A ROLE FOR BACTERIAL-DERIVED PROTEASES AND PROTEASE INHIBITORS IN FOOD SENSITIVITY.

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for the Degree Doctor of Philosophy

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DESCRIPTIVE NOTE

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ABSTRACT

Celiac disease is a chronic atrophic enteropathy triggered by the ingestion of dietary gluten in genetically predisposed individuals expressing HLA class II genes, DQ2 or DQ8. Both innate and adaptive immune mechanisms are required for the development of the disease. The adaptive immune response is well characterized and includes the development of gluten-specific T-cells and antibodies towards gluten and the autoantigen, tissue transglutaminase 2. Less is known about the initiation of the innate immune response and its triggers, which is characterized by increases in cytokines such as IL-15, leading to proliferation and cytotoxic transformation of intraepithelial lymphocytes that are not specific to gluten. Although genetic susceptibility is necessary for celiac disease and present in 30% of most populations, only about 1% will develop it. This points to additional environmental factors, which could be microbial in origin. Disruptions of the gut microbial ecosystem, termed the intestinal microbiota, have been associated with the development and severity of many gastrointestinal disorders. Indeed, compositional differences in fecal and small intestinal microbiota have been described in patients with active celiac disease compared to healthy controls. This “dysbiosis” includes increases in small intestinal Proteobacteria and reductions in Firmicutes, but mechanistic information is lacking. Our lab has previously demonstrated that patients with active celiac disease have decreased expression of the serine protease inhibitor elafin in the duodenal mucosa, suggesting that proteolytic imbalance from either host or microbial origin, could be important in disease pathogenesis. The aim of this thesis is *to study mechanisms through which the intestinal microbiota may contribute to the development of gluten sensitivity*. I

thus hypothesized that the proteolytic capacity of a dysbiotic small intestinal microbiota could promote the development of gluten sensitivity, in a genetically susceptible host. Firstly, I investigated whether the intestinal microbiota is a source of proteases in the gut lumen, that metabolize gluten *in vivo* affecting its immunogenicity. Secondly, I studied whether and how bacterial proteases stimulate innate immune mechanisms, relevant to celiac disease pathogenesis. Last, I investigated whether some of these mechanisms could be targeted to improve gluten sensitivity.

In **Chapter 3** of this thesis, I show that the small intestinal microbiota participates in gluten metabolism *in vivo* in mice. Proteobacteria and opportunistic pathogen *Pseudomonas aeruginosa,* isolated from patients with celiac disease, metabolize gluten proteins through microbial elastase. Bacterially-modified gluten peptides translocate the mucosal barrier with efficiency and retain immunostimulatory capacity when incubated with gluten-specific T-cells from patients with celiac disease. However, bacteria found in higher abundance in healthy individuals, such as Lactobacilli, metabolize *P. aeruginosa*-modified gluten peptides reducing their antigenicity. This constitutes an opportunistic pathogen-gluten-host mechanism that could modulate celiac disease risk in genetically susceptible people.

In **Chapter 4**, I show that bacterial proteases capable of extracellularly metabolizing gluten, such as *P. aeruginosa* elastase, also have the capacity to stimulate innate immune responses in the small intestine, likely through a protease activated receptor-2-dependent mechanism. This innate immune response does not require HLA-risk genes and is characterized by non-specific increases in small intestinal intraepithelial

lymphocytes. In mice transgenic for the HLA-DQ8 gene, the presence of *P. aeruginosa* elastase in the small intestine exacerbates gluten-induced pathology.

In **Chapter 5**, I use a commensal *Bifidobacterium longum* strain that naturally produces a serine protease inhibitor, with the ability to inhibit elastase activity. I show that administration of *Bifidobacterium longum* to mice expressing the HLA-DQ8 gene prevents gluten immunopathology. The effect is dependent on the production of the bacterial serine protease inhibitor, as it was not observed with a *B. longum* knock-out for the serine protease inhibitor gene. Within this thesis, I demonstrate that microbial proteases can modulate gluten sensitivity through participation in both adaptive and innate immune pathways using celiac disease as a model of gastrointestinal disease. Further, I provide pre-clinical support that this pathway can be targeted for therapeutic purposes using protease inhibitors from commensal bacterial strains. My results provide causal evidence and novel mechanisms through which small intestinal bacteria may exacerbate or attenuate gluten sensitivity.

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LIST OF ABBREVIATIONS

Ahr aryl hydrocarbon receptor

ALR absent in melanoma (AIM)-like receptor

APC antigen-presenting cell

ASF altered Schaedler flora

ATI α-amylase/trypsin-inhibitor

CD cluster of differentiation

CeD celiac disease

CLR C-type lectin receptor

CXCR1 C-X-C motif chemokine receptor 1

DNBS dinitrobenzene sulfonic acid

DSS dextran sodium sulphate

ELISA enzyme-linked immunosorbent assay

ELISPOT Enzyme-Linked ImmunoSpot

Foxp3 forkhead box p3

GALT gut-associated lymphoid tissue

GFD Gluten-free diet

GMO genetically modified organism

HLA human leukocyte antigen

HNE human neutrophil elastase

IBD inflammatory bowel disease

IBS irritable bowel syndrome

IEL intraepithelial lymphocyte

IFN-γ interferon γ

IgA immunoglobulin A

IL interleukin

ILC innate lymphoid cell

iTreg inducible Treg

LC-MS/MS Liquid Chromatography-Mass Spectrophotometry/ Mass Spectrophotometry

M cell microfold cell

MAIT mucosal-associated invariant T-cell

MHC major histocompatibility complex

MIC MHC class chain related gene

MLN mesenteric lymph node

MPO Myeloperoxidase

MyD88 myeloid differentiation primary response gene 88

NCG/WS non-celiac gluten/wheat sensitivity

NFκB nuclear factor kappa-light-chain-enhancer

NKG2D The Natural Killer Group 2D

NKT natural killer T-cell

NLR nod-like receptor

NLS natren life start

NOD/DQ8 non-obese diabetic DQ8

NOD nucleotide-binding oligomerization domain-containing protein

PAR protease activated receptor

PBMC Peripheral blood mononuclear cell

PBS phosphate buffered solution

PRR pattern-recognition receptor

Psa *Pseudomonas aeruginosa*

PT pepsin-typsin

RIG-I retinoic acid-inducible gene-1

RLR retinoic acid-inducible gene-1-like receptor

SCFA short chain fatty acid

serpin serine protease inhibitor

SFB Segmented Filamentous bacteria

SFU Spot forming unit

SLPI secretory leukocyte protease inhibitor

SPF specific pathogen free

SEM standard error of the mean

T-bet T-box transcription factor TBX21

TCR T-cell receptor

Tfc T follicular helper cell

TG2 transglutaminase 2

TGF transforming growth factor

Th T helper

TIMP tissue inhibitors of metalloprotease

TLR toll-like receptor

TNBS 2,4,6-trinitrobenzene sulfonic acid

TNF tumor necrosis factor

Tr1 type 1 regulatory T-cell

Treg T regulatory cell

TxA *C. difficile* toxin A

CHAPTER 1: INTRODUCTION

1.1 The gastrointestinal immune system

Within the gastrointestinal tract lies a collection of immune tissues and structures that participate in the recognition and differential response to commensal bacteria, innocuous antigens, and potential pathogens. These immune structures include the gut-associated lymphoid tissue (GALT), mesenteric lymph nodes (MLNs), lamina propria and epithelium. The GALT comprises sites of induction including Peyer's patches, small lymphoid aggregates (Mowat and Agace 2014) and effector cells present outside organized lymphoid tissue. These tissues are critical for the development of antigen-specific immune responses. The MLNs are also sites of induction, drain the gastrointestinal tract and are critical for immune cell development. The lamina propria and epithelial barrier are considered effector sites, composed of immune cells critical for the formation of lymphoid tissues and protection against pathogens (Mowat and Agace 2014). Collectively, these tissues have both innate and adaptive immune functions that allow for protection from invasive organisms while maintaining tolerance to innocuous antigens (Overview in **Figure 1.1**).

1.1.1 Innate mucosal immunity

The innate immune system is composed of cell types, receptors, and pathways that are fast acting or afferent, non-specific and capable of leading to more specific downstream adaptive immune responses. These functions include the detection of invariant features of microbes, recruitment of immune cells, and antigen presentation. Cell types with innate immune functions include those from myeloid lineage (monocytes, eosinophils, neutrophils, mast cells, basophil, macrophage and dendritic cells), lymphoid

lineage (innate lymphoid cells; ILCs and intraepithelial lymphocytes; IELs), as well as cells non-classical immune cells (epithelial cells).

One of the innate immune system's primary tasks is to detect pathogens, and differentiate them from commensal microbes. Due to the proximity of the mucosal immune system to bacteria and other antigens, receptors recognizing microbial components are a large component of the innate immune system. These receptors, termed pattern-recognition receptors (PRRs), include, and are not limited to, toll-like receptors (TLRs), nod-like receptors (NLRs), retinoic acid-inducible gene-1 (RIG-I)-like receptors (RLRs), C-type lectin receptors (CLRs), absent in melanoma (AIM)-like receptors (ALRs) (Munoz-Wolf and Lavelle 2016) and protease-activated receptors (PARs) (Shpacovitch, Feld et al. 2007). They are expressed in a variety of cell types, including immune and non-immune cells, and their activation leads to diverse downstream cellular events, such as the translocation of nuclear factor kappa-light-chain-enhancer (NFκB) (Newton and Dixit 2012) or assembly of inflammasome components (Latz, Xiao et al. 2013). NFκB is a critical transcriptional factor in innate immune function that under classical or alternative pathways transcribe cytokines, chemokines, enzymes, lymphoid organogenesis genes, adhesion molecules and antimicrobial peptides (Bonizzi and Karin 2004). The inflammasome is a relatively recently discovered component of the innate immune system and its expression is key at barrier surfaces, where it participates in the recognition of some commensal bacteria and pathogens (Sellin, Maslowski et al. 2015; Santana, Martel et al. 2016). Activation of the inflammasome leads to the maturation and biological relevant forms of the inflammatory mediators interleukin (IL)-1β and IL-18

(Latz, Xiao et al. 2013) that contribute to commensal mutualism (Levy, Thaiss et al. 2015) and defense against pathogens (Vladimer, Marty-Roix et al. 2013; Chen and Ichinohe 2015).

Another key component of the innate immune system is the intestinal epithelial barrier, as this is the site of first contact with luminal commensal bacteria, pathogens and dietary antigens. The epithelial barrier is comprised of a combination of different cell types with specific functions that includes enterocytes, enteroendocrine cells, Paneth cells, M cells, tuft cells, goblet cells and cup cells (Barker 2014). Through this cell differentiation, the gut mucosa produces mucus (goblet cells) and antimicrobial peptides (Paneth cells and enterocytes) that limit the exposure of the mucosal immune system to encroaching microbes. Sampling of luminal contents is ensured by M cells that deliver antigens to the underlying antigen-presenting cells (APCs). Proper functioning of the barrier is necessary for the development of protective immune responses to infectious agents and is also thought to be involved in the development of oral tolerance to innocuous antigens (Suzuki, Sekine et al. 2008). Consequently, barrier dysfunction has been proposed as a mechanism in the exacerbation of inflammatory conditions. Clinically, many gastrointestinal disorders are associated with increased gastrointestinal permeability (Arrieta, Bistritz et al. 2006). This could lead to uncontrolled access of microbial and/or dietary antigens to the lamina propria, where they can interact with the basolateral surface of epithelial cells and immune cells. This mechanism is of importance in conditions like celiac disease, where translocation of the dietary antigen, gluten, is required for the initiation of the adaptive immune response.

Granulocytes (neutrophils, eosinophils and basophils), and monocytes (precursors of macrophage and dendritic cells) arise from the myeloid cell lineage. Monocytes, macrophages, and dendritic cells play a critical role in bridging the innate and adaptive immune responses. These APCs and are located throughout the GALT, MLNs, lamina propria and can be associated with the epithelium. They express both major histocompatibility complex (MHC) I and II that are responsible for the presentation of self and non-self-antigen to facilitate downstream adaptive immune responses. They also often express the innate immune receptors described above and are thus key for pathogen recognition (Mogensen 2009). Upon sensing invading pathogens, macrophages engulf bacteria or viruses (phagocytosis) for destruction by endosomal lysozyme and trigger the production of immune mediators, such as cytokines or chemokines or proteases (Mogensen 2009). As innate immune effector mechanisms, these mediators play a role in antimicrobial pathways and immune cell recruitment, limiting spread and replication of pathogens.

There are also cells with innate functions that originate from common lymphoid progenitors. These cells include innate lymphoid cells, γδ T-cells, mucosal-associated invariant T-cells (MAITs), natural killer T-cells (NKT), natural killer cells and IELs. ILCs are split into subsets depending on the type of immune response that they confer, and by their similarity to T-cell helper subsets. The subclasses of ILCs include ILC1s, ILC2s, and ILC3s. These cell types are different from T-cells as they do not bare classic T-cell markers, meaning they are not directly involved in the activation of memory responses or B-cell help. ILC1s include cytotoxic natural killer (NK) cells and low

cytotoxic ILC1s. These cells produce substantial amounts of interferon-γ (IFN-γ) and express the transcription factor T-box transcription factor TBX21 (T-bet) (Spits, Bernink et al. 2016) and are critical for resistance to infection. ILC2s produce similar cytokine profiles as Th2 cells (eg. IL-13, IL-5), are induced by helminth infection (von Moltke, Ji et al. 2016) and exacerbate allergic responses (Halim, Steer et al. 2014). ILC3s are the innate companion of Th17 cells, producing copious amounts of IL-17 and IL-22. These cells can be induced by the intestinal microbiota (Qiu, Guo et al. 2013) and have been shown to control tolerance towards commensal bacteria and dietary antigens. ILC3s express major histocompatibility complex (MHC) II and its associated genes, allowing them to dictate adaptive immune responses (cluster of differentiation (CD)4^+ T-cell responses) towards commensal bacteria (Hepworth, Monticelli et al. 2013). Finally, they are capable of inducing Tregs that can protect against loss of tolerance to dietary antigens (Mortha, Chudnovskiy et al. 2014). We are just beginning to understand how ILCs contribute or protect against disease, and how we may be able to control their numbers and function to prevent or alleviate gastrointestinal disorders.

Since proper structure and function of the intestinal epithelial barrier is critical for innate immune regulation, there are cells that have evolved to perform surveillance roles. IELs are unconventional T-cells that are considered to have innate-like properties and are strategically located between intestinal epithelial cells (Sheridan and Lefrancois 2010; Cheroutre, Lambolez et al. 2011; Swamy, Abeler-Dorner et al. 2015; Jabri and Sollid 2017). Unlike classical T-cells, IELs are predominately located in peripheral tissues and are often considered innate, because they do not require antigen priming before obtaining

functionality (Cheroutre, Lambolez et al. 2011). IELs detect and respond to epithelial stress, damage, and infection (T-cell receptor (TCR)-independent) (Bedoui, Gebhardt et al. 2016). In addition to this function, IELs also express a TCR and can respond to antigens upon presentation by APCs (TCR-dependent).

IELs are generally split into two subtypes. The first are "inducible", express TCR$\alpha\beta^+$ and CD8$\alpha\beta^+$, and are tissue resident cells that can become activated upon recognition of antigen (external, non-self-antigens). Inducible IELs can also express TCR$\gamma\delta^+$ and be CD8$\alpha\alpha^+$ or CD8$^-$CD4$^-$. These cells are thymus derived, having gone through an alternative thymic selection and can recognize self-antigen. They are important for resistance to some infections (Cuff, Cebra et al. 1993; Chardes, Buzoni-Gatel et al. 1994), but are also associated with the destruction of host tissue and subsequent inflammation (Egan, Maurer et al. 2011), such as celiac disease. Inducible IELs can upregulate expression of cytotoxic receptors (eg. the Natural Killer Group 2D; NKG2D) and induce cell death in susceptible cells expressing the appropriate ligands (Cheroutre, Lambolez et al. 2011). The cytotoxicity of these cells can be triggered by both TCR-dependent (adaptive) and TCR-independent (innate) events. An example of the innate functionality is the one observed in celiac disease, characterized by apoptosis of the small intestinal epithelium, constituting a key event in the development of intestinal atrophy (Meresse, Chen et al. 2004).

The second IEL type are termed "natural" and express TCR$\gamma\delta$. Natural IELs are critical for maintenance of the barrier, by producing barrier supporting cytokines (eg. IL-22) (Walker, Hautefort et al. 2013) and epithelial growth factors (Yang, Antony et al.

2004). These cells play a role in resistance against infection (Bozic, Forcic et al. 1998; Takano, Nishimura et al. 1998; Nakamura, Matsuzaki et al. 1999), are capable of producing antibacterial molecules (Ismail, Severson et al. 2011) and are protective in animal models of inflammatory bowel disease (IBD) (Ismail, Behrendt et al. 2009). There is currently a gap in knowledge in the field of IEL biology as to what stimuli (either innate or adaptive) maintain their populations. However, some have shown that IEL populations are dependent on the gut microbiota, as germ-free mice have reduced numbers (Suzuki, Nakao et al. 2002; Li, Innocentin et al. 2011; Jiang, Wang et al. 2013). Because the innate immune response is non-specific, constant activation or uncontrolled continued production of pro-inflammatory mediators can lead to immunopathology of host tissues. Non-specific immune activation and inflammation, as occurs in IBD and colorectal cancer, are associated with increased IELs (Thaiss, Zmora et al. 2016). There has been some speculation on the potential triggers of small intestinal IELs in celiac disease, but they remain largely unknown (Kim, Mayassi et al. 2015).

1.1.2 Adaptive mucosal immunity

The adaptive immune system's primary role, is to create memory towards antigens and subsequently respond to and neutralize that antigen upon future encounters. The two components of the adaptive immune system are T-cells and B-cells. These cells originate from a common progenitor, lymphoid. In a naive state, these cells are contained within lymphoid follicles of the GALT or MLNs in T and B-cell zones. When APCs pick-up antigen at effector sites, such as the lamina propria, they migrate to sites of induction, such lymphoid follicles or MLNs, and present to antigen naive $CD4^+$ T-cells. Upon

presentation, naive $CD4^+$ T-cells mature into one of several Th cell subsets and migrate to effector sites. Additionally, T-cells can participate in the maturation of B-cells into antibody-producing plasma cells. In a comparative manner, plasma cells migrate to effector sites to produce antibodies (Kurosaki, Kometani et al. 2015).

In the past decade, the helper T-cell field at mucosal surfaces has grown tremendously. Many memory T-cell subsets exist, each defined by specific gene expression and characterized by the type of immune milieu they create at mucosal surfaces. Simplistically, memory cells can be characterized into subsets, such as Th1 immunity and Th2 immunity. Th1 immunity (induced by IL-12) is characterized by the production of IFN-γ and is generally responsible for protection against intracellular bacteria and viruses (Paul and Zhu 2010). On the other hand, Th2 (induced by IL-4) immunity is associated with cytokines such as IL-4, IL-5, IL-13 and are critical for protection against helminth infection. There are limitations to the classification model (Th1 vs Th2) of Th cell biology. The biggest of them being the assumption of simplicity and homogeneity, as the diversity of Th cells is a lot more complicated in mammals. Further, the emergence of new Th cell types has made classification in this model more difficult. However, applying this simplicity to disease states, helps define and expose potential candidate pathways involved in disease progression and potential therapies. For example, conditions that affect the gut mucosa such as Crohn's disease and celiac disease are characterized by a Th1 immune response (Brand 2009; Qiao, Iversen et al. 2012). On the other hand, allergy and asthma are associated with Th2 immunity (Ngoc, Gold et al. 2005).

Other Th cell types pertinent to mucosal immunity include T regulatory cells (Tregs) and Th17 cells. Tregs are the critical cell type in suppressing both Th1 and Th2 responses. These cells produce massive quantities of IL-10, giving them a suppressive capacity to alleviate inflammation, such as that driven by infections (Oldenhove, Bouladoux et al. 2009), autoimmune (Kim, Rasmussen et al. 2007) and dietary antigens (Dubois, Chapat et al. 2003; DePaolo, Abadie et al. 2011; Hadis, Wahl et al. 2011; Bouziat, Hinterleitner et al. 2017). Decreases in their numbers are associated with chronic inflammatory conditions (Maul, Loddenkemper et al. 2005), or in the case of celiac disease, decreases in their suppressive capacity due to IL-15, lead to inability to suppress responses to dietary antigens (DePaolo, Abadie et al. 2011). Th17 cells (induced by IL-6, transforming growth factor (TGF)β, IL1β and IL21), are a subclass of Th cells that produce IL-17 and IL-21, and are important for protection against extracellular pathogens (Khader and Gopal 2010). Some Th17 subsets have been shown to be involved in the exacerbation of autoimmune diseases (Emamaullee, Davis et al. 2009; Wu, Ivanov et al. 2010; Komatsu, Okamoto et al. 2014), however their role as initiators of inflammation is unclear. Increased numbers of Th17 cells have been shown in celiac disease and other inflammatory conditions, as well as in animal models of colitis (Feng, Qin et al. 2011; Coccia, Harrison et al. 2012; Natividad, Pinto-Sanchez et al. 2015; Egan, Sodhi et al. 2016). New T helper cell subsets are being uncovered and reclassified, such as Th22 (induced by IL-6 and tumor necrosis factor (TNF) α) (Eyerich, Eyerich et al. 2009), Th9 (Veldhoen, Uyttenhove et al. 2008), T follicular helper cells (Tfhs)(Crotty 2014), and type 1 regulatory T-cells (Tr1) cells (Groux, O'Garra et al. 1997). Overall, the T helper

cells subsets outlined above are just examples of an expanding field of adaptive immunity at mucosal surfaces, and their detailed description is beyond the scope of this thesis (for review see (Luckheeram, Zhou et al. 2012)).

In contrast to T helper cells, cytotoxic ($CD8^+$) T-cells have the capability of inducing apoptosis in other host cell types. Some IEL subtypes are $CD8^+$, and recognize antigens presented by MHC class I, which is partially facilitated by CD8. The antigens involved are generally host-specific and either derived from cancerous or virus infected cells. Upon recognition of these antigens, $CD8^+$ T-cells perform effector functions, including the release of cytotoxins to induce apoptosis.

1.1.3 Tolerance to luminal antigens

The mucosal immune system is constantly challenged by a large load of antigens, from bacterial, viral, protozoan, fungal, parasitic, environmental, and dietary sources that are present in the lumen of the gastrointestinal tract. Oral tolerance is the state of local and systemic unresponsiveness to innocuous antigens encountered orally (Pabst and Mowat 2012). Under homeostatic conditions the mucosal immune system is programmed to develop mucosal tolerance, and systemic ignorance, to oral antigens arising from the commensal microbiota and the diet (Macpherson and Uhr 2004). Development of pro-inflammatory immune responses, to otherwise harmless antigens, occur when the mechanisms that ensure development of tolerance are disrupted. Antigen uptake is tightly regulated and occurs through transport by epithelial cells, microfold cells (M cells) transcytosis, or the paracellular route between epithelial cells. Antigen crossing can also

be facilitated by macrophages or dendritic cells, through the capture antigen via extension of processes through the epithelial layer into the lumen (Pabst and Mowat 2012). Upon entering the lamina propria, antigens are captured by APCs, which traffic to the MLNs and present them to T or B-cells. Under tolerogenic conditions, $CD103^+$ DCs acquire antigen, and through production of TGFβ and retinoic acid (RA), promote the generation of antigen-specific, IL-10-producing forkhead box p3 $(Foxp3)^+$ Tregs (Hadis, Wahl et al. 2011). Tregs play a critical role in the suppressing inflammation and, dysfunction of Tregs, can lead to the loss of oral tolerance to dietary antigens (Dubois, Chapat et al. 2003; DePaolo, Abadie et al. 2011; Hadis, Wahl et al. 2011; Bouziat, Hinterleitner et al. 2017).

The mechanisms behind the loss of tolerance to luminal antigens, or sensitization, are multiple and vary depending on the type of antigen, its dose and the genetic background of the host. For instance, allergens induce a Th2 phenotype, and the loss of tolerance to proteins such as ovalbumin or peanut is dependent on IL-33 released from IELs and OX40L on DCs, leading to antigen-specific IgE and Th2 cytokines (IL-4, IL-13, and IL-9) that suppress Treg function and proliferation (Oyoshi, Oettgen et al. 2014). In contrast, celiac disease, which is characterized by the loss of tolerance to the dietary group of proteins called gluten, is dominated by a Th1 response. DePaolo *et al.* (2012) demonstrated that a combination of upregulated IL-15 and RA can lead to the loss of tolerance towards gluten, in genetically susceptible mice. In their model, IL-15 and RA converted tolerogenic DCs into inflammatory DCs, producing IL-12p70 and IL-23. These cytokines enhanced effector T-cell responses (IFN-γ producing) and antibody production

towards gluten proteins, contributing to the loss of suppressive Tregs (DePaolo, Abadie et al. 2011). Although this study is important for the understanding of the mechanisms behind the loss of tolerance to gluten, it utilized genetically modified mice, transgenically overexpressing IL-15. Thus, environmental factors that likely contribute to the loss of tolerance to dietary antigens are difficult to explore in this system. This has been elegantly outlined in a recent study by Bouziat *et al.* (2017), using wild-type and HLA-DQ8 mice, that show that gastrointestinal infection with a specific reovirus, could induce the loss of tolerance to dietary antigens, including gluten. This process was dependent upon the induction of type 1 interferons induced by the virus in the small intestine, which suppressed Treg function and was associated Th1 responses to dietary antigens (Bouziat, Hinterleitner et al. 2017). However, it is unlikely that all cases of celiac disease are triggered by viral infection and, other environmental factors, such as bacterial opportunistic pathogens may be involved.

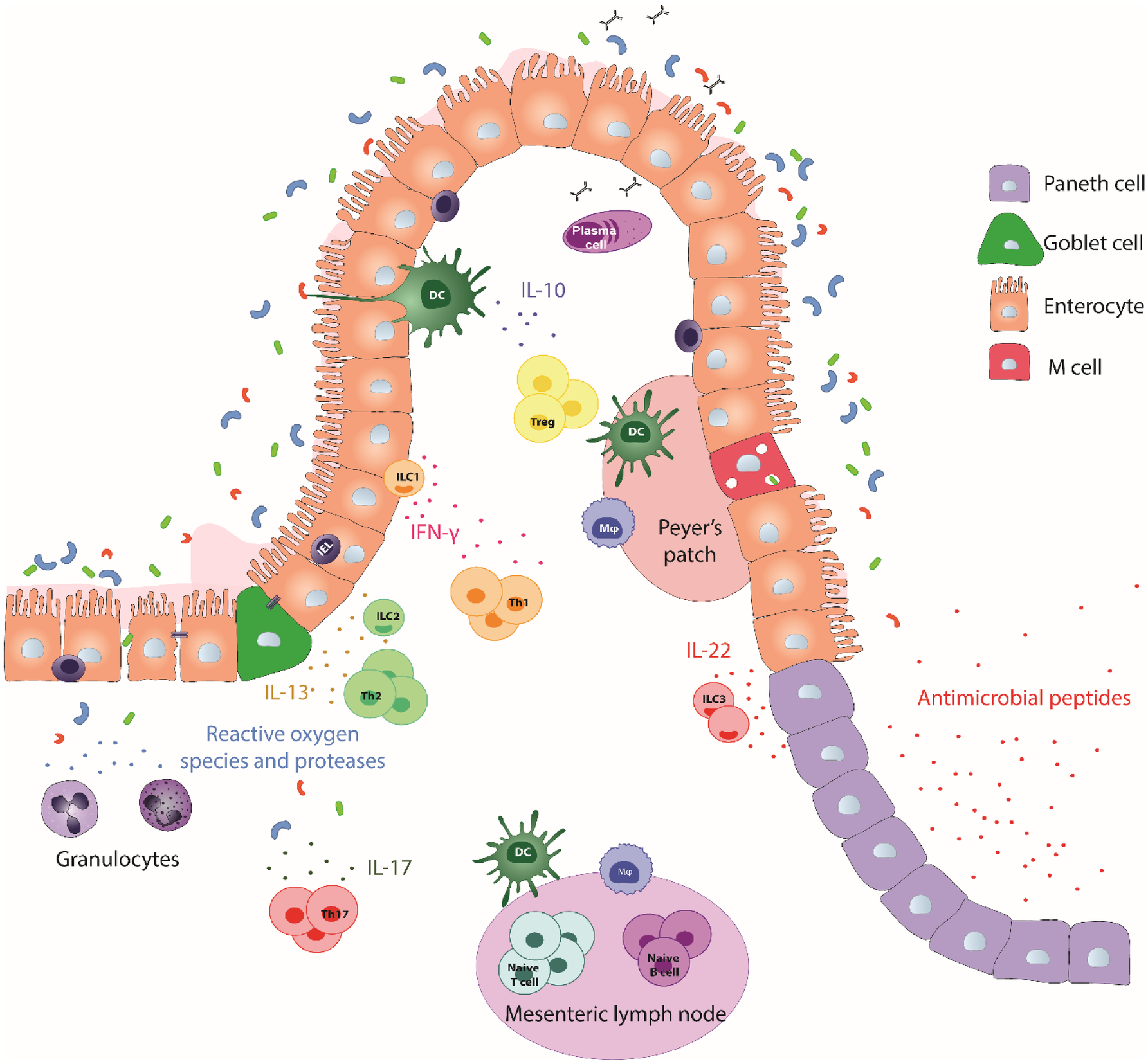

Figure 1.1. Synopsis of the mucosal gastrointestinal immune system. Depicted here are the GALT, lamina propria, epithelium and MLNs. Both innate and adaptive immune cells recognize invading microbes and respond with the production of reactive oxygen species, cytokines and chemokines supporting epithelial barrier function among other things. DCs capture antigen at both the epithelium and the underlying Peyer's patch where they

migrate to sites of the GALT to present to naïve T-cells. Mature T-cells and plasma cells migrate into the lamina propria.

1.2 The intestinal microbiota

The gastrointestinal mucosal immune system is constantly in contact with a complex array of intestinal microorganisms. These include bacterial, fungi, protists (eg. protozoa), and viruses among others. In the last decade, it has been demonstrated that the intestinal microbiota is critical for many physiological processes, including the development of the mucosal immune system. Many studies have concentrated on the bacterial component of the microbiota.

1.2.1 The landscape of the intestinal microbiota

Humans are colonized at birth with the microbiota that resides within the maternal skin and vaginal mucosa. Research has revealed that this process is critical for future immune function. The seeding of newborns by their mother's microbiota is so important that efforts to restore (enrichment with vaginal bacteria, eg. *Lactobacillus*) the intestinal microbiota in Cesarean section infants at birth have been made (Dominguez-Bello, De Jesus-Laboy et al. 2016). There is a consensus that infants are first colonized with facultative anaerobes dominated by the Actinobacteria and Proteobacteria phyla (Rodriguez, Murphy et al. 2015). During the first year of life, strict anaerobes from the Bacteroidetes and Firmicutes phyla begin to colonize the gastrointestinal tract and in time, the intestinal microbiota starts to resemble that of an adult. Disruption of the normal

colonization process has been suggested to predispose to disease later in life, such as obesity (Mueller, Whyatt et al. 2015), asthma (Thavagnanam, Fleming et al. 2008), celiac disease (Decker, Hornef et al. 2011) and allergy (Renz-Polster, David et al. 2005). Indeed, Cesarean section is associated with different intestinal microbiota profiles later in life (Dominguez-Bello, Costello et al. 2010; Dominguez-Bello, De Jesus-Laboy et al. 2016; Stokholm, Thorsen et al. 2016). Antibiotic therapy in early life, dietary practices such as formula feeding, and delivery by C-section, have all been suggested to predispose to disease later in life (Neu and Rushing 2011; Guinane and Cotter 2013). Although research has suggested the intestinal microbiota of infants is rather flexible, as time progresses, the intestinal microbiota in adulthood remains relatively stable over time (Faith, Guruge et al. 2013). However, it is still susceptible to disruptions by diet, drugs, and disease (Brown, DeCoffe et al. 2012; Rodriguez, Murphy et al. 2015).

The gastrointestinal tract harbors a vast number of microenvironments, from the stomach to the small intestine (duodenum, jejunum, and ileum), and to the large intestine (cecum and colon). These gastrointestinal sections are composed of different bacterial communities that have adapted to the specific conditions imposed by the host. For instance, the duodenum has a lower pH (around 6.1) and higher oxygen levels, compared to more distal portions of the gastrointestinal tract (Ileum 7.4). Therefore, small intestinal bacteria have evolved ways to tolerate its pH and aerobic environment (Fallingborg 1999). These features explain the lower colony forming units (CFUs) (10^5) and low diversity of the microbiota in the small intestine, compared to the more distal sections of the gastrointestinal tract. The bacteria that colonize the proximal small intestine

predominantly include Lactobacilli and Streptococci species, which are capable of tolerating oxygen. Bacteria reach up to 10^7 CFU in the ileum, and more than 10^{12} CFU in the cecum and colon (Mowat and Agace 2014). The distal portions of the intestine also have higher diversity and anaerobic organisms, which include bacteria from the phyla Firmicutes and Bacteroidetes.

Many studies have investigated the progress of the microbiota over time, its association with disease, and how environmental factors alter its composition and function. Most studies, particularly those performed in humans, analyze fecal communities. This is likely due to the ease of sample collection. However, this has left gaps in the field regarding the compositional and functional changes in other portions of the gastrointestinal tract, such as the small intestine. The dynamics of the small intestinal microbiota in response to environmental factors could help elucidate the contributions and associations with diseases such as celiac disease, or the breakdown of oral tolerance to other dietary antigens, that is likely to occur in the small intestine. Due to the many bacteria that inhabit the gastrointestinal tract, the mucosal immune system constantly interacts with these bacterial communities to sustain homeostasis.

1.2.2 Interactions between the mucosal immune system and intestinal microbiota

The commensal microbiota contributes to the maturation and maintenance of cell types and tissues within the mucosal immune system. The relationship between the commensal microbiota and the mucosal immune system is complex, therefore reductionist methods must be employed to understand the role of specific bacteria in the

development of the mucosal immune system. These reductionist models generally involve gnotobiotic mice, and most information extracted about the role of the microbiota in the development of the immune system and physiology has been revealed by experimentation with germ-free animals. Studies by Profs. John Cebra, Helena Talskalova and Andrew Macpherson have highlighted this, demonstrating that adaptive immune responses such as secretory immunoglobulin A (IgA), are almost non-existent in germ-free animals (Moreau, Ducluzeau et al. 1978). However, upon colonization, these adaptive responses can be restored and can remain elevated even upon reversing back into germ-free status (Hapfelmeier, Lawson et al. 2010). Additional research has also shown that, not only are adaptive components of the immune system dependent on bacteria, but the microbiota is also critical for innate immune cell development (Khosravi, Yanez et al. 2014). Studies have overwhelmingly shown that the microbiota contributes to immune maturation at any point in life. However, we are just beginning to understand whether some functions require the existence of a critical window of time during infancy (Gensollen, Iyer et al. 2016). Altogether, studies suggest that colonization of germ-free mice is dynamic, with both the microbiota and immune system of the host evolving over time (El Aidy, Derrien et al. 2013).

The relationship between Th17 cells and the microbiota, is one of the best-characterized examples of the key role of the microbiota in immune system development. The mouse commensal bacterium Segmented Filamentous bacteria (SFB) was shown to induce Th17 cells in the ileum of colonized mice (Ivanov, Atarashi et al. 2009). The same group showed that human commensal bacterial isolates also induce Th17 cells in mice,

and this process was dependent on their ability to attach or associate to the epithelium (Atarashi, Tanoue et al. 2015; Tan, Sefik et al. 2016). Importantly, induction of Th17 cells by the microbiota was protective in gastrointestinal infection models (Ivanov, Atarashi et al. 2009; Atarashi, Tanoue et al. 2015), but detrimental in colitis models (Feng, Qin et al. 2011) suggesting a dual role of Th17 cells under inflammatory conditions. On the other hand, other groups demonstrated IL-17 was protective capacities in the context of colitis (Maxwell, Zhang et al. 2015), which points back to the complex pro and anti-inflammatory roles of Th17 subsets. The gut microbiota can also influence systemic Th17 cell induction and contribute to the development of diabetes (Kriegel, Sefik et al. 2011) and arthritis (Wu, Ivanov et al. 2010) in animal models.

Another well studied subset is that of Tregs, which are induced in the small intestine by Clostridia strains (Atarashi, Tanoue et al. 2013), through their production of butyrate (Furusawa, Obata et al. 2013). Commensal *Clostridium* spp. have also been linked to the induction of oral tolerance to luminal antigens (Atarashi, Tanoue et al. 2013; Stefka, Feehley et al. 2014). Zaiss et al (2015) showed that shifts in the microbiota induced by helminth infection can functionally change the microbiota to produce more short-chain fatty acids (SCFA) and protect against inflammation associated with allergy. The mechanism was dependent on increased Treg suppressive capacity (Zaiss, Rapin et al. 2015). Clostridia strains can also act independently of Tregs to protect against loss of tolerance to oral antigens. An example of this was demonstrated by Stefka *et al.* (2014), showing that the microbiota could act as a factor protecting against sensitization dietary antigens (Stefka, Feehley et al. 2014). They showed that Clostridia strains could increase

barrier function through induction of IL-22 producing ILC3s, and prevent sensitization to peanut antigens.

Many diseases do not exhibit a consistent compositional signature in the microbiota, and therefore research is beginning to focus on functional changes that include production of bacterial-derived metabolites. SCFAs are just one example of dietary bacterial metabolism that may directly interact with the mucosal immune system. The metabolism of tryptophan by microbes is another important process for homeostasis in the GALT. The generated metabolites (eg. indoles) regulate multiple cell types, including IELs (Li, Innocentin et al. 2011) that support barrier function through the Ahr (Quintana, Basso et al. 2008; Li, Innocentin et al. 2011; Lamas, Richard et al. 2016). Currently, the role of known obvious metabolites such as SCFAs in the development of disease is somewhat overvalued, and more research is required to identify additional specific molecules that are derived from the microbiota and their influence on mucosal immunity.

Regulation of barrier function is also dependent on the presence of microbiota. This is highlighted by studies suggesting that the microbiota maintains IEL populations. This was showcased by a study demonstrating that germ-free mice have lower numbers of IELs (Imaoka, Matsumoto et al. 1996). Further, specific microbial components, such as nucleotide-binding oligomerization domain-containing protein (NOD) 2 agonists derived from the microbiota are critical maintaining IEL populations (Jiang, Wang et al. 2013). Lastly, IEL populations are regulated by microbiota-derived aryl hydrocarbon receptor (Ahr) agonists (Li, Innocentin et al. 2011), suggesting diet may influence these

populations. It is unknown how specific bacteria, or microbial communities, increase IEL numbers, or stimulate them to become cytotoxic. It is also unknown whether the microbiota contributes to disease through IEL modulation, which is of particular interest in celiac disease.

In summary, evidence supports the role of the microbiota in both mucosal and systemic immune system development. The role of specific bacteria in the induction or depletion of certain mucosal cell types and their influence on disease progression, or resolution, is also beginning to emerge. Even bacteria that are closely related phylogenetically, can have vastly different outcomes on immune cell development (Geva-Zatorsky, Sefik et al. 2017). To complicate matters, our intestinal microbiota is composed of thousands of species, whose complex interactions influence the outcome of mucosal parameters and homeostasis. Under normal and homeostatic physiological conditions, the relationship between the intestinal microbiota and mucosal immune system strikes a balance in which, both the host and the microbes, benefit.

1.2.3 Dysbiosis and gastrointestinal disorders

Dysbiosis is defined as a shift in the composition, or function, of the microbiota associated with the development or exacerbation of disease. Dysbiosis is often observed in patients with gastrointestinal disorders including IBD (Sartor and Wu 2017), celiac disease (Verdu, Galipeau et al. 2015) and irritable bowel syndrome (IBS) (Collins 2014). Although compositional dysbiosis has been observed in association with certain chronic conditions, evidence demonstrating that it contributes to initiation of disease, is limited. This is because of the inherent difficulty of dissecting the contributions of the dysbiotic

microbiota to disease, and the influence of the host's inflammatory response, genetics, and physiology, on the microbiota. Reductionist systems using animal models of inflammation that also harbor a dysbiotic microbiota are beginning to help dissect mechanisms that could be implicated in disease pathogenesis (Lamas, Richard et al. 2016; Chen, Wilson et al. 2017).

Pathobionts are commensal bacteria that reside in the microbiota and do not cause disease under homeostatic conditions. However, when genetic or environmental conditions are altered, these bacteria may trigger the onset or exacerbate pre-existing disease (Zechner 2017). The most studied disorder linking the role of pathobionts and exacerbation of inflammation is IBD. There is no precise definition of pathobionts, but it is understood that these are microbes harbor features that lie between a commensal and a true pathogen, and can promote damage under certain circumstances. For instance, the emergence of a pathobiont *E. coli* (Ayres, Trinidad et al. 2012) after therapy with antibiotics can exacerbate colitis in mice. Opportunistic pathogens, such as *Clostridium difficile*, may be present in the microbiota without inducing disease (carrier status). However, when conditions that favor their overgrowth are encountered (eg. antibiotics), they can trigger disease (Buffie, Bucci et al. 2015) by direct interaction with host's tissues. The presence of pathobionts and opportunistic pathogens are examples of a state of compositional dysbiosis associated with disease. Other forms of dysbiosis, unrelated to pathobiont and opportunistic pathogen presence, are also possible.

Environmental factors, such as the previously mentioned antibiotic use, can drive dysbiosis. Diet is another factor that could induce microbiota changes and, or, the

emergence of pathobionts associated with disease. This has been highlighted by Devkota *et al.* (2012), who fed IL10$^{-/-}$ mice, that spontaneously develop colitis, a diet containing saturated fat. This practice led to the blooming of *Bilophila wadsworthia*, and altered bile acid metabolism, which in turn exacerbated colitis (Devkota, Wang et al. 2012). Devkota's study describes one potential bacterial-dietary fat mechanism through which pathobionts could influence gastrointestinal disease. IBDs are without doubt, the most extensively studied models for the role of dysbiosis in gastrointestinal disease. Although dysbiosis has also been recently associated with other intestinal conditions, such as celiac disease, our knowledge of the nature of this association has been significantly less studied. This is likely due to the outdated concept that gluten is the only environmental trigger necessary for the development of the disorder, and the misconception that a gluten-free diet will always improve the condition. The interaction between bacterial small intestinal dysbiosis and gluten proteins, and how this could contribute to inflammatory mechanisms relevant to celiac disease, are addressed in **Chapter 3** and **4** of this thesis.

1.2.4 Targeting the intestinal microbiota as a therapeutic approach for gastrointestinal disorders

Due to the involvement of the intestinal microbiota in health and disease much interest has emerged to target the intestinal microbiota therapeutically. Please see **Appendix 1** for a thorough review of the emerging targets for therapeutic modulation of the gut microbiota in intestinal conditions, including celiac disease (McCarville, Caminero et al. 2016).

1.3 Celiac disease

Celiac disease is a chronic, small intestinal autoimmune enteropathy caused by ingestion of cereal gluten (wheat, barley, rye) in genetically susceptible individuals, expressing the human leukocyte antigen (HLA) -DQ2 or HLA-DQ8 (Black, Murray et al. 2002). Celiac disease is one of the most common gastrointestinal diseases, whose prevalence has steadily increased over the last 40 years and now has a worldwide prevalence of about 1% (Rubio-Tapia, Kyle et al. 2009). Therefore, it is widely accepted that there are unidentified environmental factors, other than the consumption of gluten, that modulate the expression of celiac disease (Sanz, De Pama et al. 2011; Verdu, Galipeau et al. 2015).

In all individuals, upon consumption of gluten, large gluten peptides resultant from partial digestion by mammalian proteases. In celiac patients, these peptides translocate across the intestinal epithelium into the lamina propria, where they encounter the activated form of the enzyme transglutaminase 2 (TG2), with which they form covalent bonds. TG2 deamidates gluten peptides, introducing negative charges. Individuals with the HLA-DQ2 or HLA-DQ8 haplotypes have positively-charged HLA pockets located on APCs, which bind with greater affinity to the negatively charged gluten constituents, than in DQ2/8 negative individuals (Meresse, Malamut et al. 2012). This leads to the selection and activation of gluten-specific $CD4^+$ T-cells, a predominantly Th1 phenotype, and the inflammatory properties associated with the disease (Di Sabatino, Ciccocioppo et al. 2006). Celiac disease is a multisystem disorder, and clinically manifests with a variety of gastrointestinal and/or extra-intestinal symptoms. It may

progress in a subclinical form (Rubio-Tapia and Murray 2008), that usually goes undiagnosed leading to complications and increased mortality. Biomarkers used in its diagnosis include serum antibody production against gliadin and TG2, confirmed by blunting of villi (atrophic enteropathy; >Marsh III) in duodenal biopsies and (Heyman, Abed et al. 2012). Milder forms of enteropathy exist, characterized by IEL infiltration (Marsh I) and villus-to-crypt ratio abnormalities (Marsh II), which need to be considered in conjunction with specific celiac serology to be interpreted clinically (Ludvigsson, Bai et al. 2014). It is currently accepted that both the innate and adaptive immune activation are required for the development of celiac disease.

The consumption of gluten can also induce a variety of disorders such as dermatitis herpetiformis and gluten ataxia, which are not always accompanied by enteropathy. Recently, a new condition has been described, non-celiac gluten/wheat sensitivity (NCG/WS). This clinical entity is characterized by gastrointestinal symptoms that resemble IBS, in the absence of celiac disease and wheat allergy (Catassi, Elli et al. 2015). It has been suggested that an aberrant innate immune response to gluten or other wheat proteins, in the absence of adaptive or autoimmune responses, is associated with NCG/WS (Verdu, Armstrong et al. 2009).

1.3.1 The adaptive immune response in celiac disease

The adaptive immune response in celiac disease is well characterized, primarily because the triggering antigen, and the autoantigenTG2, have been identified. The hallmarks of the adaptive immune responses in celiac disease are the development of antibodies towards gluten and TG2, as well as T-cell-specific responses towards these

antigens (Qiao, Iversen et al. 2012). The adaptive immune response is required for the development of the disease and a positive TG2 test is our primary method for diagnosis, followed by a confirmatory biopsy. It was in 1997 that TG2 was described as the autoantigen, and since then we have developed sensitive assays (TG2-specific antibody enzyme-linked immunosorbent assay (ELISA)) to identify individuals with celiac disease (Dieterich, Ehnis et al. 1997). Development of antibodies against gluten (called anti-gliadin antibodies in clinical practice) in the absence of TG2 antibodies and biopsy changes are not specific to diagnose celiac disease, but may be helpful in gluten ataxia or NCG/WS (Armstrong, Don-Wauchope et al. 2011).

One area of great interest in the field of celiac disease is the study of specific constituents or epitopes in gluten proteins, that can trigger both antibody and T-cell responses in celiac disease patients. There is heterogeneity as to what epitopes patients respond to (Camarca, Anderson et al. 2009). Gluten is made up of two main groups of structural proteins, gliadins, and glutenins. Within gliadins, there are groups of smaller proteins, α-gliadin, β-gliadins, γ-gliadins, and ω-gliadins. Of note is α-gliadin, as much of the research into pro-inflammatory gluten constituents has investigated the breakdown products of this protein. α-gliadin has a high glutamine and proline abundance, making it difficult to hydrolyze by mammalian digestive proteases. A paper in 2002 by the group of Khosla described the breakdown products of this protein, and this lead to the discovery of what is now one of the most important and well-studied peptide sequences in the adaptive immune response of celiac disease, the 33-mer (Shan, Molberg et al. 2002). This 33-amino acid long peptide, contained within the α-gliadin molecule, is thought to be the

main product of mammalian gluten digestion. It is resistant to mammalian proteases and has a great capacity to induce gluten-specific T-cell immune responses in celiac disease patients, as it contains 3 of the main epitopes most celiac disease patients react to (Qiao, Bergseng et al. 2004). Thus, despite heterogeneity in gluten T-cell responses, there is some level of hierarchy. Although there has been interest in determining the immunogenicity of peptides in α-gliadin, it is important to note that other peptides within ω-gliadins and c-hordein (from barley) also have potent immunogenic properties (Tye-Din, Stewart et al. 2010). The field of T-cell biology regarding celiac disease is still ongoing, and our understanding of the potency of specific peptide sequences of gluten and other structural proteins within cereal grains is still being unveiled. However, the 33-mer is an excellent tool to study the induction of T-cell responses in celiac disease patients (Hardy, Girardin et al. 2015) and, particularly useful for the development of therapies for celiac disease (Tye-Din, Anderson et al. 2010). The downstream response of gluten-specific T-cells is the production of Th1 type cytokines, such as IFN-γ (Nilsen, Jahnsen et al. 1998). The increase of IFN-γ within the mucosa of celiac disease patients is characteristic (Brottveit et al., 2015), and the quantification of IFN-γ from T-cell assays is a gold standard method for determining gluten-specific responses (Anderson, Degano et al. 2000) (Overview of adaptive immune response in celiac disease in **Figure 1.2**).

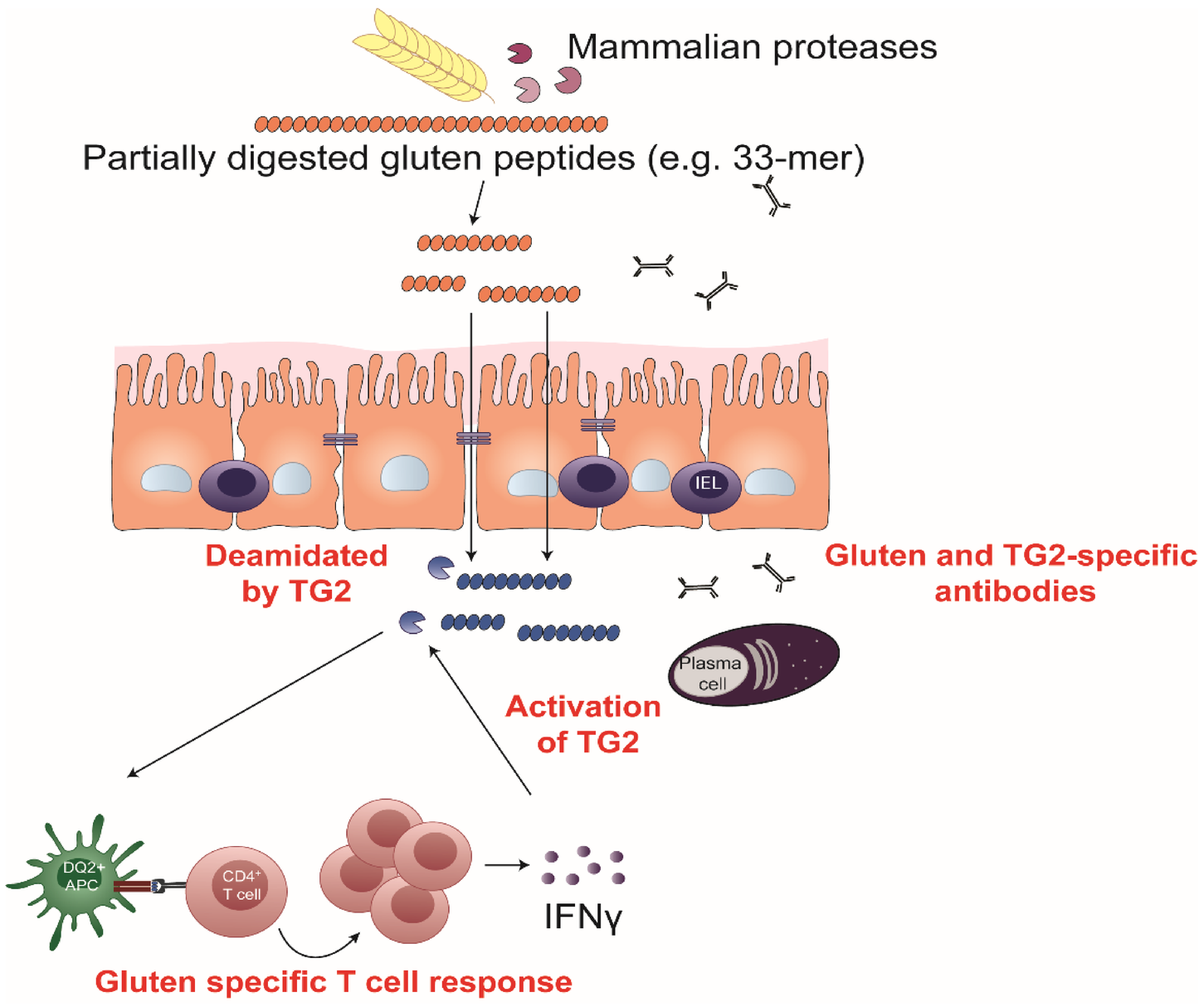

Figure 1.2. Overview of the accepted pathways for the adaptive immune response in celiac disease. Upon ingestion of gluten, mammalian proteases digest these groups of proteins into peptides such as the 33-mer. The peptides translocate the epithelial barrier where glutamine residues are transformed into glutamic acid by TG2, in a process known as deamidation. The introduced negative charges in glutamic acid allow the peptides to bind with greater affinity to HLA-DQ2/8 on APCs, leading to downstream gluten-specific T-cells and gluten and TG2 specific antibodies.

1.3.2 The innate immune response in celiac disease

Unlike the adaptive immune response in celiac disease, the innate immune response is not well characterized. There is an alteration in innate immune parameters described within the mucosa of celiac disease patients, however, the triggers for these changes are unknown. An increase in IL-15 in the celiac mucosa occurs in a great proportion of patients and is driven by unknown innate inducers (Malamut, El Machhour et al. 2010; Sarra, Cupi et al. 2013; Korneychuk, Ramiro-Puig et al. 2014). Additionally there are also increases in the "innate type" cytokine IL-18 in the celiac disease mucosa, (Salvati, MacDonald et al. 2002; Wapenaar, van Belzen et al. 2004; Lettesjo, Hansson et al. 2005; Leon, Garrote et al. 2006; Sarra, Cupi et al. 2013). Changes in both IL-15 and IL-18 are thought to be a consequence of barrier stress leading to increases in IL-21, however, the factors triggering epithelial stress, remain unknown.

Another characteristic of celiac disease is increased numbers of IELs within villi tips, which correlate with atrophy in patients with celiac disease (Kutlu, Brousse et al. 1993). These IELs do not respond to gluten and are driven by TCR-independent antigens. Once again, the triggers responsible for their induction are unknown, although IL-15 is a clear driver of IEL proliferation and cytotoxic transformation. Under homeostatic conditions, these unconventional T-cells patrol the barrier in the gut mucosa, interacting directly with IECs and inducing apoptosis when required (Cheroutre, Lambolez et al. 2011). In celiac disease, the proliferative and cytotoxic capacities of IELs are unleashed, causing villus damage, which, if not treated, will lead to atrophy and, in a small proportion of cases, to severe complications such as refractory celiac disease and

lymphoma (Ettersperger, Montcuquet et al. 2016). Both TCR (γδ and αβ) subsets of IELs are increased in the celiac mucosa (Hue, Mention et al. 2004). Research suggests that TCRαβ IELs are responsible for the mucosal damage in celiac disease (Kutlu, Brousse et al. 1993). In contrast, it has been shown that TCRγδ IELs have regulatory type characteristics (Bhagat, Naiyer et al. 2008) and are decreased in refractory celiac disease (Verbeek, von Blomberg et al. 2008). It has therefore been hypothesized that the TCRγδ IELs remain elevated in the celiac mucosa as a counter mechanism for repair of the damaged epithelium.

The mechanism by which IELs induce apoptosis in intestinal epithelial cells is through FasL (Maiuri, Ciacci et al. 2001), perforin (Buri, Burri et al. 2005), granzyme B (Oberhuber, Vogelsang et al. 1996) and NKG2D (Meresse, Chen et al. 2004). Unfortunately, studies evaluating the epithelial apoptosis in celiac disease have not investigated multiple apoptotic markers or a combination of markers on IELs. Thus, it is unknown which contributes most to cell death. We do know, however, that the increased expression of IL-15 in the celiac mucosa is critical for increases in NKG2D on IELs and, therefore, their IEC-specific cytotoxic ability (Meresse, Chen et al. 2004). As mentioned previously, it is well accepted that this aberrant IL-15 expression originates from an innate source (Kim, Mayassi et al. 2015), and the upstream mechanisms that cause the expression of IL-15, and eventual increase in killer receptors, are unclear. In celiac disease, epithelial cells also upregulate MHC class chain related gene (MIC) molecules and non-classical HLA-E molecules. This is likely a response to epithelial stress, that is recognized by IELs via NKG2D. The combination of IL-15-induced IEL cytotoxicity and

recognition of MIC molecules triggers apoptosis of the epithelium (Meresse, Chen et al. 2004).

There are developing hypotheses as to what the triggers of the innate immune response be in celiac disease. Most putative triggers fall into one of these categories: 1) gluten constituents that do not bind to HLA-DQ2 or DQ8 (triggering myeloid differentiation primary response gene 88 (MyD88)-dependent cytokine production *in vitro* and *in vivo*) (Thomas, Sapone et al. 2006; Araya, Gomez Castro et al. 2016); 2) Infection with bacterial pathogens (eg. *Campylobacter*) or viruses that increase susceptibility to celiac disease non-specifically by producing a pro-inflammatory milieu (Verdu, Mauro et al. 2007; Bouziat, Hinterleitner et al. 2017); 3) non-gluten proteins such as α-amylase/trypsin inhibitors (ATIs) (pest resistant molecules) present in wheat that induce innate immune responses via TLR4 dependent mechanisms (Junker, Zeissig et al. 2012; Zevallos, Raker et al. 2017); 4) A dysbiotic microbiota with opportunistic pathogens that provide a pro-inflammatory milieu (Wacklin, Laurikka et al. 2014). It is likely that more than one of these factors are involved (Overview of innate immune response in celiac disease in **Figure 1.3**). Understanding these triggers, and more importantly, the underlying mechanisms can help develop preventive and therapeutic approaches.

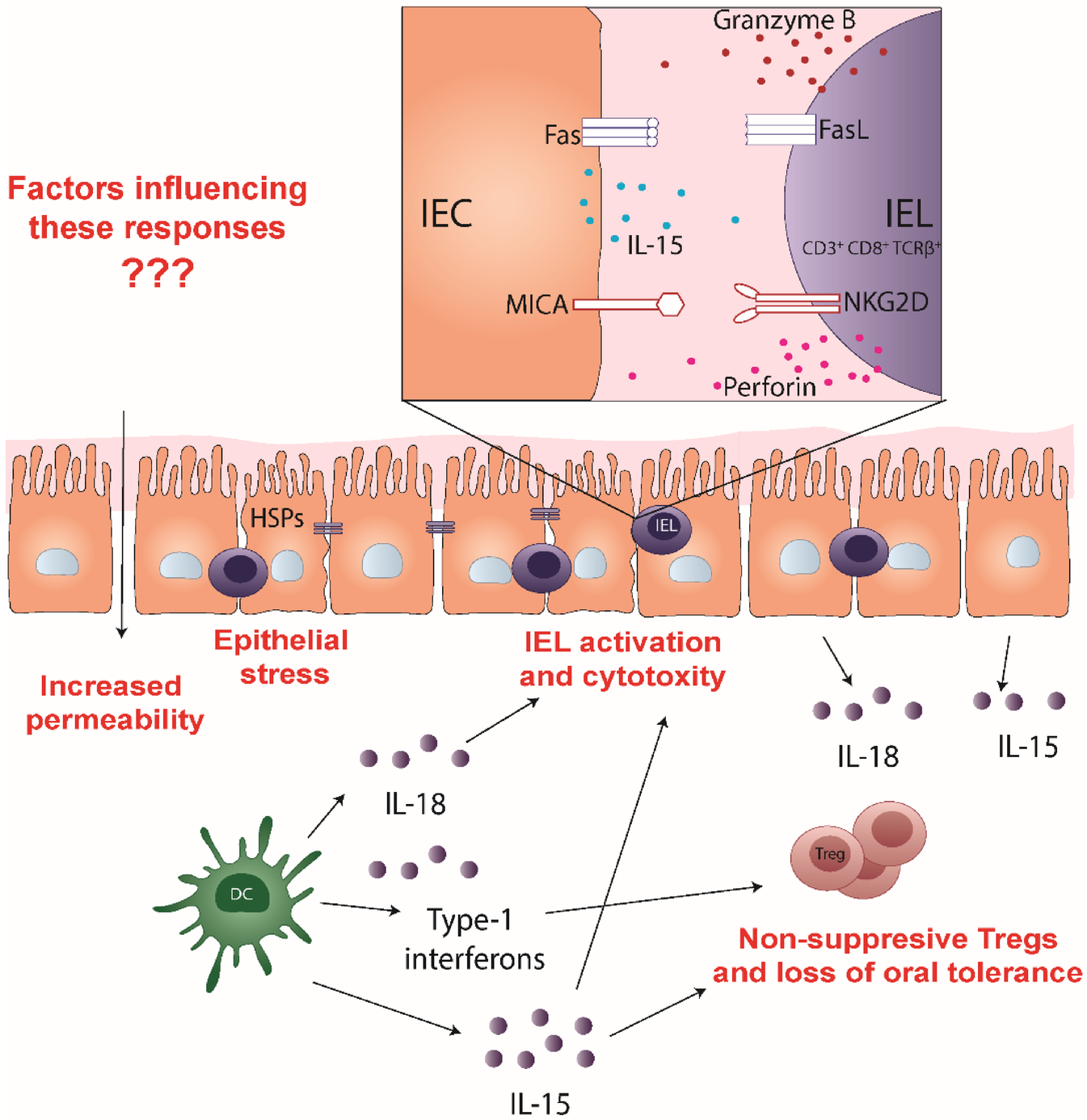

Figure 1.3. Relevant innate immune responses and pathways in celiac disease. IL-15, type-1 interferons and IL-18 are increased in the mucosa of celiac disease patients. IL-15 is responsible for the upregulation of IEL numbers and cytotoxicity. The role of IL-18 in celiac disease pathogenesis is unknown, however, it may contribute to increased IEL numbers and their cytotoxicity. Type-1 interferons support Th1 responses and loss of oral tolerance. The celiac disease mucosa is also characterized by increased permeability and epithelial stress.

1.3.3 Modulation of celiac disease development and severity

It is widely accepted that the development and severity of celiac disease are influenced by environmental factors (Ludvigsson and Green 2014), and/or by non-HLA associated genes (Zhernakova, Stahl et al. 2011). Although 30-40% of the population expresses the HLA haplotypes to develop celiac disease, only approximately 3-4% of these individuals will develop it. On the other hand, gluten-related disorders are very heterogeneous and individuals may develop many different clinical manifestations. For instance, individuals may harbor the HLA-DQ2/8 and produce gluten-specific and TG2 antibodies but have normal duodenal histology. This situation, defined as "potential celiac disease" may turn into active celiac disease later in life, or never develop at all. The factors that influence disease progression are unknown. It is currently speculated that it is likely innate factors that could tip the scale in the development of the disease in individuals with genetic risk or potential celiac disease (Kim, Mayassi et al. 2015). Some patients will develop symptoms, and/or non-specific increases in small intestinal IELs after gluten consumption (Marsh I), but have no evidence of adaptive immune responses towards gluten or TG2 (Uhde, Ajamian et al. 2016). These individuals are classified as having NCG/WS, and roughly 50% of this population does not have the genetic risk (Volta, Tovoli et al. 2012).

1.4 Treatment of celiac disease

The gluten-free diet (GFD) is the only current treatment for celiac disease. However, there are limitations to dietary management alone, and there is currently clear

consensus that adjuvant pharmacological therapies to the GFD are needed. In fact, major pharmaceutical companies are becoming involved in celiac drug development. The reasons being that this is a common disease world-wide which is increasingly being recognized, where patients are clearly dissatisfied with dietary therapy alone (Shah, Akbari et al. 2014) and where, unlike IBD, there is no pharmacological drug competitor in the market. Please see **Appendix 2** for a comprehensive review of the current therapies in celiac disease (McCarville, Caminero et al. 2015).

1.5 The gut microbiota in celiac disease

1.5.1 Clinical association between dysbiosis and presence of pathobionts in celiac disease

Caesarean delivery (Decker, Hornef et al. 2011) and antibiotic exposure (Marild, Ye et al. 2013) have been reported to increase the risk of celiac disease. Proton pump inhibitors, which cause small intestinal overgrowth, have also been reported to increase celiac disease risk (Lebwohl, Spechler et al. 2014). These clinical observations support efforts into the investigation of the role of the gut microbiota in the development and/or severity of celiac disease. Overall, some studies have revealed that there is altered microbial composition in feces and small intestine (dysbiosis) of individuals with active celiac disease, leading to an increase in the proportions of pro-inflammatory bacteria (e.g. *Bacteroides* and Proteobacteria) and decreases in anti-inflammatory bacteria (eg. *Bifidobacterium*) (Pozo-Rubio, Olivares et al. 2012; Sanchez, Donat et al. 2013).

Differences in microbiota composition are observed among patients with active (consuming gluten), and treated (on a gluten-free diet) patients with celiac disease, as

well as versus healthy controls. A study by Di Cagno *et al.* (2011) found higher levels of *Bacteroides*, *Staphylococcus*, *Salmonella*, *Shigella*, and *Klebsiella* in treated celiac disease patients compared comparison with healthy controls (Di Cagno, De Angelis et al. 2011). Sanchez *et al.* (2012) demonstrated that certain virulence genes, methicillin-resistance (*mecA*) and adhesion (*atlE*), were more prevalent in *Staphylococcus epidermidis* isolated from patients with celiac disease than from healthy people (Sanchez, Ribes-Koninckx et al. 2012). In a similar study, Sanchez *et al.* (2008) also found that virulent gene carrying (*papc*, *sfaD/E* and *hlyA*) *E. coli* were more prevalent in celiac disease patients (Sánchez, Nadal et al. 2008). Interestingly, treated celiac disease patients, with persistent symptoms (non-responders to the GFD), harbored increased Proteobacteria in their small intestine (Wacklin, Laurikka et al. 2014) compared with treated patients that became asymptomatic. These studies support the notion that celiac disease patients harbor a dysbiotic microbiota, characterized by a shift towards a higher proportion of bacteria with pathogenic characteristics. Whether this is cause or consequence, remains to be determined.

More than a decade ago, Forsberg *et al.* (2004) discovered, using scanning electron microscopy (SEM), that patients with active and treated celiac disease had rod-shaped bacteria often associated with the epithelium in the jejunum (Forsberg, Fahlgren et al. 2004). In a follow-up study by Ou *et al.* (2009), the group attempted to identify the bacteria associated with active celiac disease using 16S rDNA sequencing. They reported that in active celiac disease patients, there were significant increases in *Clostridium*, *Prevotella* and *Actinomyces* strains. All three of these bacteria are rod-shaped and they all

exhibit a high mucosal adhesion index. The group concluded that the presence of the rod-shaped bacteria, closely attached to the small intestinal mucosa, increased the incidence of the celiac disease fourfold (Ou, Hedberg et al. 2009). More recently, D'Argenio *et al.* (2016) published a study regarding pathobionts in celiac disease. This study found an increase in Proteobacteria in duodenal biopsies of celiac disease patients. *Neisseria flavescens* was shown to be the dominant member of the Proteobacteria phyla in celiac disease patients, which had the capability to induce inflammatory responses in DCs and mucosal explants *in vitro* (D'Argenio, Casaburi *et al*. 2016).

These studies have increased the interest in microbiota-targeted therapies for celiac disease (Marild, Ye et al. 2013). Smecuol *et al.* (2013) (Smecuol, Hwang et al. 2013) found that administration of the probiotic bacterium, *Bifidobacterium* Natren life start (NLS), attenuated celiac disease symptoms in untreated patients (who continued on a gluten-containing diet). In a follow-up study by Pinto-Sánchez *et al.* (2016) (Pinto-Sanchez, Smecuol et al. 2016), administration of NLS was shown to decrease small intestinal Paneth cell numbers and expression of the antimicrobial peptide α-defensin-5. Although the attenuation of the disease by bacterial strains is an important avenue of research, how specific bacterial components may function to attenuate disease remain unclear. Therefore, to study mechanisms in which the gut microbiota alters celiac disease susceptibility or severity, reductionist animal models, or *in vitro* systems of the disease are useful.

1.5.2 Experimental evidence for the role of the gut microbiota in celiac disease pathogenesis

To date, the potential causal role of the gut microbiota in the severity of celiac disease has predominantly been studied using *in vitro* systems. De Palma *et al.* (2010) investigated the inflammatory capacity of Gram-negative bacteria (*Bacteroides fragilis* DSM2451, *Escherichia coli* CBL2, and *Shigella* CBD8) isolated from individuals with celiac disease on peripheral blood mononuclear cells (PBMCs). These Gram-negative bacteria induced greater production of IL-12 and IFN-γ than control *Bifidobacterium* strains. The same pattern was observed when combining these bacteria with gliadin (De Palma, Cinova et al. 2010). One study utilizing intestinal loops in rats indicated that a specific isolated bacteria, *Shigella* CBD8 from celiac disease patients, could increase pro-inflammatory cytokine production as well as break down tight junction proteins causing the translation of gliadin into the lamina propria (Sanz, De Pama et al. 2011). These studies suggest that specific bacteria associated with celiac disease have inflammatory properties, and may contribute to disease severity.

Like most diseases, animal models of celiac disease require manipulation of genetics for disease occurrence. Most mouse models of celiac disease possess the human DQ8 (Verdu, Huang et al. 2008; DePaolo, Abadie et al. 2011; Galipeau, Rulli et al. 2011; Pinier, Fuhrmann et al. 2012) or DQ2 (de Kauwe, Chen et al. 2009) haplotypes, required to develop gluten-specific immune responses. However, other models of food-induced inflammation that resemble celiac disease exist, such as mice harboring MHC II towards ovalbumin (OTII) (Korneychuk, Ramiro-Puig et al. 2014). This model requires the loss of

oral tolerance to dietary antigens, and this does not usually occur spontaneously. Therefore, celiac disease models require either sensitization using mucosal adjuvants (Verdu, Huang et al. 2008; Galipeau, Rulli et al. 2011; Pinier, Fuhrmann et al. 2012) or transgenic overexpression of IL-15 (DePaolo, Abadie et al. 2011; Korneychuk, Ramiro-Puig et al. 2014). In all these models, increases in IELs and villus-to-crypt abnormalities are mild to moderate, and comparable in degree.

Our group performed a study that determined that gluten-induced pathology in non-obese diabetic (NOD)/DQ8 mouse model of gluten sensitivity, was dependent on colonization status (**Appendix 3**). Specific pathogen free (SPF) NOD/DQ8 mice harboring Proteobacteria and opportunistic pathogens, developed more pronounced decreases in villus-to-crypt ratios and anti-gliadin antibody levels than "clean" SPF mice (initially colonized with altered Schaedler flora; ASF, and diversified through accumulation of additional organisms for 2 generations but ensuring the absence of Proteobacteria). Additionally, germ-free NOD/DQ8 developed severe small intestinal immunopathology, suggesting that the microbiota can have a dual role, exacerbating gluten sensitization in the presence of pathobionts, but dampening immune responses in the presence of a benign microbiota, such as clean SPF. The more severe responses to gluten sensitization observed in the absence of a microbiota (germ-free mice) strengthen the notion that commensals are required for appropriate immune responses to dietary antigens. Proteobacteria phyla have been clinically associated with active celiac disease, and bacteria within this phylum were absent in our clean SPF colonies. The protective effects of the clean SPF microbiota could be reversed by supplementing these mice with

an adherent *E. coli* strain (ENT:CA15) isolated from the small intestine of a celiac disease patient (Galipeau, McCarville et al. 2015) (Overview of the role of the microbiota in the NOD/DQ8 model in **Figure 1.4**). Although this study was the first to demonstrate the role for the microbiota in modulating gluten pathology in mice, the mechanisms by which the microbiota can either protect or exacerbate disease are still unknown. Further, the role of virulence factors or adhesion of bacteria to the epithelia, although associated clinically, is unknown in celiac disease. Questions pertaining to the role of specific virulence factors carried by bacteria and their contribution to gluten immunopathology in NOD/DQ8 mice is addressed in **Chapter 4** of this thesis.

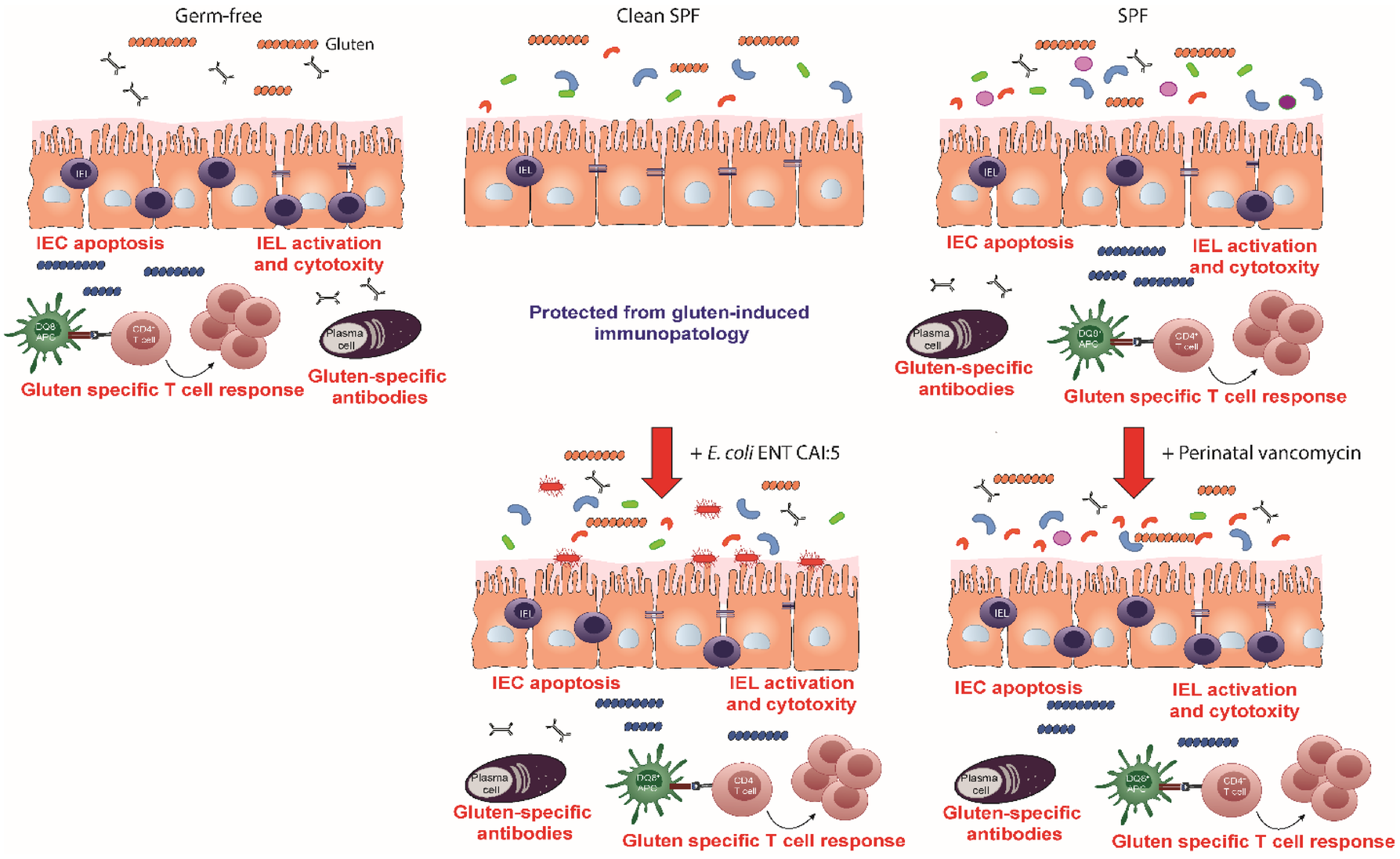

Figure 1.4. The colonization status of NOD/DQ8 mice influences the severity of gluten-induced pathology. Germ-free NOD/DQ8 mice develop marked decreases in villus-to-

crypt ratios and adaptive immune responses to gluten, characterized by gluten-specific T-cells and anti-gluten serum IgG and IgA, and small intestinal anti-gluten IgA responses. Specific pathogen-free (SPF) mice develop moderate pathology and adaptive responses (T-cell and antibody) to gluten; while clean SPF mice, devoid of Proteobacteria opportunistic pathogens, are protected from gluten-induced pathology. Perinatal antibiotic treatment of SPF NOD/DQ8 increases Proteobacteria abundance in offspring and develop enhanced gluten-pathology later life. On the other hand, supplementation of clean-SPF mice using a clinical *E. coli* isolate renders these mice susceptible to gluten pathology.

1.5.3 Digestion of gluten by gastrointestinal bacteria

The gut microbiota plays a significant role in the metabolism of dietary proteins, and under most circumstances, the host benefits due to the availability of amino acids and small peptides. Gluten is no different than other proteins in this manner. Bacteria that have the capacity to degrade gluten have been isolated from the gastrointestinal tract of humans (Caminero, Herran et al. 2014). Interestingly, intestinal contents (containing a "soup" of proteases) from celiac disease patients and healthy people, have different gluten digestion patterns (Caminero, Nistal et al. 2015). For the past 10 years there has been interest in the use of bacterial enzymes that digest gluten, as it is postulated these could help detoxify immunogenic peptides better than mammalian and host enzymes (Salden, Monserrat et al. 2015; Wei, Tian et al. 2015; Wei, Tian et al. 2016). Some clinical trials using pharmacologic enzymes and their combinations have shown promising results, in

particular *in vitro*. Phase 1 clinical trials showed a good safety profile, but consistent evidence of *in vivo* efficacy has been limited due to many factors, including protease pharmacokinetics, delivery, and stability issues, as well as clinical trial design (Tye-Din, Anderson et al. 2010; Lahdeaho, Kaukinen et al. 2014; Murray, Kelly et al. 2017).

This field continues to be of interest, as evidenced by the new enzymes discovered with more potent gluten degrading capacity, such as a recent study our lab contributed to (Rey, Yang et al. 2016) and, may eventually yield the ideal combination for use in patients with celiac disease. The approach I took in this thesis, however, was to study gluten digestion by intestinal bacteria that produce glutenases *in situ*. I, therefore, move away from a direct pharmacological bacterial protease approach and study instead, in **Chapter 3** of this thesis, the role of the small intestinal bacteria in gluten metabolism, and the immunostimulatory capacity of the produced gluten peptides.

1.6 Proteases and protease inhibitors in the gastrointestinal tract

1.6.1 Proteolytic balance in the gastrointestinal tract

The relationship between host extracellular proteases and their respective protease inhibitors are integral for maintaining homeostasis under healthy conditions. Proteases can be broken down into subsets: 1) serine, 2) threonine, 3) cysteine and 4) aspartic (Vergnolle 2016). These proteases are defined by their catalytic triad and have adapted to specific conditions, such as pH and temperature (Vergnolle 2016).

Within the gastrointestinal tract, there are many cell types that express both proteases and protease inhibitors. The primary purpose of extracellular proteases in the gastrointestinal tract is for digestion. These proteases are secreted by the pancreas and stomach and include zymogenic forms of trypsin and chymotrypsin, as well as pepsin and pancreatic elastase. However, there are many other sources of proteases in the gastrointestinal tract, including immune cells, epithelial cells, nerve cells and luminal microbes.

Proteases secreted by immune cells mediate innate immunity, direct microbial killing, as well as apoptosis of other host cells (Heutinck, ten Berge et al. 2010). Mast cells are one cell type that excretes extracellular proteases upon degranulation, including cathepsin G, granzyme B, and tryptase. They mediate smooth muscle activity, intestinal transport and immune pathways (Heutinck, ten Berge et al. 2010). Neutrophils are also a large source of proteases in the gut, secreting an elastase, proteinase-3, and cathepsin G. These proteases are critical for innate immune responses and matrix remodeling (Chua and Laurent 2006). Macrophages express matrix metalloproteases (MMPs), caspases, cathepsins L and D, important for matrix remodeling and antimicrobial activities (Edgington-Mitchell 2016). Cytotoxic cells of the adaptive immune system also express proteases, such as granzyme B, which induce apoptosis in recipient cells (Heutinck, ten Berge et al. 2010). Not surprisingly, due to their role in mediating host-microbe interactions, it has recently been demonstrated that epithelial cells express extracellular proteases, specifically trypsin-3 when stimulated with the TLR4 agonist lipopolysaccharide (LPS) (Rolland-Fourcade, Denadai-Souza et al. 2017).

Because substantial quantities of proteases are produced by the host, their overexpression can be catastrophic and has been linked to some disease states (Vergnolle 2009). Therefore, to counteract proteases the host also encodes and expresses protease inhibitors. The balance of proteases and protease inhibitors is critical for homeostasis. Like proteases, protease inhibitors are expressed throughout the gastrointestinal tract by a variety of cell types. Proteases inhibitors include, but are not limited to, serine protease inhibitors (serpins), secretory leukocyte protease inhibitors (SLPI), elafin and tissue inhibitors of metalloproteases (TIMPs) (Vergnolle 2016).

1.6.2 Recognition of proteases by protease activated receptors (PARs)

The activity of extracellular proteases is recognized by a subfamily of receptors called protease activated receptors (PARs) that resemble their substrate. They are G protein-coupled receptors that become activated upon the proteolytic cleavage of their extracellular N-terminal domain. There are four known receptors within this subfamily (PAR-1, PAR-2, PAR-3, and PAR-4). Recognition of serine proteases (eg. trypsin, elastase, chymotrypsin, subtilisin, thrombin) by PARs are the best described in the literature. These receptors are expressed on a large array of cells types and have been linked to the important physiological process involved in coagulation, visceral pain, immunity and gastrointestinal function (Bunnett 2006).

Of the four PARs, the ones with greatest interest for gastrointestinal physiology and immunity, are PAR-1 and PAR-2. These receptors are expressed throughout the gastrointestinal tract. Although they are cleaved by serine proteases, their cleavage is

protease-specific. For instance, PAR-1 recognizes thrombin, and PAR-2 recognizes trypsin. Both PAR-1 and PAR-2 have similar roles mediating endothelial and epithelial barrier integrity, immunity and physiology, and this has been reviewed extensively elsewhere (Bunnett 2006; Shpacovitch, Feld et al. 2008; Soh, Dores et al. 2010; Nieman 2016; Vergnolle 2016). Indeed, gastrointestinal luminal proteases have been demonstrated to activate PAR-1 and PAR-2 to cause barrier dysfunction (Chin, Vergnolle et al. 2003; Roka, Ait-Belgnaoui et al. 2007; Ronaghan, Shang et al. 2016), increased gastrointestinal transit (Kawabata, Kinoshita et al. 2001) and increased pro-inflammatory cytokine secretion from a variety of cell types (Vergnolle 2004; Wang, Moreau et al. 2010; Maeda, Ohno et al. 2013). Regarding immune function, these receptors are expressed on both basolateral and apical sides of epithelial cells, lymphocytes, and cells of the monocytic origin (Vergnolle 2009). Activation on epithelial cells causes reorganization of tight junctions and pro-inflammatory cytokine secretion (Bueno and Fioramonti 2008). Similarly, activation of PAR-2 on dendritic cells leads to downstream T-cell activation (Ramelli, Fuertes et al. 2010) and Th1 responses (Csernok, Ai et al. 2006).

Upon extracellular cleavage and activation of PAR-1 and PAR-2, an array of downstream cellular events can occur that are involved in inflammation and endosome formation. Downstream of PAR-2, activation pathways can be split into two classes, the canonical G-protein signaling pathway, and the β-arrestin signaling pathway. In the canonical pathway, the activation of PAR-2 is involved in the downstream activation of ERK, NFκB, AMPK and PYK2. These intracellular pathways are involved in the

transcription of pro-inflammatory cytokines, prostaglandins and adhesion molecules (Rothmeier and Ruf 2012; Nieman 2016). However, activation of the β-arrestin pathway induces the formation of endosomes, playing a significant role in cell motility (Rothmeier and Ruf 2012; Nieman 2016).

The downstream signaling events of PARs are complex and it is unclear how these receptor pathways differ from one another, feed into each other, or where redundancy lies. However, due to the importance in the balance of proteases, protease inhibitors, and their receptors, when there is disruption in their homeostasis, they can contribute to disease. Disorders where proteolytic imbalances have been demonstrated include, but are not limited to, asthma, cancer, arthritis, stroke, ischemia and gastrointestinal diseases (Vergnolle 2009; Vergnolle 2016).

1.6.3 Proteolytic imbalances in IBD and functional bowel disorders

The recognition of the contribution of proteases to the pathogenesis of gastrointestinal disorders emerged about a decade ago. These studies suggest that there is a proteolytic imbalance present in the mucosa of patients with IBS and IBD. For instance, fecal proteolytic activities (including tryptase) are elevated in biopsies from IBS patients (Cenac, Andrews et al. 2007; Tooth, Garsed et al. 2014). Further, proteases (cysteine) detected in biopsies from IBS patients contribute to neuronal excitability, a characteristic of IBS, via PAR-2 (Valdez-Morales, Overington et al. 2013). Similarly to IBS, an increase in proteolytic activity has been demonstrated in biopsies from IBD patients (Schmid, Fellermann et al. 2007). Experimental studies in mice have confirmed the

relationship behind the exacerbating effects of proteases in both IBS and IBD. For instance, administration of luminal proteases in mice (mammalian origin) increases inflammation in the colon (Cenac, Coelho et al. 2002) characterized by increases paracellular permeability, myeloperoxidase (MPO) and macroscopic damage scores. Further, proteases enhance inflammation in chemical models of IBD (Hyun, Andrade-Gordon et al. 2010; Cattaruzza, Lyo et al. 2011). The role of proteases in exacerbation of disease is concrete as PAR-$2^{-/-}$ mice and pharmacological inhibitors of PAR-2 induce resistance to chemical-induced colitis (Hyun, Andrade-Gordon et al. 2008; Hyun, Andrade-Gordon et al. 2010; Lohman, Cotterell et al. 2012). Interestingly, the role of PAR-2 in the pathogenesis of colitis in mice seems to be dependent on the methods used to induce colitis, as PAR-2 activation has shown to be protective in 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis rodent models (Fiorucci, Mencarelli et al. 2001).

Although most studies involving colitis models and proteases have used chemical injury, pathogen-induced serine proteases from the host play a significant role in damage associated with infectious colitis. This was demonstrated utilizing the *Citrobacter rodentium* infection model, showing that PAR-$2^{-/-}$ mice were resistant to the damage associated with infectious colitis (Hansen, Sherman et al. 2005). Interestingly, the resistance of PAR-$2^{-/-}$ mice to infection is not specific to *C. rodentium*, as these mice are also resistant to *C. difficile* toxin A (TxA) (Cattaruzza, Amadesi et al. 2014).

The above studies suggest that proteases may be directly involved in the pathogenesis of both IBS and IBD. Further, the receptor PAR-2 plays a critical role in the recognition of these extracellular proteases, leading to pathogenic immune responses and gut

dysfunction related to functional bowel disorders and overt inflammation. Less is known about the role of PAR-1 in gastrointestinal disease states and pathophysiology. However, mice treated with a PAR-1 activating peptide, decreased oxazolone-induced inflammation (Cenac, Cellars et al. 2005). In addition to the proteolytic imbalance in biopsies from patients with IBS and IBD, the studies also detected increased proteolytic activities in luminal intestinal secretions, suggesting that microbial contribution could be important (Steck, Mueller et al. 2012).

Due to the compelling evidence that proteases play a pathogenic role in IBS and IBD, this has been exploited for novel therapeutic development. This includes inhibitors of PAR-1 and PAR-2, as well as specific protease inhibitors to mammalian enzymes, such as tryptase (Vergnolle 2016). A luminal therapy, that targets proteolytic imbalance *in situ* is of great interest. For this reason, genetically-modified organisms for the delivery of proteases inhibitors have been developed. Delivery (adenoviral vector and recombinant *Lactococcus lactis*) of a human serine protease inhibitor, elafin, to mice treated with dextran sodium sulphate (DSS) or dinitrobenzene sulfonic acid (DNBS) protected against development of colitis (Motta, Magne et al. 2011; Motta, Bermudez-Humaran et al. 2012; Bermudez-Humaran, Motta et al. 2015). This suggests that the proteolytic imbalances in the gastrointestinal tract associated with pathophysiology can be restored and therefore may be a viable option for treatment of these disorders in the future (contribution of proteases reviewed in **Figure 1.5**).

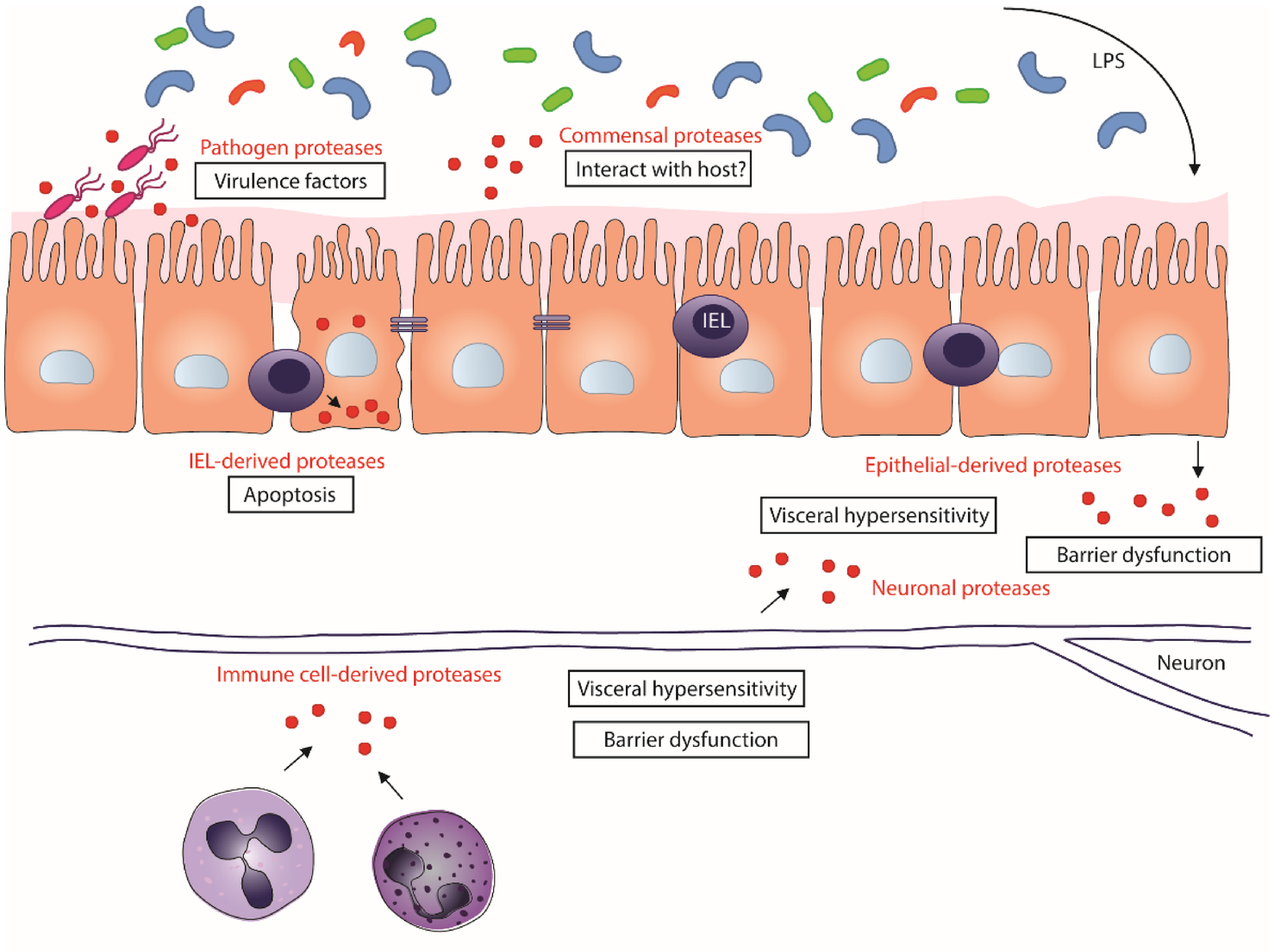

Figure 1.5. Overview of mucosal gastrointestinal proteases. Extracellular proteases can be released by pathogens and commensal bacteria in the lumen and cytotoxic T-cells, innate immune cells, epithelial cells and nerve cells in the mucosa. The effects of these proteases are vast, including apoptosis, visceral hypersensitivity, barrier dysfunction and unknown functions, such as the effects of commensal-released proteases.

1.6.4 Proteolytic imbalance in celiac disease

The theme of proteolytic imbalance in celiac disease is a newly emerging field. Our group has previously demonstrated that patients with celiac disease have decreased elafin expression within the small intestinal mucosa, in comparison to treated celiac disease and healthy individuals. Further, delivery of elafin via recombinant *L. lactis*

expressing elafin alleviated gluten-induced immunopathology in NOD/DQ8 mice (Galipeau, Wiepjes et al. 2014). This finding opened the door to the idea that targeting proteolytic imbalances in celiac disease may be efficacious help to prevent, or treat the disorder (McCarville, Caminero et al. 2015). This is a novel concept, and counterintuitive at first glance, with the approach to use gluten-degrading proteases as a treatment for celiac disease that was discussed previously. The inhibition of proteolytic imbalance should be targeted to those proteases that induce pro-inflammatory pathways. For this, the role of specific microbial proteases in the pathophysiology of celiac disease needs to be elucidated first.

1.6.5 Bacteria as a source of proteases and protease inhibitors

Bacteria are a source of extracellular proteases, some of which share homology with mammalian proteases. Proteolytic capacities attributed to specific bacterial species have been demonstrated to be both pro-inflammatory and anti-inflammatory in host organisms, from both commensal and pathogenic bacteria. The anti-inflammatory properties of proteases are likely a mechanism used by the bacterium for immune evasion (Potempa and Pike 2009). Proteases secreted by bacteria act as key components for colonization in the host for both infectious bacteria (*E. coli*, *Porphyromonas gingivalis*) (Lourbakos, Chinni et al. 1998; Harrington, Sheikh et al. 2009; Abreu, Abe et al. 2016) and commensals (eg. *E. faecalis*, *Akkermansia muciniphila*) (Del Papa, Hancock et al. 2007). However, proteases can also signal through host PARs, inducing pro-inflammatory events (Azghani, Connelly et al. 1990; Kon, Tsukada et al. 1999; Maharshak, Huh et al. 2015). The link between microbial proteases and their contribution to health and disease

is a dynamic field of research. Some examples include how these proteases contribute to host defense (LaRock, Todd et al. 2016) or, to the development of tolerance to infection and chronicity (LaFayette, Houle et al. 2015; Rangan, Pedicord et al. 2016).

Pseudomonas aeruginosa is an opportunistic pathogen and a member of the Proteobacteria phylum, which harbors a well-characterized elastase. Although present in the small intestine, much of the research into the commensal and pathogenic states of *P. aeruginosa* has been carried out in the lungs, as this bacterium is associated with exacerbation of cystic fibrosis (Hoiby, Ciofu et al. 2010). Interestingly, the role of proteases secreted by *P. aeruginosa* in infection is contrasting. For example, recent research has suggested that elastase from *P. aeruginosa* dampens host immune responses through two pathways. The first is by inhibiting TLR signals (van der Plas, Bhongir et al. 2016) and the second, by degrading pro-inflammatory cytokines from host cells (LaFayette, Houle et al. 2015). In the study by LaFayette *et al.* (2015), *P. aeruginosa* lacking *LasR* (regulator of *LasA* and *LasB*) had reduced proteolytic capacity, making the strain more virulent and inducing greater inflammation *in vitro*, and in mice and human explants. This group also discovered that proteases produced by *P. aeruginosa* degraded pro-inflammatory cytokines (IL-8 and IL-6) released from the host (LaFayette, Houle et al. 2015). This recent research apparently contradicts previous research that *P. aeruginosa* elastase exacerbates inflammation in models of infection (Azghani, Connelly et al. 1990; Kon, Tsukada et al. 1999). Indeed, targeting *P. aeruginosa* elastase is a method to reduce virulence (Cathcart, Quinn et al. 2011). Although the research around elastase from *P. aeruginosa* is controversial, it is an important mediator in inflammation. Moreover, it

constitutes an interesting research tool to investigate its role within the gastrointestinal tract.

Another emerging concept relates to the contribution of protease inhibitors from the microbiome, and how this may influence disease conditions (Guo, Chang et al. 2017). Protease inhibitors have been identified in commensal bacteria, and in vitro they show potent inhibition towards inflammatory mammalian protease, such as neutrophil elastase (Ivanov, Emonet et al. 2006; Mkaouar, Akermi et al. 2016). *In vivo* activity of these inhibitors produced by commensals is unknown, and most supportive evidence arises from the use of food-grade bacteria that transgenically express desired human-derived inhibitors, such as elafin (Motta, Bermudez-Humaran et al. 2012; Galipeau, Wiepjes et al. 2014; Bermudez-Humaran, Motta et al. 2015).

In **Chapter 4,** I address the role of proteases produced by intestinal commensals isolated from healthy subjects and patients with celiac disease and investigate their contribution to gluten-induced pathology. Further, in **Chapter 5**, I demonstrate that a protease inhibitor produced by a commensal bacterium can attenuate or prevent gluten-induced pathology.

1.6.6 Bacteria as contributors of proteolytic imbalances in gastrointestinal disorders

Both dysbiosis and proteolytic imbalance are independently proposed hypotheses in the pathogenesis of chronic intestinal disorders. Although the role of commensal bacteria contributing to the proteolytic imbalance in gastrointestinal disorders has been suggested, it has not been extensively experimentally studied. In theory, these proteases could

contribute to direct activation of host receptors, leading to immune activation and gut dysfunction (Steck, Mueller et al. 2012). An example of a protease-producing bacteria in the exacerbation of gastrointestinal disease is *Enterococcus faecalis*. Studies have shown that extracellular protease gelatinase (metalloprotease) can increase intestinal permeability and activate inflammatory cascades through PAR-2 dependent signaling (Maharshak, Huh et al. 2015). Additionally, this protease has been shown to exacerbate experimental colitis (Steck, Hoffmann et al. 2011), however direct mechanisms remain unknown.

This thesis investigates mechanisms through which the gastrointestinal microbiota exacerbates or attenuates gluten-induced pathology through both adaptive and innate immune mechanisms (**Figure 1.6**). Specifically, the role of gluten-degrading proteases from commensal gut bacteria is evaluated in producing gluten-derived immunostimulatory peptides (**Chapter 3**) and direct bacterial protease-host interactions (**Chapter 4**). Further, the influence of protease inhibitors from commensal bacteria in attenuating gluten-induced inflammation is evaluated in **Chapter 5**.

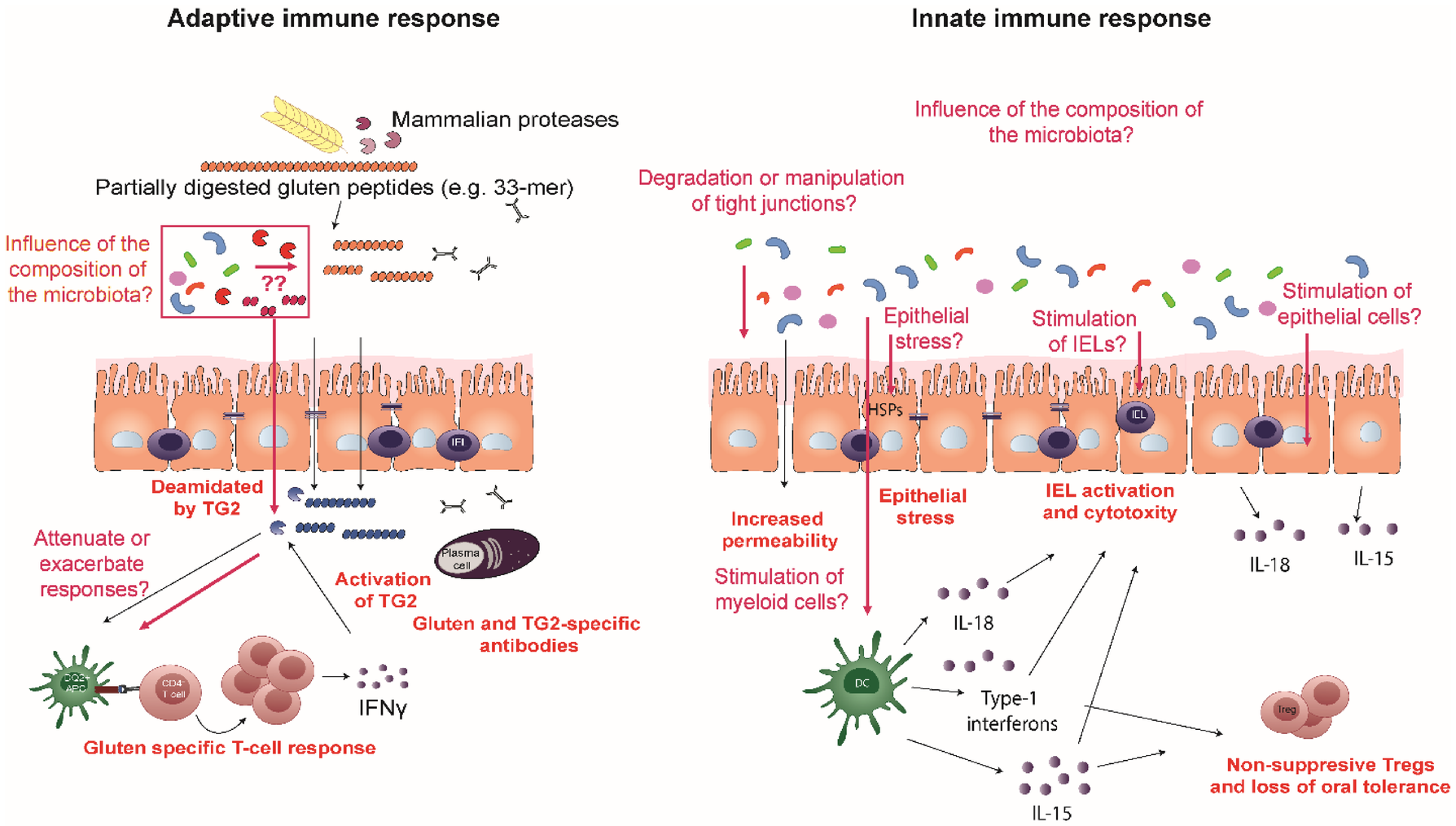

Figure 1.6. Overview of the potential mechanisms through which the microbiota may influence adaptive and innate immune mechanisms in celiac disease. When consumed, gluten is partially digested by mammalian proteases in the intestinal lumen. Peptides translocate across the epithelium, where they are deamidated by TG2, leading to downstream Th1 responses and gluten- and TG2-specific antibodies. The role of commensal, bacterially-derived proteases in influencing the metabolism of gluten, and therefore downstream adaptive responses, has not been studied before. Also, the capacity of these proteases to directly stimulate the innate immune pathway is unknown but could involve interactions with the epithelium, IELs or APCs. Elucidation of these mechanisms is key to prevent the development of celiac disease and develop better gluten-detoxifying therapies.

CHAPTER 2: THESIS OBJECTIVES

Thesis scope

It is widely accepted that additional environmental factors to gluten, influence the development and severity of celiac disease. Microbial factors, both bacterial and viral, have long-been suspected to contribute to celiac disease development. However, underlying mechanisms have remained elusive. Recently, a study by Bouziat *et al.* (2017) implicated sub-clinical viral enteric infection in celiac disease, by induction of a pro-inflammatory signature in APCs (Bouziat, Hinterleitner et al. 2017; Verdu and Caminero 2017). The study is timely and exciting, as it provides support for my thesis scope which is centered on the elucidation of adaptive and innate immune pathways through which bacterial proteases could modify celiac disease risk. Altogether the new insights arising from my studies will be key for the development of targeted microbiota-modulating therapies to prevent or treat gluten-related disorders.

Thesis Aims

General aim: *To determine the role and mechanisms through which the intestinal microbiota modulates the development and severity of immune responses to gluten.*

This general aim is accomplished throughout the work described in the three chapters of this thesis. In **Chapter 3**, intestinal bacteria from celiac disease patients and healthy subjects were used to determine their role in gluten metabolism, characterize the bacterially-modified gluten peptides and their immunogenicity. In **Chapter 4**, I studied gnotobiotic mice (wild-type and humanized mice expressing the human celiac risk gene DQ8), that were colonized with fecal slurs of celiac disease patients and healthy controls, followed by selected gnotobiotic or mono-colonizations to investigate whether and how bacteria with high glutenasic activity *in vitro* and *in vivo*, induce innate immune responses in the small intestine. In **Chapter 5**, I tested whether a commensal bacterium, *B. longum*, endogenously producing a serine protease inhibitor, prevents gluten-induced immunopathology in a mouse model of gluten sensitivity.

Specific aims:

Chapter 3: Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity.

Aim: To determine whether the gut microbiota, and specific bacterial isolates from patients with celiac disease and healthy controls, participate in gluten metabolism and modification of its immunogenicity.

Experimental approach: Intestinal bacteria from patients with celiac disease and healthy controls are used in gluten metabolism assays *in vitro*, and tested *ex-vivo* and *in vivo* using wild-type germ-free mice that are colonized with selected bacteria. Additionally, immunogenicity of bacterially-modified peptides is studied in T-cell assays from patients with celiac disease.

- **Paper published:** Caminero A, Galipeau HJ, **McCarville JL**, Johnston CW, Bernier SP, Russell AK, Jury J, Herran AR, Casqueiro J, Tye-Din JA, Surette MG, Magarvey NA, Schuppan D, Verdu EF. Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. Gastroenterology 2016;151:670-83.

Chapter 4: Microbial proteases modulate innate immunity and increase sensitivity to dietary antigen through PAR-2.

Aim: To characterize direct mechanisms, such as protease production, in which bacteria influence small intestinal innate immunity and host responses to gluten.

Experimental approach: Germ-free mice (wild-type and expressing DQ8 gene) are colonized with fecal slurs from patients with celiac disease or healthy controls, with selected communities, or, with one specific bacterium or its mutant. Bacterial proteolytic activity, and the molecular and immune pathways induced in the host with relevance to celiac disease, are investigated.

- **Paper submitted:** Alberto Caminero*, **Justin L. McCarville***, Heather Galipeau, Steve Bernier, Marlies Meisel, Joe Murray, Brian Coombes, Yolanda Sanz, Bana

Jabri, Michael G. Surette, Nathalie Vergnolle and Elena F. Verdu. Microbial proteases modulate innate immunity and increase sensitivity to dietary anitgen through PAR-2.

* indicates equal 1st author contribution

Chapter 5: A commensal *Bifidobacterium longum* strain improves gluten-related immunopathology in mice through expression of a serine protease inhibitor

Aim: To investigate whether administration of a commensal, producing a protease inhibitor, protects against gluten-induced pathology in a mouse model expressing the human DQ8 gene.

Experimental approach: A commensal-producing a protease inhibitor is tested as targeted therapy to prevent the development of pathology in mice expressing DQ8 gene that are sensitized to gluten.

- **Paper published: McCarville JL***, Dong J*, Caminero A, Jury J, Langella P. Bergonzelli G, Duboux S, Verdu EF. A commensal *Bifidobacterium longum* strain improves gluten-related immunopathology in mice through expression of a serine protease inhibitor. Applied and Environmental Microbiology 2017; 83: e01323-17.

* indicates equal 1st author contribution

CHAPTER 3:

DUODENAL BACTERIA FROM PATIENTS WITH CELIAC DISEASE AND HEALTHY SUBJECTS DISTINCTLY AFFECT GLUTEN BREAKDOWN AND IMMUNOGENICITY

Summary and Significance

Aim: To determine whether the gut microbiota and specific bacterial isolates from patients with celiac disease and healthy controls, participate in gluten metabolism and modification of its immunogenicity.

Summary: Celiac disease affects approximately 1:100 people worldwide, however, 30-40% of most populations express the risk haplotypes (DQ8/2) suggesting environmental factors, additional to gluten, influence disease pathogenesis. The gut microbiota has been hypothesized as one of the environmental factors contributing to the development of celiac disease. Clinically, it has been demonstrated that individuals with active celiac disease harbor a dysbiotic microbiota, with increased abundance of duodenal Proteobacteria. Multiple studies have demonstrated that microbial proteases, and some bacteria isolated from the human gut, can metabolize gluten *in vitro.* "Complete gluten detoxification" by these non-eukaryotic enzymes is the premise behind some therapies currently in development. However, it is unclear whether intestinal bacteria participate in gluten metabolism within the small intestine, and it is not always obvious what peptides are generated. In a collaborative work, I contributed to demonstrate that mice colonized with selected bacteria isolated from celiac patients and healthy controls metabolize gluten in the intestine differently. When analyzing the metabolic products of this bacterial gluten digestion, and specifically by the Proteobacteria member *Pseudomonas aeruginosa*, we found that the breakdown products of the 33-mer still harbored immunogenic sequences. Further, these peptides stimulated robust adaptive immune responses, as measured *ex vivo* by IFN-γ release from celiac disease patient $CD4^+$ T cells ($DQ2^+$). These immunogenic

gluten peptides were however detoxified by *Lactobacilli* from healthy individuals. Finally, we determined that a central enzyme involved in bacterial gluten metabolism was the serine protease, elastase.

Significance: The results indicate that gluten digestion in the small intestine is a complex process influenced by mammalian and resident bacteria proteases, and this process could modify the balance of peptides produced and their immunogenicity. This is a direct *bacterial-gluten-host* mechanism that provides an explanation for the known fact that some individuals that are genetically predisposed, will never develop celiac disease, while others will do it in childhood, or later in life. It is also a plausible explanation for the recent increased prevalence of celiac disease, particularly in North America.

Preface: In this Chapter, I performed gnotobiotic experiments (colonization and sample collection) and 16s sequencing (PCR and analysis).

Alberto Caminero, Heather Galipeau, **Justin L. McCarville**, Chad W. Johnston, Steve P. Bernier, Amy K. Russell, Jennifer Jury and Alexandra R. Herran performed experiments for this manuscript. Alberto Caminero, Heather Galipeau and **Justin L. McCarville** performed experiments involving gnotobiotic colonizations. Alberto Caminero and **Justin L. McCarville** carried out microbiology techniques and microbiota analysis. Jennifer Jury performed Ussing chamber experiments. Amy K. Russell carried our T-cell assays. Chad Johnson performed and analyzed LC-MS/MS experiments. Jason A. Tye-Din, Michael G. Surette, Nathan A. Magarvey, Detlef Schuppan and Elena F.

Verdu designed and contributed scientific input. Alberto Caminero, Heather Galipeau, **Justin L. McCarville** and Elena F. Verdu wrote the manuscript.

Duodenal bacteria from patients with celiac disease and healthy subjects distinctly affect gluten breakdown and immunogenicity

Short title: Role of bacteria on gluten immunogenicity

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Abbreviations- Altered Schaedler Flora, ASF; antigen presenting cells, APCs; CeD, celiac

disease; human leukocyte antigen, HLA; pepsin-trypsin, PT; Peripheral blood mononuclear cells, PBMCs; *Pseudomonas aeruginosa* (Psa); Spot forming units (SFU); Transglutaminase 2, TG2.

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Conflicts of interest- JT-D is a co-inventor of patents pertaining to the use gluten peptides in therapeutics, diagnostics, and non-toxic gluten. JT-D is a shareholders of Nexpep Pty Ltd and a consultant to ImmusanT, Inc. Full disclosure was provided to all study participants. The remaining authors have no conflicts of interest to declare.

Author Contributions- A.C., E.F.V. conceptualized the study, designed experiments, analyzed data and wrote the manuscript. J.C., A.R.H. and A.C. isolated gluten-degrading bacteria. A.C., H.J.G. and J.L.M. performed germ-free animal studies. J.A.T. and A.K.R. performed gluten-specific T-cell responses. N.A.M. and C.W.J. performed LC-MS/MS analysis. Screening of mutant library was accomplished by S.P.B. and M.G.S. J.J. performed Ussing Chamber experiments. DS contributed to scientific concepts and discussion. All of the authors discussed the results and assisted in the preparation of the manuscript.

ABSTRACT

BACKGROUND & AIMS: Partially-degraded gluten peptides from cereals trigger celiac disease (CeD), an autoimmune enteropathy occurring in genetically susceptible persons. Susceptibility genes are necessary but not sufficient to induce celiac disease and additional environmental factors related to unfavorable alterations in the microbiota have been proposed. We investigated gluten metabolism by opportunistic pathogens and commensal

duodenal bacteria and characterized the capacity of the produced peptides to activate gluten-specific T-cells from celiac disease patients.

METHODS: We colonized germ-free C57BL/6 mice with bacteria isolated from the small intestine of celiac disease patients or healthy controls, selected by their *in vitro* gluten-degrading capacity. After gluten gavage, gliadin amount and proteolytic activities were measured in intestinal contents. Peptides produced by bacteria used in mouse colonizations from the immunogenic 33-mer gluten peptide were characterized by LC-MS/MS and their immunogenic potential was evaluated using peripheral blood mononuclear cells from celiac patients after receiving a 3-day gluten challenge.

RESULTS: Bacterial colonizations produced distinct gluten degradation patterns in the mouse small intestine. *Pseudomonas aeruginosa (Psa)*, an opportunistic pathogen from celiac disease patients, exhibited elastase activity and produced peptides that better translocated the mouse intestinal barrier. *Psa*-modified gluten peptides activated gluten-specific T-cells from celiac disease patients. In contrast, *Lactobacillus* spp. from the duodenum of non-celiac disease controls degraded gluten peptides produced by human and *Psa* proteases, reducing their immunogenicity.

CONLUSIONS: Small intestinal bacteria exhibit distinct gluten metabolic patterns *in vivo*, increasing or reducing gluten peptide immunogenicity. This microbe-gluten-host interaction may modulate autoimmune risk in genetically susceptible persons and may underlie the reported association of dysbiosis and celiac disease.

KEYWORDS: celiac disease, gluten metabolism, intestinal microbiota, intestinal inflammation.

INTRODUCTION

Gluten-related disorders are increasingly prevalent conditions (Leonard and Vasagar 2014) that encompass all diseases triggered by dietary gluten, including celiac disease (CeD), a T-cell mediated enteropathy, dermatitis herpetiformis, gluten ataxia and other forms of non-autoimmune reactions (Ludvigsson, Leffler et al. 2013). Gluten proteins, predominantly gliadins in wheat, are resistant to complete degradation by mammalian enzymes which results in the production of large peptides with immunogenic sequences such as the 33-mer in alpha-gliadin. Overall, this specific peptide contains 6 copies of 3 different epitopes (PYPQPQLPY, PQPQLYPQ, PFPPQPQLPY) to which most celiac patients react (Shan, Molberg et al. 2002; Hardy, Girardin et al. 2015). Partially digested gluten peptides translocate the mucosal barrier and are deamidated by human transglutaminase 2 (TG2), the celiac disease associated autoantigen (Dieterich, Ehnis et al. 1997). This process converts glutamine residues to glutamate and increases peptide binding affinity to human leukocyte antigen (HLA)-DQ2 or DQ8 heterodimers in antigen presenting cells (APCs), initiating the T-cell mediated inflammation characteristic of celiac disease (Kim, Quarsten et al. 2004). Up to 40% of most populations express the susceptibility genes for celiac disease; however, only 2-4% will develop disease, possibly due to additional unknown environmental triggers (Verdu, Galipeau et al. 2015). As with other autoimmune and inflammatory diseases, intestinal dysbiosis characterized by abundance of Proteobacteria and decreases in *Lactobacillus* has been described in some celiac disease patients (Nadal,

Donat et al. 2007; Wacklin, Laurikka et al. 2014; D'Argenio, Casaburi et al. 2016). There is little mechanistic insight regarding the association between dysbiosis and gluten-specific T-cell responses, and thus the functional relevance of these associations in celiac disease remain unclear.

The human gastrointestinal tract is colonized by bacteria with *in vitro* gluten-degrading capacity (Fernandez-Feo, Wei et al. 2013; Caminero, Herran et al. 2014). This has prompted the hypothesis that bacteria could reduce gluten immunogenicity by producing enzymes that effectively cleave proteolytic-resistant sequences in gluten peptides (Bethune and Khosla 2012). Here we show a complex scenario in which gluten metabolism in the small intestine of gnotobiotic mice is differentially affected by opportunistic pathogens and commensal bacteria. We demonstrate that *Pseudomonas aeruginosa* (*Psa*), isolated from the duodenum of celiac disease patients, produces, through its elastase activity, a multitude of peptides that activate gluten-specific T-cells in HLA-DQ2.5^{+} celiac disease patients. Conversely, *Lactobacillus* spp. from healthy subjects, degrade *Psa*-modified peptides and decrease their immunogenic potential. We identify a microbe-dietary-host interaction that may modulate autoimmune risk in genetically susceptible persons and that could be targeted to reduce the rising incidence of these conditions.

MATERIALS AND METHODS

Mice

C57BL/6 germ-free mice were generated by axenic 2-cell embryo transfer technique, as previously described (Slack, Hapfelmeier et al. 2009), and maintained in flexible film

isolators at the McMaster University Axenic Gnotobiotic Unit. Germ-free status was evaluated weekly by a combination of culture and culture-independent techniques (Slack, Hapfelmeier et al. 2009; Galipeau, McCarville et al. 2015). We used mice colonized with an 8 strain-murine microbiota (altered Schaedler flora; ASF) (Dewhirst, Chien et al. 1999) as controls. All mice had unlimited access to a gluten-free autoclaved mouse diet (Harlan, Indianapolis, IN) and water. All experiments were carried out in accordance with the McMaster University animal utilization protocols.

Origin of bacterial strains used

We previously sequenced and isolated a collection of bacterial strains with *in vitro* gluten-degrading capacity from the small intestine of celiac disease patients and non-celiac controls (Herran 2015; Nistal, Caminero et al. 2016). Briefly, duodenal biopsies were incubated in specific gluten media (MCG-3)(Caminero, Herran et al. 2014) for 48 hr under anoxic and microaerophilic conditions. Bacteria were selected based on: i) production of a proteolytic halo and ii) lack of growth in the same media without gluten. Most of the strains were classified within the phylum *Firmicutes* (88%), mainly from the genera *Lactobacillus*. Strains were also classified into *Actinobacteria (8%)*, *Proteobacteria* (3%) and *Bacteroidetes* (1%) (Herran 2015). For the experiments in this study, three bacterial groups of interest were chosen (Supplemental Table 1). *Psa* X-46.1 was selected as an opportunistic pathogen only isolated from celiac disease patients, and a member of Proteobacteria, a group previously associated with celiac disease (Wacklin, Kaukinen et al. 2013; Wacklin, Laurikka et al. 2014). *Staphylococcus* spp. was selected because alterations in this group have been described in celiac disease patients (Sanchez, Ribes-Koninckx et

al. 2012). *Lactobacillus* spp. from healthy subjects were selected since it constitutes a core resident group in the human small intestine (de Meij, Budding et al. 2013; Nistal, Caminero et al. 2016) that is involved in gluten metabolism *in vitro* (Caminero, Herran et al. 2014) and is altered in celiac disease patients (Nistal, Caminero et al. 2012; Caminero, Nistal et al. 2015).

16S Sequencing

DNA was extracted from small intestinal samples of colonized mice as previously described (Whelan, Verschoor et al. 2014). Extracted DNA underwent amplification for the hypervariable 16S rRNA gene v3 region and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Generated data were analyzed as described previously. Briefly, sequences were trimmed using Cutadapt software version 1.2.1, aligned using PANDAseq software version 2.8, operational taxonomic units selected via AbundantOTU, and taxonomy assigned against the Greengenes reference database (Ye 2011; Masella, Bartram et al. 2012).

QPQLPY-peptide quantification

The amount of QPQLPY-peptide, a key motif in the major immunogenic epitope within the 33-mer peptide from α-gliadin, was measured with the competitive G12 ELISA GlutenTox Kit (Biomedal, Spain) according to the manufacturer's instructions (Moron, Bethune et al. 2008). For animal studies, total small intestinal content was flushed at sacrifice with 3 ml of extraction solution provided by the kit.

Degradation of QPQLPY-peptides by intestinal washes

Intestinal contents were collected from colonized mice at sacrifice and diluted 1:5 with PBS and incubated at 37°C with 7 mg of pepsin-typsin (PT)-gliadin for 30 mins, 2 hr and 4 hr. After incubations, remaining QPQLPY-peptides were quantified by G12 antibody in ELISA GlutenTox Kit (Moron, Bethune et al. 2008).

Cleavage of gluten-derived tripeptides

Peptidase activity against gluten-derived tripeptides was performed as previously described (Helmerhorst, Zamakhchari et al. 2010). Five synthetic analogs, Z-YPQ-pNA, Z-QQP-pNA, Z-PPF-pNA and Z-PFP-pNA, Z-QPQ-pNa were chosen as representative gliadin-derived substrates (Biomatik). Twenty mM of each peptide was incubated with the small intestinal washes of *Psa*-, *Lactobacillus* spp.- or *Staphylococcus* spp.-colonized mice or with single bacteria cell cultures at the same concentration found in the small intestine of mice (10^4 CFU) in 50 mM ammonium bicarbonate buffer, pH 8.0. Enzyme activity was determined by the proteolytic removal of the paranitroanilide group which was monitored spectrophotometrically at 405 nm.

Proteolytic activity in gluten media

Degradation of gluten proteins in solid media was measured using bioassays on agar plates containing 1% gluten (Caminero, Nistal et al. 2012). Small intestinal contents of mice were diluted 1:5 with PBS and incubated at 37°C in gluten-agar media for 24 hr. Plates were evaluated by measuring the diameter of the halo formed. Trypsin diluted in saline was used for construction of a standard curve.

LC-MS/MS analysis of 33-mer-derived peptides

Degradation of 33-mer peptide was performed using LC-MS/MS. The reaction mixtures, (100 µL) containing 10 µL of bacterial culture (10^4 CFU) and 60 µM of the 33-mer peptide in PBS (pH 7.3), were incubated at 37°C for 4 hr. Reactions were stopped by incubation at 100°C for 10 min, and resultant products subjected to LC-MS/MS. LC-MS/MS data was collected using a Bruker AmazonX ion trap mass spectrometer coupled with a Dionex UltiMate 3000 HPLC system, equipped with a Luna C18 column (150 mm × 4.6 mm, Phenomenex) for analytical separations, running acetonitrile with 0.1% formic acid and ddH_2O with 0.1% formic acid as the mobile phase at a flow rate of 1.2 mL/min. Putative gliadin peptides were identified by comparison of LC-MS/MS chromatograms from samples with gliadin to gliadin-free controls. Peptides were confirmed and annotated by manual MS/MS sequencing, assisted by iSNAP LC-MS/MS peptide fragmentation analysis software (Ibrahim, Yang et al. 2012).

Gluten challenge in celiac disease patients

Patients with biopsy-proven celiac disease (n = 20) expressing the most common susceptibility genotype HLA-DQ2.5$^+$ and in clinical, serological and histological remission on a gluten-free diet were recruited (Supplementary Table 2). Gluten challenge was performed as previously described (Tye-Din, Stewart et al. 2010). Briefly, four 50 g slices of standard gluten-containing wheat bread (total ~ 10 g gluten) were consumed in divided doses daily for 3 days. Blood sampling was done in the morning before (day 0) and 6 days after commencing the gluten challenge (day 6).

ELISpot assay

IFN-γ ELISpot assays (Mabtech, Cincinatti, OH) were performed as previously described (Tye-Din, Stewart et al. 2010) Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood using Ficoll density-gradient centrifugation. Fresh or cryopreserved PBMC were incubated overnight with or without native or deamidated gliadin, peptides, or with tetanus toxoid (CSL, Australia) and phytohemagglutinin-L (PHA-L; Sigma-Aldrich) as positive controls. Spot forming units (SFU) in individual wells were counted using an automated ELISPOT reader. Wells showing more than 10 SFU and >3x the SFU counted in wells containing PBMC incubated with medium alone were regarded as positive. SFU were adjusted to one million PBMC plated to enable comparisons.

Peptide translocation in mice

Permeability studies were assayed *in vitro* by Ussing chamber technique, as previously described (Natividad, Huang et al. 2009) (World Precision Instruments, Sarasota, FL). We collected jejunal tissues from specific pathogen-free C57BL/6 mice (n=10 per group), and four sections of jejunum from each mouse were assessed. Intestinal permeability was stimulated by adding prostaglandin E2 (30 μM) to the serosal side of the chamber. Tissue conductance and mucosal-to-serosal flux of the paracellular probe ^{51}Cr-EDTA were determined to check integrity of the tissue (data not shown). PT-gliadin (1 mg/ml) and PT-gliadin further degraded by bacteria (10^4 CFU 37°C for 4 hr) were added to the mucosal size of the chamber. After 2 hr, samples were collected from the serosal side of the chamber and gluten content was quantified using the G12 antibody (Moron, Bethune et al. 2008).

Deamidation by Transglutaminase (TG2)

The enzymatic activity of TG2 was checked by cross linking PT-gliadin or PT-gliadin incubated with *Psa* X-46.1 for 4 hrs (glutamine donors) with monodansyl cadaverine (glutamine acceptor). One µg of crude gliadin (Sigma-Aldrich) was incubated with 30 µmol/ml monodansyl cadaverine (CovalAb, German) and 20 µg/ml of Pig Transglutaminase (Sigma-Aldrich) in 100 µl buffer containing 0.1 mol/l Tris·HCl, 0.15 mol/l NaCl, 5 mmol/l CaCl2, pH 8.8. Cross linking was allowed for 2 hr at 37°C. Fluorescence was measured for 1 hr at λex 360 nm and λem 535 nm in the kinetic mode (Tian, Wei et al. 2014).

Screening of the non-redundant transposon mutant library of *P. aeruginosa*

An unbiased genetic strategy to identify genes associated with gluten metabolism was performed by using the available non-redundant transposon mutant library of *Psa* PA14 (Liberati, Urbach et al. 2006). Briefly, 96-well microtiter plates containing 100 µl per well of LB (with gentamicin at 15 µg/ml) were inoculated directly from each plate of the frozen library using a 96-pin replicator and incubated statically at 37°C overnight. Overnight cultures were then transferred with a 96-pin replicator (VP408, V&P Scientific) onto gluten agar plates (1% of gluten) and incubated for 16 hr at 37°C. Transposon mutants with growth on gluten-containing agar and no zone of clearing around spotted colonies were used as stringent selection criteria and reported in Supplementary Table 3.

Statistics

All the variables were analyzed with SPSS v 18.0. Categorical variables are expressed as numbers and percentages, and quantitative variables as means +/- standard error of mean

(SEM) or medians as appropriate. Data are depicted as either dot plots or bar graphs. The ANOVA test was performed to evaluate differences between various samples with a parametric distribution and a Bonferroni correction was applied. The T-student test was performed to evaluate the differences between two independent samples or paired samples as appropriate. Data with non-parametric distribution were evaluated with Kruscall-Wallis test for multiple samples, Mann-Whitney test for two independent samples or Wilcoxon test for two related samples as appropriate. A p-value <0.05 was selected to reject the null hypothesis by two-tailed tests.

RESULTS

Commensals and opportunistic pathogens contribute to gluten metabolism in the gut

To investigate the small intestinal gluten metabolic activity of the strains selected in this study, we colonized germ-free C57BL/6 mice (n=13/group) with: 1) *Psa* X-46.1, a Proteobacteria isolated from the duodenum of celiac disease patients, 2) *Staphylococcus epidermidis* X-35.1 and *Staphylococcus warneri* X-18.3 from the duodenum of celiac disease patients and, 3) *Lactobacillus rhamnosus* X-32.2 and *Lactobacillus fermentum* X-39.3 from the duodenum of non-celiac healthy volunteers (Supplementary Table 1). Mice were colonized by oral gavage with 10^7 colony-forming units (CFU) of each strain and kept on gluten-free chow for one week. Control groups included germ-free mice and ASF-colonized mice, a bacterial community selected for their dominance and persistence in the normal microbiota of mice (Dewhirst, Chien et al. 1999) (Figure 1A). 16S sequencing of ASF small intestinal contents showed 90% *Lactobacillus*, 5% *Parabacteroides* and other

minority groups (Figure 1B). One-week post-colonization, mice were gavaged with 7 mg of gliadin (n=8/group), or with saline (n=5/group), and sacrificed after 2 hr. We recovered 10^4-10^5 CFU/g of intestinal content in each group. Culture analysis (Slack, Hapfelmeier et al. 2009) confirmed the purity of colonizations (Supplemental Materials). The content of QPQLPY-peptides, a repetitive immunogenic sequence within the 33-mer peptide, was quantified by the G12 antibody (Moron, Bethune et al. 2008). Colonization with ASF or *Psa* decreased QPQLPY-peptides content compared to germ-free mice, that exhibited a range of values reaching a maximum of 12,000 ng/ml (Figure 1C).

To investigate whether these differences were due to bacterial proteolytic activity, intestinal washes from colonized mice were incubated with 7 mg of partially degraded, immunologically active PT-gliadin for 30 min, 2 hrs and 4 hrs. Small intestinal washes from ASF-colonized mice degraded 50-60% of QPQLPY-peptides within 30 min, while washes from germ-free mice only 5-10%. Intestinal washes from *Psa*-colonized mice degraded more than 50% of peptides at 2 hr and washes from *Lactobacillus*-colonized mice reached similar activity at 4 hrs (Figure 1D). Intestinal washes from *Staphylococcu*s-colonized mice also degraded QPQLPY-peptides but this did not reach statistical significance compared with germ-free. Because gluten consists of a complex mix of proteins with multiple amino acid sequences, we next tested the ability of small intestinal washes to degrade whole gluten. Unlike the G12 antibody that detects the QPQLPY sequence, the bioassay assesses global gluten degradation non-specifically. Using solid gluten media we showed that intestinal washes from *Psa*-colonized mice had 10x higher proteolytic activity than the rest of the groups (Figure 1E). Thus, gluten degradation in the

small intestine results from the combined enzymatic action of mammalian and resident bacteria.

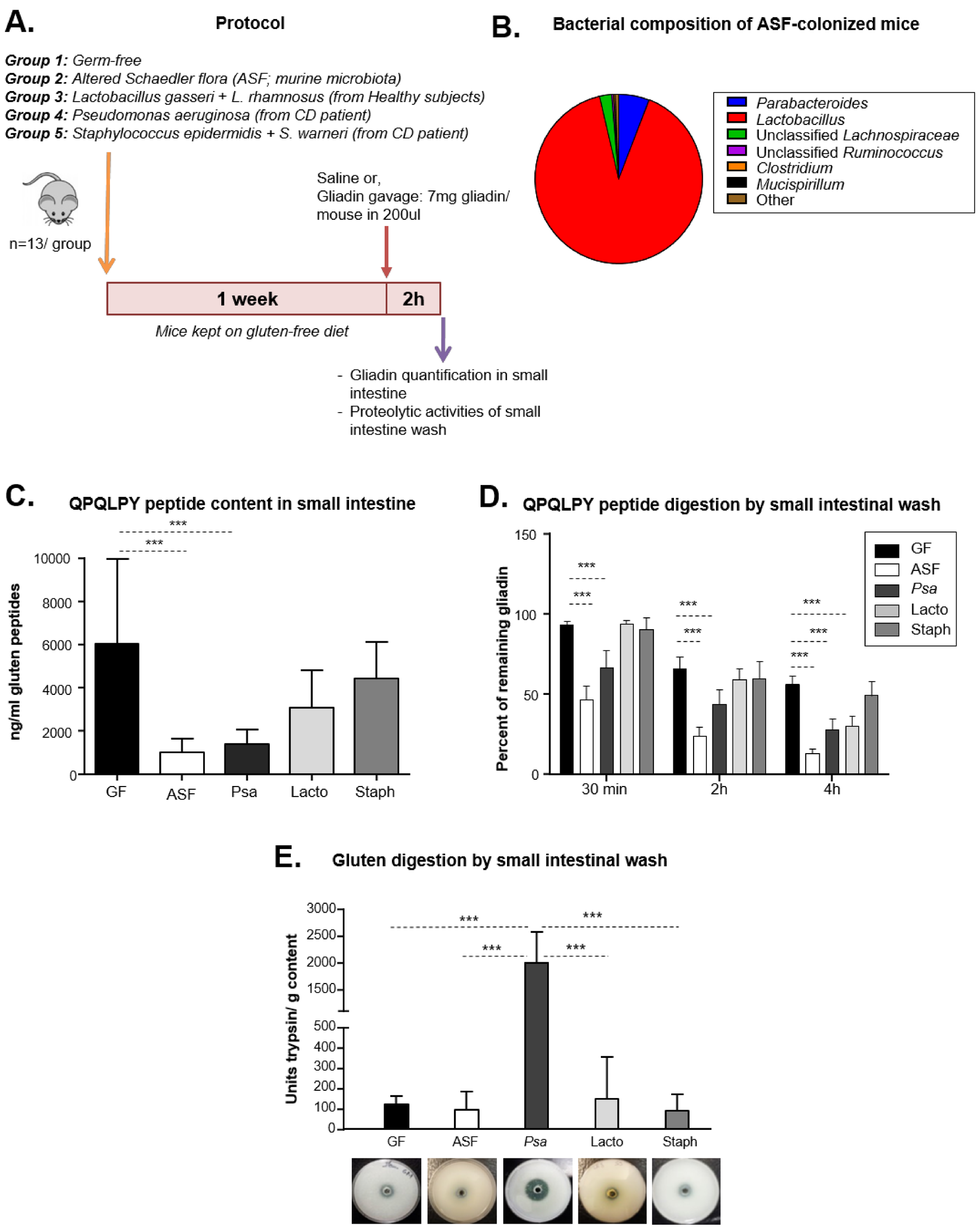

A.
Protocol
Group 1: Germ-free
Group 2: Altered Schaedler flora (ASF; murine microbiota)
Group 3: Lactobacillus gasseri + L. rhamnosus (from Healthy subjects)
Group 4: Pseudomonas aeruginosa (from CD patient)
Group 5: Staphylococcus epidermidis + S. warneri (from CD patient)
n=13/ group
Saline or,
Gliadin gavage: 7mg gliadin/ mouse in 200ul
1 week
2h
Mice kept on gluten-free diet
- Gliadin quantification in small intestine
- Proteolytic activities of small intestine wash
B.
Bacterial composition of ASF-colonized mice
Parabacteroides
Lactobacillus
Unclassified Lachnospiraceae
Unclassified Ruminococcus
Clostridium
Mucispirillum
Other
C.
QPQLPY peptide content in small intestine
ng/ml gluten peptides
10000
8000
6000
4000
2000
0
***
***
GF
ASF
Psa
Lacto
Staph
D.
QPQLPY peptide digestion by small intestinal wash
Percent of remaining gliadin
150
100
50
0
GF
ASF
Psa
Lacto
Staph
30 min
2h
4h
E.
Gluten digestion by small intestinal wash
Units trypsin/ g content
3000
2500
2000
1500
500
400
300
200
100
0
***
GF
ASF
Psa
Lacto
Staph

Figure 1: Resident intestinal bacteria participate in gliadin degradation. **(A)** Experimental design of mouse colonization study. **(B)** 16S sequencing of small-intestinal bacteria in Altered Schaedler Flora (ASF)-colonized mice at the genus level. **(C)** Amount of gliadin measured by G12 antibody, detecting the immunogenic QPQLPY sequence within the 33-mer, in the small intestine of germ-free (GF), ASF-, *Pseudomonas aeruginosa* (*Psa)*-, *Lactobacillus*(Lacto)-, and *Staphylococcus* (Staph)-colonized mice 2 hrs after gliadin gavage. Data are represented as mean ± SEM. **(D)** Degradation of 7 mg of pepsin-trypsin (PT)-gliadin by intestinal washes from germ-free, ASF-, *Psa*, Lacto-, and Staph-colonized mice by measuring QPQLPY peptides by G12 antibody. Data are represented as mean ± SEM. **(E)** Degradation of gluten in solid media by intestinal washes of GF, ASF-, *Psa*-, *Lacto*- and *Staph*-colonized. Data represented as mean ± SEM (units of trypsin/gram of intestinal content). Bioassays show non-specific degradation (clearing zone) in solid gluten media (white).

Small intestinal bacteria induce distinct gluten metabolic patterns

To investigate specific regions of gliadin cleavage by bacteria, five tripeptides, representing sequences which appear frequently in immunogenic gliadin peptides, were incubated with intestinal washes of colonized mice. Single bacterial strains that were used in colonizations were directly incubated *in vitro* with the five tripeptides as controls. Intestinal washes of all groups, including germ-free, cleaved YPQ and PPF (Figure 2A), suggesting mammalian origin. Both *Psa* X-46.1 and *Staphylococcus* strains degraded PPF tripeptide and mice

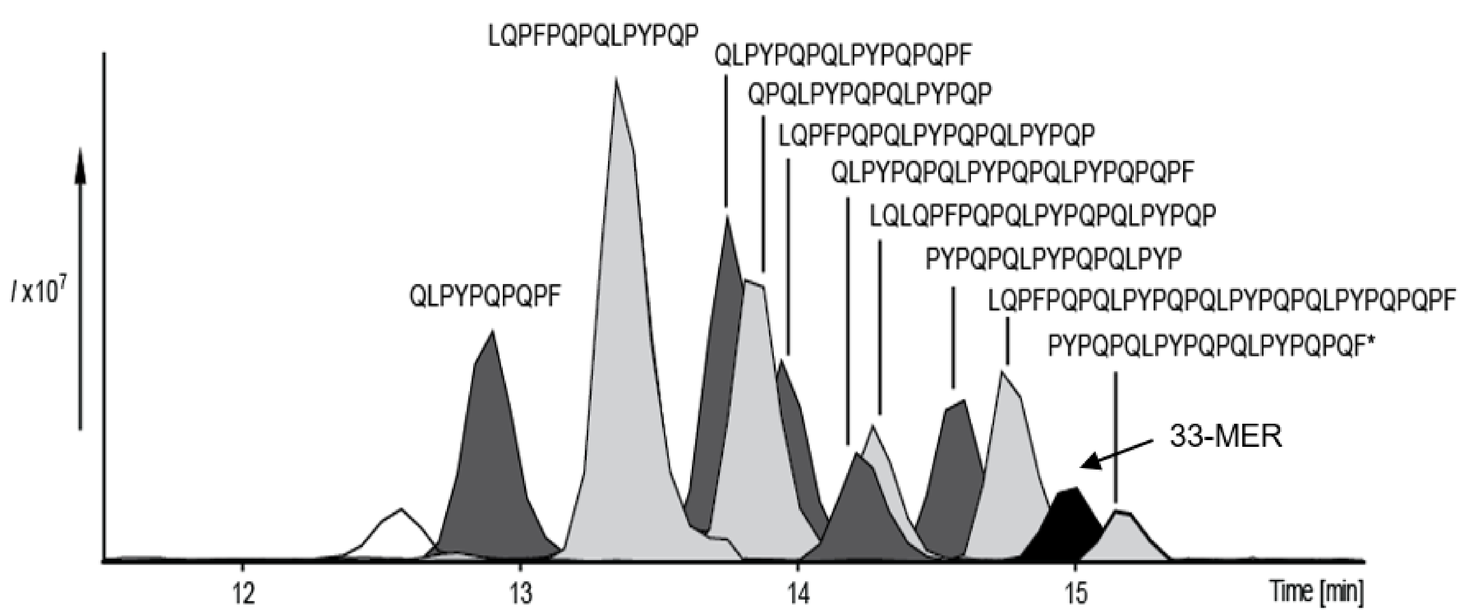
colonized with these bacteria showed higher PPF degradation than germ-free mice. Thus, different bacteria could have similar degradation profiles that are conferred to mice colonized with these strains. Furthermore, direct incubation of *L. rhamnosus* X-32.2 with tripeptides resulted in PFP breakdown. Similarly, incubation of intestinal washes of *Lactobacillus*- and ASF-colonized mice, which are dominated by *Lactobacillus*, resulted in PFP breakdown. Intestinal washes of ASF-colonized mice demonstrated PQP cleavage, not present in germ-free mice. The results suggest there are specific bacterial cleavage sites that influence the pool of gliadin peptides produced during digestion.

To identify the capacity of specific human bacterial isolates to cleave key immunogenic gliadin peptides, we incubated bacteria with the human protease resistant 33-mer peptide that encompasses six overlapping immunodominant HLA-DQ2.5-restricted 9-mer T-cell epitopes (Shan, Molberg et al. 2002; Kim, Quarsten et al. 2004). Peptides generated from the 33-mer following incubation with bacteria were determined using LC-MS/MS. Partial scission of the 33-mer was detected with all tested bacteria. In isolation, *Lactobacillus* spp. produced three peptides of 25-32 amino acids and *Staphylococcus* X-18.3 two large peptides of 28 and 32 amino acids. *Psa* X-46.1 cleaved regions recognized by G12 antibody and produced a variety of smaller 33-mer derived peptides (10-30 amino acids). *Psa* X-46.1 did not cleave QLP regions in the 33-mer, which are associated with immunogenicity (Kim, Quarsten et al. 2004) (Figure 2B and 2C). These results indicate that degradation of the 33-mer by bacteria generate gluten peptides that maintain sequences with known immunogenicity in CeD (Tye-Din, Stewart et al. 2010).

A. **Cleavage of gluten-derived tripeptides by bacteria and small intestinal washes**

	GF	ASF	*Pseudomonas*		*Lactobacillus spp.*			*Staphylococcus spp.*		
	SIW	SIW	*P. aeruginosa*	SIW	*L. rhamnosus*	*L. gasseri*	SIW	*S. epidermidis*	*S. warneri*	SIW
YPQ	+	+	-	+	-	-	+	-	-	+
QQP	-	-	-	-	-	-	-	-	-	-
PPF	+	+	++	++	-	-	+	+	++	++
PFP	-	+	-	-	+	-	+	-	-	-
PQP	-	+/-	-	-	-	-	-	-	-	-

B. **Chromatogram generated after 33-mer digestion by *P. aeruginosa***



C. **Scission of the 33-mer by bacteria**

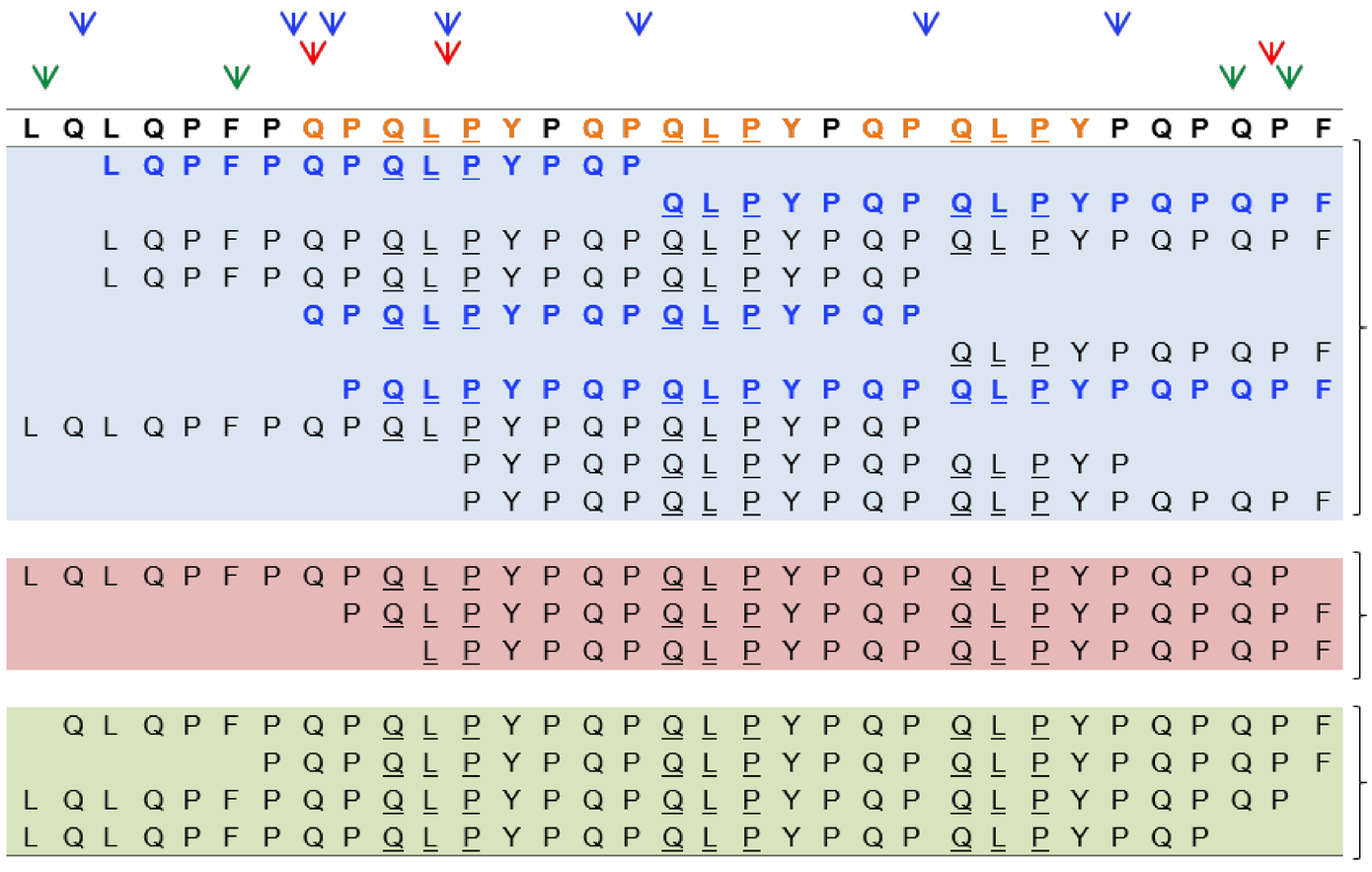


Figure 2: Intestinal bacteria induce distinct gluten metabolic patterns against gliadin peptides **(A)** Cleavage of gluten-derived tripeptides YPQ, QQP, PPF, PFP, PQP by small intestinal washes (SIW) of germ-free (GF)-, Altered Schaedler Flora (ASF)-, *Pseudomonas aeruginosa*-, *Lactobacillus*-, *Staphylococcus*-colonized mice and by individual bacterial strains. No activity:(-), activity:(+), saturated activity:(++). **(B)** Chromatogram generated after 33-mer degradation by *P. aeruginosa*. Black peak: remaining 33-mer. Grey peaks: peptides produced by *P. aeruginosa* degradation. **(C)** Degradation of the 33-mer by *P. aeruginosa* (blue arrows), *Lactobacillus* (red arrows) and *Staphylococcus* (green arrows). Yellow letters: G12 antibody epitopes. Underlined: Transglutaminase 2 epitopes. Blue letters: Selected peptides for gluten specific T-cell stimulation assays. (***p<0.005,*p<0.05).

***Psa*-modified gluten peptides are immunogenic to celiac disease patients**

The immunogenicity of 33-mer-derived peptides released by *Psa* X-46.1 was then tested using gluten-specific T-cells induced in HLA-DQ2.5+ celiac disease patients. Ten celiac disease patients underwent 3-day wheat gluten challenge to induce gluten-specific T-cells (Anderson, Degano et al. 2000; Tye-Din, Stewart et al. 2010) (Supplementary Table 2). PBMCs were isolated from blood collected before and 6 days after commencing the challenge, when circulating gluten-specific T-cells are at their peak. A panel of four gluten peptides generated following 33-mer incubation with *Psa* X-46.1 were synthesized. IFN-γ ELISpot using these PBMCs was performed to validate the immunogenicity of these peptides as well as PT-gliadin and the 33-mer peptide. Seven of ten participants mounted a significant IFN-γ ELISpot response on day 6 to the 33-mer, PT-gliadin and all four *Psa*-

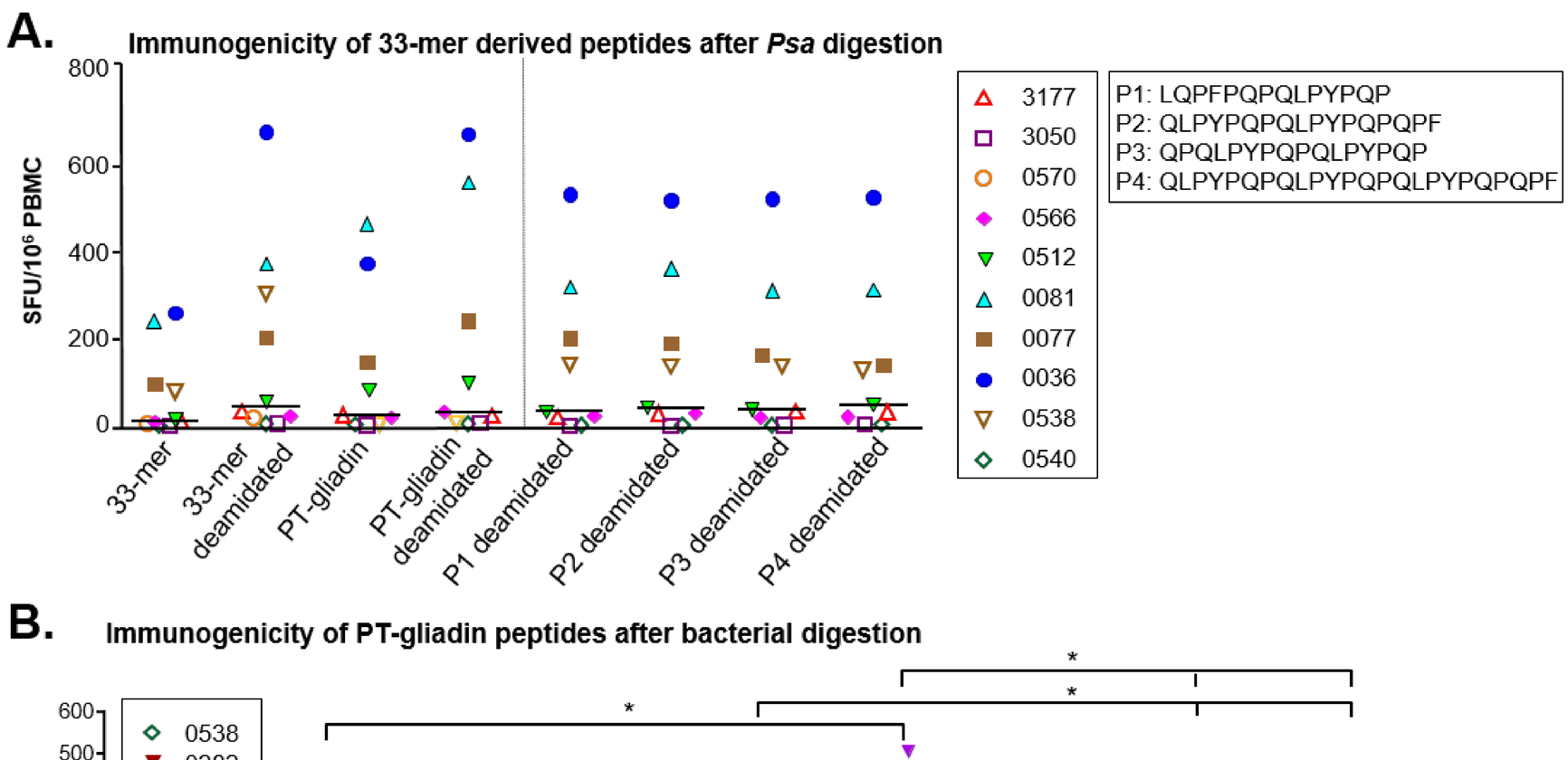
peptides (Figure 3A). Responses were detected only after gluten challenge and were generally dose-dependent and enhanced by deamidation, consistent with a disease-relevant T-cell response to deamidated gluten (Supplementary Figure 1).

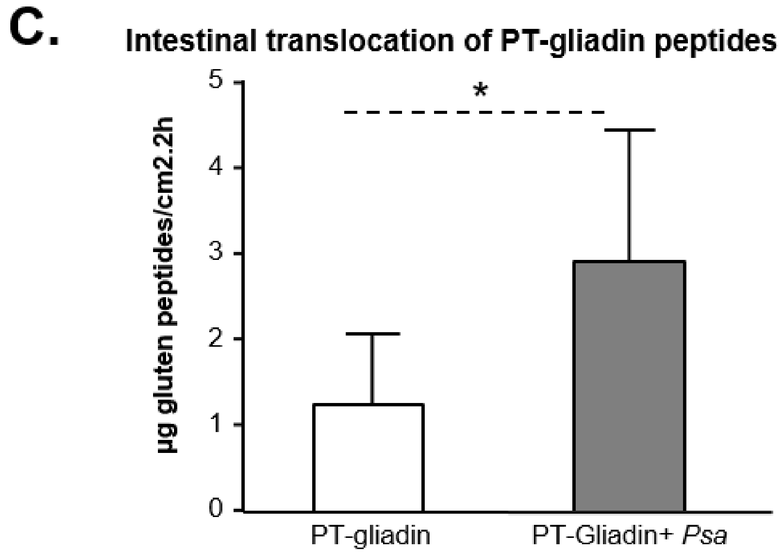
We next assessed the immunogenicity of PT-gliadin incubated with either *Psa* X-46.1 or *Lactobacillus* spp. Gluten-specific T-cell responses to these peptides were performed using blood collected from celiac disease patients (Supplementary Figure 2). The median response to PT-gliadin incubated with *Psa* X-46.1 was increased compared to deamidated PT-gliadin alone, and this was statistically significant after 8hr incubation with *Psa* X-46.1. In contrast, the median response to PT-gliadin incubated with *Lactobacillus* spp was lower than that to deamidated PT-gliadin at all incubation time points (Figure 3B).

Psa-modified gluten peptides translocate better the epithelial barrier in mice

Uptake of gluten peptides through the intestinal barrier is necessary for the adaptive gluten-specific immune response in the lamina propria (Menard, Lebreton et al. 2012). We measured translocation of immunogenic gluten peptides in mouse jejunum by Ussing chambers (Natividad, Huang et al. 2009). PT-gliadin or PT-gliadin incubated with *Psa* X-46.1 were added to the mucosal side of the chamber and QPQLPY peptide content, as measured by G12 antibody, was determined on the serosal side after 2 hr (Moron, Bethune et al. 2008). Prior incubation of PT-gliadin with *Psa* X-46.1 led to increased QPQLPY peptide transport across the intestinal barrier, compared to PT-gliadin alone (Figure 3C). This suggests that *Psa*-modified gliadin peptides crossed the mucosal barrier more efficiently and that bacterially-mediated gliadin degradation in the lumen may facilitate

immunogenic peptide translocation (Figure 3C). Gliadin peptide deamidation in the lamina propria by the celiac disease autoantigen TG2 increases peptide affinity to HLA-DQ2+ APCs (Dieterich, Ehnis et al. 1997). Different ratios of deamidation after bacterial degradation could therefore be associated with reduced immunogenicity. We found a similar TG2 deamidation ratio of PT-gliadin and PT-gliadin incubated with *Psa* X-46.1 (Figure 3D). Thus, *Psa* degradation produced shorter gluten peptides that were often highly immunogenic, inducing responses in many cases as strong as the parent 33-mer. These shorter peptides readily crossed the epithelial barrier.


A.
Immunogenicity of 33-mer derived peptides after Psa digestion
SFU/10⁶ PBMC
800
600
400
200
0
33-mer
33-mer deamidated
PT-gliadin
PT-gliadin deamidated
P1 deamidated
P2 deamidated
P3 deamidated
P4 deamidated
3177
3050
0570
0566
0512
0081
0077
0036
0538
0540
P1: LQPFPQPQLPYPQP
P2: QLPYPQPQLPYPQPQPF
P3: QPQLPYPQPQLPYPQP
P4: QLPYPQPQLPYPQPQLPYPQPQPF
B.
Immunogenicity of PT-gliadin peptides after bacterial digestion
SFU/10⁶ PBMC
600
500
400
300
200
200
150
100
50
0
0538
0282
0650
0648
0572
0081
0120
0565
0035
0570
0633
*
*
*
Negative control
PT-gliadin
PT-gliadin deamidated
PT-gliadin+Psa (2h) deamidated
PT-gliadin+Psa (4h) deamidated
PT-gliadin+Psa (8h) deamidated
PT-gliadin+Lacto (2h) deamidated
PT-gliadin+Lacto (4h) deamidated
PT-gliadin+Lacto (8h) deamidated


C.
Intestinal translocation of PT-gliadin peptides
µg gluten peptides/cm2.2h
5
4
3
2
1
0
*
PT-gliadin
PT-Gliadin+ Psa

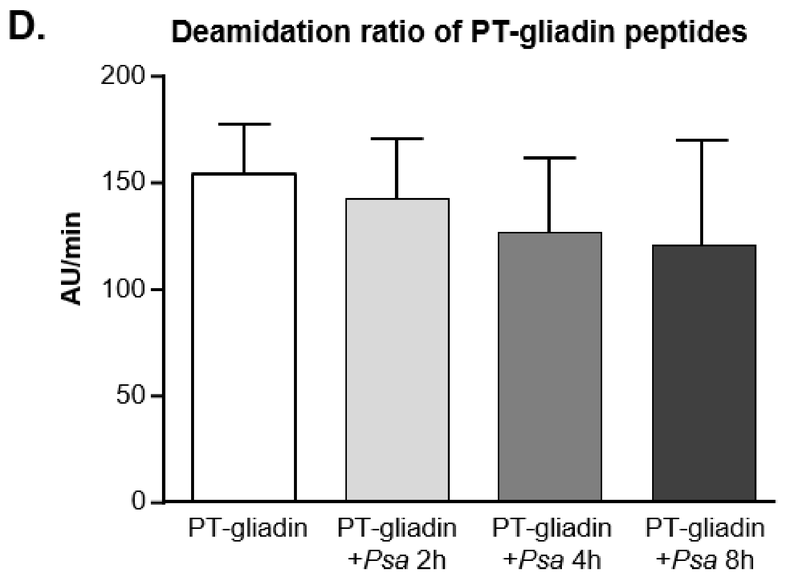

D.
Deamidation ratio of PT-gliadin peptides
AU/min
200
150
100
50
0
PT-gliadin
PT-gliadin +Psa 2h
PT-gliadin +Psa 4h
PT-gliadin +Psa 8h

Figure 3: Intestinal bacteria modify the immunogenicity of gluten peptides. **(A and B)** IFN-γ ELISpot in peripheral blood mononuclear cells (PBMC) harvested after gluten challenge of patients with celiac disease in remission and *ex vivo* stimulated with (A) Transglutaminase 2 (TG2)-deamidated 33-mer-derived peptides (P1, P2, P3, P4) produced following incubation with *Pseudomonas aeruginosa* (*Psa)* X-46.1 or (B) Pepsin-trypsin predigested (PT)-gliadin incubated with *Psa* X-46.1 or *Lactobacillus* and deamidated by TG2. Non-deamidated and TG2-deamidated 33-mer or PT-gliadin were used as controls. Results are shown as spot forming units (SFU) per 10^6 PBMC**.** The median response is represented by horizontal lines. Each patient donor response (numeric code) is represented with characteristic shape and colour dots. **(C)** Small intestinal translocation of PT-gliadin and PT-gliadin incubated with *Psa* X-46.1 as measured by Ussing chambers. Results shown as the transport of QPQLPY gliadin peptides from the mucosal to the serosal side over 2 hrs. Data are represented as mean ± SEM. **(D)** Crosslinking of PT-gliadin and PT-gliadin incubated with *Psa* for 2 hrs, 4 hrs and 8 hrs to monodansyl cadaverine by TG2. Results are shown as maximum rates of crosslinking to monodansyl cadaverine (AU/min). (***$p<0.005$, *$p<0.05$).

LasB elastase is the main protease involved in gluten metabolism by Psa

We first confirmed that *Psa* PA14*,* a human clinical isolate (Rahme, Stevens et al. 1995), had an identical degradation pattern to *Psa* X-46.1 against gluten and 33-mer (data not shown). To identify genes associated with gluten metabolism we used the available non-redundant transposon mutant library of PA14 (Liberati, Urbach et al. 2006). Approximately

6000 transposon mutants were tested for their ability to degrade gluten and 23 mutants consistently failed to generate a typical hydrolytic halo surrounding colonies on gluten-containing agar (Supplementary Table 3). These mutants included *LasB*, which encodes elastase, genes involved in the expression of *LasB* elastase, and the type II system known for the secretion of exoenzymes including *LasB* in *Psa* (Douzi, Ball et al. 2011). Our analysis supports that *LasB* was the main extracellular protease involved in gluten degradation, and consistent with these results, a *LasB* mutant had no peptidase activity against the 33-mer compared to its wild-type parent strain PA14 (Figure 4A and 4B). Colonization of germ-free mice with this mutant showed a reduction of gluten and gliadin degradation, and an increase of gliadin QPQLPY-peptides in the small intestine, compared to *Psa* X-46.1-colonized mice (Figure 4C-4E), further supporting its role in gluten metabolism *in vivo*.

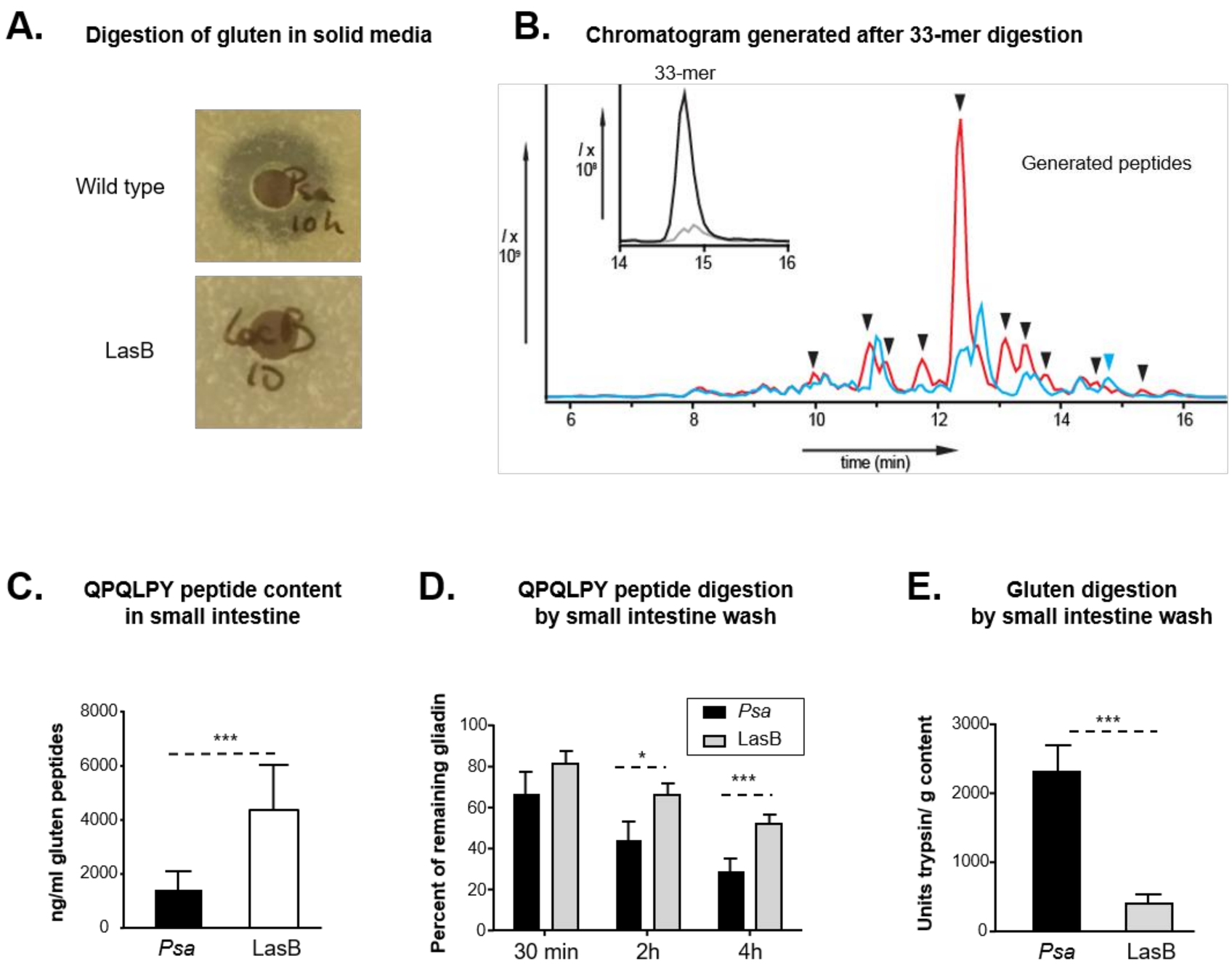


Figure 4: *LasB* elastase is involved in gluten degradation by *Pseudomonas aeruginosa* (*Psa*). **(A)** Degradation of gluten proteins in solid media of *Psa* PA14 wild-type and *LasB* mutant. Bioassays show non-specific degradation (clearing zone) in solid gluten media (white). **(B)** Cleavage of the 33-mer peptide (blue arrow) by *Psa* PA14 (red chromatogram) and *LasB* mutant (blue chromatogram). Black arrows: peptides generated after 33-mer degradation by *Psa* PA14. Inset shows 33-mer after *LasB* mutant incubation (black chromatogram) and after *Psa* PA14 incubation (grey chromatogram). **(C)** Germ-free mice were colonized with *Psa* or LasB mutant for one week, after which they were gavaged with

7 mg gliadin. The amount of the QPQLPY sequence in the small intestine of colonized mice was measured by G12 antibody 2 hrs following gliadin gavage. Data are represented as mean ± SEM. **(D)** Degradation of 7 mg of pepsin-trypsin predigested (PT)-gliadin by intestinal washes from *Psa*-colonized mice and *LasB*-colonized mice after 30 min, 2 hr, and 4 hr incubation. The amount of the QPQLPY-peptides after incubation was measured by G12 antibody. Data are represented as mean ± SEM. **(E)** Degradation of non-specific gluten proteins in solid media by intestinal washes of *Psa*-colonized mice and *LasB*-colonized mice. Bar graph shows units of trypsin per gram of intestinal washes based on a standard curve with trypsin. Data represented as mean ± SEM (***p<0.005, *p<0.05).

***Immunogenic peptides produced by Psa are detoxified by Lactobacillus* spp.**

The intestinal microbiota is a dynamic community where bacteria coexist with the host and with other bacteria (Ley, Peterson et al. 2006). Combinations of bacteria producing different proteases could further affect gliadin degradation products. To investigate this, we colonized C57BL/6 mice with *Psa* X-46.1 and *Lactobacillus* spp. Controls were colonized with *Psa* X-46.1 or with *Lactobacillus* spp. One week post-colonization, mice were gavaged with 7 mg gliadin and QPQLPY peptide content was measured in the small intestine after 2 hr (Figure 5A). Mice were successfully colonized with *Psa*+*Lactobacillus* spp. (Figure 5B) and they had lower QPQLPY gliadin peptide content compared to mice colonized with *Lactobacillus* alone (Figure 5C). Incubation of intestinal washes from colonized mice with PT-gliadin demonstrated that the combination of *Psa* X-46.1 and

Lactobacillus spp. enhanced QPQLPY degradation (70% in 2 hr) compared to *Psa*- (55%) and *Lactobacillus*-colonized mice (40%) (Figure 5D).

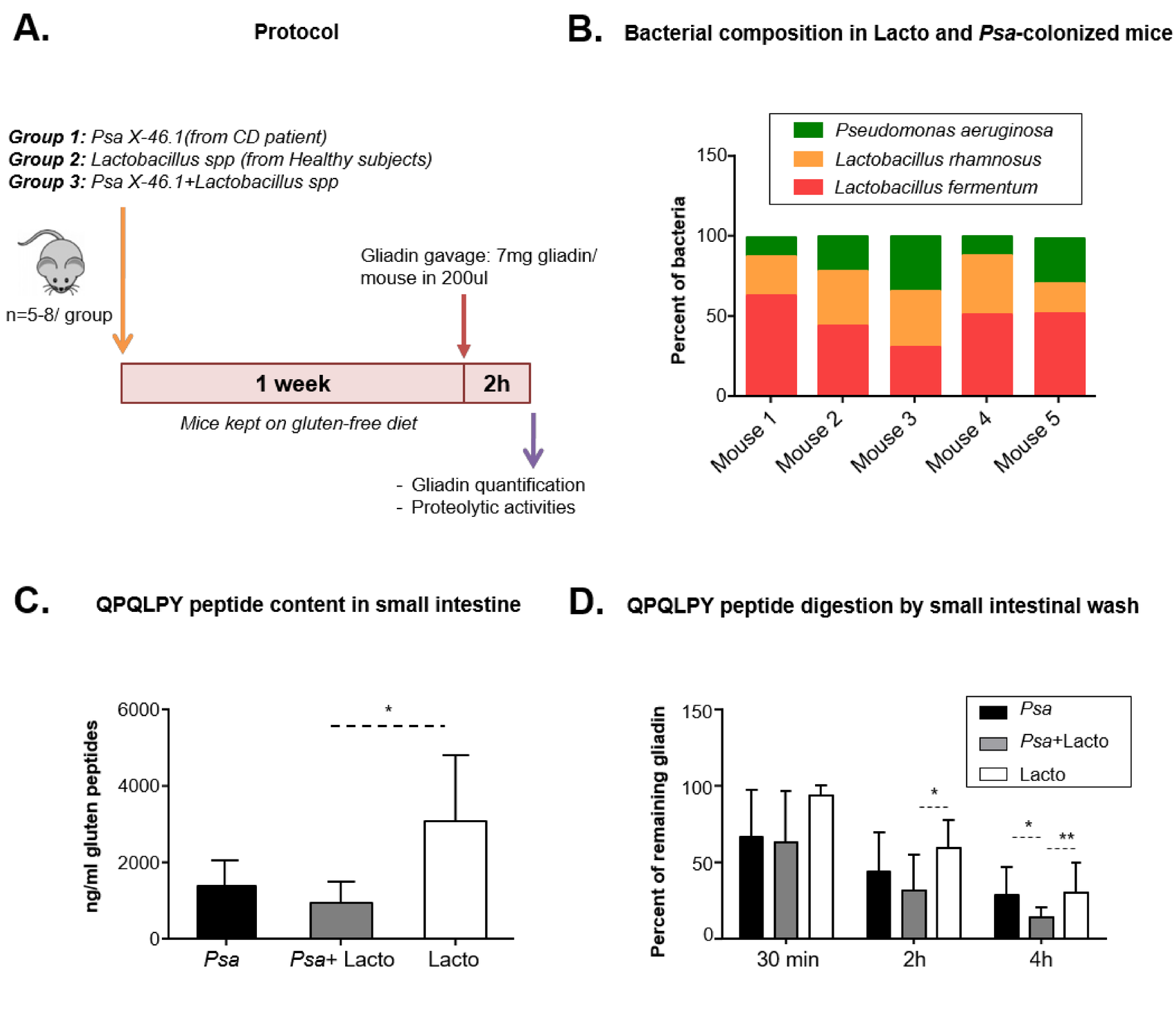
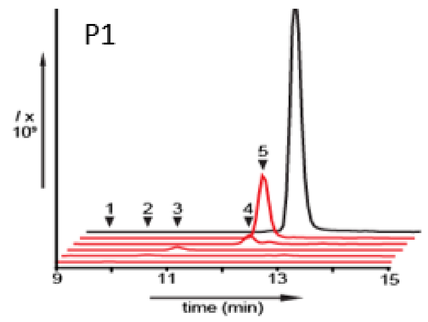


Figure 5: Peptides modified by *Pseudomonas aeruginosa* (*Psa)* can be degraded by *Lactobacillus* (Lacto). **(A)** Experimental design of mouse colonization study. **(B)** Bacterial composition in the small intestine of mice colonized with *Lactobacillus* spp. and *Psa*. **(C)** Amount of QPQLPY-peptides measured by G12 antibody in the small intestine of *Psa*-, *Lactobacillus*- and *Psa*+*Lactobacillus*-colonized mice 2 hrs after gliadin gavage (7 mg). Data are represented as mean ± SEM. **(D)** Degradation of 7 mg of pepsin-trypsin

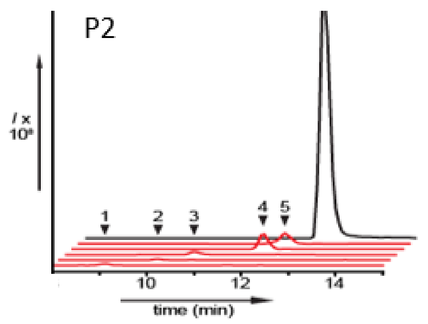
predigested (PT)-gliadin by intestinal washes from *Psa*-, *Lacto*- and *Psa*+*Lacto*-colonized mice by measuring QPQLPY-peptides. Data are represented as mean ± SEM. (***p<0.005, *p<0.05).

We then analyzed whether *Lactobacillus* reduced major immunogenic peptides generated by *Psa* X-46.1. We sequenced the peptides produced by *Psa* X-46.1. These peptides were then incubated with *Lactobacillus* spp. and analyzed by LC-MS/MS. Compared to *Lactobacillus* spp.-mediated degradation of intact 33-mer, *Lactobacillus* spp. degraded *Psa*-modified 33-mer derived peptides more efficiently, delivering peptides of 4-12 amino acids (Figure 6A). Most of these peptides are shorter than the 9 amino acids required for efficient antigen binding to HLA-DQ2 and activation of T-cells. We confirmed this by measuring gluten-specific T-cell responses to these peptides using celiac disease patients who underwent wheat challenge (Supplementary Table 2 and Supplementary Figure 3) (Anderson, Degano et al. 2000). Only a minority of *Lactobacillus* spp. degraded *Psa*-modified 33-mer-derived peptides showed immunogenicity and, overall, there was a reduction of immunogenicity compared with *Psa*-modified peptides (Figure 6B). The results suggest immunogenic peptides generated by *Psa* can be degraded to non-immunogenic peptides in the presence of *Lactobacillus* spp.

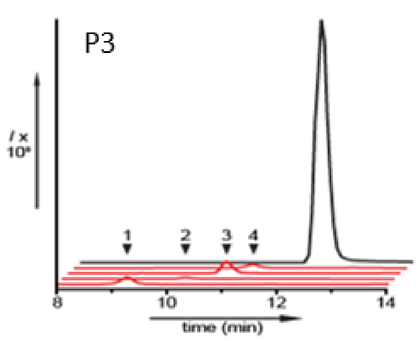
A. **Chromatograms and peptides generated after 33-mer digestion by bacteria**



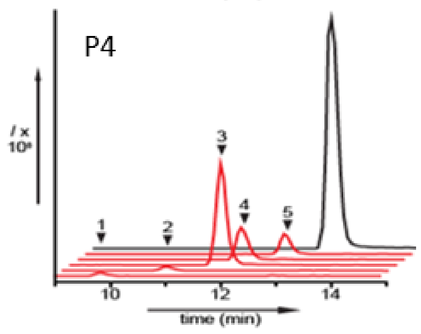
P1	L	Q	P	F	P	Q	P	Q	L	P	Y	P	Q	P
L1		Q	P	F	P	Q	P	Q	L	P	Y	P	Q	P
L2		Q	P	F	P	Q	P	Q	L	P	Y	P		
L3		Q	P	F	P	Q	P	Q						
L4		Q	P	F	P	Q	P							
L5				F	P	Q	P	Q	L	P	Y			



P2	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	F
L6	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	
L7						Q	P	Q	L	P	Y	P	Q	P	Q		
L8									L	P	Y	P	Q	P	Q	P	F
L9									L	P	Y	P	Q	P	Q		
L10			P	Y	P	Q				P	Y	P	Q				



P3	Q	P	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P
L11								Q	P	Q	L	P	Y	P	Q	P
L12											L	P	Y	P	Q	P
L10												P	Y	P	Q	



P4	P	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	F
L13		Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P	Q	P	
L14			L	P	Y	P	Q	P	Q	L	P	Y	P	Q	P										
L15																Q	L	P	Y	P	Q	P	Q	P	F
L16																			Y	P	Q	P	Q	P	F
L9			L	P	Y	P	Q	P	Q								L	P	Y	P	Q	P	Q		

B. **Immunogenicity of 33-mer derived peptides after bacterial digestion**

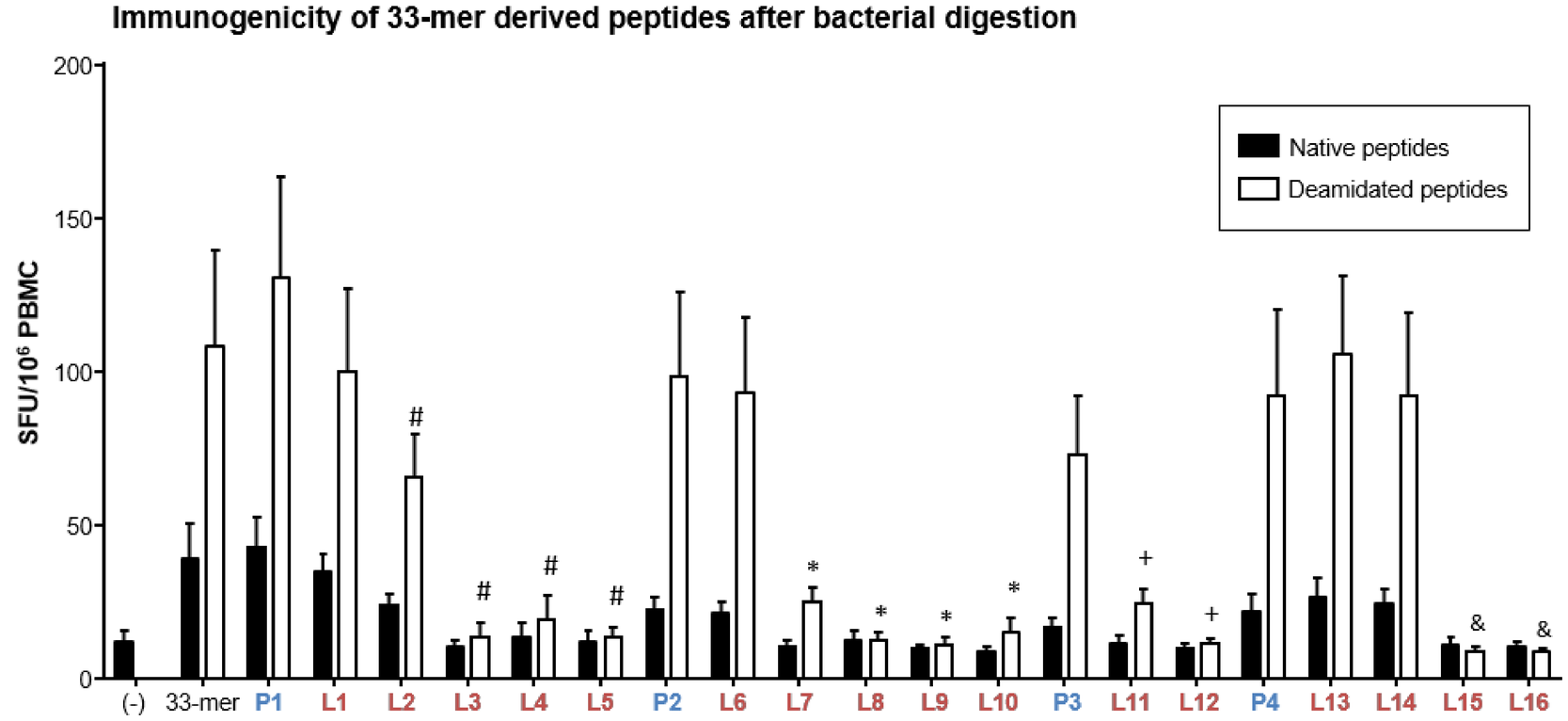


Figure 6: Immunogenic peptides produced by *Pseudamonas aeruginosa (Psa)* can be degraded to non-immunogenic peptides by *Lactobacillus* spp. **(A)** Chromatograms and sequences of peptides generated after 33-mer degradation by *Psa* (P1-P4) that were further degraded by *Lactobacillus* (L1-L16). Chromatograms (left) show *Psa*-derived 33-mer peptides (P1-P4; black peaks) generated by *Lactobacillus* degradation (red peaks). Tables (right) show sequences of *Psa*-derived 33-mer peptides (P1-P4; in blue) and peptides produced by *Lactobacillus* from *Psa*-derived 33-mer peptides (L1-L16; in red). Underlined: Transglutaminase 2 (TG2)-epitopes. **(B)** IFN-γ ELISpot in peripheral blood mononuclear cells (PBMC) harvested after gluten challenge in celiac disease patients in remission and *ex vivo* stimulated with native and deamidated peptides produced by Lacto (L1-L16) from *Psa*-derived 33-mer peptides (P1-P4). TG2-deamidated and non-deamidated 33-mer were used as controls. Results are shown as spot forming units (SFU) per 10^6 PBMC**.** Median response represented by horizontal line. Each patient donor response (numeric code) is represented with characteristic shape and colour dots. ($^{\#}p<0.05$ vs deamidated P-1; $^{*}p<0.05$ vs deamidated P-2; $^{+}p<0.05$ vs deamidated P-3; $^{\&}p<0.05$ vs deamidated P-4 peptide).

DISCUSSION

The role of intestinal microbiota in health and disease has been one of most studied areas in the past decade (Dethlefsen, McFall-Ngai et al. 2007), and its contribution to food sensitivities (Stefka, Feehley et al. 2014) and autoimmune disorders such as celiac disease (Sanz, De Pama et al. 2011) is emerging. Celiac disease represents a unique model to study

diet-induced intestinal inflammation and autoimmunity because the main environmental trigger, gluten, has been identified as well as the molecular mechanisms underlying peptide association with MHC class II and subsequent T-cell activation (Kim, Quarsten et al. 2004). Here we demonstrate that bacteria from the human small intestine participate in gluten metabolism, and we characterize the pool of peptides produced during bacterial gluten degradation. We show that the tested opportunistic pathogens and core gut commensals generate distinct breakdown patterns of gluten with increased or decreased immunogenicity which could influence autoimmune risk.

The Western diet contains about 20 g of gluten per day (Koning 2015). Gluten proteins are resistant to mammalian protease degradation, but are good substrates for bacterial metabolic activity (Caminero, Herran et al. 2014). The use of proteases produced by environmental microorganisms have been proposed as pharmacological therapy in celiac disease (Shan, Marti et al. 2004; Tack, van de Water et al. 2013; Lahdeaho, Kaukinen et al. 2014). However, the ability of these proteases to effectively degrade the amount of gluten present in a normal diet before reaching the small intestine has been questioned (Koning 2015). This may limit enzymatic therapy in celiac disease to prevention of gluten-induced effects due to inadvertent gluten consumption in patients who are already on a gluten-free diet. On the other hand, detoxification of gluten *in situ* by the metabolic activity of resident small intestinal bacteria could constitute an attractive approach. The gluten-degrading capacity of opportunistic pathogens isolated from human feces such as *Psa* has also been recently proposed (Wei, Tian et al. 2015). We found *Psa* indeed cleaves the proteolytic resistant 33-mer gluten peptide, a product of mammalian enzyme degradation, but delivers

peptides longer than 9 amino acids that strongly stimulate gluten-specific responses in disease-relevant T-cells isolated from HLA-DQ2 celiac disease patients. Moreover, incubation of *Psa* with gliadin predegraded by human proteases enhances peptide immunogenicity in celiac disease patients. The effect on bioactivity is presumed to relate to proteolytic action of the bacteria, but chemical modification via other mechanisms such as peptide deamidation by *Psa* transglutaminases (Milani, Vecchietti et al. 2012) could be important. Although our results show a similar deamidation ratio of PT-gliadin with and without bacterial incubation by human TG2, bacterial transglutaminases could deamidate proteins differently. Partial *Psa*-gluten degradation may also facilitate uptake of shorter peptides but with retained immunogenicity through paracellular or transcellular pathways, increasing their availability to APCs in celiac disease patients. Indeed, we found that *Psa*-modified peptides translocate the mucosal barrier more efficiently than peptides generated by human proteases. Finally, through a genomic approach, we identified *LasB*, a metalloprotease virulence factor which could play a pivotal role in infection, as the main bacterial protease involved in the gluten metabolic activity of *Psa*. *LasB* has been demonstrated to be important in numerous infection models and it has been a target for the development of anti-pseudomonal therapy (Cathcart, Quinn et al. 2011). We therefore propose that opportunistic pathogens, such as *Psa*, colonizing the small intestine may constitute an additional pathogenic factor in celiac disease through their gluten metabolic activity. This highlights the importance of characterizing microbial proteases involved in gluten metabolism, as well as the derived peptides released, before they are proposed as pharmacological therapy in celiac disease.

Bacterial interactions could affect gluten degradation patterns and peptide output. In mice, we found that bacterial communities dominated by *Lactobacillus,* such as ASF, showed fast and effective gluten metabolism. In agreement with this, our previous work has shown a protective role of this community in mouse models of gluten sensitivity (Galipeau, McCarville et al. 2015) and *Lactobacillus* have been previously suggested as potential beneficial organisms in CeD (D'Arienzo, Maurano et al. 2008; Sarno, Lania et al. 2014). In isolation, *Lactobacillus* spp. do not efficiently degrade the 33-mer. However, incubation of gliadin predegraded by pepsin and trypsin with *Lactobacillus* strains, reduces its immunogenicity to gluten-specific T-cells from celiac disease patients. This suggests *Lactobacillus* can detoxify gliadin peptides after partial digestion by human proteases. Moreover, we found that immunogenic peptides produced by *Psa* proteases are also further degraded and rendered less immunogenic in the presence of *Lactobacillus*. This mechanism provides an explanation linking imbalances between pathobionts and core commensals such as *Lactobacillus*, and susceptibility to autoimmune disease in a genetically predisposed host.

It is important to stress that the strains tested in this study are not the only ones which could potentially modify celiac disease risk. Several bacterial groups from the human gastrointestinal tract have been implicated in gluten metabolism *in vitro* (Fernandez-Feo, Wei et al. 2013; Caminero, Herran et al. 2014). Moreover, studies using 16S sequencing continue to identify pathobionts, particularly from Protebacteria, present and abundant in populations of celiac disease patients.[10] In addition to modification of gluten immunogenicity, it is possible some pathobionts influence celiac disease risk through non-

specific pro-inflammatory effects such as altering intestinal permeability or the innate immune response.

In summary, we identify both pathogenic and protective microbe-gluten-host interactions that may modulate autoimmune risk in HLA-DQ2 susceptible persons. We show that *Psa* elastase generates highly immunogenic gliadin peptides that translocate through the mucosal barrier. However, *Lactobacillus* further degrade the elastase products to peptides with lower immunogenicity (Figure 7). The mechanisms described in this paper could be targeted to reduce disease, by inhibiting elastase and similar proteases (Galipeau, Wiepjes et al. 2014) or increasing the protective enzymatic activity of certain bacteria.

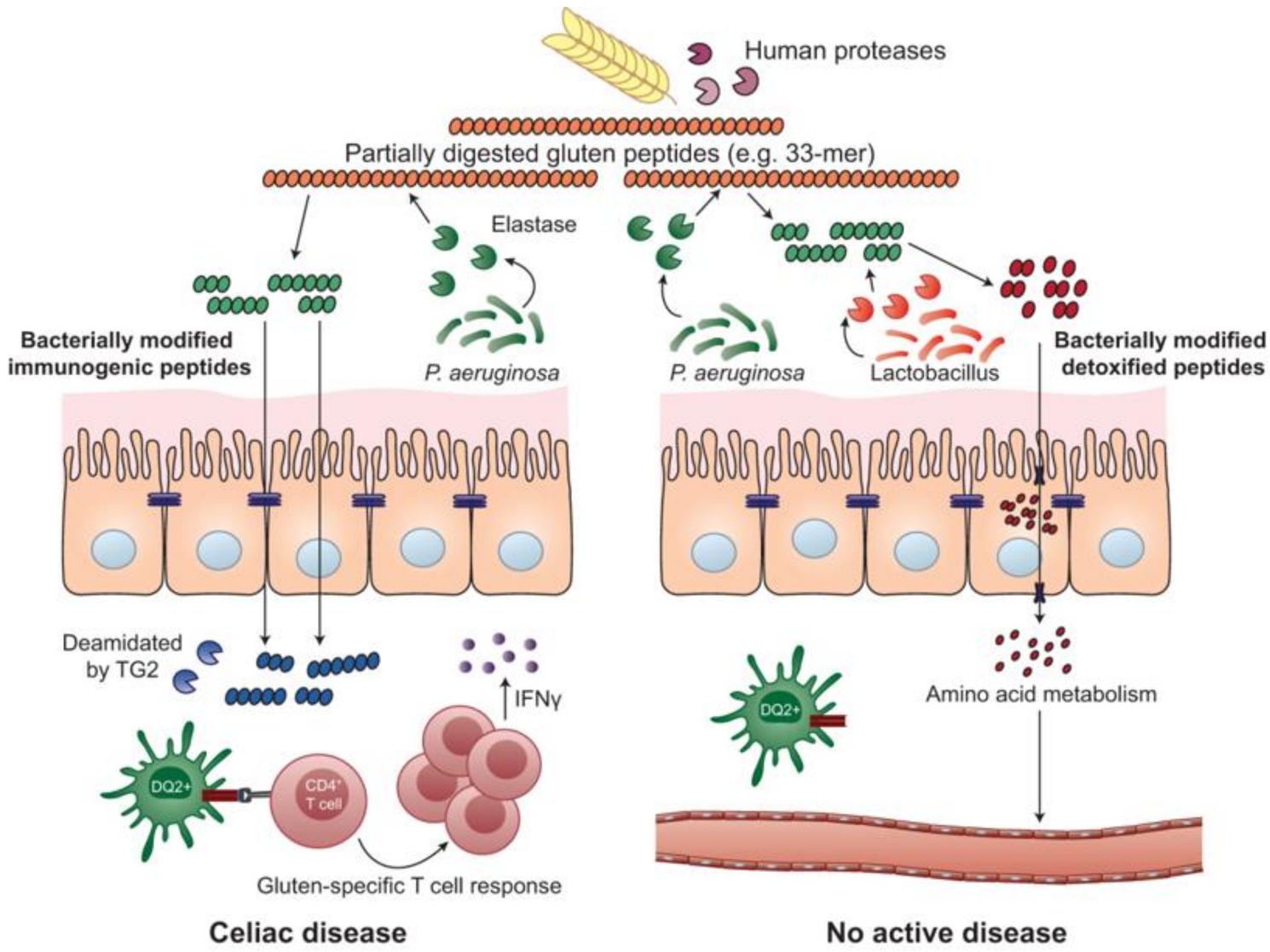


Figure 7: Model depicting how imbalances between pathobionts and core commensals in the proximal small intestine could affect susceptibility to celiac disease in a genetically

predisposed host. Gluten proteins rich in proline residues are only partially digested by human proteases generating large and immunogenic peptides such as the 33-mer peptide. Partially degraded gluten peptides (e.g. 33-mer) could be metabolized by opportunistic pathogens, such as *Pseudomonas* (*Psa*), producing slightly shorter peptides, but with retained immunogenicity. These *Psa*-modified gluten peptides translocate more efficiently the mucosal barrier to interact with antigen presenting cells expressing HLA-DQ2. On the other hand, *Psa*-modified gluten peptides can be further detoxified by other members of the duodenal microbiota such as *Lactobacillus* spp. The metabolic activity of *Lactobacillus* produces peptides shorter than the 9-amino acid-length required for efficient antigen binding to HLA-DQ2 and activation of T-cells.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1: Information on small intestinal bacteria used in this study.

Bacteria	Origin	Growth in Gluten media	Gluten Proteolysis
Pseudomonas aeruginosa X-46.1	Active celiac patient	+	+
Staphylococcus epidermidis X-35.1	Active celiac patient	+	+
Staphylococcus warneri X-18.3	Active celiac patient	+	-
Lactobacillus rhamnosus X-32.2	Healthy volunteer	+	-
Lactobacillus fermentum X-39.3	Healthy volunteer	+	-

(+) Function detected. (–) No function detected.

Supplementary Table 2. Celiac disease participants and gluten challenge details.

Subject	Age (Years)	Sex	Years since diagnosis	33-mer T-cell response[#]	Symptoms (Mild +, Moderate ++, Severe +++)							
					N	B	V	D	C	P	L	F
0570	56	M	2.2	NR								
0081	62	F	7.8	+++	++	++					+	+
0077	51	M	45.0	++								++
3177	11	F	3.0	+	+							
3050	12	F	1.7	NR						+	+	
0566	62	F	5.3	+		+		+		++	+	+
0512	56	F	5.3	+	+		+	+				
0036	36	F	9.2	+++	+++	++	+++			++++	++	+
0540	70	F	9.8	NR	+++	+++	+++	+		++++	+++	
0538	54	F	6.7	+++								
0035	35	F	9.8	+	+++	++	+++	+		++		
0570	47	F	2.5	NR								
0565	69	M	11.5	+		+				+		
0185	46	F	14.6	+							++	+
0120	43	F	13.1	+	++	+	+	+		+	++	+
0572	53	F	4.6	++		++		+		+	+	+
0633	63	F	2.0	+	+++	++	+++		+	++	+++	+++
0650	58	F	4.1	+++	+			+		+		

0648	53	F	7.1	+								
0282	32	F	14.4	+								

[#]T-cell response: < 100 SFU/10^6 PBMC +, 100-200 SFU/10^6 PBMC ++, >200 SFU/10^6 PBMC +++, NR (non-responder). Symptoms: N= nausea, B= bloating, V= vomiting, D= diarrhoea, C= constipation, P= abdominal pain, L= lethargy, F= flatulence. Dark shading denotes asymptomatic participants.

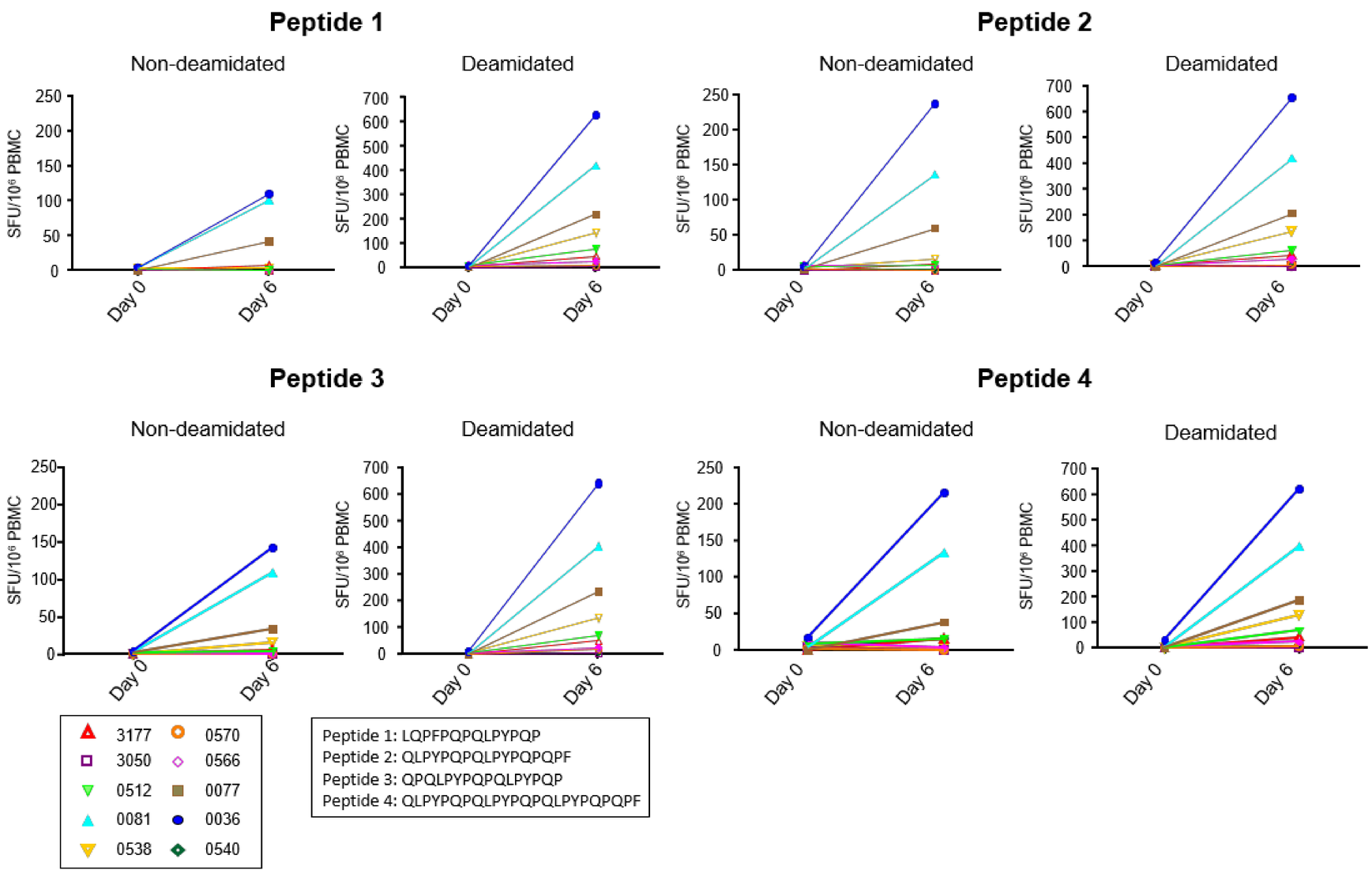
Supplementary Table 3. *P. aeruginosa* PA14 transposon mutants without zone of clearing on gluten-containing agar plates

		Gene number		
Tn mutant ID	**Gene**	**PA14**	**PAO1**	**Function**
38761	*lepA*	PA14_54370	PA0767	Translation, post-translation modification
4702	*phhB*	PA14_53000	PA0871	Amino acid metabolism
30077	*motC*	PA14_45560	PA1460	Motility and attachment
38076	-	PA14_45510	PA1463	Unknown function
46438	*oprF*	PA14_41570	PA1777	Membrane proteins, porin
35555	*pyrF*	PA14_26890	PA2876	Nucleotide metabolism
41602	*xcpZ*	PA14_24100	PA3095	Protein secretion, type II
39963	*xcpW*	PA14_24060	PA3098	Protein secretion, type II
25593	*xcpT*	PA14_24020	PA3101	Protein secretion, type II
34060	*xcpT*	PA14_24020	PA3101	Protein secretion, type II
48094	*xcpR*	PA14_23990	PA3103	Protein secretion, type II
43537	*xcpQ*	PA14_23970	PA3105	Protein secretion, type II

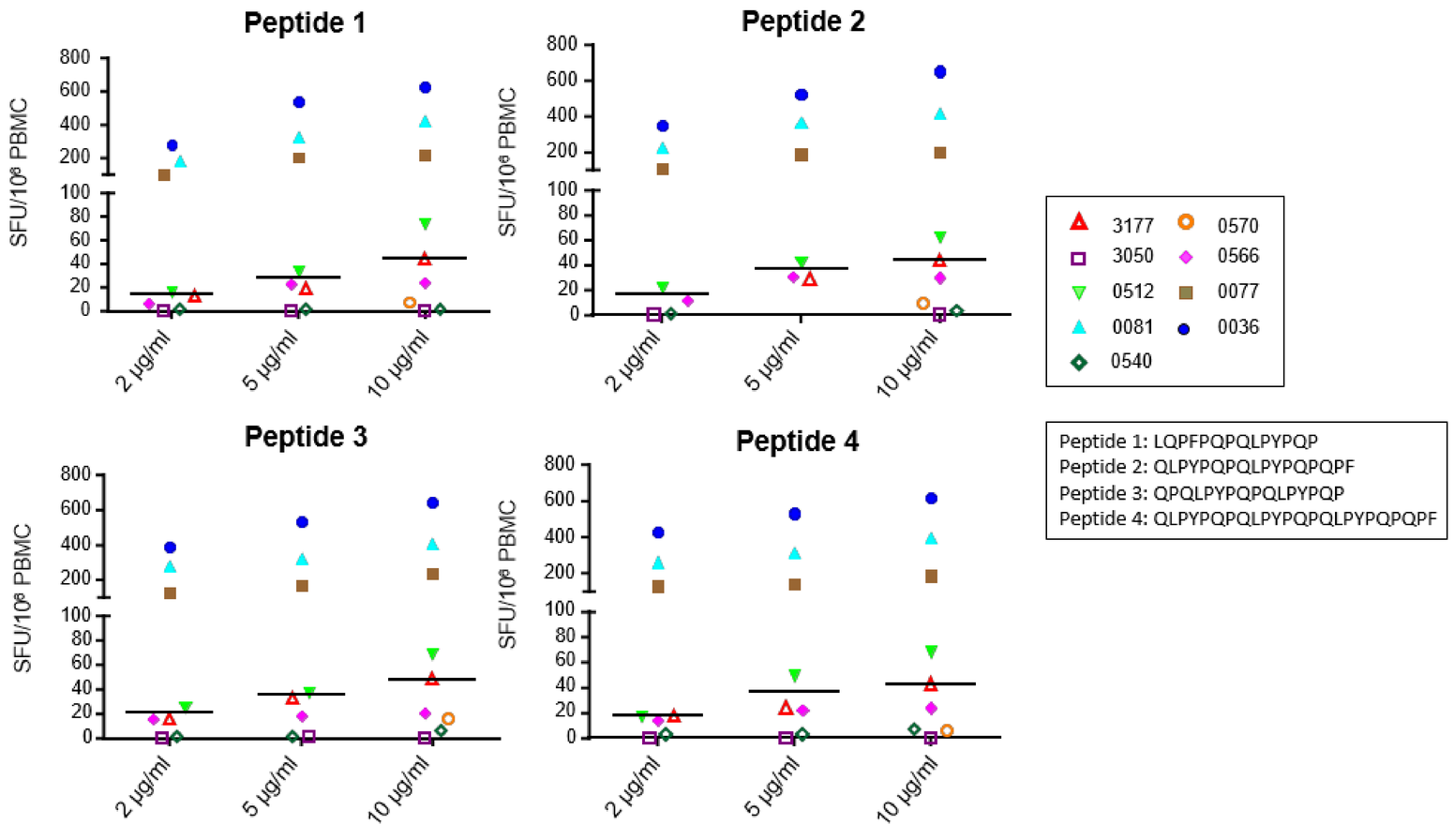
31938	*lasB*	PA14_16250	PA3724	Secreted factors, elastase
45691	*lasB*	PA14_16250	PA3724	Secreted factors, elastase
55048	*thrC*	PA14_16090	PA3735	Amino acid metabolism
32448	-	PA14_11210	PA4072	Transport of small molecules
32277	*carB*	PA14_62910	PA4756	Amino acid/nucleotide metabolism
39940	*carA*	PA14_62930	PA4758	Amino acid/nucleotide metabolism
45346	*Orn*	PA14_65410	PA4951	Transcription, RNA processing and degradation
28409	*Pyre*	PA14_70370	PA5331	Nucleotide metabolism
46326	*Pyre*	PA14_70370	PA5331	Nucleotide metabolism
54110	*soxG*	PA14_71510	PA5419	Amino acid metabolism
38036	-	PA14_72040	PA5458	Unknown function

Supplementary Figure 1.

A. **Immunogenicity of 33-mer derived peptides after *Psa* digestion, before and after gluten challenge**



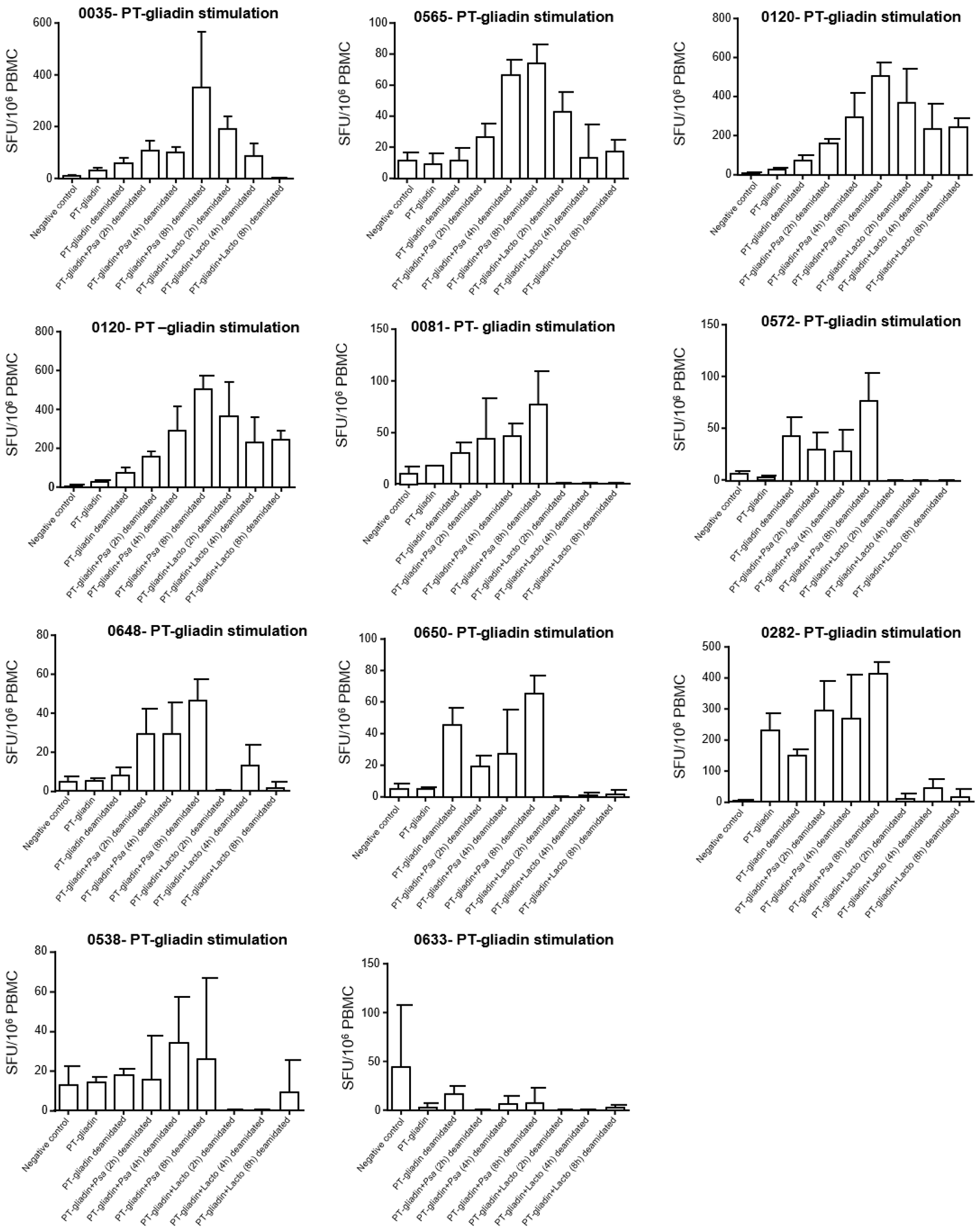
B. **Immunogenicity of 33-mer derived peptides after *Psa* digestion at different concentrations**



Supplementary Figure 1: (A) IFN-γ ELISpot in peripheral blood mononuclear cells (PBMCs) harvested before (day 0) and after gluten challenge (day 6) of celiac patients in remission and *ex vivo* stimulated with non-deamidated and deamidated gliadin peptides produced following incubation of 33-mer with *Psa* X-46 (Peptide 1-4). **(B)** IFN-γ ELISpot in PBMCs harvested after gluten challenge of celiac patients in remission and *ex vivo* stimulated with different concentrations of deamidated gliadin peptides produced following incubation of 33-mer with *Psa* X-46 (Peptide 1-4). Results are shown as spot forming units (SFU) per 10^6 PBMCs**.** Median response represented by horizontal line. Each patient donor response (numeric code) is represented with characteristic shape and colour dots.

Supplementary Figure 2.

Immunogenicity of peptides produced by bacteria in each patient



Supplementary Figure 2: IFN-γ ELISpot in peripheral blood mononuclear cells (PBMCs) harvested after gluten challenge of each celiac patient and *ex vivo* stimulated with pepsin-trypsin (PT)-gliadin pre-digested by *Psa* X-46.1 or *Lactobacillus spp*. and deamidated by transglutaminase 2 (TG2). Non-deamidated and deamidated PT-gliadin were used as positive controls. Results are shown as spot forming units (SFU) per 10^6 PBMC**.** Median response represented by horizontal line. Each patient donor response is represented with a numeric code.

Subject	0633	0650	0081	0538	0572	0120	0035	0185	0648	0282	0565
Medium (nil antigen)	22	2	5	3	3	3	5	4	2	2	6
Positive response cut-off	66	10	15	10	10	10	14	12	10	10	17
Tetanus toxoid	23	85	41	35	31	82	79	17	77	98	11
Wildtype 33mer	3	61	34	35	24	19	26	13	1	3	5
Deamidated 33mer	27	148	89	122	80	44	43	22	8	15	13
Maximum SFU	183	134	94	114	74	73	46	38	23	21	19
P1: LQPFPQPQLPYPQP	66	35	35	19	21	16	21	13	3	8	12
L1: QPFPQPQLPYPQP	43	23	31	9	17	17	17	19	3	6	12
L2: QPFPQPQLPYP	15	18	18	18	16	8	8	11	3	6	17
L3: QPFPQPQ	11	3	2	1	4	7	2	9	1	3	10
L4: QPFPQP	30	1	5	3	3	5	7	13	4	4	6
L5: FPQPQLPY	25	5	5	2	5	2	4	13	3	1	6
P2: QLPYPQPQLPYPQPQPF	6	19	30	7	9	14	14	13	3	4	12
L6: QLPYPQPQLPYPQPQP	11	18	25	8	8	8	6	14	14	3	10
L7: QPQLPYPQPQ	12	7	5	3	7	2	4	7	3	2	12
L8: LPYPQPQPF	19	5	2	4	6	2	5	12	2	2	15
L9: LPYPQPQ	5	3	4	5	2	5	5	11	6	3	4
L10: PYPQ	6	3	4	2	5	5	4	11	3	0	9
P3: QPQLPYPQPQLPYPQP	16	14	13	10	11	6	7	10	2	4	6
L11: QPQLPYPQP	21	6	4	4	5	3	7	7	2	2	6
L12: LPYPQP	7	3	5	2	4	3	5	13	3	5	6
P4: PQLPYPQPQLPYPQPQLPYPQPQPF	8	18	36	7	10	18	8	16	1	2	9
L13: QLPYPQPQLPYPQPQLPYPQPQP	36	12	35	8	12	9	16	12	4	3	10
L14: LPYPQPQLPYPQP	7	29	23	14	13	16	12	11	4	2	12
L15: QLPYPQPQPF	6	3	5	2	2	11	7	15	2	5	8
L16: YPQPQPF	5	4	3	5	4	12	7	7	4	4	7
P1: LQPFPQPQLPYPQP-Deamidated	183	134	91	95	65	73	27	38	9	18	14
L1: QPFPQPQLPYPQP-Deamidated	16	132	94	103	71	53	29	24	7	21	14
L2: QPFPQPQLPYP- Deamidated	47	63	55	60	38	36	20	21	4	14	13
L3: QPFPQPQ- Deamidated	32	3	6	3	4	3	4	6	5	4	12
L4: QPFPQP- Deamidated	53	5	7	4	11	3	3	12	5	3	11
L5: FPQPQLPY- Deamidated	14	5	5	4	10	6	5	18	2	2	10
P2: QLPYPQPQLPYPQPQPF-Deamidated	9	125	87	110	68	53	33	26	7	14	16
L6: QLPYPQPQLPYPQPQP-Deamidated	60	134	78	94	56	29	28	21	23	19	14
L7: QPQLPYPQPQ- Deamidated	30	23	12	18	15	6	6	12	3	6	10
L8: LPYPQPQPF- Deamidated	8	4	6	4	4	10	4	14	3	1	15

L9: LPYPQPQ- Deamidated	7	4	7	4	3	6	4	14	3	1	11
L10: PYPQ- Deamidated	34	4	6	4	2	5	3	10	2	2	13
P3: QPQLPYPQPQLPYPQP-Deamidated	83	81	47	77	37	14	23	20	6	11	13
L11: QPQLPYPQP- Deamidated	11	29	15	17	16	5	12	21	2	6	6
L12: LPYPQP- Deamidated	9	5	3	5	5	8	5	10	3	1	10
P4: PQLPYPQPQLPYPQPQLPYPQPQPF-Deamidated	9	122	90	110	64	37	32	26	6	16	9
L13: QLPYPQPQLPYPQPQLPYPQPQP- Deamidated	65	109	80	114	66	49	46	26	6	16	19
L14: LPYPQPQLPYPQP-Deamidated	9	120	87	106	74	44	22	24	8	12	10
L15: QLPYPQPQPF- Deamidated	8	3	4	4	2	2	5	9	5	2	8
L16: YPQPQPF- Deamidated	5	3	5	4	8	3	5	6	4	3	9

Supplementary Figure 3: IFN-γ ELISpot in peripheral blood mononuclear cells (PBMCs) harvested after gluten challenge of each celiac patient and *ex vivo* stimulated with deamidated and non-deamidated gliadin peptides produced following incubation of 33-mer with *Psa* (P1-P4) or with *Psa* then *Lactobacillus spp.* (L1-L16). Medium, tetanus toxoid, deamidated and non-deamidated 33-mer were used as controls. Results are shown as spot forming units (SFU) per 10^6 PBMC**.** Each patient donor response is represented with a numeric code. Color-coding represents a significant immune response as a percentage of the maximal peptide SFU (red: >70%, orange: 41-70%, yellow: 21-40%, green: 11-20%, purple: 6-10%). Blue: *Psa*-derived 33-mer peptides. Pink: *Lactobacillus*-derived 33-mer peptides.

Supplemental Materials and Methods

Substrates

Gliadin and gluten were purchased from Sigma Aldrich to perform our studies. Gluten was used to prepare solid media. Gliadin was sterilized by irradiation and used in mouse and *in vitro* degradation studies (Galipeau, Rulli et al. 2011). Pepsin-trypsin digested gliadin (PT-gliadin) was employed in permeability studies and for proteolytic activities (Galipeau, McCarville et al. 2015). Gluten peptides were synthesized at a purity of 95% (Biomatik, Cambridge, ON). We sequenced the immunogenic alpha gliadin 33-mer (LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF) and the following 33-mer-derived peptides that were generated after partial bacterial proteolysis: 1) LQPFPQPQPQLPYPQPL, 2) QPFPQPQPQLPYPQP, 3) QPFPQPQPQLPYP, 4) QPFPQPQ, 5) QPFPQP, 6) FPQPQLPY, 7) QLPYPQPQLPYPQPQPF, 8) QLPYPQPQLPYPQPQP, 9) QPQLPYPQPQ, 10) LPYPQPQPF, 11) LPYPQPQP, 12) PYPQ, 13) QPQLPYPQPQLPYPQP, 14) QPQLPYPQP, 15) LPYPQP, 16) PQLPYPQPQLPYPQPQLPYPQPQPF, 17) QLPYPQPQLPYPQPQLPYPQPQP, 17) LPYPQPQLPYPQP, 18) QLPYPQPQPF, 19) YPQPQPF, 20) LPYPQPQ. Substrates were individually deamidated with 50 µg/ml pig tissue transglutaminase (Sigma-Aldrich, St Louis, MO) in PBS for 2 hr at 37°C plus 1 mM $CaCl_2$ (Tye-Din, Stewart et al. 2010). Gliadin-derived paranitroanilide tripeptides were synthesized to study gliadin peptidase activity (Biomatik) (Helmerhorst, Zamakhchari et al. 2010). Gliadin-derived paranitroanilide tripeptides (Z-YPQ-pNA, Z-QQP-pNA, Z-PPF-pNA and Z-PFP-pNA, Z-QPQ-pNa) were synthesized to study gliadin peptidase activity (Biomatik).

Study subjects

Celiac disease (CeD) patients and non-CeD controls were recruited in this study. Active celiac disease was defined by positive IgA anti-tissue transglutaminase antibodies, HLA-DQ2 phenotype and a characteristic duodenal biopsy finding (> Marsh IIIa lesion) (Ludvigsson, Leffler et al. 2013). Non-celiac disease controls were first-degree relatives of celiac disease patients in whom celiac disease was ruled out and who had no HLA-DQ2 phenotype. None of the individuals included in the study had been treated with antibiotics for at least 1 month prior to the sampling time. For microbiology studies, duodenal biopsy specimens were collected at the Department of Gastroenterology at the Hospital de Leon (Spain) by upper endoscopy for small intestinal bacteria isolation. Informed consent was obtained from the adult patients. The study was approved by the local Research Ethics Committee of the Hospital de León. For PBMCs stimulation studies, celiac disease patients were recruited in The Royal Melbourne Hospital (Australia) and the study was approved by the Melbourne Health Human Research Ethics Committee.

Microbial composition in colonized mice

Bacterial composition in mice was analyzed after sacrifice by culture (Slack, Hapfelmeier et al. 2009). We used non-specific culture media (Brain Hearth Infusion, Tryptic Soy Broth, de Man Rogosa and Sharpe media) and specific culture media for gluten-degrading (MCG-1 and MCG-3)(Caminero, Herran et al. 2014) and for each bacterial group (*Pseudomonas* isolation agar-Sigma) in aerobic and anaerobic conditions. Colonies were inspected for morphological and biochemical characteristics. Bacteria in each group were able to grow in specific selective media under the reported conditions. Single bacteria isolated in each

media were sequenced by Sanger method. The isolates were also examined morphologically by Gram stain. We did not recover any contaminant bacterial strain from mono- or di-colonizations.

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CHAPTER 4: MICROBIAL PROTEASES MODULATE INNATE IMMUNITY AND INCREASE SENSITIVITY TO DIETARY ANITGEN THROUGH PAR-2

Summary and significance

Aim: To characterize direct mechanisms, mediated by protease production, through which bacteria influence small intestinal innate immunity and host responses to gluten.

Summary: Compositional alterations in gut microbiota have been hypothesized as one potential environmental factor that contributes to the development of celiac disease in genetically predisposed individuals consuming gluten. Patients with active celiac disease harbor a dysbiotic microbiota, and increased abundance of small intestinal Proteobacteria. This is also observed in patients who remain symptomatic after the gluten-free diet. We have previously published that the degree of gluten-induced immunopathology in the NOD/DQ8 mouse model of gluten sensitivity is dependent on microbial colonization status. A clean-SPF(ASF-derived) microbiota, devoid of Proteobacteria and predominantly composed of Lactobacilli and *Parabacteroides distasonis*, protected mice from developing gluten-induced immunopathology. Moreover, both supplementation of clean-SPF mice with an *E. coli* isolate from active celiac disease patients or, experimental expansion of Proteobacteria in the small intestine of SPF mice enhanced gluten immunopathology. In this chapter, I investigate direct *bacterial-host* effects that could be relevant in triggering innate immune pathways described in celiac disease. We found that the microbiota from patients with active celiac disease, induced small intestinal IELs in recipient wild-type mice exposed to gluten. We analyzed responses induced by individual human donors, and rationally selected one healthy (HD3) and one celiac disease donor (CeD2) that had induced low and high IEL counts, respectively, in the recipient mice. We found that CeD2-colonized mice developed increased IELs, independently of exposure to

gluten, increased Proteobacteria and decreased Firmicutes abundance, compared to HD3-colonized mice. CeD2-colonized mice also had high small intestinal proteolytic activities towards gluten and harbored bacteria with glutenasic activity in gluten media. We then used the Proteobacteria member, *Pseudomonas aeruginosa,* as a model organism with high elastase production, to elucidate the direct role of proteases in the severity of gluten-induced pathology. The induction of IELs and proteolytic activities within the small intestine of *P. aeruginosa*-colonized mice was associated with increased expression of PAR-2 in the intestinal epithelium. A variety of bacterial isolates from mice colonized with CeD2 and *P. aeruginosa,* cleaved the external domain of PAR-2 using a PAR-2 cleavage reporter cell assay. We determined PAR-2 signaling was involved in the generation of IELs as the increase was not observed in SPF PAR-2 knock-out (PAR-$2^{-/-}$) mice supplemented with *P. aeruginosa*, despite PAR-$2^{-/-}$ mice developing increased intestinal proteolytic activities. Finally, we found that the presence of *P. aeruginosa* producing elastase enhances gluten-induced pathology in mice expressing the human DQ8 gene.

Significance: In a small cohort of donors, we confirmed that microbiota from celiac disease patients cluster separately from the microbiota from healthy controls. The experiments support the notion that pro-inflammatory microbial-derived proteases such as elastase, have direct host innate immunostimulatory activity through PAR-2 cleavage. In a genetically susceptible host, overgrowth of Proteobacteria with bacterial-elastase activity could constitute the elusive innate immune trigger that contributes to the

development of enteropathy. This mechanism could be targeted specifically to reduce risk or treat celiac disease. This possibility is explored in **Chapter 5**.

Preface: In this chapter, I performed gnotobiotic experiments (colonization of germfree mice with donor microbiota and clean-SPF microbiota, and sample collection), SPF PAR-$2^{-/-}$ experiments (supplementation with *P. aeruginosa* and sample collection), immunohistochemistry ($CD3^{+}$ staining and IEL counts), 16s sequencing (PCR and analysis), glutenastic activity quantification and isolation of gluten-degrading bacteria.

McCarville JL, Caminero AF, Galipeau HJ, Bernier S and Meisel M performed experiments. **McCarville JL**, Caminero AF and Galipeau HJ carried out gnotobiotic experiments and analysis. **McCarville JL** performed all microbiota techniques and analysis. Caminero AF carried out PAR-2 cleavage experiments. Mesiel M performed qPCRs from celiac and healthy microbiota colonization experiments. Bernier S aided *in vitro* experiments involving *Pseudomonas aeruginosa*. **McCarville JL**, Caminero AF, Galipeau, Verdu EF, Vergnolle N designed experiments. Surette M, Murray J, Jabri B provided critical review of the manuscript.

Microbial proteases modulate innate immunity and increase sensitivity to dietary antigen through PAR-2.

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ABSTRACT

Disruption of the gut microbiota can contribute to loss of tolerance to dietary antigens. Microbiota-modulating approaches constitute an attainable goal to prevent or treat food sensitivities, providing compositional and functional mechanisms are unraveled. We show that intestinal bacteria from patients with active celiac disease, an enteropathy caused by the breakdown of oral tolerance to gluten proteins, induced proteolytic activity towards gluten, and was associated with the presence of bacteria with gluten-degrading capacity, elevated Proteobacteria abundance and distinct immune responses in the small intestine of germ-free mice. *Pseudomonas aeruginosa* elastase activity, encoded by the gene *LasB*, was critical for the development of these immune responses. The changes were not observed in PAR-2 knock-out mice. In a celiac-relevant mouse model, transgenic for the human HLA-DQ8 gene, the presence of *P. aeruginosa* elastase enhanced small intestinal enteropathy induced by gluten. These findings identify a pro-inflammatory, bacterial protease effector pathway, signaling through PAR-2, which could be targeted therapeutically to prevent food sensitivity.

INTRODUCTION

Compositional and functional alterations of the gut microbiota associated with disease (dysbiosis) have been described (Carding, Verbeke et al. 2015). Mechanistic information through which dysbiosis causes disease is limited and is needed for the development of preventative measures and targeted therapies (McCarville, Caminero et al. 2016). Commensal bacteria and opportunistic pathogens produce a large array of mediators that differentially influence the host's immune system (Rooks and Garrett 2016). These have been mainly studied in the colon, the site with highest bacterial density in the gastrointestinal tract. However, the main site of interaction between food proteins, gut microbes, and the host, occurs in the small intestine. Mechanistic understanding of microbiota-host interactions in the upper gastrointestinal tract, and how they contribute to immune responses to specific food proteins, is needed. One of the most abundant sources of protein in the Western diet is gluten, the global name given to a mix of proteins rich in glutamine and proline amino acid residues, that can trigger celiac disease in genetically predisposed people (Lebwohl, Ludvigsson et al. 2015).

Celiac disease is a common, model gastrointestinal disorder that results from the development of a gluten-specific T-cell response in conjunction with the activation of innate immune pathways in the gut mucosa (Jabri, Kasarda et al. 2005; Kim, Mayassi et al. 2015). Risk alleles (HLA-DQ2 or -DQ8) are necessary, but insufficient for the development of celiac disease, and therefore other environmental modifiers have been proposed (Ludvigsson and Green 2014). Gluten is only partially digested by mammalian proteases in the duodenum, leaving large peptides that initiate the pro-inflammatory adaptive immune

response. The innate immune response is characterized by proliferation and activation of intraepithelial lymphocytes (IELs) (Abadie, Discepolo et al. 2012; Kim, Mayassi et al. 2015). Key drivers of this process have been identified, such as increases in cytokines such as IL-15 and possibly IL-18 (Okazawa, Kanai et al. 2004; Leon, Garrote et al. 2006; Abadie and Jabri 2014). However, the underlying triggers remain unknown. Although viral infections have long been suspected (Bouziat, Hinterleitner et al. 2017; Verdu and Caminero 2017), a bacterial contribution has largely been ignored, in part due to the lower density of bacteria colonizing the small intestine, compared to the colon. Microbiome studies have changed this view indicating that there is an abundance of Proteobacteria in the duodenum of patients with celiac disease, with some species playing an important role in gluten protein metabolism (Caminero, Galipeau et al. 2016). A role for secreted proteases, as direct inducers of effector mechanisms in the small intestine in the presence and absence of gluten, is unknown.

Here we demonstrate that colonization of germ-free mice with gut microbiota, and bacteria capable of degrading gluten from patients with celiac disease, increased IEL numbers, and proteolytic activities in the small intestine of recipient mice. This effect was mediated by the presence of bacterial-derived proteases through activation of proteinase-activated receptors. We identify a novel microbial protease-host pathway that could be targeted therapeutically to prevent enteropathy in a genetically susceptible host.

METHODS

Animals.

Female and male 8 to 12-week-old C57BL/6 mice were generated by two-stage embryo transfer and bred under germ-free conditions at the axenic gnotobiotic-unit (AGU) at McMaster University or, purchased from Taconic in specific-pathogen-free (SPF) conditions, and bred subsequently at McMaster's Central Animal Facility (CAF). Germ-free female and male 8 to 12-week-old non-obese diabetic HLA-DQ8 (NOD/DQ8) transgenic mice (Galipeau, Rulli et al. 2011), and germ-free HLA-DQ8 mice that lack mouse MHC class II molecules, but express human HLA-DQ8, were generated by 2-stage embryo transfer and bred at the AGU (Marietta, Black et al. 2004; Galipeau, Rulli et al. 2011). PAR-2$^{-/-}$ breeding pairs (R38E-PAR2) were obtained from Johannes Gutenberg University Mainz provided by Wolfram Ruf and bred at McMaster's CAF under SPF conditions. In some experiments, clean-SPF (originally altered Schaedler flora (ASF)-colonized and allowed to diversify for 2 generations, but devoid of Proteobacteria phylum) were used as murine microbiota donors. All colonies were fed an autoclaved gluten-free mouse diet (Harlan, Indianapolis, IN) for 2 generations (Harlan Laboratories, Indianapolis, IN) until used in experiments. All mice had unlimited access to food and water. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Human microbiota donors.

Fecal samples from children attending the Pediatric Clinic at the Hospital Universitario La Fe were collected (n=7). Healthy children attending the clinics for regular pediatric

check-up were used as control donors (n=8). See Supplementary Table 1 for demographic details. Samples were collected anaerobically and stored at -80°C until use for mouse colonization experiments. Bacterial strains (*Pseudomonas aeruginosa* and *Enterococcus faecalis*) were isolated from patients with active celiac disease attending the gastroenterology Division at Leon Hospital, Spain (Caminero, Galipeau et al. 2016; Herran, Perez-Andres et al. 2017).

Microbiota-humanized mouse model and bacterial supplementation.

For microbiota-humanized mouse colonizations, germ-free C57BL/6 mice were inoculated with fecal slurs (1:10 in PBS) processed anaerobically, from 4 randomly selected donors in the celiac, and 4 in the healthy control group (HD). Each human donor microbiota was used to colonize 4-5 mice. Subsequent experiments used one celiac and one control based on host IEL responses induced. After colonizations, the microbiota was allowed to stabilize for 3 weeks before dietary antigen sensitization (Geuking, Cahenzli et al. 2011) (Bercik, Verdu et al. 2010). For experiments using minimally colonized (gnotobiotic) mice, germ-free C57BL/6, HLA-DQ8 and NOD/DQ8 mice were gavaged with cecal contents of a clean-SPF (ASF-derived; 1/10 in PBS) mouse colonizer. Specific bacterial supplementation of clean-SPF mice was performed by oral gavage with *Pseudomonas aeruginosa* PA14, or its knockout mutant for elastase (LasB), using 10^{10} CFU/ per mouse, twice per week for 2 weeks. Given the more complex microbiota of SPF mouse colonies compared to clean-SPF, bacterial supplementation of SPF PAR2$^{-/-}$ mice was performed daily for 2 weeks (10^{10} CFU of bacteria/mouse). The presence of *Pseudomonas* in the supplemented mice was monitored individually by plating feces in

gluten media. For mono-colonizations using *Pseudomonas aeruginosa* PA14 or its LasB mutant, germ-free mice were gavaged once with 10^8 CFU/mouse.

Bacterial culture.

Gluten-degrading bacteria from the small intestine of colonized mice were cultured using previously described gluten-selective media (Caminero, Herran et al. 2014; Herran, Perez-Andres et al. 2017). *Pseudomonas aeruginosa* strains from the available and previously described non-redundant transposon mutant library PA14 were used (Liberati, Urbach et al. 2006). These strains were grown in TSB, adding gentamycin (50 ug/ml) only in those knockout mutants carrying Tn5 transposon, to the desired concentration. Proteolytic activity and phenotype of all strains were monitored using gluten media. Luminous *Pseudomonas aeruginosa* PA14 for measuring *LasB* expression was created. The *LasB* promoter was fused to the luciferase genes and integrated into the genome of PA14 at the CTX integration site, therefore not replacing the WT *LasB* promoter. This wild-type PA14 expresses light due to *LasB* expression, wherein the insertion is located at a neutral area in the genome, therefore the native *LasB* gene and promoter are intact and functional.

Gluten sensitization and challenge.

We used a previously described sensitization protocol, where mice are gavaged weekly with a combination of cholera toxin (CT) and pepsin, trypsin digested gliadin (PT-gliadin), over a 3-week period. Following PT-gliadin sensitization, mice were challenged by oral gavage with 2 mg of sterile gliadin (Sigma-Aldrich) dissolved in acetic acid three

times a week for 2 weeks (gluten treatment). In some experiments, non-sensitized mice were used as controls that had received cholera toxin alone during the sensitization phase and acetic acid alone during the challenge phase (Galipeau, Rulli et al. 2011).

Evaluation of enteropathy.

At sacrifice, sections of proximal small intestines were collected in 10% formalin, Carnoy's fixative or formaldehyde and paraffin embedded. Using immunohistochemistry, $CD3^+$ IEL were quantified in villi tips of formalin fixed sections. Slides were examined at 20X magnification using light microscopy in a blinded fashion. The number of $CD3^+$ IELs per 20 enterocytes were counted from five randomly chosen villus tips and expressed as IELs/100 enterocytes (Biagi, Luinetti et al. 2004). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation of tissue morphology under light microscopy (Olympus, ON, Canada). Using the Image-Pro 6.3 software (Mediacybernetics, MD, USA), enteropathy was quantified in a blinded fashion by measuring villus-to-crypt ratios (V:C) as previously described (Galipeau, Rulli et al. 2011).

Quantification of luminal proteolytic activity.

Non-specific, elastolytic and glutenasic activities of fecal bacteria were measured. Proteolytic activities were measured from mice small intestinal or bacterial isolate supernatants. Non-specific proteolytic activity was determined using the azocasein method (Sigma-Aldrich). Glutenasic activity was measured by bioassay using 1% gluten (Sigma-Aldrich) as previously described (Caminero, Galipeau et al. 2016). Positive

proteolytic activity was determined by the presence of a hydrolytic halo surrounding the inoculation site on media containing respective substrates (Caminero, Galipeau et al. 2016). Elastase activity was analyzed using Suc-Ala3-pNa (Sigma-Aldrich) and FITC-elastin (AnaSpec) substrates. Briefly, small intestinal washes and bacterial supernatants were incubated in 50 mM Tris-HCl buffer pH8.2 supplemented with 1mM CaCl2, 50 mM NaCl and Triton 0.25%, at 37 C with the different substrates. Absorbance and luminescence were measured at various time points in a kinetic way. Units of enzyme were determined using standard curves of trypsin from bovine pancreas (Sigma-Aldrich) and elastase from porcine pancreas (Sigma-Aldrich).

Bioluminescent Imaging.

Bioluminescent imaging was performed for detection of luminescent *LasB* detection with an IVIS Spectrum *in vivo* imaging system (PerkinElmer). Germ-free and clean-SPF mice were supplemented with PA14 expressing light in function of *LasB* expression (10^{10} CFU of bacteria/mouse) and luminescence was performed after 2 weeks.

PAR-2 expression.

Bacterial proteases were incubated with Caco-2 cells expressing human PAR-2 tagged to luciferase. Bacteria were incubated for 16 h in OptiMEM (Gibco) and then the supernatant was added to Caco-2 cells expressing PAR-2 for 20 min. Cleavage of the external domain of PAR-2 by proteases was detected by Nanoglo luciferase assay (Promega). The reagent contains an integral lysis buffer allowing use directly on cells expressing luciferase or the culture media when luciferase is secreted. PAR-2 expression was quantified in the small

intestine of mice using the rabbit polyclonal antibody PAR-2 (H-99) 57797 (Santa Cruz Biotechnology) raised against amino acids 230-238 of PAR-2. Small intestine pieces were fixed in paraformaldehyde 4% overnight and then incubated in 30% sucrose solution. After fixation, samples were imbedded in OCT and frozen at -80°C degrees. Sections of 8 μM were cut using a cryo-microtome and incubated with H-99 antibody diluted 1/500 overnight. A fluorophen-conjugated secondary rabbit antibody was applied for 1 hr and fluorescence was measured. PAR-2 expression was quantified using ImageJ and expressed as % of positive fluorescence/total area on the epithelial surface of the villi.

RNA processing and RT-PCR.

For RNA extraction from whole tissue, a Tissue-Tearor Homogenizer (Biospec) was used. RNA was prepared using the RNeasy Mini Kit (Qiagen). cDNA synthesis was performed using GoScript (Promega) according to the manufacturer's instructions. Expression analysis was performed in duplicate via real-time PCR on a Roche LightCycler 480 using SYBR Green (Clontech). Expression levels were quantified and normalized to *Gapdh* expression (F: AGGTCGGTGTGAACGGATTTG; R: TGTAGACCATGTAGTTGAGGTCA) using the following primer pairs: Murine *IL-15*, (F: CAT CCA TCT CGT GCT ACT TGT G R: GCC TCT GTT TTA GGG AGA CCT), Murine *IL-2rb* (F: TGGAGCCTGTCCCTCTACG;R: TCCACATGCAAGAGACATTGG), Murine *IL-2rg* (F: CTCAGGCAACCAACCTCAC; R: GCTGGACAACAAATGTCTGGTAG) and Murine IFN-γ (F: ATGAACGCTACACACTGCATC; R: TCTAGGCTTTCAATGACTGTGC).

Microbiota and bacterial isolate sequencing.

Bacteria were identified using HDA1 and HDA2 PCR primers specific to 16s DNA V4 region. These products underwent sequencing via Sanger. Fecal and small intestinal contents were collected and flash frozen on dry ice. DNA was extracted from samples and amplified for the hypervariable 16S rRNA gene v3 region for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA). Analysis of data was performed as previously described (Whelan, Verschoor et al. 2014). Briefly, sequences were trimmed using Cutadapt software (version 1.2.1) and aligned through the PANDAseq software (version 2.8) (Masella, Bartram et al. 2012). Operational taxonomic units selected using AbundantOTU (Ye 2011) were assigned taxonomy according to the Greengenes reference database (DeSantis, Hugenholtz et al. 2006). B-diversity was calculated and clustered based on Bray-Curtis distance matrices and statistics performed by PERMANOVA in Qiime.

RESULTS

Microbiota from celiac patients and controls induce differential immune responses in C57BL/6 mice independently of gluten.

Fecal microbiota from all active celiac patients and controls was sequenced via 16S V3 region (demographic characteristics outlined in Supplementary Table 1). Samples from the two groups clustered separately via β-diversity on a PCoA plot (Bray-Curtis Dissimilarity, $p=0.014$) (Figure 1A). No major differences in taxa were observed between celiac patients and controls (Kruskal-Wallis, FDR; $q<0.08$) (Figure 1B and Supplementary Figure 1).

To investigate the functional capacity of microbial communities from celiac patients and controls, we colonized germ-free C57BL/6 mice with 4 randomly selected donors from each group, and subsequently sensitized them to gluten (Figure 1C). Pooled group data revealed that mice colonized with microbiota from celiac patients had higher IEL counts than mice colonized with control microbiota (Figure 1E).Differential individual IEL counts were recorded in the small intestine of recipient mice, according to celiac patient or control donor (Figure 1D). Because all colonized mice had been exposed to gluten, we selected one celiac donor (CeD2) that induced high IEL (>10 IELs/100 enterocytes) counts in the mouse small intestine, and one control donor (HD3), with low IEL counts (<5 IELs/100 enterocytes), for further colonization experiments in the presence and absence of gluten treatment. We confirmed that mice colonized with CeD2 had higher IEL counts than mice colonized with HD3, and determined this was independent of gluten (Figure 1F). We found no differences in small intestinal transcript levels of IL-15 or two of its receptor subunits IL-2Rγ and IL-2Rδ (Figure 1G). However, IFN-γ was increased in mice colonized with CeD2 compared with HD3 (Figure 1G). No changes in villus-to-crypt ratios were observed in any of the groups (Figure 1H).

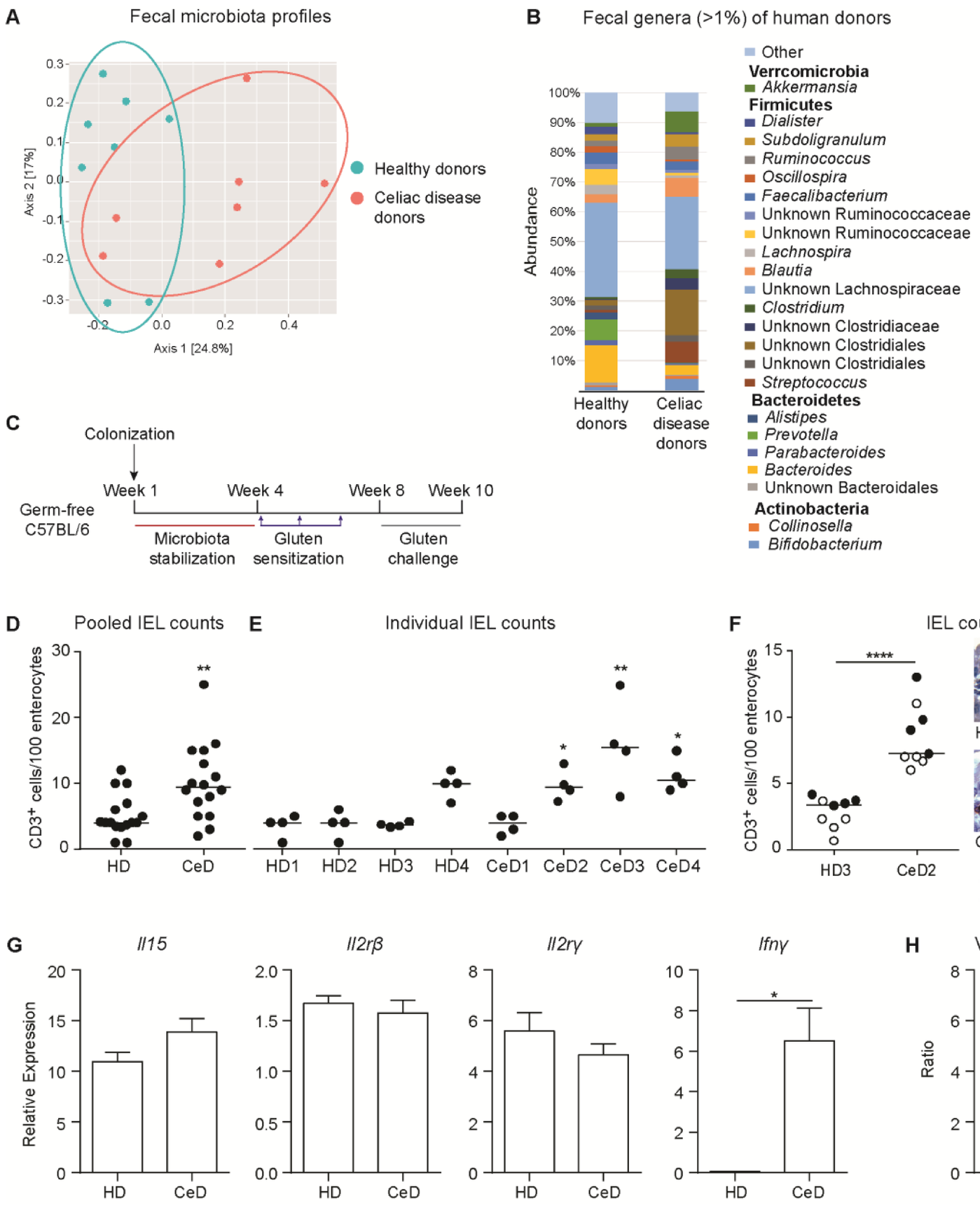

Figure 1. Celiac disease donors (CeD) and healthy donors (HD) harbor different fecal microbial communities and induce differential responses in gnotobiotic mice. (A) β-diversity Bray-Curtis dissimilarity PCoA plot of HD and celiac disease microbiota profiles. (B) HD and celiac disease profiles at the genus level. (C) Protocol for gluten sensitization and challenge in C57BL/6 mice. (D) Pooled IEL counts of colonized mice with HD and

celiac disease microbiota. (E) Small intestinal IEL counts of HD and celiac patient colonized mice. (F) Comparison of HD3 and CeD2 IEL counts with and without gliadin sensitization and challenge. (G) Quantification of gene expression of IL-15, its receptors IL-2Rβ and IL-2Rγ, and IFN-γ in the small intestine of mice colonized with HD3 or CeD2 (n=9-15). (H) Small intestinal villus-to-crypt ratios of mice colonized with HD3 or CeD2 (5-10). β-diversity statistics were performed by PERMANOVA in QIIME, in phyla, genera using Mann-Whitney followed by false discovery rate (FDR, $q<0.08$) in SPSS, Student's T-test and ANOVA with Tukey posthoc where applicable, $*p<0.05$ $**p<0.01$. Data represented as the mean ± standard error of the mean (SEM).

IEL counts associate with Proteobacteria expansion and presence of gluten-degrading bacteria in recipient mice.

We sequenced small intestinal contents from colonized gluten and control mice at the end of the experiments (Figure 2A, B and Supplementary Figure 2). 16s V3 sequencing revealed that mice colonized with CeD2 microbiota had higher proportions of Proteobacteria ($q<0.08$) and Actinobacteria ($q<0.08$), with depletions in Firmicutes ($q<0.08$), independently of gluten treatment (Figure 2C). Specifically, within this phylum, we observed high proportions of *Parasutterella* ($q<0.08$) in CeD2-colonized mice, along with differences in *Slakia* and *Holdermania* (Figure 2D) within Actinobacteria and Firmicutes, respectively.

We next examined the proteolytic capacity of the microbial communities used in mouse colonizations. Using described bioassays that employ gluten as the only protein source, we observed increased proteolytic activity in small intestinal washes of mice colonized with CeD2, then with HD3 ($p<0.01$) (Figure 2E). To determine whether the higher proteolytic activity observed was of bacterial origin, small intestinal washes from the colonized mice were plated aerobically and anaerobically on gluten-containing media. Only mice colonized with CeD2 harbored gluten-degrading bacteria in the small intestine. We then isolated 5 bacteria that were classified by 16S Sanger sequencing as *Enterococcus faecalis*, *Bacillus subtilis* and *Staphylococcus epidermidis* (Figure 2F). Some of these strains had elastase activity, a virulence factor present in opportunist pathogens that have been previously described in celiac disease patients (Sanchez, Donat et al. 2013) (Figure 2G). Thus, we screened for strains with elastase activity, from our collection of small intestinal bacteria previously isolated from patients with active celiac disease (Herran, Perez-Andres et al. 2017). *Pseudomonas aeruginosa* X-46.1 and *Clostridium perfringens* A-40 had elastase activity, with *Pseudomonas* being the most efficient at degrading elastin (Figure 2G).

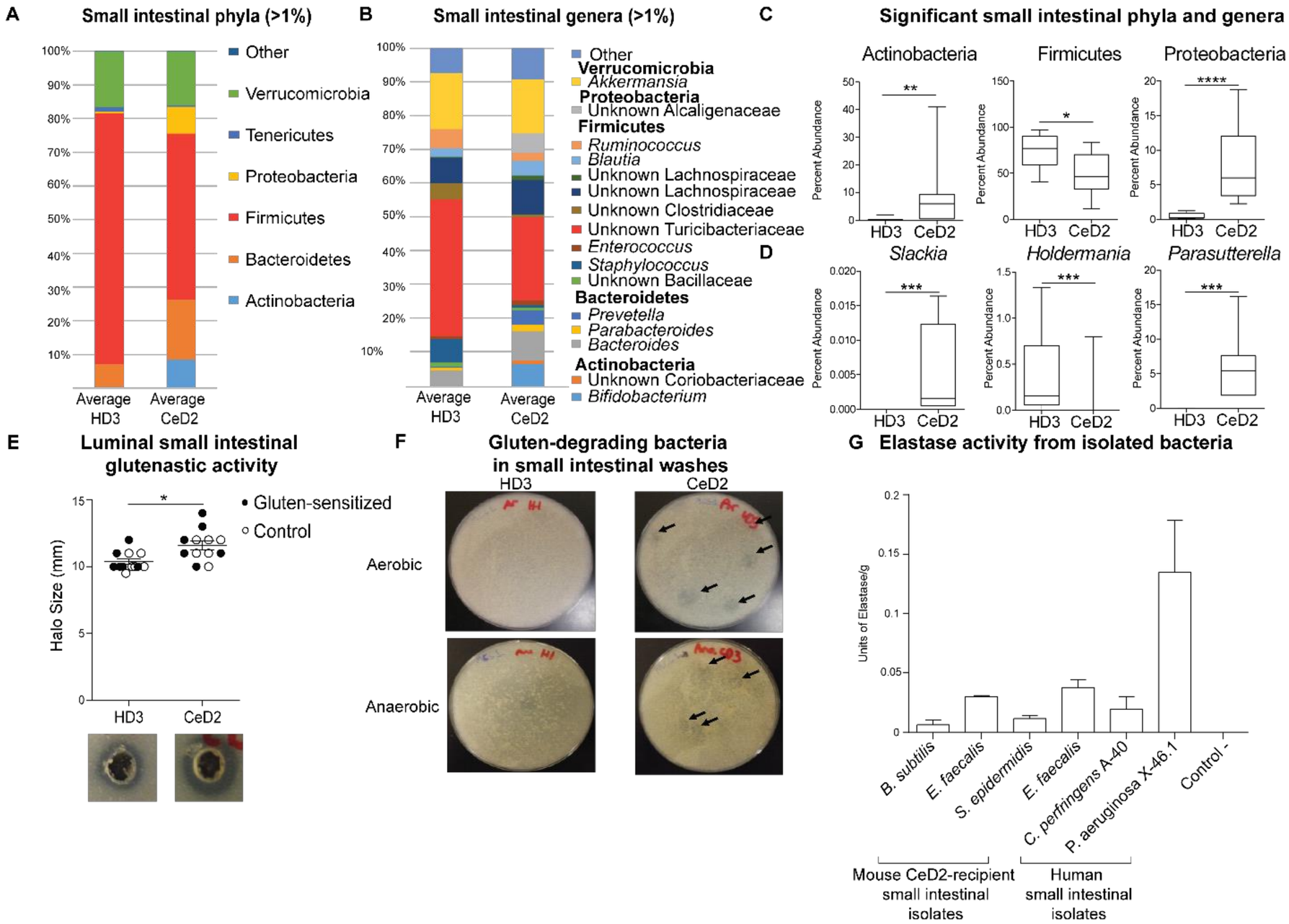

Figure 2. Characterization of small intestinal microbiota in C57BL/6 mice colonized with celiac (CeD2) or control (HD3) microbiota. (A) Microbial composition of small intestinal contents from HD3 and CeD2 donors at the phyla level (n=10/group). (B) Microbial composition of small intestinal contents from HD3 and CeD2 donors at the genus level (n=10/group). (C) Significant differences in phyla between HD3 and CeD2-colonized mice (n=10/group). (D) Significant differences in genera between HD3 and CeD2-colonized mice (n=10/group). (E) Small intestinal glutenasic activity of HD3 and CeD2-colonized mice. (F) Gluten-degrading bacteria isolated from the small intestine of mice colonized with HD3 and CeD2 donors. (G) Elastase activities of bacterial strains isolated

from the small intestine of CeD2 colonized mice and humans. Data represented as min to max or mean ± SEM. Statistical significance determined by Mann-Whitney followed by False Discovery Rate (FDR, q<0.08), and Student's T-test or Mann-Whitney where applicable, p<0.05 **p<0.01, ***p<0.001, ****p<0.0001.

Gluten promotes elastase-producing opportunist pathogen growth and increases IELs in C57BL/6 mice.

We chose *P. aeruginosa* wild-type PA14 as a model organism to further investigate the contributions of bacterial elastase to innate immune responses in the small intestine. First, we supplemented clean-SPF C57BL6 mice with a luminescent-producing mutant linked to the *LasB* promoter, to confirm PA14 colonization and expression of the elastase in the small intestine (Supplementary Figure 3A). We found that PA14 colonized the small intestine of these mice and expressed the protease gene, *LasB* (Figure 3A). Next, we utilized a *P. aeruginosa* PA14 knockout (KO) mutant of *LasB* gene encoding for bacterial elastase (*LasB*) (Liberati, Urbach et al. 2006). We confirmed by molecular techniques and gluten media plating that mutants lack proteolytic activity towards gluten substrates compared to PA14 (Supplementary Figure 3A, B). We supplemented clean-SPF mice with PA14 or, the *LasB* mutant, and then exposed them to gluten treatment (Figure 3B). *P. aeruginosa* load in the small intestine was similar between PA14 and *LasB* (Figure 3C) and bacteria maintained their *in vitro* proteolytic phenotype (Supplementary Figure 3C). PA14-supplemented mice had higher elastase activity in the small intestine than *LasB*-colonized

mice (Figure 3D) and gluten increased *P. aeruginosa* load and elastolytic activity (Figures 3C and D). PA14 also increased IEL counts compared to mice supplemented with the *LasB* mutant (Figure 3E). However, neither PA14 nor *LasB* decreased villus-to-crypt ratios in C57BL/6 mice (Figure 3F).

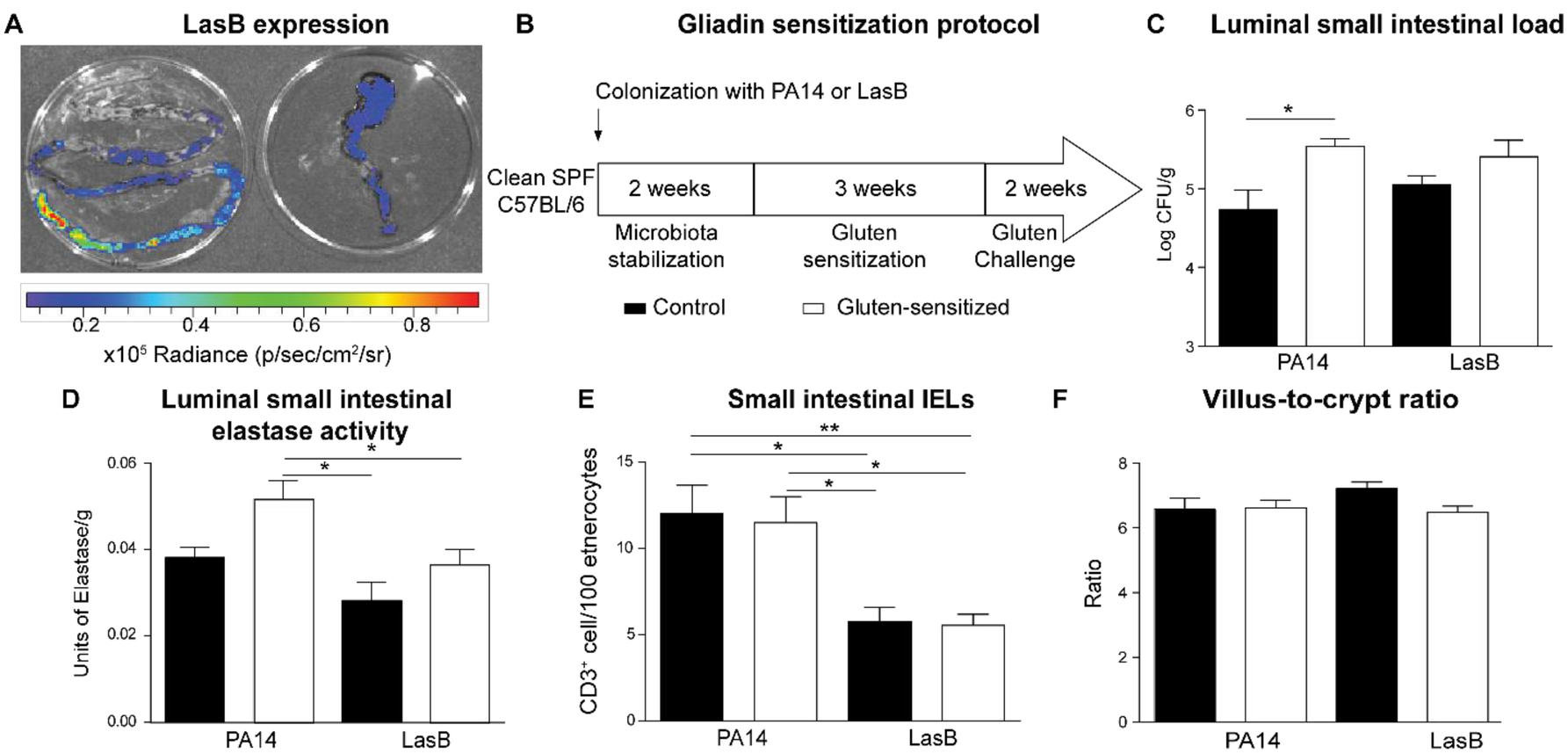

Figure 3: *P. aeruginosa*-producing elastase increases IELs in clean SPF C57BL/6 mice. (A) Visual expression of *LasB* in the gastrointestinal tract of clean SPF-colonized mice using luminescent producing mutant linked to the *LasB* promoter for 2-week colonization. (B) Protocol for gliadin sensitization and gliadin challenge of Clean SPF C57BL/6 mice colonized with PA14 or *LasB*. (C) Colonization rates of *P. aeruginosa* in the small intestine of clean SPF C57BL/6 mice colonized with PA14 or *LasB*. (D) Elastase activity measured in the small intestine of clean SPF C57BL/6 mice colonized with PA14 or *LasB*. (E) Quantitative measurement of IELs/100 enterocytes in villi tips of clean SPF C57BL/6 mice colonized PA14 or *LasB*. (F) Small intestinal villus-to-crypt ratios of clean

SPF C57BL/6 mice colonized with PA14 or *LasB*. n=4-5/group. Data represented as mean ± SEM. Statistical significance was determined by ANOVA followed by Tukey post hoc analysis, *p<0.05 **p<0.01.

Elastase activity is required for P. aeruginosa-induced increases in IELs.

To determine PA14 elastase was responsible for the increase in IEL counts, and to rule out possible secondary contributions of a dysbiotic microbiota, we mono-colonized germ-free C57BL/6 mice with various *P. aeruginosa* strains. Utilizing the luminescent-producing mutant linked to the *LasB* promoter, we confirmed expression of the elastase gene *in vivo* (Figure 4A). To investigate the role of host genetics, we also mono-colonized germ-free HLA-DQ8 mice on a C57BL/6 background with PA14, or *LasB*, for 2 weeks (Figure 4B). We recovered 10^4-10^5 CFU/g of small-intestinal content in each group at endpoint (data not shown). Small intestinal washes of PA14-colonized mice had higher elastase and glutenasic activity than *LasB*-colonized mice (Figure 4C and 4D). As previously reported, mice mono-colonized with PA14 had higher levels of IELs in the small intestine suggesting that this phenotype was produced by protease-host interaction (Figure 4E).

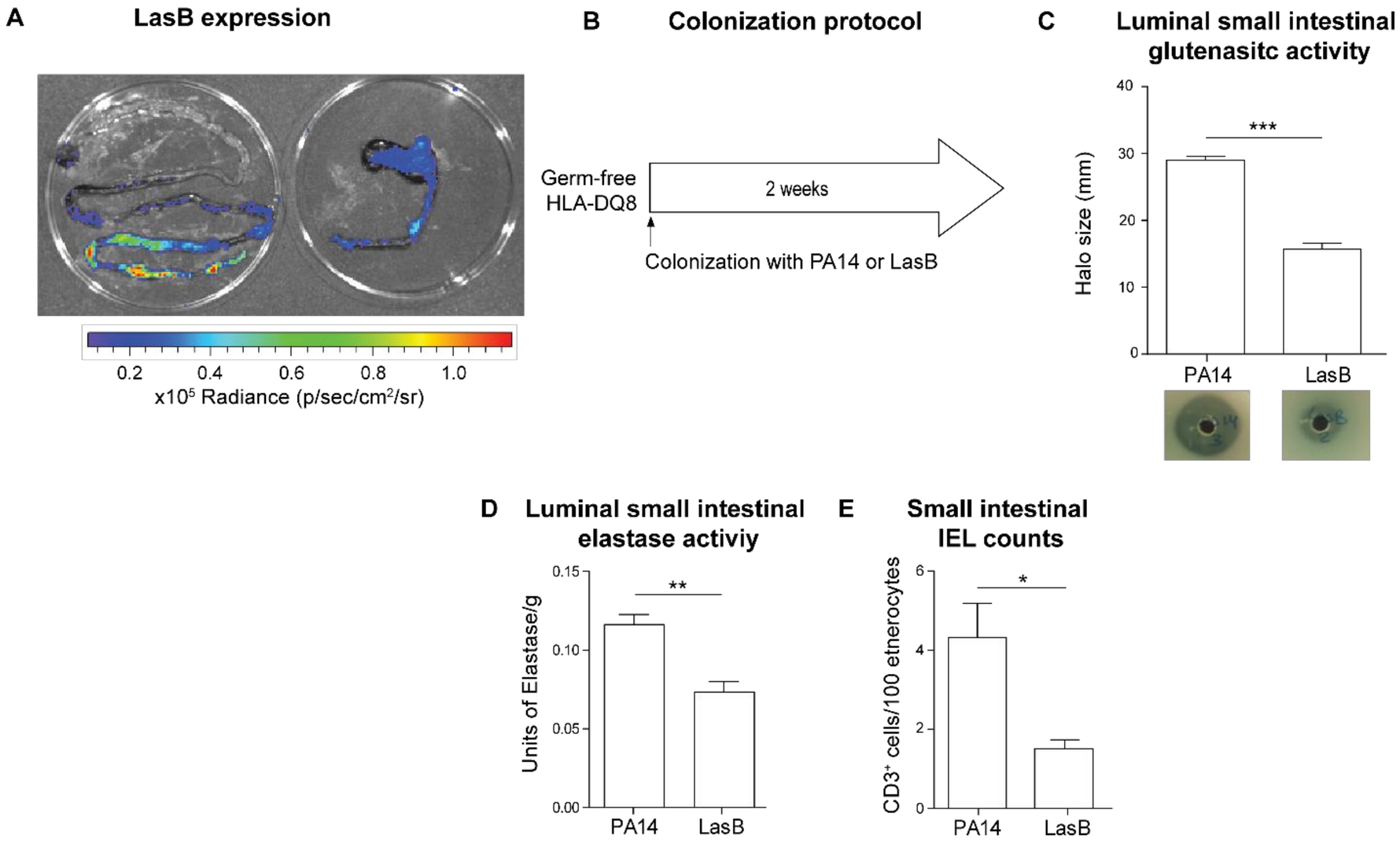

Figure 4. *P. aeruginosa* producing elastase induces IELs. (A) Monocoloinzation with luminescent producing mutant linked to the *LasB* promoter. (B) Protocol for monocolonization of GF HLA-DQ8 mice with PA14 or *LasB*. (C) Luminal small intestinal glutenastic activity from mice monocolonized with PA14 or *LasB*. (D) Luminal small intestinal elastase activity from mice monocolonized with PA14 or *LasB*. (E) Quantitative measurement of IELs/100 enterocytes in villi tips of PA14 or *LasB* monocolonized mice. Data represented as mean ± SEM (n=6/group). Statistical significance determined by Student's T-test, *p<0.05**p<0.01***p<0.001.

Small intestinal bacteria and pathobionts with proteolytic activity, cleave proteinase activated receptor-2.

Protease-activated receptors (PARs) are a subfamily of G-protein-coupled receptors that are activated by the proteolytic cleavage of part of their extracellular domain. We analyzed whether gluten-degrading bacteria isolated from the small intestine of mice colonized with CeD2 cleaved the external domain of PAR-2 using a transgenic cell line expressing these receptors. We found that *Staphylococcus*, *Bacillus*, and *Enterococcus* cleaved the external domain of PAR-2 (Figure 5A). We also found that PA14, but not *LasB* mutant, cleaved the external domain of PAR-2, suggesting that bacterial elastase activates this pathway (Figure 5A). In accordance with this, immunostaining for PAR-2 in the small intestine of mice supplemented with PA14, but not *LasB* mutant, had increased PAR-2 epithelial expression, independently of gliadin exposure (Figure 5B). Finally, PA14 supplementation of SPF PAR-$2^{-/-}$ mice (Figure 5C) showed lower IEL counts than wild-type mice (Figure 5D), although they harbored the same luminal small intestinal elastase activity (Figure 5E).

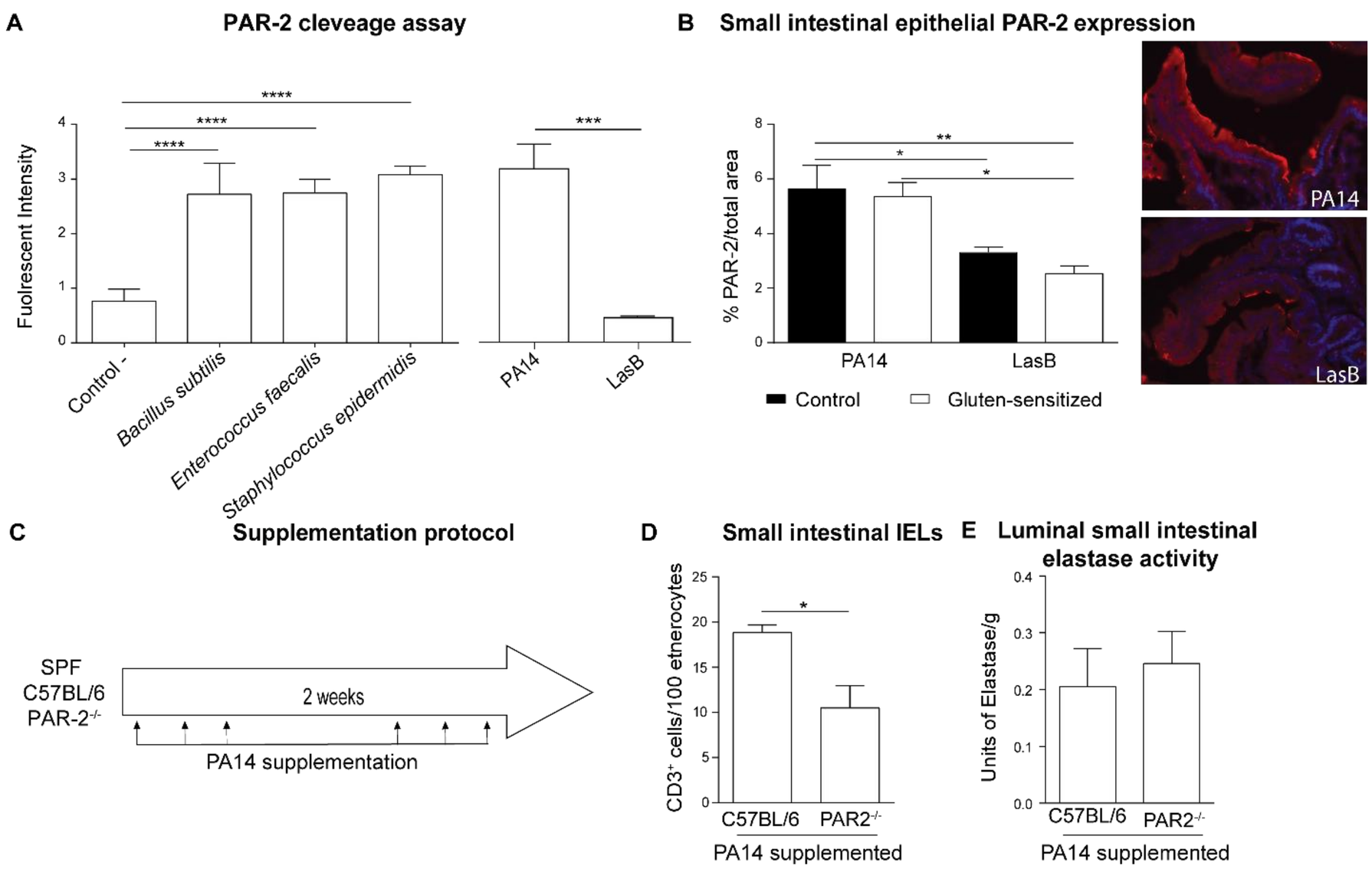

Figure 5. Gluten degrading bacteria cleave proteinase activated receptor-2 and rely on PAR-2 for IEL induction *in vivo*. (A) Cleavage of the external domain of PAR-2 by gluten-degrading bacteria isolated from CeD2-colonized mice and from PA14 and *LasB* (N=3/group). (B) Immunostaining of PAR-2 in the small intestine of clean SPF C57BL/6 mice colonized with PA14 or *LasB* (N=4-5/group). (C) Protocol for supplementation of SPF C57BL/6 and PAR-$2^{-/-}$ mice supplemented with PA14. (D) Quantitative measure of IELs/100 enterocytes in villi tips of SPF C57BL/6 and PAR-$2^{-/-}$ mice with PA14 (N=3/group). (E) Luminal small intestinal elastase activity from SPF C57BL/6 and PAR-$2^{-/-}$ mice supplemented with PA14 (N=3/group). Data represented as mean ± SEM. Statistical significance determined by ANOVA with Tukey post hoc analysis or Student's T-test where applicable, *p<0.05**p<0.01***p<0.001****p<0.0001.

Gluten enhances bacterial elastase activity and worsens immunopathology in NOD/DQ8.

We have previously shown that responses to gluten in NOD/DQ8 mice are microbiota-dependent and that clean-SPF colonized mice, are protected from developing immunopathology (Galipeau, McCarville et al. 2015). We, therefore, supplemented clean-SPF NOD/DQ8 mice with PA14, or *LasB*, and subsequently treated them with gluten (Figure 6A). *P. aeruginosa* strains successfully colonized the small intestine (Figure 6B), increased elastase activity (Figure 6C) and increased IEL counts (Figure 6D). In contrast to C57BL/6 mice, the presence of PA14 resulted in decreased small intestinal villus-to-crypt ratio after gliadin exposure in NOD/DQ8 mice (Figure 6E). *P. aeruginosa*-supplemented mice also had increased PAR-2 expression (Figure 6F).

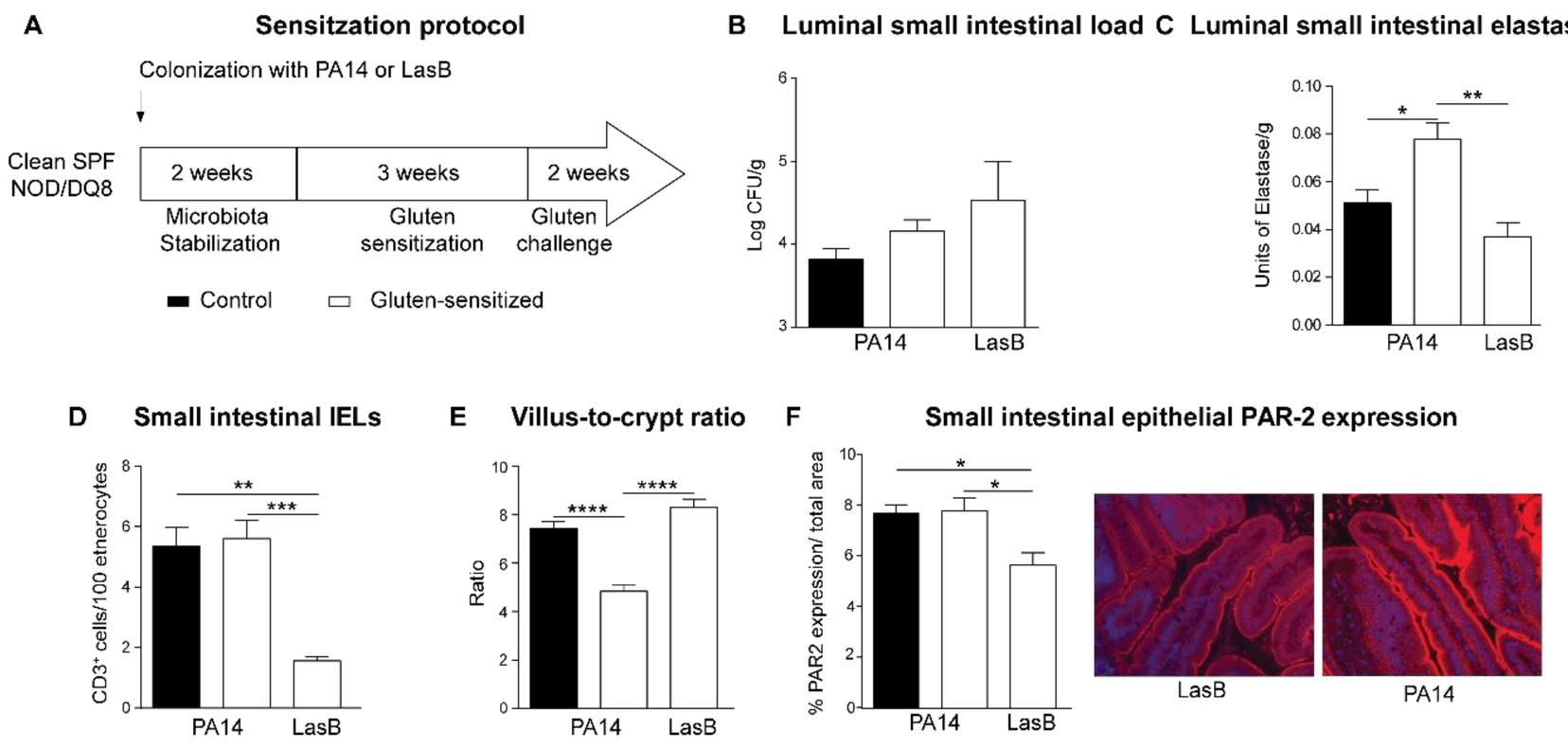

Figure 6. *P. aeruginosa* producing elastase enhances gluten sensitivity in genetically susceptible mice. (A) Protocol for gliadin sensitization and gliadin challenge of clean SPF NOD/DQ8 mice colonized with PA14 or *LasB*. (B) Colonization rates of *P. aeruginosa* in

the small intestine of clean SPF NOD/DQ8 colonized with PA14 or *LasB*. (C) Luminal small intestinal elastase activity of clean SPF NOD/DQ8 mice colonized with PA14 or *LasB*. (D) Quantitative measure of IELs/100 enterocytes in villi tips of clean SPF NOD/DQ8 colonized mice PA14 or *LasB*. (E) Villus-to-crypt ratios of clean SPF NOD/DQ8 mice with PA14 or *LasB*. (F) PAR-2 expression and quantification on epithelium of clean SPF NOD/DQ8 mice colonized with PA14 or *LasB*. N=4-10/group. Data represented as mean ± SEM. Statistical significance determined by ANOVA with Tukey post hoc analysis, *p<0.05**p<0.01***p<0.001****p<0.0001.

DISCUSSION

Microbiota-modulating approaches based on the inhibition of pro-inflammatory bacterial proteolytic activity could be developed to prevent host inflammatory reactions to food proteins like gluten. This inhibition would require knowledge of the proteases involved, and the signaling events in the host. Our results demonstrate that the small intestinal microbiota of patients with active celiac disease, a condition that develops when oral tolerance to gluten is broken, has increased proteolytic capacity and is capable of increasing IEL counts in colonized mice. We observed donor-dependent increases in $CD3^+$ IELs in individual germ-free recipient mice, with a clear overall induction of higher counts in all groups colonized with celiac microbiota. Subsequent mouse colonization experiments using selected donors (CeD2 and HD3), that increased, or not, IELs respectively, demonstrated that the effect was not induced by gluten, but associated with gluten-

degrading bacteria in the small intestine of the CeD2-recipient mice. These results provide a mechanistic link for the clinical data reporting small intestinal overgrowth and expansion of pathobionts members of Proteobacteria, in active (Wacklin, Kaukinen et al. 2013) and non-responsive celiac disease to the gluten-free diet (Wacklin, Laurikka et al. 2014).

P. aeruginosa from celiac patients can metabolize, through elastase production, non-digested gluten by human proteases into immunogenic peptides recognized by human gluten-specific T-cells (Caminero, Galipeau et al. 2016). While this constitutes a *microbe-diet-host* event that affects the adaptive pathway in celiac disease, we here demonstrate that gluten exposure also leads to blooming of *P. aeruginosa* and bacterial elastase production in the small intestine of colonized mice. Indeed, *in vitro*, PA14 can outcompete other bacteria in the presence of gluten. The ubiquitous presence of gluten in the diet, and its resistance to gastrointestinal digestion allows the presence of high molecular weight oligopeptides in the gut lumen (Hausch, Shan et al. 2002) that become substrates of microbial metabolism. Our results identify a direct *diet-microbe* interaction that supports previously described gluten-degrading bacterial overgrowth in celiac patients on a gluten-containing diet and the presence of opportunistic pathogens such as *Pseudomonas* spp (Caminero, Galipeau et al. 2016; D'Argenio, Casaburi et al. 2016).

To investigate underlying pathways, we used *P. aeruginosa* as a model Proteobacteria member with high elastolytic activity, previously isolated from the small intestine of patients with active celiac disease (Caminero, Galipeau et al. 2016). Clean-SPF mice supplemented with either PA14, or a mutant incapable of producing elastase (*LasB*), developed increased $CD3^+$ IEL counts in comparison to *LasB*, and gluten was not required

for this effect. This suggests that induction of IELs in the host is mainly dependent on the elastolytic activity of the strain. Previous work has shown that IEL numbers are reduced in the small intestine of germ-free mice and that colonization with SPF microbiota restores their numbers (Imaoka, Matsumoto et al. 1996). It has also been previously shown that mice colonized with pathobiont, such as segmented filamentous bacteria, have higher numbers of IELs than conventional SPF mice (Umesaki, Okada et al. 1995). However, the exact pathways behind this induction are unclear. Our work demonstrates the ability of small intestinal bacteria with glutenastic activity, to increase IELs, an important feature in the progression to enteropathy in celiac disease.

The receptor/s involved in the induction of innate immune responses in celiac disease are unknown. This is partly because there is great controversy on the potential triggers of this response, which, unlike the gluten-dependent adaptive immune pathway, may be gluten independent (Kim, Mayassi et al. 2015). PARs are protein-coupled receptors that are activated by the proteolytic cleavage of part of their extracellular domain and are ubiquitously expressed in the gut, the most exposed organ to host and bacterial proteases. Activation of PARs induces a wide array of pro-inflammatory and proliferative effects. Our study reveals that *P. aeruginosa* elastase cleaves the external domain of PAR-2 *in vitro*, suggesting activation of the receptor. Moreover, administration of PA14 to SPF PAR-$2^{-/-}$ mice, leads to lower IEL counts than in wild-type C57/BL6 mice, implicating PAR-2 signaling in IEL homeostasis. Thus, we demonstrate the role of microbial elastase in the induction of an innate immune response in the host that could favor the development of celiac disease in the presence of genetic risk.

We have previously shown that mice expressing the HLA-DQ8 gene in NOD background (NOD/DQ8) develop moderate gluten-immunopathology, including reductions in villus-to-crypt ratios, in SPF conditions. In contrast, gnotobiotic NOD/DQ8 mice colonized with a clean-SPF (ASF-derived) microbiota devoid of opportunistic pathogens and Proteobacteria, are protected (Galipeau, McCarville et al. 2015). The model can thus be employed to investigate microbially-driven mechanisms that influence host responses to gluten. Here we found that PA14 supplementation of clean-SPF NOD/DQ8, but not *LasB* mutant, increases IEL counts and reduces villus-to-crypt ratios after gluten. Thus, in contrast to C57BL/6 mice, that only develop increases in IELs when colonized with PA14, genetically susceptible mice that are protected from gluten immunopathology while kept under gnotobiotic conditions, progress to develop moderate villus blunting when supplemented with PA14 and exposed to gluten. These results highlight the complexity of interactions leading to the development of immunopathology and food sensitivity which includes a dietary trigger, genetic predisposition, and specific microbial-induced pathways, including activation of PAR-2 signaling and IEL induction. Microbiota-modulating therapies are currently employed empirically in clinical practice with little pathophysiological understanding. Given the current interest in the development of adjuvant therapies to the gluten-free diet, our results identify a microbial-driven mechanism, through protease production and PAR-2 signaling, that could be exploited therapeutically in gluten-related disorders and other food sensitivities.

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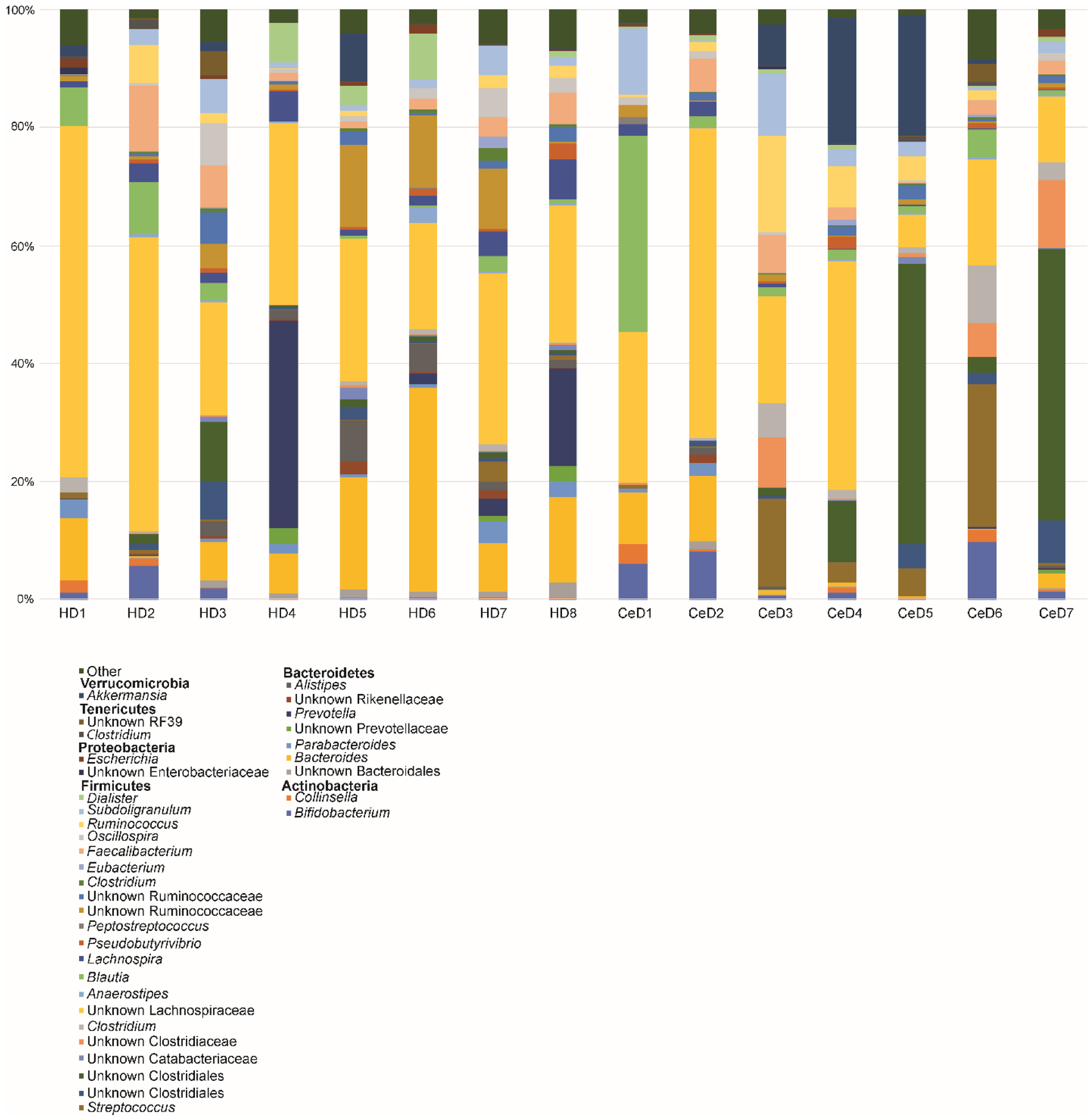
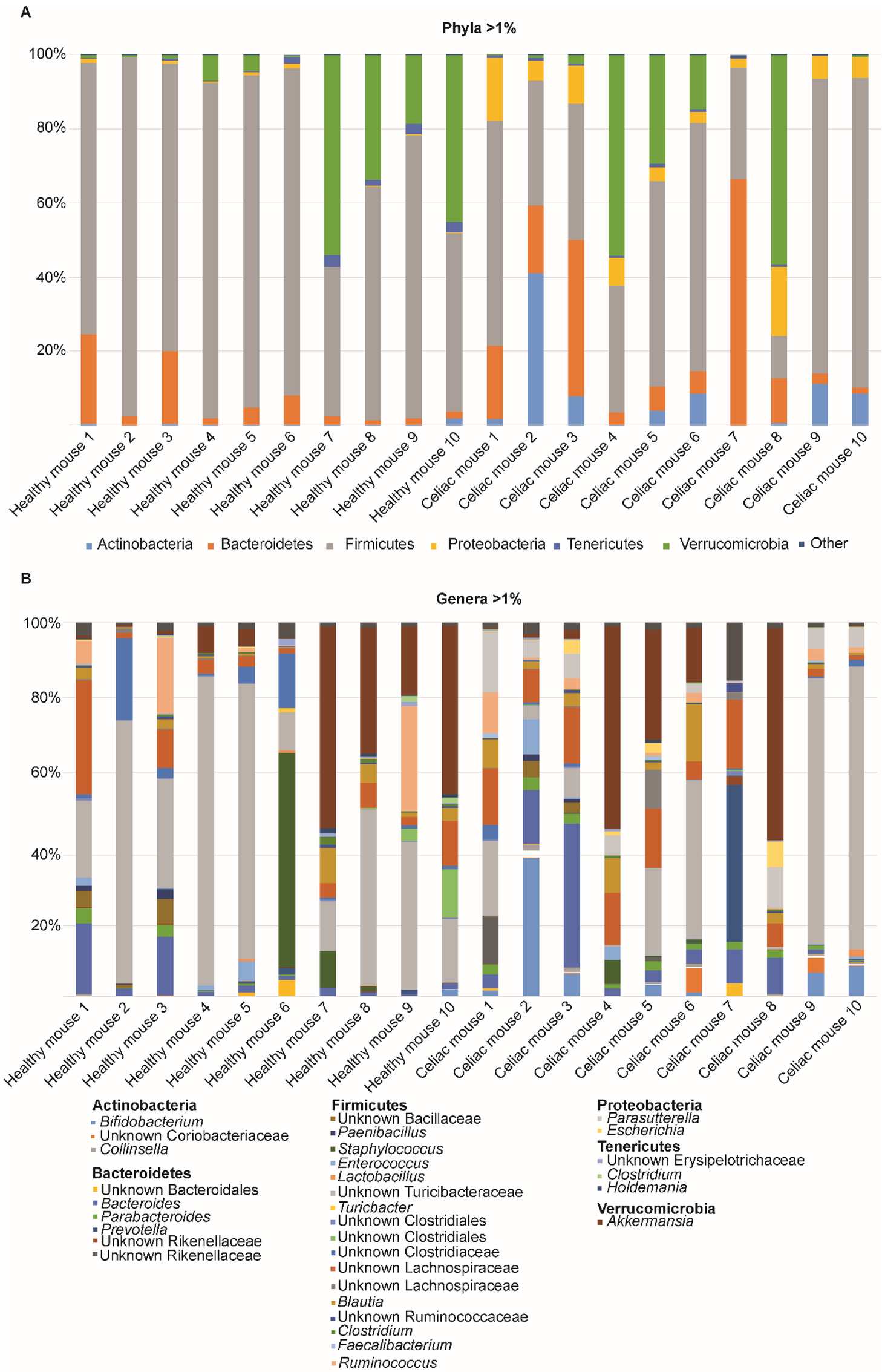
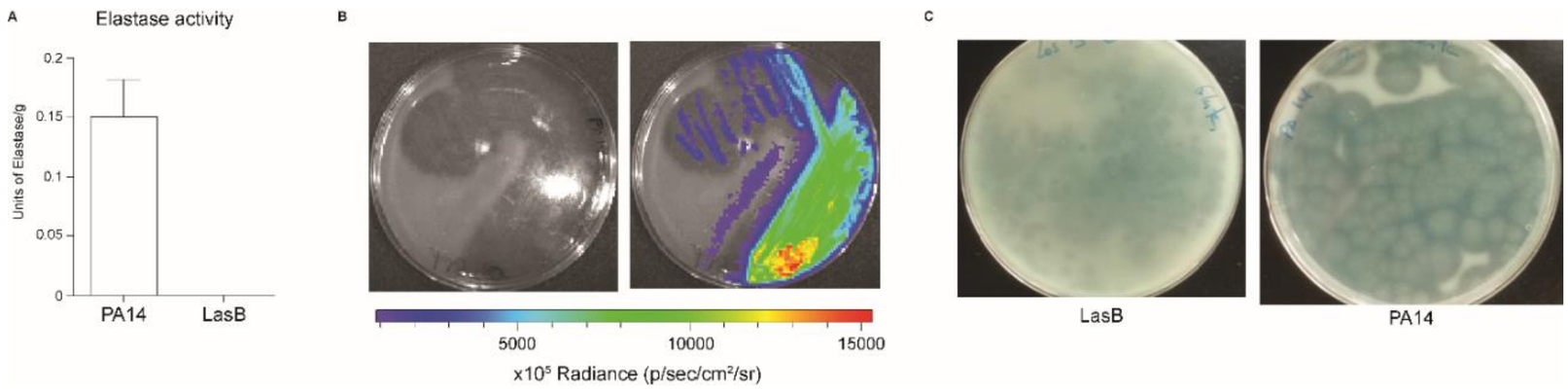
SUPPLEMENTARY MATERIALS



Supplementary Figure 1. Fecal microbiota at the genus level (>1%) of individual celiac patients and healthy controls. 16s data were produced in QIIME, genera statistics using Mann-Whitney followed by false discovery rate (FDR, $q<0.08$) in SPSS.


A
Phyla >1%
100%
80%
60%
40%
20%
Healthy mouse 1
Healthy mouse 2
Healthy mouse 3
Healthy mouse 4
Healthy mouse 5
Healthy mouse 6
Healthy mouse 7
Healthy mouse 8
Healthy mouse 9
Healthy mouse 10
Celiac mouse 1
Celiac mouse 2
Celiac mouse 3
Celiac mouse 4
Celiac mouse 5
Celiac mouse 6
Celiac mouse 7
Celiac mouse 8
Celiac mouse 9
Celiac mouse 10
Actinobacteria
Bacteroidetes
Firmicutes
Proteobacteria
Tenericutes
Verrucomicrobia
Other
B
Genera >1%
100%
80%
60%
40%
20%
Healthy mouse 1
Healthy mouse 2
Healthy mouse 3
Healthy mouse 4
Healthy mouse 5
Healthy mouse 6
Healthy mouse 7
Healthy mouse 8
Healthy mouse 9
Healthy mouse 10
Celiac mouse 1
Celiac mouse 2
Celiac mouse 3
Celiac mouse 4
Celiac mouse 5
Celiac mouse 6
Celiac mouse 7
Celiac mouse 8
Celiac mouse 9
Celiac mouse 10
Actinobacteria
Bifidobacterium
Unknown Coriobacteriaceae
Collinsella
Bacteroidetes
Unknown Bacteroidales
Bacteroides
Parabacteroides
Prevotella
Unknown Rikenellaceae
Unknown Rikenellaceae
Firmicutes
Unknown Bacillaceae
Paenibacillus
Staphylococcus
Enterococcus
Lactobacillus
Unknown Turicibacteraceae
Turicbacter
Unknown Clostridiales
Unknown Clostridiales
Unknown Clostridiaceae
Unknown Lachnospiraceae
Unknown Lachnospiraceae
Blautia
Unknown Ruminococcaceae
Clostridium
Faecalibacterium
Ruminococcus
Proteobacteria
Parasutterella
Escherichia
Tenericutes
Unknown Erysipelotrichaceae
Clostridium
Holdemania
Verrucomicrobia
Akkermansia

Supplementary Figure 2. Small intestinal microbiota of mice colonized with CeD2 (celiac mice) or HD3 (healthy mice). (A) Small intestinal microbiota of colonized mice at the phyla level. (B) Small intestinal microbiota of colonized mice at the genera level. 16s data were produced in QIIME, genera statistics using Mann-Whitney followed by false discovery rate (FDR, $q<0.08$) in SPSS.



Supplementary Figure 3. Degradation of gluten by *P. aeruginosa* via elastase activity.

(A) Elastase activity from *P. aeruginosa* lab strain PA14 and mutant *LasB*. (B) Luminescence linked to expression of *LasB* corresponding with degradation of gluten by *P. aeruginosa*. (B) Degradation of gluten by *P. aeruginosa* with intact *LasB* expression.

Supplementary Table 1. Celiac disease donor characteristics

	CeD1	CeD2	CeD3	CeD4	CeD5	CeD6	CeD7
Genetics	DQ2.5	DQ2/8	DQ2	DQ2	DQ2	DQ2	DQ2
Family history 1st or 2nd degree relative or negative	HLA compatible	HLA compatible	No	Yes 1st degree	No	No	Yes 2nd degree
Marsh grade	3b	3c	3b	3b	3b	3b	3b
Age of diagnosis	1 year and 10 months	4 years	12 years	8.5 years	6 years	9 years	6 years
Gender	Female	Male	Female	Male	Male	Male	Male
Antibodies	TG2+ >100, EMA+	AGA +, TG2+ >100	TG2+ >100	TG2+ >100	TG2+ >100	TG2 >100	TG2+ >100

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CHAPTER 5:

A COMMENSAL *BIFIDOBACTERIUM LONGUM* STRAIN IMPROVES GLUTEN-RELATED IMMUNOPATHOLOGY IN MICE THROUGH EXPRESSION OF A SERINE PROTEASE INHIBITOR

Summary and Significance

Aim: To investigate whether administration of a commensal producing a protease inhibitor protects against gluten-induced pathology in a mouse model expressing the human DQ8 gene.

Summary: Our lab has previously shown a protease-anti-protease imbalance in celiac disease. Specifically, individuals with celiac disease have decreased levels of the serine protease inhibitor, elafin, within their mucosa. Further, the results presented thus far in this thesis have demonstrated a role for bacterial-derived proteases in the pathogenesis of celiac disease (innate and adaptive mechanisms). Utilizing a recombinant *Lactococcus lactis* delivering elafin, we have previously demonstrated that delivery to NOD/DQ8 mice prevents gluten-induced immunopathology. Although promising, issues have been raised as to the clinical applicability of the *L. lactis* producing elafin as it is a genetically modified organism (GMO). For this reason, we tested a *Bifidobacterium longum* (*Bl*) (NCC2705) endogenously producing a serine protease inhibitor (serpin). We demonstrate that equal ability to protect against gluten-induced pathology in the NOD/DQ8 model of gluten sensitivity compared to *L. lactis* producing elafin. Further, using two transgenic strains, a *Bl* serpin knockout strain, and a *Bl* serpin constitutive expressing strain, we demonstrate that this protective capacity is due to the endogenous serpin. Interestingly, we observed shifts in the microbiota between the different *Bl*-treated groups, which may partially contribute to its protective effects.

Significance: A role of extracellular proteases in gastrointestinal disorders has been demonstrated, and for the first time we show that a human commensal bacterium can

prevent the onset of immunopathology via its ability to inhibit proteases. This data could provide grounds for the next steps into human clinical trials for a supportive therapy for gluten-related disorders or other gastrointestinal disorders in which proteolytic imbalances have a role in pathogenesis.

Preface: In this chapter, I performed SPF NOD/DQ8 experiments (administration of *B. longum* strains and sample collection), 16s sequencing (PCR and analysis), immunohistochemistry ($CD3^+$ staining and IEL counts) and histological analysis.

McCarville JL, Dong J, Caminero A, Bermudez-Brito M and Jury J performed experiments. **McCarville JL** and Dong J carried out all animal work. **McCarville JL** performed all microbiota work and analysis. Bermudez-Brito M performed elastase inhibition experiments. Murray JA provided the NOD/DQ8 mice and contributed critical evaluation of the manuscript. Langella P provided the *L. lactis* elafin producing strain and contributed critical evaluation of the manuscript. Duboux S, Steinmann M, Delley M, Tangyu M, Langella P, Mercenier A, Bergonzelli G, Verdu EF designed experiments and contributed scientific input. **McCarville JL**, Dong J and Verdu EF wrote the manuscript.

A commensal *Bifidobacterium longum* strain improves gluten-related immunopathology in mice through expression of a serine protease inhibitor

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Running Title: A commensal *B. longum* prevents gluten pathology through expression of a serpin

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ABSTRACT

Microbiota-modulating strategies, including probiotic administration, have been tested for the treatment of chronic gastrointestinal diseases despite limited information regarding their mechanisms of action. We previously demonstrated that patients with active celiac disease have decreased duodenal expression of elafin, a human serine protease inhibitor, and supplementation of elafin by a recombinant *Lactococcus lactis* prevents gliadin-induced immunopathology in the NOD/DQ8 mouse model of gluten sensitivity. The commensal probiotic strain *Bifidobacterium longum* NCC2705 produces a serine protease inhibitor (Srp) that exhibits immune-modulating properties. Here, we demonstrate that *B. longum* NCC2705, but not a *srp* knockout mutant, attenuates gliadin-induced immunopathology and impacts intestinal microbial composition in NOD/DQ8 mice. Our results highlight the beneficial effects of a serine protease inhibitor produced by commensal *B. longum* strains.

IMPORTANCE:

Probiotic therapies have been widely used to treat gastrointestinal disorders with variable success and poor mechanistic insight. Delivery of specific anti-inflammatory molecules has been limited to the use of genetically modified organisms, which has raised some public and regulatory concerns. By examining a specific microbial product naturally expressed by a commensal bacterial strain, we provide insight into a mechanistic basis for the use of *B. longum* NCC2705 to help treat gluten-related disorders.

INTRODUCTION

Microbiota-modulating therapies have been tested for the treatment of chronic gastrointestinal diseases and disorders with inconsistent findings. Probiotics are live microorganisms, which when administered in adequate amounts, confer a health benefit on the host (Food and Agriculture Organization and World Health Organization, 2002). Specific strains have shown modest efficacy in irritable bowel syndrome (IBS) (Moayyedi, Ford et al. 2010), complications of inflammatory bowel disease (IBD), such as pouchitis (Mimura, Rizzello et al. 2004), and celiac disease (CeD), a chronic enteropathy caused by ingestion of gluten-containing cereals in genetically susceptible individuals (Pinto-Sanchez, Smecuol et al. 2016). In particular, a number of strains belonging to genus *Bifidobacterium* have been proposed as beneficial supplements for a wide range of health conditions (Tojo, Suarez et al. 2014). Depletions in bifidobacteria have been noted in patients with CeD (Golfetto, de Senna et al. 2014), and attempts have been made to supplement some strains as a therapy for CeD (Pinto-Sanchez, Smecuol et al. 2016; Quagliariello, Aloisio et al. 2016). However, despite great public interest in the clinical use of specific probiotic strains for intestinal disorders, there is insufficient mechanistic insight to rationalize consistent recommendations. Investigating therapeutic effects of specific molecules produced by probiotic strains may help bridge this gap.

Dysregulated proteolytic balance has been described in several gastrointestinal disorders (Bustos, Negri et al. 1998; Róka, Rosztóczy et al. 2007; Gecse, Roka et al. 2008; Annaházi, Molnár et al. 2012; Motta, Bermúdez-Humarán et al. 2012). We have previously shown that the expression of the human serine protease inhibitor (serpin),

elafin, is decreased in the duodenum of patients with active CeD (Motta, Bermúdez-Humarán et al. 2012; Galipeau, Wiepjes et al. 2014). Recombinant *Lactococcus lactis* expressing elafin has been shown i) to be protective in several murine colitis models (Motta, Bermúdez-Humarán et al. 2012) and ii) to prevent gluten immunopathology in the NOD/DQ8 mouse model of gluten sensitivity (Galipeau, Wiepjes et al. 2014). However, given the concerns raised with the clinical application of such genetically modified organisms (GMOs), we investigated the effect of a commensal bacterium that naturally expresses an elafin-like serpin. The role of serpins produced by bacteria is unknown, but they are thought to be a contributor to host-commensal mutualism as these serpins likely provide protection from host proteases (Ivanov, Emonet et al. 2006) (Turroni, Foroni et al. 2010). Although eukaryotic serpins such as elafin are known to possess anti-inflammatory properties (8), bacterially produced serpins have not been explored for their therapeutic capacity *in vivo*. The infant-derived commensal probiotic strain *Bifidobacterium longum* NCC2705 (*B. longum srp*$^{+}$) produces a serpin (Srp) encoded by the gene *BL0108* (*srp)*, in a non-constitutive manner. Expression of *srp* is induced in the murine intestinal tract, and Srp may exhibit anti-inflammatory properties as it inhibits both pancreatic and neutrophilic elastase *in vitro* (Ivanov, Emonet et al. 2006). We tested the hypothesis that administration of the commensal *B. longum srp*$^{+}$ prevents immunopathology in the NOD/DQ8 mouse model of gluten sensitivity.

We show that both the wild-type *B. longum srp*$^{+}$ and a recombinant strain constitutively expressing *srp* (*B. longum srp* (Con)), prevents gliadin-induced immunopathology in NOD/DQ8 mice, while the *srp* knockout strain (*B. longum* Δ*srp*)

does not. These results clearly suggest that the beneficial effect of *B. longum srp*$^{+}$ is mediated by Srp. This warrants clinical investigation of commensal *B. longum srp*$^{+}$ in managing CeD and non-celiac gluten/wheat sensitivity (NCG/WS), or chronic gastrointestinal conditions associated with proteolytic imbalance.

MATERIALS & METHODS

Construction of bacterial strains.

B. longum NCC2705 (*B. longum srp*$^{+}$) was isolated at the Nestlé Research Center from the feces of a healthy infant (Schell, Karmirantzou et al. 2002). The strain is well-characterized at the molecular and biochemical levels. The full genome of 2.26 Mb has been sequenced, and it was demonstrated that *srp*, previously known as *Bl0108*, encodes a *bona fide* serine protease inhibitor with affinity and inhibitory activity to eukaryotic elastases (Ivanov, Emonet et al. 2006).

Upstream and downstream sequences (3kb) of the *srp* gene of *B. longum srp*$^{+}$ were amplified by PCR and cloned into the pJH101 vector. The pJH101 vector is available at the German Collection of Microorganisms and Cell Cultures (DSMZ) and was initially designed for the construction of integrable plasmids in *B. subtilis.* pJH101 contains a chloramphenicol resistance gene and does not contain an origin of replication for *B. subtilis* nor *B. longum.* The resulting plasmid pMDY24 containing no coding sequences of *srp* was introduced into *B. longum srp*$^{+}$. Transformation was performed as described previously (Argnani, Leer et al. 1996). Five transformants were obtained by

plating on MRS medium supplemented with 0.05% cysteine (MRS-cys) containing chloramphenicol, and integration was confirmed by Southern blot. Transformants were cultivated for 100 generations on MRS-cys without chloramphenicol to clear the antibiotic resistance gene. Twelve chloramphenicol-sensitive isolates were confirmed to be *srp* knockout strains. One isolate was included in the Nestlé Culture Collection under *B. longum* NCC 9035 (*B. longum* Δ*srp*).

The plasmid pMDY25 was constructed by inserting a constitutive promoter from *B. longum* NCC 2705, pr-BL1363 (promoter of gene *Bl1363*, coding for a glyceraldehyde 3-phosphate dehydrogenase), in front of the *srp* gene in the pMDY23 plasmid which encodes spectinomycin resistance (Klijn, Moine et al. 2006). In this recombinant strain, the level of synthesis of Srp no longer depends on any kind of induction (*B. longum srp*(Con)).

The *L. lactis* food-grade strain was engineered to express recombinant human elafin (*L. lactis*-elafin), whose expression was driven by a nisin-inducible promoter, as described in detail previously (Motta, Bermúdez-Humarán et al. 2012). Strains and plasmids used in this manuscript are outlined in Table 1.

Supplementary Table 1. Bacterial strains used and plasmids employed to construct them.

Strains	
L. lactis-elafin	Recombinant *Lactococcus lactis* strain expressing elafin (Motta, Bermudez-Humaran et al. 2012).
B. longum srp^{+}	Commensal probiotic strain *Bifidobacterium longum* NCC 2705 expressing serpin (Schell, Karmirantzou et al. 2002).
B. longum srp(Con)	Recombinant *Bifidobacterium longum* strain derived from NCC 2705 (NCC 2705 pMDY25), constitutively producing serpin (this work).
B. longum Δ*srp (*Δ*srp)*	Genetically modified *Bifidobacterium longum* strain derived from NCC 2705 (NCC 9035) without serpin coding sequences (this work).
Plasmids	
pJH101	Commercially available plasmid (DSMZ) initially designed for the construction of integrable plasmids in *B. subtilis*. Contains a chloramphenicol resistance gene and does not contain origin of replication for neither *B. subtili*s nor *B. longum*

pMDY24	Plasmid derived from pJH101 containing upstream and downstream sequences of the BL108 serpin gene and containing chloramphenicol resistance gene (this work).
pMDY23	Versatile reporter plasmid based on *B. longum* cryptic plasmid and the *Escherichia coli* gusA gene and containing spectinomycin resistance gene (Klijn, Moine et al. 2006).
pMDY25	Plasmid derived from pMDY23 containing a constitutive promoter in front of the BL108 serpin gene (this work).

Supplementary Table 1. Bacterial strains used and plasmids employed to construct them.

Preparation of *B. longum* biomass.

B. longum srp^{+} and *B. longum* Δ*srp* strains were inoculated at 2% from a fresh overnight culture in MRS-cys and grown anaerobically at 37°C for 16h. Bacteria were harvested by centrifugation, resuspended in sterile PBS containing 20% glycerol (PBS-20% glycerol) and stored in aliquots at -80°C. Viable counts for the *B. longum srp*$^{+}$ and *B. longum* Δ*srp* preparations were equal to $6.6x10^{9}$ cfu/ml and $4.4x10^{9}$ cfu/ml, respectively. *B. longum srp*(Con) was cultured and further processed as described above

in the presence of 100 μg/ml of spectinomycin and grown for 48 h at 37°C. Levels of viable bacteria were equal to $1.5x10^9$ cfu/ml.

***In vitro* inhibitory activity of *B. longum* strains against elastase.**

Enzymatic activity of human neutrophil elastase (HNE) was determined by cleavage of FITC-labeled elastin (FITC-elastin). Concentrations of HNE (1.5, 3.125, 6.25, 12.5, 25, and 50 mU/mL) were incubated with 10^8 cfu of *B. longum srp*$^+$, *B. longum srp*(Con) or *B. longum* Δ*srp* and 40 μL of buffer solution (50 mM Tris-HCL, 1mM $CaCl_2$, 50 mM NaCl, 0.25% Triton X100; pH 8.0) at 37°C for 30 min. 50 μL FITC-elastin substrate was added and fluorescence was measured at an excitation wavelength of 530 nm using a spectrophotometer (SpectraMax, Molecular Devices, San Leandro, CA).

Animals.

All experiments were conducted with approval from the McMaster University Animal Care Committee. Female and male 8 to 12-week-old NOD/DQ8 transgenic mice (Galipeau, Rulli et al. 2011) were fed a gluten-free diet for 2 generations (Harlan Laboratories, Indianapolis, IN) and housed in a specific pathogen-free colony at McMaster University. These mice lack all endogenous mouse MHC II molecules and express the DQ8 human transgene on a NOD background (Galipeau, Rulli et al. 2011). Oral sensitization of NOD/DQ8 mice with peptic-tryptic (PT) digest of gliadin, one of the main protein fractions in gluten, and subsequent gliadin challenge induces moderate enteropathy, intraepithelial lymphocytosis, and barrier dysfunction (Figure 1A) as described previously (Galipeau, Rulli et al. 2011).

Mucosal delivery of *B. longum* and gliadin sensitization.

Mice were orally gavaged with 500 μg PT-gliadin plus 25 μg cholera toxin as adjuvant (Sigma-Aldrich) once a week for 3 weeks. Non-sensitized mice (control) were gavaged with PBS plus 25 μg cholera toxin. Sensitized mice were then treated daily by oral gavage (10^9 cfu, 200μl/mouse) for two weeks with either *B. longum srp*$^+$, *B. longum* Δ*srp*, or *B. longum srp* (Con) suspended in PBS-20% glycerol. During the probiotic treatment period, sensitized mice were orally challenged with gliadin (2 mg/mouse) dissolved in 0.02 M acetic acid (vehicle) three times per week. Vehicle-treated mice were simultaneously gavaged PBS-20% glycerol during the challenge period. Control mice were maintained on a gluten-free chow diet and gavaged with PBS-20% glycerol and 0.02 M acetic acid (Figure 1A).

Detection of *B. longum* strains.

Primers used for specificity towards *B. longum srp*$^+$ and derivatives, targeting the insertion sequence IS*Blo1b described previously (Berger, Moine et al. 2010),* were used to amplify DNA extracted from proximal small intestinal tissue and contents (Figure 6B). The primers were as follows: forward, 5'-TCCAGATCATTTCCGATTCC-3'; reverse, 5'-CGGCGTATTTCTATCGCATC-3' and amplified as previously described (Berger, Moine et al. 2010).

***srp* mRNA expression in *B. longum* strains.**

B. longum srp$^+$, *B. longum srp*(Con) and *B. longum* Δ*srp* were cultured as above for 8 h and cells were collected by centrifugation. Total RNA was extracted using the

RNeasy mini kit (Qiagen) with additional DNAse treatment. Purity and quality was checked using QIAxcel RNA Quality Control Kit v2.0 (Qiagen). RNA level was quantified using the SuperScript III Platinium SYBR Green One-Step qRT-PCR kit (Invitrogen) and using standard PCR conditions described in the kit. *srp* primers were as follows: forward 5'-ACCAATCGCTGCTAAGTTCG-3', reverse 5'-TCGCTGGCAAGAGAGTAGTC-3'. The lactate dehydrogenase (*ldh*) housekeeping gene was used for standardization. The following primers were used for *ldh*: forward, 5'-CGAACGCCATCTACATGCTC-3' and reverse, 5'-AAGATCTGGTTCTCTTGCAG-3'. Fold change of *srp* mRNA was calculated using the Pfaffl method (Pfaffl 2001).

srp detection _in vivo_.

srp mRNA was measured in small intestinal contents and feces *of B. longum srp*$^{+}$-treated mice. Samples were collected fresh and flash frozen in liquid nitrogen. RNA was extracted from these samples using the PowerMicrobiome^{TM} RNA Isolation kit (cat N° 26000-50, MoBio Laboratories Inc., Carlsbad, CA). RNA quality was checked using the Agilent 2100 Bioanalyzer system with the Agilent RNA 6000 nano kit. RNA was quantified using the Quant-it Ribogreen RNA kit. *srp* mRNA was measured by qRT PCR in two steps. RNA transcription to single stranded cDNA was performed using 1 µg of RNA in 20 µl of total reaction using qScript cDNA supermix and the following PCR conditions: 25°C 5 min; 42°C 30 min; 85°C 5 min and 4°C hold. *srp* was further amplified by real time PCR using the following primers: forward 5'-ACCAATCGCTGCTAAGTTCG-3', reverse 5'-TCGCTGGCAAGAGAGTAGTC-3'and probe FAM 5'-CCGAGATGAGCGCCGCGAACT-3'BHQ (Microsynth). cDNA (100 ng) was used in a

total reaction of 20 µl with the TaqMan Universal PCR Master Mix and the following cycle: 50°C for 2 min; 95°C for 10 min; 95°C for 15 sec; 60°C for 1 min; repeated for 40 cycles.

Evaluation of small intestinal immunopathology.

Small intestinal cross-sections were fixed in 10% buffered formalin for 48 hours and embedded in paraffin, as previously described (Galipeau, Rulli et al. 2011).

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded sections of proximal small intestine to visualize $CD3^+$ cells as described previously (Biagi, Luinetti et al. 2004; Galipeau, Rulli et al. 2011). Slides were examined at 20X magnification using light microscopy in a blinded fashion. The number of $CD3^+$ IELs per 20 enterocytes were counted in five randomly chosen villous tips by a blinded observer as described, and expressed as IELs/100 enterocytes (Biagi, Luinetti et al. 2004). Paraffin-embedded sections were stained with hematoxylin and eosin (H&E) for histological evaluation of tissue morphology under light microscopy (Olympus, ON, Canada). Using the Image-Pro 6.3 software (Mediacybernetics, MD, USA), enteropathy was quantified in a blinded fashion by measuring villus-to-crypt ratios (V:C) as previously described (Galipeau, Rulli et al. 2011). Intestinal paracellular permeability was evaluated *ex vivo* by Ussing Chamber technique as previously described (World Precision Instruments, Sarasota, FL) (Galipeau, Rulli et al. 2011). Paracellular permeability of proximal small intestinal samples was evaluated by measuring the mucosal-to-serosal flux of the inert paracellular probe ^{51}Cr-EDTA. ^{51}Cr-EDTA was quantified in samples using a liquid scintillation counter and expressed as % recovery/cm^2/hour, or ^{51}Cr-EDTA flux.

Microbiota compositional analysis.

Fecal and small intestinal contents were collected and flash frozen on dry ice. DNA was extracted from samples as previously described (Whelan, Verschoor et al. 2014) and amplified for the hypervariable V3 region of the 16S rRNA gene for sequencing on the Illumina MiSeq platform (Illumina, San Diego, CA). Analysis of data was performed as previously described (Whelan, Verschoor et al. 2014). Briefly, sequences were trimmed using Cutadapt software (version 1.2.1) (Martin 2011) and aligned through the PANDAseq software (version 2.8) (Masella, Bartram et al. 2012). Operational taxonomic units selected using AbundantOTU (Ye 2011) were assigned taxonomy according to the Greengenes reference database (DeSantis, Hugenholtz et al. 2006). Principal coordinate analysis (PCoA) plots were generated using R (R Foundation for Statistical Computing, Vienna, Austria). Pairwise UniFrac distances were calculated among microbial communities, and both relative abundance data (weighted) and presence/absence information (unweighted).

Statistical Analysis.

Data were analyzed in GraphPad Prism 6.0, QIIME, R and SPSS software. Normal data were analyzed by ANOVA followed by Bonferroni Post-hoc analysis. HNE inhibition statistics were performed using Mann-Whitney test compared to buffer. Microbiota β-diveristy statistics were performed using PERMANOVA. Microbiota abundances were analyzed in SPSS via Kruskal-Wallis followed by FDR ($q<0.05$). All significant genera presented passed FDR.

RESULTS

B. longum srp$^+$ and L. lactis-elafin are equally effective in preventing gliadin immunopathology in mice.

We initially compared the efficacy of *B. longum srp*$^+$ with elafin delivery by recombinant *L. lactis*, previously shown to prevent intraepithelial lymphocytosis in NOD/DQ8 mice sensitized with gliadin (Galipeau, Wiepjes et al. 2014). Mice treated with *L. lactis*-elafin or *B. longum srp*$^+$ had lower CD3$^+$ IEL counts in the small intestine compared with mice receiving vehicle and gliadin ($p<0.05$) (Figure 1B and 1C).

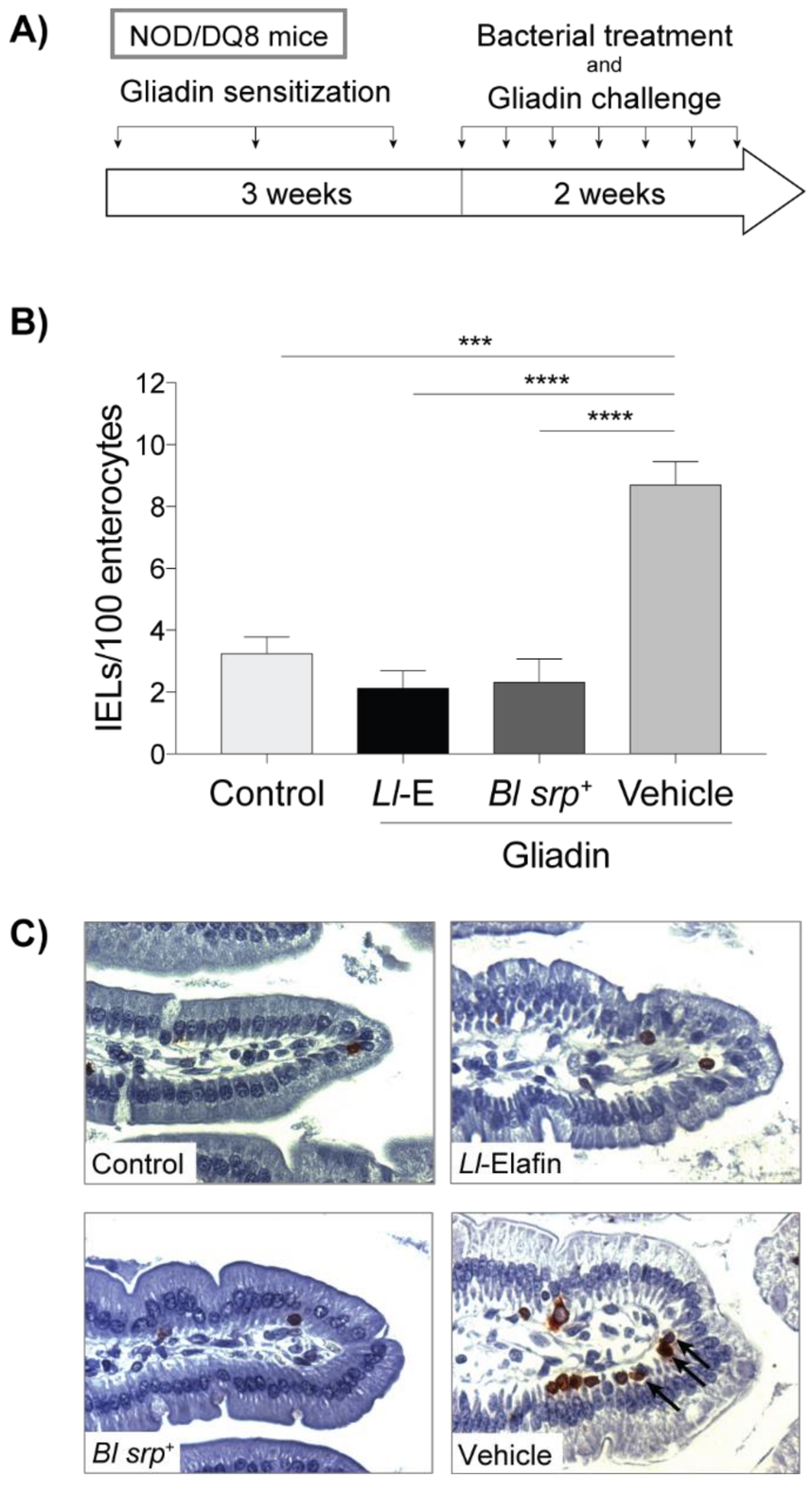

Figure 1. *B. longum srp*$^{+}$ and *L. lactis*-elafin are equally effective in preventing gliadin immunopathology in mice.

A) NOD/DQ8 mice were sensitized with cholera toxin and pepsin-trypsin digested gliadin 1x per week for 3 weeks. Non-sensitized mice (controls) received cholera toxin alone. Subsequently, mice were treated daily with *B. longum srp*$^{+}$*, L. lactis*-elafin, or PBS-20% glycerol and simultaneously challenged with gliadin 3x per week for 2 weeks. Control

mice received no bacterial treatment. **B)** $CD3^+$ intraepithelial lymphocytes in small intestinal villi tips were quantified and expressed as IELs per 100 enterocytes. Mice treated with *srp*-expressing *B. longum srp*$^+$ had significantly lower numbers of IELs compared to gliadin-sensitized mice receiving no bacterial treatment. Further, *B. longum srp*$^+$ treatment resulted in similar numbers of IELs as those treated with *L. lactis* expressing elafin. **C)** Representative images were captured at 40X magnification. Data shown as mean ± standard error of the mean (SEM). Statistical significance was performed by ANOVA followed by Bonferroni post-hoc analysis, *$p<0.05$. Non-sensitized, no treatment (Control); gliadin + WT *B. longum* NCC2705 (*Bl srp*$^+$); gliadin + *L. lactis* expressing elafin (*Ll*-E); gliadin, no treatment (Vehicle) (n=3-6/group).

B. longum constitutively expressing srp exhibits an increased inhibitory capacity towards human neutrophil elastase in vitro.

To characterize *B. longum srp*$^+$, *B. longum* Δ*srp,* and *B. longum srp*(Con), we first measured the expression of *srp in vitro*. *B. longum srp*(Con) expressed 452-fold more *srp* mRNA *in vitro* than *B. longum srp*$^+$, and no *srp* mRNA was detected in *B. longum* Δ*srp* (Figure 2A). We then quantified the ability of *B. longum srp*$^+$ and *B. longum srp*(Con) to inhibit human neutrophil elastase (HNE) activity *in vitro,* as pure Srp from *B. longum srp*$^+$ was previously shown to inhibit HNE. *B. longum* Δ*srp* did not inhibit proteolysis of elastin by HNE, as RFU produced from cleavage of FITC-elastin was similar at all concentrations of HNE added. Compared to *B. longum* Δ*srp, B. longum srp*$^+$ inhibited elastin degradation by HNE at 1.5 mU/ml ($p<0.01$) resulting in a lower RFU

value. *B. longum srp*(Con) inhibited HNE activity at all concentrations of HNE compared to *B. longum* Δ*srp* ($p<0.01$ at 3.125, 6.25, 50 mU/ml; $p<0.05$ at 1.5, 12.5, 25 mU/ml) (Figure 2B).

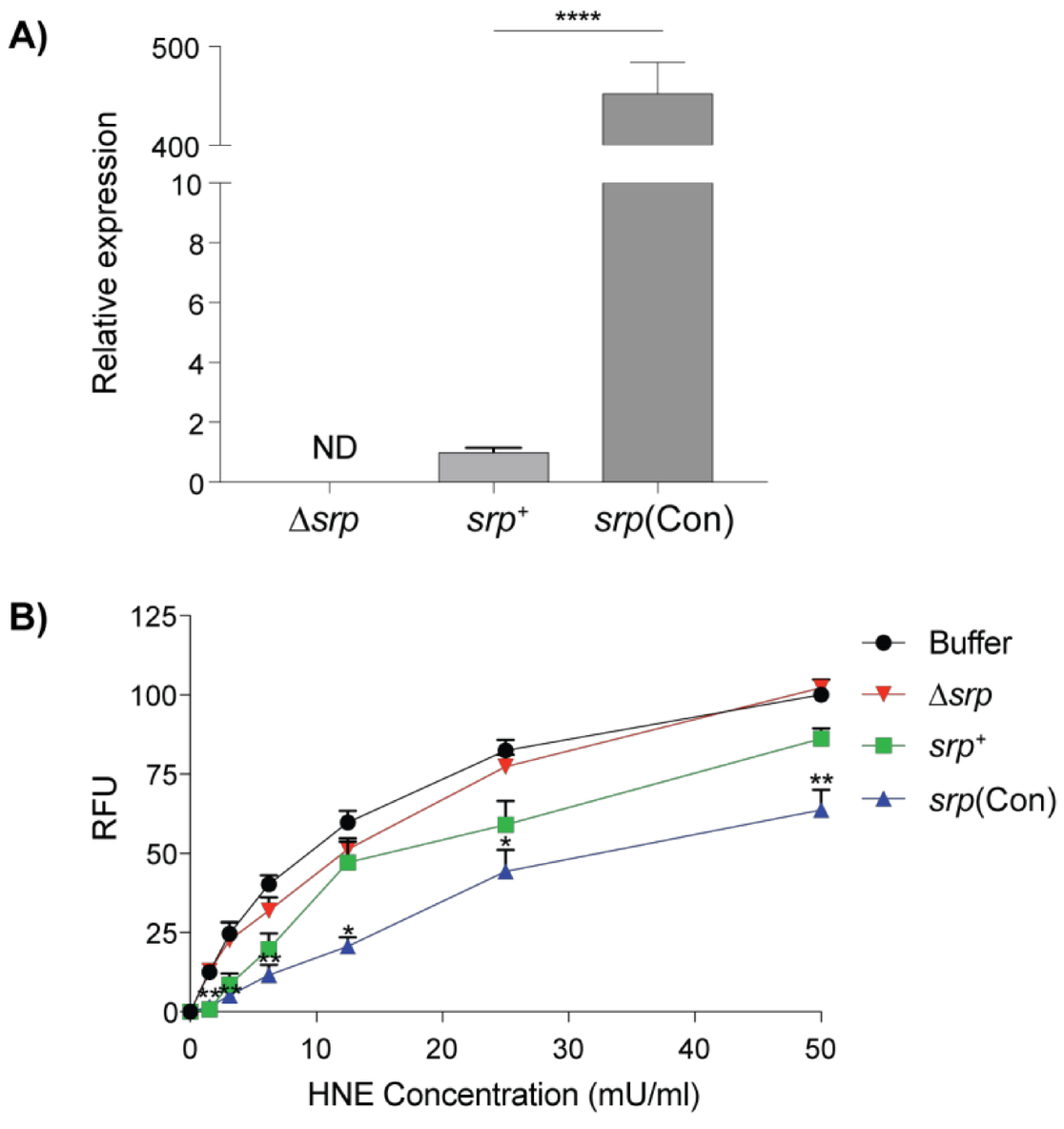

Figure 2. *B. longum* constitutively expressing *srp* inhibits human neutrophil elastase activity *in vitro*.

A) *srp* mRNA levels were quantified from various *B. longum* strains. Expression of *srp* was higher in *B. longum srp*(Con) than *B. longum srp*$^+$(**** $p<0.0001$). No *srp* mRNA was detected in *B. longum* Δ*srp* (n=4). **B)** Inhibitory capacities of various *B. longum* strains were tested *in vitro*, as measured by fluorescence produced via cleavage of FITC-elastin substrate at 1.5, 3.125, 6.25, 12.5, 25, and 50 mU/mL, expressed as relative fluorescence units (RFU) (n=3/group). Compared to *B. longum* Δ*srp, B. longum srp*$^+$

inhibited cleavage of elastin by human neutrophil elastase (HNE) *in vitro* at 1.5 mU/mL ($p<0.01$), resulting in lower RFU. In the same assay, *B. longum srp*(Con) further inhibited HNE across all concentrations of HNE compared to *B. longum* Δ*srp*, resulting in lower RFU. As well, *B. longum* Δ*srp* did not inhibit HNE, as cleavage of FITC-elastin determined by RFU produced was not significantly different between *B. longum* Δ*srp* and buffer alone at any concentration of HNE added. HNE alone (buffer); *B. longum* srp^+ (srp^+); *B. longum* Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)). ND, not detectable. Data shown as mean ± SEM. Statistical significance was performed using Kruskal-Wallis, * $p<0.05$ vs. *B. longum* Δ*srp*; ** $p<0.01$ vs. *B. longum* Δ*srp*.

B. longum srp mediates the protective effect observed in mice.

We tested the capacity of *B. longum* strains to prevent gliadin immunopathology using *B. longum* srp^+, *B. longum* Δ*srp* (positive control), and *B. longum srp*(Con), NOD/DQ8 mice sensitized to gliadin and treated with *B. longum* Δ*srp* had higher IEL counts compared to non-sensitized mice (controls; $p<0.0001$) or to mice receiving *B. longum* srp^+ ($p<0.0001$) or *B. longum srp*(Con) ($p<0.0001$) (Figure 3A). Mice receiving *B. longum* Δ*srp* had reduced V:C ratios compared with controls ($p<0.05$) and *B. longum srp*(Con)-treated mice ($p<0.05$) (Figure 3B). Lastly, mice treated with *B. longum* Δ*srp*, but not mice receiving *B. longum* srp^+ or *B. longum srp*(Con), had increased paracellular permeability in the proximal small intestine compared with controls ($p<0.05$) (Figure 3C).

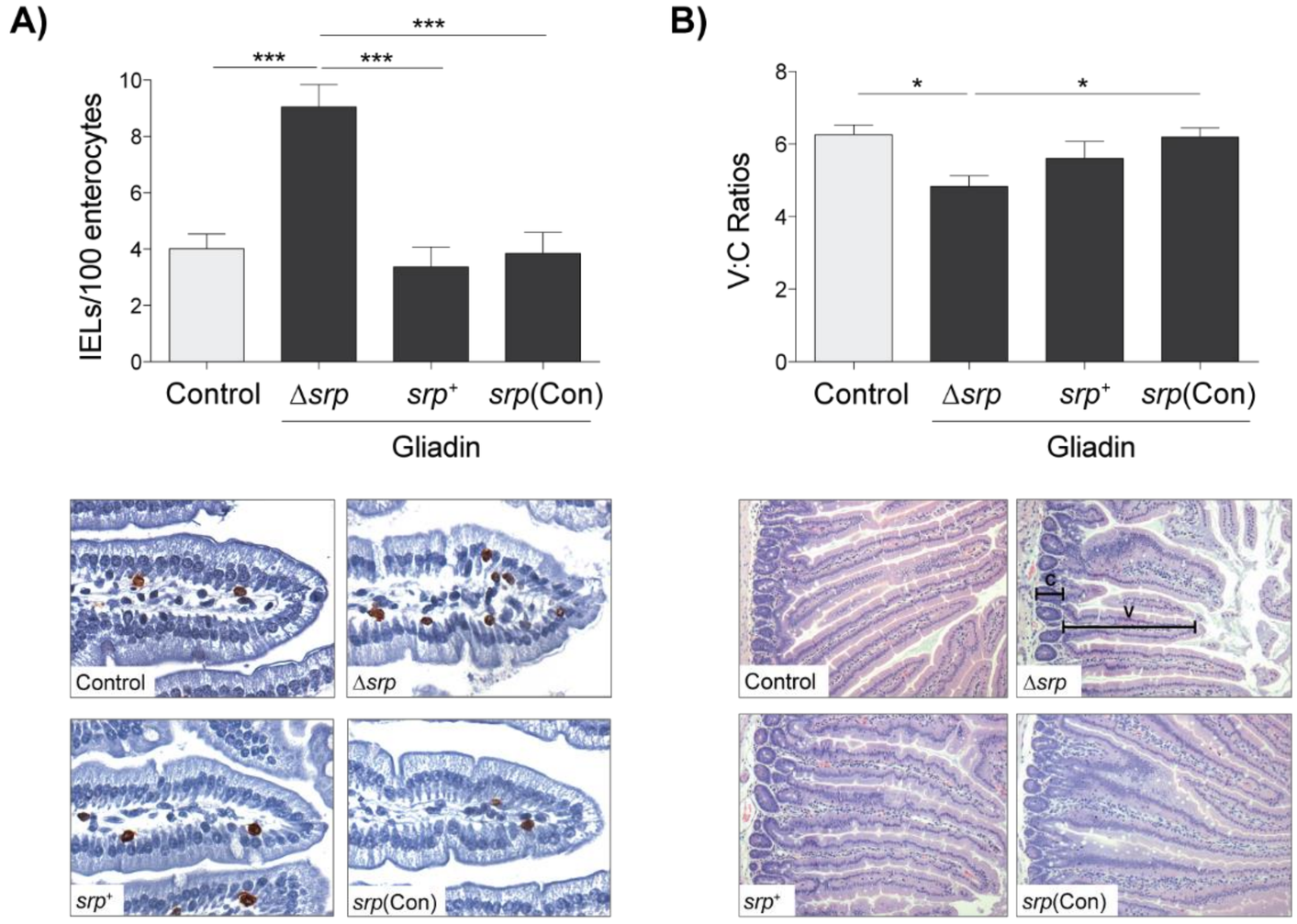

Figure 3. *B. longum srp* mediates the protective effect observed in mice.

NOD/DQ8 mice were sensitized with cholera toxin and pepsin-trypsin digested gliadin 1x per week for 3 weeks. Non-sensitized mice (controls) received cholera toxin alone. Subsequently, sensitized mice were treated daily with either *B. longum srp*$^+$ (*srp*$^+$), *B. longum* Δ*srp* (Δ*srp*), *B. longum srp*(Con) (*srp*(Con)), and simultaneously challenged with gliadin 3x per week for 2 weeks. Control mice received PBS-20% glycerol. **A)** CD3$^+$ intraepithelial lymphocytes in small intestinal villi tips were quantified and expressed as

IELs per 100 enterocytes. Mice treated with *srp*-expressing *B. longum srp*$^{+}$ or *B. longum srp*(Con) had significantly lower numbers of IELs than *B. longum* Δ*srp*-treated mice. Representative images were captured at 40X magnification (n=10-11/group). **B)** Small intestinal sections were H&E-stained, and villus (V) and crypt (C) lengths were measured via light microscopy, expressed as V:C ratios. *B. longum srp*(Con) treatment in gliadin-sensitized mice resulted in significantly higher V:C ratios than *B. longum* Δ*srp* treatment. Representative images were captured at 10X magnification (n=10-11/group). **C)** Paracellular permeability was restored in sensitized NOD/DQ8 mice treated with *B. longum* strains expressing *srp*, *B. longum srp*$^{+}$ and *B. longum srp*(Con). Proximal small intestinal sections were mounted on Ussing chambers to measure *ex vivo* paracellular permeability, expressed as ^{51}Cr-EDTA flux (n=7-8/group). Data is shown as mean ± SEM. Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum* Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)). Statistical significance was performed by ANOVA followed by Bonferroni post-hoc analysis, ***p<0.001, *p<0.05.

Gliadin and B. longum srp expression shift fecal microbiota profiles in mice.

Both the small intestinal and fecal contents of controls and gliadin-sensitized *B. longum*-treated NOD/DQ8 mice were sequenced using 16s Illumina technology. The small intestinal microbiota profiles were similar between all groups (Supplementary Figures 1, 2). However, in both weighted (Bray-Curtis Dissimilarity) and unweighted (Unifrac) β-diversity parameters, shifts in fecal microbiota profiles were observed between controls and all gliadin-sensitized mice (Figure 4, Supplementary Figure 3.). Moreover, gliadin-sensitized mice treated with *B. longum srp*$^{+}$ and *B. longum srp*(Con)

clustered separately from mice receiving *B. longum* Δ*srp*, and this difference in β-diversity was significant (Figure 4).

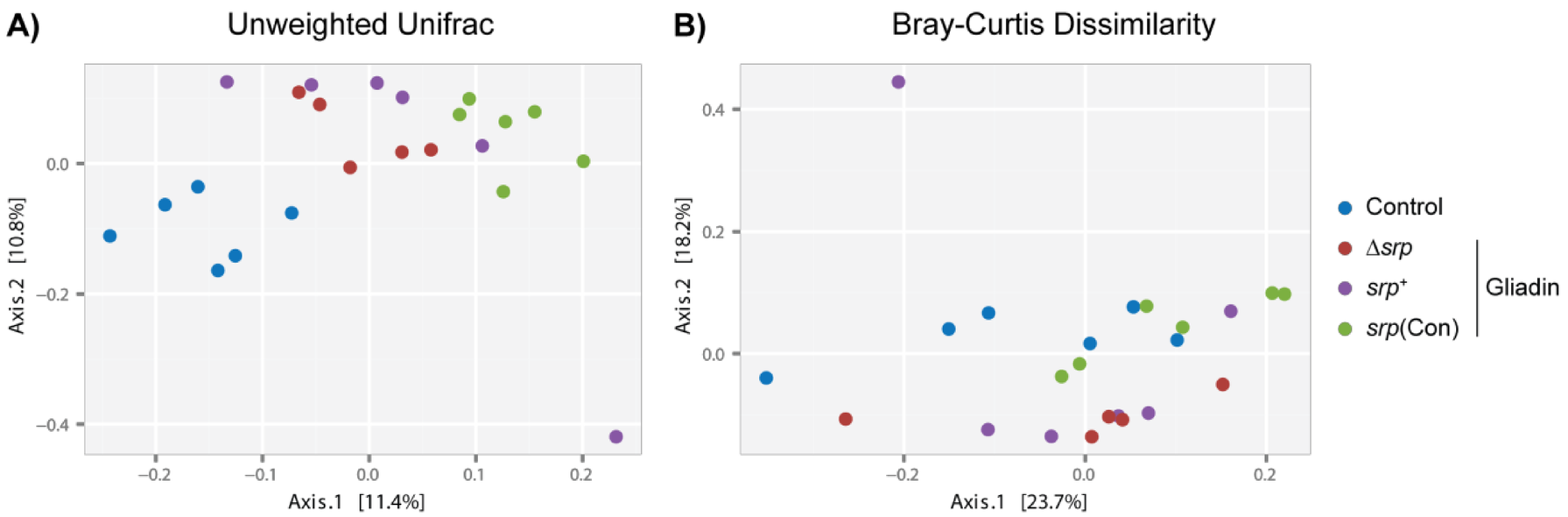

Figure 4. Gliadin and treatment with *srp*-expressing *B. longum* shift fecal microbiota profiles.

Principal coordinate analysis plots of 16S data in NOD/DQ8 mice. **A)** Gliadin induces a shift in β-diversity calculated using Unifrac unweighted distance ($p<0.001$). Microbial compositions are different between mice receiving *B. longum* Δ*srp* and *B. longum* srp^+ ($p<0.05$); *B. longum* Δ*srp* and *B. longum srp*(Con) ($p<0.001$); and *B. longum* srp^+ and *B. longum srp*(Con) ($p<0.001$) (n=5-6/group). **B)** Gliadin also shifts β-diversity when assessed using Bray-Curtis dissimilarity parameters ($p<0.005$). Microbial compositions are significantly different between *B. longum* Δ*srp* and *B. longum* srp^+ ($p<0.05$); *B. longum* Δ*srp* and *B. longum srp*(Con) ($p<0.005$); *B. longum* srp^+ and *B. longum srp*(Con) ($p<0.005$). Each circle represents an individual fecal sample (n=5-6/group). Non-sensitized, no treatment (Control); *B. longum* srp^+ (srp^+); *B. longum* Δ*srp* (Δ*srp*); *B.*

longum srp(Con) (*srp*(Con)). Statistics were performed via PERMANOVA in QIIME. Plots were constructed in R.

Relative abundances of Actinomycetales were lower in all gliadin-sensitized mice compared with controls. *B. longum srp*(Con) administration was associated with elevated levels of *Akkermansia*. An unknown Clostridiaceae was increased in mice treated with *B. longum srp*(Con) compared with those given *B. longum srp*$^{+}$ or no probiotic. Relative abundance of an unknown Clostridiales Family XIII was increased in gliadin-treated mice given *B. longum* Δ*srp* and *B. longum srp*$^{+}$ (Figure 5B).

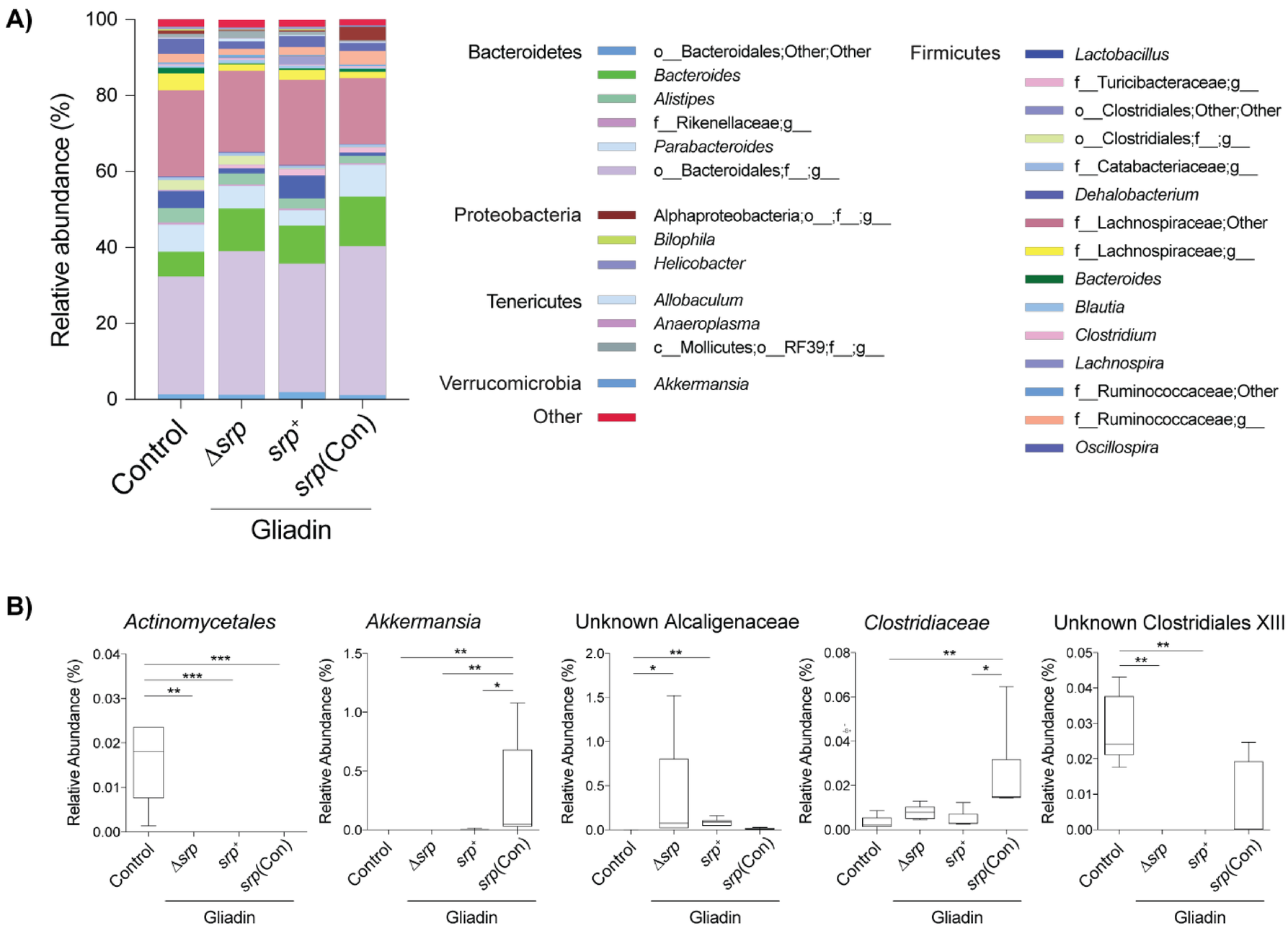

Figure 5. Fecal genera affected by *B. longum* expressing *srp*.

A) Genus-level composition of fecal microbiota after *B. longum* treatment in NOD/DQ8 mice are depicted as an average percentage of each group in stacked column charts. **B)** Genera significantly differing in relative abundances between groups. Data shown as box and whisker plots. (*p<0.05; **p<0.01; ***p<0.001). Non-sensitized, no treatment

(Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum* Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)) (n=5-6/group). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).

B. longum strains and B. longum srp are detected in the gastrointestinal tract of treated mice.

We next determined whether *B. longum srp*$^{+}$ and the mutant strains, *B. longum srp*(Con) and *B. longum* Δ*srp,* were present in the small intestinal lumen of treated mice via PCR amplification. There was no difference in relative abundances of total Bifidobacteria in the small intestine between mice receiving vehicle or any of the *B. longum* strains, as measured by 16 Illumina sequencing (Figure 6A). However, strain-specific primers for *B. longum srp*$^{+}$ revealed that *B. longum srp*$^{+}$ and its derivatives were present in the small intestine of treated mice (Figure 6B). Furthermore, *srp* mRNA was detected in the feces and/or intestinal content of 2/4 mice treated with *B. longum srp*$^{+}$ and 3/4 mice treated with *B. longum srp*(Con). In contrast, *srp* mRNA was not detected in any sample from controls or *B. longum* Δ*srp*-treated mice.

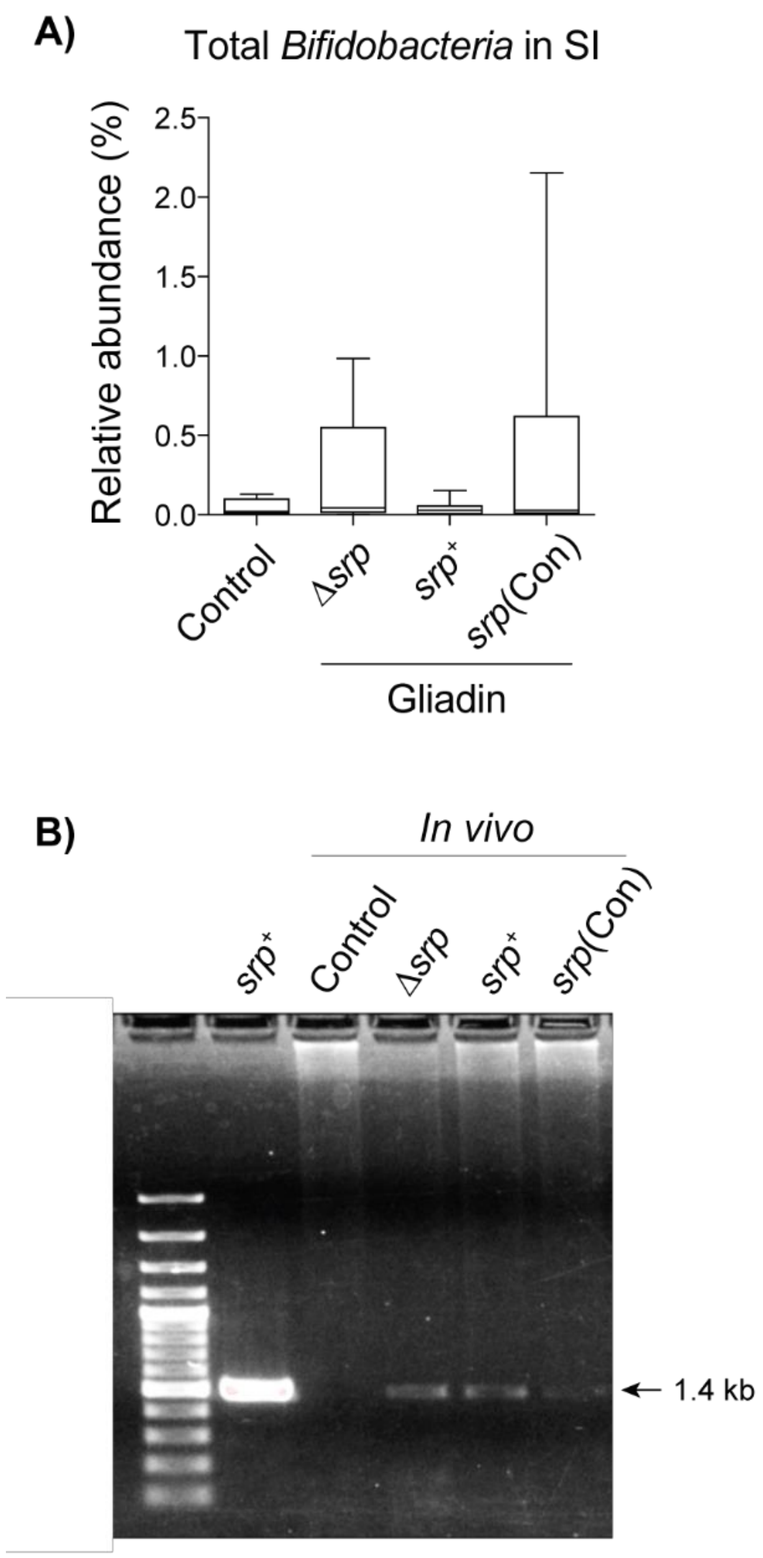

Figure 6. *B. longum* NCC2705 is detected in the gastrointestinal tract of treated mice. **A)** Relative abundance of Bifidobacteria genus members as determined by 16S rRNA sequencing is similar between all groups of NOD/DQ8 mice (n=5-6/group). **B)** Strain-specific primers detected *B. longum* Δ*srp*, *B. longum srp*$^{+}$, and *B. longum* srp(Con) in small intestinal DNA extracted from all bacterially-treated mice. Non-sensitized, no

treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum* Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)) (n=5/group). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).

DISCUSSION

There is a spectrum of clinical conditions caused by adverse reactions to gluten, and its constitutive proteins, such as gliadin. These include the well-characterized autoimmune enteropathy CeD, wheat allergy, as well as NCGS/NCWS which overlaps symptomatically with IBS (Verdu, Armstrong et al. 2009). The only effective management for CeD is a life-long gluten-free diet (GFD), which has several limitations including poor compliance, accidental contaminations and slow resolution of mucosal inflammation (Wacklin, Kaukinen et al. 2013). Patients with NCGS/NCWS also improve symptomatically on a GFD, but it is unknown whether these patients will tolerate less restrictive avoidance or could be successfully treated with other therapies. Since patients with active CeD and non-responders to the GFD, have been found to harbor dysbiotic intestinal communities (Decker, Engelmann et al. 2010; Marild, Ye et al. 2013; Golfetto, de Senna et al. 2014; D'Argenio, Casaburi et al. 2016), probiotics have been proposed as potential candidates to restore gut microbial homeostasis. Smecuol *et al.* (2013) found that administration of the *Bifidobacterium* Natren life start (NLS) attenuated symptoms in CeD patients on a gluten-containing diet (Smecuol, Hwang et al. 2013), and administration of NLS was shown to modulate innate immunity in a follow-up study

(Pinto-Sanchez, Smecuol et al. 2016). In another clinical trial, children with newly diagnosed CeD that received encapsulated *B. longum* CECT 7347, showed moderate changes in inflammatory markers and microbiota, but no symptomatic improvement beyond those achieved with the concomitant GFD (Olivares, Castillejo et al. 2014). Although these studies raise the possibility that certain probiotics may be beneficial, adjuvant to the GFD, in CeD and perhaps other gluten-related disorders, their use was not guided by pathophysiological rationale and the mechanisms of action remain unclear.

Our study addresses the efficiency of a specific bacterial serpin (Srp), expressed naturally by *B. longum srp*$^{+}$, in the prevention of inflammation induced by gliadin in a genetically susceptible mouse model with previously determined well-defined endpoints (Galipeau, Rulli et al. 2011; Galipeau, Wiepjes et al. 2014; Galipeau, McCarville et al. 2015). We have previously shown that the severity of gluten immunopathology in NOD/DQ8 mice is influenced by the microbiota with which these mice are colonized, and that administration of recombinant *L. lactis* expressing elafin can attenuate the inflammatory response of the host towards gluten (Galipeau, Wiepjes et al. 2014; Galipeau, McCarville et al. 2015; Caminero, Galipeau et al. 2016). Serpins are produced by a wide range of organisms and play a key role in maintaining immune homeostasis (Kaiserman, Whisstock et al. 2006; Heit, Jackson et al. 2013). In the gut, serpins are expressed at mucosal surfaces and are involved in regulating barrier function (Hasnain, McGuckin et al. 2012; Uchiyama, Naito et al. 2012). Srp inhibits eukaryotic serine proteases *in vitro*, including both neutrophilic and pancreatic elastase. The inhibition of neutrophil elastase, which is a driver of intestinal tissue damage and a biomarker of

intestinal inflammation (Langhorst, Elsenbruch et al. 2008), represents an immunomodulatory capacity for Srp that may be relevant in treating gastrointestinal inflammatory conditions (Ivanov, Emonet et al. 2006). We confirmed that the recombinant *B. longum srp*(Con) expresses higher levels of *srp* than *B. longum srp*$^{+}$ *in vitro* (Figure 2A), and that *srp* expression is undetectable in the mutant strain *B. longum* Δ*srp in vitro*. Since purified Srp from *B. longum srp*$^{+}$ has been demonstrated to inhibit human neutrophil elastase (HNE) (Ivanov, Emonet et al. 2006), we tested HNE inhibition by the three strains expressing *srp* at various levels *in vitro*. Indeed, *B. longum* strains expressing *srp*, but not *B. longum* Δ*srp*, inhibited HNE, suggesting that *B. longum* Srp has potential anti-inflammatory properties. Compared to *B. longum* Δ*srp*, *B. longum srp*(Con) inhibited HNE across all concentrations. Despite significant differences in *srp* expression between *B. longum srp*$^{+}$ and *B. longum srp*(Con) both strains inhibited HNE in our method of elastase activity quantification. More significant inhibition of HNE by *B. longum srp*(Con) may be observed using alternative incubation times, concentration of elastase and substrate. Further, it was previously shown that elastase was capable of inducing serpin mRNA levels in wildtype *B. longum* strains (Turroni, Foroni et al. 2010). Such induction could explain why only a limited difference between the two strains (*srp*$^{+}$ and *srp*(Con)) is observed in this experiment. The innate immune response is a key component in the development of atrophy in CeD (Jabri and Sollid 2009; Sollid and Jabri 2013), and has been proposed to be involved in the pathogenesis of NCGS/NCWS (Junker, Zeissig et al. 2012; Araya, Gomez Castro et al. 2016). The influx and release of neutrophil components is increased in patients with CeD (Hällgren, Colombel et al.

1989), and by inhibiting HNE activity, Srp may specifically target a mechanism that contributes to gluten-related disorders.

Using the NOD/DQ8 model of gluten sensitivity, we examined the therapeutic potential of *B. longum srp*$^{+}$ *in vivo*. As a quality control, we confirmed the presence of *B. longum* in the small intestine of probiotic-treated mice (Figure 6B), and confirmed *srp* expression only in mice receiving *B. longum srp*$^{+}$ and *B. longum srp*(Con). Oral administration of *B. longum srp*$^{+}$ and *B. longum srp*(Con) for 2 weeks protected mice from developing gliadin-induced immunopathology. Because these effects were not achieved in mice receiving *B. longum* Δ*srp*, *srp* expression is important for the protective mechanism. This may be related to immune regulation, maintenance of barrier function, overall beneficial shifts in gut microbiota or inhibition of elastase released during inflammation (Nikolaus, Bauditz et al. 1998; Kessenbrock, Fröhlich et al. 2008).

Probiotic-based therapies have been advocated to restore the balance of a "dysbiotic" or disease-promoting microbiota (Quagliariello, Aloisio et al. 2016). Proteobacteria overgrowth in the small intestine has been reported in patients with active CeD and in those with persistent symptoms after gluten withdrawal (Wacklin, Kaukinen et al. 2013; Galipeau, McCarville et al. 2015). We have shown that experimental Proteobacteria expansion in the small intestine of NOD/DQ8 worsens gluten immunopathology (Wacklin, Kaukinen et al. 2013; Galipeau, McCarville et al. 2015). We therefore measured small intestinal and fecal microbial β-diversity and relative abundances of bacterial groups (Figures 4-5, Supplementary figures 1-2). We found no significant shifts in the small intestinal microbiota between the separate groups,

suggesting that Srp from *B. longum* is unlikely to act through modification of compositional changes of the upper gastrointestinal tract microbiota (Behnsen, Deriu et al. 2013; Eloe-Fadrosh, Brady et al. 2015; Grimm, Radulovic et al. 2015; Kristensen, Bryrup et al. 2016). On the other hand, mild shifts in β-diversity were observed in fecal microbiota of mice treated with *B. longum srp*$^{+}$ and *B. longum srp*(Con) compared to *B. longum* Δ*srp*. Although most differences in relative abundances of genera between groups are difficult to interpret, *Akkermansia* spp. were exclusively increased in *B. longum srp*(Con)-treated mice compared to all other groups. The commensal *Akkermansia muciniphila* is considered to be anti-inflammatory and beneficial for the intestinal mucus layer and barrier integrity in some models of inflammatory disorders (Reunanen, Kainulainen et al. 2015; Derrien, Belzer et al. 2017), and decreased levels of *A. muciniphila* have been observed in patients with IBD and metabolic disorders (Png, Lindén et al. 2010; Rajilic-Stojanovic, Shanahan et al. 2013). This raises the hypothesis that a significant level of Srp delivery, as that provided by *B. longum srp*(Con), may improve the overall mucosal barrier and immune function of the gut, in part, through increases in *Akkermansia* species. Because the role of *Akkermansia* is somewhat controversial based on a recent study (Seregin, Golovchenko et al. 2017), the implications of this finding in our model must be further tested to draw conclusions.

In conclusion, *B. longum srp*$^{+}$ is a commensal bacterium that expresses a serpin, in a non-constitutive manner, that is effective in preventing gliadin-induced immunopathology in NOD/DQ8 mice. As a commensal bacterium, *B. longum srp*$^{+}$ circumvents controversy surrounding the use of GMOs for the delivery of anti-

inflammatory molecules, which may facilitate its translation for human consumption. This study provides mechanistic insight and pathophysiological rationale to explore the efficacy of *B. longum srp*$^{+}$ as an adjunctive therapy in gluten-related disorders or other gastrointestinal inflammatory conditions associated with proteolytic imbalance. Future studies should address the host mechanisms behind protection of gluten-induced pathology by protease inhibitors, or exacerbation due to excess luminal proteases.

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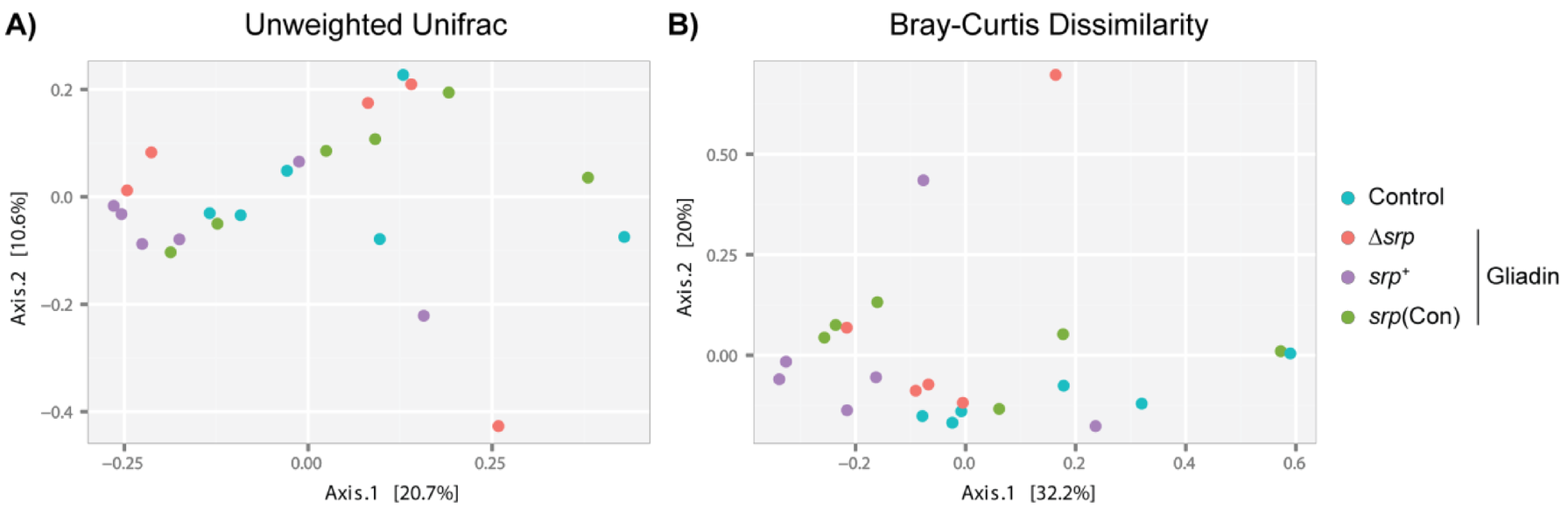
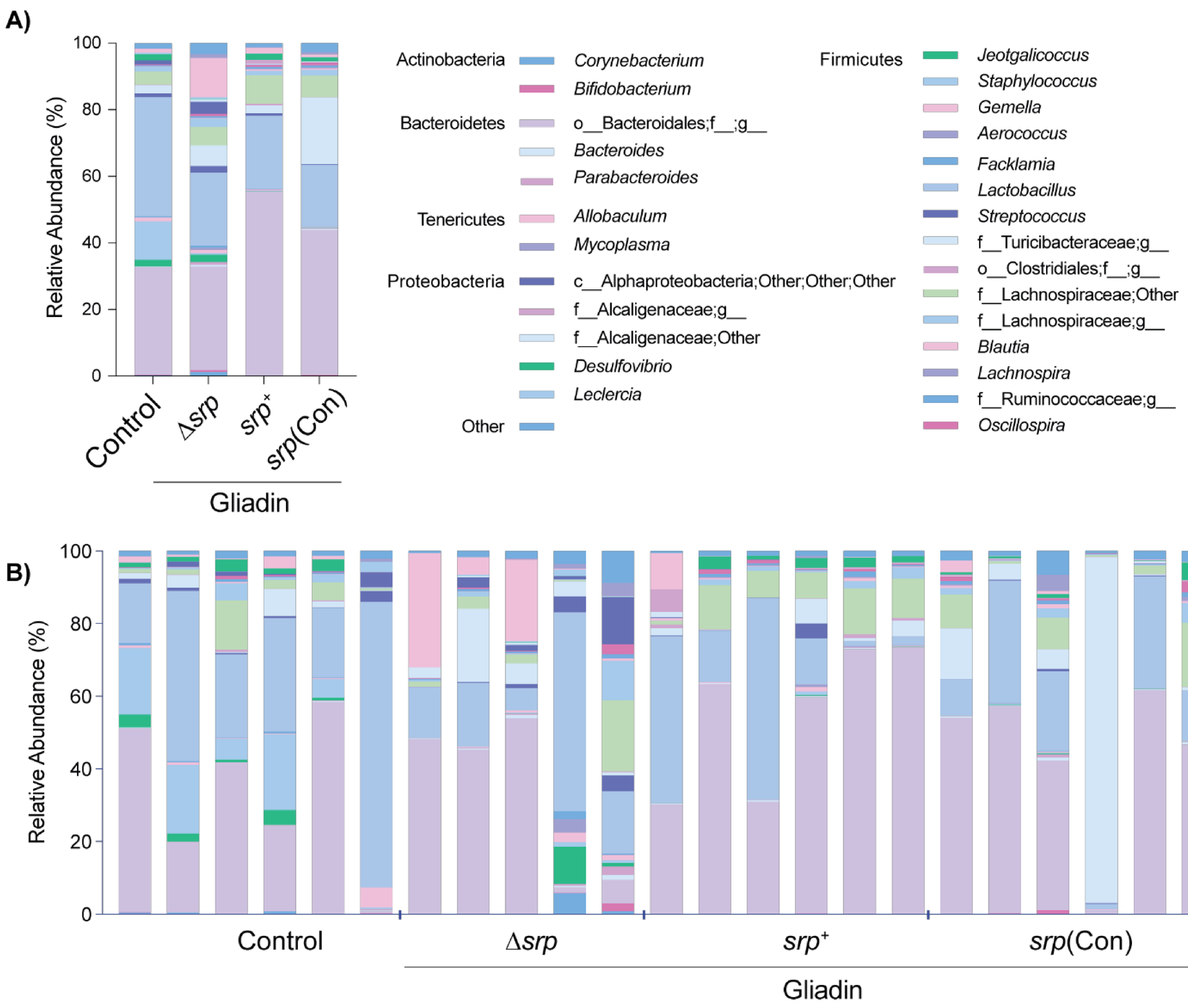
SUPPLEMENTARY MATERIALS



Supplementary figure 1. Small intestinal microbiota profiles.

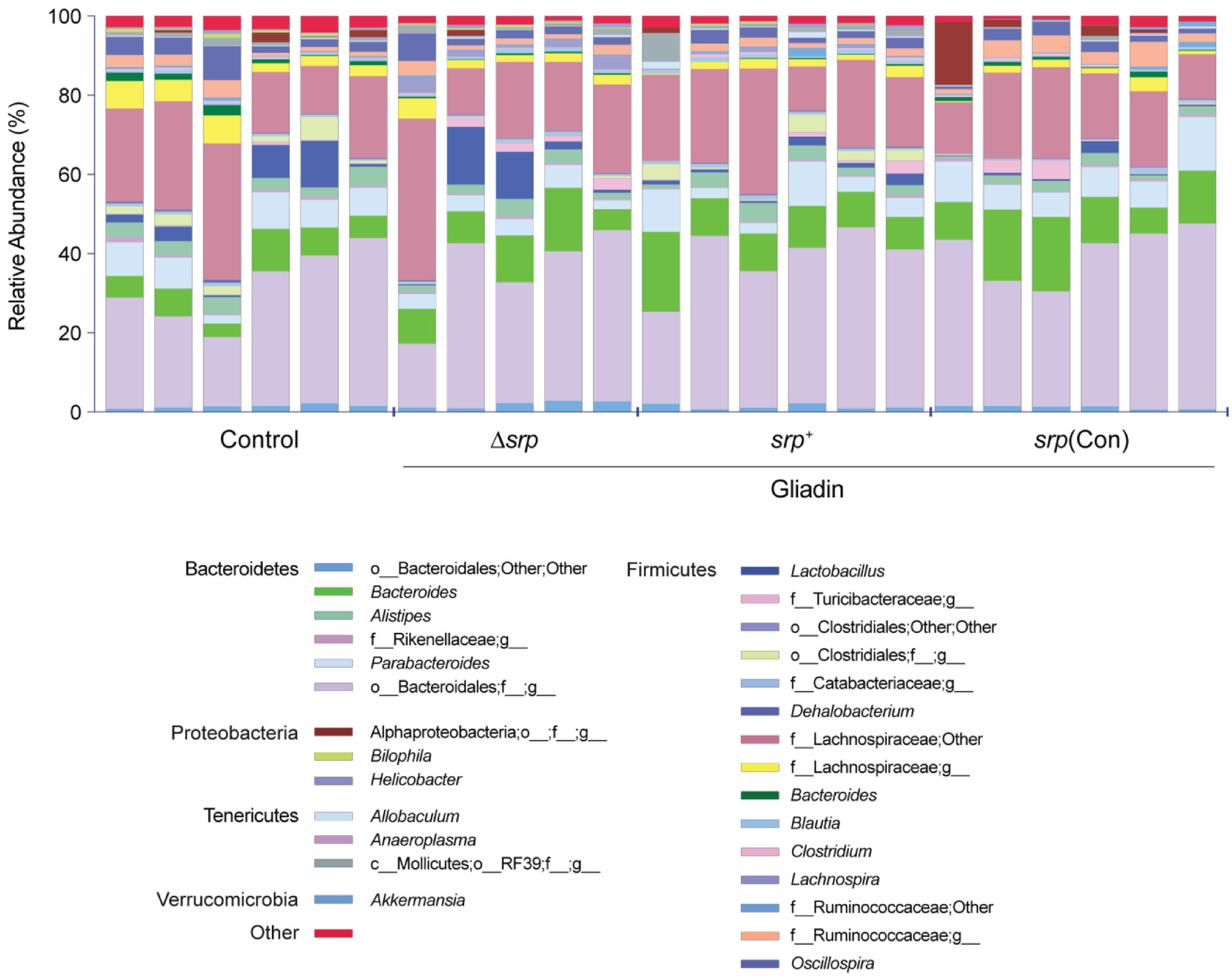
Principal coordinate analysis plots representing β-diversity using both A) Bray-Curtis Dissimilarity and B) Unifrac Unweighted parameters revealed no significant differences in small intestinal microbiota (n=5-6). Non-sensitized, no treatment (Control); *B. longum* srp^+ (srp^+); *B. longum* Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via PERMANOVA in QIIME. Plots were constructed in R.



Supplementary figure 2. Relative abundances in small intestinal microbiota profiles at genus level.

Small intestinal microbiota was sequenced via 16s miSeq Illumina technology. Operational taxonomic units at relative abundances ≥ 1% are presented as A) average of each group and B) per mouse (n=5-6). No changes in relative abundances were found. Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B. longum* Δ*srp* (Δ*srp*); *B.*

longum srp(Con) (*srp*(Con)). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).



Supplementary figure 3. Relative abundances of fecal microbiota for individual mice at genus level.

Fecal microbiota was sequenced via miSeq Illumina technology by amplification of the 16S rRNA gene, and relative abundances are represented at a genus level in stacked column charts (n=5-6). Non-sensitized, no treatment (Control); *B. longum srp*$^{+}$ (*srp*$^{+}$); *B.*

longum Δ*srp* (Δ*srp*); *B. longum srp*(Con) (*srp*(Con)). Statistics were performed via Kruskal-Wallis followed by FDR ($q<0.05$).

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CHAPTER 7: DISCUSSION

6.1 Summary

Celiac disease is a chronic autoimmune enteropathy in which environmental and genetic factors are required for its expression. The most important risk factor is genetic, and given by the carriage of HLA-DQ2 or -DQ8. The environmental triggering agent is dietary gluten, however, in recent years the role of additional factors that modulate disease risk have emerged. Clinical studies have suggested that individuals with celiac disease harbor a dysbiotic gut microbiota (Wacklin, Kaukinen et al. 2013; D'Argenio, Casaburi et al. 2016), with differences in their ability to metabolize gluten (Caminero, Nistal et al. 2015; Tian, Faller et al. 2017). The relationship is thus far, based on association, and detailed immunogenic and biochemical analysis of the combined microbial and host protease gluten digestion has not been evaluated. Our group addressed this issue initiating studies using mice genetically predisposed to react to gluten, through transgenic expression of HLA-DQ8. We showed that the composition of the gut microbiota is critical for either the development or protection of immunopathology induced by gluten (Galipeau, McCarville et al. 2015). However, the mechanisms through which microbial factors contribute to either protection or exacerbation of disease are unknown. *The aim of this thesis was to identify the role and mechanisms through which the intestinal microbiota modulates the development and severity of immune responses to gluten*. In **Chapter 3**, we determined the contribution of the gut microbiota in gluten metabolism *in vivo* in mice, and indirectly, in the consequences of this metabolism on the host. The rationale for this was based on the known, key crucial step that partial gluten digestion by mammalian enzymes plays in the pathogenesis of celiac disease. This led to

the discovery that bacteria can differentially metabolize gluten into peptides that retain high immunogenicity for celiac patients and translocate with ease the mucosal barrier, or, into much smaller peptides, many of which that have lost their immunogenic capacity. This is the first study to highlight that intestinal commensals contribute to the metabolism of gluten in the small intestine and that provides a mechanism, related to the adaptive immune arm of celiac disease, that could modify its risk. In **Chapter 4**, we investigated how microbiota from active celiac disease patients contribute to direct (independent of gluten metabolism) pro-inflammatory events in a murine host. These studies revealed that immune responses relevant to celiac disease, such as IEL induction, are associated with the presence of gluten-degrading bacteria and increases in Proteobacteria in the small intestine. Further, using *P. aeruginosa* (Proteobacteria member with high elastase producing potential) as a model organism, we showed that proteases that metabolize gluten contribute to the severity of gluten-induced pathology in NOD/DQ8 mice. To support the concept that luminal proteases exacerbate gluten pathology, and examine the therapeutic potential of inhibiting this mechanism, in **Chapter 5** we studied the therapeutic potential of a protease inhibitor (serpin) by a commensal bacterium, *B. longum* in NOD/DQ8 mice. Overall the results suggest that gluten metabolism by intestinal bacteria is a mechanism that could contribute to modulate celiac disease risk. Specific protease inhibitors could be developed as adjuvant therapy to the gluten-free diet to treat celiac disease and other gluten-related disorders.

6.2 Commensal-derived proteases metabolize gluten, producing peptides with different immunogenic potential

In **Chapter 3** we demonstrated that the small intestinal microbiota participates in gluten metabolism and plays a key role in determining the immunogenicity of the gluten constituents. We showed that elastase produced by *P. aeruginosa* degrades gluten into peptides that can stimulate Th1 immunity (IFN-γ production by $CD4^+$ T-cells) in patients with celiac disease. Further, the *P. aeruginosa*-derived gluten products can be metabolized by *Lactobacilli* species into peptides incapable of inducing Th1 immunity. This suggests a cooperation between mammalian and bacterial enzymes contributing to gluten metabolism, that is specific to an individual's microbiota and can lead to different abundances of pro-inflammatory or non-inflammatory luminal gluten peptides in humans.

Until the work presented in this thesis it has been generally accepted that large gluten peptides, such as the 33-mer, were the final product of gluten degradation in the gastrointestinal tract, by mammalian enzymatic digestion (Shan, Molberg et al. 2002). For a decade, the existence of non-commensal microbes that produce gluten-degrading enzymes has been known. This has led to the synthesis and production of microbial enzymes with the objective to aid in the digestion of gluten, for pharmacological development in celiac disease (Lahdeaho, Kaukinen et al. 2014; Murray, Kelly et al. 2017). Although recent studies have shown that human intestinal bacteria (commensals) are also capable of digesting gluten *in vitro* (Zamakhchari, Wei et al. 2011; Caminero, Herran et al. 2014; Wei, Tian et al. 2015), the exact *in vivo* significance has remained unknown. For instance, Wei *et al.* (2015) identified *P. aeruginosa* as a bacterium with high gluten-degrading capacity. They also identified the major protease capable of doing

so, elastase. However, their conclusion centered in the traditional "enzymatic therapeutic hypothesis" and the authors concluded that "*P. aeruginosa* elastase would be a suitable candidate for therapeutic intervention in celiac disease" (Wei, Tian et al. 2015). We raised concerns regarding the safety of using a pro-inflammatory protease from an opportunistic pathogen for human consumption. We, therefore, engaged in a detailed analysis of gluten metabolism *in vivo*, by bacteria isolated from celiac patients that exhibited gluten metabolic activity *in vitro*. Our results support a different conclusion from Wei et al. (2015), that *P. aeruginosa* elastase is likely to have negative consequences for individuals with celiac disease, as the products of gluten metabolism are highly immunogenic. Our results raise a new dimension in the understanding of gluten metabolism by bacteria that have implications for celiac disease pathogenesis and the development of enzymatic therapies.

Studies have shown that celiac disease patients harbor a dysbiotic microbiota (Wacklin, Kaukinen et al. 2013; D'Argenio, Casaburi et al. 2016). Some studies propose that patients with celiac disease have less abundance of Lactobacilli (Lorenzo Pisarello, Vintini et al. 2015). Our results suggest that at least one mechanism through which the described dysbiosis could contribute to modulate celiac disease risk may be through impaired gluten metabolism by some Lactobacilli. This is in agreement with a study in which supplementation of mice with *Lactobacillus* was effective in alleviating gluten-induced pathology (D'Arienzo, Bozzella et al. 2011). Further, our group has previously shown that in the NOD/DQ8 model of gluten sensitivity the development of gluten immunopathology is dependent on the gut microbiota composition (Galipeau, McCarville

et al. 2015). Specifically, NOD/DQ8 mice colonized with clean SPF (ASF-derived) were protected from developing gluten immunopathology. Interestingly, in **Chapter 3** we show that the composition of the small intestinal microbiota is dominated by *Lactobacillus* in clean SPF-colonized mice. This suggests that the protection in clean SPF NOD/DQ8 mice may be, in part, due to the capacity of Lactobacilli to degrade pro-inflammatory gluten peptides. Further studies must address how the metabolism of gluten by the microbiota and downstream adaptive immune responses may influence gluten-immunopathology in NOD/DQ8 mice.

There is great controversy on how large gluten fragments, such as the well-described 33-mer, assumed to be a main product of mammalian enzyme digestion, translocate the epithelial barrier. This is a critical point in celiac disease pathogenesis as it will allow these peptides to interact with TG2 to trigger downstream adaptive immune responses. Multiple mechanisms have been proposed to explain gluten peptide translocation, including a paracellular route through altered tight junctions (Lammers, Lu et al. 2008) and a transcellular route that becomes active in later stages of the disease through ectopic expression of the tranferrin receptor in epithelial cells (Matysiak-Budnik, Moura et al. 2008; Lebreton, Menard et al. 2012). The transcellular route has been criticized, based on the fact the 33-mer peptide and other peptides resulting from mammalian protease digestion are very large. In **Chapter 3** we show that peptides generated from a digestion of gliadin treated with pepsin and trypsin (PT-gliadin) and further modified by *P. aeruginosa*, translocate the mucosa more readily than PT-gliadin alone. This suggests that the degradation of gluten constituents by bacterial enzymes may

have a critical role in determining which peptides translocate the intestinal barrier. We thus uncover a new mechanism by which the microbiota may contribute to further reduce the size of some gluten peptides, enhancing their capacity for translocation, while retaining immunogenic sequences. This mechanism may be targeted therapeutically by either luminal degradation of pro-inflammatory gluten by beneficial bacteria (detoxification) or, by inhibiting the enzymatic activity of opportunistic pathogens that enhance peptide translocation. These concepts bring new insight to the understanding of gluten peptide metabolism in the small intestine and of specificity of enzymatic therapy in the treatment of celiac disease.

6.3 Commensal-derived proteases trigger intestinal inflammation and exacerbate gluten pathology

The role of proteases in mediating inflammation at mucosal surfaces has been previously evaluated. However, most of these studies have investigated the role of host proteases in these processes. In the previous study evaluating the role of the microbiota in the severity of gluten-induced pathology in NOD/DQ8 mice, there were no clear microbial mechanisms established in the NOD/DQ8 model of gluten sensitivity that mediate gluten immunopathology. In **Chapter 4**, we establish that, 1) consuming gluten may increase the abundance of gluten-degrading bacteria and their associated proteases; 2) The same bacterial proteases involved in the metabolism of gluten can also interact with the host leading to low-grade inflammation; 3) These proteases contribute to the severity of gluten immunopathology in a host at risk.

Interestingly, we have previously shown that clean SPF NOD/DQ8 mice become susceptible to gluten immunopathology upon colonization with a virulent *E. coli* strain isolated from a celiac disease patient. This strain of *E. coli* harbored virulence factors (*fimA* and *kfiC*) allowing attachment of epithelial cells (Galipeau, McCarville et al. 2015). This raises the possibility that there may be common mechanisms through which this *E. coli* strain and *P. aeruginosa* predispose NOD/DQ8 mice to more severe gluten immunopathology. Both bacteria contain virulence factors that are recognized by host immune pathways and provide them with a competitive advantage. One possibility is that proteases released by *P. aeruginosa* degrade mucin and enhance microbial encroachment to the epithelium. Thus, closer contact or attachment of opportunistic pathogens to the duodenal mucosa, may be a required step for the initiation of direct microbe-host signals (Ou, Hedberg et al. 2009).

A role of bacterial-derived proteases in the weakening of mucosal barrier that is responsible for segregation of host from luminal bacteria has previously been established in a colitis model. Steck *et al.* (2011) demonstrated that *E. faecalis* gelatinase (GelE) predisposes IL-$10^{-/-}$ to a greater occurrence of spontaneous colitis. Using mono-colonized mice, they show there are no specific T-cell responses to GelE, suggesting that exacerbation of colitis is likely degradation of the tight junction E-cadherin (Steck, Hoffmann et al. 2011). However, this study did not identify host receptors and signaling pathways that GelE could activate or disarm to trigger downstream inflammation. We determined that bacterial proteases cleave PAR-2 *in vitro*, and using animal models we showed that this correlated with higher IEL counts in the small intestinal mucosa, as the

changes did not occur in PAR-2$^{-/-}$ mice. Further, PAR-2 was highly expressed on the intestinal epithelium of mice supplemented with *P. aeruginosa* producing elastase, suggesting the presence of the opportunistic pathogen was the source of PAR-2 activation. We do not, however, know whether bacterial pro-inflammatory proteases reach the lamina propria, where they could also directly interact with immune cells. This mechanism of action could be important as PAR-2 signaling has previously been shown to be involved in antigen transport and T-cell activation (Ramelli, Fuertes et al. 2010). We cannot rule out the possibility that the elastase from *P. aeruginosa* disrupts the clean-SPF structure, which could exacerbate immune responses and pathology further. Indeed, neutrophil elastase has been shown to trigger dysbiosis (Gill, Ferreira et al. 2012). We also cannot rule out that gluten-degrading proteases directly degrade tight junction proteins in a similar way as described for GelE, as elastase has been shown to do this *in vitro* (Azghani 1996). This would constitute an additional mechanism that could contribute to disease pathogenesis through the facilitation of gluten peptide translocation to the lamina propria.

The interaction between diet-microbiota-host is a common theme that is unraveling in the literature. Complex interactions between diet, increased pathobiont burden and the inflammatory state of the host (Devkota, Wang et al. 2012), as well as microbially-derived anti-or pro-inflammatory metabolites (Lamas, Richard et al. 2016), have been described. Signaling pathways and mechanistic details are needed as well as the consequences of these interactions in health and disease. In **Chapter 4**, we show that the addition of gluten to microbial communities *in vitro* and *in vivo* leads to the expansion

of *P. aeruginosa* and its pro-inflammatory elastase. We identify a bacterial-gluten interaction that may contribute to host inflammation and susceptibility to disease through proteases that can directly act and induce inflammatory cascades within the host. This may be a common mechanism in disorders when consuming substrates incapable of digestion by mammalian proteases. This mechanism allows for targeted therapy, such as the delivery of luminal protease inhibitors.

Although the contents of this thesis evaluate the role of proteases and protease inhibitors in the context of celiac disease, these molecules and the concepts arising from this thesis could also be applied to other gluten-related disorders or food sensitivities. NCG/WS is a clinical condition believed to be triggered by ingestion of wheat components that induce innate immune activation once celiac disease or wheat allergy have been ruled out. Our data suggest that, under some circumstances, consuming gluten may increase the abundance of gluten-metabolizing bacteria and their associated proteases, triggering innate immunity, independently of host genotype. NCG/WS is not necessarily associated with the typical celiac risk alleles, and therefore a gluten-pathobiont-host mechanism may contribute to its pathophysiology and warrant further investigation. The same mechanism could apply to other proposed triggers in wheat for NCG/WS, such as amylase trypsin inhibitors (ATIs), a group of proteins with potent innate immune stimulation. We have preliminary unpublished data (McCarville *et al.* and Zevallos *et al.*, DDW 2017) suggesting intestinal bacteria also participate in ATI metabolism.

6.4 Delivery of a protease inhibitor by a commensal bacterium prevents gluten-induced pathology

Our group has previously shown that patients with celiac disease have decreased expression of the human serine proteases inhibitor elafin in their small intestinal mucosa. To confirm a role of proteolytic imbalance in celiac disease, recombinant delivery of elafin via oral treatment with *Lactococcus lactis* expressing elafin alleviated gluten-induced pathology in NOD/DQ8 mice (Galipeau, Wiepjes et al. 2014). Interestingly, the alleviation of the gluten-induced pathology appeared to be independent of changes in mucosal (host) protease activity (trypsin-like or elastase-like). The study showed that elafin can block deamidation of gluten *in vitro* by competitive binding to TG2, suggesting a possible mechanism of action that would directly affect a pathogenic step in celiac disease. Moreover, a direct barrier protecting effect was observed *in vivo* after administration of *L. lactis*-elafin. However, concerns were raised regarding the use of genetically-modified organism (GMO) in human consumption. Thus, we turned out attention to commensal organisms that could naturally produce elafin-like molecules.

In **Chapter 5**, I establish a role for a commensal serpin produced by a human-derived *B. longum* strain in preventing gluten-immunopathology in NOD/DQ8 mice. This *B. longum*-derived serpin prevented increases in IELs, decreases in V/C ratios and barrier dysfunction in NOD/DQ8 treated with gluten. Although the mechanism by which the serpin acts in our model is unknown, we hypothesize that it involves blocking pathways of either innate or adaptive immune responses relevant to celiac disease, in an analogous way as described in our previous work using *L. lactis*-elafin. The study described in **Chapter 5** aimed at providing proof of concept that a commensal organism naturally

producing a molecule of interest (serpin) could be used therapeutically as an alternative to a GMO like *L. lactis*-elafin. The results of both **Chapter 3** and **Chapter 4** suggest that gastrointestinal commensal-derived proteases can modify adaptive and innate mechanisms involved in celiac disease pathogenesis. I thus hypothesize that the *B. longum* serpin inhibits host, or commensal pro-inflammatory proteases. For instance, the serine protease granzyme B is involved in the pathogenesis of celiac disease (Korneychuk, Ramiro-Puig et al. 2014) and may be a target of the *B. longum*-derived serpin. The *B. longum* serpin may also prevent gluten-immunopathology through modulation of gluten metabolism by both host and bacterial proteases. The prevention of gluten degradation by serine proteases such as elastase may reduce the formation of pro-inflammatory gluten peptides as described in **Chapter 3**. Further, it could inhibit activation of PAR-2 or other receptors by microbial or host proteases as described in **Chapter 4**. The results of this study have triggered significant interest in our industrial partners, and a pilot study to test *B. longum* serpin in celiac patients that remain symptomatic despite the gluten-free diet, in being planned.

Proteolytic imbalances are not just dependent on increases in proteolytic activities, but also involve decreases in protease inhibitors. No changes in proteolytic activities have been shown in the NOD/DQ8 mice when sensitized with gluten. However, a decrease in host or microbial protease inhibitors, as previously observed in patients with active celiac disease (Galipeau, Wiepjes et al. 2014) is possible. Therefore, the protective effects of *B. longum* serpin may be due to a correction of a deficiency in protease inhibitors. We do not know if *B. longum* serpin blocks deamination of TG2 in an equivalent way to elafin,

and this should be determined in future studies. However, previous work from our lab suggest that the NOD/DQ8 mouse model is independent of deamidation of gluten, as antibody formation in these mice is specific to non-deamidated gluten (Galipeau, Rulli et al. 2011). Therefore, this should be studied in humans or *in vitro*, using human TG2.

6.5 Future Directions

The contents of this thesis evaluate the role of commensal microbial-derived proteases and protease inhibitors in influencing responses to dietary antigens, through adaptive and innate immune mechanisms. However, questions remain that should be addressed in future studies. Firstly, the contributions of commensal microbial proteases to the total protease profile present in the gastrointestinal tract, is unknown. The role of bacterial-derived proteases to the total proteolytic balance in SPF conditions should be evaluated in comparison with other microbial colonizations. As a clean SPF colonization is protective in the NOD/DQ8 model of gluten sensitivity, it would be important to evaluate the proteolytic contributions of this defined microbiota in comparison to SPF colonization.

Secondly, in the NOD/DQ8 model of gluten sensitivity, mice are sensitized to gluten with a combination of PT-gliadin and cholera toxin. The contents of this thesis evaluate the role of proteases and protease inhibitors in influencing the immunopathology to gluten (from oral gluten challenge) after this sensitization process. Future directions must address if proteases could be involved in the breakdown of oral tolerance towards

gluten (sensitization phase) in NOD/DQ8 mice. This would reveal the role of bacterial proteases in instigating the disease and therefore, may be more clinically relevant.

Lastly, future studies should evaluate how human commensal-derived proteases or protease inhibitors from small intestinal bacteria, in combination with genetics, and gluten exposure are associated with the development celiac disease. For instance, of interest to me is whether there is a difference in the way an individual at risk for celiac disease, or a potential celiac, metabolizes gluten in comparison to an individual without celiac disease risk. This could unravel clinical associations between the presence of certain bacteria in the small intestine and the probabilities of developing celiac disease. This could also reveal whether the dysbiosis observed in celiac disease patients is associated with differential metabolism of gluten. Further, within this thesis, we observed that some bacteria capable of metabolizing gluten (ie. *P. aeruginosa*), bloom in the small intestine of gluten challenged mice. Thus, future directions should investigate the connection between gluten ingestion and changes in gut microbiota structure and proteolytic balance in patients with NCG/WS.

6.6 Conclusions

The data encompassed in this thesis provide evidence that the gastrointestinal microbiota can exacerbate gluten-related disorders through two distinct mechanisms. The first being the metabolism of gluten constituents into peptides capable of evoking potent adaptive immune responses in patients with celiac disease. The second is the production of proteases that generate direct innate immune responses in the small intestinal mucosa.

When these proteases are present in a host at risk of developing celiac disease, they could constitute the second hit required for the development of enteropathy. These mechanisms can be therapeutically targeted by delivery of a commensal-derived serpin that would protect genetically-susceptible individuals from developing celiac disease.

Together these results suggest that a combination of 1) Host genetics; 2) Gluten peptide metabolism by both bacterial and mammalian enzymes (adaptive arm) and 3) Microbes capable of directly activating innate immune pathways and signaling through PARs, are critical to the development of celiac disease. These results may help explain the gap between the number of individuals who develop celiac disease and those that have the genetic susceptibility haplotypes associated with celiac disease. Like other diseases, it appears the “three-hit” model applies to the development of celiac disease in genetically susceptible individuals, which includes microbial signals influencing innate immune processes (eg. IELs and epithelial stress) and adaptive gluten-specific responses. Further, the combination of bacterial-derived components may work in unison with other microbial factors, such as viruses (Bouziat, Hinterleitner et al. 2017) that imprint pro-inflammatory phenotypes in APCs that may be required for the development of celiac disease or other food sensitivities. In conclusion, commensal-derived proteases, and protease inhibitors, are factors that affect both adaptive and innate immune pathways relevant in celiac disease. These pathways could be targeted to prevent or treat gluten-related disorders.

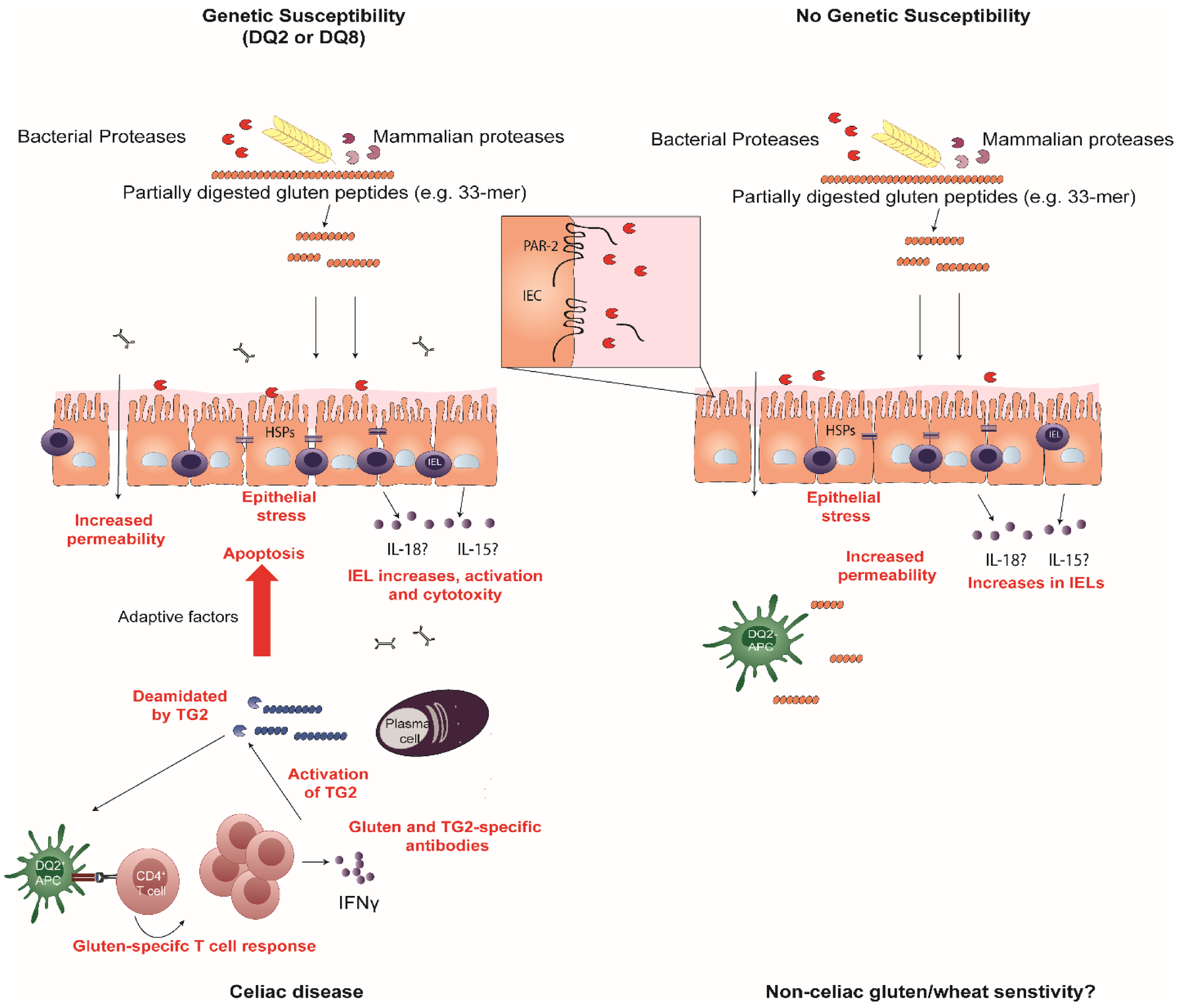

Figure 6.1. Overview of the potential contribution of bacterial proteases in celiac disease. When consumed, gluten is digested by a combination of microbial and mammalian proteases. These proteases can cleave PARs, leading to downstream innate immune responses, which in an individual without genetic risk for celiac disease, could cause gluten-sensitive conditions such as NCG/WS. On the other hand, partial digestion of gluten by some elastase-producing opportunistic pathogens, facilitates immunogenic peptide translocation. In an individual with genetic susceptibility this could lead to

downstream adaptive immune responses. The combination of innate and adaptive immune mechanisms described, could promote development of enteropathy in a genetically-susceptible host.

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APPENDIX I: NOVEL PERSPECTIVES ON THE HERAPEAUTIC MODULATION OF THE GUT MICROBIOTA

Therapeutic Advances in Gastroenterology *Review*

Novel perspectives on therapeutic modulation of the gut microbiota

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***Abstract*:** The gut microbiota contributes to the maintenance of health and, when disrupted, may drive gastrointestinal and extragastrointestinal disease. This can occur through direct pathways such as interaction with the epithelial barrier and mucosal immune system or indirectly *via* production of metabolites. There is no current curative therapy for chronic inflammatory conditions such as inflammatory bowel disease, which are complex multifactorial disorders involving genetic predisposition, and environmental triggers. Therapies are directed to suppress inflammation rather than the driver, and these approaches are not devoid of adverse effects. Therefore, there is great interest in modulation of the gut microbiota to provide protection from disease. Interventions that modulate the microbiota include diet, probiotics and more recently the emergence of experimental therapies such as fecal microbiota transplant or phage therapy. Emerging data indicate that certain bacteria can induce protective immune responses and enhance intestinal barrier function, which could be potential therapeutic targets. However, mechanistic links and specific therapeutic recommendations are still lacking. Here we provide a pathophysiological overview of potential therapeutic applications of the gut microbiota.

Keywords: fecal microbiota transplant, inflammatory bowel disease, irritable bowel syndrome, microbiota, prebiotics, probiotics

Introduction

During birth we are colonized with a plethora of microorganisms that will play a crucial role in defining our future physiology and immunity, impacting health and disease. The key role of bacteria in shaping immunity and gut structure emerged over the last decades [Logan *et al.* 1991; Shroff and Cebra, 1995; Round and Mazmanian, 2009; El Aidy *et al.* 2012]. These studies have provided the rationale to develop procedures that reconstitute the microbiota to a state that would prevent or treat disease [Carding *et al.* 2015]. Gut dysbiosis can be defined as a stable, perturbed ecosystem that has reduced capacity for protection and is associated with disease. The most studied gastrointestinal clinical condition in association with dysbiosis is inflammatory bowel disease (IBD) [Dalal and Chang, 2014], although meta-analyses, studies that combine multiple trials and provide the highest clinical evidence, have not been able to reproduce consistently the alterations that are described within single studies [Walters *et al.* 2014]. One of the key questions relates to causality of these associations. Some animal models have shown a primary role of dysbiosis in models of IBD [Natividad *et al.* 2015; Schaubeck *et al.* 2015; Seo *et al.* 2015], but it has been difficult to translate this to the clinical setting [Abraham and Cho, 2009; Kaser *et al.* 2010]. Another common gut disorder is celiac disease, a chronic autoimmune enteropathy, genetically linked to human leucocyte antigen (HLA) DQ2/8 and triggered by dietary gluten. Additional environmental factors to gluten are believed to play a role in disease progression, given the fact that 30% of the population carry genetic risk but about 3–4% will develop the disease. Some studies have demonstrated dysbiosis in patients with celiac disease [De Palma *et al.* 2010], with increases in bacteria with virulence factors [Sanchez *et al.* 2008] and persistence of dysbiosis after treatment with the gluten-free diet in association with clinical symptoms [Wacklin *et al.* 2014]. More recently, evidence for a modulatory role of gut microbiota

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in host responses to gluten has been described in animal models of gluten sensitivity [Galipeau *et al.* 2015]. The microbiota has also been implicated in one of the most common categories of gastrointestinal disease, functional bowel disorders, and its prototype irritable bowel syndrome (IBS) as well as its psychiatric comorbidities [Bercik *et al.* 2011; Collins, 2014; De Palma *et al.* 2015]. In addition to gut disorders, studies are beginning to show how the microbiota can influence systemic diseases such as arthritis and psoriasis [Scher *et al.* 2015a], type 1 diabetes [Sun *et al.* 2015a], cardiovascular disease [Koeth *et al.* 2013] and conditions such as obesity [Cox *et al.* 2015]. Overall, studies in both animals and humans suggest that the microbiota could constitute a worthy target for preventative or therapeutic modulation (overviewed in Figure 1). Many studies have demonstrated the roles of specific microbes in contributing to either protection or exacerbation of disease. However, harnessing the power of these microbes has not yet been fully elucidated.

Potential targets of microbiota modulation

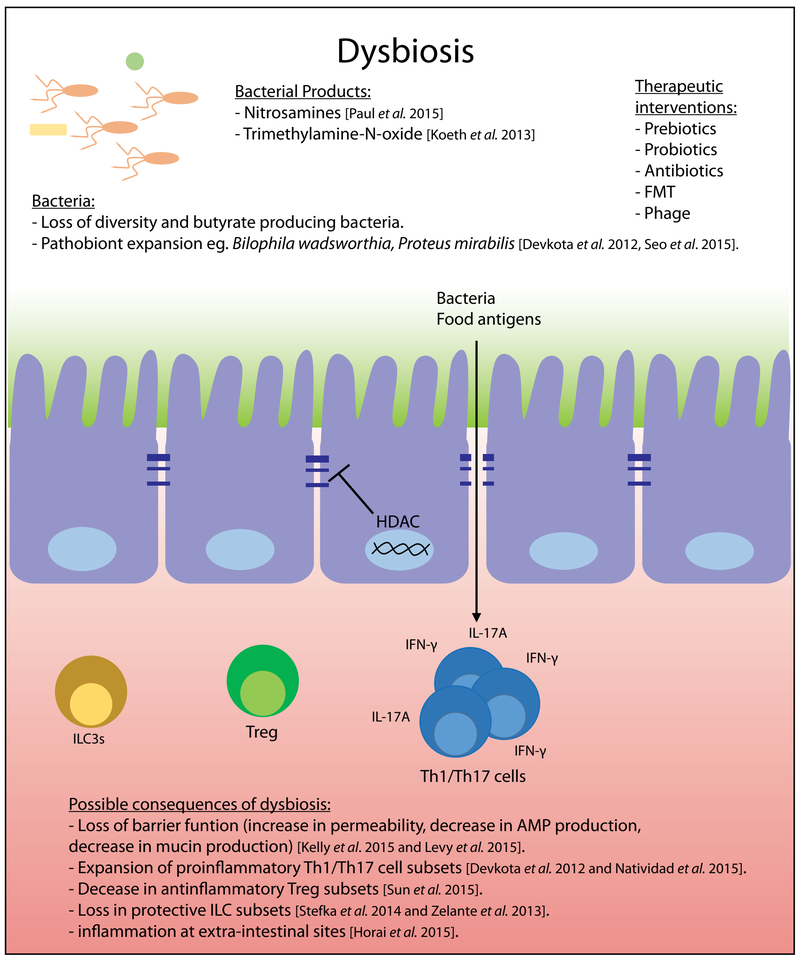
T-cell modulation

The development and maintenance of T cells is directly influenced by the microbiota as demonstrated in seminal studies investigating $CD4^+$ T-cell induction in germ-free mice colonized by commensal microbes [Macpherson and Harris, 2004]. Indirect interactions through changes in microbiota induced by diet have been demonstrated. A diet high in milk-derived saturated fats led to blooming of the pathobiont, *Bilophila wadsworthia*, and enhanced colonic T helper 1 (Th1) responses [interferon (IFN)-γ-producing $CD4^+$ cells] in interleukin (IL)-$10^{-/-}$ mice [Devkota *et al.* 2012]. Targeting the expansion of a pathobiont that induces proinflammatory responses could be one approach to treat disease. Indeed, antibiotics decrease both IFNγ and IL-17 producing $CD4^+$ cells [Hill *et al.* 2010] and studies using genetic models of IBD have demonstrated that treatment of mice with antibiotics decreases disease severity [Gkouskou *et al.* 2014]. However, antibiotics have also been linked to the development of disease later in life [Vangay *et al.* 2015]. But as recently shown, mice colonized with human bacteria rich in Firmicutes, specifically *Lachnospiricheae* and *Ruminocaceae*, were less susceptible to colitis, and had reduced pathogenic Th17 cells [Natividad *et al.* 2015]. Thus, expansion of beneficial bacteria that decrease proinflammatory responses may also constitute a viable approach to treat IBD. This may also apply to extraintestinal disorders that involve the proinflammatory Th17 pathway [Horai *et al.* 2015], which could be driven by gut dysbiosis.

T-regulatory cells (Tregs) are essential for homeostasis in the gastrointestinal tract for suppression of responses to nonpathogenic bacteria [Sun *et al.* 2015b] and food antigens [Pabst and Mowat, 2012]. Induction of Tregs improves colitis in animal models of IBD [Mayne and Williams, 2013], autoimmune diseases [Wing and Sakaguchi, 2010] and allergy [Palomares *et al.* 2010]. Specific microbial inducers of Tregs have been identified, such as those belonging to Clostridia [Atarashi *et al.* 2011, 2013] and possibly *Parabacteroides* [Kverka *et al.* 2011]. The mechanism likely depends on the production of the short-chain fatty acid (SCFA) butyrate [Furusawa *et al.* 2013], as it has been shown to expand Tregs *in vivo* [Arpaia *et al.* 2013] *via* an epigenetic mechanism; acetylation of Foxp3 locus. Demonstrating this in the context of disease is a recent study indicating protection of offspring from allergic airway disease *via* Treg induction by supplementing a maternal diet with high fiber that increases acetate production [Thorburn *et al.* 2015]. Additionally, polysaccharide A from *Bacteroides fragilis* has been shown to induce IL-10-producing Tregs, likely through a different mechanism [Round and Mazmanian, 2009], protecting against experimental autoimmune encephalomyelitis [Ochoa-Reparaz *et al.* 2009]. More recently, a distinct Treg population, expressing Rorγ (defined as $Ror\gamma^+$ $Helios^-$ $FoxP3^+$ $CD4^+$ $TCR\beta^+$) has been shown to be controlled by microbiota, specifically *Clostridium ramosum*, *Bacteroides thetaiotaomicron*, *Peptostreptococcus magnus* and *Bacteroides fragilis*. This Treg subset was shown to improve 2, 4, 6 trinitrobenzenesulfonic acid model of colitis [Sefik *et al.* 2015]. Thus, mechanisms of Treg induction could be targeted to prevent of disease.

Dendritic cell modulation

Dendritic cells (DCs) control downstream T-cell responses, and thus are potential targets of therapeutic manipulation. The mechanisms by which microbiota affects DC subsets has been less explored than those of T cells. However, a few studies have demonstrated a microbiota–DC axis. For instance, mice colonized with segmented filamentous bacteria produce more serum amyloid A (SAA) which act on DCs to skew toward a Th17



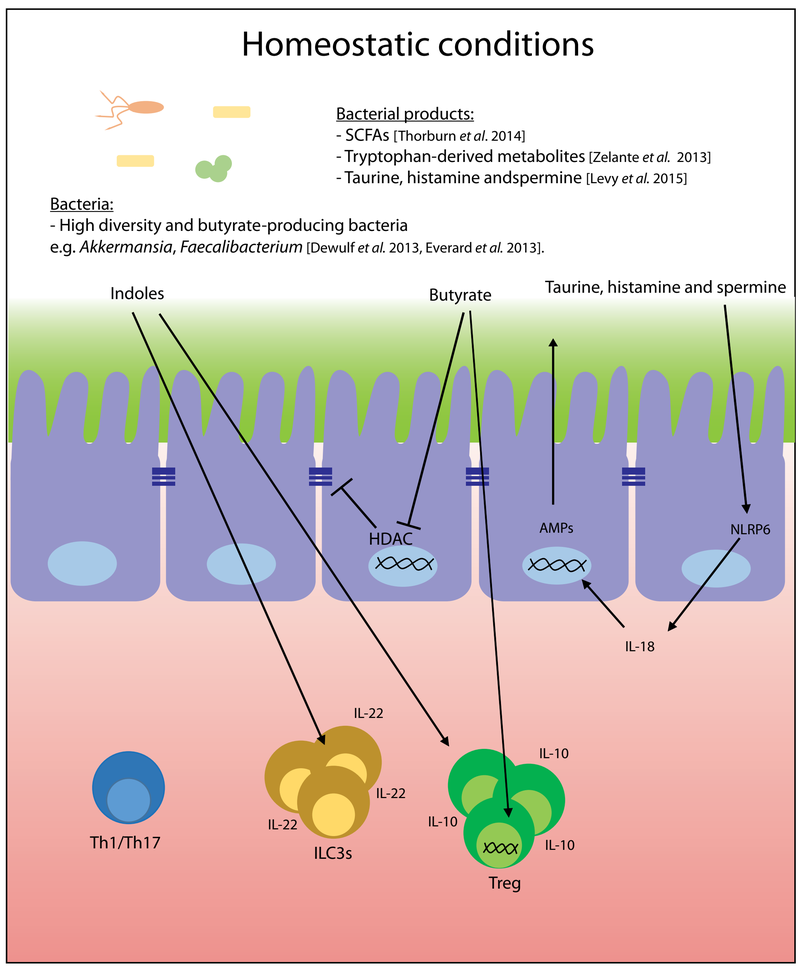


Figure 1. Proposed homeostatic and dysbiotic mechanisms of the microbiota and possible therapeutic interventions. AMP, antimicrobial peptides; FMT, fecal microbiota transplant; HDAC, histone deacetylase; IFN, interferon; IL, interleukin; ILC, innate lymphoid cell; NLRP6, NOD-like receptor family pyrin domain containing 6; SCFA, short chain fatty acid; Treg, T-regulatory cell.

phenotype [Ivanov *et al.* 2009]. Microbiota can induce SAA, leading to neutrophil migration, which may also be partly mediated by DC activity [Kanther *et al.* 2014]. Further, it has been demonstrated that induction of SAA is mediated by bacterial adhesion to epithelial cells, leading to downstream Th17 cell responses *via* $CD11c^+$ cells, which were critical for the response [Atarashi *et al.* 2015]. Recent data suggest that manipulating the microbiota may modulate cancer immunotherapy through DCs. Intestinal microbes, such as *Bifidobacterium*, may cause DCs to enhance $CD8^+$ T-cell responsiveness to checkpoint blockade therapies, although the mechanism of this effect remains to be elucidated [Sivan *et al.* 2015]. More research is needed to identify how pro- and anti-inflammatory DC subsets interact with the microbiota and their metabolites.

Intestinal barrier function

Many diseases of the gastrointestinal tract and extraintestinal conditions are associated with intestinal barrier alterations. This includes both functionality (permeability) and immune or structural parameters of the barrier such as secretion of antimicrobial peptides and mucins, in which the microbiota can play a central role [Vaishnava *et al.* 2008; Natividad and Verdu, 2013]. Alterations in barrier function by bacteria has been associated in animal studies with increases in translocation of bacteria, uptake of microbial products and dietary antigens, and it has been proposed that this could promote a proinflammatory state. Clinically, small intestinal bacterial overgrowth is associated with changes in barrier function in humans, however mechanisms and bacterial culprits or constituents are unknown [Bures *et al.* 2010]. It has been previously shown that mice fed a high-fat diet have increased paracellular permeability in the ileum. This change correlated with a decrease in the class Clostridia [Hamilton *et al.* 2015]. The class Clostridia was also implicated in the development of allergy. Stefka and colleagues demonstrated that mice colonized with Clostridia are protected from allergy due to IL-22-mediated enhanced barrier function [Stefka *et al.* 2014] and induction of innate lymphoid cells (ILCs). Although, the implications of supplementing Clostridia for enhancement of barrier function have not been explored, the same group has recently found that supplementing children with a probiotic *Lactobacillus rhamnosus* GG induced members of the Clostridia class, *Lachnospiraceae*, which are butyrate producers [Berni Canani *et al.* 2016]. Low-dose SCFAs have also been shown to enhance barrier function *in vitro* [Peng *et al.* 2007], reduce translocation of bacteria [Lewis *et al.* 2010] and affect tight

junction assembly [Peng *et al.* 2009], and be protective in animal models of colitis *via* regulation of barrier function [Macia *et al.* 2015]. Specifically, in animal models, butyrate is critical for many aspects of barrier function including permeability, mucin and antimicrobial peptide production, by inducing HIF1α [Kelly *et al.* 2015]. Barrier function is controlled epigenetically and butyrate, being a histone deacetylase inhibitor *via* suppression of histone deacetylases, can increase tight junction proteins [Bordin *et al.* 2004]. The bacterium *Faecalibacterium prausnitzii* has also demonstrated the ability to increase barrier function in dextran sodium sulfate (DSS) colitis, which may be mediated through butyrate [Carlsson *et al.* 2013]. These studies suggest that a diet targeting the expansion or the introduction of butyrate-producing bacteria, such as Clostridia, may enhance barrier function. Antimicrobial production is also important in shaping barrier function in the gut. Bacterial metabolites and constituents have a critical role in induction of antimicrobials. It has been recently demonstrated by Levy and colleagues that microbial-derived taurine, histamine and spermine are important in NOD-like receptor family pyrin domain containing 6 (NLRP6) inflammasome activation, critical for antimicrobial production in the epithelia of the colon and protective in DSS-induced colitis [Levy *et al.* 2015]. Utilizing these metabolites in the clinic may be a future therapy for restoring microbial homeostasis in the gut of diseased individuals.

New players

Interest in the role of ILCs has emerged in the past few years since their populations are regulated by the microbiota [Sonnenberg and Artis, 2015]. ILCs play a role in an array of inflammatory and autoimmune conditions, including asthma, psoriasis and IBD [McKenzie *et al.* 2014; Marafini *et al.* 2015]. ILC3s have been shown to be affected by the microbiota and to modify the microbiota in turn. The expansion and maintenance of ILC3s is dependent on the aryl hydrocarbon receptor (AHR) [Qiu *et al.* 2013]. It has been demonstrated *in vitro* that indole, tryptamine and indole-3-acetate modulated AHR-mediated responses in Caco-2 cells, and indole is produced by the microbiota [Jin *et al.* 2014]. Specifically, it has been shown that *Lactobacillus reuteri* and *L. acidophilus* can produce indole-3-aldehyde from dietary tryptophan, inducing the production of IL-22 from ILC3s [Zelante *et al.* 2013]. This may have implications for allergy [Leung and Loke, 2013] and IBD [Zenewicz *et al.* 2008, 2013], thus more research is needed on the role of specific microbes that induce ILCs in mammalian hosts.

Manipulation of the gut microbiota

Basic research studies have demonstrated important roles of the gut microbiota in the development of immune, metabolic and host function, as well as provided evidence that disruptions in host–microbiota interactions may impact disease. Strategies to manipulate the gut microbiota with the aim to treat inflammatory disorders, such as probiotics, prebiotics and FMT have therefore been proposed.

Diet as a strategy to modulate microbiota and prevent or treat disease

Diet is considered one of the main driving forces shaping intestinal bacterial communities. Several studies highlight the effects of diet on gut microbiota composition and repertoire of microbial metabolites [Wu *et al.* 2011; David *et al.* 2014]. Sonnenburg and colleagues have shown that a modern diet low in fiber contributes to the loss of taxa over generations, and may be responsible for the lower-diversity microbiota observed in the industrialized world. Humans have experienced major dietary changes from gathered to farmed foods during the agricultural revolution, and more recently to the mass consumption of processed foods in the industrialized world. Each dietary shift was most likely accompanied by a concomitant adjustment in the microbiota [Sonnenburg *et al.* 2016]. This could explain the increasing rate of intestinal disorders in western countries. Altered diet–intestinal microbiota interactions may play a role in IBD, IBS and metabolic disorders such as obesity, type 2 diabetes, insulin resistance, and nonalcoholic fatty liver disease. Despite many unsubstantiated claims, the exact characteristics of what the ideal 'healthy microbiota' constitutes, or the 'ideal diet' promoting it, have not been completely elucidated. Several studies support the concept that diet should be viewed as a means to prevent alteration of symbiosis (beneficial host–bacteria interactions) observed in some intestinal and extraintestinal diseases [Thorburn *et al.* 2014; Cani and Everard, 2016; Lee *et al.* 2015].

Human intervention studies have demonstrated that dietary consumption of certain food products can result in changes in the composition of the

gut microbiota in line with the prebiotic concept [Roberfroid *et al.* 2010]. Prebiotics typically refer to selectively fermented nondigestible food ingredients or substances that specifically support the growth and activity of health-promoting bacteria that colonize the gastrointestinal tract [Bindels *et al.* 2015]. The general consensus is that low bacterial richness is associated with IBD and altered host metabolic markers such as increased body weight fat mass, insulin resistance and inflammation [Hansen *et al.* 2010; Le Chatelier *et al.* 2013]. Microbiota gene richness was predictive of a reduced response to low-caloric diet intervention in terms of weight loss and improvement of metabolism and inflammatory traits [Le Chatelier *et al.* 2013]. Cotillard and colleagues showed that energy restrictions and supplementation with dietary fibers may increase microbial diversity up to 25% in people with low diversity [Cotillard *et al.* 2013]. It has also been shown that resistant starches affect the composition of the microbiota and enhance metabolic health, as assessed by circulating inflammatory mediators, innate immune cells, and the bile acid cycle [Bindels *et al.* 2015]. Dietary components such as fructo-oligosaccharides, galacto-oligosaccharides, soya-oligosaccharides, xylo-oligosaccharides, and pyrodextrins have also been proposed to increase bacterial diversity in the gut [Gibson and Roberfroid, 1995; Bindels *et al.* 2015]. Independently of an increase in bacterial richness, nondigestible foods are fermented by bacteria producing important metabolites in gut homeostasis such as SCFAs, ω-3 fatty acids or tryptophan derivate metabolites [Furusawa *et al.* 2013; Thorburn *et al.* 2014]. Dietary metabolites have a key role in gut homeostasis: promoting epithelial integrity, regulatory T cell responses and tissue repair, as discussed previously. *Faecalibacterium prausnitzii* and *Akkermansia muciniphila* have been associated with SCFA production, bacterial richness, metabolic markers and anti-inflammatory effects. Specific prebiotic feeding increases the abundance of these bacteria and improves metabolic markers of disease [Dewulf *et al.* 2013; Everard *et al.* 2013]. However, recent observations suggest that this concept may be too simplistic due the strong individuality of responses to nutritional modulation and its dependence on the initial microbiota composition [Dore and Blottiere, 2015]. Moreover, advances in our understanding of diet–microbiome–host interactions challenge important aspects of the current concept of prebiotics, and especially the requirement for effects to be 'selective' or 'specific' [Bindels *et al.* 2015]. With this in mind, Bindels and colleagues proposed a new definition of prebiotic as a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and activity of the gut microbiota, thus conferring a beneficial physiological effect on the host [Bindels *et al.* 2015].

Gut function parameters such as intestinal motility, visceral perception, permeability or low-grade inflammation have also been linked to bacteria–diet interactions [Verdu *et al.* 2006; Kau *et al.* 2011; Dey *et al.* 2015]. A recent study in a novel murine model reported that malnutrition changes the small intestinal microbiota composition and their metabolites, intraepithelial lymphocyte phenotype, and the susceptibility to enteric infection [Brown *et al.* 2015]. Observational studies have attempted to identify dietary patterns that contribute to the risk of specific diseases. Diets high in red meat and fat, particularly polyunsaturated fatty acids, induce large-scale changes in the microbiota composition and bacterial metabolites that may be involved in the pathogenesis of metabolic disorders, IBD or cancer [Daniel *et al.* 2014; Lee *et al.* 2015]. For instance, bacteria can metabolize meat proteins and produce nitrosamines, which may be linked to carcinogenesis [Hughes *et al.* 2001; Paul *et al.* 2015]. Consumption of red meat delivers L-carnitine to certain gut bacteria converting it to trimethylamine-N-oxide which has been shown to cause atherosclerosis in mice [Koeth *et al.* 2013]. In contrast, cruciferous vegetables such as cabbage, broccoli, kale, and cauliflower are rich sources of fiber, lutein, flavonoids, phytosterols, folic acid, sulphur-containing glucosinolates, and vitamin C, each of which have been associated with reduced risk of various kinds of cancer [Paul *et al.* 2015]. Sulphur-containing glucosinolates can be converted to biologically active compounds such as indoles, nitriles, and isothiocyanates by β thioglucosidases produced by the gut microbiome. Epidemiological studies suggest a lower risk of IBD among people with diets high in fiber, fruits and vegetables. Specifically, the Mediterranean diet has been suggested to beneficially impact the gut microbiota and associated metabolome [De Filippis *et al.* 2015]. Some dietary nutrients have been shown to help regulate mucosal immune functions, such as vitamins, amino acids, and SCFAs, many of which are influenced by the gut microbiota [Brestoff and Artis, 2013].

Thus, defined diets may become attractive tools in the personalized treatment of inflammatory and metabolic disorders, but more research is needed to characterize this. Dietary intervention studies and their effects on the microbiota and health, in humans, are limited by the difficulty in accurately capturing dietary intake, as well as the potential for complex interactions between foods and bacteria, which are characteristic of each individual. However, studies are beginning to emerge, as recently demonstrated by Dey and colleagues showing how a single food ingredient interacts with a functional microbiota trait to regulate host physiology [Dey *et al.* 2015].

Some diets have also been proposed to worsen symptoms in patients with IBS, which is a functional gastrointestinal disorder characterized by abdominal pain or discomfort in conjunction with altered bowel habits, in the absence of organic pathology [Longstreth *et al.* 2006; Drossman *et al.* 2011]. Approximately 60% of patients with IBS identify food as a trigger for their symptoms, and there has been interest in exclusion diets for the management of IBS. However, no specific food item has conclusively been implicated in the overall IBS population, and these patients improve modestly to a variety of food restrictions as well as traditional dietary advice [Bohn *et al.* 2015]. IBS is a heterogeneous condition and multifactorial and thus many dietary components may trigger symptoms, and not all necessarily through alterations in the microbiota. Dietary components which stimulate bacterial fermentation in the colon have been related to IBS symptoms such as bloating. Foods high in fermentable oligosaccharides, disaccharides and polyols (FODMAPs), which are poorly digested carbohydrates, may cause IBS symptoms in a subgroup of patients with IBS through their osmotic effect and fermentation by colonic bacteria, leading to gas production, as well as their direct effects on gastrointestinal motility [Halmos *et al.* 2014; Bohn *et al.* 2015; Halland and Saito, 2015]. It has been shown that diets differing in FODMAP content can affect gut microbiota composition [Halmos *et al.* 2015]. It is important to remember that IBS is a complex syndrome, multifactorial, and that recent studies have shown that traditional dietary advice is as effective as selective restrictive diets to improve symptoms [Bohn *et al.* 2015]. Microbiota and dietary manipulation in IBS may be one of many options of treatment, and not necessarily effective in all IBS subtypes.

Probiotics in the treatment of intestinal disorders

Probiotics are live microorganisms which, when administered in sufficient quantity, provide a health benefit to the host. There are numerous probiotic strains commercially available currently, many of which lack strong clinical evidence to support their use in specific diseases. It has been suggested that probiotic bacteria should be preferably isolated from the human intestine, proven to be safe for the host, genetically stable and capable of surviving passage through the gastrointestinal tract. Among the many health claims for probiotics, beneficial immunomodulation, restoration of barrier function, modulation of metabolic parameters, and modification of intestinal microbiota have been proposed [Wolvers *et al.* 2010; Rijkers *et al.* 2011]. The growing skepticism regarding the use of probiotics in clinics is based on poorly substantiated health claims, lack of reproducibility and standardization of probiotic strains, doses and delivery methods in the different studies. However, a few studies have been well conducted and powered, suggesting we may be able to separate wheat from chaff and some probiotic intervention strategies may become an important element to prevent or attenuate intestinal disorders.

Meta-analyses suggest the use of probiotic formulations in different diseases such as IBS or necrotizing enterocolitis [Moayyedi *et al.* 2010; Wang *et al.* 2012]. Some Bifidobacteria strains have beneficial effects on global IBS, abdominal pain, bloating, and flatulence [O'Mahony *et al.* 2005]. Some patients with IBS display altered composition of the gut microbiota and the use of probiotics would be beneficial at the gastrointestinal and psychological levels *via* gut–brain axis [De Palma *et al.* 2014]. In the specific case of necrotizing enterocolitis, oral supplementation of *Bifidobacterium breve* and *Lactobacillus casei* reduced the occurrence of the disease while there was no evidence of benefit for other specific probiotics such as *B. breve* BBG-001 [Braga *et al.* 2011; Costeloe *et al.* 2016].

Although several probiotic formulations have been proposed as a treatment for IBD, results are controversial. Studies in animal models of colitis have been promising. *Lactobacillus* strains such as *L. plantarum* 299v, *L. reuteri* (R2LC), *L. salivarius* UCC118 or formulations like VSL#3 prevented onset of colitis [Mao *et al.* 1996; Madsen, 2001; O'Mahony *et al.* 2001; Schultz *et al.* 2002]. Unfortunately, there has been poor clinical

correlation. One confounding factor relates to the fact that clinical studies have been performed combining drugs with different probiotics such as *L. casei GG*, *L. salivarius* UCC118, *Saccharomyces boulardii, Escherichia coli Nissle* 1917 [Malchow, 1997; Venturi *et al.* 1999; Gupta *et al.* 2000; Guslandi *et al.* 2000; Prantera *et al.* 2002]. Therefore, no clear recommendation on dose or probiotic strain can be given for patients with active IBD, and more research is needed. A different paradigm in the search for anti-inflammatory strains emerged with the identification of *Faecalibacterium praustnizii* depletion, a core member of the intestinal microbiota, in IBD. This bacterium may protect the host from mucosal inflammation by several mechanisms, including the downregulation of inflammatory cytokines or stimulation of IL-10 [Sokol *et al.* 2008]. Specific mechanisms of action of probiotic strains, relevant to IBD pathophysiology, will need to be identified and then these strains tested in patients with IBD before formal recommendations can be made. Prevention, maintenance of remission, *versus* active therapy will be important aspects of future research.

Intestinal bacteria play a dual role in the response to dietary components, either generating harmful or beneficial metabolites for the host. Some bacteria are implicated in the production of nutrients and metabolites such as the previously mentioned SCFAs. Intestinal microbiota can also synthesize several vitamins and affect the absorption of key minerals, such as iron [Kau *et al.* 2011]. However, observational studies have reported greater abundance of *Firmicutes* and lower abundance of *Bacteroidetes* in people with obesity compared with their lean counterparts. The basis for this may relate to the capacity of gut microbiota components to harvest energy differentially for the host through microbial fermentation of dietary polysaccharides that cannot be digested by the host, and by microbial regulation of host genes that promote deposition of the lipids in adipocytes [Backhed *et al.* 2004; Turnbaugh *et al.* 2006]. In this context, certain bacteria could be used as probiotics with targeted pathways to restore health in metabolic disorders. Intestinal bacteria such as *Akkermansia muciniphila*, several *Lactobacillus* strains and probiotic formulations as VSL#3 have been proposed as modulators of metabolism [Le Barz *et al.* 2015], but more research is needed to understand what subsets will benefit from these strains and the short- and long-term benefits, as well as cost–benefit of such supplementation.

Fecal microbiota transplant

Fecal microbiota transplants (FMTs) have been used in the last decade for severe cases of *Clostridium difficile*. For mild cases, specific antibiotics are the primary care, particularly vancomycin and metronidazole [Rohlke and Stollman, 2012], however the rates of success for FMT in *C. difficile* infection are thought to be greater, roughly 90% [Gough *et al.* 2011]. Although FMT has been shown to be an effective option for *C. difficile*, the mechanisms as to why exactly it works are unknown. The leading hypothesis being that certain bacteria in the microbiota of healthy individuals contribute to colonization resistance, inhibiting the growth or outcompeting *C. difficile* for space and nutrients. To support this notion, there are taxa which have been shown to be protective or provide resistance to *C. difficile* infection and antibiotics can make an individual more susceptible to *C. difficile* infection [Buffie *et al.* 2012, 2015; Deshpande *et al.* 2013; Theriot *et al.* 2014; Collins *et al.* 2015]. Bacteria that provide resistance include, but are not limited to, *B. longum*, and species belonging to *Lachnospiraceae* and *Porphyromonadaceae*. Lawley and colleagues found that treating mice infected with *C. difficile* with healthy stool resolved the infection and they further created a therapeutic synthetic mixture of six strains of bacteria (novel *Bacteroidetes* species, *Lactobacillus reuteri*, *Enterococcus hirae*, novel *Anaerostipes* species, *Straphylococcus warneri* and a novel *Enterorhabdus* species) [Lawley *et al.* 2012]. More recently, Buffie and colleagues demonstrated that *Clostridium scindens* provides resistance to mice from *C. difficile.* The mechanism being that this bacterium is 7-dehydroxylating, producing secondary bile acids that inhibit *C. difficile* expansion and can treat already present *C. difficile* infection [Buffie *et al.* 2015]. Due to the high success of treating *C. difficile* infection with FMT, there is great interest in evaluating FMT in other gastrointestinal disorders where the microbiota has a key role in disease development or severity. Particularly in ulcerative colitis (UC), a recent trial suggests success of treatment [Moayyedi *et al.* 2015]. One of the suggested mechanisms include the abundance of the butyrate producer *Lachnospiraceae* in the donor microbiota [Moayyedi *et al.* 2015; Natividad *et al.* 2015]. However, due to the rates of success in UC compared with treating *C. difficile*, it is likely that the underlying mechanism of success of FMT is different. Thus far FMT also seems safe as studies have not reported serious adverse effects even in immunocompromised patients [Kelly *et al.* 2015].

One important regulatory issue for the future relates to transmission of pathogens or other morbidity phenotypes by FMT, given the bulk of animal work indicating effects of microbiota in the gut–brain axis [Bercik *et al.* 2011; De Palma *et al.* 2015]. Additionally, there is a documented case of a female patient with *C. difficile* infection receiving a FMT from an obese individual and subsequently becoming obese herself [Alang and Kelly, 2015]. Thus, there are concerns that need to be addressed before this form of therapy is approved in clinical management of IBD.

Phage therapy for microbiota manipulation

Bacteriophage are the most abundant organic entities on the planet, intimately controlling microbial populations. Therefore, their role in modulating microbial communities in the gut has arisen. Until recently, the role of phage in the gastrointestinal tract had been greatly understudied. It was only in 2003, when an estimated 1200 viral genotypes in fecal contents were reported in healthy humans [Mills *et al.* 2013]. Most studies investigating bacteriophage in the gut have utilized healthy individuals, and their role in diseases has not been investigated in depth. A few studies have demonstrated that individuals with Crohn's disease or UC have increased bacteriophage quantities, some suggesting decreased diversity overall [Lepage *et al.* 2008; Perez-Brocal *et al.* 2013; Wagner *et al.* 2013] and another suggesting increases in diversity [Norman *et al.* 2015]. The changes occurring in the virome during disease seem to be disease specific, however increases in *Caudovirales* have been demonstrated in both Crohn's disease and UC [Norman *et al.* 2015]. The same large-scale study also provided data supporting the notion that increases in phage populations were not due to decreased bacterial diversity, suggesting phage may contribute to dysbiosis in IBD and therefore inflammation. The potential application of phage is vast, from developing communities of phage to skew whole microbial dysbiosis to phage-bacterial-specific therapies or mining for compounds that block phage induction. For instance, specific bacteria have shown to contribute to IBD in animal models, such as adherent-invasive *E. coli* [Rolhion and Darfeuille-Michaud, 2007], which could be targeted by a specific single phage therapy. In addition, certain bacteria use phage to remain viable in a niche (including some pathobionts) [Duerkop *et al.* 2012], blocking phage induction could be an alternative to modulating whole microbiota. An additional noteworthy role of phage is that the interactions of phage with mammalian hosts is greatly understudied, but it has been demonstrated that phage are capable of inducing immune responses, having documented anti-inflammatory and proinflammatory attributes [Mills *et al.* 2013]. The dynamics of phage in the gut is intriguing and holds great potential. Currently, therapeutic applications using phage therapy remain exploratory and at a discovery phase.

Conclusion

The role of a perturbed microbiota in triggering inflammation and disease progression has been proposed largely based on animal studies. Clinical studies have and continue to be conducted to test the hypotheses generated in basic research studies. Several clinical studies have confirmed an association between chronic gut inflammatory, functional disorders, and metabolic conditions with an altered microbiome. Also some, but not all studies, have identified specific probiotic interventions with beneficial effects in particular clinical conditions. The results using a given probiotic are not applicable to all disease conditions or to all probiotic products. Basic studies are performed in controlled environments that include defined microbiota, dietary control, and genetic background. They are key to provide mechanistic insight and hypothesis generation, but their results are not always directly translatable to humans. There is growing interest to manipulate the gut microbiome for preventative and therapeutic purpose. The area shows great promise, however more research is needed before conclusive clinical recommendations can be given. Specifically, we need to understand how certain microbiota components or metabolic activities influence specific disease conditions, and decipher the complex interactions between microbes and dietary components. Combined basic and clinical research will continue to provide insight for the ultimate strategies on how to manipulate the microbiota to protect against onset and progression of disease.

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APPENDIX II: PHARMACOLOGICAL APPRAOCHES IN CELIAC DISEASE

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Current Opinion in Pharmacology

Pharmacological approaches in celiac disease

Justin L McCarville, Alberto Caminero and Elena F Verdu

Celiac disease is an autoimmune enteropathy triggered by the ingestion of gluten, characterized by immune responses toward gluten constituents and the autoantigen transglutaminase 2. The only current treatment available for celiac disease is a gluten-free diet, however there are a plethora of therapies in development for the treatment of celiac disease (e.g. vaccine), management of symptoms while consuming gluten (e.g. *Necator americanus*) or adjuvant therapies in conjunction with the gluten-free diet (e.g. larazotide acetate). Current approaches in development target barrier function, immune responses, detoxifying gluten or sequestering gluten. Developing therapies include those targeting environmental factors, such as the microbiota or proteases.

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Introduction

Celiac disease (CeD) is a chronic autoimmune enteropathy triggered by exposure to gluten in genetically susceptible individuals expressing the human-leukocyte antigen (HLA)-DQ2.5 or DQ8 [1]. Gluten proteins are partially digested in the intestinal lumen, creating large peptides which cross the small intestinal barrier, and are deamidated by transglutaminase 2 (TG2), introducing negative charges [2]. These peptides bind to the positively charged binding pockets of HLA-DQ2.5/8 on antigen presenting cells (APCs). This leads to a cascade of events, primarily characterized by the formation of gluten-specific T cells and the production of antibodies, specific to the gluten constituents and TG2 (auto-antibodies) [3,4]. Induction and activation of intraepithelial lymphocytes (IELs) is a key initial step in CeD [5]. The increase in IEL numbers and activation in CeD is mediated in part by high expression of IL-15 in the CeD mucosa [6]. Recently it has been demonstrated that IL-15 in combination with signals, likely IL-2, from gliadin/TG2-specific T cells are critical for atrophy in CeD [7]. It is widely accepted that there is an unknown environmental trigger in CeD as the HLA DQ2.5/8 haplotypes alone (prevalence of 30%) are not sufficient to cause the disease.

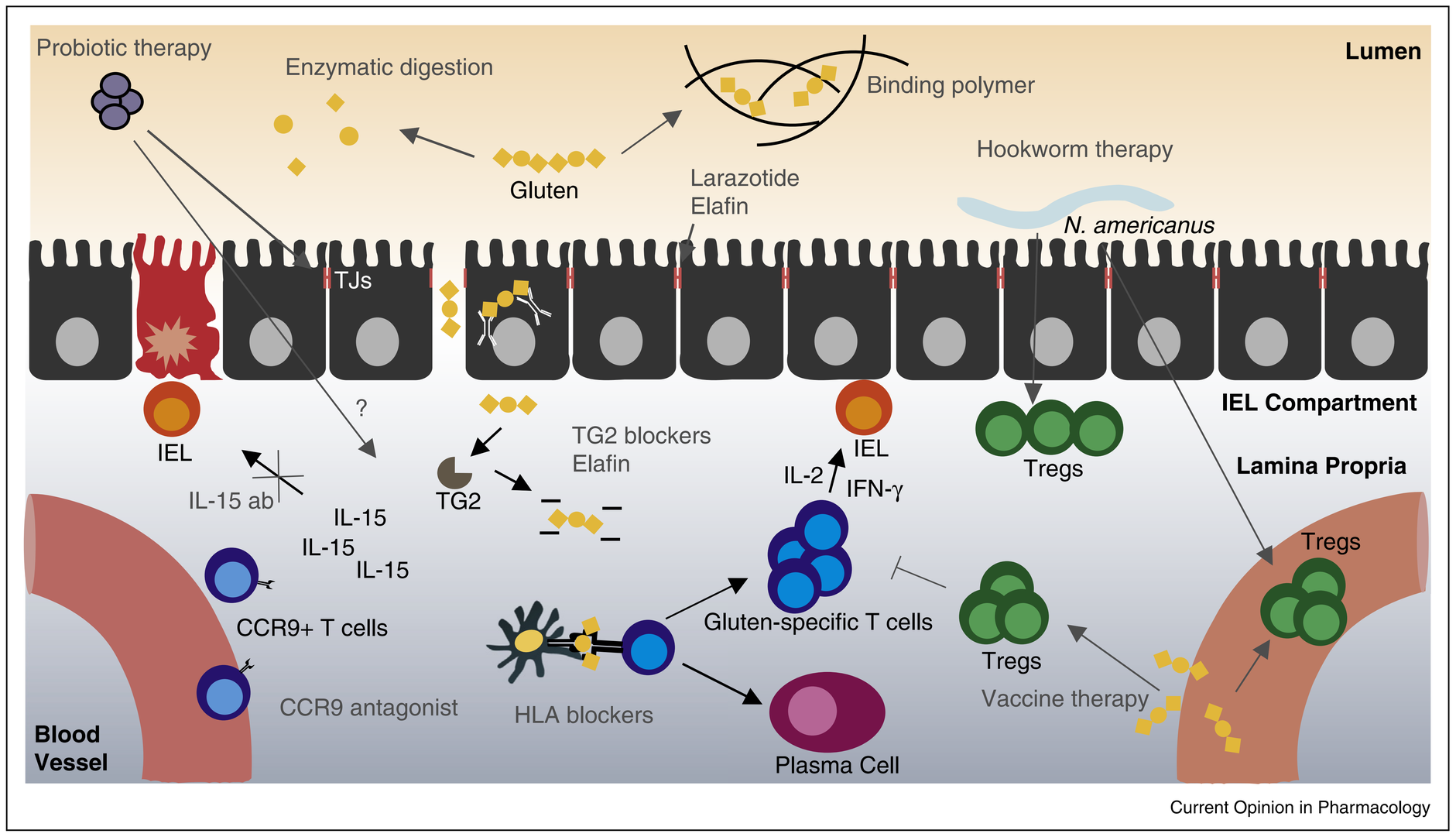
The only current therapy for CeD is a lifetime of gluten-free diet (GFD). However, it has been demonstrated that mucosal recovery is not immediate and a large proportion of patients exhibit persistent low-grade inflammation despite a GFD [8,9]. A GFD installs an aspect of social burden, nutritional deficiencies and issues of compliance [10,11]. A small proportion of CeD patients (10–18%) do not respond to the GFD. These patients are at risk for complications and require additional therapy, a disorder termed refractory celiac disease (RCD) [9,12]. Thus, there has been intense research into the development of adjuvant therapies for the GFD. Developing therapies fall into the following categories (i) degradation or neutralization of gluten, (ii) barrier enhancing therapies, (iii) immune targeted therapies, (iv) protease inhibitors, (v) microbiota-modulating and nematode, all of which will be discussed in this review (Figure 1).

Gluten detoxification and prevention of mucosal interactions

Oral enzymatic therapy to complement gluten luminal digestion

Gluten is resistant to complete digestion by human digestive enzymes due to its high proline content. Consequently, high molecular weight oligopeptides persist in the lumen of the small intestine, some of which trigger the inflammatory cascade in CeD. Thus, the focus of oral enzymatic therapy is to inactivate immunogenic gluten peptides in the human gastrointestinal tract [13]. The most commonly studied enzymes are proteases from the prolyl endopeptidase family (PEPs), however there are limitations to their use *in vivo* [14]. These enzymes must be resistant to human proteases and be efficacious at low stomach pH. AN-PEP is a prolyl endoprotease derived from *Aspergillus niger* which can break down gluten at acidic pHs and is resistant to digestion by pepsin [15]. AN-PEP was evaluated in a double-blind, placebo controlled, randomized trial in 16 patients with diagnosis of CeD, who adhered to a strict GFD, resulting in normalized antibodies and mucosal recovery. AP-PEP has a good safety profile in CeD patients, although clinical improvements were not clear [16].

Figure 1



Overview of current approaches in development for the treatment or adjuvant therapy of celiac disease.

ALV003 is an orally administered mixture of two recombinant gluten-specific proteases (ALV001 and ALV002) which degrade gluten *in vitro*. ALV001 is a glutamine-specific cysteine endoprotease derived by germinating barley seeds and ALV002 is a PEP from *Sphingomonas capsulate* [17,18]. A phase 2a clinical trial has been performed in adult CeD patients receiving ALV003 ($n = 20$) or placebo ($n = 20$), demonstrating that ALV003 can attenuate both gluten-induced small intestinal mucosal injury and gluten-specific immune responses. However, no differences in symptomatology were resolved by ALV003 intake [19••]. On the basis of these promising results, a Phase 2b has been conducted (ClinicalTrials.gov Identifier: NCT01917630), evaluating the safety and efficacy of ALV003 at varying dosages over a twelve-week period in 500 CeD patients who are symptomatic despite attempting a GFD [20]. STAN 1 is a third protease mixture (two common food-grade enzymes) being tested in a Phase 2 clinical trial (ClinicalTrials.gov Identifier: NCT00962182) [21]. The primary outcome of this trial is to determine whether STAN 1 cocktail supplements decrease serum activity markers in CeD patients insufficiently treated by previous gluten exclusion.

Luminal sequestration of gluten by gluten binding polymers

Sequestering gluten in the lumen, is another way of avoiding contact of gluten with the mucosa. This is the mode of action of the gluten binding polymer, BL-7010 or copolymer (P(HEMA-co-SS)). Consumption of this polymer, when co-administered with gluten, would block the ability of enzymes to cleave and avoid production of inflammatory peptides. This polymer has been tested *in vitro* [22] and animal models of gluten sensitivity [23,24•], avoiding induction of IELs, intestinal atrophy and decreased barrier function. This polymer is non-absorbable and is safe in animal use. Phase 1 clinical trials are currently underway (ClinicalTrials.gov Identifier: NCT01990885).

Modulation of permeability and epithelial uptake

The precise pathways by which gluten crosses the small intestinal barrier to the lamina propria, where it interacts with APCs, is unknown. Trancellular and paracellular pathways (receptor or antibody mediated) have been suggested. Both are altered in CeD and likely contribute to disease severity, in conjunction to gluten uptake [25,26]. Some studies have shown that gliadin or its constituents can directly cause barrier dysfunction through recognition of gliadin by the receptor CXCR3 in intestinal epithelial cells, which regulate tight junction (TJ) function [27]. Larazotide acetate, or AT-1001, is a TJ modulator, being developed as a barrier enhancer in CeD. *In vitro*, this molecule has shown to prevent disassembly of TJs due to gliadin and block translocation of gliadin

constituents [28]. Larazotide acetate has shown efficacy in animal models, normalizing TJ protein levels and decreased macrophage recruitment induced by gliadin [28]. A recent study showed that symptomatic CeD patients on larazotide acetate had fewer symptoms after individuals had been on a GFD for more than 12 months. Specifically, a 26% decrease in patient reported symptomatic days, a 31% increase in improved symptom days and a 50% decrease in abdominal pain score was noted [29••]. Targeting transcellular pathways, such as CD71 antibody mediated pathways have not yet been explored, however may be attempted in the future.

Modulation of antigen presentation and immune response

TG2 blockers

Deamidation of gluten by active TG2 allows for efficient antigen presentation. Most research with TG2 inhibitors has been *in vitro*, with the exception of ERW1041E [30]. This molecule was shown to inhibit TG2 in mice. However, its efficiency to prevent proinflammatory events related to gluten has not yet been examined. The most applicable study to humans was carried out with two irreversible inhibitors, R281, a cell impermeable molecule and R283, a cell-permeable molecule. This study found that R281 and R283 were capable of reversing gliadin-induced barrier dysfunction in Caco-2 cells. Further, R281 was capable of decreasing gliadin-induced $CD25^{-}$ $IL15^{+}$ T cells and $FoxP3^{+}$ cells from biopsies, as well as reverse crypt-cell proliferation [31]. Additionally, TG2 is also involved with CD71, required for antibody mediated translocation of gliadin constituents *via* the transcellular route [26], however investigation of inhibitors in this context has not been explored.

HLA blockers

The purpose of HLA blockers is to prevent the binding of deamidated gluten peptides to the HLA binding pocket, thereby preventing antigen presentation and production of gluten-specific T cells. Kapoerchan *et al.*, developed a gluten peptide in which the proline residues were replaced with azidoprolines, decreasing T cell immune responses from CeD patients, specific to HLA-DQ2.5 [32]. Xia *et al.* developed a similar therapy with cyclic and dimeric peptides, blocking T cell proliferation and antigen presentation [33].

T cell mediated and vaccine therapies

Drugs affecting T cell infiltration and being developed for diseases such as inflammatory bowel disease (IBD), may be applied to CeD. As with IBD, CeD exhibits intestinal T cell infiltration. CCR9 antagonists, such as Vercirnon (Traficet-EN), target cells that co-express $\alpha_4\beta_7$ and CCR9, decreasing infiltration of proinflammatory T cells into the intestinal lamina propria [34]. Phase II clinical trials for Vercirnon for treatment of Crohn's disease show that it is effective at a dose of 500 mg daily at 8 weeks (60% response rate compared to 49% placebo) and 12 weeks (61% response rate compared to 47% placebo) [35]. T cell-specific therapy in CeD is being attempted in vaccine development, with the aim to induce oral tolerance to gluten. Tye-Din *et al.* discovered 3 peptides in wheat, barley and rye, of 16,000, specific for the majority of immune responses induced by isolated T cells from HLA-DQ2.5 individuals [36]. These peptides have been incorporated into a vaccine, NEXVAX2, for intradermal injection and is currently in phase 1b clinical trials (ClinicalTrials.gov Identifier: NCT00879749).

Specific inhibition of proinflammatory cytokines: IL-15

Therapies that target IL-15 are being developed for CeD and its complications as well as other diseases. Particularly, the development of the antibody Hu-Mik-β-1 for both CeD and lymphocytic leukemia, has been successful [37]. In an animal model of IL-15 over-expression and small intestinal atrophy, this antibody (TM-beta1), specific for IL-2/IL-15Rβ, proved to reverse destructive effects of IL-15 over-expression and could prove useful in RCD [38]. In another model of IL-15 over-expression-induced atrophy, a human IL-15 antibody (AMG714), blocked IEL accumulation in the small intestine of treated mice. Further, this antibody blocked anti-apoptotic pathways in IELs involved in RCD from human biopsies [39].

Inhibition of proinflammatory proteases: elafin

The balance of proteases and anti-proteases is important in inflammatory disorders [40]. Protease inhibitors have been an effective treatment in animal models of IBD [41], however, their role in CeD is less understood. Elafin is a serine proteases inhibitor and is decreased in the mucosa of IBD [41] and CeD patients [42••]. In gluten sensitive mice, administration of elafin by the recombinant *Lactococcus lactis* was able to prevent gluten-induced pathology, including induction of IELs and disruption of barrier function. Elafin was demonstrated to be a competitive antagonist of TG2 *in vitro*, blocking the deamination of the 33-mer, a proinflammatory gluten constituent. Although promising, the therapy is only in discovery phase, more research must be conducted to elucidate mode of action of elafin, or other protease inhibitors in the context of CeD.

Microbiota and nematode based therapies

Intestinal dysbiosis has been described in some patients with active CeD and those following a GFD and thus microbiota-modulating therapy using probiotics has been suggested in CeD [43,44]. However, there is currently no evidence-based recommendation for specific probiotic formulations in CeD. Caution has recently been raised after the demonstration of gluten contamination in some probiotic preparations, raising the importance of quality control for over the counter preparations [45]. VSL#3, a probiotic preparation, was shown to hydrolyze gliadin

proteins *in vitro*, however this was not confirmed *in vivo* [46]. Other bacterial strains belonging to *Bifidobacterium* and *Lactobacillus* spp. have been proposed as potential probiotics in CeD based on modulation of epithelial and immune function *in vitro* and in mouse models [47,48]. An exploratory, randomized, double-blind, placebo-controlled study suggested that *Bifidobacterium infantis* Natren Life Start (NLS) superstrain may alleviate symptoms in untreated CeD and produce some immunologic changes [49•]. A double-bind, randomized, placebo-controlled intervention trial in children with CeD showed that *Bifidobacterium longum* CECT 7347 improved the health status of CeD patients on a GFD who showed persistent alterations in gut microbiota composition and a biased immune response [50]. Recent studies support that the human digestive tract is colonized with bacteria involved in gluten metabolism capable of degrading toxic gluten peptides [51,52]. *Rothia mucilagenosa* from the oral cavity and *Pseudomonas aeruginosa* from fecal samples have demonstrated hydrolysis properties of immunogenic peptides [53,54].

Helminth therapy has been suggested as a treatment for autoimmune and inflammatory disorders. Parasitic helminths have evolved together with the mammalian immune system over many millennia and as such they have become remarkably efficient down regulators of host immune responses in order to promote their own survival [55]. *Necator americanus* is a hookworm that persists in the small intestine of mammalian hosts which has shown beneficial effects in CeD, attenuating immune responses to gluten *in vitro* and *in vivo* [56–58]. Recently, a clinical study was performed involving 12 CeD adults, inoculated with *N. americanus* and followed by escalating gluten challenges. Results demonstrated that the helminth therapy promoted tolerance and stabilized indices of gluten toxicity by improving quality of life, resisting changes in marsh scores and IEL counts, reducing intestinal IFNγ-expressing T cells and increasing Tregs [59•]. However, some side effects associated with this therapy, such as anemia, may be anticipated [58].

Conclusions

In the past few years, therapies for CeD have advanced tremendously. However, these therapies are still in preclinical development. Consequently they are not yet available for their use in the clinical practice, and recommendations are in progress. For instance, some therapies may be recommended for patients without complications but who remain symptomatic despite following a GFD. It is possible that some of these therapies can be applied preventively in subjects with genetic risk. New insight and a fertile crescent of research is expected in the next decade as the first pharmacological adjuvant therapies are approved and released in the market, together with new discoveries related to environmental modulators of CeD development.

Conflict of interest statement

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Individuals with celiac disease were inoculated with 20 *Neacator americanus* laravae and received 3 phases of escalating gluten challenges. Although the primary endpoints were not met due to lack of enteropathy at set time points in positive controls, inoculation improved quality of life and decreased intestinal inflammation (decreased IELs and IFNγ-producing T cells and increased regulatory T cells).

APPENDIX III: INTESTINAL MICROBIOTA MODULATES GLUTEN-INDUCED IMMUNOPATHOLOGY IN HUMANIZED MICE

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GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Intestinal Microbiota Modulates Gluten-Induced Immunopathology in Humanized Mice

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Celiac disease (CD) is an immune-mediated enteropathy triggered by gluten in genetically susceptible individuals. The recent increase in CD incidence suggests that additional environmental factors, such as intestinal microbiota alterations, are involved in its pathogenesis. However, there is no direct evidence of modulation of gluten-induced immunopathology by the microbiota. We investigated whether specific microbiota compositions influence immune responses to gluten in mice expressing the human *DQ8* gene, which confers moderate CD genetic susceptibility. Germ-free mice, clean specific-pathogen-free (SPF) mice colonized with a microbiota devoid of opportunistic pathogens and Proteobacteria, and conventional SPF mice that harbor a complex microbiota that includes opportunistic pathogens were used. Clean SPF mice had attenuated responses to gluten compared to germ-free and conventional SPF mice. Germ-free mice developed increased intraepithelial lymphocytes, markers of intraepithelial lymphocyte cytotoxicity, gliadin-specific antibodies, and a proinflammatory gliadin-specific T-cell response. Antibiotic treatment, leading to Proteobacteria expansion, further enhanced gluten-induced immunopathology in conventional SPF mice. Protection against gluten-induced immunopathology in clean SPF mice was reversed after supplementation with a member of the Proteobacteria phylum, an enteroadherent *Escherichia coli* isolated from a CD patient. The intestinal microbiota can both positively and negatively modulate gluten-induced immunopathology in mice. In subjects with moderate genetic susceptibility, intestinal microbiota changes may be a factor that increases CD risk. *(Am J Pathol 2015, 185: 2969—2982; http://dx.doi.org/10.1016/j.ajpath.2015.07.018)*

Celiac disease (CD) is an immune-mediated enteropathy triggered by gluten in individuals with genetic risk. Proteolytic-resistant gluten peptides are deamidated by transglutaminase 2 (TG2) in the small-intestinal lamina propria, increasing their binding affinity to the CD-associated HLA-DQ2 or DQ8 molecules, leading to T-cell activation.[1,2] CD also requires an innate immune response, characterized by up-regulation of stress markers on epithelial cells as well as up-regulation and activation of intraepithelial lymphocytes (IELs).[3,4] There has been a rapid rise in CD prevalence over the past 50 years.[5] This, in conjunction with the fact that only 2% to 5% of genetically susceptible individuals will develop CD, argues for environmental modulators of CD expression.[6]

The intestinal microbiota plays an important role in mucosal immune maturation and homeostasis as evidenced from seminal studies using germ-free and gnotobiotic mice.[7,8] Clinical and animal studies also suggest that altered

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colonization early in life increases susceptibility to chronic inflammatory diseases and food sensitivities.[9–11] Indeed, alterations in intestinal microbial composition have been described in CD patients, some of which normalize after treatment with a gluten-free diet.[12] Clinical studies have also proposed a link between antibiotic use and elective caesarean section and CD development.[13–15] However, recent studies in families with high genetic risk for CD (positive family history or homozygous for HLA-DQ2.5) have not been able to identify an environmental determinant, including the timing and dose of gluten introduction to an infant's diet.[16,17] Microbial factors were not directly investigated, and results may not apply to the general population or individuals with moderate genetic risk for CD (HLA-DQ2 heterozygous or HLA-DQ8).

Whether altered colonization instigates CD in an individual at moderate risk of developing CD remains unclear.[18] Therefore, we investigated whether microbial colonization modulates host responses to gluten using transgenic HLA-DQ8 mice on the nonobese diabetic background (NOD/DQ8).[19] We used complementary strategies to investigate host responses to gluten under different microbial conditions. Clean specific-pathogen-free (SPF) mice, strictly monitored for the absence of a variety of pathobionts and Proteobacteria, were protected from gluten-induced immunopathology when compared to germ-free and conventional SPF mice. Perinatal disruption of the microbiota leading to Proteobacteria expansion in conventional SPF mice further enhanced severity of responses to gluten; whereas specific pathobiont supplementation to clean SPF mice reversed the protective effect of the benign microbiota.

Materials and Methods

Mice and Colonization Procedures

Female and male germ-free, clean SPF and conventional SPF NOD AB° DQ8 (NOD/DQ8) transgenic mice maintained on a gluten-free diet were used for experiments.[20] Germ-free mice were generated by two-stage embryo transfer, as previously described,[21] and bred and maintained in flexible film isolators in McMaster's Axenic Gnotobiotic Unit. Clean SPF mice originated from germ-free mice that were naturally colonized by co-housing with female mouse colonizers harboring altered Schaedler flora and bred for three generations in individually ventilated cage racks. Pathogen contamination and microbiota diversification in mouse cecum contents of clean SPF mice was evaluated every 2 weeks in cage sentinels and at the end of the study in the experimental mice by PCR for *Helicobacter bilis*, *H. ganmani*, *H. hepaticus*, *H. mastomyrinus*, *H. rodentium*, *Helicobacter spp.*, *H. typhlonius*, and *Pneumocystis murina*. Mouse serum was also tested for murine viral pathogens by multiplexed fluorometric immunoassay/enzyme-linked immunosorbent assay (ELISA)/indirect fluorescent antibody tests. Germ-free status was monitored in sentinels and, at the end of the study in the experimental mice, by immunofluorescence (SYTOX Green; Invitrogen, Burlington, ON, Canada), anaerobic and aerobic culture, as well as PCR technique.

Additional experiments were performed in germ-free and clean SPF C57BL/6 mice. For pathobiont supplementation experiments, 8- to 12-week-old clean SPF NOD/DQ8 mice were orally fed with 10^8 colony-forming units of *Escherichia coli* ENT CAI:5, isolated from fecal microbiota of a CD patient,[22] three times a week, 1 week before the start of sensitization and once a week during the sensitization and challenge period. Conventional SPF mice were bred and maintained in a conventional SPF facility at McMaster University. All experiments were conducted with approval from the McMaster University Animal Care Committee.

Gluten Sensitization and Challenge

NOD/DQ8 mice were sensitized with 500 μg of sterilized pepsin-trypsin digest of gliadin (PT-gliadin) and 25 μg of cholera toxin (Sigma-Aldrich, St. Louis, MO) by oral gavage once a week for 3 weeks, as previously described.[19] PT-gliadin was prepared as previously described.[19] In antibiotic experiments, mice were sensitized at 3 weeks of age, following weaning. For all other experiments, 8- to 12-week-old mice were used for sensitizations. Following PT-gliadin sensitization, gluten-treated mice were challenged by oral gavage with 2 mg of sterile gluten (Sigma-Aldrich) dissolved in acetic acid three times a week for 2 weeks. Nonsensitized control mice received cholera toxin alone during the sensitization phase and acetic acid alone during the challenge phase. NOD/DQ8 mice were weaned and maintained on a gluten-free diet. In additional experiments, C57BL/6 mice were sensitized with PT-zein and cholera toxin, once a week for 3 weeks, and challenged with zein dissolved in acetic acid three times a week for 2 weeks. All preparations were tested for lipopolysaccharide contamination using the E-Toxate kit (Sigma-Aldrich). Mice were sacrificed 18 to 24 hours following the final gluten or zein challenge.

Microbial Analysis

Fecal and cecal samples were collected and flash frozen on dry ice. DNA was extracted from samples as previously described.[23] Extracted DNA underwent amplification for the hypervariable 16S rRNA gene v3 region and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA). Generated data were analyzed as described previously.[23] Briefly, sequences were trimmed using Cutadapt software version 1.2.1,[24] aligned using PANDAseq software version 2.8,[25] operational taxonomic units selected via AbundantOTU,[26] and taxonomy assigned against the Greengenes reference database.[27] α-Diversity was calculated using Quantitative Insights Into Microbial Ecology (QIIME),[28] and heat maps were generated using R (R Foundation for Statistical

Computing, Vienna, Austria), clustered based on Bray-Curtis dissimilarity.[29]

Histology and Immunohistochemistry

Cross sections of the jejunum were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin for histological evaluation by light microscopy (Olympus, Richmond Hill, ON, Canada) using Image-Pro Plus software version 6.3 (Media Cybernetics, Rockville, MD). Enteropathy was determined by measuring villus-to-crypt (V/C) ratios in a blinded fashion, as previously described.[19] Intraepithelial lymphocytosis was determined by counting $CD3^+$ IELs per 20 enterocytes in five randomly chosen villus tips, as previously described, and expressed as IELs/100 enterocytes. $CD3^+$ immunostaining was performed on paraffin-embedded sections of the jejunum as previously described.[19]

Enterocyte cell death was determined by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining using the ApopTag Peroxidase *in Situ* Apoptosis Detection Kit (Millipore, Billerica, MA) according to the manufacturer's instructions. Slides were viewed by light microscopy (Olympus). The percentage of TUNEL-positive enterocytes in 20 villi was determined for each mouse.

Small-Intestinal Lamina Propria and IEL Preparation

Small intestines were removed from mice, and IELs and lamina propria lymphocytes isolated by established protocols. Briefly, small intestines from mice were flushed to remove intestinal contents, Peyer's patches and mesentery were removed, intestines opened longitudinally, and cut into 3- to 5-mm pieces. Intestinal pieces were incubated five to six times in EDTA/HEPES/Dulbecco's phosphate buffered saline for 15 minutes in a 37°C shaker. After each 15-minute incubation, intestines were vigorously vortexed and the IELs were collected by passing the supernatants through a 40-μm cell strainer. Intestinal pieces were further digested with DNase I (Roche, Mississuaga, ON, Canada) and Collagenase Type VIII (Sigma-Aldrich) to collect lamina propria lymphocytes. IELs and lamina propria lymphocytes were enriched on a Percoll gradient and resuspended in fluorescence-activated cell sorting buffer for cell staining.

Single-cell suspensions of lamina propria preparations were stained with fluorochrome-labeled cell-surface antibodies including CD4-APC (RM4-5), CD8a-PerCP (53-6.7), and CD25-PE (7D4) purchased from BD Biosciences (San Jose, CA). IEL cell suspensions were stained with fluorochrome-labeled cell-surface antibodies including CD3-Alexa Fluor-700 (ebio500AZ; eBioscience, San Diego, CA), CD3-Pacific Blue (RM4-5; BD Biosciences), CD8-PerCP (53-6.7; BD Biosciences), β T-cell receptor (βTCR) (H57-597; eBioscience), TCRγδ-APC (eBioGL3; eBioscience), NKG2D-PE (CX5; eBioscience), and CD69-PE-CF594 (H1.2F3; BD Biosciences). For intracellular staining, cells were permeabilized using the Foxp3 staining buffer set (eBioscience). Lamina propria cells were incubated with fluorescein isothiocyanate–conjugated antibodies to Foxp3 (FJK-16s; eBioscience), and IELs were incubated with PE-Cy7–conjugated Granzyme-B (NGZB; eBioscience) for 90 minutes at 4°C. Stained cells were acquired using the LSR II (BD Biosciences) and analyzed with FlowJo software version 7.2.4 (TreeStar, Ashland, OR).

T-Cell Proliferation and Cytokine Analysis

Single-cell suspensions of mesenteric lymph nodes were prepared in RPMI 1640 (1% penicillin/streptomycin, 10% fetal calf serum, 2 mmol/L L-glutamine). $CD4^+$ T cells were isolated from mesenteric lymph nodes using the EasySep Mouse $CD4^+$ T cell Enrichment Kit (StemCell, Vancouver, BC, Canada), and labeled with carboxyfluorescein succinimidyl ester (CFSE; Life Technologies, Grand Island, NY). $CD11c^+$ cells were isolated from spleens using the Easysep Mouse CD11c Selection Kit (StemCell). A total of 5×10^4 $CD11c^+$ cells were co-cultured with 2×10^5 $CD4^+$ T cells in the presence of 500 μg/mL PT-gliadin, 500 μg/mL PT-zein, or medium alone in a round-bottom 96-well plate for 3 days at 37°C, 5% CO_2. Cells were resuspended in fluorescence-activated cell sorting buffer and stained with fluorochrome-conjugated antibodies to CD3 and CD4 and a viability stain. CFSE-labeled cells were acquired using the LSR II (BD Biosciences). Viable cells were gated on $CD4^+$ T cells. CFSE intensity for this population was determined using FlowJo software (TreeStar) and the percentage of divided cells determined for each condition (PT-gliadin, PT-zein, medium). Proliferation of cells in response to PT-gliadin or PT-zein stimulation were normalized to the proliferation of medium alone and expressed as a proliferation index.

Anti-Gliadin ELISA

Serum IgA and IgG antibodies to gliadin were measured by ELISA as previously described,[30,31] with minor modifications. In addition, intestinal wash IgA antibodies to gliadin were measured similarly. Intestinal wash IgG antibody reactivity was too low to be detected reliably and was not measured. One hundred mg of the US hard red spring wheat *Triticum aestivum* cv Butte 86 variety flour was suspended in 1 mL of phosphate-buffered saline and mixed for 1 hour at 4°C. The suspension was centrifuged at 10,000 × *g* for 20 minutes. The supernatant containing mostly non-gluten proteins, was removed. The pellet was washed with phosphate-buffered saline, resuspended in 50% isopropanol, and mixed for 1 hour at room temperature. The suspension was centrifuged at 10,000 × *g* for 20 minutes, and the supernatant, containing gliadin and glutenin proteins, was stored at −20°C. The 96-well Maxisorp round-bottom polystyrene plates (Nunc, Roskilde, Denmark) were coated with 50 μL/well of a 0.01 mg/mL solution of the gliadin gluten extract in 0.1 mol/L carbonate buffer (pH 9.6) or were left uncoated to serve as control wells. Wells were blocked by incubation with 1% bovine serum albumin. Serum samples were

Table 1 Microbial Composition of Clean SPF and Conventional SPF NOD/DQ8 Mice

Phylum	Class	Order	Family	Genus	Clean SPF, %	Conv SPF, %
Bacteroidetes	Bacteroidia	Bacteroidales	Unclassified	Unclassified	0	3.7
			Bacteroidaceae	*Bacteroides**	0	62.2
			Porphyromonadaceae	Unclassified	0	0.5
				Parabacteroides	73.9	14.3
			Rikenellaceae	Unclassified	0	0.3
				Alistipes	0	0.1
Deferribacteres	Deferribacteres	Deferribacterales	Deferribacteraceae	*Mucispirillum*	1.9	0
Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus**	0	0.1
		Gemellales	Gemellaceae	*Gemella*	0	0.2
		Lactobacillales	Aerococcaceae	*Aerococcus*	0	0.1
			Lactobacillaceae	*Lactobacillus*	0.1	1.7
			Streptococcaceae	*Streptococcus**	0	0.1
		Turicibacterales	Turicibacteraceae	Unclassified	2.4	0.1
	Clostridia	Clostridiales	Unclassified	Unclassified	0.2	0
			Clostridiales Family XIII. Incertae Sedis	Eubacterium	0	0.1
			Lachnospiraceae	Unclassified	21	13.1
				Blautia	0.2	0.2
				Clostridium	0	0.1
			Ruminococcaceae	Unclassified	0.2	0.2
				Anaerotruncus	0	0.1
Proteobacteria	Betaproteobacteria	Burkholderiales	Unclassified	Unclassified*	0	0.1
	Epsilonproteobacteria	Campylobacterales	Helicobacteraceae	*Helicobacter**	0	0.1
	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Escherichia**	0	2.0
Tenericutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	*Allobaculum*	0	0.4
				Coprobacillus	0	0.1

Numbers indicate relative proportions at the genus level. Cutoff: 0.1% abundance.
*Contains pathogenic or opportunistic bacteria associated with inflammation.
Conv, conventional; SPF, specific pathogen free.

diluted at 1:100, whereas intestinal wash samples were diluted at 1:10. The samples were added at 50 μL per well in duplicates and incubated for 1 hour. Each plate contained a positive control sample. After washing the wells, they were incubated with a 1:2000 dilution of either horseradish peroxidase–conjugated anti-mouse IgG (GE Healthcare, Piscataway, NJ) or IgA (Abcam, Cambridge, MA) secondary antibodies. The plates were washed, and 50 μL of developing solution was added to each well; absorbance was measured at 450 nm after 20 minutes. Absorbance values were corrected for nonspecific binding by subtraction of the mean absorbance of the associated bovine serum albumin–coated control wells. The corrected values were first normalized according to the mean value of the positive control duplicate on each plate. The mean antibody level for the clean SPF control group was then set as 1.0 arbitrary units, and all other results were normalized accordingly.

Anti-Gliadin Western Blots

Antibody reactivity to gluten in sera was confirmed by Western blot. The Butte 86 gluten extract was dissolved in sample buffer, heated for 10 minutes at 75°C, and separated by SDS-PAGE (0.66 μg of protein per lane) using NuPAGE 4% to 20% bis-tris gels (Life Technologies). Protein transfer onto nitrocellulose membranes was performed with the iBlot Dry Blotting System (Life Technologies). The membrane was incubated for 2 hours in blocking solution (5% milk + 0.5% bovine serum albumin) in tris-buffered saline containing 0.05% Tween-20 (TBS-T). Serum specimens (1:500) were incubated in dilution buffer (10% blocking solution + 10% fetal bovine serum in TBS-T) for 1 hour. The secondary antibody used was horseradish peroxidase–conjugated anti-mouse IgG or IgA. Bound antibodies were detected by the ECL system (Millipore) and autoradiography film (Fuji, Valhalla, NY).

Cytokine Measurement

Sections of the jejunum were collected 18 to 24 hours following the final gluten challenge, homogenized, and tissue supernatants collected. Supernatants were also collected from T-cell proliferation assays after 3 days of stimulation. Cytokines were measured in tissue supernatants and cell culture supernatants using the Mouse Inflammatory CBA kit (BD Biosciences), and then analyzed using FACSarray Bioanalyzer System (BD Biosciences).

Antibiotic Treatment

Pregnant conventional SPF NOD/DQ8 mice were placed on 200 mg/L vancomycin (Sigma-Aldrich) in sterile

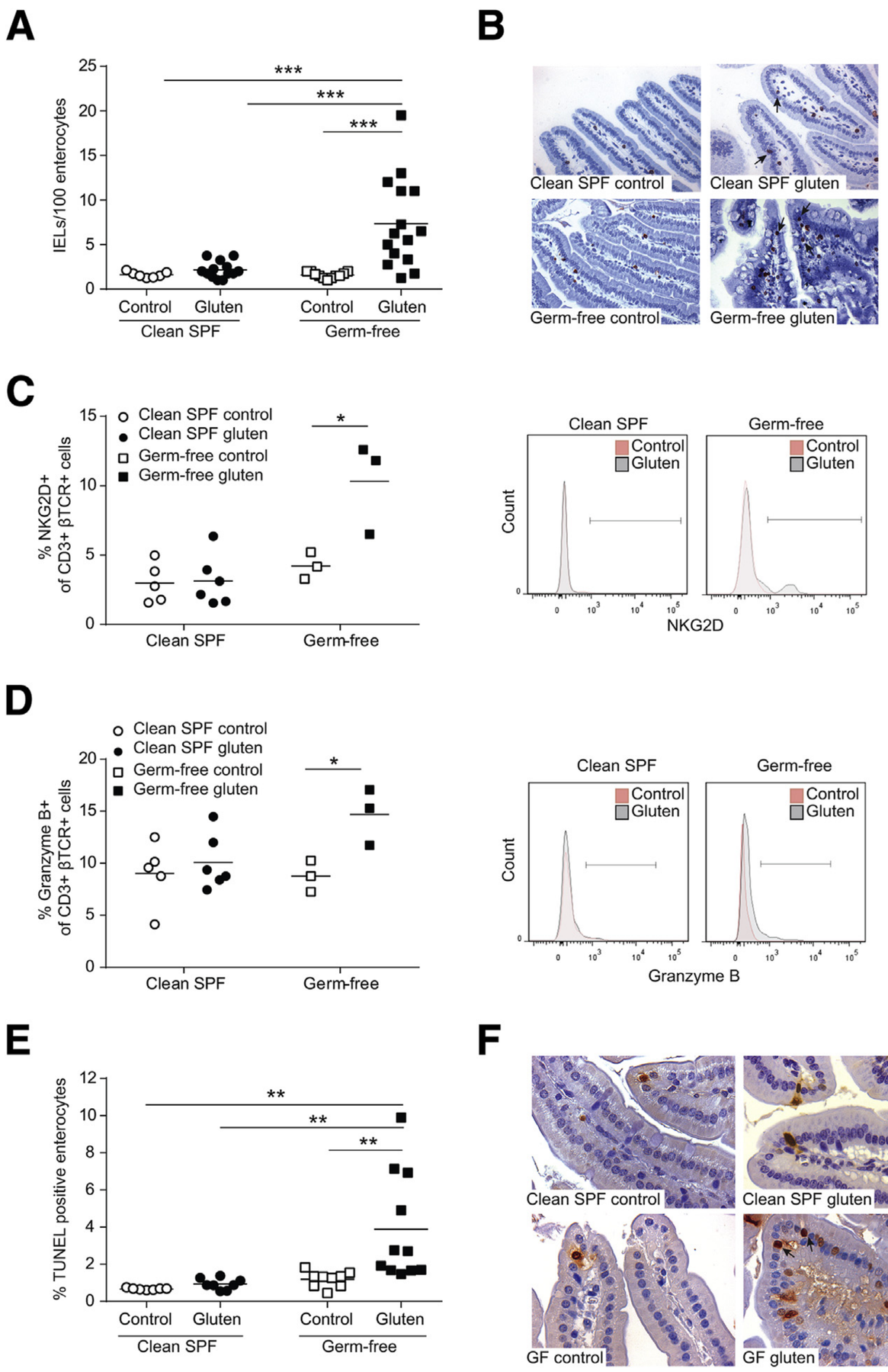

Figure 1 Colonization with a clean specific pathogen free (SPF) microbiota attenuates gluten-induced intraepithelial lymphocytosis, markers of intraepithelial lymphocyte (IEL) cytotoxicity, and enterocyte cell death in germ-free NOD/DQ8 mice. **A:** Quantification of $CD3^+$ cells in villi tips in sections of jejunum, expressed as IELs per 100 enterocytes. **B:** Representative $CD3^+$ stained sections of jejunum. **Arrows** indicate examples of IELs. **C** and **D:** Quantification and corresponding histograms of $NKG2D^+$ (**C**) and granzyme B^+ (**D**) cells gated on $CD3^+$ β T-cell receptor $(TCR)^+$ small-intestinal IELs. Open circles represent clean SPF controls, closed circles represent clean SPF gluten-treated mice, open squares represent germ-free controls, and closed squares represent germ-free gluten treated mice. **E:** Quantification of the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive enterocytes in the villi of jejunum sections. **F:** Representative TUNEL-stained sections of the jejunum. **Arrows** indicate examples of positive cells. Each dot represents an individual mouse. $*P < 0.05$ (**C** and **D**), $**P < 0.01$ (**E**), and $***P < 0.001$ (**A**). Original magnification: ×20 (**B**); ×40 (**F**).

drinking water and continued after birth until pups were weaned at 3 weeks of age. Vancomycin-containing water was replaced every 3 days. Fecal pellets were collected at 3 weeks of age for microbial analysis by 16S rRNA gene sequencing. Additional mice originating from non–antibiotic-treated NOD/DQ8 mice served as controls.

Statistics

Data were evaluated by analysis of variance with the Bonferroni post-hoc test for multiple comparisons when comparing more than two groups. Unpaired *t*-test was used to compare two groups. For microbial analysis, the *U*-test was used. $P < 0.05$ was considered statistically significant. All statistical analysis were performed in GraphPad Prism software version 6 (GraphPad Software, San Diego CA).

Results

Colonization with a Clean Microbiota Attenuates Gluten-Induced Markers of IEL Cytotoxicity and Enterocyte Cell Death

Proliferation and activation of IELs is a hallmark of CD.[32] To test the hypothesis that the background microbiota modulates host responses to gluten, we first compared IEL numbers and phenotype in germ-free and clean SPF

NOD/DQ8 mice following gluten sensitization and challenge (gluten treatment). The microbiota of clean SPF mice was dominated by members of the Bacteroidetes and Firmicutes phyla, and was free from any pathogens, opportunistic bacteria, or bacteria from the Proteobacteria phylum (Table 1). IEL counts increased following gluten treatment in germ-free, but not in clean SPF mice, and were greater in gluten-treated germ-free versus clean SPF mice (Figure 1, A and B). To test whether the germ-free condition enhances mucosal sensitivity to cholera toxin or to other non-gluten antigens such as zein, we determined IEL counts in germ-free and clean SPF C57BL/6 mice following sensitization and challenge with zein. No alterations in IEL counts were observed in germ-free or clean SPF C57BL/6 mice sensitized to zein (Supplemental Figure S1A).

Consistent with the literature,[33] naive germ-free mice had a higher proportion of $\gamma\delta TCR^+$ IELs compared to clean SPF mice (Supplemental Figure S2A). The proportions of $\gamma\delta TCR^+$ IELs or βTCR^+ IELs did not change following gluten treatment in germ-free or clean SPF conditions (Supplemental Figure S2, A–C). However, gluten-treatment in germ-free mice, but not clean SPF mice, led to an increase in NKG2D and granzyme B expression in $CD3^+\beta TCR^+$ IELs (Figure 1, C and D). No changes in NKG2D or granzyme B were detected on $\gamma\delta TCR^+$ IELs in germ-free or clean SPF mice following gluten treatment (data not shown). The increase in IEL activation markers was accompanied by an increase in the percentage of TUNEL-positive enterocytes in gluten-treated germ-free mice, but not in gluten-treated clean SPF mice (Figure 1, E and F). IL-15 mRNA was expressed at low levels in small-intestinal tissues in all groups, and did not change following gluten treatment (Supplemental Figure S2D). IL-21 mRNA levels were undetectable in all groups (data not shown). The data suggest that in the absence of commensal colonization, gluten induces a cytotoxic IEL response associated with increased enterocyte cell death.

Colonization with a Clean Microbiota Attenuates Gluten-Induced Pathology

Gluten treatment in germ-free but not clean SPF NOD/DQ8 mice led to a reduction in V/C ratios (Figure 2, A and C). Under baseline conditions, germ-free mice have longer and thinner villi compared to colonized mice (Figure 2A and Supplemental Figure S3).[34] Thus, to account for baseline differences under different microbial conditions, V/C ratios for gluten-treated mice were also expressed as a percentage of their respective controls for all experiments. When normalized to their controls, V/C ratios in germ-free mice were significantly lower compared to clean SPF mice following gluten treatment (Figure 2B). No alterations in V/C ratios were observed in zein-treated mice (Supplemental Figure S1, B–D). Thus, in the presence of susceptibility genes, commensal colonization with a benign microbiota attenuates gluten-induced enteropathy.

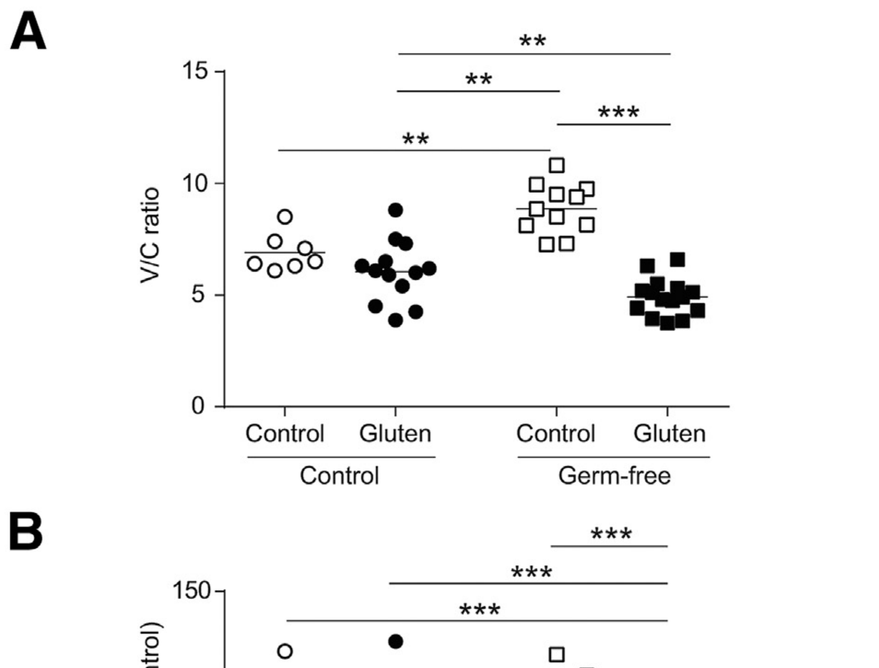

Figure 2 Colonization with a clean specific pathogen free (SPF) microbiota attenuates gluten-induced enteropathy in germ-free NOD/DQ8 mice. **A:** Quantification of villus-to-crypt (V/C) ratios in jejunum sections. **B:** V/C ratios for clean SPF and germ-free control and gluten-treated mice, expressed as a percentage of controls. Each dot represents an individual mouse. **C:** Representative hematoxylin and eosin–stained sections of jejunum. $^{**}P < 0.01$, $^{***}P < 0.001$. Original magnification, ×10 (**C**).

Colonization with a Clean Microbiota Attenuates Gliadin-Specific Responses

Serum and intestinal washes were tested for the presence of anti-gliadin IgA and IgG antibodies (AGA). No clean SPF or germ-free control mouse developed AGA (Figure 3, A–C). In germ-free conditions, 4 of 10 gluten-treated mice developed positive AGA in serum (IgG and IgA) and intestinal washes (IgA); by contrast, only 1 of 16 clean SPF mice developed AGA (Figure 3, A–C).

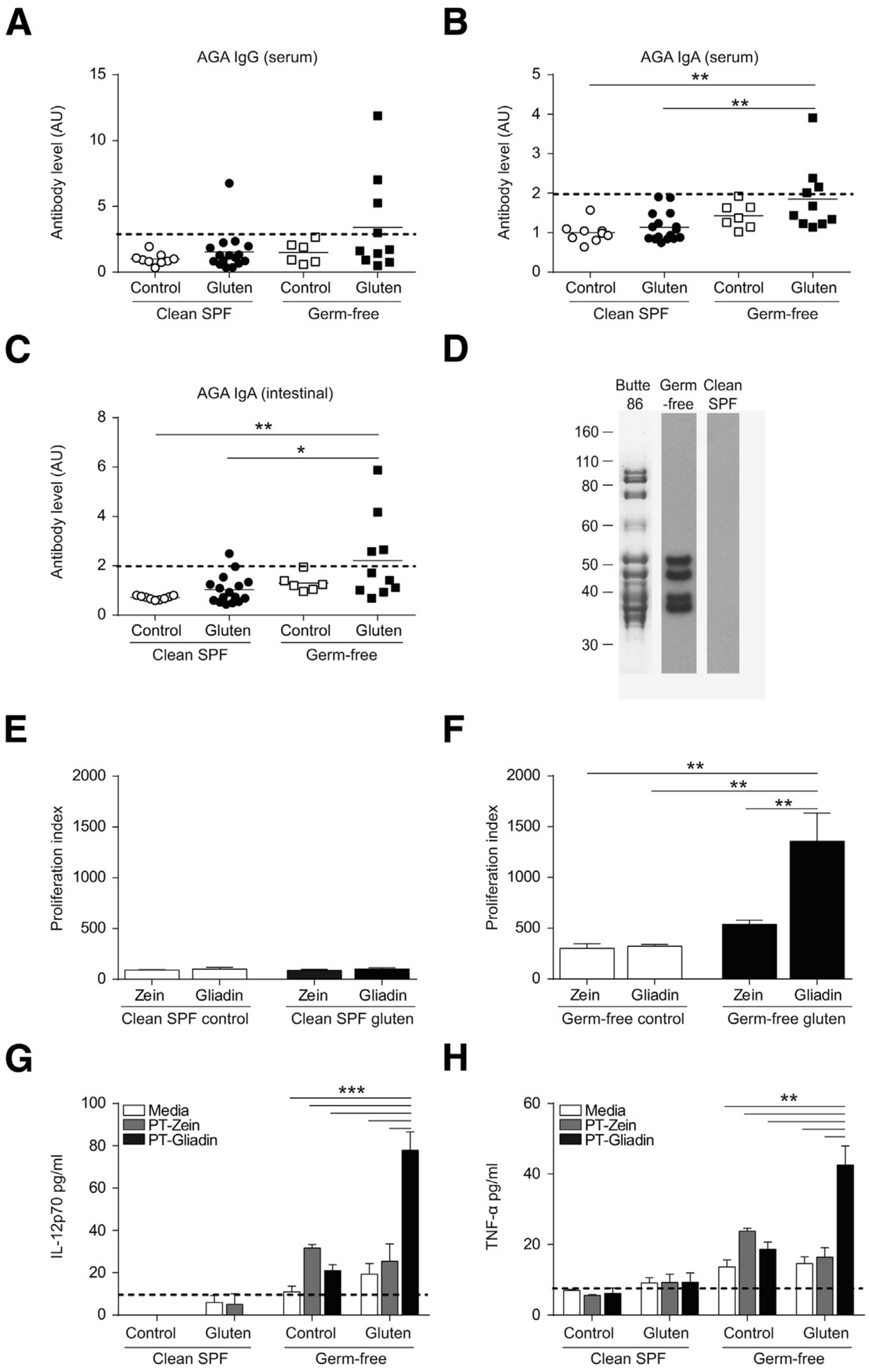

Figure 3 Colonization with a clean specific pathogen free (SPF) microbiota attenuates gliadin-specific immune responses in germ-free NOD/DQ8 mice. **A**–**C:** Serum and small-intestinal washes were collected for determination of serum anti-gliadin IgG (**A**), serum anti-gliadin IgA (**B**), and intestinal anti-gliadin IgA (**C**). Each dot represents an individual mouse. Positive reactivity was determined by using a positive cutoff value of $\geq$2 standard deviations above the mean of control mice, as indicated by the dotted line. **D:** Confirmation of antibody reactivity to gluten proteins by Western blotting. SDS-PAGE profile of the gluten extract from the Butte 86 cultivar used for immunoblotting assays. Western blot reactivity of serum antibodies from a representative germ-free gluten-treated mouse and a representative clean SPF control mouse. Molecular weight markers on the left are in kiloDaltons. **E** and **F:** $CD4^+$ T cells were isolated from mesenteric lymph nodes of non-sensitized control and gluten-treated clean SPF (**E**) and germ-free NOD/DQ8 (**F**) mice and stimulated with pepsin-trypsin (PT)-gliadin, PT-zein, or medium. The percentage of divided cells was determined for each stimulation condition (PT-gliadin, PT-zein), normalized to the proliferation of medium only wells, and expressed as a proliferation index. **G** and **H:** Production of IL-12p70 (**G**) and tumor necrosis factor (TNF)-α (**H**) in cell culture supernatant. Black dotted line represents the limit of detection. Data presented as means $\pm$ SEM. $n = 3$ (**G** and **H**, per group). $^*P < 0.05$ (**C**), $^{**}P < 0.01$ (**B**, **C**, **F**, and **H**), and $^{***}P < 0.001$ (**G**). AGA, anti-gliadin antibodies.

Antibody reactivity to specific gluten proteins of wheat was confirmed by Western blot analysis (Figure 3D). No differences in anti-TG2 IgA antibodies were detected between groups (data not shown). To further assess the immune reactivity to gliadin, T-cell proliferation in response to PT-gliadin or PT-zein stimulation was determined using $CD4^+$ T cells isolated from the mesenteric lymph nodes of germ-free and clean SPF control and gluten-treated mice. No response to PT-gliadin or PT-zein was observed in T cells isolated from clean SPF mice (Figure 3E). $CD4^+$ T cells isolated from gluten-treated germ-free mice had a greater response to PT-gliadin stimulation than to PT-zein stimulation (Figure 3F). Increased reactivity to PT-gliadin in cultures from gluten-treated germ-free mice was accompanied by increased production of IL-12p70 and tumor necrosis factor-α (TNF-α) in culture supernatant (Figure 3, G and H) and mild increases in IL-6 and interferon-γ (IFN-γ) (Supplemental Figure S4, A and B). No response to gluten or zein was observed in germ-free NOD/DQ8 controls (Figure 3F) or in zein-treated mice (Supplemental Figure S1E), indicating the responses observed in germ-free mice are gluten-specific and are attenuated by colonization with clean SPF microbiota.

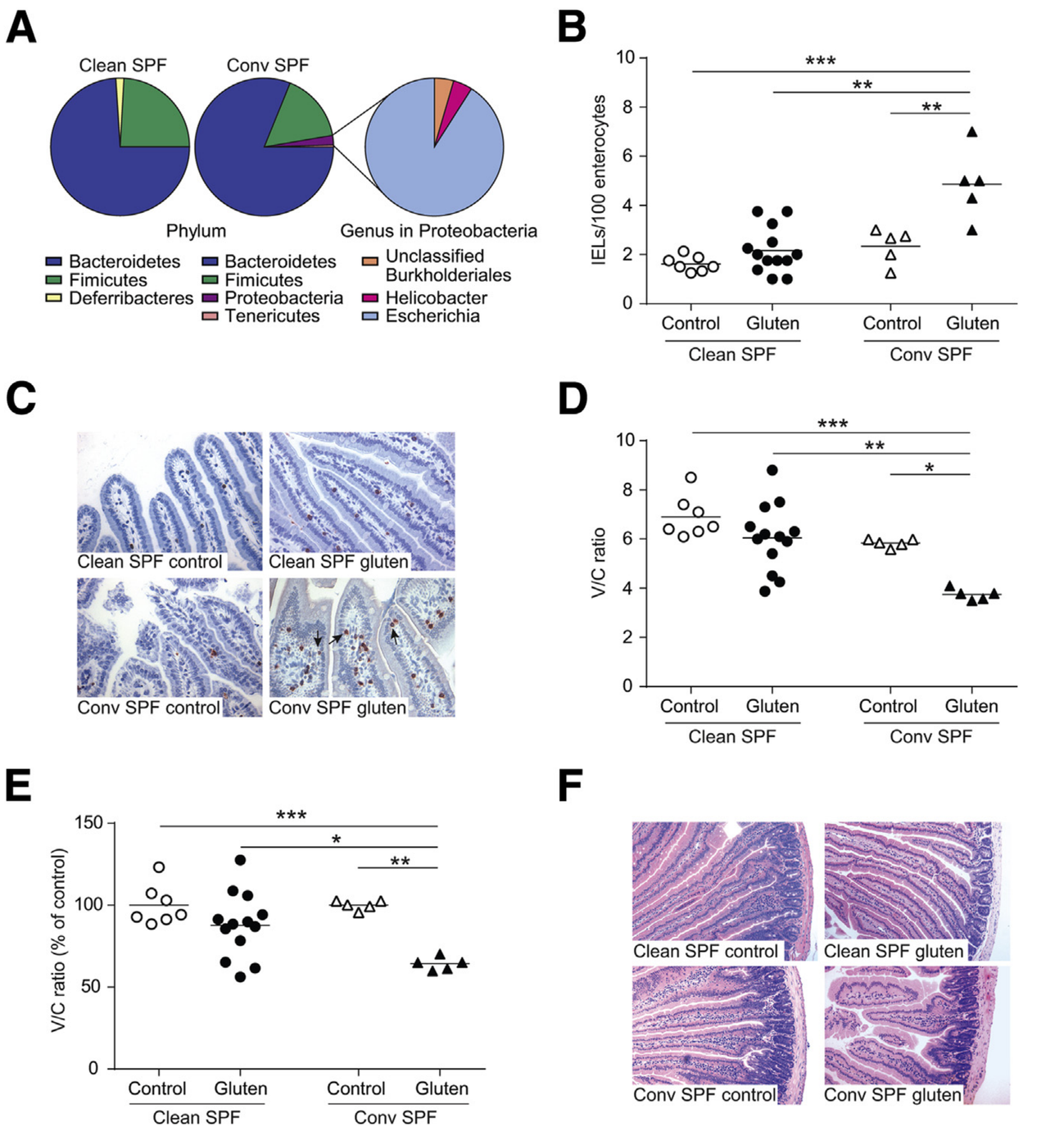

Figure 4 Conventional specific pathogen free (SPF) mice harbor opportunistic bacteria and develop more severe gluten-induced pathology compared to clean SPF NOD/DQ8 mice. **A:** Microbial composition of cecal contents from clean SPF and conventional (conv) SPF NOD/DQ8 mice by 16s rRNA sequencing at the phylum and genus level within the Proteobacteria phylum. **B:** Quantification of $CD3^+$ cells in villi tips of jejunum sections, expressed as intraepithelial lymphocytes (IELs) per 100 enterocytes. **C:** Representative $CD3^+$-stained sections of the jejunum. **Black arrows** indicate examples of IELs. **D:** Quantification of villus-to-crypt (V/C) ratios in jejunum sections. **E:** V/C ratios for clean SPF and conventional SPF control and gluten-treated mice, expressed as a percentage of controls. Each dot represents an individual mouse. **F:** Representative hematoxylin and eosin–stained sections of jejunum. $^*P < 0.05$ (**D** and **E**), $^{**}P < 0.01$ (**B**, **D**, and **E**), and $^{***}P < 0.001$(**B**, **D**, and **E**). Original magnification: ×20 (**C**); ×10 (**F**).

Attenuation of Gluten-Induced Responses in Clean SPF Mice Is Independent of Increased T Regulatory Cells

Regulatory T cells (Tregs), defined as $CD4^+CD25^+Foxp3^+$ T cells, were increased in the small intestine of naive germ-free NOD/DQ8 mice compared to naive clean SPF mice (Supplemental Figure S4, C and E). Furthermore, gluten treatment had no effect on Treg proportions or small-intestinal IL-10 levels (Supplemental Figure S4, D–F). In addition, no changes in TNF-α, IFN-γ, monocyte chemotactic protein 1, IL-6, or IL-12p70 cytokine levels were detected in small-intestinal tissues of clean SPF or germ-free gluten-treated mice (data not shown).

Conventional SPF Mice Develop More Severe Gluten-Induced Pathology Compared to Clean SPF NOD/DQ8 Mice

Unlike clean SPF, conventional SPF mice harbor several members from the Proteobacteria phylum such as *Helicobacter* and *Escherichia* species (Table 1 and Figure 4A). We found that gluten treatment in conventional SPF mice increased IELs in villi tips (Figure 4, B and C) and decreased V/C ratios (Figure 4, D–F) to a greater extent than in clean SPF mice.

Perinatal Antibiotic Treatment of Conventional SPF NOD/DQ8 Mice Worsens Gluten-Induced Pathology

To evaluate whether expansion of the Proteobacteria phylum in conventional SPF mice further exacerbated gluten-induced pathology, we treated NOD/DQ8 mice perinatally with vancomycin (Figure 5A). Antibiotic treatment resulted in an increase in the relative abundance of Proteobacteria and Firmicutes; a decrease in the relative abundance of Actinobacteria, Bacteroidetes, and Tenericutes; and lower fecal microbiota diversity (Figure 5, B–D). At the genus level, antibiotic-treated mice had greater abundances of *Escherichia*, *Helicobacter*, *Pasteurella*, an unclassified Betaproteobacteria, and *Lactobacillus* (Figure 5E). Additionally, the family Lachnospiraceae was reduced ($P = 0.056$) and the genera *Bacteroides* and *Parabacteroides* were significantly reduced in antibiotic-treated mice (Figure 5E).

Gluten treatment increased IEL counts in mice treated with or without antibiotics. However, IEL counts were greater in gluten-treated mice that received antibiotics compared to non–antibiotic-treated mice (Figure 6, A and B). Furthermore, antibiotic treatment increased the proportion of βTCR^+ IELs following gluten treatment

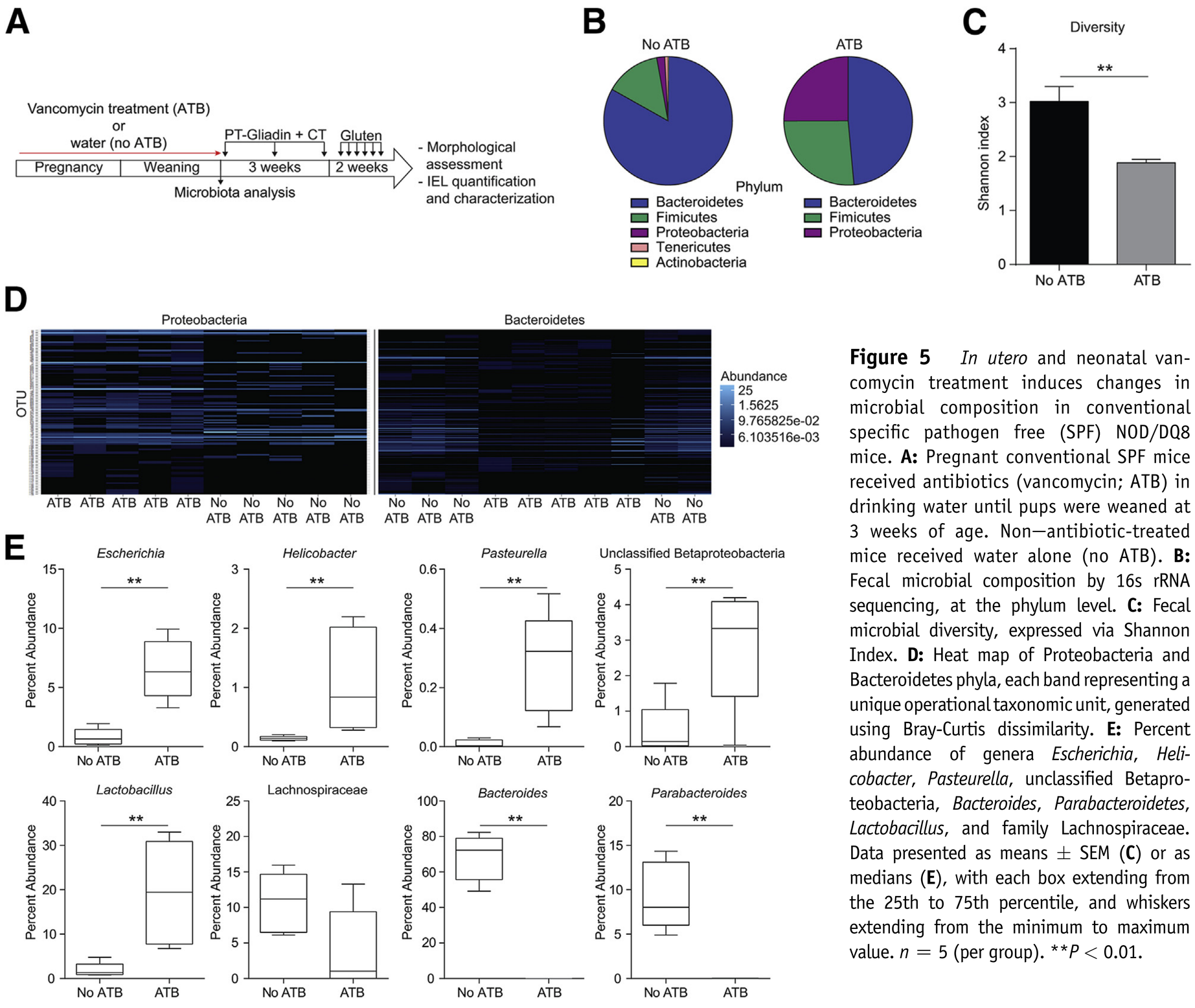

Figure 5 *In utero* and neonatal vancomycin treatment induces changes in microbial composition in conventional specific pathogen free (SPF) NOD/DQ8 mice. **A:** Pregnant conventional SPF mice received antibiotics (vancomycin; ATB) in drinking water until pups were weaned at 3 weeks of age. Non—antibiotic-treated mice received water alone (no ATB). **B:** Fecal microbial composition by 16s rRNA sequencing, at the phylum level. **C:** Fecal microbial diversity, expressed via Shannon Index. **D:** Heat map of Proteobacteria and Bacteroidetes phyla, each band representing a unique operational taxonomic unit, generated using Bray-Curtis dissimilarity. **E:** Percent abundance of genera *Escherichia*, *Helicobacter*, *Pasteurella*, unclassified Betaproteobacteria, *Bacteroides*, *Parabacteroidetes*, *Lactobacillus*, and family Lachnospiraceae. Data presented as means ± SEM (**C**) or as medians (**E**), with each box extending from the 25th to 75th percentile, and whiskers extending from the minimum to maximum value. $n = 5$ (per group). $^{**}P < 0.01$.

(Figure 6C). No changes in NKG2D or granzyme B expression were detected following gluten treatment in either group (data not shown). Gluten treatment also decreased V/C ratios, with or without antibiotics (Figure 6, D—F). Together, these studies demonstrate that perturbation of early colonization and induction of dysbiosis, characterized by increased Proteobacteria, enhances the severity of gluten-induced responses in NOD/DQ8 mice.

Administration of *E. coli* ENT CAI:5 to Clean SPF Mice Renders Mice Susceptible to Gluten-Induced Pathology

Finally, we investigated whether protection against gluten-induced immunopathology in clean SPF mice could be reversed through administration of a Proteobacteria member. Given the expansion of *Escherichia* associated with gluten-induced pathology in antibiotic-treated mice, and the clinical association between CD and increased abundance of Proteobacteria,[35—37] we supplemented clean SPF microbiota with a noninvasive, enteroadherent *E. coli* ENT CAI:5 strain carrying multiple virulence genes (*fimA* and *kfiC*),[22] originally isolated from a CD patient (Figure 7A). *E. coli*—supplemented clean SPF mice developed increased IEL counts in villi tips (Figure 7, B and C), reduced V/C ratios (Figure 7, D—F), and gliadin-specific $CD4^+$ T-cell responses (Figure 7G) after gluten treatment. *E. coli* ENT CAI:5 supplementation alone had no effect (Figure 7, B—G), supporting the notion that the presence of specific groups of bacteria, combined with genetic susceptibility and gluten, influences the development of immunopathology.

Discussion

The incidence of CD has risen dramatically over the last 5 decades, suggesting an important role for environmental factors in disease development.[5,38] Studies investigating environmental modulators of CD risk have not confirmed a protective role of feeding practices in infants at high risk.[16,17,39] A role for the

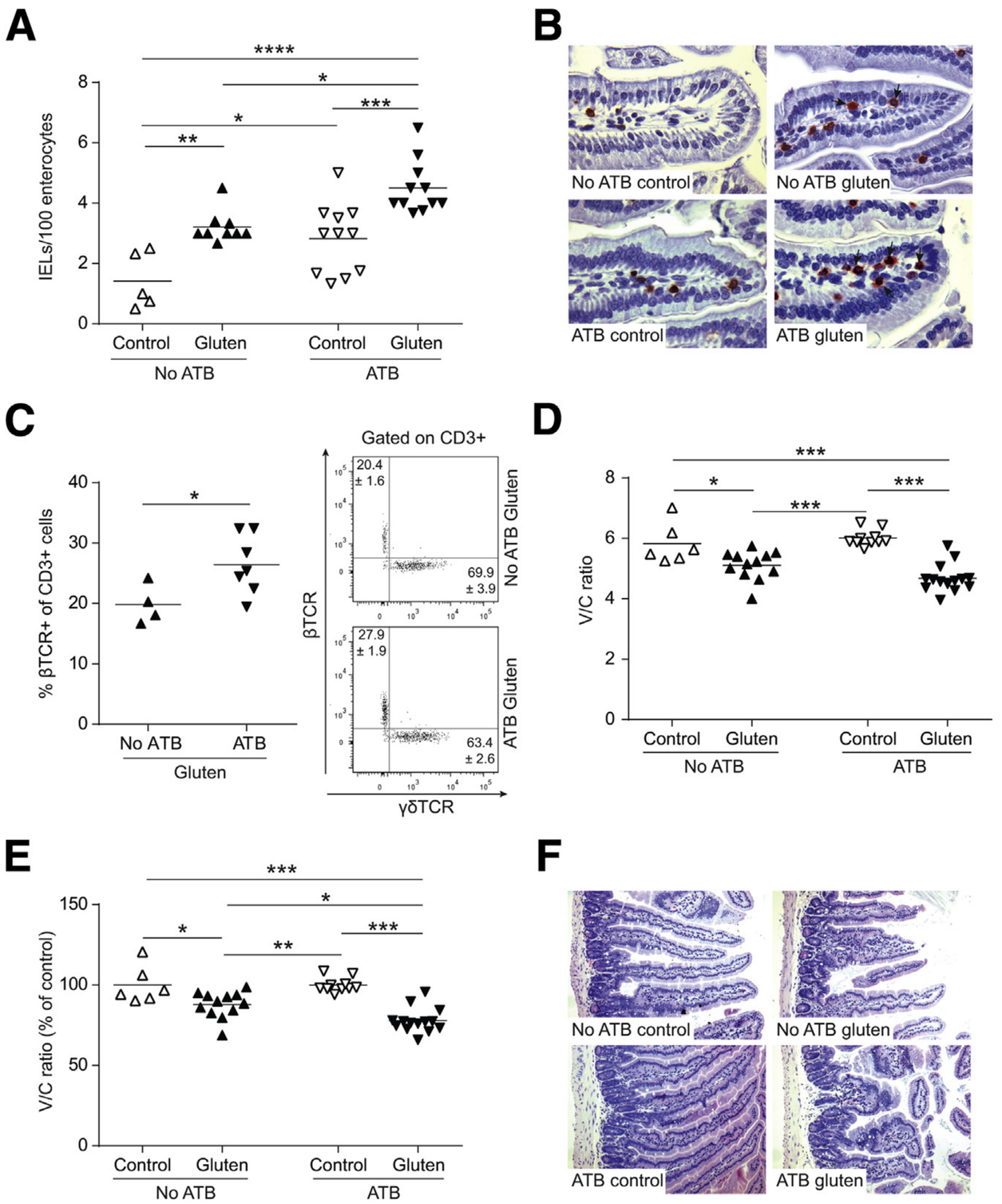

Figure 6 Perturbation of the colonization process in NOD/DQ8 mice increases severity of gluten-induced pathology. **A:** Quantification of $CD3^+$ cells in villi tips of jejunum sections, expressed as intraepithelial lymphocytes (IELs) per 100 enterocytes. **B:** Representative $CD3^+$-stained sections of jejunum. **Arrows** indicate examples of IELs. **C:** Quantification of the percentage of β T-cell receptor $(TCR)^+$ cells gated on $CD3^+$ small-intestinal IELs and representative flow cytometry plots for $CD3^+$ $\gamma\delta TCR^+$ and $CD3^+$ βTCR^+ IELs are shown with the means ± SEM indicated. **D:** Quantification of villus-to-crypt (V/C) ratios in jejunum sections. **E:** V/C ratios for control and gluten-treated mice, expressed as a percentage of controls. Each dot represents an individual mouse. **F:** Representative hematoxylin and eosin–stained sections of the jejunum. $^*P = 0.05$ (**A**, **C**, **D**, and **E**), $^{**}P < 0.01$ (**A** and **E**), $^{***}P < 0.001$ (**A**, **D**, and **E**), and $^{****}P < 0.0001$ (**A**). Original magnification: ×40 (**B**); ×10 (**F**). ATB, antibiotics.

intestinal microbiota as a contributing factor to CD has been suggested in some clinical studies.[13,14] However, a modulatory role of the microbiota on gluten-induced responses has remained elusive. We used a gnotobiotic approach to test the hypothesis that the background microbiota constitutes an environmental factor that modulates host responses to gluten in NOD/DQ8 mice. Colonization with a microbiota free from any opportunistic bacteria and bred in gnotobiotic conditions (clean SPF) prevented the development of gluten-induced immunopathology compared to the germ-free status, or to conventional SPF mice that harbor a diverse microbiota containing opportunistic pathogens belonging to the Proteobacteria phylum. Perinatal antibiotic disruption of the microbiota, leading to Proteobacteria expansion, further enhanced gluten-induced immunopathology in conventional SPF mice. When clean SPF mice were supplemented with an *E. coli* isolated from a CD patient (*E. coli* ENT CAI:5), the protection conferred by the benign microbiota was suppressed.

Gluten-treated germ-free mice developed decreased V/C ratios, a cytotoxic IEL phenotype and increased enterocyte cell death compared to clean SPF mice. The finding is in agreement with an earlier study, which demonstrated that long-term gliadin feeding to germ-free wild-type rats induced moderate small-intestinal damage.[40] However, interpretation of that study was limited by the lack of appropriate colonized controls. Expanding on that study, we show through several different strategies, that the composition of the microbiota can modulate gluten-induced immunopathology in the context of the HLA-DQ8 gene. Moreover, we demonstrate these effects with short-term gluten challenge. IELs from gluten-treated germ-free mice in our study had increased expression of NKG2D and granzyme B, which mediate epithelial cell death and are increased in IELs from active CD patients.[4,41] In addition to increased markers of IEL cytotoxicity, germ-free mice treated with gluten developed gliadin-specific antibodies and a proinflammatory gliadin-specific T-cell response, which were absent in clean SPF conditions. The underlying mechanisms could relate to the absence of homeostatic regulation by the commensal microbiota in germ-free mice. It is known that commensal bacterial colonization induces maturation of intestinal structure as well as immune gut function.[42] The microbiota of our

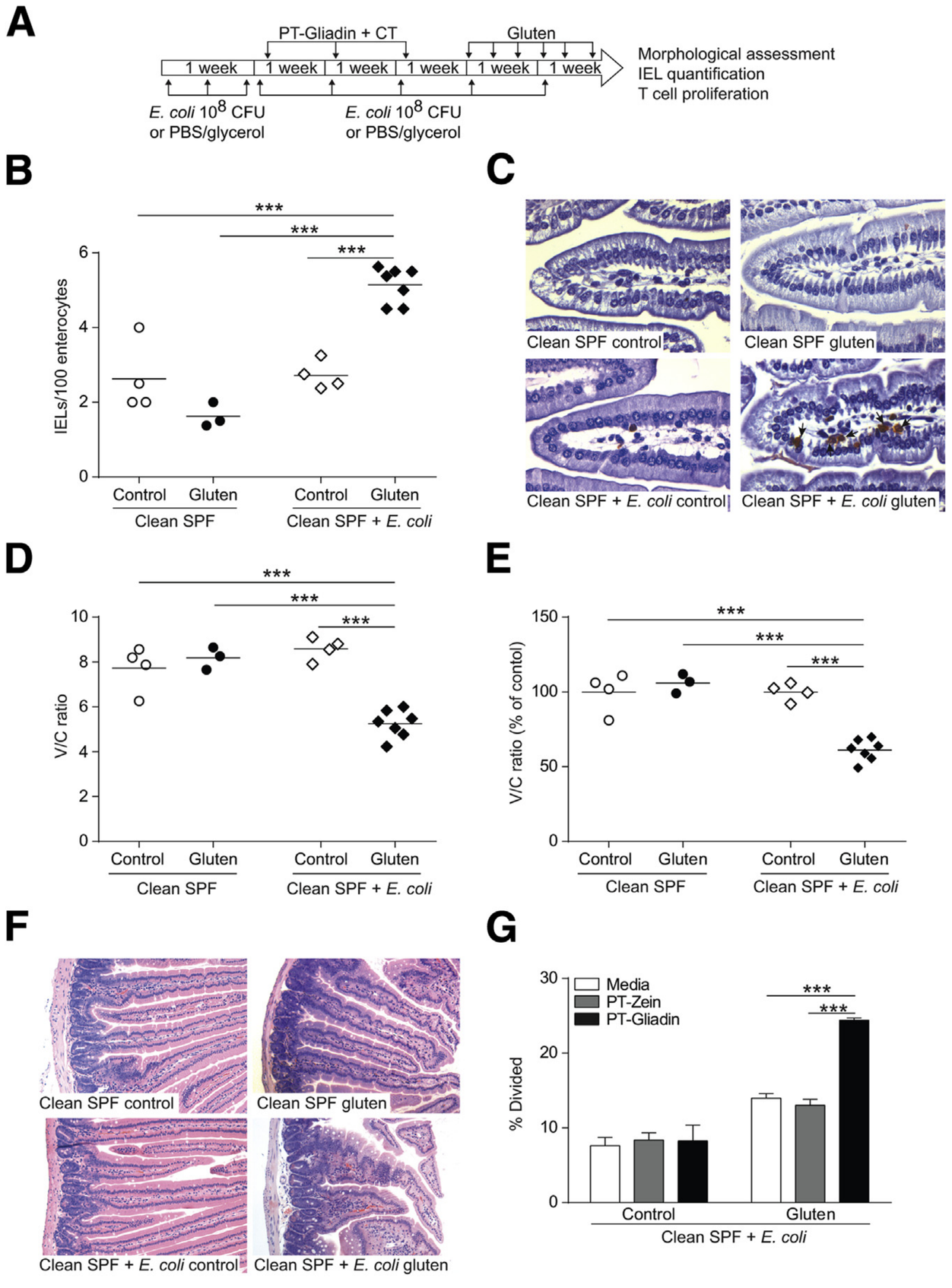

Figure 7 Supplementation of clean specific pathogen free (SPF) microbiota with *Escherichia coli* ENT CAI:5 increases severity of gluten-induced pathology in NOD/DQ8 mice. **A:** *E. coli* ENT CAI:5 supplementation and gluten treatment protocol. **B:** Quantification of $CD3^+$ cells in villi tips of jejunum sections, expressed as intraepithelial lymphocytes (IELs) per 100 enterocytes. **C:** Representative $CD3^+$-stained sections of the jejunum. **Arrows** indicate examples of IELs. **D:** Quantification of villus-to-crypt (V/C) ratios in jejunum sections. **E:** V/C ratios for control and gluten-treated mice, expressed as a percentage of controls. Each dot represents an individual mouse. **F:** Representative hematoxylin and eosin—stained sections of the jejunum. **G:** Mesenteric lymph node $CD4^+$ T-cell proliferation in response to pepsin-trypsin (PT)-zein or PT-gliadin stimulation in *E. coli* ENT CAI:5-supplemented clean SPF mice. Data presented as means ± SEM. $n = 3$ per group. $^{***}P < 0.001$. Original magnification: ×40 (**C**); ×10 (**F**). CFU, colony-forming units.

clean SPF mice is primarily composed of bacteria that are generally considered important for inducing maturation of the immune system[7,21] and down-regulating adverse inflammatory responses.[10,43] At the genus level, the microbiota was dominated by *Parabacteroides* which has been shown to protect against experimental colitis through several pathways, including induction of regulatory mechanisms.[43]

Tregs can be modulated by the microbiota, and they play a central role in oral tolerance.[7,44] However, the role of the microbiota in modulating oral tolerance is controversial.[45,46] Unlike what has been previously reported for colonic Tregs,[7] our study suggests the proportion of small-intestinal lamina propria Tregs was higher in naive germ-free NOD/DQ8 mice compared to colonized mice, indicating that a decrease in Treg proportions does not explain the higher reactivity to gluten in germ-free mice. Although we did not detect changes in small-intestinal levels of the regulatory cytokine IL-10, the results do not rule out that differences in Treg function underlie this observation. However, studies have suggested that small-intestinal Tregs from patients with CD[47] or IL-15 transgenic mice[48] are functional and suppressive, and that the defective response resides in effector T cells becoming unresponsive to Tregs due to dysregulated IL-15 signaling.[49] Increased expression of IL-15 has been found in a proportion of CD patients,[50] and animal models have reported IL-15–mediated gluten- or ovalbumin-induced enteropathy.[48,50] There is also evidence that IL-15 can induce IEL activation.[4,51] However, we found that IL-15 mRNA expression was low, and no changes were detected between germ-free or clean SPF groups at the transcriptional level. Although methodological issues have been raised regarding IL-15 measurement,[52] NOD mice have been reported to have reduced *IL15* gene expression, which explains our results.[53] This suggests that

the increased IEL numbers and markers of cytotoxicity observed in germ-free NOD/DQ8 mice following gluten treatment is mediated through an IL-15−independent pathway.

Conventional SPF NOD/DQ8 mice have previously been shown to respond to gliadin sensitization and challenge, developing a mild decrease in V/C ratios, increased IEL counts in villi tips, increased intestinal permeability, as well as gliadin-specific antibody and T-cell responses compared to nonsensitized controls.[19,54] However, mice with distinct colonization conditions have never been compared with respect to the degree of gluten-induced immunopathology. We found more severe gluten-induced responses in conventional SPF compared to clean SPF mice. Together with the germ-free studies, the data reveal a complex modulatory role of the microbiota to gluten, which may also include exacerbation of responses due to the presence of opportunistic pathogens within the conventional SPF microbiota. To test this hypothesis, we performed experiments using perinatal vancomycin treatment of conventional SPF NOD/DQ8 mice to deliberately perturb the normal colonization process and expand the Proteobacteria phylum.[55] Early-life antibiotic treatment led to significant changes in microbial profiles at 3 weeks of age with increases in Proteobacteria and Firmicutes. Interestingly, infants with a high genetic risk for CD have a higher relative abundance of Proteobacteria, including *Escherichia*.[56] Antibiotic-treated mice had increased IELs, with or without gluten, as well as increased proportions of βTCR^+ IELs in adult gluten-treated mice, an IEL subset that has been shown to be responsible for small-intestinal enteropathy associated with CD,[57] whereas $\gamma\delta$TCR IELs may play a protective role.[41] Microbial signaling also modulate IEL number and phenotype,[58] and thus, combinatory effects of both changes in microbial composition and presence of gluten likely explain the higher IEL numbers in antibiotic- and gluten-treated mice.

Increased abundance of Proteobacteria, including *E. coli*, has been reported in CD children,[35,36] and increased abundance of Proteobacteria has been associated with persistent symptoms in CD patients following a gluten-free diet.[37] Furthermore, *E. coli* isolated from CD children have been shown to carry a higher number of virulence genes[22] and induce proinflammatory cytokine production and activation markers in response to gluten stimulation in peripheral blood mononuclear cell cultures, dendritic cell cultures, and intestinal loops.[59−61] To further explore the role of pathobionts in modulation of responses to gluten, we supplemented clean SPF mice with *E. coli* ENT CAI:5. This rendered clean SPF mice, otherwise protected, susceptible to gluten sensitization as evidenced by increased IEL counts and increased T-cell proliferation to gliadin *in vitro*. Although the findings highlight a potential disease-modifying role of Proteobacteria in CD, there could be additional microbial differences between clean, conventional, and antibiotic-treated mice that may contribute. Thus, additional basic and clinical studies are needed to define the exact contribution of Proteobacteria versus other microbes in the modulation of gluten-induced responses.

In summary, we show that distinct changes in microbiota structure can either ameliorate or enhance IEL and $CD4^+$ T-cell responses to gluten in NOD/DQ8 mice. Our results support the concept that alterations in microbiota recently reported in active or symptomatic CD patients who are on a gluten-free diet could be causally related. Importantly, the data argue that the recognized increase in CD prevalence in the general population is causally driven, at least in part, by perturbations in intestinal microbial ecology. Specific microbiota-based therapies may aid in the prevention or treatment of CD in subjects with moderate genetic risk.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.ajpath.2015.07.018*.

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