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Original Article

The Multinutrient Fortasyn Connect Influences Gut Microbiota and Intestinal Function in Early Alzheimer's Disease

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ABSTRACT: Nutritional supplementation is emerging as a promising strategy to support clinical management of early Alzheimer's disease (AD), partly through modulation of the intestinal microbiome via the microbiota–gut–brain axis. This study investigated the impact of Fortasyn Connect (Souvenaid®), a multinutrient formulation, on gut microbiota using a dual approach: i) a dynamic gastrointestinal simulator (simgi®) inoculated with feces from AD patients, and ii) an observational study involving early-stage AD patients (n = 22) receiving or not the supplement. The *in vitro* model provided a direct, host-independent assessment of microbiota responses, showing increased *Bifidobacterium* and *Lactobacillus* levels, alongside enhanced short-chain fatty acid (SCFA) production. In patients, supplementation was associated with higher fecal abundance of *Bifidobacterium* and *Christensenellaceae,* reduced inflammatory markers (calprotectin and myeloperoxidase), and increased butyrate levels. Fecal lipidomic and proteomic analyses indicated improved lipid digestion, increased secretory IgA, and modulation of host proteins linked to gut–brain homeostasis. Systemically, elevated levels of iron, folate, and vitamin B12 were also observed. For the first time, this study shows that supplements such as Fortasyn Connect can beneficially modulate the gut ecosystem and related immune–metabolic pathways in early AD, thereby targeting disease-relevant mechanisms through the gut–brain axis, in the context of aging.

Keywords: Alzheimer's disease; nutritional supplementation; Fortasyn Connect (Souvenaid®); gut microbiota; simgi® gastrointestinal simulator; fecal lipidomics and proteomics

INTRODUCTION

Alzheimer's disease (AD) is the most common disorder associated with cognitive impairment, and the main cause of dementia globally [1, 2]. AD is categorized by the World Health Organization as a public health priority; it is estimated that by 2050, it will affect more than 100 million people worldwide. Thus, early detection and action through preventive or therapeutic strategies is crucial. The multifactorial nature of the disease, marked by multiple factors inside and outside the central nervous system, supports the notion that AD is a syndrome of many etiologies in which the defining pathology of amyloid-β (Aβ) plaques and tangles composed of hyperphosphorylated tau protein coexists with many others, including presynaptic protein α-synuclein and oxidative stress. Additionally, several environmental and metabolic factors have been identified as contributing to the development of the disease, with the emerging consideration of AD as a multi-organ disorder [3]. This complexity highlights the need to explore multimodal therapeutic targets and unconventional interventions, particularly in the early stages of the disease and in the broader context of aging.

Over the past decade, growing evidence has pointed to the gut microbiome as a key player in the pathogenesis

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and progression of AD through the microbiota–gut–brain axis. This bidirectional communication links gut microbes with the central nervous system via endocrine, immune, neural, and microbial metabolite pathways [4, 5]. Among these, microbial metabolites such as short-chain fatty acids (SCFAs) -including acetate, propionate, and butyrate- have shown potential to modulate brain function beneficially [6]. Nevertheless, how microbial signaling along the gut–brain axis influences cognitive function, mood, and behavior remains to be fully elucidated. This knowledge gap has fueled growing interest in diet-based interventions targeting the gut microbiota as a strategy to preserve brain health and delay cognitive decline [7, 8].

Diet is one of the most powerful modulators of the gut microbiome, and specific dietary patterns and ingredients, such as omega-3 polyunsaturated fatty acids (-3 PUFA) and certain vitamins, have shown neuroprotective effects in preclinical models [9], with emerging clinical evidence in mild cognitive impairment (MCI) patients, suggesting a role mediated through gut-brain interactions [10–12]. Among nutrition-based interventions, Fortasyn Connect is a specific multinutrient formulation designed to support synaptic function and brain health in the early stages of Alzheimer's disease. It contains a synergistic combination of docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), uridine monophosphate, choline, phospholipids, and several essential vitamins and cofactors (such as B12, folate, and vitamin E) [13]. This formulation is the basis of the medical food Souvenaid®, developed for dietary management of patients with prodromal or mild AD. Clinical trials have shown that regular intake of Souvenaid® can help slow cognitive decline and brain atrophy in early-stage AD and MCI [14–19]. Importantly, the interplay between lipids, the gut microbiota, and lipid metabolism is increasingly recognized as a critical axis in AD progression and prevention [20]. However, the effects of multinutrient interventions such as Fortasyn Connect on the gut microbiota and its metabolic activity remain unexplored.

The present study aimed to evaluate the impact of regular Fortasyn Connect (Souvenaid®) intake on the composition and functionality of the gut microbiota in patients with mild AD. Additionally, we sought to combine fecal lipidomics and proteomics for a more comprenhensive understanding of the complex mechanism underlying the interaction among host metabolism, immune responses and ultimately, cognitive health.

MATERIALS AND METHODS

In vitro gut model

The simgi® system (www.cial.uam-csic.es/simgi/) is a computer-controlled dynamic *in vitro* model of the human gastrointestinal tract consisting of five successive reactors simulating the stomach, small intestine (SI), and three colonic regions: ascending (AC), transverse (TC), and descending colon (DC). It mimics physiological digestive conditions and hosts a metabolically active human colonic microbiota [21]. The experimental procedure is shown in **Figure 1**. Parameters such as pH, temperature, flow rates, and compartment volumes were fully automated [21].

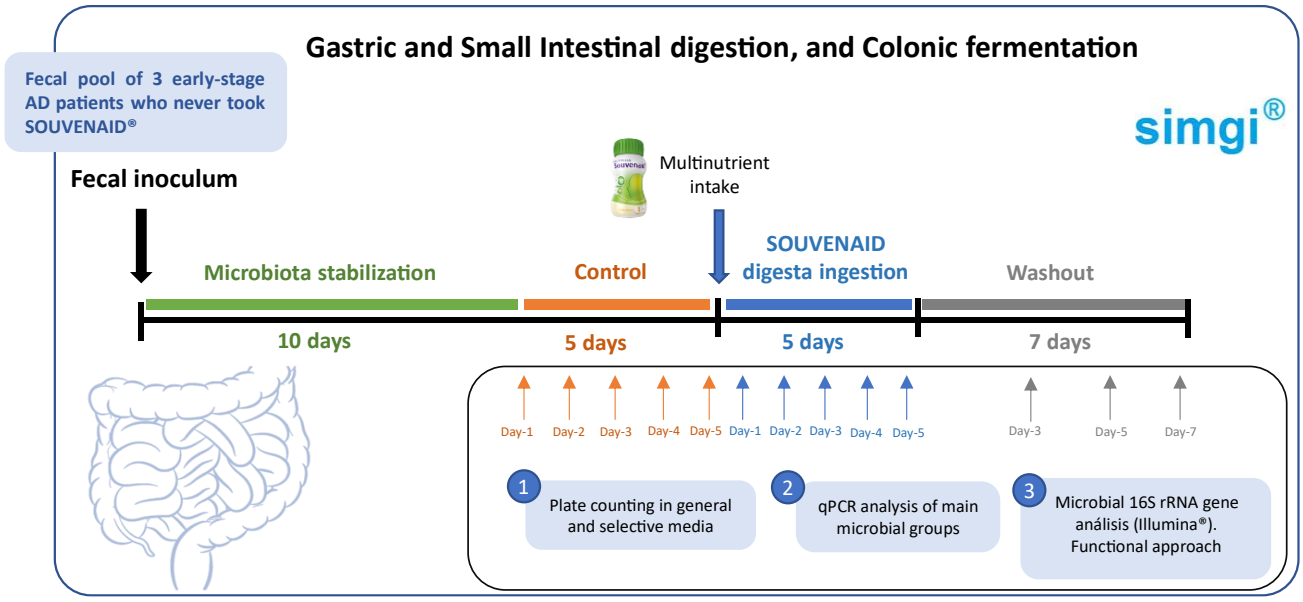

Figure 1. Experimental set-up of the *in vitro* gastrointestinal simulation study on simgi® model.

Initially, the colonic microbiota was stabilized over ten days in the AC, TC, and DC reactors, using a 20% (w/v) fecal slurry from three volunteers with mild AD who had never consumed Fortasyn Connect (Souvenaid®). During this period, compartments were fed three times daily with gut nutrient medium (GNM, pH 6), including arabino-galactan (1 g/L), to promote microbial adaptation. After stabilization, the experiment proceeded through three stages: (i) Control stage: 5 days of feeding with GNM; (ii) Multinutrient feeding stage: 5 days with a daily dose of 125 mL of Fortasyn Connect-Souvenaid® (recommended clinical intake), previously homogenized from six commercial bottles, and (iii) Washout stage: 7 days of GNM-only feeding to return the system to baseline. The detailed composition of the nutritional supplement is presented in Supplementary Table 1. Simulated digestion began with a gastric phase, where pH was reduced from 5.6 to 2 using HCl (1 M), and gastric juice was infused at 3.9 mL/min to a final volume of 15 mL. This was followed by intestinal digestion at 37 °C for 2 hours, with the addition of 40 mL pancreatic juice (5 mL/min). The resulting chyme was then sequentially transferred through the colonic compartments.

Samples (2 mL, in triplicate) were collected from AC, TC, and DC during the control (days 1–5), multinutrient feeding (days 2–5), and washout stages (days 3, 5, and 7). Microbial plate counts were conducted using serial dilutions on different selective media [22], and results were expressed as log CFU/mL. For each sample, aliquots were centrifuged (10,000 rpm, 10 min, 4 °C), and supernatants were filtered (0.22 µm) and frozen at –80 °C for ammonium and SCFA analysis. Pellets were also stored at –80 °C for further metataxonomic microbial characterization.

Study design and early AD patient's assessment

This pilot study lasted from January to December 2023 and involved 22 mild cognitive impairment (MCI) Alzheimer's disease (AD) patients (mean age: 72.38 ± 4.78 years) selected from the patient cohort of the Neurology Department of the Infanta Sofía University Hospital (HUIS) (Madrid, Spain). Subjects underwent clinical and neuropsychological assessment, lumbar puncture MRI scanning, and cerebrospinal fluid (CSF) biomarkers (Aβ-amyloid 40-42, tau protein, and tau phosphorylated at threonine position 181 [tau-p181]) at the HUIS laboratory. The diagnosis of MCI due to AD was based on defined clinical criteria and cognitive status questionnaires, the Mini Mental State Examination (MMSE), and the Free and Cued Selective Reminding Test (FCSRT) [23]. The diagnosis of MCI was further confirmed by the DSM-V criteria: i) self- or informant-reported cognitive complaint; ii) objective cognitive impairment; iii) preserved independence in functional abilities; iv) no dementia. Apolipoprotein E genotyping was performed using two TaqMan allele discrimination assays (C_3084793_20 and C_904973_10, Thermo Fisher Scientific, CA, USA) on the Quantstudio 5 Real-Time PCR Instrument (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Genomic DNA was extracted from peripheral blood using a QIAamp DNA blood minikit (Qiagen AG, Basel, Switzerland). APOE genotyping was performed with polymerase chain reaction amplification and HhaI restriction enzyme digestion.

This prospective study included two patient groups: those who had received Fortasyn Connect-Souvenaid® (125 mL drink, once-a-day, Supplementary Table 1) daily for at least three months beforehand (n = 11), called the AD-SOUV group; and those who had never received it (n = 11), referred to as AD-CONTROL. The administration of the multi-ingredient was supervised by the Neurology Service and nutritionists at the hospital, in accordance with its prescription as a medical food [24]. In terms of exclusion criteria, participants should not have received antibiotics for at least six months before the study, or suffer from type I diabetes, have severe cardiac, endocrine, gastrointestinal disorders, or follow exclusive diets (vegan or vegetarian). In addition, individuals consuming probiotic or prebiotic supplements within the previous six months were also excluded to avoid potential confounding effects on gut microbiota composition. Moreover, subjects were not allowed to use, within 1–2 months before participating in the study and during the study, fatty acid-containing supplements or vitamins B, C, and/or E. The study was approved by the Ethics Committee of the Infanta Sofía University Hospital (Madrid) (Ref 048-2022) and complied with the Declaration of Helsinki and applicable local and European legislation on nutritional products and personal data protection. Written informed consent was obtained from the participants and their caregivers before they participated in the study. Biological samples (blood, stools) were collected from all the participants. Stool samples were stored anaerobically (Anaerocult, Merck Millipore) in hermetically sealed bags at -80 °C until analysis.

In relation to the sample size for this human study, due to the lack of similar studies and the difficulty of finding a confidence interval in microbiome studies – with multiple variables involved – we do not have objective data to make a detailed calculation of the sample size required for each study objective. However, in light of the available studies and interindividual variability, and based on the formula $n = (z^2 x\, \hat{p}(1-\hat{p}))/\varepsilon^2$, where z is the z score, ε is the margin of error, N is the population size, and $\hat{p}$ is the population proportion, for a statistical power

of at least 80%, an absolute error of 15%, and a population percentage of 60% (given that approximately 60% of AD patients take Fortasyn Connect-Souvenaid® in Spain), it is estimated that a minimum of 18 participants is necessary to complete the different objectives of the study. Thus, our sample size of 22 fulfills the minimum necessary conditions to meet the desired minimum statistical power requirements.

Table 1. Clinical and demographic characteristics of early-stage Alzheimer's disease (AD) patients according to Fortasyn Connect-Souvenaid® intake.

Parameter	AD-CONTROL group	AD-SOUV group	*q-value*
General parameters			
Women (n), %	n = 6 (55%)	n = 7 (64%)	-
Men (n), %	n = 5 (45%)	n = 4 (36%)	-
Age (years)	73.82 ± 6.80	70.92 ± 5.34	0.260
Height (cm)	165.72 ± 6.85	163.53 ± 8.15	0.456
Weight (kg)	65.55 ± 10.70	73.09 ± 12.66	0.167
Abdominal circumference (cm)	90.57 ± 14.41	97.00 ± 16.46	0.520
BMI (kg/m^2)	23.79 ± 3.09	26.60 ± 5.10	0.179
Heart frequency (beats/min)	70.29 ± 9.85	70.50 ± 5.95	0.552
Systolic pressure (mmHg)	135.18 ± 8.72	138.62 ± 15.45	0.136
Diastolic pressure (mmHg)	78.45 ± 6.83	82.38 ± 7.39	0.321
Parameters related to AD			
MMSE score	23.14 ± 3.83	24.40 ± 3.23	0.794
FCSRT immediate score	12.57 ± 6.54	14.60 ± 13.21	0.970
FCRST different score	3.57 ± 2.32	6.44 ± 4.79	0.424
APOE ε4 genotype (n (%))	n = 6 (54.5%)	n = 6 (54.5%)	1.000
- ε2, ε3, ε3- ε3 (noncarriers)			
- ε3- ε4 (heterozygote genotype)	n = 5 (45.5%)	n = 5 (45.5%)	1.000
- ε4- ε4 (homozygote genotype)	n = 1 (9%)	n = 1 (9%)	1.000
Lifestyle features			
Work retirement (years)	8.92 ± 7,970	8.83 ± 9,950	0,650
Smoker (n (%))	12 (52,2%)	8 (34,8%)	0,202
Cigarettes/day	8.65 ± 11,250	7 ± 11,930	0,311
Sleep disorders (n (%))	10 (45,5%)	12 (54,4%)	0,763
Weekly frequency of physical exercise			
- Never (n (%))	3 (13,6%)	5 (22,7%)	0,770
- 1 day/week (n (%))	5 (22,7%)	5 (22,7%)	1,000
- 2 times/week (n (%))	2 (9,1%)	3 (13,6%)	1,000
- 3 days or more/week (n (%))	6 (27,3%)	4 (18,3%)	0,770
- Daily (n (%))	6 (27,3%)	5 (22,7%)	1,000
Biochemical parameters			
Glucose (mg/dL)	105.67 ± 30.87	100.10 ± 12.38	0.442
Triglycerides (mg/dL)	86.67 ± 35.25	94.70 ± 21.02	0.352
Total cholesterol (mg/dL)	166.33 ± 44.69	192.50 ± 43.11	0.313
HDL cholesterol (mg/dL)	62.83 ± 18.03	54.70 ± 9.56	0.392
LDL cholesterol (mg/dL)	86.33 ± 35.31	118.90 ± 38.05	0.140
GOT_AST (U/L)	**32.50 ± 18.30**	**20.60 ± 4.98**	**0.079 #**
GPT_ALT (U/L)	32.67 ± 21.83	19.80 ± 5.08	0.187
Gamma_GT (U/L)	15.00 ± 3.56	21.10 ± 9.92	0.376
Alkaline phosphatase (U/L)	64.33 ± 16.98	76.63 ± 17.82	0.315
Calcium (mg/dL)	9.80 ± 0.42	9.64 ± 0.36	0.420
Phosphorus (mg/dL)	3.33 ± 0.19	3.30 ± 0.55	0.991
Iron (µg/dL)	**65.20 ± 14.23**	**93.20 ± 24.01**	**0.040 ***
Proteins (g/dL)	6.85 ± 0.34	7.00 ± 0.37	0.391
Hb A1c (%)	5.48 ± 0.18	10.10 ± 11.66	0.276
Reactive C protein (mg/L)	2.75 ± 4.64	1.89 ± 2.01	0.448
Folic acid (ng/mL)	15.95 ± 6.11	19.21 ± 5.43	0.133
Vitamin B12 (pg/mL)	493.17 ± 160.62	568.2 ± 374.16	0.792
25-OH Vitamin D (ng/mL)	24.24 ± 4.55	32.53 ± 14.7	0.329
Insulin (µUI/mL)	6.68 ± 2.54	8.24 ± 2.47	0.388
Fecal calprotectin	**298.05 ± 127.52**	**141.76 ± 152.01**	**0.020 ***

Data are presented as mean ± standard deviation (SD) or number of individuals (percentage). Statistically significant differences between the AD-SOUV and AD-CONTROL groups were assessed using the Mann-Whitney U test and Benjamini-Hochberg multiple testing correction for multiple comparisons (FDR = 0.05). Symbols indicate significance levels: *q-value* < 0.05 (*), *q-value* < 0.1 (#).

General evaluations, blood biochemistry and faecal inflammatory biomarkers in AD patient's

For all participants, serum and plasma biomarkers were measured using an automated biochemical auto-analyzer. The test included measuring glucose levels, cholesterol and triglycerides, biomarkers of liver inflammation, folic acid, and vitamins D and B12, and insulin, among others (Table 1). Fecal calprotectin was determined by quantitative enzyme-linked immunosorbent assay (ELISA) (Bühlmann Laboratories) immediately after collection. All determinations were carried out at least in duplicate. Participants also completed a comprehensive questionnaire to gather data on socio-demographics, polypharmacy and medical history.

Diet assessment

The participants provided detailed data on their diet via a food frequency questionnaire (FFQ) and a three-day complete food record. Based on data from the three-day complete food questionnaire, concentrations of macro- and micronutrients were obtained, using PCN Pro©UB CESNID 1.0 software (University of Barcelona), and the calculations of the MIND diet adequacy scores were performed [25]. The adherence to a Mediterranean diet was assessed using the Mediterranean Diet Adherence Screener (MEDAS) questionnaire. The questionnaire comprises 12 questions on food consumption frequency and two questions on food intake habits that are considered characteristic of the Spanish Mediterranean diet [26]. Each question/answer was scored either 0 or 1. The following criteria were used to evaluate the participants' diets; if a participant did not meet the criteria, 0 points were recorded for that category.

Fecal microbiota analysis

Total DNA extraction

Human fecal solutions were prepared with 0.8 gram of fecal samples diluted in 1/10 mL of PBS. After a vigorous homogenization, samples were centrifuged at 800 rpm for 10 min at 4 °C to remove fecal debris, and the resulting supernatant was then centrifuged at 10000 rpm for 7 min at 4 °C to pellet bacterial content. The remaining fecal supernatant was filtered by 0.22 µm filter and stored at -80 °C until metabolite analysis. Total genomic DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen, Germany), following the manufacturer's recommendations, with some modifications as described [27]. The DNA concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA) and by fluorimetry using Qubit 4 (Invitrogen, Thermo-Scientific). After extraction, the DNA was stored at -20 °C until metataxonomic analysis.

16S gene sequencing and processing

Fecal samples were analysed by 16S rRNA gene sequencing, as previously described [28], with an Illumina® MiSeq instrument (Illumina®, USA). RStudio 2023.06.2 software was used to process the files with raw reads. The fastqc files were filtered for low-quality reads and the presence of alien DNA using DADA2. The DADA2 algorithm was also employed to denoise, join paired-end reads, and filter out chimeras in the raw data, following standard pipeline published on (https://benjjneb.github.io/dada2/tutorial.html). This algorithm enables the differentiation of even a single nucleotide, helping to form amplicon sequence variants (ASVs). Taxonomic assignment was performed using the naïve Bayesian classifier implemented in DADA2 using Silva v.138.1 as a reference database [29]. A total of 1695 ASVs were found. Biodiversity, expressed in terms of alpha diversity, was estimated using ASVs by calculating the Observed, Shannon, and Simpson indices through the "Phyloseq" package. Beta diversity was evaluated using a Bray-Curtis dissimilarity matrix represented by principal coordinates analysis (PCoA), based on weighted unifrac distances, or nonmetric multidimensional scaling (NMDS) as suitable.

qPCR analysis for quantitative assessment of main microbial populations

Samples from simgi® colonic compartments were also evaluated by qPCR quantitative assay, in order to obtain a quantitative and a qualitative image of the colonic microbial populations and their changes during the process. qPCR analysis was performed using a ViiA7 Real-Time PCR System (Applied Biosystems). Primers for different microbial groups were evaluated and the conditions are shown in Supplementary Table 2 [30, 31]. To generate the standard curve for each group, a representative type strain was selected for each group, and serial dilutions (1/10) of the complete sequence of the 16S rDNA gene were performed (obtained from the NCBI database and purchased from Twist Bioscience). Type strains used for the standard curves are shown in Supplementary Table 3. All qPCR reactions were performed in sealed 384-well optical plates with MicroAmp optical coverslips (Applied Biosystems, USA). The final volume of each reaction was 10 µL, containing 5 µL of SYBR Green PCR Master Mix (Applied Biosystems), 0.3 µL of each 10 µM primer (Sigma-Aldrich, United States), 3.4 µL of Sigma water (Sigma-Aldrich, United States), and 1 µL of template

DNA. The amplification program consisted of a first cycle at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 1 min at the appropriate temperature of each primer. Results were obtained in 16S rRNA gene copy number/mL. In order to compare the data with the microbial plate count data, this value was converted to log10 bacteria/mL using the mean number of 16S rRNA gene copies for each group analyzed, with the information being obtained from the EzBioCloud database.

Fecal microbial metabolism

As an approximation of microbiota functionality, levels of SCFAs in fecal samples from patients and simgi® colonic compartments were evaluated. Specifically, the levels of acetic, butyric, propionic, iso-butyric, valeric, and iso-valeric acids were quantified by GC–MS as previously described [27]. However, since no significant differences or interesting trends were obtained with isobutyric, isovaleric, valeric, caproic, and heptanoic acids, only acetic, propionic, and butyric acids were reported. SCFA analysis was performed in triplicate, and the results were averaged.

To assess the protein metabolism levels of the microbiota, ammonium ion production in simgi® colonic compartments was analyzed using a Spectroquant Ammonium Test Kit (Merck, Germany) [21].

Faecal lipids analysis

Lipids extraction

Lipids were extracted from the thawed sample of fecal material of the patients according to previously described [32], using methanol: dicloromethane: water (2:2:1), then vortexed and recovered into the organic part. Fat content was gravimetrically determined.

Lipid classes analysis and cholesterol metabolites

The analysis of lipid classes and cholesterol metabolites was firstly performed by Thin Layer Chomatography [33]. Briefly, TLC plates (Silica gel on TLC plates, 20 cm x20 cm, Merck) were prewashed with methanol and subsequently with the mobile phase (hexane: ether: AcOH, 80:20:2). The plates were activated in an oven at 120ºC for one hour. Lipids classes were revealed into a 10% copper sulfate in 8% phosphoric acid solution and then, heated at 160 ºC for 10 minutes. Calibration graphs were determined for each lipid classes. Cholesterol and their metabolites coprostanol, 24- and 25-hydroxycholesterol, cholesteryl stearate, 1,2,3-trihexadecanoylglycerol (TG standard) and oleic acid (FFA ester), were acquired from Larodan (Larodan, Sweden).

Medium and Long Chain Fatty Acids composition

Fatty acids were analysed by GC-FID. Lipids extracted were transesterified according as stated [34]. Briefly, lipids were methylated according to Lepage and Roy and analysed by gas chromatography (GC/FID, Clarus 500, Perkin–Elmer) [32, 35]. The separation of fatty acid methyl esters was achieved on a SP-2330 fused silica capillary column (30m x 0.25mm i.d., 0.20µm; Supelco Inc., Bellefonte, USA). The oven temperature was set at 140 ºC and programmed to 205 ºC at 1 ºC/min rate. The injector and detector temperatures were maintained at 275 and 260 ºC, respectively. All chemicals used were of analytical grade and water was purified on a Milli-Q system (Millipore, Billerica, MA).

Analysis of fecal lipid peroxidation

The resulting lipid organic fraction (5 µg per sample) from Bligh and Dyer extraction described above was used to measure fecal levels of 4-HNE, 4-HHE, MDA and oxPL via dot-blot immunoassays, as previously reported [32]. The oxPL antibody was obtained from Avanti® Polar Lipids, Inc. (Croda International Plc, Birmingham, AL, USA), while all other primary and secondary antibodies were purchased from Antibodies.com (Europe AB, Stockholm, Sweden).

Analysis of fecal proteome

Fecal Protein Extraction and fractionation in SDS-PAGE

Fecal proteins were extracted from post-lipid-extraction pellets using urea buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 0.4% DTT), sonicated on ice, centrifuged, and quantified by the Bradford method. Ten micrograms of protein were separated by 15% SDS-PAGE and Coomassie-stained. Each Coomassie-stained gel lane, corresponding to an individual sample, was divided into six fractions. Each gel fration was diced into 1 mm^3 cubes and digested with trypsin overnight for LC-MS/MS analysis [32].

LC-MS/MS Analysis of human fecal proteome

Tryptic-digested fractions (six per individual) were analyzed using a nano-LC ESI-IT-MS/MS system consisting of a Dionex UltiMate 3000 chromatograph coupled to an LTQ Velos Pro mass spectrometer equipped with electrospray ionization (Thermo Fisher Scientific, Rockford, IL, USA). Prior to separation, peptides were

concentrated and washed on a µ-Precolumn C18 PepMap using 0.1% formic acid in water as the mobile phase. Peptide separation was achieved on an analytical C18 column (Acclaim PepMap RSLC C18) with a 90-minute linear gradient from 5% to 35% acetonitrile containing 0.1% formic acid, at a flow rate of 300 nL/min. Mass spectrometric analysis was performed in data-dependent acquisition mode, with MS1 scans acquired over a 400–2000 Da range, followed by MS2 analysis of the ten most intense precursor ions (charge ≥ +2) using collision-induced dissociation at 35% normalized collision energy, a 2 Da isolation width, and a 30-second dynamic exclusion. Protein identification was carried out with the PEAKS Studio software suite, searching against the UniProt/Swiss-Prot Homo sapiens database. Search parameters included trypsin specificity, oxidation of methionine, histidine, and tryptophan as variable modifications, allowance for up to two missed cleavages, and mass tolerances of ±1 Da, for precursor ions, and ±0.6 Da for product ions. Proteins were accepted when the false discovery rate was below 1%. The qualitative fecal proteome of each individual was obtained by combining results from all six fractions, and comparative analyses were performed between the AD-CONTROL and AD-SOUV groups. For relative quantification, a label-free spectral counting approach was employed, comparing the number of MS/MS spectra assigned to each protein detected in at least 80% of individuals in both group

Statistical analysis

Data derived from *in vitro* gastrointestinal simulation (plate counts, qPCR counts, 16S rRNA gene profiling, and SCFAs) were analyzed using XLSTAT software. Due to the nature of the data, a two-way ANOVA test and the Games-Howell post hoc test were used for all statistical analyses to study differences between samples at each time point and in different colonic compartments. Significant differences were determined considering an adjusted *p-value* (*q-value*) of < 0.05 with the XLSTAT Statistic software. Further, from a microbiological perspective, plate counting values were considered significantly different when $\Delta\log(\text{CFU/mL}) \geq 1$, due to plate counting limitations [31].

For clinical observational study, different approaches were used. Data from blood biomarkers and lifestyle questionnaires were analyzed with the Mann-Whitney U test and a Z-test statistic for proportions, respectively, using XLSTAT Statistic software for Microsoft Excel, 2022.3.1 (Addinsoft-SARL, USA).

All statistical analysis to evaluate potential changes in the gut microbiota of AD patients related to Souvenaid® intake (AD-CONTROL vs. AD-SOUV), as well as on lipidomic and proteomic profiles were performed in RStudio v.2023.06.2 with R v.4.3.1. Non-parametric Mann-Whitney U analysis was employed to detect significant differences between alpha diversity values, with Benjamini–Hochberg correction applied (False Discovery Rate, FDR = 0.05). Compositional differences between samples (beta diversity) were assessed by permutational multivariate analysis (PERMANOVA) belonging to the "vegan" (v.2.6-4) package, with Holm's multiple comparison testing correction applied by default. Differential abundance analysis of bacterial taxa were conducted using "Microbiomemarker" (v0.99.7) and "yingtools2" (v0.0.3) R packages, applying three complementary statistical approaches: ANCOM (analysis of composition of microbiomes)[36], metagenomeSeq [37] and LefSE [38], each implemented with its recommended normalization procedure (Cummulative Sum Scaling (CSS) for metagenomeSeq, Total Sum Scaling (TSS) for LefSE followed by a log10 transformation, and TSS for ANCOM). Multiple testing correction was performed using the Benjamini-Hochberg method with a false discovery rate (FDR) of 0.05. Taxa were considered differentially abundant if they were significant (*q-value* < 0.05) in at least two of the three methods and exhibited an absolute $\log_2$ fold change (log2FC)> 2. Prior to analysis, features with prevalence below 10% across samples were removed to reduce sparsity. All analyses were conducted on the original count data without rarefaction. Differences between AD-SOUV and AD-CONTROL patients in lipidomic, proteomic and SCFAs profiles were assessed using Mann-Whitney U with Benjamini-Hochberg correction for multiple comparisons (FDR = 0.05), considering *q-value* < 0.05 as statistically significant.

Data availability

The FASTQ files for the human fecal samples used in this study were uploaded to the Sequence Read Archive (SRA) and are included in a larger project, under the BioProject accession number PRJNA1099418. Data can be accessed through the following link: https://dataview.ncbi.nlm.nih.gov/object/PRJNA1099418?reviewer=6qa040hcn6pod4jvr8ch7oskbl. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [39] partner repository with the dataset identifier PXD069669 and 10.6019/PXD069669.

RESULTS

Gastrointestinal digestion of the supplement in the simgi® simulator

Initially, using the *simgi*® simulator, we assessed the direct effects of Fortasyn Connect on the gut microbiota, excluding systemic host interactions. After microbiota stabilization, multinutrient feeding induced compartment- and time-dependent changes in microbial counts. Notably, *Bifidobacterium* spp. levels increased across all colon regions, with additional shifts observed in the ascending colon (AC). No significant changes were found in the transverse (TC) and descending colon (DC) compartments, except for the consistent rise in *Bifidobacterium* spp (Supplementary Table 4).

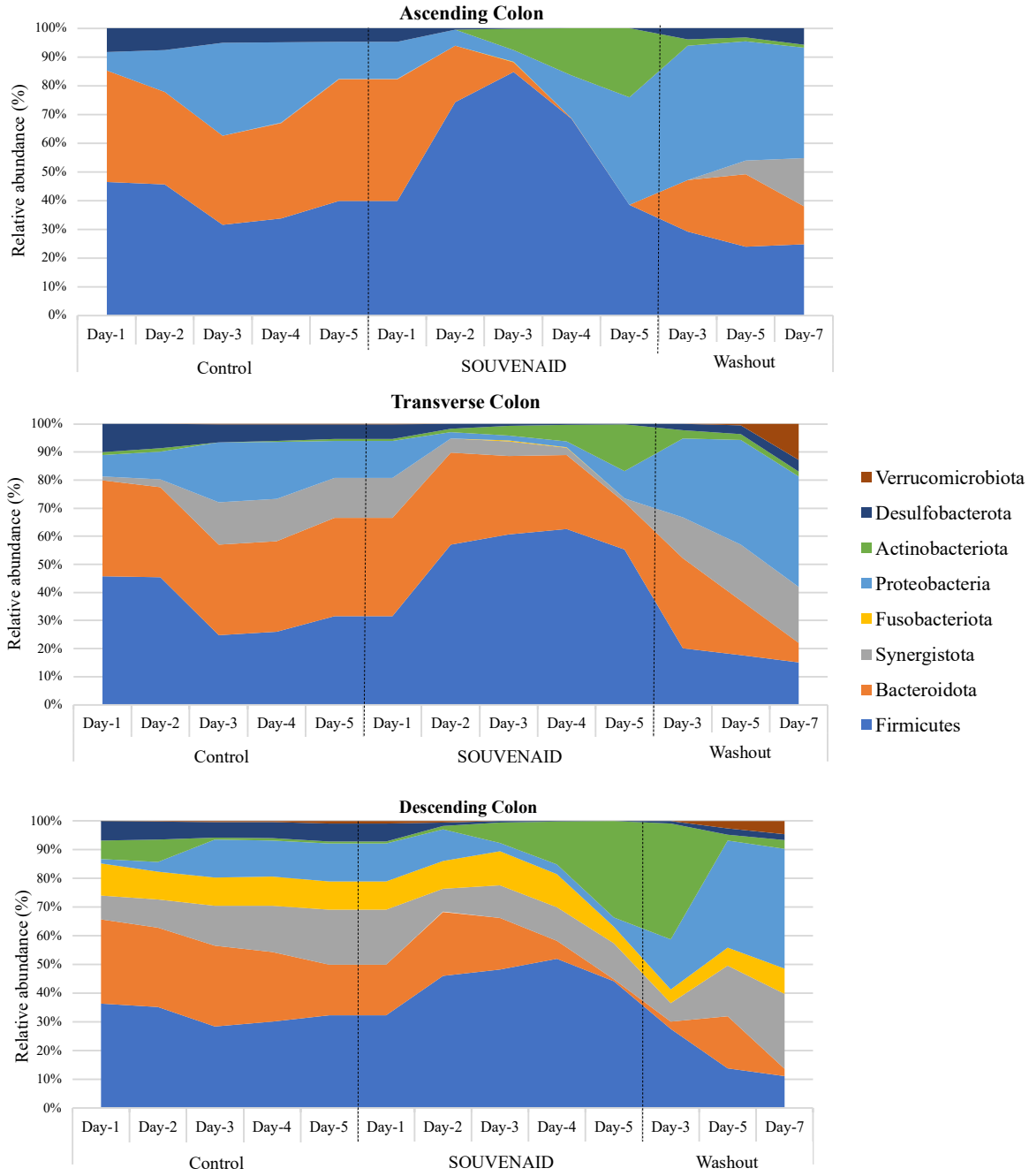

Figure 2. Phylum-level microbial changes in response to Fortasyn Connect-Souvenaid®. Evolution of the relative abundance (%) at phylum level for the different simgi® colonic compartments during stages of colonic fermentation: control, Fortasyn Connect feeding, and washout. Graphs include the taxa with a relative abundance > 0.5% in at least one of the time points of one simgi® colonic compartment.

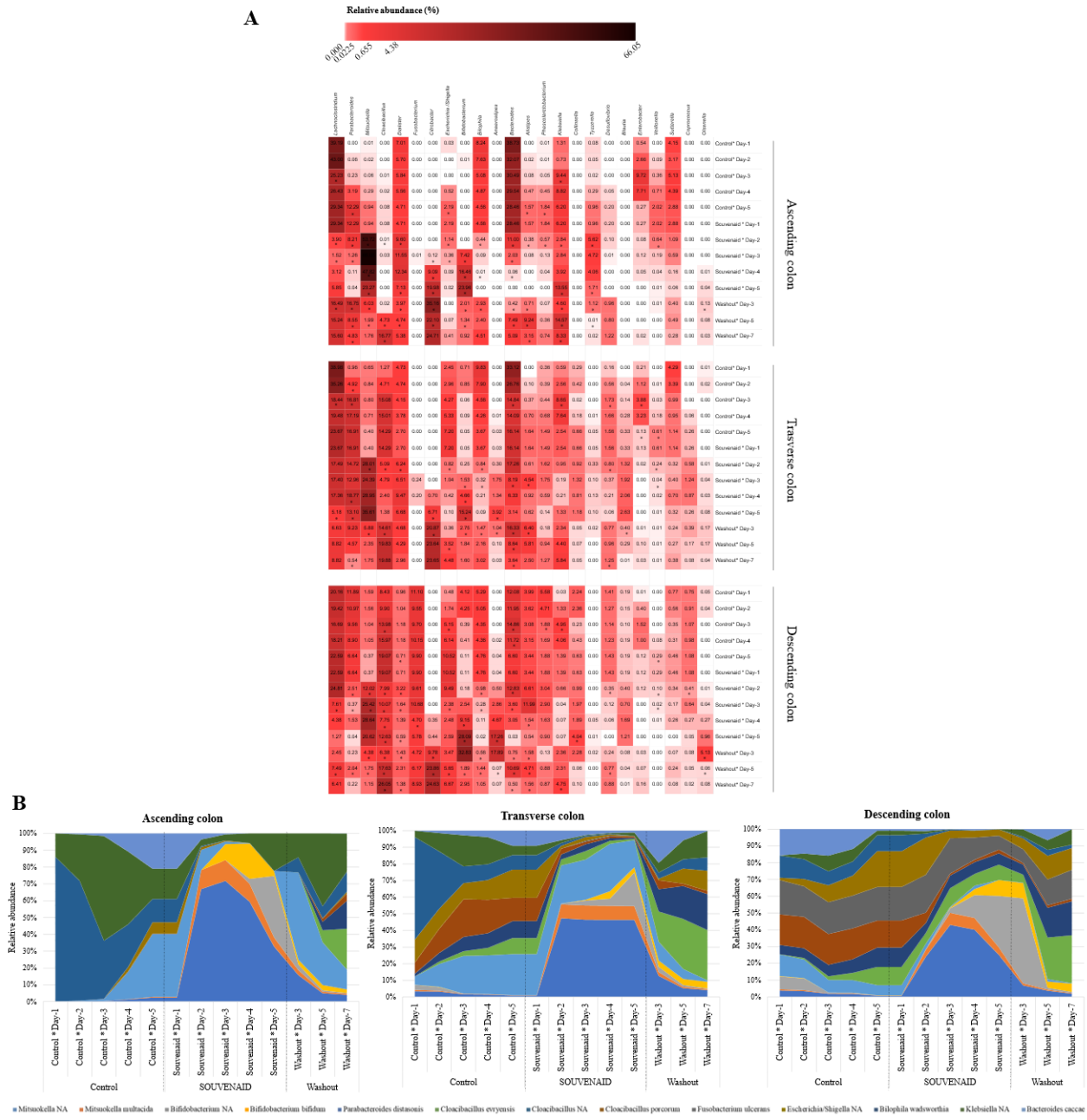

Figure 3. **Genus- and species-level shifts during multinutrient colonic fermentation. (A)** Heatmap representing evolution of the relative abundance (%) at genus level for the different simgi® colonic compartments during stages of colonic fermentation: control, Fortasyn Connect feeding, and washout. The graph includes taxa with a relative abundance > 0.5% at any time point in at least one simgi® compartment. Statistically significant differences with respect previous timepoint are highlighted with an asterisk (*; adjusted *p-value* < 0.05). **(B)** Evolution of the relative abundance (%) at species level for the different simgi® colonic compartments during stages of colonic fermentation: control, feeding, and washout stages. Only species properly identified and/or especially relevant due to their relative abundance > 5% are shown.

Metataxonomic analysis through 16S rRNA gene profiling confirmed the effects on colonic microbial communities by Fortasyn Connect. While alpha diversity remained unchanged across the control, intervention, and washout phases (Supplementary Fig. 1A), beta diversity revealed compartment-specific shifts in microbial composition (Supplementary Figure 1B). In the ascending colon (AC), the multinutrient increased Firmicutes and Actinobacteriota phyla while reducing Bacteroidota and Proteobacteria, with a concurrent decline in Fusobacteriota in the descending colon (DC) (Fig. 2, Supplementary Table 5). These effects diminished after washout, although Bacteroidota levels remained low in TC and DC. At the genus level, the medical food reduced

potentially proinflammatory taxa (e.g., *Klebsiella, Escherichia/Shigella, Bilophila, Bacteroides*), and increased beneficial genera such as *Bifidobacterium, Anaerostipes, Mitsuokella* and *Dialister*. Also, *Fusobacterium* levels showed a decrease in the DC compartment. Partial reversion occurred post-intervention, with a rise in *Citrobacter, Olsenella, Desulfovibrio* and *Alistipes* (Fig. 3A, Supplementary Table 6).

At the species level, multinutrient intervention in the simgi® model led to a significant increase in *Mitsuokella multacida*, *Bifidobacterium bifidum*, and unidentified species of *Bifidobacterium* and *Mitsuokella*, along with decreased levels of *Parabacteroides distasonis*, *Bacteroides caccae*, and *Bilophila wadsworthia* in the AC compartment (Fig. 3B). Similar trends were observed in TC and DC, including reductions in *Cloacibacillus spp.*, *Escherichia/Shigella* (NA) and *Fusobacterium ulcerans*. These effects largely diminished during the washout phase.

Quantitative qPCR confirmed increased absolute abundance of *Bifidobacterium* and *Lactobacillus,* especially in AC, and a reduction in *Enterobacteriaceae*, *Enterococcaceae*, *Bacteroides*, and *Clostridium cluster XIVa* after suplementation. These microbial shifts were partially reversed during the seven-day washout, underscoring that the observed effects are directly dependent on the continued intake of the multinutrient intervention (Supplementary Fig. 2).

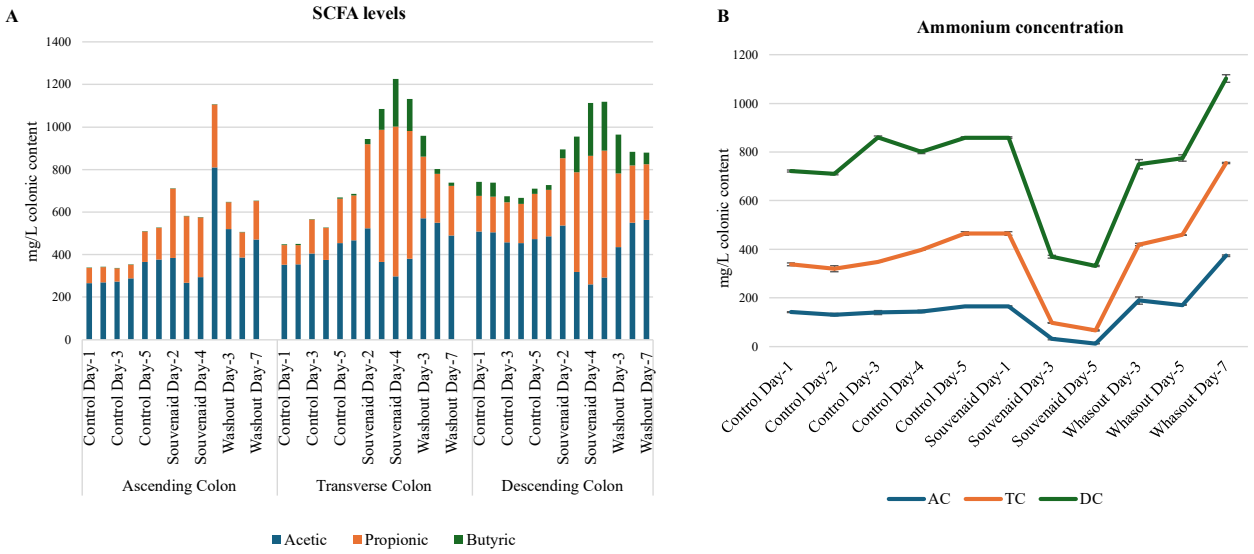

Figure 4. Evolution of the concentration of main short-chain fatty acids (SCFAs) and ammonium production in colonic fermentation compartments of simgi®. (A) SCFA concentration (mM) in the different colon compartments at each time point; **(B)** Ammonium concentration (mg/L) in the different colon compartments at each time point.

It is worth noting that multinutrient administration also modulated microbial metabolic activity in the *simgi®* model. Specifically, it increased the production of key short-chain fatty acids (SCFAs), acetate and propionate in the AC, and butyrate in TC and DC, reaching levels >100 µM after several days (Figure 4A). Concurrently, ammonium production, a marker of protein fermentation, was notably reduced during the intervention across compartments (Fig. 4B). These functional effects were progressively reversed during the washout phase, further confirming that the metabolic modulation observed is dependent on the sustained intake of Fotasyn Connect. Together, these *in vitro* findings indicate that multinutrient supplementation influences both the composition and functionality of the colonic microbiota, independent of interactions with the host.

Exploring differences between supplemented and non-supplemented early AD patients

Clinical and lifestyle factors of AD patients

Clinical evaluation of early-stage AD patients (Table 1) revealed no major differences between the Fortasyn Connect-Souvenaid® (AD-SOUV) and control (AD-CONTROL) groups, except for higher iron levels in the AD-SOUV group (adjusted *p-value (q-value)* < 0.05). Additionally, AD-SOUV patients showed significantly lower fecal calprotectin levels (*q-value* < 0.05), suggesting reduced intestinal inflammation, and a trend toward lower aspartate aminotransferase (AST/GOT) levels (*q-value* < 0.1), indicative of possible attenuation of liver inflammatory markers. No significant differences in the presence of comorbilities or in medication use were

observed between patients from both groups (Supplementary Table 7).

Dietary intake was assessed for both groups using a 115-item semiquantitative food frequency questionnaire (FFQ) that had been previously validated [40]. Consumption frequencies were measured in nine categories (ranging from "never or almost never" to "more than six servings daily"), with each item accompanied by a standard portion size. Daily intake for each food was estimated by multiplying the portion size by its reported frequency of consumption. No statistically significant differences between groups were found in the daily consumption of most food items (Supplementary Table 8). Adherence to the MIND diet (Mediterranean-DASH Intervention for Neurodegenerative Delay) [25] and the Mediterranean diet [26] was also assessed using validated scoring systems. While no significant differences in nutrient intake were found between groups (Table 2), a trend toward higher MIND and MEDAS scores was observed in the AD-SOUV group (*q-value* < 0.1), although the absolute differences were minimal.

Table 2. Daily intake of macro- and micronutrients, and adherence scores to the MIND and MEDAS diets in early stage AD patients according to Fortasyn Connect-Souvenaid® intake.

Food group	AD-CONTROL group	AD-SOUV group	*q-value*
Energy (kcal/day)	2479.58 ± 287.37	1975.82 ± 603.43	0.669
Edible portion (g/g raw food)	22.7 ± 4.52	19.51 ± 5.68	0.417
Total dietary fiber (g/day)	40.12 ± 28.41	20.41 ± 10.53	0.417
Total protein (g/day)	119.25 ± 29.7	91.41 ± 25.71	0.740
Animal protein (g/day)	79.61 ± 26.07	65.07 ± 20.82	0.740
Vegetal protein (g/day)	39.64 ± 11.25	26.35 ± 10.11	0.270
Total carbohydrates (g/day)	225.07 ± 32.58	189.33 ± 64.44	0.740
Digestible polysaccharides (g/day)	131.85 ± 17.29	93.98 ± 27.46	0.270
Digestible sugars (g/day)	93.26 ± 28.93	95.38 ± 40.85	0.230
Total carotenoids (µ/day)	2802.55 ± 844.56	3395.62 ± 2759.81	0.813
Total retinoids (µg/day)	387.71 ± 86.58	295.63 ± 162.35	0.601
Cholesterol (mg/day)	572.23 ± 231.93	393.82 ± 250.45	0.417
Monounsaturated fatty acids (g/day)	53.81 ± 9.8	41.59 ± 16.33	0.536
Polyunsaturated fatty acids (g/day)	18.18 ± 4.51	14.51 ± 6.99	0.813
Saturated fatty acids (g/day)	34.52 ± 11.52	27.08 ± 11.27	0.417
Total lipids (g/day)	116.39 ± 24.14	90.90 ± 34.92	0.887
Ethanol (g/day)	5.16 ± 8.51	2.69 ± 3.57	0.984
Calcium (mg/day)	1109.67 ± 345.43	978.78 ± 593.89	0.669
Iron (mg/day)	17.79 ± 3.3	14.74 ± 8.97	0.475
Magnesium (mg/day)	495 ± 92.3	390.09 ± 184.24	0.601
Phosphorus (mg/day)	1830.91 ± 198.01	1509.34 ± 670.64	0.740
Potassium (mg/day)	4326.25 ± 860.11	3646.43 ± 1841	0.813
Sodium (mg/day)	7484.58 ± 640.47	6821.14 ± 5167.95	0.161
Zinc (mg/day)	11.99 ± 1.26	9.50 ± 2.87	0.270
Folic acid (µg/day)	357.63 ± 114.01	253.48 ± 109.56	0.475
Total Vitamin A (µg/day)	854.09 ± 193	1694.6 ± 2182.94	0.887
Vitamin B1 (mg/day)	2.31 ± 1.19	2.56 ± 1.27	0.740
Vitamin B2 (mg/day)	2.28 ± 0.39	2.93 ± 1.15	0.740
Vitamin B12 (µg/day)	6.57 ± 2.64	7.05 ± 2.88	0.601
Vitamin B6 (mg/day)	2.32 ± 0.25	2.50 ± 1.61	0.813
Niacin (vit. B) (mg/day)	28.99 ± 7.9	29.97 ± 11.33	0.962
Vitamin D (mcg/day)	10.11 ± 9.53	10.18 ± 10.89	0.962
Vitamin E (mg/day)	19.35 ± 9.55	17.28 ± 10.62	0.740
Vitamin C (mg/day)	117.02 ± 45.66	123.85 ± 62.91	0.669
Water (g/day)	1823.11 ± 461.02	1635.33 ± 508.92	0.364
Adherence to MIND score	**7.28 ± 1.16**	**7.71 ± 1.36**	**0.092 #**
Adherence to MedDiet (MEDAS) score	**8.00 ± 2.81**	**9.11 ± 2.23**	**0.085 #**

Dietary intake was assessed using a validated 115-item semiquantitative food frequency questionnaire and complemented with three-day food records. Nutrient intake estimations were obtained using PCN Pro©UB CESNID 1.0 software. Statistical differences between AD-CONTROL and AD-SOUV groups were assessed with the Mann–Whitney U test and corrected for multiple comparisons using the Benjamini–Hochberg method (FDR = 0.05). Symbols indicate significance levels: *q-value* < 0.05 (*), *q-value* < 0.1 (#).

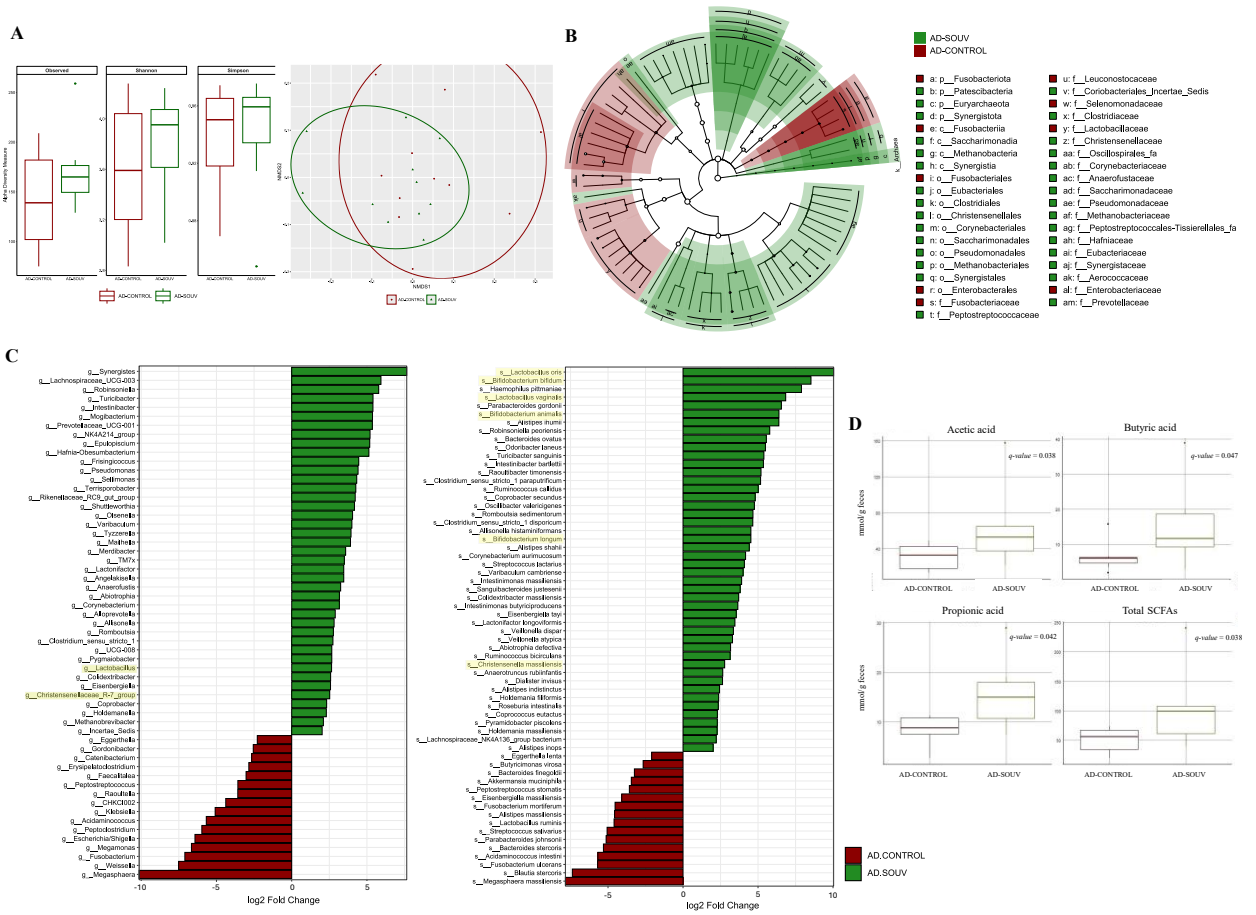

Figure 5. Gut microbiota diversity and functional shifts in early AD with Fortasyn Connect-Souvenaid®. (A) Biodiversity indexes, in terms of alpha and beta diversity (NMDS), in fecal samples from AD-CONTROL and AD-SOUV groups. Statistical significance was assessed by Mann-Whitney U test and Benjamini-Hochberg correction (FDR = 0.05) for alpha-diversity metrics, and by a permutational multivariate analysis of variance (PERMANOVA) test for beta-diversity analysis. **(B)** Cladogram representing differential abundant taxa at phylum, class, order and family levels. **(C)** Differential abundance analysis at genus and species level. Differential analysis was assessed by ANCOM, MetagenomeSeq, and LEFSe methods (*q-value* < 0.05, log2FC < 2 or < -2), considering significant those taxa statistically different by at least two methods. **(D)** Boxplots representing SCFA levels (mmol/g feces) in AD patients depending on multinutrient intake. Statistical significance was assessed by Mann-Whitney U test, considering significant results with a *q-value* < 0.05 after correcting by Benjamini-Hochberd posthoc (FDR=0.05).

Gut microbiota composition and functionality of AD patients

To evaluate the impact of multinutrient intake on the gut microbiota, fecal samples from AD patients were analyzed by 16S rRNA sequencing. Differential microbial patterns were observed between the AD-SOUV and AD-CONTROL groups. Comparison of biodiversity parameters did not show significant differences (*q-value* < 0.05) (Fig. 5A and B) (PERMANOVA *p-value* = 0.714, R^2 = 0.0434). However, a trend towards lower levels of alpha-diversity metrics was observed in AD-CONTROL patients, together with a clustering among AD-SOUV participants, indicating a more homogeneous microbial profile (Fig. 5B).

A differential abundance analysis was performed using ANCOM, MetagenomeSeq, and LEFSe approaches, identifying 231 differential abundant taxa between groups, of which 166 were accurately classified across the three approaches at the taxonomic level (*q-value* < 0.05 and log2FC > 2 or < -2, statistically significant by at least two methods). With regard to AD-SOUV patients, the results revealed higher levels of *Christensenellaceae* members such as *Christensenella massiliensis* or the *Christensenellaceae* R-7 group, as well as of different species from the *Bifidobacterium* and *Lactobacillus* genus, especially *Bifidobacterium bifidum*, *B. animalis*, and *B. longum* (Fig. 5C). Moreover, *Lachnospiraceae* members and other related SCFA producers such as *Ruminococcus* and UCG-008 were also

prominent in AD-SOUV patients. Conversely, AD-CONTROL patients exhibited higher relative abundances of *Streptococcus salivarius*, as well as *S. parasanguinis* and *Dialister succinatiphilus*, two species of the *Fusobacterium* genus, and members of *Escherichia/Shigella*, *Weisella*, *Megamonas* and *Peptostreptococcus*, among others (Fig. 5C). SCFAs, as key microbial metabolites, in faeces were also quantified. Patients receiving the multinutrient showed higher levels of acetic, propionic, and butyric acids (*q-value* < 0.05), as well as higher total SCFA levels (Fig. 5D). Therefore, based on these results, we conceived a more in-depth study of the fecal lipid and protein profiles to investigate how all these changes associated with Fortasyn Connect-Souvenaid® supplementation may relate to the host physiological state.

Addressing fecal lipid and protein profiles in supplemented and non-supplemented early AD patients

To explore the potential intestinal effects of multinutrient supplementation in early AD, we conducted a targeted analysis of fecal lipid and protein profiles. The lipid assessment included total fat content, fatty acid composition, and key lipid classes, as well as markers of lipid peroxidation. In parallel, the fecal proteome was characterized both qualitatively and quantitatively to investigate host-derived proteins linked to intestinal function.

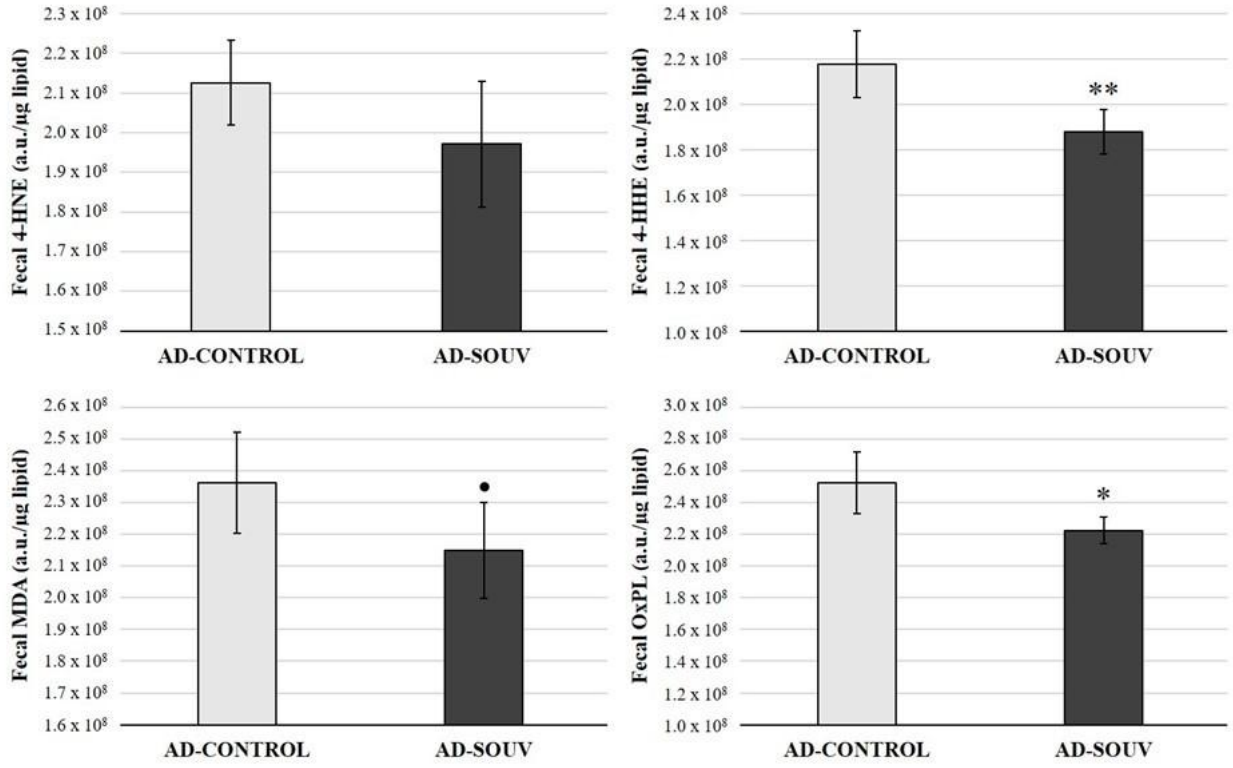

Figure 6. Fecal levels of 4-hydroxynonenal (HNE), 4-hydroxyhexenal (HHE), malondialdehyde (MDA) and oxidized phospholipids (oxPL) in AD-CONTROL and AD-SOUV groups. Data are expressed in arbitrary units (a.u.). Statistical significance was assessed by Student's t-test. • *p-value* < 0.1; * *p-value* < 0.05; ** *p-value* < 0.01. a.u.: arbitrary units.

Lipids in faeces

No statistically significant differences were observed between AD-SOUV and AD-CONTROL groups (p = 0.518), although a trend towards lower fat excretion was detected in the supplemented group (4.64 ± 1.79% vs. 5.17 ± 2.00% of fat content in feces, respectively). Similarly, there were no significant differences in the fatty acid composition of fecal lipids between the two groups (Supplementary Table 9). However, a general trend toward lower amounts of fecal fatty acids was observed in AD-SOUV patients (23.71 ± 19.60 in the AD-SOUV group vs. 24.62 ± 13.80 mg fatty acids/g feces in the AD-CONTROL group), particularly for several ω-3 PUFAs, including alpha-linolenic acid (ALA, 18:3 ω3), eicosapentaenoic acid (EPA, 20:5 ω3), docosapentaenoic acid (DPA, 22:5 ω3), and docosahexaenoic acid (DHA, 22:6 ω3). These findings suggest improved absorption and/or metabolic utilization of these components in the group receiving the multinutrient supplementation.

The main fecal lipid classes were triglycerides (TG), free fatty acids (FFA), cholesteryl esters (ChEst), cholesterol and coprostanol (Supplementary Table 10). A strong positive correlation was found between the amount of excreted fat and the total saponifiable lipid content (TG + FFA + ChEst, *p-value* < 0.001), as well as with the absolute amount of fecal fatty acids (*p-value* < 0.001). Conversely, a significant negative correlation was observed between fat content and cholesterol (*p-value* < 0.003) and coprostanol (*p-value* < 0.002). These results indicate that increased fecal lipid content is associated with higher levels of free and esterified fatty acids, consistent with fat malabsorption caused by impaired digestion and/or absorption. Notably, the percentage of FFA relative to total fecal lipids was lower in AD-SOUV patients (34.9 ± 16.3%) compared to AD-CONTROL (40.4 ± 23.6%). A similar trend was observed for cholesteryl esters, with higher values in the control group than in the supplemented group (7.80 ± 1.25% vs. 5.63 ± 1.26%, respectively), further suggesting more efficient lipid digestion and absorption in AD-SOUV patients. Moreover, the coprostanol/cholesterol ratio was higher in the control group (8.28 ± 0.82 µg coprostanol/µg cholesterol) than in patients receiving the multinutrient (6.65 ± 0.72 µg coprostanol/µg cholesterol), indicating greater microbial conversion of cholesterol to coprostanol in the control group.

Lipid peroxidation products in faeces

Lipid peroxidation products were quantified in fecal samples to evaluate the extent of oxidative stress occurring in the intestinal tract. As shown in Figure 6, several markers derived from the non-enzymatic oxidation of PUFAs were analyzed, including 4-hydroxynonenal (4-HNE), 4-hydroxyhexenal (4-HHE), malondialdehyde (MDA), and oxidized phosphatidylcholines (oxPLs). The results revealed that AD patients receiving the multinutrient intervention exhibited lower levels of lipid peroxidation products in feces compared to controls. This reduction was particularly significant for 4-HHE and oxidized phospholipids (*p-value* < 0.05), with a clear trend toward reduced MDA levels as well (*p-value* < 0.1). These findings suggest that the multinutrient supplementation may help reduce intestinal oxidative stress in early Alzheimer's disease, potentially through improved antioxidant status or modulation of lipid metabolism.

Fecal proteome and functional intestinal outcomes

A proteomic approach was additionally employed to evaluate the intestinal environment in AD patients, providing further insights into the metabolic and functional state of the gut. The overall fecal protein profiles from both AD-CONTROL and AD-SOUV individuals are visualized in Supplementary Figure 3. In general, the lanes corresponding to AD-SOUV individuals (lanes 6–13) appear more homogeneous and suggest improved protein digestion, as evidenced by the absence of distinct protein bands throughout the lanes. The fecal proteome analysis of the entire studied individuals yielded a total of 437 proteins of human origin, with 239 proteins identified in the AD-CONTROL group and 359 proteins identified in the AD-SOUV group. The average of proteins identified in each fecal sample was 98.60 ± 14.08 in patients belonged to the AD-CONTROL group and 118.00 ± 38.64 proteins in patients included in the AD-SOUV group.

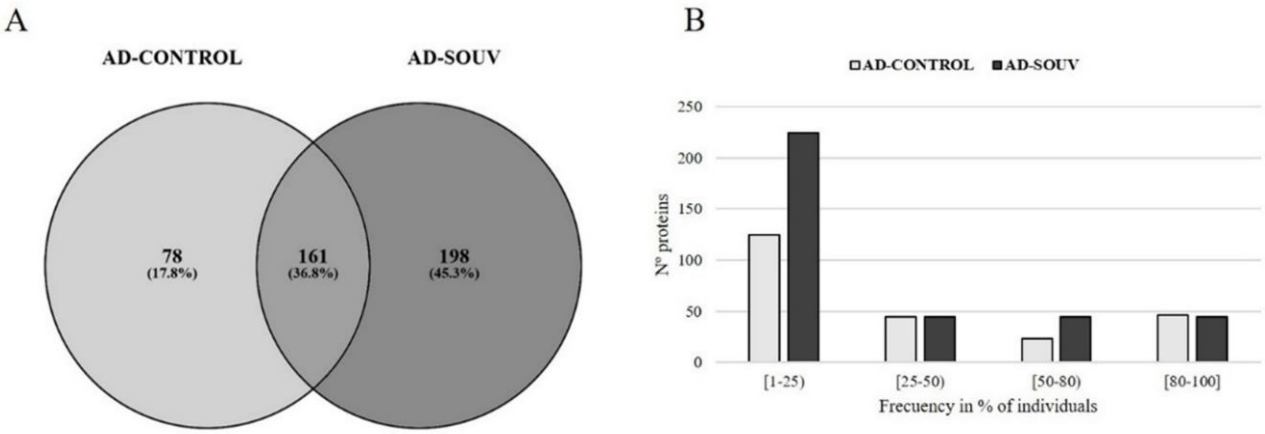

Figure 7. Human proteins identified in fecal samples. (A) Venn diagram showing the number of common and unique proteins identified in the two groups, AD-CONTROL and AD-SOUV. **(B)** Frequency histogram showing the distribution of proteins based on their occurrence across individuals within each group, expressed as the percentage of individuals in whom each protein was detected.

As shown in Figure 7A, nearly 40% (161 proteins) were common to both groups, while approximately 18% (78 proteins) were exclusively found in AD-CONTROL and 45% (198 proteins) were unique to AD-SOUV. Figure 7B presents a histogram showing the distribution of proteins according to the percentage of individuals in each experimental group in which they were detected. A total of 45 proteins in AD-CONTROL and 46 in AD-SOUV were detected in at least 80% of the individuals in their respective groups, representing 19.25% and 12.53% of the total proteins identified in each group, respectively. Interestingly, 28 proteins (11.72%) were found in all AD-CONTROL individuals, while 18 proteins (5.01%) were found in all AD-SOUV individuals.

To thoroughly analyze the impact of Fortasyn Connect supplementation on the human fecal proteome, both qualitatively and quantitatively, changes in the abundance of human-derived proteins in fecal samples were assessed. The yield of total extracted fecal protein was higher in Souvenaid-supplemented patients (0.97 ± 0.20 arbitrary units) compared to controls (0.87 ± 0.39), although differences did not reach statistical significance. In line with the reduction in ammonium production observed in the simgi® model, these findings suggest that the multinutrient may limit microbial proteolytic degradation. To ensure robustness and biological relevance, only proteins detected in at least 80% of individuals in each group (61 proteins) were included in the analysis. The list of these proteins, along with their detection frequency and relative abundance, is presented in Supplementary Table 11, whereas those showing significant differences or trends (*q-value* < 0.1) are presented in Table 3.

Table 3. Proteins of human origin identified in the fecal proteome from AD-CONTROL and AD-SOUV.

	Family	Change in AD-SOUV vs. Control	Functional category	Main biological implication
Digestive enzymes				
Q99895|CTRC_HUMAN	Chymotrypsin-C OS=Homo sapiens OX=9606 GN=CTRC PE=1 SV=2	↑	Digestive enzyme	Contributes to protein hydrolysis; supports digestive efficiency.
P35030|TRY3_HUMAN	Trypsin-3 OS=Homo sapiens OX=9606 GN=PRSS3 PE=1 SV=2	↑	Digestive enzyme	Serine protease enhancing intestinal protein digestion and nutrient absorption.
Innmunoglobulins				
P01877|IGHA2_HUMAN	Immunoglobulin heavy constant alpha 2 OS=Homo sapiens OX=9606 GN=IGHA2 PE=1 SV=5	↑	Mucosal immunity	Reinforces gut immune defense and host–microbiota homeostasis.
P0DOX2|IGA2_HUMAN	Immunoglobulin alpha-2 heavy chain OS=Homo sapiens OX=9606 PE=1 SV=2	↑	Mucosal immunity	Reinforces gut immune defense and host–microbiota homeostasis.
P01876|IGHA1_HUMAN	Immunoglobulin heavy constant alpha 1 OS=Homo sapiens OX=9606 GN=IGHA1 PE=1 SV=3	↑	Mucosal immunity	Reinforces gut immune defense and host–microbiota homeostasis.
P01833|PIGR_HUMAN	Polymeric immunoglobulin receptor OS=Homo sapiens OX=9606 GN=PIGR PE=1 SV=4	↑	Mucosal immunity	Mediates IgA transport to the intestinal lumen, supporting barrier protection.
Fecal biomarkers of intestinal inflammation				
P05164|PERM_HUMAN	Myeloperoxidase OS=Homo sapiens OX=9606 GN=MPO PE=1 SV=1	↓	Inflammation	Indicates reduced intestinal oxidative and inflammatory activity.
Cytoskeletal proteins				
P04264|K2C1_HUMAN	Keratin type II cytoskeletal 1 OS=Homo sapiens OX=9606 GN=KRT1 PE=1 SV=6	↑	Cytoskeletal / epithelial integrity	Reflects epithelial turnover.
P13647|K2C5_HUMAN	Keratin type II cytoskeletal 5 OS=Homo sapiens OX=9606 GN=KRT5 PE=1 SV=3	↑	Cytoskeletal / epithelial integrity	Reflects epithelial turnover
P13645|K1C10_HUMAN	Keratin type I cytoskeletal 10 OS=Homo sapiens OX=9606 GN=KRT10 PE=1 SV=6	↑	Cytoskeletal / epithelial integrity	Reflects epithelial turnover
P13533|MYH6_HUMAN	Myosin-6 OS=Homo sapiens OX=9606 GN=MYH6 PE=1 SV=5	↑	Cytoskeletal / transpot	Reflects epithelial turnover

P35609|ACTN2_HUMAN	Alpha-actinin-2 OS=Homo sapiens OX=9606 GN=ACTN2 PE=1 SV=1	↑	Cytoskeletal	Reflects epithelial turnover
P35527|K1C9_HUMAN	Keratin type I cytoskeletal 9 OS=Homo sapiens OX=9606 GN=KRT9 PE=1 SV=3	↑	Cytoskeletal / epithelial integrity	Reflects epithelial turnover
	Miscellaneous			
Q9NR71|ASAH2_HUMAN	Neutral ceramidase OS=Homo sapiens OX=9606 GN=ASAH2 PE=1 SV=2	↓	Sphingolipid metabolism	Modulation of intestinal sphingolipid pathways
O14983|AT2A1_HUMAN	Sarcoplasmic/endoplasmic reticulum calcium ATPase 1 OS=Homo sapiens OX=9606 GN=ATP2A1 PE=1 SV=1	↑	ER stress / βAPP fragment generation in AD	Reflects epithelial turnover, systemic expression, or degradation processes
Q08380|LG3BP_HUMAN	Galectin-3-binding protein OS=Homo sapiens OX=9606 GN=LGALS3BP PE=1 SV=1	↑	Immune regulation	Modulates immune and inflammatory processes; may support gut barrier maintenance

Statistical analysis was assessed through Mann-Whitney U t-test and adjusted by Benjamini-Hochberg post-hoc with a FDR = 0.05. Only changes with statistically significant differences or trends (*q-value*<0.1) are shown. Proteins detected in higher proportions in the AD-SOUV group are highlighted in green, whereas those detected in higher proportions in the AD-CONTROL group are highlighted in red.

Proteins were classified into five functional categories: digestive enzymes, immunoglobulins, keratins and cytoskeletal proteins, mucosal proteins, and miscellaneous. A quality assessment of the dataset based on the distribution of protein abundance across individual samples demonstrated good consistency. Statistical comparison of protein abundance between AD-CONTROL and AD-SOUV groups revealed several significant differences. Specifically, in the category of digestive enzymes, a clear trend toward increased abundance was observed in the AD-SOUV group. This included proteins such as trypsin-3, chymotrypsin-C, intestinal-type alkaline phosphatase, and to a lesser extent, pancreatic triacylglycerol lipase. Additionally, significant changes were detected in the abundance of immunoglobulins. Notably, levels of IgAs, including immunoglobulin alpha-2 heavy chain, immunoglobulin heavy constant alpha 2, and immunoglobulin heavy constant alpha 1, were significantly higher in the AD-SOUV group compared to AD-CONTROL. This finding is particularly relevant, as it may suggest that Fortasyn Connect supplementation enhances the production of secretory IgA in these patients.

Regarding fecal proteins associated with inflammation, the fecal proteome analysis supports previous findings of reduced inflammation in AD-SOUV patients, as indicated by the lower fecal calprotectin levels (Table 1). Although no statistically significant differences were found in the average abundance of the two proteins that form the calprotectin heterodimer (*S100-A8* and *S100-A9*) [41], likely due to high inter-individual variability, both proteins showed approximately twofold higher mean abundance in the AD-CONTROL group compared to AD-SOUV (Supplementary Table 11). Further, myeloperoxidase (MPO), another protein biomarker of intestinal inflammation, was only detected in the AD-CONTROL group and was absent in all patients receiving the multinutrient (Table 3). Results also showed a significant elevation of several cytoskeletal proteins in fecal samples from the AD-SOUV group, including keratins type I cytoskeletal 10, keratins type II cytoskeletal 1 and 5, alpha-actinin-2, and myosin-6. In addition, higher levels of sarcoplasmic/endoplasmic reticulum calcium ATPase 1 (ATP2A1) were also detected in the AD-SOUV group (Table 3). This pattern may reflect increased epithelial turnover, cytoskeletal remodeling, or gut barrier activity associated with the intervention. Finally, fecal levels of neutral ceramidase (ASAH2) were reduced in AD-SOUV, reflecting modulation of intestinal sphingolipid metabolism.

DISCUSSION

Cognitive health effects of Fortasyn Connect-Souvenaid® have been clinically documented over the last few decades [13, 17, 18, 42]. Originally designed to provide brain-specific precursors and cofactors that support synapse formation and neuronal membrane integrity, its use has been prioritized for the early stages of AD, when interventions are most likely to modify disease progression [13]. However, the bioactive components of this multinutrient formulation are also involved in broader physiological processes, including those occurring in the gastrointestinal tract. The gut–brain axis has emerged as a central framework in neurodegenerative research, with the gut microbiota acting as a key intermediary in the bidirectional communication between the intestine and the central nervous system [43]. Despite this, no previous studies had assessed the potential of Fortasyn Connect to modulate gut microbial ecosystems and intestinal function, representing an important knowledge gap in understanding its full therapeutic potential. To address this, we first employed the simgi® dynamic gut model, using *ex-vivo* fecal samples from AD patients, to simulate gastrointestinal digestion and assess the direct effects of Fortasyn Connect on the colonic microbiota. The

multinutrient supplementation markedly increased the abundance of *Bifidobacterium* and *Lactobacillus*, while reducing taxa associated with gut dysbiosis and inflammation, including *Enterobacteriaceae*, *Enterococcus*, *Klebsiella* and *Fusobacterium*. The fact that the two most popular probiotics used in studies linked to cognition benefits are *Lactobacillus* and *Bifidobacterium* [4, 44], suggests a relationship between Fortasyn Connect and healthy gut-brain effects. Gut microbiota was differently modulated depending on the colon segment, with the transverse (TC) and descending (DC) colon regions showing the strongest increases in health-associated metabolites such as acetate and butyrate. These results indicate that the availability of cofactors and nutrients of Fortasyn Connect-Souvenaid® may lead to bacteria producing more SCFAs when they were supplied within the dietary supplement. Additionally, a trend toward reduced ammonium production was observed in the distal colon, suggesting decreased proteolytic fermentation, which is typically associated with inflammation and microbial imbalance [45].

Although these beneficial shifts appeared rapidly, they diminished during the washout period, with microbial profiles returning toward baseline. This is consistent with human studies showing that the gut microbiome can revert to pre-intervention states following cessation of dietary supplementation [46, 47]. The relatively short intervention period may partly explain this transient response, underscoring the need for longer-term or sustained supplementation strategies to determine whether these microbial effects can be sustained over time or dissipate once supplementation ceases. In addition, the influence of baseline gut microbiota composition and interindividual variability in AD patients warrants further investigation. It should also be noted that dynamic colonic fermentation systems, such as simgi®, may retain partial microbial adaptation between experimental periods, a phenomenon often referred to as a 'memory effect'. Although stabilization and washout phases were extended according to validated protocols [48, 49] to ensure steady-state conditions prior to treatment exposure, some residual carryover effects cannot be completely ruled out. This limitation is intrinsic to continuous *in vitro* gut models and should be considered when extrapolating results to *in vivo* conditions.

Taken together, these results support the usefulness of simgi® as a model to explore gut microbial responses to multinutrient intake under controlled conditions. To assess the relevance of these findings *in vivo*, we next examined gut microbiota and intestinal function in early-stage AD patients receiving Fortasyn Connect as part of routine clinical practice.

The results of the clinical observational study in patients were consistent with the initial effects observed in the *in vitro* gut model. Indeed, Fortasyn Connect taken daily in doses used in the clinical management of the disease for more than 12 weeks significantly modulated specific beneficial gut taxa in early AD patients compared to non-supplementation controls, despite similar dietary patterns between groups. Although no global β-diversity shifts were observed, a bifidogenic effect was noted, characterized by increased abundance of *Bifidobacterium*, *Lactobacillus* and *Christensenellaceae*—taxa typically associated with prebiotic supplementation [50–52]. These changes were accompanied by reduced levels of oral and potentially pathogenic bacteria, such as *Streptococcus salivarius*, *Escherichia/Shigella* and *Fusobacterium*, previously linked to gut dysbiosis and cognitive decline [53–56]. Notably, SCFA-producing species, including *B. bifidum*, *B. longum*, *Lachnospiraceae* and *Christensenellaceae*, were detected in higher proportion in supplemented patients, alongside increased levels of acetate and butyrate. These metabolites are key mediators in the gut–brain axis, contributing to neuroinflammation modulation and synaptic plasticity [43, 57].

The observed microbial shifts may be partly attributed to specific components of Fortasyn Connect. Several *Bifidobacterium* species detected in higher abundance are known to biodegrade omega-3 fatty acids [58, 59]. Moreover, omega-3 PUFAs and vitamins included in the formulation have been shown to influence microbial communities in both human and *in vitro* colonic models [60–62]. However, it is more likely that the synergistic combination of ingredients, not isolated components, underlies the modulation of beneficial taxa and suppression of potential pathobionts. These findings appear to be independent of baseline characteristics, as no differences were observed between groups for potential confounding variables, including sex, age, BMI, dietary patterns, physical activity, comorbidities, and medication use, among others. Consequently, these variables were not incorporated as covariates in the statistical models employed. Nonetheless, the possible influence of other unmeasured confounders cannot be entirely excluded. Future studies with larger sample sizes should incorporate covariate-adjusted models to further validate these findings and better account for residual confounding effects.

Beyond microbiota composition, the multinutrient intervention seems to be also associated with modulation of intestinal function. Lipids contained in Fortasyn Connect, such as omega-3 PUFAs, phospholipids, and cholesterol precursors, may influence digestion and absorption processes. In our study, fecal lipid profiling revealed a higher overall lipid content in AD patients not receiving the multinutrient, a difference primarily

associated with increased levels of excreted free fatty acids and other esterified lipid species. This finding is consistent with the notion that lipid malabsorption leads to greater fecal excretion of triglyceride breakdown products, particularly free fatty acids [63]. Moreover, the intestinal absorption of cholesteryl esters depends on pancreatic cholesterol esterase activity, and its deficiency has been linked to increased fecal excretion of these compounds [64]. These changes may reflect both a direct effect of Fortasyn Connect on digestive capacity and an indirect effect mediated by gut microbiota. Indeed, taxa enriched in the AD-SOUV group, such as *Bifidobacterium* and *Lactobacillus,* are known to participate in PUFA metabolism, including degradation of omega-3 and omega-6 fatty acids [58, 59]. Although the lipid metabolism of the gut microbiome remains hardly understood, microbial lipid catabolites have been proposed to exert neuroactive and anti-inflammatory effects, potentially relevant for neurodegenerative diseases [65]. However, systematic studies on lipid–microbiota–brain interactions are still lacking.

Lipid peroxidation products also differed according to multinutrient intake, with lower levels detected in the AS-SOUV group, suggesting reduced oxidative stress in these patients. These products act as important signaling molecules involved in the regulation of inflammation and other pathological processes, such as AD [20], and their formation is influenced by gut microbiota [66]. Consequentely, their reduction suggests that Fortasyn Connect may help mitigate intestinal and systemic oxidative stress, further supporting its anti-inflammatory potential. Additionally, the fecal levels of **coprostanol**, the main microbial metabolite derived from cholesterol, showed to be lower in the AD-SOUV group. Coprostanol is a non-absorbable sterol excreted in feces, and its production varies according to diet and microbiota composition. Elevated levels have been associated with aging and a higher risk of colonic disorders [67–69]. In our study, Fortasyn Connect intake seems to modulate cholesterol metabolism in the gut, possibly by reducing the abundance or activity of cholesterol-converting bacteria. Although intestinal cholesterol conversion was first described over a century ago, only a few bacterial strains capable of converting cholesterol to coprostanol have been isolated and characterized [69]. The enzymatic pathways involved, and the broader microbial diversity responsible for this function, remain poorly defined. In our study, higher levels of *Eggerthella lenta* and *Lactobacillus ruminis* were observed in the control group; both species are traditionally linked to bile salt and steroid metabolism [70, 71]. While they have not been directly associated with coprostanol production, their enzymatic repertoire or interactions within microbial consortia may contribute indirectly [72]. Indeed, cholesterol-to-coprostanol metabolism is likely driven by cooperative microbial networks, involving species that encode cholesterol oxidases and *ismA*, a gene implicated in this transformation [72, 73]. Altogether, our findings suggests that the beneficial effects of Fortasyn Connect may, at least in part, be mediated through targeted modulation of the gut microbiome, including the taxa and metabolic functions involved in lipid processing, reinforcing its potential role in targeting the gut–brain axis in early AD.

Furthermore, fecal proteomic analysis revealed that the multinutrient intervention modulated key metabolic, digestive, and immune functions in AD patients, supporting a link between these physiological changes and gut microbiota modulation. Notably, the AD-SOUV group showed increased levels of several human digestive enzymes. These enzymes may enhance lipid digestion and absorption, consistent with our findings, while also shaping microbial composition by generating metabolites that serve as substrates for specific bacterial populations. Previous studies have shown that administration of exogenous enzymes, such as lipases, proteases, or amylases, can modulate gut microbiota and improve digestive efficiency in both healthy individuals and clinical populations (e.g., cystic fibrosis or pancreatic insufficiency) [74]. Enhanced lipid hydrolysis and absorption may further influence host lipid and cholesterol homeostasis, key factors in AD pathophysiology.

Interestingly, our results also revealed that AD patients receiving the multinutrient showed increased levels of secretory IgAs (sIgA), a key component of the gastrointestinal immune system and the first line of defense of the mucosal barrier. Although elevated IgA levels have been reported in the serum and brain of AD patients [75], it is important to distinguish serum IgA (associated with systemic immune responses) from secretory IgA, whose function is more localized to the gut and involves modulating host–microbiota interactions [76]. Despite the importance of sIgA, the precise mechanisms by which it regulates microbial populations remain incompletely understood [77]. In parallel, inflammatory markers in feces, such as calprotectin and myeloperoxidase (MPO), further supported a reduction in gut inflammation in the AD-SOUV group. MPO, a neutrophil-derived enzyme, contributes to oxidative stress by generating reactive oxidants [78]; its absence in the AD-SOUV group aligns with the lower levels of lipid peroxidation products observed, suggesting a broader attenuation of oxidative and inflammatory processes. Beyond the gut, MPO has also been implicated in AD pathophysiology, with elevated plasma MPO levels and MPO-immunoreactive cells reported in the brains of AD patients [79, 80], highlighting its potential as a biomarker of gut–brain axis dysfunction [81]. Consistent with these

findings, the increased concentrations of SCFAs following multinutrient intervention, observed both in patients and in the *in vitro* gut model, may further contribute to the observed anti-inflammatory effects and the maintenance of gut barrier integrity. Taken together, the reduction in inflammatory biomarkers, changes in gut and systemic health, and favorable modulation of the gut microbiota suggest that Fortasyn Connect may exert its beneficial effects in part through immunomodulatory actions within the gut environment, complementing its systemic benefits. However, increased levels of certain cytoskeletal proteins in feces, including keratins, suggest a potential shedding of epithelial cells or contamination during sample handling. While some keratins (e.g., keratin 19) have been proposed as plasma biomarkers of AD progression [82], their significance in the intestinal environment remains uncertain. Similarly, the increased presence of sarcoplasmic/endoplasmic reticulum calcium ATPase 1, an enzyme implicated in ER stress and βAPP fragment generation in AD [83], may reflect altered epithelial turnover, systemic expression, or degradation processes.

Finally, beyond the intestinal and microbiota-related findings already discussed, our results also revealed differences in some blood parameters in early-stage AD patients consuming Fortasyn Connect. Specifically, significantly higher serum iron levels in were observed in the AD-SOUV group, constrasting with a different pattern in the AD-CONTROL cohort. Previous studies have linked low iron and hemoglobin levels to cognitive impairment and AD progression [84], suggesting that iron homeostasis may play a role in the disease's pathophysiology. Also, serum levels of vitamin B12 and folate were elevated in the AD-SOUV group, in agreement with earlier reports showing that Fortasyn Connect-Souvenaid® improves plasma micronutrient status in patients with mild cognitive impairment [24]. These findings are particularly relevant given recent evidence that folate supplementation can promote brain rejuvenation and cognitive improvement in aged animal models [85], as well as results from a meta-analysis of over 20 clinical trials involving 3604 participants, which concluded that folic acid supplementation significantly improves cognitive function in patients with MCI or AD [86]. In this context, and beyond the well-documented micronutrient effects of Souvenaid®, an important question arises: to what extent can nutritional strategies, through coordinated actions on gut microbiota, host metabolism, and micronutrient status, contribute to slowing or preventing cognitive decline?. In other words, to what extent might the gut microbiota mediate the clinical benefits observed with Fortasyn Connect in AD patients?. Exploring this interplay may yield novel mechanistic insights and support the development of refined nutritional strategies targeting both microbiota composition and micronutrient balance in the early stages of Alzheimer's disease. Although no direct associations were observed between gut microbiota modulation and cognitive or neurofunctional parameters (e.g., MMSE scores or circulating biomarkers), this should be interpreted in the context of the study's primary objective and the known pathophysiology of AD. Once neurodegenerative biomarkers and cognitive decline are established, these parameters tend to remain relatively stable and are unlikely to be reversed by nutritional or microbiota-targeted interventions in the short term. Consistent with previous studies on Fortasyn Connect [14, 15, 17], which reported improvements in synaptic function and cognition without changes in core biomarkers, our findings suggest complementary peripheral mechanisms. The observed modulation of gut microbial taxa, SCFA production, and intestinal immune–metabolic markers supports the notion that Fortasyn Connect may exert beneficial effects through gut-mediated functional pathways, contributing to neuroprotection and systemic homeostasis, and warranting further longitudinal studies integrating gut and brain endpoints.

In summary, our findings provide novel and integrative evidence that Fortasyn Connect-Souvenaid® may beneficially modulate key taxa in the gut microbiome and intestinal function in early-stage AD patients. Through a combined *in vitro* and clinical approach, we observed favorable shifts in microbial composition and metabolism—particularly increased butyrate production and gut–brain-associated taxa—alongside changes in digestive, immune, and systemic markers towards a more favorable profile. The novel inclusion of fecal proteomics and lipidomics further supports a mechanistic link between microbiota regulation, host metabolism, and gut–brain axis function. Nevertheless, the relatively small sample size of the observational arm represents a limitation in terms of statistical power and generalizability. This study should therefore be interpreted as exploratory and hypothesis-generating. Despite this constraint, the consistency of the effects observed across independent analytical layers supports the robustness and biological plausibility of our findings. Larger, well-powered studies are needed to confirm these associations and assess their clinical implications.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Materials

The Supplementary data can be found online at: www.aginganddisease.org/EN/10.14336/AD.2025.1176.

References

[1] (2023). 2023 Alzheimer's disease facts and figures. Alzheimer's Dement, 19:1598–1695.

[2] Livingston G, Huntley J, Sommerlad A, Ames D, Ballard C, Banerjee S, et al. (2020). Dementia prevention, intervention, and care: 2020 report of the Lancet Commission. Lancet, 396:413–446.

[3] Eiser AR, Fulop T (2023). Alzheimer's Disease Is a Multi-Organ Disorder: It May Already Be Preventable. J Alzheimers Dis, 91:1277–1281.

[4] Molinero N, Antón-Fernández A, Hernández F, Ávila J, Bartolomé B, Moreno-Arribas MV (2023). Gut Microbiota, an Additional Hallmark of Human Aging and Neurodegeneration. Neuroscience, 518:141–161.

[5] Cryan JF, Mazmanian SK (2022). Microbiota-brain axis: Context and causality. Science, 376:938–939.

[6] Silva YP, Bernardi A, Frozza RL (2020). The Role of Short-Chain Fatty Acids From Gut Microbiota in Gut-Brain Communication. Front Endocrinol (Lausanne), 11:25.

[7] Schneider E, O'Riordan KJ, Clarke G, Cryan JF (2024). Feeding gut microbes to nourish the brain: unravelling the diet–microbiota–gut–brain axis. Nat Metab, 6:1454–1478.

[8] Livingston G, Huntley J, Liu KY, Costafreda SG, Selbæk G, Alladi S, et al. (2024). Dementia prevention, intervention, and care: 2024 report of the Lancet standing Commission. Lancet, 404:572–628.

[9] Labrousse VF, Nadjar A, Joffre C, Costes L, Aubert A, Grégoire S, et al. (2012). Short-term long chain omega3 diet protects from neuroinflammatory processes and memory impairment in aged mice. PLoS One, 7:e36861.

[10] Chang J, Liu M, Liu C, Zhou S, Jiao Y, Sun H, et al. (2024). Effects of vitamins and polyunsaturated fatty acids on cognitive function in older adults with mild cognitive impairment: a meta-analysis of randomized controlled trials. Eur J Nutr, 63:1003-1022.

[11] Fan C, Xu J, Tong H, Fang Y, Chen Y, Lin Y, et al. (2024). Gut-brain communication mediates the impact of dietary lipids on cognitive capacity. Food Funct, 15:1803-1824.

[12] Tamayo, M., Olivares, M., Ruas-Madiedo, R., Margolles, A., Espín, J.C., Medina, I.; Moreno-Arribas, M.V., Canals, S., Mirasso, C.; Ortín, S.; Beltrán-Sanchez, H.; Palloni, A.; Tomás-Barberán, F.A., Sanz Y (2024). How Diet and Lifestyle Can Fine-Tune Gut Microbiomes for Healthy Aging. Annu. Rev. Food Sci. Technol. 15: 283-305.

[13] Van Wijk N, Broersen LM, De Wilde MC, Hageman RJJ, Groenendijk M, Sijben JWC, et al. (2014). Targeting synaptic dysfunction in Alzheimer's disease by administering a specific nutrient combination. J Alzheimers Dis, 38:459–479.

[14] Scheltens P, Twisk JWR, Blesa R, Scarpini E, Von Arnim CAF, Bongers A, et al. (2012). Efficacy of Souvenaid in mild Alzheimer's disease: results from a randomized, controlled trial. J Alzheimers Dis, 31:225–236.

[15] Scheltens P, Kamphuis PJGH, Verhey FRJ, Olde Rikkert MGM, Wurtman RJ, Wilkinson D, et al. (2010). Efficacy of a medical food in mild Alzheimer's disease: A randomized, controlled trial. Alzheimers Dement, 6:1-10.e.1.

[16] Olde Rikkert MGM, Verhey FR, Blesa R, Von Arnim CAF, Bongers A, Harrison J, et al. (2015). Tolerability and safety of Souvenaid in patients with mild Alzheimer's disease: results of multi-center, 24-week, open-label extension study. J Alzheimers Dis, 44:471–480.

[17] Soininen H, Solomon A, Visser PJ, Hendrix SB, Blennow K, Kivipelto M, et al. (2021). 36-month LipiDiDiet multinutrient clinical trial in prodromal Alzheimer's disease. Alzheimers Dement, 17:29–40.

[18] Soininen H, Solomon A, Visser PJ, Hendrix SB, Blennow K, Kivipelto M, et al. (2017). 24-month intervention with a specific multinutrient in people with prodromal Alzheimer's disease (LipiDiDiet): a randomised, double-blind, controlled trial. Lancet Neurol, 16:965–975.

[19] Hendrix SB, Soininen H, Solomon A, Visser PJ, van Hees AMJ, Counotte DS, et al. (2023). Combined Evidence for a Long-Term, Clinical Slowing Effect of Multinutrient Intervention in Prodromal Alzheimer's Disease: Post-Hoc Analysis of 3-Year Data from the LipiDiDiet Trial. J Prev Alzheimer's Dis, 10:464–470.

[20] Luo Y-X, Yang L-L, Yao X-Q (2024). Gut microbiota-host lipid crosstalk in Alzheimer's disease: implications for disease progression and therapeutics. Mol Neurodegener, 19:35.

[21] Gil-Sánchez I, Cueva C, Tamargo A, Quintela JC, de la Fuente E, Walker AW, et al. (2020). Application of the dynamic gastrointestinal simulator (simgi®) to assess the impact of probiotic supplementation in the metabolism of grape polyphenols. Food Res Int, 129:108790.

[22] Tamargo A, Cueva C, Álvarez MD, Herranz B, Bartolomé B, Moreno-Arribas MV, et al. (2018).

Influence of viscosity on the growth of human gut microbiota. Food Hydrocoll, 77:163–167.

[23] Dubois B, Villain N, Frisoni GB, Rabinovici GD, Sabbagh M, Cappa S, et al. (2021). Clinical diagnosis of Alzheimer's disease: recommendations of the International Working Group. Lancet Neurol, 20:484–496.

[24] Rijpma A, Meulenbroek O, Van Hees AMJ, Sijben JWC, Vellas B, Shah RC, et al. (2015). Effects of Souvenaid on plasma micronutrient levels and fatty acid profiles in mild and mild-to-moderate Alzheimer's disease. Alzheimers Res Ther, 7:51.

[25] Morris MC, Tangney CC, Wang Y, Sacks FM, Barnes LL, Bennett DA, et al. (2015). MIND diet slows cognitive decline with aging. Alzheimers Dement, 11:1015–1022.

[26] Schröder H, Fitó M, Estruch R, Martínez-González MA, Corella D, Salas-Salvadó J, et al. (2011). A short screener is valid for assessing Mediterranean diet adherence among older Spanish men and women. J Nutr, 141:1140–5.

[27] Jiménez-Arroyo C, Molinero N, Sabater C, Margolles A, Terrón-Camero LC, Andrés-León E, et al. (2024). Gut microbiome and clinical and lifestyle host factors associated with recurrent positive RT-PCR for SARS-CoV-2. Front Cell Infect Microbiol, 14:1494193.

[28] Jiménez-Arroyo C, Tamargo A, Molinero N, Reinosa JJ, Alcolea-Rodriguez V, Portela R, et al. (2023). Simulated gastrointestinal digestion of polylactic acid (PLA) biodegradable microplastics and their interaction with the gut microbiota. Sci Total Environ, 902:166003.

[29] Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res, 41:D590-6.

[30] Nogacka AM, Salazar N, Arboleya S, Ruas-Madiedo P, Mancabelli L, Suarez A, et al. (2020). In Vitro Evaluation of Different Prebiotics on the Modulation of Gut Microbiota Composition and Function in Morbid Obese and Normal-Weight Subjects. Int J Mol Sci, 21:906.

[31] Gil-Sánchez I, Cueva C, Sanz-Buenhombre M, Guadarrama A, Moreno-Arribas MV, Bartolomé B (2018). Dynamic gastrointestinal digestion of grape pomace extracts: Bioaccessible phenolic metabolites and impact on human gut microbiota. J Food Compos Anal, 68:41–52.

[32] Moreno F, Méndez L, Fernández I, Miralles-Pérez B, Giralt M, Romeu M, et al. (2024). Influence of the Degree of Unsaturation in Fish Oil Supplements on Oxidative Stress and Protein Carbonylation in the Cerebral Cortex and Cerebellum of Healthy Rats. Antioxidants, 13:1408.

[33] Christie WW Lipid analysis: isolation, separation, identification, and structural analysis of lipids, 2nd ed. Pergamon Press, Oxford; 1982.

[34] Lepage G, Roy CC (1986). Direct transesterification of all classes of lipids in a one-step reaction. J Lipid Res, 27:114-120.

[35] Dasilva G, Pazos M, García-Egido E, Gallardo JM, Rodríguez I, Cela R, et al. (2015). Healthy effect of different proportions of marine ω-3 PUFAs EPA and DHA supplementation in Wistar rats: lipidomic biomarkers of oxidative stress and inflammation. J Nutr Biochem, 26:1385–1392.

[36] Lin H, Peddada S Das (2020). Analysis of compositions of microbiomes with bias correction. Nat Commun 2020 111, 11:1–11.

[37] Paulson JN, Talukder H, Bravo HC (2017). Longitudinal differential abundance analysis of microbial marker-gene surveys using smoothing splines. bioRxiv, 099457.

[38] Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. (2011). Metagenomic biomarker discovery and explanation. Genome Biol, 12:R60.

[39] Perez-Riverol Y, Bandla C, Kundu DJ, Kamatchinathan S, Bai J, Hewapathirana S, et al. (2025). The PRIDE database at 20 years: 2025 update. Nucleic Acids Res, 53:D543–D553.

[40] Fernández-Ballart JD, Piñol JL, Zazpe I, Corella D, Carrasco P, Toledo E, et al. (2010). Relative validity of a semi-quantitative food-frequency questionnaire in an elderly Mediterranean population of Spain. Br J Nutr, 103:1808–1816.

[41] Jukic A, Bakiri L, Wagner EF, Tilg H, Adolph TE (2021). Calprotectin: from biomarker to biological function. Gut, 70:1978.

[42] Calderon Martinez E, Zachariah Saji S, Salazar Ore JV, Borges-Sosa OA, Srinivas S, Mareddy NSR, et al. (2024). The effects of omega-3, DHA, EPA, Souvenaid® in Alzheimer's disease: A systematic review and meta-analysis. Neuropsychopharmacol Reports, 44:545–556.

[43] Aburto MR, Cryan JF (2024). Gastrointestinal and brain barriers: unlocking gates of communication across the microbiota-gut-brain axis. Nat Rev Gastroenterol Hepatol, 21:222–247.

[44] Cryan JF, O'riordan KJ, Cowan CSMM, Sandhu K V., Bastiaanssen TFSS, Boehme M, et al. (2019). The Microbiota-Gut-Brain Axis. Physiol Rev, 99:1877–2013.

[45] Frame LA, Costa E, Jackson SA (2020). Current explorations of nutrition and the gut microbiome: a comprehensive evaluation of the review literature. Nutr Rev, 78:798–812.

[46] Liu F, Li P, Chen M, Luo Y, Prabhakar M, Zheng H, et al. (2017). Fructooligosaccharide (FOS) and Galactooligosaccharide (GOS) Increase Bifidobacterium but Reduce Butyrate Producing Bacteria with Adverse Glycemic Metabolism in healthy young population. Sci Rep, 7:11789.

[47] Burton KJ, Rosikiewicz M, Pimentel G, Bütikofer U, Von Ah U, Voirol MJ, et al. (2017). Probiotic yogurt and acidified milk similarly reduce postprandial inflammation and both alter the gut microbiota of healthy, young men. Br J Nutr, 117:1312–1322.

[48] Minekus M, Smeets-Peeters M, Havenaar R, Bernalier A, Fonty G, Marol-Bonnin S, et al. (1999). A computer-controlled system to simulate conditions of the large intestine with peristaltic mixing, water absorption and

absorption of fermentation products. Appl Microbiol Biotechnol, 53:108–114.

[49] Haindl R, Engel J, Kulozik U (2021). Establishment of an In Vitro System of the Human Intestinal Microbiota: Effect of Cultivation Conditions and Influence of Three Donor Stool Samples. Microorg, 9:1049.

[50] Waters JL, Ley RE (2019). The human gut bacteria Christensenellaceae are widespread, heritable, and associated with health. BMC Biol 2019 171, 17:1–11.

[51] Gibson GR, Roberfroid MB (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. J Nutr, 125:1401–1412.

[52] Nagy DU, Sándor-Bajusz KA, Bódy B, Decsi T, Van Harsselaar J, Theis S, et al. (2023). Effect of chicory-derived inulin-type fructans on abundance of Bifidobacterium and on bowel function: a systematic review with meta-analyses. Crit Rev Food Sci Nutr, 63:12018–12035.

[53] Molinero N, Taladrid D, Zorraquín-Peña I, Celis M de, Belda I, Mira A, et al. (2022). Ulcerative Colitis Seems to Imply Oral Microbiome Dysbiosis. Curr Issues Mol Biol, 44:1513–1527.

[54] Pan Q, Li YQ, Guo K, Xue M, Gan Y, Wang K, et al. (2021). Elderly Patients with Mild Cognitive Impairment Exhibit Altered Gut Microbiota Profiles. J Immunol Res, 5578958.

[55] Nagpal R, Neth BJ, Wang S, Craft S, Yadav H (2019). Modified Mediterranean-ketogenic diet modulates gut microbiome and short-chain fatty acids in association with Alzheimer's disease markers in subjects with mild cognitive impairment. EBioMedicine, 47:529–542.

[56] Li B, He Y, Ma J, Huang P, Du J, Cao L, et al. (2019). Mild cognitive impairment has similar alterations as Alzheimer's disease in gut microbiota. Alzheimer's Dement, 15:1357–1366.

[57] Qian XH, Xie RY, Liu XL, Chen S Di, Tang HD (2022). Mechanisms of Short-Chain Fatty Acids Derived from Gut Microbiota in Alzheimer's Disease. Agind Dis, 13:1252-1266.

[58] Miyamoto J, Igarashi M, Watanabe K, Karaki S ichiro, Mukouyama H, Kishino S, et al. (2019). Gut microbiota confers host resistance to obesity by metabolizing dietary polyunsaturated fatty acids. Nat Commun. doi: 10.1038/S41467-019-11978-0.

[59] Gorissen L, Raes K, Weckx S, Dannenberger D, Leroy F, De Vuyst L, et al. (2010). Production of conjugated linoleic acid and conjugated linolenic acid isomers by Bifidobacterium species. Appl Microbiol Biotechnol, 87:2257–2266.

[60] Rehman A, Pham V, Seifert N, Richard N, Sybesma W, Steinert RE (2024). The Polyunsaturated Fatty Acids Eicosapentaenoic Acid and Docosahexaenoic Acid, and Vitamin K1 Modulate the Gut Microbiome: A Study Using an In Vitro Shime Model. J Diet Suppl, 21:135–153.

[61] Costantini L, Molinari R, Farinon B, Merendino N (2017). Impact of Omega-3 Fatty Acids on the Gut Microbiota. Int J Mol Sci, 18:2645.

[62] La Rosa F, Clerici M, Ratto D, Occhinegro A, Licito A, Romeo M, et al. (2018). The Gut-Brain Axis in Alzheimer's Disease and Omega-3. A Critical Overview of Clinical Trials. Nutr, 10:1267.

[63] Khouri MR, Ng SN, Huang G, Shiau YF (1989). Fecal Triglyceride Excretion Is Not Excessive in Pancreatic Insufficiency. Gastroenterology, 96:848-852.

[64] Howles PN, Carter CP, Hui DY (1996). Dietary free and esterified cholesterol absorption in cholesterol esterase (bile salt-stimulated lipase) gene-targeted mice. J Biol Chem, 271:7196-7202.

[65] Brown EM, Clardy J, Xavier RJ (2023). Gut microbiome lipid metabolism and its impact on host physiology. Cell Host Microbe, 31:173–186.

[66] Zhuge A, Li S, Yuan Y, Han S, Xia J, Wang Q, et al. (2023). Microbiota-induced lipid peroxidation impairs obeticholic acid-mediated antifibrotic effect towards nonalcoholic steatohepatitis in mice. Redox Biol, 59:102582.

[67] Benno P, Midtvedt K, Alam M, Collinder E, Norin E, Midtvedt T (2005). Examination of intestinal conversion of cholesterol to coprostanol in 633 healthy subjects reveals an age- and sex-dependent pattern. Microb Ecol Health Dis, 17:200-204.

[68] Norin E (2008). Intestinal cholesterol conversion in adults and elderly from four different European countries. Ann Nutr Metab, 52:12-14.

[69] Juste C, Gérard P (2021). Cholesterol-to-coprostanol conversion by the gut microbiota: What we know, suspect, and ignore. Microorganisms, 9:1881.

[70] Ridlon JM (2020). Conceptualizing the vertebrate sterolbiome. Appl Environ Microbiol, 86:e00641-20.

[71] Zhang M, Hang X, Fan X, Li D, Yang H (2008). Characterization and selection of Lactobacillus strains for their effect on bile tolerance, taurocholate deconjugation and cholesterol removal. World J Microbiol Biotechnol, 24:7-14.

[72] Molinero N, Ruiz L, Sánchez B, Margolles A, Delgado S (2019). Intestinal Bacteria Interplay With Bile and Cholesterol Metabolism: Implications on Host Physiology. Front Physiol, 10:185.

[73] Kenny DJ, Plichta DR, Shungin D, Koppel N, Hall AB, Fu B, et al. (2020). Cholesterol Metabolism by Uncultured Human Gut Bacteria Influences Host Cholesterol Level. Cell Host Microbe, 28:245-247.e6.

[74] Menden A, Hall D, Hahn-Townsend C, Broedlow CA, Joshi U, Pearson A, et al. (2022). Exogenous lipase administration alters gut microbiota composition and ameliorates Alzheimer's disease-like pathology in APP/PS1 mice. Sci Rep, 12:4797.

[75] Knecht L, Dalsbøl K, Simonsen AH, Pilchner F, Ross JA, Kristian Winge K, et al. (2024). Autoantibody profiles in Alzheimer´s, Parkinson´s, and dementia with Lewy bodies: altered IgG affinity and IgG/IgM/IgA responses to alpha-synuclein, amyloid-beta, and tau in disease-specific pathological patterns. J Neuroinflammation, 21:317.

[76] Catanzaro JR, Strauss JD, Bielecka A, Porto AF, Lobo FM, Urban A, et al. (2019). IgA-deficient humans exhibit gut microbiota dysbiosis despite secretion of compensatory IgM. Sci Rep, 9:13574.

[77] Pabst O, Izcue A (2022). Secretory IgA: controlling the gut microbiota. Nat Rev Gastroenterol Hepatol, 19:149-150.

[78] Prokopowicz Z, Marcinkiewicz J, Katz DR, Chain BM (2012). Neutrophil myeloperoxidase: Soldier and statesman. Arch Immunol Ther Exp (Warsz), 60:43-54.

[79] Tzikas S, Schlak D, Sopova K, Gatsiou A, Stakos D, Stamatelopoulos K, et al. (2014). Increased myeloperoxidase plasma levels in patients with alzheimer's disease. J Alzheimer's Dis, 39:557-564.

[80] Gellhaar S, Sunnemark D, Eriksson H, Olson L, Galter D (2017). Myeloperoxidase-immunoreactive cells are significantly increased in brain areas affected by neurodegeneration in Parkinson's and Alzheimer's disease. Cell Tissue Res, 369:445-454.

[81] Oriá RB, Costa DVS, de Medeiros PHQS, Roque CR, Dias RP, Warren CA, et al. (2023). Myeloperoxidase as a biomarker for intestinal-brain axis dysfunction induced by malnutrition and Cryptosporidium infection in weanling mice. Brazilian J Infect Dis, 27:102776.

[82] Duggan MR, Gomez GT, Joynes CM, Bilgel M, Chen J, Fattorelli N, et al. (2024). Proteome-wide analysis identifies plasma immune regulators of amyloid-beta progression. Brain Behav Immun, 120:604–619.

[83] Bussiere R, Oulès B, Mary A, Vaillant-Beuchot L, Martin C, El Manaa W, et al. (2019). Upregulation of the sarco-endoplasmic reticulum calcium ATPase 1 truncated isoform plays a pathogenic role in Alzheimer's disease. Cells, 8:1539.

[84] Li DD, Zhang W, Wang ZY, Zhao P (2017). Serum copper, zinc, and iron levels in patients with Alzheimer's disease: A meta-analysis of case-control studies. Front Aging Neurosci, 9:261619.

[85] Antón-Fernández A, Cuadros R, Peinado-Cahuchola R, Hernández F, Avila J (2024). Role of folate receptor α in the partial rejuvenation of dentate gyrus cells: Improvement of cognitive function in 21-month-old aged mice. Sci Reports 2024 141, 14:1–14.

[86] Xu M, Zhu Y, Chen J, Li J, Qin J, Fan Y, et al. (2024). Effects of folic acid supplementation on cognitive impairment: A meta-analysis of randomized controlled trials. J Evid Based Med, 17:134–144.