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Link between ultra-processed foods and drinks intake, gut microbiota and inflammation: an exploratory analysis in adult volunteers

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Abstract

Background: Ultra-processed foods and drinks (UPFD) have been acknowledged to promote metabolic disturbances and inflammation. Knowing that both events can be associated with gut microbiota alterations, this observational longitudinal analysis aimed to explore the link between UPFD intake, faecal microbiota composition and activity, and inflammatory biomarkers.

Methods: MICROBOOST-1 study included 20 adults for 3 testing days spaced at least 7 days apart. Dietary intakes were collected using three non-consecutive 24h recalls, including a weekend day, and performed the week preceding each study visit. Foods and drinks were categorized in a double-blind manner as ultra-processed following the NOVA classification. Mean energy-adjusted UPFD consumption was then divided into quartiles. Stool samples were collected at most four days before each study visit. Faecal microbiota composition was analysed using 16S-rDNA Illumina sequencing, and exhaled breath hydrogen – reflecting gut microbial fermentation - was measured. Inflammatory cytokines were quantified in saliva. Mixed models were applied accounting for multiple time points per participant. Overall UPFD, "meat, fish, and cheese" (MFC) and "sugar-sweetened beverages" (SSB) categories were investigated.

Results: In comparison with the lowest consumption quartile of UPFD categories, the highest quartile showed no change in α- and β-diversity, but a lower relative abundance of *Faecalibacterium*, *Desulfovibrio*, *Veillonella* and a higher relative abundance of *Hominimerdicola* and *Phocaeicola* for all UPFD (q-value <0.05). A decrease in *Agathobacter* and *Coprococcus* and an increase in *Actinomyces* relative abundances characterized the MFC category, whereas higher *Actinomyces* and lower *Flintibacter* relative abundances were observed for SSB category (q-value <0.05). SSB intake was also related to lower exhaled hydrogen and to higher salivary level of IL-6 (*p*-value <0.05). Total UPFD intake was linked to increased salivary IL-1β (*p*-value <0.05). This cytokine was positively associated with *Flintibacter* and *Desulfovibrio and* negatively associated with *Faecalibacterium*. In addition, *Phocaeicola*, *Desulfovibrio*, *Veillonella*, *Actinomyces* and *Coprococcus* were positively associated with IL-6, while *Desulfovibrio* and *Veillonella* were positively and *Actinomyces* and *Agathobacter* negatively related to IL-8 (*p*-value <0.05).

Conclusion: In our study, using non-invasive tools, we observed associations between the level of UPFD intake and components of the gut microbiota and inflammation.

Trial Registration: The protocol is recorded at « clinicaltrials.gov » (NCT05949411).

Keywords: ultra-processed food; gut microbiota; inflammation; exhaled breath hydrogen

Introduction

An unhealthy diet represents one of the main health risk factors, contributing to the global increase in overweight, obesity, and diet-related non-communicable diseases [1]. The current shift towards poor-quality diet is driven by the substantial availability of processed, often ready-to-eat, nutrient-unbalanced products available [2]. These features are typical of ultra-processed foods and drinks (UPFD), which are known for their excessive content of fat, saturated and trans fatty acids, added sugar, salt, and their low levels of fibre and micronutrients [2–4].

Different classification systems have been developed to identify UPFD, with NOVA being the most widely used in the literature as well as in reports from the Food and Agriculture Organization of the United Nations and World Health Organization [5,6]. According to the refined NOVA classification, UPFD include "*snacks, drinks, ready meals and many other products created mostly or entirely from substances extracted from foods or derived from food constituents with little if any intact food, which often contain flavours, colours and other additives that imitate or intensify the sensory qualities of foods or culinary preparations made from foods*" [7]. Their consumption has become a worldwide concern, reaching more than 50% of the daily energy intake in some populations [8]. In Europe, using data from 22 countries, the proportion of energy derived from UPFD varies widely, ranging from 14% to 44% across countries [9]. In Belgium, where our study was conducted, the most recent food consumption survey revealed that 36% of the diet and 50% of total energy intake were derived from ultra-processed food [10].

UPFD have been associated with deleterious health outcomes in meta-analyses of longitudinal studies [11], and following a dose-response relationship in a recent umbrella review, notably a significant higher risk of mortality, cardiovascular disease, cancer, overweight, obesity and type 2 diabetes [12]. They have also been identified as a risk factor for gastrointestinal disorders such as inflammatory bowel diseases [13–15]. UPFD encompass a broad range of foods and beverage types, leading to diverse health effects. Among the subcategories, two — namely 'animal-based products' and 'artificially and sugar-sweetened beverages' — have been significantly associated with increased risks of cancer and cardiometabolic multimorbidity, highlighting the need for further investigation into their specific health impacts [8].

At the intersection of diet and health, the gut microbiota has been proposed as a potential mediator [16,17]. Both nutritional and non-nutritional (i.e., additives and contaminants) characteristics of UPFD have been suggested to alter the intestinal barrier function, the gut microbiota composition, the short-chain fatty acids production, and to trigger inflammation [5,15,18,19]. Chronic low-grade inflammation is recognised as a common pathway leading to the adverse health effects of UPFD [20]. Evidence suggests that inflammation may be involved both upstream, as a potential mediator between UPFD and gut microbiota, and downstream, as a response to microbiota alterations.Therefore, characterising the role of the gut microbiota in the relationships between UPFD and health may help elucidate the mechanisms leading to chronic diseases.

Although recent studies have investigated the association between UPFD and the gut microbiota, the evidence remains limited as it has relied mainly on cross-sectional data or has focused on specific populations [16,21–23]. Building on these recent findings, the present study aimed to explore the associations between UPFD consumption using the NOVA score, gut microbiota composition, and the contribution of biomarkers of inflammation in a cohort of adults recruited in Belgium, using prospective repeated measurements. In addition, this study incorporated novel non-invasive markers of inflammation, and examined specific UPFD subcategories to investigate whether distinct UPFD groups exhibit differentiated relationships with gut microbiota measures, which represent a major gap in the literature [5].

Based on the existing literature, we hypothesised that higher UPFD intake would be associated with differences in gut microbiota composition and diversity, and higher levels of inflammatory biomarkers. We further hypothesised that nutritional factors and inflammation may contribute to the associations observed between UPFD intake and gut microbiota features. Accordingly, the primary objective was to study the associations between UPFD consumption and (1) gut microbiota related characteristics and composition, and (2) inflammation biomarkers. The secondary objective was to evaluate the contribution of nutritional factors and inflammation to the relationship between UPFD and the gut microbiota features.

Methods

Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology (STROBE-nut) reporting guidelines were followed for the report of the present study [24].

Study design

The present study is a post-hoc analysis of the interventional study MICROBOOST-1 (MB1), performed on the observational data collected throughout the study. Briefly, the MB1 study was composed of 3 visits, with a minimum interval of 7 days between them (median time between two study visits of 11 days, with an interquartile range of 9 to 14 days), to avoid carry-over effects on the gut microbiota between each visit. The first visit was to determine each participant's fermentative profile, whether participants were hydrogen or methane producers after lactulose ingestion, following a procedure previously described [26]. More specifically, the participants came for three visits organized minimum 7 days apart (median time between two study visits of 11 days, with an interquartile range of 9 to 14 days), for saliva and breath sampling collected in the morning in the fasting state. Observational data related to the habitual diet were collected upon three days (two in the week and one in the weekend) during the week preceding each visit. Stool samples were collected maximum 4 days prior each visit. The study protocol was approved by the institutional Ethics Committee (Comité d'Ethique Hospitalo-Facultaire UCLouvain/Cliniques Universitaires Saint-Luc, registration code 2023/13AVR/183) and all participants gave the written informed consent. The protocol is registered on ClinicalTrials.gov (NCT05949411).

Participants

A total of 20 volunteers participated in the MB1 study and were further included in the present analyses. The sample size was determined for the original intervention trial based on previous studies, from the Fibertag project, where 15 participants were sufficient to detect differences in breath volatile metabolites production following either acute or chronic ingestion of an insoluble dietary fiber (chitin-glucan), an effect that was associated with gut microbiota composition [26,27]. Inclusion criteria included (1) being aged between 18 and 65 years old, (2) having a body mass index between 18 and 30 kg/m^2, (3) not smoking, (4)

speaking French, and (5) providing informed consent to participate and comply with all study procedures. The exclusion criteria were : (1) Presence of severe systemic disease (renal or hepatic failure, cancer, pancreatic cancer, chronic pancreatitis, asthma, …); (2) History or current chronic or severe gastrointestinal disorder such as duodenal ulcer, chronic colitis, or chronic inflammatory disease of the digestive tract disease (Crohn's disease, ulcerative colitis), celiac disease or irritable bowel syndrome; (3) History of digestive tract surgery (except appendectomy) or any surgery performed within the two months prior to the study; (4) Psychiatric problems and/or the use of antipsychotics; (5) Current or recent (less than 4 weeks) intake of antibiotics, fibre supplement, and/or any products modulating gut transit (laxative, anti-diarrheal, …) or gut microbiota composition (these products were also avoided for the duration of the study); (6) Chronic medication use, except contraceptives; (7) Following a low-calorie diet and being followed by a doctor or dietician in progress or recently (less than 6 weeks); (8) Pregnancy or breastfeeding or intention to become pregnant during the study period or not using an effective method of contraception (e.g. oral contraception, IUD, abstinence, ...); (9) Drinking more than 3 glasses of alcohol per day (30 g of alcohol per day); (10) Participation in another clinical trial two weeks prior to screening; (11) Engaging in intense physical activity more than 10 hours per week; (12) Language barriers, mental or legal incapacity, or unwillingness/inability to understand and comply with the study procedures.

Data collection and treatment

Among the various data collected throughout the study period, this study will focus on (1) the dietary intake collected the week before each study visit, (2) the gut microbiota composition analysed from the stool samples collected within 4 days prior each study visit, (3) the hydrogen level in the breath and (4) the level of inflammatory cytokines from the saliva sample collected fasted in the morning of study visit (Figure 1).

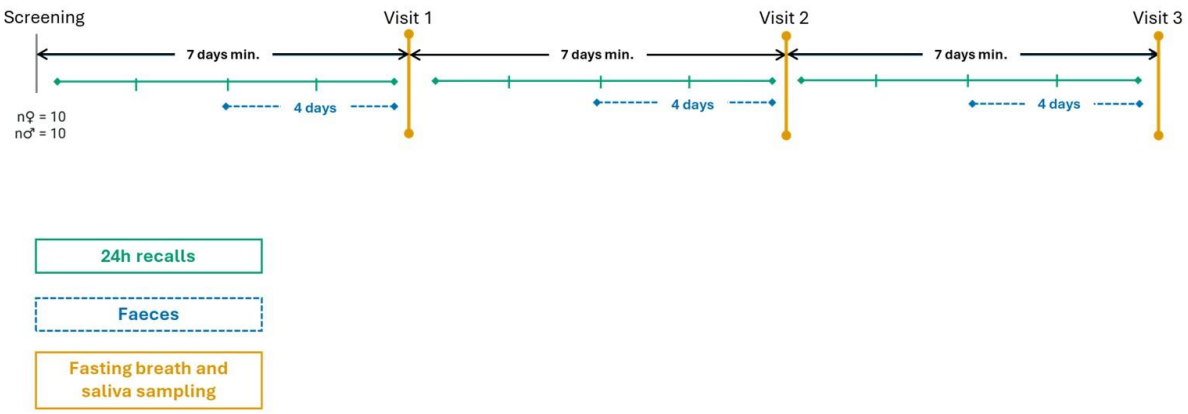

Figure 1. Study protocol and data used in the present analysis.

20 participants (10 women and 10 men) were included to take part to three visits set at least 7 days apart (median time between two study visits of 11 days, with an interquartile range of 9 to 14 days). Three 24h recalls were collected the week before each study visit for the dietary intake, stool samples were obtained maximum 4 days before the study visit for the gut microbiota composition, and the breath and salivary samples were collected in fasting state for the analysis of hydrogen and inflammatory cytokines levels, respectively.

Dietary data

The detailed dietary intake of the week prior to each study visit was assessed using a computerized self-reported 24h recall (Automated Self-Administered 24-hour Dietary Assessment Tool (ASA24®), Canadian version 2018, developed by the National Cancer Institute) on three non-consecutive days including one weekend day. The three 24h recalls collected and used for the present study did not include the day before the study visit in which specific dietary requirements were asked to the participants. This validated tool utilizes the "Automated Multiple-Pass Method", which improves the precision and completeness of dietary data collection [28,29]. Portion size estimation was enabled by photographs of an extensive range of food items or meals. Additionally, the inclusion of food items and recipes relevant and specific to the participants' dietary habits were allowed by manual entry to ensure the adaptation to the study population. Then, all entries and responses from the participants were manually reviewed to ensure relevance and accuracy. To prevent participants from anticipating and adapting their dietary intake, the recall days were kept confidential until the day of reporting their intake from the previous day.

Nutrient intake was then calculated using the Food and Nutrient Database for Dietary Studies (FNDDS) 2021-2023 (U.S. Department of Agriculture, Agricultural Research Service, 2020), offering comprehensive nutrient coverage and extensive food item catalogue. Indeed, the FNDDS includes more than 5400 food items, enabling a detailed analysis of the dietary intake. Also, as it is regularly updated, freely available, and one of the most comprehensive, it is used at the international level, allowing more consistent between-country comparison [30]. The increased level of detail and completeness was considered to outweigh the potential limitations related to regional bias.

Ultra-processed food identification and categories classification

The food or drink items collected from the 24h recalls were then classified following the NOVA score [7] depending on their processing degree into four distinct categories:

- Group 1: unprocessed or minimally processed, where no substance is added to the original food.
- Group 2: processed culinary ingredients (i.e., plant or animal fat, sugar, honey, salt).
- Group 3: processed, which include canned, pickled, smoked, cured or fermented foods from group 1.
- Group 4: ultra-processed, identifying industrial formulations following processes such as extraction and chemical modification, with additives added to increase the sensory quality of the product and comprising very small quantity of the original foods.

Complex meals were decomposed into individual food items following their standard recipe. To ensure a more robust methodology, the NOVA classification was performed on all dietary items independently by two researchers (LL and EB) and the concordance between the two was then evaluated with the Cohen's kappa coefficient at two levels: on the classification in the four categories (Cohen's kappa of 0.898 [95% confidence interval between 0.885 and 0.911]) and on the classification of the item being ultra-processed or not (Cohen's kappa of 0.862 [95% confidence interval between 0.843 and 0.882]). In both levels, the Cohen's kappa coefficient was above 0.81 indicating an almost perfect agreement between the two evaluators. The residual discordance was then examined to reach consensus, and a final score was attributed. All foods and drinks from the participants recalls were thus classified.

The dietary items classified as ultra-processed were further subdivided into 10 different categories based on our data and on pre-existing categories in the literature , with a focus on

the “Meat, fish and cheese” (MFC) and the “sugar-sweetened beverages” (SSB) categories in the present study for their higher cardio-metabolic risk [8]. The other categories were not part of the objectives of the present study, while considered in the total UPFD intake. A detailed list of all categories is provided in Supplementary Table S1.

To obtain the UPFD consumption on each study visit, the mean over the three 24h recalls the week preceding each study visit was calculated and then adjusted for total energy intake using the residual method [31]. Briefly, UPFD intake in grams were regressed against energy intake, and the resulting residuals were used in the statistical analyses. The residual method can be interpreted as the difference in the observed compared to the theoretical UPFD intake expected for an individual with similar total energy intake. Quartiles of energy-adjusted UPFD consumption were subsequently defined by pooling data from the whole study period (three distinct weeks per participant), resulting in 15 observation per quartile. This approach accounts for day-to-day variability, ensures consistent quartile classification, and prevents misclassification due to isolated high or low intake on a single day, therefore allowing for a more robust comparison of interquartile differences across the study period. The flow-chart of this process is shown in Figure 2.

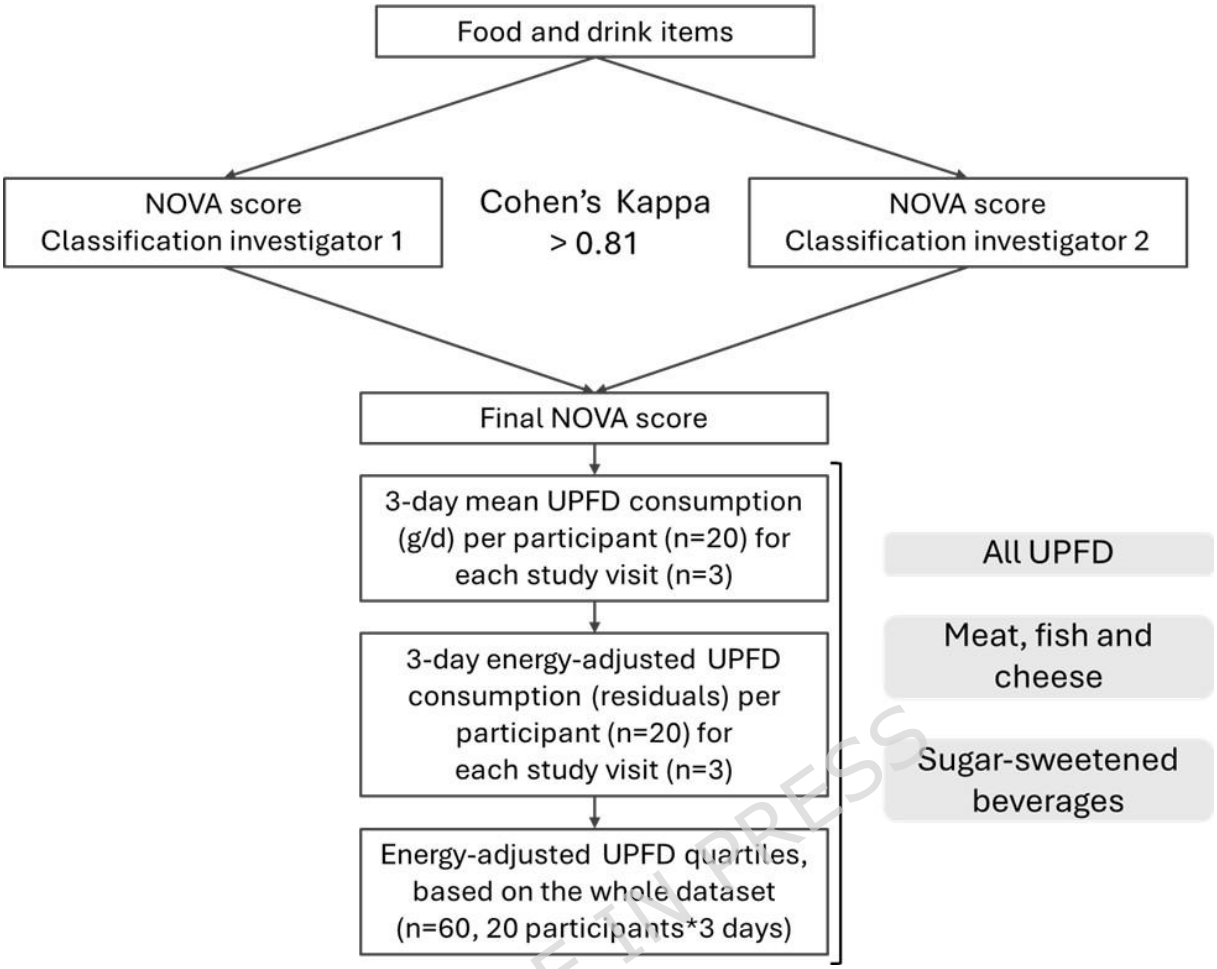

Figure 2. Flow-chart of the ultra-processed foods and drinks (UPFD) consumption calculation.

The food or drink items collected from the 24h recalls were classified following the NOVA score independently by two researchers with an almost perfect agreement (Cohen's kappa coefficient over 0.81 across the entire foods and drinks database). For each participant, the mean UPFD intake from the three 24h recalls prior to each study visit was adjusted on energy intake based on the residual method. Then, energy-adjusted UPFD intake was divided into quartiles considering date from the whole study period aggregated together.

Gut microbiome analyses

The stool samples of the participants were collected within four days before each study visit (the median number of days between stool sample collection and the study visit was 1 (interquartile range of 1–2 days)), using the stool collection tubes (Sarstedt, Germany) and following the detailed instructions given by the investigators. They were transported in isothermal boxes with gel packs to maintain frozen conditions (approximately −20°C) until

delivery to the laboratory, where they were then stored at -80°C at the UCLouvain biobank. Bacterial DNA was extracted using the QIAamp DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The gut microbiota composition was evaluated by bacterial DNA sequencing (V5-V6 region of the gene encoding 16S rRNA Illumina). All samples of this study were sequenced in the same run. Sequencing procedures, as well as bioinformatics and biostatistics analyses are detailed in the Supporting Information. Raw sequences obtained from Illumina sequencing of the 16S rRNA gene will be made public upon acceptance in the Sequence Read Archive (SRA) database.

Breath hydrogen

Exhaled breath samples were collected into bags (FMONP.SBAG1.01 from MEC R&D SPRL, Belgium) at the beginning of each study visit, while participants were fasting and after rinsing their mouths with water. Hydrogen concentration in the breath was measured using the Lactotest 202 (Medical Electronic Construction, MEC). To account for variability in exhaled air volume, the hydrogen level was adjusted on the CO_2 concentration (measured at the same time and by the same tool than hydrogen) following the formula: H_2 (ppm) × (5% / CO_2%).

Salivary inflammatory parameters

The level of inflammation was assessed through the measure of inflammatory cytokines in saliva as a non-invasive tool to reflect serum concentrations [44]. In fasted participants and after they had rinsed their mouths with water, saliva samples were collected using a sterile foam swab placed in the participant's mouth for approximately 2 min without mastication. If salivary flow was insufficient, the swab was retained longer to ensure sufficient sample volume. After collection, the samples were immediately placed on ice and centrifuged (1000g, 2 min, 4°C) to extract the saliva. The supernatant was aliquoted and stored at –80 °C at the UCLouvain biobank.

Interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-1β (IL-1β) were quantified using a multiplex immunoassay (custom U-PLEX multiplex assay, Meso Scale Discovery, Maryland, USA). After thawing, the samples were centrifuged briefly and analyzed according to the

manufacturer protocol. Of note, salivary samples were available for 19 of the 20 participants, as one participant did not provide enough saliva during collection, rendering the sample unusable. This sample was thus discarder from the analyses about inflammation markers.

Statistical analysis

The normality of the variables was checked following the Shapiro-Wilk test. As none of the variables followed a Gaussian distribution, results are expressed as median and interquartile range [Percentile 25 – Percentile 75].

The variability across study visits for UPFD consumption, relative-abundances (%) of genera, and inflammation levels through IL6, IL-8 and IL-1β was investigated applying a non-parametric analysis of variance using the Extended Mantel-Haenszel (EMH) test with rank transformation. This non-parametric method, conceptually related to the Friedman test, allows for stratification by participant and evaluates day-to-day variability using ranked data in a repeated measures framework.

The association between the UPFD consumption quartiles, as the independent variable, and the nutrient intake, the faecal microbiota related characteristics (i.e., exhaled hydrogen, α-diversity, and β-diversity) and composition (i.e., genus-specific relative abundances (%)), and the inflammation level, as dependant variables, was explored through mixed-effects models with random effects specified for study visit nested within participants, accounting for repeated measures. Residual normality was assessed using the Shapiro–Wilk test, and heteroskedasticity using the Breusch–Pagan/Cook–Weisberg test. To account for non-normality and heteroscedasticity, we used robust maximum likelihood method of estimation, which provides reliable estimates under violations of distributional assumptions. An autoregressive correlation structure of order 1 (AR1) was specified for the residuals to account for temporal autocorrelation across repeated study visits within participants, as correlation between observations was expected to decrease with increasing time lag. Quartiles of UPFD consumption were modelled as categorical variables, with the lowest quartile (Q1) used as the reference category. The models were adjusted for total energy intake and underreporting. The identification of inaccurate diet reports was performed

following the McCrory method [45]. No overreporting was identified and a total of 8% of data was classified as underreported.

The significant associations from the mixed models examining the relationship between UPFD consumption quartiles and faecal microbiota composition were visualised using Estimated Marginal Means (EMM). EMMs represent the model-adjusted means for each level of the studied factor (i.e., UPFD quartiles), considering the covariates from the models. EMMs for UPFD consumption quartiles were computed using Stata's margins command, with robust standard errors adjusted for clustering by participant. Results were then exported and visualized in R using the ggplot2 package (version 3.5.2) [46]. Of note, the values from the EMMs are intended for graphical representation and do not fully reflect the inferential results of the mixed models.

To explore further the influence of diet intake on the relationship between UPFD and gut microbiota, mixed models were applied to genera previously identified as significantly associated with UPFD, and were additionally adjusted for nutrients that were also significantly linked to UPFD. To investigate the influence of inflammation, mixed models were performed to assess the associations between genera significantly associated with UPFD consumption and inflammatory cytokines, adjusting for UPFD intake, total energy intake and underreporting. When inflammation was considered as a potential mediator between UPFD consumption and gut microbiota, only the cytokines significantly associated with UPFD were included in the analysis. When inflammation was considered as the outcome, all inflammatory cytokines and genera significantly associated with UPFD were included, depending on the UPFD category.

All the statistical analyses were performed on all UPFD, on the MFC and on the SSB categories. For descriptive visualisations (including heatmap) as well as for the EMH test and mixed-effects models, genus-level relative abundances (%) were used without additional scaling or transformation, as our aim was to illustrate within-individual variation.

Statistical significance was set at p-values lower than 0.05.Because the models exploring the association between UPFD consumption and its subcategories and faecal microbiota composition at the genus-level served as the basis for selecting genera to be included in subsequent analyses, only for these were q-values inferior to 0.05 considered significant after false discovery rate correction for multiple testing following the Benjamini-Hochberg

method [47]. . The non-parametric analysis of variance and the mixed-effects models were conducted using the Stata Software package (StataCorp LLC, College Station, Texas, USA) version 18.5 and the Beta-diversity analyses and the visual representations (PCA, EMM) were performed in the R software version 4.5.1.

Results

A total of 20 participants were included in the MB1 study, comprising 10 women and 10 men, and all were hydrogen-producers. The participants were aged between 19 and 26 years old (median of 23.5 years old) and their body mass index was between 19.3 and 28.4 kg/m² (median of 22.2 kg/m²).

Of all the food items, 30% were classified as ultra-processed with a NOVA score of four. Of these, 32% were SSB and 12% were in the MFC category (Figure 3). The median consumption of UPFD across the three study visits was not different in terms of energy, grams or energy-adjusted residuals (*p*-value >0.05) (Table S2).

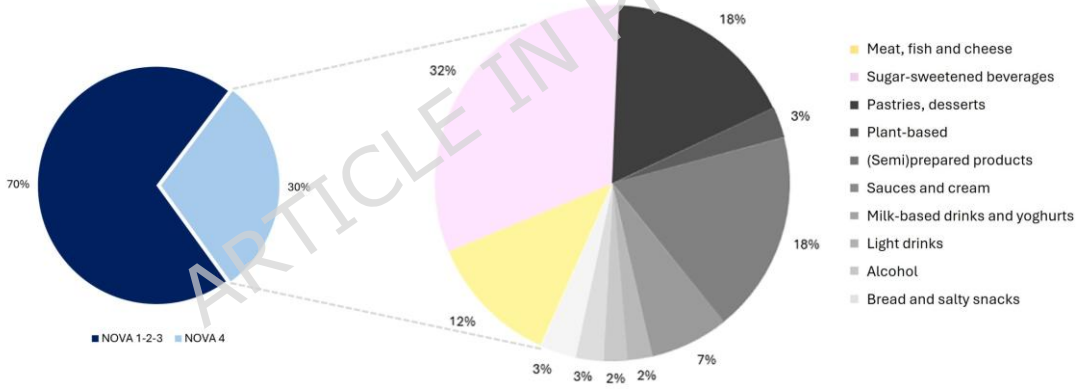

Figure 3. Identification of ultra-processed foods and drinks (UPFD) and repartition into the different subcategories.

Out of the 30% of the items classified as ultra-processed according to the NOVA score 4, 32% were sugar-sweetened beverages and 12% belonged to “the meat, fish and cheese” category.

Nutrient intake was then compared across quartiles of UPFD consumption. After adjusting for total energy intake using the residual method, the highest quartile of total UPFD consumption was associated with lower fibre intake when compared with the lowest

quartile. A significantly higher sodium intake was also observed in quartile 3 compared with quartile 1. An increased SSB intake was significantly related to higher sugar intake for quartile 4, a higher lipid intake for quartile 2, and a lower carbohydrates intake for quartile 2, when compared to quartile 1. For the MFC category, using quartile 1 as reference, an increased consumption was significantly associated with lower fibre intake (quartiles 3 and 4), lower carbohydrate intake (quartile 4), and higher protein intake (quartiles 2 and 4) (Table 1).

Table 1. Associations between UPFD and nutrient intake.

		All UPFD		Sugar-sweetened beverages		Meat, fish, cheese	
	Energy-adjusted intake quartiles	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*
Energy total (kcal)	1 (reference)	1776.8 [1589.9-2416.7]	/	2316.7 [1792.8-2570.1]	/	2082.6 [1589.1-2416.7]	/
	2	2198.2 [1677.8-2614.2]	117.4	1624.0 [1188.2-1696.5]	-231.8	1708.3 [1176.7-2215.2]	-141.5
	3	1785.6 [1642.3-2120.9]	-177.9	2035.8 [1708.3-2199.8]	-86.7	2015.7 [1792.8-2487.1]	35.8
	4	2089.5 [1899.9-2316.7]	73.91	2082.6 [1739.7-2265.9]	-114	1986.9 [1589.3-2214.5]	-169.5
Fibre (g/day)	1 (reference)	15.6 [11.9-17.5]	/	17.4 [14.5-20.3]	/	16.3 [14.5-19.8]	/
	2	15.9 [12.3-22.2]	-0.6	12.8 [9.0-15.8]	-2.6	14.5 [10.0-15.9]	-1.5
	3	15.7 [10.2-18.6]	-1.1	15.8 [10.0-19.3]	-1.6	17.0 [12.3-19.1]	**-2.7***
	4	14.9 [9.9-17.4]	**-2.8***	14.9 [12.4-17.4]	-2.2	12.4 [9.0-17.4]	**-3.5***
Carbohydrate (g/day)	1 (reference)	177.5 [147.3-234.8]	/	224.7 [183.6-281.7]	/	224.7 [151.0-257.1]	/
	2	217.3 [162.6-315.7]	11.2	150.4 [118.7-164.1]	**-20.5***	164.1 [132.4-241.7]	-17.2
	3	178.7 [140.6-222.2]	4.8	207.1 [140.6-236.4]	-8.5	228.2 [183.6-262.3]	-10
	4	235.5 [170.7-257.1]	12.9	235.5 [170.7-251.2]	7.5	170.7 [150.4-242.1]	**-21.0***
Protein (g/day)	1 (reference)	71.4 [62.6-87.6]	/	87.6 [65.3-111.0]	/	71.2 [54.2-85.2]	/
	2	81.9 [65.4-111.0]	1.9	71.2 [45.0-77.5]	-0.8	75.0 [63.9-101.4]	**16.5***
	3	82.6 [64.6-99.6]	2.1	85.2 [66.1-109.3]	1.4	82.8 [62.6-99.6]	1.7
	4	79.6 [57.3-98.9]	-3.8	79.8 [57.3-98.3]	-3.2	82.6 [75.1-119.2]	**20.5***
Lipid (g/day)	1 (reference)	77.1 [68.9-95.1]	/	95.1 [75.2-110.0]	/	80.5 [76.6-97.8]	/
	2	96.7 [77.2-111.1]	-1.6	77.1 [68.9-78.5]	**9.1***	76.5 [64.4-89.3]	2
	3	80.5 [68.5-94.5]	3.2	89.3 [68.5-105.5]	5.7	95.1 [84.0-110.0]	6
	4	87.6 [79.4-97.8]	1.1	87.5 [76.5-94.5]	1.7	83.5 [64.4-105.5]	0.7
Saturated fatty acids (g/day)	1 (reference)	26.8 [20.5-32.9]	/	31.9 [26.8-37.0]	/	30.2 [22.0-37.0]	/
	2	32.9 [29.2-41.2]	0.4	24.9 [18.8-30.8]	4.1	27.8 [18.8-35.5]	1.9
	3	28.6 [22.8-35.5]	1.1	33.3 [22.8-41.2]	2.4	32.9 [29.9-36.0]	3.4
	4	34.1 [27.2-38.5]	0.1	30.2 [27.2-36.3]	-0.03	29.2 [24.1-36.3]	1.7
Sugar (g/day)	1 (reference)	40.4 [32.1-48.0]	/	64.8 [45.3-117.5]	/	85.4 [48.0-115.0]	/
	2	64.8 [41.9-118.7]	3.9	40.4 [20.2-48.0]	-7.1	44.7 [32.1-82.2]	-12.1
	3	50.7 [44.3-82.2]	7.3	65.8 [44.3-104.2]	13.3	84.5 [45.9-117.5]	-11.5
	4	92.3 [72.6-115.0]	16.9	92.3 [50.7-115.0]	**21.1***	46.3 [33.9-88.4]	-14.4
Sodium (mg/day)	1 (reference)	2965.7 [2608.1-3456.3]	/	3348.7 [2871.7-4082.1]	/	3008.0 [2349.0-3366.9]	/
	2	3252.6 [2396.7-4174.3]	171.5	2825.7 [2223.3-3173.2]	25.2	2825.7 [2223.3-3175.7]	-80.5
	3	3220.3 [2809.3-3825.9]	**398.6***	3153.3 [2608.1-3825.9]	-88	3303.9 [2815.8-3896.1]	47.2
	4	3089.8 [2349.0-3630.2]	4.7	3141.3 [2349.0-3630.2]	-4.9	3333.4 [2703.6-4082.1]	289.2

***P-values <0.05.** Mixed-effects models adjusted for energy intake and underreporting. The coefficients are the estimated effect of UPFD consumption quartiles (quartile 1 as reference) on the outcome. Positive values indicate an increase compared to the reference quartile, while negative values indicate a decrease. P, Percentile; UPFD, Ultra-processed foods and drinks.

UPFD and gut microbiota characteristics

From the different gut-microbiota related characteristics (Table 2), including the exhaled hydrogen, the α-diversity indexes and β-diversity, only the exhaled hydrogen was significantly associated with the adjusted-consumption of SSB. A lower hydrogen level in the breath was observed in the highest consumption quartile of SSB versus the lowest. None of the α-diversity indices and β-diversity were significantly associated with UPFD in either category. Regarding β-diversity assessed at the genus level, total UPFD and the MFC category

explained each 7% of the variance in the gut microbiota composition between the quartiles of consumption, and 4% was explained by SSB (pseudo-R^2 from the Adonis test, *p*-value>0.05). These results were illustrated using PCA plots that were explored until components 3 (9% variance explained) and 4 (8% of variance explained). In all components, no clear separation between quartiles was observed (Supplementary Figure S1, only components 1 and 2 shown).

Table 2. Gut microbiota-related characteristics according to energy-adjusted intake quartiles of the different UPFD categories.

		All UPFD		Sugar-sweetened beverages		Meat, fish, cheese	
	Energy-adjusted intake quartiles	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*
Exhaled hydrogen (ppm)	1 (reference)	6.25 [4.00-14.55]	/	14.00 [4.24-20.63]	/	5.66 [3.92-11.11]	/
	2	5.93 [2.63-13.89]	3.7	7.50 [2.77-16.07]	-8.6	11.11 [5.26-16.36]	4.7
	3	8.82 [4.46-15.45]	6.8	5.26 [3.92-10.00]	-9.5	7.50 [2.90-14.17]	2.1
	4	8.04 [4.55-15.52]	5.0	5.88 [3.51-11.11]	**-11.0***	7.50 [3.13-16.07]	-1.7
Heip's Evenness Index	1 (reference)	0.06 [0.06-0.08]	/	0.07 [0.06-0.08]	/	0.07 [0.06-0.08]	/
	2	0.07 [0.06-0.08]	-0.001	0.06 [0.05-0.07]	-0.0003	0.06 [0.06-0.07]	-0.001
	3	0.06 [0.05-0.08]	0.0001	0.06 [0.06-0.07]	-0.002	0.07 [0.06-0.08]	-0.002
	4	0.07 [0.06-0.08]	-0.001	0.07 [0.06-0.08]	-0.002	0.07 [0.06-0.08]	-0.003
Observed ASV	1 (reference)	2305 [1821-2399]	/	1944 [1815-2382]	/	2012 [1913-2329]	/
	2	2311 [2012-2424]	-40.0	2329 [2085-2424]	62.4	2228 [1974-2352]	-50.0
	3	2090 [1888-2331]	-5.8	2090 [1913-2314]	-34.5	2106 [1860-2456]	-17.6
	4	1994 [1924-2249]	-90.1	2220 [1959-2333]	39.1	2290 [1994-2333]	13.4
Pielou Index	1 (reference)	0.64 [0.63-0.66]	/	0.66 [0.64-0.67]	/	0.65 [0.64-0.66]	/
	2	0.65 [0.63-0.66]	-0.003	0.64 [0.63-0.66]	0.001	0.64 [0.62-0.66]	-0.003
	3	0.64 [0.62-0.67]	-0.002	0.63 [0.62-0.66]	-0.005	0.65 [0.63-0.66]	-0.005
	4	0.65 [0.64-0.66]	-0.004	0.65 [0.63-0.67]	-0.005	0.65 [0.63-0.67]	-0.005
Shannon diversity index	1 (reference)	7.14 [6.84-7.32]	/	7.18 [6.92-7.32]	/	7.17 [7.05-7.24]	/
	2	7.18 [6.99-7.41]	-0.1	7.14 [6.99-7.41]	0.05	7.11 [6.84-7.31]	-0.1
	3	7.05 [6.75-7.44]	-0.03	6.95 [6.83-7.35]	-0.1	7.18 [6.83-7.32]	-0.1
	4	7.12 [7.01-7.31]	-0.1	7.16 [7.01-7.44]	-0.03	7.22 [6.91-7.50]	-0.04
Simpson Index	1 (reference)	0.98 [0.97-0.98]	/	0.98 [0.98-0.99]	/	0.98 [0.98-0.99]	/
	2	0.98 [0.97-0.99]	-0.003	0.98 [0.97-0.98]	-0.002	0.98 [0.97-0.98]	-0.004
	3	0.98 [0.97-0.99]	0.001	0.98 [0.97-0.98]	-0.003	0.98 [0.97-0.99]	-0.002
	4	0.98 [0.98-0.99]	-0.0003	0.98 [0.97-0.99]	-0.003	0.98 [0.97-0.99]	-0.004
Simpson's Evenness	1 (reference)	0.02 [0.02-0.03]	/	0.03 [0.02-0.04]	/	0.03 [0.02-0.04]	/
	2	0.03 [0.01-0.03]	-0.0005	0.02 [0.01-0.03]	-0.001	0.02 [0.01-0.03]	-0.002
	3	0.02 [0.02-0.03]	0.003	0.02 [0.02-0.03]	-0.002	0.03 [0.02-0.03]	-0.001
	4	0.03 [0.03-0.03]	0.0002	0.03 [0.02-0.03]	-0.001	0.03 [0.02-0.03]	-0.004
Beta-diversity (genus)	Anosim R	0.03		-0.03		0.02	
	Adonis – Model	0.07		0.04		0.07	

***P*-values <0.05**. Mixed-effects models (except Beta-diversity) adjusted for total energy intake and underreporting. The coefficients are the estimated effect of UPFD consumption quartiles (quartile 1 as reference) on the outcome. Positive values indicate an increase compared to the reference quartile, while negative values indicate a decrease. P, Percentile; UPFD, Ultra-processed foods and drinks.

When examining the genus level, *Faecalibacterium* was the most abundant with a relative abundance between 8.7 and 7.9%, whereas *Desulfovibrio* was the least abundant with a relative abundance between 0.0005 and 0.0007% across all study visits. Several genera varied significantly between study visits, such as *Phascolarctobacterium*, *Vescimonas*,

Adlercreutzia, *Streptococcus*, *Segatella*, *Faecalibaculum*, *Flavonifractor*, and *Victivallis* (Figure 4).

Figure 4. Median relative abundance (%) of the genera between study visits.

*Significant difference (*p*-values <0.05) between days using the Extended Mantel-Haenszel test with rank transformation.

The associations between all genera and UPFD categories are presented in Supplementary Table S3. Comparing to the lowest energy-adjusted consumption quartile, at the q-value level, significant associations were observed for *Faecalibacterium* and *Hominimerdicola* in the third and fourth quartiles of total UPFD category. *Phocaeicola*, *Desulfovibrio*, and *Veillonella* were significantly associated in the fourth quartile, whereas *Ruminococcoides*, *Hydrogeniiclostridium*, and *Paraprevotella* were associated only in the second or the third quartiles. *Faecalibacterium*, *Desulfovibrio*, *Paraprevotella*, and *Veillonella* showed a decreased relative abundance, while *Phocaeicola*, *Hominimerdicola*, *Ruminococcoides*, and *Hydrogeniiclostridium* showed increased relative abundance (Figure 5A).

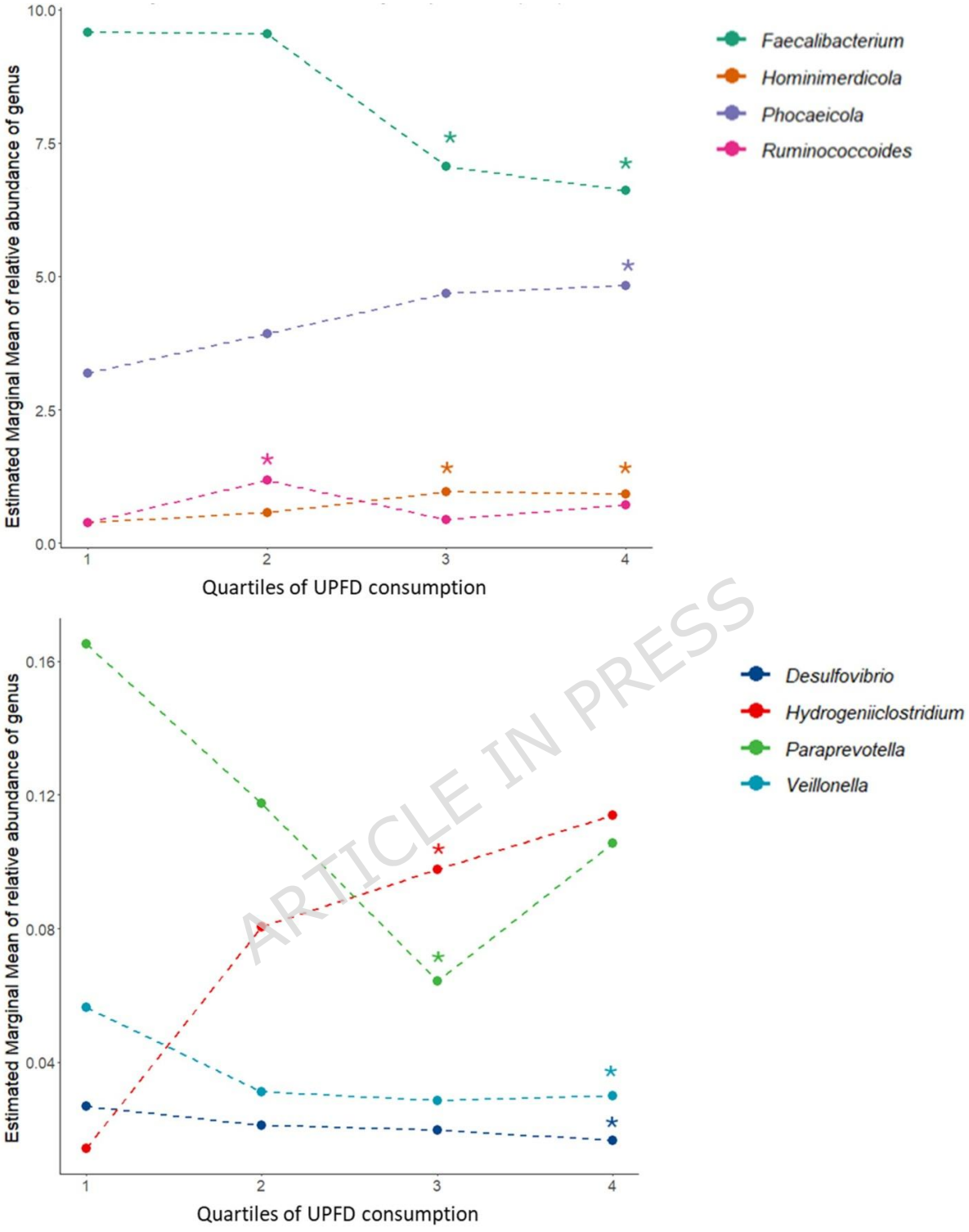

Figure 5A. Estimated Marginal Means of genera's relative abundance by ultra-processed foods and drinks (UPFD) consumption quartiles.

*q-values from the main mixed models <0.05, quartile 1 used as reference. Only the significant adjusted associations from the mixed-effects models are visualized. The Estimated Marginal Means of genera's relative abundance corresponds to the model-adjusted means for each UPFD consumption quartile, after accounting for the covariates included in the mixed models.

The highest consumption quartile of SSB was significantly associated with an increased and a decreased relative abundance of *Actinomyces* and *Flintibacter*, respectively, compared to the lowest quartile. In addition, *Agathobaculum*, *Akkermansia*, and *Streptococcus* were significantly increased in the third quartile of SSB intake versus the first quartile (Figure 5B).

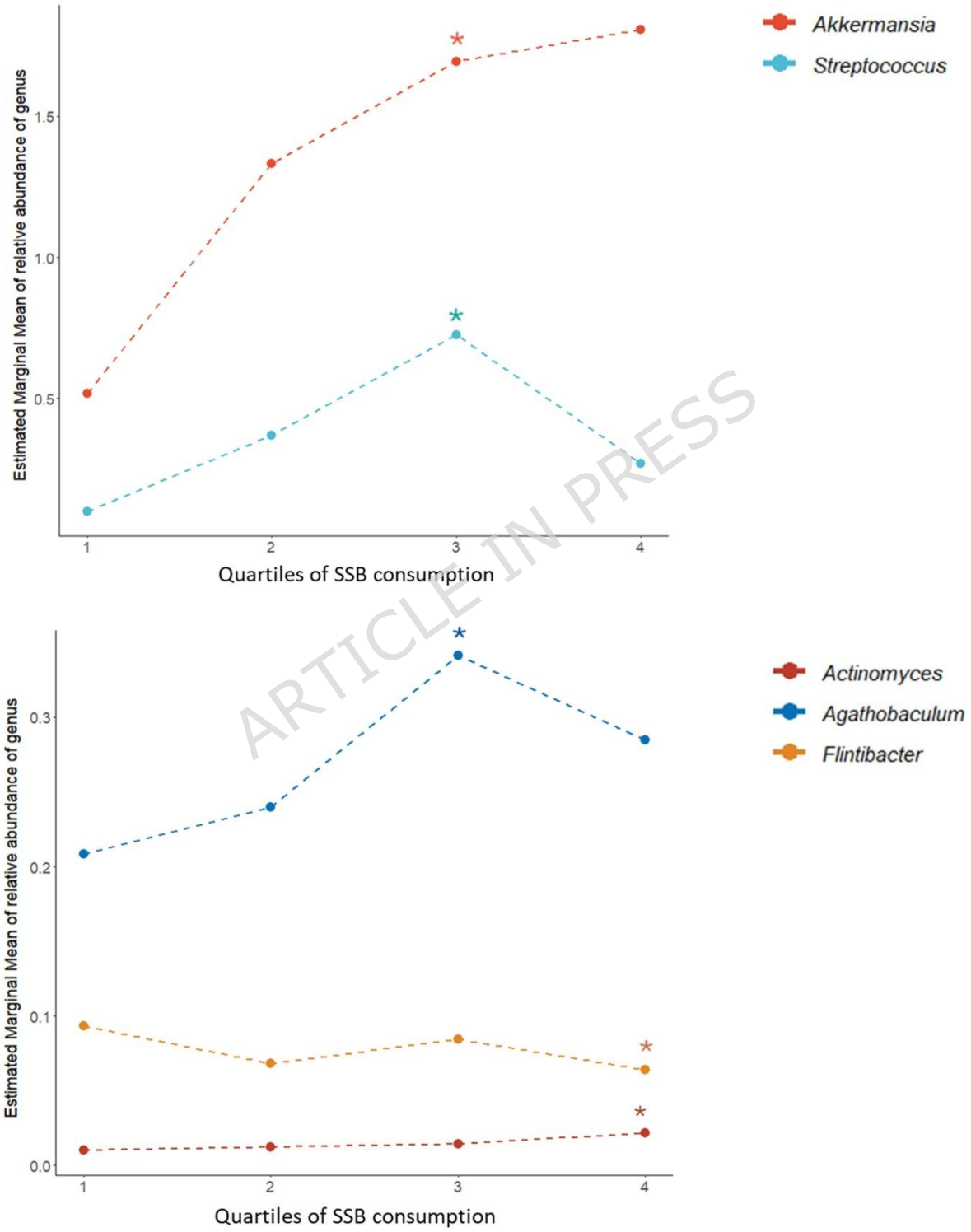

Figure 5B. Estimated Marginal Means of genera's relative abundance by sugar-sweetened beverages (SSB) consumption quartiles.

*q-values from the main mixed models <0.05, quartile 1 used as reference. Only the significant adjusted associations from the mixed-effects models are visualized. The Estimated Marginal Means of genera's relative abundance corresponds to the model-adjusted means for each SSB consumption quartile, after accounting for the covariates included in the mixed models.

Regarding the MFC category, the highest consumption quartile was significantly associated with an increased relative abundance of *Actinomyces* and a decreased relative abundance of *Coprococcus* and *Agathobacter* (also significant at quartiles 2 and 3*)*, whereas *Escherichia/Shigella* was significantly increased at quartiles 2 and 3, and *Flintibacter* decreased at quartile 3as compared to the lowest energy-adjusted quartile of consumption (Figure 5C).

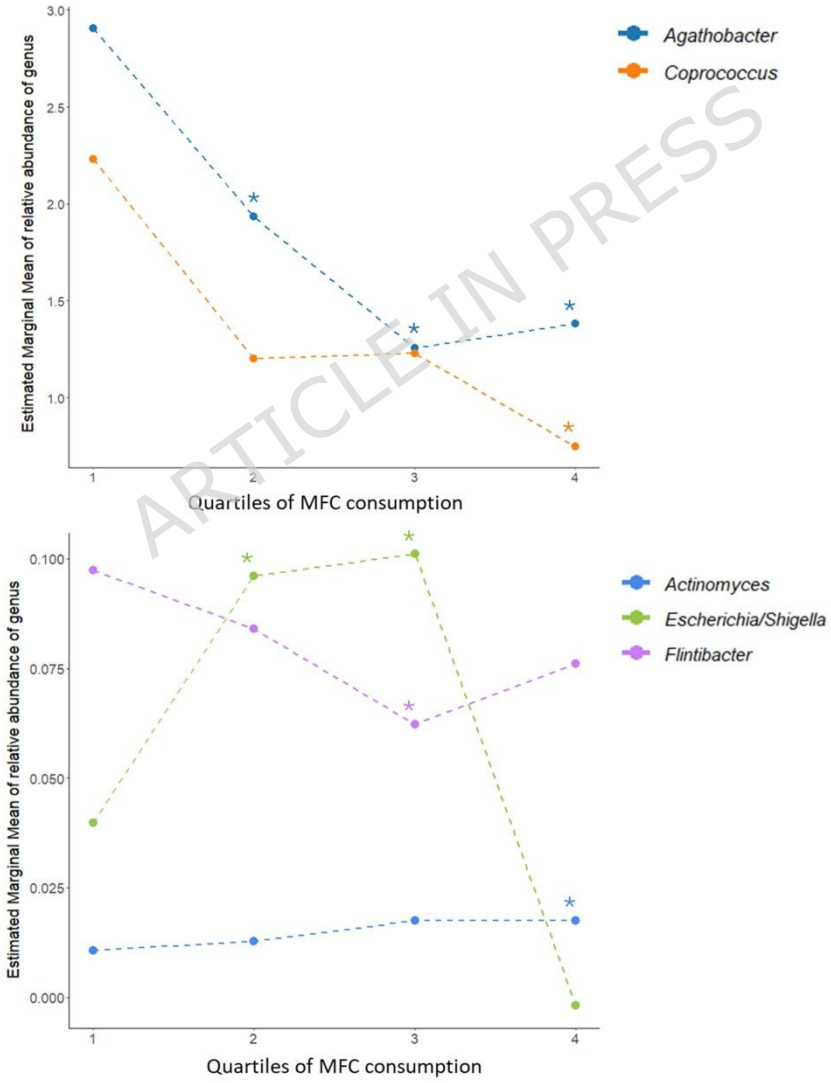

Figure 5C. Estimated Marginal Means of genera's relative abundance by "Meat, fish, cheese" (MFC) consumption quartiles.

*q-values from the main mixed models <0.05, quartile 1 used as reference. Only the significant adjusted associations from the mixed-effects models are visualized. The Estimated Marginal Means of genera's relative abundance corresponds to the model-adjusted means for each MFC consumption quartile, after accounting for the covariates included in the mixed models.

To assess whether nutrient intake mediated the association between UPFD and gut microbiota, the significant mixed models assessing the associations between UPFD categories and the genera at the p-values level were further adjusted for nutrients significantly associated with UPFD consumption (Supplementary Tables S4A, S4B and S4C). The results remained similar at the highest quartile consumption, except for the genus *Desulfovibrio* for which the association with all UPFD was no longer significant after adjustment for fibre intake, and *Hominimerdicola* for the all UPFD model adjusted for sodium intake, suggesting that these nutrients may have mediated the association between UPFD and these genera. For SSB consumption, the highest quartile was no longer significantly associated with *Streptococcus* when adjusted for sugar intake.

Link between inflammation, faecal microbiota and UPFD

Salivary inflammatory cytokines were analysed in participants with available saliva samples at different time points during the study period (19 out of 20 participants). The inflammatory cytokines in saliva did not vary significantly between study visits (Table S5). Compared to quartile 1, quartile 2 of all UPFD and quartiles 2 and 4 of SSB were significantly associated with IL-6, and quartiles 2 and 4 of all UPFD and quartile 3 of SSB with IL1-β. No significant association was observed between the MFC category and any inflammatory cytokines (Table 3).

Table 3. Inflammatory cytokines in saliva according to energy-adjusted intake quartiles of the different UPFD categories (n=19).

		All UPFD		Sugar-sweetened beverages		Meat, fish, cheese	
	Energy-adjusted intake quartiles	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*	*Median [P25 – P75]*	*Coefficient*
IL-6 (pg/ml)	1 (reference)	0.8 [0.7 – 1.2]	/	0.9 [0.7 – 1.0]	/	1.0 [0.7 – 1.4]	/
	2	0.9 [0.7 – 1.0]	**-0.3***	0.8 [0.7 – 1.3]	**0.7***	0.8 [0.7 – 1.2]	0.3
	3	0.9 [0.5 – 1.1]	-0.3	0.9 [0.7 – 1.0]	0.1	0.8 [0.6 – 1.0]	-0.1
	4	1.1 [0.8 – 1.4]	-0.02	1.0 [0.8 – 1.4]	**0.6***	1.0 [0.7 – 1.3]	0.3
IL-8 (pg/ml)	1 (reference)	32.8 [21.6 – 60.2]	/	14.9 [5.3 – 42.4]	/	21.9 [14.9 – 31.6]	/
	2	9.1 [5.0 – 29.6]	-8.9	31.4 [9.1 – 39.4]	12.8	31.2 [9.1 – 39.4]	4.2
	3	18.8 [11.3 – 29.6]	-1.7	21.6 [16.6 – 31.6]	-4.8	11.4 [5.3 – 33.2]	-0.4
	4	15.8 [9.7 – 36.6]	-10.2	15.0 [7.8 – 34.4]	0.9	18.8 [11.3 – 36.6]	3.1
IL1-β (pg/ml)	1 (reference)	47.6 [23.0 – 82.4]	/	23.4 [10.9 – 46.2]	/	33.6 [22.0 – 53.4]	/
	2	23.7 [15.5 – 45.2]	**-15.1***	47.0 [15.9 – 59.2]	4.2	43.2 [24.6 – 68.4]	-6.3
	3	37.0 [10.9 – 59.2]	-7.9	43.4 [35.6 – 70.0]	**15.0***	34.8 [15.5 – 47.0]	-14.2
	4	28.4 [22.0 – 36.6]	**-15.8***	28.0 [24.2 – 31.0]	0.6	24.2 [13.9 – 41.0]	-10.8

***P-values <0.05**. Mixed-effects models adjusted for total energy intake and underreporting. The coefficients are the estimated effect of UPFD consumption quartiles (quartile 1 as reference) on the outcome. Positive values indicate an increase compared to the reference quartile, while negative values indicate a decrease. P, Percentile; UPFD, Ultra-processed foods and drinks.

Among cytokines significantly associated with the highest consumption quartile (in comparison to the first quartile), IL-6 was significantly and positively associated with *Actinomyces* after adjusting for SSB intake. IL-1β was negatively associated with *Faecalibacterium* and positively with *Desulfovibrio* after adjusting for total UPFD intake (Supplementary Table S6).

When inflammatory cytokines were considered as dependant variables (Supplementary Table S5), IL-6 was significantly positively associated with *Phocaeicola*, *Desulfovibrio*, *Veillonella* after adjusting for UPFD intake, and with *Actinomyces* and *Coprococcus* after adjustment for MFC intake. IL-8 was positively associated with *Desulfovibrio* and *Veillonella* when considering total UPFD, and negatively associated with *Actinomyces* and *Agathobacter* in the MFC subcategory. Finally, IL-1β was negatively related to *Faecalibacterium* and positively to *Flintibacter* after adjusting for the total UPFD or the SBB consumption, respectively.

Discussion

The aim was to explore the relationships between UPFD consumption, gut microbiota, and inflammation in healthy volunteers. Regarding gut microbiota, none of the three categories was associated with changes in α or β- diversity, and only SSB category was significantly associated with lower exhaled hydrogen in quartiles 4 compared to 1. Specific genera were also linked to UPFD intake in the different categories, and these associations remained after adjustment for the significant UPFD-associated nutrients. Inflammation appeared to play a role at different levels in the relationship between the UPFD categories and gut microbiota.

In the studied sample including healthy volunteers with a mean age of 22 years, UPFD accounted for 30% of all the reported food items consumed, which is slightly lower than the proportion observed at the Belgian population level, where ultra-processed represents 36% of the total food intake considering children, adolescents and adults [10]. Throughout the study period, the total UPFD intake did not significantly vary, either in absolute terms or when adjusted for energy intake values. The main contributor was SSB, accounting for 32% of all UPFD in the present study. This finding is consistent with the European data in adults [9].

The nutritional composition of UPFD is described in the literature as being low in fibre, energy-dense, and high in sodium, SFA, and sugars [2]. Among these characteristics, only a decrease in fibre intake was significantly associated with the highest consumption of all UPFD and MFC category, and an increased intake of sugars for the SSB category. Interestingly, although a downward trend in fibre intake was also observed for SSB, it was not statistically significant, in contrast to the results obtained for the other categories. The lack of a marked effect on fibre intake could be explained by the specific nature of the sugary drinks, which, unlike other categories of solid UPF, have a limited impact on satiety and can even decrease it [48]. They provide no fibres, nor a feeling of fullness, and therefore do not appear to reduce the consumption of other foods potentially rich in fibres. Their nutritional impact may thus be limited mainly to increasing sugar intake, without significantly influencing other components of the diet. Higher protein and lower carbohydrate intake were observed in the highest quartile of MFC consumption compared with the lowest quartile, which is consistent with the nutritional characteristics of these

products. Some nutrients showed significant associations with some UPFD categories, such as sodium in quartile 3 for all UPFD or carbohydrates and lipids in quartile 2 only for SSB. Since these associations did not persist for higher intake levels (in quartile 4), this suggests random or transient variations in the distribution of food intake, rather than a consistent link to the consumption of UPFD.

In terms of gut microbiota characteristics, lack of significant α-diversity differences is coherent considering the stable UPFD intake across study visits. . For β-diversity, although the associations were not statistically significant, a modest R^2 of 7 and 4% were observed for the models. Notably, the proportion of the variance explained by diet in our models is higher than what has been reported [16,49]. For comparison, a meta-analysis investigating diet more broadly showed that the increase of fibre intake explained on average 1.5% of the variance observed in the microbiota composition [49]. To compare with results on UPFD specifically, one-year UPFD consumption accounted for less than 1% in the differences in faecal microbiota composition [16]. Together with the lack of statistical significance, these findings may indicate high inter-individual faecal microbiota variability across and within the UPFD consumption quartiles, a more diffuse effect of UPFD on different taxon, or an indirect effect after other potential parameters including environmental, genetic, or metabolic factors.

Breath hydrogen, a functional marker of the gut microbiota fermentation activity [50], was significantly associated with SSB consumption. A lower exhaled hydrogen level related to increased intake of sugar may appear counterintuitive if one considers acute sugar malabsorption episodes. However, in our study, the daily sugar intake reported in each SSB quartile reflects the mean consumption over the week. It is therefore unlikely that large amounts of sugars reached the gut microbiota at once to be fermented. This supports the interpretation that the relationship between SSB consumption and exhaled hydrogen is more likely indirect, rather than a direct fermentative effect of SSB. SSB are sugar-dense, with a high glycaemic index and these nutritional characteristics have been shown to trigger a low-grade inflammation [51]. Consistently, higher SSB intake was associated with higher IL-6 levels in our results. Interestingly, low levels of hydrogen in breath have been identified in the literature as a marker of metabolic dysfunction and oxidative stress [52] and have been related to post-prandial glycemia dysregulation [50]. Furthermore, inflammation has been

linked to alterations in the gut microbiota functions, change in composition, and reduced short-chain fatty acid levels [17,51].

The potential role of inflammation can be supported in our data by a higher relative abundance of *Actinomyces* related to SSB consumption, which has already been pointed out in a pro-inflammatory state [53]. Furthermore, increased IL-6 associated with higher consumption of SSB was related to higher relative abundance of *Actinomyces*, when considered as the independent variable. Also, a significant decrease in relative abundance of *Flintibacter* was observed in the SSB category, which is a butyrate-producer bacteria. Low levels of butyrate have been linked to inflammation [54] and inflammation-related diseases such as inflammatory bowel disease, and its production has been observed to be affected by hydrogen levels [55]. However, here, lower *Flintibacter* was associated with lower IL-1β as an outcome. Among the three investigated inflammatory cytokines, IL-6 is recognised as the most accurate reflection of the concentration in the serum [44]. Furthermore, lower relative abundance of *Flintibacter* can be the result of an increased abundance of *Actinomyces*.

Similar effects on faecal microbiota composition were observed for the MFC category, with an increased relative abundance of *Actinomyces*, and a decreased relative abundance of *Agathobacter* and *Coprococcus* in quartile 4 compared with quartile 1. For *Agathobacter*, the increase in relative abundance observed after quartile 3, together with the similar median relative abundance in quartiles 3 and 4, may suggest a plateau effect of UPFD intake on this genus at higher consumption levels. *Agathobacter* and *Coprococcus* are both butyrate-producer bacteria, and their depletion was already reported in several intestinal inflammation-related diseases [56,57]. In this subcategory, inflammation was significant only when assessed as an outcome. However, the influence of the genera on the inflammatory cytokines was non-homogeneous with higher *Actinomyces* associated with higher IL-6 levels but also lower IL-8, lower *Coprococcus* with lower IL-6 and lower *Agathobacter* with increased IL-8.

Regarding the category including all UPFD, while a decrease of *Faecalibacterium* was observed in the highest consumption quartile, of which reduced levels were reported to be associated with inflammatory status and metabolic alterations such as type 2 diabetes [58–60], decreased relative abundance of *Desulfovibrio* and *Veillonella* were also found. These two being considered harmful bacteria [61–63], lower levels tend to be a positive effect.

Indeed, while lower abundance of *Faecalibacterium* was associated with higher IL-1β, lower *Desulfovibrio* and *Veillonella* was related to lower inflammatory levels in IL-6 and IL-8. Also, higher relative abundance of *Hominimerdicola* and *Phocaeicola* were observed for quartile 4. Not much is known and described in the scientific literature about these two bacteria, except that *Phocaeicola* can adapt to diverse conditions, even its metabolism depending on the substrate [64], influencing then the microbiota functions. Here, *Phocaeicola* was associated with higher levels of IL-6. The diverse results regarding inflammation can be explained by the heterogeneity of food items included when all UPFD are considered, not all considered unhealthy (i.e., Pre-packaged wholegrain sliced bread) and a diet including UPFD can even comply with nutritional recommendations (Dickens). This reinforces the idea that investigating UPFD subcategories is essential.

The inflammatory cytokines investigated were measured in saliva rather than in blood directly. Saliva represents a novel, non-invasive medium for assessing inflammation, as its high degree of vascularization allows for the exchange of compounds between blood and saliva, and it is therefore thought to reflect serum concentrations [44]. Among the three cytokines investigated, IL-6 is considered the most precise indicator of the serum level [44], and salivary IL-8 has already been observed increased in patients in an inflammatory state [65]. In comparison with circulating inflammatory biomarkers, a recent scoping review highlighted that most studies in adult populations observed a positive association between IL-6 and UPFD consumption (in 5 included studies on a total of 6), but no significant association was reported for IL-1β, whereas no studies were found about IL-8 [66].

The low nutritional quality of UPFD was one aspect highlighted in the influence observed on gut microbiota. However, in our data, the significant nutrients associated with UPFD did not influence the results observed between UPFD and the genera. UPFD are also characterized by the addition of food additives for cosmetic function (colours, flavours, emulsifiers, thickeners, etc) or for preservation [17]. Beyond food additives, UPFD are also prone to alterations in the food matrix [67] and to other contaminants through food processing or packaging [19]. Accumulating evidence suggests that these food additives and contaminants affect the gut microbiota, mostly in *in vitro* and animal studies [18]. In particular, the mechanisms through which they alter gut microbiota composition and function and trigger intestinal inflammation has been depicted in two recent reviews of Whelan et al. [21] and

Bevilacqua et al. [19]. Although concerns are raised on the effects of some additives and contaminants on the gut microbiota, there is still lack of research to fully elucidate their role in the gut microbiome and health.

Limitations can be mentioned, suggesting caution when interpreting the results. A first limitation is the sample size (20 participants) which may have limited the statistical power of our analyses and increased the risk of missing relevant effects. Indeed, although adequate for the original aims of the MB1 study, based on a previous study with similar design and targeted outcomes, it may be limited for the associations performed in the present post-hoc analysis. However, the used of a repeated model design enabled us to increase the amount of data collected. In addition, the homogeneous profile of our included population, composed of young and healthy individuals, all hydrogen-producers can be an advantage for analysing the gut microbiota to limit the effects of variability in the individual characteristics. Nevertheless, variability in dietary intake was also reduced which may limit the generalizability of the findings. This could have lowered our ability to highlight potential effects linked to UPFD consumption. Second, the 24h recall, like other dietary report methodologies, brings the recall and the social desirability bias, where participants tend to embellish their true intakes. The ASA24® follows the "AMPM" methods that structures the questionnaire process in steps to increase the details of the diet report and emphasize the items frequently forgotten (i.e., snacks, drinks, etc) [28,29]. Furthermore, photographs of portion size were used. The recalls were auto administered, limiting the potential influence of the investigator on the social desirability bias. The under-reporting was assessed in our data, and only 8% of the individual recalls were considered inaccurate, which is lower than what is reported in other studies around 20-30%. Finally, the days on which the participants were asked to complete the 24h recalls were kept secret to avoid the participants to adapt their diet these specific days for the study. The ASA24® was used as a 24h recall questionnaire and while it is validated, it was not for the Belgian population specifically. Because no 24-h dietary recall tool has been developed and validated specifically for use in Belgium, we used a structured recall format in which participants could manually add recipes, brand names, preparation methods, and additional details. This approach allowed us to adapt food items to the Belgian food environment and to capture consumption with greater accuracy despite the absence of a fully validated national recall tool. Third, the UPFD

in our database were identified following the NOVA classification. It is the most used in epidemiological, Public Health policies, and nutritional recommendations [5,6]. It is a classification system that is already used internationally, in low-middle, upper-middle, and high-income countries [68] More recently, it has been applied in Europe, including in Belgium [69,70]. At the same time, this classification has received criticism regarding the criteria used, as it relies solely on the degree of processing rather than on the nutritional profile, leading to a heterogeneous variety of products in the ultra-processed category. In our case, subcategories were identified and investigated. Also, the criteria are considered ambiguous, with subjective interpretation for certain items [5]. To reduce subjectivity bias for the classification, it was done independently by two evaluators. Furthermore, a Cohen's kappa of 0.88 indicated an almost perfect agreement between the two. Fourth, while genus-level analyses are appropriate for 16S rRNA sequencing, shotgun metagenomic sequencing would have allowed the capture of within-genus diversity and enabled fine-scale functional investigation. Fifth, non-parametric tests were used because of the distribution and nature of the data. While this approach reduces the risk of inflated type-I error and increases the robustness of our results, we acknowledge that it may also reduce statistical power and increase type-II error, potentially leading to false-negative findings. Moreover, reverse causation and residual confounding cannot be excluded.

Conclusions

Depending on the UPFD category concerned, the present study highlighted different associations with the gut microbiota at the genus level. Furthermore, higher consumption of sugar-sweetened beverages was linked to lower exhaled hydrogen, a potential marker of the fermentative activity of the gut microbiota and of metabolic and inflammatory alterations. In addition, salivary inflammatory cytokines emerged as relevant biomarkers when exploring the interplay between UPFD and gut microbiota. While certain nutrients were related to UPFD, some hypothesised mechanisms could also involve non-nutritional characteristics—such as additives, contaminants, and changes in the food matrix—that may contribute to these relationships, reinforcing the need for further studies on this issue. Larger populations are needed to consider the inter-individual heterogeneity in terms of dietary patterns and microbiota.

List of abbreviations

AMPM: Automated Multiple-Pass Method

AR1 : Autoregressive correlation structure of order 1

ASA24®: Automated Self-Administered 24-hour Dietary Assessment Tool

CLR : Euclidean distance of the Centered Log-Ratio

EMH : Extended Mantel-Haenszel

EMM : Estimated Marginal Means

FNDDS : Food and Nutrient Database for Dietary Studies

IL-(6, 8, 1β) : Interleukin (6, 8, 1β)

MB1 : MICROBOOST-1

MFC: Meat, fish and cheese

PCA: Principal Component Analysis

PERMANOVA : Permutational multivariate analysis of variance

SSB: Sugar-sweetened beverages

STROBE-nut : Strengthening the Reporting of Observational Studies in Epidemiology-Nutritional Epidemiology

UPFD: Ultra-processed foods and drinks

Declarations

Ethics approval and consent to participate

The study protocol was approved by the institutional Ethics Committee (Comité d'Ethique Hospitalo-Facultaire UCLouvain/Cliniques Universitaires Saint-Luc, registration code 2023/13AVR/183). It is published at « clinicaltrials.gov » (NCT05949411).

Consent for publication: Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

PDC is inventor on patent applications dealing with the use of specific bacteria and components in the treatment of different diseases. PDC was cofounder of Enterosys. The other authors declare that they have no competing interests.

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Authors' contributions

L.L., M.A., A.M.N., N.M.D. contributed to the conception or design of the work; L.L., M.A., A.M.N., E.B., M.R. acquired the data; L.L. analysed the data; L.L., A.M.N., E.B., L.B.B., and N.M.D. participated in the interpretation of data; L.L. wrote the paper. All authors critically revised the manuscript for important intellectual content and approved the final version of the manuscript.

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