2. REVIEW OF LITERATURE

CD is a disease caused due to interplay of 3 main factors i.e. genetics, environmental factors (eg. gluten) and intestinal microbiome. For each particular individual, trigger of disease may vary. In CD, gluten leads to an immune activation to trigger inflammatory events at intestinal mucosa thereby causing destruction of tight junction barrier function. Disturbed tight junction barrier leads to increased permeability of intestine to the dietary antigens and infections. Activated immune pathways further cause cascades of immune activations leading to production of antibodies against gluten proteins and several self antigens (anti tTG antibodies, Anti endomysial antibodies). These antibodies are primarily used to screen the CD patients based on serum anti tTG antibody titters. Patients positive for these antibodies are confirmed for disease based on histopathological report of duodenal biopsy sample. Till date, biopsy sample of duodenum or small intestine is considered gold standard for confirmation of CD. In biopsy sample, disease confirmation is decided based on destruction of intestinal villi (villous atrophy), elongated crypts (crypt hyperplasia) (1) and altered intestinal barrier (2). CD is a T-cell mediated disease in which gliadin-derived peptides affect lamina propria and T lymphocytes that lead to the release of proinflammatory cytokines IFN-γ and IL-15, responsible for the activation of the cytotoxicity in intraepithelial lymphocytes (33, 34). Studies suggest about interaction of intestinal bacteria with immune system to direct the differentiation of both pro-inflammatory and anti-inflammatory T cell populations (17). Role of Tregs has become clearer with the efforts of Serena et. al.(2017) helping out to understand the pathogenic role of gut microbiota and their metabolites in CD through epigenetic processes. Regulatory T cells (Treg) are subset of CD4 T cells responsible for maintaining immune response to foreign antigens. Treg cells mediate suppression of responder cells by different mechanisms. Deficiencies of Treg cells population is associated with various autoimmune diseases such systemic lupus erythematosus (28). Previous studies have reported an increase in the number of Treg cells in CD patients and suggested that functional impairments in their suppressive function may be related to the onset of the disease. The exact mechanisms that cause the altered regulatory activity of Treg
cells in CD is not known. FoxP3 is a transcription factor fundamental for the suppressive function and differentiation of Treg cells. They reported a higher expression of an alternatively spliced isoform of FOXP3 in the intestine of active CD patients as compared to non-celiac controls while no differences were seen in the expression of FOXP3 FL thereby suggesting that altered intestinal ratio between FoxP3 isoforms may be an important indeces in the pathogenesis of CD. Furthermore, higher expression of isoform FOXP3 D2 is associated with inflammation. Ex-vivo cultures of intestinal biopsies treated with butyrate triggers a balance between FoxP3 isoforms in HC subjects, while the same does not occur in CD patients. That shows the role of microbial metabolites in modulation of splicing, thus triggering to an inflammatory state with some role in pathogenesis of CD.

Pathogens are important factors in pathogenesis of some gastrointestinal diseases. Intestinal microbes interact with immune cells and activate the inflammatory cascades through TLR pathway (17, 18) that regulates the intestinal barrier function (19). The interaction of intestinal epithelial cells takes place with microbes residing in the gut lumen via pattern recognition receptors such as TLRs and Nod-like receptors that induces antibacterial molecules and chemokines to promote adaptive immune responses in the intestine (18). Moreover, TLR2 pathways have been reported to be involved in intestinal barrier function as mice lacking TLR2 expression are more susceptible to microbial-induced colitis (19). In addition, duodenal TLR2 expression is lower in CD subjects and higher for TLR9 as compared to healthy controls (35) thereby suggesting the involvement of microbes in inducing impairment of intestinal barrier function and immune dysregulation through TLR dysfunction. Furthermore, microorganisms stimulate IFN-γ, TNFα production and activate T-cell macrophage-associated activity may trigger a TH1-type cytokine profile (36). Such pro-inflammatory response can contribute to an increase in the epithelial permeability that favors the access of higher antigen loads to the submucosa (36-38). Furthermore, intestinal bacteria can also regulate the ability of monocytes recruited to the mucosa to respond to gliadins and IFN-γ in CD subjects, thereby influencing the course of the disease (39). The process of autoimmunity might have resulted in epithelial stress triggered by infections, gluten peptides and inflammation. Some experimental studies further affirm the interactions between gluten, microbiota and mucosal immune dysregulation.
The composition of the intestinal microbiota affects the permeability of the intestinal mucosa and could be involved in the early stages of CD development (40). Some CD infections like *Pseudomonas aeruginosa* directly trigger the process of inflammation as reported by Alberto Caminero *et. al.* (41). *P. aeruginosa* possess elastase properties to cleave gluten peptides in such manner making them highly immunotoxic. These peptides were more potent to be translocated through the intestinal barrier and to cause activation of T-cells from CD patients (41). In context to pathogenic role of some intestinal microbes in CD, when colorectal adenocarcinoma (Caco-2) cells were exposed to *Bacteroides fragilis* and gliadin, increase in intestinal permeability as well as increased production of TNFα and IL-1β18 was reported (42). After *ex-vivo* treatment of duodenal biopsy with gluten digest and CD associated bacteria, IL-17A responses were induced (43) that is known as pro-inflammatory cytokine in autoimmune and inflammatory diseases (44). Sequencing technologies provide the basis of host microbe interactions. Whole genome sequencing of microbes has enabled to predict pathogenic behavior of microbes (45-47) whereas D'Argenio V *et. al.* (47) confirmed the pathogenic role of CD gut microbes by combination of *in-vitro* approaches and revealed that *Neisseria flavescens* species were most abundant in the symptomatic patients of CD. Genetic composition of virulence determinant genes was different in the strains of healthy controls compared to the diseased ones, as showed by whole genome sequence of the microbes. In addition, these strains isolated from patients, activated inflammatory responses in DCs and in *ex-vivo* culture of duodenal biopsies (47).

As the process of immune dysregulation in CD is looking to be derived from intestinal dysbiosis and infections while antibiotics act as trigger giving rise to a disease prone dysbiosis. The following sections describe the types of dysbiosis caused in CD and its impact on commensal organisms.

### 2.1. Intestinal Dysbiosis in CD

In some studies, dysbiosis in CD subjects was represented by outnumbered of *Bacteroides* spp. and lower numbers of *Bifidobacterium* spp. and *B. longum*, that was unchanged after GFD (20-22). Other studies reported increased prevalence of *B. dentium* and *B. vulgatus* while decreased prevalence of *B. catenulatum* and *Bacteroides* species (21, 48, 49). During analysis of fecal microbiota in active (Symptomatic) CD children, the levels of *Bacteroides, Clostridium, Staphylococcus,
Clostridium histolyticum, Prevotella, Eubacterium rectal, C. coccoides, Atopobium, and sulfate reducing bacterial groups were reported higher (50). Greater prevalence of infectious microbes and gram-negative bacteria in the duodenum of celiac children was found responsible for symptomatic presentation of the disease (20). Analyzing duodenal microbiota, T-CD patients with persistent symptoms were occupying different texture of intestinal microbes in comparison with those without symptoms. Patients with persistent symptoms comprised of higher relative abundance of Proteobacteria and a lower abundance of Bacteroidetes and Firmicutes. Comparative microbial richness in these patients was lesser along with persistent symptoms even though being on strict GFD (51).

As per a recent report, when infants at high genetic risk were compared to those with lower risk, lower proportions of duodenal Actinobacteria and Bifidobacterium were observed in the former whereas higher proportions of Firmicutes and Proteobacteria were found in the later (24). Some studies showed direct relationship of commensal bacterial groups indicating the potential therapeutic implications as described in the next sections.

2.1.1. Associations of Lactobacilli and Bifidobacterium with CD

Based on the recent advancements in the molecular studies, we can clearly find a strong association of some commensal bacterial groups with CD that may guess about the modulation of the disease pathogenesis by them.

In some new findings, significant lower counts of Lactobacilli were found in feces of celiac children on GFD as compared to healthy children, whereas Enterobacteria were found increased in celiac children (52). Compared to the healthy subjects, fecal counts of Bifidobacteria were significantly higher in T-CD patients of Brazil (28). While studying the duodenal microbiota, counts of Lactobacillus were found significantly reduced as compared to the healthy individuals. CD patients were possessing Streptococcus, Bacteroides and E.coli species whereas lower counts of Streptococcus and Bacteroides were observed and numbers of Bifidobacterium, Lactobacillus and Acinetobacter were found higher in controls (29).

The ratio of Lactobacilli and Bifidobacterium to Bacteroides and Enterobacteria was lesser in T-CD subjects as compared to Healthy Controls (HCs) and this difference was more when compared with U-CD subjects (30, 53).
In contrast the GFD fed CD subjects also shown a reduction in the diversity of *Lactobacillus* spp. and *Bifidobacterium* spp. while presence of *Bifidobacterium bifidum* was higher in normal diet fed CD subjects than healthy adult (54). The ratio of *Lactobacillus/Bifidobacterium* to *Bacteroides/E. coli* has been known to be significantly lower in symptomatic and asymptomatic diseased subjects compared with controls (20). *Leuconostoc mesenteroides, Lactobacillus curvatus,* and *Leuconostoc carnosum* species were reported as characteristic of CD subjects, while *Lactobacillus casei* group was characteristic of healthy controls while species diversity for *Bifidobacterium* was significantly higher in healthy children than that of CD (31). It can be concluded that number and types of *Lactobacilli* and *Bifidobacterium* strains are associated with CD and may have influence in the disease outcome. The results derive their implications towards therapeutic use because *Lactobacilli* are recently well proved for their glutenase activity and anti-inflammatory actions (55-57).

### 2.2. INTERACTION OF MICROBIOTA AND GLUTEN

Digestion of gluten proteins (gliadins and glutenins) is difficult by human proteolytic enzymes (58). These proteins have been reported to possess differential immune targets that make them immunotoxic in nature.

During proteolytic digestion in intestine, proline and glutamine rich gluten polypeptides are produced that are immunogenic and have the potential to stimulate T cells (59). These peptides are resistant to further hydrolysis due to enrichment of proline residues in the amino acid sequences (60). As most of the peptides are immunogenic in nature, some of them (p10-mer, QQPQDAVQPF) possess protective effects whereas others prevent the gliadin-dependent dendritic cell maturation.

Some new studies further highlighted the value of gut microbes in determination of gluten immunogenicity. *Pseudomonas aeruginosa* isolated from CD patients cleaved 33 mer peptide in such a manner that has activated gluten-specific T-cells in CD patients. On the other hand, *Lactobacillus* spp. isolated from the non-CD controls had potential to reduce the immunogenicity of the peptides produced by *Pseudomonas aeruginosa* (41). Looking at the necessity of degrading such peptides, an era of glutenases seems an evergreen field of research for years.
2.2.1. Trends in Glutenases

Some studies were targeted to explore the proteolytic enzymes from sources other than bacteria that could degrade the immunogenic gliadin. An enzyme from barley, EP-B2 (glutamine-specific endoprotease) degrades complex gluten bread proteins whereas prolyl endopeptidase (PEP) from *Sphingomonas capsulata* detoxifies the residual oligopeptide products of EP-B2 prolyl endopeptidase. AN-PEP (*Aspergillus niger* prolyl endopeptidase) also known to enhance the gluten digestion in such an excellent manner that only traces of gluten were detected in small intestine (61, 62). AN-PEP degrades T cell stimulatory peptides and gluten (63). For still better digestion of gluten, proline and glutamine specific endopeptidases from barley, fungi and bacteria were taken into account collectively by different scientific communities (60, 63-66).

The use of PEPs proved extensively effective in *in-vivo* (animal models) and *in-vitro* studies (67-69). Encouraged from these preliminary studies, the present microbiota research is more emphasized on discovery of gluten degrading bacteria that might help in enzyme therapy or directly as live culture against CD.

2.2.2. Role of intestinal microbiota in gluten degradation

The oral cavity occupies protease producer microorganisms capable to hydrolyze proline and glutamine rich peptides (70, 71). Salivary microorganisms exhibit glutamine endopeptidase activity to degrade most of the gliadins (70).

*Rothia aeria* HOT-188, *Rothia mucilaginosa* HOT-681, *Streptococcus mitis* HOT-677, *Streptococcus* sp. HOT-071, *Actinomyces odontolyticus* HOT-701, *Neisseria mucosa* HOT-682 and *Capnocytophaga sputigena* HOT-775 were the other gluten degrading bacteria identified by Fernandez-Feo et al (72). *Rothia mucilaginosa* and *Rothia aeria* are highly active towards gluten and are able to cleave 33-mer and 26-mer immunogenic peptides. Interestingly, the enzyme produced by *Rothia aeria* is active over a wide pH range (pH 3-10) of intestinal pH (71).

In addition, salivary microbiota and metabolome are reported to be associated with CD (73). Despite of significance of oral microbiota in gluten degradation, Caminero et al. (74), gained attention by certifying the significance of intestinal/duodenal microbiota in metabolism of proteins that is also admirable work of its kind. Although, *Lactobacillus helveticus* alone is efficient to cleave the long immunogenic...
peptides (75) but Caminero et. al. (74), reported 144 strains of 35 bacterial species exhibiting the property of gluten metabolism. Most of these strains were from phyla Firmicutes and Actinobacteria (74). Exploring the role of duodenal microbes in gluten degradation, their group discovered 31 strains with extracellular proteolytic activity against gluten and 27 other strains had peptidolytic activity against the 33-mer peptide. A mixture of Lactobacilli and fungal proteases were studied to demolish the immunogenicity of wheat by long-time fermentation and 33-mer peptide was efficiently hydrolyzed by Lactobacilli (76).

On the footsteps of traditional trends in glutenases, modern research is promoting to commercialize the glutenases into action. Savvateeva LV et al. (77), proved that recombinant wheat cysteine protease triticain-α exhibited glutenase and collagenase activities that is stable at intestinal pH and was potent to hydrolyze immunotoxic gluten peptides. In a phase 2 trial, glutenase ALV003 successfully attenuated mucosal injury in CD patients consuming upto 2g gluten daily along with GFD (78). Approaches to screen gluten degrading bacteria are still in fast progression. Lactobacillus ruminis, Lactobacillus johnsonii, Lactobacillus amylovorus, Lactobacillus salivarius bacteria comprises of high peptide-degrading properties. All the strains possessed different degradation rates and cleavage patterns capable of reducing immunotoxic gluten peptides but were not efficient for complete removal of peptides (79).

2.3. GUT FLORA MODULATION: IMPACT ON CD

Now CD is known for overgrowth of pathogenic microbes that dominates to symbionts thereby excluding the later from intestinal ecosystem (32). Administration with some probiotics either alters the intestinal microbiome composition or brings about some health beneficial immunomodulations. As described here, the gut flora modulation proved to be beneficial when trials were conducted in different in-vitro and in-vivo models and somehow in human trials also.

2.3.1. Probiotics

Probiotics are live microorganisms that provide health beneficial impact on host when administered in adequate amounts. Reports showing probiotic induced beneficial effects in animal models of CD certify that probiotics have a positive influence on disease pathology through different mechanisms. Down-regulation of
pro-inflammatory biomarkers, expression of NF-kB, TNFα, and IL-1β was modulated in cell culture experiments after administration with *Bifidobacteria* (80).

### 2.3.2. Probiotic based approaches and tolerance induction

A strategy used to induce suppression of immune responses to an antigen to develop tolerance for the same antigen is known as tolerance induction. Different approaches for tolerance induction are used. Stefania Senger *et al.* (81), showed the potential usefulness of recombinant alpha-gliadin protein for the immunomodulation of this disease. Intranasal administration of recombinant alpha-gliadin in DQ8 transgenic mice induced downregulation of immune responses against gliadin.

Another study was emphasised on oral tolerance that is the induction of antigen-specific suppression of immune responses to an antigen by its prior oral feeding. *Lactobacillus lactis* was engineered to secrete a deamidated DQ8 gliadin epitope. Oral administration of this bacteria suppressed DQ8 restricted T-cell responses in NOD ABα Q8 transgenic mice (82). This might be a promising therapeutic approach for treatment of CD and may prove to be helpful to prevent CD in DQ8 associated genetically susceptible individuals.

*Saccharomyces boulardii* KK1 strain had capability to hydrolyse the 28-kDa alpha-gliadin fraction, and when fed to mice, attenuated enteropathy development as well as decreased expression of epithelial cell CD71+ cells and cytokines (83). However, the administration of the *Bifidobacterium longum* CECT 7347 to rats fed gliadins ameliorated the inflammation caused by gliadin feeding alone (84). Recently, Alvarez-Sieiro *et al.* (85), designed two food-grade *Lactobacillus casei* strains by genetic engineering that could deliver PEP. Out of these two, one was capable to secrete PEP into the extracellular medium whereas another was able to maintain PEP in the intracellular environment (85). The strain was most effective to degrade 33-mer peptide and was resistant to simulated gastrointestinal stress. Although pre-clinical trials of these strains are still pending that need to confirm their actions against gluten.

### 2.3.3. Probiotics and clinical trials in CD patients

Apart from a number of *in-vitro* and *in-vivo* preclinical studies on probiotics in CD, studies are scarcely available on human trials. An exploratory trial of probiotic *Bifidobacterium* natren life start strain was proved to alleviate symptoms in untreated
CD but was not beneficial to strengthen intestinal permeability (86). The effect of this probiotic proved helpful for U-CD patients as far as gastrointestinal symptoms and serological markers are concerned. Similarly, a recent double blind randomised-placebo controlled intervention trial of *Bifidobacterium longum* CECT 7347 improved growth related parameters in Spanish children under study thereby improving the efficacy of GFD (87). This strain affected lymphocyte subsets which might contribute to recovery from mucosal inflammation. Administration of this probiotic modulated the intestinal microbiota with decrease in total copy number of microbes and decrease in *Bacteroids fragilis* that further correlated with decrease in secretory IgA evaluated from stool samples. These correlations presented the recovery of intestinal mucosa after administration of *Bifidobacterium longum*. Probiotic intervention with two strains, *Bifidobacterium breve* BR03 and *B. breve* B632 was given to CD patients in a very recent study (88). This 3 months intervention depicted a positive effect in decreasing TNFα production in CD children on GFD and the effect got reversed after 3 months of trial. Along with advancement in probiotic therapies, helminth therapy is also emerging as a fruitful outcome of research on host microbe interactions (89). During this trial of experimental hookworm infection in subjects, intestinal microbiota structure was preserved with increase in microbial species richness even when challenged with moderate gluten. This study revealed that gluten-induced inflammation can be regulated by hookworms (89).

### 2.4. FUNCTIONAL FOODS

Since probiotic bacteria are known to have protective effects in different manners, thus these are expected to ferment the foods that can be safe for CD patients. Functional foods are the foods with health-promotion or disease prevention effects that include processed food or foods prepared with health-promoting additives (90). Fermented foods with live cultures are also considered functional foods with probiotic benefits (91).

Lactic acid bacteria (LAB) under specific processing conditions have the capacity to effectively hydrolyse the major gluten protein gliadin (55, 56). Selected bacteria *Lactobacillus sanfranciscensis* 7A, *Lactobacillus alimentarius* 15M, *Lactobacillus hilgardii* 51B and *Lactobacillus brevis* 14G completely hydrolyzed gliadins during fermentation of a mixture of millet and buckwheat flours, wheat and non-toxic oat
(55). Forty-six strains of sourdough LAB were also screened for their proteolytic activity and the sourdough cultures with *Lactobacillus sanfranciscensis* LS40, LS41, *Lactobacillus plantarum* CF1 were used for the manufacture of gluten free bread (92). *In-vitro* studies on intestinal tissue of CD subjects showed that the proteolytic action of bacteria not only eliminate traces of gluten but also remains of the baked products (93). Several strains worked differently to degrade 31-43 and 62-75 alpha-gliadin fragments, whereas the 57-89 peptide degradation depends upon their genetic information to produce peptidases (94). Trials of fermented foods in CD subjects also proved safe with recovery from intestinal inflammation (95, 96). A 60-day baked diet made from hydrolyzed wheat flour with sourdough *Lactobacilli* and fungal proteases was not toxic to subjects with CD (97). The digestion of protein increased when fermentation is followed by use of intermediate content of gluten rather than whole gluten (98). The probiotic VSL#3 also decreased the toxicity of wheat flour when fermented for a longer duration (56). Gliadins predigested by VSL#3 lead decreased intestinal mucosal permeability by decreasing reorganization of the F-actin (99, 100). The development of gluten free cereals or use of pseudocereals will be most relevant if one is concerned to maintain nutritional values and safety for T-CD subjects whereas, use of enzyme therapies or functional foods may prove to be harmful because even traces of gluten peptides, if left undigested from a fermented product taken up by CD patient may lead to chances of reoccurrence.

### 2.5. ALTERNATIVE FOODS

Quinoa, a pseudo-cereal, thought to be suitable for celiac subjects because it is highly nutritive plant with low content of polyamines and has no gluten. As quinoa is a naturally occurring gluten-free seed, it can be used for formulating new bakery products suitable for celiac subjects. Fermentation of quinoa by LAB is now considered as a good alternative for production of bakery products of high nutritional value suggesting the use of LAB strain to produce baked goods for celiac patients (101). Zevallos *et al.* (102), proved that GFD added with quinoa was tolerated with improved histological and serological parameters in CD subjects and suggesting that 50g of quinoa fed daily for 6 weeks can be well tolerated. However, its long-term effects are more important to evaluate. The option to replace gluten containing foods or gluten derived fermented products with gluten free foods and fermented products of pseudocereals, is safer. But the fermented products of gluten containing cereals
may not be entirely free of gluten due to incomplete degradation of gluten during fermentation and this may prove as harmful for CD patients. In contrast, the positive results in some fermentation experiments for use of gluten derived fermented products proved complete digestion of toxic peptides in *in-vitro* and also proved safe in patients (56, 95-98), but long term use of such products remain unclear and still need to be explored thoroughly.

### 2.6. GENETICALLY MODIFIED WHEAT AND WHEAT INTOLERANCE

Wheat intolerance is a commonly noticed problem in certain individuals. Gluten free diet (GFD) is the only option for all wheat associated disorders (WADs). The elimination of immunogenic proteins from the wild wheat is the most appropriate approach to ameliorate the sufferings of affected individuals and also meet their nutritional requirements. RNA interference (RNAi) technology can be exploited to silence the expression of gliadins so as to produce a wheat variety lacking immunogenic proteins of WADs but there are challenges before implementation of transgenic varieties in market.

Wheat is the causal factor for a number of diseases, which include CD, Wheat Dependent Exercise Induced Anaphylaxis (WDEIA), Wheat allergy, Dermatitis Herpetiformis and Non-celiac Gluten Sensitivity (NCGS). The literature is scarce about the prevalence of most of the (WADs) except for CD. Recently, prevalence of wheat allergy in a birth cohort (10/11 year olds) was reported as 0.48% in United Kingdom (103), whereas prevalence is between 1.2 and 75.3 per 100,000 people for Dermatitis Herpetiformis (104-110). Studies conducted in United States reported prevalence of NCGS to be 6% in a hospital based study (111), but with the exception of one study which reported 0.55% prevalence of NCGS in general population of United States (112). CD is the most studied and prevalent amongst all wheat related diseases. CD was first reported to have prevalence 1: 184 in an Italian scholar age children population (3). Prevalence of CD varies from 0.006-5.6% in different populations across the world (4-12). Saharawi population of Africa accounts for 5.6% of cases afflicted with CD and is considered as the highest prevalent wheat related disease, the world over (13). Prevalence of CD is around 11% in Type 1 diabetes mellitus population in India (113). CD is the cause of other diverse health problems also (114, 115). CD is an autoimmune disorder of the small intestine that leads to destruction of intestinal villi as a consequence of inflammation (1, 2). Though, there are some genetic and environmental factors associated with disease (14, 25, 45, 46, 116). Wheat gluten (wheat proteins) remains the antigenic trigger and its
withdrawal from the diet would improve clinical conditions of the patients. The extent of gluten intake is strongly associated with severity and prevalence of CD (12). Even after some decades, there is no successful remedy available to alter the option of GFD. However, strict lifelong adherence to GFD always remains a challenge for CD patients and even occasional ingestion of gluten-containing food facilitates reoccurrence of gluten induced inflammation. Moreover, some of the patients afflicted with CD never recovered from symptoms even after remaining on GFD for a long time that is due to intestinal dysbiosis (51). Although, GFD is beneficial for patients but as per some clinical reports its effectiveness seems compromised in malnourished patients (117). Therefore, exclusion of wheat from diet may lead to secondary problems which may persist along with altered intestinal microbiota (118).

In the current scenario, trials of the ongoing therapies in CD patients still evoke different opinions with regard to eliciting beneficial response. Thus, a wheat variety lacking immunogenic components of the gluten may prove to be beneficial in this condition. In the similar context, RNAi technology is being used by scientific community to produce a wheat variety devoid of immunogenic proteins. Though, metabolomics in this field is in a stage of infancy, this review elucidates the utility of metabolomics in the development and validation of safety measures with regard to GM plants. Some other studies are also discussed in which metabolomics is used as a marker of biosafety for crop plants with an aim to elucidate the effect of fertilizers on metabolome of plants. Hence, metabolomics approaches have potential of elucidating better safety measures from the health point of view.

2.6.1. Immunogenic gluten peptides: the causal factor for CD

Gluten is the protein component of wheat that contains α-gliadin, γ-gliadins and glutenins as immunogenic components and ω5-gliadins as allergenic component. Digestion of gluten proteins is difficult by human proteolytic enzymes (58). These proteins have been reported to possess differential immune targets that make them immunotoxic in nature. During proteolytic digestion in intestine; immunogenic proline and glutamine rich gluten polypeptides are produced that can stimulate T cells (59). These peptides are resistant to further hydrolysis due to enrichment of proline residues in the amino acid sequences (60). The peptides, 33-mer peptide and 26-mer peptide derived from α-gliadin and γ-gliadin, subsequently trigger immunological responses in intestine of CD subjects (1, 68, 119, 120). Peptide 31–49 of α-gliadin was reported to be a potent activator of innate immune processes in CD mucosa when tested on T-cell lines established from CD intestinal
Another similar peptide of α-gliadin was tested in Caco-2 cell line model and was reported as resistant to digestive enzymes with potential to penetrate across a Caco-2 monolayer (122). Immunogenic potential of some peptides i.e. α-9 (57–68) and α-2 (62–75) was confirmed when they were recognized by the T- Cell line derived from CD patients of Norwegian patients (123). Gliadin peptide 31–43 promotes an endoplasmic reticulum (ER)-stress pathway by inducing Ca2+ mobilization from the ER, whereas peptides 31–43 and 57–68 can induce immune dysfunction (124). In addition, peptides 31–43 and 57–68 can alter immune regulators and induce deamidation of gluten peptides and gliadin–tTG crosslinking in enterocytes (59, 123). As most of the peptides are immunogenic in nature, some of them (p10-mer, QQPQDAVQPF) have protective effects that prevent the gliadin-dependent dendritic cell maturation (125) (Table 1).

**Table 1.** Immunotoxic gluten peptides and their diverse targets.

<table>
<thead>
<tr>
<th>Immunotoxic peptides</th>
<th>Protein name</th>
<th>Study model</th>
<th>Key findings</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>31–43</td>
<td>α2-gliadin</td>
<td><em>In-vitro</em> study on duodenal samples.</td>
<td>Induce anti-endomysial antibodies production.</td>
<td>(59)</td>
</tr>
<tr>
<td>31–49</td>
<td>Prolamins of α-gliadin</td>
<td><em>In-vivo</em> study on CD patients</td>
<td>Patients with CD display variable sensitivity.</td>
<td>(126)</td>
</tr>
<tr>
<td>Peptide corresponding to residues 56–75</td>
<td>α-gliadin</td>
<td><em>In-vivo</em> study on CD patients</td>
<td>Celiac specific intestinal morphology and intraepithelial lymphocyte count increased significantly after</td>
<td>(127)</td>
</tr>
<tr>
<td><strong>57–89, 33-mer epitope</strong></td>
<td>α 2-gliadin</td>
<td>CD patients</td>
<td>These peptides induced gut-derived human T cell lines derived from 14 celiac sprue patients</td>
<td>(68)</td>
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<tr>
<td><strong>α-9(57–68) and α-2(62–75) Peptides</strong></td>
<td>α-gliadin</td>
<td>T-cell line derived from CD patients</td>
<td>These are the common α-gliadin epitopes recognized by T cells in Norwegian patients of CD.</td>
<td>(123)</td>
</tr>
<tr>
<td><strong>31–49</strong></td>
<td>α-gliadin</td>
<td>T-cell lines established from CD intestinal mucosa</td>
<td>Potent activator of the innate immune activation in CD mucosa.</td>
<td>(121)</td>
</tr>
<tr>
<td><strong>31–55</strong></td>
<td>α-gliadin</td>
<td>Caco 2 Cell line model</td>
<td>The peptide is resistant to digestive</td>
<td>(122)</td>
</tr>
</tbody>
</table>
enzymes and could penetrate across a Caco-2 monolayer.

<table>
<thead>
<tr>
<th>31–43 and 57–68</th>
<th>α-gliadin</th>
<th>Caco 2 Cell line model</th>
<th>Promote an ER-stress pathway that may be relevant in CD pathogenesis and involved in inflammation.</th>
<th>(124)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Decapeptides QQPQRPQQ PF (pRPQ) and its homologous QQPQDAVQ PF (pDAV), and from human thyroid peroxidase (hTPO) (LDPLIRGIL LARPAKLQ V)</td>
<td>Gliadin peptides</td>
<td>Human monocyte-derived DC</td>
<td>These peptides significantly prevent the gliadin induced maturation of DC.</td>
<td>(125)</td>
</tr>
</tbody>
</table>
2.6.2. RNAi and Wheat

A conserved biological response to double-stranded RNA, known variously as RNA interference (RNAi) or post-transcriptional gene silencing, mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes. RNAi has been cultivated as a means to manipulate gene expression experimentally and to probe gene function on a whole-genome scale (128). RNAi technology has been used to produce GM plants to provide benefits to CD patients also. Gil-Humanes et al. (129), designed hairpin constructs which are expressed in the endosperm of bread wheat and have the potential in down regulating gliadin proteins in the transgenic lines. Three of their transgenic wheat lines did not elicit T-cell responses during in-vitro treatment with T-cell clones derived from intestinal lesion of CD patients. Another study by the same group, described that downregulation of γ-gliadins proved beneficial for quality of dough. The subsequent transgenic clones resulted in stronger dough quality that had tolerance to over-mixing as per industrial point of view. In a recent study, all gliadin fraction of wheat was successfully down regulated by using RNAi, so that new wheat line exhibited stability and tolerance to over-mixing, thereby showing better bread making qualities. Continuing their work on previous wheat lines they evaluated the physical properties and contents of gliadins, thus further predicting the amount of safe bread intake possible for the celiac patients. Hence, Gil-Humanes et al. (130), claimed their wheat line to possess similar baking and sensory properties like normal flour but lacked 97% of gliadin content unlike normal flour. Interestingly, wheat so developed also had better nutritional properties because of higher content of lysine, an important essential amino acid. Although, no clinical trial was performed in this study but a safe consumption of 67 grams of their bread per day by the celiac patients was claimed as per descriptions of per day maximum safe limits of gluten intake by Catassi et al. (131).

The ω5-gliadins are the major sensitizing allergens in WDEIA, a disorder in which a patient gets allergic response during exercise. To contain such a response, Altenbach et al. (132), generated transgenic lines of wheat which were knocked down for ω-5 gliadins. Further, in a set of additional experiments, they revealed that protein contents of flour are determined by the fertilizer regime in both transgenic samples as well as normal wheat samples. Subsequently, ω-5 gliadin was also indicated to have a negative effect on flour quality, thereby suggesting that transgenic wheat lines produce better flour quality than wild type wheat.
Altenbach et al. (133), further observed that the allergenic response of ω5-gliadins knocked down wheat lines was appreciably reduced in patients as determined by serum immunoglobulin E (IgE) reactivity in a clinical trial. Two transgenic wheat lines were assessed for their allergenic potential in which ω5-gliadin genes were silenced by RNAi. Sera from 7 of 11 WDEIA patients showed greatly reduced levels of IgE reactivity to ω5-gliadins for both transgenic lines. However, the sera also showed low levels of reactivity to other gluten proteins but sera from three patients showed the greatest reactivity to proteins other than ω5-gliadins, that included either high-molecular-weight glutenin subunits (HMW-GSs), α-gliadins, or non-gluten proteins. The complexity of immunological responses among these patients suggests that flour from the transgenic lines would not be suitable for individuals diagnosed with WDEIA. To best of our knowledge, transgenic wheat varieties knocked down for α-gliadins have not yet entered clinical trials for CD in humans. However, the present trial of ω5-gliadins revealed that its administration in WDEIA population could reduce the incidence of this food allergy. The current clinical trial has also raised the questions on the applicability of transgenic lines thereby pointing towards the complexity of WADs i.e., WDEIA, CD, wheat allergy and complex status of overall wheat proteins. As the wide range of complex immunogenic proteins of wheat is still a challenge in developing immune-tolerant wheat line, therefore the wheat proteins as a whole should be targeted while trying to give an alternative to GFD. This study also raised questions on the use of such silenced GM plants even after a number of quality checks. The effect of gene knocked-down on other proteins and metabolites that remain untraceable by current molecular techniques, is the another challenge in a field of GM plants. Further, what makes other proteins immunogenic for the WDEIA population is still not evident and whether it is because of changed proportion of a targeted protein component, or a consequence of the gene silencing on plant’s metabolic control or because of imbalanced proportion of proteins is a subject of further investigations. A study by Gil-humanes (134), reported that silencing induced strong reduction in all the gliadins but caused a compensatory effect on the synthesis of non-gluten proteins by up-regulation. Furthermore, the effect of gliadin knocked down on nutritional values of wheat remained contradictory but to some extent Barro F. et. al. (134), tried to solve by validating that silencing WADs associated proteins did not affect total protein and starch content of wheat. The authors designed a combination of seven plasmids, containing RNAi fragments to mask all major components of WADs i.e. α-, γ-, ω-gliadins, and LMW glutenin subunits. Out of these combinations, two of them provided
more than 90% reduction in gluten content in comparison with wild type, when measured by anti-gliadin 33-mer monoclonal antibody. However, total protein and starch contents remained unaffected in all types of combinations. Gel electrophoresis, reversed phase-high performance liquid chromatography and liquid chromatography–mass spectrometry were used to measure the extent of silencing. Though, promising results were observed in experiments by Altenbach SB et.al (133) but questions would still remain until pre-clinical and clinical trials likewise yield convincing results.

2.6.4. Fertilizers as predominant factors for accumulation of risk proteins in wheat

Currently, prevalence and incidence of food intolerances are increasing. The reason for widespread food intolerance is not known. The data presented in this section reports the adverse effects of sulphur fertilizers on gene regulation of wheat plant thereby increasing the content of risk proteins (immunogenic proteins i.e. gliadins). These adverse effects cause due to genomic level changes need validations by using metabolomics and other platforms of OMICS approaches integrated with clinical practices/trials in suitable models. Further, the dysregulations at the genetic levels modulate the metabolic profiles of plants is a concern of food safety.

Altenbach et. al. (135), in their study revealed that environmental factors including fertilizers affect the composition of specific flour proteins and their regulation. While conducting experiments, Triticum aestivum was grown with or without postanthesis fertilization (PAF) followed by quantitative 2-dimensional gel electrophoresis of the subsequent flour. Subsequently, the proteomics profiling clarified that the proportions of 54 unique proteins were appreciably altered in the treatment group. PAF treatment resulted in increased proportion of most ω-gliadins, HMW-GS, serpins and some α-gliadins.

Further, Hurkman et. al. (136), while conducting studies to observe the differential effects of temperature and fertilizer treatment on the development and yield of grain, reported that both the treatments elicited the accumulation of gluten proteins in wheat flour. Though, under high temperature conditions, gluten protein accumulation was not observed by PAF treatment, but majority of HMW-GS, ω-gliadins and some α-gliadins were found to be elevated thereby affirming that environmental stimuli do influence the accumulation of risk proteins of CD. The studies presented provide evidence that the man made means for increasing productivity of crops can be a prominent cause for incidence of food intolerances because of lack of food safety measures. The above studies, evidently showed the adverse effects of sulphur in terms of food safety, that need to be validated by
targeting studies to evaluate the effect of sulphur induced metabolome change and its effect on food safety in relation to human health. Although, there is lack of literature in this regard but there are some evidences in support of side effects of sulphur fertilizer in GM wheat as described in the next section.

2.6.5. Cross talk on RNAi, food safety and metabolomics in wheat

It is worthwhile to elucidate the unwarranted side-effects of knocked-down wheat lines of α-gliadins on plant metabolites, as α-gliadins contain sulphur-rich amino acids which include cysteine and methionine. Further, in wheat line knocked down for α-gliadin genes, sulphur amino acids to be used up in synthesis of α-gliadin might remain accumulated in the plant. The accumulation of sulphur is supposed to induce its incorporation into other plant metabolites that may become a food safety concern by but the same was not observed in case of metabolomics studies in transgenic verses wild lines. Therefore, with support of metabolomics, the recent studies advocated that transgenic varieties are safe, as no change in the metabolomic signature in transgenic verses wild wheat lines was observed.

RNAi produced wheat lines lack antigenic components but it leaves diverse side-effects on plants. Initially, evaluation was done by proteomics analysis of different parts of plant (grains, leaves, straw, husk). Comparison of proteomics profiles of transgenic lines with that of normal gives an overview of disturbed gene function or description of knocked down genes. RNAi-induced silencing of 75 α-gliadin genes that completely eliminated all gliadin proteins as conferred by proteome analysis, raises the safety issues, of such varieties that need to be considered a priority, especially the ones that are caused by disturbance in single plant metabolite or whole metabolome, with and without treatment of fertilizers. In the recent past, metabolomics has helped in better understanding of the effects of any external stimuli (fertilizer) or genetic modifications (gene knockdown) on plant metabolism and the associated issues of bio-safety.

Christian Zorb et. al. (137), performed gas chromatography mass spectrometry (GC MS)-based metabolite profiling of flour of wheat line silenced for 75 α-gliadin genes and reported that in comparison to the wild type plants, no appreciable difference in 109 metabolites was seen when plants were grown without sulphur. No unintended side-effects of RNAi induced gene silencing were observed. Conversely, the effect of fertilization/ single nutrient (sulphur) availability in disturbing metabolomic status was much higher as compared to RNAi-induced silencing. The concentration of metabolites
was also found to increase with increasing sulphur supply. Zorb *et al.* (137), also revealed that variable amounts of sulphur supply influenced the yield aspects of grain metabolome in both, the wild type and transgenic wheat line. Plants grown with higher sulphur content showed elevated grain metabolite concentration in comparison to the plants grown without sulphur treatment. Moreover, principal component analysis showed that the levels of β-amino isobutyric acid are affected the most by variation in sulphur supply in wild type wheat. Further, when α-gliadins were knock-down, the amino acids alanine, glycine, serine, homoserine and tyrosine were found to be associated with sulphur induced differences in the grains. These studies confirm the effects of sulphur fertilizers in GMO wheat but seeing the concerns expressed in the last section, it is pertinent to elucidate the effects of sulphur alone on gene regulation and also the associated metabolomics in order to make the food safety concerns relevant.

Collectively, it can be concluded from the above two sections that sulphur was not only potent to induce genetic dysregulations in wheat (136, 138), but it might have produced metabolic changes that were reflected in metabolomics studies (137).

The results of various experiments related to proteomics and metabolomics are in accordance with each other and reveal that fertilizers affect the proteome and metabolome of wheat flour thereby raising the issues related to food safety. Though, RNAi-induced gene silencing does not affect the metabolomic signature of wheat flour but fertilizers do. Sulphur fertilizers remain the determining factors for disturbance in plant metabolism, followed by dysregulation in proteome. Moreover, proteomics studies demonstrate that sulphur fertilizers induce accumulation of gliadins in wheat that make the flour more immunogenic. Thus, the techniques adopted are useful for validating the fertilizers induced metabolic disturbances in plants. However, clinical trials of wheat line knocked down for ω-gliadin showed reduction in disease specific markers in wheat-dependent exercise-induced anaphylaxis (WADEIA) patients but reactivity to other wheat protein components could not be ruled out. This raises controversy on the gene expression status of other gluten proteins that may be dysregulated as a consequence of interference exerted by RNAi. Therefore, though OMICS techniques are useful to validate the global metabolic control or gene expression status but initial *in-vitro* or *in-vivo* pre-clinical studies are needed to guide better future clinical output. Further, future research in this
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field should be attributed to validation of food safety issues through improved platforms of transcriptome, proteome, metabolome and immunome.

2.7. Prospective and conclusions
CD is known to be triggered and maintained by gluten in genetically predisposed individuals. Children possessing HLA haplotype DR3–DQ2 or DR4–DQ8 are considered at increased risk of CD but in children possessing these risk genes, the disease risk was reported more in Sweden than in United States, Finland and Germany that highlight the importance of environmental factors in CD (139). On the same conscience, in a recent study from different regions of India, gluten intake correlated to prevalence of CD instead of HLA genetics (12). It can be concluded that there might be the additive effects of gluten and infection or antibiotic induced dysbiosis that interfere with mucosal immune homeostasis to develop this complex disorder (Fig 2). HLA and non-HLA genes are best used to identify individuals at risk of CD (140), but genetics in CD, either defined by HLA or non-HLA genes seems secondary to the environmental factors. Therefore, the environmental factors that influence gut barrier integrity might have primary role in incidence, progression and management of CD rather than genetics (Fig 1). Apart from this, gluten is considered as environmental trigger of the disease whereas infections and use of antibiotics need to be addressed equally as important factors in pathogenesis of CD.

2.8. Clinical implications
Infections and antibiotic treatment in the early stages of life is the contributing factor for dysbiosis, as a trigger for CD (25-27, 32, 40), that may be reversed by modulating gut microbiota. Decreased number of Lactobacilli and Bifidobacteria were reported in CD as compared to healthy individuals, where the former has potential to degrade immunotoxic peptides of gliadin while later is well known for its protective effects exerted on gliadin induced inflammation. Therefore, imbalance with these bacterial species can be supposed to influence gluten digestion and inflammatory state of intestine.

Apart from intestinal microbiome, some reports about oral microbiome prove its crucial role in the digestion of dietary gluten (70-72). Oral microbiome might be a determining factor for composition of intestinal microbiome. In addition, very
recently Francavilla et al. (73) reported that salivary microbiota is associated with CD (73), so the role of oral microbiota as the gluten degraders cannot be ignored.

CD is now well studied for oral and intestinal dysbiosis. In such condition, it is advisable that along with GFD, administrating the subjects with probiotics may help in improving intestinal barrier function by immunomodulations as well as gut microbiota modulations, improving quality of life because GFD itself cannot restore intestinal microbiome (51) and small-intestinal mucosa (141). In contrast, probiotics may suppress disease complications in U-CD subjects also because LABs may act to degrade immunotoxic gluten peptides, thereby creating tolerance to gluten. Another aspect, that short chain fatty acids produced by LABs possess anti-inflammatory potential, potential of intestinal barrier function restoration and potential to modulate regulatory T cell function at intestinal mucosa (142-144). At present, instead of enzyme therapies and other approaches, probiotic therapy seems as the most applicable and safe biotherapy like a multipurpose sword against CD.

2.9. FUTURE RESEARCH TARGETS

Microbes, now an important part in pathology of U-CD and T-CD patients but rare efforts have been made to modulate the intestinal microbiota in a health favorable manner (145). Diet is the modulator of gut microbiota (146, 147), but except several reports on pre and post GFD examination of gut microbiota, lesser is known about the modulation in dietary therapy and its impact on intestinal microbes. Although, sequencing technologies has been frequently manipulated to understand diet-microbe interactions and its effect on host physiology (148) but in CD it need more advancements. For example according to a very recent report effect of an Italian-style GFD was observed in the patients who were following African-style GFD for at least two years. Salivary microbiota and metabolome was observed with significant differences suggesting about metabolic dysfunction after switching to Italian-style dietary habits. This switching had lead to enrichment of Granulicatella, Porphyromonas and Neisseria while decreased counts of Clostridium, Prevotella and Veillonella (149). At present, as GFD is the only option for patients, therefore, efforts should be made to modulate the GFD in such a manner that it should promote the growth of beneficial microbes and should promote a better host metabolic control rather than growth of pathogens leading to metabolic deregulation. By modulating the
GFD, post-GFD complications may also be best managed and GFD may remain as the best treatment option of CD ever.

A combination of probiotics having specific properties can be recommended because as per the reported human trials, patients were administered with strains of *Bifidobacteria* only (86-88), but different microbes possessing specific beneficial properties and glutenase producing microbes should be included in a panel of probiotic supplement to achieve desired goals.

As microbial dysbiosis is reported by several studies in CD (23) with an aim of finding dysbiosis as a cause or consequence of disease, from a subset of 22 at risk individuals (23), it has been concluded that HLA-DQ2 genotype selects early intestinal microbiota composition in infants at high risk of developing CD (24) but a recent Indian study on 23,331 adults supported the importance of other factors rather than genetics, in which HLA genes were not associated with prevalence of disease (12). To better understand better the controversial status and contribution of interacting environmental factors, microbiota and host genetics in CD, future research in this area should focus on the impact of particular type of microbiota change on disease specific markers in individuals with and without genetics susceptibilities in which whole genome sequencing of microbes (137), metagenomics and transcriptomics would be helpful (150). In addition, prevalence and types of infections associated with CD need to be explored, their impact on outcome of host-microbe interactions (151), impact of different type of antibiotics in creating disease susceptible environment, role of specific biologically active dietary components in ameliorating the effect of dysbiosis (152) followed by subsequent changes at mucosal surface should also be taken under consideration for future research strategies, thereby emphasizing the use of suitable disease models or gnotobiotic animal models to accomplish such an advancements in this field.