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Faecal Concentrations of Short-chain Fatty Acids and Selected Bacteria in Healthy and Celiac Children

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Abstract *Background*: Knowledge about the interplay between diet, microbiota and short-chain fatty acids (SCFAs) so far exists. Moreover, raising evidence suggests their influence on the pathogenesis of the celiac disease (CD). *Objective*: Our aim was to study and evaluate differences in the composition of selected bacterial groups and SCFAs in faeces of healthy and CD children. *Methods*: The study included 41 children with CD, 8 newly discovered, not treated children (ND) and 33 children on gluten-free diet for more than 1 year (GFD) and 17 healthy children as a Control group. Bacterial communities and SCFAs in faecal samples were determined by real-time PCR and HPLC analysis, respectively. *Results*: There were no statistically significant differences between GFD and ND patients. GFD patients compared to Controls had significantly lower *Lactobacillus* spp. (p = 0.027) and Enterobacteriaceae family group (p = 0.003), but higher propionic acid (p = 0.034). Acetic (p = 0.027) and propionic acid (p = 0.014) were significantly higher in ND patients compared to Controls. *Lactobacillus* spp. negatively correlated with total SCFAs in the Control and the ND group. In ND and GFD patients, *Lactobacillus* spp. negatively correlated with *Clostridium sensu stricto* cluster I. A very strong positive correlation (p = 0.002) between Enterobacteriaceae family and *Bacteroides fragilis* was found in GFD patients. *Conclusions*: Changes in microbiota and SCFAs are clearly related to the pathogenesis of CD. As being potential pro-inflammatory agents in CD, acetic and propionic acid may serve as important disease-related markers. Their origin in relation to *Lactobacillus* and *Bifidobacterium* is debatable and still need to be further investigated. Enterobacteriaceae family might not be directly addressed to pathogenesis of CD.

Keywords: *celiac disease, children, gluten-free diet, microbiota, short-chain fatty acids*

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1. Introduction

The main role in the development of intestinal microbiota has been addressed to food, even at birth, when the first phase of intestinal colonization of microorganisms occurs [1]. Microbial colonization is a complex process, depending on the interactions between intestinal microbiota, endogenic (host) and exogenic factors, which concomitantly affect the quantity and the composition of the microbiota itself [2,3,4,5,6]. Despite some speculations about the actual diet effect on infant gastrointestinal microbial composition [7,8], by the age of three, microbiota achieves its peak in changing the composition and structure and further it remains relatively stable [1,6,9].

Worldwide increasing frequency of the disease has put celiac disease (CD) on the top of the most common food-related disorders, affecting children as well as adults [10,11]. In fact, a complex mixture of genetic and environmental factors contribute to the disease pathology characterized by chronic immune-mediated inflammation and abnormal microbiota composition [11,12,13,14]. Gluten-free diet (GFD) has so far been the only available effective CD treatment, but doubts about its effectiveness have arisen [15,16,17]. Moreover, the restoration of the microbiota after GFD may not be completed, even after some time, as it has been shown in in vitro [18] and in vivo [19] studies, which led to the conclusion that GFD itself also seems to have an effect on the microbiota by changing its composition [20]. There is an ongoing debate about the role of microbiota in CD patients, whether it is the

cause or the consequence of the disease [10,19,21,22,23,24]. Nevertheless, it is believed that CD patients tend to accumulate higher numbers of Gram negative to Gram positive bacteria compared to healthy subjects [19].

SCFAs are organic acids, produced as end products in the microbial fermentation of non-digestible carbohydrates. Highest concentration of SCFAs are found in caecum and large intestine. SCFAs production clearly depends on the diet, i.e. quantity of available fibers, as well as upon the microbiota composition. Increasing amount of evidence suggests profound effects of SCFAs on host health and homeostasis (reviewed in den Besten et al. [25] and Sun et al. [26]). However, SCFAs quantification and correlation to microbiota in CD studies has been rare and consequently provided limited insight into the disease pathology.

Therefore, our aim was to study differences in the composition of SCFAs and some representative bacterial groups between ND, GFD and healthy children. By statistical comparison between the measured parameters we aimed to evaluate significance of microbiota and SCFAs as prediction parameters for the disease characterization and progress during the GFD treatment.

2. Materials and Methods

The research study was conducted within a group of patients that were included in another study (for details see Klemenak et al. [27]. The study was registered at https://www.clinicaltrials.gov (registration number: NCT02244047).

Based on the available faecal samples obtained at the beginning of the study (baseline), three different groups of patients were selected and their faeces was analysed: healthy patients (Control; n = 17), newly discovered (not treated) CD patients (ND; n = 8) and patients, who were on gluten – free diet for more than 1 year (GFD; n = 33).

2.1. Inclusion / Exclusion Criteria

Patients within this study were children aged between 1 and 19 years. The GFD group included children that strictly adhered to the GFD ranging from 1 to 15 years, 7.8 years on average. For the detailed inclusion / exclusion criteria please refer to the study of Klemenak et al. [27].

2.2. Collection of Faecal Samples

Children's parents or caregivers were given instructions about faecal sampling and transportation procedure. Immediately after defecation, faecal samples were put into a standard laboratory collecting cup for faecal samples, transported in iced cold bags to the laboratory, aliquoted and stored at -80 °C until the analysis.

2.3. Derivatization of SCFAs and HPLC Analysis

Derivatization and HPLC analysis of SCFAs was performed according to Torii et al. [28]. Briefly, faeces were weighed (0.2 – 0.5 g) and put into a 15 mL centrifuge tube. 5.0 mL of 70% ethanol was added, homogenized and centrifuged at 2500 rpm for 10 min at room temperature. The supernatant was collected for further analysis. SCFAs were derivatized into acid hydrazides and separated by YMC-Pack FA 250 x 6 mm ID column (YMC, Kyoto, Japan) under isocratic conditions using Waters 2695 XE Separation Module (Waters Corporation, Milford, MA 01757, US). SCFAs derivatives were detected by diode-array detector at 400 nm and quantified by peak area integration. Four SCFAs were measured during the HPLC analysis (acetic, propionic, n-butyric) and 2-ethylbutyric acid was used as an internal standard. All consumables used during derivatization and solvents for HPLC analysis were from Sigma-Aldrich Co. LLC (St. Louis, MO, US). Results were expressed for acetic, propionic and n-butyric acid in μmol / g of wet weight faeces.

2.4. DNA Extraction

DNA extraction of 200 mg of faeces was carried out using the QIAamp DNA Stool Mini Kit (Qiagen, West Sussex, UK), according to manufacturer's instructions with a slight modification. An additional incubation at 95°C for 10 min of the faecal sample with the lysis buffer was added to the standard protocol, to improve the bacterial cell rupture [29]. The purity and the concentration of the extracted DNA were determined by measuring the ratio of the absorbance at 260 and 280 nm (Infinite®200 PRO NanoQuant, Tecan, Mannedorf, Switzerland).

2.5. Quantitative PCR (qPCR)

The quantification of selected microbial groups of intestinal microbiota (*Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis*, *Clostridium sensu stricto* or cluster I and the total Enterobacteriaceae) was determined by real-time PCR. The assays were performed using Fast SYBR® Green Master Mix (Applied Biosystems, according to manufacturer's instructions). Standard curve construction and data elaboration expressed in Log CFU/g faeces were performed according to Aloisio et al. [29]. Optimized concentrations of primers, primer sequences and annealing temperature for the quantification of *Bidobacterium* spp., *Lactobacillus* spp., *Bacteroides fragilis* were reported in Aloisio et al. [29]. Primers have been chosen by evaluating the best Ct/ΔRn ratio and their specificity has been verified before the analysis. Enterobacteriaceae family were evaluated using 400 nM of each primers – Eco 1457F (CATTGACGTTACCCGCAGAAGAAGC) and Eco 1652R (CTCTACGAGACTCAAGCTGC) – at 60°C as annealing temperature [30]. *Clostridium sensu stricto* were evaluated using 200 nM of each primers – CI-F1 (TACCHRAGGAGGAAGCCAC) and CI-F2 (GTTCTTCCTAATCTCTACGCAT) – at 60°C as annealing temperature [31].

2.6. Statistical Analysis

Data were analyzed by SPSS Statistics 22.0 software (IBM Inc., Armonk, New York) using non-parametrical Mann-Whitney U-Test after Shapiro-Wilk test of normality. Correlations of variables were determined using Spearman correlations. P value ≤ 0.05 or 0.01 in correlation analyses was considered statistically significant.

3. Results

Due to the insufficient amount of faecal material, only a subset (58 children) from the study of Klemenak et al. [27] were included in this study and grouped as mentioned before in the Methodology section.

Faeces was collected from ND, GFD and Control children, followed by quantification and statistical analysis of SCFAs and selected bacterial groups (Figure 1. and Figure 2., respectively). There were no statistically significant differences between any of the parameters in the ND patients compared to the GFD patients. In the GFD group compared to the Controls, propionic acid values were significantly higher ($p = 0.034$), in contrast to *Lactobacillus* spp. ($p = 0.027$) and Enterobacteriaceae ($p = 0.003$) that were significantly lower. Acetic and propionic acid values were significantly higher ($p = 0.027$ and $p = 0.014$, respectively) in the ND patients compared to the Controls. Moreover, *Bifidobacterium* spp. values were almost statistically significantly higher ($p = 0.059$) in the ND patients compared to the Controls (data not shown).

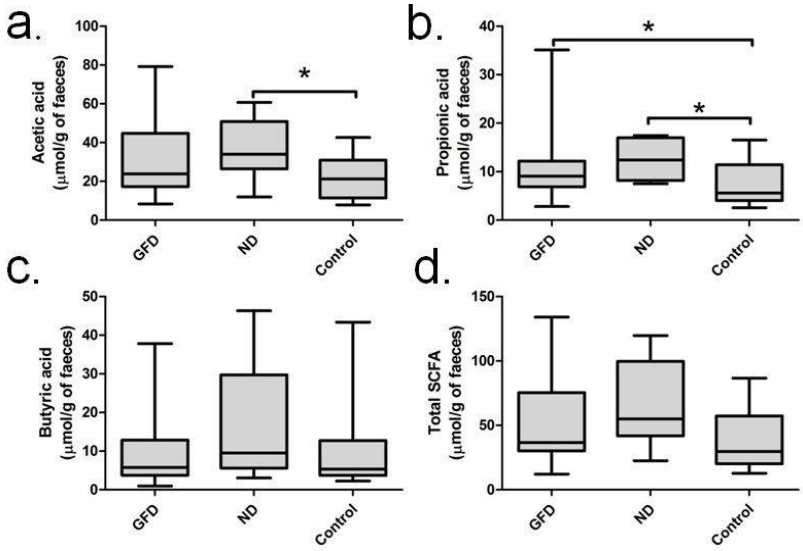

Figure 1. Analysis of faecal short-chain fatty acids in gluten-free diet (GFD), newly discovered (not treated – ND) and healthy (Control) patients. a. Acetic acid; b. Propionic acid; c. Butyric acid; d. Total SCFAs. Bars represent min, median, max, lower and upper quartile. Statistically significant differences between the groups were determined using Mann-Whitney U-test (*$p \leq 0.05$)

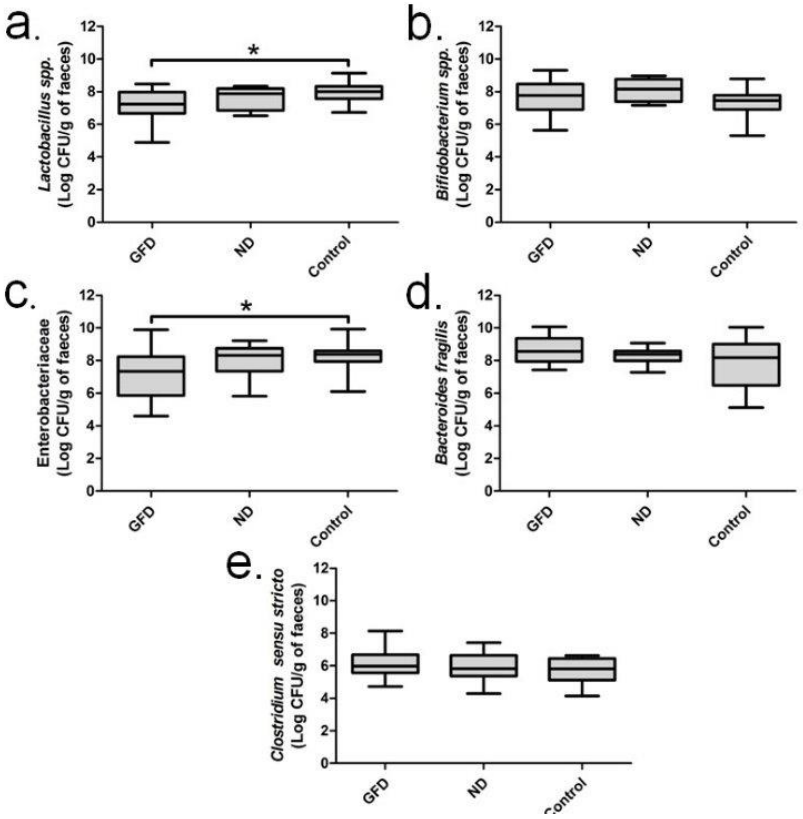

Figure 2. Analysis of different bacterial groups in gluten-free diet (GFD), newly discovered (not treated – ND) and healthy (Control) patients. a. *Lactobacillus* spp.; b. *Bifidobacterium* spp.; c. Enterobacteriaceae; d. *Bacteroides fragilis*; e. *Clostridium sensu stricto*. Bars represent min, median, max, lower and upper quartile. Statistically significant differences between groups were determined using Mann-Whitney U-test (*$p \leq 0.05$)

Furthermore, potential correlations between SCFAs and bacteria in each individual group were separately examined. In healthy controls, total SCFAs strongly positively correlated with acetic, propionic and butyric acid (all p = 0.000). Propionic acid strongly positively correlated with acetic (p = 0.001) and n-butyric acid (p = 0.000). The latter had a positive correlation (p = 0.049) with acetic acid. *Lactobacillus* spp. had a negative correlation (p = 0.02) to acetic acid and total SCFAs (p = 0.01). Moreover, a strong negative correlation (p = 0.002) between *Lactobacillus* spp. and propionic acid was observed (Table 1).

Table 1. Correlation coefficients and *p* values between SCFAs and bacteria in healthy patients (Control group)

		AA	PA	BA	tSCFAs	*Bi*	*L*	E	B	Cl
AA	C		0.76**	0.48*	0.85**	0.22	-0.62*	-0.26	-0.47	-0.01
	P		0.001	0.049	0.000	0.41	0.02	0.32	0.06	0.96
PA	C	0.76**		0.87**	0.89**	0.20	-0.79**	0.00	-0.04	0.05
	P	0.001		0.000	0.000	0.46	0.002	0.99	0.87	0.85
BA	C	0.48*	0.87**		0.82**	0.19	-0.46	0.15	011	-0.24
	P	0.049	0.000		0.000	0.46	0.11	0.55	0.69	0.35
tSCFAs	C	0.85**	0.89**	0.82**		0.20	-0.68*	-0.09	-0.21	0.01
	P	0.000	0.000	0.000		0.43	0.01	0.73	0.43	0.96
Bi	C	0.22	0.20	0.19	0.20		-0.27	-0.07	0.24	-0.22
	P	0.41	0.46	0.46	0.43		0.37	0.79	0.35	0.39
L	C	-0.62*	-0.79**	-0.46	-0.68*	-0.27		-011	-0.27	-0.16
	P	0.02	0.002	0.11	0.01	0.37		0.72	0.37	0.59
E	C	-0.26	0.00	0.15	-0.09	-0.07	-0.11		0.46	0.31
	P	0.32	0.99	0.55	0.73	0.79	0.72		0.06	0.22
B	C	-0.47	-0.04	0.11	-0.21	0.24	-0.27	0.46		0.12
	P	0.06	0.87	0.69	0.43	0.35	0.37	0.06		0.64
Cl	C	-0.01	0.05	-0.24	0.01	-0.22	-0.16	0.31	0.12	
	P	0.96	0.85	0.35	0.96	0.39	0.59	0.22	0.64	

Footnotes:
(AA) acetic acid; (PA) propionic acid; (BA) n-butyric acid; (tSCFAs) total short chain fatty acids; (*Bi*) *Bifidobacterium* spp.; (L) *Lactobacillus* spp.; (E) Enterobacteriaceae; (B) *Bacteroides fragilis*, (Cl) *Clostridium sensu stricto*; (C) correlation coefficient; (P) *p* value. Correlations were calculated using Spearman correlations.
*Correlation is significant at the 0.01 level (2-tailed).**Correlation is significant at the 0.05 level (2-tailed).

In ND patients, n-butyric acid had a positive correlation to acetic and propionic acid (both p = 0.01). Total SCFAs had a strong positive correlation to n-butyric and propionic acid (p = 0.002 and 0.000, respectively), followed by acetic acid (p = 0.03). *Lactobacillus* spp. negatively correlated with propionic acid, total SCFAs and *Clostridium sensu stricto* (all p = 0.04) (Table 2).

Table 2. Correlation coefficients and *p* values between SCFAs and bacteria in newly discovered not treated CD patients (ND group)

		AA	PA	BA	tSCFAs	*Bi*	*L*	E	*B*	*Cl*
AA	C		0.55	0.81*	0.76*	-0.77	-0.54	0.43	0.54	0.14
	P		0.16	0.01	0.03	0.07	0.27	0.40	0.27	0.79
PA	C	0.55		0.81*	0.95**	-0.31	-0.83*	0.14	0.60	0.54
	P	0.16		0.01	0.000	0.54	0.04	0.79	0.21	0.27
BA	C	0.81*	0.81*		0.91**	-0.20	-0.77	0.31	0.77	0.49
	P	0.01	0.01		0.002	0.70	0.07	0.54	0.07	0.33
tSCFAs	C	0.76*	0.95**	0.91**		-0.31	-0.83*	0.14	0.60	0.54
	P	0.03	0.000	0.002		0.54	0.04	0.79	0.21	0.27
Bi	C	-0.77	-0.31	-0.20	-0.31		0.09	-0.37	-0.09	0.26
	P	0.07	0.54	0.70	0.54		0.87	0.47	0.87	0.62
L	C	-0.54	-0.83*	-0.77	-0.83*	0.09		0.26	-0.31	-0.83*
	P	0.27	0.04	0.07	0.04	0.87		0.62	0.54	0.04
E	C	0.43	0.14	0.31	0.14	-0.37	0.26		0.77	-0.31
	P	0.40	0.79	0.54	0.79	0.47	0.62		0.07	0.54
B	C	0.54	0.60	0.77	0.60	-0.09	-0.31	0.77		0.26
	P	0.27	0.21	0.07	0.21	0.87	0.54	0.07		0.62
Cl	C	0.14	0.54	0.49	0.54	0.26	-0.83*	-0.31	0.26	
	P	0.79	0.27	0.33	0.27	0.62	0.04	0.54	0.62	

Footnotes:
(AA) acetic acid; (PA) propionic acid; (BA) n-butyric acid; (tSCFAs) total short chain fatty acids; (*Bi*) *Bifidobacterium* spp.; (L) *Lactobacillus* spp.; (E) Enterobacteriaceae; (B) *Bacteroides fragilis*, (Cl) *Clostridium sensu stricto*; (C) correlation coefficient; (P) *p* value. Correlations were calculated using Spearman correlations.
*Correlation is significant at the 0.01 level (2-tailed).**Correlation is significant at the 0.05 level (2-tailed).

In GFD patients, a strong positive correlation between all SCFAs and consequently total SCFAs (all $p = 0.000$) was observed. Total SCFAs were close to negative correlation with *Lactobacillus* spp. ($p = 0.051$). There was a positive correlation ($p = 0.02$) between acetic acid and *Clostridium sensu stricto*. The later had a negative correlation ($p = 0.02$) to *Lactobacillus* spp. A strong positive correlation ($p = 0.002$) between Enterobacteriaceae family and *Bacteroides fragilis* was found (Table 3).

Table 3. Correlation coefficients and *p* values between SCFAs and bacteria in patients on GFD for more than 1 year (GFD group)

		AA	PA	BA	tSCFAs	*Bi*	*L*	E	*B*	*Cl*
AA	C		0.74**	0.73**	0.96**	-0.12	-0.36	0.03	-0.26	0.46*
	P		0.000	0.000	0.000	0.55	0.06	0.86	0.18	0.02
PA	C	0.74**		0.77**	0.83**	-0.21	-0.31	0.20	-0.04	0.17
	P	0.000		0.000	0.000	0.29	0.11	0.31	0.85	0.41
BA	C	0.73**	0.77**		0.87**	-0.13	-0.24	0.03	-0.13	0.20
	P	0.000	0.000		0.000	0.50	0.22	0.88	0.51	0.32
tSCFAs	C	0.96**	0.83**	0.87**		-0.17	-0.37*	0.08	-0.20	0.35
	P	0.000	0.000	0.000		0.40	0.05	0.69	0.31	0.07
Bi	C	-0.12	-0.21	-0.13	-0.17		0.15	0.09	0.20	0.03
	P	0.55	0.29	0.50	0.40		0.45	0.66	0.30	0.88
L	C	-0.36	-0.31	-0.24	-0.37	0.15		0.07	0.23	-0.45*
	P	0.06	0.11	0.22	0.051	0.45		0.73	0.24	0.02
E	C	0.03	0.20	0.03	0.08	0.09	0.07		0.56**	-0.01
	P	0.86	0.31	0.88	0.69	0.66	0.73		0.002	0.96
B	C	-0.26	-0.04	-0.13	-0.20	0.20	0.23	0.56**		0.18
	P	0.18	0.85	0.51	0.31	0.30	0.24	0.002		0.36
Cl	C	0.46*	0.17	0.20	0.35	0.03	-0.45*	-0.01	0.18	
	P	0.02	0.41	0.32	0.07	0.88	0.02	0.96	0.36	

Footnotes:
(AA) acetic acid; (PA) propionic acid; (BA) n-butyric acid; (tSCFAs) total short chain fatty acids; (*Bi*) *Bifidobacterium* spp.; (L) *Lactobacillus* spp.; (E) Enterobacteriaceae; (B) *Bacteroides fragilis*, (Cl) *Clostridium sensu stricto*; (C) correlation coefficient; (P) *p* value. Correlations were calculated using Spearman correlations.
*Correlation is significant at the 0.01 level (2-tailed).**Correlation is significant at the 0.05 level (2-tailed).

4. Discussion

The active phase of the CD is mainly described as a non – treated disease or a disease not responding to the GFD treatment. In contrast, the non-active phase of the disease is illustrated as a disease in remission, mostly due to the GFD adherence [32,33]. Clearly, to study the pathogenesis of gastrointestinal diseases, knowledge about the microbiota composition and its relationship to the immune system is crucial.

The most consistent findings in active CD disease patients, when compared to healthy subjects, are lower levels of *Lactobacillus* [20,34] and *Bifidobacterium* [32,35,36]. Nevertheless, it should be taken into consideration, that these two genera should not be generalized, as some of the *Lactobacillus* [22] and *Bifidobacterium* [20,36,37] species may be specific for CD patients and / or healthy subjects. In fact, the diversity in *Bifidobacterium* and *Lactobacillus* species in CD patients has also been illustrated by contradictory results (reviewed in de Sousa Moraes et al. [10]). Moreover, in our results, the ND compared to the Control group did not reveal any statistically significant difference between bacterial groups, except for *Bifidobacterium* spp., which in our case was surprisingly almost significantly higher ($p = 0.059$) in the ND than in the Control group. However, a limitation in the number of ND patients in our study should also be taken in consideration.

Unlike other researchers, we did not find any statistically significant differences in *Bacteroides* [32,34,36,38,39], Enterobacteriaceae family group [13] and *Clostridium sensu stricto* between the ND and the Control group. Next, we evaluated differences between the GFD in relation to the ND and Control groups. By comparing the GFD patients with the ND patients, we found no statistically significant differences between the measured bacteria. However, differences were observed between the GFD and the Control group. The GFD group had significantly lower levels of *Lactobacillus* spp. than the Control group, which is in concordance with other studies [19,40]. In our study, the GFD group had significantly lower levels of Enterobacteriaceae family, compared to the healthy Control, but not the ND group. Many controversies can be found in the literature about the role of Enterobacteriaceae in CD. While in one study [40] this bacterial family tended to increase in the symptom free GFD patients relative to the healthy subjects, another study [13] identified members of this family to be abundant only in patients with active disease on a normal diet. Since other research groups also found Enterobacteriaceae to be higher in active [41], non-active (treated with GFD) [19] disease or even in both cases [34], this may lead to the conclusion that this family might not be directly related to the pathogenesis of the CD. Namely, healthy subjects increased the number of Enterobacteriaceae (*Escherichia coli*) [18,42] during the shift from normal to the GFD diet. Concomitantly, the number of so called potentially harmless and beneficial bacteria (*Bifidobacterium* spp. and

Lactobacillus spp.) in both studies decreased, which may lead to some open questions about what really causes the changes in microbiota.

Furthermore, an interesting observation was found in the ND and the GFD patients. In both groups, there was a negative correlation between *Lactobacillus* spp. and *Clostridium sensu stricto*, cluster I, suggesting diet independent relationship. In fact, *Clostridium* spp. is a very large group of bacteria that has also been detected in human gastrointestinal samples. Some of them belong to the *Clostridium sensu stricto*, cluster I. Although someone may speculate about it, their members are generally illustrated as pathogenic [43]. A very strong positive correlation between Enterobacteriaceae and *Bacteroides fragilis* was found in the GFD group. Since the previous studies have found out increased levels of possibly harmful *Bacteroides* [19,32,34,35,36,38] and Enterobacteriaceae family [13,32,34,40] in CD patients (active and / or non-active disease), these two groups of bacteria may actually correlate with each other and their correlations could have an important role, at least in the gastrointestinal tract of CD patients. Further investigations, regarding previously mentioned observations and potential relationship between the two bacterial groups are needed.

Gut microbiota is responsible for the production of SCFAs. Since microbiota dysbiosis occurs in CD patients, SCFAs profile is generally expected to change. In terms of SCFAs, acetic and propionic acid were significantly higher in the ND compared to the Control group, which is in agreement with previous studies [21,44,45]. According to the study of Tjellström et al. [45], acetic acid may have pro-, in contrast to butyric acid, which may have anti-inflammatory effects. However, butyric acid in our study was not recognized to differentiate ND patients from healthy children. Furthermore, propionic acid was significantly higher in the GFD group compared to the healthy Controls, which was also a case in two other studies [21,44]. Unlike some researchers [19,21,45,46], who found total SCFAs to be higher in CD patients compared to healthy subjects, we did not observe any statistically significant differences in total SCFAs between all patient groups. Interestingly, *Lactobacillus* spp. negatively correlated with total SCFAs in the Control and the ND group and almost negatively correlated ($p = 0.051$) in the GFD group. Moreover, in our study *Lactobacillus* spp. also had a negative correlation to propionic acid in the Control and the ND group, which altogether may not support the suggestion of Di Cagno et al. [46] about *Lactobacillus* and *Bifidobacterium* being responsible for an increase in large intestine SCFAs synthesis.

5. Conclusions

A complex interplay between diet, microbiota, immune system and individual factors may synergistically contribute to the disease pathology. Acetic and propionic acid are pro-inflammatory-related agents in CD and could be as useful marker of stage activity of the disease and prosperity of the treatment. Nevertheless, their origin in relationship to *Lactobacillus* and *Bifidobacterium* need to be further assessed. Enterobacteriaceae family might not be directly connected to the pathogenesis of CD. We believe that, apart from SCFAs and microbiota, a more complex input data model is needed to elucidate the trigger-response relationships in CD.

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Statement of Competing Interests

The authors have no competing interests.

Ethical Approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

List of Abbreviations

CD – celiac disease
SCFAs – short-chain fatty acids
ND - newly discovered, not treated children
GFD – gluten-free diet.

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