2018. Briefly, urine samples were diluted 1:50 with acetonitrile/water (50:50) and centrifuged at 21100g × 10 min at 4 °C. Thereafter, supernatants were added with D-Mannitol ^{13}C as internal standard (5 μg/mL) and filtered with regenerated cellulose membrane filters (0.2 μm pore size) before being injected onto LC-MS/MS. All the standards were purchased from Sigma-Aldrich (Italy).

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 TSKgel amide 80, 3 μm column (2 × 150 mm) (TOSOH BIOSCIENCE, Germany) with a setting temperature of 45 °C and a flow rate of 0.2 mL/min and the injection volume was 5 μL. Monitored compounds were separated by using a binary gradient mobile phase composed of mobile phase A (13 mM ammonium acetate in distilled water) and mobile phase B (50% acetonitrile) and programmed as follows: 75 % B (2 min), 75–5 % B (6

min), 5 % B (8 min), 5–75% B (12 min), constant 75%B (15 min). The acquisition was performed in negative ion mode on an API 2000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a TurboIonSpray source in MRM (Multiple Reaction Monitoring). All the acquisition parameters are summarised in Supplementary Table 2.

2.6. Milk-load test

Participants consumed a diet free of milk and dairy products, including a standardized lunch and dinner (boiled rice and roasted chicken/fish) on the day before the test, for two days. Then, following an overnight fast, participants underwent a blood drawing and measurement of blood glucose in capillary blood. A urine sample was also collected before the participants were instructed to drink 250 mL of ultrahigh temperature (UHT) processed semi-skimmed cow milk (provided by Lactalis). Blood samples were collected by venipuncture into serum separator, EDTA-containing tubes, and EDTA aprotinin-containing tubes at 0.5, 1, 2, 4, and 6 h in parallel with measurements of blood glucose. Urine samples were collected in urine pots at 1, 2, 4, 6, 8, 12, and 24 h after milk consumption; participants also rated their GI symptoms and appetite on visual analogue scale (VAS) questionnaires. Once prepared, serum and plasma samples were aliquoted and immediately frozen at -80 °C until analysis. Participants were asked to avoid milk and dairy products up to 24 h after milk ingestion and provided with a standardized lunch (bread and lactose-free ham) and dinner (bread and tuna). Compliance with the protocol was assessed by a 24-h self-recorded food diary. Blood samples were analyzed to assess the following: the concentration of milk-derived BAPs and AAs by liquid chromatography–high resolution mass spectrometry (LC-HRMS); the serum DPPIV concentration and activity by Bio-Plex Pro immunoassay kits; plasma ECs and NAEs by LC-HRMS; GI hormones (such as ghrelin, insulin, C-peptide, and gastric inhibitory peptide [GIP]) by Bio-Plex Pro immunoassay kits and blood glucose levels by a finger prick and a bedside glucometer (OneTouch Sure Step; Life Scan Inc.). Urine samples were analyzed by LC–MS/MS to assess the concentration of indoxyl sulfate.

2.6.1. Analysis of plasma amino acids by LC-HMRS

Simultaneous quantification of AAs from plasma were performed using the method by Shin et al. (2019). Briefly, 100 μL of internal standard L-Proline-2,5,5-d3 (25 μg/mL) were added to 100 μL of plasma. Methanol 800 μL was then added to allow protein precipitation, then, the mixture was centrifuged at 21,100 g for 10 min, 4 °C. The supernatant was diluted (1:5) in a solution acetonitrile/water (80:20) with 0.2% formic acid before injection onto liquid chromatography- high resolution mass spectrometry (LC-HMRS).

The LC–HMRS system consisted of Accela U-HPLC system coupled to an Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The Accela system consisted of a quaternary pump, a thermostated autosampler (10 °C) and a column oven heated at 35 °C. The mobile phase consisted of 0.1% formic acid water (A) and 0.1% formic acid acetonitrile (B), the flow rate was set to 300 μL/min and the injection volume was 5 μL. The analytes were separated on a Syncronis 3 μm HILIC column (50 × 2.1 mm), (Thermo Fisher Scientific, Bremen, Germany) with SecurityGuard Cartridge Kit (Phenomenex) with setting temperature at 45 °C and eluted by a linear gradient of a 95% B (0–1.5 min), 95–10% B (1.5–4 min), 10% B (4–7 min), 10–95% B (7–9 min) and then constant at 95% B (9–12 min). The analytical standards of the L-amino acids and L-Proline-2,5,5-d3 were purchased from Sigma-Aldrich (Italy).

MS detection was performed in positive-ion mode in the *m/z* 65–500 mass range: spray voltage was 4.8 KV, capillary voltage 25 V, heater temperature 250 °C, capillary temperature at 295 °C, sheath gas 30 and auxiliary gas 5 arbitrary units, respectively.

Supplementary Table 3 reports the molecular formula, theoretical

and experimental mass, the mass accuracy and the retention time of identified compounds.

2.6.2. Analysis of plasma milk-derived bioactive peptides by LC-HMRS

Simultaneous extraction and quantification of BAPs from plasma were performed adapting the method by Aristoteli et al. (2007). BCM7 standard was purchased from Bachem, Switzerland. Plasma samples (200 μL) previously diluted 1:5 with water with 0.1% formic acid (Solvent A) were added with 10 μL of the internal standard 2.5 μg/mL solution of ^{13}C-labeled BCM7 (Bachem, Switzerland). Then, the samples were vortexed and centrifuged 21,000 g for 5 min at 4 °C. Strata C18-E (55 μm, 70 Å) cartridges (50 mg/1mL) (Phenomenex, USA) were preconditioned with 1 mL of methanol and equilibrated using 1 mL of solvent A. Samples were introduced onto the cartridges and were washed with 1 mL of solvent A, and the BAPs were eluted in 1 mL of acetonitrile/water (80:20) with 0.1% formic acid (Solvent B). The eluate was dried under nitrogen flow and reconstituted in 100 μL of solvent A before the LC–HMRS analysis.

BAPs were separated on a Luna Omega 1.6 μm Polar C18 100 (50x2.1 mm) column (Phenomenex, USA) with setting temperature at 40 °C. The mobile phases consisted of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and the gradient elution was linearly programmed as follows: 5% di B (0.5 min), 5–80 % B (0.5–9 min), constant at 80% B (3 min), 80–5% B (12–15 min). The flow rate was set at 100 μL/min, and the injection volume was 5 μL.

MS detection was performed in positive-ion mode in the m/z 75–1500 mass range: spray voltage was 4.2 KV, capillary voltage 25 V, heater temperature 250 °C, capillary temperature at 295 °C, sheath gas 30 and auxiliary gas 5 arbitrary units, respectively.

Supplementary Table 4 reports the molecular formula, theoretical and experimental mass, the mass accuracy of the tentatively identified compounds.

Full scan data processing was performed using Thermo Scientific™ ExactFinder™ and peptide identifications were obtained using the milk bioactive peptide database (MBPDB) (Nielsen et al., 2017) and BIOPEP-UWM™ Database of Bioactive Peptides (Minkiewicz et al., 2008). The tolerance range for mass accuracy of BAPs was fixed at ± 5 ppm. Peptides were expressed as equivalents of BCM7.

2.6.3. Identification and confirmation of plasma milk-derived BAPs by LC-MS/MS

Plasma milk-derived BAPs identified at LC-HMRS were further confirmed by LC–MS/MS.

Supplementary Fig. 1 displays chromatograms of all BAPs confirmed by LC–MS/MS. 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 summarised in Supplementary Table 5. The analyses were performed using the following settings: drying gas (air) was heated to 400 °C, capillary voltage (IS) was set to + 5500 V. Fragment assignments were accomplished by Protein Prospector, allowing the confirmation of BAPs. Chromatographic separation was performed using an HPLC apparatus provided with two micropumps, Perkin-Elmer Series 200 (Norwalk, CT, USA). The chromatographic conditions used for the analysis were the same reported in the LC-HMRS analysis.

2.6.4. Analysis of plasma endocannabinoids and N-acylethanolamines by LC-HMRS

Plasma samples were treated and analyzed for ECs and NAEs as previously described (Tagliamonte et al., 2021).

2.6.5. Blood glucose, gastrointestinal hormones, dipeptidyl peptidase-IV concentration and activity

Glycaemia was measured at baseline and after milk consumption immediately before the blood collection by finger pricking and using a bedside glucometer (OneTouch Sure Step; Life Scan Inc.). Accuracy of

the glucometer was evaluated by the manufacturer by using least squares linear regression analysis and it was found to be 97% "clinically accurate" compared with reference (YSI2700) results. Blood samples were collected into EDTA aprotinin-coated tubes, a DPPIV inhibitor. They were centrifuged at 2400 g per 10 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) in plasma samples, was achieved by Bio-Plex Pro immunoassay kits as described by Vitaglione et al., (2015). The sensitivity levels of the assay (pg/mL) were for C-peptide 14.3, for ghrelin 1.2 and for GIP 0.8. The inter-assay variation (% CV) was 4%, and the intra-assay variation (% CV) was 5%.

The serum DPPIV concentration was measured by using an R&D Systems immunoassay kit and an xMAP technology-based system (Bio-Plex 200; Bio-Rad) (Daan et al., 2016); the enzymatic activity of serum DPPIV was determined with the "direct photometric method" adapted to 96-well plates (Jarmołowska et al., 2007).

2.6.6. Indoxyl sulfate analysis in urine by LC-MS/MS

Urine samples at baseline and pooled urine samples within two intervals 0–6 and 6–24 h were analyzed for indoxyl sulfate content by adapting the Zhu et al. (2011) method. Briefly, urine specimens were diluted 1:10 with distilled water, centrifuged at 21,100 g × 10 min at 4 °C and filtered with regenerated cellulose membrane filters (0.2 μm pore size) prior to LC-MS/MS analysis.

Chromatographic separation was performed using an HPLC apparatus coupled to an API 2000 MS equipped with a TurboIonSpray source as already described above. The compounds were separated on a Kinetex 2.6 μ C18 100 Å column (100 mm × 2.1 mm) (Phenomenex, Torrance, CA) with setting temperature at 40 °C and eluted by a linear gradient of a water (0.1% formic acid) (solvent A) and acetonitrile (0.1% formic acid) (solvent B) with a flow rate of 200 μL/min and volume injection of 10 μL. According to Chen et al. (2018), eluting gradient was adapted as follows: 5% B from 0 to 0.5 min, 5–70% B from 0.5 to 1.5 min, 70–95% B from 1.5 to 3.5 min, 95% B from 3.5 to 5 min, 95–5% B from 5 to 6 min and kept at 5% B until 11 min. Calibration curves in urine were built in the linearity range 1–15 μg/mL. Indoxyl sulfate showed a $[M-H]^-$ ion at 212 m/z, and the daughter ion m/z 132 generated with collision energy (CE) of 33 V. Indoxyl sulfate potassium salt standard was purchased from Sigma-Aldrich (Italy).

2.7. Statistical analysis

The sample size was calculated considering the primary endpoints and levels of postprandial plasma BAPs, GI hormones, ECs and NAEs. According to a previous study, a sample size of 19 participants per group could detect a 40% change in plasma BAP levels (Deth et al., 2015).

Concerning postprandial circulating concentrations of GI hormones, ECs and NAEs, a sample size of 13 volunteers could detect a significant postprandial change, according to Mennella et al., (2015). Therefore, 19 participants in each group would allow detection of significant differences in the selected biomarkers with an α error of 0.05, 80% power and two-tailed testing.

Statistical analysis and visualization were carried out in R version 4.0.3 (https://www.r-project.org). After variables were checked for normality, significantly skewed variables were natural-log transformed [ln(x + k), with k values zeroing the skewness]. For variables with a normal distribution according to the Shapiro–Wilk test, an independent-samples t test was performed to assess differences between groups; for variables that included potential confounding factors, an ANCOVA was performed including the covariates in the analysis. For non-parametric variables, the Mann–Whitney test was conducted to detect between-group differences. The chi-square test of independence was performed using the chisq.test function (stats package) to analyse the frequency table formed by two categorical variables.

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 total postprandial area under the curves (AUCs) were estimated using the linear trapezoidal rule, and differences in AUCs between the groups were assessed by parametric or nonparametric analyses, as appropriate, including the potential confounding variables measured at baseline as covariates. Two-tailed P values lower than 0.05 were considered significantly different. Data are expressed as the means ± standard errors (SEMs).

To explore differences in gut microbiome profiles, a linear discriminant analysis (LDA) effect size (LEfSE) was applied (Segata et al., 2011).

3. Results

3.1. Participants suffering from milk-induced GID had distinctive diets and gut microbiome compositions to those without milk-induced GID but similar gut permeability

Table 1 shows the general, anthropometric, lifestyle and psychological characteristics of the 19 (10F/9 M, average BMI: 23.5 ± 0.7 kg/m^2, age: 24.2 ± 0.9 years) NHMC and 20 (10F/10 M, average BMI: 25.6 ± 0.8 kg/m^2, age: 25 ± 0.7 years) HMC participants; 9 participants in each group showed lactose malabsorption assessed by the lactose breath test.

The two groups were similar on all the characteristics assessed except for anxiety level, which was higher in NHMCs, and for some dietary aspects. NHMCs consumed significantly less lactose-containing cow milk (the selection criterion), resulting in a lower amount of overall dairy products (including milk) consumed. The intake of fibres and proteins was similar between the two groups, but NHMCs had a higher dietary-fibre-to-protein ratio and a trend towards higher-plant-protein-to-animal-protein ratio.

These differences in diet did not affect gut microbial diversity and gene richness, which were similar between the groups (Fig. 2**a, b**). However, some differences in gut microbiome composition between NHMC and HMC groups were observed. NHMCs had a significantly lower abundance of the Actinobacteria phylum and higher abundance of *Paraprevotella* and *Prevotella*; additionally, they exhibited a higher abundance of *Bacteroides clarus*, *Coprococcus eutactus* and *Ruminococcus lactaris*. Conversely, HMCs had a higher abundance of *Bifidobacterium adolescentis*, *Bifidobacterium longum*, and *Dialister invisus* than NHMCs (Fig. 2**c**). Moreover, the gut microbiome in NHMC exhibited a lower abundance of protease gene families (Fig. 2**d** and Supplementary Fig. 2), along with decreased enrichment in several Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways (Supplementary Fig. 3).

Gut permeability did not differ in participants suffering from milk-related GID. Indeed, NHMCs and HMCs showed similar 24-h urinary excretions of lactulose, mannitol, and sucralose after ingesting the sugar solution (Fig. 3**a, b**).

3.2. Participants suffering from milk-induced GID experienced slower and lower protein digestion

The plasma profiles of the 31 BAPs and 20 AAs we monitored are reported in Supplementary Fig. 4 and Supplementary Fig. 5.

After 2 days of consuming a dairy product-free diet followed by 10 h of fasting, NHMCs exhibited significantly lower concentrations of 10 out of 31 milk-derived BAPs (Supplementary Fig. 4) and total BAP concentrations at baseline compared to HMCs (Fig. 4**a**), consistent with a significantly lower habitual intake of dairy products.

Compared to HMCs, NHMCs exhibited a slower overall BAP response and lower overall BAP concentration in the plasma (Fig. 4**b**). The plasma profile of all BAPs in NHMCs did not change until 4 h after milk

Table 1
General, anthropometric, lifestyle, and psychological characteristics of habitual milk consumers (HMCs) and nonhabitual milk consumers (NHMCs).

	NHMCs (n = 19)	HMCs (n = 20)	P-value
Sex (M/F)	9/10	10/10	
Age	24.16 ± 0.90	25.00 ± 0.65	0.30
Body weight (kg)	66.53 ± 2.88	72.44 ± 3.12	0.17
BMI (kg/m^2)	23.50 ± 0.65	25.56 ± 0.82	0.06
Total METs [a]	2723.89 ± 556.86	2590.42 ± 562.52	0.77
King's stool chart score	3.74 ± 0.50	3.82 ± 0.52	0.84
TFEQ RESTRAINT	10.32 ± 1.42	9.85 ± 1.02	0.79
TFEQ DISINHIBITION	5.89 ± 0.96	7.35 ± 0.90	0.22
TFEQ HUNGER	5.05 ± 0.86	5.25 ± 0.74	0.61
QUALITY OF LIFE-PCS [b]	56.53 ± 1.06	56.92 ± 0.73	0.76
QUALITY OF LIFE-MCS [c]	41.06 ± 2.33	45.51 ± 2.12	0.11
Depression			
normal/mild, n (%)	16 (84.2%)	15 (75.0%)	0.75
moderate/severe/extremely severe, n (%)	3 (15.8%)	5 (25.0%)	
Anxiety			
normal/mild, n (%)	10 (52.6%)	19 (95%)	**0.008***
moderate/severe/extremely severe, n (%)	9 (47.4%)	1 (5%)	
Stress			
normal/mild, n (%)	14 (73.7%)	16 (80.0%)	0.93
moderate/severe/ extremely severe, n (%)	5 (26.3%)	4 (20.0%)	
Habitual diet			
Milk intake (mL/week)	80.52 ± 26.11	1197.75 ± 150.13	**<0.001***
Lactose-free milk intake (mL/week)	276.32 ± 77.74	11.17 ± 10.00	**0.012***
Dairy products intake (g/week)	1070.38 ± 157.45	1995.40 ± 136.98	**<0.001***
Daily energy intake (kcal/day)	1942.33 ± 194.12	2133.56 ± 140.80	0.43
% Energy from proteins (%)	17.90 ± 0.52	18.63 ± 0.61	0.37
% Energy from fats (%)	37.27 ± 1.98	36.39 ± 1.02	0.73
% Energy from carbohydrates (%)	40.93 ± 1.92	41.74 ± 1.32	0.75
% Energy from fibres (%)	2.74 ± 0.27	2.21 ± 0.15	0.10
% Energy from alcohol (range/mean) (%)	1.19 ± 0.30	0.98 ± 0.23	0.52
Proteins (g/day)	86.7 ± 8.72	97.5 ± 5.83	0.30
Fats (g/day)	78.9 ± 8.60	86.7 ± 6.43	0.47
Carbohydrates (g/day)	204.0 ± 23.35	223.23 ± 16.67	0.50
Dietary fibre (g/day)	24.17 ± 2.60	23.50 ± 2.44	0.90
Italian Mediterranean Index (range/mean)	5.68 ± 0.31	5.75 ± 0.33	0.97
Plant protein/animal protein intake ratio	0.55 ± 0.06	0.43 ± 0.04	0.06
Dietary Fibre/Protein intake ratio	0.31 ± 0.03	0.24 ± 0.02	**0.016***

* p < 0.05 HMC *vs* NHMC assessed by Independent sample T test or Mann-Whitney test. Data are expressed as means ± SEM.
[a] Metabolic equivalent of tasks;
[b] Physical component summary;
[c] Mental component summary.

consumption, when BAP concentrations weakly peaked and then returned to baseline within 6 h after milk consumption. In contrast, in HMCs, the plasma BAP concentrations peaked after 30 min, returned to baseline after 4 h and peaked again 6 h after milk consumption. These profiles indicate a lower circulating level of overall BAPs in NHMCs compared to HMCs (Fig. 4**c**).

On the other hand, despite differences in the profile of some AAs (Supplementary Fig. 5), the postprandial plasma profile and total level of AAs did not differ between NHMC and HMC participants (Fig. 4**d, e**). Therefore, the plasma BAP findings indicate that a less efficient digestion of milk proteins occurs in NHMCs compared to HMCs. This fact, combined with the lower proteolytic activity of the gut microbiome, suggests the presence of undigested proteins/oligopeptides in the large intestine of NHMCs; the NHMC gut microbiome was unable to deliver
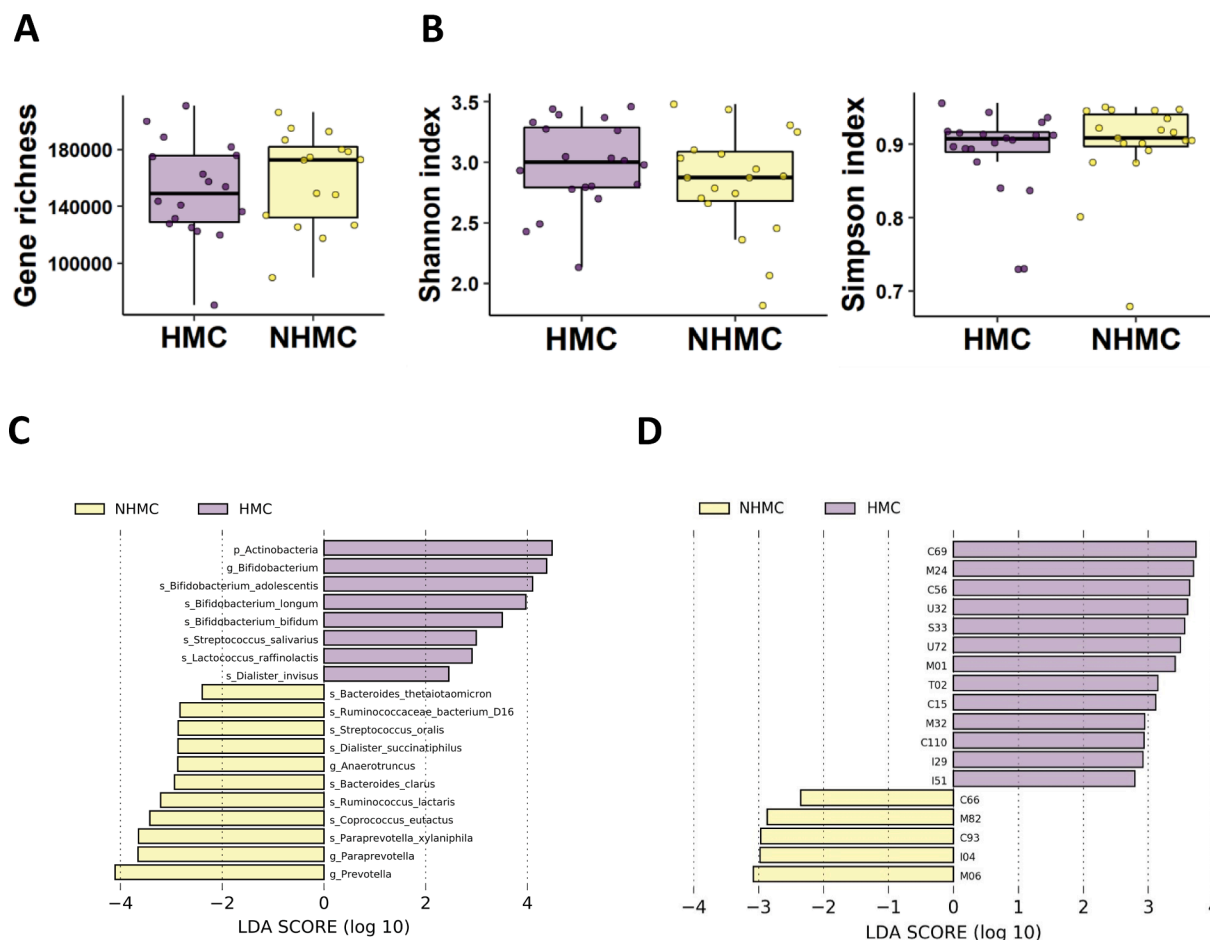
Fig. 2. (A) Gene richness of the gut microbiome of habitual milk consumers (HMCs) and nonhabitual milk consumers (NHMCs), (B) Shannon and Simpson diversity indices of the gut microbiome of habitual milk consumers (HMCs) and nonhabitual milk consumers (NHMCs). (C) Linear discriminant analysis effect size (LEfSe) showing the differentially abundant species between habitual milk consumers (HMCs; light violet) and nonhabitual milk consumers (NHMCs; yellow). The bacterial taxa shown exhibited a statistically significant change (p < 0.05) when the logarithmic linear discriminant analysis (LDA) score threshold was set to 2. The name of the taxon level is abbreviated as p-phylum, g-genus and s-species. (D) Linear discriminant analysis effect size (LEfSe) showing differentially abundant protease families between habitual milk consumers (HMCs; light violet) and nonhabitual milk consumers (NHMCs; yellow). The proteases shown exhibited a statistically significant change (p < 0.05) when the logarithmic linear discriminant analysis (LDA) score threshold was set to 2. Proteases families: C, cysteine; I, inhibitor; M, metallo; S, serine; T, threonine; U, unknown.
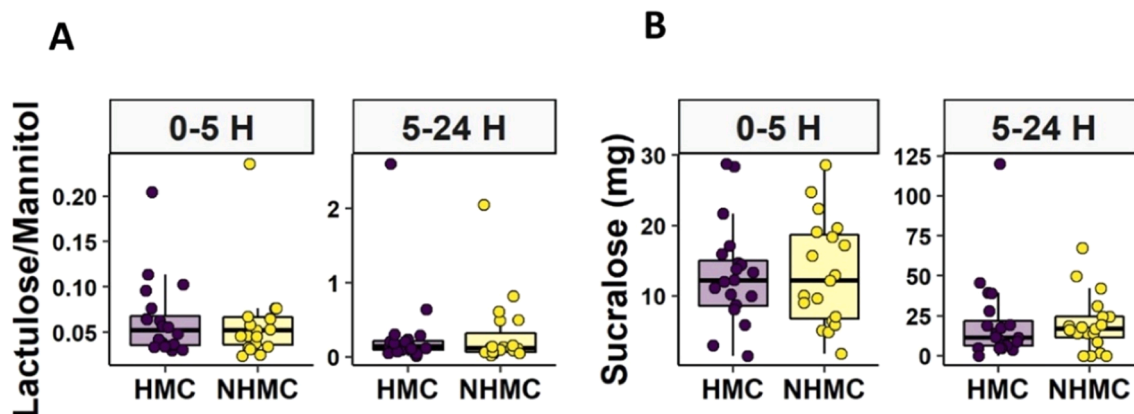

Fig. 3. (A) Urinary excretion of lactulose/mannitol and (B) urinary excretion of sucralose in habitual milk consumers (HMC) and nonhabitual milk consumers (NHMC) during intervals of 0–5 h and 5–24 h after the gut permeability test. The box plots show the data distribution based on the first quartile, median and third quartile.

tryptophan for conversion into indole, explaining the lower amount of the hepatic metabolite indoxyl sulfate in urine 6 h or longer after milk consumption (Fig. 4f). Consistent with the presence of undigested proteins in the large intestine, NHMCs reported less hunger 6–24 h after milk consumption (Fig. 4g).
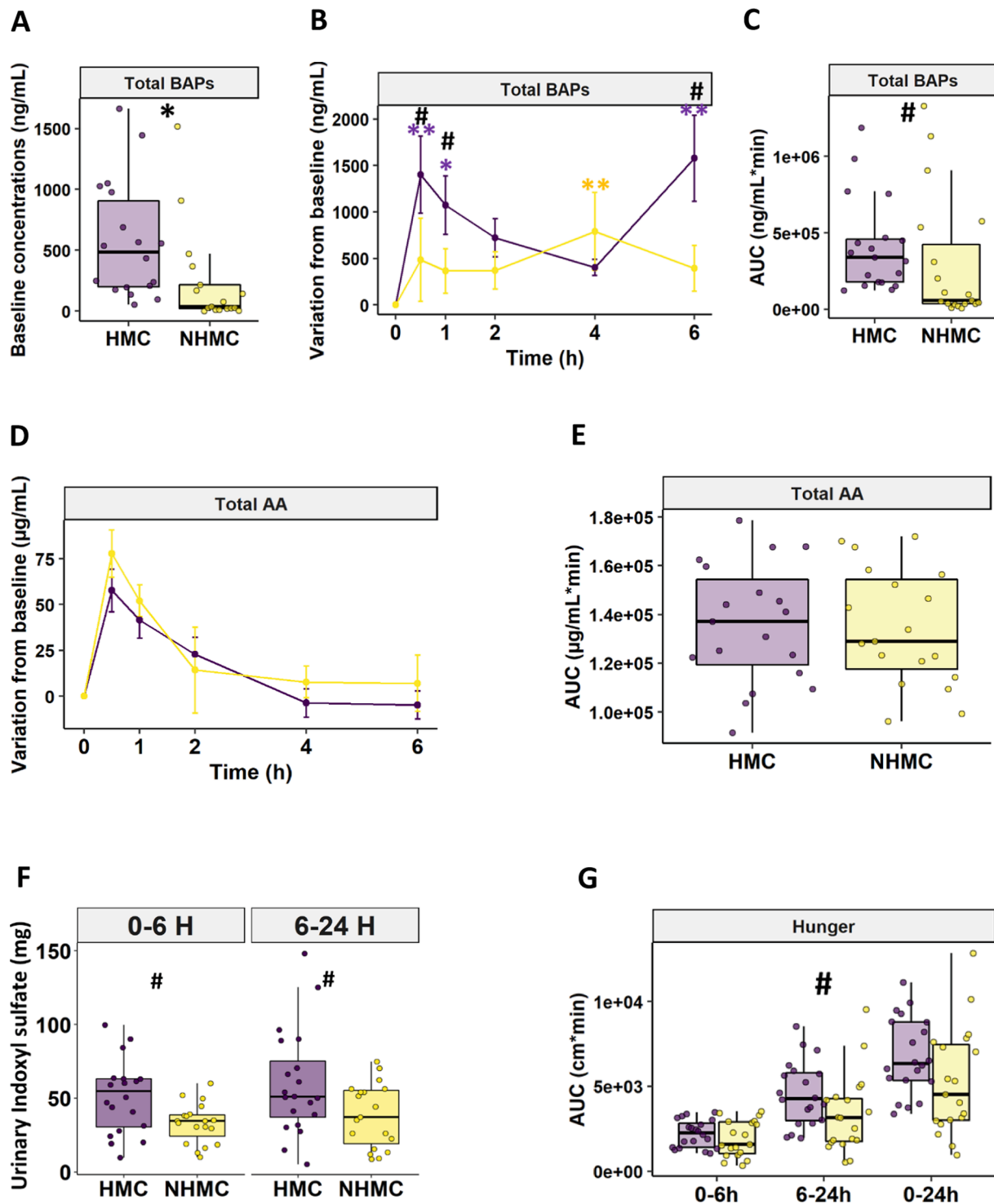
Fig. 4. (A) Plasma baseline concentrations of overall circulating BAPs in habitual milk consumers (HMCs) and nonhabitual milk consumers (NHMCs). # p-value < 0.05, HMC *vs* NHMC between-group difference assessed by an independent-samples *t* test. The box plots show the data distribution based on the first quartile, median and third quartile; (B) Plasma total milk-derived bioactive peptides (BAPs) in habitual milk consumers (HMCs; light violet) and nonhabitual milk consumers (NHMCs; yellow) after consuming 250 mL of milk. Data are shown as the means ± standard errors (SEMs). * p value < 0.05; ** p–value < 0.01, within-group difference versus baseline assessed by one-way repeated-measures ANOVA; # p < 0.05, between-group difference assessed by one-way repeated-measures ANOVA; (C) Areas under the curves (AUCs) of overall circulating BAP levels in HMCs and NHMCs after consuming 250 mL of milk. # p value < 0.05, HMC *vs.* NHMC between-group difference assessed by ANCOVA adjusted for baseline values. The box plots show the data distribution based on the first quartile, median and third quartile; (D) Plasma time-concentration curves of total amino acid levels in HMCs and NHMCs after consumption of 250 mL of milk. Data are shown as the means ± SEMs; (E) Areas under the curves (AUCs) of overall circulating amino acid levels in HMCs and NHMCs after consumption of 250 mL of milk. The box plots show the data distribution based on the first quartile, median and third quartile; (F) Urinary indoxyl sulfate excretion in HMCs and NHMCs during the intervals of 0–6 h and 6–24 h (b) after milk consumption. # p < 0.05, HMC *vs.* NHMC between-group difference assessed by independent-samples *t* test. The box plots show the data distribution based on the first quartile, median and third quartile; (G) Violin plots representing the hunger AUC sensation monitored by visual analogue scales (VAS) in HMCs and NHMCs after consuming 250 mL of milk. # p–value < 0.05, HMC *vs.* NHMC between-group difference assessed by ANCOVA adjusted for baseline values.
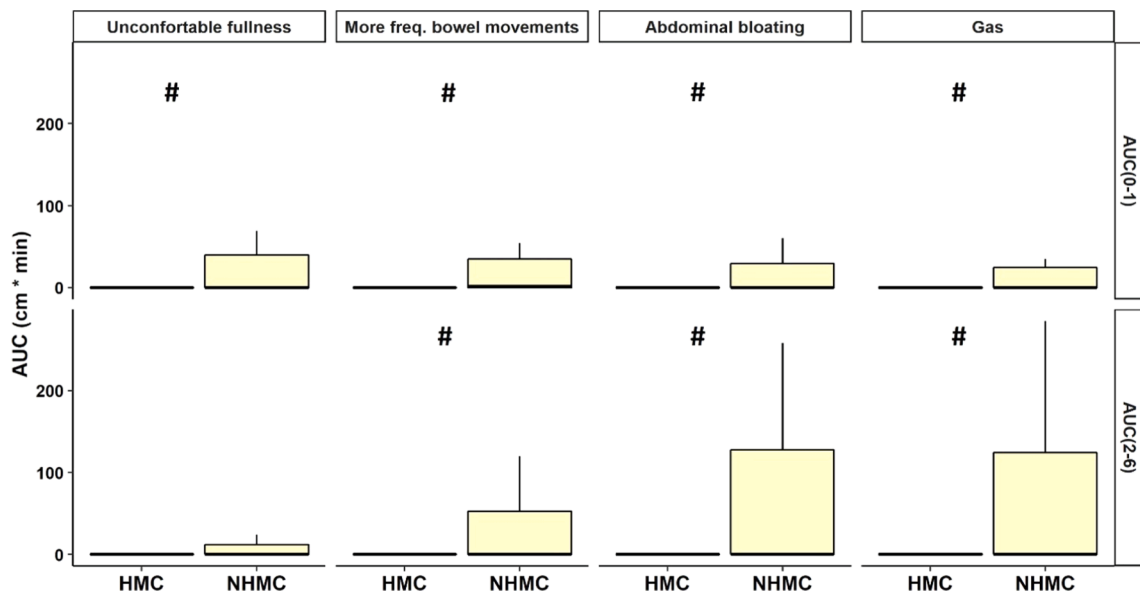
Fig. 5. Most reported gastrointestinal discomfort of HMCs and NHMCs during the intervals of 0–1 h and 2–6 h after consuming 250 mL of milk. # p value < 0.05, HMC *vs.* NHMC between-group difference assessed by one-way ANOVA.
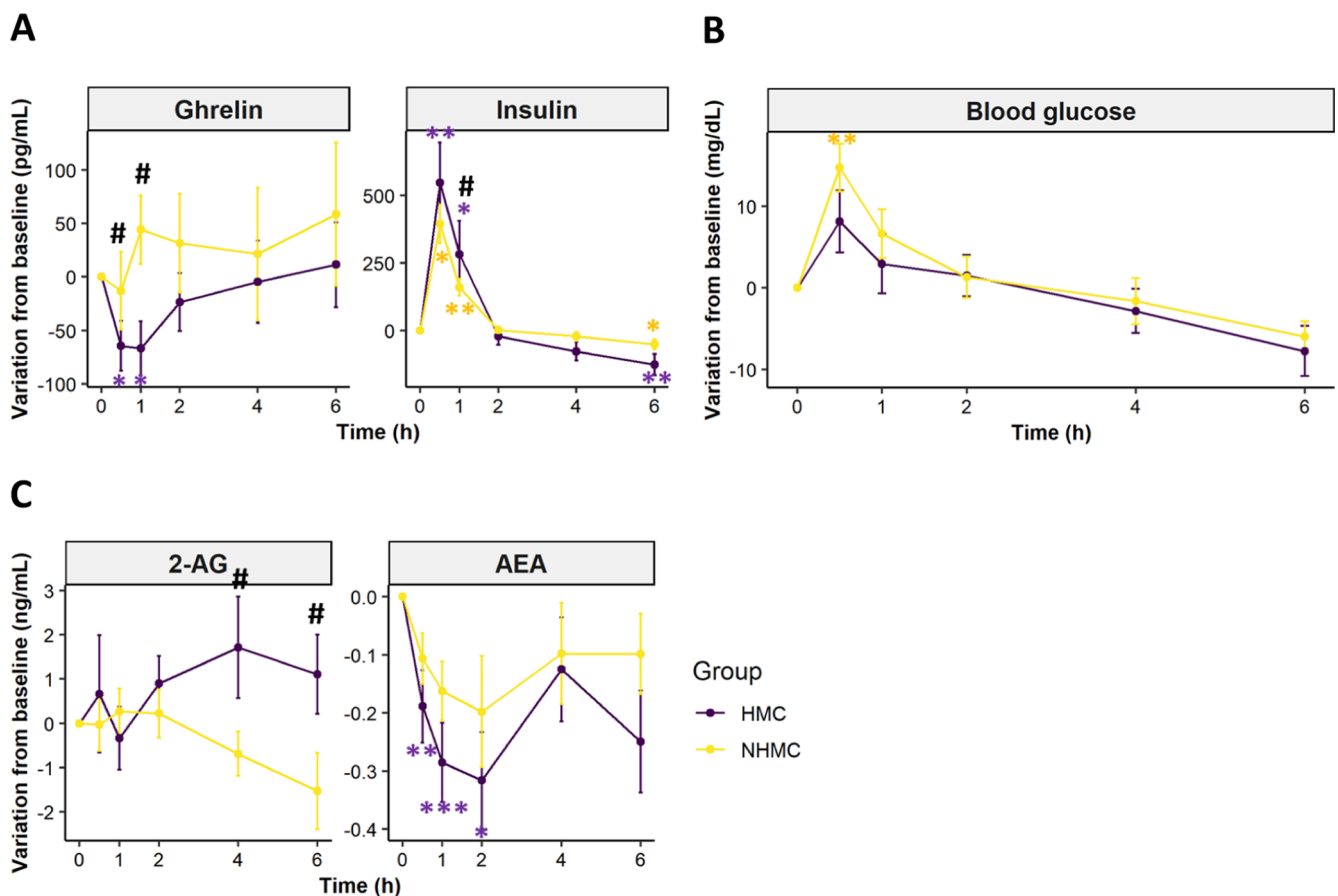

Fig. 6. (A) Time-concentration curves of plasma ghrelin and insulin levels in habitual milk consumers (HMCs; light violet) and nonhabitual milk consumers (NHMCs; yellow) after consuming 250 mL of milk. Data are shown as the means ± SEMs. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001, within-group difference versus baseline with Bonferroni adjustment for multiple comparisons; # p < 0.05, between-group difference assessed by a one-way repeated-measures ANOVA controlling for BMI; (B) Time-concentration curves of blood glucose in HMCs and NHMCs after consuming 250 mL of milk. Data are shown as the means ± SEMs. **p < 0.05 for the measured time point compared with baseline within each group, as assessed by one-way repeated-measures ANOVAs and a Bonferroni adjustment for multiple comparisons; C) Plasma time-concentration curves of 2-arachidonoylglycerol (2-AG) and arachidonoylethanolamide (AEA) in HMCs and NHMCs after consuming 250 mL of milk. Data are shown as the means ± SEMs. * p-value < 0.05; ** p-value < 0.01; *** p-value < 0.001, within-group difference *vs.* baseline with Bonferroni adjustment for multiple comparisons. # p < 0.05, HMC *vs.* NHMC between-group difference assessed by Mann–Whitney test.

3.3. Digestive processes occurring soon after milk consumption affect GID evolution in NHMCs

The most frequently reported symptoms of GID in NHMCs were uncomfortable fullness, gas, abdominal bloating, and frequent intestinal movements (Supplementary Table 6). Uncomfortable fullness was mainly reported during the first hour after milk consumption, whereas the other GID symptoms persisted in the following hours (Fig. 5).

The lower circulating levels of BAPs (Fig. 4b) and perceived uncomfortable fullness were accompanied by a weaker response of ghrelin and insulin soon after milk consumption in NHMCs compared to that in HMCs. NHMCs did not exhibit a postprandial change in plasma levels of ghrelin, while HMCs exhibited decreases in ghrelin concentration 30 and 60 min after milk consumption (Fig. 6a). A significantly higher concentration of plasma branched chain AAs was found in NHMCs 30 min after milk consumption (Supplementary Fig. 5), likely explaining the concomitant relative increase in insulin, which remained at a lower concentration than that in HMCs at 1 h after milk consumption (Fig. 6a). The lower insulin response and the sustained ghrelin levels explain the blood glucose peak at 30 min after milk consumption that was found

only in NHMCs (Fig. 6b), independent of C-peptide and GIP levels (Supplementary Fig. 6).

Consistent with the ghrelin response, arachidonoylethanolamide (AEA) levels did not exhibit a postprandial change in NHMCs, while these levels decreased in HMCs (Fig. 6c). As expected, no changes were found in circulating levels of 2-arachidonoylglycerol (2-AG) within the groups; however, 2-AG levels were significantly lower at 4 h and 6 h after milk consumption in NHMCs. A quicker reduction in circulating NAE levels was also found in NHMCs (Supplementary Fig. 7), suggesting that the two groups differ in fatty acid uptake.

Taken together, these observations suggest that some physico-chemical features of NHMC chyme affect nutrient sensing by GI receptors lining the mucosa, modulating hormonal responses and individual sensations along with digestion of milk.

3.4. Circulating levels of BAP opioid antagonists and agonists modulate GI transit and GID evolution after milk consumption

To clarify the relationship between protein digestion and GID, the milk-derived BAPs were grouped for their known bioactivity as follows:
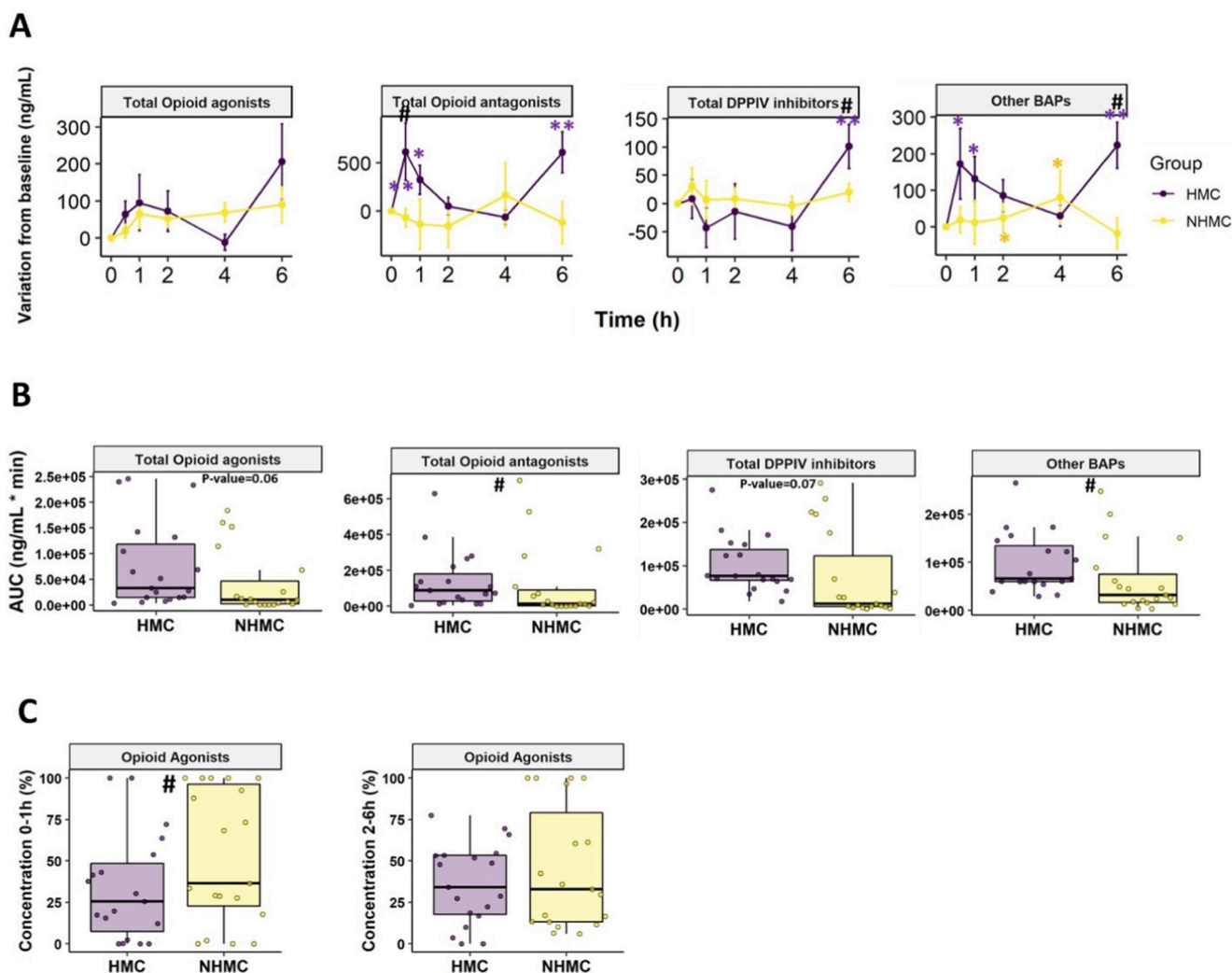

Fig. 7. A) Plasma time-concentration curves of milk-derived bioactive peptides (BAPs) grouped according to their biological properties in habitual milk consumers (HMCs; light violet) and nonhabitual milk consumers (NHMCs; yellow) after consuming 250 mL of milk. * p-value < 0.05; ** p-value < 0.01, within-group difference *vs.* baseline with Bonferroni adjustment for multiple comparisons. # p value < 0.05, HMC *vs.* NHMC between-group difference; B) Areas under the curve (AUCs) of BAPs grouped according to their biological properties in HMCs and NHMCs after consuming 250 mL of milk. # p value < 0.05, HMC *vs.* NHMC between-group difference assessed by ANCOVA adjusted for baseline values; C) Circulating amount of BAP opioid agonists relative to antagonists during a 0–1 h interval in HMCs and NHMCs after consuming 250 mL of milk. # p value < 0.05, HMC *vs.* NHMC between-group difference assessed by ANCOVA adjusted for baseline values; D) Circulating amount of BAP opioid agonists relative to antagonists during a 2–6 h interval in HMCs and NHMCs after consuming 250 mL of milk.

opioid receptor agonists, opioid receptor antagonists, DPPIV inhibitors and peptides with other activities, including antioxidant, anti-inflammatory, antihypertensive, and anxiolytic activities.

Circulating levels of BAP opioid receptor antagonists were significantly lower in NHMCs than HMCs (Fig. 7a, b), possibly slowing postprandial GI transit due to a postabsorptive effect on opioid receptors. Compared to HMCs, this effect was more evident 1 h after milk consumption and induced the perception of uncomfortable fullness in NHMCs when the circulating amount of BAP opioid agonists was higher than opioid antagonists (Fig. 7c). Participant perceptions of other symptoms (gas, abdominal bloating, and more frequent bowel movements) 2–6 h after milk consumption were consistent with the presence of undigested proteins/oligopeptides in the intestine as described above.

Interestingly, the circulating levels of DPPIV-inhibiting BAPs inversely correlated with serum DPPIV activity (r = -0.455, p = 0.004) (Fig. 8a). Indeed, concomitant with a lower circulating level of DPPIV-inhibiting BAPs at baseline, a trend towards higher DPPIV activity (p = 0.058) was found in NHMCs compared to HMCs, independent of

serum DPPIV concentration (Supplementary Fig. 8). DPPIV activity was significantly higher (Fig. 8b) when BAP DPPIV inhibitor concentrations were lower in NHMCs (i.e. 6 h after milk consumption) (Fig. 7a); coherently, it was greater over 6 h after milk consumption (Fig. 8c).

4. Discussion

This study showed that healthy people suffering from GID after cow milk consumption presented a slower and lower digestion of milk proteins from early steps of digestion. The phenomenon persisted in the lower intestinal tract, where undigested proteins/oligopeptides likely accumulated due to the lower proteolytic activity of the gut microbiome. The postprandial circulating levels of BAPs and the undigested proteins in the gut lumen slowed GI transit and induced GID.

The postprandial ghrelin and insulin responses suggested that participants with GID experience reduced detection of milk nutrients in the stomach (Vancleef et al., 2015) and explained the blood glucose peak, as ghrelin increases hepatic glucose production and decreases the glucose
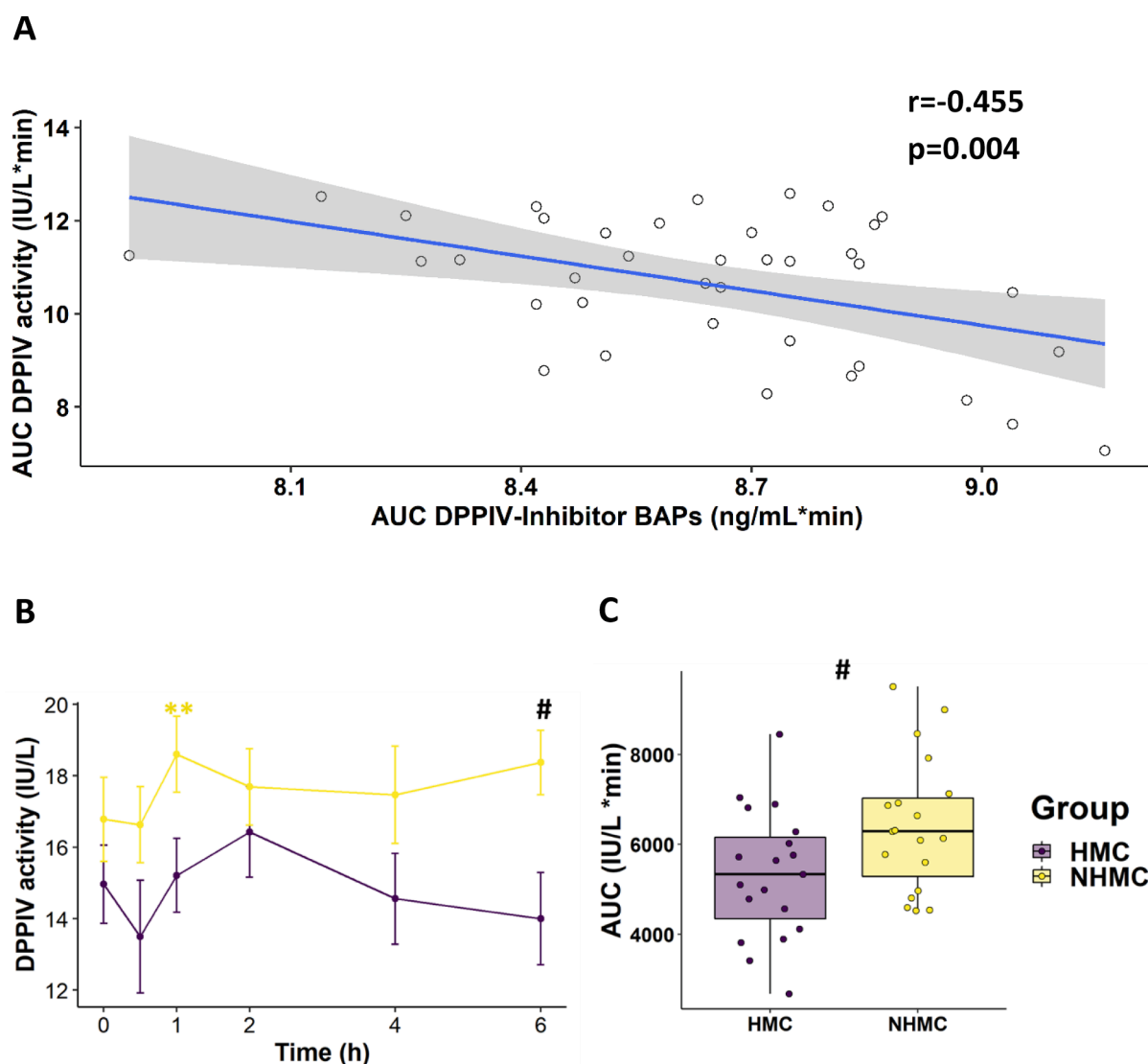

Fig. 8. (A) Correlation between serum concentrations of DPPIV and plasma DPPIV-inhibiting peptides. R and p values are assessed by Pearson correlation analysis of natural-log transformed variables; (B) Serum dipeptidyl peptidase IV (DPPIV) activity profile in HMCs and NHMCs after consuming 250 mL of milk. Data are shown as the means ± SEMs. **$p < 0.05$, comparison of the measured time point with baseline within each group via one-way repeated-measures ANOVAs and Bonferroni adjustment for multiple comparisons. # $p < 0.05$, between-group difference assessed by one-way repeated-measures ANOVA; C) Areas under the curve (AUCs) of serum dipeptidyl peptidase IV (DPPIV) in HMCs and NHMCs after consuming 250 mL of milk. # p value < 0.05, HMC *vs.* NHMC between-group difference assessed by ANCOVA adjusted for baseline values. The box plots show the data distribution based on the first quartile, median and third quartile.

disposal rate (Sun et al., 2006; Vestergaard et al., 2008). Conversely, peptone sensing and/or increased osmolarity in the lumen of the small intestine as well as protein-induced postabsorptive signals resulted in the expected postprandial reduction in ghrelin and the absence of a blood glucose peak in the participants without GID (Dranse et al., 2018; Overduin et al., 2014).

We hypothesize that the slower GI transit in participants with GID was sustained by opioid agonists and caused the feeling of uncomfortable fullness over 1 h after milk consumption (Holzer, 2009). In addition, we suspect that undigested proteins/polypeptides accumulated in the gut lumen were sensed by enterochromaffin cells that acted as chemo- and mechano-sensors and slowed down the intestinal transit thus eliciting GID (Berthoud et al., 2021; Linan-Rico et al., 2016; Van Avesaat et al., 2015). These phenomena were supported by the lower proteolytic activity of the gut microbiome in participants with GID (Peled and Livney, 2021). Indeed, they exhibited a lower abundance of proteases in the gut microbiome, along with a lower abundance of *Streptococcus* spp. (*S. salivarius*) and a higher abundance of *Ruminococcus* spp. (namely, *R. bacterium* D16 and *R. lactaris)*, consistent with previous associations found between these bacteria and fecal protease activity (Carroll et al., 2013). In other words, in people with GID, undigested proteins or peptones that enter the colon are not further hydrolysed by bacterial proteases and cannot be absorbed (Freeman, 2015), contrary to what occurs in participants without GID, who experience a second plasma peptide peak 6 h after milk consumption along with higher urinary excretions of indoxyl sulfate, a marker of microbial protein fermentation (Agus et al., 2018).

Some differences in the gut microbiome of NHMCs and HMCs might be explained by differences in their habitual diets. Due to the higher intake of dairy products (including milk), the participants without GID had a higher gut level of potentially probiotic *Bifidobacterium* species, namely, *B. bifidum, B. adolescentis,* and *B. longum*, which is consistent with several studies showing that milk proteins increase the abundance of lactobacilli and bifidobacteria (Zhang et al., 2020). In addition, a tendency towards a higher intake of plant proteins over animal proteins in participants with GID was consistent with the higher gut abundance of the genus *Prevotella*, traditionally associated with agrarian diets (Gorvitovskaia et al., 2016). The higher fibre-to-protein ratio might reflect lower dietary protein metabolism of the gut microbiome, as dietary fibres can increase bowel motility, thus reducing, over a long period, the abundance of microorganisms requiring dietary proteins to survive (Diether and Willing, 2019; Korpela, 2018).

Although we enrolled healthy participants, those with GID showed specific gut microbiome signatures resembling those of gut inflammatory conditions. A low fecal abundance of the Actinobacteria phylum is also found in patients with functional abdominal bloating/distention (FABD) (Noh and Lee, 2020), and a high abundance of the *Paraprevotella* and *Prevotella* genera is a feature associated with irritable bowel syndrome (IBS) as well as FABD (Wang et al., 2019; Noh and Lee, 2020). Similarly, high abundances of *Bacteroides clarus*, *C. eutactus* and *R. lactaris* have been previously found in patients with IBS/IBD, FABD or Crohn's disease (El Mouzan et al., 2018; Rajilić-Stojanović et al., 2011), whereas low abundances of *B. adolescentis*, *B. longum*, and *D. invisus* were shown in patients with FABD or Crohn's disease compared to healthy subjects (Noh and Lee, 2020; Joossens et al., 2011; Vich Vila et al., 2018). Interestingly, participants with GID exhibited significantly higher levels of anxiety, which is in line with the co-occurrence of GID and psychosocial symptoms in patients with intestinal diseases and in those with DGBIs (Barberio et al., 2021; Mukhtar et al., 2019).

Another important finding of this study is the significant difference in fasting plasma concentrations of ten BAPs in HMC and NHMC participants, who exhibit different dairy product (including milk) intake. Three out of the ten BAPs that were lower in NHMC participants were DPPIV inhibitors, and plasma levels of these BAPs were inversely correlated with serum DPPIV activity after milk consumption. This association may be clinically relevant, as higher DPPIV activity is typical

of people suffering from metabolic diseases (Nargis and Chakrabarti, 2018), due to the involvement of this enzyme in the degradation and inactivation of numerous hormones, chemokines, growth factors, and neuropeptides implicated in the pathophysiological pathways (Mentlein, 1999). Therefore, we cannot rule out the implication of a higher DPPIV activity in the development of milk-related GID, especially given its role in degrading and inactivating endomorphin and substance-P, thus weakening analgesia pathways in the body and increasing pain perception (Guieu et al., 2006). This is a limitation of our study: we did not assess mediators of pain signalling, such as neuropeptides that could be affected by DPPIV activity. Exploration of these mediators would clarify the implication of DPPIV in the development of milk-related GID.

Another study limitation is that despite the lactulose/mannitol gut permeability test is the gold standard method for determining the small intestinal permeability, the test is not indicative of the transport of macromolecules; therefore, we cannot exclude a different intestinal permeability of bigger peptides between the two groups.

In addition, the inclusion of participants with lactose malabsorption may be considered as a study limitation because that condition affects gastrointestinal motility and food digestion (Misselwitz et al., 2019). However, the equal distribution of subjects with that characteristic between the two groups nullified the possible bias in results' interpretation that such choice might have caused. Moreover, since lactose malabsorption is very common among general population (Misselwitz et al., 2019), the inclusion of those subjects in the study may be seen as a strength as it made the two groups highly representative of the reality.

In conclusion, we demonstrated that milk-related GID in healthy people co-occurred with a slower and less efficient digestion of milk proteins in the intestinal tract. We showed that the circulating levels of milk-derived BAPs were lower in people suffering from milk-related GID than in people without GID and were concomitant with altered metabolic and hormonal responses. The less efficient protein digestion led to the accumulation of undigested proteins/oligopeptides in the intestine; this accumulation would explain the symptoms of increased gas, abdominal bloating and more frequent bowel movements. Our hypothesis is supported by the reduced proteolytic activity of the gut microbiome observed in individuals with milk-related GID as a possible result of decreases in the daily consumption of dairy products (including milk). Differences in the gut microbiome and habitual diet affected plasma levels of milk-derived BAPs and serum DPPIV activity in fasting subjects.

As a future perspective, in the era of precision medicine and personalized nutrition results of the present study advantage the definition of personalized therapies and dietetic approaches (De Filippis et al., 2018). Indeed, study results suggest that healthy people suffering from milk-related GID may benefit from proteolytic enzymes-based therapies or *ad hoc* microbiome-targeted intervention. Moreover, in personalized dietetic approach, milk-related GID may be managed by consuming milk-based foods containing pre-digested milk proteins, as recently shown by Laatikainen and colleagues (2020) in patients with DGBIs (Laatikainen et al., 2020). Finally, it could be speculated that the consumption of probiotics known to exert proteolytic activity in the gut may further mitigate both GID and anxiety in sensitive individuals. Randomized controlled trials in the target population will be crucial for assessing these hypotheses.

Data Sharing

The raw sequence reads generated in this study have been deposited in the Sequence Read Archive (SRA) of the NCBI under accession number PRJNA832737.

Other dataset supporting the current study are available from the

corresponding author on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2023.112953.

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