Probiotics Applications in Autoimmune Diseases

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Additional information is available at the end of the chapter

1. Introduction

An autoimmune disorder (AD) is a condition in which the immune system mistakenly attacks its own body cells through the production of antibodies that target certain tissues. Such attack triggers further inflammation that result in more attacks and a significant inflammatory response leading to tissue destruction and cessation of functionality [1]. ADs include diabetes, rheumatoid arthritis, Graves' disease, systemic lupus and inflammatory bowel disease (IBD) [2]. ADs are on the rise worldwide and have major health implications from the diseases themselves as well as complications. Even though the causes of AD have been postulated to be genetic and environmental, the actual triggers remain poorly defined [3]. Genetic predisposition contribute to about 30% of AD while 70% to environmental factors such as infections (e.g., virus, bacteria) and lifestyle-associated factors such as food.

Recent data show that AD has prevalence of 6-8% and are currently affecting 400 million people worldwide, with the majority of all those affected being women. Previous figures underestimated the scope of the problem, while even the most pessimistic predictions fell short of the current figure. It is predicted that the total number of people living with AD will increase drastically within the coming thirty years if no new and substantially more effective drugs are produced [4]. On 2009, estimated health costs of autoimmune disorders have exceeded 100 billion dollars only in the US. This adds to the cost generated from higher rate of hospitalization, higher mortality rate, and impaired performance of workers with the disease [5]. AD is a condition that incorporates various metabolic disturbances and inflammatory physiological and biochemical reactions including blood dyscrasias and endocronological and pathophysiological imbalances. Of recently, gastrointestinal abnormalities have been directly linked to the initiation and progression of autoimmune diseases especially slower gut movement (gastroparesis) and microfloral overgrowth (especially of fermentation bacteria and yeasts due to the slightly more acidic gut contents). Improving AD complications, reducing prevalence and restoring normal

physiological patterns should significantly optimise treatment outcomes and the quality of life for patients.

In healthy individuals, the immune system prevents self-attack by two main routes. Firstly, by neutralizing dysfunctional lymphocytes in the thymus before they start attacking own body cells. This results in preventing the initiation of inflammation and progression of the autoimmune symptoms. Secondly, when dysfunctional lymphocytes are released into the mainstream, the immune system minimizes their ability to interact with triggers (antigens) through direct and indirect effects [6-8]. This results in a significant reduction in the severity of potential inflammatory response. Accordingly, treating AD can be achieved by either replacing the function of the damaged tissues (e.g. through injecting insulin when treating Type 1 diabetes, T1D) or suppressing the dysfunctional immune cells (e.g. through steroid therapy) [9-11].

Generally, clinical and laboratory research has suggested that certain immune cells called B-cells may have a stronger influence on the development and progression of various autoimmune diseases than previously thought [12]. Inflammatory cells attack different organs in different autoimmune disorders. In T1D, the autoimmune system attacks the β-cells of the pancreas triggering an inflammatory reaction, which results in the destruction of these cells and the cessation of insulin production [13]. In rheumatoid arthritis, rheumatoid factor antibodies are produced by the immune system and are interact with γ globulin (blood proteins) forming a complex that triggers inflammation that targets muscles and bones [14]. In Graves's diseases, an autoimmune disease of the thyroid gland, antibodies are produced against the thyroid protein thyroglobulin. These antibodies are called Thyroid Stimulating Hormones Receptors (TSHR) antibodies results in the increase in thyroid synthesis and section and thyroid growth as well as all accompanying symptoms [15-17]. In some autoimmune blood disorders, antibodies are produced against the body red and white blood cells, while in other autoimmune disorders, antibodies attack a wide range of tissues and organs resulting in more debilitating symptoms [18]. In systemic lupus, antibodies target antigens that are present in nucleic acids and cell organelles such as ribosomes and mitochondria. Lupus can cause dysfunction of many organs, including the heart, kidneys, and joints [19]. IBDs include two main conditions, ulcerative colitis and Crohn's disease. The inflammation in both conditions can affect the small and large intestine and sometimes other parts of the digestive system. Generally, ulcerative colitis is limited to the colon, primarily affecting the mucosa and the lining of the colon. Extensive inflammation gives rise to small ulcerations and microscopic abscesses that produce bleeding which exacerbate further the inflammatory response and worsen symptoms. Crohn's disease affects the small and large intestine, and rarely the stomach or oesophagus.

Many ADs have been characterized by a compromised gut movement which has been linked to the disturbed immune system and can result in substantial gut bacterial and yeast overgrowth [20-24]. Such an overgrowth is postulated to disturb body physiological and biochemical reactions and exacerbate the autoimmune-associated inflammation. This has also been linked to long term complications and weaker prognosis resulting in poor drug response and worsening quality of life [25, 26].

Diagnosing autoimmune diseases can be particularly difficult, because these disorders can affect any organ or tissue in the body and produce a wide variety of signs and symptoms. Many early symptoms of these disorders — such as fatigue, joint and muscle pain, fever or weight change — are nonspecific. Symptoms are often not apparent until the disease has reached a relatively advanced stage. Accordingly, prevention in most susceptible individuals and early diagnosis are two most important approaches, when researching the future therapy for autoimmune diseases.

ADs include wide range of inflammatory disease models that are characterized by the presence of a colossal inflammatory response. The trigger of the inflammation is versatile and complex with many hypotheses ranging from ingested toxins to idiopathic causes [9, 18, 27]. However, genetic influence remains a strong cause and is considered a contributing factor for the development and progression of these diseases. AD-associated inflammation can cause chemical unbalance that has been linked to poor tissue sensitivity to drug stimulation, rise in the levels of reactive radicals in the blood, poor enterohepatic recirculation and negatively affecting liver detoxification and performance. The level and extent of tissue damage depend on the severity of the inflammatory response and varies in different disease models. Accordingly, future therapy should focus not only on symptomatic relief, but also on rectifying the disturbances in body physiology and associated short and long term complications. These disturbances may affect the whole body and have been strongly linked to inflammatory lymph nodes in the gut walls. Thus, future therapy should also focus on normalizing gut disturbed immune response, which can be achieved through normalizing the composition of bile acids and microflora, gut immune-response and microflora-epithelial interactions towards maintaining normal biochemical reactions and healthy body physiology.

Of recently, the applications of probiotics in autoimmune diseases have gained great interest due to the feasibility of their administration and also their safety. A good example is hypoglycemic effect of probiotics in a rat model of Type 1 diabetes [28]. Possible mechanisms of actions include their anti-inflammatory effect resulting in a significant reduction in diabetes progression and complications [24]. This can be brought about through the normalization of gut disturbed-microflora by the administered probiotic-bacteria. Interesting, probiotic co-administration with a sulphonylureas antidiabetic drug has shown to reduce inflammation and ameliorate diabetes complications suggesting a significant role and great potential of probiotic applications as anti-inflammatory adjunct therapy.

Probiotics are dietary supplements containing bacteria which, when administered in adequate amounts, confer a health benefit on the host. Combinations of different bacterial strains can be used but a mixture of Lactobacilli and Bifidobacteria is a common choice. Probiotics have been shown to be beneficial in a wide range of conditions including infections, allergies, metabolic disorders such as diabetes mellitus, ulcerative colitis and Crohn's disease.

This chapter aims to explore the changes in gut microflora, physiology and metabolic pathways which are associated with the autoimmune diseases. A great focus will be on the potential application of probiotics on rectifying the disturbed gut composition associated with these diseases and whether such intervention can prevent or even treat these diseases.

2. Autoimmune-associated disturbances in gut microflora

The initial set of gut microfloral composition in human starts during birth. The physical structure of the gut is altered by the presence of microorganisms during growth. Once matured, the integrity of the epithelial barrier is maintained by the presence of these same microbes. Accordingly, the mother's microflora is considered a source of the infant own initial gut bacterial colonization, which is then influenced by the mother's milk, tissues' growth, the maturation of the immune system, as well as other factors. Gut motility and contents have been emerging as an important area of research when investigating the origin and potential therapeutics of autoimmune disease. Many patients with autoimmune disease have shown strong evidence of disturbances in the composition of gut microflora and the subsequent toxin buildup and other associated physiological and biochemical abnormalities [29]. A good example is Type 1 diabetic patients. Although the pathogenesis of T1D remains unclear, there is strong evidence supporting the hypothesis that the trigger leading to T1D, starts in the gut of genetically susceptible individuals [30, 31]. This inflammation causes major disturbances in both, the gut microfloral composition and bile acids ratios. This results in ongoing inflammatory response that brings about the destruction of pancreatic tissues and subsequent cessation of insulin production leading to clinical signs and symptoms of Type 1 diabetes. Another good example showing disturbed microfloral composition is IBD. Patients with IBD have shown clear shift of the gut microfloral composition towards less lactic acid-producing bacteria. In addition, the relative load of some species of colon-associated bacteria such as Bifidobacteria shows little presence in the gut of IBD patients indicating less bacterial-synchronization and disturbed quorum sensing processes in such patients. Interestingly, antibiotics are used in IBD to treat infective complications and to improve symptoms through altering the gut microfloral composition [32].

Maintenance of the physical integrity of the gut is essential to limit penetration of harmful bacteria. Dorsal to the epithelial layer in the gastrointestinal tract is a protective mucous gel layer which is altered by the existing microbial colonies. The neutral pH of the epithelium is preserved by the mucin, which creates a gradient to the acidic contents of the gut. It acts as a physical barrier to block microorganisms from adhering to the underlying epithelium and prevents sheer stress on the gut. The spread of harmful xenobiotics through the gut is limited by the mucin, which is normally a thick and viscous layer. In a germ-free environment the mucous layer is thinner and has a different mucin content and composition. Recent literature has shown that in ulcerative colitis and, to a lesser extent, Crohn's disease are associated with a significant reduction of the protective gut-mucus layer, however, the role of this alteration in the pathogenesis of both diseases remain unclear [33].

Localized inflammatory responses are modulated by the gut microfloral bacteria that seek to establish an ideal environment for their growth. The gut microfloral bacteria also alter inflammatory mediators which utilize the lymphatic system for transport, altering sites of inflammation outside the gut.

Intercellular interactions can also change gut permeability and are influenced by gut microflora. Zonula occludens are proteins that provide a structural framework to cells and seal the space between them, preventing the movement of ions across the barrier. A number of pathogenic bacteria and parasites target these epithelial cell membranes to increase the gut vulnerability to penetration. Comparatively, the presence of some beneficial bacteria can increase the expression of zonula occludens at tight junctions, improving epithelial integrity and cell-cell adhesiveness.

It is important to stress the fact that both, the complexity and versatility of gut microflora, remain major challenges to precisely be able to measure the changes in bacterial composition in diseases patients and compare that to healthy ones. In addition, the effect of food, drug consumption, gender and age may also influence gut microfloral composition adding complexity when comparing healthy versus disease states. To complicate this further, the interaction between bile acids and gut microflora has a significant effect on the density, composition, type, colonization and quorum sensing processes of various strains of gut bacteria, thus, making bile acids (BA) a major component of the bacterial-ecosystem that exists in the gut. This necessitates including bile acids, with when investigating autoimmune-associated disturbances in gut microbiota.

BAs are naturally produced in human. They are known to provide human with health benefits through their endocronological, microfloral, metabolic and other known and unknown effects. Disturbances in bile acids composition and functionality may cause tissue damage and eventual necrosis due to higher than normal concentrations of potent bile acids such as lithocholic acid compared with less potent bile acids such as chenodeoxycholic acid [34]. The nature of the interaction between gut microflora and bile acids is based on the fact that secondary bile acids are solely produced by the action of gut microflora. Gut microflora activates primary bile acids to secondary bile acids. This interaction between bile acid composition and the composition of gut microflora represents the base of the hypothesized linking between bile acid, gut microflora and energy balance. However, even though the compositions of bile acids and gut microflora are reported to be different in diabetic patients [35], it is still not clear how these changes directly affect the development and progression of diabetes or its complications. These complications include cardiovascular, tissue necrosis and ulcerations, and metabolic disturbances.

T1D is a good example of a common autoimmune disease which is on the rise worldwide. Even though the composition of gut microflora has been reported to be different in T1D patients, it may be difficult to quantify or qualify such a difference. Gut microflora interacts closely with the body immune system and has shown to control the immune response to various inflammatory stimuli. The mechanism of action of probiotics could be one or more of the following. Firstly, by competitive exclusion, where gut microfloral bacteria resist

colonization of other 'foreign' bacteria. Secondly, by barrier formation where the microflora forms a physical barrier reducing bacterial translocation by forming a wall surrounding the outside part of the gut enterocytes. Thirdly, gut bacteria can produce bacteriocins and change the pH to create a harsher environment for other invading bacteria to settle in the gut. Fourthly, gut microflora can influence the immune system through its effect on gut enterocytes (quorum sensing) and the innate and adaptive immune system [36, 37]. To understand better the autoimmune-associated disturbances in the gut microflora, there is a definite need to understand the mechanism by which gut microflora interacts with the epithelial mucosa lining up the intestinal tract. Over the last decade, there have been growing interests in studying the mechanism by which enterocytes interact with gut microflora.

The epithelial mucosa is inhabited by significant number of various immune cells that work as a link between the gut epithelia and lumen-contents [38]. One of these immune cells is lymphocytes such as T helper cells. These cells play an important role in the adaptive immune response. Thus, T helper cells have a more administrative role where it comes to neutralizing infected cells. Accordingly, they do not have direct cytotoxic or phagocytic effect. This role covers activating and directing other immune cells to destroy xenobiotics. They are essential in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing the antibacterial activity of phagocytes such as macrophages [39-41]. After a period of time, T helper cells start expressing CD4 which is a specialized surface protein. So when a body-cell is infected with an antigen, and this cell expresses this antigen on MHC class 2, a CD4 cell will promote the cell interactions and elimination. The lamina propria is a layer of connective tissue that lies adjacent to the epithelium of a mucous membrane. The intestinal epithelial mucosa consists of the lamina propria and the mucus. Many T helper cells, macrophages and IgA-producing plasma cells are present in the lamina propria [4].

Specialized microfold (M) cells of the lymph tissues can be found in the epithelial mucosa in the gut. M cells play a crucial role in the genesis of systemic immune response by delivering antigenic substrate to the underlying lymphoid tissue where immune responses start. Although it has been shown that dendritic cells also have the ability to sample antigens directly from the gut lumen, M cells certainly remain the most important antigen-sampling cell and are affected in the autoimmune diseases. M cells transport bacteria and antigen to the lymphatic tissue. Dendritic cells are bone marrow-derived antigen-presenting cells that essentially influence all aspects of innate and acquired immunity (Figure 2). These cells sense the microbes in their milieu through TLRs, and by signalling via different TLRs, generate biological reactions which produce variable responses from excitatory to suppressive. Dendritic cells are heterogeneous inhabitants of the intestine found scattered in all lymphoid compartments and can enter between epithelial cells to taster lumenal bacteria which they can then present to immune cells in the mucosa.

In healthy individuals, cytokines and mature T cells suppress 'exaggerated' T cell response that may result in unwanted cell damage, apoptosis and death. Thus, gut microflora in each individual, works as a finger print and exerts a significant control over the immune response to various 'antigenic' stimuli. In addition to the gut microfloral control on the

intestinal immunoregulatory system and the mucosal barrier, it is also involved in the pathogenesis of symptoms related to metabolic interactions of the microflora with intestinal contents or intestinal functions such as peristaltic movement [25, 26, 42-44]. As a result, many gastrointestinal disorders can be benefited from probiotic treatments. This includes travel diarrhoea, bloating and irritable bowel disease. Changes in the permeation of the intestine have been strongly associated with various autoimmune diseases such as T1D and IBD. However, the efficacy of probiotic treatment in autoimmune diseases is still under scrutiny and despite excellent progress in studying changes in gut microfloral composition associated with many autoimmune diseases, probiotic therapy has still not shown clear clinical efficacy in treating such conditions. The reported changes of intestinal permeation seem to indicate weakness of enterocytic tight junctions as well as the integrity of the epithelial mucosa as a whole. During the autoimmune process, inflammation becomes sound resulting in increased mucosal permeability (**Figure 1**). This may result in antigens reaching the lamina propria (from the lumen) triggering an autoimmune response. This starts through activation of the T cells and proinflammatory cytokines release. This results in further increase to the mucosal permeability and exacerbates the immune response [45-48].
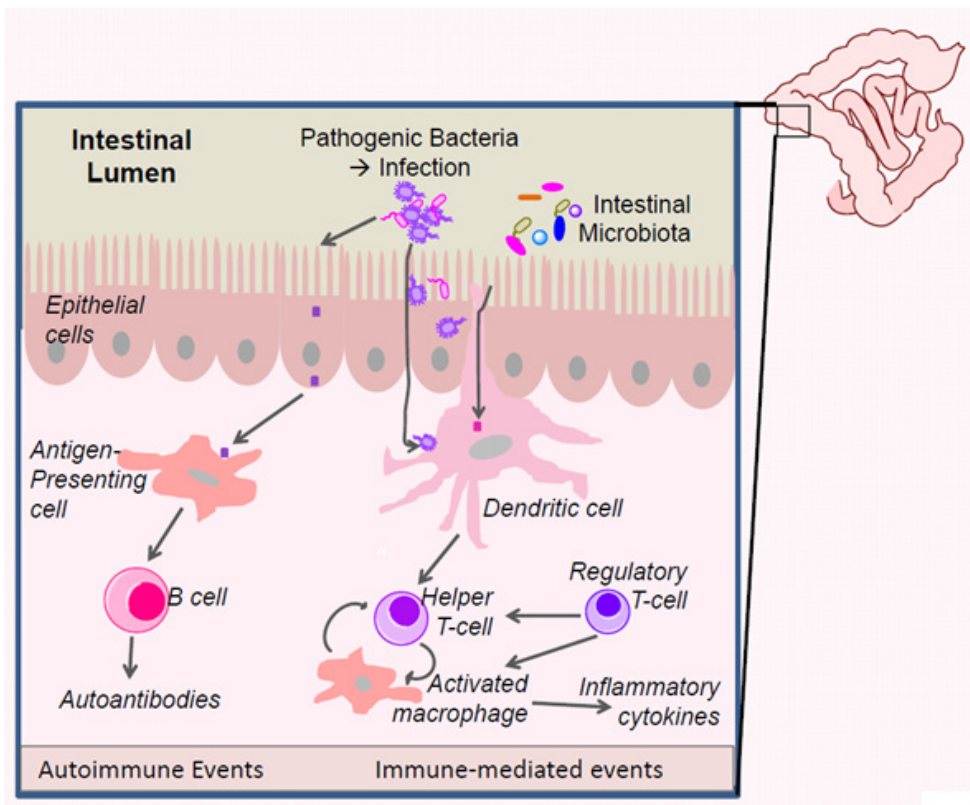
Figure 1. Intestinal permeability during an autoimmune response

3. Animal models suitable for investigating probiotic applications in autoimmune diseases

During the process of drug development, various *in vivo*, *ex vivo*, *in situ* and *in silico* methods can be used. Each method has advantages and disadvantages, and so using more than one method can provide better confirmation of findings. *In silico* methods can provide an initial insight into a potential drug candidate with predicted high pharmacological activity and good stability, while *ex vivo* methods can provide more information about a drug's interaction with living tissue, and are more cost-effective compared with *in vivo* animal models [49]. *In situ* methods can better predict drug absorption compared with *ex vivo* models but *in vivo* models can provide more comprehensive pharmacokinetic profiles and give a better understanding of drug-tissue interactions [50]. I*n vivo* studies are usually carried out where drug therapeutic formulations are administered to animals in order to investigate short and long term safety, to explore various clinical effects and to study different physicochemical parameters before confirming suitability of the formulation to a disease condition(s). Various animal models are used to represent various diseases [51].

In vivo studies on specialized animal models have allowed a great progress in tailoring research questions towards individualized gene contributions and their effect on the pathogenesis of these diseases. This has been done using standard inflammatory disease models in transgenic animals and by identifying novel models through the induction of the disease using chemicals. Although there is a surplus of animal models (spontaneous and induced) to study various autoimmune diseases, there is no ideal or standard model for studying the effect of probiotics on each condition [52-55]. Rats, mice and hamsters have been used to study probiotics applications in Ads. However, future research is needed, to compare the effect of probiotics on various animal models of ADs.

An ideal animal model should represent a specific medical condition in terms of disease development, pathophysiology, biological disturbances and short & long term complications [56-58].

If we are to create a better model of human AD, we should carefully consider the disease effect on the following:

- Relevant end points including primary, secondary and tertiary.
- The relevant speed and stages of disease development and progression.
- Disease complications, their progression and the relevant clinical end point(s).
- Symptomatic/nonsymptomatic signs of the disease.
- Feasibility of sample collections in terms of tissue site and sample volume.
- The incidence in males vs. females.

The current therapeutics for ADs are inadequate, which necessitates further drug development and *in vivo* trials. Clinical translation of AD's pathophysiology and clinical manifestations, from animal to human, has been limited and rather difficult. This is because very little is known about the pathophysiology and prognosis of such conditions; the extent of heterogeneity, polymorphism, genetic distance, the exact site of initial immune response

(gut, lymph nodes, blood, brain or?), and 'potential' triggering antigens. To complicate this further, different Ads have different signs and symptoms and thus, one animal model is unlikely to be always suitable for all conditions. Creating a suitable animal model for ADs requires the ability to accurately translate the findings to human. These findings include therapeutic efficacy (prevention/treatment), safety and PK/PD profiles. With regards to different ADs, various animal models have been proposed. In fact, many ADs have more than one animal model representing the disease. For example, T1D has many animal models. The nonobese diabetic (NOD) mouse is considered the 'standard' animal model of the disease. Other models are induction models of rats, mice and hamsters using alloxan or streptozotocin to destroy pancreatic beta cells and induce T1D. The NOD mouse represents the best spontaneous model for a human autoimmune disease, in particular, T1D. NOD mouse model allows the investigation of various immunointerventions that can be used in human T1D. Similar to T1D in human, NOD mice have higher levels of macrophages, dendritic cells, CD4+ and B cells. The induction of T1D in NOD mouse can be achieved through environmental conditions, mimicking the development of T1D in human. However, the development of T1D in NOD mouse takes place quickly and can produce a significant inflammatory condition that may over-respond to immunomanipulation and exaggerate the effect of a treatment. Also, the incidence of T1D is different between males and females in this model while the incidence is the same in males and females in human. This can further limit the applications and the findings of this animal model [59]. Many therapeutics that showed good efficacy in this model failed to achieve similar results in T1D human subjects [60]. Having said that and regardless of how different this model is, from the 'true' human TID, NOD mouse remains the most representative of human T1D. Interestingly, in a recently published study, the incidence of T1D was much higher, when the mice were maintained in a germ-free environment suggesting direct connection between gut microflora and the development of T1D [61, 62].

Overall, a suitable animal model for human AD should ideally be easy to breed and handle, and can accommodate various medical conditions that may come about or be associated with the condition it is representing. Thus, extrapolation of its findings to human should be easily done, and with great accuracy and precision.

4. The influence of gut microflora on the development of autoimmune diseases

In many autoimmune diseases, the gut microfloral composition is different than that of healthy individuals. However, the cause of this change of composition and whether this change is a contributing factor to the development of the disease remain unclear. Probiotic treatment has demonstrated potential benefits in many Ads, assumingly, through normalizing such changes in the gut microfloral composition. Interestingly, the literature suggests that the effect of probiotic treatment on ADs' development and progression may be brought about through the effect on the expression and functionality of certain protein transporters. Recent publications suggest that many transporters have their expression and functionality altered in the autoimmune disease; T1D [23, 27, 72]. The exact mechanism

associating the change in transporters and diabetes' development is still unknown but there are few assumptions to explain such an interaction. The first assumption is that some ADs, start with a direct insult in the gut, initiating a disturbance in the gut microflora and a consequent disturbed bile flow. This results in an altered bile feedback mechanisms and a change in the expression of protein transporters responsible for bile enterohepatic recirculation. The second assumption is that disturbance in protein transporters expression and functionality, caused by a genetic mutation, produces a disturbance in enterocytic-microfloral interactions triggering an inflammatory response. This response is further exacerbated by the resulted increase in gut permeability and ileal lymph/tissue necrosis. The third assumption is that the functionality of the immune system is altered (due to either an insult in the gut or genetic mutation). This alters the composition of gut microflora resulting in initiating of inflammation reaching various body tissues causing systemic inflammatory response triggering an autoimmune disorder and eventuating in autoimmune systematic response. In all these assumptions, genetic susceptibility is expected, and contributes further to the disease development and progression. The above assumptions were based on the work of the authors as well as careful evaluation of the literature.

In recent publications, alterations in the functionality of some transporters have been linked directly to the development of some autoimmune diseases such as diabetes. In addition, the enterohepatic recirculation of bile acids has also been related, by association, since secondary bile acids are solely produced by the action of gut microflora [13]. Bile salts' output in diabetic animals was high compared with healthy, and the expression of Mdr2 was also high after STZ treatment [63]. In another study, a mutation in Zinc transporter 8 (ZT8) located in beta cells, is implicated in the dysregulation of insulin transport and release, and an exacerbation of the inflammatory response leading to T1D. In this study, ZT8 was considered as an autoantigen resulting in the stimulation and production of beta cells autoantibodies and T1D development [64]. Moreover, streptozotocin (STZ) had different but significant effect on the expression of Na/Cl/glucose cotransporters, and the administration of insulin reduced such an effect [65]. Hyperglyemia itself directly reduced the activity of Mdr1 suggesting a clear association between pre-T1D hyperglycemia and disturbances in protein transporters [66]. In another recent study, the effect of STZ on cation protein transporters was reported, interestingly, at different levels of protein synthesis; transcriptional and posttranscriptional depending on the type of the transporters affected [67]. However, some studies suggest a diabetic influence is stronger on enzymatic activities than on protein transporters with the enzymatic influence being the cause of exacerbation of inflammation and development of the disease [68]. The impairment of protein transporters functionality, reported in the diabetic animals can take place either by reduced protein expression or reduced action. When glucose protein transporters in the blood brain barrier were studied under chronic hyperglycemia, their concentrations remain constant but functionality and glucose intake were impaired [69]. However, under acute hyperglycemia induced by STZ, their concentration decreased suggesting different response at different stages of the disease [70-72]. Accordingly, protein transporters have shown strong association with diabetes development and progression as well as diabetic complications.

Although there is some evidence suggesting that unrelated infections can result in the induction of organ specific autoimmunity [73], there is abundant epidemiological, clinical, and experimental evidence linking similar and closely related infectious agents with autoimmune diseases. Accordingly, the most acceptable hypothesis explaining how infectious agents cause autoimmunity is "molecular mimicry". Molecular mimicry directly invokes the specificity of the immune response to the resultant breakdown of tolerance. It proposes that microbial peptides have structural similarities to self-peptides and are therefore involved in the activation of autoreactive immune cells [74, 75]. Peptides, primarily, heat shock proteins (HSPs), have been implicated in autoimmunity [76, 77].

HSPs are a highly conserved family of proteins with significant structural homology between humans and bacteria. HSPs are located on almost all subcellular and cellular membranes and their numbers are induced in response to high temperatures and stress. HSPs function as molecular chaperons which are instrumental for signalling and protein trafficking. HSPs induced synthesis is implicated in autoimmunity. HSPs are believed to act through the activation of Toll-like receptors (TLRs) which trigger the expression of several genes that are involved in immune responses.

TLRs are only present in vertebrates and at least 11 TLRs are currently known. Distinct TLRs are differentially distributed within cells: TLR1, TLR2, TLR4, TLR5, TLR6, TLR10 and TLR11 are transmembrane proteins expressed on cell surfaces that contain extracellular domains rich in leucine that interact with pathogenic peptides, whereas TLR3, TLR7, TLR8 and TLR9 are primarily distributed on the membranes of intracellular compartments such as endosomes [78, 79]. Accordingly, TLRs are another potential target to bacterial manipulation. They are proteins on intestinal membranes that bind to pathogen-associated molecular patterns (PAMPs). After binding they release nuclear factor-kappa B (NF-kB) which moves into the cell nucleus and stimulates the release of pro-inflammatory mediators to target pathogens [80, 81]. Gut microfloral bacteria can directly trigger TLRs through adhering to the epithelial mucosa. As the human gut contains such large volumes of beneficial bacteria, they constantly trigger the TLRs. This leads to an eventual attenuation in the TLR response [82-84], (see **Figure 2**).

Although both pathogenic and probiotic bacteria regulate immunity via activation of TLRs, they do not usually trigger the same pathogenic inflammatory responses. Different probiotic bacteria stimulate distinct TLRs on host cells. Therefore, it is of biological and clinical importance to understand how very similar molecular proteins (HSPs) released by both commensal and pathogenic bacteria can trigger different responses by stimulating the same cellular receptors. One of the reasons for this may be that although the proteins are very similar they are not identical and thus they may stimulate the receptors in different ways to either produce a pro-inflammatory or an anti-inflammatory response. Another possibility is that the slight differences in the peptides allow them to bind to different TLRs leading to dissimilar responses. A third reason might be that more than one TLR is involved and that the effects seen are a synergistic effect depending on which TLRs are involved. TLR2 recognizes a variety of microbial components which include lipopeptides and peptidoglycan as well as lipopolysaccharides (LPS) from non-enterobacteria. TLR4 is an essential receptor

for (LPS) recognition [85-87] and it has been shown to be involved in the recognition of endogenous heat shock proteins, eg HSP60 and HSP70. Microbial recognition by TLRs facilitates dimerization of these receptors. TLR2 appears to form a heterophilic dimer with TLR1 or TLR6 but other TLRs are believed to form homodimers. TLR1 and TLR6 that are functionally associated with TLR2 allow for the discrimination between diacyl and triacyl lipopeptides. Dimerisation of TLRs triggers activation of signalling pathways through the cell and into the nucleus. However, different gene expression profiles are triggered depending on which TLRs and TLR combinations are activated.
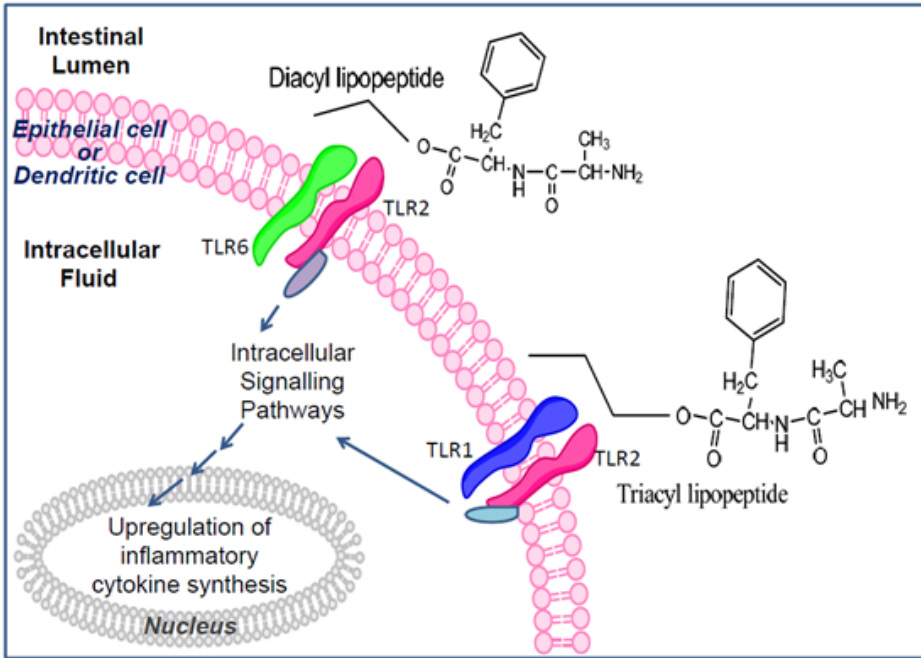
Figure 2. Molecular mimicry as a proposed cause of autoimmune diseases through the induction of 'mistaken-identity' immune response.

Loss of tolerance of the immune system to the body's own tissues can be caused by a number of factors including infection, excessive dendritic cell stimulation by intestinal microbiota, inadequate regulatory T-cell function or genetic factors. Dendritic cells are believed to be critical to the balance between tolerance and active immunity. Intestinal Dendritic cells are excessively activated in IBD as well as other autoimmune diseases which indirectly links the gut microfloral disturbances with the initiation or the progression of the disease (see Figure 2). Thus, the influence of disturbances in normal gut microflora may be indirectly linked to the initiation, development, progression and prognosis of many of the autoimmune disease. Such disturbances have been linked to changes in the expression and functionality of protein transporters in and outside the gastrointestinal tract. These disturbances have also been linked to changes in the composition and functionality of bile

acids and many physiological and biochemical feedback mechanisms that showed clear impact on the stability, performance and efficiency of the immune system and its associated lymph tissues. However, many studies may show a significant impact or the lack of it, when trying to rectify these disturbances through the treatment with probiotics, making the influence of gut microflora on the development and progress of autoimmune disease difficult to clearly explain. Consequently, a direct influence of normal microfloral composition on the body's inflammatory response has been demonstrated in the literature. This directs further research towards investigating how the gut microflora can potentially control the immune system to the extent where its manipulation may delay or even prevent the initiation of the inflammatory response leading to the clinical signs and symptoms of the immune disease.

5. The effect of probiotics on autoimmune-associated inflammation

Bacterial gut-microflora live in an ecosystem, where each bacterial colony is part of a bacterial strain that colonizes the gut, and interacts with each other, as well as, with other gut-bacterial strains. The nature of this interaction is being currently studied at many scientific labs worldwide, and evidence of cross-talking continues to emerge. Bacterial cross-talking process involves polypeptide-based signals being secreted by various bacteria that influence the protein expression and functionality in other bacteria [25, 88]. This means that bacteria can influence the expressions and functionality of various proteins and membrane-transporters of other bacteria, via changing the gut concentrations of certain polypeptides. This can be brought about through the induction or suppression of membrane-transporters or through the process of direct-signalling [38]. In matter of fact, sequencing of human faecal samples has identified over 5000 different active gut-bacteria, with known metabolic activities [24]. This exceeds the average number of mammalian cells present in the body! Infants in the womb are mainly germ-free with the exception of some microbes that may be acquired through the swallowing of the amniotic fluid. The type and variance of these microbes and the role each gut-bacterial strain plays in initial gut-ecosystem development is still not completely understood. The next exposure to microflora takes place during birth when infants inherit a bacterial profile from their mother that shapes the composition of the matured gut. This profile of bacteria differs with type of delivery (vaginal or caesarean), time taken for the membrane of the amniotic sac to rupture, gestational age and use of antibiotics during labour. The human gut undergoes continuous maturation over many years, and has a shifting microbe population that varies between individuals and their exposure to family members, especially siblings, the sanitation of living conditions, and food and drink. The balance of different bacteria stabilises as people age but is still affected by factors including diet, location, antibiotic use and radiation exposure in adults. Gut composition seems to become more unstable again as people age, as the faecal microbial profiles of those 65 years and older show considerably more variability between individuals [89].

Compromised gut movement associated with autoimmune disease can result in substantial bacterial and yeast overgrowth which is postulated to disturb bile acids composition and exacerbate the disease-associated inflammation [105-107]. Autoimmune disease such as

diabetes, show substantial inflammatory response, and bile acids disturbances can cause chemical unbalance that has been linked to poor tissue sensitivity to insulin [108], rise in the levels of reactive radicals in the blood [109], poor enterohepatic recirculation and dysfunctional protein-transporters in the gut that is negatively affecting liver detoxification and performance [110]. Accordingly, future AD-therapy should not only focus on rectifying physiological imbalance but also in targeting the disturbances in bile acids composition, protein transporters and overall the inflammation cascade initiated in the gut. This can be achieved through normalizing the composition of gut microflora and bile acids, gut immune-response and microflora-epithelial interactions towards maintaining normal biochemical reactions and healthy body physiology. Physiological features of human development including the innate and adaptive immunity, immune tolerance, bioavailability of nutrients, and intestinal barrier functions, are directly related to the composition and functionality of the human microflora. This includes the percentages of what is currently known as good and bad gut microflora. Good microflora includes two main species, Lactobacillus and Bifidobacteria. Microflora modifications may take place due to antibiotics consumption, prebiotic and probiotics administration and the use of drugs which affect gastric motility resulting in changes in gastric pH and gut-emptying rate. These modifications have been shown to be significantly profound in diabetic subjects resulting in the reduction of the percentage of good bacteria, the increase of the percentage of bad bacteria and yeasts and the consequent increase in the percentage of toxic bile salts such as lithocholic acid. This can also contribute to the higher incidence of gall stones and liver necrosis reported in diabetic patients. Accordingly, probiotics can introduce missing microbial components with known beneficial functions for the human host, while prebiotics can enhance the proliferation of beneficial microbes or probiotics, resulting in sustainable changes in the human microflora. Symbiotic relationship between probiotics and prebiotic administration is expected to exert a synergistic effect and in the right dose, may normalize and even reverse dysbiosis-associated complications.

Continuous exposure to bacteria can induce mucin secretion and change the structure of the mucous layer which can play a role in maintaining mucus thickness and its protective effects. In a recent *in vivo* study, Wistar rats were administered a probiotic formulation (VSL#3) daily for seven days. After probiotic treatment, basal luminal mucin content increased by 60% which has been linked to better protective effect and substantial stimulation of mucin secretion at the level of DNA-gene expression [90-93].

The significance and magnitude of the effect of host genetics on gut microfloral composition and functionality is difficult to accurately determine [94, 95]. It is generally agreed on that initial colonisation has the greatest effect on the lifelong bacterial types and functionality. Accordingly, it is expected that family members with shared genetic factors are likely to share the same initial colonisation similarities between their bacterial types. However, when the similarity of bacterial populations was compared between identical twins, non-identical twins and siblings, it was found that identical twins had significantly closer microflora compositions while others did not [96]. Other studies have observed bacteria modification after changes in host allele types, which also indicates some genetic effects but evidence remains controversial. Thus, it is clear that genetics do influence bacterial types in the gut, as

does diet, environment and a multitude of other factors. Accurate definition to the contribution of each factor to the types and functionality of gut microflora remains to be studied. Microfloral bacteria in the gut play a number of beneficial roles [97]. They ferment and break down otherwise indigestible food components, thus, making additional nutrients available to the human host. The presence of gut bacteria is protective against pathogens; the multitude of bacteria reduce the amount of available nutrients for invading pathogens, adhesion of pathogens to epithelial walls is restricted and commensal bacteria may produce bacteriocins that have an inhibitory effect of pathogenic bacterial growth.

Gut microflora is reported to influence the formation of cells essential to the immune system. Gut-associated lymphoid tissues are collections of immune cells in lymphoid tissue in the gastrointestinal tract [98]. They play an essential role in the localised immune defence of the gut. While small accumulations of lymphoid tissue occur throughout the gastrointestinal tract, the majority is found in Peyer's patches, mesenteric lymph nodes and dendritic cells [99] (see Figure 3).
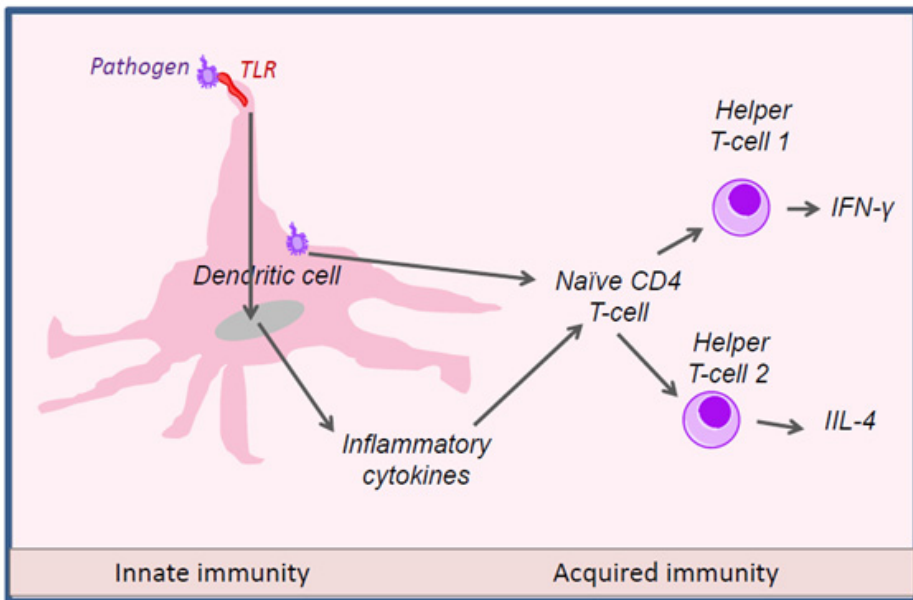
Figure 3. The influence of gut microflora on the activation of intestinal epithelial immune cells.

Peyer's patches store the inflammatory mediators, of a localised immune response including naive T-cells. Dendritic cells function as messengers which present endocytosed antigens to the Peyer's patches or mesenteric lymph nodes to prime T-cells into effector cells [100]. If the antigens are presented to the mesenteric lymph nodes, the effector cells are released into systemic circulation via the efferent lymphatic system, leading to an inflammatory response from central lymph nodes. Through effects on the dendritic cell intermediary, bacteria can modulate T-cell regulators which can lead to alter systemic inflammation via lymphatic

systems. Gut growth in animal studies where mice are raised in a microbe free environment shows a different intestinal structure compared to normal gut growth and the amount of gut-associated lymphoid tissue is reduced [101, 102]. This results in reduced gut microfloral differentiation between beneficial and pathogenic bacteria, bringing about a significant reduction in the area of the gut which can launch an innate immune response and decreases the communication of antigen information to central lymph nodes. This makes the entire body more vulnerable to harmful bacteria passing through the gut epithelium unnoticed [103-105].

In mice, a disturbed TLR-pathway results in compromised TLR signalling which results in any intestinal injury being met with an exaggerated response [81, 106-108]. A down-regulated TLR pathway caused by dysbiosis could cause a similar inflammatory process, making commensal bacteria potentially protective against IBD [109, 110]. This indicates the necessity of the TLR conditioning to develop an immune tolerance to bacterial threats in the gut. Bacteria in the gut can also bind to PAMPs to deliberately initiate an inflammatory response to signal the presence of invading pathogens.

Overall, these changes to inflammatory signalling and response based on interactions with gut microfloral bacteria are numerous and varied in mechanism. This indicates a complex relationship between the innate immune system and gut microflora where both parties are adaptive to the other, rather than static in response.

Many autoimmune and inflammatory diseases have shown positive response to probiotic and prebiotic treatments. The composition of the intestinal microflora may even affect mammalian physiology outside the gastrointestinal tract [111]. Recent studies have shown significant changes in gut microfloral and bile acid compositions in T1D [28, 43]. Thus, it is clear that our symbiotic microflora award many metabolic capabilities that our mammalian genomes lack [112], and so therapeutics that target microfloral modulation may prove rewarding. When the new born baby leaves the germ free uterus, she/he enters a highly contaminated extra-uterus environment. This requires the activation of her/his immune system to prevent infection. Over the period of the first year, the new born's intestinal microflora develops and its composition becomes her/his gut microfloral fingerprint! Gut microflora has been shown to play a major rule in controlling the inflammatory response of the host immune system through direct and indirect bacteria-bacteria and bacteria-host interactions. These interactions include physical and metabolic functions of the gut microfloral bacteria, which protect the intestinal tract from foreign pathogenic bacteria, eliminate the presence of unwanted bacteria through producing bacteriocins and other chemicals, and inform the gut epithelium and the host immune system about whether a local inflammatory response is needed [37, 113]. Gut microflora can control the host immune system through four main actions. The induction of IgA secretion to protect against infection, triggers localized inflammatory responses, neutralizing T-helper (Th) cell response and also contributing to the induction or inhibition of generalized mucosal immune responses. Recent studies have shown that in autoimmune diseases and gut inflammation disorders, there is a significant disturbances in the ratios of Th cells such as the increase in the Th-2/Th-1 ratio associated with inflammatory bowel diseases, which has been linked to exacerbation of the gut inflammation and the development of the disease. In recent studies, gut-associated dendritic cells in the lamina propria can extend their appendices reaching the

gut mucosa and using their Toll-like receptors (TLR) 2 and 4, to sample bacterial metabolites [114, 115]. This may result in dendritic cells releasing certain cytokines that stimulate the activation of naive Th-0 into active Th- cells such as 1, 2 and 3/1 [115]. Interestingly, some microfloral bacteria can actually cross enterocytic microfolds and interact with antigen presenting immune cells in mesenteric lymph nodes to activate naive plasma cells into IgA-producing B cells [116]. IgA coats the intestinal mucosa and control further bacterial penetration thus protecting the host from potential pathogenic bacteria. Even more interestingly, gut microflora bacteria have shown ability to not only initiate an inflammatory response but also to control and inhibit such a response. Some microfloral bacteria or their metabolites can interact with the intracellular receptor TLR-9, to which the bacteria activates T cells through the production of potent anti-inflammatory cytokines such as IL-10 [117, 118]. Microfloral bacteria can also produce small molecules that can enter intestinal epithelial cells to inhibit activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) [119]. Moreover, prolonged exposure to bacterial endotoxins, in particular, LPS (which interacts with TLR 2 and 4) can activate intracellular anti-inflammatory associated proteins that result in an overall anti-inflammatory effect [120]. Such gut bacterial-host interactions are critical in maintaining a balanced and effective immune response to various infections while maintaining control over prolonged or chronic inflammation and reducing the overstimulation of the host immune system.

Recent evidence suggests that a particular gut microfloral community may favour occurrence of the metabolic diseases. It is well know that the composition of gut microflora changes with diet and also as we age [121, 122]. In one study, a high fat diet was associated with higher endotoxaemia and a lowering of bifidobacterium species in mice cecum [123-125]. In a follow up study, the administration of prebiotics, in particular, oligofructose, to mice given high fat diet, restored the reduced quantity of bifidobacterium. This also resulted in reducing metabolic endotoxaemia, the inflammatory tone and slowing the development of diabetes. In this study and compared with control mice on chow diet, high fat diet significantly reduced intestinal Gram negative and Gram positive gut bacteria, increased endotoxaemia and diabetes-associated inflammation. However, when diabetic mice on high fat diet were given oligofructose, metabolic normalization took place including the quantity of gut bifidobacteria. In these mice, multiple correlation analyses showed that endotoxaemia negatively correlated with bifidobacteria quantity [126, 127]. By the same token, bifidobacterium quantity significantly and positively correlated with improved glucose tolerance, glucose-induced insulin secretion and normalised inflammatory tone (decreased endotoxaemia and plasma and adipose tissue proinflammatory cytokines) [123-125]. In general, the level of microfloral diversity and gut bifidobacteria in human, relate to health status and both decrease with age [128, 129].

6. The potential applications of probiotics in autoimmune diseases

Probiotics have been shown to be beneficial in wide range of conditions including infections, allergies, and metabolic disorders such as diabetes mellitus, ulcerative colitis and Crohn's disease [130-132].

When discussing therapeutic applications in AD, the use of probiotics is an area of growing interest, not just as an adjunct therapy but also as a mainstream treatment aiming at normalizing the disturbed gut-microfloral composition, as well as, directly relieving signs and symptoms of the disease. In order to design a probiotic formulation that targets disease-associated disturbances in gut microflora, a better and more detailed understanding of these disturbances is necessary. Better understanding of microfloral composition in the gut can be achieved through cell-culturing and protein-based assays that analyse the nature, type and quantity of various bacteria that exist in the gut.

However, beneficial effects of probiotics in ADs are modest, bacterial-strain and disease-state specific and limited to certain manifestations of disease and duration of use of the probiotic.

6.1. Type 1 diabetes and probiotics

Probiotic administration in animal models of Type 1 diabetes has shown great potentials. Combinations of different bacterial strains can be used [133] but a mixture of *Lactobacilli* and *Bifidobacteria* is a common choice [20-23, 26, 42, 92, 134-136]

There are reports in the literature that probiotic treatment can be useful in diabetes [28] but there is little explanation of the mechanisms involved. The initial site of diabetogenic cells has been hypothesized to be in the gut whereas pancreatic lymph nodes serve as the site of amplification of the autoimmune response [137]. This autoimmune response may disturb the composition of the normal gut flora. Treatment with *Bifidobacteria* and *Lactobacilli* has been shown to normalize the composition of the gut flora in children with T1D [131, 138]. In addition, the administration of *Lactobacilli* to alloxan-induced diabetic mice prolonged their survival [139, 140] and administration to non-obese diabetic (NOD, a rodent model of T1D) mice inhibited diabetes development possibly by the regulation of the host immune response and reduction of nitric oxide production [140]. Furthermore, the administration of a mixture of *Bifidobacteria*, *Lactobacilli* and *Streptococci* to NOD mice was protective against T1D development postulated to be through induction of interleukins IL4 and IL10 [141].

Slowing of peristalsis (gastroparesis) has been reported in T1D patients. This can result in a bigger population of bacteria in the gut and a subsequent rise in the concentration of secondary bile acids [142, 143] such as lithocholic acid [144, 145]. In addition, the disturbed bile acid composition in T1D (8) is strongly linked with autoimmune and liver diseases. The administration of *Lactobacilli* and *Bifidobacteria* may restore the bile acid composition [146, 147]. It is important to select the right probiotic species based on efficacy, stability in the gut (bile and pH tolerability) and long term safety. For example, some probiotic-bacterial cells have been examined for stability as well as efficacy in various autoimmune diseases. *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *Bifidobacterium lactis* show good bile and pH tolerability under normal conditions of pH (1.5-8) and bile acid concentration (0.8 – 3 %), in addition to long term safety [148-150].

6.2. Inflammatory bowel diseases and probiotics

In IBD such as UC colitis, there is a substantial inflammatory component with atypical type 2 T-helper cell (Th2) activation. Th2 are activated by the presence of antigens and then direct

other immune cells in the body. In UC they can become overly sensitised and secrete interleukin-13, an inflammatory mediator [151]. This drives T-cells not normally present in the colon to migrate there and makes the colon mucosa more sensitive to commensal bacteria which drives further inflammatory responses [152].

Naïve CD4 T cells differentiate into Th1 or Th2 effector T cells on activation by antigen-presenting cells (see Figure 4). Th1 and Th2 cells carry out distinct antigen specific adaptive immune functions; Th1 cells mediate cellular immunity against intracellular pathogens, whereas Th2 cells enable humoral immunity and immunity against extracellular pathogens. The effector functions of Th1 cells are exerted in part by production of interferon (IFN)-γ and those of Th2 cells by interleukins including IL4. Inappropriate regulation of Th1 and Th2 cell functions can cause autoimmune diseases.

In IBD, UC in particular, as with other inflammatory conditions, the production of immunoglobulins is elevated. Immunoglobulins, or antigens, bind to antibodies to encourage an immune response to the antigen while limiting the harm the antigen can do. UC displays an increased production of IgA, IgM, IgF but also has a disproportionately high level of IgG1. IgG1 binds to a colonic epithelial antigen in an autoimmune response. That antigen is also present in the eyes, skin and joints and inflammatory responses there can cause the extraintestinal symptoms associated with UC, including peripheral arthritis, erythema nodosum, iritis, uveitis and thromboembolism [153].

The identification of a causative UC pathogen would greatly simplify diagnosis and new treatment identification. Three broad studies used sequenced bacteria from the human gut to try and identify a healthy gut microbial profile. When the bacteria strains were divided by phylogenetic type it was found that 98% of bacteria were part of four phyla [154-156]. Another study compared this control data to samples from patients with Crohn's disease and UC. Two-thirds and three-quarters of the diseased samples, respectively, had the same bacterial balance as healthy controls. In the other IBD samples there was no consistency in the atypical bacterial groups, indicating that although dysbiosis is present there are no single causative bacteria [154]. Unfortunately, it is still unknown whether the dysbiosis precipitates gut inflammation or if another cause initiates the disease and dysbiosis occurs due to the inflammatory changes [157]

It has been shown that patients with UC display an increased microflora density [151] meaning the total population of bacteria in the colon is increased. In one study the number of bacteria in colon biopsies taken during endoscopy from newly diagnosed and untreated UC patients was double that of healthy controls [158]. The samples from UC patients also showed a thinner and less sulphated mucosal layer of the gut epithelium [159] which could support the increased bacterial levels through a lessened mucus flow to dislodge bacteria or an improved nutritional role from less sulphate.

VSL#3 is a high dose probiotic mixture that shows how information from multiple trials and *in vitro* studies can be brought together. Considering how new data fits into the probiotic profile established from previous investigations can help highlight any challenges to existing assumptions. Alternatively, when study results are replicated by different research

centres the significance of the findings is increased. This reflective process should develop an understanding of the probiotic that is based on clinical evidence. VSL#3 contains a combination of three strains of bifidobacterium, four strains of lactobacilli and one strain of streptococcus salivarius. A trial in 1999, shortly after the probiotic was developed, tested faecal samples of 20 UC patients to determine changes in bacterial concentrations when VSL#3 was administered with no other treatment. An increase in the bacterial numbers of strains found in the probiotic was observed in all patients from the 20th day of treatment and remained stable. This established that the probiotic could colonise the gut and encouraged further clinical trials [160]. VSL#3 was then trialled repeatedly in small studies which had similar conclusions regarding safety and efficacy. The studies showed a low number of reported side effects which were consistently mild, so safety in the trialled patient types was assumed. The outcomes from the trials were encouraging as the probiotic treated groups usually showed an improvement in disease state [92, 161-166]. This identified VSL#3 as a feasible new UC treatment but a large, randomised, placebo controlled study was needed to verify results [167]. Two studies have provided the additional clinical evidence needed to substantiate the conclusions from earlier trials. The first was conducted on patients in India in 2009 over a 12 week treatment regime. The second trial, in 2010, had a shorter treatment time of 8 weeks and was carried out in Italy. Both trials were multicentre, randomised and placebo controlled and were conducted on 144 patients. Information on the safety of VSL#3 was definitely supported by both trials. The only side effects reported by the probiotic treatment group were mild, primarily abdominal bloating and discomfort. Additionally, there were no patient withdrawals from the VSL#3 group due to worsening of symptoms [167-169]. As both trials were on patients with mild to moderate UC as determined by the Ulcerative Colitis Disease Activity Index (UCDAI) score, safety in this demographic can be seen to have been established. The safety of VSL#3 in more severe disease stages were not assessed by these trials and remains unknown. The primary outcome from both trials was a 50% reduction in the patient UCDAI score. When the results of the group receiving probiotics were compared to the group not receiving probiotics it was shown that a significantly greater the percentage of VSL#3 treated patients achieved the outcome compared to the placebo. This was consistent between the two trials. One of the secondary outcomes was the achievement of disease remission, which was the reduction in UCDAI to 2 or less. It is interesting that this was only a secondary outcome as remission is often considered the main goal of treatment of UC by patients. Both trials achieved remission in approximately 50% of patients on VSL#3. This was statistically significant in the 2009 Indian trial as the placebo remission rate was only 15% [168]. The second trial, based in Italy, had an unusually high placebo remission rate of 40% which meant that 50% remission in the VSL#3 was not significant [169]. This placebo rate weakens the evidence for VSL#3 inducing disease remission when adjunctive treatments are unchanged. However, these results do support the role of VSL#3 as an effective UC treatment to reduce symptom severity.

Despite promising treatment outcomes with VSL#3, exact mechanisms of action and the extent and significance of synergism remain to be clearly identified. The mechanism of action has been investigated a number of times and these studies suggest alteration of intestinal integrity is likely to be central to VSL#3 activity. Intestinal epithelial cells

incubated in media with VSL#3 show increased transepithelial resistance. This may be mediated by specific elements of the Mitogen-activated protein kinase (MAPK) pathway, which was activated by VSL#3. Pathogen-induced reduction in transepithelial resistance was diminished by VSL#3, probably due to the prevention of cell structure dysfunction at tight junctions [170]. VSL#3 may also alter mucin secretion, which makes up the mucous layer in the gastrointestinal tract. Of the nine identified genes, MUC2 is the predominant gel-forming mucin. MUC2 was induced in a concentration dependant manner by the exposure of the probiotic mixture to cells in media. It was postulated that this would correlate with an increase in mucin secretion. Rats fed with VSL#3 for seven days had an increase in MUC2 gene expression leading to an increase in the total mucin pool [159] When rat colonic loops were exposed to live VSL#3 an increase in mucin secretion was observed immediately without the need for a change in the mucin pool. Separate colonisation of the bacterial strains in VSL#3 identified that Lactobacilli is most likely to be responsible for mucin changes. Mucin secretion is known to effect bacterial adhesion and colonisation, so lactobacilli may upregulate MUC2 to improve colonisation. This implies that the benefits to intestinal structure are coincidental. One murine model of colitis, dextran-sodium sulphate-induced colitis, showed no mucin response to VSL#3 treatment. Mucous barrier thickness and expression of mucin genes were unchanged and inflammation did not decrease. The inactivity of VSL#3 may be a result of the colitis model used, which may have altered probiotic mediated effects as VSL#3 did adhere and change the microflora population. Trials on intestinal biopsies with ulcerative colitis could aid in supporting or invalidating the effect of VSL#3 on mucin.

Inflammatory mediators also play an important role in the reduced inflammation reported after treatment with VSL#3. The expression of TLR2 by dendritic cells is down regulated, which lessens the potential for TLR signalling for pro-inflammatory processes. An increase in production of IL-10, an anti-inflammatory cytokine, was also observed. This may be as a result of the changes to TLR2 or the overall reduction in inflammation. VSL#3 exerts multiple direct and indirect effects on gut inflammation which have not been fully elucidated, but can be observed in patient trials. While some studies suggest limitations to VSL#3 usefulness in UC treatment, further research is needed before they can be confirmed. Current information suggests that VSL#3 holds great promise as a low risk adjunctive treatment for mild to moderate UC to reduce symptom severity.

Strains that are identified for use as probiotics should not be pathogenic or carry antibiotic resistance as their use would be potentially harmful. There may be other consequences from treatment that can lead to physiological harm. As probiotic treatments often utilise bacterial strains found in the healthy human gut there is an assumption that probiotic treatment is without risks. Low withdrawal rates due to side effects from clinical trials support this notion, even in critically ill patients [171]. However, probiotic sepsis, a potentially deadly complication, has occasionally been reported [172]. Sepsis may be more likely in individuals with severe illness as they may be immunologically compromised.

HLA-DR is a MHC class 2 surface receptor responsible for identifying and binding to an antigen before presenting to the immune system to educate T and B-cells. There are more

than a dozen major subtypes of HLA-DR, some of which have been associated with specific diseases. The prevalence of serotypes DR2, DR9, and DRB1*0103 is significantly higher in people with active UC when compared to a healthy population. This could be a genetic factor that indicates a susceptibility to UC [173].Alternatively, the more common strains may be created by the body in response to the mucosal damage in the colon as a reparative effort [174]. As the prevalence of HLA-DR subtypes differs between populations the implications of these results are complex to apply. For example, the DR2 subtype showed a definite increased occurrence in UC patients from Japanese, Finn and Siscilian populations. In other culturally heterogenous populations the association is less strong or even absent, even though the association with DR2 is still significant when considered over all populations. DR9 is also more prevalent in Japanese populations, so it may be more important when assessing factors of disease susceptibility then in other ethnic groups. DRB1*0103 may be applied more specifically as it may be an indicator for how extensive UC could be. DR4, though, seems to be protective against UC, as the frequency that is occurs at is much lower in people with UC [173].

Another potential genetic factor in the development of UC is the expression of transcription factor XPB1 which regulates secretory and other stress-responsive cells in the endoplasmic reticulum stress response. In mice where the factor is absent, intestinal epithelial cells are more susceptible to potential colitis inducers and displayed spontaneous enteritis [175]. In humans, a variance in XPB1 has been associated with both Crohn's disease and UC. The activity of peroxisome proliferator-activated receptor-gamma (ppar-gamma) is an inflammatory system change that is unique to ulcerative colitis. In healthy individuals ppar-gamma modulates inflammation by attenuating nuclear factor-kappa B (NF-kB), a protein present in almost all cells that responds to harmful cell stimuli. Ppar-gamma activity in colonic epithelial cells of UC patients is reduced, but gene expression of ppar-gamma is normal. This indicates that bacteria present in the gut affect the activity of ppar-gamma in UC [176].

Bacterial imbalance may indicate more aggressive disease progression. The intestinal samples for the study were taken during surgery required to treat IBD or other conditions (primarily colonic cancer), not especially for the study. The age of the patients with atypical bacterial balances was on average 8 years younger than that of the control group. The need for surgery at a younger age could demonstrate a more aggressive disease. Alternatively, the changes in bacteria may be secondary to (not causative of) severe disease. The samples with Crohn's disease in the atypical group were also more likely to have abscesses [154]. Whether an imbalanced gut microflora was a contributing factor to the development of the abscess, or if the development of the abscess encouraged the growth of bacteria normally atypical to the human gut is difficult to discern.

When the microbial composition in the rectum was compared between patients with UC and normal patients, it was found that levels of Bifidobacterium were reduced in the samples with the inflammatory disease [177]. This is in keeping with a theory that post-operative pouchitis after surgical resection of the colon to manage UC is linked to a reduction in levels of Lactobacillus lactis and Bifidobacterium *[178]* Pouchitis occurs when the illeoanal pouchy becomes inflamed and passes diarrhoea, sometimes bloody, and causes

fever. After up to 10% of surgeries pouchitis becomes recurrent although the cause is unknown [179].

Even with these changes in microbial balance it has been found that use of antibiotics has no effect on the development or progression of UC. This is a marked point of difference compared to Crohn's disease where certain antibiotic therapies have been known to complete remission [180]. This may be associated with the absence of serum bacterial antibodies in patients with UC. While Crohn's disease has numerous elevated bacterial antibodies, indicating that particular bacteria may play a specific role in the disease, there is only one that has been identified in UC; perinuclear antineutrophil antibody. This antibody identifies bacterial antigens that have cross-reacted with nuclear antigens and it responds in tests to enteric bacterial antigens [181]. This shows a generalized overactive immune response targeting much of the gut bacteria resulting in wide spread exacerbation of the immune system and damaging further the intestinal tissues including the gut-associated lymphoid system. Thus, probiotic treatment poses great potential in treating IBD and further research is needed to investigate whether normalizing the gut microfloral composition will result in preventing the disease or ameliorating its severity and long term complications.

7. Lupus and probiotics

Systemic Lupus (SL) is an autoimmune disease which shares a significant inflammatory response and overactive and hypersensitive Th2 cells. A study of the autoimmune response in SL has found that one type of T cells is commonly found among SL patients. Cytotoxic CD8+ T-cell is found to be initially activated at the early stages of the disease and results in wide spread generalized activation of a long inflammatory cascade that brings about a full SL symptoms.

Similar to that of T1D, there are clear disturbances in gut microflora in SL, and, similar to other autoimmune diseases, a direct link between such changes and the initiation of the disease remains unclear. The literature suggests that gut microflora participates in the progression and complications of SL. This is brought about through an initial antigenic trigger that results in immune system 'confusion' which brings about an inflammatory response that attacks and destroys body's own tissues. The role of gut microflora in the initiation and development of SL is complex. This starts with a trigger that initiates a shift in gut microfloral composition which results in a formation of specific DNA-targeting antibodies directed towards specific pathogenic bacterial cells e.g. burkholderia bacteria [182]. This antibodies production is exacerbated through wider inflammatory response which brings about symptomatic SL and further complications of the disease. In theory and similar to the potential beneficial effect of probiotic administration on other autoimmune diseases, probiotic treatment, in particular, long term, is anticipated to neutralize gut-microfloral disturbances that brings about a stabilization of antibody production and eventual cessation of the inflammatory response which results in less severity and reduced signs and symptoms of the disease. In one study, authors measured the resistance of normal gut microflora to the colonization of pathogenic bacteria. This was done by a comprehensive

biotyping technique in healthy individuals and patients with inactive and active SL. Colonization resistance was found to be lower in active SL patients than in healthy individuals (P = 0.09, Wilcoxon one sided, with correction for ties) suggesting that in patients with SL, various types and more bacteria are translocating across the gut wall than in healthy individuals, due to lower colonization resistances in these patients. Some of these may serve as polyclonal B cell activators or as antigens cross-reacting with DNA [183]. Thus, administering probiotic bacteria such as bifidobacteria which may restore normal gut-microflora and reduce the inflammatory response and production of such antibodies should be beneficial. However, the use of probiotics in the prevention or treatment of SL remains doubtable due to many challenges including dose and frequency required to exert a clinical beneficial effect, targeted delivery to live bacteria to the large intestine, bacterial loading and bacterial interaction with other drugs.

Overall, the therapeutic applications of probiotics in autoimmune diseases can be summarized in three main mechanisms covering preventative measures as well reliving the signs and symptoms of the diseases. This focuses on the role of probiotic 'long-term' treatment of the gut aiming at manipulating and neutralizing the gut-microfloral bacteria to restore healthy body physiology and biochemical reactions, as well as minimizing symptoms through ameliorating the inflammatory response. In addition, probiotics have been shown to increase non-specific host resistance to pathogenic bacteria. Probiotics are believed to deliver their effects via three main mechanisms: (1) competitive exclusion, (2) production of anti-bacterial substances and (3) regulation of immune responses.

7.1. Competitive exclusion

Probiotics compete with pathogens and toxins for adherence to the intestinal epithelium. This concept describes the manner by which probiotic bacteria populate, overtake the pathogenic bacteria and go on to completely colonize and 'crowd' the gut.

7.2. Production of anti-bacterial substances

Probiotics exert anti-bacterial effects on pathogenic bacteria by producing bactericidal substances including bacteriocins and acid which work synergistically or alone to inhibit pathogenic bacterial growth. Bacteriocins are antimicrobial peptides which are produced by some gram positive bacteria while acetic, lactic and propionic acid are produced by a wide range of probiotic bacteria leading to a decrease in pH and inhibition of growth of many pathogenic gram negative bacteria.

7.3. Regulation of immune responses

Infections can disrupt T-cell tolerance [Rocken et al, 1992] due to the enormous bacterial load of the intestinal lumen. It appears that sustained exposure to bacterial antigens can result in impaired T-cell function [Bronstein-Sitton et al, 2003]. An inadequate function of immunoregulatory cells can lead to loss of tolerance.

Probiotics regulate immune responses by modulating pathogen induced inflammation caused by TLR-mediated signalling pathways. Probiotic bacteria have been shown to skew the Th1/Th2 balance toward Th1, which helps down-regulate overactive Th2-mediated allergic responses. Effects on the Th1/Th2 balance have been observed in some animal models of allergy [184]; however not all strains stimulated Th1 immunity [185, 186]. Nonetheless, stimulation of Th1 immunity has been reported in clinical trials [187-191] and clinical efficacy has been demonstrated in adults, children and infants for diseases including IBS and IBD [192, 193], see Figure *4*.
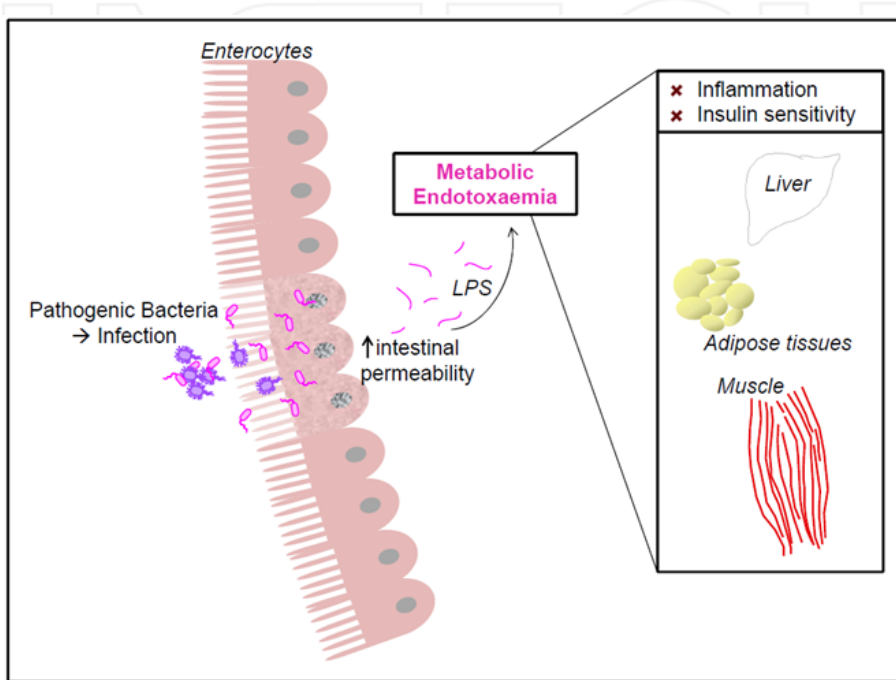
Figure 4. The relationship between LPS endotoxins and inflammation pathology in some autoimmune diseases. This figure adapted with modification from Cani P & Delzenne NM [105].

8. Safety and toxicology of probiotics

The World Health Organisation has guidelines for the evaluation of probiotic health claims. The guidelines begin by emphasising the importance of identifying the genus and species of the probiotic bacteria, as effects are strain specific. The WHO report also outlines assessment of probiotic storage, safety and evidence used to substantiate health claims [194].

Strains that are identified for use as probiotics should not be pathogenic or carry antibiotic resistance as their use would be potentially harmful. There may be other consequences from treatment that can lead to physiological harm. As probiotic treatments often utilise bacterial strains found in the healthy human gut there is an assumption that probiotic treatment is

without risks. Low withdrawal rates due to side effects from clinical trials support this notion, even in critically ill patients [171]. However, probiotic sepsis, a potentially deadly complication, has occasionally been reported [172]. Sepsis may be more likely in individuals with severe illness as they may be immunologically compromised.

The mechanism of immune system modulation through gut microflora may change during certain disease states. A large trial on patients with acute pancreatitis found that 16% patients in the probiotic group died compared with 6% of the placebo group, indicating an increase in mortality with prophylactic probiotic treatment in such immunocompromised patients [195]. This highlights the need for caution when treating a disease state or severity that safety has not been established with.

A range of probiotics have been used to treat mild to moderate UC without severe side effects. However, probiotic safety in severe UC has not been established. While patients with symptoms that are unresponsive to current therapies may benefit greatly from new treatments, until the mechanisms of action of probiotics are better understood the risk to patients is also unknown. Accordingly, probiotic administration has shown good safety profile in individuals with overall good health status, and may be suffering from mild infections or GI disorders [196, 197]. Probiotic safety stems from the fact that many strains are of human origin and present in large numbers in human GIT [131]. Accordingly, the reported incidences of probiotics inducing bacterial infection and bacteremia are very low (18). The only major concern with probiotic administration is the potential of bacterial translocation resulting in the induction of antibiotic-resistance strains that may lead to pathogenesis and haemodyscrasia [198, 199]. Having said that and as previously explained, the risks of infections caused by probiotic treatment is expected to be significant in immunocompromised patients [200-204].

Clinical trials of new treatments for many Ads vary greatly in trial length, inclusion criteria and *in vivo* models used. The diversity of these trials makes meaningful comparison of probiotic treatments difficult. For example there is no standard index for UC, with variety of different symptom based evaluations, composite scores and patient evaluated scoring systems used in clinical trials [205]. Patient inclusion in the trial, response to a treatment, and whether remission is induced, is usually determined by a disease activity index score of a pre-specified value being met. Comparison of different definitions of success is complex, as a patient could be considered in remission by one trial but in a state of active disease by another. In addition, clinical trials of treatments of UC are known to have a diverse and unpredictable placebo response rate [206]. A 2007 meta-analysis of 40 clinical trials found that placebo induced remission rates ranged from 0-40% while placebo response was as high as 67% [207]. An unpredictable placebo response can interfere with the perceived usefulness of new treatments making findings hard to interpret. On the other hand, clinical trials that evaluated outcomes based on subjective scores (physician impression of disease severity, patient reported quality of life, etc.) were associated with higher placebo rates of response and remission. Use of objective assessments, e.g. the presence of inflammatory markers or sigmoidoscopy score, can reduce placebo values and make comparison of clinical trials simpler. The patient acceptability and cost of invasive tests like colonoscopies and blood

sampling limit their use. Objective scores also do not quantify changes in time off work and symptoms like urgency and tenesmus, which are reported to be most important to patients.

The length of the clinical trial can change both rates of success and placebo responses. Shorter trials with fewer study visits lessen the cost of the study and reduce placebo values [206]. Long term trials may document a decrease in clinical effectiveness as relapses occur, the treatment ceases working and symptoms return. This may be due to the nature of disease rather than the treatment, as e.g. 67% of UC patients experience a relapse within the first ten years [208].

Risk of relapse makes withdrawal of existing therapy prior to commencing clinical trials undesirable. As a result, most probiotic treatments are initiated as adjunctive therapy to a stable oral dose of 5-aminosalicylic acid or an immunosuppressant. The period of time the dosage of other medications must have been stable for prior to the trial varies. The effect of these existing medications on the mechanism and efficacy of probiotics is unknown.

The adoption of a standardised disease activity index and trial endpoints would allow for comparison and combination of data from multiple trials. Until then, the value of an individual probiotic trial should be assessed with an understanding of how the trial characteristics may have influenced the reported results.

Commercially available probiotics often contain more than one bacterial type. The careful selection and administration of multiple strains of bacteria in combination has the potential to be more effective than any strain on its own. This concept is supported by a small review of 16 studies which found the multiple strain products was more effective than the composite single strains 75% of the time. Additionally, a study that did ex vivo screening of probiotic strains for beneficial changes in the regulation of T-cells and pro-inflammatory cytokines identified that multistrain combinations were more potent, adding to the theory that the use of multiple bacterial strains allows for better therapeutic effects.(37)

Doses may play a role in the comparative effectiveness of a probiotic mixture. The number of bacteria in a dose can be as high as the combined quantity from a therapeutically effective dose of each composite strain assuming no synergism. The higher combined dose may have a greater effect, making the multistrain probiotic therapy more likely to be effective especially if synergistic interaction exists between used bacterial strains [209]. Countering this as the sole mechanism influencing efficacy are studies where animals were administered single strain and multiple strain probiotics to protect against pathogens. Although the total dose of each probiotic was the same, the mixtures still had a greater protective effect or survival rate, indicating the presence of bacterial synergism [210-212].

A number of potential mechanisms for additive and synergistic interactions between probiotic strains exist. Some are probably the result of fortunate coincidence, while others are likely to be due to bacterial adaptation. The mechanism for the synergy may be simple, e.g. a byproduct of one bacteria increasing another strains' rate of growth. Other mechanisms may be more complex, involving more than two strains or using intermediaries to alter signalling pathways. The potential intricacy of these bacterial interactions prevents

any single strain from a multi strain probiotic being identified as the sole cause of a therapeutic effect without detailed additional research. Using more strains of bacteria in a probiotic preparation does not guarantee a better therapeutic response. Multiple strains of bacteria can have an antagonistic effect on each other through the production of agents that inhibit growth or competition for resources and adhesion sites. Other bacterial interactions could mask the influence of the antagonism on patient response, to the point where it may not be identified at all. This means bacteria with no clinical benefit could be included in probiotics unnecessarily.

Given that the effects of probiotics are strain specific, it is not possible to determine whether multiple strain probiotics are 'better' than single strain probiotics or vice versa. It does seem that some bacterial strains do have an increased clinical efficacy in one preparation over the other. Additional strain specific research could develop a reference to aid in determining if a probiotic bacterial strain is likely to benefit more from the reduced competition when administered alone or the potential synergism when multiple strains interact.

The mechanism of immune modulation through gut microfloral bacteria change during certain disease states. A large trial on patients with acute pancreatitis found that 16% patients in the probiotic group died compared with 6% of the placebo group, indicating an increase in mortality with prophylactic probiotic treatment [195]. This highlights the need for caution when treating a disease state or severity that safety has not been established with.

If the use of probiotics is to become part of autoimmune disease therapy, their safety concerns may be overcome by thoroughly studying appropriate dosing and frequency, their short and long term effect on mucosal membranes and the variation of their effect in different populations.

9. Conclusion

It is becoming more evident that the initiation, modulation and exacerbation of the inflammatory response resulting in ADs, is associated with disturbances of the gut microflora, as well as other biophysiological and biochemical processes inside and outside the gastrointestinal tract. *In vitro* studies have elucidated some of the complex proposed mechanisms associating gut microfloral disturbances with the development and progress of many ADs. Clinical trials have also provided evidence implicating probiotic intake to some health benefits noticed in ADs such as UC and T1D. However, significant clinical applications of probiotics as first line treatment for ADs have not been demonstrated or clearly proven, despite limited success in alleviating signs and symptoms of the diseases. As they are safe, probiotics are easily available to patients interested in trialling their effects. Many probiotics can be taken only once or twice a day which makes dosing convenient. Human trials have, so far, had a low incidence and severity of side effects. However, until trials are done using a broader range of disease severities with multiple bacterial strains, probiotic use may be limited to mild to moderate disease state and efficacy remains limited and at times controversial.

Main limitations to probiotic efficacies include formulation challenges, survival rate, cell-forming-bacterial-units required to exert a clinical effect and the versatility of gut microflora in different individuals and different stages of the disease. This makes selection of the bacterial strains, dosing volume and frequency and safety of AD patients, challenging. In addition, direct comparison of multiple clinical trials is complicated by the variability in study endpoints, disease severity assessment and other medication usage.

Ultimately, the primary treating physician, alongside the patient and the health care team, needs to assess whether a patient may benefit from probiotic treatment. If probiotics are to be used, trials on populations with a similar disease state to the patient can provide some guidance in strain selection. Clinical evidence should be used to determine if probiotic treatment is to be adjunctive or not, whether remission or symptom improvement is possible and to manage expectations. Disease state activity index scoring can monitor patient improvement or deterioration. For the patient, though, it is likely that the only monitoring that is meaningful is whether probiotic treatment has improved their perceived quality of life, thus, patient perception should always be taken into account when probiotic intake is considered.

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Acknowledgement

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