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The link between ultra-processed food consumption, fecal microbiota, and metabolomic profiles in older mediterranean adults at high cardiovascular risk

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Abstract

Background Ultra-processed food (UPF) consumption has been linked to adverse metabolic outcomes, potentially mediated by alterations in gut microbiota and metabolite production.

Objective This study aims to explore the cross-sectional and longitudinal associations between NOVA-classified UPF consumption, fecal microbiota, and fecal metabolome in a population of Mediterranean older adults at high cardiovascular risk.

Methods A total of 385 individuals, aged between 55 and 75 years, were included in the study. Dietary and lifestyle information, anthropometric measurements, and stool samples were collected at baseline and after 1-year follow-up. Fecal microbiota and metabolome were assessed using 16 S rRNA sequencing and liquid chromatography-tandem mass spectrometry, respectively.

Results At baseline, higher UPF consumption was associated with lower abundance of *Ruminococcaceae incertae sedis* ($\beta = -0.275$, $P=0.047$) and lower concentrations of the metabolites propionylcarnitine ($\beta = -0.0003$, $P=0.013$) and pipercolic acid ($\beta = -0.0003$, $P=0.040$) in feces. Longitudinally, increased UPF consumption was linked to reduced abundance of *Parabacteroides* spp. after a 1-year follow-up ($\beta = -0.278$, $P=0.002$).

Conclusions High UPF consumption was associated with less favorable gut microbiota and metabolite profiles, suggesting a possible link to reduced short-chain fatty acid (SCFA) production, altered mitochondrial energy

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metabolism, and impaired amino acid metabolism. These findings support the reduction of UPF consumption and the promotion of dietary patterns rich in fiber for better gut health. Further research is needed to confirm these associations and clarify the underlying mechanisms.

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Keywords Ultra-processed foods, Fecal microbiota, Fecal metabolites, Mediterranean diet, Cardiovascular disease

Introduction

Ultra-processed foods (UPF), categorized as Group 4 in the NOVA food classification system [1], are industrial formulations primarily composed of substances derived from foods, along with additives and cosmetic ingredients. These products typically contain minimal whole-food content and are designed for convenience, enhanced palatability, and long shelf life [2].

The global increase in UPF consumption has emerged as an important public health concern, as it has been linked to several adverse health outcomes [3], including obesity [4], cardiovascular disease, and metabolic disorders [5, 6]. To understand the impact of UPF consumption on human physiology and metabolism, nutrition research has primarily focused on the dietary characteristics of UPF, such as energy density and the content of added fats, sugars, and salt. However, other aspects introduced during food processing could play an equally substantial role, particularly by triggering inflammation-related processes through interactions between the diet, microbiome, and host [7].

The gut microbiota, a complex ecosystem influenced by dietary and lifestyle factors, has emerged as a potential mediator of diet-related health outcomes [7–10]. Recent evidence suggests that UPF consumption may negatively impact the diversity and abundance of beneficial bacteria, potentially disrupting metabolic pathways crucial for health [11, 12]. In contrast, dietary patterns like the Mediterranean diet (MedDiet), characterized by a higher intake of whole and minimally processed foods, have been shown to induce changes in gut microbiota composition linked to better metabolic health [13, 14].

Despite these associations, the relationship between UPF consumption and gut microbiota remains insufficiently explored, particularly in the context of long-term dietary changes. A previous study from our group suggested an association between gastrointestinal inflammation-related taxa and high UPF consumption in a cohort of older adults at high cardiovascular risk. However, this study only demonstrated cross-sectional associations [12]. A recent review article highlights the limited availability of research and the need for further human and animal studies to better understand the effect of UPF on the gut microbiome [15]. To address this research gap, our study aims to provide new insights into the complex

interplay between diet, gut microbiota, and metabolic health by examining how baseline and long-term changes in UPF consumption influence fecal microbial composition and metabolic profiles.

Utilizing the large-scale randomized clinical trial PRE-DIMED (PREvención con DIeta MEDiterránea)-Plus [16] as a platform, and integrating 16 S rRNA sequencing and metabolomics, we explore both cross-sectional and longitudinal associations between NOVA-classified UPF consumption, fecal microbiota composition, and fecal metabolome in a cohort of 385 individuals. We hypothesize that higher UPF consumption may be associated with a less favorable gut microbiota composition and metabolic profiles, both at baseline and after one year of follow-up. The study offers the opportunity to explore how diets rich in UPF might influence gut health.

Methods

Study design and participants

This study was conducted as part of the PREDIMED-Plus randomized clinical trial, using an observational cross-sectional and longitudinal design.

The PREDIMED-Plus trial is a 6-year, multicenter, parallel-group, randomized, single-blind intervention designed to evaluate the long-term effects of a lifestyle intervention—including an energy-reduced Mediterranean diet, physical activity promotion, and behavioral support for weight loss—compared with a traditional Mediterranean diet with *ad libitum* caloric intake, on cardiovascular disease and mortality. Eligible participants were men and women aged between 55 and 75 years without cardiovascular disease at baseline, with a baseline body mass index (BMI) between 27 and 40 kg/m², who met at least three criteria for metabolic syndrome [17]. Participants were randomized in a 1:1 ratio to either the intervention or control group. Additional details are provided elsewhere [16].

Detailed information regarding participant selection is available elsewhere [14]. A subsample of 400 participants, recruited from centers in Alicante, Barcelona, Reus, and Valencia, had fecal microbiota 16 S rRNA sequencing and fecal metabolomics data available at both baseline and after a 1-year follow-up. Of these 400 participants, 15 were excluded since they were outside the pre-defined range for total energy intake.

General assessments and anthropometric measurements

Participants completed a comprehensive questionnaire to collect data on sociodemographic and medical history. Leisure-time physical activity was estimated using the validated REGICOR Short Physical Activity Questionnaire [18]. Waist circumference was measured twice at the midpoint between the lowest rib and the iliac crest using an anthropometric measuring tape. Body weight and height were also measured twice, using calibrated electronic scales and a wall-mounted stadiometer, respectively. The mean of both measurements was calculated and used.

Dietary assessments

Dietary intake was assessed by trained dietitians using a validated 143-item semi-quantitative Food Frequency Questionnaire (FFQ) [19] during face-to-face visits at baseline and at the 1-year follow-up. Detailed information is available elsewhere [16]. Nutrient intake, including sodium, saturated and trans fatty acids, fiber, alcohol (g/day), and total energy (kcal/day), was calculated using Spanish food composition Table [20]. The intake of specific food groups, such as fruits and vegetables (g/day), was also determined.

Participants who did not complete the FFQ at baseline or after the 1-year follow-up, or whose total energy intake fell outside the pre-specified limits (women: <500 or >3500 kcal/day; men: <800 or >4000 kcal/day), were excluded from the analysis ($N=15$) [21].

The NOVA Food Classification system [2] was used to categorize food and beverage items from the FFQ into four groups based on their degree of processing. Using the FFQ data, we calculated the intake of foods and beverages in the different NOVA groups, expressed in g/day. In the present study, we focused on food items classified as NOVA Group 4, which includes ultra-processed food and drink products, considered as a continuous variable.

Stool sample collection

Participants were instructed to collect stool samples and to keep them frozen until delivery to the laboratory within 12 h after excretion, as previously reported [14]. Specifically, participants were provided with a stool collection kit containing a disposable cardboard urinal, a 50 mL sterile container with a spatula, a portable cooler, cold packs, and a sealed plastic container. Detailed instructions were given to ensure proper sample collection, storage, and transport. Participants collected a stool sample (approximately the size of a walnut) using the provided spatula, sealed the container, and immediately placed it in their home freezer. On the day of delivery, the sample was transported in the portable cooler with frozen cold packs to maintain a frozen state. Upon receipt, researchers processed the samples in a refrigerated environment,

dividing each sample into 250 mg aliquots, which were stored in labeled cryovials at -80°C until analysis.

Fecal metabolomics analyses

Metabolomic profiling of stool samples collected at baseline and at the 1-year follow-up was conducted using a liquid chromatography-tandem mass spectrometry platform. Detailed information is available elsewhere [14]. The fecal metabolomics data included a total of 532 identified metabolites.

Fecal bacterial DNA extraction and 16 S amplicon sequencing

Microbial DNA was extracted using the QIAamp PowerFecal DNA Kit (Qiagen, Hilden, Germany), and DNA quality was assessed with the Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The V4 region of the 16 S rRNA gene was amplified in triplicate PCR reactions, followed by purification and quantification of the PCR products. Sequencing libraries were constructed from purified PCR products and sequenced on an Illumina NovaSeq platform, with mock communities and negative controls included for quality assurance. Detailed information on these analytical steps can be found elsewhere [14].

Amplicon sequence variants (ASV) were determined using the DADA2 pipeline [22], and taxonomy was assigned based on the Silva database [23], version 138.1. A total of 218 genera were identified after filtering the raw ASV counts to include only features with a total relative abundance ≥ 0.001 in at least 10% of the samples.

Statistical analyses

All statistical analyses were conducted using the R programming language and software environment (version 4.4.1) along with the RStudio integrated development environment (version 2023.6.0.421).

The baseline characteristics of the study population and 1-year changes were summarized using means and standard deviations for continuous variables and using numbers and percentages for categorical variables. Differences between timepoints for continuous variables were tested using paired t-tests. UPF consumption (in g/day) was adjusted for total energy intake using the residual regression method [24]. This approach allows UPF consumption to be expressed in g/day rather than as a percentage of energy intake, to account for foods with little or no caloric content (e.g., artificially sweetened beverages) and considering non-nutritional factors related to food processing (e.g., presence of food additives).

Fecal microbiota alpha diversity indices, including Chao1, Shannon, and Simpson [25–27], were calculated from absolute ASV counts. For the cross-sectional assessment, the association between calculated indices and

baseline UPF consumption was examined by fitting linear regression models and adjusting for selected covariates: age, sex, center, smoking status, education level, prevalence of diabetes, hypertension and hypercholesterolemia, BMI, physical activity, alcohol intake, fiber intake, and consumption of food and beverages categorized by other NOVA groups. Before fitting the linear models, numerical covariates were standardized using z-scores. In a similar manner, for the longitudinal assessment, we explored the association between changes in alpha diversity and changes in the UPF consumption over one year. These changes were calculated by subtracting baseline values from the values at 1 year. The PREDIMED-Plus study arm was also included as a covariate. Results with $P < 0.05$ were reported as significant.

Beta diversity was evaluated by calculating Euclidean distance on centered log-ratio transformed genus counts (Aitchison distance) [28], including only features with a total relative abundance of ≥ 0.001 in at least 10% of observations. Permutational multivariate analysis of variance (PERMANOVA) [29] was implemented using the “adonis2” function from the R package *vegan* (version 2.6–6.1) (available at <https://CRAN.R-project.org/package=vegan>) to test whether differences in fecal microbiota composition were significantly associated with the given predictors. Results with $p < 0.05$ were reported as significant.

For the cross-sectional analysis, the association between differences in Aitchison distance and UPF consumption at baseline was tested, accounting for covariates. For the longitudinal analysis, the association between differences in Aitchison distance, calculated between baseline and 1-year counts, and the interaction term UPF 1-year change $\times$ time was tested. In this analysis, participant IDs were specified as a variable within which permutations were constrained, and the PREDIMED-Plus study arm of the trial was included as an additional covariable.

Differential abundance analysis was performed using the *MaAsLin2* R package [30], utilizing the same input data as the beta diversity analysis. General linear models were fitted, adjusting for the same covariates considered in the alpha and beta diversity association analyses, with the total number of reads included as an additional covariate. No further filtering was applied in the model. Continuous covariates were standardized to the same scale. Results were subset based on the exposure of interest, and Benjamini-Hochberg adjusted P -values were calculated, focusing comparisons solely on the predictor of interest while controlling for covariates. Findings with adjusted P -value < 0.05 were reported.

In the cross-sectional assessment, the association between fecal microbiota genera abundance and UPF consumption at baseline was tested. For the longitudinal

assessment, the association between the 1-year change in fecal microbiota genera abundance and the 1-year change in UPF consumption was evaluated. The time between baseline and follow-up, as well as the PREDIMED-Plus study group, was included as fixed effects along with the other covariates. Participant IDs were specified as random effects to account for the non-independence of samples from the same individual.

Output files generated with DADA2 (ASV table and representative sequences) were parsed and used as input for PICRUSt2 [31] to generate a table of inferred per-sample abundances of KEGG orthologs. The omixer-Rpm package [32] (version 0.3.3) was used to reconstruct microbial functionality by computing the abundance of predefined gut metabolic modules (GMM) [33] based on the KO abundances obtained. Differential abundance analysis was performed on GMM abundance tables, following the same approach used for taxonomic counts, to assess cross-sectional and longitudinal associations with UPF consumption.

In relation to fecal metabolomics, rank-based inverse normal transformation (INT) [34] was applied to the data prior to analysis to account for the typically skewed distribution of metabolites and to ensure robustness in subsequent analyses. This method has been previously used in metabolomic studies [35] and performs well with other nonnormal data [36]. The INT involved a two-step procedure: first, the observations were transformed into the probability scale using the empirical cumulative distribution function; second, the observations were transformed into z-scores on the real line.

We used a penalized regression model with the Elastic Net regularization technique [37] as the primary approach to identify metabolites most strongly associated with UPF consumption. This method applies regularization to select only the most stable and reproducible features across multiple iterations. Specifically, we assessed the cross-sectional relationship between UPF consumption and fecal metabolite concentrations at baseline, as well as the longitudinal association between 1-year change in UPF consumption and 1-year changes in fecal metabolite concentrations.

Training and validation procedures were performed. Model training and hyperparameter tuning for alpha and lambda were executed using the *caret* R package [38]. A resampling strategy of 10-fold cross-validation (10-fold CV) was implemented, with the process repeated 10 times to enhance model reliability. This resampling was conducted on 90% of the dataset designated as the training set, with the remaining 10% held out for final model validation. The best model accuracy was achieved with alpha and lambda values of 0.6 and 36.5, respectively, for the cross-sectional assessment, and with alpha and lambda values of 1 and 34.6, respectively, for the

Table 1 Baseline characteristics of the study population

Characteristics		N = 385
Women, n (%)		166 (43.1)
Age (years), mean $\pm$ SD		64.57 $\pm$ 4.93
Education, n (%)	Primary	214 (55.6)
	Secondary	103 (26.8)
	Tertiary	68 (17.7)
Center, n (%)	Reus	218 (56.6)
	Alicante	84 (21.8)
	Barcelona	44 (11.4)
	Valencia	39 (10.1)
Smoking status, n (%)	Never	185 (48.1)
	Former	152 (39.5)
	Smoker	48 (12.5)
Diabetes prevalence, n (%)		88 (22.9)
Hypertension prevalence, n (%)		314 (81.6)
Hypercholesterolemia prevalence, n (%)		258 (67.0)

longitudinal assessment. In a subsequent robustness check, 10-fold cross-validation Elastic Net models were run using the defined alpha and lambda values with different random seeds. Only metabolites with coefficients consistently different from zero across all 10 runs were retained, ensuring the selection of stable predictors for the outcome of interest.

To examine the directionality and effect sizes of these pre-selected metabolites, we conducted linear regression analyses adjusting for covariates. Since the metabolites were already pre-selected based on their association with UPF consumption, applying additional FDR correction at this stage would not meaningfully improve statistical rigor and could introduce artificial bias. Instead, we relied on the prior selection process of Elastic Net to ensure robust feature selection.

Linear regression models were fitted using the same approach as that employed to explore the association between alpha diversity and exposure. Results with $P < 0.05$ were reported as statistically significant.

Results

General characteristics of the study population

The general baseline characteristics of the study population are presented in Table 1.

The baseline and 1-year changes in selected covariates are summarized in Table 2. Participants had a significant decrease in BMI (-1.00 ± 5.09 kg/m²) and waist circumference (-3.17 ± 14.51 cm) after 1-year follow-up. In addition, there was a significant improvement in physical activity (106.70 ± 477.35 METs min/day). After one year, the fiber intake and the NOVA Group 1 consumption significantly increased (3.16 ± 10.92 and 107.06 ± 484.81 g/day, respectively), while the NOVA Group 3 and UPF consumption significantly decreased (-71.55 ± 300.68 and -61.43 ± 172.51 g/day, respectively).

Results of fecal microbiota analysis

We did not observe any significant cross-sectional associations between calculated alpha diversity indices and UPF consumption at baseline (Supplementary Tables 1, 2, 3), nor any longitudinal association between 1-year changes in calculated alpha diversity indices and 1-year change in UPF consumption (Supplementary Tables 4, 5, 6). We did not observe significant variance in gut microbiota composition explained by UPF consumption at baseline (Supplementary Table 7), neither explained by 1-year change in UPF consumption (Supplementary Table 8). Differential abundance analysis revealed a negative cross-sectional association between the abundance of *Ruminococcaceae incertae sedis* and the UPF consumption ($\beta = -0.275$, adjusted $P = 0.047$) (Fig. 1). The longitudinal analysis showed a significant decrease in the abundance of *Parabacteroides* spp. associated with increased UPF consumption after 1-year follow-up ($\beta = -0.278$, adjusted $P = 0.002$) (Fig. 2).

Differential abundance analysis of GMM did not yield significant results, either cross-sectionally or longitudinally.

Table 2 Baseline and 1-year changes in selected anthropometric, physical activity and dietary variables

Characteristics	Baseline	1-year change	P value
BMI (kg/m ²)	32.83 $\pm$ 3.51	-1.00 ± 5.09	< 0.001
Waist circumference (cm)	107.75 $\pm$ 9.99	-3.17 ± 14.51	< 0.001
Physical activity (METs min/day)	368.29 $\pm$ 324.39	106.70 ± 477.35	< 0.001
Alcohol intake (g/day)	10.83 $\pm$ 13.45	-1.19 ± 18.31	0.204
Fiber intake (g/day)	26.74 $\pm$ 7.99	3.16 ± 10.92	< 0.001
NOVA G1 consumption (g/day)	1349.27 $\pm$ 355.99	107.06 ± 484.81	< 0.001
NOVA G2 consumption (g/day)	60.67 $\pm$ 18.49	-1.93 ± 25.48	0.137
NOVA G3 consumption (g/day)	432.34 $\pm$ 219.68	-71.55 ± 300.68	< 0.001
UPF (NOVA G4) consumption (g/day)	155.89 $\pm$ 135.80	-61.43 ± 172.51	< 0.001

BMI, body mass index; MET, metabolic equivalent of task; G1, Group 1; G2, Group 2; G3, Group 3; G4, Group 4; UPF, ultra-processed food. Differences between timepoints tested with paired t-test. Significant $P < 0.05$

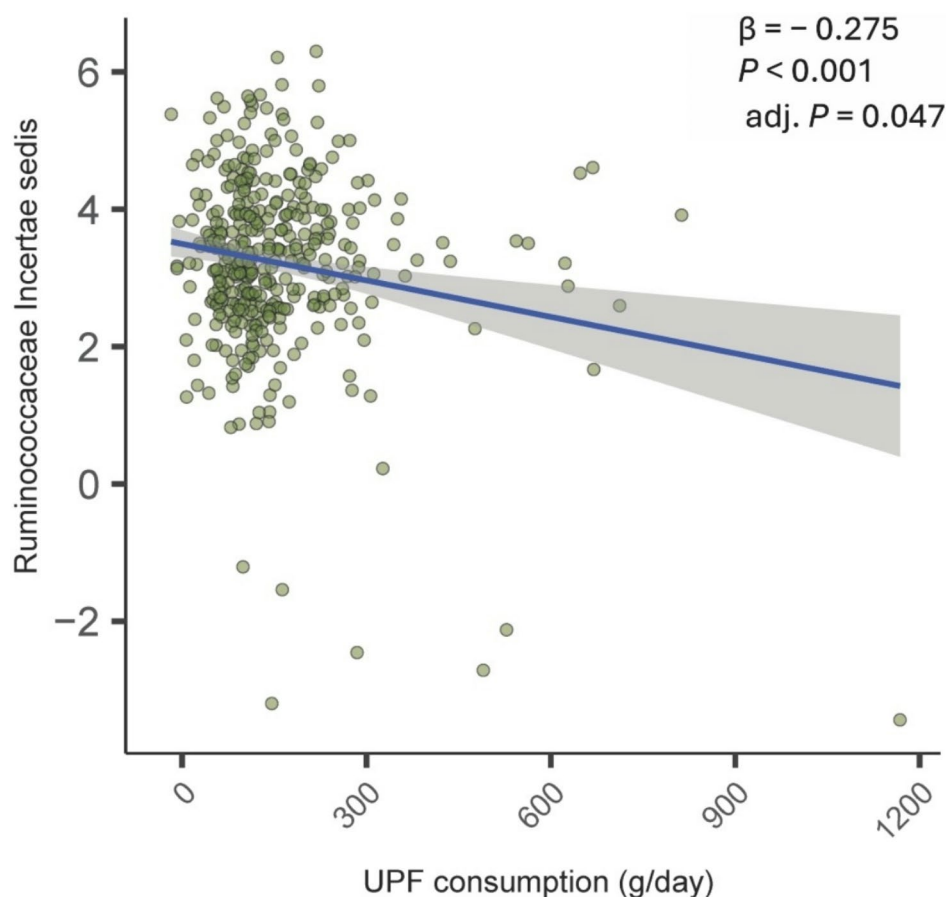


Fig. 1 Differentially abundant taxa associated with baseline ultra-processed foods (UPF) consumption. Multivariable association tested with generalized linear model adjusted for sex, age, education (primary, secondary, tertiary), recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (never, former, smoker), diabetes, hypertension, hypercholesterolemia prevalence, body mass index, waist circumference, physical activity, alcohol intake, fiber intake, NOVA Group 1, Group 2, and Group 3 foods consumption. Values in x axis indicate UPF consumption in g/day, values in y axis indicate genera centered log-ratio relative abundance with Benjamini-Hochberg adjusted $P < 0.05$

Results of fecal metabolomics analysis

A total of 21 metabolites were selected across all 10 iterations of the binomial elastic net regression cross-validation for the baseline UPF consumption (Fig. 3). Specifically, 11 metabolites presented negative coefficients, and 10 metabolites presented positive coefficients. The linear regression analysis adjusted for covariates returned two metabolites significantly associated with UPF consumption (Supplementary Table 9). Specifically, we observed a negative association with propionylcarnitine ($\beta = -0.0003$, $P = 0.013$) and L-Pipecolic acid ($\beta = -0.0003$, $P = 0.040$) (Fig. 4).

Six metabolites were selected when analyzing the 1-year change in UPF consumption using binomial elastic net regression across all 10 iterations of the cross-validation. (Fig. 5). Specifically, four metabolites presented negative coefficients, and two metabolites presented positive coefficients. However, the linear regression analysis did not show any statistically significant association between these metabolites and the

1-year change in UPF consumption (Supplementary Table 10).

Discussion

Our study explored the link between UPF consumption, the fecal microbiota composition and the fecal metabolites concentrations in a population of Mediterranean older adults at high cardiovascular risk, within the framework of the PREDIMED-Plus trial. We found that higher UPF consumption was associated with alterations in both fecal microbiota and metabolites, suggesting additional insights into how UPF consumption may impair metabolic health.

At baseline, higher UPF consumption was associated with lower abundance of *Ruminococcaceae incertae sedis*. While some members of the *Ruminococcaceae* family are known to contribute to short-chain fatty acid (SCFA) production, the role of *Ruminococcaceae incertae sedis* remains unclear. Therefore, the observed reduction suggests a potential link to reduced short-chain fatty acids

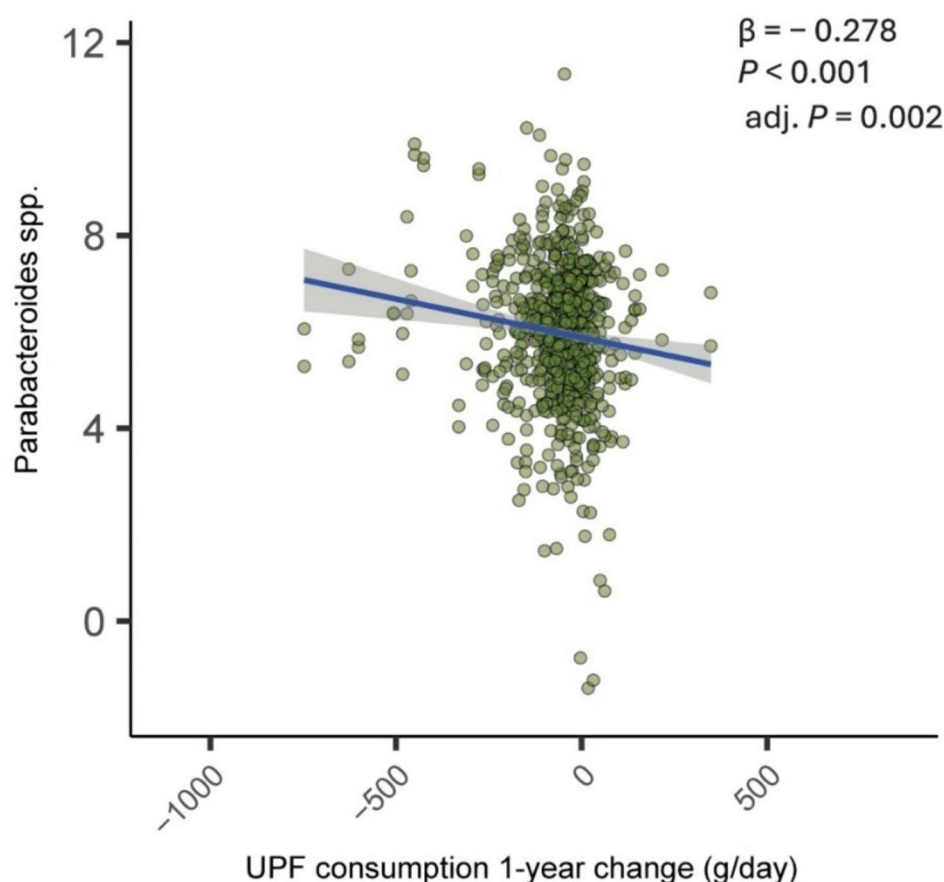


Fig. 2 Differentially abundant taxa 1-year change associated with ultra-processed foods (UPF) consumption 1-year change. Multivariable longitudinal association tested with generalized linear model adjusted for time (baseline, 1 year), sex, age, education (primary, secondary, tertiary), recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (never, former, smoker), diabetes, hypertension, hypercholesterolemia prevalence, body mass index, waist circumference, physical activity, alcohol intake, fiber intake, NOVA Group 1, Group 2, and Group 3 foods consumption. Participants' ID was specified as random effect. Values in x axis indicate UPF consumption 1-year change in g/day, values in y axis indicate genera centered log-ratio relative abundance with Benjamini-Hochberg adjusted $P < 0.05$

(SCFA) production [39], although this interpretation remains speculative and requires further investigation. This finding aligns with previous research indicating that UPF are typically low in fiber [40], a key substrate for SCFA production [40, 41]. However, the relationship between UPF consumption and *Ruminococcaceae* may involve mechanisms beyond fiber intake alone. Reduced SCFA production has been shown to negatively affect host energy metabolism and immune function [42, 43], as SCFA have anti-inflammatory properties and serve as an energy source for colonic epithelial cells [44].

Additionally, higher UPF consumption was associated with a lower fecal concentration of propionylcarnitine. This could potentially be explained by reduced fiber intake and the consequent decrease in microbial propionate production [45]. Propionyl-CoA, a precursor of propionylcarnitine, is an intermediate in microbial propionate production [46, 47]. The propanediol pathway relies on specific gut microbes, including members of

the *Ruminococcaceae* family, to convert dietary fiber into propionate [47, 48]. The observed reduction in both fecal *Ruminococcaceae incertae sedis* abundance and propionylcarnitine concentration suggest that UPF consumption may impair pathways related to SCFA production, potentially leading to lower SCFA availability. Propionylcarnitine also contributes to mitochondrial energy metabolism by maintaining the mitochondrial acyl-CoA/CoA ratio and potentially stimulating the tricarboxylic acid cycle [49]. It indirectly supports mitochondrial function and energy production [49]. Reduced fecal concentrations of propionylcarnitine may indicate impaired mitochondrial function and altered energy metabolism due to UPF consumption [50], though further mechanistic studies are needed to confirm these mechanisms.

We also observed that higher UPF consumption was linked with lower fecal concentrations of pipecolic acid, a non-proteinogenic amino acid involved in lysine catabolism [51]. Pipecolic acid is partially produced by

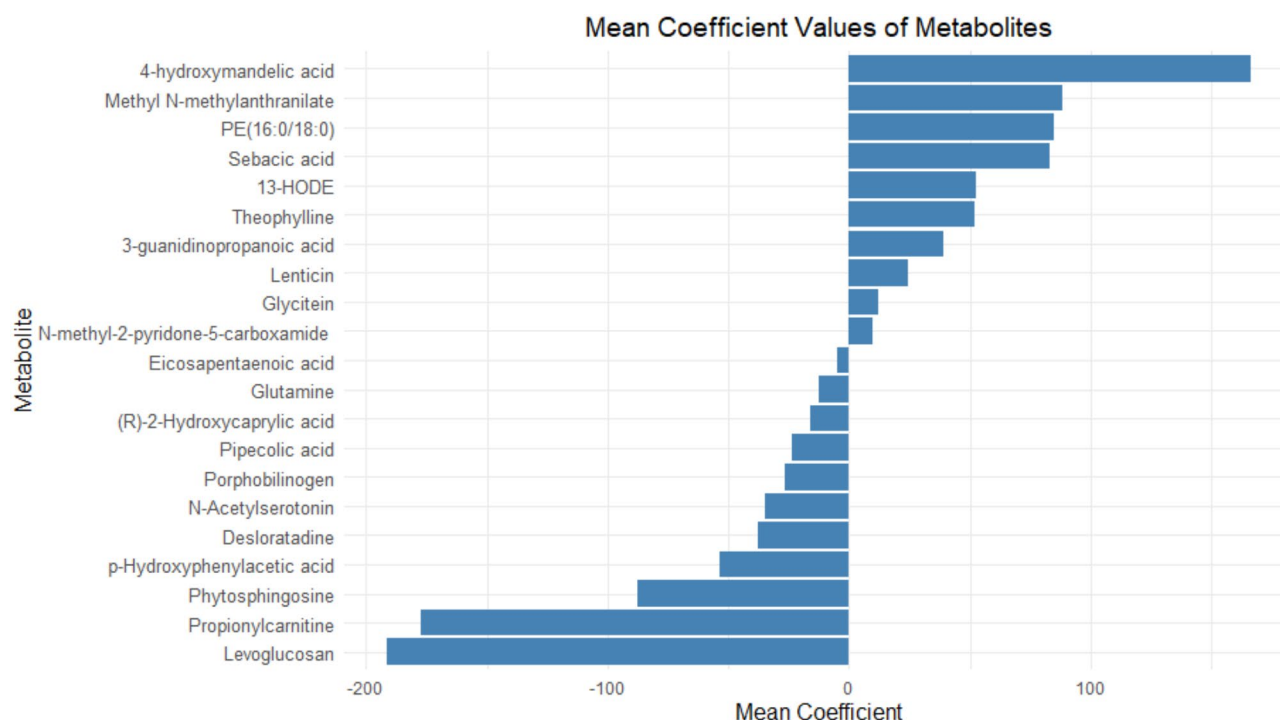


Fig. 3 Mean coefficients of baseline fecal metabolites concentration selected 10 times in the 10-fold cross-validations of the binomial elastic net regression for the baseline ultra-processed foods consumption

certain gut bacteria through lysine breakdown, and a disrupted microbiota could result to reduced production of this metabolite [52]. UPF are often characterized by lower overall nutritional quality, including reduced protein content [53, 54], which could potentially limit lysine availability. However, the relationship between UPF consumption and pipecolic acid levels may not be fully explained by protein intake alone, as other factors, such as inflammation or gut barrier dysfunction, could also play a role [55–57], potentially affecting the conversion of lysine to pipecolic acid. Given its role in immune modulation, reduced pipecolic acid levels may contribute to inflammatory processes [58].

Our findings further suggest that UPF consumption may disrupt amino acid metabolism, as indicated by the observed reduction in pipecolic acid. This disruption could be linked to negative effect of UPF on mitochondrial function, given the central role of mitochondria in amino acid metabolism [50]. UPF often contain advanced glycation end-products (AGE), which can impair mitochondrial function and lead to oxidative stress [50, 59]. These AGE, along with other food additives found in UPF, may exacerbate gut dysbiosis and inflammation, particularly through the accumulation of reactive metabolites that can damage cellular structures, including mitochondria [59]. The observed reduction in pipecolic acid could be indicative of this mitochondrial dysfunction, as mitochondria are involved in its production. However, the

direct relationship between UPF consumption and fecal pipecolic acid concentrations requires further investigation. Future studies should explore the links between UPF consumption, lysine intake, gut microbiome alterations, and pipecolic acid production and excretion.

Over one year, increased UPF consumption was associated with reduced abundance of *Parabacteroides* spp., a genus linked with beneficial metabolic effects, dietary fiber intake, and SCFA production [60, 61]. Our findings suggest that long-term UPF consumption may promote gut dysbiosis, potentially worsening metabolic health. However, longitudinal research on specific microbial genera remains limited, and longer follow-up periods are needed to confirm these findings.

Binomial elastic net regression identified six metabolites associated with changes in UPF consumption over one year. Four metabolites (allopurinol riboside, sphingomyelin, xanthine, 7-dehidrodesmosterol) showed negative coefficients, while two metabolites (glyceric acid, tartaric acid) showed positive coefficients, indicating distinct metabolic responses. However, linear regression analyses did not confirm significant relationships, highlighting the need for cautious interpretation.

Our study benefits from a well-characterized cohort with diverse Spain geographic representation, enhancing the generalizability of our findings within large at-risk populations. The integration of 16 S rRNA sequencing and metabolomics provides a comprehensive

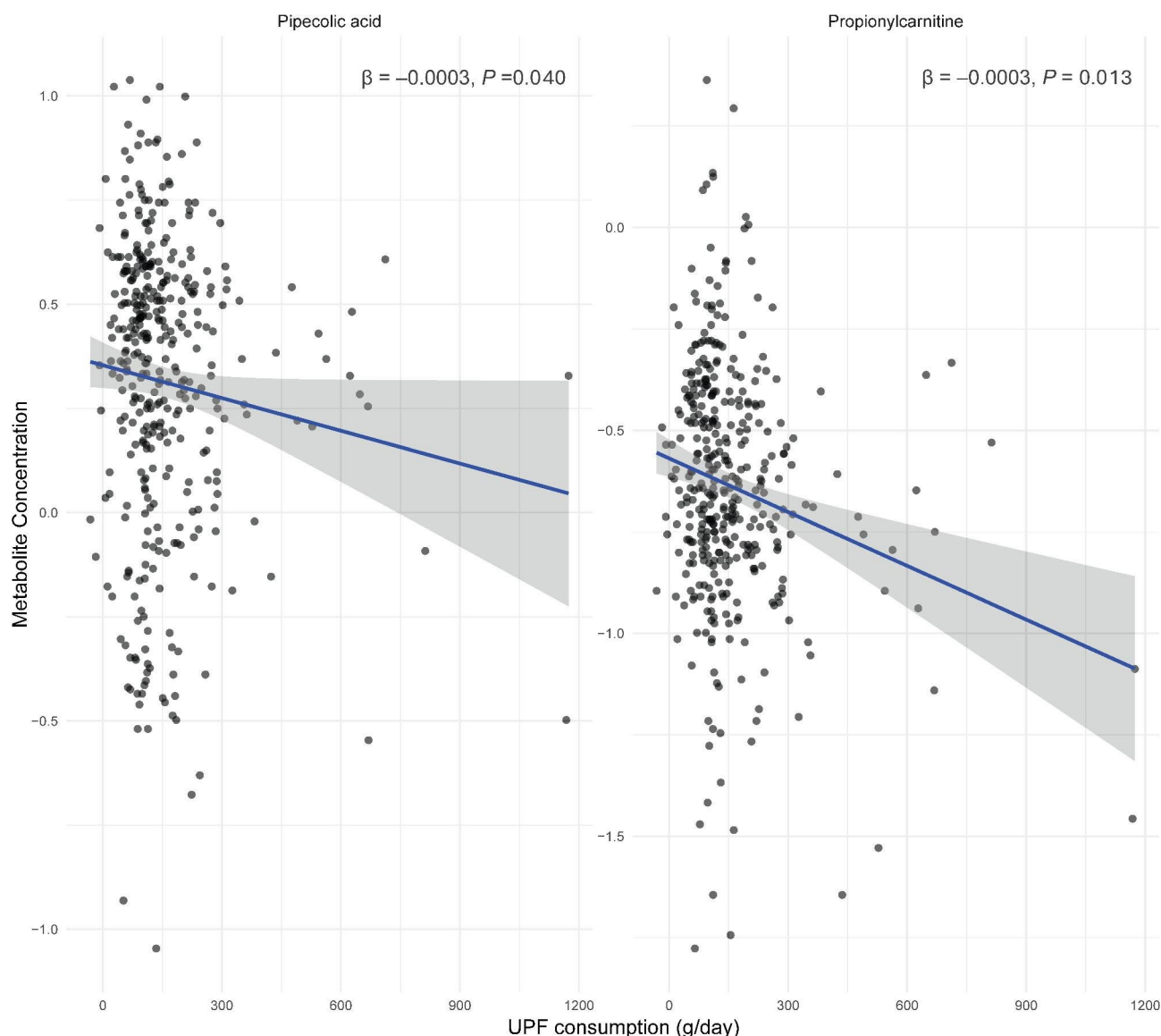


Fig. 4 Baseline fecal metabolites selected by the binomial elastic net regression significantly associated with baseline ultra-processed foods (UPF) consumption. Association tested with linear regression adjusted for sex, age, education (primary, secondary, tertiary), recruiting center (Alicante, Barcelona, Reus, Valencia), smoking status (never, former, smoker), diabetes, hypertension, hypercholesterolemia prevalence, body mass index, waist circumference, physical activity, alcohol intake, fiber intake, NOVA Group 1, Group 2, and Group 3 foods consumption. Values in x axis indicate UPF consumption in g/day, values in y axis indicate rank-based inverse normal transformation of metabolite concentration with $P < 0.05$

multi-dimensional view of both microbial and metabolic alterations linked to UPF consumption. Furthermore, the longitudinal design provides a unique opportunity to examine temporal associations, enhancing our understanding of how UPF consumption might influence gut microbiota composition and metabolic outcomes over time.

The NOVA classification provides a widely used framework for assessing food processing levels, however, it does not differentiate between nutritionally distinct UPFs, such as fiber-rich vs. nutrient-poor products. This heterogeneity may complicate the interpretation of the

findings. Our statistical models were adjusted for fiber intake to account for this potential confounding, and the consistency of our findings suggests that fiber content alone does not fully explain the observed associations. Future studies should subclassify UPFs based on their nutritional quality to better understand their impact on gut health and metabolism.

The observational nature of the study limits causal inference, and the lack of longitudinal associations consistent with the cross-sectional findings underscores the need for caution when interpreting these results. Future randomized dietary interventions are needed to confirm

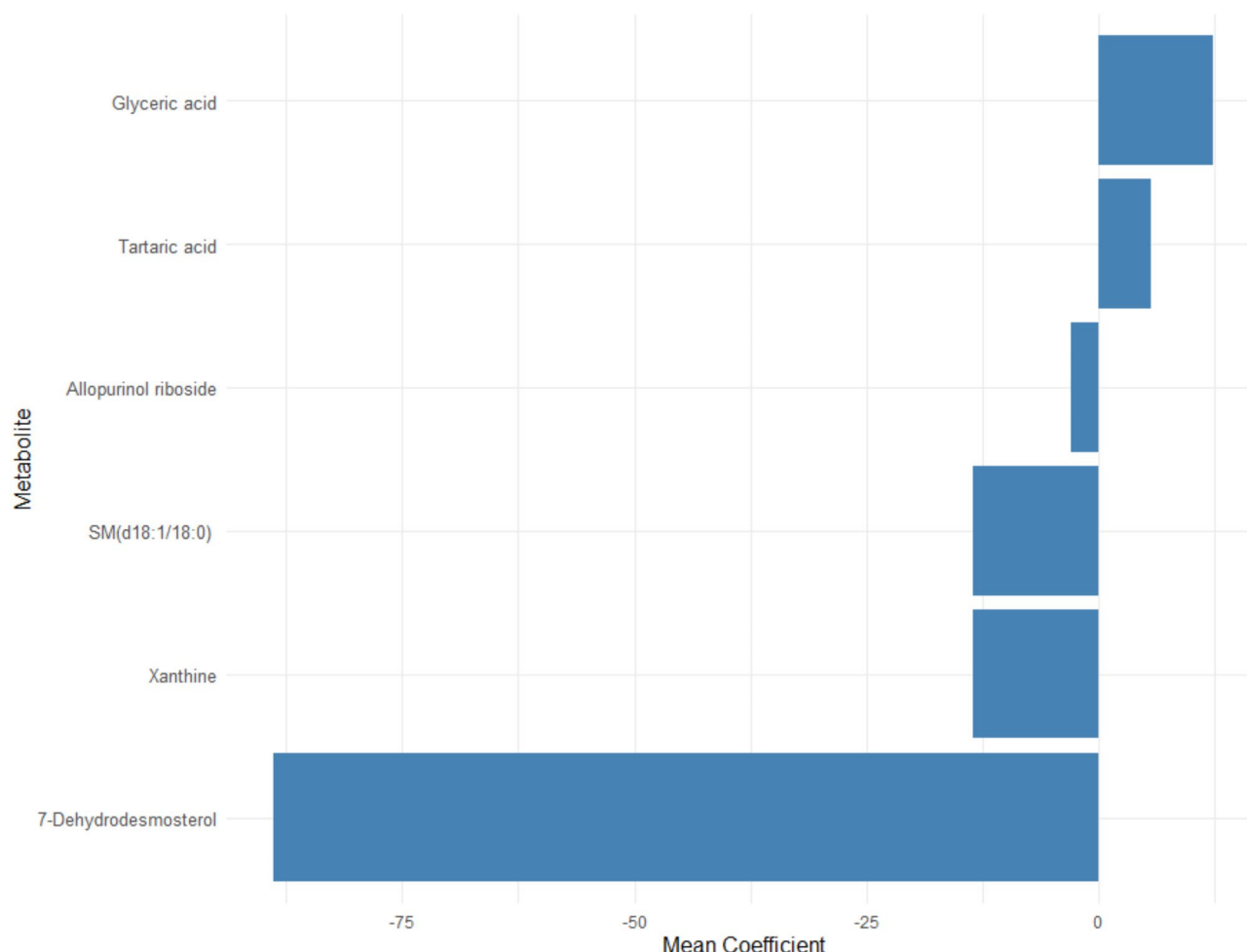


Fig. 5 Mean coefficients of fecal metabolites concentration 1-year change selected 10 times in the 10-fold cross-validations of the binomial elastic net regression for the ultra-processed foods consumption 1-year change

the observed associations and clarify the temporal relationships between UPF consumption and gut health.

The inclusion of numerous covariates, while justified by their theoretical relevance, may introduce bias due to collinearity or unmeasured confounders. Although some covariates did not significantly contribute to microbiota variability in PERMANOVA analyses, they were retained to ensure consistency and control for potential confounding. Additional limitations include the limited generalizability of the findings to other populations and the use of 16 S rRNA sequencing, which provides only genus-level taxonomic profiling. Future research should employ multi-omics approaches in more diverse populations.

The results related to the fecal metabolome profile should be interpreted with caution, as gut metabolites can arise from the interaction between dietary metabolites, secondary metabolites produced by gut microbiota, and endogenous metabolites secreted by enterocytes and associated immune cells.

Finally, FFQ may misclassify UPF intake due to self-report bias, leading to potential over- or underestimations across NOVA categories. Future studies should incorporate alternative dietary assessment tools (e.g., 24-hour recalls, food diaries) for improved accuracy.

Conclusions

Our study highlights significant associations between UPF consumption and alterations in fecal microbiota composition and metabolite concentrations, providing valuable insights into the impact of UPF consumption on gut health.

While our study is observational, it suggests that UPF consumption may contribute to metabolic dysregulation and inflammation, particularly in vulnerable populations, warranting further investigation into the underlying mechanisms. Future analyses should explore the potential relationships between these microbial and metabolic alterations to further elucidate the link between diet, gut health, and cardiometabolic outcomes.

From a dietary perspective, our findings suggest that interventions targeting the gut microbiota, such as increasing fiber intake or following a Mediterranean-style diet, may help mitigate the negative metabolic and inflammatory effects of UPF consumption. Choosing whole, minimally processed foods rich in fiber and essential nutrients could potentially counteract some of the negative effects associated with UPFs.

Abbreviations

UPF	Ultra-processed foods
MedDiet	Mediterranean diet
PREDIMED	PREvención con DietaMEDiterránea
BMI	Body mass index
FFQ	Food frequency questionnaire
ASV	Amplicon sequence variants
PERMANOVA	Permutational multivariate analysis of variance
GMM	Gut metabolic modules
INT	Inverse normal transformation
SCFA	Short-chain fatty acid
AGE	Advanced glycation end-product

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12937-025-01125-5>.

Supplementary Material 1

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Author contributions

All the principal PREDIMED-Plus investigators contributed to the study concept and design and to data extraction from the PREDIMED-Plus participants. A.A. and J.S-S contributed to the concept and design of the present study. A.A. drafted the manuscript and performed the statistical analyses with the support of A.H-C and N.K., and the supervision of J.S-S. All authors reviewed the manuscript for important intellectual content and approved the final version to be published.

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Data availability

The datasets generated and analysed during the current study are not publicly available due to data regulations and for ethical reasons, considering that this information might compromise research participants' consent because our participants only gave their consent for the use of their data by the original team of investigators. However, collaboration for data analyses can be requested by sending a letter to the PREDIMED-Plus steering Committee (predimed_plus_scom-mittee@googlegroups.com). The request will then be passed to all the members of the PREDIMED-Plus Steering Committee for deliberation.

Declarations

Ethics approval and consent to participate

This trial was approved by the institutional review board of all participating institutions and was registered on the ISRCTN registry (ISRCTN89898870) on July 24, 2014. All participants provided written informed consent, and the procedures were implemented in accordance with the ethical standards of the Declaration of Helsinki.

Consent for publication

Not applicable.

Competing interests

J.S-S reports serving on the board of and receiving grant support through his institution from the International Nut and Dried Fruit Council, serving on the board of the Instituto Danone Spain and the International Danone institute. None of the other authors declare competing interests.

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