Reviewer ID: 00343118

The authors would like to thank for review and for valuable comments and suggestions, which were involved in the manuscript. All changes are highlighted in red. The language was improved, and the manuscript was corrected by English language specialist.

A detailed description of the Reviewer's comments:

Lane 73 added "e.g lymphocytes....".

It was added. The current sentence is following:

"In CD individuals, some of these peptides can bind to HLA-DQ2 or -DQ8 heterodimers expressed on the surface of the antigen presenting cells (e.g. macrophages, lymphocytes and dendritic cells) and, after triggering T cell responses, lead to local tissue damage."

Lane 79...tissue damage direct cytotoxicity (is missing).

The given phrase was incorporated into the text, and sentence now is following (now lines 77-80):

"Gluten-activated T cells release pro-inflammatory cytokines (mainly interferon gamma (IFN-), interleukin (IL)-21, IL-17), which induce mucosal inflammation and have a direct cytotoxic effect on the epithelium, all of which finally leads to villous atrophy in the small intestine."

Lane 84 "of innate immunity dependent"...while innate and not both innate and adaptive ?? please explain better your concept.

In the corrected version we emphasized the importance of both adaptive (specific) and innate (non-specific) immunity.
The current sentence(s) is as follows (now lines 81-83):

…." Thus, this adaptive (specific) T-cell response is a requirement for CD development. Nonetheless, innate (non-specific) immunity also plays an important role in CD development…"

Lane 91, "NK receptor" Which ?

It was added. Now it is: ……. "and the increased expression of NK receptors, such as NKG2D and CD94/NKG2C, on the surface of IELs."

Lane 93.” permeability to immunogenic gluten peptides”...but not only.

We corrected this sentence. Now it is:

“Finally, IL-15 activation leads to innate cytotoxic disruption of epithelial cells, resulting in increased intestinal permeability to different luminal macromolecules, including immunogenic gluten peptides.”

Lane 96. “Gluten is introduced into the diet at the 17 weeks of age”...also in the mother milk ?

This section was changed as the other Reviewer wanted to specified exact time of gluten introduction into the diet. The data concerning gluten introduction in breastfed infants now is included. The corrected section is following (now lines 97-110):

“The role of both breastfeeding and the time when gluten is first introduced into the diet in the risk of CD has long been debated. Retrospective data from Sweden indicated that introducing gluten in small amounts to breastfed infants at the age between 4 and 6 months reduced the risk of CD compared with introducing gluten in larger amounts at older ages[7,8]. However, a recently published systematic review with meta-analysis of studies that assessed the effect of gluten consumption on CD development showed that for infants at high genetic risk of CD, gluten introduction at the age of 4, 6 or 12 months, resulted in similar rates of CD diagnosis in childhood, and neither breastfeeding as such (at any time during an infant’s life) nor breastfeeding during gluten introduction were shown to reduce the risk of CD[9]. Also the recently published prospective PreventCD cohort study showed that neither the gluten consumption pattern nor the amount of gluten consumed at the age of 11–36 months influenced CD development in children with a genetic risk[10]. Thus, the time of gluten introduction into the diet seems not to play a key role in CD development”.

Lane 106. and “antimicriobial peptides”

It was corrected (now lines 119-120):

……. “the epithelium, with its specialized mucus-producing cells and cells producing antimicrobial peptides;…..”

Intrahepitheial...

The mistake in the word “interepithelial” was corrected through the whole manuscript.

Lane 158. “The zonulin was identified as pre-haptoglobin 2 and structural analysis of this protein revealed similarities with several growth factors”....examples ?

The examples were added. Now it is (lines 171-172):

….” several growth factors, such as hepatocyte growth factor or epidermal growth factor (EGF),…..”

Lane 161. “Increased zonulin release can be triggered by both, gluten peptides and dysbiosis of the intestinal microbiota” AS ?? like ??

The release of zonulin in the small bowel was shown to be triggered by enteric bacteria, and recent study present that the level of serum zonulin is dependent on composition of the gut microbiota. Thus, we apologize for the shortcut. Now we describe this association in more details following (now lines 184-190):

“In vitro studies showed that increased zonulin release in the small intestine can be triggered by both gluten peptides[38,39] and enteric bacteria[40]. Zonulin secretion has been demonstrated to be independent of either the species or the virulence of the microorganisms tested[40]. However, recently an association of low serum zonulin levels with lower quantities of Bacteroidaceae and Veillonellaceae and higher quantities of Faecalibacterium has been found in overweight pregnant women[41]. Thus, this in vivo study suggests that zonulin release could be affected by changes in gut microbiota composition.”

DYSFUNCTIONS OF INTEREPITHELIAL JUNCTIONS IN CD PATIENTS

Lane 174. “Interepithelial” or intraepithelial ??? junctions

It was changed to “interepithelial”.

The gut....

Lane 234. “GAL”...initial , indicate the abbreviation words.

The abbreviation words [GALT (gut associated lymphoid tissue)] were indicated earlier in Introduction section (line 120).

Lane 240. Subsequently .."and directly affect the composition of the gut microbiota"

We changed this section. Now it is (lines 265-267):

"SIgAs are natural antibodies that constitute the first line of defense by react with a wide spectrum of microorganisms and toxic molecules, which directly affects the composition of the gut microbiota[61]."

Lane 250. " Thus, the gut microbiota protects the epithelium and strengths its barrier functions". This is an hypothesis.

Now we stressed that this is hypothesis following (lines 275-276):

"Thus, the gut microbiota seems capable of protecting the epithelium and strengthening its barrier function."

Lane 306. Which volatile organic compounds?

It was added. Now it is (line 338) ……. volatile organic compounds (e.g. phenols, ketones)……..,

Lane 321. Please complete your conclusions/sentence.

This section was rewritten as following (now lines 361-364):

"Thus, dysbiosis, which can follow viral or bacterial infections or antibiotic therapy, may activate innate immunity leading to pro-inflammatory changes, with the resulting IEL infiltration, epithelial barrier disruption, and increased transfer of immunogenic gluten peptides, which in turn activate inflammation leading to CD development (Figure 3)."

Lane 326. "Since low dose of pro-inflammatory cytokines are sufficient to induce bacterial endocytosis by epithelial cells". Why??,

We explained the mechanism, and rewritten the sentence following (now lines 352-355):

“Low doses of pro-inflammatory cytokines, such as IFN-γ were shown not to affect TJ protein expression but to activate bacterial endocytosis by epithelial cells[95]. This process is dependent on extracellular signal-regulated kinase (ERK) 1/2 and ADP-ribosylation factor (ARF)-6 signaling[96].”

Lane 331. “.....Increased activity and expression of inducible nitric oxide synthase in human duodenal enterocytes is characteristic for CD patients “ The relationship is not obvious.

We changed the sentence following (now lines 358-359):

“An increased activity and expression of inducible nitric oxide synthase in human duodenal enterocytes has been reported in CD patients[97].“

Lane 336. “The gut microbiota is responsible not only for the immune homeostasis and functioning of the epithelial barrier, but also can have direct impact on gluten digestion in the intestinal tract. There is evidence that certain bacterial strains isolated from feces, e.g. Bifidobacterium and Bacteroides fragilis, are capable to digest immunogenic gliadin peptides rich in proline residues, which are resistant to human enzymes” This sentence is important but not in this contest. Please introduce the concept or change the position of this sentence in the text.

This part was moved to the section **THE GUT MICROBIOTA: THE MAINSTAY OF EPITHELIAL AND IMMUNE HOMEOSTASIS (lines 313-317).** It is marked in red in the manuscript.

The role of gut

Lane 400. “To date we have limited evidence that HLA-DQ2/-DQ8 genotype found in CD patients could selectively influence colonization of the gut microbiota, but recent microbiome analyses using the next generation sequencing ...” While the next generation technique would be added more information?

You are right that the information concerning the technique does not matter in the context of this sentence. We wanted only to emphasised that in this study complex microbiome was analysed, not cultivation or FISH methods were used. Finally we changed this sentence into (lines 421-422):

"Recent microbiome analyses performed on 22 infants demonstrated that certain HLA genes predisposing to CD could affect microbiota composition"

Lane 418. "It cannot be excluded that this CD associated microbiota ??? is more sensitive to infections agents, antibiotics, life style factors in the future life, and..."

Lane 432. "first clinical studies presenting such possibility" ...with the results ??? Idem lane 431.

Lane 436. HLA could also predispose to anti-Ig TTG2 that could further increase tissue damage.

Suggestions lines 418, 432, 436:
As the other reviewer suggested to rewrite the section Conclusions and Future strategies we have changed this section. Now there is no selected sentences (line 432 and 436). The section Conclusions and Future Strategies was changes following (now lines 440-453):

"Although gluten is necessary in order to activate the processes leading to CD, there is evidence that an imbalance in the gut microbiota and intestinal epithelium can precede the specific gluten-dependent immune response. Under certain conditions affecting the intestinal microbiota, e.g. after infections or antibiotic therapy, an increased translocation of dietary macromolecules (including gluten peptides) via the opening of epithelial junctions triggers a cascade of events in genetically susceptible individuals, leading to overt CD. Microbiota disturbances are observed not only in untreated CD patients, but also in potential CD patients and those following a GFD as well as in infants at high genetic risk of CD. The microbial fingerprint associated with CD is likely dependent on specific genetic factors, including (but not exclusively) the HLA-DQ2/DQ8 genotype. Future strategies should include prospective, birth cohort studies involving comprehensive genome, microbiome, and metabolome analyses. Such an approach could help identify a "CD-specific" microbial/metabolic fingerprint, which would become the target for both primary prevention and management of CD."

REVIEWER ID 01944824

Authors would like to thank for review and for valuable comments, which all were included in the manuscript. All changes are highlighted in red. The language was improved, and the manuscript was corrected by English language specialist.

A detailed description of the Reviewer's comments:

Lines 30-31: The implication here is that gluten appears in genetically predisposed individuals.
We corrected the sentence following:
"Celiac disease (CD) is a chronic immune-mediated disorder, which appears in genetically predisposed patients, triggered by the ingestion of gluten."
Line 33: Use "deamidated" only once in a sentence.
It was done.
Line 65: see lines 30-31
It was done.
Line 67: Delete "It is known that"
It was done.
Lines 82-85: Run-on sentence
It was done.
Line 86: Delete "It is known that"
It was done.
Lines 95-97: Please reference. The introduction of gluten by 17 weeks of age is not universal. Nevertheless, recent data suggest the CD risk may be increased in subjects delaying gluten exposure until after one year of age. The authors must consider this phenomenon in their exposition.
This section was rewritten and more information were included. Now the section is following(lines 97-110):
"The role of both breastfeeding and the time when gluten is first introduced into the diet in the risk of CD has long been debated. Retrospective data from Sweden indicated that introducing gluten in small amounts to breastfed infants at the age between 4 and 6 months reduced the risk of CD compared with introducing gluten in larger amounts at older ages[7,8]. However, a recently published systematic review with meta-analysis of studies that assessed the effect of gluten consumption on CD development showed that for infants at high genetic risk of CD, gluten introduction at the age of 4, 6 or 12 months, resulted in similar rates of CD diagnosis in childhood, and neither breastfeeding as such (at any time during an infant's life) nor breastfeeding during gluten introduction were shown to reduce the risk of CD[9]. Also the recently published prospective PreventCD cohort study showed that neither the gluten consumption pattern nor the amount of gluten consumed at the age of 11–36 months influenced CD development in children with a genetic risk[10]. Thus, the time of gluten introduction into the diet seems not to play a key role in CD development."

Lines 156-157: The authors should be more descriptive in discussing the zonulin pathway. Increased permeability (via paracellular movement) is induced by the opening of tight junctions, thus permitting macromolecular uptake.

The zonulin pathway was discussed in greater detail. Now the section is following(lines 173--182):

"Zonulin transactivates the EGF receptor through proteinase-activated receptor 2 (PAR2), and then activates phospholipase C, which hydrolyzes phosphatidyl inositol (PPI) to release inositol 1, 4, 5-tris phosphate (IP-3) and diacylglycerol (DAG)[38, 39]. Protein kinase C α (PKCα) is then activated, either directly (via DAG) or through the release of intracellular calcium ions (via IP-3). Membrane-associated, activated PKCα catalyzes the phosphorylation of target proteins, including ZO-1 and myosin 1C, as well as polymerization of soluble G-actin in F-actin. This polymerization results in actin filament rearrangement and subsequent displacement of proteins (including ZO-1) from the junctional complex. As result, intestinal TJs become looser, which increases the paracellular transport of luminal molecules."

Line 162-170: How does a polarity change influence permeability? The unfortunate implication here is that the Dutch study represented a population of genetically unique (and homogeneous) subjects. While the study was performed in the Netherlands, I doubt whether this conclusion was either stated or implied. Please re-write, with special attention to grammar and sentence structure.

We rewritten the section concerning proteins regulating the cell polarity and Dutch results following (now lines 191-201):

"Recently, epithelial polarity regulators, especially Par-3 protein, have been reported to be likely involved in regulating TJ permeability[42]. Par-3 and other proteins regulating cell polarity, such as Par-6 and atypical protein kinase C, form the apical polarity complex that orchestrates the formation of AJC. In addition, Par-3 located in the junctional complex together with ZO-1 and catenins is able to affect TJs by rearranging the actin cytoskeleton. Shuman et al (2012) found a reduced level of Par-3 and a defect in performing lateral exclusion of Par-3 in the epithelial cells of CD patients[43]. In this context, genetic studies on non-HLA gene candidates associated with CD seem to be very interesting. Wapenaar et al. (2008) found two candidate genes: Par-3 and Magi2, encoding the proteins regulating of epithelial polarity[44]. However, this study involved a homogenous Dutch population, and further genome-wide association studies (GWAS) did not confirm this association[45]."

Lines 213-221: Poorly written, difficult to interpret, with run-on sentences. The last sentence is unintelligible. Please review and re-write.

The paragraph was rewritten and simplified as following (now lines 244-248):

"Overall, the presented results show that epithelial barrier impairment occurring in CD patients can play an important role in CD development. Because epithelial function is regulated by microorganisms colonizing the intestines[56], there is a hypothesis that dysbiosis, i.e. disturbances in both the quantity and composition of the gut microbiota, is a critical factor for the activation of innate immunity, leading to epithelial barrier dysfunctions"

Lines 227-228: This statement is incorrect. The microbiota:cell ratio is much closer to 1:1 (Sender R, Fuchs S, & Milo R. (2016) Revised estimates for the number of human and bacteria cells in the body. bioRxiv doi: http://dx.doi.org/10.1101/036103)

Thank you for this correction. The reference was included and the mistake was improved (now lines 254-257) following:
"The number of bacteria in the gut microbiota is similar to the number of cells making up the human body[58], and microbiota genes (microbiome) outnumber those in the human genome by approximately 100-fold"
Lines 385-388: Please clarify the risk determination in these situations (?increased, ?decreased).
We explained that the risk increased (now line 417):
"…….there have been studies showing that cesarean sections and antibiotic treatment in infancy increased the risk of CD[113–115]"
Lines 391-397: Please re-write. This is extremely confusing and difficult to interpret. The authors must be careful in implying that the HLA DQ2/DQ8 genotype "found in CD patients" is associated with a specific microbial fingerprint. Since 25-30% of the general population exhibits the same HLA alleles, other factors must be prescient (assuming the microbial population in CD is unique).
We rewritten this section following (now lines 431-437):
"As CD is strongly associated with HLA genes – almost 100% individuals with CD are carriers of alleles encoding HLA-DQ2/DQ8 molecules, these findings suggest that children with the CD risk genotype have a different microbiota profile than those without genetic predisposition. However, it must be emphasized that about 25–30% of the general population exhibits the same HLA genotypes as CD patients[121]. In addition, there are also non-HLA genes associated with CD."

Lines 405-411: Convoluted, run-on sentences, without a clear focus. Furthermore, data either "suggest" or "may indicate" (the term "could suggest" is not acceptable).
Concluding Remarks: If this reviewer has interpreted the manuscript correctly, the authors are stating the following:
Patients with latent CD manifest a unique microbial fingerprint. This "CD specific" distribution of microbial species is likely dependent upon specific genetic factors, including (but not exclusively) the HLA DQ2/DQ8 phenotype. Under conditions of altered gut permeability, for example during acute infection, macromolecular uptake (via the opening of parcellular tight junction gates, cf. Fasano et al) leads to translocation of dietary macromolecules and microbial elements. This phenomenon triggers a cascade of events in genetically susceptible individuals, leading to overt CD.
At least, that is my reading of this section, which must be rewritten to address: 1. A review of data presented and the derived conclusions; and, 2. A suggested pathway for future research.

The section "Conclusions Remarks and Future Strategies" was rewritten following (now lines 440-453):
"Although gluten is necessary in order to activate the processes leading to CD, there is evidence that an imbalance in the gut microbiota and intestinal epithelium can precede the specific gluten-dependent immune response. Under certain conditions affecting the intestinal microbiota, e.g. after infections or antibiotic therapy, an increased translocation of dietary macromolecules (including gluten peptides) via the opening of epithelial junctions triggers a cascade of events in genetically susceptible individuals, leading to overt CD. Microbiota

disturbances are observed not only in untreated CD patients, but also in potential CD patients and those following a GFD as well as in infants at high genetic risk of CD. The microbial fingerprint associated with CD is likely dependent on specific genetic factors, including (but not exclusively) the HLA-DQ2/DQ8 genotype. Future strategies should include prospective, birth cohort studies involving comprehensive genome, microbiome, and metabolome analyses. Such an approach could help identify a “CD-specific“ microbial/metabolic fingerprint, which would become the target for both primary prevention and management of CD”.

REVIEWER ID 01552044

The authors would like to thank for the review of the manuscript. The language was improved, and the manuscript was corrected by English language specialist.