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Impact of biological treatment on intestinal microbiom in children with Crohn's disease

**Agnieszka Krawczyk^{1,*}, Agnieszka Sroka-Oleksiak², Kinga Kowalska-Duplaga³,
Krzysztof Fyderek³, Tomasz Gosiewski², Dominika Salamon²**

¹Faculty of Biotechnology and Horticulture, University of Agriculture in Cracow,
Al. 29 Listopada 54, 31-425, Cracow, Poland

²Department of Molecular Medical Microbiology, Chair of Microbiology, Faculty of Medicine,
Jagiellonian University, Medical College, Cracow, Poland

³Department of Pediatrics, Gastroenterology and Nutrition,
Jagiellonian University, Medical College, Cracow, Poland

*E-mail address: a.krawczyk993@gmail.com

ABSTRACT

Crohn's disease (CD) is a chronic, inflammatory illness of the digestive tract, characterized by alternating periods of remission and recurrence. The pathogenesis of CD is still unclear but probably is a result of a complex interaction between immunological, genetic and microbiological disorders. In recent years, there has been an increasing extent of evidence that gut microbiota plays a very important role in the pathogenesis of CD. Currently, the most effective treatment is biological therapy using anti-TNF monoclonal antibodies. It is interesting whether biological drugs resulting in fast remission, contributes to the normalization of the gut microbiota. Due to the fact that the children's population is a significant percentage of all patients with CD, it is important to pay close attention to the problem of microbiological disorders in this age group. The aim of this study was to investigate whether there are quantitative changes of chosen bacteria species and fungi of the genus *Candida* in children with Crohn's disease relative to healthy children and assesment of quantitative changes in patients after biological treatment. In the group of children with Crohn's disease, the numbers in *Candida* were significantly higher (9.74×10^{17} CFU/g) than in the control group (9.35×10^{10} CFU/g, $p = 0.011$). Biological therapy led to a significant reduction in the amount *Candida* (5.91×10^{11}) and was comparable with the number in the control group. In the case of bacteria, we observed an increase in *S. marcescens* ($3,4 \times 10^8$) in the patients group compared to the controls ($1,85 \times 10^8$) and an increase in *L.*

fermentum ($2,34 \times 10^{10}$) in relation to healthy children ($3,31 \times 10^8$, $p = 0,048$) Biological treatment had an impact on the decrease in *L. fermentum* ($4,76 \times 10^9$, $p = 0.05$)

Keywords: Crohn's disease, children, gut microbiota, biological treatment

1. INTRODUCTION

Crohn's disease (CD) is a chronic, inflammatory illness of the digestive tract, characterized by alternating periods of remission and recurrence. Symptoms of CD range from chronic, persistent diarrhea, nausea, abdominal pain and emesis to complications outside the gastrointestinal tract including arthritis, erythema nodosum, weight loss or delayed sexual maturation and growth in children [1].

The pathogenesis of this disease is still unclear but it is probably a result of a complex interaction between immunological, genetic and microbiological disorders. In recent years, there has been an increasing extent of evidence that gut microbiota plays a very important role in the pathogenesis of CD [2-4]. The first investigations focused on involving individual pathogens which could participate in the initiation of inflammation of the digestive tract. In particular, the contribution of *Mycobacterium paratuberculosis* (MAP), *Bacteroides fragilis* and adherent-invasive *Escherichia coli* (AIEC) pathotype have been considered [5-7].

Although the above mentioned bacteria are often found in people with CD and have a predisposition to initiate inflammation, so far reliable evidence has not been found confirming the participation of a specific microorganism in promoting the disease. Recently, it has been suggested that CD may not be caused by pathogenic microorganism but rather by intestinal dysbiosis leading to the growth of proinflammatory microbial species which constantly stimulate the immune system and interfere with its action or it is the effect of abnormal immune response directed against commensal microbes colonizing the gastrointestinal tract [8].

Crohn's disease is incurable so the aim of medical treatment is to reduce the inflammation which triggers the symptoms and which may lead to remission. The main drugs used in CD are 5-aminosalicylic acid (5-ASA) preparations and corticosteroids, but they are not always effective. In severe cases, the most effective treatment is biological therapy with anti-TNF antibodies, such as infliximab or adalimumab, which causes the healing of the inflammation of the intestinal mucosa [9].

These agents bind to TNF- α with high affinity thereby neutralizing biological activity of this proinflammatory cytokine. It is interesting whether the biological treatment resulting in fast remission, contributes to the normalization of the gut microbiota and if it is similar to the intestinal flora in healthy people. Due to the fact that the children's population accounts for a significant percentage of all patients with CD, it is important to pay close attention to the problem of microbiological disorders in this age group. Therefore, the aim of this study was to investigate whether there are any quantitative changes of chosen microorganisms in children with Crohn's disease in relation healthy children and assessment of quantitative changes in patients after biological treatment.

2. MATERIAL AND METHODS

Patients and samples: The study included 14 children hospitalized in the Department of Pediatrics, Gastroenterology and Nutrition, University Hospital, Kraków, Poland; whose diagnoses were based on clinical data, endoscopy, radiology and histology. Disease activity was scored by the Pediatric Crohn's Disease Activity Index (PCDAI) [10]. The control group consisted of 18 healthy volunteers. The subjects were required to be children up to 18 years old. Children who were taking probiotics or antibiotics in the 4 weeks before sampling were excluded. The subjects provided informed consent for participation in the study. The study was performed according to the Declaration of Helsinki, and was approved by the Bioethical Committee of the Jagiellonian University No. 122.6120.67.2015.

The control and patients were asked to deliver stool samples. Children with CD provided their stool samples twice: before anti-TNF- α (Remsima®) biological treatment and 2 weeks after the end of treatment which lasted 6 weeks. Samples were stored at -70 °C until analysis.

Table 1. Characteristics of the studied population

	Children with CD n = 14	Healthy children n = 18
Girls	7	9
Boys	7	9
Age (\pm SD)	13 (\pm 4,51)	11 (\pm 3,45)

DNA extraction and PCR amplification: Fecal bacterial and fungal DNA was extracted from 200-300 mg of feces. Isolation was carried out using Genomic mini AX Stool (A&A Biotechnology, Gdynia, Poland) along with pretreatment including enzymatic lysis with lysozyme (A&A Biotechnology), lysostaphin (A&A Biotechnology), lyticase (Sigma) and mechanical lysis using a FastPrep homogenizer (MP Biomedicals). Briefly, 100 μ l NaCl, 20 μ l of lysozyme and 10 μ l of lysostaphin were added into the tubes containing 200-300 mg of feces and glass beads.. The samples were homogenized (30 s, speed 4,0 m/s) using FastPrep and incubated for 30 min at 37 °C. Next, 200 μ l NaOH (Sigma) was added and left for 10 min at 95 °C.

After incubation, the tubes were centrifuged at 12 000 rpm for 10min (MPW-215 Adverti, Lodz, Poland). The supernatant was discarded and 500 μ l buffer with β -mercaptoethanol (A&A Biotechnology) and lyticase were added into the precipitate. The samples were incubated at 37 °C for 30 min. The consecutive steps of DNA isolation were performed according to the Genomic mini AX Stool protocol pursuant to the producer's instructions.

The extracted DNA was quantitatively examined for the following selected CD microbiota constituents: *Bacteroides fragilis*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Serratia marcescens* and *Candida spp.* by quantitative Real Time Polymerase Chain Reaction (qPCR) using the BioRad CFX96 thermal cycler. The reaction was prepared in a volume of 10µl. The mixture used in qPCR in the case of bacteria contained: 2,8µl of water, 5,0 µl of Real Time PCR Mix SYBR® C reaction mixture (A&A Biotechnology), 0,1 µl of forward primer (20 mM), 0,1 µl of reverse primer (20 mM) and 2 µl of DNA. TaqMan probe FAM-5'-TTAACCTACTAAATAGTGCTGCTAGC-BHQ1-3' (Genomed) [14] was used to detect *Candida* DNA and the composition of the reaction mixture was as follows: 2 µl of water, 5,0 µl of Real Time PCR Mix Probe (A&A Biotechnology) reaction mixture, 0,2 µl of forward primer (20 mM), 0,2 µl of reverse primer (20 mM) and probe (10 mM). The applied primers specific for the above-mentioned microorganisms are shown in Table 2. Oligonucleotides were synthesized by Genomed. The amplification programs are described in Table 3. Total microbial concentration was determined using standard curves which were generated from 10-fold serial dilutions of known concentrations of reference strain genomic bacterial or fungal DNA (range: 10¹ – 10⁷). The number of selected microorganisms was calculated per gram of stool by interpolating the cycle threshold (Ct) values obtained from the samples relative to appropriate standard calibration curve.

Table 2. Primers used in the qPCR reaction

Microorganisms	Primers	Sequence 5'→3'	Melting temperature (°C)	References
<i>Bacteroides fragilis</i>	Forward	5'-TCRGGGAAGAAAGCTTGCT-3'	45,8	[11]
	Reverse	5'-CATCCTTTACCGGAATCCT-3'	48,9	
<i>Lactobacillus rhamnosus</i>	Forward	5'-CGGCTGGATCACCTCCTTT-3'	53,2	[12]
	Reverse	5'-GCTTGAGGGTAATCCCCTCAA-3'	54,4	
<i>Serratia marcescens</i>	Forward	5'- TGCCTGGAAAGCGGCGATGG-3'	57,9	[13]
	Reverse	5'- CGCCAGCTCGTTCGTTGTGGT-3'	57,9	
<i>Lactobacillus fermentum</i>	Forward	5'-AACCGAGACCACCGCATTAT-3'	51,8	[14]
	Reverse	5'-ACTTAACCTTACTGATCGTAGATCAGTC-3'	57,3	
<i>Candida spp.</i>	Forward	5'-TTGGTGGAGTGATTTGTCTGCT-3'	53	[15]
	Reverse	5'-TCTAAGGGCATCACAGACCTG-3'	54,4	

To compare differences between the patients subjected to biological treatment the control group, as well as to check whether this difference is statistically significant, the non-parametric Mann–Whitney *U* test was used. The value of $p < 0.05$ was considered statistically significant.

Table 3. Thermal profile of the qPCR reaction

<i>Bacteroides fragilis</i>	95 °C – 5 min 95 °C – 30 sec 45 °C – 30 sec 72 °C – 30 sec	50 x
<i>Lactobacillus fermentum</i>	95 °C – 5 min 95 °C – 30 sec 51 °C – 30 sec 72 °C – 30 sec	40 x
<i>Lactobacillus rhamnosus</i>	95 °C – 5 min 95 °C – 30 sec 52.4 °C – 30 sec 72 °C – 30 sec	50 x
<i>Serratia marcescens</i>	95 °C – 5 min 95 °C – 20 sec 55 °C – 30 sec 72 °C – 1 sec	39 x
<i>Candida</i> spp.	95 °C – 2 min 95 °C – 15 sec 55 °C – 30 sec 72 °C – 30 sec	50 x

3. RESULTS

In this study we quantified a selected microorganisms in stool samples taken from children with Crohn’s disease in relation to control group and for the first time we examined the influence of biological treatment on the intestinal microflora. The average bacterial and fungal quantifications of stool in each group are presented in Table 4. The comparisons of the fecal microorganisms in all groups is shown in Fig. 1.

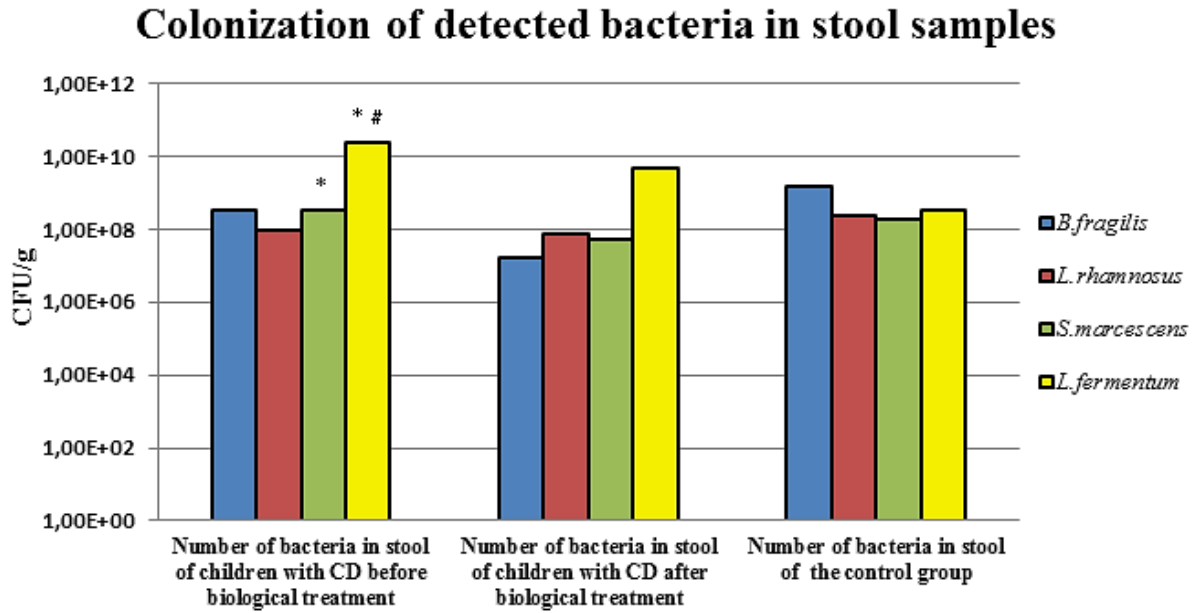


Fig. 1. Quantitative evaluation of bacteria detected in the feces of patients with CD before biological treatment, after treatment and control using qPCR.
 * significant differences between children with CD and the control group;
 # significant differences between children with CD before biological treatment and after therapy

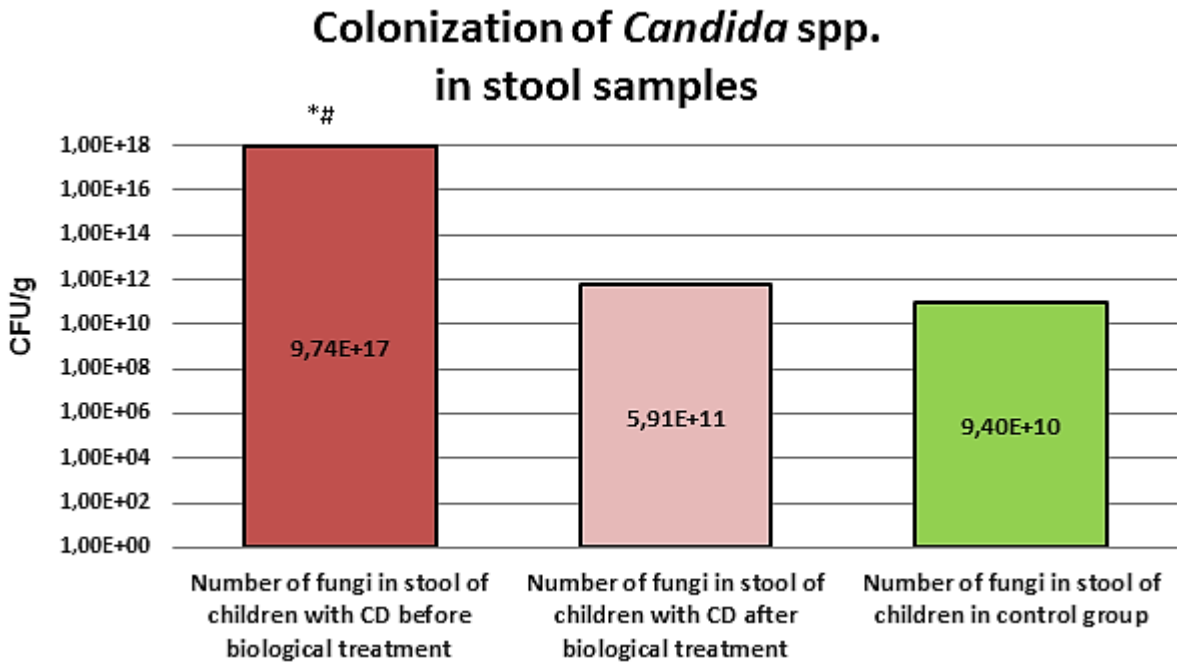


Fig. 2. Quantitative assessment of fungi of the genus *Candida* in the feces of patients with CD before biological treatment, after TNF- α therapy and control using qPCR.
 *significant differences between children with CD and the control group;
 # significant differences between children with CD before biological treatment and after therapy

We observed an increase in *Candida* in the group of children with Crohn's disease. The number of fungal cell was a significantly higher compared to the control group ($p=0.011$). Moreover, biological therapies led to a substantial reduction in the number of fungi in the digestive tract of children with Crohn's disease which were comparable with healthy control ($p=1.0$). In the case of bacteria, we observed an increase in *S. marcescens* in the patients group compared to the controls ($p=0.048$) and an increase in *L. fermentum* in relation to healthy children ($p=0.001$) Biological treatment had an impact on the decrease in *L. fermentum* ($p=0.05$) and caused reduction in number of *S.marcescens* but in this case, it was not statistically significant. There were no quantitative changes in *L. rhamnosus* among the patients relative to the healthy group.

Table 4. Median number of microorganisms isolated per gram in the studied groups

Microorganism	Number of microorganisms in stool of children with CD before biological treatment [CFU/g]	Number of microorganisms in stool of children with CD after biological treatment [CFU/g]	Number of microorganisms in stool of children in the control group [CFU/g]
<i>Candida</i> spp.	$9.74 \times 10^{17a,b}$	5.91×10^{11}	9.40×10^{10}
<i>Bacteroides fragilis</i>	3.49×10^8	1.68×10^7	1.45×10^9
<i>Lactobacillus fermentum</i>	$2.34 \times 10^{10a,b}$	4.76×10^9	3.31×10^8
<i>Lactobacillus rhamnosus</i>	9.25×10^7	7.23×10^7	2.46×10^8
<i>Serratia marcescens</i>	3.4×10^{8a}	5.1×10^7	1.85×10^8

^a $p < 0.05$ differences between children with CD and the control group;

^b $p < 0.05$ differences between children with CD before biological treatment and after therapy

4. DISCUSSION

4 types of bacteria constitute more than 90% of the natural gut microbiota: Firmicutes (64%), Bacteroidetes (24%), Proteobacteria (8%) and Actinobacteria (3%) among which the following types are predominant: *Bifidobacterium*, *Lactobacillus*, *Streptococcus*, *Enterobacteriaceae* and *Bacteroides* [16]. Their occurrence brings many positive aspects such as: neutralization of toxins, supporting digestive processes, sealing of the intestinal barrier or protection against pathogenic bacteria. Maintaining a balance between specific strains is a necessary condition for preserving homeostasis and appropriate functioning of the body.

Among the people suffering from CD, an imbalance can be found in the amount, composition and functioning of individual microorganisms, which is referred to as dysbiosis. Scientific data show that quantitative changes of gut microorganisms may cause excessive stimulation of the immune system - which in genetically predisposed people, results in autoimmune diseases, including CD [8].

One of the several bacteria widely suspected of pathogenesis of Crohn's disease is *Bacteroides fragilis*. A lot of studies have shown a significant abundance of these bacteria in people with CD [17,18]. A potential virulence factor of *B.fragilis* is the production of enterotoxin - fragilysin, which hydrolyzes cadherin peptide bonds, a protein that is part of tight junctions. Disruption of the intestinal mucosa barrier by division of these intercellular proteins between enterocytes leads to an increase in intestinal permeability, which allows the passage of undesirable substances such as toxin, bacterial or food antigens from inside the gastrointestinal tract into the bloodstream, leading to the development of chronic inflammation [19]. In this study, we did not observe a significant abundance of this bacterium in stool samples of patients. Also, biological treatment did not affect the quantitative changes as regards this microorganism. A lot of studies confirming an increase in *B.fragilis* in CD patients were carried out with the participation of adults. Our studies in children did not show an increase in the number of these bacteria in a pediatric group with CD, which can suggest that quantitative abnormalities of *Bacteroides* which were found in the previous research, may be a secondary effect of the disease and be a result of a long-lasting inflammatory process of the digestive system, but they are not the primary cause of Crohn's disease.

A new subject for research in the pathogenesis of Crohn's disease is *S. marcescens*. Until now, the only study taking into account the participation of this bacterium in the pathogenesis, carried out by Hourau et al. showed a significant abundance of *S.marcescens* cells in patients with CD. Moreover, the researchers showed that the abundance of this bacterium was correlated with excessive numbers of *Escherichia coli* and *Candida tropicalis*. Interestingly, it was noted that these microorganisms cooperate together to form a thick layer of biofilm tightly adhering to the intestine, thus inducing an inflammatory reaction that could result in symptoms of Crohn's disease [20]. In our study, we also observed statistically significant increased numbers of *S.marcescens* in patients. Moreover, we proved a significant abundance of *Candida* spp. in stool samples of children with Crohn's disease. Interestingly, anti-TNF- α therapy had an impact on reducing the amount of fungal cells and after treatment they were comparable to healthy children. These results may suggest that among patients with CD there is a loss of tolerance towards commensal fungi residing in the digestive tract, which results in hyper-activity of the gastrointestinal tract with release excessive amounts of TNF- α , leading to non-specific intestinal tissue damage. With the reduction of inflammation and healing of the intestinal mucosa, the number of fungi is reduced and is similar to the one in healthy people. The confirmation of this hypothesis may be the results of study carried out by Iliev et al., which showed that mice with polymorphisms within the Dectin-1 receptor (involved in innate immune responses to fungal pathogens), do not tolerate intestinal fungi of the genus *Candida* and release excessive amounts of TNF- α and IFN- γ cytokines against them which results in inflammatory bowel disease [21]. When secretion of TNF- α is stopped after the administration of biological drugs, the number of *Candida* decreases and patients receive long-term remission even after the therapy.

There are varied reports about changes in the abundance of *Lactobacillus* in people with CD. Some studies have reported an increased number of *Lactobacillus* among patients [22] while others claim that there is a significantly reduced number of these bacteria [23].

The strong interest of researchers in this bacterium is primarily due to the fact that the majority of *Lactobacillus* strains have a beneficial effect on the human body and are used as probiotic preparations which have a preventive effect against the invasion of pathogenic microorganisms and also have a soothing effect on diarrhea or abdominal pain. Therefore, some researchers assumed that in Crohn's disease too low amounts of the individual strains of the genus *Lactobacillus* may be present resulting in a weakened protective barrier against pathogenic strains inducing an inflammatory response and manifested by frequent diarrhea. The best-documented health benefits are demonstrated by *L. rhamnosus*, which is commonly used in the form of probiotic preparations and has antidiarrheal properties and inhibitory effects on the growth of pathogenic bacteria [24, 25], therefore, we wanted to examine the amount of this strain in the patient population. In our study, there were no statistically significant differences in the colonization of the intestine by *Lactobacillus rhamnosus* in children compared to the healthy control.

Biological treatment did not influence the amount of these microorganisms either. Considering the fact, that previous studies (which showed different results from ours) were carried out on adults whose disease processes have been ongoing for many years, it may be assumed that in older people with CD, quantitative changes within commensal bacteria are most likely caused by environmental factors such as long-term use of antibiotics like metronidazole or ciprofloxacin, which are often used as an auxiliary therapy of Crohn's disease or may be the result of diet (poor in dairy products which are a source of *Lactobacillus*), hence decreased levels of these bacteria are caused by long-term influence of external factors and most probably, that the participation of *L. rhamnosus* does not play a direct role in the initiation and pathogenesis of this disease entity. In addition, this thesis can be confirmed by the fact that attempts at probiotic application of the bacteria from the genus *Lactobacillus* to people with Crohn's disease do not contribute to relieving symptoms or inducing remission [26, 27]. It should be noted that *Lactobacillus* is a huge group of bacteria, which includes many species with extremely different life requirements and various effects on the human body, so it can not be ruled out that the inflammatory process in the gastrointestinal tract in the course of CD, determines in some way the amount of certain species of lactobacilli. In our study, for the first time we examined the participation of *L. fermentum* in Crohn's disease in people.

One should pay attention to the fact that in stool samples of children with Crohn's disease an increase in this strain was observed. Moreover, biological therapy led to reduced levels of *L. fermentum*. It must be noted that this species of *Lactobacillus* produces H₂O₂ and thereby may induce inflammation in the intestine, which would explain the increased number of these bacteria in patients [28, 29]. When the inflamed tissue is healing due to the biological treatment, the amount of bacteria is reduced. Moreover, previous studies on the potential risks associated with these probiotic bacteria - suggested that some strains of *L. fermentum* can have a negative influence on intestinal barrier integrity. Anderson et al. demonstrated that some human oral isolates of this microorganism caused an increased expression of genes and an abundance of proteins responsible for loosening the tight junction between epithelial cells [30] which is an important disorder in the pathogenesis of CD. Furthermore, other independent studies involving rats showed strong proinflammatory properties of a certain

strain of *L. fermentum*. Anderson et al. proved that this bacterium has an impact on the activity of the Toll - like receptor (TLR) belonging to pattern recognition receptors (PRRs). Due to TLR, the human immune system is able to distinguish non-self antigens from self-antigens. Unexpectedly, *L. fermentum*, belonging to the commensal microflora, induced excessive expression of PRR, resulting in the release of a significant amount of proinflammatory cytokines such as TNF-alpha and IL-6 [31]. These data show that, among the patients with CD, there may be genetic disorders within the TLR resulting in an excessive immune response to the abundance of *L. fermentum* resulting in intestinal inflammation. Further studies are needed regarding the participation of this bacterium in the pathogenesis of CD.

5. CONCLUSION

In the group of children with Crohn's disease, the numbers of *Candida*, *S. marcescens* and *L. fermentum* were a significantly higher than in the control group. Biological therapy led to a significant reduction in the amount of the *Candida* fungi and *L. fermentum* in the stool sample from children with Crohn's disease. Biological treatment did not impact the reduction in the number of *S. marcescens*. Differences in the numbers of *B. fragilis* or *L. rhamnosus* were not observed.

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