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Sila, Sara

UNIVERSITY OF ZAGREB
FACULTY OF FOOD TECHNOLOGY AND BIOTECHNOLOGY

Sara Sila

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THE IMPACT OF DIET ON INTESTINAL MICROBIOTA OF PAEDIATRIC PATIENTS WITH INFLAMMATORY BOWEL DISEASE

Sara Sila, MSc Nutrition

Thesis performed at the Children's Hospital Zagreb

Supervisor: Assistant Professor *Iva Hojsak*, **MD, PhD**

Short abstract
The diet has a profound effect on the gut microbiota and development of inflammatory bowel disease (IBD). Characteristics of the gut microbiota were determined in children with IBD at the time of diagnosis and on the 2nd and the last day of exclusive enteral nutrition (EEN). Children with IBD demonstrate significant differences in dietary intake (lower intake of energy, calcium and fruits) and lower lean mass-for-age z-scores at diagnosis. Moreover, lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in patients compared to healthy siblings and healthy controls at the time of diagnosis. EEN leads to similar changes in the microbiota composition in children with CD and their healthy siblings.

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UTJECAJ PREHRANE NA CRIJEVNU MIKROBIOTU U PEDIJATRIJSKIH BOLESNIKA S UPALNIM BOLESTIMA CRIJEVA

Sara Sila, mag. nutr.

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Kratki sažetak disertacije
Prehrana ima značajan utjecaj na crijevnu mikrobiotu i razvoj kroničnih upalnih bolesti crijeva (IBD). Karakteristike crijevne mikrobiote u djece s IBD-om određene su u vrijeme dijagnoze, te na drugi i posljednji dan isključive enteralne prehrane (EEN). Djeca s IBD-om pokazuju značajnu razliku u prehrambenom unosu (niži unos energije, kalcija i voća), te niži z-score za nemasnu tjelesnu masu u vrijeme dijagnoze u usporedbi sa zdravim kontrolama. Nadalje, utvrdili smo manju zastupljenost bakterija iz reda Firmicutes, te veću zastupljeno iz reda Proteobacteria kod djece s IBD-om u vrijeme dijagnoze. EEN dovodi do sličnih promjena u sastavu mikrobiote u djece sa CD-om i njihovih zdravih braće i sestara.

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THE IMPACT OF DIET ON INTESTINAL MICROBIOTA OF PAEDIATRIC PATIENTS WITH INFLAMMATORY BOWEL DISEASE

It has been confirmed in previous studies that the diet has a profound effect on the gut microbiota, and consequently development of inflammatory bowel disease (IBD). This has become especially evident with the use of exclusive enteral nutrition (EEN) as a first line therapy for induction of remission in children with Crohn's disease (CD). Mechanism of action of EEN includes modification of the gut microbiota, which in turn leads to reduction of inflammation and consequently remission of the disease. The aim of this dissertation was to compare characteristics of the gut microbiota in children with IBD at the time of diagnosis with that of healthy unrelated controls, but also to that of their healthy siblings (who share a common genetic origin and live in the same environment). Moreover, impact of EEN on the microbiota of both CD patients and their healthy siblings has been investigated. We have demonstrated that children with IBD have significantly lower intake of energy, some micronutrients and fruits compared to healthy controls, along with lower lean mass-for-age z-scores at diagnosis. Moreover, children with IBD demonstrate significant differences in microbiota composition at the time of diagnosis, with reduced presence of genus *Eubacterium*, *Lactobacillus*, *Enterobacter* and *Clostridium*, and increased presence of genus *Streptococcus*, *Prevotella* and *Escherichia*, compared to healthy siblings and healthy controls. Nevertheless, EEN leads to similar changes in the gut microbiota composition of both children with CD and their healthy siblings, which is, interestingly, occurring more rapidly in healthy siblings. In conclusion, microbiome change could have a crucial role in the remission induction of the disease.

Keywords: children; diet; exclusive enteral nutrition; gut microbiota; inflammatory bowel disease

UTJECAJ PREHRANE NA CRIJEVNU MIKROBIOTU U PEDIJATRIJSKIH BOLESNIKA S UPALNIM BOLESTIMA CRIJEVA

Prethodna istraživanja su potvrdila da prehrana igra važnu ulogu u etiopatogenezi mnogih bolesti, uključujući i kronične upalne bolesti crijeva (eng. inflammatory bowel disease – IBD). Navedeno je potvrđeno korištenjem isključive enteralne prehrane (eng. exclusive enteral nutrition – EEN) kao prve linije indukcije remisije u djece s Crohnovom bolesti (eng. Crohn's disease – CD). Mehanizam djelovanje EEN uključuje, između ostalog, i modifikaciju crijevne mikrobiote, što dovodi do smanjenja upale i posljedično remisije bolesti. Cilj ove disertacije bio je usporediti karakteristike crijevne mikrobiote u djece s IBD-om u vrijeme dijagnoze sa zdravim kontrolama, ali i zdravom braćom i sestrama koji dijele genetsko podrijetlo i žive u istom okruženju. Nadalje, ispitan je utjecaj EEN-a na mikrobiotu kod bolesnika s CD-om, ali i njihovih zdravih braće i sestara. Pokazali smo da djeca s IBD-om imaju značajno manji unos energije, nekih mikronutrijenata i voća u odnosu na zdrave kontrole, te da imaju manji z-score za nemasnu tjelesnu masu u vrijeme dijagnoze. Nadalje, potvrdili smo da djeca s IBD-om pokazuju značajne razlike u sastavu mikrobiote u vrijeme postavljanja dijagnoze u usporedbi sa zdravim kontrolama, sa smanjenim udjelom bakterija iz rodova *Eubacterium*, *Lactobacillus*, *Enterobacter* i *Clostridium,* te povećanim udjelom bakterija iz rodova *Streptococcus*, *Prevotella* i *Escherichia*. Osim toga, EEN dovodi do sličnih promjena u sastavu mikrobiote kod djece s CD-om i njihovih zdravih braće i sestara, no taj je učinak brže uočljiv kod zdravih braće i sestara. Zaključno, promjene u mikrobioti mogle bi imati ključnu ulogu u uvođenju bolesti u remisiju.

Ključne riječi: crijevna mikrobiota; djeca; isključiva enteralna prehrana; prehrana; upalne bolesti crijeva

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GENERAL INTRODUCTION

Inflammatory bowel disease (IBD), comprising of Crohn's disease (CD), ulcerative colitis (UC) and IBD-unclassified (IBD-U), is an immune mediated chronic inflammatory disorder of gastrointestinal (GI) tract. CD and UC are two very distinct diseases with regard to their clinical manifestations and the part of the GI tract they are affecting. CD can involve any part of the GI tract from the mouth to the anus and can present with inflammatory, penetrating, stricturing or combination phenotype. Ulcerative colitis is characterized by diffuse, continuous inflammation of the colon.

About ¼ of patients with IBD present before the age 20 years old (Baldassano and Piccoli, 1999) in whom the disease can have significant effect on growth and development, but also their quality of life. In the last 20 to 30 years, a striking increase in the incidence of IBD has been observed especially in this group of patients (Ruel et al., 2014). Many different factors are involved in the development of this disease. Among them, significant attention has been given to the environment.

Different environmental factors have been shown to be associated with development of IBD, including exposure to sunlight/vitamin D, smoking, exposure to pets, exposure to antibiotics, consumption of dietary fibers, processed foods, breastfeeding, just to name a few (Shouval and Rufo, 2017). It is clear today that all the factors could influence immune system development and affect human gut microbiome. These environmental factors, especially affecting in early life, might be associated with profound and long-lasting changes and possibly could contribute to the rising incidence of IBD.

Since paediatric patients with IBD are prone to impaired growth and inadequate nutritional status, importance of the adequate diet and nutritional support has been recognized long ago. Effect of the diet on the disease is even more emphasized by the effect of the use of exclusive enteral nutrition (EEN) for remission induction in children with CD. Not only does it lead to reduction in inflammation, but it also supports growth and improves nutritional status, along with mucosal healing.

How diet effects and modifies immune reaction is still not clear, but increasing evidence is showing that the diet causes significant changes in the microbiota composition of patients with CD (Assa and Shamir, 2017a; MacLellan et al., 2017). Therefore, the importance of microbiota in the pathogenesis of IBD has been well recognized. However, to this date, it is not clear whether dysbiosis is a consequence or cause of the intestinal inflammation.

Throughout this thesis the following questions were examined:

- Does nutritional status differ between newly diagnosed children with IBD and healthy controls?
- How does dietary intake differ between newly diagnosed children with IBD and healthy controls?
- Is there a difference in intestinal microbiota in children with newly diagnosed IBD and their siblings and healthy unrelated controls?
- Does the route of EEN delivery (orally vs NG tube) and type of polymeric formula (with taste vs tasteless and isocaloric vs hypercaloric) impact the disease outcome and nutritional status in children with CD?
- What is the effect of EEN therapy on the intestinal microbiota in children with CD and how do they compare to the changes caused by EEN in healthy individuals?

CHAPTER I

Theoretical background

- Epidemiologic features
- Pathogenesis
- Clinical presentation
- Treatment

1. THEORETICAL BACKGROUND

1.1. Epidemiologic features

Approximately 25% of patients with IBD present before age of 20 years (Baldassano and Piccoli, 1999). Compared to IBD in adult age, IBD in children presents with more extensive disease phenotype and more severe clinical course (de Bie et al., 2013; Duricova et al., 2014). Furthermore, the disease in children shows tendency to spread over time, and recurrent exacerbations in CD change the disease behavior from inflammatory to stricturing and penetrating form (Duricova et al., 2014; Malmborg and Hildebrand, 2016). Additionally, in children the disease has significant consequences on their growth and development, as well as their quality of life.

The latest data shows broad variation in the incidence rates in paediatric population, ranging from 0.5 to 23/100000 for IBD, 0.1 to 13.9/100000 for CD, 0.3 to 15.0/100000 for UC and 0.0 to 3.6/100000 for IBD-U (Sýkora et al., 2018). The incidence of IBD greatly varies based on the geographical region. The regions with the highest IBD burden are Europe (0.2-23.0/100000) and North America (1.1-15.2/100000), whereas Oceania, Asia, Latin America and Africa have the lowest reported IBD incidence (Sýkora et al., 2018).

In the past 20 to 30 years, a striking increase in the incidence of IBD has been observed in patients <20 years of age (Ruel et al., 2014). Moreover, increase in IBD mostly concerns paediatric-onset CD, while UC incidence has been reported to be stable (Ruel et al., 2014). The data published during the previous two decades demonstrated the plateauing incidence of IBD in the Western countries (Sýkora et al., 2018) after a previously documented increase. However, the incidence still remains high. In newly industrialized countries in Asia, the Middle East and Africa, the incidence is approaching the rates reported in westernized countries. Based on the data from the newly established national pediatric IBD registry, annual incidence of PIBD in Croatia was 7.05/100000 children under 18 years of age/year, with the predominance of UC over other two forms of IBD (Ivković et al., 2020).

1.2. Pathogenesis

It has been observed that in IBD, host genetic, environmental and microbial factors combine and lead to a dysregulated mucosal immune response against the gut content/intestinal microbiota

(Khor et al., 2011). Dramatic lifestyle changes in the last century have, along with improved quality of life, led to increased risk of various diseases. Since only a small proportion of IBD can be explained by genetics, these lifestyle changes have been increasingly explored in connection to IBD development. Microbiota, influenced by many lifestyle factors, has also been emphasized as having an important role in the etiology of IBD. Today it is considered that IBD is in fact a group of related complex diseases which results from a confluence of genetic, microbial and environmental factors, all leading to dysregulation in the mucosal immune system (Shouval and Rufo, 2017) (Figure 1). In genetically predisposed individuals, there is an aberrant immune response to the microbiome that leads to the development of intestinal inflammation (Glassner et al., 2019). Indeed, data from a number of animal studies have shown that colonization of mice with intestinal microbiota from donors with IBD exacerbates colitis by altering immune responses (Britton et al., 2019). Moreover, transfer of naïve $CD4^+$ lymphocytes from healthy mice into mice that lack T and B lymphocytes can induce colitis (Powrie, 1995; Powrie et al., 1994). Nevertheless, many of the genetic mutations associated with IBD are related to immune function and interactions between the immune system and the microbiome (Glassner et al., 2019).

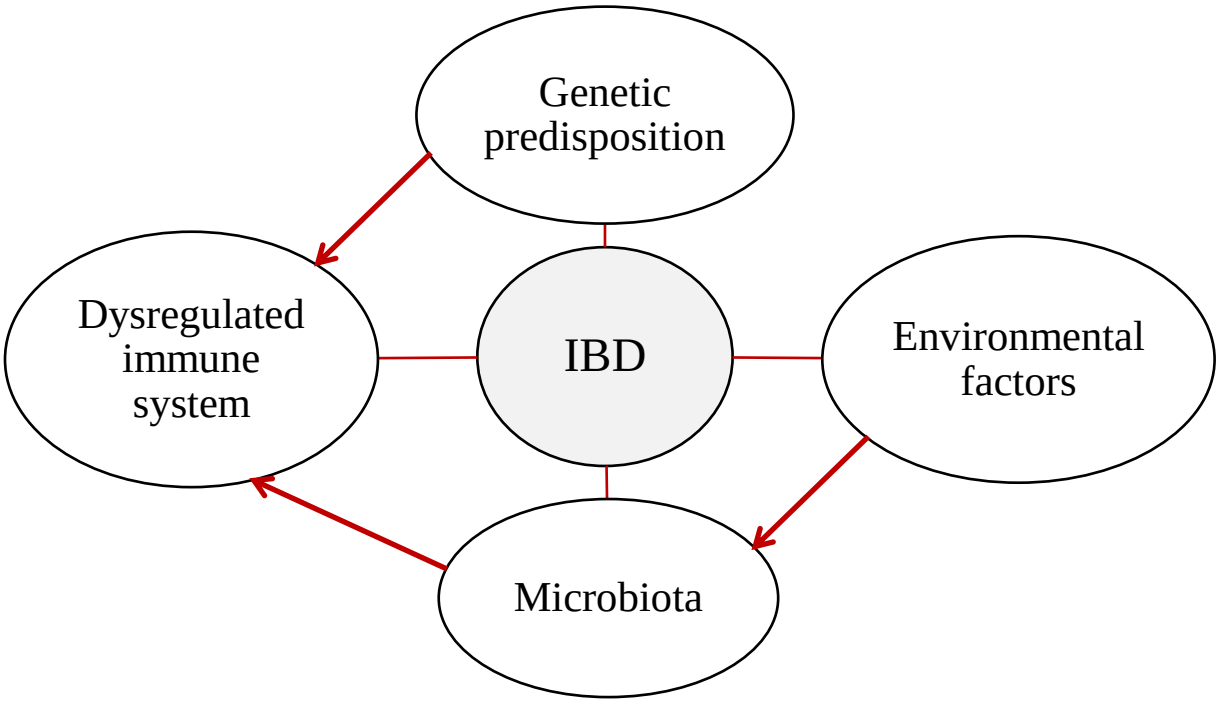

Figure 1. The pathogenesis of inflammatory Bowel Disease (IBD)

1.2.1. Genetic factors

The most important risk factor in etiopathogenesis of IBD is the presence of the disease in the family. Positive family history of IBD is present in 19-41% of children compared to only 5-10% of patients in whom disease started after the age of 18 (Gower-Rousseau et al., 2013). Children whose parent has been diagnosed with IBD have 2-13 times higher risk of being diagnosed with IBD compared to the general population (Peloquin et al., 2016). However, the risk of being diagnosed with IBD inside the family refers to other factors additional to hereditary predisposition, such as being exposed to the same environmental factors. More than 163 genes have been discovered to be correlated with higher risk of IBD. Genetic mutations of genes, of which the most important ones are nucleotide oligomerization domain 2 (*NOD2*), autophagy-related 16-like 1 (*ATG16L1*), caspase recruitment domain-containing protein 9 (*CARD9*), and C-type lectin domain family 7 member A (*CLEC7A*) lead to changes in the immune function and are associated with development of IBD (Cohen et al., 2019; Jostins et al., 2012; Lavoie et al, 2019; Liu et al., 2015). However, only about 7.5% of incidence of CD and 13.6% of incidence of UC can be explained by genetics (Jostins et al., 2012) (with the exception of monogenic diseases). Therefore, current emphasis is on different environmental (such as the diet) and microbial factors, which could play a crucial role in the etiopathogenesis of IBD. Importance of the environment on the development of IBD is becoming more apparent with the raising research in the field of pathogenesis of IBD.

1.2.2. Environmental factors

Rapidly changing epidemiology of IBD suggests that environmental factors are playing a critical role in the development of IBD as well as in the modulation of disease phenotypes over time. Today, a new term – *exposome* – encompasses the composite of accumulated environmental exposures that start *in utero* and continue through childhood into adulthood (Wild, 2012). These factors provide understanding into the pathogenesis of the IBD, but also provide new insights into potential dietary, lifestyle and pharmacologic interventions which could help change the course of the disease, or lower the risk of acquiring the disease. Many different factors have been shown to be correlated with IBD, as shown in Figure 2. However, it is not yet clear to which extent and at what time of the exposure they could affect individuals' risk for IBD.

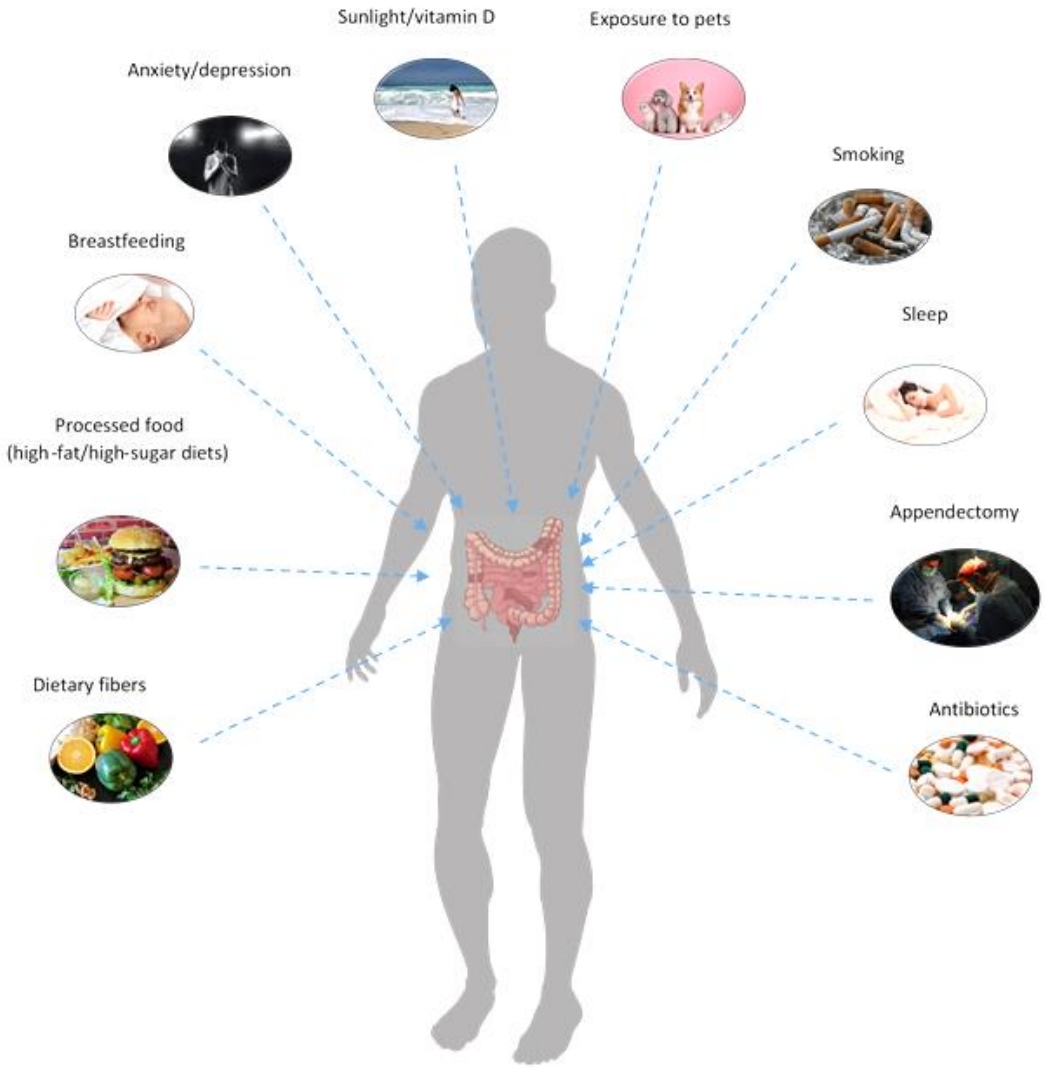

Figure 2. Environmental factors that possibly modulate the risk of developing inflammatory bowel disease (Shouval and Rufo, 2017).

Three important observations have been made, which confirm the significant role that environment plays on the disease development: 1) the concordance rate for CD in monozygotic twins is only 50% and even less for UC (Tysk et al., 1988); 2) the rising incidence of IBD in the last 60 years could not be explained by changes in our genetic makeup, which did not change for thousands of years; 3) the incidence of IBD rises as the country becomes more developed (Loftus, 2004). Additionally, children whose parents immigrated from developing countries to developed country exhibit the same incidence of IBD as native population (Carr and Mayberry, 1999). Moreover, many environmental factors have been implicated in the development of IBD, such as mode of

delivery, type of feeding during first years of life and early exposure to antibiotics (Ananthakrishnan et al., 2018).

1.2.2.1.Early life factors

It has been established in recent years that different early-life events might explain the development and clinical severity of IBD. Studies have shown that the functional maturation of gut microbiome occurs at the first 3-5 years of life, after which it remains stable despite different environmental factors (Yatsunenko et al., 2012). This observation emphasized potential importance of influences in that period on subsequent risk of IBD. Early-life risk factors that have shown the most significant association with the disease development are mode of delivery (cesarean vs vaginal delivery), early exposure to antibiotics and breastfeeding (Ananthakrishnan et al., 2018; Kolaček and Hojsak, 2017; Shouval and Rufo, 2017).

Babies born by caesarian section are deprived of contact with the maternal gut or vaginal microbiota, therefore, show different microbiota composition compared to children born vaginally (Azad et al., 2016, 2013). Further studies indicate that abnormal microbiota reported after caesarian section delivery could continue even beyond infancy (Salminen et al., 2004). Even though data have clearly demonstrated that mode of delivery can have a major effect on the composition of the intestinal microbiota, data as to whether mode of delivery affects the subsequent risk of developing IBD later in life are conflicting. A population-based study which determined whether mode of delivery affects risk of IBD showed no difference in the percentage of individuals with IBD born by caesarian section (11.6%) versus controls (11.7%, P=0.93). Furthermore, individuals with IBD were no more likely to have been born by caesarian section than were their siblings without IBD (11.6% vs 11.3%; P=0.79) (Bernstein et al., 2016). In a large Danish cohort individuals delivered via caesarean birth had a modest but significant risk in the subsequent development of IBD (Bager et al., 2012). Study in Norway showed that patients with IBD were less likely to be delivered by caesarean (Bengtson et al., 2010).

Another early-life event that was shown to be protective of later development of IBD in some studies, is breastfeeding. Breast milk contains a large number of anti-microbial, anti-inflammatory and immunomodulatory compounds that could potentially modify the risk of the development of chronic disease (Rodriguez-Palmero et al., 1999). Furthermore, breastmilk-fed infants develop

markedly different patterns of gut colonization in comparison with formula-fed infants (Orrhage and Nord, 1999). Two meta-analysis were performed to assess the role of breastfeeding in the development of early onset IBD. One meta-analysis showed a strong inverse association between breastfeeding and IBD in both CD and UC (Klement et al., 2004). Another study similarly demonstrated a strong inverse association with early-onset diseases (Barclay et al., 2009). Moreover, a duration response effect for both UC and CD was observed, whereby protective effect was only significant when duration of breast feeding was greater than 12 months (Ng et al., 2015). However, some studies have shown opposite results. A new case-control study found that breastfeeding for more than 3 months was associated with an increased risk for CD (Strisciuglio et al., 2017). Furthermore, it was shown that mother's degree, duration of breast feeding more than 3 months, fathers' employment, early gluten introduction at the time of weaning, number of siblings less than 2 were significant risk factors for CD, while owning of pets and bed sharing were protective factors for CD. For UC, early gluten introduction and number of siblings less than 2 were significant risk factors, whereas owning of pets and family parasitosis were protective factors for UC.

Additionally, several studies have examined whether antibiotic use early in life predisposed to IBD and have consistently shown this association in Western population (Ungaro et al., 2014). Studies have shown that individuals who were receiving one or more dispensations of antibiotics in their first year of life were nearly three times as likely to be diagnosed with IBD (Shaw et al., 2010). Similarly, another study showed that individuals with otitis media by the age of 5, who were given antibiotics as a treatment, were nearly three times as likely to have IBD (Shaw et al., 2013). Furthermore, a dose-dependent relationship between the number of antibiotics dispensations and risk of IBD was observed (Shaw et al., 2011).

Causality studies are difficult to be performed and could have many cofounding factors which could at least partially explain why results are not uniform. However, they, if taken together could point to same direction - hygiene hypothesis - which states that areas with better sanitation and higher hygiene standards, where the exposure to different microbes is often reduced, are in higher risk of acquiring IBD later in life. Hygiene hypothesis can also explain why some areas have shown that breastfeeding was associated with an increased risk of developing IBD – in developed countries, with higher sanitation and hygiene standards, highly educated women are more likely

to breastfeed (Strisciuglio et al., 2017). It is now being confirmed by other studies that lower exposure to microbes leads to changes in microbiota which could potentially increase the risk of acquiring IBD later in life in genetically predisposed individuals (Fofanova et al., 2016).

1.2.2.2.Dietary factors

It was already emphasized that diet is considered as the most important risk factor. It is known today that the complex interaction of dietary components and the diet itself with the host's immune system, most probably indirectly via intestinal microbiota, is a key part in the development of chronic inflammation and consequently IBD, but also other diseases. Researchers have been focusing on the diet in two different aspects when linking it to IBD: 1) to identify dietary habits and components of the diet that are associated with higher risk of acquiring IBD in susceptible individuals (pre-illness diet) and 2) to identify dietary habits and components of the diet that can affect the course of the disease and potential dietary treatment of IBD.

It has been discussed earlier in the text that western life style, characterized by high intake of fat, read meat and sugar along with low intake of fruits and vegetables, has been positively correlated with the risk of developing IBD in epidemiologic studies (Hou et al., 2011; Persson et al., 1992). The most convincing results exist for high intake of fruits and dietary fiber, which were shown to be associated with lower risk of CD (Hou et al., 2011). Results were conflicting for vegetables. As for UC, data have shown decreased risk of UC with high fiber intake, however, results were not statistically significant (Hou et al., 2011). In a prospective study of 170,000 women enrolled in the Nurses' Health Study, higher omega-3/omega-6 long chain polyunsaturated fatty acids (LC PUFA) ratio was associated with lower risk of UC (Ananthakrishnan et al., 2014). One case-control study has also demonstrated protective effect of higher intake of docosahexaenoic acid on risk of CD (Chan et al., 2014).

Contrary, studies reported increased risk of developing UC with high intake of total fat, LC PUFA, omega-6 fatty acids, and meats, and increased risk of CD with high intake of PUFAs, omega-6 fatty acids, saturated fats, and meat (Hou et al., 2011). In the EPIC study, large European prospective study which investigated the relationship between diet as a whole and IBD risk, it was demonstrated that the diet characterized by high sugar and soft drinks intake and low intake of vegetables and non-processed seafood was associated with higher UC risk. Interestingly, when

high intake of sugar and soft drinks was coupled with high intake of vegetables, association was lost. Authors speculate that vegetable intake could modulate a deleterious effect of high soft drink consumption in UC, thus neutralizing the harmful effects of soft drinks in UC. Authors haven't found association between a priori or a posteriori dietary patterns and CD risk (Racine et al., 2016).

There has been growing interest in the last 10 years in links between CD and vitamin D deficiency. Although it was originally thought that the vitamin D deficiency is merely a consequence of the disease due to both malabsorption and low sun exposure in active disease, recent studies have pointed that vitamin D deficiency might also contribute to the pathogenesis of IBD. Interestingly, CD has been linked to low sun exposure and studies have confirmed that incidence of CD rises with increasing latitude within North America and Europe (Khalili et al., 2012; Lim et al., 2005; Loftus, 2004). In the Nurses' Health study cohort of 72 719 individuals, women with a predicted highest vitamin D levels had a significantly lower risk of incident CD (Ananthakrishnan et al., 2012). Moreover, studies have shown that vitamin D metabolism and signaling play key role in immune system function, especially in relation to innate immunity (White, 2018). The discovery that vitamin D might additionally have distinct immunological functions has initiated a huge interest in its possible pathogenic influence on the clinical course of IBD (Nielsen et al., 2018). Recent studies have shown that vitamin D is involved in cell proliferation and differentiation, immunomodulation and that it can influence gut microbiome (Gominak, 2016; Holick, 2007; Rosen, 2011; Theodoratou et al., 2014). Moreover, a number of studies have linked vitamin D levels with meaningful clinical outcomes in patients with IBD (Kabbani et al., 2016).

Lately, much attention has been given to food additives, especially emulsifiers and food thickeners, in the etiopathogenesis of IBD. In vitro and in vivo analysis have demonstrated that emulsifiers alter the mucosal epithelial barrier directly or via change in microbial diversity. Indeed, Roberts et al. recently demonstrated clear correlation between annual emulsifier consumption in food and beverages and the incidence in IBD (Roberts et al., 2013). Similarly, high intake of margarine (which is rich in emulsifying agents and hydrogenated fats) was positively correlated with development of CD and UC (Cashman and Shanahan, 2003; Maconi et al., 2010). Experimental data also shows that very small concentrations of the emulsifier polysorbate 80 enhance bacterial translocation across intestinal epithelia (Roberts et al., 2013).

In conclusion, the external environment offers particular promise as a modifiable risk factor for both incident disease and for outcomes in those with established disease (Ananthakrishnan, 2015). Taken together, it seems that no specific dietary component, but rather inadequate dietary patterns, mostly characterized by high intake of fat, sugar, meat and low intake of fiber from vegetables and fruits and omega-3 fatty acids, are associated with higher risk of IBD. Additionally, high intake of processed foods rich in food additives is given significant attention recently since it seems to be correlated with IBD development in some individuals. Indeed, a diet rich in fruits and vegetables, high in n-3 fatty acids and low in n-6 fatty acids is associated with decreased risk of CD and UC and has therefore been recommended by the European Society for Clinical Nutrition and Metabolism (ESPEN) in patients with IBD (Forbes et al., 2017).

1.2.3. Microbiota

Most humans live in a close harmony with the 100 trillion different microbial organisms, including bacteria, viruses, fungi, and protozoa. Moreover, more than 1000 different species reside in the gastrointestinal tract and the collective genome of intestinal microbes is estimated to contain approximately 100 times more genes than the human genome (Qin et al., 2010). At the same time, only a single layer of intestinal epithelial cells separates these organisms from patrolling immune cells. The most abundant bacteria phyla found in the large intestine of healthy adults are gram-negative Bacteroidetes and gram-positive Firmicutes (Human Microbiome Project Consortium, 2012).

The gut microbiota in healthy individuals is known to promote a range of health benefits to the host, such as pathogen protection, nutrition, metabolism, and the immune system. A variety of symbiotic interactions between the host and the microbiota is necessary to maintain human health. An unfavorable alteration of the composition and function of the microbiota is known as dysbiosis, which alters host-microbiota interaction and the host immune system. There is growing evidence that dysbiosis of the gut microbiota is associated with human diseases such as IBD, irritable bowel syndrome, allergy, asthma, metabolic syndrome and cardiovascular disease (Nishida et al., 2018).

As discussed in the previous chapter (3.2. Environmental factors), it is clear today that the effects of rapid urbanization are likely to be reflected in the human gut microbiome. Environmental factors are, both directly and indirectly, affecting the gut microbiome. Exposure to a multitude of

environmental factors, such as the diet, antibiotics and pollution in early life might be associated with the loss of specific bacterial species of our ancestral microbiota and hence might contribute to the rising incidence of IBD.

1.2.3.1. Gut microbiota in IBD

Multiple studies have reported that the composition of microbiota in IBD patients is altered compared with that in healthy subjects (Kostic et al., 2014). Reduction in microbiota diversity is the single most reproducible finding of studies on microbes in IBD (Sigall-Boneh et al., 2017). Reduced diversity results in less flexibility and adaptation leading to impaired microbial functional capacity. Reduced diversity can be the result of certain bacterial taxa elimination and/or increase in certain taxa that displace others (Wine, 2014). However, it is not clear whether dysbiosis is a consequence or cause of the intestinal inflammation. Both expansion of potential pathogens and global changes in composition have been described. For example, what is usually considered as "beneficial" phylum Firmicutes is often reduced in proportional abundance in the stool of patients with IBD, while member of Proteobacteria phylum, who are considered "detrimental", are commonly increased in patients with IBD relative to healthy individuals (Ni et al., 2017). However, the division of certain bacterial species into either beneficial or detrimental has recently been challenged. Paradoxically, in patients treated with exclusive enteral nutrition (EEN) (Gerasimidis et al., 2014a, 2014b; Pigneur et al., 2019; Sokol and Langella, 2014) or Crohn's disease (CD) treatment-with-eating diet (CD-TREAT) (Svolos et al., 2019), a decrease in proportion of potentially beneficial bacteria has been described. Contrary, Levine et al. (Levine et al., 2019) have recently demonstrated that exclusion of dietary components by EEN or Crohn's Disease Exclusion Diet (CDED) reduced potentially harmful Proteobacteria while increasing potentially beneficial Firmicutes.

As mentioned before, the best evidence on the efficacy of a dietary intervention in treatment of CD is available for EEN (Assa and Shamir, 2017a). Even though the mechanism of action of EEN has not been fully elucidated, studies have shown that EEN causes significant changes in the microbiota composition of patients with CD, leading to modification of microbial-based gut inflammation and consequently – remission of the disease (Assa and Shamir, 2017b; MacLellan et al., 2017). Current evidence supports the notion that CD is associated by community-level

imbalances in gut microbiota, rather than presence of certain bacterial species (Alhagamhmad et al., 2016), therefore its modification could have an influence on disease course. However, even though one would expect that EEN causes gut microbiota to change in a way that would more closely resemble the microbiota composition of heathy controls, studies have shown mixed results (Assa and Shamir, 2017b; Dziechciarz et al., 2007; Heuschkel et al., 2000; MacLellan et al., 2017; Zachos et al., 2007). More specifically, studies have shown that EEN, at least initially, may increase microbial dysbiosis in patients with CD (Gerasimidis et al., 2014a; Kaakoush et al., 2015; Lewis et al., 2015; Quince et al., 2015). It was proposed by MacLellan et al. (MacLellan et al., 2017), that EEN perhaps disrupts established dysbiotic microbial communities, and allows for recolonization and formation of a „heathier“ microbiota.

EEN-induced remission in CD is transient in nature, since approximately 42-67% of patients relapse within 12 months of EEN cessation (Faiman et al., 2014; Frivolt et al., 2014; Hojsak et al., 2014; Lambert et al., 2012; Rodrigues et al., 2007). Moreover, there is limited data into changes in microbiota after the return of patients to the regular diets following EEN. To our knowledge, only two studies performed a follow-up microbiota composition identification in children treated with EEN - Leach et al. found that while microbiome profiles were only 15–38% similar to pre-treatment profiles after eight weeks of EEN, profiles four months after EEN showed 31–41% similarity to pre-treatment profiles, indicating a partial reversion (Leach et al., 2008). Likewise, Gerasimidis et al. observed a regression of major EEN-induced microbiome changes upon return to habitual free diet. Specifically, microbiome diversity and *F. prausnitzii* levels, which had been depleted during EEN, increased significantly along with concentrations of fecal SCFAs and sulfide (Gerasimidis et al., 2014a).

1.3. CLINICAL PRESENTATION

1.3.1. Most frequent symptoms

Clinical presentation of IBD in children does not differ notably from adults. In CD most common symptoms are anorexia, fever, abdominal pain, chronic/reoccurring diarrhea. In UC, the most prominent symptoms are bloody diarrhea and tenesmus. Because CD can present with atypical symptoms, up to 20% of children are diagnosed one year after the development of CD, which is one of the important reasons of delayed growth in those patients (Sawczenko and Sandhu, 2003).

There are many factors that lead to inadequate nutritional status and impaired growth in these children, which will be discussed in more detail in the next chapters.

1.3.2. Nutritional status

Malnutrition is the extra-intestinal manifestation of IBD, comprising both undernutrition and overnutrition. Factors leading to malnutrition in paediatric IBD patients are multifactorial, and include suboptimal nutritional intake, malabsorption, alteration in nutrient requirements and metabolism, excessive gastrointestinal losses and medication (Gerasimidis et al., 2011). Higher metabolic rate:fat free mass (FFM) ratio has been reported in IBD patients compared to healthy controls (Azcue et al., 1997; Capristo et al., 1998; Gasparetto and Guariso, 2014). Impaired gastric acid and pancreatic enzymes have also been reported in adult undernourished CD patients (Winter et al., 2004). Moreover, the effect of proinflammatory cytokines on energy and nutrient requirements, bone and development can also lead to further undernutrition (Wong et al., 2006).

1.3.2.1. Growth and development

The most important presentation of the disease in children is impaired growth, inadequate nutritional status and delayed sexual maturation. Approximately 60% of patients with CD and 35% of patients with UC present underweight at the time of diagnosis (Gerasimidis et al., 2011). Even so, children with IBD are affected by current population trends towards overweight and obesity (Kugathasan et al., 2007). It was reported that up to 1/5 of children with CD and 1/3 of children with UC included in a multi-center registry in the United States are overweight or obese during the follow up (Long et al., 2011).

In every other child delayed linear growth precedes other disease symptoms and persists even after the therapy introduction (Cezard et al., 2002). The faltering is temporary in 40-50% of cases but can be prolonged in up to 10-20% in CD, in whom final height is more than 8.0 cm below target height (Sawczenko et al., 2006). However, being underweight decreased dramatically from 35% to 2% (Cameron et al., 2013).

Moreover, alterations in body composition in children with IBD have been consistently reported, with increased fat mass and depleted lean mass (Bryant et al., 2013). More importantly, even after

normalization of BMI in IBD patients, an increment in FFM was not proportional to increase in BMI (Sylvester et al., 2009), which could have clinical implication in paediatric patients, but which, to this date, have not been fully evaluated.

1.3.2.2. Micronutrient deficiencies

IBD patients are prone to micronutrient deficiencies because of their gut loss through diarrhea and inadequate dietary intake due to anorexia, as well as because multiple medications used for IBD can interfere with normal micronutrient absorption (Hwang et al., 2012). Moreover, IBD patients are prone to avoidance of specific foods, with as much as 53% of paediatric IBD patients avoiding foods in relation to abdominal symptoms (Diederen et al., 2018). Anemia, impaired linear growth and poor bone health are just some of the consequences of inadequate micronutrient status. Micronutrient deficiencies also have important implications for outcomes – patients with anemia have a poorer quality of life and cognitive function (Wells et al., 2006); vitamin D deficiency is associated with an increased risk of relapse (Gubatan et al., 2017) and increase disease activity (Torki et al., 2015) while normalization of vitamin D status is associated with a reduction in the risk of CD-related surgery (Ananthakrishnan et al., 2013). The most common micronutrient deficiencies in IBD include iron, vitamin D, folic acid, zinc, magnesium, calcium, vitamin A, B12, D, E and K deficiency (Peter Irving et al., 2011).

Interpreting blood results of micronutrients and trace elements is challenging since many markers of status are positive or negative acute phase reactants, meaning that they rise or fall as a part of inflammatory response. Indeed, during acute phase of the disease ferritin and copper increase but folate, selenium and zinc decrease (Gerasimidis et al., 2013). However, micronutrient status in patients in clinical remission seems to be impaired nevertheless (Filippi et al., 2006; Geerling et al., 1998), even in apparently well-nourished individuals (Vagianos et al., 2007).

Iron deficiency is common in IBD with up to 95% of patients having been reported to be iron deficient at diagnosis, while up to 70% of patients may have iron deficiency even 2 years after diagnosis (Fritz et al., 2019). There are multiple factors that could lead to iron deficiency in paediatric IBD patients, such as poor disease control or insufficient oral intake and/or inadequate supplementation. Indeed, studies have shown iron deficiency even in patients who were receiving oral supplementation (Dohil et al., 1998). As recommended by the Porto IBD Group of ESPGHAN

(European Society for Paediatric Gastroenterology, Hepatology and Nutrition), patients with CD and UC should be screened for iron status at diagnosis as well as regularly throughout the course of the disease, regardless of disease activity, location and supplement use (Miele et al., 2018).

Vitamin D insufficiency and deficiency is common in IBD patients, with as many as 98% of patients with IBD found to be vitamin D deficient or insufficient (Fritz et al., 2019). It is known that distance from the equator is important factor in vitamin D status, with patients living more than 35 degrees from the equator being at a higher risk of poor vitamin D status. Moreover, even patients living less than 35 degrees from the equator may have poor vitamin D status (Hartman et al., 2009; Levin et al., 2011; Middleton et al., 2013). Notably, even with high observed deficiency of vitamin D in children with IBD, it is unclear whether patients with IBD are more likely than healthy children to have vitamin D deficiency (Laakso et al., 2012; Middleton et al., 2013; Sentongo et al., 2002). Therefore, there is no overall consensus on the vitamin D status and necessary actions in children and adolescents with IBD. Nevertheless, according to ESPEN guidelines (Forbes et al., 2017) vitamin D deficiency should be treated and regular evaluation for vitamin D deficiency should be performed.

Iron and vitamin D deficiency are particularly common in paediatric IBD, while other deficiencies include folic acid, zinc, magnesium, calcium, vitamins A, B12, D, E, and K (Peter Irving et al., 2011). According to ESPEN guidelines, a careful account of nutrition intake, anthropometric measurements, including history of growth with plotting of previous measurements of weight and height and assessment of growth rate are essential, along with laboratory work up to identify and treat nutrient deficiencies. A daily multivitamin supplement is recommended even though it is no guarantee of adequacy, with iron, zinc and vitamin D likely to require specific replacement regimens. Poor compliance, particularly in adolescents, is common with multivitamin supplements and patient education about the rationale behind their use is important (Forbes et al., 2017).

1.4. TREATMENT

The current goals of treatment of IBD in children and adolescents include elimination of symptoms and improved quality of life, restoration of normal growth and elimination of complications. Based on the guidelines of the ESPGHAN and the European Crohns and Colitis Organization (ECCO), treatment of CD and UC differs (Ruemmele et al., 2014; Turner et al., 2012).

The main specificity of treatment of CD in children is the use of EEN as a first line therapy to induce remission in paediatric luminal CD (Turner et al., 2012). The benefits of EEN in IBD were first described in the surgical literature when CD patients unexpectedly improved after being administered EEN to optimize their nutritional status (Voitk et al., 1973). Its efficacy has been confirmed in many studies (Miele et al., 2018; Zachos et al., 2007). It shows the same efficacy as corticosteroids, but without corticosteroid-associated adverse effects. Additionally, EEN leads to mucosal healing, supports growth and improves nutritional status of a child and promotes growth and mineralization of bones (Borrelli et al., 2006). EEN is defined as the provision of essentially 100% of caloric needs by liquid enteral formula (elemental or polymeric) for 6 to 12 weeks orally or via nasogastric (NG) tube. Studies have demonstrated that the protein source (polymeric vs elemental formula) does not affect the efficacy of EEN (Ludvigsson et al., 2004; Verma et al., 2000) and therefore, polymeric formula is preferred due to better palatability and lower cost (Miele et al., 2018; Rodrigues et al., 2007). Moreover, guidelines recommend that EEN should be taken orally, unless there is a failure to achieve adequate oral intake in which case NG tube should be used (Miele et al., 2018), but evidence for this recommendation is lacking. Recently, very promising alternative to strict EEN has been offered. This new diet, called the CD exclusion diet (CDED) is a whole-food diet coupled with partial enteral nutrition (PEN), designed to reduce exposure to dietary components that have adverse effects on the microbiome and intestinal barrier (Pigneur and Ruemmele, 2019). The study showed that CDED plus PEN was effective in inducing remission by week 6 and was better tolerated than EEN in children with mild to moderate CD.

In children with CD in whom EEN is not effective or in whom it cannot be used, corticosteroid therapy is given. However, due to its' numerous side effects, primarily regarding its effect on growth and maturing, its usage should be limited. In cases of severe presentation of the disease, biologic agents can be used to treat CD (de Bie et al., 2012). Its usage is usually limited in corticosteroid-resistant form of the disease and active disease that doesn't respond to the immuno-modulatory therapy (de Bie et al., 2012). Immunomodulators and biologic therapy are also used for maintenance of remission (Rosen et al., 2015).

In UC, as a first line therapy aminosalicylates are used, which exert a topical anti-inflammatory effect on the intestinal mucosa. They can be administered orally or topically via enema or suppository (Rosen et al., 2015). Alternatively, in patients with more severe form of the disease,

corticosteroids are used. Immunomodulators are used as a maintenance therapy in patients who don't respond to aminosalicylates. In case of active disease despite appropriate immunomodulatory therapy, biologic therapy can be used (Rosen et al., 2015). Currently, there are no dietary therapies available for the induction or maintenance of remission in UC patients (Miele et al., 2018).

1.4.1. Nutritional team

In clinical practice, nutritional status assessment is of utmost importance since it helps to decide on the treatment course. The discovery of the importance of nutritional status for the clinical outcome of the disease, as well as for the growth and development of the child has contributed to the recognition of the importance of adequate nutritional support for patients. Since nutrient requirements in children are increased due to growth and development (Koletzko, 2004), malnutrition in children can lead to impaired growth, delayed cognitive development, decreased educational and social achievement, long-term health problems, and, finally, poor quality of life in adulthood (Emond et al., 2007; Galler et al., 1983; Galler and Ramsey, 1989; Rudolf and Logan, 2005).

The main goal of nutritional support in paediatric patients is to sustain or restore adequate nutritional status by providing adequate energy and nutrients to the patients. Nutritional support will help to preserve body composition, avoid nutrition-related complications, and promote developmentally adequate feeding habits and skills (Kolaček, 2009). To be able to achieve these goals, nutrition support team, consisting of a dietitian/nutritionist, paediatrician with an expertise in clinical nutrition, a nurse, and a pharmacist, needs to be established.

The main modalities of treating malnutrition include: 1) nutritional counseling; 2) oral nutritional supplements; 3) different enteral feeding protocols; 4) parenteral nutrition (PN) with or without enteral intake. The choice of nutrition support depends on the age, clinical situation, gastrointestinal status (digestive and absorptive), possibilities of oral intake, as well as on dietary habits and costs (Kolaček, 2009).

Today, the goal of nutritional support in IBD patients is not only to maintain or establish normal nutrition status and enable adequate growth and development, but also to support prolonged remission of the disease.

1.5. OBJECTIVES AND HYPOTHESIS

The goals of this research are:

- To identify differences in anthropometric measurements and body composition of children with IBD at the time of diagnosis and healthy unrelated controls – Paper 1
- To identify differences in dietary intake of children with IBD at the time of diagnosis and healthy unrelated controls – Paper 1
- To identify differences in intestinal microbiota in children with IBD compared to their healthy siblings and healthy unrelated controls at the time of diagnosis – Paper 2
- To identify if the route of EEN delivery (orally vs NG tube) and type of polymeric formula (with taste vs tasteless and isocaloric vs hypercaloric) have an effect on the disease course and nutritional status of children with CD – Paper 3
- To identify differences in intestinal microbiota after 2 days of EEN in children with IBD and their healthy siblings – Paper 4
- To identify the impact of EEN on the microbiota of children with CD – Paper 4

The hypotheses of this research are:

- Anthropometric measurements and body composition significantly differs between newly diagnosed children with IBD and healthy controls.
- Dietary intake significantly differs between newly diagnosed children with IBD and healthy controls.
- Intestinal microbiota in children with IBD significantly differs from the intestinal microbiota of their siblings and healthy unrelated controls.
- Route of delivery and/or type of enteral formula have an effect on the disease outcome and/or nutritional status in children with CD.
- EEN therapy has a significant effect on the intestinal microbiota in children with CD and changes are similar to the changes after EEN in healthy individuals.

CHAPTER II

Methods

- Participants
- Anthropometric measurements and body composition
- Dietary intake
- Characterization of intestinal microbiota
- Ethical approval
- Statistical analysis

2. METHODS

2.1.Participants

Three groups of participants were included in this study:

- Newly diagnosed patients with IBD who were diagnosed and treated in tertiary medical center (Children's Hospital Zagreb)
- Patients who were diagnosed with CD and followed up for at least one year (Children's Hospital Zagreb)
- Control group I: healthy siblings of newly diagnosed IBD patients, who were included in the study since they live in the same household, eat the same food and share a common genetic origin as IBD patients
- Control group II: healthy unrelated controls

All participants were 18 years of age or younger. Newly diagnosed patients with IBD were recruited at the Children's Hospital Zagreb. Healthy siblings of newly diagnosed IBD patients were recruited after the acceptance of participation of newly diagnosed IBD patients. Healthy unrelated controls were recruited from randomly selected elementary and high schools in urban and rural area in Croatia that responded positively to the invitation to participate in the study – in those children anthropometric measurements and dietary intake were estimated. Additionally, in children of hospital staff who have accepted the invitation to participate in the study, stool samples were collected. Moreover, retrospective data of children who were diagnosed with CD for a year or longer were collected in order to assess effect of different formula of EEN and route of delivery on nutritional status and disease outcome. Written consent was obtained from the patients and healthy controls who were 9 years of age or older and one of their parents. In patients and healthy controls who were younger than 9 years of age, written consent was obtained only from their parents or caregivers.

The diagnosis of IBD was established according to the revised Porto criteria (Levine et al., 2013), while disease location was defined using the Paris classification (Fell, 2012). Severity of the disease was estimated by Pediatric Crohn's disease activity index (PCDAI) (Hyams et al., 1991) and Pediatric ulcerative colitis activity index (PUCAI) (Turner et al., 2007). Exclusion criteria in healthy siblings and healthy unrelated controls included unintentional weight loss in the last 6

months, changes in stool frequency or consistency or other symptoms suggestive of undiagnosed IBD.

In all three groups the following parameters were evaluated:

- Anthropometric measurements and body composition
- Dietary intake
- Characterization of intestinal microbiota

2.2.Anthropometric measurements and body composition

Anthropometric measurements and body composition were assessed in newly diagnosed children with IBD, healthy siblings and healthy unrelated controls. In newly diagnosed children with IBD the measurements were made within the 24 hours from the diagnosis. Anthropometric assessment included measurements of body weight (BW), body height (BH), middle upper arm circumference (MUAC), triceps skinfold thickness (TST), subscapular skinfold thickness (SST) and handgrip strength (HS) and were measured by the same trained person. BW was measured on an electronic scale with subjects being dressed in a light-weight gym clothes. BH was measured with a portable stadiometer. TST and SST were estimated using a Holtain skinfold caliper. Jamar hydraulic handgrip dynamometer was used to estimate both right and left handgrip strength in children older than 6 years old.

Bioelectrical impedance was used to estimate subjects' body composition (Maltron BF906, Maltron International Ltd, Rayleigh, Essex, United Kingdom). Z-scores for BW, BH, body mass index (BMI) and MUAC were estimated by the Growth Analyzer software. TSF and SSF were estimated using CDC (Center for disease control and prevention) reference curve (Addo and Himes, 2010). Z-scores for lean mass (LM) were estimated using UK reference data for pediatric body composition (Wells et al., 2012).

Nutritional status of participants was determined using World Health Organization (WHO) Growth reference data for children and adolescents (5-19 years) (WHO, 2006).

Estimated energy requirements for each child were calculated using Schofield equation. Since data about physical activity hasn't been collected, it was presumed that most of participants had "light" physical activity level (PAL) (Firouzbakhsh et al., 1993).

Retrospective data on nutritional status was assessed in all children who were diagnosed with CD and followed up for at least one year at the Children's Hospital Zagreb (tertiary medical center) from October 2007 to November 2017 in order to assess effect of composition and route of EEN delivery on disease outcome and nutritional status in patients with CD

2.3. Dietary intake

Dietary intake was estimated in newly diagnosed patients with IBD and healthy unrelated controls. Information about food consumption was obtained with a validated Food Frequency Questionnaire (FFQ). FFQ is a tool that estimates frequency of consumption of different foods as well as their quantity. FFQ that was used contained 87 different items divided into 8 different food groups: „Milk and milk products", „Cereals and grains", „Juices and sodas", „Fruits", „Vegetables", „Snacks", „Meat, poultry, eggs and fat" and „Fast food". FFQ included frequently consumed national foods and estimated frequency and quantity of consumption of food items in the last month. Available frequencies of food consumption were: „never", „1-3 times a month", „once a week", „2-4 times per week", „5-6 times per week", „once a day", „2-3 times per day", „4-5 times per day" or „ 6+ times per day". Available portion sizes were small, medium and large and participants were able to distinguish their usual portion size using three portion size photos. Frequency of consumption was obtained in a form of personal interview with trained interviewers.

The individual food records data obtained by the FFQ were analyzed by Microsoft Office Excel 2007 worksheet that was generated by using a combination of the Bundeslebensmittelschlüssel 3.01 (BLS 3.01), Fachmann-Kraut-Nährwerttabellen (FKN, Stuttgart, 2005), USDA (FoodData Central) and Kaić-Rak et al. (Kaić-Rak and Antonić, 1990) food composition databases, in order to achieve the most accurate estimation of nutritional values of most frequently consumed foods. The frequency of consumption of food items was multiplied by the portion size to calculate the amount of nutrients consumed in a 30-day period, from which an average daily energy and nutrient intake per each participant were calculated. Intake of 24 nutrients were analyzed: total protein, plant protein, total fat, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA),

polyunsaturated fatty acids (PUFA), cholesterol, total carbohydrates, mono- and disaccharides, polysaccharides, dietary fiber, sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, retinol equivalent, vitamin B1 and B2, niacin, vitamin B6 and vitamin C.

2.4.Characterization of intestinal microbiota

Stool samples were collected in newly diagnosed patients, healthy siblings of IBD patients and 20 healthy unrelated controls. Stool samples of IBD patients were collected at 3 time points:

- Prior to therapy introduction (at the time of diagnosis)
- The second day of EEN therapy
- The last day of EEN therapy (6-8 weeks from the time of diagnosis)

In healthy siblings of children with CD who accepted the invitation to participate in the study and who consented to taking EEN for two days, stool samples were collected at two time points

- Before the introduction of EEN (close to the time of diagnosis of CD in their siblings)
- The second day of EEN

In healthy unrelated controls stool samples were collected only at one-time point.

EEN was given as a polymeric formula. The choice of formula depended on the taste preference of the child/adolescent. Healthy siblings used the same EEN formula as the one provided to patient with CD.

All stool samples were stored in the hospital or at home at -20°C for a maximum of 24 hours, after which they were transferred in the cold packs to the Department of Clinical Microbiology at the University Hospital for Infectious Diseases and stored at -80°C.

2.4.1. PCR amplification and T-RFLP analysis

PCR amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis were performed according to Andoh et al with slight modifications (Andoh et al., 2012). 6'-carboxyfluorescein (6-FAM) labeled 27-F (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers (Thermo Fisher Scientific, USA) were used

for the amplification of the 16S rRNA gene from the human fecal DNA (Andoh et al., 2012). The PCR amplification (20 ng of DNA) was performed in 50 µL reactions, in triplicates, according to previously described protocol (Matsumoto et al., 2005). Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Germany) and diluted in 50 µL of elution buffer.

HhaI and *MspI* enzymes were used for the restriction of amplified 16S rRNA genes (Andoh et al., 2012). 120 ng of purified PCR product was digested separately in 30 µL reaction volumes, using 1 µL of FastDigest *HhaI* and FastDigest *MspI* (Thermo Fisher Scientific, USA) at 37°C for one hour. Restriction products were purified by ethanol/ sodium acetate/EDTA precipitation and resuspended in 12 µL deionized formamide (Thermo Fisher Scientific, USA) to a final concentration of 10 ng/ µL (Li et al., 2007). 3 µL of restriction digest product (~36 ng) was mixed with 11 µL of deionized formamide and 0.5 µL of fourfold diluted GS2500ROX (Thermo Fisher Scientific, USA). The length of the terminal restriction fragments (T-RFs) was determined with an ABI PRISM 310 genetic analyzer in GeneScan mode (20s injection time; 15 kV, and 60°C for 48 min for each sample) (Thermo Fisher Scientific, USA) (Matsumoto et al., 2005).

Fragment sizes were estimated by using the Local Southern Method GeneMapper 3.7 software (Thermo Fisher Scientific, USA). T-RFs in the range of 50–810 bp with a peak height greater than 25 fluorescence units were included in the analysis. Alignment of T-RFs was performed by T-REX software (http://trex.biohpc.org/) (Li et al., 2007). Binning treshold of 2 bp was used for assignment of T-RFs to operational taxonomic units (OTUs) (Andoh et al., 2012). The OTUs were quantified as the percentage values of an individual OTU per total OTU area, and this was expressed as the % area of the underpeak curve (% AUC) (Sakamoto et al., 2003).

Assignment of OTUs to bacterial taxa was performed in silico using the web-based analysis tool (PAT+) provided by MiCA3 (http://mica.ibest.uidaho.edu/pat.php), based on the RDP (Ribosomal Database Project) release 10 16s rRNA gene database (Culman et al., 2009).

2.5.Ethical approval

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

Study was approved by Ethics Committee Children's Hospital Zagreb (IRB number: 21102014). Informed consent was obtained from all individual participants and at least one of their parents included in the study.

2.6.Statistical analysis

2.6.1. Paper 1 (Sila et al., 2019)

The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed by one-way ANOVA followed by Bonferroni test for post hoc analysis. Pearson correlation was performed in order to assess correlation between nutritional status and body composition and food intake. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (IBM Corporation, Chicago, Illinois, United States of America) statistical software.

2.6.2. Paper 2 (Sila et al., 2020a)

The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed based on distribution and number of groups by ANOVA or t test and Kruskal-Wallis or Mann-Whitney U test. The relative abundance of OTUs was used to calculate Shannon-Wiener diversity index in order to compare diversity between different sample groups. Cluster analyses were performed using BioNumerics software (Applied Maths, Belgium) based on the HhaI or MspI T-RFLP patterns. A dendrogram representing calculated similarity distances was generated using Pearson's similarity coefficient analysis and the unweighted pair-group methods with arithmetic means (UPGMA). P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (Chicago, IL) statistical software.

2.1.1. Paper 3 (Hojsak et al., 2019)

The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed by two tailed Student t test for independent samples. Binary logistic regression model was used to assess characteristics of polymeric formula (isocaloric vs hypercaloric and with taste vs tasteless), mode of EEN delivery (orally vs NG tube),

volume of EEN, and disease location have an influence on EEN failure. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (Chicago, IL) statistical software.

2.1.2. Paper 4 (Sila et al., 2020b)

The differences between categorical variables were assessed by chi-square test. The Kolmogorov–Smirnov test was applied to test whether the data have a normal distribution. As quantitative variables were not normally distributed difference between healthy controls and patients with CD were determined by Mann-Whitney U test. Repeated measures for CD patients were analyzed by Friedman test; post hoc analysis was performed by Wilcoxon test (with Bonferroni adjustment where p-values from the Wilcoxon tests were multiplied by the number of tests being carried out). Repeated measures for heathy siblings were analyzed by Wilcoxon test. Due to high number of variables in OTU comparisons, p values were adjusted for multiple comparison and values less then 0.01 were considered significant.

The relative abundance of OTUs was used to calculate Shannon-Wiener diversity index in order to compare diversity between different sample groups. Cluster analyses were performed using BioNumerics software (Applied Maths, Belgium) based on the HhaI or MspI T-RFLP patterns. Statistical analysis was performed using SPSS 2319.0 (Chicago, IL) statistical software.

CHAPTER III

Results

- Nutritional status and dietary intake
- Microbiota composition of newly diagnosed IBD patients – comparison with healthy siblings and healthy controls
- The route of EEN delivery and type of polymeric formula
- Microbiota composition in CD patients and healthy siblings during EEN

3. RESULTS

3.1.Nutritional status and dietary intake (Paper 1)

Overall, there were 89 patients with newly diagnosed IBD and 159 healthy controls included in this study. Of those, 49 (55%) patients were affected by CD (mean age: 14.6 ± 2.6 years) and 40 (45%) by UC (mean age: 14.0 ± 3.7 years for UC). Based on PCDAI/PUCAI scoring, 54 patients (63.5%) had mild disease and 31 patients (36.5%) had moderate to severe disease at the time of diagnosis.

BMI z-score was used to estimate nutritional status of all participants. Nutritional status of newly diagnosed IBD patients and healthy controls is presented in Figure 3.

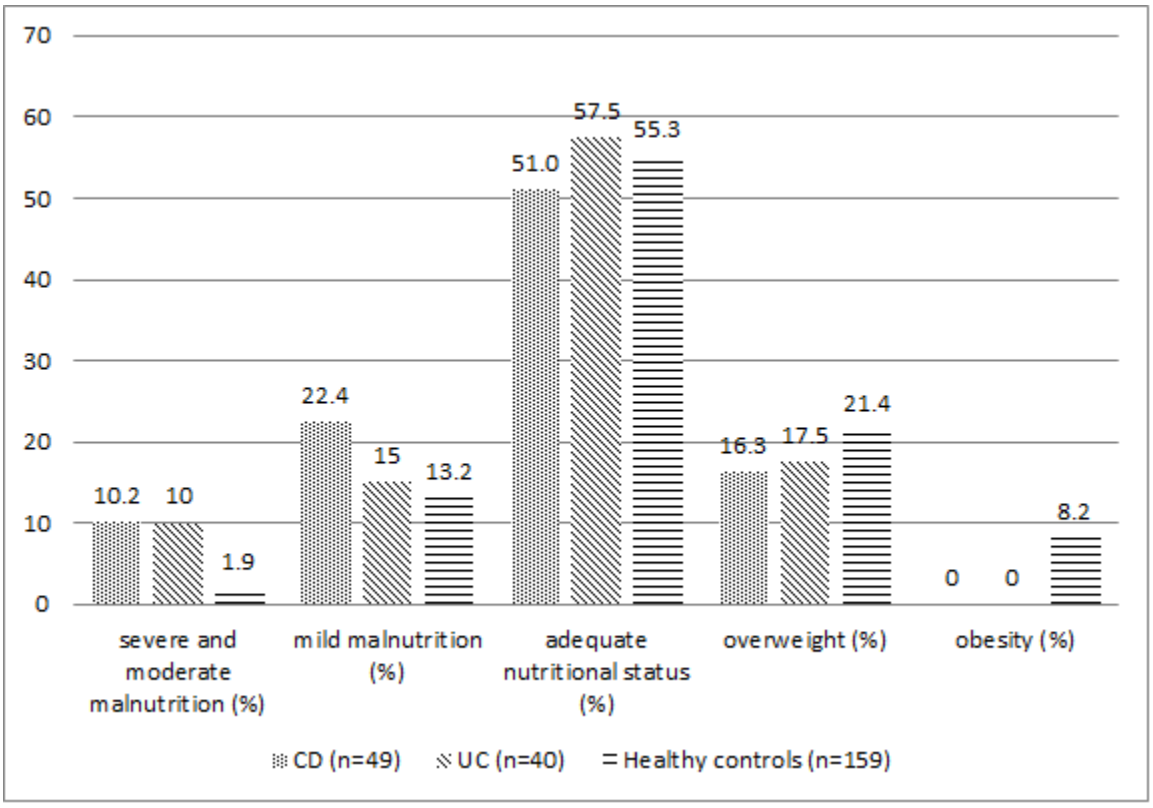

Figure 3. Comparison of nutritional status according to WHO criteria between IBD patients and healthy controls (Sila et al., 2019).

There was a significant difference in BW-for-age, BMI-for-age and basal metabolic rate (BMR) but not for triceps- and subscapular skinfold thickness between both CD patients and UC patients vs. healthy controls. Significant difference in BH-for-age z-score and MUAC-for-age z-score was found only between CD patients vs. healthy controls. There was no significant difference in any of anthropometric measures between CD and UC patients. Both CD and UC patients had significantly lower lean mass-for-age z-scores compared to healthy controls.

Mean energy intake differed significantly between UC patients and healthy controls (7780.9 ± 2774.5 kj; 10198.3 ± 4409.5 kj; p=0.003), but not CD patients (8915.4 ± 3377.6 kj; 10198.3 ± 4409.5 kj; p=0.202).

Difference in intake of macronutrients, micronutrients and different food groups between CD patients, UC patients and healthy controls is shown in Table 1.

Table 1. Difference in dietary intake between CD patients, UC patients and healthy controls (Sila et al., 2019).

	CD (n=49)	UC (n=40)	Healthy controls (n=159)	p-value
Energy intake (kj), mean (SD)	8915.4 (3377.6)	7780.9 (2774.5)	10198.3 (4409.5)	0.002***
% EER (%), mean (SD)	90.0 (52.8)	86.6 (39.6)	109.3 (45.9)	0.004*
Total proteins (g), mean (SD)	92.4 (33.3)	83.5 (31.6)	107.1 (43.8)	0.002***
Vegetable proteins (g), mean (SD)	25.7 (11.1)	23.6 (8.7)	29.0 (14.3)	0.043
Animal proteins (g), mean (SD)	46.0 (21.5)	43.7 (20.7)	57.2 (27.4)	0.002*
Total fat (g), mean (SD)	102.1 (45.9)	81.1 (31.1)	108.1 (52.9)	0.010***
Saturated fat (g), mean (SD)	35.9 (17.3)	30.3 (13.8)	43.6 (22.0)	0.001***
Unsaturated fat (g), mean (SD)	31.9 (14.5)	25.6 (10.7)	54.0 (27.2)	0.006***

Total carbohydrates (g), mean (SD)	252.7 (100.8)	228.7 (85.1)	292.9 (133.9)	0.007***
Mono- and disaccharides (g), mean (SD)	107.8 (62.3)	98.5 (51.8)	131.3 (59.3)	0.002***
Fibers (g), mean (SD)	21.8 (9.2)	20.8 (8.0)	25.8 (12.3)	0.014***
Calcium (mg), mean (SD)	765.1 (399.7)	766.6 (385.1)	987.4 (446.2)	0.001*
Magnesium (mg), mean (SD)	247.3 (193.3)	219.1 (123.5)	279.5 (160.4)	0.091
Phosphorus (mg), mean (SD)	1455.7 (521.1)	1364.9 (508.3)	1742.2 (674.1)	0.001*
Iron (mg), mean (SD)	13.2 (5.5)	11.3 (4.5)	14.7 (6.4)	0.005***
Zinc (mg), mean (SD)	8.6 (3.9)	7.3 (3.5)	8.9 (3.6)	0.047***
Proteins (% EI), mean (SD)	17.3 (2.8)	17.9 (2.8)	17.7 (2.5)	0.622
Carbohydrates (% EI), mean (SD)	46.5 (7.1)	48.8 (5.6)	47.6 (7.5)	0.355
Fat (% EI), mean (SD)	41.8 (7.0)	38.7 (5.7)	39.0 (6.8)	0.044
Fruits (%EI), mean (SD)	3.3 (3.9)	5.2 (5.3)	6.2 (4.7)	0.002**
Vegetables (%EI), mean (SD)	3.7 (1.7)	4.2 (2.0)	3.8 (2.3)	0.535
Juices and sodas (%EI), mean (SD)	5.2 (4.0)	5.2 (3.9)	4.9 (4.0)	0.814
Snacks (%EI), mean (SD)	10.8 (9.9)	8.7 (6.2)	9.9 (6.6)	0.444
Fast food (%EI), mean (SD)	13.5 (6.9)	11.3 (4.2)	13.0 (6.3)	0.246

EI – energy intake

Post hoc analysis: *$p<0.05$ for CD and UC vs. healthy controls; **$p<0.05$ for CD vs. healthy controls; ***$p<0.05$ for UC vs. healthy controls.

3.2. Microbiota composition of newly diagnosed IBD patients – comparison with healthy siblings and healthy controls (Paper 2)

In this part of study, 19 IBD patients (n=13 CD patients; 63.2% male; mean age 14.77±0.65 years), 20 healthy siblings (30% male; mean age 12.84±0.85 years) and 19 healthy controls (47.4% male; mean age 10.72±0.84 years) were included.

The fecal microbiota profiles of all 3 groups are illustrated by a dendrogram (Figure 4).

A setting of similarity generated 2 major clusters. Most of healthy controls (17/19 with *HhaI* and 18/19 with *MspI*) and healthy siblings (20/20 by *HhaI* and 20/20 by *MspI*) were classified in cluster I. In IBD patients, 57.9% (*HhaI* digestion) and 52.6% (*MspI* digestion) were classified into cluster II.

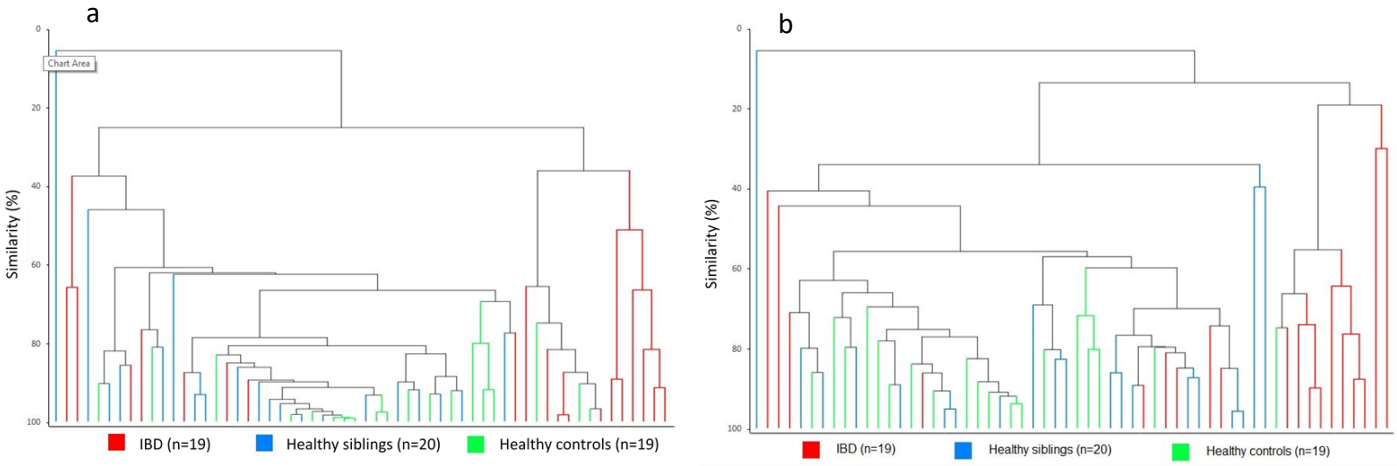

Figure 4. Dendrogram of the fecal microbiota profiles of IBD patients, healthy unrelated controls and healthy siblings (a. *HhaI*-digestion, b. *MspI*-digestion) (Sila et al., 2020a).

Based on the result of *HhaI*/*MspI*-digested T-RF patterns, microbial diversity of IBD patients was reduced compared to that of healthy siblings and healthy unrelated controls (Table 2).

Table 2. Comparison of fecal bacterial diversity between IBD patients, healthy siblings and healthy controls. The Shannon diversity index was calculated from the *HhaI*- and *MspI*-digested T-RF patterns (Sila et al., 2020a).

Shannon index	IBD (n=19)	Healthy siblings (n=20)	Healthy controls (n=19)	p-value
MspI digestion, mean (SD)	2.11 (0.12)	2.45 (0.85)	2.44 (0.74)	0.018**
HhaI digestion, mean (SD)	1.75 (0.12)	2.14 (0.81)	1.99 (0.66)	0.013*

Post-hoc analysis: *p<0.05 for IBD vs. healthy siblings, **p<0.05 for IBD vs. healthy siblings and IBD vs. healthy controls. IBD – inflammatory bowel disease

All *HhaI*- and *MspI*-associated OTUs predicting the genus *Clostridium,* among others, were significantly decreased in IBD patients at the time of diagnosis compared to healthy siblings and healthy controls. Some other *HhaI*- and *MspI*-associated OTU-s representing phylum Firmicutes, which include bacteria from the genera *Paenibacillus*, *Bacillus*, *Lactobacillus*, *Blautia*, *Eubacterium*, *Roseburia* and *Ruminoccocus* were significantly reduced in IBD patients compared to healthy siblings and healthy controls. On the contrary, the genera *Streptococcus*, *Lactococcus* and *Enterococcus* predicted by the *MspI*-associated 555-bp and 563-bp were significantly increased in IBD patients. The same has been noticed for the phylum Proteobacteria, represented by genera *Enterobacter*, *Citrobacter*, *Escherichia* and *Klebsiella* (495-bp *MspI* OTUs).

3.3.The route of EEN delivery and type of polymeric formula (Paper 3)

A total of 92 CD patients were included in the study (54.3% male, mean age 13.6 ± 3.0 years).

A total of 71 (77.2%) children treated with EEN achieved remission. Overall 42 (45.7%) patients that received EEN via NG tube until the end of the EEN period. Moreover, 42 (45.7%) patients received formula with taste, from which 12 patients received a hypercaloric formula (1.5 kcal/ml), 30 patients received an isocaloric formula (1 kcal/ml), and 50 received a standard isocaloric, tasteless, polymeric formula.

None of the assessed factors, including age, disease location, characteristics of the formula (with taste vs tasteless and isocaloric vs hypercaloric), and mode of delivery (orally vs trough NG tube for the whole duration of EEN) were associated with EEN failure (Table 3).

Table 3. Risk factors at diagnosis evaluated with respect to exclusive enteral nutrition (EEN) failure. Binary logistic regression multivariable model (Hojsak et al., 2019).

	HR	95% CI
Age	1.011	0.742-1.378
Disease location		
L1	0.254	0.045-1.429
L1	1.518	0.423-5.449
Enteral formula with taste	0.412	0.086-1.960
Hypercaloric (1.5 kcal/ml) enteral formula	2.5	0.377-16.588
NG tube for the duration of EEN	1.001	0.286-3.504
Energy intake via EEN (kcal/kg body weight)	0.964	0.907-1.025

NG - nasogastric

3.4.Microbiota composition of CD patients and healthy siblings during EEN (Paper 4)

Seventeen newly-diagnosed children with CD (52.9% male; mean age 15.98±1.46 years) and 10 of their healthy siblings (30% male; mean age 14.2±3.02 years) participated in this part of the study.

Difference in microbial diversity estimated by *HhaI*/*MspI*-digested T-RF patterns between all children with CD and healthy siblings at the start of EEN and on the second day of EEN is presented in Figure 5. There was no significant difference in microbial diversity between children with CD and healthy siblings before EEN treatment (p=0.127 for *HhaI*-digestion; p=0.604 for

MspI-digestion). There was a significant difference in microbial diversity on the second day of EEN between children with CD and healthy controls (p=0.006 *HhaI*-digestion; p=0.023 *MspI*-digestion; Figure 5). As for microbial diversity change during course of EEN, there was no difference in microbial diversity in CD children between start, second day and the end of EEN (p=0.319 *HhaI*-digestion; p=0.257 *MspI*-digestion).

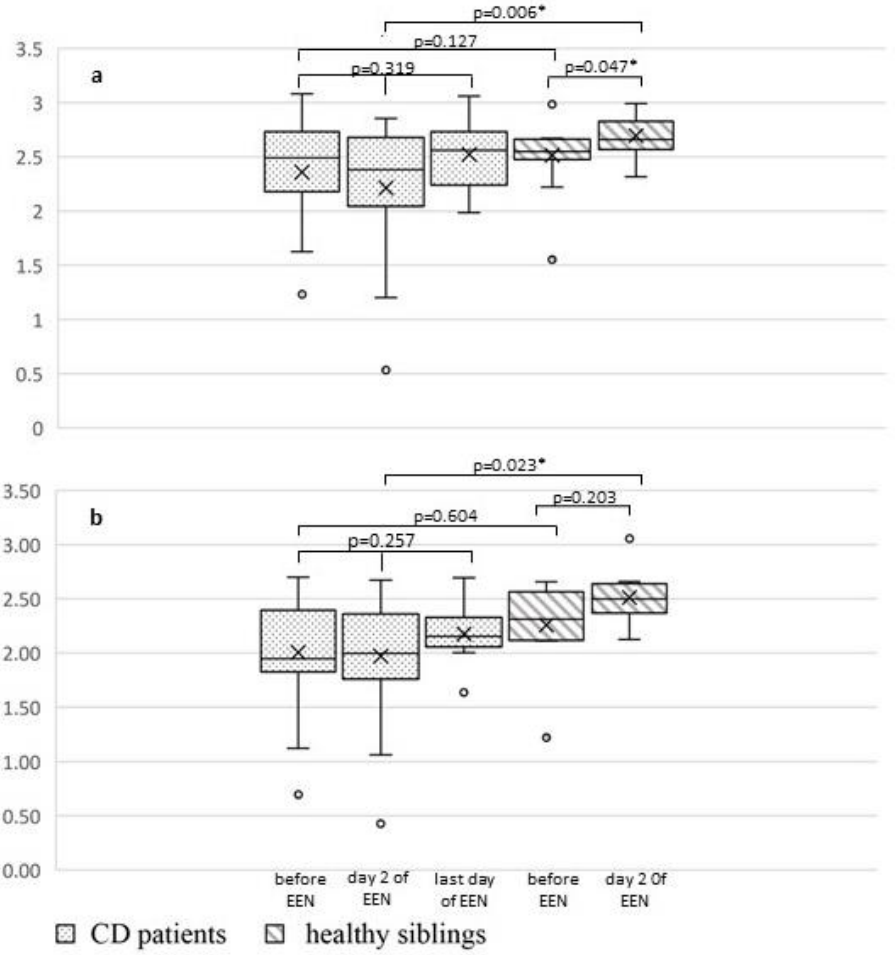

Figure 5. Shannon diversity index for each EEN sample time for a) HhaI-digestion and b) MspI-digestion (Sila et al., 2020b).

Figure 6 demonstrates the fecal microbiota profiles of patients with CD and healthy siblings before the start of EEN and on the second day of EEN. The fecal microbiota profiles of patients with CD and healthy siblings before the start of EEN show a tendency for clustering, however not reaching

significance (Figure 6a). Figure 6b demonstrates the fecal microbiota profiles of patients with CD and healthy siblings on the second day of EEN, showing that two major clusters were formed (one for healthy siblings and other for patients with CD).

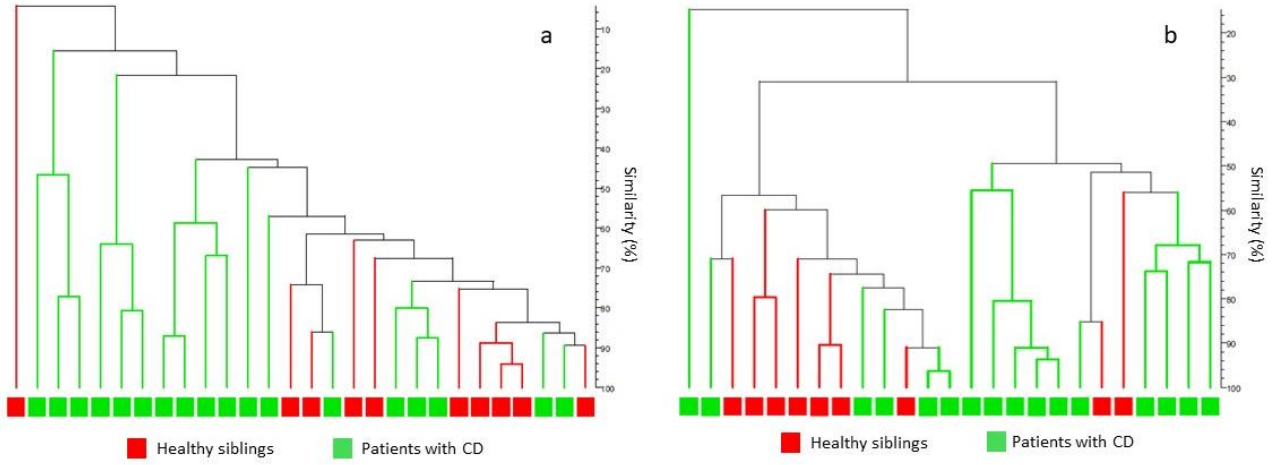

Figure 6. Dendrograms of the fecal microbiota profiles of CD patients and healthy siblings (a. before the introduction of EEN; b. the second day of EEN) (Sila et al., 2020b).

CHAPTER IV

Discussion

- Dietary intake of IBD patients
- Microbiota composition of children with IBD
- Impact of route of delivery and type of EEN formula on nutritional status and course of the disease in children with CD
- Impact of EEN on the microbiota composition of CD patients and their healthy siblings

4. DISCUSSION

These studies together have demonstrated that children with IBD display significant differences in nutritional status and dietary intake at the time of diagnosis compared to healthy children. Moreover, we have shown significant differences in microbiota diversity and composition at the time of diagnosis in children with IBD, compared to both healthy unrelated children and healthy siblings. In healthy siblings, some microbiota composition patterns similar to those in children with IBD had been observed. Furthermore, we have shown that the diet, with regard to EEN used as a primary therapy for induction of remission in children with CD, leads to significant changes in microbiota composition in both children with CD and their healthy siblings. However, these changes are delayed in children with CD compared to their healthy siblings.

4.1. Dietary intake of IBD patients (Paper 1)

Significant differences in intake of macronutrients and some micronutrients were present in IBD patients at the time of diagnosis compared to healthy population. Estimating dietary intake of IBD patients is not only important for its impact on the nutritional status of the child, but it has been shown that the diet is an important factor in the pathogenesis of IBD since it impact the microbial composition, function, the gut barrier and host immunity (Levine et al., 2018). Moreover, poor nutrition in childhood IBD contributes to disrupted pubertal development and impaired growth velocity which may lead to short stature in adulthood (Forbes et al., 2017). Most studies looking into dietary intake of children with IBD have assessed intake in children with established disease (Diederen et al., 2018; Hartman et al., 2009; Pons et al., 2009; Thomas et al., 1993; Tsiountsioura et al., 2014). Therefore, looking into the diet of children before the disease has occurred is important to estimate potential nutrient deficiencies as well as to set up appropriate nutritional intervention.

Surprisingly, we have found that energy intake of CD patients was appropriate and did not significantly differ from that of healthy individuals. However, intake of certain nutrients and food groups was altered compared to healthy controls, with regard to intake of calcium, phosphorus and fruit which was lower in these patients. In UC patients, low intake of energy, all macronutrients and dietary fiber was observed, as well as lower intake of phosphorus, calcium, iron and zinc. Low

intake of energy and, consequently all macronutrients in UC patients is of no surprise, since these children present with acute symptoms (bloody diarrhea and abdominal cramps) at the time diagnosis. The acute symptoms which are especially prone in UC (severe bloody diarrhea) could cause the restriction in food intake and increase the likelihood of restrictive diet introduction. Interestingly, we haven't found that children with CD had lower intake of energy compared to healthy children, while they did present with deteriorated nutritional status and body composition. However, in IBD, the pathogenesis of malnutrition is multifactorial. The mechanisms involved include not only limited food intake, but also malabsorption of nutrients and increased nutrient losses, among others (Forbes et al., 2017). Interestingly, resting energy expenditure is not associated with disease activity in children with IBD (Hart et al., 2005; Wiskin et al., 2012, 2009). Therefore, even though children with CD did not have lower energy intake compared to healthy controls, lower BMI z-score was probably caused by other factors like malabsorption and increased nutrient losses.

Low intake of calcium in IBD patients (756.1 ± 399.7 mg for CD patients; 766.6 ± 385.1 mg for UC patients) is an important observation since it can lead to poor health outcomes like malnutrition and abnormal bone mineralization. Although we did not investigate it, low intake of calcium in our cohort is most probably related to low intake of milk and dairy products, as was previously demonstrated in IBD patients (Pituch-Zdanowska et al., 2019). Low intake of milk products at the time of diagnosis is most probably associated with secondary lactose intolerance. Indeed, secondary lactose malabsorption and intolerance are caused by damage of the small intestinal mucosa in CD leading to reduced lactase expression with the risk of lactase deficiency (Wiecek et al., 2014). In one meta-analysis, the overall odds ratio for lactose malabsorption in patients with IBD was 1.6 (95% CI: 1.0 to 2.6, p=0.048), being highest in CD affecting the small bowel (Szilagyi et al., 2016). Therefore, it is not surprising that one of the most commonly avoided foods in children with IBD are dairy products, especially in active disease, with as many as 30-41% of paediatric IBD patients excluding dairy products from their diets (Pituch-Zdanowska et al., 2019). Moreover, around 1/3 of parents of paediatric patients with IBD believe that the diet could be the cause of the disease, and 67.1% believe that diet/different type of food can trigger an IBD flare (Pituch-Zdanowska et al., 2019). Even though we do not know if this was the case in our cohort, early recognition of different dietary beliefs, especially in newly diagnosed patients with IBD, is

important in order to prevent unnecessary self-prescribed dietary restrictions. Moreover, IBD is already a condition associated with bone mineral loss, development of osteoporosis, and increased risk of fracture, with the underlying inflammatory process, as well as the low body mass, corticosteroid use and malabsorption, all commonly recognized factors in contributing to bone disease (Even Dar et al., 2019). Therefore, unnecessary dietary restrictions such as elimination of dairy products containing calcium and vitamin D should be avoided in these patients. Early recognition and intervention are of outmost importance.

Lower intake of fruits and dietary fiber in our cohort could be the result of active symptoms of the disease. However, no difference was observed in the intake of other food groups such as intake of vegetables, fast food, soda and juices or snacks, so that is unlikely. Indeed, few studies have found similar results to ours, where both patients with active and inactive IBD had lower intake of dietary fiber in comparison with healthy children (Hartman et al., 2016), which means that even children in remission with no underlying symptoms reduce the intake of these important foods. Indeed, up to 41.3% and 32.3% of parents of children with IBD report that their children avoid consumption of fruits and vegetables, respectively (Pituch-Zdanowska et al., 2019). Moreover, we have observed high intake of snacks, fast food, juices and sodas in both IBD patients and healthy children, together contributing to even 29.5% and 25.2% of total energy intake in CD and UC patients, respectively. These observations taken together - low intake of fruit and dietary fiber along with high intake of industrial processed food - are important for two reasons. First is that in IBD patients in whom body composition is already impaired due to inflammation, low levels of physical activity and use of different medicine (Beattie et al., 2006), special attention should be given to appropriate nutrition in order to improve body composition. Secondly, this type of diet itself may irreversibly reduce bacterial diversity and lead to the disappearance of specific bacterial species in the digestive system (Sonnenburg et al., 2016). Moreover, fibers and starches found in fruits and vegetables are a vital substrate for the production of butyrate and other short chain fatty acids, which play an important role in downregulating inflammation, as well as enhancing innate immunity (Furusawa et al., 2013; Roberts et al., 2013). Furthermore, depriving colonic microbes from fiber can lead to depletion of the mucus layer, disruption of the barrier, immune activation and tissue damage (Desai et al., 2016). All these factors have been recognized as risk factors for

IBD development, and therefore, early nutritional interventions and educations in newly-diagnosed children with IBD should be employed.

4.2.Microbiota composition of children with IBD (Paper 2)

We have shown in our studies that microbiota diversity and composition of IBD patients significantly differs from healthy controls and their siblings at the time of diagnosis. Furthermore, lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in IBD patients compared to healthy siblings and healthy controls. This is of no surprise, since studies in both adults and children have shown that paediatric IBD patients have significantly less diverse microbial composition compared to both healthy controls and healthy siblings (Sheehan and Shanahan, 2017). However, to this day, it is not clear if that is the cause or a consequence of the disease. Therefore, comparing the microbiota of healthy siblings with that of IBD patients, who share both the genetic background and environmental exposures that predispose to IBD, but in whom cumulative effect of different environmental triggers is insufficient to produce the full-blown disease phenotype, could provide explanation into potential mechanism of IBD development.

In our cohort, we have found microbiota composition of IBD patients to be significantly different from that of healthy siblings – which would lead us to conclude that dysbiosis is purely the consequence of the inflammation in the bowel. Interestingly, even though we didn't find statistically significant differences, we did show that some differences observed between healthy siblings and healthy controls were similar to the differences observed when comparing IBD patients to healthy controls. More specifically, most OTUs representing bacteria from phylum Firmicutes ("beneficial" bacteria), but not from phylum Proteobacteria ("detrimental" bacteria) that were observed in healthy siblings, differed from that of healthy controls and were approaching values observed in IBD patients. Indeed, reduced abundances of "beneficial" bacteria in siblings of IBD patients have been observed in other studies as well (Hedin et al., 2017; Joossens et al., 2011). As shown in the study by Joosens et al. (Joossens et al., 2011), the dysbiosis in relatives of patients was not characterized by a lack of butyrate-producing capacity, which was the case in IBD patients, but bacterial species that are known to degrade gastrointestinal mucin. Degradation of the mucosal barrier might enhance bacterial translocation and lead to increased permeability of the

gut. Authors have concluded that enhanced mucin degradation capacity found in relatives of CD patients might precede or predispose further to development of CD. Furthermore, they speculate that dysbiosis observed in relatives of the patients with CD might be an intermediate step towards CD and disease-associated dysbiosis. Moreover, in study by Hedin et al. (Hedin et al., 2017), increased *E. coli* contributed to the dissimilarity between patients and healthy controls but not to the siblings and healthy controls. Thus, authors speculate, it may be that the CD dysbiosis comprises microbial factors that contribute to pathogenesis (such as lower abundances of "beneficial" bacteria), overlaid with microbial alterations that are consequent of inflammation (such as higher abundance of *E. coli* – "detrimental" bacteria). These observations taken together could pinpoint to the hypothesis that some "predisposition", in a sense that there is certain dysbiosis already present before the presentation of the full-blown disease, potentially does pave the road to the development of the disease later in life, probably triggered by some environmental factor(s). Just one example of such trigger could be low consumption of dietary fiber along with high consumption of industrially processed foods, which could further accentuate the dysbiosis, and in genetically predisposed individuals eventually lead to the development of IBD. However, we cannot confirm if this was the case in our cohort.

Even more so, rather than focusing on the microbial composition at the time of diagnosis when the disease is already established, it may be that the influence of the gut microbiota develops long before the occurrence of the disease. Even though we haven't found differences in duration of breastfeeding, time of weaning, number of siblings, mode of delivery and owning of pets in our cohort, shared exposures to different risk factors in early perinatal and postnatal life when the microbiota is developing and becoming stabilized could lead to permanent changes in microbiota, which could in turn influence the risk of developing IBD later in life (Bianco-Miotto et al., 2017).

4.3. Impact of route of delivery and type of EEN on nutritional status and course of the disease in children with CD (Paper 3)

Guidelines for the treatment of CD in children suggest an oral route of administration of enteral formula should be employed (Miele et al., 2018). However, this recommendation almost solely relies on the expert opinion, and no study to date has demonstrated if oral route should be preferred (Gailhoustet et al., 2002). Therefore, this retrospective study aimed to assess if the route of delivery

of EEN (orally vs NG tube) and characteristics of enteral formula (isocaloric vs hypercaloric and with taste vs tasteless) impact the effectiveness of EEN in remission induction, disease course, and nutritional status. We have demonstrated that the route of EEN delivery and the characteristics of the polymeric formula have no effect on the outcome of treatment and nutritional status in pediatric patients with CD.

Moreover, our study has shown that there was no difference in weight change or BMI change during EEN in children receiving EEN orally or via NG tube. Importantly, there was no difference in energy intake between children receiving ENN orally or via NG tube. The only other study that has compared oral to continuous EEN via NG tube (Rubio et al., 2011) found no difference in remission induction, however, opposite to our study, they did find significant difference in weight gain, which was significantly higher in the continuous EEN group – which could partially be explained by the fact that patients in our study received EEN as a bolus treatment.

The type and the amount of formula could both potentially impact the extent of change of microbiota composition. Even though the amount of EEN formula (and energy intake) ingested by CD patients was not estimated in this retrospective study, we assume it did not differ between the two groups, since there was no difference in weight gain between the two groups. Moreover, we have demonstrated that the route of delivery and characteristics of the polymeric formula have no impact on the remission of the disease – which we know is related to microbiota alterations caused by EEN. Therefore, we hypothesize that the extent of microbiota composition change caused by EEN is not dependent on the route of delivery or type of formula used.

4.4. Impact of EEN on the microbiota composition of CD patients and their healthy siblings (Paper 4)

Perhaps the strongest evidence for the role of diet in CD is the therapeutic effect of EEN in the treatment of active CD (Ruemmele et al., 2014). This effect does not depend on the protein source (type of formula) (Hojsak et al., 2019), but is very dependent on exclusion of ordinary table food (Johnson et al., 2006; Lee et al., 2015). The mechanism by which EEN leads to reduction of inflammation and mucosal healing is not well understood, however studies have shown that EEN modifies the microbiota of CD patients, which in turn supports intestinal homeostasis and the

immune system function (MacLellan et al., 2017). No study to date has investigated if EEN has a similar effect on the microbiota of healthy children, as compared to children with CD.

Therefore, our study investigated, for the first time, the differences in the stool microbial content in the response to EEN in children with CD compared to their healthy siblings. Furthermore, we have investigated changes in microbiota composition in children with CD during the course of the disease. In children with CD, we have demonstrated that EEN causes significant changes in microbiota composition, however, those changes are delayed and become apparent on the last day of EEN. In healthy siblings, these changes were already observed on the second day of EEN.

Similarly, other studies have demonstrated significant changes in microbiota composition during EEN, indicating that CD is not associated with a single bacteria change, but rather with community-level imbalances in the gut microbiome (MacLellan et al., 2017). We have shown lower abundance of the phylum Firmicutes in CD patients compared to healthy siblings before the start of EEN. Moreover, we have shown higher abundances of the phylum Proteobacteria in CD patients compared to healthy siblings, however, we have failed to reach statistical significance. As discussed in the previous chapters, two important factors in pathogenesis od CD have been discussed in the literature – the loss of symbionts with anti-inflammatory properties (Takahashi et al., 2016) along with expansion of potentially pathogenic symbionts that can cause activation of the immune response and consequently cause inflammation when this activation is overstimulated (Zechner, 2017). We could speculate that in our cohort, altered microbiota composition of CD patients this is possibly consequence of both the disease (inflammation) and altered dietary intake, characterized by high intake "Western diet" food and low intake of fruits, which predisposed to the diagnosis of the disease, as discussed in the previous chapters. With that in mind, therapy that would result with less "dysbiotic" microbiota, i.e. the one that would be more similar to the microbiota of healthy controls, might lead to remission of disease in children with CD. Our results have confirmed this hypothesis and have shown a tendency towards the increase in abundance of phylum Firmicutes and a decrease in abundance of phylum Proteobacteria, similar to what was observed in a study by D'Argenio et al (D'Argenio et al., 2013) and Schwerd et al. (Schwerd et al., 2016). These studies, along with ours, would suggest that EEN "normalizes" previous dysbiosis, leading to reduction of inflammation. However, other studies have shown opposing results (Gerasimidis et al., 2014a; Kaakoush et al., 2015; Lewis et al., 2015; Quince et al., 2015)

and have suggested that EEN leads to even more dysbiotic state and have even suggested that reduction in relative abundance of families within the Firmicutes phylum correlated with clinical improvement. Interestingly, Lewis et. al (Lewis et al., 2015) have demonstrated that there was a marked difference in microbiome composition between patients who ultimately responded to EEN and those who did not, with those patients who have responded to treatment exhibiting microbiota profiles that were more similar to healthy controls.

Moreover, we wanted to see if similar changes occur in both patients with CD and their healthy siblings during the 2-day course of EEN. A time period of two days of EEN was chosen as not to be overly demanding for otherwise healthy children and because limited data in the literature showed that intestinal microbiota changes are already present one day after initiation of EEN (Meister et al., 2002). Moreover, a dramatic and rapid rearrangement in the human microbiome within 24 hours after reduction of carbohydrate (including fiber) was observed in a recent study (Mardinoglu et al., 2018), showing that changes in the diet lead to rapid changes in microbiota composition. We have found that EEN has caused significant changes in microbiota composition in both children with CD and healthy siblings. However, microbiota of healthy siblings responded "more rapidly" to EEN introduction, with significant changes being observed already on the second day of EEN, similar to those that were observed in children with CD on the last day of EEN. Furthermore, significant increase in microbial diversity was already apparent on the second day of EEN in healthy siblings while diversity did not significantly change throughout the EEN course in diseased children. Since children with CD might have dysbiosis that is both a cause and a consequence of the disease, they tend to present with slower change in microbiota composition caused by persistent severe gut inflammation that could not be rapidly changed by day 2. Moreover, it could be that "healthier" microbiota, present in healthy siblings, responds more rapidly and uniformly to the diet change, whereas CD patients exhibited divergent microbiome changes in response to EEN. Therefore, though our data could help in clarifying the role of different microbiota in CD patients, the major conundrum of whether it is the cause or the consequence of inflammation remains.

Interestingly, newer studies have demonstrated that specific changes in the diet, with regard to the exclusion of potentially proinflammatory ingredients, coupled with partial enteral nutrition (PEN) can lead to similar changes in microbiota composition as those observed with the use of EEN. This

diets were developed as an answer to a challenge that EEN poses to physicians and patients, mostly related to monotony of food and taste fatigue, which limits access and availability of the therapy. The most promising approach to this date is CD exclusion diet (CDED), whose mechanism of action is based on the exclusion of foods that have been associated with altered host barrier or bacterial clearance, dysbiosis and virulence factors that may allow bacteria to become mucosa-associated and enable translocation (Levine and Wine, 2013). This diet has recently been used for induction and maintenance of remission along with partial enteral nutrition (PEN) in CD patients (Levine et al., 2019). In the recent multinational randomized controlled trial, CDED diet was compared with EEN in inducing and sustaining remission in children with mild to moderate CD (Levine et al., 2019). Investigators have demonstrated that both diets were associated with high and comparable rates of clinical remission and a significant and similar decrease in inflammation by week 6. Furthermore, both groups had similar changes in the microbiome induced by diet by week 6. The initial preliminary analyses of CDED study indicate that there is a clear difference in the modulation of the intestinal microbiota in patients coming into and maintaining remission on CDED compared with those failing remission (Pigneur and Ruemmele, 2019).

Our studies have some limitations. They are mainly related to small sample size, however, it is comparable to other studies and adds important information to the still scarce literature. Moreover, we have investigated dietary intake in the previous month using FFQ, since 3-day food diary was not feasible in the case of patients who were referred to the hospital once the patient was suspected of having IBD. Moreover, FFQ used in this study was compared and correlates strongly with the results of a 3-day nutritional intake diary (Mocic Pavic et al., 2015). As for the analysis of microbiota, by using other sequencing methodology such as strain-level shotgun metagenomics with deep sequencing, we would have been able to provide strain-level taxonomic classification. Nevertheless, an advantage of this methodology is hypothesis-free approach, non-selective of certain bacterial species. Moreover, the period of two days of EEN in healthy siblings might not have been long enough to detect more profound changes in microbiota composition. Nonetheless, data in the literature have shown that intestinal microbiota changes already within one day after initiation of EEN which was also confirmed by our results (Meister et al., 2002). Moreover, we do not know if changes in the microbiota of CD patients were apparent already before the end of EEN since we did not analyze stools between the day 2 and the last day of EEN.

Strengths of our studies include that all controls, healthy unrelated and siblings, were younger than 18 years of age, ensuring that age was not a confounder in this cohort. Second, all the patients were treatment naïve and recruited at the time of diagnosis, which excludes the effect of treatment on the microbiota profiles. Moreover, important strengths of our study are in the unique design which included healthy siblings receiving EEN that was not investigated before.

CHAPTER V

Conclusions

5. CONCLUSIONS

The most important results of this dissertation thesis are:

- Lower intake of energy, macronutrients and various micronutrients in patients with UC and lower intake of fruits, calcium and animal protein in patients with CD compared to healthy controls was observed.

- IBD patients have altered body composition, with significantly lower lean mass-for-age z-scores at diagnosis compared to healthy controls.

- Altered microbiota composition, with reduced presence of genus *Eubacterium*, *Lactobacillus, Enterobacter* and *Clostridium*, and increased presence of genus *Streptococcus, Prevotella* and *Escherichia* was observed in IBD patients as compared to healthy siblings and healthy controls.

- None of the factors including age, disease location, type of formula (with taste vs tasteless and isocaloric vs hypercaloric) and mode of delivery (orally vs through NG tube for the whole duration of EEN) are associated with EEN failure in children with CD.

- Significant difference in microbial diversity between children with CD and healthy siblings was observed the second day of EEN, but not before the start of EEN and on the last day of EEN.

- In healthy controls, significant changes in microbiota composition were apparent already on the second day of EEN, contrary to children with CD in whom similar changes in microbiota composition were apparent on the last day of EEN.

Our studies build on the existing literature and confirm that children with IBD exhibit significant difference in microbiota composition already at the time of diagnosis of the disease. We have also confirmed that EEN leads to significant changes in microbiota composition of children with CD, probably being one of the leading mechanisms involved in the remission of the disease. Moreover, for the first time, we have shown that the similar changes occur in the siblings of children with CD. Opposite to other studies, we haven't detected significant changes in microbiota diversity as an effect of EEN in our cohort. We have also demonstrated that children with IBD show some

important differences in nutritional intake as opposed to healthy children, which emphasizes the importance of early nutritional intervention in these children. Moreover, we have confirmed that children with IBD often present malnourished and have altered body composition compared to healthy children.

Our studies confirm the importance of the diet in microbiota composition in children with IBD but also nutritional status (including micronutrients). Although we do not know if the diet that predisposed to diagnosis of the disease was associated with dysbiosis that was observed in our cohort, results of other studies have confirmed that the diet plays significant role in both the development, but also in the treatment of the diseases, and therefore, special attention to the nutrition of these children should be given.

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Paper 1

Nutritional status and food intake in pediatric patients with inflammatory bowel disease at diagnosis significantly differs from healthy controls

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Nutritional status and food intake in pediatric patients with inflammatory bowel disease at diagnosis significantly differs from healthy controls

Sara Sila[1*], Ivana Trivić[1*], Ana Močić Pavić[1*], Tena Niseteo[1], Sanja Kolaček[1,2], Iva Hojsak[1,2,3]

[1]Children's Hospital Zagreb, Zagreb, Croatia

[2]University of Zagreb, School of Medicine, Zagreb, Croatia

[3]University J.J. Strossmayer, School of Medicine Osijek, Osijek, Croatia

* All authors contributed equally and share first authorship

Corresponding author

Iva Hojsak, MD, PhD,

Referral center for pediatric gastroenterology and nutrition

Children's Hospital Zagreb

Klaićeva 16, 10000 Zagreb

Croatia

Phone: +38514600130; Fax: +38514600160

e-mail: ivahojsak@gmail.com

ABSTRACT

Nutritional status and dietary intake in pediatric-onset inflammatory bowel disease are complex and need to be further explored. Therefore, we have assessed anthropometric measures, body composition, and dietary intake of newly diagnosed pediatric patients and compared them with healthy controls. This was a prospective cross-sectional study including newly diagnosed patients with inflammatory bowel disease (n=89) and healthy controls (n=159). Mean energy intake was significantly higher in healthy controls compared to patients with ulcerative colitis, but not in patients with Crohn's disease. Intake of all macronutrients, dietary fiber and calcium was significantly lower in patients with ulcerative colitis, whereas only intake of animal protein, fruit, and calcium differed significantly in patients with Crohn's disease. There were no significant differences in the body fat percentage between patients with ulcerative colitis or Crohn's disease vs. controls, however, lean mass-for-age z scores were significantly lower in patients with both disease in comparison to controls.

Conclusion*:* Food intake of newly diagnosed pediatric patients with inflammatory bowel disease significantly differed from healthy controls. Altered anthropometry and body composition are present already at the time of diagnosis.

Key words inflammatory bowel disease, children, adolescents, diet, anthropometry

INTRODUCTION

Inflammatory bowel disease (IBD) is a group of chronic relapsing and remitting inflammatory diseases of the alimentary tract, consisting of Crohn's disease (CD), ulcerative colitis (UC) and, if undistinguishable, IBD-unclassified (IBD-U). Approximately a quarter of patients with IBD present in childhood [1] and, according to a recent systematic survey, the incidence and prevalence of pediatric-onset IBD is on the rise while the overall incidence of IBD has stabilized [2]. The exact etiology of IBD has yet to be elucidated. Continued progress has been made in identifying potential genetic risk factors. However, the genetic contribution seems to be low (with the exception of monogenic IBD) compared to environmental risk factors, among which dietary factors are receiving considerable attention [3]. Dietary factors play a key role in child's growth and development, but they may also have an important role in altering gut microbiota, metabolome, host barrier function and innate immunity, thus contributing to the disease development [4].

In IBD malnutrition is a well-recognized condition, especially in children with CD in whom linear growth failure often precedes gastrointestinal symptoms [5] and around two thirds of them are underweight at the time of diagnosis [1]. Malnutrition is not only found in the acute phases of CD [6] but has also been documented during clinical remission [7]. Symptoms of IBD include, but are not limited to, abdominal pain, change in bowel habits, impaired general well-being and malnutrition. The latter is often caused by suboptimal energy and nutrient intake, malabsorption and increased energy requirements [8]. Even so, children with IBD are affected by current population trends towards overweight and obesity [9]. A recent study reports that up to 1/5 of children with CD and 1/3 of children with UC included in a multi-center registry in the United States are overweight or obese during the follow up [10]. Abnormal nutritional status is a result of complex pathophysiological processes such as, but not limited to, genetic predisposition, inappropriate food intake, malabsorption, increase in basal

metabolism due to present inflammatory processes, disturbances of the growth hormone/insulin-like growth factor axis and the use of drugs such as corticosteroids [11].

Current evidence across studies regarding the diet of adults with IBD has been rather inconsistent [3] and the number of studies on the diet of children with IBD is even smaller [12-16]. Moreover, the relationship between nutritional status and dietary intake in pediatric-onset IBD is complex and needs to be further explored. Therefore, the aim of this study was to assess the anthropometric measurements, body composition and data regarding dietary intake in children and adolescents with newly diagnosed IBD and to compare that data to the age and sex matched healthy controls.

METHODS

Study design

This was a prospective cross-sectional study. Newly diagnosed patients with IBD who were diagnosed and treated in tertiary medical center (Children's Hospital Zagreb) and healthy controls were included into the study, provided they were 18 years of age or younger. Healthy controls were matched by age, gender and area of residence. They were recruited from randomly selected elementary and high schools in urban and rural area in Croatia that responded positively to the invitation to participate in the study. Written consent was obtained from the patients who were 9 years of age or older and one of their parents. In patients who were younger than 9 years of age, written consent was obtained only from their parents or caregivers. As for healthy controls, permission for the study was obtained from appropriate authorities, parents were informed about the survey from the school principals, and their written consent was obtained. Study was approved by Ethics Committee Children's Hospital Zagreb (IRB number: 21102014).

The IBD diagnosis has been established according to the Porto criteria [17] and localization was determined based on Paris classification [18]. Severity of the disease has been

estimated by the Pediatric Crohn's disease activity index (PCDAI) and the Pediatric ulcerative colitis activity index (PUCAI) [19,20]. Patients in whom, based on the dietary intake interview, it was recognized that there was a recent significant change in the diet were excluded from the study. Exclusion criteria in healthy controls included chronic illness or family history positive for chronic intestinal diseases (celiac disease, IBD, gastrointestinal carcinoma).

Anthropometric measurements, body composition and food intake were measured in patients and healthy controls.

Anthropometric measurements and body composition

Anthropometric measurements and body composition were assessed for each participant. In children with IBD the measurements were made within 24 hours of the diagnosis. The anthropometric assessment included measurements of body weight (BW), body height (BH), middle upper arm circumference (MUAC), triceps skinfold thickness (TST), subscapular skinfold thickness (SST) and handgrip strength (HS) and were measured by the same two trained persons. BW was measured on an electronic scale with subjects being dressed in a light-weight gym clothes. BH was measured with a portable stadiometer. TST and SST were estimated using a Holtain skinfold caliper. Jamar hydraulic handgrip dynamometer was used to estimate both right and left handgrip strength in children older than 6 years old.

Bioelectrical impedance was used to estimate subjects' body composition (Maltron BF906, Maltron International Ltd, Rayleigh, Essex, United Kingdom). Z-scores for BW, BH, body mass index (BMI) and MUAC were estimated by the Growth Analyzer software. TSF and SSF were estimated using CDC (Center for disease control and prevention) reference curves [21]. Z-scores for lean mass (LM) were estimated using UK reference data for pediatric body composition [22].

The nutritional status of participants was determined using the World Health Organization (WHO) Growth reference data for children and adolescents (5-19 years) [23].

Estimated energy requirements for each child were calculated using Schofield equation. Since data about physical activity hasn't been collected, it was presumed that most of participants had "light" physical activity level (PAL) [24].

Dietary intake

Information about food consumption was obtained at the time of diagnosis for all included patients. In healthy controls, food consumption was estimated during late winter and early spring. Food consumption was obtained using a Food Frequency Questionnaire (FFQ) version which was previously validated for Croatian children and adolescents [25]. FFQ is a tool that estimates frequency of consumption of different foods as well as their quantity. FFQ that was used contained 87 different food items divided into 8 different food groups: 'Milk and milk products', 'Cereals and grains', 'Juices and sodas', 'Fruits', 'Vegetables', 'Snacks', 'Meat, poultry, eggs and fat' and 'Fast food'. FFQ included frequently consumed national foods and estimated frequency and quantity of consumption of food items in the last month. Available frequencies of food consumption were: 'never', '1-3 times a month', 'once a week', '2-4 times per week', '5-6 times per week', 'once a day', '2-3 times per day', '4-5 times per day' or „'6+ times per day'. Available portion sizes were small, medium and large and simple portion sizes photos were used to distinguish the former. The frequency of consumption was obtained in a form of a personal interview with trained interviewers, with the presence of the caregivers (in children younger than 12 years of age) or without their presence in older participants.

The individual food records data obtained by the FFQ were analyzed by Microsoft Office Excel 2007 worksheet that was generated by using a combination of USDA [26] and Kaić-Rak et al. [27] food composition databases. Approximately 10 minutes were taken to administer the FFQ for each participant by a trained interviewer. The frequency of consumption of food items was multiplied by the portion size to calculate the amount of nutrients consumed

in a 30-day period, from which an average daily energy and nutrient intake per each participant were calculated.

Statistics

The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed by one-way ANOVA followed by Bonferroni test for post hoc analysis. Pearson correlation was performed in order to assess correlation between nutritional status and body composition and food intake. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (IBM Corporation, Chicago, Illinois, United States of America) statistical software.

RESULTS

Patient characteristics

Table 1 summarizes baseline characteristics of enrolled participants. Overall, there were 89 patients with newly diagnosed IBD and 159 healthy controls included into the study. Of those, 49 (55%) patients were affected by CD and 40 (45%) by UC. No significant difference in age was found between the CD patients, UC patients and healthy controls (mean age: 14.6 ± 2.6 years for CD; 14.0 ± 3.7 years for UC; 14.7 ± 2.3 years for healthy controls; p=0.349). Based on PCDAI/PUCAI scoring, 54 patients (63.5%) had mild disease and 31 patients (36.5%) had moderate to severe disease at the time of diagnosis.

Table 1. Baseline characteristics of enrolled patients.

	CD (n=49)	UC (n=40)	Healthy controls (n=159)
Age, mean (SD)	14.6 (2.6)	14.0 (3.7)	14.7 (2.3)
Male, n (%)	29 (59.2)	23 (57.5)	80 (50.3)
BW, mean (SD)	51.5 (14.6)	51.0 (16.8)	59.0 (14.2)

BH, mean (SD)	163.2 (14.7)	161.1 (18.7)	167.4 (11.9)
BMI, mean (SD)	19.6 (2.8)	18.2 (2.8)	20.7 (3.2)
Duration of symptoms in months, mean (SD)	7.6 (2.1)	4.2 (1.0)	-
Localization of the disease*	L1 (ileal): 12 (24.5%) L2 (colonic): 6 (12.2%) L3 (ileocolonic): 31 (63.3%) L4 (additional upper gastrointestinal disease): 18 (36.7%)	E1 (proctitis): 1 (2.5%) E2 (left sided): 8 (20%) E3 (extensive): 5 (12.5%) E4 (pancolitis): 26 (65%)	-

BM – body weight; BH – body height; BMI – body mass index; SD-standard deviation

*Paris classification of the inflammatory bowel disease [17].

Anthropometric measurements and body composition

BMI z-score was used to estimate nutritional status of all participants. Nutritional status of newly diagnosed IBD patients and healthy controls is shown in Figure 1. Overall, 32.6% of CD-patients and 25% of UC-patients were malnourished according to the WHO criteria (22). Overweight was found in 16.3% and 17.5% of CD and UC patients, respectively. None of the patients were obese. Only 4 (4.5%) IBD patients were stunted (BH-for-age z-score <-2SD), of whom 3 were CD patients.

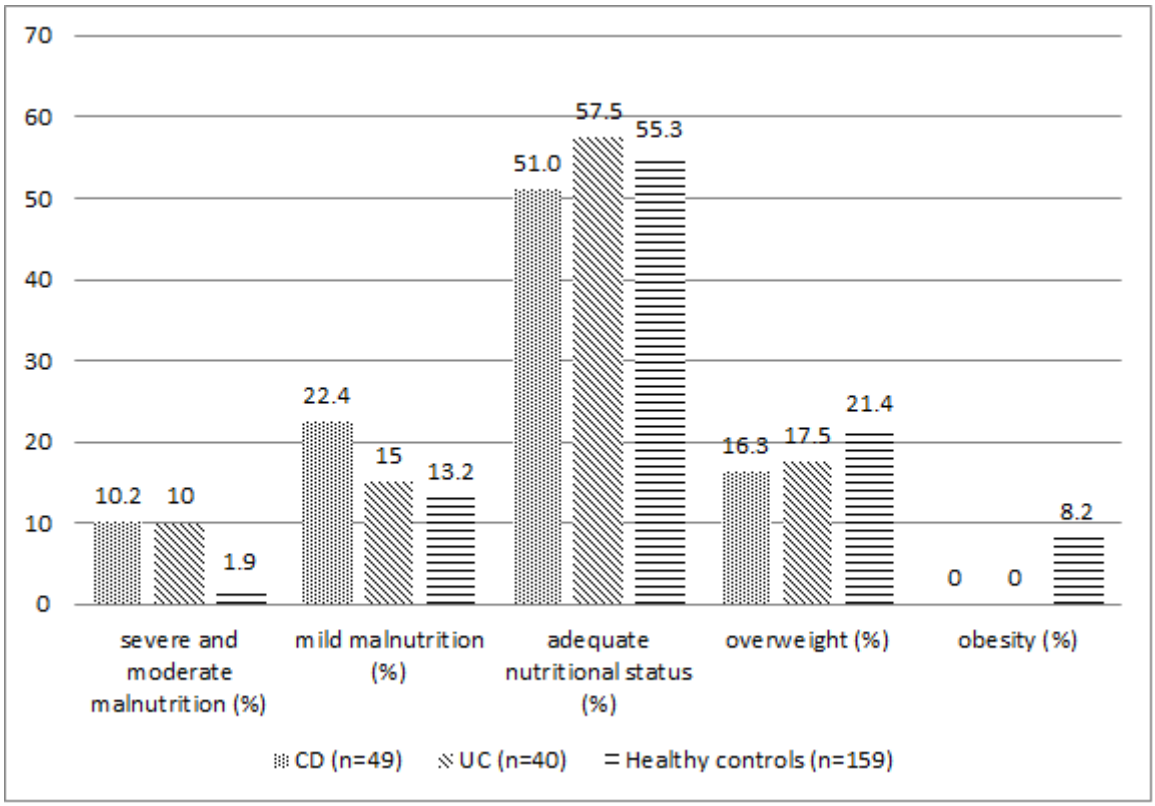

Figure 1. Comparison of nutritional status according to WHO criteria between IBD patients and healthy controls.

There was a significant difference in BW-for-age, BMI-for-age and basal metabolic rate (BMR) but not for triceps- and subscapular skinfold thickness between both CD patients and UC patients vs. healthy controls (Table 2). Significant difference in BH-for-age z-score and MUAC-for-age z-score was found only between CD patients vs. healthy controls. There was no significant difference in any of anthropometric measures between CD and UC patients.

As for body composition estimated by bioelectrical impedance, there was no statistically significant difference in body fat percentage between CD and UC patients vs. healthy controls. However, both CD and UC patients had significantly lower lean mass-for-age z-scores compared to healthy controls (Table 2.)

Table 2. Difference in anthropometric characteristics between CD patients, UC patients and healthy controls.

	CD (n=49)	UC (n=40)	Healthy controls (n=159)	p-value
BW-for-age, mean (SD)	-0,5 (1,2)	-0,2 (1,1)	0.3 (1.1)	<0.001*
BH-for-age, mean (SD)	-0,5 (1,0)	-0,2 (0,9)	0.0 (1.0)	0.013**
BMI-for-age, mean (SD)	-0,4 (1,3)	-0,2 (1,2)	0.4 (1.1)	<0.001*
EER (kj), mean (SD)	8743.0 (1524.8)	8694.7 (1744.3)	9451.3 (1602.6)	0.003*
MUAC-for-age, mean (SD)	-0,7 (1,0)	-0,5 (0,8)	-0.3 (0.8)	0.009**
Triceps skinfold thickness-for-age, mean (SD)	0,8 (0,8)	0,7 (0,9)	0.8 (0.7)	0.871
Subscapular skinfold thickness-for-age, mean (SD)	0,7 (0,8)	0,7 (0,6)	0.7 (0.8)	0.998
Handgrip strength				
Right hand (kg), mean (SD)	20.2 (9.9)	20.7 (14.1)	25.3 (9.3)	0.006**
Left hand (kg), mean (SD)	17.9 (9.9)	16.3 (9.7)	23.2 (8.6)	<0.001*
Body fat %, mean (SD)	19.3 (7.6)	18.5 (7.6)	20.7 (7.7)	0.239

BMR (kcal), mean (SD)	1438.1 (315.3)	1469.4 (301.9)	1598.4 (237.3)	0.001
Lean mass-for-age, mean (SD)	0.2 (1.4)	0.4 (1.2)	1.2 (1.4)	<0.001*

BM – body weight; BH – body height; BMI – body mass index; EER – estimated energy requirements; MUAC – middle upper arm circumference; BMR – basal metabolic rate; SD-standard deviation

Post hoc analysis: *p<0.05 for CD and UC vs. healthy controls; **p<0.05 for CD vs. healthy controls

There was no association between duration of symptoms and anthropometric measures or body composition. Similarly, nutritional status did not significantly differ depending on the disease severity estimated by PUCAI/PCDAI score.

Dietary intake

The mean energy intake differed significantly between UC patients and healthy controls (7780.9 ± 2774.5 kj; 10198.3 ± 4409.5 kj; p=0.003), but not CD patients (8915.4 ± 3377.6 kj; 10198.3 ± 4409.5 kj; p=0.202). On average, CD patients satisfied 90 ± 52.8%, UC patients 86.6 ± 39.6% and healthy controls 109.3 ± 45.9% of their estimated energy requirements.

Lower intake of all macronutrients and dietary fiber has been found between UC patients vs. healthy controls (Table 3). In CD patients, significant difference was found only for animal proteins when compared to healthy controls (46.0 ± 21.5 g in CD; 57.2 ± 27.4 g in healthy controls; p=0.037). There was no difference in energy and macronutrient intake between UC and CD patients.

Table 3 shows the mean intake of micronutrients. When compared to healthy controls, there was no significant difference in intake of most micronutrients, except for intake of phosphorus and calcium between CD and UC patients vs. healthy controls and for iron and zinc in UC patients vs. healthy controls.

When contributions of different food groups to total energy intake were estimated, a difference was found for intake of fruits in CD patients compared to healthy controls. The contribution of other food groups to total energy intake did not differ significantly (Table 3).

Table 3. Difference in nutritional intake between CD patients, UC patients and healthy controls.

	CD (n=49)	UC (n=40)	Healthy controls (n=159)	p-value
Energy intake (kj), mean (SD)	8915.4 (3377.6)	7780.9 (2774.5)	10198.3 (4409.5)	0.002***
% EER (%), mean (SD)	90.0 (52.8)	86.6 (39.6)	109.3 (45.9)	0.004*
Total proteins (g), mean (SD)	92.4 (33.3)	83.5 (31.6)	107.1 (43.8)	0.002***
Vegetable proteins (g), mean (SD)	25.7 (11.1)	23.6 (8.7)	29.0 (14.3)	0.043
Animal proteins (g), mean (SD)	46.0 (21.5)	43.7 (20.7)	57.2 (27.4)	0.002*
Total fat (g), mean (SD)	102.1 (45.9)	81.1 (31.1)	108.1 (52.9)	0.010***
Saturated fat (g), mean (SD)	35.9 (17.3)	30.3 (13.8)	43.6 (22.0)	0.001***
Unsaturated fat (g), mean (SD)	31.9 (14.5)	25.6 (10.7)	54.0 (27.2)	0.006***
Total carbohydrates (g), mean (SD)	252.7 (100.8)	228.7 (85.1)	292.9 (133.9)	0.007***
Mono- and disaccharides (g), mean (SD)	107.8 (62.3)	98.5 (51.8)	131.3 (59.3)	0.002***
Fibers (g), mean (SD)	21.8 (9.2)	20.8 (8.0)	25.8 (12.3)	0.014***

Calcium (mg), mean (SD)	765.1 (399.7)	766.6 (385.1)	987.4 (446.2)	0.001*
Magnesium (mg), mean (SD)	247.3 (193.3)	219.1 (123.5)	279.5 (160.4)	0.091
Phosphorus (mg), mean (SD)	1455.7 (521.1)	1364.9 (508.3)	1742.2 (674.1)	0.001*
Iron (mg), mean (SD)	13.2 (5.5)	11.3 (4.5)	14.7 (6.4)	0.005***
Zinc (mg), mean (SD)	8.6 (3.9)	7.3 (3.5)	8.9 (3.6)	0.047***
Proteins (% EI), mean (SD)	17.3 (2.8)	17.9 (2.8)	17.7 (2.5)	0.622
Carbohydrates (% EI), mean (SD)	46.5 (7.1)	48.8 (5.6)	47.6 (7.5)	0.355
Fat (% EI), mean (SD)	41.8 (7.0)	38.7 (5.7)	39.0 (6.8)	0.044
Fruits (%EI), mean (SD)	3.3 (3.9)	5.2 (5.3)	6.2 (4.7)	0.002**
Vegetables (%EI), mean (SD)	3.7 (1.7)	4.2 (2.0)	3.8 (2.3)	0.535
Juices and sodas (%EI), mean (SD)	5.2 (4.0)	5.2 (3.9)	4.9 (4.0)	0.814
Snacks (%EI), mean (SD)	10.8 (9.9)	8.7 (6.2)	9.9 (6.6)	0.444
Fast food (%EI), mean (SD)	13.5 (6.9)	11.3 (4.2)	13.0 (6.3)	0.246

EI – energy intake

Post hoc analysis: *$p<0.05$ for CD and UC vs. healthy controls; **$p<0.05$ for CD vs. healthy controls; ***$p<0.05$ for UC vs. healthy controls.

There was no association between duration of symptoms and dietary intake in both CD and UC patients.

Pearson correlation revealed no significant correlation in BMI z-score, lean mass-for-age z-scores, and TST and dietary intake (total energy and micronutrient intake). A positive correlation was found for SST and total energy (correlation coefficient 0.282, p=0.015), total carbohydrates (correlation coefficient 0.252, p=0.026), total protein (correlation coefficient 0.282, p=0.012) and total fat (correlation coefficient 0.275, p=0.015) intake.

DISCUSSION

This study is, best to our knowledge, the first study which investigated food intake at diagnosis of pediatric patients with IBD compared to healthy controls and showed lower intake in energy and all macronutrients in IBD patients. This difference was mainly contributed to UC patients.

Energy and macronutrient intake

Previously, studies have looked into dietary intake of IBD patients [12-16,28]. However, these studies have assessed dietary intake in children and adolescents with already established disease, which was both active and in relapse [14,12,13,15,16]. One study estimated consumption of food items of newly diagnosed IBD patients over a past year (using a yearly FFQ) and did not include a control group [28]. Results of all previously published studies are not unified; study by Hartman et al. [13], found that patients with active and inactive IBD had lower intake of energy, carbohydrates and dietary fiber in comparison with healthy Israeli children. However, this study did not stratify patients by disease type. Diederen et al. [15] included CD, UC and IBD-U patients, of which 46% had active disease. Energy intake and total carbohydrate intake in all IBD patients was lower, while intake of vegetable proteins and total fat intake was higher compared to the reference population. When distributed by the type of IBD, energy intake of CD patients was lower compared to that of general population, while UC patients did not significantly differ from the reference population. These results are opposite to ours, where patients with UC had lowest energy and macronutrients consumption. This difference could be at least partially explained by the acute symptoms (bloody diarrhea and abdominal cramps) at the time of the UC diagnosis. The acute symptoms could cause the restriction in the food intake and increase the likelihood of restrictive diet introduction. However, it should be noted that in the study by Diederen et al. [15], dietary intake of IBD patients was estimated using FFQ, while dietary intake of reference population was estimated

by 24-hour recalls. Interestingly, studies mentioned above did not find difference in macronutrient intake when stratified by the disease activity, meaning that restriction was noted also in patients who were in the clinical remission [13,15]. Opposite to that, in an older study by Pons et al. [12], total energy and all macronutrient intake were lower in children with active CD, compared with children with CD in remission and controls. In our cohort, neither disease activity nor duration of symptoms was associated with dietary intake. This could be related to our relatively coherent population of patients, in whom PCDAI/PUCAI scoring was fairly similar as well as duration of the disease.

Micronutrient intake

As for micronutrients, lower intakes of calcium, iron and zinc in UC patients and of calcium in CD patients were detected in our study. Results of other studies have shown mixed results. In a study by Thomas et al. [14] where food diary records were used to estimate dietary intake, intake of iron and zinc in CD patients in relapse was significantly lower compared to controls. Using the same methods as Thomas et al. [14], Hartman et al. [13] detected lower intakes of calcium in Israeli IBD patients. Pons et al. [12] have used FFQ to estimate nutritional intake and haven't detected lower intakes of calcium in CD patients compared to controls. In addition to the IBD itself, low calcium intake has been described as a risk factor for fractures among adult IBD patients [29]. Additional to low calcium intake, risk of developing osteoporosis in IBD patients is related to malnutrition, altered absorption of nutrients, lower physical activity and due to administration of corticosteroids amongst others [30]. IBD patients are prone to reduce the intake of milk and milk products for fear of lactose consumption [31]. Although this could be a plausible explanation, we do not know whether this was a case in our cohort.

Intake of different food groups

Although this study was not designed to assess causality of different diets and IBD, we confirmed a lower contribution of fruits to total energy intake in CD patients compared to

healthy controls. Opposite to ours, Diederen et al. [15] have found higher intakes of vegetable and fruit in children with IBD, but as previously mentioned, those were the patients with established diagnosis and, thus, could be advised during their treatment to increase the intake of "healthy foods" like fruits and vegetables. Regarding our study, we could speculate that patients with CD avoided fruit intake due to active symptoms of their disease, however this is highly unlikely since intake of other nutrients and food groups (vegetables, soda and juices, snacks and fast food) did not differ from that of healthy population.

Anthropometry measurements and body composition

As reported by others, our study found significant difference in anthropometric measurements and lean mass between CD and UC patients vs. healthy controls. One of the most prominent presenting features of IBD includes malnutrition [32]. In our cohort, 10.2% of CD and 10% of UC patients were undernourished (Z-score <-2 SD) at the time of diagnosis. Compared to ours, in the French cohort, even 32% of pediatric patients with CD had BMI z-score <-2 SD at the time of diagnosis [33], similar to the results of El Mouzan et al. [34] where 35% of CD and 24% of UC patients had BMI z-score <-2 SD. Contrary to undernutrition, 16.3% of CD and 17.5% of UC patients in our cohort had BMI z-score >1 SD, similar to that of general population [35]. Our results are comparable to the results of other countries [34,9]. Whether the lower proportion of undernourished patients in our cohort is a result of overall increase in the BMI of general population that can influence the BMI at the diagnosis is yet to be determined. Some studies suggested a possible shared environmental link between the increase in the IBD and obesity incidence. Moreover, it was also observed that obesity might impact disease development and response to therapy in patients with IBD [36]. It has been emphasized recently that assessing body composition, rather than simple anthropometric changes, gives a much better indication of nutritional status in all chronic diseases as well as in IBD [37]. In our cohort, despite having lower BMI-for-age z-scores compared to healthy controls, both CD and UC

patients had significantly lower z-scores for lean body mass while having same body fat percentage. That clearly confirms that BMI is not an ideal marker of body composition in children with IBD. Similarly, Burnham et al. [37] reported fat mass adjusted for age and fat mass adjusted for height that was not significantly different from controls. Furthermore, in our cohort, no difference in skinfold thickness has been found in IBD patients compared to healthy controls, which could be explained by higher body fat percentage in relation to total body mass in these children. Comparable to ours, in their study, Wiskin et al. [38] found lower values of proxies for lean tissue but not for fat only in children with CD, and not in those with UC. Our results together with previously published studies indicate that nutritional assessment should not be solely based on anthropometric measures and should include estimation of body composition. Interestingly, in our cohort, symptoms duration and disease activity were not associated with anthropometric measures and body composition, which could be related to our relatively coherent population of patients. Furthermore, this study has found that only SST, but not TST, BMI and lean mass-for-age z score, positively correlates with total energy and all macronutrients intake in IBD patients. This could indicate that higher energy intake was associated with more truncal deposition of body fat even in children with IBD.

Our study has several limitations mainly related to the relatively small sample size and usage of FFQ for estimation of dietary intake. For more accurate nutritional intake, 3-day food diary would be more appropriate. However, FFQ used in this study was compared and correlates strongly with the results of a 3-day nutritional intake diary [25]. Nevertheless, this study compared newly diagnosed patients with IBD and healthy matched controls, and as previously mentioned, for the first time provides an insight into the nutritional intake in patients with newly diagnosed IBD.

In conclusion, this study showed significantly lower intake of energy, macronutrients and various micronutrients in patients with UC compared to healthy controls, while patients with

CD had lower intake of fruits, calcium and animal protein. Furthermore, altered anthropometry, and more importantly, body composition in both CD and UC patients, at the diagnosis has been shown. This study indicates that specific nutritional interventions should be implemented early after diagnosis, since malnutrition and its consequences are often seen in pediatric IBD patients. Furthermore, IBD patients are prone to unnecessary restrictions in their diets, which can further deteriorate their nutritional status. This study contributes to the still scarce literature on diet and anthropometry in pediatric patients with IBD.

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Conflict of Interest: Iva Hojsak received payment/honorarium for lectures or consultation from BioGaia, Nutricia, Nestle, GM pharma, Chr Hansen, Sanja Kolaček received fees for lectures from Abbott, AbbVie, Fresenius, Mead and Johnson, Nestle, Nutricia, Oktal Pharma. Tena Niseteo received fee for lectures from 4U Pharma. Sara Sila, Ivana Trivić and Ana Močić Pavić declare no conflict of interest.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Study was approved by Ethics Committee Children's Hospital Zagreb (IRB number: 21102014).

Informed consent: Informed consent was obtained from all individual participants and at least one of their parents included in the study.

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Paper 2

Altered gut microbiota is present in newly diagnosed pediatric patients with inflammatory bowel disease

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Altered gut microbiota is present in newly diagnosed pediatric patients with inflammatory bowel disease

Sara Sila, MSc[1], Marko Jelić, PhD[2], Ivana Trivić, MD[1], Arjana Tambić Andrašević[2], MD; PhD[2,3], Iva Hojsak, MD, PhD[1,4,5*], Sanja Kolaček, MD, PhD[1,5*]

[1]Children's Hospital Zagreb, Zagreb, Croatia

[2]University Hospital for Infectious Diseases, Zagreb, Croatia

[3] University of Zagreb, School of Dental Medicine, Zagreb, Croatia

[4]University J.J. Strossmayer, School of Medicine Osijek, Osijek, Croatia

[5]University of Zagreb, School of Medicine, Zagreb, Croatia

[*]Authors contributed equally and share last authorship

Corresponding author

Iva Hojsak, MD, PhD,

Referral center for pediatric gastroenterology and nutrition

Children's Hospital Zagreb

Klaićeva 16, 10000 Zagreb

Croatia

Phone: +38514600130; Fax: +38514600160

e-mail: ivahojsak@gmail.com

ABSTRACT

Background and aims: Clinical and experimental data suggest that gut microbiota plays an important role in the pathogenesis of inflammatory bowel disease. The aim of this study was to determine intestinal microbiota in newly diagnosed patients with inflammatory bowel disease and to compare it to patients' healthy siblings who share same genetic and environmental background and to healthy unrelated controls.

Methods: Molecular approach targeting 16S ribosomal RNA was employed for analyzing the gut microbiota of participants' stool samples. Terminal restriction fragment length polymorphphism analysis was performed.

Results: Newly diagnosed pediatric patients with inflammatory bowel disease (n=19, 68.4% CD, mean age 14.8 ± 0.65 years), their unaffected healthy siblings (n=20, mean age 12.8 ± 0.85 years) and unrelated healthy controls (n=19, mean age 10.7 ± 0.8 years) were included. Microbial diversity differed significantly between inflammatory bowel disease patients, healthy siblings and healthy controls (p=0.018 for *MspI*-digestion, p=0.013 for *HhaI*-digestion). No significant difference in microbial diversity was found between healthy siblings and healthy controls. In patients reduced presence of genus *Eubacterium, Lactobacillus, Enterobacter* and *Clostridium,* and increased presence of genus *Streptococcus, Prevotella* and *Escherichia,* compared to healthy siblings and healthy controls, was found.

Conclusion: Newly diagnosed pediatric patients with inflammatory bowel disease show significantly less diverse microbiota and microbial composition compared to healthy siblings and healthy controls.

Key words: microbiology; pediatrics; Crohn's disease; ulcerative colitis; children; microbiome

What is known

- Clinical and experimental data suggests that microbiome plays an important role in the development of inflammatory bowel disease.
- Both increased and decreased abundance of specific bacterial taxa, as well as difference in microbial diversity has been described in patients with inflammatory bowel disease.

What is new

- This study found significant difference in microbiota composition in newly diagnosed, treatment naive pediatric patients with inflammatory bowel disease compared to healthy siblings and healthy controls.
- Lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in patients compared to healthy siblings and healthy controls.

INTRODUCTION

The etiopathogenesis of inflammatory bowel disease (IBD) is unclear, but clinical and experimental data suggests the crucial role of a microbiome, intestinal mucosal barrier and of the immune system (1, 2). Therefore, it is proposed that in IBD there is an unrestrained abnormal immune response to gut microbiota / content occurring in genetically predisposed individuals (2). The largest pediatric study to date by Gevers et al. (3) has confirmed the result of other smaller studies (4-6) that composition of microbiota in treatment-naïve pediatric IBD patients is altered compared to healthy subjects. Both increased and decreased abundance of specific bacterial taxa, as well as difference in microbial diversity has been described (3). A reduced diversity of microbiota, the lower abundance of "beneficial" bacteria, mainly Firmicutes and increases in abundance of "detrimental" bacteria, such as Proteobacteria (mainly *E. coli*), was observed in patients with IBD (7). However, the division of certain bacterial species into either beneficial or detrimental has recently been challenged. Paradoxically, in patients treated with exclusive enteral nutrition (EEN) (8-11) or Crohn's disease (CD) treatment-with-eating diet (CD-TREAT) (12), a decrease in proportion of potentially beneficial bacteria has been described. Contrary, Levine et al. (13) have recently demonstrated that exclusion of dietary components by EEN or Crohn's Disease Exclusion Diet (CDED) reduced potentially harmful Proteobacteria while increasing potentially beneficial Firmicutes. Not only that the results of different studies differ, moreover, it is not clear whether dysbiosis in IBD patients is merely a consequence of the disease, or it has a role in the disease development.

The gut microbiota has multiple functions, including supplying energy and nutrients to the host, such as vitamin K and water-soluble vitamins synthesized by human commensal bacteria (14). Furthermore, short-chain fatty acids (SCFA) produced by the phyla Firmicutes and Bacteroidetes serve as a primary energy source for colonic epithelial cells (15). It was observed that IBD patients have diminished ability to produce SCFA, which further alters microbiota

composition and consequently influences intestinal and immune homeostasis (16). Finally, microbial products released into the bowel lumen can epigenetically influence the long-term function of both, intestinal immunity and the mucosal barrier (17, 18).

In approximately a quarter of patients, disease is diagnosed before the age of 18 years with the significant increase in incidence being observed in this specific age group (19, 20). Thus, it is of utmost importance to understand the contributing factors for disease development, which may provide the possibility for disease prevention and/or treatment that is more efficient. With the exception of rare monogenic diseases, most commonly occurring in the first years of life (21), only about 7.5% of incidence of CD and 13.6% of ulcerative colitis (UC) can be explained by genetics (22). Therefore, the current emphasize is on different environmental factors, which could play a crucial role in the etiopathogenesis of IBD (23). Studying currently healthy siblings of patients with IBD, who share both, the genetic background and environmental exposures, may provide further insights into IBD pathogenesis.

To our knowledge, only few studies have compared fecal microbiota of IBD patients to their unaffected siblings/relatives (24-29). The results of these studies have not been conclusive. Some studies have shown that dysbiosis was present in both IBD patients and healthy siblings, suggesting that dysbiosis in IBD patients is not merely a consequence of intestinal inflammation (24, 28). However, not all studies have confirmed these results (26, 29).

The aim of this study is, therefore, to determine the composition of intestinal microbiota in newly diagnosed IBD patients and to compare it to patients' healthy siblings and healthy unrelated controls. To our knowledge, this is the first study that compared microbiota of newly diagnosed, treatment naive pediatric IBD patients with that of healthy pediatric siblings and healthy unrelated pediatric controls.

MATERIALS AND METHODS

Patients and study design

Newly diagnosed pediatric IBD patients and their unaffected healthy siblings were recruited at the Referral Centre for Pediatric Gastroenterology and Nutrition at the Children's Hospital Zagreb from June 2016 to April 2019. Unrelated healthy controls were recruited by circular e-mail sent to hospital staff who accepted their children's participation. All participants older than 9 years of age and their parents gave written informed consent. The diagnosis of IBD was established according to the revised Porto criteria (30), while disease location was defined using the Paris classification (31). Severity of the disease was estimated by Pediatric Crohn's disease activity index (PCDAI) and Pediatric ulcerative colitis activity index (PUCAI) (32, 33). Exclusion criteria in healthy controls included chronic illness or family history positive for chronic intestinal diseases (celiac disease, IBD, gastrointestinal carcinoma). In healthy siblings, exclusion criteria included unintentional weight loss in the last 6 months, changes in stool frequency or consistency or other symptoms suggestive of undiagnosed IBD. For each participant, retrospective data on type of delivery (vaginal birth or C-section), months of total breastfeeding, months of exclusive breastfeeding, time of weaning, number of siblings and owning of pets were collected. Stool samples of all participants were collected in sample containers, prior to therapy introduction. They were stored in the hospital or at home at -20°C for a maximum of 24 hours, after which they were transferred in the cold packs to the Department of Clinical Microbiology at the University Hospital for Infectious Diseases and stored at -80°C. Stool samples of newly diagnosed patients were collected prior to therapy introduction.

Total fecal bacterial DNA extraction, from ~150 mg of stool samples, was performed using Quick-DNATM Fecal/Soil Microbe Miniprep Kit (Zymo Research, USA) according to manufacturer's instructions.

PCR amplification and T-RFLP analysis

PCR amplification and terminal restriction fragment length polymorphphism (T-RFLP) analysis were performed according to Andoh et al 2012 with slight modifications(34) 6'-carboxyfluorescein (6-FAM) labeled 27-F primer (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Thermo Fisher Scientific, USA) were used for the amplification of the 16S rRNA gene from the human fecal DNA (34). The PCR amplification (20 ng of DNA) was performed in 50 µL reactions, in triplicates, according to previously described protocol (35). Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Germany) and diluted in 50 µL of elution buffer.

HhaI and *MspI* enzymes were used for the restriction of amplified 16S rRNA genes (34). 120 ng of purified PCR product was digested separately in 30 µL reaction volumes, using 1 µL of FastDigest *HhaI* and FastDigest *MspI* (Thermo Fisher Scientific, USA) at 37°C for one hour. Restriction products were purified by ethanol/ sodium acetate/EDTA precipitation and resuspended in 12 µL deionized formamide (Thermo Fisher Scientific, USA) to a final concentration of 10 ng/ µL (36). 3 µL of restriction digest product (~36 ng) was mixed with 11 µL of deionized formamide and 0.5 µL of fourfold diluted GS2500ROX (Thermo Fisher Scientific, USA). The length of the terminal restriction fragments (T-RFs) was determined with an ABI PRISM 310 genetic analyzer in GeneScan mode (20s injection time; 15 kV, and 60°C for 48 min for each sample) (Thermo Fisher Scientific, USA) (37).

Fragment sizes were estimated by using the Local Southern Method GeneMapper 3.7 software (Thermo Fisher Scientific, USA). T-RFs in the range of 50–810 bp with a peak height greater than 25 fluorescence units were included in the analysis. Alignment of T-RFs was performed by T-REX software (http://trex.biohpc.org/) (38). Binning treshold of 2 bp was used

for assignment of T-RFs to operational taxonomic units (OTUs) (36). The OTUs were quantified as the percentage values of an individual OTU per total OTU area, and this was expressed as the % area of the underpeak curve (% AUC) (39).

Assignment of OTUs to bacterial taxa was performed in silico using the web-based analysis tool (PAT+) provided by MiCA3 (http://mica.ibest.uidaho.edu/pat.php), based on the RDP (Ribosomal Database Project) release 10 16s rRNA gene database (40).

Statistics

The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed based on distribution and number of groups by ANOVA or t test and Kruskal-Wallis or Mann-Whitney U test. The relative abundance of OTUs was used to calculate Shannon-Wiener diversity index in order to compare diversity between different sample groups. Cluster analyses were performed using BioNumerics software (Applied Maths, Belgium) based on the *HhaI* or *MspI* T-RFLP patterns. A dendrogram representing calculated similarity distances was generated using Pearson's similarity coefficient analysis and the unweighted pair-group methods with arithmetic means (UPGMA). P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (Chicago, IL) statistical software.

Study was approved by Ethics Committee of the Children's Hospital Zagreb (IRB number: 21102014).

RESULTS

Baseline characteristics of enrolled participants (IBD patients, healthy siblings and healthy controls) are summarized in Table 1. Overall, with the exception of age (healthy controls being significantly younger compared to IBD patients and healthy siblings) there were no differences in study populations in respect to gender, duration of breastfeeding, time of weaning, number of siblings, mode of delivery and owning of pets. At the time of assessment,

12 CD patients (92.3%) had mild disease according to PCDAI scoring (PCDAI score between 10 and 30), while 1 patient (7.7%) had moderate to severe disease (PCDAI score higher than 30). In UC patients, 3 patients (50%) had mild disease (PUCAI score between 10 and 34) and 3 patients (50%) had moderate disease (PCDAI score between 35 and 64) according to PUCAI score.

Table 1. Baseline characteristics of study population.

	IBD (n=19)	Healthy siblings (n=20)	Healthy controls (n=19)	p-value
Male, n (%)	63.2%	13/7	10/9	0.212
Crohn's disease, n (%)	13 (68.4%)			
Age (years), mean (SD)	14.77 (0.65)	12.84 (0.85)	10.72 (0.84)	0.005
Breastfeeding (months), mean (SD)	13.29 (2.59)	13.7 (3.39)	10.0 (1.57)	0.606
Weaning (months), mean (SD)	5.4 (0.3)	5.35 (0.27)	5.26 (0.21)	0.994
Number of siblings, mean (SD)	1.4 (0.11)	1.4 (0.11)	1.05 (0.21)	0.055
Vaginal delivery, n (%)	15 (79.0%)	16/4	14/5	0.881
Owning of pets, n (%)	12 (63.2)	8/12	7/12	0.973
PCDAI, mean (SD)	21.4 (7.5)			
PUCAI, mean (SD)	36.7 (21.4)			

Localization of the disease (CD patients)*	L1 (ileal): 4 (30.8%) L2 (colonic): 1 (7.7%) L3 (ileocolonic): 8 (61.5%)			
Localization of the disease (UC patients)*	E1 (proctitis): 0 (0.0%) E2 (left sided): 0 (0.0%) E3 (extensive): 0 (0.0%) E4 (pancolitis): 5 (100.0%)			

IBD – inflammatory bowel disease, CD – Crohn's disease, UC – ulcerative colitis, PCDAI - Pediatric Crohn's disease activity index, PUCAI - Pediatric ulcerative colitis activity index.
*Paris classification of the inflammatory bowel disease [31]

The fecal microbiota profiles of all 3 groups are illustrated by a dendogram (Figure 1). A setting of similarity generated 2 major clusters. Most of healthy controls (17/19 with *HhaI* and 18/19 with *MspI*) and healthy siblings (20/20 by *HhaI* and 20/20 by *MspI*) were classified in cluster I. In IBD patients, 57.9% (*HhaI* digestion) and 52.6% (*MspI* digestion) were classified into cluster II (Table 2). In CD patients there was a significant difference between cluster and disease localization (p=0.002); all patients with CD classified in cluster I (n=4) had L1 localization of the disease while none of the patients in cluster II had L1 localization (8 patients had L3 and one patient L2 localization).

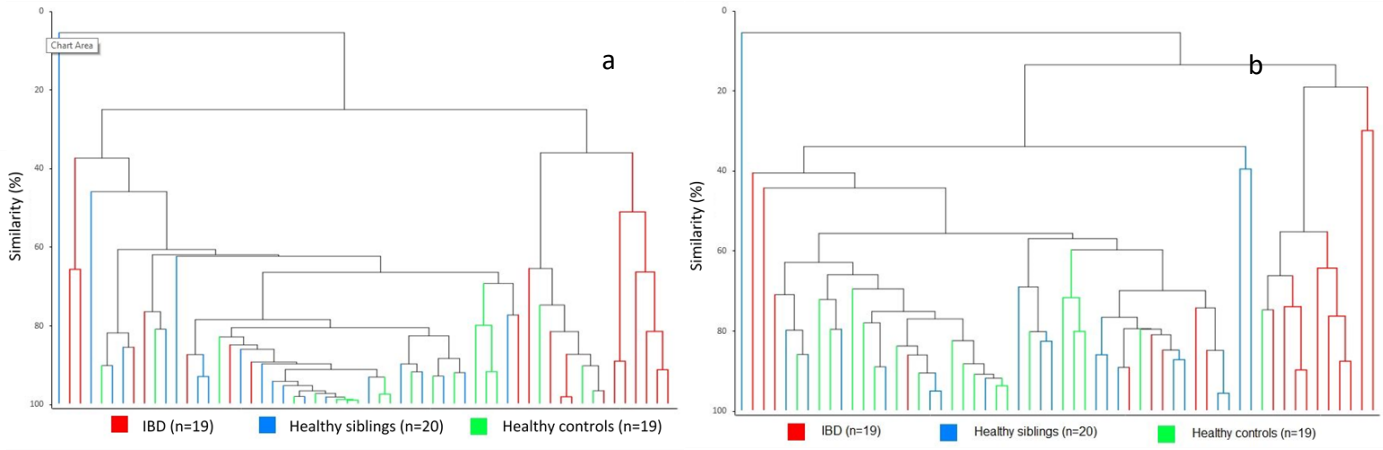

Figure 1. Dendrogram of the fecal microbiota profiles of IBD patients, healthy unrelated controls and healthy siblings (A. *HhaI*-digestion, B. *MspI*-digestion).

Table 2. Distribution of fecal microbiota profiles in IBD patients, healthy siblings and healthy controls.

	Cluster I (HhaI-digestion/MspI-digestion)	Cluster II (HhaI-digestion/MspI-digestion)
IBD patients (n=19)	8 (42.1%) / 9(47.4%)	11 (57.9%) / 10 (52.6%)
CD patients (n=13)	5 (38.5%) / 5 (38.5%)	8 (61.5%) / 8 (61.5%)
UC patients (n=6)	3 (50%) / 3 (50%)	3 (50%) / 3 (50%)
Healthy siblings (n=20)	20 (100%) / 20 (100%)	0 (0%) / 0 (0%)
Healthy controls (n=19)	17 (89.5%) / 18 (94.7%)	2 (10.5%) / 1 (5.2%)

IBD – inflammatory bowel disease, CD – Crohn's disease, UC – ulcerative colitis

There was no association between cluster distribution and disease severity based on PUCAI (p=0.10) or PCDAI score (p=0.825), age (p=0.503 for CD and p=1.0 for UC),

symptoms duration (p=0.414 for CD and p=1.0 for UC), presence of perianal disease in patients with CD (p=0.098) and disease localization in UC (p=1.0).

Based on the result of *HhaI*/*MspI*-digested T-RF patterns, microbial diversity of IBD patients was reduced compared to that of healthy siblings and healthy unrelated controls (Table 3). Post-hoc analysis revealed the difference was significant only for IBD-patients vs. healthy siblings when estimated by *HhaI* digestion (p=0.011), and in IBD patients vs. healthy siblings and healthy controls (IBD vs healthy siblings, p=0.035; IBD vs. healthy controls, p=0.05) when estimated by *MspI* digestion. No significant difference in microbial diversity has been observed between healthy siblings and healthy controls.

Table 3. Comparison of fecal bacterial diversity between IBD patients, healthy sibings and healthy controls. The Shannon diversity index was calculated from the HhaI- and Mspi-digested T-RF patterns.

Shannon index	IBD (n=19)	Healthy siblings (n=20)	Healthy controls (n=19)	p-value
MspI digestion, mean (SD)	2.11 (0.12)	2.45 (0.85)	2.44 (0.74)	0.018**
HhaI digestion, mean (SD)	1.75 (0.12)	2.14 (0.81)	1.99 (0.66)	0.013*

Post-hoc analysis: *p<0.05 for IBD vs. healthy siblings, **p<0.05 for IBD vs. healthy siblings and IBD vs. healthy controls. IBD – inflammatory bowel disease

Supplemental Table 1 and Supplemental Table 2 show OTUs with significant changes after *HhaI* and *MspI* digestion. The relative abundance of 37 of 149 (24.8%, *MspI* digestion) and 27 of 169 (16.0%, *HhaI* digestion) OTUs differed significantly between the 3 groups. All

HhaI- and *MspI*-associated OTUs predicting the genus *Clostridium,* among others, were significantly decreased in IBD patients at the time of diagnosis compared to healthy siblings and healthy controls. There was no significant difference between OTUs predicting the genus *Clostridium* between healthy siblings and healthy controls. However, all mentioned OTUs were lower in healthy siblings compared to healthy controls and were approaching OTU values of IBD patients.

Some other *HhaI*- and *MspI*-associated OTU-s representing phylum Firmicutes which include bacteria from the genus *Paenibacillus, Bacillus, Lactobacillus, Blautia, Eubacterium, Roseburia* and *Ruminoccocus* were significantly reduced in IBD patients compared to healthy siblings and healthy controls (Supplemental tables 1 and 2). Although the difference was not significant, the same bacteria were lower in healthy siblings compared to healthy controls and were approaching values of IBD patients. Only one *MspI* associated OTU (128-bp *MspI* OTU) representing the genus *Citrobacter*, *Collinsella* and *Paenibacillus* differed significantly between healthy siblings and healthy unrelated controls, with healthy siblings having lower values, similarly to that in IBD patients.

On the contrary, the genus *Streptococcus*, *Lactococcus* and *Enterococcus* predicted by the *MspI*-associated 555-bp and 563-bp were significantly increased in IBD patients. The same has been noticed for the phylum Proteobacteria, represented by genus *Enterobacter*, *Citrobacter*, *Escherichia* and *Klebsiella* (495-bp *MspI* OTUs). For one *HhaI*-associated OTU (374-bp OTU) representing phylum Proteobacteria, abundance was significantly lower in patients with IBD.

DISCUSSION

In this study we have replicated previous findings that newly diagnosed pediatric patients with IBD have significantly less diverse microbial composition compared to healthy controls, but also to healthy siblings. Furthermore, lower abundance of the phylum Firmicutes and higher abundance of the phylum Proteobacteria was observed in IBD patients compared to healthy siblings and healthy controls, while no significant difference in microbiota composition was observed in healthy siblings and healthy controls. To our knowledge, this was the first study that compared microbiota of newly diagnosed, treatment-naive pediatric IBD patients with healthy pediatric siblings and healthy unrelated controls.

We identified six studies that have compared microbiota composition of IBD patients with that of healthy siblings/relatives (24-29). Of those, four have included healthy unrelated controls (24, 26, 29) and only three have included pediatric patients with IBD (25-27). Methodologies of these studies have differed by the type of IBD patients (both CD and UC patients, only CD patients), age of control group (healthy minor or adult siblings/relatives), disease activity (patients with active or inactive disease) and methods used for microbiota analysis (DNA extraction, sequencing methodology and data analysis).

Previous studies have consistently shown that *de novo* pediatric IBD is strongly associated with microbiota alterations (7). Microbial communities of new pediatric IBD patients could be differentiated with high accuracy from those of healthy unrelated controls (3, 41). In our study, dendrograms comparing the gut microbiota separated patients and controls into two major clusters. Almost all healthy siblings and healthy controls were included in one cluster, while about 55% or IBD patients were included in a second cluster. Remaining IBD patients were included in the same cluster with healthy siblings and healthy unrelated controls. In CD group of patients, all patients that were in cluster I (together with healthy siblings and controls) had L1 localization of the disease (ileal/ileocecal disease), while all CD patients with

colonic involvement were in cluster II. In a study by Andoh et al. (34) almost all healthy individuals were included in one cluster, while 74.6% of adult CD patients (active disease, remission-achieved, remission-maintained patients) were forming two separate clusters. Similarly, in a study by Ijaz et al. (26) the gut microbiota community structure (β-diversity) of pediatric CD patients was different to the microbiota of the unaffected adult relatives of CD children and adult healthy unrelated controls. Additionally, similar to our results, no difference in the gut microbiota community structure between the healthy relatives and healthy unrelated controls was seen (26).

Dysbiosis, besides reduced microbial diversity, involves also changes in abundances of potentially pathogenic and/or beneficial taxa (7, 42). Consistent with the results of other studies (24-29), in our study, microbial diversity of IBD patients was significantly lower compared to healthy siblings and healthy controls. Furthermore, abundance of specific taxa has been increased/reduced in IBD patients. In their review, Ni et al. (43) have reported that the phylum Firmicutes is often reduced in adult IBD patients (44-48). Our study confirmed these results and has shown lower abundance of the phylum Firmicutes in IBD patients, more specifically OTUs representing bacteria from the genus *Paenibacillus*, *Bacillus*, *Lactobacillus*, *Blautia*, *Eubacterium*, *Roseburia* and *Ruminoccocus.* OTUs representing phylum Proteobacteria have been increased in patients with IBD, observation which has also been reported previously (44, 49). Previously mentioned changes in the composition of the gut microbiota could lead to metabolite alterations, primarily reduction in amino acid biosynthesis and carbohydrate metabolism pathways and increase in expression of genes related to oxidative stress, that are likely to have a role in the IBD pathogenesis (43). However, there are studies in pediatric patients showing different results, implicating that the abovementioned pathogenesis may not be valid – at least not in pediatric patients (10, 13).

Comparing microbiota profiles of healthy siblings with that of healthy unrelated controls, we have not found significant differences neither in microbiota diversity, nor in specific bacteria genus, which is not in accordance with previous research findings (24, 25, 27). In our study, only one *MspI* associated OTU (representing genus *Citrobacter*, *Collinsella* and *Paenibacillus*) differed significantly between healthy siblings and healthy unrelated controls. However, some differences observed between healthy siblings and healthy controls were similar to the differences observed when comparing IBD-patients to healthy controls. More specifically, most OTUs representing bacteria from phylum Firmicutes, but not from phylum Proteobacteria observed in healthy siblings, differed from that of healthy controls and were approaching values observed in IBD patients. Those differences were not statistically significant, but we might have been underpowered to find a significant difference in our cohort. The former may indicate that, to some extent, dysbiosis does exists in the microbiota of healthy siblings of IBD patients, but only with regard to reduced 'beneficial' bacteria, which has been observed in other study as well (24). However, as speculated by Ijaz et al. (26) dysbiosis reported in paeditric IBD patients occurs to a much lower extent in their healthy genetically linked counterparts. Similarly, in a study by Joossens et al. (29) dysbiosis signature found in adult patients with CD was markedly characteristic for the disease as it was not observed in unaffected relatives. The described pattern of decrease in “beneficial” bacteria in microbiota of siblings could be attributed to shared genetics and environmental exposures in siblings. Even more so, shared exposures to different risk factors in early perinatal and postnatal life when the microbiota is developing and becoming stabilized could lead to permanent changes in microbiota, that could influence the risk of developing IBD later in life (18).

The main limitation of this study is small number of subjects, which is nevertheless, comparable to other studies. Furthermore, by using other sequencing methodology such as strain-level shotgun metagenomics with deep sequencing, we would have been able to provide

strain-level taxonomic classification. There are several strengths to our study. First, all controls, healthy unrelated and siblings, were younger than 18 years of age, ensuring that age was not a confounder in this cohort. Second, all the patients were treatment naïve and recruited at the time of diagnosis, which excludes the effect of treatment on the microbiota profiles.

In conclusion, significantly different microbiota composition is present already at diagnosis, in treatment naive patients with IBD. Although the conundrum on the causes and consequences cannot be resolved by our findings, we have also identified the differences in microbiota composition between healthy siblings and healthy unrelated controls, but the extent of them is small.

Supplemental Table 1. OTUs with significant changes (*MspI*-digestion).

OTU	Representative bacteria predicted by T-RF length	IBD-patients (n=19)	Healthy siblings (n=20)	Healthy controls (n=19)	p-values
128	*Citrobacter* *Collinsella* *Paenibacillus*	2.19±1.57	1.99±0.6	4.16±0.85	0.001**
139	*Paenibacillus*	0.36 ± 0.16	2.04 ± 0.48	2.28 ± 0.48	0.000*
142	*Bacillus* *Paenibacillus*	1.05±0.36	2.1±0.47	3.61±0.95	0.004*
190	*Lactobacillus*	1.20 ± 0.89	2.39 ± 0.61	3.38 ± 0.65	0.000*
458	*Clostridium*	0.77±0.29	2.69±0.86	4.14±0.98	0.005*
495	*Enterobacter* *Citrobacter* *Escherichia* *Klebsiella*	16.61±5.24	0.85±0.55	3.04±2.10	0.000*
521	*Clostridium*	0.31±0.15	1.16±0.30	1.37±0.41	0.003*
555	*Streptococcus* *Lactococcus* *Bacillus*	9.38 ± 4.24	3.58 ± 1.11	2.01 ± 0.89	0.022***
563	*Streptococcus* *Enterococcus* *Lactobacillus*	4.63 ± 2.50	0.54 ± 0.53	0.00 ± 0.00	0.000*

Each value indicates the percentage of individual operational taxonomic unis (OTU) area per total OTU area after Mspl digestion. Values are expressed as means ± SD.

Post hoc analysis: *p<0.05 for IBD vs. healthy siblings and IBD vs. healthy controls; **p<0.05 for IBD vs. healthy controls and healthy siblings vs. healthy controls, *** p<0.05 for IBD vs. healthy controls.

Supplemental Table 2. OTUs with significant changes (*HhaI*-digestion).

OTU	Representative bacteria predicted by T-RF length	IBD patients (n=19)	Healthy siblings (n=20)	Healthy controls (n=19)	p-values
189	*Clostridium* *Blautia* *Eubacterium* *Roseburia* *Ruminococcus*	0.76± 0.31	9.83± 2.62	13.27 ± 2.94	0.000*
225	*Lactobacillus* *Clostridium* *Bacillus* *Paenibacillus*	2.51± 1.04	3.99± 0.88	2.15± 0.45	0.014*
230	*Clostridium* *Bacillus*	0.23± 0.13	0.93± 0.33	1.27± 0.47	0.013**
374	*Citrobacter* *Enterobacter* *Escherichia* *Klebsiella*	2.34± 2.03	4.59± 1.85	7.32± 2.48	0.001*

Each value indicates the percentage of individual operational taxonomic unis (OTU) area per total OTU area after HhaI digestion. Values are expressed as means ± SD.

Post hoc analysis: *$p<0.05$ for IBD vs. healthy siblings and IBD vs. healthy controls;

**$p<0.05$ for IBD vs. healthy controls.

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Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Study was approved by Ethics Committee Children's Hospital Zagreb (IRB number: 21102014).

Informed consent: Informed consent was obtained from all individual participants and at least one of their parents included in the study.

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Paper 3

Characteristics of polymeric formula and route of delivery of exclusive enteral nutrition have no effect on disease outcome and weight gain in pediatric Crohn's disease

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Characteristics of polymeric formula and route of delivery of exclusive enteral nutrition have no effect on disease outcome and weight gain in pediatric Crohn's disease

Iva Hojsak[a,b,c], Karlo Matic[b], Sara Sila[a], Ivana Trivić[a], Zrinjka Mišak[a,b], Sanja Kolaček[a,b]

Affiliation:

[a]Referral Center for Pediatric Gastroenterology and Nutrition, Children's Hospital Zagreb, Zagreb, Croatia

[b]University of Zagreb, School of Medicine, Zagreb, Croatia

[c]University J.J. Strossmayer, School of Medicine, Osijek, Croatia

Corresponding author:

Iva Hojsak, MD, PhD

Referral Center for Pediatric Gastroenterology and Nutrition,

Children's Hospital Zagreb

Klaićeva 16, Zagreb, Croatia

Phone:+385-1-4600130, Fax: +385-1-4600160

e-mail: ivahojsak@gmail.com

ABSTRACT

Background and aims: This study aimed to evaluate the effect of the route of exclusive enteral nutrition (EEN) delivery (orally or via nasogastric (NG) tube) and type of polymeric formula (with taste vs tasteless and isocaloric vs hypercaloric) on the disease outcome and nutritional status in children with Crohn's disease (CD).

Methods: This was a single center retrospective study which included all CD patients whose active disease at diagnosis was treated with EEN in the period from October 2007 to November 2017. All patients received polymeric formula orally or through a NG tube, which was based on the physicians and child's preference.

Results: A total of 92 CD patients were included in the study (mean age 13.6±3.0 years; 45.7% female). Overall, 42 (45.7%) patients received EEN via NG tube until the end of the EEN period. Remission was achieved in 71 (77.2%) children. There was no difference in the EEN failure status, remission duration, inflammatory markers, and weight gain at the end of the EEN period between oral intake and NG tube groups.

None of the factors including age, disease location, type of formula (with taste vs tasteless and isocaloric vs hypercaloric) and mode of delivery (orally vs through NG tube for the whole duration of EEN) demonstrated an association with EEN failure.

Conclusion: This study failed to demonstrate an effect of the route of EEN delivery and the characteristics of the polymeric formula on the outcome of treatment in pediatric patients with CD.

Key words: Crohn's disease; enteral nutrition; naso-gastric tube; polymeric feeds;

INTRODUCTION

Crohn's disease (CD) is a lifelong, chronic inflammatory disease mainly involving the gastrointestinal tract which is characterized by periods of remission and relapses (1). It has been known for years that exclusive enteral nutrition (EEN) is efficacious in inducing remission, along with improving nutritional status in children with CD (2). The overall combined remission rate for EEN is 73% (relative risk (RR) 0.95, 95% confidence interval (CI) 0.67–1.3428) (3) and has the same efficacy in the induction of remission as corticosteroids, but with much less side effects (4). EEN is, therefore, recommended as a first line therapy for remission induction in children/adolescents with active luminal CD (5). There are studies demonstrating that the protein source does not affect the efficacy of EEN (6-8), therefore, polymeric formula is preferred due to the better palatability and the lower costs (4, 9).

The recently published position paper of the European Society of Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) recommends that EEN should comprise of polymeric formula taken orally, while nasogastric (NG) tube may be used when there is failure to achieve adequate oral intake (4). However, it was recognized that there is no strong evidence whether the route of delivery and characteristics of polymeric formula have an influence on disease course and weight gain in children with CD.

Therefore, this study aimed to evaluate whether the treatment outcome and nutritional status differ in respect to the route of EEN delivery (orally or via NG tube) and characteristics of polymeric formula (with taste *vs* tasteless and isocaloric *vs* hypercaloric).

MATERIALS AND METHODS

We retrospectively analyzed data on all children who were diagnosed with CD and followed up for at least one year at the Children's Hospital Zagreb (tertiary medical center) from October 2007 to November 2017. Only children in whom EEN was used as a first remission induction therapy were included. All patients were seen and followed by 3 pediatric gastroenterologists, from which two preferentially prescribed EEN by oral route, while one preferred prescribing EEN by NG tube. Patient assignment to one of the senior pediatric gastroenterologists was arbitrary and determined only by the initial physician contact. EEN was given as a polymeric formula. All patients who received EEN via NG tube received standard, isocaloric, flavorless enteral formula (Osmolite®, Abbott), while patients receiving EEN orally usually received palatable oral enteral formula that was either isocaloric (PediaSure®, Abbott or Modulen®, Nestle) or hypercaloric (1.5 kcal/ml) (Ensure Plus®, Abbott or Resource

Junior®, Nestle). The choice of formula depended on the taste preference of the child/adolescent.

The caloric requirements were calculated for every specific patient and the optimal volume of EEN was determined. EEN was initiated during hospital stay and the amount of formula was step-wisely increased over a period of 2 to 5 days. Patients were advised to distribute enteral formula into 4-5 even bolus feeds per day, but the method of intake was left to their discretion. EEN was given over a period of 6 to 8 weeks as induction therapy and was only prematurely ceased if the therapy was not tolerated or if remission was not achieved.

Methodological review of each chart was performed, and the following data was extracted: type of the disease, age, gender, weight and height for age standard deviation score (SDS) and body mass index (BMI) SDS, inflammatory markers, disease location, type of EEN, route of EEN delivery, and maintenance treatment. All data was extracted from the diagnosis (the time of introduction of EEN) and during the follow up at the end of EEN therapy. The diagnosis of CD was established according to the revised Porto criteria (10), while disease location was defined using the Paris classification (11). Relapse was defined as a Pediatric CD Activity Index (PCDAI) score of >10 (12) and the need for other remission induction therapy and remission as PCDAI ≤10 (12). EEN failure was defined as the inability to reduce symptoms and normalize inflammatory markers during EEN use, along with the need for step up to corticosteroid therapy. Malnutrition was defined using World Health Organization (WHO) criteria and the BMI for age <−2 SDS was classified as undernutrition and BMI for age of >2 SDS as overweight (13).

As for the treatment, ECCO/ESPGHAN guidelines were followed whenever applicable (5).

The primary outcome was to assess whether the route of EEN delivery (oral or via NG tube) had an effect on EEN failure and remission duration. The secondary outcomes were to assess whether route of EEN delivery (oral or via NG tube) had an impact on weight gain, need for escalation in maintenance treatment, and the need for corticosteroid use in the first year of diagnosis. Furthermore, it was assessed whether characteristics of polymeric formula (isocaloric vs hypercaloric and with taste vs tasteless), route of EEN delivery (orally vs NG tube), volume of EEN, and disease location have an influence on EEN failure.

Statistics. The differences between categorical variables were assessed by chi-square test. The differences for non-categorical variables were assessed by two tailed Student t test for independent samples. Binary logistic regression model was used to assess characteristics of

polymeric formula (isocaloric vs hypercaloric and with taste vs tasteless), mode of EEN delivery (orally vs NG tube), volume of EEN, and disease location have an influence on EEN failure. P values less than 0.05 were considered significant. Statistical analysis was performed using SPSS 19.0 (Chicago, IL) statistical software.

RESULTS

A total of 92 CD patients were included in the study, majority were male (50, 54.3%). Epidemiological data at the diagnosis is presented in Table 1. Majority of patients (n=70, 76%) were well nourished at diagnosis, 19 (20.7%) were undernourished and 3 (3.3%) were overweight.

Table 1. Epidemiological data at diagnosis; SD – standard deviation;

Age (mean ±SD, years)	13.6±3.0
Gender, female (%)	42 (45.7)
Duration of symptoms before diagnosis (mean ±SD, months)	4.6±4
Weight for age Z score (mean ±SD)	-0.8±1.1
Height for age Z score (mean ±SD)	-0.5±1.0
BMI for age Z score (mean ±SD)	-0.6±1.4
Disease location (n, %) L1 L2 L3	 20 (21.7) 18 (19.6) 54 (58.7)
Upper gastrointestinal disease (n, %)	40 (43.5)
Perianal disease (n, %)	13 (14.1)

A total of 71 (77.2%) children treated with EEN achieved remission. Overall, 44 (47.8%) patients had a NG tube for the provision of EEN at the beginning of EEN, but 2 patients reverted to oral intake, leaving 42 (45.7%) patients that received EEN via NG tube until the end of the EEN period. None of the patients receiving oral EEN switched to a NG tube. Overall, 42 (45.7%) patients received formula with taste, from which 12 patients received a hypercaloric

formula (1.5 kcal/ml), 30 patients received an isocaloric formula (1 kcal/ml), and 50 received a standard isocaloric, tasteless, polymeric formula.

There was no significant difference in the group receiving EEN orally vs via NG tube in gender, age, disease location, presence of upper gastrointestinal or perianal disease, provided energy, weight gain, laboratory indices and concomitant therapy (Table 2).

Table 2. Difference between patients treated with exclusive enteral nutrition (EEN) orally and via nasogastric (NG) tube at diagnosis.

	Oral (n=50)	NG tube (n=42)	p
Sex (female/male)	20/30	22/20	0.295
Age (mean ± SD, years)	13.5 ± 3.3	13.4 ± 2.7	0. 968
Location (n, %) L1 L2 L3	 12 (24) 8 (16) 30 (60)	 8 (19.1) 10 (23.8) 24 (57.1)	0.606
Upper gastrointestinal disease (n, %)	26 (52)	14 (33.3)	0.092
Perianal disease (n, %)	4 (8)	9 (21.4)	0.078
Duration of EEN (mean ± SD, weeks)	5.2 ± 2.5	5.4 ± 2.4	0.46
Energy provided by EEN (kcal/kg, mean ± SD)	47.7 ± 1.96	51.6 ± 1.93	0.166
CRP change after EEN (mg/L, mean ± SD)	-21.6 ± 5.8	-36.3 ± 6.8	0.1
Hemoglobin change after EEN (g/L, mean ± SD)	1.5 ± 1.4	2.8 ± 3.3	0.707
Weight change after EEN (kg, mean ± SD)	0.1 ± 2.8	0.4 ± 2.8	0.252
BMI change after EEN (kg/m^2, mean ± SD)	-0.1± 1.0	0.1 ± 1.1	0.553

Concomitant maintenance therapy (n, %) None Azathioprine Methotrexate Mesalamine	 7 (14) 36 (72) 1 (2) 6 (12)	 3 (7.1) 28 (66.7) 1 (2.4) 10 (23.8)	0.403
EEN failure (n, %)	12 (24)	9 (21.4)	0.808
Number of the relapses in the first year	0.86 ± 0.12	0.86 ± 0.14	1.0
Need for corticosteroids in the first year from diagnosis (n, %)	8 (16)	11 (26.2)	0.303
Duration of remission after EEN (time to first relapse, months) (mean ± SD)	12.3 ± 2.3	12.9 ± 3.1	0.877
Need for escalation in the maintenance treatment in the first year (n, %)	28 (56)	27 (64.3)	0.523

BMI – body mass index, CRP - C reactive protein, SD – standard deviation.

Binary logistic regression multivariable analysis failed to connect any of the assessed factors, including age, disease location, characteristics of the formula (with taste vs tasteless and isocaloric vs hypercaloric), and mode of delivery (orally vs trough NG tube for the whole duration of EEN) with EEN failure (Table 3).

Table 3. Risk factors at diagnosis evaluated with respect to exclusive enteral nutrition (EEN) failure. Binary logistic regression multivariable model; NG- nasogastric.

	HR	95% CI
Age	1.011	0.742-1.378
Disease location L1 L2	 0.254 1.518	 0.045-1.429 0.423-5.449
Enteral formula with taste	0.412	0.086-1.960
Hypercaloric (1.5 kcal/ml) enteral formula	2.5	0.377-16.588

NG tube for the duration of EEN	1.001	0.286-3.504
Energy intake via EEN (kcal/kg body weight)	0.964	0.907-1.025

DISCUSSION

This retrospective study suggests that the route of delivery of EEN, as well as the characteristics of the polymeric formula, have no influence on its effectiveness in remission induction, disease course, and nutritional status. Current guidelines recommend EEN as a first line therapy for luminal CD (4, 5). EEN has several beneficious effects. It induces remission, decreases mucosal inflammation, and promotes weight gain (14-23). The mechanisms of action for EEN are not yet fully elucidated, but include suppression of proinflammatory mediators in the intestinal mucosa (15, 19, 24), reduced production of IL-1, IL-6, IL-8, and TNF-alpha (25), and a significant impact on intestinal microbiota (26).

A recent meta-analysis (27) confirmed the effectiveness of EEN in the pediatric population, contrary to adult patients. The reason as to why EEN is very effective in pediatric patients is not completely clear. It could be partially explained by the lower tolerance and compliance to EEN in adults, which is mainly related to the necessity for adults to wear a NG tube (27).

Since EEN was introduced as a treatment strategy for children with CD, many different protocols were evaluated, including the composition of enteral formula, mode of EEN delivery, as well as duration of EEN. For some of the choices, there is reasonable evidence guiding the clinical practice. First off, it was shown that there is no additional benefit if protein in the formula was hydrolyzed (6-8). Therefore, current guidelines recommend the use of polymeric formula (5). Otherwise, guidelines prefer an oral route of administration. The main reason why oral intake is preferred in children and adolescents with CD, is the psychological impact linked to the wearing of a NG tube (28). However, the recommendation to prefer oral intake is almost solely based on expert opinions, as worldwide practices differ. There is only one retrospective pediatric study which compared oral to continuous EEN via NG tube (20). That study found no difference in remission induction, PCDAI, as well as the individual clinical and biological parameters between groups at the end of an EEN period of 8 weeks. The only significant difference found was in weight gain, which was significantly higher in the continuous EEN group. Our study provided bolus feeding via oral route or via NG tube, but failed to show any difference in the outcomes, including weight gain. The results involving weight gain was of a much lower proportion in our study compared to a study reported by Rubio et al (20), but the

weight for age Z score at diagnosis in our cohort was significantly higher compared to the above mentioned study (mean value of -1.2 and -1.3 SD in Rubio et al study vs -0.8 SD in this study). The difference in weight gain could be partially explained by the fact that patients in our study received EEN as a bolus treatment. Therefore, regardless of whether they had a NG tube or oral EEN, they could decrease the volume of EEN per day without revealing this to their physician. On the contrary, the study by Rubio et al, provided EEN via NG tube, but as a continuous feed delivered by volumetric enteral pump. The volume was predefined and therefore less prone to the patient's influence.

In our study, EEN was able to induce remission in 77% of patients, which is similar to previous reports (29, 30). None of the investigated factors related to EEN (route, characteristics of the formula or volume of EEN), disease location and age were associated with treatment failure.

There are several limitations to our study. It is a single-center retrospective study and therefore, patients were not randomized, but rather inclusion into the group was dependent solely on the recommendation of the physician and acceptance of the child/adolescent and their family. Furthermore, caloric intake that was actually taken could not be measured. The total duration of EEN also varied and therefore, nutritional status was not measured in the same time points for all patients. Finally, it is important to emphasize that this study was initiated due to an observation made in several patients who limited their oral bolus intake to the extent of deterioration of their nutritional status. The main reason for this was the inability to drink the prescribed/required amount of the EEN.

However, regardless of the limitations, to our knowledge, this is the first study that looked at the role of EEN route on disease outcomes. In conclusion, our results support the current recommendation that oral intake should be first choice together with palatable enteral formula.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional ethics committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.
Study was approved by Ethics Committee Children's Hospital Zagreb (IRB number: 21102014).

Informed consent: Informed consent was obtained from all individual participants and at least one of their parents included in the study.

Conflict of interest statement: Iva Hojsak received honorarium for lectures or consultation for BioGaia, Nutricia, Nestle, GM pharma, Chr Hansen. Sanja Kolaček received lecture fees from Abbott, Abbvie, Fresenius, Nestle, Shire, Nutricia. Other authors have stated that they have no conflict of interest.

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Paper 4

Healthy siblings of children with Crohn's disease exhibit more rapid changes in microbiota composition as a response to exclusive enteral nutrition

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Healthy siblings of children with crohn's disease exhibit more rapid changes in microbiota composition as a response to exclusive enteral nutrition

Sara Sila, MSc[1], Marko Jelić, PhD[2], Ivana Trivić, MD[1], Arjana Tambić Andrašević, MD, PhD[2,3], Sanja Kolaček, MD, PhD[1,5*], Iva Hojsak, MD, PhD[1,4,5*]

Affiliation:

[1]Children's Hospital Zagreb, Zagreb, Croatia

[2]University Hospital for Infectious Diseases, Zagreb, Croatia

[3] University of Zagreb, School of Dental Medicine, Zagreb, Croatia

[4]University J.J. Strossmayer, School of Medicine Osijek, Osijek, Croatia

[5]University of Zagreb, School of Medicine, Zagreb, Croatia

[*]Authors contributed equally and share last authorship

Corresponding author

Assistant Professor Iva Hojsak, MD, PhD,

Referral center for pediatric gastroenterology and nutrition

Children's Hospital Zagreb

Klaićeva 16, 10000 Zagreb, Croatia

Phone: +38514600130; Fax: +38514600160

e-mail: ivahojsak@gmail.com

ABSTRACT

Background: The impact of exclusive enteral nutrition (EEN) on the microbiota of healthy siblings of children with Crohn's disease (CD) is not known. The aim of this study was to determine the impact of EEN on the microbiota composition of the newly-diagnosed CD patients and to determine the effect of EEN received for two days in siblings of patients with CD.

Materials and Methods: Newly-diagnosed paediatric CD patients (n=17) and unaffected healthy siblings (n=10) participated in the study. In CD patients stool samples were collected at three time points: prior to therapy introduction, the second day and the last day of EEN therapy. In healthy siblings stool samples were collected before the introduction of EEN and the second day of EEN. Molecular approach targeting 16S ribosomal RNA was employed for analyzing the gut microbiota of participants' stool samples.

Results: There was no significant difference in microbial diversity between children with CD and healthy siblings before EEN (p=0.127 for *HhaI*-digestion; p=0.604 for *MspI*-digestion) as opposed to the second day of EEN (p=0.006 HhaI-digestion; p=0.023 MspI-digestion). In healthy controls, significant changes in microbiota composition were apparent already on the second day of EEN, contrary to children with CD in whom similar changes in microbiota composition were apparent on the last day of EEN.

Conclusion: EEN leads to significant microbiota changes in both healthy and children with CD. Changes in microbiota composition occur more rapidly in healthy children, while in children with CD significant changes were detected at the end of EEN.

Keywords: Crohn's disease, microbiota, exclusive enteral nutrition

CLINICAL RELEVANCY STATEMENT

Studies have shown that exclusive enteral nutrition (EEN) causes significant changes in the microbiota composition of patients with Crohn's disease (CD). However, no study to date explored the impact of EEN on the microbiota of healthy children. Therefore, it is not known if EEN would have the same effect on the microbiota of healthy individuals, and, more specifically, on the microbiota of genetically related individuals. Studying microbiota of healthy siblings of children with CD would give new insights into the mechanism of action of EEN in children with CD.

INTRODUCTION

The etiopathogenesis of Crohn's disease (CD) is unclear, however, it has been proposed that environmental, genetic and immune factors are interacting and triggering abnormal immune response to gut microbiota/content in genetically predisposed individuals[1]. It could be hypothesized, therefore, that gut microbial content has an important role in the development of CD, particularly as it has already been shown that patients with CD have different microbiota composition compared to healthy controls [2]. However, whether this is a cause or a consequence of the disease remains speculative. With the diet being one of the most prominent environmental factors associated with changes in the gut microbiota, strong emphasis in the recent years has been put on the influence of diet on the microbiota of CD patients [3].

The best evidence on the efficacy of a dietary intervention in treatment of CD is available for exclusive enteral nutrition (EEN) [4]. EEN administered for 6-8 weeks is as effective as corticosteroid therapy [5,6], moreover it improves nutritional status, initiates mucosal healing, and, as such, is recommended as a first line treatment in the newly-diagnosed paediatric patients [7]. Although the mechanism of action of EEN has not been fully elucidated, studies have shown that EEN causes significant changes in the microbiota composition of patients with CD, which

may lead to modification of microbial-based gut inflammation and consequently – remission of the disease [4,8]. Current evidence supports the notion that CD is associated with community-level imbalances in gut microbiota, rather than presence or absence of certain bacterial species [9,10], therefore community level changes could have an influence on disease course.

Even though one would expect that EEN causes gut microbiota to change in a way that would more closely resemble the microbiota composition of heathy controls, studies have shown mixed results [4,8]. More specifically, studies have shown that EEN, at least initially, may increase microbial dysbiosis in patients with CD [11–14]. It was proposed by MacLellan et al. [8], that EEN perhaps disrupts established dysbiotic microbial communities, and allows for recolonization and formation of a „heathier" microbiota. However, no study to date explored the impact of EEN on the microbiota of healthy children. Therefore, it is not known if EEN would have the same effect on the microbiota of healthy individuals, and, more specifically, on the microbiota of genetically related individuals, sharing the same environment, of whom only one has developed the disease.

The aim of this study was to determine the impact of EEN on the microbiota of the newly-diagnosed CD patients, and to determine the effect of EEN in siblings of patients with CD who consent to receiving it for two days.

MATERIALS AND METHODS

Patients and study design

Newly diagnosed paediatric CD patients and their unaffected healthy siblings were recruited at the Referral Centre for Pediatric Gastroenterology and Nutrition at the Children's Hospital Zagreb. All parents and participants older than 9 years of age gave written informed consent. The diagnosis of inflammatory bowel disease (IBD) was established according to the revised Porto criteria [15], while disease location was defined using the Paris classification [16]. Severity of the disease was estimated by Pediatric Crohn's disease activity index (PCDAI) [17].

EEN failure was defined as the inability to reduce symptoms and normalize inflammatory markers during EEN use, along with the need for step up to corticosteroid therapy. Exclusion criteria in healthy siblings included unintentional weight loss in the last 6 months, changes in stool frequency or consistency or other symptoms suggestive of undiagnosed IBD.

In CD patients in whom the first line of treatment was EEN, stool samples were collected at three time points: prior to therapy introduction (in suspected patients, before the endoscopy was performed), the second day of EEN therapy (between 24 and 48 hours after starting EEN) and the last day of EEN therapy before the introduction of normal food (the last stool sample was not collected only in those patients who haven't finished the full course of EEN). The duration of EEN depended on the choice of the physician, and lasted from 6 to 8 weeks. In healthy siblings who accepted the invitation to participate in the study and who consented to taking EEN for two days, stool samples were collected at two time points: before the introduction of EEN (close to the time of diagnosis of CD in their siblings) and the second day of EEN (before the introduction of normal food). EEN was given as a polymeric formula. The choice of formula depended on the taste preference of the patient (in 6 children the formula used was Osmolite, Ensure + was used in 5 children, Pediasure was used in 4 children, Modulen IBD was used in 1 child and Resource Junior in 1 child). Healthy siblings used the same EEN formula as the one that was provided to the patient with CD (except in the case of Osmolite when another isocaloric formula was given to healthy siblings due to unpleasant taste). Quantity of consumed formula was recorded in all patients and healthy siblings.

All stool samples were stored in the hospital or at home at -20°C for a maximum of 24 hours, after which they were transferred in the cold packs in the interval of 15 min to the Department of Clinical Microbiology at the University Hospital for Infectious Diseases and stored at -80°C.

Additionally, for each CD patient, type of the disease, age, gender, weight and height for age standard deviation score (SDS) and body mass index (BMI) SDS, inflammatory markers, disease location, type of EEN, and maintenance treatment were collected at the diagnosis and at the end of EEN.

Total fecal DNA extraction, from ~150 mg of stool samples, was performed using Quick-DNATM Fecal/Soil Microbe Miniprep Kit (Zymo Research, USA) according to manufacturer's instructions.

PCR amplification and T-RFLP analysis

PCR amplification and terminal restriction fragment length polymorphism (T-RFLP) analysis were performed according to Andoh et al with slight modifications [18]. 6'-carboxyfluorescein (6-FAM) labeled 27-F (6-FAM-5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') primers (Thermo Fisher Scientific, USA) were used for the amplification of the 16S rRNA gene from the human fecal DNA [18]. The PCR amplification (20 ng of DNA) was performed in 50 µL reactions, in triplicates, according to previously described protocol[19]. Amplified DNA was purified using QIAquick PCR Purification Kit (Qiagen, Germany) and diluted in 50 µL of elution buffer.

HhaI and *MspI* enzymes were used for the restriction of amplified 16S rRNA genes [18]. 120 ng of purified PCR product was digested separately in 30 µL reaction volumes, using 1 µL of FastDigest *HhaI* and FastDigest *MspI* (Thermo Fisher Scientific, USA) at 37°C for one hour. Restriction products were purified by ethanol/ sodium acetate/EDTA precipitation and resuspended in 12 µL deionized formamide (Thermo Fisher Scientific, USA) to a final concentration of 10 ng/ µL [20]. 3 µL of restriction digest product (~36 ng) was mixed with 11 µL of deionized formamide and 0.5 µL of fourfold diluted GS2500ROX (Thermo Fisher Scientific, USA). The length of the terminal restriction fragments (T-RFs) was determined with

an ABI PRISM 310 genetic analyzer in GeneScan mode (20s injection time; 15 kV, and 60°C for 48 min for each sample) (Thermo Fisher Scientific, USA) [19].

Fragment sizes were estimated by using the Local Southern Method GeneMapper 3.7 software (Thermo Fisher Scientific, USA). T-RFs in the range of 50–810 bp with a peak height greater than 25 fluorescence units were included in the analysis. Alignment of T-RFs was performed by T-REX software (http://trex.biohpc.org/) [20]. Binning threshold of 2 bp was used for assignment of T-RFs to operational taxonomic units (OTUs) [18]. The OTUs were quantified as the percentage values of an individual OTU per total OTU area, and this was expressed as the % area of the underpeak curve (% AUC) [21].

Water suspension of equivalently mixed *E. coli, Lactobacillus* and *Staphylococcus aureus* bacterial cells was used as a positive control and Microbial DNA-Free Water (Qiagen, Germany) was used as a negative control for DNA extraction.

Assignment of OTUs to bacterial taxa was performed in silico using the web-based analysis tool (PAT+) provided by MiCA3 (http://mica.ibest.uidaho.edu/pat.php), based on the RDP (Ribosomal Database Project) release 10 16s rRNA gene database [22].

Statistics

The differences between categorical variables were assessed by chi-square test. The Kolmogorov–Smirnov test was applied to test whether the data have a normal distribution. As quantitative variables were not normally distributed difference between healthy controls and patients with CD were determined by Mann-Whitney U test. Repeated measures for CD patients were analyzed by Friedman test; post hoc analysis was performed by Wilcoxon test (with Bonferroni adjustment where p-values from the Wilcoxon tests were multiplied by the number of tests being carried out). Repeated measures for heathy siblings were analyzed by Wilcoxon test.

We have also performed a paired analysis between patients and their own siblings (only patients who had sibling that participated) using a paired Wilcoxon sign rank to assess the variation between the sample diversity.

The relative abundance of OTUs was used to calculate Shannon-Wiener diversity index in order to compare diversity between different sample groups. Cluster analyses were performed using BioNumerics software (Applied Maths, Belgium) based on the *HhaI* or *MspI* T-RFLP patterns. Due to high number of variables in OTU comparisons, p values were adjusted for multiple comparison (based on two groups and 3 comparisons in time, p value was decreased by 5 times), values less than 0.01 were considered significant.

Difference in abundance of OTUs between patients and their sibling pairs was analyzed with permutational multivariate analysis of variance (PERMANOVA) taking sampling time into account (fixed effect, using type III sum of squares and unrestricted permutation of data with 999 permutations). Data were transformed (square root), Bray-Curtis measure was used to assess dissimilarities and centroids were determined for every group (patients and controls) and sample time point (baseline, day 2 and day 42). Principal coordinates ordination analysis (PCO) was used to visually present dissimilarities between conditions (patients and controls) at each sampling time (baseline, day 2 and day 42).

Statistical analysis was performed using SPSS 23.0 (Chicago, IL) and Primer 7 software (Auckland, New Zealand).

Study was approved by Ethics Committee of the Children's Hospital Zagreb (IRB number: 21102014).

RESULTS

Seventeen newly-diagnosed children with CD and 10 of their healthy siblings participated in the study. Baseline characteristics of study participants are shown in Table 1. Ten of the children with CD started with concomitant azathioprine treatment during EEN (at

the 3rd week of EEN treatment). The average quantity of formula in patients was 1900 ± 316.23 ml and in healthy siblings 1890 ± 433.21 ml per day (p=0.824).

In total, 48 samples were collected from children with CD. In 14 patients all 3 stool samples were collected, while the last sample at the end of EEN was not collected in 3 patients who failed EEN before the end of an EEN treatment. In 3 patients who have failed EEN before the end of EEN course, pre-treatment sample and 2nd day of EEN sample were included into the analysis. In two patients who have finished the whole course of EEN, but were not in full remission by the end of EEN, stool samples on the last day of EEN were collected and included into the analysis. On average, PCDAI decreased by 15.15 points (p=0.001) and remission was achieved in 70.6% of patients using EEN.

Ten healthy siblings participated in the study (epidemiological data presented in Table 1). In total, 20 samples were collected from healthy controls, with all healthy controls providing two stool samples – before EEN and on the second day of EEN. Since not all patients have siblings which are under 18 years of age, the number of included siblings is lower than the number of CD patients.

Table 1. Demographic characteristics of participants. BMI body mass index; CD Crohn's disease; IQR – interquartile range (25th to 75th percentile); SD- standard deviation;

	CD (n=17)	Healthy siblings (n=10)	p-value
Male, n (%)	9 (52.9%)	3 (30.0%)	0.247
Age (years), mean (SD)	15.98 (1.46)	14.20 (3.02)	0.127
Body weight (z-score), median (min, max) [IQR]	-0.12 (-2.42, 1.36) [-0.42-0.19]	0.72 (-1.83, 1.84) [-0.31-0.96]	0.170
Body height (z-score), median (min, max) [IQR]	-0.33 (-1.4, 1.81) [-0.92-0.12]	0.17 (-1.68, 0.87) [-0.87-0.66]	0.414
BMI (z-score), median (min, max) [IQR]	0.24 (-3.59, 1.54) [-0.39-0.65]	0.55 (-1.39, 2.18) [0.22-1.11]	0.505
Duration of symptoms before diagnosis (months), median (min, max) [IQR]	3 (0, 19) [3-6]		
Duration of exclusive enteral nutrition (weeks), median (min, max) [IQR]	6 (2, 8) [6-6]		
Disease location, n (%) L1 – ileal L2 - colonic L3 - ileocolonic	 6 (35.29) 3 (17.65) 8 (47.10)		
Upper gastrointestinal disease, n (%)	5 (29.41)		
Perianal disease, n (%)	3 (17.65)		
PCDAI at the beginning, median (min, max) [IQR]	27.5 (15,60) [20-30]		
Calprotectin at the diagnosis (mg/kg), median (min, max) [IQR]	1415.5 (327, 3670) [907.25-2000]		
Calprotectin after 6 weeks of EEN (mg/kg), median (min, max) [IQR]	742 (23, 1374) [374.8-936]		

BMI, body mass index; CD, Crohn’s disease

Start of EEN

Difference in microbial diversity estimated by *HhaI/MspI*-digested T-RF patterns between all children with CD and healthy siblings at the start of EEN is presented in Figure 1.

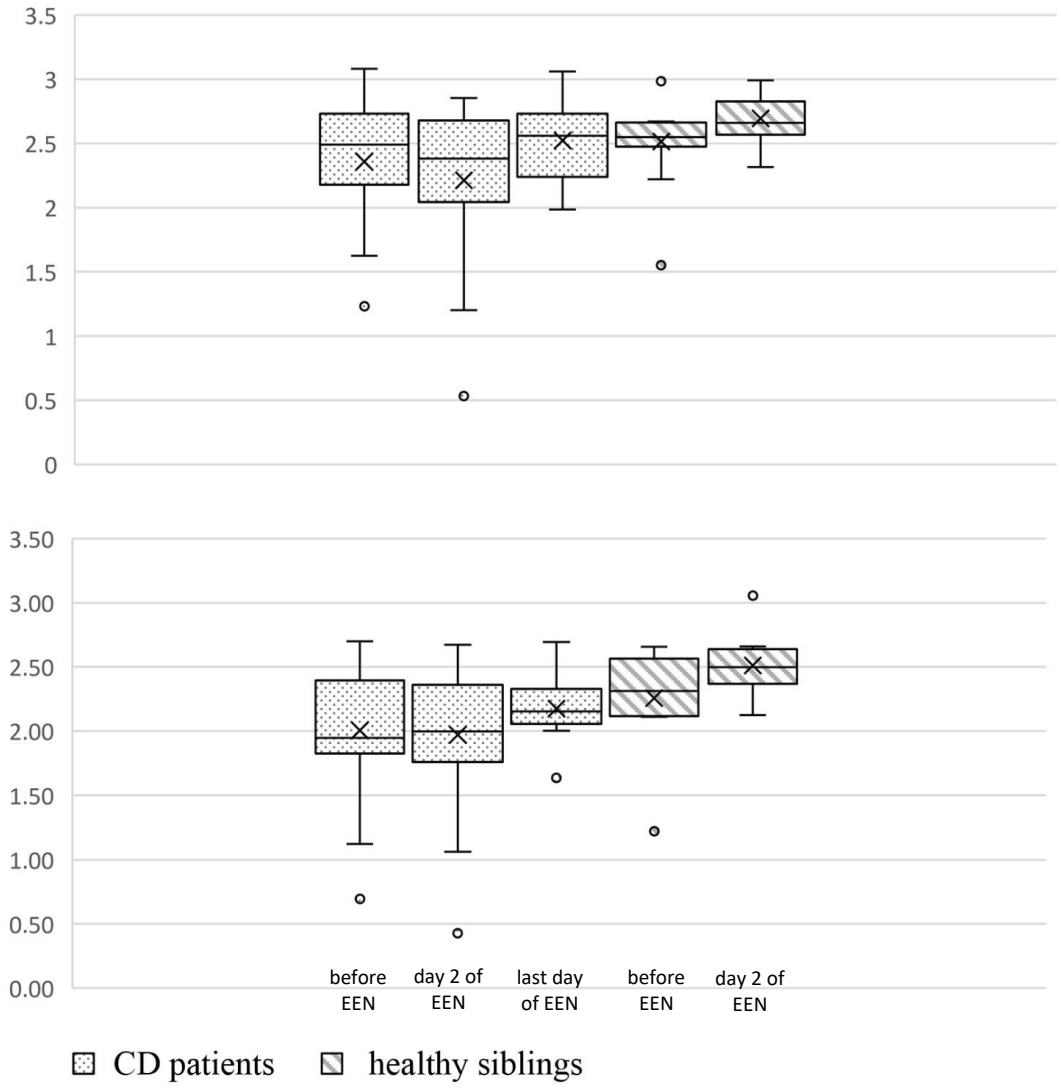

Figure 1. Shannon diversity index for each EEN sample time for a) *HhaI*-digestion and b) *MspI*-digestion. There was no difference in microbial diversity between healthy siblings and children with CD before EEN. There was a significant difference between the two groups on the second day of EEN. Diversity was not impacted during EEN in children with CD. In healthy siblings, there was a significant increase in microbial diversity on the second day of EEN for *HhaI*-digested TR-F patterns.

CD, Crohn's disease; EEN, exclusive enteral nutrition; T-RF, terminal restriction fragment

There was no significant difference in microbial diversity between children with CD and healthy siblings before EEN treatment (p=0.127 for *HhaI*-digestion; p=0.604 for *MspI*-digestion). Paired analysis (included only patients who had siblings that participated in the study) analyzing Shannon index before EEN showed no significant difference between patients and their siblings (p=0.374 for *HhaI*-digestion; p=0.314 for *MspI*-digestion). Figure 2a demonstrates the fecal microbiota profiles of patients with CD and healthy siblings before the start of EEN.

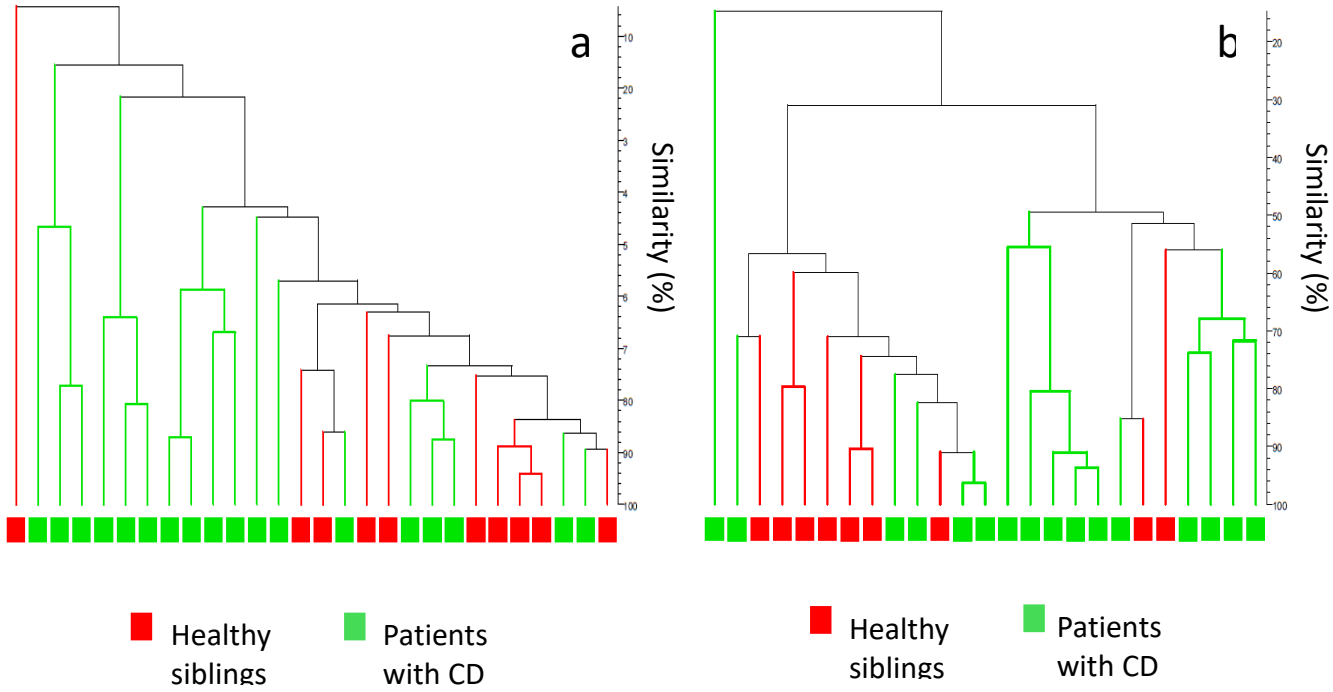

Figure 2. Dendrograms of the fecal microbiota profiles of CD patients and healthy siblings (a. before the introduction of EEN; b. the second day of EEN).

CD, Crohn's disease; EEN, exclusive enteral nutrition

Table 2 shows OTUs that differed significantly between children with CD compared to their siblings before EEN treatment. The relative abundance of 5 out of 191 (2.6%, *HhaI* digestion) and 2 out of 162 (1.2%, *MspI* digestion) OTUs differed significantly (with adjusted p value of ≤ 0.01) between the two groups before EEN treatment. Overall, OTUs predicting

bacteria from the genera *Clostridium, Blautia, Eubacterium, Bacillus and Lactobacillus,* were significantly lower in IBD patients compared to healthy siblings. On the contrary, OTUs predicting bacteria from the genera, *Desulfotomaculum, Burkholderiales and Streptococcus* were significantly higher in IBD patients compared to healthy siblings. Moreover, OTUs predicting bacteria from the genera *Roseburia, Ruminococcus, Fusobacterium* and C*oprobacillus* were lower, while bacteria from the genera *Alistipes, Enterobacter* and *Parabacteroides* were higher in abundance in children with CD compared to healthy siblings, however, those OTUs did not reach statistical significance. Generally, OTUs presenting bacteria from the phylum Firmicutes were lower in abundance in CD patients compared to healthy siblings.

Table 2. OTUs with significant differences before the start of EEN.

OTU	Representative bacteria predicted by T-RF length	Healthy siblings (n=10)	CD patients (n=17)	p-value
HhaI digestion				
65B	*Blautia, Clostridium, Eubacterium*	2.43 ± 2.45	0.51 ± 0.76	0.001*
171B	*Desulfotomaculum*	4.13 ± 6.28	0.86 ± 1.92	0.036
203B	*Eubacterium*	0.75±1.36	0.12±0.32	0.006*
206B	*Bacillus, Burkholderiales*	0.06±0.16	3.39±5.91	0.007*
214B	*Paenibacillus*	1.94±6.10	7.14±13.63	0.019
230B	*Clostridium, Bacillus*	1.24±1.45	0.32±1.12	0.005*
381B	*Desulfotomaculum, Firmicutes*	0.01±0.04	10.29±22.61	0.005*
MspI digestion				
102	*Bacillus*	0.08±0.19	0.00±0.00	0.019

146	*Bacillus, Clostridium, Paenibacillus, Staphylococcus*	0.39±0.70	0.05±0.11	0.049
190	*Clostridium, Desulfotomaculum, Lactobacillus*	3.35±3.59	1.01±2.79	0.001*
211	*Desulfotomaculum, Lachnospiraceae*	0.05±0.10	0.74±1.38	0.038
222	*Blautia, Clostridium, Desulfotomaculum, Eubacterium, Roseburia, Ruminococcus*	6.06±9.42	0.54±1.53	0.028
235	*Eubacterium*	0.10±0.10	0.02±0.05	0.021
268	*Fusobacterium*	4.83±9.77	1.05±2.98	0.033
485	*Clostridium, Eubacterium*	1.80±1.44	1.28±1.97	0.043
536	*Eubacterium*	0.08±0.12	0.82±1.02	0.043
540	*Coprobacillus*	0.57±0.61	0.17±0.33	0.022
561	*Streptococcus*	0.00±0.00	3.77±10.87	0.004*

CD, Crohn's disease; OTU, operational taxonomic units; T-RF, terminal restriction fragment

Each value indicates the absolute value of individual operational taxonomic units (OTU) area per total OTU area. Values are expressed as means ± SD at the order of magnitude 10^{-2}.

*Due to multiple comparisons p values ≤0.01 were considered significant.

Change in microbial diversity and composition during EEN

There was a significant difference in microbial diversity on the second day of EEN between children with CD and healthy controls (p=0.006 *HhaI*-digestion; p=0.023 *MspI*-

digestion; Figure 1). Difference in microbial diversity was still significant for both *HhaI*-digested (p=0.006) and *MspI*-digested (p=0.043) T-RF patterns when only children who achieved remission were included into the analysis. Paired analysis (included only patients who had siblings that participated in the study) analyzing Shannon index showed that patients after 2 days of EEN had lower index comparing to controls (median 1.96 (range 0.43-2.67) vs 2.42 (range 2.12-3.05); p=0.011 for *HhaI*-digestion; p=0.604 and median 2.42 (range 1.2 -2.79) vs 2.62 (range 2.31-2.99); p=0.051 for *MspI*-digestion). Figure 2b demonstrates the fecal microbiota profiles of patients with CD and healthy siblings on the second day of EEN, forming two major clusters.

As for microbial diversity change during course of EEN, there was no difference in microbial diversity in CD children between start, second day and the end of EEN (p=0.319 *HhaI*-digestion; p=0.257 *MspI*-digestion; Figure 1). When compared for EEN failure, there was no difference in microbial diversity before the start of EEN or on the second day of EEN between children who have failed EEN compared to children in whom EEN was successful. There was no difference in the microbial diversity between children in whom immunosuppressive (azathioprine) was introduced during treatment of EEN and children who did not receive immunosuppressive treatment. Moreover, sub-analysis showed that there was no association between EEN failure and different disease location groups (p=0.093), stenosis (p=0.870), fistula (p=0.506) and upper GI involvement (p=0.536).

To the contrary, median Shannon index in healthy siblings before EEN was significantly lower compared to day 2 of EEN for *HhaI*-digested T-RF patterns (p=0.047, Figure 1). No difference in microbial diversity was found for *MspI*-digested T-RF patterns.

PERMANOVA analysis showed significant difference in microbial communities between patients and their paired siblings in different time points for *HhaI*-digestion (df = 1, MS = 8722.1, pseudo F = 4.197, p = 0.001) and sampling time (df = 2, MS = 5027.1, pseudo F

= 2.419, p = 0.001) and for *MspI* – digestion for groups (df = 1, MS = 8034, pseudo F = 4.2537, p = 0.001) and sampling time (df = 2, MS = 8993.2, pseudo F = 2.3808, p = 0.002). Figures 3 and 4 represent PCO on the basis of dissimilarity matrices of OUTs for *HhaI* (Figure 3) and *MspI* digestion (Figure 4); there is a separation between groups (patients and their siblings) and sampling time on baseline and day 2, while sample on day 42 from CD group shows shift towards controls on days 2.

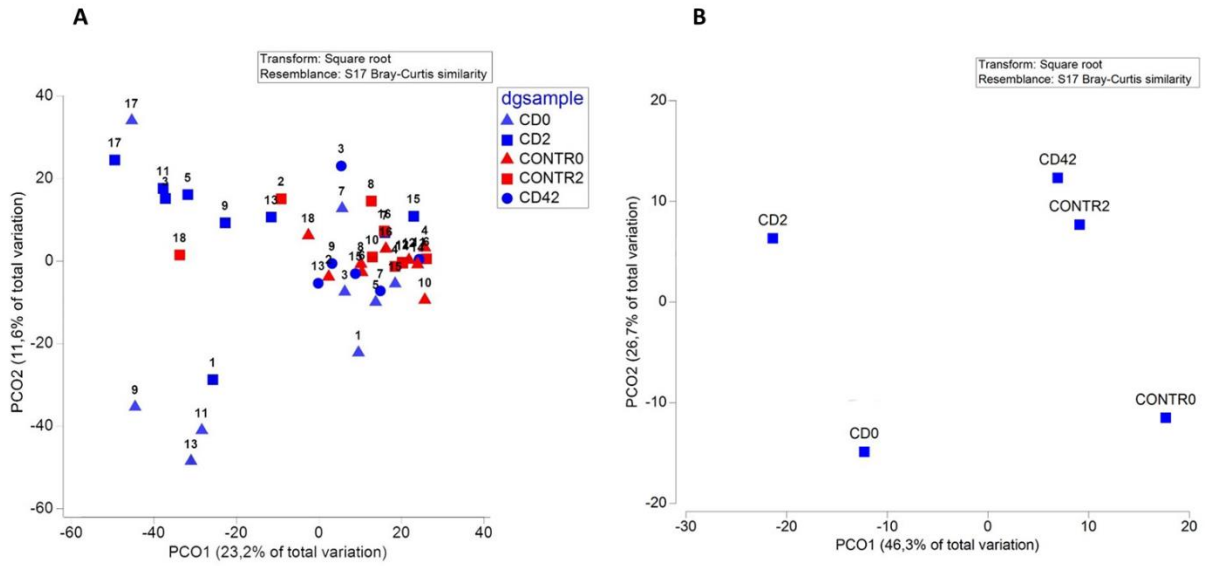

Figure 3. Principal coordinate ordination analysis (PCO) based on the OTU for *HhaI*-digestion. Each data point represents an individual sample. PCO was analyzed using Bray-Curtis distances (3a) and with centroids (3b). Color/shape is indicative of cohort and sampling time (baseline, day 2 and day 42) and each symbol is accompanied with specific number of the patient.

CD0 – Crohn's disease group at baseline; CD2 - Crohn's disease group on day 2; CD42 Crohn's disease group on day 42; CONTR0 – healthy siblings group at baseline; CONTR2 - healthy siblings group on day 2.

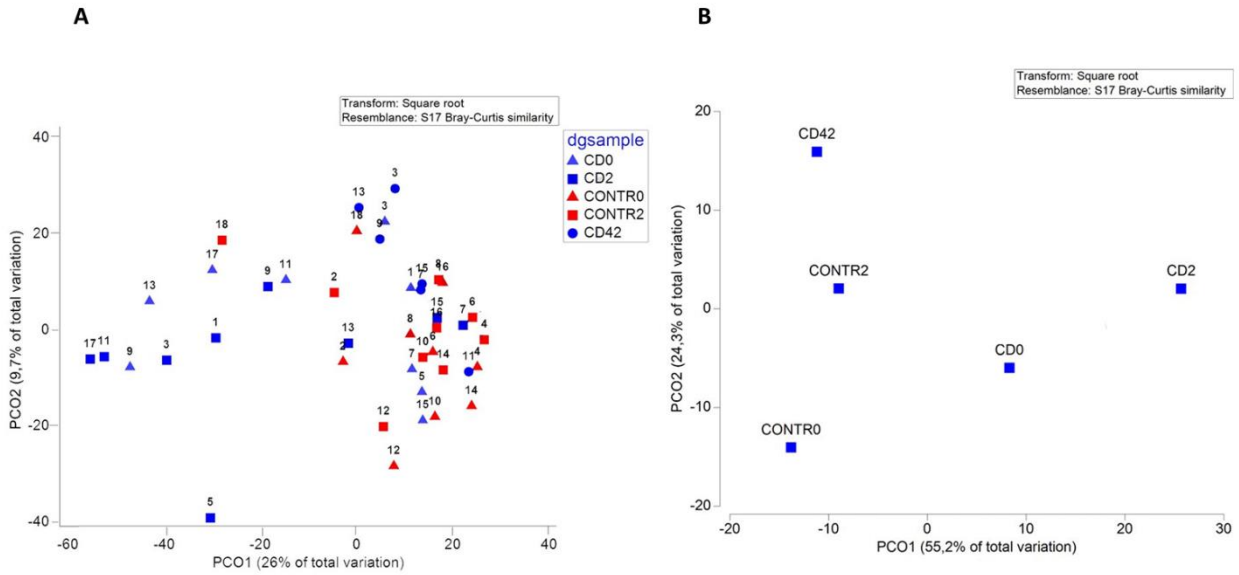

Figure 4. Principal coordinate ordination analysis (PCO) based on the OTU for *MspI*-digestion. Each data point represents an individual sample. PCO was analyzed using Bray-Curtis distances (3a) and with centroids (3b). Color/shape is indicative of cohort and sampling time (baseline, day 2 and day 42) and each symbol is accompanied with specific number of the patient.
CD0 – Crohn's disease group at baseline; CD2 - Crohn's disease group on day 2; CD42 Crohn's disease group on day 42; CONTR0 – healthy siblings group at baseline; CONTR2 - healthy siblings group on day 2.

There was a significant change (with adjusted p value of ≤0.01) in abundance of one out of 191 OTUs (0.5%, *HhaI*-digestion) and 3 out of 162 OTUs (1.8%, *MspI* – digestion) in CD children during the course of EEN (Table 3). OTUs representing bacteria from genera *Bacillus, Clostridium, Desulfotomaculum, Desulfovibrio Lactobacillus, Paenibacillus, Eggerthella and Olsenella* tended to increase during the course of EEN. Only OTU 485 (*MspI*-digestion) (representing bacteria from genera *Clostridium* and *Eubacterium)* showed significant decrease on the second and the last day of EEN compared to values before the start of EEN. In general, most OTUs representing bacteria from the phylum Frimicutes tended to increase during the

course of EEN, while only some representatives of the phylum Proteobacteria (*Citrobacter, Enterobacter, Escherichia* and *Klebsiella)* tended to decrease during the course of EEN, although we did not reach statistical significance for all OTUs. Moreover, post-hoc analysis revealed that most of the changes observed refer to the difference between the day 0 vs. the last day of EEN and day 2 vs. the last day of EEN.

Table 3. OTUs with significant changes in children with CD during EEN course.

OTU	Representative bacteria predicted by T-RF length	Before EEN (n=17)	2nd day of EEN (n=17)	Last day of EEN (n=14)	p-value
HhaI-digestion					
230	*Clostridium, Bacillus*	0.32±1.12	0.19±0.57	3.61±7.02	0.031
372	*Bifidobacterium, Citrobacter, Clostridium, Enterobacter, Escherichia, Klebsiella*	0.12±0.35	2.80±5.82	0.01±0.04	0.043
581	*Bacillus, Enterococcus, Lactococcus, Paenibacillus, Streptococcus*	4.51±8.49	0.01±0.03	0.08±0.32	0.020
812	*Bacillus, Clostridium, Desulfovibrio, Tannerella*	0.38±0.62	0.29±1.19	7.03±11.35	0.005
MspI-digestion					
102	*Bacillus*	0.00±0.00	0.01±0.03	0.02±0.04	0.018
133	*Bacillus, Bifidobacterium, Desulfovibrio, Eggerthella, Olsenella, Paenibacillus*	0.52±1.17	0.43±0.97	1.93±2.64	0.009**
162	*Bacillus, Clostridium, Desulfovibrio, Lachnospiraceae, Paenibacillus, Propionibacterium*	0.95±1.50	0.66±0.68	1.06±0.90	0.036
190	*Clostridium, Desulfotomaculum, Lactobacillus*	1.01±2.79	0.25±0.48	4.63±5.22	0.001**,***
214	*Desulfotomaculum*	0.00±0.00	0.06±0.18	0.37±0.57	0.012
286	*Desulfovibrio*	0.05±0.13	0.02±0.06	0.00±0.00	0.050
299	*Clostridiacease, Clostridiales*	0.29±0.81	0.89±3.48	5.27±8.30	0.020
302	*Clostridiales, Clostridium, Megasphaera, Veillonella*	0.88±1.91	1.03±2.96	0.78±2.50	0.012
432	*Clostridium*	0.02±0.08	0.09±0.15	0.02±0.07	0.029
457	*Clostridium*	1.70±3.70	0.48±1.00	4.30±4.99	0.017
485	*Clostridium, Eubacterium*	1.28±1.97	0.04±0.18	0.01±0.03	0.001*,**
515	*Clostridium*	0.00±0.00	2.25±5.64	1.02±3.84	0.023

557	*Bacillus, Lactococcus, Streptococcus*	6.69±16.97	0.00±0.00	0.00±0.00	0.050

CD, Crohn's disease; EEN, exclusive enteral nutrition; OTU, operational taxonomic units; T-RF, terminal restriction fragment

Each value indicates the absolute value of individual operational taxonomic units (OTU) area per total OTU area. Values are expressed as means ± SD at the order of magnitude 10^{-2}.

Due to multiple comparisons p values ≤0.01 were considered significant.

Post hoc analysis (Wilcoxon test with Bonferroni adjustment): *p<0.05 for day 0 vs day 2; **p<0.05 for day 0 vs the end of EEN, *** p<0.05 for day 2 vs the end of EEN

In healthy controls, changes in microbiota composition were already apparent on the second day of EEN (Table 4). Four OTUs changed significantly during the course of EEN in healthy siblings, with OTUs representing bacteria from genera *Bacillus*, *Desulfovibrio*, *Streptococcus* and *Staphylococcus* increasing in abundance, while bacteria from genera *Clostridium* and *Eubacterium* tended to decrease significantly during EEN course. Only OTU 485 (*MspI*-digestion) changed significantly in both healthy siblings and CD patients andshowed similar decreases in bacterial abundance in both children with CD and healthy siblings. Generally, abundance of different genera tended to increase for all but 3 OTUs presented in the Table 4, although statistical significance was not reached for all presented OTUs.

Table 4. OTUs with significant changes in healthy siblings during EEN course.

OTU	Representative bacteria predicted by T-RF length	Before EEN (n=10)	2nd day of EEN (n=10)	p-value
HhaI-digestion				
58	*Desulfovibrio*	0.06±0.12	1.68±2.28	0.008*
199	*Bacillus, Clostridium, Fusobacterium*	0.04±0.10	0.32±0.54	0.028
206	*Bacillus, Burkholderiales*	0.06±0.16	0.11±0.12	0.050
222	*Bacillus, Clostridium, Lactobacillus, Klebsiella, Enterococcus, Streptococcus*	0.01±0.04	0.15±0.20	0.043
241	*Bacillus, Paenibacillus, Clostridium*	0.01±0.05	0.12±0.15	0.012
372	*Bifidobacterium, Citrobacter, Clostridium, Enterobacter, Escherichia, Klebsiella*	0.88±2.05	2.94±3.20	0.017
578	*Bacillus, Streptococcus, Staphylococcus*	0.09±0.20	4.10±4.42	0.008*
581	*Bacillus, Enterococcus, Lactococcus, Paenibacillus, Streptococcus*	3.74±5.22	0.00±0.00	0.011
MspI-digestion				

86	*Alistipes, Desulfotomaculum, Enterobacter, Parabacteroides, Streptococus*	0.04±0.10	0.09±0.16	0.043
138	*Bacillus, Bifidobacterium, Desulfotomaculum, Eubacterium, Paenibacillus*	1.35±2.48	2.03±2.58	0.047
206	*Clostridium, Ruminococcus*	0.58±0.83	1.18±1.61	0.017
485	*Clostridium, Eubacterium*	1.80±1.44	0.01±0.02	0.005*
499	*Citrobacter*	0.00±0.00	5.09±10.99	0.043
511	*Clostridiaceae, Clostridium*	0.00±0.00	0.11±0.16	0.028
520	*Clostridiaceae, Clostridium, Eubacterium*	1.34±1.42	0.00±0.01	0.008*

EEN, exclusive enteral nutrition; OTU, operational taxonomic units; T-RF, terminal restriction fragment

Each value indicates the absolute value of individual operational taxonomic units (OTU) area per total OTU area. Values are expressed as means ± SD at the order of magnitude 10^{-2}.

*Due to multiple comparisons p values ≤0.01 were considered significant.

DISCUSSION

Our study investigated, for the first time, the differences in the stool microbial content in the response to EEN in children with CD compared to their healthy siblings, i.e. children having similar genetic background and sharing the same environment. The results obtained confirmed that healthy siblings exert significant changes in microbiota composition already on the second day of EEN, in contrast to children with CD where most of the changes were delayed and became apparent on the last day of EEN. Microbiota composition tended to change in the same direction in both children with CD and healthy siblings on EEN. Importantly, microbiota diversity increased significantly in healthy siblings on EEN, while no change in microbiota diversity was observed in children on EEN during the course of EEN.

Up to this day, most of the studies [11–14,23–28] have demonstrated significant changes in microbiota composition during EEN, indicating that CD is not associated with a single bacteria change, but rather with community-level imbalances in the gut microbiome [8]. It is considered that the mechanism of action of EEN involves exclusion of many dietary components which might have had a deleterious effect on intestinal permeability [4]. Therefore, different diets have been developed given the limitation of EEN on the quality of life of the patients due to its exclusivity, i.e. not allowing any additional food for the prolonged period (8-12 weeks) [29]. The

two most promising approaches include Crohn's disease exclusion diet (CDED) [30], and the CD-TREAT diet [31.]

In our cohort microbial communities of patients with CD couldn't have been differentiated from that of healthy siblings before the introduction of EEN, as presented by dendrogram (Figure 2). However, similar to what was previously demonstrated [2], we have confirmed significant difference in microbiota composition in children with CD compared to healthy controls, with overall lower relative abundance of the phylum Firmicutes [10]. In our cohort, we have shown that the relative abundance of the phylum Proteobacteria was higher in CD patients, however it was not statistically significant at the significance level 0.01. The loss of symbionts with anti-inflammatory properties [32] along with expansion of potentially pathogenic symbionts that can cause activation of the immune response and consequently cause inflammation [33] are considered to be the main factors in pathogenesis of CD. Therefore, the therapeutic effect of EEN was attributed to its effect on both the intestinal immune system and intestinal microbiota [4].

With that in mind, therapy that would result with less "dysbiotic" microbiota may lead to remission of disease in children with CD. In that regard, studies of limited number, have been conducted to investigate how EEN affects the microbiota composition of children [4,8]. Our results have shown a tendency towards the increase in relative abundance of phylum Firmicutes and, although not statistically significant, overall tendency of decrease in relative abundance of phylum Proteobacteria, similar to what was observed in a study by D'Argenio et al [23] and Schwerd et al. [27]. However, other studies have shown opposing results [11–14] and have suggested that EEN leads to even more dysbiotic state and have even suggested that reduction in relative abundance of families within the Firmicutes phylum correlated with clinical improvement [12]. Moreover, Lewis et. al [13] have demonstrated that there was a marked difference in microbiome composition between patients who ultimately responded to EEN and those who did not, with

those patients who have responded to treatment exhibiting microbiota profiles that were more similar to healthy controls. However, our study didn't confirm these results.

Recent studies have also focused on the difference in microbiota composition of children with CD compared to their healthy siblings who share both the genetic background and environmental exposures. Some studies have demonstrated that dysbiosis was present in siblings of children with CD compared to healthy controls [34,35], however, results were conflicting[36,37]. In our cohort we have recently shown that, while we did find the stool microbiota composition of IBD patients to significantly differ from that of healthy controls and healthy siblings, no significant difference in microbiota composition was observed between healthy siblings and healthy controls [10]. Interestingly, in this study, while we did find significant differences in microbiota composition of children with CD compared to healthy siblings, cluster analysis showed similar microbial community structures between the two groups (Figure 2).

In the present study we have identified, for the first time, gut microbiota changes in healthy siblings receiving EEN in comparison to patients with CD. A time period of two days of EEN was chosen as not to be overly demanding for otherwise healthy children and because limited data in the literature showed that intestinal microbiota changes are already evident one day after initiation of EEN [38]. We have found that EEN has caused significant changes in microbiota composition in both children with CD and healthy siblings. While microbial communities of patients with CD couldn't have been differentiated from that of healthy siblings before the introduction of EEN, on the second day of EEN two major clusters separating CD patients from healthy siblings were formed (Figure 2). Moreover, microbiota of healthy siblings responded "more rapidly" to EEN introduction, with significant changes being observed already on the second day of EEN (Table 4), as compared to children with CD where no significant differences have been observed on the second day of EEN (Table 3). However, by the last day of EEN, microbiota composition of children with CD was changed in a way to

resemble that of healthy siblings (Figures 3 and 4), with an overall statistically significant increase in the phylum Firmicutes and a tendency for a decrease of the phylum Proteobacteria.

Furthermore, significant increase in microbial diversity was already apparent on the second day of EEN in healthy siblings while diversity did not significantly change throughout the EEN course in children with the disease. Our speculation is that the slower change in microbiota composition in patients with CD are caused by persistent severe gut inflammation that could not be rapidly changed by day 2. However, it could be that "healthier" microbiota, present in healthy siblings, responds more rapidly and uniformly to the diet change, whereas CD patients exhibited divergent microbiome changes in response to EEN. Therefore, though our data could help in clarifying the role of different microbiota in CD patients, the major conundrum of whether it is the cause or the consequence of inflammation remains.

We are aware of the main limitation of this study, which is primarily a small number of subjects, which is, however, comparable to other published studies [11–14,23–28]. Moreover, the period of two days of EEN might not have been long enough to detect more profound changes in microbiota composition. Nonetheless, data in the literature have shown that intestinal microbiota changes already within one day after initiation of EEN [38] which was also confirmed by our results. Moreover, we do not know if changes in the microbiota of CD patients were apparent already before the end of EEN since we did not analyze stools between the day 2 and the last day of EEN. Finally, by using molecular approach targeting 16S ribosomal RNA gene we weren't able to detect strain-level taxonomic classification, which can make interpretation of the results challenging. However, an advantage of this methodology is hypothesis-free approach, non-selective of certain bacterial species. Important strengths of our study are in the unique design which included healthy siblings receiving EEN that was not investigated before, as well as the treatment naivety of the patients who were recruited at the time of diagnosis, which excludes the effect of treatment on the microbiota profiles.

CONCLUSION

This study demonstrated that EEN leads to significant microbiota changes in both healthy children and children with CD. Furthermore, changes seem to be more uniform and occur rapidly in healthy children, while in children with CD significant changes in the direction similar to those found in healthy siblings, were detected only 6 weeks after EEN introduction.

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CURRICULUM VITAE

Sara Sila, MSc, was born in Zagreb where she finished elementary and grammar school. She graduated at the Faculty of Food technology and biotechnology, University of Zagreb in 2016. Since 2016 she is working as an assistant on the project of Croatian Science Foundation named “Pediatric inflammatory bowel disease: incidence and natural history, and the role of diet and gut flora in etiopathogenesis” at the Children’s Hospital Zagreb. As a part of her assistant position, she has enrolled Postgraduate University Doctoral Study at the Faculty of Food Technology and Biotechnology, University of Zagreb.

During her work, she took part in various educational programs organized by the European Society for Pediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) in Croatia and abroad such as "Beyond Nutrients Nutrition School" in Italy, "Young Investigator Forum" in the Netherlands and "Allied Health Professionals Summers School "in Croatia. She was a project associate on 3 scientific projects and 5 educational and professional projects. She was the main author or a coauthor of 12 scientific papers. Of the mentioned papers, 11 are indexed in SCI-Expanded Base. She has attended 8 international congresses and on 6 of mentioned congresses she has presented her work as abstract presentation.