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Article

Role of Simulated *Nannochloropsis gaditana* Digests in Shaping Gut Microbiota and Short Chain Fatty Acid Levels

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Abstract: The connection between gut microbiota and factors like diet is crucial for maintaining intestinal balance, which in turn impacts the host's overall health. *Nannochloropsis gaditana* microalgae is a sustainable source of bioactive compounds, mainly known for its use in aquaculture and extraction of bioactive lipids, with potential health benefits whose effects on human gut microbiota are still unknown. Therefore, the goal of this work was to assess the impact *N. gaditana* on human gut microbiota composition and derived metabolites by combining the INFOGEST protocol and *in vitro* colonic fermentation process to evaluate potential effects on human gut microbiota conformation through 16S rRNA gene sequencing and its metabolic functionality. The results have demonstrated the ability of the digests from *N. gaditana* to significantly modify gut microbiota composition, promoting an increase in beneficial bacterial genera such as *Akkermansia*, *Butyrivibrio*, *Eisenbergiella*, *Lachnospirillum*, and *Marvinbryantia* in contrast to inulin after 48 h of colonic fermentation. Additionally, the digests increased the levels of both major and minor short-chain fatty acids (SCFAs), particularly butyric and valeric acids, considered as intestinal biomarkers, and increased ammonium production. This research has demonstrated, for the first time, the potential of *N. gaditana* microalgae as a sustainable agent for influencing the composition and functionality of human gut microbiota.

Keywords: *Nannochloropsis gaditana*; inulin; *in vitro* gastrointestinal digestion; colonic fermentation; metagenomic analysis

1. Introduction

Nowadays, the relationship between human health and gut microbiota is considered a key target in the study and treatment of many diseases [1]. The community of thousands of highly diverse microorganisms that live throughout the entire gastrointestinal tract has shown to be involved in numerous trophic functions by being a regulatory element of epithelial cell proliferation and differentiation, enhancing the intestinal mucosal barrier and collaborating with the immune system in the protection against antigens and pathogenic germs, among others [2]. In addition, one of its most attractive roles is the regulation of metabolic and nutritional functions such as the fermentation of indigestible fiber from the diet, the production of short chain fatty acids (SCFA) and vitamins, and the absorption of ions, which makes the metabolites and soluble factors resulting from its activity have a special interest for human health [3]. Moreover, its multifunctionality and complex nature has expanded the number of factors that could be involved in maintaining its homeostasis with the host, as well as the number of strategies that attempt to modulate or influence its behavior [4]. In fact, a

dysbiosis state or imbalances in its composition have been hypothesized as contributing factors in the development of chronic inflammatory diseases such as ulcerative colitis, Crohn disease and obesity, among others [5]. Therefore, the interaction between gut microbiota and its influencing factors such as diet, plays an important role to maintain intestinal homeostasis, which ultimately affects the host's health.

The current interest of consumers in sustainable and health-beneficial products as well as the growth of the world's population and impact of the food industry on the environmental resources, has led global health organizations such as the Food and Agriculture Organization (FAO) and the World Health Organization (WHO) to recommend a transition of the existing eating pattern to one more focused on the consumption of sustainable functional foods. This circumstance is prompting an ongoing worldwide search for alternative sources of bioactive compounds [6]. Among the different options being evaluated, microalgae stand out due to their sustainable nature and associated health benefits attributed to the presence of potential bioactive compounds capable of exhibiting antioxidant, antitumor, regenerative, antihypertensive, neuroprotective and immunostimulant effects [7,8]. The consumption of some microalgae species such as *Arthrospira platensis*, *Chlorella vulgaris* and *Tetraselmis chunii* has been already approved by the European Food Safety Authority (EFSA) and the United States Department of Agriculture (USDA). However, there are other species like *Nannochloropsis gaditana* that are not yet approved, but their promising potential makes them a new niche to explore.

N. gaditana is a green, unicellular, subspherical, marine microalgae that has been mainly used for wastewater treatment, animal feeding, aquaculture and the extraction of bioactive lipids, mostly omega-3 polyunsaturated fatty acids (PUFAs) [9]. Nevertheless, the studies focused on the health benefits of other compounds present in *N. gaditana* are still scarce. To date, the studies that demonstrate the prebiotic and postbiotic capacity of microalgae are limited to the effects of oligosaccharides isolated from these organisms on the growth of specific probiotic cultures. In the study carried out by de Medeiros et al. 2021 [10], the digests of different microalga species showed selective prebiotic effects, stimulating the proliferation of *Lactobacillus-Enterococcus* spp. and *Bifidobacterium* spp. and inhibiting the growth of Prevotellaceae-Bacteroidaceae, *Clostridium histolyticum*, *Eubacterium rectale*, and *Clostridium coccooides*. A more recent study carried out by these researchers demonstrated the stimulating effect of *A. platensis* on the growth and metabolic activity of *Lb. acidophilus* to a greater extent than that exerted by traditional fructooligosaccharides [11]. Additionally, a pre-clinical study with *Phaeodactylum tricornutum* as a suitable diet for mouse feed, showed positive effects on microbiota composition and SCFA production, suggesting beneficial effects on gut health [12].

In case of *Nannochloropsis* sp., it has demonstrated that the fecal microbiota composition of dogs fed with *N. oculata*-supplemented diets was modified by promoting the beneficial *Turicibacter* and *Peptococcus* genera associated with gut health and activation of the immune system [13]. Furthermore, research on European seabass (*Dicentrarchus labrax*) demonstrated that the microalga *N. oceanica* could modulate gut bacterial communities, with beneficial effects on gut homeostasis when combined with other algae like *Gracilaria gracilis* [14]. Specifically, regarding *N. gaditana*, inclusion of its hydrolyzate in the *Sparus aurata* fish diet, resulted in a beneficial effect on intestinal microbiota beyond its nutritional role without causing significant alterations in intestinal morphology or function [15,16]. Although scarce, these results suggest the potential of this microalga and its metabolites to modulate the gut microbiota. Therefore, the main goal of our work was to assess the role of *N. gaditana* simulated gastrointestinal digests on human gut microbiota composition and derived metabolites to more comprehensively explore the potential of this microalga as a sustainable modulator agent of human gut microbiota.

2. Materials and Methods

2.1. Samples and Reagents

Commercial microalga *N. gaditana* biomass was kindly supplied by AlgaEnergy S.A. (Madrid, Spain). Pepsin (EC 232-629-3; 3,200 units/mg protein), pancreatin (232-468-9; 8X USP), sodium phosphate (NaH_2PO_4), disodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), calcium chloride (CaCl_2), hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium chloride (NaCl), yeast extract, dipotassium phosphate (K_2HPO_4), sodium bicarbonate (NaHCO_3), magnesium sulfate heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), calcium chloride hexahydrate ($\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$), Tween-80, hemin, vitamin K, L-cysteine and phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Simulated Gastrointestinal Digestion

The simulated gastrointestinal digestion of microalgae biomass was conducted following the static *in vitro* INFOGEST protocol [17] with some modifications. A pool of saliva was obtained from 7 healthy volunteers and the simulated gastrointestinal fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the protocol [17] and preheated at 37°C. Pepsin from porcine gastric mucosa was mixed with ultrapure water to achieve a final concentration of 33 mg/mL. Pancreatin from porcine pancreas was prepared by dissolving it in SIF to a final concentration of 111.1 mg/mL. Finally, bovine bile was mixed with SIF to a final concentration of 12.9 mg/mL. For the recreation of the oral phase, 1 g of dried microalga biomass was dissolved in 500 μL of Milli-Q water, and 4.5 mL of saliva were added. The mixture was then incubated at 37°C with 120 rpm of agitation for 2 min in the Environmental Shaker – Incubator ES 20/60 (Biosan Medical-biological Research & Technologies, Warren, MI, USA). After completing the oral phase, 4.8 mL of SGF were added to the bolus, adjusting the pH to 3.0 with 1 M HCl. Next, 3 μL of 0.3 M CaCl_2 solution and 300 μL of pepsin (ratio enzyme:substrate, E:S, 1:100, w/w) were added to reach a final volume of 12 mL, and the mixture was incubated at 37°C with agitation for 2 h. Moreover, the orogastric chyme was mixed with 5.1 mL of SIF and the pH was adjusted to 7.0 with 1 M NaOH. Then, 1.5 mL of bile (1:50, w/w), 24 μL of 0.3 M CaCl_2 solution, and 3 mL of pancreatin (E:S 1:3, w/w) were added. The resultant homogenate was incubated at 37°C with agitation for 2 h. At the end of this phase, the enzymes were inactivated at 95°C for 15 min in a Memmert thermostated bath (Schwabach, Germany), and the digest was then centrifuged in an Eppendorf™ Centrifuge 5804R (Hamburg, Germany), at 2000 g for 30 min at 4°C. Two fractions were obtained, the supernatant corresponding to the absorbable fraction that was discarded, and the precipitate corresponding to the non-absorbable fraction (NAF) that was collected. Five digestion replicates were conducted, and the NAFs obtained were pooled and stored at -20°C until the colonic fermentation assays were carried out. 1 g of inulin, a well-known prebiotic carbohydrate [18,19], was also digested in quintuplicate, and used as control.

2.3. Static Colonic Fermentation

For the static colonic fermentation, the fecal inoculum was obtained by combining, in a Stomacher 400 Circulator (Seward, USA), 1 g of feces (from a healthy volunteer) with 10 mL of 0.1 M PBS (pH 7.0) for 5 min to prepare the fecal slurry [20]. Then, 3 g of the NAF, 6 mL of the fecal inoculum and 54 mL of colon nutrient medium [peptone water (2 g/L), yeast extract (2 g/L), NaCl (0.1 g/L), K_2HPO_4 (0.04 g/L), KH_2PO_4 (0.04 g/L), NaHCO_3 (2 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.01 g/L), $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01 g/L), Tween 80 (2 mL/L), hemin (0.05 g/L), vitamin K (10 $\mu\text{L}/\text{L}$), L-cysteine (0.5 g/L), bile salts (0.5 g/L), and distilled water were mixed as previously described by Tamargo et al. 2023 [21]. The fermentation process was carried out for 72 h in triplicate. Anaerobic conditions were followed at 37°C, pH 6.8, continuous agitation (120 rpm), while taking aliquots at 0, 24, 48, and 72 h. From each 5 mL-aliquot, 1 mL was used for microbial counts. Other 4 mL were centrifuged at 10,000 rpm for 10 min at 4°C using the Eppendorf™ Centrifuge 5804R (Hamburg, Germany), separating the supernatants that

were stored at -20°C for SCFA and ammonium analysis. The pellets were stored at -80°C until the metagenomic analysis.

2.4. Microbial Composition Analysis

2.4.1. Analysis of Microbial Communities

Bacterial counts were performed on general and selective media. Serial dilutions (1/10, 1/100, 1/1000) of the colon digests samples in sterile physiological saline solution (0.9% NaCl) were prepared. Then, spot inoculations (10 μ L in triplicate) of each dilution were made on the selected media (BD Difco, NJ, USA): Tryptic Soy Agar (total aerobes), Wilkins Chalgren agar (total anaerobes), MacConkey agar (*Enterobacteriaceae* family), *Enterococcus* agar (*Enterococcus* sp.), De Man, Rogosa and Sharpe agar (lactic acid bacteria), Tryptose Sulfite Cycloserine agar (*Clostridium* sp.), BBL CHROMAgar (*Staphylococcus* sp.), Bifidobacterium agar modified by Beerens (*Bifidobacterium* sp.), and LAMVAB (*Lactobacillus* sp.). The plates were incubated at 37°C for 24 to 72 h, depending on the culture medium. All media, except Tryptic Soy Agar and BBL CHROMAgar, were incubated in an anaerobic chamber (BACTRON Anaerobic/Environmental Chamber, Shel Lab, Sheldon Manufacturing Inc., Cornelius, OR, USA). Lastly, the resultant colonies were counted with a colony counter SC6PLUS (Stuart, UK) and the results were expressed as the logarithm of colony-forming units (CFU) per mL. The differences in values were considered significant when they were higher or lower than 1 log (CFU/mL) compared to the control (inulin).

2.4.2. Metagenomic Analysis of Gut Microbiota

Extraction and Quantification of Microbial DNA

The isolation and extraction of the microbial DNA from the precipitates obtained at 0 h and 48 h-fermentation were carried out using the QIAamp Fast DNA Stool mini kit (Quiagen, Hilden, Germany). DNA integrity was evaluated with the BioAnalyzer 2100 (Agilent, Santa Clara, CA, USA), and the concentration was quantified with the fluorimeter Qubit 3.0 using the dsDNA HS assay (Life Technologies S.A., Alcobendas, Madrid, Spain).

2.4.2.2 16S Ribosomal RNA Gene Sequencing and Bioinformatics Analysis

All the samples underwent amplification of the 16S ribosomal RNA gene via polymerase chain reaction (PCR) using the Ion 16S Metagenomics kit (Life Technologies S.A.), able to amplify seven hypervariable regions (V2, V3, V4, V6-7, V8, and V9). Then, libraries were generated by repairing the ends of the amplicons with the Ion Plus Fragment Library kit (Life Technologies S.A.) and attaching DNA molecular identifiers using the Ion Express Barcode Kit Adapters (Life Technologies S.A.). Furthermore, libraries were diluted and underwent clonal amplification by emulsion PCR in the Ion OneTouch™ 2 System (Thermo Fisher Scientific, Waltham, MA, USA) and each sample was sequenced using an Ion S5™ System with an Ion 520™ Chip (Life Technologies S.A.).

2.4.2.3. Bioinformatics Analysis

Samples were filtered on the platform-specific pipeline incorporated in the Torrent Suite v5.8 (ThermoFisher Scientific). Then, the primers were removed and trimmed the sequences to 165 bp in length using a self-developed Python. FASTQ files were analyzed using QIIME 2 platform and by following Bolyen et al. [22] and Segata et al. [23] methodology. Reference-based clustering of operational taxonomic units (OTUs) at 99% similarity was performed using SILVA 16S database (v138). Relative OTU abundances were computed as percent proportions based on the total number of reads per sample.

α -diversity was assayed calculating four metrics: Observed OTUs, Chao1, and Shannon and Simpson indexes. To study β -diversity, Jaccard dissimilarity index was computed. Principal

coordinate analysis (PCoA) was then applied to the resulting dissimilarity matrices, and two-dimensional PCoA plots were used for the visualization of the results.

Taxonomic assignment of the identified OTUs was performed using QIIME2 and SILVA 16S taxonomy. PICURSt2 software package (v2.2.0-b) was used to predict the functional content of microbial communities as Kyoto Encyclopedia of Genes and Genomes (KEGG) ortholog profiles. PICRUST2 inference relies on 16S rRNA gene sequences and associates OTUs with gene contents. Linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to identify differentially abundant taxa and functional pathways among groups. Taxa with a relative abundance less than 0.01% were filtered for this analysis.

2.5.1. SCFA Analysis

The SCFA analysis was carried out following García-Villalba et al. [24] methodology. The supernatants were acidified with 0.5% phosphoric acid and mixed with the internal standard (1.97 mM, 2-methylvaleric acid, Sigma-Aldrich). After extracting the mixture with n-butanol, the analysis was performed using a gas chromatograph (GC) equipped with an autoinjector, a DB-WAX capillary column (Agilent Technologies, Palo Alto, CA, USA) and a flame ionization detector. Quantitative data were obtained by calculating the area of each compound relative to the internal standard. The analyses were carried out in triplicate.

2.5.2. Ammonium and Protein Content

The protein concentration of supernatants was carried out by the bicinchoninic acid (BCA) method, using the Pierce BCA kit (Thermo Fisher Scientific), following the manufacturer's instructions [25]. Bovine serum albumin (BSA) at concentrations ranging from 50 to 1000 µg/mL was used as standard. The ammonium ion (NH_4^+) production was measured using the Photometric Spectroquant® ammonium reagent test (Merck & Co., Kenilworth, NJ, USA), measuring the absorbance at 690 nm using the Biotek Synergy™ HT plate reader (Winooski, VT, USA). A standard curve (2-75 mg NH_4^+ /L) was used to calculate the content that was expressed as mg NH_4^+ /L. Both ammonium and protein analyses were carried out in triplicate.

2.6. Statistical Analysis

The statistical analysis of data from cultured microbial communities, SCFAs, protein and ammonium content were performed with GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA) using a one-way analysis of variance (ANOVA) followed by a Tukey test. For the metagenomic analysis, differences among groups were analyzed with a Kruskal–Wallis test or a paired t-test as appropriate. The differences between groups regarding the β diversity were analyzed with a permutation analysis and multiple ANOVA (PERMANOVA) with 999 permutations. LEfSe consisted on the application of a Kruskal-Wallis test to identify taxa with significantly different relative abundances, followed by logarithmic Linear discriminant analysis (LDA) score to determine an effect size of each taxon. Finally, the scipy PYTHON package and IBM® SPSS® Statistics software package version 27 (IBM Inc., Armonk, NY, USA) were used and, for all analyses, differences were considered statistically significant when $P < 0.05$.

3. Results and Discussion

3.1. Microbial Composition Analysis

3.1.1. Analysis of Microbial Communities

The effect of the NAFs of *N. gaditana* and inulin digests on the total aerobes and anaerobes microbial counts are shown in Figure 1. In the case of the total aerobes (Figure 1A), an increase was observed at 24 h in both samples, obtaining values of 8.07 CFU/mL and 8.04 CFU/mL for inulin and *N. gaditana*, respectively. After that time, the values measured in the inulin sample remained constant until the end of colonic fermentation (8.25 CFU/mL), while a significant decrease was observed in the

microalga sample, reaching 5.34 CFU/mL at 72 h-fermentation. Regarding total anaerobes population, both samples experienced an increase all through the fermentation and the differences between inulin and microalga samples were not significant (Figure 1B).

In relation to the specific microbial plate counts, the NAF from *N. gaditana* also exerted multiple effects. Thus, the *Enterobacteriaceae* population decreased significantly from 6.42 CFU/mL at 24 h to 4.32 CFU/mL at the end of the fermentation process (Figure 1C). However, the count reached after 24 h-fermentation with the NAF from inulin digest (7.58 CFU/mL) remained constant until the end of the fermentation (7.84 CFU/mL). Moreover, the *Staphylococcus* sp. population increased after 24 h (6.20 CFU/mL) when inulin was added to the medium, remaining constant until the fermentation was completed (6.53 CFU/mL) (Figure 1D). Nevertheless, when the NAF from *N. gaditana* digest was added, a significant decrease in the microbial population was observed, decreasing to a value of 5.06 CFU/mL at 72 h.

Differences between the effects of microalga and inulin NAF were also observed for lactic acid bacteria. In *N. gaditana*, there was a slight increase in the microbial population at 24 h (6.61 CFU/mL) that decreased at 48 and 72 h, with values of 5.31 and 5.13 CFU/mL, respectively. However, in the case of inulin, the lactic acid bacteria increased during the fermentation until reaching a final value of 6.29 CFU/mL. No differences were observed for *Enterococcus* sp., *Clostridium* sp. and *Bifidobacterium* sp. microbial counts between *N. gaditana* and inulin at any of the times studied.

The information available on the effects of microalgae *N. gaditana* digests on microbial counts was limited. Only, in the work of Medeiros et al. 2021 [10], a significant decrease of the relative abundances of intestinal inflammation related bacteria like *Eubacterium rectale*, *Clostridium histolyticum*, *Prevotellaceae*, *Bacteroidaceae*, and some *Porphyromonadaceae* was observed in the colonic media when fructooligosaccharides and different microalgae digests were tested, suggesting microalgae potential ability to difficult the growth of inflammation related bacteria groups like *Enterobacteriaceae* and *Staphylococcus* sp.

Figure 1. Impact of the non-absorbable fractions (NAF) of *Nannochloropsis gaditana* and inulin on gut microbiota microbial counts (log Colonies Forming Units (CFU)/mL) after 0, 24, 48 and 72 h of colonic fermentation. (A) Total Aerobes, (B) Total Anaerobes, (C) *Enterobacteriaceae* (D) *Staphylococcus* sp. *differences in values were considered significant when they were higher or lower than 1 log (CFU/mL) compared to the inulin (control).

The discrepancies found with our results could mainly be related to the microalgae strain, the growing conditions, and/or the experimental design. Furthermore, studies on the growth of *N. gaditana* have shown that both, the microalga and its microbiome can adapt to low oxygen environments [26,27]. During *in vitro* fermentation, a low-oxygen environment is established, and factors such as the native microbiological load of *N. gaditana*, that could have resisted the digestive process should not be discarded. Moreover, the phenolic compounds bound to the undigested polysaccharides and proteins in the microalga-NAF could interact with gut microbiota after digestion. This interaction could produce energy that supports primary microbial consumers and their syntrophic partners, also exhibiting the well-known gut-modulating effects of phenolic compounds [28–30].

3.1.2. Metagenomic Analysis of Gut Microbiota

The effects of simulated gastrointestinal digests from *N. gaditana* on gut microbiota composition were investigated by assayed bacterial community changes after 48 h of colonic fermentation, using 16S rRNA gene sequencing. Initially, α -diversity, a measurement that considers the internal biodiversity of each sample considering richness and evenness (Figure 2A-D), was calculated. For this purpose, the sequence depth in all samples was rarefied. As expected, no significant differences were found neither in terms of richness (observed OTUs and Chao1) nor evenness (Shannon, and Simpson indexes) at the baseline sample (zero time) between *N. gaditana* and inulin (Figure 2A-D). However, after 48 h of fermentation with NAFs from *N. gaditana* and inulin, all α -diversity indexes decreased significantly, indicating a reduction in both bacterial richness and evenness. Previous

studies have shown a decline in bacterial abundance during fermentation likely due to the competitive inhibition of less dominant strains by others [31,32]. Notably, samples treated with *N. gaditana* NAFs exhibited higher observed OTUs and Chao1 indexes compared to inulin-treated samples, though their evenness was similar. This suggests that *N. gaditana* would have a distinct impact on α -diversity compared to inulin.

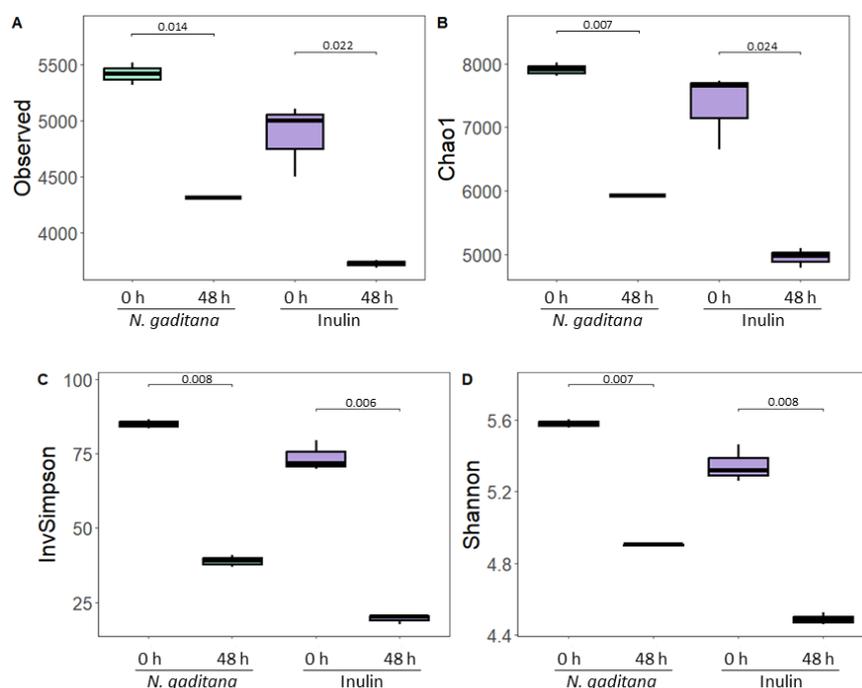


Figure 2. Gut microbiota diversity of NAFs from *N. gaditana* and inulin at zero time and after 48 h. α diversity is presented by observed OTUs (A), Chao1 (B), Simpson richness index (C), and Shannon diversity index (D). The results are shown in boxplots.

Regarding β -diversity, which accounts for differences between groups, all samples clustered at the beginning of the fermentation, indicating no initial differences (Figure 3). However, after 48 h of fermentation, both *N. gaditana* and inulin samples displayed changes in bacterial composition relative to the initial time, with significant differences observed between NFA from *N. gaditana* compared to that from inulin. The first two principal coordinates explained over 72% of the variation in bacterial communities (Figure 3). Overall, these data would indicate that fermentation of *N. gaditana* had a specific influence on the gut microbiota consisting in the reduction of α -diversity and alteration of microbial composition. The influence of *N. gaditana* on the fecal microbiota was then evaluated by analyzing changes in the taxonomic composition of gut microbiota. Both, at the beginning of the experiment and at 48 h, Firmicutes, Bacteroidota, and Proteobacteria were the most abundant phyla, accounting for around the 95% of bacterial taxa (Figure 4). After fermentation, *N. gaditana* and inulin showed a notable increase in Proteobacteria which is consisted with the production of acetic acid that lowers the pH [33]. Regarding the less abundant phyla, there was an increase in the Fusobacteriota and Verrucomicrobiota phyla in samples treated with *N. gaditana* digest, but not in those treated with inulin digest. These data, joined to a less increment in Proteobacteria phylum showed a differential effect of *N. gaditana* compared to inulin in the gut microbiota composition.

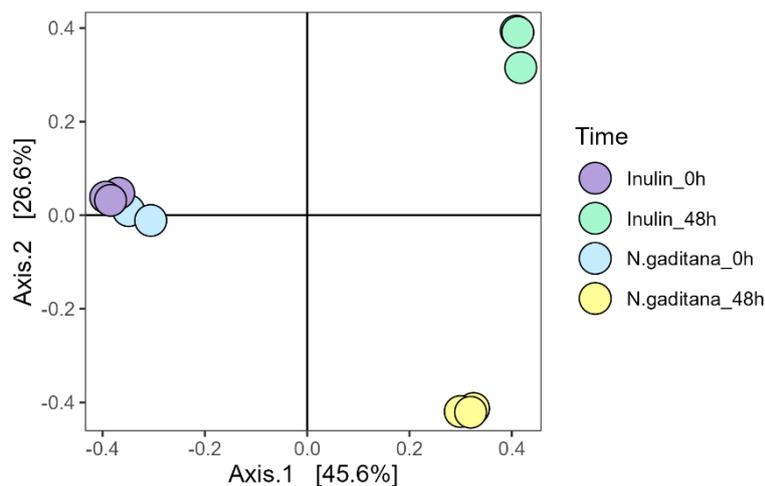


Figure 3. β diversity presented by PCoA plot of Jaccard dissimilarity index of NAFs from *N. gaditana* and inulin at zero time and after 48 h. PCo1 and PCo2 values for each sample are plotted with the percentage of explained variance shown in parentheses.

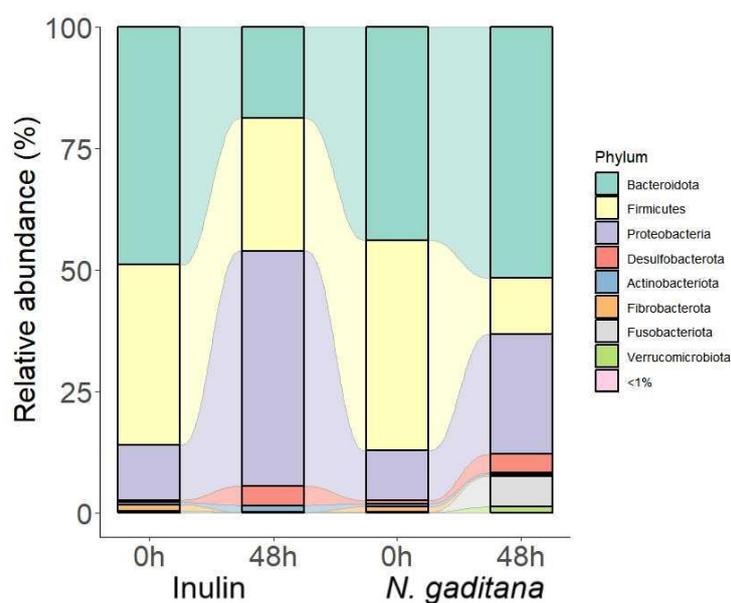


Figure 4. Gut microbiota composition at phylum level of NAFs from *N. gaditana* and inulin at zero time and after 48 h.

Furthermore, we investigated whether the NAFs from *N. gaditana* were associated with more specific changes of the gut microbiota composition. Thirty-six genera differed significantly in abundance in comparison with the beginning of the fermentation (FDR-corrected $P < 0.05$) (Figure 5). Twelve genera were significantly increased and 18 decreased in both *N. gaditana* and inulin samples (Figure 5). However, NAFs from *N. gaditana*, but not from inulin, induced an increment of the SCFA-producing bacteria *Akkermansia*, *Butyricoccus*, *Eisenbergiella*, *Lachnoclostridium*, and *Marvinbryantia* (Figure 5).

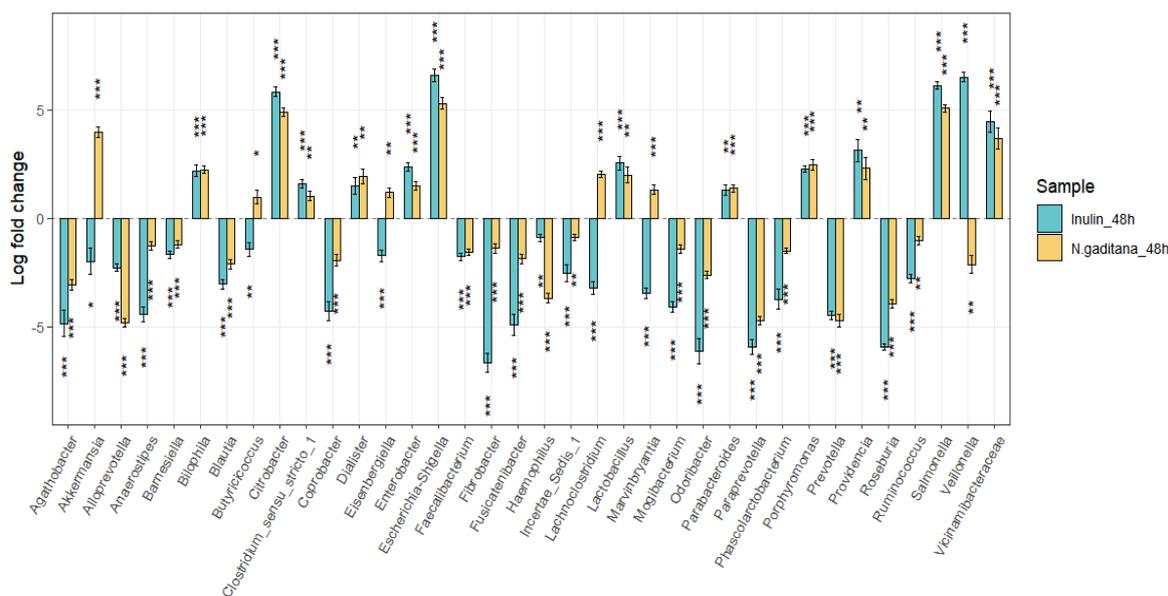


Figure 5. Changes in the abundance of bacteria genera differentially expressed on NAFs from *N. gaditana* and inulin after 48 h. Data are expressed as log fold changes with respect to zero time. *** $P < 0.0001$ vs zero time.

Two health-related bacteria were identified as key biomarkers in the *N. gaditana* fermentations. A significant increase in *Akkermansia* was observed, which is known to influence host metabolism by producing SCFAs and regulating energy balance, contributing to protective effects against conditions like obesity and diabetes [34]. Additionally, *Akkermansia's* ability to metabolize mucin may help maintain gut barrier integrity and modulate immune responses. The bacterial genera *Butyricoccus*, *Eisenbergiella*, *Lachnospirillum*, and *Marvinbryantia* are key members of the gut microbiota involved in metabolism and inflammation regulation, largely through the production of SCFAs and other metabolites that benefit colonic health and immune modulation. *Butyricoccus* is a key producer of butyrate, an SCFA that strengthens the gut barrier, reduces inflammation, and supplies energy to colonic epithelial cells [35,36]. *Eisenbergiella* and *Lachnospirillum* are linked to the fermentation of complex carbohydrates and may indirectly support butyrate production by supplying metabolites that other bacteria can convert into SCFAs [37,38]. *Lachnospirillum* also participates in the regulation of the immune system. In addition, *Marvinbryantia* is a cellulose-degrading bacterium that produces propionate and acetate, SCFAs known to positively influence glucose and lipid metabolism [39]. Importantly, in human gut microbiota, acetate is the predominant butyrate-producing pathway [40]. Overall, an increment in these genera implies that *N. gaditana* might act as a prebiotic, creating an intestinal environment that promotes SCFA production, which has beneficial effects on gut health and potentially on systemic metabolism.

3.2. Microbial Functionality Analysis

In order to study the putative functional profiles of the microbial communities, a LEfSe analysis was performed to compare the KEGG metabolic pathways between *N. gaditana* and inulin after 48 h of colonic fermentation (Figure 6). *N. gaditana* samples were more enriched in metabolic pathways related to bile acid metabolism (primary and secondary synthesis), sphingolipid metabolism and α -linolenic acid metabolism, which could be related to the abundance in *Bilophila* and *Akkermansia* since these bacteria are known to interact with bile acids, suggesting differential regulation of these metabolic pathways [41,42]. In contrast, the inulin samples were more active in pathways related to glycerophospholipid metabolism [43], unsaturated fatty acid biosynthesis and fatty acid elongation, among others. These samples showed a higher abundance of bacteria such as *Butyricoccus*, *Clostridium_sensu_stricto*, and *Roseburia*, which are associated with fatty acid metabolism.

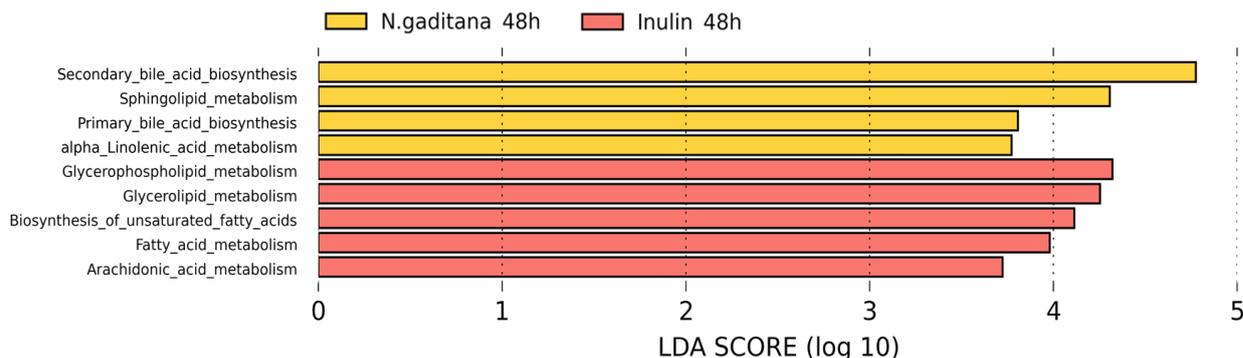


Figure 6. LEfSe analysis showing discriminative KEGG metabolic pathways between *N.gaditana* and inulin after 48 h of colonic fermentation. All KEGG pathways showed a statistically significant change ($P < 0.05$), with an LDA score threshold set to 2.5.

3.2.1. SCFA Profile

The concentration of the total, major and minor SCFAs production after *N. gaditana* and inulin NAFs fermentation over time are shown in Figure 7A-H. *N. gaditana* digest was able to significantly increase the total SCFAs concentration up to 46.84 mM at 72 h, surpassing inulin SCFAs production (30.98 mM, Figure 7A). It would be related to the increase in SCFA-producing bacteria from the NAF of *N. gaditana*, such as *Akkermansia*, *Butyrivibrio*, *Eisenbergiella*, *Lachnoclostridium*, and *Marvinbryantia* (Figure 5). The concentrations of the major SCFAs, acetic acid (Figure 7B) and propionic acid (Figure 7C), over time were also higher when colonic medium was treated with NAF from *N. gaditana* digest than with inulin-NAF, reaching final concentrations of 24.36 and 13 mM, respectively. Similar results were obtained for butyric acid, which concentration at the end of the fermentation with the microalga was 2.7-fold higher than that determined when inulin-NAF was added to the medium (Figure 7D).

Several biological functions have been attributed to these major SCFAs, acting as key signaling molecules in human metabolic health [44]. Acetate and propionate have important roles in human metabolism being utilized in lipid synthesis, acetylation reactions, and source of energy [45]. Most obesity animal models and human trials have suggested a beneficial effect of both SCFAs, favoring weight loss and improving insulin sensitivity [46–48]. On the other hand, butyric acid stands out as a key metabolite of the colonic microbiome. Recent studies have explored its chemistry, cellular signaling mechanisms, and clinical benefits, particularly for the colonic mucosa, where it serves as the main fuel source for mature colonocytes [49]. Beyond the colon, butyric acid also functions as a local and systemic microbial metabolite with significant anti-inflammatory activity, making it a widely recognized biomarker of intestinal health [50].

Our findings correlate well with previous results from other microalgae species and suggest the potential of the green microalgae *N. gaditana*, as an important inducer of gut SCFAs production. Jin et al. [19] demonstrated prebiotic effects of the digests of different microalgae on microbial species involved in propionic acid metabolism, which resulted in an increase of this SCFA. Moreover, de Medeiros et al. [10,11] determined the major SCFAs produced by different microalgae species like *A. platensis*, *C. vulgaris*, and *D. maximus*. Regarding acetic acid, its production was increased when the medium was treated with *C. vulgaris* or *A. platensis* biomass, while butyric acid was highly increased in the fermentation of *D. maximus* biomass after 48 h. A more recent study focused on *A. platensis* and *P. tricornutum* extracts subjected to *in vitro* digestion and fermentation, showed beneficial effects because of the increased production of acetic, propionic, and butyric acids [51].

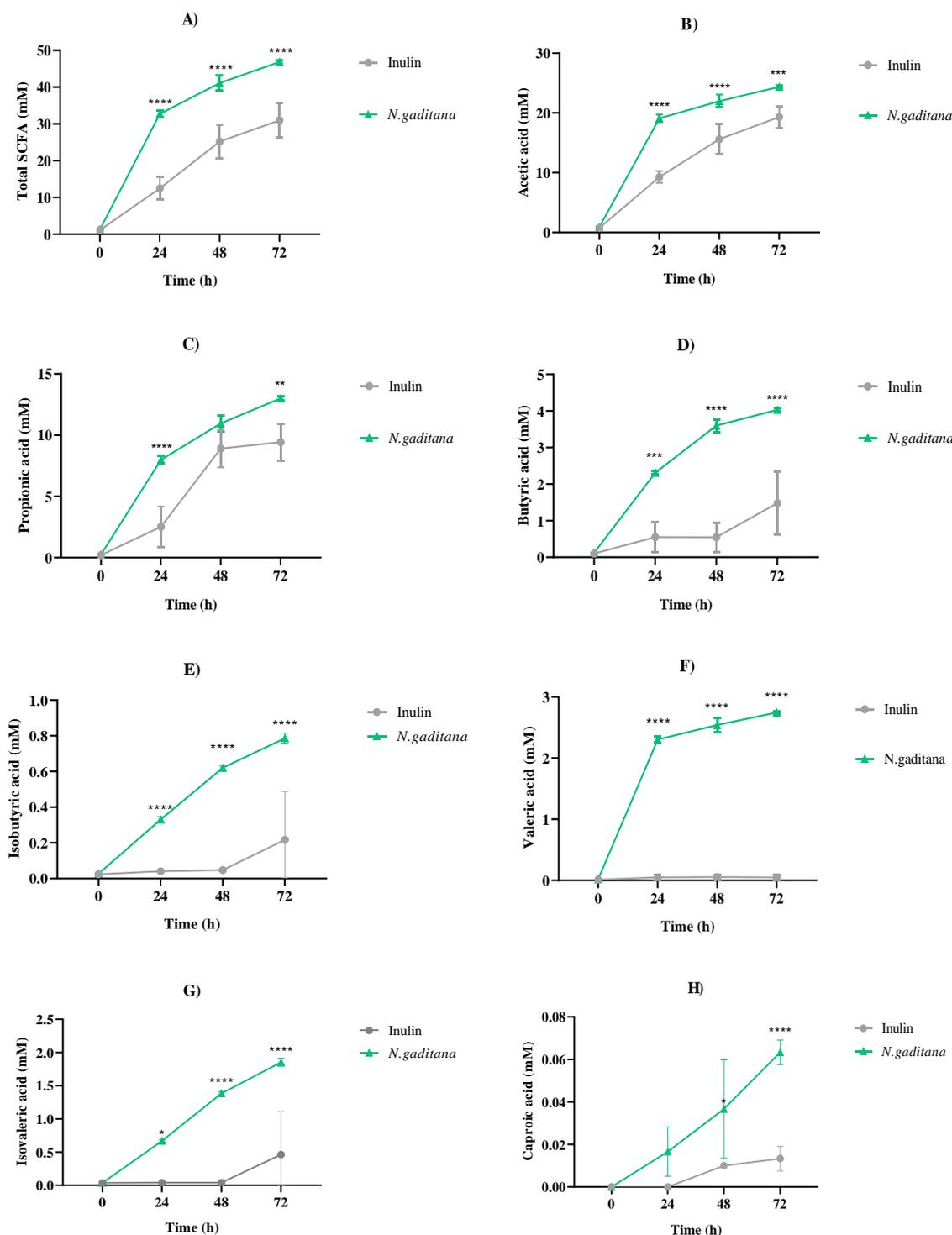


Figure 7. Major and minor short chain fatty acids (SCFAs) production after the colonic fermentation of the non-absorbable fractions (NAF) from *Nannochloropsis gaditana* and inulin (control) at 0, 24, 48 and 72 h. (A) Total SCFAs, (B) Acetic acid, (C) Propionic acid, (D) Butyric acid, (E) Isobutyric acid, (F) Valeric acid, (G) Isovaleric acid, (H) Caproic acid. Level of significance each time compared to the inulin (control): * $0.01 < P\text{-value} < 0.05$; ** $0.001 < P\text{-value} < 0.01$; *** $0.0001 < P\text{-value} < 0.001$; **** $P\text{-value} < 0.0001$.

N. gaditana-NAF also exerted a stimulatory effect over the production of minor SCFAs, when compared to inulin-NAF (Figures 7E-7H). A potent stimulating effect was observed for valeric acid production up to 2.74 mM at 72 h-fermentation, in contrast to the level reached by inulin-NAF (0.05

mM) (Figure 7F). Recently, valeric acid has been identified as one of the most potent histone deacetylase inhibitors, demonstrating anti-cancer, anti-diabetic, antihypertensive, anti-inflammatory, and immunomodulatory activities in different *in vitro* and *in vivo* studies [52]. Dietary fiber oat β -glucans, soybean isoflavones and other plant derived polysaccharides have been mainly reported to induce the generation of valeric acid by gut microbiota [53,54]. *N. gaditana* is considered one of the microalgae with the thickest cell walls with polysaccharides mainly composed of glucose (68%) along with rhamnose, mannose, ribose, xylose, fucose, and galactose (4-8%) [55]. Hence, our findings suggested that during simulated gastrointestinal digestion, this polysaccharide rich cell wall could be partially degraded, being susceptible to the action of gut microbiota and the consequent release of major and minor SCFAs during the fermentation process.

3.2.2. Protein Degradation and Ammonium Production

The protein and ammonium levels throughout the colonic fermentation of the NAF of *N. gaditana* and inulin are shown on Figure 8.

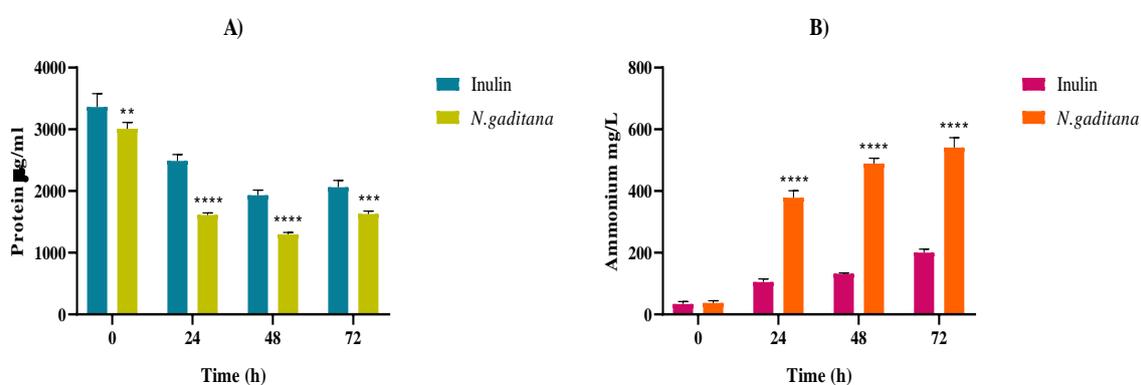


Figure 8. Protein and ammonium levels throughout the colonic fermentation of the non-absorbable fractions (NAF) of *Nannochloropsis gaditana* and inulin (control) at 0, 24, 48 and 72 h. (A) Protein content ($\mu\text{g/mL}$) (B) Ammonium content (mg/mL). Level of significance each time compared to the inulin (control): * $0.01 < P\text{-value} < 0.05$; ** $0.001 < P\text{-value} < 0.01$; *** $0.0001 < P\text{-value} < 0.001$; **** $P\text{-value} < 0.0001$.

N. gaditana protein content decreased during the first 48 h of fermentation (Figure 8A) and inversely, the ammonium content increased over time in both samples, being significantly higher in the case of *N. gaditana* (540.91 mg/mL) than that of inulin (200.15 mg/mL) at the end of the fermentation (Figure 8B). Proteins contained in the microalgal biomass together with peptides and amino acids released during the gastrointestinal digestion could be a nitrogen source for the growth of gut microbiota, releasing ammonium as a protein metabolism indicator [56]. Ammonium is known to play a significant role in human health by influencing the composition and functionality of the gut microbiota. The study by Regueiro et al. [57] examined the microbiome's response to controlled shifts in ammonium and long-chain fatty acids in anaerobic co-digestion systems. They found that high ammonium levels were correlated with shifts in microbial community composition, increasing the abundance of certain bacteria linked to SCFA production, while also leading to volatile fatty acid accumulation. This highlights the complex relationship between ammonium levels and SCFA production, suggesting that the higher SCFA stimulation observed with *N. gaditana*, compared to the inulin control, may also be linked to the increase in ammonium levels.

4. Conclusions

Our findings support the modulatory role of *N. gaditana* on human gut microbiota composition and derived metabolites after its simulated gastrointestinal digestion. After 48 h of colonic fermentation, NAFs from *N. gaditana* significantly changed gut microbiota bacterial composition

favoring the rise of health-related bacteria genera such as *Akkermansia*, *Butyricoccus*, *Eisenbergiella*, *Lachnoclostridium*, and *Marvinbryantia* in contrast with inulin. Moreover, compared to this prebiotic, *N. gadihana*'s simulated gastrointestinal digests increased the production of major and minor SCFAs, especially the key bioactive SCFAs butyric acid and valeric acid, as well as the ammonium production after complete protein digestion. Consequently, this work highlights for the first time the potential of *N. gadihana* microalgae as new sustainable modulator agent of human gut microbiota composition and functionality that could become a practical food ingredient to enhance health by improving the intestinal microbial environment. Additional studies should be carried out to elucidate the molecular mechanisms of the compounds responsible for the observed effects.

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