CHAPTER 5

Composition and diversity of the duodenal mucosa-associated microbiome in children with untreated coeliac disease

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ABSTRACT

Background
Intestinal microbiome may play a role in the pathogenesis of coeliac disease (CD). Studies comparing intestinal microbiome in children with and without CD are contradictory.

Aim
To compare the composition and diversity of the duodenal mucosa-associated microbiome in children with untreated CD and control children without CD and to identify specific gut bacteria associated with CD at diagnosis.

Methods
Total microbiome profile in small bowel biopsies of 42 children (21 with untreated CD and 21 age-matched controls) were analysed by means of IS-pro, a 16S-23S interspacer (IS) region based profiling method.

Results
Both groups showed a similar mucosa-associated microbiome pattern and diversity, with high concentrations of the genera Streptococcus, Lactobacillus and Clostridium.

Conclusion
Mucosa-associated duodenal microbiome composition and diversity did not differ between children with untreated CD and control children. Duodenal mucosa associated bacteria do not seem to play an important role in the pathogenesis of CD.
INTRODUCTION

Coeliac disease (CD) is an auto-immune disorder caused by a T-cell response to gluten epitopes, resulting in damage to the small bowel mucosa.\(^1\)\(^2\) The disease can manifest itself at any age, but typical cases present in early childhood.\(^3\) CD is characterised by histological alterations in the small bowel, consisting of elevated intraepithelial lymphocyte counts, crypt hyperplasia and villous atrophy. Circulating CD antibodies at the time of diagnosis, especially IgA anti endomysium (EMA) and anti transglutaminase type 2 antibodies (TG2A), and their disappearance after the introduction of a gluten-free diet support the diagnosis of CD.\(^4\) Over the last decades, significant progress has been made in the understanding of the aetiology and pathogenesis of CD. In addition to genetics and the aberrant immune response to deaminated gluten peptides, microbial infections and imbalances in the composition of the gut microbiome have been suggested to be associated with the pathogenesis of CD.\(^5\)-\(^10\)

The commensal microbiome at epithelial surfaces has an important function in the establishment and maintenance of immune homeostasis in the gut, with epithelial cells emerging as key players.\(^11\) Infections could be involved in CD pathogenesis both by impairing mucosal barrier function, thus favouring the access of dietary gluten to subepithelial lymphoid tissue, and by promoting a defensive Th1 mediated immune response with the production of pro-inflammatory cytokines.\(^12\) In recent years, multiple studies have focused on the identification of bacterial species potentially playing a role in the aetiology of CD. Since the majority of bacterial species inhabiting the human gut are refractory to culturing, microbiome analysis based on culture leads to a distorted and incomplete view of its composition.\(^13\)

Molecular microbial methods have been developed in the past decades based on the analysis of bacterial DNA, thus circumventing the need of culturing. In-depth analysis of the gut microbiome and identification of species is now largely performed with sequencing-based techniques, which however are laborious and expensive. Furthermore, none of the currently available techniques have been specifically designed to analyse the complex gut microbiome. In this study IS-pro, a recently validated 16S-23S interspace (IS) region-based profiling method, is used to analyse the gut microbiome of children with CD compared to age-matched healthy subjects.\(^14\) The aim of this study was to determine and compare the composition and diversity of the microbiome of the proximal small intestinal mucosa of children with CD and age-matched children without CD by means of IS-pro and to identify specific gut bacteria associated with CD.
MATERIALS AND METHODS

Subjects

**CD patients**

We took advantage of the small bowel biopsies collected during mass screening for CD performed in 1998 in children 2 to 4 years of age attending the Community Child Health Care Centres in the Dutch province of Zuid-Holland. In this study, a total of 57 children with positive IgA EmA had undergone gastroduodenoscopy during which diagnostic small bowel biopsy specimens were harvested. CD was identified in 31/57 children based on the histological alterations characteristic for CD. Intestinal biopsies of 9 of these untreated CD patients, chosen at random, were included in our patient group. In addition, we studied another 12 small bowel specimens from randomly selected children (median age 6.8 years; range 3.0-15.2 years) with untreated CD in the period January 1998 until May 2002.

**Controls**

The control group consisted of 21 age-matched children without CD (median age 8.1 years; range 2.4-13.6 years), who had undergone upper endoscopy because of clinical suspicion of CD or other gastrointestinal disorders, but who all had normal histology of the small bowel and normal specific TG2A and EmA. Indications for endoscopy in the control group were (suspicion of) gastro-oesophageal reflux disease (4), Helicobacter pylori gastritis (4), chronic abdominal pain and positive family history for CD (3), gastric ulceration (1), failure to thrive (4), chronic abdominal pain (4) and IgA deficiency (1). Characteristics of the patients in both groups are shown in Table 5.1. The harvest of all biopsy specimens was approved by the medical ethical review board of the Leiden University Medical Centre. All small bowel biopsy specimens were analysed by IS-pro and the results of both groups were compared.

**Sampling preparation, DNA extraction and PCR**

Mucosal biopsy specimens were collected from the duodenum with a flexible video endoscope (Olympus, Hamburg, Germany) and a flexible biopsy forceps and transferred into a sterile plastic recipient, immediately frozen at -20°C until further processing. Bacterial DNA was extracted from mucosal biopsy specimens by a first step consisting of lysis of tissue and bacteria. 360 μl ATL buffer from the QIAamp DNA mini Kit (QIAGEN, Hilden, Germany) and 40 μl proteinase K were added to samples in an Eppendorf container. This
mixture was vortexed and incubated at 56°C whilst shaking at 100 RPM for 1 h. Subsequently
the mixture was centrifuged at 16,200 g for 10 s to remove droplets from the inside of the
container. 400 μl of AL buffer (QIAGEN) was added, and the mixture was incubated at 70°C
whilst shaking at 100 RPM for 10 min. An easyMAG automated DNA isolation machine
(Biomérieux, Marcy l’Etoile, France) was used for further DNA extraction. The entire mixture
(approximately 800 μl) was used and DNA was eluted in 110 μl NucliSens easyMAG extraction
buffer 3 as provided by the manufacturer (Biomérieux), choosing the machine’s regular
program with internal lysis, according to the manufacturer’s instruction. DNA was amplified
with universal bacterial forward primer DZU16Sf (5’ GGATTAGATACCCBGGTAGTCC 3’)
and reverse primers DZU23Sr1 (5’ CTTTTCACCTTTCCTACAGTAC3’), DZU23Sr2
(5’CTTTTGCTTTCCCCAGTAC3’), DZU23Sr3 (5’CTTTTGCGCTTTCCCCAGTAC3’),
DZU23Sr4 (5’CTTTTGACCTTTCCCTACGATC3’). The resulting PCR products were
subsequently amplified by means of IS-pro, as described previously.14

Data analysis

DNA fragment analysis was performed on an ABI Prism 3130XL Genetic Analyzer (Applied
Biosystems). Data were further analysed with the BioNumerics (Applied Maths, Sint-Martens-
Latem, Belgium) and Spotfire (TIBCO, Palo Alto, USA) software packages. Where correlation

Table 5.1 Characteristics of the untreated CD children and controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Untreated CD</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>Number of patients</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Age (years)</td>
<td>Median (range)</td>
<td>6.8 (3.0-15.2)</td>
</tr>
<tr>
<td>Sex</td>
<td>Male/female</td>
<td>12/9</td>
</tr>
<tr>
<td>Duodenal biopsy</td>
<td>Marsh* 0-1</td>
<td>0</td>
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<tr>
<td></td>
<td>Marsh 2</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Marsh 3A</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Marsh 3B</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Marsh 3C</td>
<td>6</td>
</tr>
<tr>
<td>Serology</td>
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<td>21</td>
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<tr>
<td></td>
<td>IgA TG2A +</td>
<td>21</td>
</tr>
<tr>
<td>HLA genetic typing</td>
<td>DQ2 +</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>DQ8 +</td>
<td>0</td>
</tr>
</tbody>
</table>

* Modified Marsh classification of CD.16
between profiles was determined, a log2 transformation was performed on peak heights for improved consistency of estimated correlation coefficient and improved detection of variation in less prominent species. Correlations were calculated with Pearson’s product-moment correlation coefficient on log2 transformed data. Fragments were assigned a taxonomic classification based on fragment length. Lengths were compared to a database consisting of interspace lengths of 576 bacterial species. This classification was confirmed by separating fragments on an agarose gel and excising and sequencing these fragments. Sequences were compared to the GenBank database with the BLASTn algorithm. Taxonomic classification to species level was based on >97% sequence identity.14

RESULTS

DNA analysis of the duodenal biopsies of the untreated CD and control group showed similar profiles of IS fragments, indicating no differences in mucosa-associated microbiome. A correlation matrix calculated by performing a Pearson’s product-moment correlation on all versus all samples did not reveal a disease specific clustering. This absence of clustering between the two subgroups is displayed in a similarity dendrogram (Figure 5.1). The bacterial material of all duodenal biopsies consisted mainly of Streptococcus species (297 and 321 nucleotides (nt)), Lactobacillus reuteri (250 nt) and Clostridium species (271 nt). In addition, species with IS-length of 362 nucleotides were dominant in both groups, an IS-length not yet linked to known species. Figure 5.2 displays the cumulative mucosa-associated IS-profiles of untreated CD children and controls, showing a similar microbial pattern of Firmicutes, Actinobacteria and Bacteroidetes, both in composition and quantity. Diversity of the duodenal mucosa-associated microbiome, represented by the number of bands present in each profile, showed virtually no clustering between the untreated CD and control group (Figure 5.3). Mean diversity was similar in both groups (18 bands).

DISCUSSION

Currently available information on the composition of the intestinal microbiome and its role in the etiology of CD is highly heterogeneous and contradictory. Studies analysing the intestinal microbiome in CD patients were previously mainly performed in faecal samples,9,18-22 whereas more recent studies have focussed on the duodenal mucosa-associated bacteria for the obvious reason that CD is primarily a disease of the small intestine. Comparison of
Figure 5.1 Dendogram displaying IS-profiles (on Y-axis, expressed in nucleotides) of untreated CD children (red) and controls (green), showing absence of clustering between the two groups. Red signals represent dominant IS-fragment lengths, grey signals represent less prevalent IS-fragment lengths.
Figure 5.2 Cumulative duodenal mucosa-associated IS-profiles of untreated CD children and controls, showing similar microbial patterns. Peak length, expressed in nucleotides (nt), corresponds to IS-fragment length. Peak height, expressed in relative fluorescence units (RFU), reflects quantity of fragments. Blue peaks represent Firmicutes/Actinobacteria, red peaks represent Bacteroidetes. Profiles in both groups consisted mainly of Streptococcus species (297 and 321 nt), Lactobacillus reuteri (250 nt) and Clostridium species (271 nt). IS-length of 362 nt is not yet linked to known species.
Microbiome in children with coeliac disease

these data is unreliable as the composition of jejunal, ileal and colonic mucosa-associated bacteria differ from that of the faecal microbiome. Furthermore, studies analysing the duodenal mucosa-associated microbiome in coeliac disease were all performed on biopsies harvested by gastroduodenoscopy. A possible confounding factor in the analysis of duodenal biopsies obtained by endoscopy is contamination with bacterial species from the densely colonized oropharynx, but alternatives to endoscopic sampling to circumvent this potential sampling bias are not easily employed in a routine clinical setting.

Significant differences have been reported in mucosa-associated duodenal microbiome between children newly diagnosed with CD and healthy controls. In 2004 large rod-shaped bacteria were observed by scanning electron microscopy in 40% of the jejunal biopsies of Swedish CD children but not in control biopsies. These micro-organisms have recently been identified as Clostridium spp., Prevotella spp. and Actinomyces graevenitzii by means of 16S rRNA sequencing. In this study, 26 of 28 CD patients with duodenal microbiome containing these rod-shaped bacteria were born between 1985 and 1996, during the

Figure 5.3 Diversity index of untreated CD children (red bars) and controls (green bars) represented by the number of bands present in each profile (Y-axis).
Mean diversity in both groups is 18.
time of the Swedish CD epidemic in which a fourfold increase in CD incidence was seen in children under 2 years of age. It has been suggested that the presence of these rod-shaped bacteria was in some way related to the Swedish CD epidemic, but it has to be taken into account that the start of the CD epidemic coincided with new national feeding recommendations for infants, postponing the introduction of gluten-containing foods from 4 unto 6 months of age. Therefore, a larger percentage of these children received gluten without breastfeeding. Another consequence of these recommendations was a twofold increase in the average gluten consumption from commercially available milk cereal drinks in children younger than 2 years of age. These recommendations were later changed to the introduction of small and gradually increasing portions of gluten-containing food into the infant’s diet between 4 and 6 months of age under the protection of breastfeeding. The exact role of these dietary recommendations in the CD epidemic is not yet known; a large prospective study investigating the optimal age of introduction of gluten is on its way. Although it is imaginable that changes in infant-feeding practices play a role in the composition of the adherent microbiome of the small intestine, this cannot explain the observed microbial differences between CD children and healthy age-matched controls. It is also unclear whether the differences in microbiome reflect a primary role in the aetiology of CD or should rather be considered a consequence of the disease.

In addition to the results of Ou et al., significant differences in duodenal and faecal microbiome of CD children as compared to healthy age-matched controls have been described in a study from Collado. Clostridium leptum and Bacteroidetes spp. were more abundant in faeces and biopsies of CD patients as measured by quantitative real-time PCR (qPCR), regardless of the stage of the disease. Another finding in this study was the significantly lower levels of Bifidobacterium spp. in the duodenal biopsies of untreated CD children as compared to control children. The authors suggest that either Bifidobacterium protects against CD, or inherent features of the CD intestine influence Bifidobacterium colonisation. The same group analysed the biodiversity in duodenal biopsies by means of fluorescent in situ hybridization (FISH) coupled with flow cytometry and reported significant more Bacteroides spp. and Escherichia coli in CD patients as compared to controls. A possible pathophysiological role for bacteria in the etiology of CD was also suggested in a recent study from Schippa et al, showing major differences in the mucosa-associated microbiome between 20 children with CD and 10 controls. Bacteroides vulgatus (85% vs. 20%) and Escherichia coli (95% vs. 20%) were significantly more abundant in CD compared to controls. In this study only 8 16S rDNA gene-targeted primers were utilized to detect
those particular species considered potentially involved in the etiology of CD (Escherichia coli, Bacteroides vulgatus, Clostridium coccoides, Bifidobacterium spp.).

The detecting methods used in these former studies are qPCR, FISH, and 16S rDNA sequencing, making it unreliable to compare the results. qPCR and FISH are restricted methods using a set of predetermined primers to detect and quantify micro-organisms, whereas 16S rDNA sequencing is an unrestricted method which has been described to have relatively low phylogenetic power at species level and limited discriminatory power for some genera.27

Our study is the first one to compare mucosa-associated microbiome of the duodenum of children with CD and age-matched healthy children by means of IS-pro, a validated 16S-23S interspacer region-based profiling method. This PCR-based method distinguishes and identifies bacteria by phylum-specific sequence polymorphisms of 16S rDNA combined with the length of the 16S-23S IS region. These regions are extremely variable in size and sequence, even within closely related taxonomic groups. Fluorescently labelled phylum-specific primers in the 16S rDNA sorts species as members of the phyla Firmicutes/Actinobacteria, Bacteroidetes and Proteobacteria, together accounting for almost the entire (>95%) human intestinal microbiome.28 Amplification of the IS region leads to further identification of the bacteria at the species level. Different from previously used methods, IS-pro gives a unrestricted, virtually complete insight in the composition of bacterial consortia, including quantification, and is therefore extremely suitable for analysis of the highly complex human gut microbiome.14 By means of this highly sensitive method we did not find significant differences between the CD and control group. The mucosal microbiome of both groups were dominated by Streptococcus spp., Lactobacillus reuteri, Clostridium spp. and an as yet unknown species with IS-length of 362 nt. Moreover, the diversity index of the mucosa-associated species was similar in both groups. Our findings are in agreement with the recent study of Ou et al. analysing the duodenal microbiome of 45 Swedish children with CD in the period between 2004 and 2007, describing a predominance of Streptococci and Neisseria spp. with only marginal differences as compared to controls.11 A recent study comparing small intestinal microbiome of 10 children with coeliac disease and 9 healthy controls by means of quantitative polymerase chain reaction (qPCR) also showed similar profiles in patients and controls.29

In conclusion, using a highly sensitive method we did not find any relevant differences in small bowel mucosal microbiome composition and diversity index between children with...
untreated CD and control children without CD. This suggests that bacteria inhabiting the duodenal mucosa do not play a significant role in the pathophysiology of CD. The fact that these results are discordant with those of several previous studies could be explained by different microbial identification methods and different harvesting locations, as several of the previous studies were done in faeces and therefore do not adequately reflect the duodenal microbiome. We suggest that studies regarding the events preceding the development of active coeliac disease should concentrate on different factors than the duodenal microbiome.

REFERENCES
