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Microbial transglutaminase: A new potential player in celiac disease

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ABSTRACT

Microbial transglutaminase is heavily used in the food processing industries to improve food qualities. Being a protein's glue, by cross-linking it creates neoepitope complexes that are immunogenic and potentially pathogenic in celiac disease. Despite low sequence identity, it imitates functionally its family member, the endogenous tissue transglutaminase, which is the autoantigen of celiac disease. The present comprehensive review highlights the enzyme characteristics, endogenous and exogenous intestinal sources, its cross-talks with gluten and gliadin, its immunogenicity and potential pathogenicity and risks for the gluten induced conditions. If substantiated, it might represent a new environmental inducer of celiac disease. The present findings might affect nutritional product labeling, processed food additive policies and consumer health education.

1. Introduction

For the last decades celiac disease (CD) incidence is increasing. While genetic accumulations of susceptible gene require generations [1], environmental changes are more dynamic and may explain those surges [2,3]. One of the major changes that occurred in the contemporary nutrition with the transition to Western diet, is the industrial food processing. In fact, 63% of the calories, at least in the USA, originate from processed food [4]. Even in the Asian, Eastern, Latin American and African countries the nutritional transition is following the Western trend [5–7]. In this regards, enzymes are heavily used in the nutrition industries, a major representative being the microbial transglutaminase (mTg), mounting to 21.9%/year increase in consumption [8–12]. A substantial source of those enzymes is coming from the modern, evolving microbial engineering technologies and biocatalysation, aiming for a cost-effective, large scale production of various enzymes [13]. The mTg enzyme is a prototype of several bacterial manipulations to secret massive amount for the industrial usage, including the processed food industries [14,15]. Its production was significantly improved along the advances made in bioprocess and genetic engineering during the last decades [14]. The present comprehensive review will update on this enzyme, its functions, food industrial applications, sources, the mutual cross-talks with gluten and its immunogenicity, as well as its potential pathogenic involvement in CD development.

1.1. mTg characteristics, functions and applications

mTg is a member of the extended transglutaminases family [16], first isolated from Streptomyces mobaraense [17] and ever since has been isolated and characterized from numerous microbial strains [15,18–20]. The Streptomyces mobaraense one consists of 331 amino acids with a molecular weight of 37.9 kDa. A list of additional bacterial strains that secrete mTg and the corresponding enzyme yields was recently published [20]. In opposite to human tissue transglutaminase (tTg), mTg is a calcium and nucleotide independent enzyme. It has a single structural domain and a much lower molecular weight. mTg has a less restricted pH optimum and a wider variety of substrates, therefore being less specific. Those enzyme characteristics are advantageous for many industrial applications [21]. Despite lacking sequence homology to tTg, mTg has substantial functional similarity with his family member, due to their active site performances [10,20,21]. In fact, both enzymes can deamidate and transamidate (crosslink) peptides, pending on physico-chemical reaction's conditions [22].

Like his family members, mTg catalyzes the formation of an isopeptide bond between an amine group (peptide-bound lysine) and the acyl group at the end of the side chain of peptide-bound glutamine. Gluten is extremely rich in glutamine (~ 30%) and contains lysine (< 2%) [23]. Having an acyl donor and acyl acceptor, it represents an ideal substrate for posttranslational modification of gluten, by deamidation or transamidation, thus imitating functionally the endogenous tTg [20,21,24]. It should be emphasized that bonds formed by

Abbreviations: CD, Celiac disease; mTg, microbial transglutaminase; tTg, tissue transglutaminase

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Table 1
Food processing industrial applications and effects.

Source	Product	mTg effect	Reference
Wheat	Backed foods	Low calorie, improved texture and elasticity, better dough characteristics	[30]
Meat	Restructured meats	Improved texture and appearance, increased hardness, elasticity and persistency	[31–33]
Milk	Creams,deserts, yoghurt dressings, drinks	Improved quality and texture	[34,35]
Fish	Fish paste, restructured products	Increased hardness	[36]
Casein	Crosslinked proteins	Reduced allergenicity	[34,37]
Gelatin	Sweet foods	Lower calories, improved texture and elasticity, increased gelation	[38]
Surimi	Crab, sea food sticks	Enhanced elasticity, persistency and mechanical properties	[39]
Myosin + actin	Muscle products	Increase rheological and textural properties	[40]

Adapted from [10,18,21,27–29,40].

transglutaminases exhibit high resistance to proteolytic degradation, the enzyme has a higher reaction rate, broad substrate specificity, higher transamidation compared to lower deamidation activity resulting in crosslinking capacity. mTg affects solubility, improves gelation and changes emulsification, foaming, viscosity and water-holding capacity, which all depend on protein solubility. It is considered as a polymerization agent, resulting in changes in the molecule's hydrophobicity.

mTg has many industrial applications, not only as a peptide linker in the processed food industries [25,26]. It can crosslink proteins to DNA, antibodies for radioimmunoassay, drugs and many other non-nutrient proteins [27,28]. However, its main application is crosslinking proteins during the food processing. The enzyme is used in a variety of nutritional industries, including: dairy, bakery, meat, sausage, fish, tofu, confection, oil, coffee, beverage, confection and convenience food production [27–29]. Table 1 summarizes the effects of mTg industrial applications in various processed food products and its effects. Innovative food processing technologies to improve mTg functionality are continuously described [41,42]. High pressure processing, ultrasonication, microwave application and ultraviolet irradiation are such examples. Applying those technologies can increase mTg affinity to cross link proteins and potentially to use the products as delivery vehicles for a wide spectrum of bioactive compounds. No doubt that those thermal and non-thermal technologies impact the cross linked product's characteristics, including their physicochemical structure and potential antigenicity [20–22,25,41].After summing up the mTg functions, its industrial applications and its avidity to gluten and before linking the two transglutaminases to CD, the potential sources of the enzyme that exists in or enters the human intestinal lumen will be briefed.

1.2. Potential mTg intestinal sources

The human gut is developing in a way that is tolerant to, and even supportive of, commensurate microorganisms [43]. In its efforts to keep the microbes at bay, numerous protective mechanisms evolved counteracting the bacterial protective ones. In this regards, mTg is essential for gut bacterial survival. It exerts anti protease activity, suppresses anti-microbial peptides, has emulsifying activity, is anti-phagocytic and affects Th1/Th2 balance [10,11,19,21]. Using sequence similarity search programs [44], hundreds of mTgs-encoding bacteria can be detected, most of them belong to the Firmicutes phylum [20]. Multiple chronic human diseases and autoimmune conditions including CD are characterized by microbiota transformation to dysbiota [24,45–47]. This microbial cargo represents a major source for intra-luminal mTg secretion.

1.3. Potential mTg extra-intestinal sources

Several, non-endogenous extra luminal sources for mTg secretion or consumption can be foreseen, including: processed food additives, ingested probiotics, gastrointestinal pathobionts and consumed vegetables.

1.3.1. mTg added to processed food products

As mentioned above, mTg is heavily consumed in a large variety of processed food products [10,18,21,27–29,40]. According to the enzyme manufacturer it improves texture, elasticity, appearance, persistency, quality, hardness, gelation and rheology and shelf-time while decreasing caloric density and allergenicity (Table 1). Based on the literature, daily intake of mTg used in the processed food's product can range up to 15 mg where every kg of mTg processed product contains around 50–100 mg of mTg [10,11,18,48]. Interestingly, a positive correlation exists between the increased annual consumption of commercial enzymes added to processed bakery products and the surge in CD incidence, in the last 4 decades [21]. It is an association, but no causality was established.

Despite the manufacturers' claims that the enzyme activity doesn't reach the supermarket shelves, two observations should be mentioned [26]. Fish, meat and meat products, collected from the supermarket shelves, were found to contain variable amounts of mTg [49]. Secondly, when duodenal fluid was checked for transglutaminase activity, the fluid was positive. Regrettably, the authors did not differentiated between the endogenous tTg and the microbial one, but to our knowledge, no tTg activity was reported in the duodenal lumen, so far, in contrast to the intestinal mucosa [50].

1.3.2. Probiotics

Like other microbes, they harbor the mTg gene, the product which is important for their luminal survival [20]. Probiotics are frequently used in the nutritional industries to improve food qualities, but it is important to remind the readers on their pathogenic potential. Gene acquisition/loss within or between various probiotic strains, by horizontal gene transfer, was reported extensively and brought up some safety concerns [51–54]. The probiotic L. *reuteri* can transfer antibiotic resistance to human microbiome [55]. Rosander et al. deleted the antibiotic resistance gene-carrying plasmids from the commercial strain of L. *reuteri* ATCC 55730 [56]. But, many more detrimental genes can be transferred, including from pathobiont's secreting mTg [19,57]. Intriguingly, secretion of an antiphagocytic mTg was lately described in *Streptococcus suis* [19]. After setting the stage for the probiotics horizontal gene transfer, it is interesting to know the extent of the probiotics use in the nutritional industry and their potential of transferring virulent genes. Global concern on the transfer of antibiotic resistances via the food chain exists and many of those bad genes are carried by probiotics [58]. An update on antibiotic resistance in foodborne *Lactobacillus* and *Lactococcus* species was recently reported [54], and being prokaryotes, the probiotics have the capacity to secret the mTg, with its pathogenic potential. The factors causing the transition from benign inhabitant of the gut microbiome or ingested probiotics to virulent pathogen are not well understood, but a combination of horizontal gene exchange of virulence genes and differential transcription of endogenous genes are clearly involved [59].

1.3.3. Pathobionts

Multiple human and animal commensals, emerging opportunistic

pathogens and food fermentative organisms are known. The *Streptococcus bovis/Streptococcus equinus* complex, *Streptococcus suis*, and other *Streptoccocus* strains, including *S. thermophiles* are some of them [57,60,61]. Evidence of widespread genetic exchanges is expending, probably involving a natural competence system and the presence of exchangeable mobile elements. Enrichment and wide dissemination of microbial virulent factors in the environment, including surface water, sewage treatment plant effluents, soils, animal wastes and urban rivers is continuously reported, thus threatening planetary health [62–64].

Taken together, in addition to their virulent cargo, the prokaryotic pathogens are potential secretors of the mTg with their pathogenic capabilities, as will be detailed bellow [20,24,51].

1.3.4. Plants and vegetables

An entirely new domain is plant's transglutaminases. Being a part of the global transglutaminases family, they are posttranslational modifiers of proteins. More so, gluten is a good substrate for them and they were implicated as a possible player in CD pathogenesis, starting the process in the intestinal lumen [65]. Soybean, fodder beet, rosemary leaves, apple, Jerusalem artichoke, bean sprouts, spinach leaves and green peas, routinely used plants, fruits and vegetables, have transglutaminase activities [42,65]. Expanding the canvas, vegetables at harvest like lettuce and endive and even organic lettuce harbor high abundance of virulent factors, like antibiotic resistant genes [66,67]. With the capacity to be transferred horizontally, those deleterious genes, including the mTg, can spread and present potential threat to human health. Fig. 1 summarizes the various potential sources and the wheat constituents' crosslinking capacities of the mTg. Gluten, glutenins and amylase-trypsin inhibitors contain glutamines and lysines, presenting good substrate for mTg cross linking. Polyols, heavily used in the processed food industry in protein-based coating and biofilm, gelatination and bio-packaging, were shown to improve thermal stability and half-live of the enzyme, thus potentiating its cross linking capacity [68].

In summary, multiple extra intestinal and luminal sources of mTg exist that are in potential contact with gluten containing proteins. This enzymatic cargo can cross linked those glutamine riched peptides, resulting in posttranslated, modified immunogenic epitopes, potentially driving autoimmunogenesis [20,21,24].

1.4. mTg and gliadin crosslinking relationship

Gluten, having an acyl donor and acceptor, being rich in glutamine and having lysine amino acids, is an ideal substrate for deamidation and transamidation by the enzymatic autoantigen of CD, namely tTg and the exogenous mTg. The transformation of naïve tolerable proteins to non-self-immunogenic ones represents the posttranslational modification of the gluten peptides by the mTg [20,24].

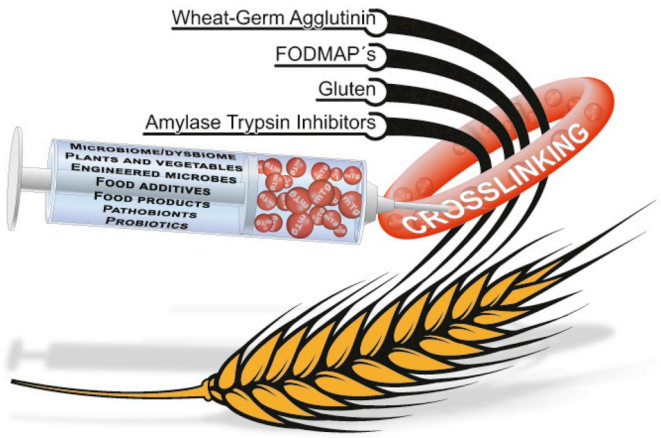

Fig. 1. The various potential sources and the wheat constituents' crosslinking capacities of the mTg. mTg = microbial transglutaminase, FODMAP's = Fermentable Oligo-, Di-, Mono-saccharides And Polyols.

According to the most accepted pathophysiological pathway in CD, this essential step occurs below the epithelium, following the passage of the toxic/immunogenic gluten peptides intra and inter the enteric enterocytes, encountering the tTg. But, the mTg is abundant in the luminal compartment and can imitate the sub-epithelial event. It can be deducted that the molecular transformations of the ingested gluten start in the gut lumen, much ahead of the common thoughts. More interesting is the hypothesis that CD events of gluten crosslinking by mTg start even in the bakeries, when mTg is added to the dough. In fact, numerous publications praise mTg usage to improve texture, elasticity and appearance of the bakery products tables [1, 21, 30].

1.5. Gluten: A wheat wolf in human clothing

Finally, since gluten peptides are docked on the mTg, thus creating a new complex with neoepitopes, it is worthwhile to expend on the gluten part of the complex. More and more data is accumulating on the detrimental effects of gluten to human health. Based mainly on human cell lines, animal models and ex-vivo intestinal biopsies and less on in-vivo human studies, numerous side effects of gluten, in non-celiac conditions, can be listed. “Gluten affects the microbiome and increases intestinal permeability. It boosts oxidative stress and affects epigenetic behavior. It is also immunogenic, cytotoxic, and pro-inflammatory. Gluten intake increases apoptosis and decreases cell viability and differentiation.” [69]. It is not known if the mTg transamidated gliadin exposes comparable drawbacks, a topic that should be further investigated.

2. Gliadin docked mTg complexes is immunogenic in pediatric celiac disease

Several observation exist for the formation of complexes when tTg or mTg are incubated with crude gluten or the 33mer gliadin peptide. Running the products on a SDS gel, a smear of peptides appears below the loaded well, of 200–400 Kd size, that disappears upon adding the corresponding inhibitor of the two enzyme (unpublished, personal communication, Dr. Ramesh Ajay). Moreover, when a dynamic competition assay was performed between various single or complexed CD associated antigens, identical outcomes of the competitive ELISA of the mTg neoepitope and tTg neoepitope antibodies, using sera of selected celiac patients, compared to several CD-associated single antigens in increasing concentrations, was depicted. Interestingly, the rest of the antibodies against tTg, gliadin, diamidated gliadin peptide and uncomplexed mTg behaved similarly. Only the antibodies against the gliadin docked mTg and tTg complexes present a separated competition graph, most probably competing on shared epitopes [70].

Based on the above the immunogenicity of the mTg-gliadin docked neoepitope complexes were most recently investigated in celiac patients/controls. High activity of IgG anti-neoepitope mTg antibodies (against the neocomplex created when gliadin is docked on the mTg), were found in celiac affected children [70,71].

Based on the gluten induced intestinal tight junction breach, the mTg induced posttranslational modifications of gluten peptides and the enhanced immunogenicity of the mTg-gliadin neocomplex, the hypothesis was forwarded that mTg might represent a new environmental factor in CD initiation and progression, [20,21,24–26,70,71]. Its place in non-celiac gluten-dependent conditions is not known, representing a challenge for further explorations.

3. mTg is a potential driver of gut-remote organ's autoimmunity

Nutrients and processed food additives can impact the intestinal

ecosystem and breach tight junction integrity [72]. Taken together, certain nutritional compounds, increased intestinal permeability, disease specific dysbiotic or pathobionts and their enzymatic capacity to posttranslate and modify naïve proteins are luminal originated events that can enhance the autoimmune pathways centrifugally, driving autoimmunity to remote organs [73]. Those irradiating luminal eco-processes can explain the extra-intestinal manifestations of CD [74]. The mucosal enriched and committed immune cells, mTg induced post-translation modified gluten peptides, proinflammatory cytokines and lymphokines have the capacity to circulate via the enteric vascular network, to bring the auto-inflammatory message to remote organs, thus establishing the gut-extraintestinal organ axes [71–74].

4. Potential risks of processed food mTg additive to gluten sensitive populations

Followed are some potential pathogenic pathways of crosslinked relationship between the mTg and gluten peptides that might be detrimental to CD patients:

1. The immunogenic pathway: mTg docked gliadin complexes are immunogenic and induce neoepitope mTg antibodies and reflect intestinal injury only in naïve CD patients and not in controls [70]. Additionally, when multiple non-celiac autoimmune diseases were checked, their neoepitope mTg activity was comparable to controls (unpublished, personal communications, Mahler A, Matthias T, 2018). As for today, it seems that neoepitope mTg antibodies are specific for gluten dependent conditions.
2. The translational modification pathway: Being a transglutaminase, the luminal mTg can posttranslate and modify gluten peptides, thus breaking gluten tolerance and, by molecular mimicry, driving CD autoimmunity [20,24].
3. The intra-enterocyte mTg-gliadin endocytic transport pathway: Several gliadin peptides are not digested by the enteric proteases. They are internalized into the enterocytes by an active process of endocytosis and are transcytosed to the sub-epithelial space [75,76]. Most recently, Zimmer P, Stricker P et al. (Justus-Liebig-University Giessen, Germany, unpublished personal communication), by applying tagged mTg and gliadin to the apical membrane of CD/control human intestinal biopsies and human originated cell line, have observed:
4. Simultaneous uptake of mTg and gliadin into the endoplasmic reticulum of the RACE-cells, much more than controls.
5. mTg and gliadin co-localization at the basolateral membrane and the lamina propria of CD duodenal biopsies.

If substantiated, those results reinforce the potential pathogenic played by the exogenous mTg-gliadin complex in CD development. It is possible that the sub-epithelial deposits of tTg-gliadin, described as an early pathological finding in CD intestinal biopsies, contain mTg-gliadin complexes.

5. mTg enhanced gliadin uptake pathways by increasing intestinal permeability

Potential mechanisms, by which mTg crosslinked gliadin or other compounds might breach tight junction integrity, were recently summarized [8,21]:

1. mTg facilitates luminal microbial survival [24] and additionally to their secreted mTg infectious agents, by themselves are known enhancers of intestinal permeability [77].
2. Glutamine and sulfur-containing amino acids deprivation and glutamine synthetase inhibition increase intestinal permeability [78,79]. mTg mediated nonspecific linking of these amino acids containing peptides to other molecules can induce a state of deprivation at the intestinal epithelial level, thus indirectly affecting tight junction performance.
3. By crosslinking gliadins/gluten, the mTg complexes can directly increase permeability, since gluten by itself is known to offend intestinal permeability [8,69].
4. mTg has emulsifying activity by crosslinking different proteins [80–82]. Emulsifiers are known to breach tight junction integrity. Interestingly, hydrolyzed gluten by itself improves emulsification, regardless of mTg treatment [83].

6. mTg can crosslink nanoparticles [84], and nanoparticles are known to increase intestinal permeability [85,86]

6. mTg prokaryotic virulent factor pathway: Most recently, a novel virulence factor, namely mTg secreted by *Streptococcus suis* serotype 2, was shown to have antiphagocytic properties, benefiting its survival [19,87]. More so, despite its pathogenicity, this novel microbial mTg was suggested as a potential source to be applied as a new biocatalyst in the biomedical and biotechnology fields, including as a food additive to improve nutritional products [19,21].

7. Several mTg features that might enhance gliadin peptides crosslinking in CD development

The widely used food industrial mTg characteristics can promote proteins or other compound crosslinking. Its wider substrate repertoire, large pH functional range and operative temperature [21,88], its independency on Ca++ and nucleotides, higher antigenicity when heated and its enterocytic co-localization/migration with gliadin [89] (Zimmer P, Stricker P, Justus-Liebig-University Giessen, Germany, unpublished personal communication), its immune reactivity to wheat treated products [21,90–93], its potential to modify surface proteins, making them more resistant to proteolytic digestion [94] and finally, its gliadin crosslinked complex immunogenicity in celiac patients [70], make mTg an ideal potential candidate affecting celiac development.

8. mTg as an allergen and immunogen and potentially unsafe

We would not have mentioned the allergic feature of the mTg, if not mentioned by the mTg biotechnology mass producers being not allergenic [95,96]. More and more data is accumulated on mTg being an occupational allergen [88]. Additionally, mTg deamidated gluten/gliadins induce Th2 response, mediated by strong IgE binding to them [97,98]. Like with the allergenicity, the manufacturers claim mTg to be safe and not immunnogenic to the consumers, including in CD [95–97]. On the contrary, they suggest that “food-added transglutaminase in the presence of an amine donor can actually decrease the immunestimulatory activity of gluten in CD patients” [96]. Those statements contradict what is known in the literature. mTg is allergenic [88,98,99], immunogenic [21,26,70,71,100], the mTg treated wheat/gluten products are immunoreactive [21,25,70,90–93,101–104], and its safety is doubtful. Concerning safety, as per the producers, it was checked only in vitro in cell-lines, on animal models like mice, guinea pigs and rats [96] and not on normal or gluten depended human conditions. In fact, the burden of the proof is on the mTg manufacturers and suppliers. They have to explain the gap between “Transglutaminase is not active in the stomach, which is characterized by acidic pH, and that is completely degraded by pepsin” or “mTg…..is a safe food enzyme” or “yet no side effects have ever been reported after ingestions of the above mentioned foods” or “marketed broadly in Europe for more than 15 years without any reported incidence of CD” [96] and the fact that transglutaminase is detected in the human intestinal lumen. Intriguingly, meat products on the shelves of Suis supermarkets contain mTg and the incidence of CD prevalence and incidence is parallel to the surge in the industrial enzyme usage in the bakeries [2,3,21,49]. Food manufacturers are not required to inform the public they've glued

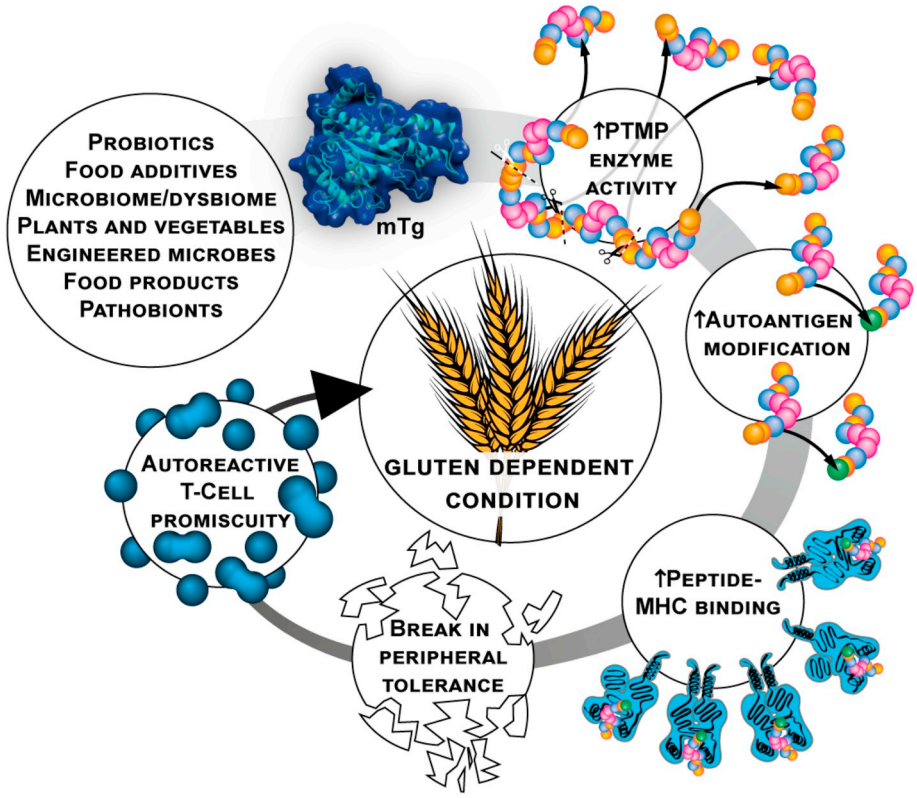

Fig. 2. Chain of mTg potential events of wheat component break of tolerance resulting in gluten induced conditions. The multi-intestinal and extraintestinal sources of mTg induce posttranslational modification of wheat proteins, creation of antigenic neoepitope complexes, taken by the antigen presenting cells, processed and presented by the MHC to the autoreactive T-cells, thus breaking their tolerance to induce gluten dependent conditions.
mTg = microbial transglutaminase, PTMP = posttranslational modification of proteins, MHC = Major Histocompatibility Complex.

processed food together, which raise an unethical and potentially dangerous aspect. Officially, mTg is regarded as a processing aid, thus escaping the definition of a food additive. mTg is not labeled and according to the manufacturer's claims, “The production process of the final product (which is normally heated) inactivates the enzyme or depletes the substrates, meaning that transglutaminase is not present in the final product, and acts as a processing aid (89/107/EEC) and labelling is therefore unnecessary.” or” All those ingredients in the … technical file should be listed, apart from transglutaminase.” [95,97]. It appears that, those statements are not going along some regulatory organizations: “The usage of transglutaminase as a food additive is permitted in some countries. However, its utilization has to be declared to ensure transparency for consumers.” [49]. Other authors have a more definitive conclusions: “Therefore mTg can enhance the immunogenicity of gluten and should not be used in food products intended for consumption by CD patients.” [104]. In fact, multiple authors are worried and warned against the mTg added to the industrial processed food, doubting its safety and bringing up it possible role in CD pathogenesis [48,49,65,93,105,106]. At least in Europe some regulatory authorities decided to warn the public about mTg food safety and recommended labeling of the enzyme [49,107,108]. In Switzerland transglutaminase is a product that requires labeling. Transglutaminase treated food has to be subjected to authorization and the product labeling has to include reconstitution and nature of the used “glue” [108]. In Germany, the BFR Bund authorities declared that a clinically relevant risk through mTg is possible for CD sufferers.” “Suitable labeling of foods produced using mTg would enable these patients to avoid the uncertainties that the scientific community has yet to clarify” [108].

9. Conclusions

mTg is heavily consumed by the general and the celiac populations and its products are not labeled. It imitates functionally tTg and crosslink gluten, gliadin and other white proteins. Fig. 2 describes the sequential chain of events, initiated by mTg cross linking of white proteins, neo peptide formation that are exposed to the MHC, resulting in break of tolerance and autoreactive t cells selection and activation, to induce the tissue damage in gluten dependent conditions.

Despite advertisements and publications on its complete safety, the enzyme is a known occupational allergen, immunogenic and potentially pathogenic in CD. If substantiated, it might present a new environmental inducer of CD. Until further studies provide additional information, it is recommended that any use of mTg in the commercial processing or baking of food be disclosed on the packaging labels to ensure transparency for consumers. Gluten-free products are known for their potential contamination. Adding mTg can further increase the risk for gluten-sensitive populations.

Conflict of interest

Not grant supported nor conflict of interest.

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