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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their Roman numerals (I–III):

I Simre K, Uibo O, Peet A, Tillmann V, Kool P, Hämäläinen AM, Härkönen T, Siljander H, Virtanen S, Ilonen J, Knip M, Uibo R; DIABIMMUNE Study Group. Exploring the risk factors for differences in the cumulative incidence of coeliac disease in two neighbouring countries: the prospective DIABIMMUNE study. Dig Liver Dis 2016; 48(11): 1296–301. doi: 10.1016/j.dld.2016.06.029.

II Simre K, Uibo O, Peet A, Puustinen L, Oikarainen S, Tamminen K, Blazevic V, Tillmann V, Hämäläinen AM, Härkönen T, Siljander H, Virtanen SM, Ilonen J, Hyöty H, Knip M, Uibo R; DIABIMMUNE Study Group. Early-life exposure to common virus infections did not differ between coeliac disease patients and controls. Acta Paediatr 2019; 108(9):1709–16. doi: 10.1111/apa.14791.

III Štšepetova J*, Simre K*, Tagoma A, Uibo O, Peet A, Siljander H, Tillmann V, Knip M, Mändar R, Uibo R. Maternal breast milk microbiota and immune markers in relation to subsequent development of celiac disease in offspring. Sci Rep 2022; 12(1):6607. doi: 10.1038/s41598-022-10679-x.
* Contributed equally.

Author's contribution:

Paper I: Collecting data from the DIABIMMUNE database, data analysis and writing the paper.

Papers II and III: Performing ELISA tests using serum samples (Paper II) or breast milk samples (Paper III), data analysis and writing the paper.

ABBREVIATIONS

AGA	anti-gliadin antibody
aOR	adjusted odds ratio
BC	birth cohort
CD	coeliac disease
CI	confidence interval
CMV	cytomegalovirus
DGGE	denaturing gradient gel electrophoresis
DGP	deamidated forms of gliadin peptide
EIU	enzyme immunoassay unit
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
EMA	endomysium autoantibody
ESPGHAN	The European Society for Paediatric Gastroenterology Hepatology and Nutrition
HLA	human leukocyte antigen
IEL	intraepithelial lymphocytes
IgA	immunoglobulin A
IgG	immunoglobulin G
IL	interleukin
NA	not available
OD	optical density
OR	odds ratio
PCoA	principal coordinate analysis
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	real-time reverse transcription–polymerase chain reaction
SD	standard deviation
sIgA	secretory immunoglobulin A
T1D	type 1 diabetes
TG2	transglutaminase 2
TGF-β1	transforming growth factor beta 1
tTG	tissue transglutaminase antibody
YCC	cohort of young children

1. INTRODUCTION

Coeliac disease (CD) is a dietary gluten and gluten-related prolamines induced immune-mediated systemic disorder that occurs in genetically predisposed people. It is characterized by variable clinical manifestations, CD-specific auto-antibodies, human leukocyte antigen (HLA) DQ2 and/or HLA-DQ8 haplotypes and enteropathy (Husby *et al.* 2012). Traditionally, the diagnosis of CD was based on CD-specific antibodies and biopsies from the small intestine to assess villous atrophy (Kelly *et al.* 2015). CD-specific antibodies include autoantibodies against autoantigens (tissue transglutaminase antibody (tTG) and endomysium autoantibody (EMA)) and antibodies targeting the offending agent (deamidated forms of gliadin peptide (DGP)).

In recent decades the prevalence of CD has increased worldwide (Kang *et al.* 2013; Makharia *et al.* 2022). Now it is estimated to range between 1% (Rubio-Tapia *et al.* 2012; Choung *et al.* 2017) and 3% (Myléus *et al.* 2009) in the Western countries. The frequency has also increased in Estonia (Ress *et al.* 2012) and in Finland (Lohi *et al.* 2007), the two neighbouring countries. It is known that until the early 1990s there were marked differences in the socio-economic background between these countries.

In the pathogenesis of CD a great role is played by genetic, environmental and immunological factors (Kagnoff 1992). Some of them are well known such as HLA-DQ2 and HLA-DQ8, autoantigen transglutaminase 2 (TG2) and gluten and gluten-related prolamines (Caio *et al.* 2019). It is known that most people with the genetic risk for CD and exposure to gluten do not develop CD, so different environmental factors must be involved. As the pathogenesis of CD involves many factors, there are yet no good tools to predict which children with the genetic risk will develop CD. Focus has been placed on different environmental risk factors, the best investigated among them being early infant feeding, infections and microbiome.

Earlier epidemiological studies (Ivarsson *et al.* 2000) have indicated that early infant feeding practices, particularly duration of breastfeeding and timing of gluten introduction, might be related to the incidence of CD. At the moment there is not enough evidence (Szajewska *et al.* 2016) to support breastfeeding versus no breastfeeding as well as breastfeeding at the time of gluten introduction reduces the risk of developing CD (Vriezinga *et al.* 2014; Lionetti *et al.* 2014). In the light of this, the amount of gluten at introduction has stimulated more interest in recent studies (Aronsson *et al.* 2019; Lund-Blix *et al.* 2019; Mårild *et al.* 2019). Additionally, it has been suggested that infections may be involved in the pathogenesis of CD, as some authors have reported seasonal variation in the birth date of patients with CD (Lewy *et al.* 2009). Different studies have proposed several viral and bacterial infections (Stene *et al.* 2006; Lebwohl *et al.* 2013; Kahrs *et al.* 2019). Possible relationships with CD have been found with gastrointestinal infections, for example rotavirus (Stene *et al.* 2006), enterovirus (Kahrs *et al.* 2019; Lindfors *et al.* 2020) or reovirus (Bouziat

et al. 2017), but also with herpes viruses (Jansen *et al.* 2016) or *Helicobacter pylori* colonization (Lebwohl *et al.* 2013). Also intestinal microbiota (Verdu *et al.* 2015) has been found to be involved in the pathogenesis of CD. Early microbial changes may already occur in infants from risk groups who later progress to CD (Olivares *et al.* 2018). Thus it is important to find out what causes these changes in the intestinal microbiota.

The general aim of the present thesis was to study the development of CD in two paediatric populations with different environmental backgrounds, Estonia and Finland, and to find out the role of different risk factors in this process.

2. REVIEW OF THE LITERATURE

2.1. Epidemiology of coeliac disease

In recent decades the incidence and prevalence of CD have increased worldwide (Kang *et al.* 2013; King *et al.* 2020; Roberts *et al.* 2021; Makharia *et al.* 2022). In the 1970s, when the diagnosis of CD was based mainly on clinical symptoms, the prevalence was estimated to be as low as 0.03% globally (Green & Jabri, 2003). Now the total prevalence is estimated to be around 1% in the Western countries (Bingley *et al.* 2004; Rubio-Tapia *et al.* 2012; Choung *et al.* 2017). According to studies based on the Finnish adult population, the prevalence has increased from 1.05% in 1978–1980 to 1.99% in 2000–2001 (Lohi *et al.* 2007). In the North American population the incidence increased from 11.1 per 100,000 person-years in 2000–2001 to 17.3 per 100,000 person-years in 2008–2010 (Ludvigsson *et al.* 2013). An increase has also been seen in China and India, which are traditionally rice based cultures, but where 'westernization' of diet has taken place (Wu *et al.* 2010; Ramakrishna *et al.* 2016). The prevalence varies between sexes, ages and geographic locations (Singh *et al.* 2018). As in several other autoimmune conditions, CD is more prevalent among women than in men, with up to twice as many women affected (Hin *et al.* 1999). The global biopsy-confirmed prevalence of CD is significantly greater among children than among adults (0.9% versus 0.5%) (Singh *et al.* 2018), but when looking countries separately this isn't always so (Table 1). The child-specific incidence is also almost twice as high (21.3 per 100,000 person-years versus 12.9 per 100,000 person-years) (King *et al.* 2020). There is also a significant difference between countries, for example, among 30–64-year old subjects the prevalence of CD varied from 2.4% in Finland to 0.3% in Germany (Mustalahti *et al.* 2010). The global prevalence and incidence of CD are shown in Table 1.

The frequency of the disease has also increased in Estonia and Finland, the two neighbouring countries. There were marked differences in the socio-economic background of these countries until the early 1990s. At the moment these differences have equalized. It was shown that the rate of age-standardized incidence of childhood CD in Estonia increased dramatically during a 35-year period: from 0.10 in 1976–1980 to 3.14 per 100,000 person-years in 2006–2010 (Ress *et al.* 2012). However, it is still lower than in many Western countries, for example 27.4 per 100,000 person-years in children under 19 years of age in Campania, Italy (Zingone *et al.* 2015) or 11.9 per 100,000 person-years in the United Kingdom (Zingone *et al.* 2015). As described above, in Finland the total prevalence of CD doubled in recent decades and was as high as 2% (Lohi *et al.* 2007).

The exact reasons why the frequency of CD is increasing are unknown. The increase is partly due to the improved serological testing and awareness of CD, but this alone cannot explain the overall increase.

Table 1. Global prevalence of CD based on Dieli-Crimi *et al.* 2015 and incidence of CD based on King *et al.* 2020. (NA – not available)

Country	Prevalence among children	Prevalence among adults	Prevalence (children and adults together)	Incidence among children (per 100,000 person-years)	Incidence among adults (per 100,000 person-years)
Estonia	0.34%	NA	NA	1.1	NA
Finland	1%	2.4%	NA	44.0	33.1
Sweden	3%	NA	NA	25.9–28.2	8.7
United Kingdom	0.16%	1.13%	NA	2.3–12.2	14.6
Italy	1.2%	0.7%	NA	18.9–34.5	3.8–9.3
Russia	0.2%	NA	NA	NA	NA
India	1%	0.56%	NA	NA	NA
Australia	NA	0.39%	NA	NA	NA
Egypt	0.53%	NA	NA	NA	NA
Brazil	0.54%	0.35%	NA	NA	NA
United States of America	NA	NA	0.79%	17.4	18.3

2.2. Pathogenesis of coeliac disease

Coeliac disease is an autoimmune disorder whose pathogenesis entails genetic, environmental and immunological factors (Kagnoff 1992; Uibo *et al.* 2011). The genetic factors HLA-DQ2 and HLA-DQ8 and the predominant autoantigen TG2 are well defined. The major environmental factors are gluten and gluten-related prolamines (Caio *et al.* 2019). The pathogenesis of CD involves both the innate and adaptive immune systems (Figure 1).

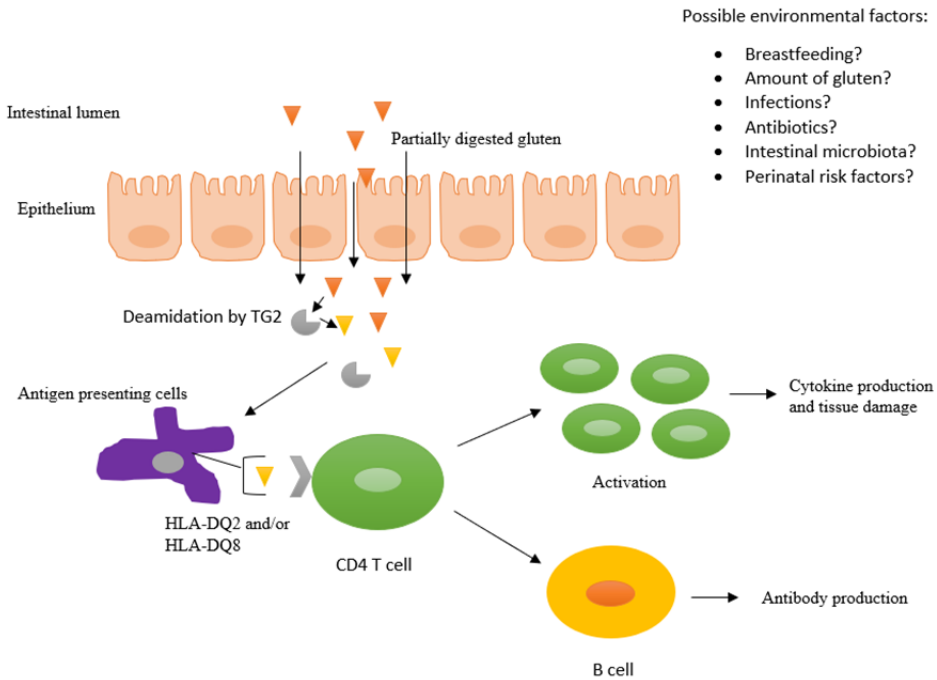

Figure 1. Partially digested gluten peptides (up to 33 amino acids long large peptides) access the lamina propria actively through the transepithelial route or passively by the paracellular flux. These peptides are modified by TG2, which deamidates distinct glutamine residues into glutamic acid. These modified epitopes are taken up by antigen presenting cells that present them to gluten-specific CD4 T cells in the context of HLA-DQ2 and/or HLA-DQ8 molecules. Upon the interaction of HLA-DQ2 and/or HLA-DQ8, gliadin peptides and distinct T cell receptors, both the T cells and the B cells, will be activated. T cell activation results in cytokine production and tissue damage and B cell activation results in antibody production.

2.2.1. Genetic factors

Genetic factors are thought to play a prominent role in the pathogenesis of CD. Familial recurrence in first-degree CD relatives is high (~15–20%) (Fasano & Catassi, 2012; Singh *et al.* 2015). There is also high concordance among monozygotic twins (75–80%) and less than 20% among dizygotic twins (Greco *et al.* 2002; Nisticò *et al.* 2006). HLA and non-HLA genes have been identified. The first are thought to contribute 40% and the latter, 14% to the risk of the disease (Trynka *et al.* 2011). The association with HLA molecules was first described in 1974 (Sollid 2002). HLA genes, HLA-DQ2 (HLA-DQ2.5 and HLA-DQ2.2) and HLA-DQ8, are located on chromosome 6p21 (Dieli-Crimi *et al.* 2015). These HLA class II molecules are responsible for binding specific gluten peptides that activate T cells (Sollid *et al.* 1989) (Figure 1). The frequency of subjects positive for either HLA-DQ2 and/or HLA-DQ8 in the general popu-

lation may be as high as 40% (Hadithi *et al.* 2007). Most of the patients (90–96%) are positive for the HLA-DQ2.5 haplotype (encoded by the DQA1*05:01 and DQB1*02:01 alleles) and almost all of the remainders are positive for HLA-DQ8 (encoded by the DQA1*03:01 and DQB1*03:02 alleles) or HLA-DQ2.2 alone (encoded by the DQB1*02:02 allele) (Wolters & Wijmenga, 2008). Exceptions are rare and mostly due to misdiagnosis (Anderson *et al.* 2013). The risk of the disease depends on the dosage effect: individuals homozygous for HLA-DQ2.5 have the highest risk and the risk decreases in individuals with HLA-DQ8, or with only the HLA-DQB1*02 allele (Tollefsen *et al*. 2006; Fallang *et al.* 2009; Bodd *et al.* 2012). Very few patients express HLA-DQ7.5 (encoded by the DQA1*05:01 allele) and have a very low risk for CD (Karell *et al.* 2003). HLA-DQ2 haplotypes may be associated with development of CD in childhood, while HLA-DQ8 haplotypes may be associated with onset in adulthood rather than in childhood (Schirru *et al.* 2011). In addition to these genes, there have been identified more than 100 non-HLA genes associated with CD (Dieli-Crimi *et al*. 2015; Lundin & Wijmenga, 2015). The non-HLA genes that play a role in the pathogenesis of CD are interleukin(*IL)2* and *IL21* genes, which are involved in T-cell maturation and differentiation (Leonard *et al.* 2015).

2.2.2. Gluten and related prolamines

Gluten is the main environmental trigger of CD, and people who are not exposed to gluten do not develop CD. “Gluten” is a general term for alcohol-soluble proteins present in various cereals. Actually, real gluten is the main storage protein of only wheat grains. It is a complex mixture of hundreds of related, but distinct proteins, mainly monomeric gliadins and polymeric glutenins, which together are referred to as prolamins. Gliadin is the main trigger of CD. There exist similar storage proteins as secalin in rye, hordein in barley, and avenins in oats (Wieser 2007; Biesiekierski 2017).

Gluten is incompletely digested by the gastric, pancreatic and brush border peptidases (Shan *et al.* 2002). These up to 33 amino acids long large peptides cross the intestinal epithelium through transcellular or paracellular routes (Matysiak-Budnik *et al.* 2008; Visser *et al.* 2009; Schumann *et al.* 2017). Then gliadin peptides are deamidated by the predominant autoantigen of CD - TG2 (Dieterich *et al.* 1997). Antigen presenting cells which express the HLA class II DQ2 and/or DQ8 molecules on their surface interact with CD4 T cells and activate adaptive immune responses (Lundin *et al.* 1993). The innate immune response to deamidated gliadin occurs in the epithelial component of the intestinal mucosa (Green & Jabri, 2003) and results in the differentiation of intraepithelial lymphocytes into cytotoxic CD8 T cells that express the natural killer cell marker NK-G2D (Schuppan *et al*. 2009). Altogether, the damage manifests itself as villous atrophy of the small intestinal mucosa and crypt hyperplasia.

2.2.3. Other environmental factors of coeliac disease

Most people with the genetic risk for CD and exposure to gluten and gluten-related prolamines do not develop CD. There are also significant differences in the prevalence of CD between people living geographically close to each other and receiving equal exposure to grain products (for example Finland and Russian Karelia) (Kondrashova *et al.* 2008). According to their study, the prevalence of CD was 1 in 107 children in Finland and 1 in 496 children in Russian Karelia. This finding indicates evident involvement of other factors.

2.2.3.1. Role of infant feeding practices

According to epidemiological studies (Ivarsson *et al.* 2000), early infant feeding practices might be related to development of CD, particularly, if breastfeeding was or was not ongoing when gluten was introduced, depending on the timing of gluten introduction.

Previous studies have shown that exclusive or any breastfeeding, or breastfeeding at the time of gluten introduction may reduce the risk of developing CD during childhood (Fälth-Magnusson *et al.* 1996; Ivarsson *et al.* 2002; Akobeng *et al.* 2006). This was thought to be related with many benefits that derive from breast milk. For example, by passive immunity breastfeeding reduced the risk of gastroenteritis by 64% and the risk of otitis media by 23% (Ip *et al.* 2007). Infections are considered to play an important role in development of CD. Moreover, some authors have reported that the time of gluten introduction may also affect the risk for developing CD (Peters *et al.* 2001; Norris *et al.* 2005).

Based on the earlier studies, the European Society for Paediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) recommended in 2008 avoiding early gluten introduction before 4 months of age and late introduction after 7 months of age, and introducing gluten while the infant is still being breastfed to decrease the risk of CD, type 1 diabetes (T1D), and wheat allergy (Agostoni *et al.* 2008).

These results have been refuted by more recent randomized prospective and cohort studies. Vriezinga and colleagues found that introduction of small quantities of gluten at 4 to 6 months of age in at-risk children did not reduce the incidence of CD by the age of 3 years (Vriezinga *et al.* 2014). Lionetti and colleagues reported that neither delayed introduction of gluten nor breastfeeding reduced the rate of CD in high-risk children, whereas later introduction of gluten was associated with delayed manifestation of the clinical disease (Lionetti *et al.* 2014). The limitation of these interventional studies was that neither of them was designed to investigate the effect of breastfeeding on development of CD. As the study by Vriezinga and colleagues lasted only 3 years, it cannot establish if breastfeeding significantly impacts the incidence of CD later in life. A large prospective birth cohort study including over 100,000 children in Norway found that gluten introduction after 6 months compared with introduction between 5 and 6 months was associated with a borderline significantly in-

creased risk of CD (Størdal *et al.* 2013). Another study demonstrated that higher prevalence of CD may be related to later gluten introduction (Ivarsson *et al.* 2013). Other authors have not found clear associations (Jansen *et al.* 2014; Aronsson *et al.* 2015) .

The findings that breastfeeding and age at gluten introduction might not affect the risk of developing CD have also been supported by systematic reviews and meta-analyses (Szajewska *et al.* 2015; Silano *et al.* 2016).

In the light of this data, ESPGHAN has published a new position papers on gluten introduction and CD risk (Szajewska *et al.* 2016; Fewtrell *et al.* 2017). These state that there is no evidence to support that breastfeeding compared with no breastfeeding reduces the risk of developing CD in children and that breastfeeding at the time of gluten introduction does not affect the risk of CD. Gluten can be introduced between 4 and 12 completed months, as its introduction in this age range does not appear to influence the risk of developing CD or CD autoimmunity.

As breastfeeding and age at gluten introduction do not seem to be related with the risk for CD development, there has grown more interest in the amount of gluten at introduction. According to earlier studies based on the information from the Swedish CD epidemic from 1984–1996, children who consume large amounts of gluten at introduction have a greater risk of developing CD (Ivarsson *et al.* 2002). According to the ESPGHAN position paper, large amounts of gluten consumption should be avoided in the first months after introduction (Szajewska *et al.* 2016). Recent papers by Aronsson and colleagues, by Mårild and colleagues and by Lund-Blix and colleagues indicate that the amount of gluten plays a role in CD (Aronsson *et al.* 2019; Lund-Blix *et al.* 2019; Mårild *et al.* 2019). These studies report that one extra slice of bread (some 2 g of gluten) per day for children seems to be linked to a 20%-50% increased risk of CD. A limitation of the first two studies is that they are only based on an at-risk child population, which might raise the question if these recommendations can be extrapolated to the whole population (Ludvigsson & Lebwohl, 2019). In contrast, the previous open-label RCT EAT Study found that early consumption of high-dose gluten can be preventive against CD development (Logan *et al.* 2020). The latter study was conducted on a general population cohort. To make this issue more complex, a recent study has suggested that enterovirus infections early in life can be potentiated by high gluten intake, which may trigger the development of CD autoimmunity in genetically predisposed children (Lindfors *et al.* 2019).

2.2.3.2. Infections and use of antibiotics as risk factors

Seasonal variation in the birth date of patients with CD has been reported (Lewy *et al.* 2009). According to that study, girls whose CD was diagnosed before age 24 months (peak July-August) showed a different seasonality of the month of birth compared to those whose CD was diagnosed after age 24 months (no rhythm) and also a different seasonality compared to boys whose CD was

diagnosed after age 24 months (peak July) (Lewy *et al.* 2009). This can indicate that infections may be involved in the pathogenesis as an environmental factor. Different studies have proposed several viral and also bacterial infections as part of the pathogenesis of CD. Most of these studies have been epidemiological (Beyerlein *et al.* 2017) and based on parental reports (Mårild *et al.* 2015; Kemppainen *et al.* 2017). Also, very few experimental (Bouziat *et al.* 2017) and laboratory (Jansen *et al.* 2016) investigations have reported this relationship. It is also possible that the pathogenesis of childhood CD is influenced by the cumulative effect of early-life infections rather than by one specific microbial or viral agent (Mårild *et al.* 2015; Beyerlein *et al.* 2017).

Many authors have found relationship between gastrointestinal infections and increased risk of CD (Stene *et al.* 2006; Kemppainen *et al.* 2017; Kahrs *et al.* 2019; Tapia *et al.* 2021). Gastrointestinal infections may initiate the secretion of intracellular tTG and lead to increased intestinal permeability, which is frequently seen in CD (Koning *et al.* 2005). The TEDDY Study has shown that gastrointestinal infections increase the risk of CD autoimmunity within the following 3 months by 33% in children with genetic susceptibility (Kemppainen *et al.* 2017). Rotavirus is one of the most common causes of gastroenteritis among children (Kapikian *et al.* 2001). As it is so common, a single rotavirus infection is probably not enough to cause CD, but recurrent infections in genetically predisposed individuals may increase the risk of CD autoimmunity in childhood (Stene *et al.* 2006). Recent prospective studies by Kahrs *et al.* and Oikarainen *et al.* also found that higher frequency of enterovirus during early childhood was associated with later CD (Kahrs *et al.* 2019; Oikarinen *et al.* 2021). In addition, an interaction between the amount of ingested gluten and enterovirus exposure might be of importance (Lindfors *et al*. 2019). Early-life parechovirus infections are also thought to be associated with development of CD in genetically at-risk children (Tapia *et al.* 2021). Earlier studies (Kagnoff *et al.* 1987; Mahon *et al.* 1991) have obtained conflicting results about adenovirus and CD, however, a recent large prospective study found that adenovirus was not associated with later CD *(Kahrs et al.* 2019). An experimental work by Bouziat *et al.* suggests that a nonvirulent pathogen, reovirus, may be related to development of CD (Bouziat *et al.* 2017). Using a viral infection model, these authors claim that reovirus can disrupt intestinal immune homeostasis at the inductive and effector sites of oral tolerance by suppressing peripheral regulatory T cell conversion and by promoting type 1 helper T cells immunity to dietary antigen (Bouziat *et al.* 2017).

More potential interactions between different types of infections and CD have been investigated in single studies. As species of the human herpesvirus family have been implicated in the pathogenesis of autoimmune diseases in adulthood (Barzilai *et al.* 2007), these viruses have been investigated in relation to CD. Herpes viruses may have a protective effect on development of CD (Jansen *et al.* 2016). Auricchio *et al.* found that during the first 24 months of life the frequency of respiratory tract infections was higher among CD patients (Auricchio *et al.* 2017). The higher frequency of respiratory syncytial virus

infection or any viral bronchiolitis has been reported also by Tjernberg and Ludvigsson (Tjernberg & Ludvigsson, 2014). *Helicobacter pylori* colonization may decrease the risk of CD (Lebwohl *et al.* 2013). Borrelia infection does not seem to be related with development of CD (Alaedini *et al.* 2017).

Previous studies have reported conflicting results about antibiotic use and CD (Mårild *et al.* 2013; Canova *et al.* 2014; Kemppainen *et al.* 2017; Duong *et al.* 2022). A large observational nationwide study in Denmark and Norway showed that exposure to systemic antibiotics in the first year of life could be a risk factor for CD (Dydensborg Sander *et al.* 2019), while no specific type of antibiotic or age at exposure was prominent.

An earlier study by Sandberg-Bennich *et al.* indicates that neonatal infections may increase the risk of later CD development (Sandberg-Bennich *et al.* 2002). On the other hand, a study involving both inpatients and outpatients with CD did not confirm this (Mårild *et al.* 2012). It has been thought that early life infections may play a role in development of CD through induction of pro-inflammatory interferons (Sollid & Jabri, 2013). At the same time, there need not be clear association between maternal infections and use of antibiotics in pregnancy and the child's risk of CD later in life (Mårild *et al.* 2014; Mårild *et al.* 2017).

A recent systematic review and meta-analysis, although not including the latest data, confirms that infections and antibiotic use seem to be an important factor in development of CD (Jiang *et al.* 2019).

2.2.3.3. Intestinal microbiota and breast milk components as risk factors

In addition to infections, the intestinal microbiota is thought to be involved in the pathogenesis of CD (Verdu *et al.* 2015). Studies based on duodenal biopsies and faecal samples have shown increased abundance of many different bacteria – for example *Bacteroidetes* (Collado *et al.* 2009), *Proteobacteria* phyla (Verdu *et al.* 2015) and *Clostridium* (Ou *et al.* 2009). The abundance of *Lactobacillus* and *Bifidobacterium* spp. appears to be decreased (Di Cagno *et al.* 2011). Earlier research has shown that infants with an increased genetic risk for CD were colonized with a lower proportion of *Actinobacteria* and *Bacteroidetes*, but a higher proportion of *Firmicutes* and *Proteobacteria* compared to infants with a low genetic risk for development of CD (Sellitto *et al.* 2012). Dysbiosis preceding the onset of the disease in at-risk infants has been confirmed (Olivares *et al.* 2018). In the first years of life the intestinal microbiota is constantly changing (Palmer *et al.* 2007). It is known that breast milk components promote oral tolerance of dietary antigens by modulating immune development and composition of the infant gut microbiota (Sepp *et al.* 2000; Bezirtzoglou *et al.* 2011; Pozo-Rubio *et al.* 2012; Ivarsson *et al.* 2013; Drell *et al.* 2017). Human breast milk contains various essential immunological components, which protect against infections and which modulate and promote immune system (for example, tumor necrosis factor-α, IL-1β, IL-6, IL-8, IL-10, interferon-γ, and transforming

growth factor-β (TGF-β)). There are several whey proteins in breast milk: α-lactalbumin, lactoferrin, lysozyme, and secretory immunoglobulin A (sIgA) (Ballard *et al.* 2013). de Palma *et al.* demonstrated that the type of milk feeding in relation to the HLA-genotype played a role in establishing infant gut microbiota (de Palma *et al.* 2012). The HLA-DQ genotype may specifically influence the colonization process of *Bacteroides* species (Sanchez *et al.* 2011). A pilot study from the PreventCD Cohort established that certain microbial species were more abundant in milk samples from mothers whose children developed CD (Benitez-Paez *et al.* 2020). These included *Methylobacterium komagatae* and *Methylocapsa palsarum*, as well as the species such as *Bacteroides vulgatus*. Theoretically, these microbiota components could be vertically transmitted from mothers-to-infants during breastfeeding, thereby influencing the CD risk. By highlighting alterations in the microbiome composition prior to the onset of CD, these studies strongly suggest that dysbiosis can be considered an additional environmental contributor (Serena *et al.* 2019). It has been observed that breast milk from CD mothers have lower levels of TGF-β1, sIgA and a reduced abundance of *Bifidobacterium* spp. and *B. fragilis* compared with healthy women (Olivares *et al.* 2015). sIgA is the main immunoglobulin in breast milk, which promotes long-term intestinal homeostasis by regulating the gut microbiota and host gene expression (Rogier *et al.* 2014). TGF-β belongs to a growth factor family that is responsible for development and maturation of the mucosal immune system (Oddy & Mcmahon, 2011). Regarding the development and homeostasis of intestine, important breast milk components are also sCD14 and MFG-E8 (lactadherin). Milk sCD14 is immune modulator in homeostasis and in the defence of the neonatal intestine (Vidal *et al.* 2001) and is preventing gastrointestinal Gram-negative infections (Vidal *et al.* 2002). MFG-E8 has been shown to have antiviral effects and protects against rotavirus, which is one of the most common causes of gastroenteritis among children (Newburg *et al.* 1998; Kvistgaard *et al.* 2004; Stene *et al.* 2006).

2.3. Diagnosis of coeliac disease in children

The diagnosis of CD is based on a combination of clinical, serological and histopathological data. The clinical picture of CD can be very variable. Common classical symptoms are chronic or intermittent diarrhoea, distended abdomen, weight loss and failure-to-thrive, because of malabsorption. There can be different gastrointestinal symptoms (for example abdominal pain, nausea, vomiting or even constipation), but also extraintestinal symptoms (for example delayed puberty, arthritis/arthralgia, aphthous stomatitis, rash, dental enamel defects, abnormal liver biochemistry). Patients can also be asymptomatic and found in screening of different risk groups (Husby *et al.* 2020). Traditionally, diagnosis of CD is based on CD-specific antibodies and small intestinal biopsies from the duodenum to assess villous atrophy, crypt epithelial hyperplasia and number of intraepithelial lymphocytes (IEL) (Kelly *et al.* 2015). In recent years there have

been some changes in paediatric guidelines regarding in whom what kind of antibodies to test and in whom gastroscopy with small intestinal biopsies to perform (Husby *et al.* 2012; Husby *et al.* 2020). In 2012 it was allowed to diagnose CD in symptomatic paediatric patients without small intestinal biopsies if immunoglobulin A (IgA)-tTG was positive over 10 times normal, and if also EMA and HLA-DQ2/DQ8 were positive (Husby *et al.* 2012). The last guideline, published in 2020, allows to diagnose CD without small intestinal biopsies also in asymptomatic children. It is not nessecary any more to test for HLA-DQ2/DQ8 (Husby *et al.* 2020).

2.3.1. Screening tests in coeliac disease

CD-specific antibodies include autoantibodies against autoantigens (tTG and EMA) and antibodies targeting the offending agent (anti-gliadin antibodies (AGA) and DGP). Both tTG and EMA have very high sensitivity (90–100%) and specificity, close to 100% for CD (Lewis & Scott, 2006; Giersiepen *et al.* 2012). CD-related autoantibodies can belong to the IgA or immunoglobulin G (IgG) classes, but only the IgA class is highly sensitive and specific for CD (Volta *et al.* 2010). IgG antibodies should be used in patients with IgA deficiency (Villalta *et al.* 2010; Husby *et al.* 2012; Husby *et al.* 2020).

Enzyme-linked immunosorbent assay (ELISA) based AGA tests (using α-gliadin as the antigen) were the first ones used for identifying CD patients (O'Farrelly *et al.* 1983). AGA tests are no longer recommended in routine clinical practice in diagnosing CD and their role is now confined to the possible identification of a subset of cases with non-celiac gluten/wheat sensitivity (Caio *et al.* 2017).

EMA test is considered to be the gold standard for detecting CD autoantibodies. These autoantibodies are specific to the smooth muscle endomysium of the gastrointestinal tract (Kaur *et al.* 2017). It is based on indirect immunofluorescence, which makes it is very subjective, labour consuming and expensive (Lindfors *et al.* 2019). This test was first described by Chorzelski *et al.* in 1983 (Chorzelski *et al.* 1983).

The tTG is the best test for initial screening in clinical practice, as it is based on operator-independent ELISA and radiobinding assays; there are also many commercial tests (Lindfors *et al.* 2019). IgA-tTG testing alone was found to be more sensitive for CD than IgA-tTG combined with EMA testing (Hopper *et al.* 2008). So this test should be done as the first-line screening test (Husby *et al.* 2012; Husby *et al.* 2020). It is known that concentration of IgA-tTG correlates with degree of villous atrophy (Singh *et al.* 2015). As the first-line screening test is IgA-tTG, also total IgA concentration should always be measured to identify patients with IgA deficiency.

Previously, DGP was recommended for diagnosing CD in children younger than 2 years (Husby *et al.* 2012), but isolated increases in the concentration of this antibody have a low positive predictive value (Hoerter *et al.* 2017). So this test should only be used in selected cases (Husby *et al.* 2020).

2.3.2. Role of small intestinal biopsy and histology finding

The normal intestine has finger-like villi on its surface. In the case of CD, the normal small intestinal mucosa is damaged. Mucosal damage may vary in different degrees, may be patchy and may appear only, for example, in the duodenal bulb (Taavela *et al.* 2016). For describing mucosal damage, the Marsh-Oberhuber classification is used (Marsh 1992; Oberhuber *et al.* 1999; Marsh *et al.* 2015) (Table 2). Small intestinal changes that should be considered have different stages.

In type 0 small intestinal histology is normal with less than 40 IEL per 100 enterocytes. CD is highly unlikely. Type 1 is seen in patients who are on gluten free diet (suggesting the minimal amounts of gluten or gliadin are being ingested), in patients with dermatitis herpetiformis, and in some family members of CD patients (potential CD); it may also be seen in the case of infections. This stage is not diagnostic for CD. Type 2 is very rare and is seen occasionally in dermatitis herpetiformis. Type 3 is divided into subclasses depending on the degree of villous atrophy and is seen in symptomatic CD patients (Oberhuber e*t al.* 1999).

Table 2. Modified Marsh Classification of histologic findings in CD (IEL – intraepithelial lymphocytes; IEL/100 enterocytes – IEL per 100 enterocytes) (Oberhuber e*t al.* 1999)

Marsh Type	IEL / 100 enterocytes – jejunum	IEL / 100 enterocytes – duodenum	Crypt hyperplasia	Villi
0	<40	<30	Normal	Normal
1	>40	>30	Normal	Normal
2	>40	>30	Increased	Normal
3a	>40	>30	Increased	Mild atrophy
3b	>40	>30	Increased	Marked atrophy
3c	>40	>30	Increased	Complete atrophy

2.4. Summary of the literature review

In recent decades the prevalence of this immune-mediated systemic disease has increased. Significant progress has been made in understanding the pathogenesis of CD. HLA and non-HLA genes have been identified. Well known is the predominant autoantigen TG2. The major environmental factors are gluten and gluten-related prolamines.

As there are significant differences in the prevalence of CD between people living geographically close to each other, big effort has been made to understand possible environmental risk factors.

There have been many studies about infant feeding. At the moment there is no evidence to support that breastfeeding compared with no breastfeeding reduces the risk of developing CD in children and breastfeeding at the time of gluten introduction does not affect the risk of CD. Gluten can be introduced between 4 and 12 completed months.

As seasonal variation in the birth date of patients with CD has been reported, there have been interest in different infections. Several viral and bacterial infections have been thought to be part of the pathogenesis of CD. Addition to infections, the intestinal microbiota is thought to be involved in the pathogenesis of CD.

More longitudinal international studies are needed to understand possible risk factors better and their relation to CD development. Important are close follow-up of patients and also laboratory analyses of different types of biomarkers.

3. AIMS OF THE THESIS

The general aim of the present thesis was to study the development of CD in two paediatric populations with different environmental backgrounds and to find out the role of different risk factors in this process.

Specific aims

1. To compare the cumulative incidence of CD between Estonian and Finnish children up to 5 years of age.
2. To analyse the role of early infant feeding and infections reported by parents in modulation of the incidence of CD.
3. To detect viruses in stool and nasal swab samples before the diagnosis, and virus-specific antibodies in serum samples at the diagnosis of CD. To compare the frequency of infections in the disease group and disease-free controls, and thereby to assess their potential involvement in the development of CD.
4. To characterize the microbiota composition of the breast milk and its possible relationship with immunological markers of the breast milk in mothers whose offspring presented or did not present CD during the first 3 years of life.

4. SUBJECTS AND METHODS

The study is based on the data and biomaterials collected during the international prospective observational DIABIMMUNE Study (Principal Investigator Prof. Mikael Knip, University of Helsinki, Finland). The DIABIMMUNE Study set out to assess the role of the hygiene hypothesis in the development of T1D and other immune-mediated diseases. The study was carried out in Estonia, Finland and Russian Karelia from September 2008 to October 2013. As there were no biopsy confirmed CD cases in Russia, that country was not included in this study. The study included two cohorts: a birth cohort (BC) observed from birth up to the age of 3 years and a cohort of young children (YCC) examined for the first time at the age of 3 years and followed up to the age of 5 years.

4.1. Background studies

Initially, during the DIABIMMUNE Study, 2714 infants from maternity hospitals in Estonia and 3105 in Finland were recruited for the BC (Figure 2). Cord blood samples were taken from all children whenever it was technically feasible and their parents gave written informed consent. All children were analysed for T1D/CD associated HLA DR/DQ alleles (Peet *et al.* 2012). The HLA DR/DQ genotypes were analysed with the polymerase chain reaction (PCR)-based lanthanide labelled oligonucleotide hybridisation method at the Immunogenetics Laboratory, University of Turku, Finland, using time-resolved fluorometry for detection as described previously (Hermann *et al*. 2003). Four hundred and twenty-six Estonian children and 713 Finnish children carried the eligible HLA genotype (DR3-DQ2 (DQA1*05-DQB1*02)/DR4-DQ8 (DRB1*04:01/2/4/5-DQA1*03-DQB1*03:02) heterozygosity, or either DR4-DQ8 or DR3-DQ2 haplotype alone without any haplotypes protecting for T1D).

Children with eligible HLA were invited for a follow-up study. In total, 258 children in Estonia (61% of the eligible) and 305 in Finland (43% of the eligible) completed the 3-year follow-up programme with planned visits at the age of 3, 6, 12, 18, 24 and 36 months (± 1 month). Non-fasting blood samples were collected by venipuncture from all participants at every visit. Serum was separated by centrifugation and stored in aliquots at -70 ºC for further use. The parents were asked to collect monthly stool samples at home, starting from the age of 1 month. The samples were stored in home freezers at -20ºC immediately after collection until they were transported frozen to the laboratory for long-term storage at -80ºC. Nasal swabs were collected at study visits by the research personnel, starting from the age of 3 months, and stored immediately at -20ºC and later at -80ºC for long-term storage. Breast milk samples were collected from the mothers 3 months after delivery. Milk samples were obtained by manual expression into sterile bags. The samples were immediately placed in a -20ºC freezer and further stored at -80ºC until processing. At the first study visit the parents were given a diary in which they were asked to report data about

infections, use of medications, allergies, vaccinations and environmental exposures. Data on breastfeeding and on the introduction of complementary foods and their character was also registered. All diary data was transferred to a central database at each visit.

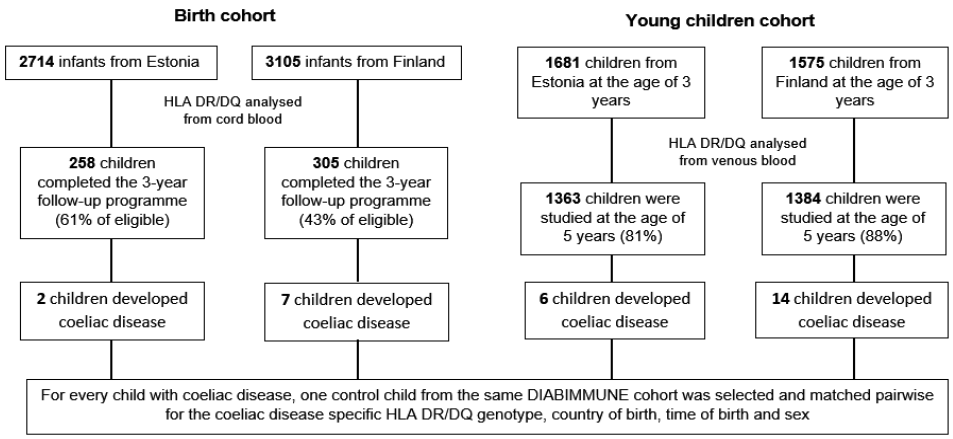

Figure 2. Study outline with relevant data about the subjects.

In the YCC children with suitable age were invited to the study. 1681 children from Estonia and 1575 children from Finland were studied at the age of 3 years, and 1363 (81%) and 1384 (88%) children, respectively, attended the follow-up visit at the age of 5 years (Figure 2). In addition, some of these children (250 children from Estonia and 448 children from Finland) were also studied at the age of 4 years. All participants were genotyped for HLA, but no selection was based on the genotype. Non-fasting blood samples were collected by venipuncture from all participants at every visit. Serum was separated by centrifugation and stored in aliquots at -70 ºC for further use. A study diary was given to the parents at the initial visit with the request that they should fill in information on food intake, infections, use of medications, vaccinations, allergies and environmental exposures. Information about the history of breastfeeding was collected at the first visit.

Both cohorts were screened regularly for CD antibodies to identify children with possible CD. Autoantibody analyses were made at one laboratory – the Institute of Biomedicine and Translational Medicine, University of Tartu, Estonia. IgA-tTG were measured using a fully automated solid-phase fluoroenzyme immunoassay technology (ImmunoCAP EliA, Phadia AB, Uppsala, Sweden). According to the manufacturer, the clinical sensitivity of ImmunoCAP EliA is 96% and specificity is 99%. The IgA-tTG values higher than 10 EliA U/ml were considered positive, and the values lower than 7 EliA U/ml were consi-

dered negative. When a IgA-tTG test result was equivocal (7 to 10 EliA U/ml), an in-house ELISA for IgA-tTG (Teesalu *et al.* 2009) and an in-house immunofluorescence assay for IgA-EMA (Uibo *et al.* 2006) were applied. In the case of a low serum volume, an in-house IgA-tTG ELISA with recombinant human tissue transglutaminase was used. If a serum sample was haemolytic, or if the IgA-tTG assay could not detect any IgA („low RU" as a sign of possible IgA deficiency), the sera were retested for IgG-DGP and IgG-tTG antibodies by ImmunoCAP EliA, using the same cut-off values for positivity as in the case of IgA-tTG EliA.

When antibody analyses were positive, children with suspected CD were referred to a paediatric gastroenterologist at Tartu University Hospital or at Helsinki Children's Hospital. The diagnosis of CD was made in accordance with the ESPGHAN guidelines (Husby *et al.* 2012) – positive IgA-tTG antibody test results and a biopsy of the small intestine with an abnormal microscopic finding. The small intestine biopsy was done by paediatric gastroenterologist. The microscopic findings of the small intestine were classified in accordance with the Marsh classification modified by Oberhuber (Oberhuber *et al.* 1999). All children with biopsy-confirmed CD were included in the current analysis.

During the DIABIMMUNE Study period 29 children developed CD: nine children (two Estonian and seven Finnish children) in the BC and 20 children (six Estonian and 14 Finnish children) in the YCC.

4.2. Study subjects

All study subjects of this thesis were drawn from the DIABIMMUNE Study.

4.2.1. Children who developed coeliac disease and their controls (Papers I–II)

For every child with CD (n=29), one control child was selected (except for one child in the YCC who had two controls – one at the age of 3 and another at the age of 5 years). The control children were selected from the BC or YCC of the DIABIMMUNE Study and matched for the CD-specific HLA DR/DQ genotype, country of birth, age and gender (Figure 2).

4.2.2. Children who were selected for breast milk sample analysis (Paper III)

For breast milk sample analyses, six children of the BC were selected. The criteria for selection were that breast milk was collected from all of their mothers at the age of 3 months, the children were followed up from birth to the age of 3 years and they all developed later CD. From the same DIABIMMUNE cohort, we selected 18 controls based on the CD-specific HLA DR/DQ genotype, country of birth, time of birth and sex of each patient.

4.3. Methods

4.3.1. Analysis of viruses in stool and nasal swab (Paper II)

Enterovirus, norovirus, parechovirus and rhinovirus RNA were analysed in the BC from the stool samples collected at the age of 3, 4, 5 and 6 months. In addition, enterovirus, parechovirus and rhinovirus RNA were analysed from the nasal swab samples collected at the age of 3 and 6 months in the same cohort. Enterovirus and rhinovirus RNA were screened from stool and nasal swab samples using real-time reverse transcription (RT) PCR. In addition, parechovirus and norovirus RNA were tested in the stool samples using specific RT-PCR methods (parechovirus also from nasal swab). Stool samples were suspended in a concentration of 10% in the HANKS solution (Sigma-Aldrich, Missouri, USA) and RNA was extracted using the viral RNA kit (Qiagen, Hilden, Germany). QuantiTect Probe reagents (Qiagen, Hilden, Germany) were used according to the manufacturer's instructions for amplification of virus RNA. All samples were run in triplicate and, if any of the reactions was positive, that sample was regarded as positive for the tested virus. Primer and probe sequences, as well as the concentrations in the reactions, were reported by Krogvold *et al.* (Krogvold *et al.* 2015). That study recorded two primer pairs for enterovirus detection, while our study used the primer pair of fwd 636 and rev 4-. The analysis was carried out at the Virus Laboratory, University of Tampere, Finland.

4.3.2. Analysis of virus' antibodies from serum (Paper II)

The analysis of norovirus, adenovirus and enterovirus IgA and IgG antibodies in serum was carried out at the Virus Laboratory, University of Tampere, Finland, using ELISA. IgA and IgG antibodies against norovirus, adenovirus, enterovirus and IgG antibodies against Epstein-Barr virus (EBV) and cytomegalovirus (CMV) were measured from the serum sample of the children with CD at the time point of seroconversion to positivity for autoantibodies associated with CD in both cohorts. The corresponding serum samples from the control children were collected at the same age. All samples were tested in pairs. Samples from the case and control childen were included in the same test run, without knowledge of the case-control status of the child. The level of anti-viral IgA and IgG is expressed in enzyme immunoassay units (EIU).

Briefly, 96-well half-area polystyrene plates (Corning Incorporated, New York, USA) were used for norovirus antibody analyses and Nunc-Immuno MaxiSorp plates (Thermo Fisher Scientific, Massachusetts, USA) for adenovirus and enterovirus antibody analyses. For norovirus antibody analyses, the plates were coated with norovirus virus-like particle antigens at a concentration of 1 μg/ml in phosphate-buffered saline (pH 7.2), as previously described (Nurminen *et al.* 2011). For adenovirus antibody analyses, an adenovirus hexon antigen was used at a concentration of 0.32 μg/ml in a phosphate-buffered saline (pH 7.2) (Lönnrot *et al.* 2000) and for enterovirus a sucrose gradient purified

coxsackievirus B4 was used at a concentration of 0.76 µg/ml in a carbonate buffer (Hyoty *et al.* 1995). Norovirus antibodies were analysed using 1/200 (IgA) and 1/4000 (IgG) serum dilutions in 1% milk + 0.05% Tween 20 (MP Biomedicals, Illkirch, France) in a phosphate-buffered saline, and adenovirus and enterovirus antibodies were analysed using 1/100 (IgA) and 1/2000 (IgG) dilutions in a phosphate-buffered saline supplemented with 1% bovine serum albumin (Sigma-Aldrich, Missouri, USA), 2% sodium chloride and 0.05% Tween 20. For norovirus IgA, the detection antibodies were goat anti-human IgA-horseradish peroxidase (Pierce cat PA1-74395, Thermo Fisher Scientific, Massachusetts, USA) and for IgG, goat anti-human IgG-HRP (Invitrogen cat 627120, Invitrogen, California, USA). For adenovirus and enterovirus IgA, Dako P0216 rabbit anti-human IgA-HRP (Dako, Glostrup, Denmark) and for IgG, Dako P0214 rabbit anti-human IgG-HRP (Dako, Glostrup, Denmark) were used. Absorbance at 490 nm (norovirus, adenovirus) or 492 nm (enterovirus) was measured on a Victor2 microplate reader (PerkinElmer, Massachusetts, USA).

The cut-off limits for the virus antibody analyses were determined by arrangement of the optical density results in ascending order to generate a histogram (with Supplementary material of Paper II). The abrupt increase in the optical density values was used to separate positive and negative values. The arbitrary negative values were used to calculate the final cut-off using the formula: the mean of the arbitrary optical density (OD) negative value + three standard deviations (SD). The cut-off is shown with an arrow on the histograms. All the values above the set cut-off value were considered positive.

For CMV IgG and EBV viral capside antigen IgG detection, we used ELISA in accordance with the manufacturer's protocol, by employing the LUCIO-Sero ELISA Cytomegalovirus IgG and the LUCIO-Sero ELISA Epstein-Barr VCA IgG (nal von minden GmbH, Moers, Germany). The results were calculated according to the manufacturer's instructions. Equivocal CMV IgG results were demonstrated in a child with CD in the BC from Estonia and this child was excluded from final analysis.

4.3.3. Analysis of breast milk (Paper III)

Breast milk samples were thawed and centrifuged for 15 min at 1000 g at 4ºC and thereafter the aqueous fraction was collected. The latter was used for further analyses. Centrifugation was repeated twice.

4.3.3.1. Analysis of immune markers (Paper III)

We used ELISA to measure TGF-β1, TGF-β2, sIgA, MFG-E8 (lactadherin), and sCD14. For measuring TGF-β1, TGF-β2, MFG-E8, and sCD14, we used commercial kits from R&D Systems (Minneapolis, MN, USA) according to the manufacturer's instructions. For sIgA, we used a commercial kit from Bethyl Laboratories (Montgomery, AL, USA) according to the manufacturer's instructions.

4.3.3.2. Molecular analysis (Paper III)

DNA extraction

Bacterial DNA from human milk was extracted using the PureLinkTM Microbiome DNA Purification Kit (Invitrogen, using an ELMI SkyLine instrument [ELMI Ltd., Riga, Latvia]). Human milk samples were centrifuged at 4,000 g for 30 min, after which the supernatant was removed. Cell pellets were washed with a phosphate-buffered saline, centrifuged at 13,000 g × 3 min at room temperature and the pellets were resuspended in a kit-specific lysis buffer. The protocol was then continued as described by the manufacturer (Invitrogen, USA).

Illumina sequencing

16S library preparation was performed using an in-house sequencing protocol with the V4 (F515/R806) primer pair (Zeller *et al.* 2014). Sequencing was performed using an iSeq 100 (SN FS10000643, Illumina) and an iSeq 100 i1 Reagent kit (Illumina) in the 2x150 bp mode and using the dual index setup with two-read sequencing protocol. 16S rDNA sequence data were analysed using BION-meta (www.box.com/bion), according to the author's instructions and in-house scripts. First, sequences were cleaned at both ends using a 99.5% minimum quality threshold for at least 18 of 20 bases for the 5′-end and 28 of 30 bases for 3′-end. The reads were then joined, followed by the removal of read pairs shorter than 150 bp. Then, the sequences were cleaned from chimeras and clustered using a 95% oligonucleotide similarity (k-mer length of 8 bp and a step size of 2 bp). Lastly, consensus reads were aligned to the SILVA reference 16S rDNA database (v123) using a word length of eight and similarity cut-off of 90%. Bacterial designation was analysed at different taxonomic levels down to the species when applicable.

Primers and probes

Denaturing gradient gel electrophoresis (DGGE), sequencing of DGGE amplicons and RT-PCR were performed with the primers and probes listed in supplementary Table S1 (with Supplementary material of Paper III).

RT-PCR

In order to establish a quantitative assay, plasmid standards were generated using the method described in Bartosch *et al.* (Bartosch *et al.* 2004). The amplified 16S rRNA gene region (amplified with specific primers) from *B. longum* DSM20219 and *L. acidophilus* ATCC4356 was cloned into chemically component E. coli JM109 cells using the pGEM-T vector system (Promega, Madison, WI, USA). Plasmids were purified with the NucleoSpin Plasmid QuickPure kit according to the manufacturer's instructions (Macherey-Nagel, Düren, Germany). Multiple dilutions of purified plasmids were quantified by spectrophotometry (NanoDrop ND-1000, Thermo Fisher Scientific, Waltham, MA, USA). Quantification of target DNA was achieved by using serial tenfold dilution from 105 to 101 plasmid copies of previously quantified plasmid standards.

Multiplex TaqMan assay PCR reactions were performed in a total volume of 25 µl using TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Each reaction included 5 µl of template DNA, 12.5 µl of TaqMan® Universal PCR Master Mix (Applied Biosystems), 400 nM of (F_allbif_IS and R_alllact_IS primers), 600 nM of (F_alllact_IS and R_alllact_ IS primers), 900 nM of (F_Eub and R_Eub primers) and 100 nM of both (P_alllact_IS, P_alllbif_IS) and 100 nM (P_Eub) of probes. The RT-PCR conditions consisted of an initial denaturation step at 50°C for 2 min and then 95°C for 10 min, continued with an amplification step followed by 40 cycles including denaturation at 95°C for 15 s, and an annealing-elongation step at 60°C for 1 min. Amplification and detection of DNA samples by RT-PCR were performed with a 7500 Fast Real-Time PCR System (Applied Biosystems Europe BV, Zug, Switzerland) using optical-grade 96-well plates. Data from triplicate samples were analysed using Sequence Detection Software version 1.6.3 (Applied Biosystems).

Denaturing gradient gel electrophoresis

PCR was performed in a reaction volume of 50 µl containing 25 µl 2X DreamTaq Hot Start PCR Master Mix (Thermo Fisher Scientific), 200 ng of a DNA solution and primers at a concentration of 10 µM. The DGGE cycling parameters were 5 min at 94°C, followed by 35 cycles of 30 s at 57°C (for primer Im-3 and Im-26), 30 s at 94°C, 30 s at 62°C (for primers Bif 164 and Bif 662+GC), and 45 s at 56°C (for primer Lac-1 and Lac-2+GC), and a final extension at 72°C for 10 min.

The DGGE analysis of PCR amplicons was performed using a DcodeTM System apparatus (Bio-Rad, Hercules, CA, USA). Polyacrylamide gel 8% [wt/vol], involving acrylamide-bisacrylamide [37.5:1] in 0.5X Tris-acetic acid-EDTA buffers with a denaturing gradient, was prepared with a gradient mixer and Econopump (Bio-Rad). Gradients from 30 to 60% were employed for the separation of the products amplified with specific primers for *Lactobacillus spp*. and from 45 to 60% for the products amplified with primers specific for *Bifidobacterium spp.*

Sequencing of DGGE amplicons

PCR amplicons (Bif164-r and Bif662-f) and (Lac-1 and Lac-2) were purified and concentrated with a QIAquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified amplicons were then cloned into the *E. coli* JM 109 strain using the pGEM-T vector system (Promega, Madison, WI, USA). Colonies of ampicillin-resistant transformants were randomly picked from each sample and were subjected to PCR with the pGEM-T specific primers T7 and SP6 (Supplementary Table S1, with Supplementary material of Paper III) from lyzed cells to check the size of inserts. Plasmid DNA from selected transformants was isolated using a QIAprep Spin Miniprep kit (Qiagen).

Sequencing reactions were performed using the BigDye Terminator CA v3.1 Cycle Sequencing kit (Applied Biosystem) according to the manufacturer's instructions. The sequences obtained were analysed using an automatic LI-COR DNA Sequencer 4000L (Licor, Lincoln, NE, USA) and were corrected manually. All of the sequences were thereafter identified using BLASTN and the NCBI nucleotide database.

4.3.4. Ethics (Papers I–III)

The study was conducted in accordance with the Declaration of Helsinki and local ethical committees approved this study accordingly. Written informed consent was obtained from all parents for the participation of their child in this study.

4.3.5. Statistical analysis (Papers I–III)

Statistical analysis was performed with the R software for Windows (The R Foundation for Statistical Computing, Vienna, Austria). The Shapiro-Wilk normality test was used to determine if the data had a normal distribution. A *p-value* less than 0.05 was considered significant.

Paper I
In Paper I the cumulative incidence of CD was calculated by dividing the number of new histologically proven CD cases by the number of the study population during the study period. The incidence rate was calculated by dividing the number of new histologically proven CD cases by the sum of the time each person was observed, totalled for all persons. For statistical analysis of the cumulative incidence, the Fisher's exact test or the Chi square test was applied in Paper I. Comparisons between the CD group and the control group were performed with the Mann-Whitney-Wilcoxon test in Paper I.

Paper II
To analyse differences in antibody levels between the CD and control groups, we used the paired t-test or the Wilcoxon signed-rank test. The paired t-test was used for comparison of norovirus and enterovirus IgA antibodies in the BC and for comparison of enterovirus IgG antibodies in both cohorts in cases where IgA-tTG was higher than 100 U/ml. The Wilcoxon signed-rank test was used for all other comparisons between the CD and control groups. Odds ratios (OR) were used to assess the risk of CD. Conditional logistic regression was applied to calculate adjusted ORs (aOR). The 95% confidence interval (CI) was used to estimate the precision of the aORs. Correlations between level of virus IgA or IgG antibodies and level of IgA-tTG were analysed with the non-parametric Spearman test. We compared antibody levels between the Estonian and Finnish children employing the Mann-Whitney-Wilcoxon test. To compare the number

of cases with antibodies or viruses between the different study groups, the Fisher's exact test or the chi-square test was applied.

Paper III

Comparison between the CD group and the control group was made using the t-test for normally distributed data and with the Mann-Whitney-Wilcoxon test for data with a skewed distribution. To compare the number of positive cases between groups, the Fisher's exact test was used. Correlations were analysed with a non-parametric Spearman's rank-order correlation test. Alpha diversity was based on the Shannon index of OTU level. Analysis of the beta-diversity of the CD and control groups, including principal coordinate analysis (PCoA) of weighted UniFrac distances, was performed and visualized using the PAST 4.0 version.

5. RESULTS

5.1. General characteristics of the children who developed coeliac disease and their matched controls (Paper I)

During the DIABIMMUNE Study period 29 children developed CD: nine children (two Estonian and seven Finnish children) in the BC and 20 children (six Estonian and 14 Finnish children) in the YCC. None of them had IgA deficiency and all 29 were positive for IgA-tTG and EMA. In addition, two Finnish children in the YCC tested positive for both IgA-tTG and EMA, however, the small intestine biopsy did not confirm the diagnosis of CD. All children with CD and those of the control group had been born full-term (gestational age between 37 and 42 weeks). Twenty-three children with CD (79%) were autumn-, winter- or spring-born (from September to May). Five children in the CD group (17%) and six children in the control group (20%) had been delivered by Caesarean section, while all the remaining children had been born vaginally. There were no statistical differences in mean birth weight either between the CD and the control groups in the BC (3632 g versus 3695 g) or the YCC (3520 g versus 3734 g), or between the Estonian and Finnish children. There were altogether more girls than boys with CD (19 versus 10, p=0.04). When looking study groups separately, there were more girls than boys with CD in the YCC (15 versus five), but not in the BC (four versus five) (p=0.20). In the CD group, 28 children carried the DR3-DQ2 haplotype (97%) and one child in the YCC carried the DR4-DQ8 haplotype. The mean age at initial seroconversion to IgA-tTG positivity for the BC was 3.0 (95% CI 2.5–3.6) years in Estonia and 2.3 (95% CI 1.6–2.9) years in Finland (p=0.12). In the YCC, five out of six Estonian children (83%) and eleven out of fourteen Finnish children (79%) showed positive IgA-tTG at their first visit at the age of 3 years. There was no statistically significant difference in mean IgA-tTG level at the time of the diagnosis between the Estonian and Finnish children with CD either in the BC (68.5 EliA U/ml (95% CI 0–608.5) versus 261.2 EliA U/ml (95% CI 0–619.1), p=0.67) or in the YCC (1045.9 EliA U/ml (95% CI 0–2749.9) versus 812.8 EliA U/ml (95% CI 87.8–1537.8), p=0.78). The IgA-tTG level of 18 out of 29 children with CD was more than 10 times as high as the cut-off level for antibody positivity (>100 EliA U/ml). This was seen in 63% of the Estonian and in 62% of the Finnish children with CD.

5.2. Cumulative incidence of coeliac disease (Paper I)

The cumulative incidence of CD in the two countries was not significantly different for the BC or for the YCC during the study period. For the BC, cumulative incidence was 0.12% (95% CI 0.02–0.49) in Estonia and 0.53% (95% CI 0.23–1.13) in Finland (p=0.10) (incidence rate 0.4 cases per 1000 person-years and 1.8 cases per 1000 person-years, respectively), while for the YCC it was

0.4% (95% CI 0.18–1.0) and 1% (95% CI 0.58–1.7) (incidence rate 2.2 cases per 1000 person-years and 5.1 cases per 1000 person-years, respectively), (p=0.12). However, when the cohorts were combined the cumulative incidence of childhood CD was significantly higher in Finland than in Estonia (0.77% versus 0.27%; p=0.01) (incidence rate 1.0 cases per 1000 person-years and 3.1 cases per 1000 person-years, respectively).

5.3. Association of environmental risk factors with coeliac disease (early feeding, infections, antibiotics, vaccinations) (Paper I)

In the BC, information about the duration of breastfeeding in weeks was available for all children, while for the YCC this information (in months) was available for 18 out of the 20 children (Table 3). There was no statistical difference in the total duration of breastfeeding between children with CD and control children of the BC or the YCC (Table 3). Nor was there any significant difference in the duration of exclusive breastfeeding between the groups in the BC. In the BC, the age at which wheat, barley or rye was introduced was not statistically different between the CD and the control groups (Table 4). The mean age when complementary food was introduced was similar in both groups (4.4 months). For the YCC, information about age at the introduction of cereal or complementary food was not collected.

Table 3. Mean duration of breastfeeding for the study subjects (BC – birth cohort, YCC – young children's cohort, CD – coeliac disease, N – number of children with available data).

	N	Mean duration	Minimum and maximum	95% CI	p-value
BC CD	9	35.7 weeks	6 ... 96 weeks	13.9–57.4	0.45
BC Control	9	43.7 weeks	1 ... 84 weeks	24.6–62.7	
YCC CD	18	8.5 months	0 ... 24 months	5.7–11.4	0.12
YCC Control	18	11.6 months	2 ... 22 months	8.9–11.4	

Table 4. Mean age (months) of the study subjects in the birth cohort at the time of cereal introduction (N – number of children with available data).

	CD (N)	95% CI	Control (N)	95% CI	p-value
Wheat	6.4 (9)	5.6–7.2	5.8 (8)	4.9–6.7	0.28
Barley	6.3 (8)	5.5–7.2	5.6 (8)	4.8–6.5	0.20
Rye	7.4 (7)	5.8–8.9	5.9 (8)	4.7–7.1	0.10

In the BC, children with CD had significantly more episodes of infections with fever by the age of 12 months compared to controls (3.4 versus 1.4; p=0.04). During the following months these differences remained, but were statistically not significant (Figure 3). In the BC there was no significant difference in the number of episodes of gastroenteritis between the CD and the control groups during the first 3 years of life (1 versus 0.9, p=0.82). No difference was seen in the use of antibiotics between the groups, either.

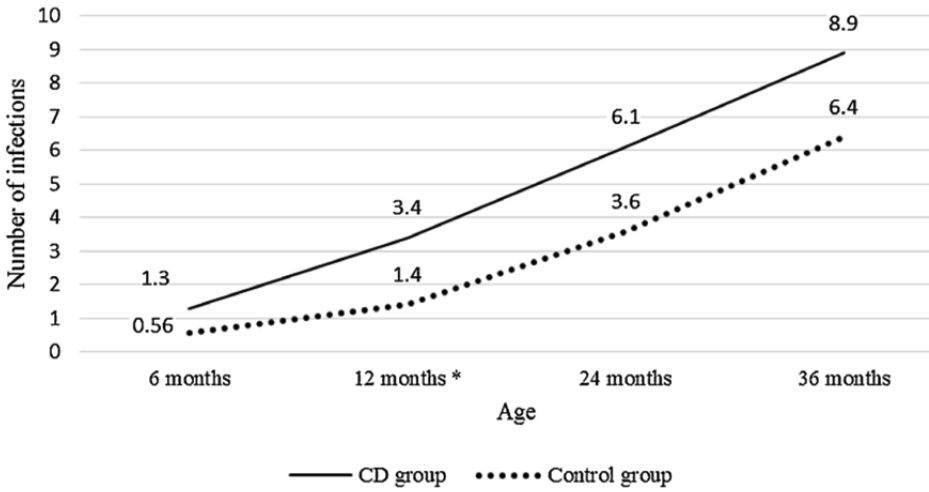

Figure 3. Mean cumulative number of infection episodes with fever during the study period for the birth control subjects (* – statistically significant difference).

For the YCC, there were no statistical differences in the number of infection episodes with fever (2.2 versus 3.3) or antibiotic therapy (1.1 versus 1.0) between the groups. Data for the first 3 years of life was collected retrospectively. By the age of 3 years children both in the CD group and in the control group had had 3.2 episodes of otitis (p=0.69) and 0.5 and 0.8 episodes of gastroenteritis, respectively (p=0.73).

For the BC and the YCC, all children in both groups were at least partly vaccinated according to the local vaccination schedule, except for one Finnish control child in the BC, and one Finnish child with CD and one Finnish control child in the YCC, who were not vaccinated.

5.4. Laboratory confirmed viral infections (Paper II)

5.4.1 Viruses in stool and nasal swab samples

In the BC, 13/120 (11%) of the serial stool samples from children who developed CD were virus positive, compared to 14/120 (12%) of the stool samples from controls (p=1.0). For the cases, 5/43 (12%) nasal swabs were virus positive compared to none of the 36 for the controls (p=0.06). At least one viral agent was detected in either the stool or nasal swab samples of 7/9 (78%) children in the CD group and in 6/9 (67%) children in the control group (p=1.0). Rhinovirus was the one that was detected most frequently in both stool and nasal swab samples. At the age of 3 and 6 months, 8/33 (24%) samples from children with CD and 7/32 (22%) samples from control children contained some rhinovirus when stool and nasal swab samples were combined. There were 5/18 (28%) rhinovirus positive nasal swabs among the cases, but none among the 16 controls (p=0.05). In stool samples, the respective figures were 3/15 (20%) and 7/16 (44%) (p=0.25). Enterovirus and parechovirus were not detected in any sample (Table 5).

Table 5. Virus test results in stool and nasal swab samples in the birth cohort at the age of 3 and 6 months (En – enterovirus, Pa – parechovirus, Rh – rhinovirus, NoV – norovirus, CD – coeliac disease).

		Nasal swab samples at the age of 3 months			**Stool samples at the age of 3 months**				**Nasal swab samples at the age of 6 months**			**Stool samples at the age of 6 months**			
		En	Pa	Rh	En	Pa	NoV	Rh	En	Pa	Rh	En	Pa	NoV	Rh
Proportion of positive cases	**CD**	0/9	0/3	3/9	0/8	0/8	2/8	2/8	0/9	0/4	2/9	0/7	0/7	1/7	1/7
	Control	0/8	0/2	0/8	0/8	0/8	1/8	4/8	0/8	0/2	0/8	0/8	0/8	1/8	3/8

5.4.2. Differences in viral antibodies between the coeliac disease and control group

There were no significant differences in the level of viral antibodies or the number of positive cases between the case and control children in the BC or the YCC, or when the two cohorts were combined (Figure 4). When the norovirus, adenovirus and enterovirus IgA positive cases were combined in the BC, the case children had a mean of 1.6 infections and the controls had a mean of 1.2 infections (p=0.55). The mean number of positive tests for IgG antibodies to these viruses was 1.4 and 1.0 (p=0.33), respectively. In the YCC the mean number of positive tests did not differ between the CD and control groups (1.7 versus 1.8 for IgA, p=0.67, and 1.5 versus 1.7 for IgG, p=0.38). There was no difference in the CMV and EBV IgG antibodies between the groups. When the aOR and 95% CI were used none of the measured virus antibodies affected the risk of CD in the BC, in the YCC or in the combined cohort (Table 6). There was no association between the level of virus IgA or IgG antibodies and the level of IgA-tTG (Table 7).

When we only took CD cases into account, where IgA-tTG was higher than 100 U/ml, there were no differences in viral antibodies between the CD and the control groups (data not shown).

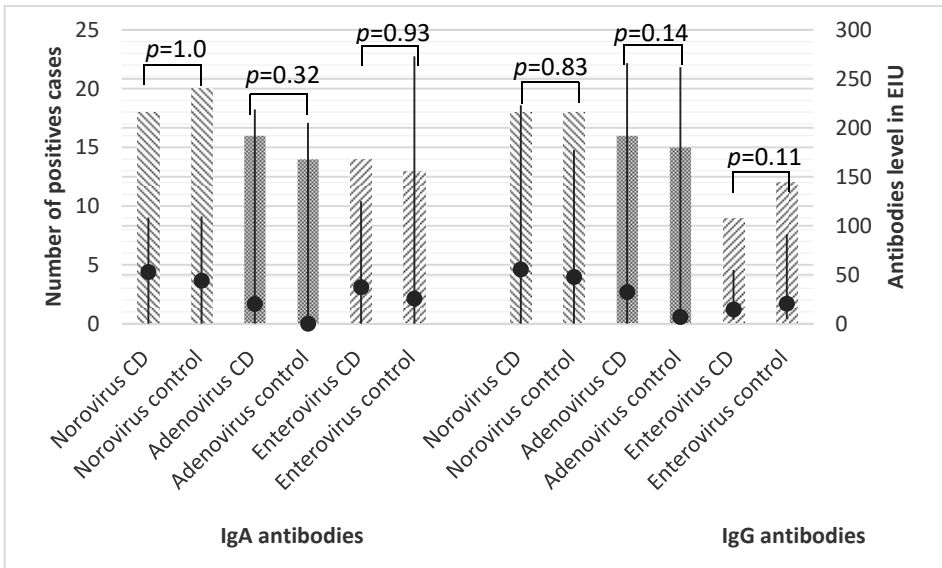

Figure 4. Number of positive cases and the median (shown as a dot), minimum (minimum point of the line) and maximum (maximum point of the line) levels of IgA and IgG antibodies in the coeliac disease and control groups (CD – child with coeliac disease).

Table 6. The risk of CD in the BC, in the YCC or in the combined cohort.

Norovirus IgA	**OR**	**95%CI**
BC	1.0	0.16 to 6.42
YCC	0.62	0.16 to 2.43
BC+YCC	0.74	0.25 to 2.18
Norovirus IgG		
BC	2.5	0.37 to 16.89
YCC	0.62	0.16 to 2.43
BC+YCC	1.0	0.35 to 2.89
Adenovirus IgA		
BC	4.38	0.56 to 33.95
YCC	0.81	0.23 to 2.86
BC+YCC	1.32	0.47 to 3.70
Adenovirus IgG		
BC	2.5	0.37 to 16.89
YCC	0.81	0.23 to 2.86
BC+YCC	1.15	0.41 to 3.23
Enterovirus IgA		
BC	1.0	0.16 to 6.42
YCC	1.22	0.35 to 4.24
BC+YCC	1.15	0.41 to 3.23
Enterovirus IgG		
BC	1.0	0.14 to 7.1
YCC	0.52	0.14 to 1.92
BC+YCC	0.64	0.22 to 1.88
CMV IgG		
BC	0.25	0.02 to 3.04
YCC	2.25	0.63 to 7.97
BC+YCC	1.33	0.47 to 3.79
EBV IgG		
BC	3.35	0.12 to 93.84
YCC	1.0	0.18 to 5.67
BC+YCC	1.39	0.28 to 6.83

Table 7. Association between the level of virus IgA or IgG antibodies and the level of IgA-tTG.

	Rho	***p*-value**
IgA-tTG and norovirus IgA	0.13	0.32
IgA-tTG and norovirus IgG	0.04	0.75
IgA-tTG and adenovirus IgA	0.14	0.29
IgA-tTG and adenovirus IgG	0.17	0.2
IgA-tTG and enterovirus IgA	0.03	0.81
IgA-tTG and enterovirus IgG	-0.16	0.22

5.4.3. Differences in viral antibodies between Estonia and Finland

The study included 42 children from Finland and 16 children from Estonia. When the different virus antibodies were compared between the two countries, enterovirus IgG class antibodies were found to be much more frequent in Estonian children (63% versus 23%, p=0.02). A comparable difference was seen in average enterovirus IgG antibody levels (34.5 EIU versus 19.0 EIU, p=0.01) (Figure 5). A similar, but non-significant, trend was also seen in enterovirus IgA antibodies (56% versus 38%, p=0.54 and 61.2 EIU versus 35.3 EIU, p=0.31). The other virus antibodies did not differ between the countries.

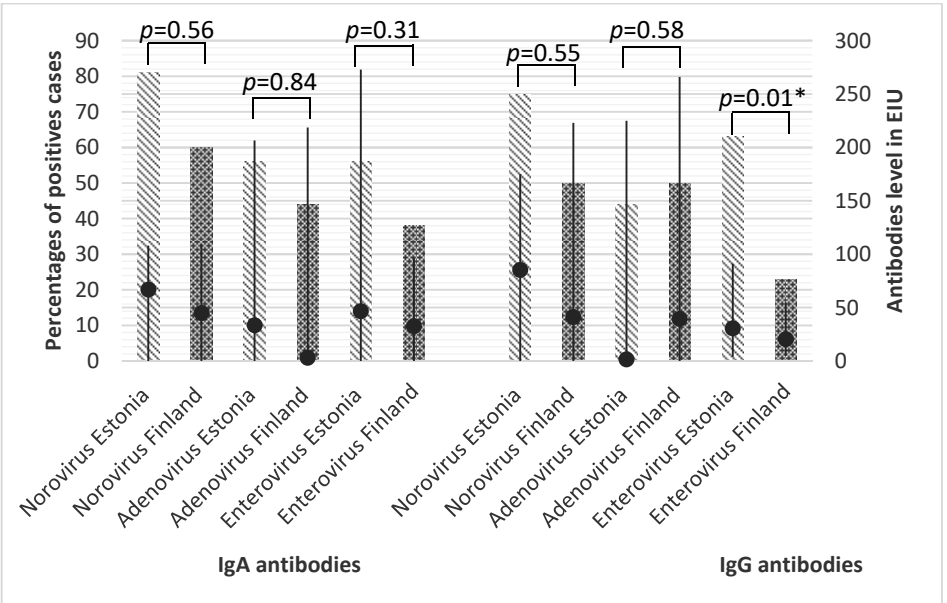

Figure 5. Number of positive cases and the median (shown as a dot), minimum (minimum point of the line) and maximum (maximum point of the line) levels of IgA and IgG antibodies between Estonian and Finnish children (* – statistically significant difference in average antibody levels).

5.5. Maternal breast milk microbiota and immune markers (Paper III)

5.5.1. General characterization of breast milk samples

Five children in the CD group were exclusively breastfed, while one child was mostly bottle-fed during the time of breast milk collection. The respective figures for the control group were 15 and 3. One CD patient and his controls were from Estonia, while all others were from Finland. The proportions of boys and

girls were equal in the CD and control groups. All offspring were HLA DQ 2.5 positive (two CD patients were HLA DQ 2.5 homozygotes). Each infant was full-term (gestational age 37–42 weeks) and born as a singleton. The mothers of the CD group were younger than the mothers of the control group (26 versus 32.8 years, p=0.002). None of the mothers or fathers of the offspring had CD. The mean age was 2.4 years (range 1.5–3.0 years) at seroconversion to positivity for IgA-tTG in the CD group. The general characteristics of the children of the CD group and the control group are summarized in Table 8.

Table 8. General characteristics of the coeliac disease (CD) group and the control group in breast milk studies (* - data missing for one child; ** p=0.002)

Characteristics	CD group (n=6)	Control group (n=18)
Sex:		
Male (%)	3 (50%)	9 (50%)
Female (%)	3 (50%)	9 (50%)
Birth weight (g) mean, (range)	3703 (3200–4075)	3661 (2700–4530)
Birth length (cm) mean, (range)	50.8 (48–53.5)	51.3 (48–54.5)*
Offspring HLA status		
HLA DQ 2.5 homozygotes (%)	2 (33%)	4 (22%)
HLA DQ 2.5 (%)	4 (67%)	14 (78%)
Mode of delivery:		
Vaginal (%)	6 (100%)	15 (83%)
Caesarean (%)	0	3 (17%)
Mean maternal age in years at delivery (range)	26 (23–30)**	32.8 (23–42)**

5.5.2. Immune markers in breast milk from mothers of coeliac disease and control offspring

There were no significant differences between TGF-β1, TGF-β2, sIgA, MFG-E8 or sCD14 levels in the breast milk from mothers in the CD group and the breast milk from mothers in the control group (Table 9).

Table 9. Immune markers in breast milk from mothers of coeliac disease (CD) and control children (median, quartiles Q1, Q3).

Immune marker	CD group	Control group	*p-value*
TGF-β1 (pg/ml)	102.3 (15, 229.6)	157.5 (59.73, 270.0)	0.42
TGF-β2 (pg/ml)	1603 (1170, 3516)	1817 (1176, 2463)	0.97
sIgA (mg/ml)	0.69 (0.05, 1.68)	0.80 (0.05, 1.45)	0.82
MFG-E8 (mg/ml)	0.014 (0.009, 0.027)	0.013 (0.008, 0.017)	0.69
sCD14 (pg/ml)	232200 (46940, 1118000)	323900 (0, 1285000)	0.92

5.5.3. Microbiome of maternal breast milk

Illumina sequencing of the 16S *r*RNA V4 region was applied to reveal the full microbiome of these investigated samples. A total of 6,475,320 high quality reads were obtained, or 269,805 ± 239,377 reads per milk sample. The OTUs were classified into known taxa (7 phyla, 17 classes, 44 genera, and more than 90 species) and unclassified groups.

Phylotype abundance and Shannon 'H' diversity index were significantly higher in milk samples from the mothers of the CD group than in maternal samples of the control group (p=0.016; p=0.008, respectively) (Table 10).

Table 10. Average number (± SD) of sequences, phylotype abundance (OTUs) and Shannon 'H' diversity index in the samples analysed in the coeliac disease (CD) and control groups ($^{*}p$=0.016; $^{**}p$=0.008)

Samples	Number of reads	Phylotype abundance (OTUs)	Shannon 'H' index (diversity)
CD group	334,403 ± 410,283	276.6 ± 28.6*	3.80 ± 0.41**
Control group	248,272 ± 161,407	219.5 ± 65.6*	2.88 ± 1.03**

A PCoA plot based on different taxonomic levels (phylum, class and genus) was generated to assess the relationships between the community structures of these samples. Phylum and class abundance data indicated significant inter-individual variability (Figure S1A and S1B, with Supplementary material of Paper III), while the PCoA plot of relative genus abundance demonstrated weighted clustering in the CD group (Figure S1C, with Supplementary material of Paper III).

Phyla

In both study groups, the phylum *Firmicutes* displayed the highest relative abundance (medians of 48.9% and 60.2% for the CD group and the control group, respectively) (Figure 6, Supplementary Table S2, with Supplementary material of Paper III). In addition, *Proteobacteria* and *Actinobacteria* were also quite abundant (median 29.0% and 9.8% for the CD group *vs*. 23.6% and 8.1% for the control group). Borderline statistical significance was detected for the relative abundance of *Bacteroidetes* and *Fusobacterium* phyla (p = 0.056 and p = 0.048; Figure 6, Supplementary Table S2, with Supplementary material of Paper III).

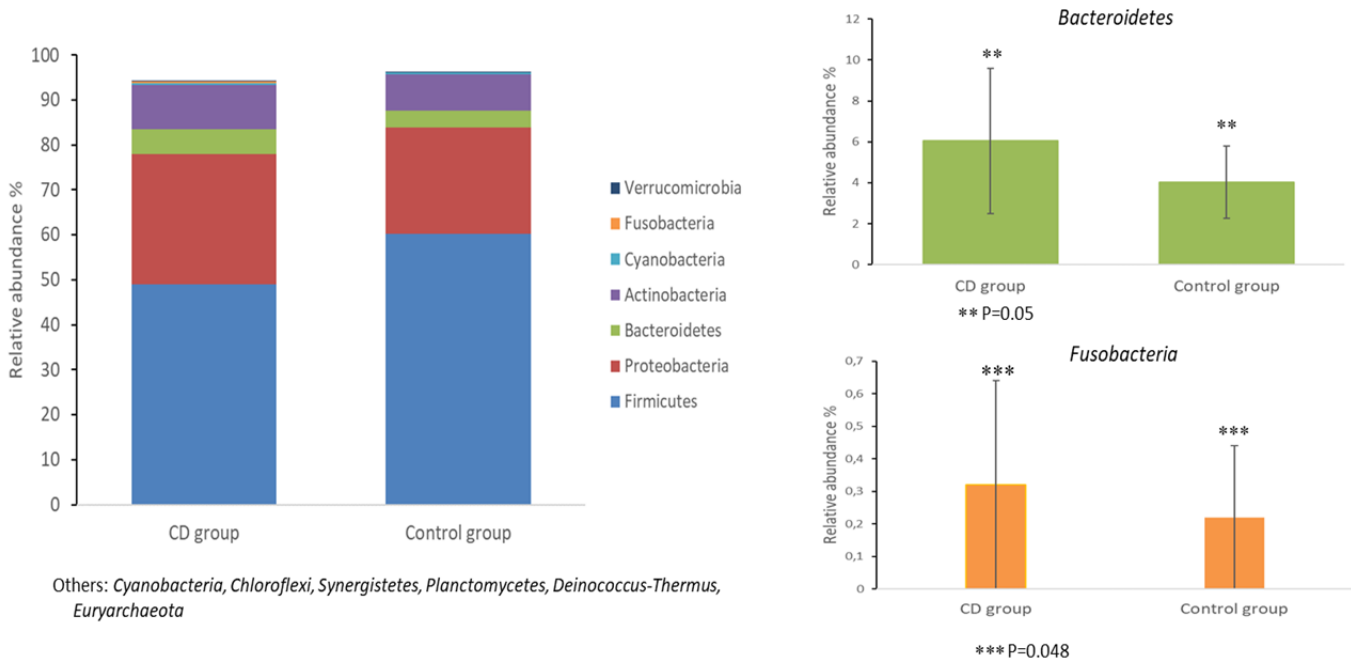

Figure 6. Relative abundance (median) of the most frequent phyla of microbial communities in maternal breast milk samples (coeliac disease (CD) group versus control group).

Classes

At the class level, *Bacilli* displayed the highest relative abundance both in the CD group (median 30.9%) and in the control group (median 56.1%), followed by *Gammaproteobacteria* (20.8% and 14.5%), *Actinobacteria* (8.9% and 8%) and *Alphaproteobacteria* (4% and 3.7%) (Figure 7, Supplementary Table S3, with Supplementary material of Paper III). Statistically significant differences were only found for the relative abundance of the classes *Clostridium* (p=0.02) and *Fusobacteriia* (p=0.05) (Figure 7, Supplementary Table S3, with Supplementary material of Paper III).

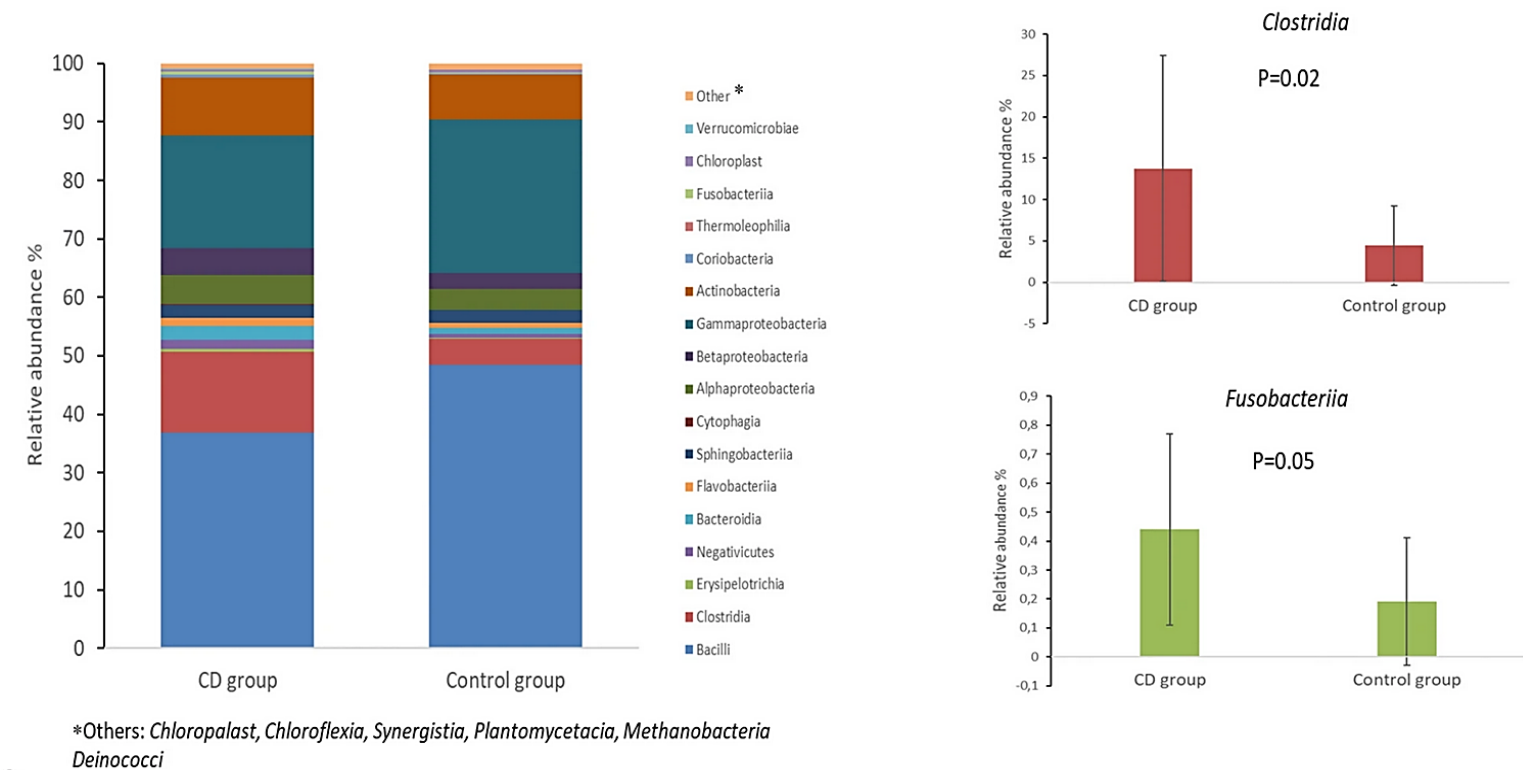

Figure 7. Relative abundance (median) of the most frequent classes of microbial communities in maternal breast milk samples (coeliac disease (CD) versus control group).

Genera

The most abundant genera of bacteria both in the CD and the control groups was *Lactobacillus* (median 10.3% and 34%, respectively), followed by *Streptococcus* (10.9% and 7.5%), *Staphylococcus* (4.9% and 4.9%), *Buttiauxella (*4.8% and 4.4%), and *Rothia* (2.8% and 2.6%) (Figure 8, Supplementary Table S4, with Supplementary material of Paper III). The prevalence of the genera *Anaerococcus* (p=0.006), *Shpingomonas* (p=0.01), *Actinomyces* (p=0.006), *Leptotrichia* (p=0.005), and *Akkermansia* (p=0.01) were significantly higher in the CD group (Supplementary Table S4, with Supplementary material of Paper III). Moreover, the relative abundances of *Sphingomonas* (p=0.04) and *Akkermansia* (p=0.05) were significantly higher in the CD group (Figure 8, Supplementary Table S4, with Supplementary material of Paper III).

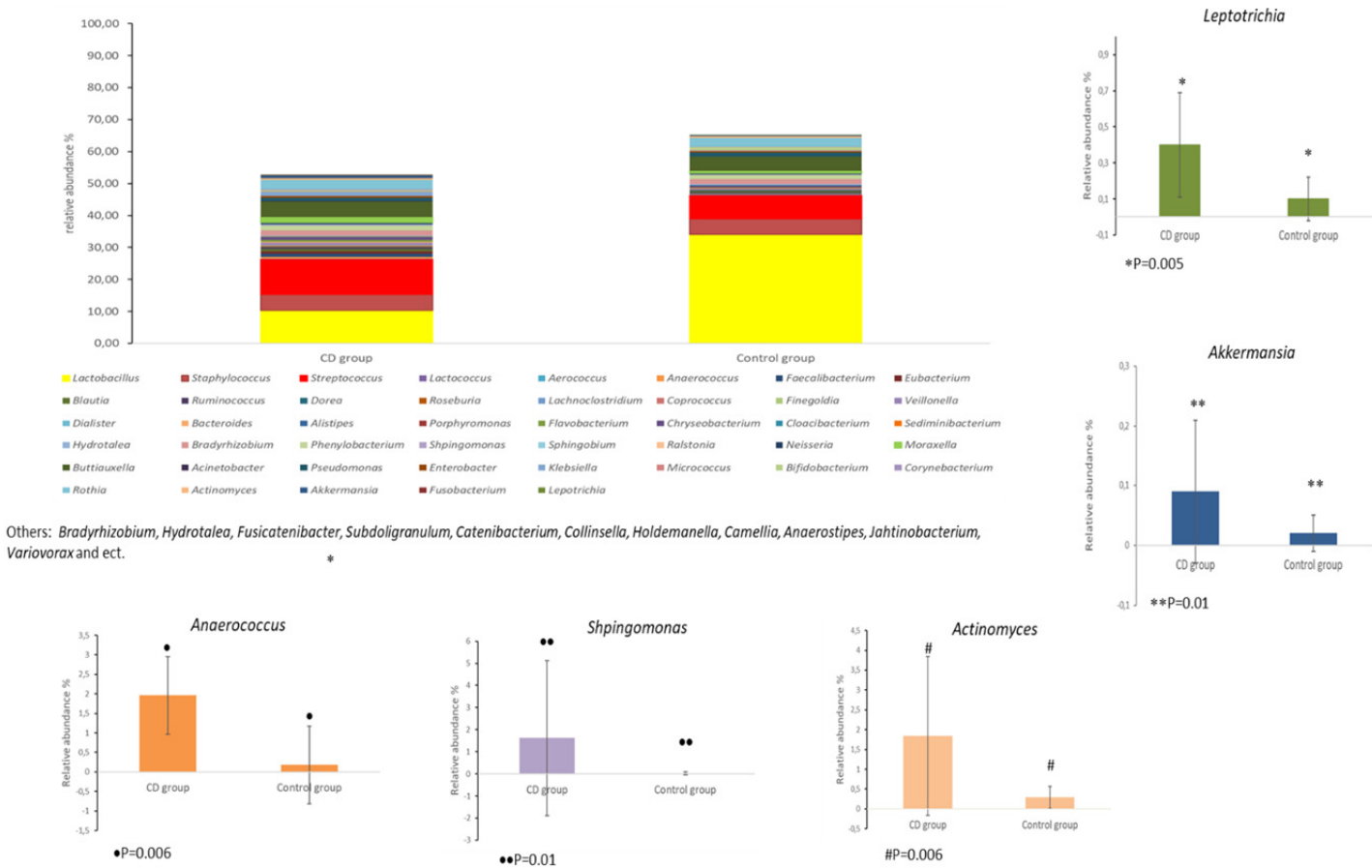

Figure 8. Relative abundance (median) of the most frequent genera of microbial communities in maternal breast milk samples (coeliac disease (CD) versus control group).

Species

The most abundant species in the CD group versus the control group were *Actinomyces odontolyticus* (p=0.03), *Anaerococcus hydrogenalis* (p=0.04), and *A. octavius* (p=0.01) (Supplementary Table S5, with Supplementary material of Paper III). The prevalence of *A. muciniphilia* was also increased in the CD group (p=0.002). It is noteworthy that milk samples from the control group had lower relative abundance of *Faecalibacterium prausnitzii* (p=0.049) (Supplementary Table S5, with Supplementary material of Paper III), while unclassified *Leptotrichia* and *Akkermansia* were detected only in the CD group. *Lactobacillus salivarius* (83 and 78%) and *Bifidobacterium animalis* (both 50%) were the most prevalent species among the lactic acid bacteria in both study groups, although their mean counts were less than 1% (Supplementary Table S5, with Supplementary material of Paper III).

A total of nineteen *Lactobacillus* species (*L. plantarum*, *L. curvatus*, *L. iners, L. mucosae*, *L. casei, L. fermentum, L. salivarius, L. reuteri, L. rhamnosus, L. crispatus, L. hominis, L. sakei, L. zeae, L. jensenii, L. senmaizukei, L. paracasei, L. oligofermentas, L. gasseri, and L. acidophilus*) (Figure 9B) and four species of *Bifidobacterium* (*B. bifidum*, *B. animalis*, *B. adolescentis*, and *B. longum*) were detected by whole genome sequencing (Figure 10B). Additionally, *L. johhansonii* (Figure 9C) and *B. pseudocatenulatum, B. infantis and B. breve* were detected using DGGE (Figure 10C, Table S9, with Supplementary material of Paper III).

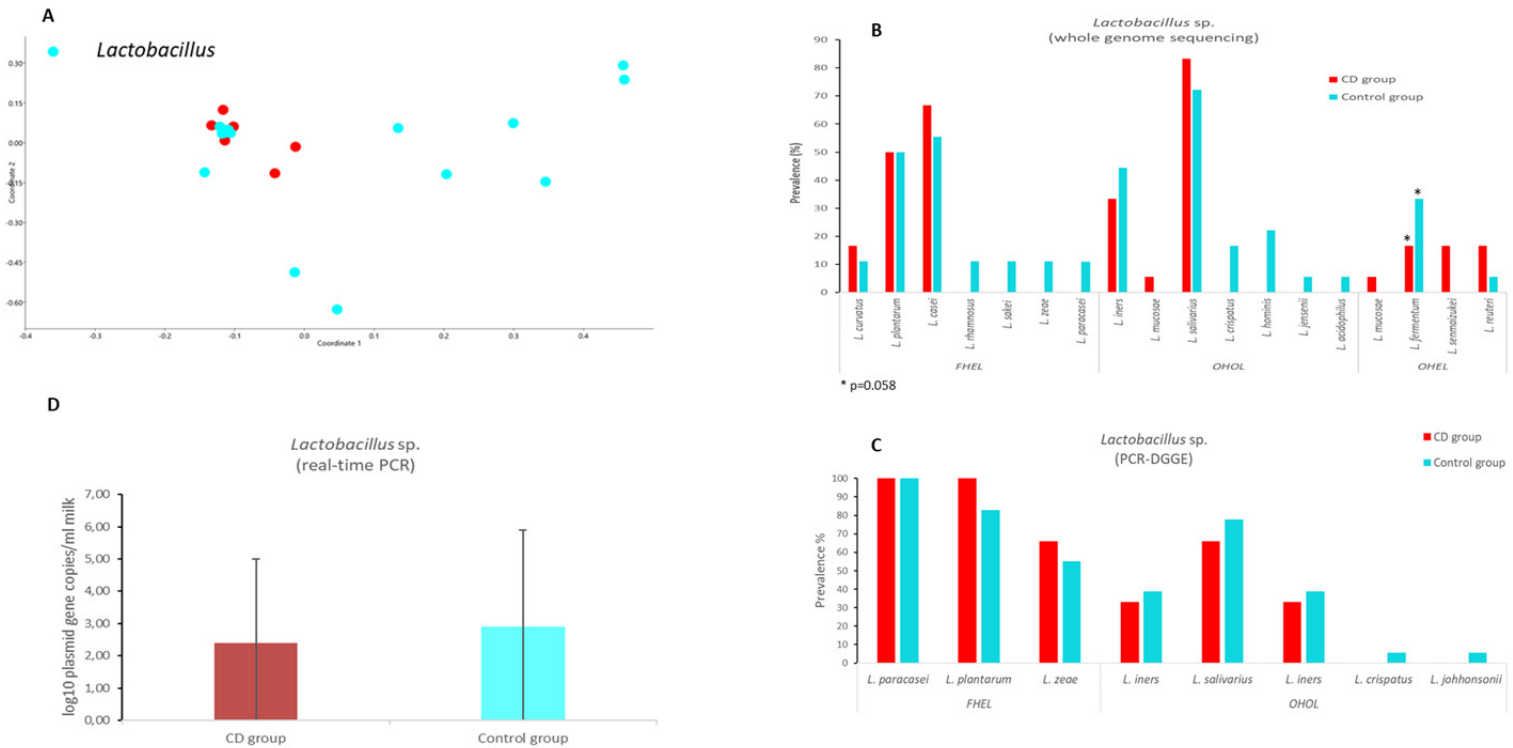

Figure 9. (A) Principal coordinate analysis (PCoA) of the lactobacilli communities in the studied samples. The plot demonstrates different clustering of different breast milk specimens (CD group [red] versus control group [blue]). Prevalence (%) of different *Lactobacillus* sp. in breast milk samples according to whole genome sequencing (B) and denaturing gradient gel-electrophoresis (C). FHEL, facultative heterofermentative lactobacilli; OHOL, obligate homofermentative lactobacilli; OHEL, obligate heterofermentative lactobacilli. (D) Total counts (log10plasmid gene copies/ml milk) of *Lactobacillus* sp. in breast milk samples from the CD and control groups by real-time PCR (mean±SD).

The PCoA plot of relative abundance data of *Lactobacillus* and *Bifidobacterium* species indicated quite different results, with weighted clustering of *Lactobacillus* in the CD group (Figure 9A) and *Bifidobacterium* in the control group (Figure 10A). No differences were found between the groups in quantitative counts for *Lactobacillus* or *Bifidobacterium* (Figure 9D, 10D; Table S9, with Supplementary material of Paper III). Milk samples from the CD group showed borderline significance for the lower relative abundance of *L. fermentum* compared to the control group (p=0.058, Supplementary Table S5, with Supplementary material of Paper III).

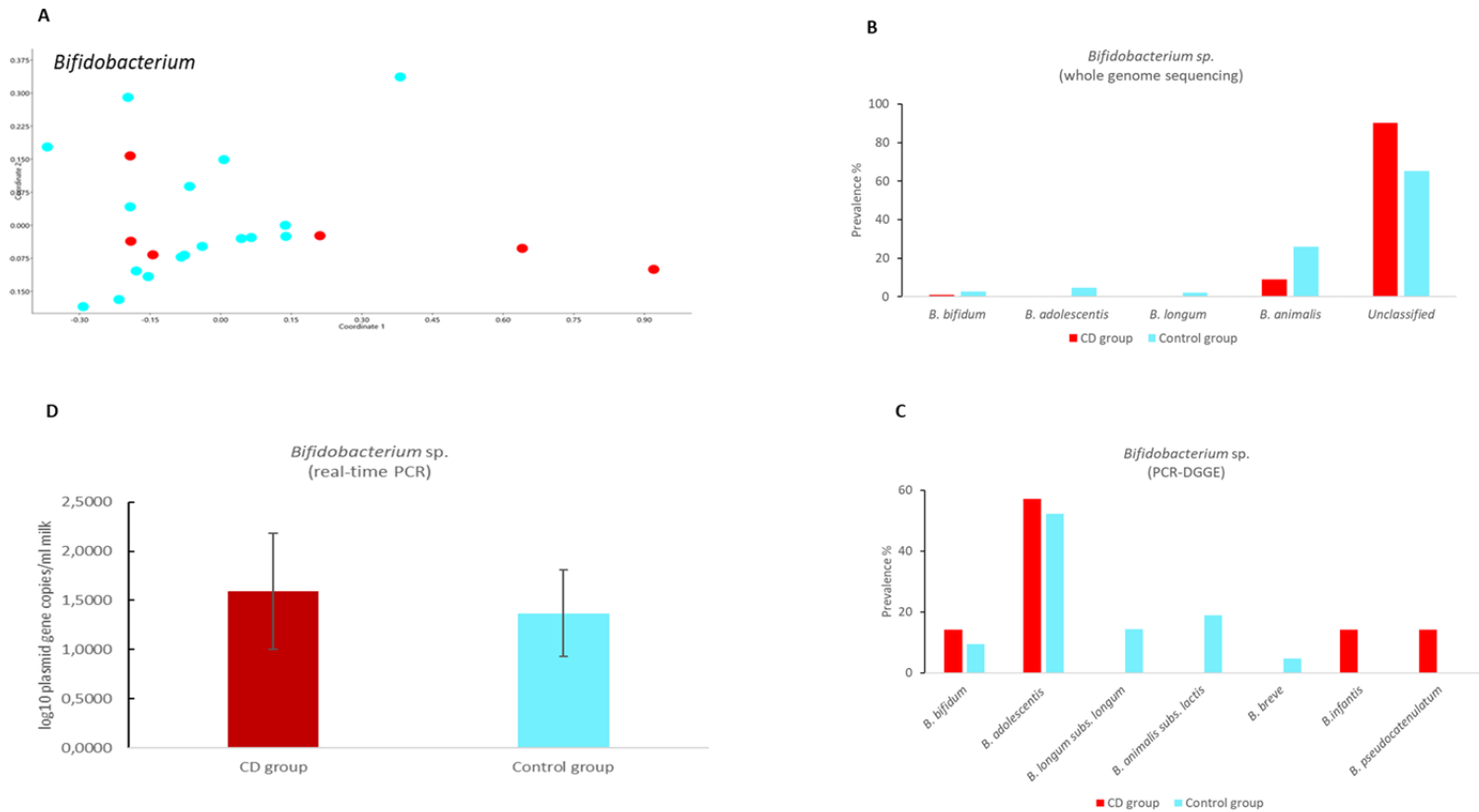

Figure 10. (A) Principal coordinate analysis (PCoA) of the bifidobacteria communities in the analysed samples. The plot demonstrates clustering of different breast milk specimens (CD group [red] versus control group [blue]). Prevalence (%) of different *Bifidobacterium* sp. in breast milk samples according to whole genome sequencing (B) and denaturing gradient gel-electrophoresis (C). (D) Total counts (log10plasmid gene copies/ml milk) of *Bifidobacterium* sp. in breast milk samples from the CD and control groups by real-time PCR (mean±SD).

5.5.4. Associations between breast milk microbiota and immune markers

A. muciniphila showed positive association with TGF-β1 (p=0.04) and TGF-β2 (p=0.007) (Table 11). Higher counts of the genera *Chryseobacterium* (p=0.04) and *Sphingobium* (p=0.01) were seen in milk samples with lower TGF-β2 levels. MFG-E8 correlated positively with bacterial classes *Flavobacteriia* (p=0.03) and *Bacilli* (p=0.05) and inversely with species *B. animalis* (p=0.03). In addition, species *L. reuteri* was positively (p=0.04) and *B. animalis* was inversely (p=0.02) correlated with sCD14 levels (Table 11). All the correlation coefficients are presented in Supplementary Tables S6–S8, with Supplementary material of Paper III.

Table 11. Spearman's rank-order correlations between bacteria present in human milk and immune markers.

Bacteria	Immune marker	R^2	*p*-value
A. muciniphila (species)	TGF-β1	0.43	0.04
Chryseobacterium (genus)	TGF-β2	-0.42	0.04
Sphingobium (genus)		-0.51	0.01
A. muciniphila (species)		0.54	0.007
Flavobacteriia (class)	MFG-E8	0.45	0.03
Bacilli (class)		0.40	0.05
B. animalis (species)		-0.44	0.03
L. reuteri (species)	sCD14	0.43	0.04
B. animalis (species)		-0.47	0.02

6. DISCUSSION

6.1. Differences in the cumulative incidence of coeliac disease and antibody positivity between Estonia and Finland (Paper I)

Previous studies have revealed significant unexplained differences in the prevalence of adulthood CD across Europe (Mustalahti *et al*. 2010). Some differences have been detected also in the prevalence of childhood CD between European countries (Kondrashova *et al.* 2008; Myléus *et al.* 2009; Laass *et al.* 2015). In the DIABIMMUNE Study the same strategy for two adjacent countries was used to identify new patients with CD and the obtained results are well comparable. Since the antibody assays for CD screening were performed in a single laboratory, the theoretical likelihood of revealing new cases was equal for both populations. Using this data, we found that the cumulative incidence of CD in children from birth to the age of 5 years was almost three times lower in Estonia than in Finland (0.27% vs 0.77%). A similar difference between Estonia and Finland has also been noted for another immune-mediated disease, T1D (Karvonen *et al.* 2006).

Additionally, the Finnish children of the BC tended to develop positive tTG antibodies earlier compared to their Estonian peers. The IgA-tTG became positive at a mean age of 3.0 years in Estonia and at 2.3 years in Finland. Although no earlier studies have compared CD-associated autoantibodies between Estonia and Finland, it has been found that anti-reticulin autoantibody frequency is lower for Estonian young adults compared to Swedish young adults (Uibo *et al.* 1998). Reticulin antibodies are directed against the reticular fibres of the endomysium, a layer of connective tissue which sheathes smooth muscle fibres (Nandiwada *et al.* 2013). These antibodies are less reliable in diagnosing CD compared to other autoantibodies (Lock *et al.* 1999). It is known that Sweden is a country with an economic and social background similar to that of Finland and thereby until the early 1990s there were also marked differences in the socio-economic background between Estonia and Sweden.

The above findings support the lower prevalence of autoimmunity and autoimmune diseases in Estonia compared to the Nordic countries. The potential reasons for this may be related to different environmental factors, since differences in the risk associated genetic factors between the two populations can hardly explain the difference. The frequency of the major CD risk haplotype was actually slightly higher among the Estonian than among the Finnish population (Nejentsev *et al.* 1998).

6.2. Environmental risk factors of coeliac disease (Paper I)

As the incidence of childhood CD for the two countries and earlier antibody development in Finland cannot be explained with genetics alone, it is evident that several environmental factors must be involved here. Of the greatest interest for us were early feeding and early infections.

The earlier ESPGHAN recommendations and the European Food Safety Authority recommended avoiding gluten introduction to children less than 4 months of age and more than 7 months of age and introducing gluten while the infant is still breastfed (Agostoni *et al.* 2008, EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)). According to the American Academy of Pediatrics, complementary food should be introduced between 4 and 6 months of age (Kleinman, 2009). In the light of recent studies these recommendations have been reviewed (Lionetti *et al.* 2014; Vriezinga *et al.* 2014; Szajewska *et al.* 2015; Szajewska *et al.* 2016). According to current recommendations in Estonia and Finland, complementary food should be introduced when the infant has reached the age of 6 months, although small tasting portions may be given after the age of 4 months. In our study we did not note any significant difference in the mean age at the time of cereal introduction between the CD and the control groups. Nor was there any difference in the total duration of breastfeeding between children with CD and control children either in the BC or in the YCC. These findings support the data that differences in early feeding may not be an important risk factor in modulating the development of CD.

There are important risk factors that may be associated with differences in cereal consumption and in the quantity and character of ingested gluten or gluten-related prolamines. Unfortunately, we could not calculate exact cereal consumption or evaluate the quantity of gluten consumed by the children of our study population.

Previous epidemiological studies have suggested that neonatal infections and infections during the first years of life may increase the risk of CD (Stene *et al.* 2006; Myléus *et al.* 2012). Myléus *et al.* reported that three or more infectious episodes during the first 6 months increased the risk of CD later in life (Myléus *et al.* 2012), while the type of infection was irrelevant. Also a more recent prospective large-scale population-based cohort study by Mårild *et al.* (Mårild *et al.* 2015) found that early life infections may play a role in development of CD. Our study supports this finding. In the BC there were more infections with fever in the CD group. Like in a study by Myléus *et al.* (Myléus *et al.* 2012), in our study the episodes of infections were reported by the parents. In the YCC the number of infections did not differ between children with CD and controls. However, in the YCC the parents were asked to retrospectively report all their children's infections from birth up to the age of 3 years at the initial study centre visit. This approach may be associated with recall bias, as parents might not accurately remember the details of all infections during this period. In the BC, where follow-up visits were conducted with shorter intervals, the parents were asked to report infections in the study diary in real time. In contrast to

Stene *et al.* (Stene *et al.* 2006), we did not find any difference in the frequency of gastroenteritis between the CD and the control groups. So far, the exact molecular mechanisms by which infections increase the risk of CD have not been defined. CD patients could have increased mucosal permeability of not only the intestine but also of the respiratory tract (Robertson *et al.* 1989; Sapone *et al.* 2011). The latter circumstance may increase susceptibility to respiratory tract infections (Mårild *et al.* 2010; Tjernberg & Ludvigsson, 2014). Infections may also imprint on the developing immune system and induce secretion of pro-inflammatory interferons and expression of tissue transglutaminase (Wessels *et al.* 2022).

According to our data, it is not likely that differences in vaccination between the study groups and the two countries would cause differences in autoimmunity.

6.3. Differences in laboratory confirmed viral infections between patients and controls and between two neighbouring countries (Paper II)

To investigate more specifically the role of infections in development of CD, we analysed enterovirus, norovirus, parechovirus and rhinovirus RNA from stool and nasal swab samples. Additionally, norovirus, adenovirus and enterovirus IgA and IgG antibodies were measured in serum. Also IgG antibodies against CMV and EBV were measured in serum.

We found that the overall level and frequency of the gastrointestinal virus (norovirus, adenovirus and enterovirus) antibodies did not differ between the patients and controls. Some previous studies have reported possible relationships between different gastrointestinal infections and CD. Kemppainen *et al.* found that gastrointestinal infections might increase the risk of CD in genetically susceptible children (Kemppainen *et al.* 2017). Stene *et al.* (Stene *et al.* 2006) reported in their prospective study that rotavirus infections might be involved here. However, we did not add rotavirus to the analyses in this study, since the rotavirus vaccination coverage was very different between the cases and controls and there were very few pairs with the same vaccination status. So far there have been no studies on potential association between norovirus and CD. Some earlier studies showed association between adenovirus type 12 and CD (Lähdeaho *et al.* 1993), but this was not confirmed in other studies (Lawler *et al.* 1994). A recent prospective study confirms the finding that adenovirus is not associated with increased risk of CD (Kahrs *et al.* 2019). Carlsson *et al.* studied the cord blood of the mothers whose offspring later developed CD, but did not find any association between enterovirus infection during pregnancy and development of CD during childhood (Carlsson *et al.* 2002). On the other hand, one study reported enterovirus genomes in intestinal biopsies collected from CD patients (Oikarinen *et al.* 2008). Although our results do not fully support this,

recent studies confirm that enterovirus may be important in development of CD (Kahrs *et al.* 2019, Lindfors *et al*. 2019). According to the Generation R Study, herpesviruses may have a protective effect in development of CD (Jansen *et al.* 2016). We failed to find this association in our study by assessing IgG antibody positivity against CMV and EBV.

However, when we combined seropositivity for at least one of the tested viruses in serum, these tended to be more common in CD patients than in controls, with a mean of 1.6 versus 1.2 infections per child, in the BC. This may even more clearly indicate that several infections early in life can increase the risk of CD, as demonstrated in previous epidemiological studies (Mårild *et al.* 2015; Beyerlein *et al.* 2017).

In nasal swab samples, rhinoviruses were detected slightly more frequently in CD patients than in controls, but this was not the case with stool samples. Rhinoviruses replicate in the respiratory tract at relatively low temperatures, but they are not known to be able to replicate in the intestinal mucosa where temperature is substantially higher. Therefore, respiratory samples are considered to be the most reliable sample type for rhinovirus detection. We cannot exclude the possibility that certain rhinovirus species, such as species C, could also replicate at higher temperatures, such as those present in the intestinal mucosa (Tapparel *et al.* 2013). Notably, the main rhinovirus receptor intercellular adhesion molecule-1 (Staunton *et al.* 1989) is also expressed in the intestinal mucosa in CD (Sturgess *et al.* 1990). Intercellular adhesion molecule-1 is involved in immunological processes such as cellular extravasations and cellular migration during inflammation, as well as in T-cell activation (van De Stolpe *et al.* 1996). One could hypothesize that rhinovirus infection may increase the expression of intercellular adhesion molecule-1 in the intestinal mucosa and thereby facilitate leukocyte migration towards infection sites, including gliadin sensitized T cells.

Comparison of the Finnish and Estonian children revealed that the latter were more likely to test positive for enterovirus IgG. Importantly, the mean levels of the antibodies were significantly higher in the Estonian cohort than in Finland. This finding was in line with previous studies (Viskari *et al.* 2004). It has been reported that T1D is more frequent in Finland (Harjutsalo *et al.* 2013) than in Estonia (Teeäär *et al.* 2010). Enteroviruses may play a role in the pathogenesis of autoimmune beta-cell destruction in T1D (Yeung *et al.* 2011). Accordingly, both CD and T1D are more frequent in Finland than Estonia, but enterovirus antibodies are less frequent. The low prevalence of enterovirus infections in Finland may cause deficient immune protection by transferred maternal antibodies in early childhood, which might make Finnish children more susceptible to early enterovirus-induced diseases, as proposed in earlier studies (Viskari *et al.* 2004). When we considered the relationship between the low prevalence of enterovirus infection and the high prevalence of T1D and CD, we presumed that this may reflect the hygiene hypothesis. This was because in countries where enterovirus infections are frequent, children had strong immune responses to these viruses, and the incidence of T1D and CD was lower

(Bach, 2005). This is consistent with the so-called polio hypothesis reported in a previous study (Viskari *et al.* 2000). Namely, in the case of poliomyelitis, a well-known disease caused by an enterovirus, severe paralytic complications were more common in countries where the circulation of the virus in the background population was less frequent. The basis for this phenomenon was a delay in the age of initial infection; some children were not exposed to polioviruses until later in childhood when they were protected no more by maternal antibodies and were thus more severely affected by the infection (Nathanson *et al.* 1997). There may be similarity in the epidemiology of T1D, poliomyelitis and CD – in countries where enterovirus infections are frequent the incidence of T1D and CD may be lower.

6.4. Maternal breast milk microbiota and immune markers from mothers of coeliac disease and control offspring (Paper III)

It is known that breast milk is a major source of bacterial diversity for the neonatal gut, including gut-associated obligate anaerobes. Therefore, it significantly influences gut colonization and maturation of the immune system (Jost *et al.* 2013; Olivares *et al.* 2015). The intestinal microbiota is thought to be involved in the pathogenesis of CD (Verdu *et al.* 2015). Different genetic and epigenetic factors may affect host microbiome interactions and shift the immune balance in subjects with CD (Cenit *et al.* 2015). In case of the intestinal microbiome, higher microbial richness and diversity values are considered to be more health-supporting (Mosca *et al.* 2016). Regarding breast milk, Benítez-Páez *et al.* have found that the microbiota of CD samples was more diverse in controls. They found that facultative methylotrophs such as *Methylobacterium komagatae* and *Methylocapsa palsarum,* as well as species such as *Bacteroides vulgatus*, which consumes fucosylated-oligosaccharides present in human milk, were more abundant in milk samples from mothers whose children developed CD compared to those that remained healthy (Benítez-Páez *et al.* 2020). We demonstrated that, compared to milk samples from mothers of unaffected offspring, the human milk microbiota of the mothers of the CD group showed higher phylotype abundance and diversity, with higher abundance of the phyla *Bacteroidetes* and *Fusobacteria*, the classes *Clostridia* and *Fusobacteria*, as well as the genera *Leptotrichia*, *Anaerococcus*, *Sphingomonas*, *Actinomyces*, and *Akkermansia*. Increased relative abundance of species such as *A. odontolyticus*, *A. hydrogenalis*, *A. octavius*, and *F. prausnitzii*, and decreased abundance of *L. fermentum* were also noted in the CD group.

Breast milk does not contain abundant culturable microbiota. However, some next generation sequencing studies have detected *Streptococcus*, *Staphylococcus*, *Gemella*, *Veillonella*, *Rothia*, *Lactobacillus*, *Propionibacterium*, *Corynebacterium*, and *Pseudomonas* in milk samples from healthy women

(Drell *et al.* 2017; Williams *et al.* 2017). In the current study, the most abundant genus in all milk samples was *Lactobacillus*, followed by *Streptococcus*, *Staphylococcus*, *Moraxella*, *Acinetobacter*, *Enterobacter*, and *Corynebacterium*. Some studies have indicated that primary bacterial taxa in milk may vary across the population, suggesting that geographic, genetic, and dietary factors could influence the microbial diversity of breast milk. In Finland, *Leuconostoc*, *Weisella*, and *Lactococcus* were the most predominant genera in milk samples (Cabrera-Rubio *et al.* 2012), while in Mexican-American mothers the predominance of *Streptococcus*, *Staphylococcus*, *Xanthomonadaceae*, and *Sediminibacterium* was observed (Dave *et al.* 2016).

In the present study we noted a significantly increased relative abundance of the class *Clostridia* in milk samples from the CD group. We also found differences in the relative abundance of the genus *Anaerococcus* (incl. *A. hydrogenalis*, *A. octavius*) and *F. prausnitzii*. The presence of *A. octavius* was reported previously in breast milk samples from healthy Spanish women. In the same study, association was shown between the amount of proteins in milk and *Anaerococcus* (Boix-Amorós *et al.* 2016). *F. prausnitzii* is the major butyrate producer in the gastrointestinal tract. It has anti-inflammatory properties important for gastrointestinal microbiota homeostasis and is associated with a range of metabolic processes in the human mucosa (Sokol *et al.* 2008). A Spanish study showed that adherence to a gluten-free diet resulted in slight microbiota shifts in adults, including decreased abundance of *F. prausnitzii* (de Palma *et al.* 2009). As *F. prausnitzii* produces the anti-inflammatory short-chain fatty acid butyrate (Lopez-Siles *et al.* 2012, Ouyang *et al.* 2020), this species may have a key role in maintaining the integrity and function of the mucus barrier, representing an important aberrant mechanism in development of CD.

We also found that the prevalence of the genus *Akkermansia* was higher in the samples of the CD group. Interestingly, unclassified *Akkermansia* species were only found in that group. A study by Huang *et al*. observed the *Akkermansia* genus only in the control group and not in CD progressors (Huang *et al.* 2020). However, they analysed faecal samples but not breast milk samples. Some experiments on mice have demonstrated that *Akkermansia* may be related to gluten in their diet. A gluten-free diet can induce changes in the intestinal microbiota by increasing the number of *Akkermansia* (Marietta *et al.* 2013; Hansen *et al.* 2014). It can be speculated that this species and development of CD can be related.

An increased relative abundance of the genus *Actinomyces* and of a species of this genus, *A. odontolyticus*, was observed in the CD group. Mucosal membranes of the oropharynx, gastrointestinal tract and female genital tract represent the normal habitat of *Actinomyces* (Smego *et al.* 1998). They have also been observed to associate with breast infection (Edmiston *et al.* 1990; Könönen *et al.* 2015). Although being low in virulence, they may rely on the presence of co-pathogens, such as anaerobic bacteria, to enhance pathogenicity (Bing *et al.* 2015). Previously, *A. graevenitzii* was identified in biopsy specimens from the

proximal small intestine of children with CD, and an unidentified *Actinomyces* sp. was shown to be attached to the epithelial lining (Ou *et al.* 2009).

For detection of *Lactobacillus* and *Bifidobacterium*, we applied two methods, next generation sequencing and PCR-DGGE, since Smidt *et al*., demonstrated the partial concordance of different molecular methods for detection of lactic acid bacteria (Smidt *et al.* 2015). In our study, PCR-DGGE appeared to be more sensitive for identification of *Bifidobacterium* sp., while more than 80% of *Bifidobacterium* sp. detected by Illumina sequencing were unclassified.

In line with our findings, other studies have also shown presence of *Lactobacillus* sp. and *Bifidobacterium* sp. in breast milk (Martin *et al.* 2003; Olivares *et al.* 2015). Low relative abundance of *L. fermentum* was observed in breast milk in the CD group. The expected pathway by which lactobacilli may get into milk is entero-mammary transport through dendritic cells (Lyons *et al.* 2020). Production of short-chain fatty acids by lactobacilli strains may play a role in the pathogenesis of CD. *L. fermentum* produces both lactic and succinic acids, which in turn modulate the key functions of many main players in the innate immune response (Blad *et al.* 2012). They mediate macrophage dependent anti-inflammatory effects (Garrote *et al.* 2015) and possess antioxidative capacities (Mikelsaar *et al.* 2009). Additionally, it was demonstrated that *L. fermentum* isolated from breast milk may have an immunostimulatory effect by inducing pro-inflammatory cytokines in rodent bone marrow derived macrophages (Martin *et al.* 2003). *In vivo* assays in mice revealed that the ingestion of *L. fermentum* enhanced the production of Th1 cytokines by spleen cells and increased IgA concentration in faeces (Diaz-Ropero *et al.* 2007). Likewise, *Lactobacillus* strains have high amylase trypsin inhibitor-metabolizing capacity to ameliorate many adverse effects induced by wheat immunogenic proteins (Caminero *et al.* 2019). Accordingly, one can assume that *L. fermentum* might protect against development of CD.

There were no significant differences in the levels of the immunological markers (TGF-β1, TGF-β2, MFG-E8, and sCD14) analysed between the CD and control groups. TGF-β belongs to a growth factor family that is responsible for development and maturation of the mucosal immune system (Oddy & Mcmahon, 2011). Vidal *et al.* demonstrated that milk sCD14 acts as a "sentinel" molecule and immune modulator in homeostasis and in the defence of the neonatal intestine (Vidal *et al.* 2001). MFG-E8 has been shown to have antiviral effects and protects against rotavirus both in a cultured cell model as well as in infants (Newburg *et al.* 1998; Kvistgaard *et al.* 2004; Stene *et al.* 2006). Furthermore, it prevented the tissue damage caused by prolonged inflammation through clearing apoptotic cells and thereby facilitating immune resolution (Hanayama *et al.* 2002). We observed association between certain immune markers and some Gram-negative (*Chryseobacterium*, *Sphingobium*, and *A. muciniphila*) and Gram-positive bacteria (*L. reuteri* and *B. animalis*). Presence of *Chryseobacterium* and *Sphingobacterium* correlated inversely with breast milk TGF-β2 levels, while *A. muciniphila* (anaerobic mucin-degrading bacterium) showed positive correlation with both TGF-β1 and TGF-β2. In our study, we

also noted that higher counts of *L. reuteri* and lower counts of *B. animalis* in breast milk were associated with higher sCD14 concentrations. The latter bacterial species were also associated with higher concentrations of MFG-E8. Supplementation of maternal breast milk with the probiotic *L. reuteri* increased TGF-β2 concentration level in breast milk and decreased sensitization in breastfed infants (Böttcher *et al.* 2008). In addition, *Bacilli* and *Flavobacteriia* classes correlated positively with the concentration of MFG-E8.

6.5. Strengths of the study

The major strengths of our study are the prospective design (with part of data obtained retrospectively), the availability of demographically well characterized children tested for specific HLA haplotypes and CD antibodies, and the fact that both centres in the two countries involved used identical protocols for follow-up and diagnosis. Another strength is the careful matching of the children with CD and the control subjects. In addition, all CD antibody analyses were carried out at one laboratory and in all CD cases the diagnosis was confirmed by a biopsy of the small intestine. In microbiota studies two different methods (next generation sequencing and PCR-DGGE) were applied in parallel for investigating *Lactobacillus* sp. and *Bifidobacteria* sp. in breast milk.

6.6. Limitations of the study

The study has also some limitations. The number of cases is relatively modest, limiting the statistical power of the study. The relatively short follow-up period (up to 3 years in the BC and from 3 to 5 years in the YCC) prevented us detecting all childhood CD cases. Some data (early feeding and early infections in the YCC, information on mothers' infections and fever episodes during pregnancy) was collected retrospectively, which may have affected its quality. In addition, we did not have the possibility to study serial stool and nasal swab samples from each child whom we studied at all time points. The limited number of study samples reflects the problems related to collecting samples from young children. Furthermore, the present study did not cover all possible viruses that could be linked to CD. In breast milk analysis the mothers of the CD group were younger than the mothers of the control group. When interpreting the results this may be important because it is known that maternal age may influence milk composition and its microbiota (Moossavi *et al.* 2019).

7. CONCLUSIONS

1. There is a significant difference in the 5-year cumulative incidence of childhood CD between Estonia and Finland, which is higher in the latter country (0.27% versus 0.77%). The children from Finland tended to develop IgA-tTG antibodies earlier than the children from Estonia.
2. No difference was seen in the age at cereal introduction or in the duration of breastfeeding between the children with CD and the control children. Sequential infections early in life may increase the risk for developing CD.
3. We did not find a marked difference in laboratory confirmed common viral infections, namely enterovirus, adenovirus, EBV, CMV, norovirus and parechovirus infections, between the children who developed CD and their matched controls. However, rhinovirus infections were found to be slightly more frequent in the CD patients than in the controls. In addition, our study confirms earlier findings of a significantly higher prevalence of enterovirus infections in Estonian children than in their Finnish peers.
4. The breast milk microbiota of the mothers of children who developed CD differed in terms of higher bacterial phylotype abundance and diversity, as well as in relation to bacterial composition when compared to the mothers of the unaffected children. The immunological markers (TGF-β1, TGF-β2, MFG-E8, sCD14) were differently associated with bacterial composition and could also influence the risk of developing CD in later life.

8. FUTURE DIRECTIONS

It is important to continue collecting data about CD incidence and prevalence worldwide and to continue analysing reasons behind the increase. It is critical to find out why some people with genetic risk develop CD and others not. The role of feeding, infections and microbiome before the development of CD should be clarified. All this could help to find preventive strategies in the future.

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SUMMARY IN ESTONIAN

Tsöliaakia teke kahes erineva keskkonna-taustaga naaberriigis

Sissejuhatus

Tsöliaakia on immuunvahendatud haigus, mis tekib geneetilise eelsoodumusega isikutel gluteeni ja gluteeniga seotud prolamiinide toimel. Tsöliaakiat võivad vallandada nisuvalgu gluteeni laguprodukt gliadiin, rukkivalk sekaliin ja odravalk hordeiin. Kaeratoodetesse võib gluteen sattuda ristsaastumise teel. Tsöliaakiale on iseloomulik varieeruv kliiniline pilt, tsöliaakia spetsiifiliste autoantikehade teke, inimese leukotsüütide antigeenide HLA-DQ2 ja/või HLA-DQ8 olemasolu ning enteropaatia (Husby *et al.* 2012). Traditsiooniliselt põhineb tsöliaakia diagnoos tsöliaakia spetsiifiliste autoantikehade olemasolul veres ning peensoole biopsiatükkides hinnataval hattude atroofial (Kelly *et al.* 2015). Tsöliaakia spetsiifiliste autoantikehade hulka kuuluvad koe transglutaminaasi vastased antikehad (anti-tTG), endomüüsiumi vastased antikehad ja deamideeritud gliadiini vastased antikehad.

Viimastel aastakümnetel on tsöliaakia esinemissagedus tõusnud ülemaailmselt (Kang *et al.* 2013; Makharia *et al*. 2022). Tsöliaakia esinemissagedus nn läänemaailmas on hinnanguliselt 1% (Rubio-Tapia *et al.* 2012; Choung *et al.* 2017) kuni 3% (Myléus *et al.* 2009). Haigus on sagenenud ka kahes naaberriigis – Eestis (Ress *et al.* 2012) ja Soomes (Lohi *et al.* 2007). On teada, et nende kahe riigi vahel esines suur sotsiaalmajanduslik erinevus kuni 1990ndate aastate alguseni.

Tsöliaakia patogeneesis on olulised nii geneetilised, keskkondlikud kui ka immunoloogilised faktorid (Kagnoff 1992). Mõned neist faktoritest on hästi tuntud – HLA-DQ2 ja HLA-DQ8, koe transglutaminaas ja gluteen ning gluteeniga seotud prolamiinid (Caio *et al.* 2019). Kuna enamikel inimestel, kellel esineb geneetiline eelsoodumus tsöliaakia tekkeks ning on olnud kokkupuude gluteeniga, ei teki haigust, siis peavad haiguse tekkega olema seotud ka erinevad keskkondlikud tegurid. Kõige rohkem on keskkondlikest faktoritest uuritud imiku ja väikelapse toitu ja toitmist, infektsioone ning soole mikrobioomi.

Varasemad epidemioloogilised uuringud (Ivarsson *et al.* 2000) on leidnud, et rinnapiimaga toitmise kestus ja gluteeni menüüsse lisamise aeg võiksid olla seotud tsöliaakia tekkega. Viimaste andmete põhjal ei ole tõestust, et rinnapiimaga toitmine kaitseks tsöliaakia tekke eest (Szajewska *et al.* 2016) ning samuti ei ole leitud, et gluteeni menüüsse lisamise erinev algusaeg võiks tsöliaakia riski vähendada (Vriezinga *et al.* 2014; Lionetti *et al.* 2014). Viimasel ajal on rohkem tähelepanu pööratud sellele, kas pigem ei võiks oluline olla gluteeni kogus imiku ja väikelapse menüüs (Andrén Aronsson *et al.* 2019; Lund-Blix *et al.* 2019; Mårild *et al.* 2019). Kuna on leitud, et erinevatel aastaaegadel sündinud inimeste tsöliaakiasse haigestumine erineb, siis arvatakse, et tsöliaakia tekkega

on seotud ka infektsioonid (Lewy *et al.* 2009). Haiguse vallandajana on uuritud erinevaid nii viiruslikke kui ka bakteriaalseid infektsioone (Stene *et al.* 2006; Lebwohl *et al.* 2013; Kahrs *et al.* 2019). On leitud võimalikke seoseid seedetrakti infektsioonidega, näiteks rotaviiruse (Stene *et al.* 2006), enteroviiruse (Kahrs *et al.* 2019; Lindorfs *et al.* 2020) ja reoviirusega (Bouziat *et al.* 2017). Seoseid on leitud herpesviiruste (Jansen *et al.* 2016) ning *Helicobacter pylori* (Lebwohl *et al.* 2013) ja tsöliaakia tekke vahel. Tsöliaakia tekkega on seotud ka soolestiku mikrobioom (Verdu *et al.* 2015). Arvatakse, et juba tsöliaakia geneetilise eelsoodumusega imikutel võivad olla mikrobioomis muutused (Olivares *et al.* 2018).

Tsöliaakia esinemissagedus on ülemaailmselt tõusnud, kuid selle täpsed põhjused ei ole selged. Antud doktoritöö peamine eesmärk oli uurida lapseea tsöliaakia teket kahes erineva sotsiaalmajandusliku tasutaga naaberriigis – Eestis ja Soomes ning uurida täpsemalt tsöliaakia erinevaid riskifaktoreid.

Töö eesmärgid

1. Uurida tsöliaakia kumulatiivset haigestumust Eestis ja Soomes kuni 5-aastaste laste hulgas.
2. Analüüsida erinevaid riskifaktoreid, mis võiksid olla seotud tsöliaakiasse haigestumuse erinevusega – peamine huvi oli imikuea ja väikelapse toitumisel ja infektsioonidel.
3. Määrata erinevaid viiruseid tsöliaakia diagnoosi eelselt rooja- ning ninalimaskesta proovides ning viirusspetsiifilisi autoantikehi diagnoosimise hetkel tsöliaakiahaigetel ja kontrollgrupi lastel ning seeläbi viiruste seotamine haiguse tekkega.
4. Analüüsida rinnapiima mikrobiootat ning uurida selle seost rinnapiima immunoloogiliste markeritega emadel, kelle lastel tekkis tsöliaakia esimese kolme eluaasta jooksul ning neil emadel, kelle lastel ei tekkinud tsöliaakiat.

Uuritavad ja meetodid

Uuritavad ja uuringumaterjal pärinevad rahvusvahelisest DIABIMMUNE uuringust (juhtivuurija professor Mikael Knip, Helsingi Ülikool, Soome), mille eesmärgiks oli hinnata 1. tüüpi diabeedi ja teiste immuunvahendatud haiguste teket. Uuring viidi läbi Eestis, Soomes ja Venemaal Karjalas 2008. aasta septembrist kuni 2013. aasta oktoobrini. Käesolevasse töösse kaasati Eesti ja Soome patsientidelt kogutud materjal. Uuring koosnes nn sünnikohordist ja nn lastekohordist. Esimeses kohordis jälgiti lapsi sünnist kuni 3. eluaastani ja teises kohordis 3. eluaastast 5. eluaastani. Sünnikohorti kaasati algselt 2714 last Eestist ja 3105 last Soomest. Eestis tekkis uuringu perioodi jooksul tsöliaakia kahel lapsel ja Soomes seitsmel lapsel. Lastekohorti kaasati Eestis 1681 last ja Soomes 1575 last. Eestis tekkis uuringuperioodi jooksul tsöliaakia kuuel lapsel ja Soomes 14 lapsel. Tsöliaakia diagnoositi vastavalt Euroopa Laste Gastroenteroloogia, Hepatoloogia ja Toitmisravi Ühingu juhendile (Husby *et al.* 2012) – veres

esinevad positiivsed tTG-IgA antikehad ning peensoole hattude atroofia leid peensoole biopsiatükkides. Igale tsöliaakia diagnoosiga lapsele valiti kontrollgrupi laps samast DIABIMMUNE uuringust vastavalt tsöliaakia spetsiifilisele HLA genotüübile, sünnimaale, sünnikuupäevale ja soole (I ja II artikkel).

Rinnapiima analüüsideks valiti nn sünnikohordist kuus last, keda oli jälgitud 3. eluaastani ning kelle kõigi emadelt oli kogutud rinnapiim lapse 3. elukuul ja lastel oli tekkinud tsöliaakia. Kontrollgruppi valiti 18 last samast DIABIMMUNE uuringu sünnikohordist vastavalt tsöliaakia spetsiifilisele HLA genotüübile, sünnimaale, sünnikuupäevale ja soole (III artikkel).

Rooja- ja nina limaskesta proove analüüsiti ainult sünnikohordi lastel. Roojaproovidest määrati enteroviiruse, noroviiruse, parehhoviiruse ja rhinoviiruse RNA laste 3., 4., 5. ja 6. elukuul kogutud proovidest. Nina limaskesta proovidest määrati enteroviiruse, parehhoviiruse and rhinoviirus RNA laste 3. ja 6. elukuul kogutud proovidest. Viiruste RNA määramiseks kasutati reaalaja polümeraasi ahelreaktsiooni (II artikkel).

Viiruste vastaseid antikehi seerumist määrati nii sünnikohordi kui ka lastekohordi patsientidel. Antikehad määrati seerumist ajahetkel, millal olid esmakordselt esinenud positiivsed tTG-IgA autoantikehad. Kontrollgrupi lastel määrati viiruste vastased antikehad samale ajahetkele vastavast proovist. Seerumist määrati noroviiruse, adenoviiruse ja enteroviruse IgA ja IgG antikehi ning CMV ja EBV IgG antikehi. Määramiseks kasutati immunoensüümmeetodit (II artikkel).

Sünnikohordi laste 3. elukuul kogutud rinnapiimadest määrati immunoensüümmeetodi abil TGF-β1, TGF-β2, sIgA, MFG-E8 ja sCD14 tasemed. Rinnapiima mikrobioomi määramiseks kasutati järgmise põlvkonna sekveneerimist ja reaalaja polümeraasi ahelreaktsiooni. Lactobacilluste ja Bifidobacterite puhul kasutati lisaks denatureeriva gradiendi geelelektroforeesi (III artikkel)

Tulemuste analüüsimiseks kasutati R statistika programmi (The R Foundation for Statistical Computing, Vienna, Austria).

Tulemused

Sünnikohordis tekkisid tTG-IgA antikehad Soome lastel esmakordselt 2,3 aastaselt (95% CI 1,6–2,9) ja Eesti lastel 3,0 aastaselt (95% CI 2,5–3,6) (*P*=0,12). Lastekohordis esinesid postiivsed tTG-IgA antikehad uuringus 3-aastaselt 11-l lapsel 14-st tsöliaakia diagnoosiga lapsest (79%). Eestis olid vastavad arvud viis ja kuus (83%). Sünnikohordis ja lastekohordis ei olnud kumulatiivne haigestumine uuringuperioodil kahe riigi vahel statistliselt erinev kui kahte kohorti hinnati eraldi (sünnikohordis oli kumulatiivne haigestumine Eestis 0,12% ja Soomes 0,53% (*P*=0,10) ning lastekohordis oli Eestis 0,4% ja Soomes 1% (*P*=0,12)). Kahe kohordi kombineerimisel oli kumulatiivne haigestumine statistiliselt oluliselt kõrgem Soomes võrreldes Eestiga (Eestis 0,27% ja Soomes 0,77% (*P*=0,01)) (I artikkel).

Tsöliaakiaga ja kontrollgrupi laste osas ei esinenud statistiliselt olulist erinevust rinnapiimaga toitmise kestuse ega terviljade (nisu, rukkis, oder) menüüsse lisamise aja osas. Sünnikohordis esines tsöliaakiaga lastel esimesel eluaastal

statistiliselt oluliselt rohkem palavikuga haigestumisi võrreldes kontrollgrupiga (3,4 vs 1,4; *P*=0,04). Esimese kolme eluaasta jooksul ei olnud sünnikohordis kahe grupi vahel erinevust gastroenteriitide põdemise ja antibiootikumide kasutamise osas. Laste kohordis ei olnud gruppide vahel erinevusi infektsioonide ja antibakteriaalse ravi osas (I artikkel).

Sünnikohordis esines nii rooja- kui ka ninakaape proovides kõige sagedamini rhinoviirust. 3. ja 6. elukuul oli rhinoviirus positiivne ninakaapest viiel tsöliaakiaga lapsel ning mitte ühelgi kontrollgrupi lapsel (*P*=0,046). Kolmandal ja 6. elukuul oli rhinoviirus positiive rooja analüüsist kolmel tsöliaakiaga lapsel ning seitsmel kontrollgrupi lapsel (*P*=0,25). Entero- ega parehhoviirust ei tuvastatud üheski roojaproovis. Sünnikohordi ega lastekohordi kuuluvate laste seerumistes ei esinenud erinevusi viiruste antikehade tiitris – noroviiruse, adenoviiruse ja enteroviruse IgA ja IgG antikehad ning CMV ja EBV IgG antikehad. Kahe riigi omavahelises võrdluses, kui kaks kohorti kombineeriti, esines enteroviiruse IgG antikehi rohkem Eesti laste hulgas (63% vs 23%; *P*=0,02) (II artikkel).

Rinnapiimast määratud immuunmarkerite (TGF-β1, TGF-β2, sIgA, MFG-E8 ja sCD14) tasemete osas ei olnud tsöliaakiaga laste ja kontrollgrupi laste vahel statistiliselt olulisi erinevusi. Liikide arvukus ja Shannoni indeks olid oluliselt kõrgemad tsöliaakiaga laste emade grupis. Tsöliaakiaga laste grupis esines kõrgem levimus *Bacteroidetes* ja *Fusobacteria* hõimkondades, *Clostridia* ja *Fusobacteriia* klassides ning *Leptotrichia, Anaerococcus, Sphingomonas, Actynomyces* ja *Akkermansia* perekondades. Nii mõnede Gram-positiivsete kui ka Gram-negatiivsete bakterite levimuse ja immuunmarkerite tasemete osas esines statistiliselt olulisi seoseid, mille lõplikuks tõlgendamiseks on vajalikud suuremate uuritavate arvuga ning täiendavad uuringud (III artikkel).

Järeldused

1. Oluline erinevus esines tsöliaakia kumulatiivses haigestumises Eesti ja Soome laste vahel esimese viie eluaasta jooksul. Tsöliaakia kumulatiivne haigestumine on kõrgem Soomes (0,27% vs 0,77%) ning seal tekivad lastel varem ka tTG-IgA antikehad.
2. Tsöliaakiaga ning kontrollgrupi laste vahel ei olnud erinevust rinnapiimaga toitmise kestuses ega teraviljade (nisu, rukkis, oder) menüüsse lisamise vanuses. Korduvad infektsioonid varajases eas võivad suurendada riski haigestuda tsöliaakiasse tuginedes prospektiivselt kogutud andmetele.
3. Tsöliaakiaga ning kontrollgrupi laste vahel ei olnud erinevust laboratoorselt kinnitatud enteroviirus, adenovirus, CMV, EBV, noroviirus ega parehhoviirus infektsioonide vahel. Rhinoviirus infektsiooni esines tsöliaakiaga lastel mõnevõrra rohkem. Sarnaselt varasemate uuringute tulemustega esines Eesti laste hulgas enteroviiruse IgG antikehi võrreldes Soome lastega rohkem.
4. Emadel, kelle lastel tekkis tsöliaakia, oli rinnapiima mikrobioomis erinevusi võrreldes emadega, kelle lastel tsöliaakiat ei tekkinud. Erinevused esinesid nii liikide arvukuses kui ka Shannoni indeksis. Bakterite levimuse ja immuun-

markerite (TGF-β1, TGF-β2, MFG-E8, sCD14) tasemete osas esines statistiliselt olulisi seoseid ning leitud muutused võivad olla olulised hilisemas elus tsöliaakia kujunemise riski mõjutamisel.

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1. Simre K, Uibo O, Peet A, Tillmann V, Kool P, Hämäläinen AM, Härkönen T, Siljander H, Virtanen S, Ilonen J, Knip M, Uibo R; DIABIMMUNE Study Group. Exploring the risk factors for differences in the cumulative incidence of coeliac disease in two neighbouring countries: the prospective DIABIMMUNE study. Dig Liver Dis 2016; 48(11):1296–301. doi: 10.1016/j.dld.2016.06.029.
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1. Simre K, Uibo O, Peet A, Tillmann V, Kool P, Hämäläinen AM, Härkönen T, Siljander H, Virtanen S, Ilonen J, Knip M, Uibo R; DIABIMMUNE Study Group. Exploring the risk factors for differences in the cumulative incidence of coeliac disease in two neighbouring countries: the prospective DIABIMMUNE study. Dig Liver Dis 2016; 48(11):1296–301. doi: 10.1016/j.dld.2016.06.029.
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