Adding Probiotics And Prebiotics To A Stable Obese Microbiota In Vitro Alters Microbial Composition

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Abstract

Microbiota, defined as the ecological community of microorganisms living on and in a host, is comprised of hundreds of species that perform functions that are important to human health. The taxonomic composition of microbiota can vary dramatically through a person's life, reflecting changes in lifestyle, nutrition, and medication usage. Changes in the microbiota have been linked to a range of diseases states including gastrointestinal and metabolic disorders, and even Alzheimer's disease. Obesity is also believed to be linked to microbiota, so there is growing interest in treating obesity by altering microbiota composition through the administration of antibiotics, probiotics and prebiotics. In this work, we assessed the effects of administration of probiotic bacteria and prebiotic fiber on the composition of gastrointestinal microbiota cultured in an in vitro system that simulates the distal part of the human colon. We hypothesized that adding probiotics and prebiotics to cultured microbiota obtained from an obese person's feces would alter the microbial composition. We assessed changes in the microbiome using 16S rRNA gene sequencing. Results indicate that the addition of both probiotic organisms and prebiotic fiber changes the composition of gastrointestinal microbiota, as assessed by the Before-After ControlImpact design analysis. We also observed a reduction of bacterial biodiversity during the 72 hr period of the experiment in the *in vitro* system.

Keywords: obesity; gastrointestinal microbiota; probiotic; prebiotic; bioreactor

Sažetak:

Dodavanje probiotika i prebiotika stabilnoj pretiloj mikrobioti in vitro mijenja mikrobni sastav

Mikrobiota, definirana kao ekološka zajednica mikroorganizama koji žive na i u domaćinu, sastoji se od stotina vrsta koje obavljaju funkcije važne za ljudsko zdravlje. Taksonomski sastav mikrobiote može dramatično varirati tijekom života osobe, odražavajući promjene u načinu života, prehrani i korištenju lijekova. Promjene u mikrobioti povezuju se s nizom bolesti uključujući gastrointestinalne i metaboličke poremećaje, pa čak i Alzheimerovu bolest. Također se vjeruje da je pretilost povezana s mikrobiotom, pa raste interes za liječenje pretilosti promjenom sastava mikrobiote primjenom antibiotika, probiotika i prebiotika. U ovom smo radu procijenili učinke primjene probiotičkih bakterija i prebiotičkih vlakana na sastav gastrointestinalne mikrobiote uzgojene u in vitro sustavu koji simulira distalni dio ljudskog debelog crijeva. Pretpostavili smo da bi dodavanje probiotika i prebiotika kultiviranoj mikrobioti dobivenoj iz fecesa pretile osobe promijenilo mikrobni sastav. Procijenili smo

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promjene u mikrobiomu pomoću sekvenciranja gena 16S rRNA. Rezultati pokazuju da dodavanje probiotičkih organizama i prebiotičkih vlakana mijenja sastav gastrointestinalne mikrobiote, kako je ocijenjeno analizom prije-poslije kontrole utjecaja. Također smo primijetili smanjenje bakterijske bioraznolikosti tijekom 72 sata eksperimenta u in vitro sustavu.

Ključne riječi: pretilost; gastrointestinalna mikrobiota; probiotik; prebiotik; bioreactor

Introduction

The human microbiota comprises all prokaryotic and eukaryotic microorganisms that reside in and on the human body. It is numerically dominated (>99%) by bacteria in the colon [1]. Intestinal bacteria comprise 500-1000 distinct species distributed among seven bacterial phyla, including
Firmicutes (64% of species), Bacteroidetes (23%), Proteobacteria (8%), Actinobacteria (3%), and Verrucomicrobia, Cyanobacteria and Fusobacteria (< 1% each) [2]. The intestinal microbiota is a complex and dynamic ecosystem that co-exists within the host organism and has a variety of effects on metabolism, and on the immune and endocrine systems. Quantitative and qualitative changes in intestinal microbiota can result in intestinal diseases, a phenomenon referred to as "dysbiosis" [3].
Along with other factors that may contribute to obesity, such as genetics and poor diet, new research suggests that intestinal microbiota can also influence a person's propensity to become obese. In the human intestine, the biodiversity of microbiota, and the ratio of Bacteroidetes to Firmicutes, is sometimes found to be higher for people having body weight in normal range compared to obese people [4]. The taxonomic composition of intestinal microbiota also changes in response to weight loss and changes in diet [5]. The mechanisms by which intestinal microbiota affect obesity in humans are still largely unknown. Hypothesized mechanisms include effects on energy release in the body, levels of fermented short-chain fatty acids that promote adipogenesis, obesity-related gene expression, and plasma production lipopolysaccharide (LPS) that are correlated with inflammation [6].
The goals of this study were two-fold. First, we investigated the potential of an *in vitro* system to maintain a stable composition of microbiota obtained from the feces of a human subject. Second, we used this system to investigate the effects of introducing the probiotic *Lactobacillus plantarum D13* and the prebiotic Nutriose® FB06 fiber on the composition of microbiota maintained in vitro, following a before-and-after-control-impact (BACI) experimental design. We hypothesized that adding probiotics and prebiotics to an obese person's stable microbiota would alter the microbial composition.

Materials and methods

Fecal samples

Fecal samples of a healthy obese female were used as the source of microbial culture for the inoculation of the nutrient medium. The 2 control fecal samples (receiving no probiotics or prebiotics) and 2 treatment fecal samples (receiving probiotics and prebiotics) were obtained from the volunteer 3 days apart. The volunteer had a body mass index (BMI) of 31 kg m^{-2} and was therefore classified as moderately obese. She had not consumed any drugs or other substances that may interfere with the intestinal microbiota composition prior to the experiment. And she provided written informed consent to participate in this study. The study was approved by the Ethics Committee of the Srebrnjak Children's Hospital and accepted by the Ethics Committee of the Faculty of Food Technology and Biotechnology, University of Zagreb, with the approval number 7/2016.

Bioreactor

Each fresh 30g fecal sample was homogenizing with a 300 mL salt solution in the Erlenmeyer flask, and then transferred to a 2L volume anaerobic bioreactor (Jupiter 2.0, Solaris Biotechnology, Mantova, Italy) using a peristaltic pump. Three different growth medium solutions were used for cultivation: a SIEM (Simulated Ileal Efflux Medium) nutrient medium [7], a salt solution, and a vitamin solution (see Supplementary Materials (SI) Table S1). SIEM aims to mimic the substrate reaching the colon from the terminal ileum through the ileal-caecal valve. The nutrient characteristics of this substrate are typical of the so-called western diet. Salt solution was sterilized separately from the SIEM substrate to which it was added after sterilization in the autoclave. The vitamin solution was stored in a refrigerator (4°C) and added to the SIEM substrate immediately before use.

Treatments

To account for factors unrelated to the treatments of interest, the cultivations treated with probiotics and prebiotics were paired with untreated cultivations [22], which we refer to as controls. The two control fecal samples and the two treatment fecal samples (which received probiotics and prebiotics at 24 h and 48 h, respectively) were cultured sequentially using the same bioreac-

tor. The probiotic used was *Lactobacillus plantarum D13*. This strain was cultured using the current MRS (Man-Rogosa-Sharpe) nutrient medium at the Laboratory for Antibiotic Technology, Enzymes, Probiotics and Starter Cultures of the Faculty of Food Technology of the University of Zagreb. The prebiotic used was the commercial product NUTRIOSE® FB06 (Roquette, USA), a soluble dietary fiber source with sugar-free bulking agent [8,9].

The first phase (0 – 24 h) involved an initial 4-hour period of adjustment under controlled temperature (37°C) and pH conditions (pH 6.8) that simulated a downward colon (see SI for further details). This adjustment period was done under anaerobic conditions using a low-speed agitator at 30 rpm. This was followed by a 20-hour period of establishment, which was initiated by mixing the base nutrient medium of 300 mL volume with microbiota suspended in a salt solution of the same volume. The second phase (24 – 72 h) involved adding the probiotic ($1x10^8$ CFU of *Lactobacillus plantarum D13* at 24 hr) and prebiotic (21 g/L [10] at 48 hr) to the two treatment cultivations, and monitoring changes in microbial communities. During each 72-hour cultivation, all essential process parameters (mixing speed, temperature, pH value, nitrogen flow, oxidation-reduction potential) were monitored using the program "Leonardo" [11].

Sampling

Microbial communities were monitored by collecting bioreactor samples using a sterile exclusion system at a 12-hr time interval and immediately storing them at -80°C. Samples from each of the 4-bioreactor cultivations were taken, starting at time 0 h, over a 72-hour period, yielding 7 samples per cultivation and 28 samples in total. Samples were processed using the Maxwell® 16 Instrument (Promega, United States [12]) for automated isolation and purification of DNA. Variable regions 3 and 4 of the gene coding for 16S rRNA were amplified using PCR, and fragments were sequenced using the Illumina MiSeq platform paired-end protocol. Raw data sequencing of the 16S rRNA gene fragments were clustered into Operational Taxonomic Units (OTUs) with a minimum 97% coverage and a minimum 97% identity using the UCLUST algorithm in QIIME 2 [13]. The representative sequence of each cluster was aligned with the SILVA taxonomic database using the PyNAST algorithm [14,15].

Statistical analysis

The statistical significance of differences in taxonomic compositions between the control and treatment cultivations was assessed using a before-after-control-impact (BACI) design [16,17].

The taxon composition of each sample was first characterized as proportions of cells for 11 taxonomic classes. BACI analyses were then separately performed using relative abundance data for each taxonomic class because previous work demonstrates that classes within phyla often differ in their responses to obesity [19]. Relative abundance data were expressed as logittransformed proportions, replacing 0 counts with values of 1 (the smallest non-zero count was 4) to avoid undefined transformed values. It is important to note that the different BACI analyses performed were not entirely independent because the 11 class proportions for a given sample must sum to 1 [18]. Nevertheless, we adopted this approach for ease of interpretation, recognising that increases in the relative abundance of any given taxon may in part reflect decreases in the abundances of other taxa in response to treatments.

Log-ratio transformed relative abundance measures for each class were separately submitted to two-way factorial ANOVA in order to assess the significance of changes in composition through time for the controls versus the treatments using the BACI approach [19]. Two types of BACI analyses were conducted, one for the probiotic treatment and one for the prebiotic treatment. For the probiotic treatment, the 3 samples collected from each cultivation between 0 and 24 hr were classified as "before", and the remaining 4 samples were classified as "after", because probiotics were added 24 hr after collecting samples. For the prebiotic treatment, the first 5 samples collected between 0 and 48 hr were classified as "before". A significant interaction between the two factor variables in the ANOVA, Before/After and Control/Treatment, would represent evidence of a treatment effect.

Results

Four fecal samples of a healthy obese woman (BMI of 31) were used as a source of microbial community for the inoculation of the nutrient medium for the two control cultivations and treatment cultivations receiving probiotics and prebiotics. These four samples were similar in microbial composition (Figure 1).

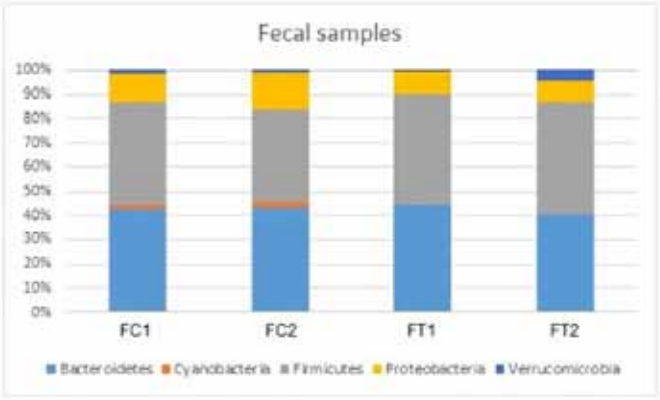

Figure 1. Initial phylum compositions (class relative abundances) for the two control fecal samples (FC1, FC2) and two treatment fecal samples (FT1, FT2).

Taxonomic diversity

Extracted DNA (Table 1) was sequenced to calculate total alpha diversity across the fourbioreactor cultivations. There was a ~50% decline in microbial diversity over the first 48 hr, with little change between 48 and 72 hr (Figure 2).

Taxonomic composition

Changes in the relative abundances of taxa were observed in each of the four cultivations over the 72-hour period at both the phylum (Figure 3) and class levels (Figure 4). The two most common bacterial phyla at the start of the experiment were Bacteroidetes (42 – 49%) and Firmicutes (32 – 44%) (Tables S2 – S3), consistent with expectations [21].

Table 1. DNA extraction from samples for the two controls, and two treatments receiving probiotic (at 24 hr) and prebiotic (at 48 hr).

	DNA Extraction (ng/uL); 2 uL			
Time(hr)	Control 1	Control 2	Treatment 1	Treatment 2
0	42.24	52.78	22.79	53.29
12	373.99	141.71	65.45	100.09
24	278.82	59.35	22.55	44.09
36	281.45	17.01	16.5	10.54
48	114.63	9.36	15.22	36.75
60	180.14	9.21	11.83	55.05
72	109.35	5.11	16.57	28.39

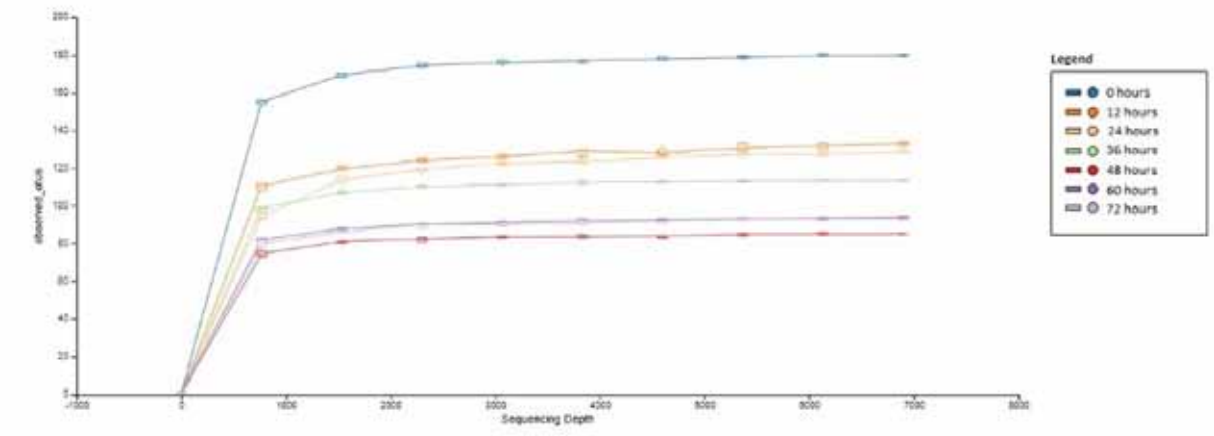

Figure 2. Total alpha diversity across the four bioreactors trials as a function of sequencing depth at 12hour intervals from 0-72 h.

At the class level (Figure 5), relative abundance exhibited a consistent decline for the control and treatment cultivations throughout most of the 72-hr experimental period for Alphaproteobacteria, Bacteroidia, Deltaproteobacteria, and Erysipelotrichi, suggesting that the bioreactor environment was relatively inhospitable for these classes.

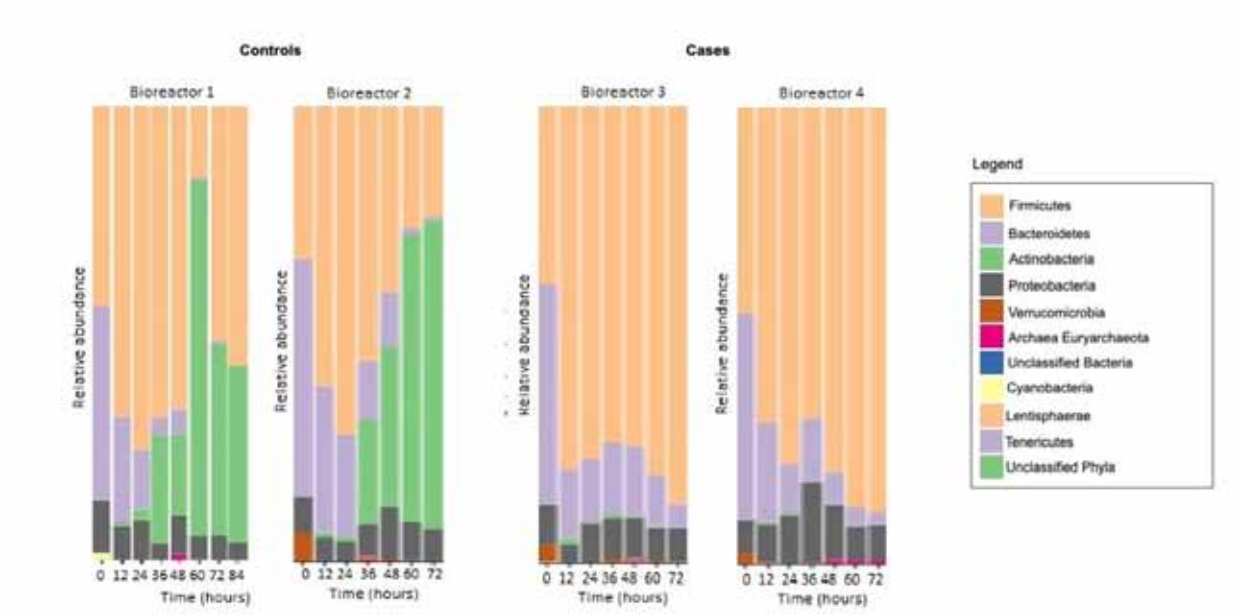

Figure 3. Changes through time in the proportional abundances of bacterial phyla for each of 4 model runs. Bioreactor runs 1 and 2 served as controls and Bioreactor runs 3 and 4 (labelled cases) received probiotics after 24 hours and prebiotics after 48 hr.

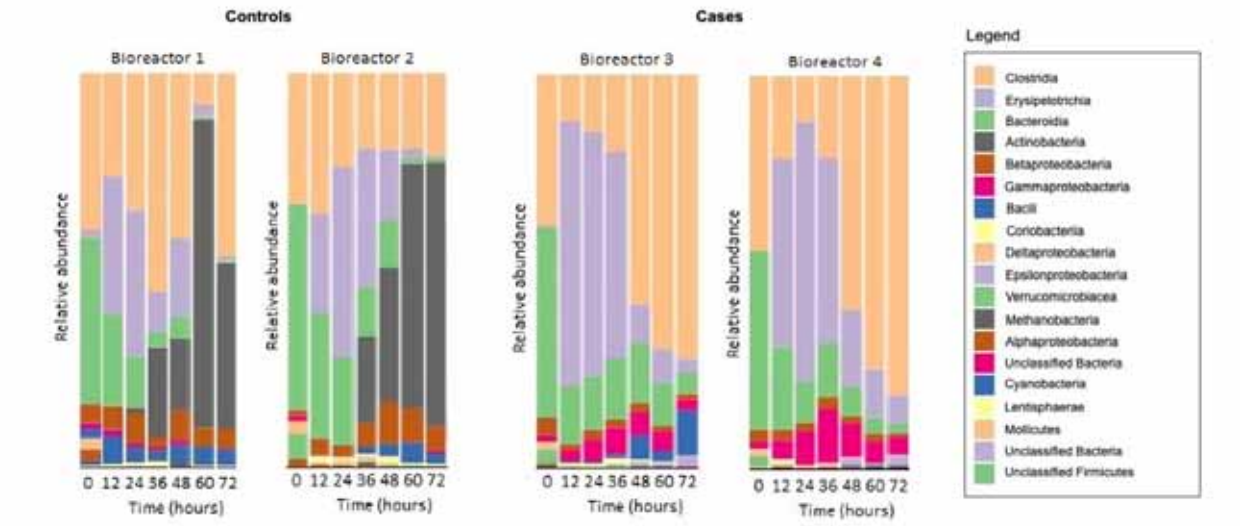

Figure 4. Changes through time in the proportional abundances of bacterial classes for each of 4 model runs. Bioreactor runs 1 and 2 served as controls and Bioreactor runs 3 and 4 (labelled cases) received probiotics after 24 hr and prebiotics after 48 hr.

Assessing treatment effects

A visual and statistical assessment of differences in trends through time for 11 selected bacterial classes indicate that Actinobacteria exhibited a statistically significant increase in abundance through time for the two control cultivations relative to the two treatment cultivations following the probiotic treatment at 24 hr and prebiotic treatment at 48 hr (significance is denoted by red vertical lines in Figure 5a; see also Table 1). The addition of probiotics at 24 hr was significantly associated with an increase in the *Clostridia* class, when comparing the control and treatment cultivations (Figure 5f). Although BACI analyses also indicated a significant prebiotic effect for this group, interpretation is challenging given that the two treatments were added sequentially to the same cultivations 24 hr apart. On the other hand, for Bacteroidia, only the prebiotic BACI analysis was significant (Figure 5d), albeit marginally (p = 0.03; Table 1), suggesting a prebiotic treatment effect. Betaproteobacteria revealed significant BACI results for both treatments, but the calculated p values are questionable given the existence of a severe outlier (72 hr treatment sample in Figure 5e), which violates the normality assumption of ANOVA.

Class	probiotic	prebiotic
Actinobacteria	< 0.001	0.026
Alphaproteobacteria	0.838	0.357
Bacilli	0.754	0.879
Bacteroidia	0.128	0.034
Betaproteobacteria	0.048	0.008
Clostridia	< 0.001	0.004
Coriobacteriia	0.250	0.262
Deltaproteobacteria	0.538	0.354
Erysipelotrichi	0.954	0.528
Gammaproteobacteria	0.118	0.506
Other	0.392	0.131

Table 2. P values for the significant of the interaction for BACI analyses conducted using factorial ANOVA on logit-transformed relative abundance data for 11 bacterial classes. P values were calculated separately for the responses to the probiotic (at 24 hr) and prebiotic (at 48 hr) treatments.

BACI analyses conducted using the full set of log-ratio transformed values as the response in MANOVA indicating significant overall effects of both treatments on class composition (Tables 3 and 4).

MANOVA results –		probiotics impact				
	Df	Pillari	Approx F	Num DF	Den DF	Pr(>F)
Before/After	1	0.87861	10.8750	10	15	3.498e-05***
Treatment/ Control	1	0.90639	14.5246	10	15	5.548e-06***
Before/After *Treatment/ Control	1	0.77078	5.0439	10	15	0.002634***
Residuals	24					

Table 3. MANOVA results for Before-After-Control-Impact probiotic treatment on overall taxonomic composition. The full set of 11 relative abundance measures was log-ratio transformed prior to analysis, yielding 10 statistically independent variables. Bacteroidia arbitrarily served as the reference level in the denominator. The interaction was significant (p = 0.003), indicating an overall significant effect of probiotics on taxon composition.

MANOVA results – prebiotics impact

	Df	Pillari	Approx F	Num DF	Den DF	Pr(>F)
Before/After	1	0.75361	4.588	10	15	0.0041975**
Case/Control	1	0.85332	8.7193	10	15	0.0001314***
Before/ After *Case/ Control	1	0.66869	3.0274	10	15	0.0260858*
Residuals	24					

Table 4. MANOVA results for Before-After-Control-Impact probiotic treatment on overall taxon composition. The full set of 11 relative abundance measures was log-ratio transformed prior to analysis yielding 10 statistically independent measures. Bacteroidia arbitrarily served as the reference level in the denominator. The interaction was marginally significant (p = 0.03), suggesting an overall significant effect of prebiotics on taxon composition.

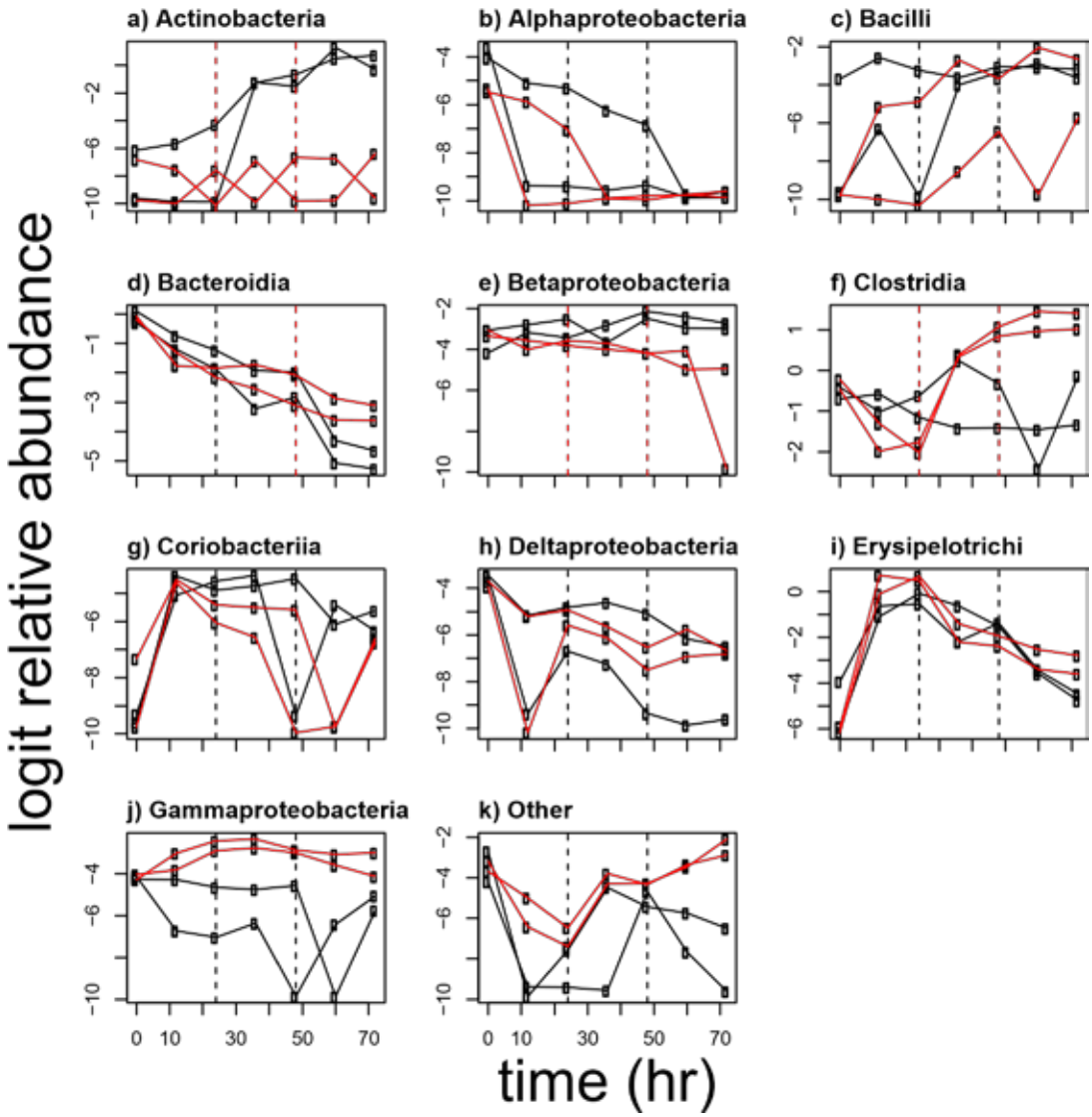

Figure 5. Changes in logit-transformed relative abundance for the control (black lines) and treatment (red lines) cultivations for selected bacterial classes. For each class, the significance of differences in abundance in relation to the time of probiotic (vertical dashed line at 24 hr) and prebiotic (vertical dashed lines at 48 hr) additions were assessed using a Before-After-Control-Impact design for the two control cultivations (black lines) and treatment cultivations (red lines). The dashed lines are highlighted red if there was a significant difference with respect to the specified treatment ($p<0.05$).

Discussion

Most in vitro microbiota studies have focus on assessing how growing conditions and substrates affect the concentrations of various short chain fatty acids. Ours is one of relatively few in vitro studies that have directly measured response of the microbiome to changes in substrates. Overall, our results strongly support the hypothesis that in vitro addition of probiotic bacteria *Lactobacillus Plantarum* and prebiotic NUTRIOSE® FB06 results in rapid changes in microbial composition at the class level. The initial fecal samples and samples extracted from the bioreactor immediately after the inoculation were very similar in taxonomic composition at the start of the experiment (Figures 1, 3-5), so we can discount this as a possible explanation for differences in the compositions in the control and treatment cultivations.

Responses to the probiotic treatment were strongest for Actinobacteria (negative) and Clostridia (positive). Obesity is associated with a decreased abundance of Clostridia and an increase in Actinobacteria [19], so the probiotic responses of these groups are consistent with expectations. The addition of probiotics *Lactobacillus plantarum* significantly influenced the dynamics of microbial composition change. Obesity has been correlated with increases in the abundance of Bacilli and Gammaproteobacteria, and declines in Bacteroidetes [19], but as shown in Figure 5, the addition of probiotics appeared to have at most weak effects on relative abundances of these taxa, contrary to expectations.

Four classes exhibited a general decline in relative abundance (Alphaproteobacteria, Bacteroidia, Deltaproteobacteria, Erysipelotrichi; Figure 5) during the 72-hr period of the experiment for both the control and treatment cultivations, suggesting that incubation conditions were not appropriate these taxa. Alpha diversity also exhibited a general decline (Figure 2), consistent with previous in vitro experiments [26]. One possible contributing factor is nutrient insufficiency of the SIEM. Bacteroidia requires nutrients of animal origin, which are not included in the SIEM nutrient base used in this study design [25]. Additionally, due to the batch-fed nature of the cultivation, continuous depletion of nutrients from the medium would have occurred, perhaps putting some taxa at a relative disadvantage.

In future research, it will be necessary to optimize the nutrient medium, particular for abundant bacterial phyla like Bacteroidetes, whose loss is most pronounced in the in vitro growing conditions. It will also be necessary to extend the time of cultivation to assess whether the microbiota composition eventually achieves a stable equilibrium that could later be used as a standard initial inoculum for future in vitro experiments.

Data Availability Statement

The datasets presented in this study can be found in the following online repositories: https://github.com/enmelvan/Chapter2.

Ethics Statement

The study is approved by the Ethics Committee of the Srebrnjak Children's Hospital and accepted by the Ethics Committee of the Faculty of Food Technology and Biotechnology, University of Zagreb. The patient provided their written informed consent to participate in this study.

Author Contributions

Ena Melvan: Conceptualization, Methodology, Validation, Formal Analysis, Data Curation, Writing – Original Draft Preparation, Visualization; **Damir Oros**: Conceptualization, Validation, Data Curation, Resources; **Jurica Zucko**: Methodology, Validation, Resources, Project Administration; **Andrew P. Allen**: Methodology, Writing – Review & Editing, Supervision; **Antonio Starcevic**: Conceptualization, Writing – Review & Editing, Supervision, Funding
acquisition.

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Author Declarations

The authors report no conflict of interest relevant to this article.

References

1. Sender R, Fuchs S, Milo R. Revised estimates for the number of human and bacteria cells in the body. PLoS Biol. 2016; 14(8):e1002533. doi: 10.1371/journal.pbio.1002533
2. D'Argenio V, Salvatore F. The role of the gut microbiome in the healthy adult status. Clin. Chim. Acta. 2015;451:97-102. doi: 10.1016/j.cca.2015.01.003
3. Clemente JC, Ursell LK, Parfrey LW, Knight R. The impact of the gut microbiota on human health: an integrative view. Cell J. 2012; 148(6):1258-70. doi: 10.1016/j.cell.2012.01.035
4. Ley RE, Turnbaugh PJ, Klein S, Gordon JI. Human gut microbes associated with obesity. Nature. 2006; 444(7122):1022-3. doi: 10.1038/4441022a
5. Turnbaugh PJ, Hamady M, Yatsunenko T, Cantarel BL, Duncan A, Ley RE et al. A core gut microbiome in obese and lean twins. Nature. 2009; 457(7228):480-4. doi: 10.1038/nature07540
6. Chen J, He X, Huang J. Diet effects in gut microbiome and obesity. J. Food Sci. 2014; 79(4):R442-51. doi: 10.1111/1750-3841.12397
7. Polzin S, Huber C, Eylert E, Elsenhans I, Eisenreich W, Schmidt H. Growth media simulating ileal and colonic environments affect the intracellular proteome and carbon fluxes of enterohemorrhagic Escherichia coli O157:H7 strain EDL933. Appl. Environ. Microbiol. 2013; 79(12):3703-15. doi: 10.1128/aem.00062-13
8. Lefranc-Millot C, Wils D, Roturier JM, Bihan CL, Saniez-Degrave MH. Fiber ingredients: food applications and health benefits. Section I: Soluble Fiber. SS cho, P. Samuel. 2009: 19-38. doi: 10.1201/9781420043853-c3
9. Lefranc-Millot C, Guérin-Deremaux L, Wils D, Neut C, Miller LE, Saniez-Degrave MH. Impact of a resistant dextrin on intestinal ecology: how altering the digestive ecosystem with NUTRIOSE®, a soluble fibre with prebiotic properties, may be beneficial for health. Int. J. Med. Res. 2012; 40(1):211-24. doi: 10.1177/147323001204000122
10. Guérin-Deremaux L, Pochat M, Reifer C, Wils D, Cho S, Miller LE. The soluble fiber NUTRIOSE induces a dose-dependent beneficial impact on satiety over time in humans. Nutr. Res. 2011; 31(9):665-72. doi: 10.1016/j.nutres.2011.09.004
11. Solaris Biotech Software, https://solarisbiotech.com/software/lab-pilot/
12. Krnajski Z, Geering S, Steadman S. Performance verification of the Maxwell 16 Instrument and DNA IQ Reference Sample Kit for automated DNA extraction of known reference samples. Forensic Sci Med Pathol. 2007; 3(4):264-9. doi: 10.1007/s12024-007-0034-1
13. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK et al. QIIME allows analysis of high-throughput community sequencing data. Nat. methods. 2010; 7(5):335-6. doi: 10.1038/nmeth.f.303
14. SILVA rRNA Database Project, https://www.arb-silva.de/
15. Caporaso JG, Bittinger K, Bushman FD, DeSantis TZ, Andersen GL, Knight R. PyNAST: a flexible tool for aligning sequences to a template alignment. Bioinformatics. 2010; 26(2):266-7. doi: 10.1038/nmeth.f.303
16. Miles J. Wiley StatsRef: Statistics Reference Online. R squared, adjusted R squared, edited by N. Balakrishnan, T. Colton, B. Everitt, W. Piegorsch, F. Ruggeri, and JL Teugels. Chichester, UK: Wiley. 2014. doi: 10.1002/9781118445112.stat07659
17. El-Shaarawi AH, Piegorsch WW, Piegorsch WW, editors. Encyclopedia of environmetrics. John Wiley & Sons; 2002. doi: 10.1002/9780470057339
18. Aitchison J. The statistical analysis of compositional data. J R Stati Soc: Series B (Methodological). 1982; 44(2):139-60. doi: 10.1111/j.2517-6161.1982.tb01195.x
19. Peters BA, Shapiro JA, Church TR, Miller G, Trinh-Shevrin C, Yuen E et al. A taxonomic signature of obesity in a large study of American adults. Sci. Rep. 2018; 8(1):1-3. doi: 10.1038/s41598-018-28126-1
20. Massey Jr FJ. The Kolmogorov-Smirnov test for goodness of fit. J AM STAT ASSOC. 1951; 46(253):68-78. doi: 10.1080/01621459.1951.10500769
21. Voreades N, Kozil A, Weir TL. Diet and the development of the human intestinal microbiome. Microbiol. 2014; 5:494. doi: 10.3389/fmicb.2014.00494
22. Eberhardt LL. Quantitative ecology and impact assessment. J. Environ. Manage.;(United Kingdom). 1976; 4(1). doi: 10.1016/0378-1127(76)90035-9
23. Cordonnier C, Thévenot J, Etienne-Mesmin L, Denis S, Alric M, Livrelli V, Blanquet-Diot S. Dynamic in vitro models of the human gastrointestinal tract as relevant tools to assess the survival of probiotic strains and their interactions with gut microbiota. Microorganisms. 2015; 3(4):725-45. doi: 10.3390/microorganisms3040725
24. Aguirre M, Jonkers DM, Troost FJ, Roeselers G, Venema K. In vitro characterization of the impact of different substrates on metabolite production, energy extraction and composition of gut microbiota from lean and obese subjects. PLoS one. 2014; 9(11):e113864. doi: 10.1371/journal.pone.0113864
25. German Collection of Microorganisms and Cell Cultures, https://www.dsmz.de. doi: 10.1016/0144-8617(89)90064-7
26. Aguirre M, Venema K. Challenges in simulating the human gut for understanding the role of the microbiota in obesity. Benef Microbes. 2017; 8(1):31-53. doi: 10.3920/bm2016.0113