Human Microbiota, Blood Group Antigens, and Disease

By: D. Rose Ewald and Susan C.J. Sumner

Abstract:

Far from being just "bugs in our guts," the microbiota interacts with the body in previously unimagined ways. Research into the genome and the microbiome has revealed that the human body and the microbiota have a long-established but only recently recognized symbiotic relationship; homeostatic balance between them regulates body function. That balance is fragile, easily disturbed, and plays a fundamental role in human health—our very survival depends on the healthy functioning of these microorganisms. Increasing rates of cardiovascular, autoimmune, and inflammatory diseases, as well as epidemics in obesity and diabetes in recent decades are believed to be explained, in part, by unintended effects on the microbiota from vaccinations, poor diets, environmental chemicals, indiscriminate antibiotic use, and "germophobia." Discovery and exploration of the *brain-gut-microbiota axis* have provided new insights into functional diseases of the gut, autoimmune and stress-related disorders, and the role of probiotics in treating certain affective disorders; it may even explain some aspects of autism. Research into dietary effects on the human gut microbiota led to its classification into three proposed enterotypes, but also revealed the surprising role of blood group antigens in shaping those populations. Blood group antigens have previously been associated with disease risks; their subsequent association with the microbiota may reveal mechanisms that lead to development of nutritional interventions and improved treatment modalities. Further exploration of associations between specific enteric microbes and specific metabolites will foster new dietary interventions, treatment modalities, and genetic therapies, and inevitably, their application in personalized healthcare strategies.

This article is categorized under:
- Laboratory Methods and Technologies > Metabolomics
- Translational, Genomic, and Systems Medicine > Translational Medicine
- Physiology > Mammalian Physiology in Health and Disease

Article:

1 INTRODUCTION

The human intestinal microbiome has emerged as an important research frontier with profound implications for understanding disease pathogenesis. As technology has advanced, research has expanded from simply identifying these microorganisms, to understanding their functions and interactions within the body, to correlating these findings with human health and disease states. Genomics, transcriptomics, metagenomic sequencing, proteomics, and metabolomics technologies have profoundly transformed the field of microbiology just as the invention of the microscope transformed the science of biology.

The Human Microbiome Project (HMP), which investigated the structure, function, breadth, and diversity of the microbiome in healthy adults, found that there were substantial taxonomic variations in the composition of the microbial community at different anatomical locations in the same person (intraindividual), as well as substantial variations at the same anatomical site in different people (interindividual) (Proctor, 2011). The eight anatomical sites chosen for taxonomic classification were the hair, skin, nostrils, oral cavity, esophagus, stomach, colon, and vagina (Proctor, 2011). The intestinal microbiome is perhaps the most complex of the eight sites studied.

The term *microbiota* refers to the collection of eukaryotic microbes and viruses, as well as bacteriophages, archaea, and bacteria which live in the human gut, while the term *microbiome* refers to the genomes of the microbiota, both the microbial genes and gene products (Proctor, 2011). Although the human microbiota is dominated by only 4 bacterial phyla (Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria) out of more than 50 known phyla (Blaser & Falkow, 2009), it has been estimated that the average human gut contains trillions of bacteria and archaea (Lepage et al., 2013). This vast bacterial biomass contains many unique or minimally redundant bacterial genes (Cho & Blaser, 2012), but because different bacterial species share functional traits, there is a high degree of functional redundancy (Lepage et al., 2013).

2 BACKGROUND

One of the biggest limitations for researchers has been the inability to identify the vast array of intestinal microbiota using laboratory culturing methods (Cho & Blaser, 2012), because it is extremely difficult to successfully maintain anaerobic culture conditions, which are required by the majority of intestinal microbes (Biedermann & Rogler, 2015). This limitation has been largely eliminated by the speed, ease, and accuracy of gene sequencing (Methé et al., 2012). Using 16S ribosomal RNA and DNA, researchers have been able to quickly detect, identify, and classify most of the microbes found in the healthy human gut (Methé et al., 2012), although reference sequences are still unknown for about one-third of the metagenome (Cho & Blaser, 2012).

Culture-independent methods have their own limitations. Organisms considered to be of the same species based on 16S ribosomal RNA gene sequencing can have large differences in other DNA sequences, and often have different sets of gene clusters that regulate the production of specialized metabolites (Ursell et al., 2014). Further, even if microbial species membership and abundance remain constant, changes in available dietary or xenobiotic substrates can alter the expression of metabolic functions (Ursell et al., 2014).

Although genomic sequencing of intestinal microbes can verify their presence or absence in the gut, neither function nor biological activity can be inferred simply from their presence, because the intestinal ecosystem is complex, interdependent, and not fully understood (Cho & Blaser, 2012; Kinross, Darzi, & Nicholson, 2011). In addition, the symbiotic relationship between the gut microbiome and the host results in extensive modulation of the metabolism and physiology of the interacting genomes, which therefore cannot be studied in isolation from each other (Martin et al., 2008).

As with any new technology, there have been challenges to overcome, and new challenges identified. Gene sequencing and cataloging has been hampered by differences in donor recruitment, protocols, and methodologies; human DNA contamination of specimens; as well as errors and artifacts generated during data production and processing (Methé et al., 2012). These issues have been resolved for the most part, yet despite much effort, research using genomic techniques has not been able to establish a "core microbiome" common to all healthy people (Huttenhower et al., 2012; Kinross et al., 2011; Proctor, 2011). Metagenomic sequencing categorized by gene function may ultimately prove more satisfactory for this purpose than whole-genome sequencing (Cho & Blaser, 2012). From preliminary studies, it appears that each human being has a virtually unique microbiome (Blaser & Falkow, 2009), but despite individual variances in its taxonomy and microbial composition, the metabolic capabilities and functions carried out by the microbiota are fairly constant and remarkably stable (Cho & Blaser, 2012; Proctor, 2011).

Recent metagenomic sequencing of the human intestinal microbiome, in fecal samples from a cohort of 124 people in the MetaHIT project, has proposed the existence of a functional core or minimum human gut metagenome necessary for the normal functioning of the gut ecosystem; this functional core is composed of about 3,364 nonredundant genes and between 1,000 and 1,150 bacterial species (Lepage et al., 2013; Qin et al., 2010). In the study population, each individual in the cohort harbored at least 160 of these bacterial species; although there was a high level of functional similarity between individuals, there was also a high interindividual specificity of intestinal microbiota, with 38% of an individual's bacterial genes shared by 50% of the population, but only 9% shared by 80% of the population (Lepage et al., 2013; Qin et al., 2010).

3 THE INTESTINAL MICROBIOTA

A robust, diverse intestinal microbiota is needed to develop and maintain the immune system (Blaser & Falkow, 2009; Proctor, 2011), regulate immunological function, as well as establish and maintain physiological homeostasis (Blaser & Falkow, 2009; Wang & Yang, 2013). The microbiota stimulates the production of cross-reactive antibodies, primarily immunoglobulin A

(IgA), which are secreted into the gut lumen, and profoundly influences the development of lymphatic structures in the gastrointestinal (GI) tract and differentiation of lymphocytes (Biedermann & Rogler, 2015). The presence of beneficial bacteria is critically important to prevent excessive colonization by pathogenic organisms, which occurs through competition for mucosal attachment sites, depletion of essential nutrients and/or oxygen, and by secretion of peroxides, antimicrobials, or bacteriocidins to inhibit other bacteria (Alonso, Vicario, Pigrau, Lobo, & Santos, 2014; Biedermann & Rogler, 2015; Jarchum & Pamer, 2011; Ouwerkerk, de Vos, & Belzer, 2013). This protective effect is known as "colonization resistance" (Jarchum & Pamer, 2011).

The intestinal microbiota has coevolved with its human host, and although within the lumen, it actually exists outside of the human body and functions independently from it but in close cooperation with it (Proctor, 2011). However, the intestinal microbiota could be considered an organ within an organ (Biedermann & Rogler, 2015), because it mediates various metabolic effects, directly influences epithelial cell proliferation, induces IgA production, stimulates lymphoid tissue development (Biedermann & Rogler, 2015; Bocci, 1992; Lepage et al., 2013), and strongly influences the host's metabolic response to environmental factors, drugs, and disease (Kinross et al., 2011). It is also intimately involved in digestion and metabolism of food, biosynthesis of vitamins and hormones that are absorbed by the host, as well as production of other compounds that the host cannot generate (Alonso et al., 2014; Biedermann & Rogler, 2015; Blaser & Falkow, 2009; Guzman, Conlin, & Jobin, 2013). Intestinal bacteria produce highly specific metabolic products and critical growth factors, which support epithelial growth and metabolism, and induce genes in epithelial cells to produce digestive enzymes that are required for physiological digestive processes (Biedermann & Rogler, 2015).

Perhaps most importantly, the nutritional value of the food that is eaten—what can be absorbed and utilized from it—depends on the composition of the microbiota itself, which is directly affected by the type of long-term diet that is consumed (Wu et al., 2011). It should be noted as well that dietary components and xenobiotic agents are metabolized in different ways by different microbial communities (Ursell et al., 2014). However, the process has layers of complexity: microbial metabolites may interfere with host metabolism of xenobiotics; diet-derived nutrients can regulate microbial metabolism of xenobiotics; and xenobiotics can modulate the expression and activity of the gut microbiome (Ursell et al., 2014).

3.1 Composition of the microbiota

The microbial community of the intestines is composed of both beneficial and pathogenic bacteria, and can include fungi and parasites, as well as viruses (Kelly & Mulder, 2012). Pathogenic microorganisms have the inherent ability to cross anatomical barriers, resist host defenses, and induce host responses (Blaser & Falkow, 2009). Infection with a pathogen sometimes causes disease, which is a function of the host/pathogen interaction (Blaser & Falkow, 2009); however, some people can be carriers of pathogenic microorganisms without ever developing symptoms.

Commensal and *mutualistic* bacteria are resident microorganisms that have, respectively, a benign or mutually beneficial relationship with their host; generally, these symbionts benefit the

host by preventing colonization by pathogens, and the genes of these resident microorganisms are included in the gene pool of the host (Blaser & Falkow, 2009). However, if these normally harmless bacteria enter the body or overpower host defenses, they become pathogenic and the consequent opportunistic infection can lead to serious or fatal illness (Biedermann & Rogler, 2015).

We interact mostly with symbionts on a daily basis, which have not been associated with significant pathology (Ohnmacht et al., 2011). It has been proposed that the immune system is primarily shaped by symbionts, and that proinflammatory immunity may be a necessary component of establishing and maintaining a state of homeostasis between host and microbiota (Ohnmacht et al., 2011). Further, it has been suggested that the pathogenicity of microbes is defined by the context in which the microbe interacts with its host: mutualists can become pathogens if penetrating the wrong niche, and notorious pathogens can become mutualists when restricted to the right niche (Ohnmacht et al., 2011).

3.2 Changes in composition

Changes in the composition of the intestinal microbiota have been associated with the development of diseases of the upper GI tract (ranging from gastritis, peptic ulcers, gastroesophageal reflux, and Barrett's esophagus, to stomach and esophageal cancers, and celiac disease) as well as the lower intestine (including inflammatory bowel disease [IBD], ulcerative colitis, Crohn's disease, and colorectal cancer) (Cho & Blaser, 2012; Lepage et al., 2013; Wang & Yang, 2013). There appears to be a characteristic microbiota community associated with many of these diseases, indicated by both taxonomic and functional markers, some of which may precede the disease state (Blaser & Falkow, 2009; Cho & Blaser, 2012; Proctor, 2011). In addition, the immune status of the host can influence the composition of beneficial communities (Artis, 2008). Age-related changes in the microbiota can have harmful effects, including changes in the ratio between Firmicutes and Bacteroidetes species, progressive development of gastric atrophy, and cancer-related somatic mutagenesis (Cho & Blaser, 2012).

The composition of the microbiota has also been changed by the use of antibiotics, vaccines, improvements in healthcare, and changes in lifestyle that have lowered infant mortality rates and increased lifespans, but which have also resulted in reduced horizontal bacterial transfer and greatly increased selection against existing ancestral microorganisms (Blaser & Falkow, 2009; Cho & Blaser, 2012). It is thought that changes in human ecology, such as clean water, more frequent bathing, widespread use of antibiotics and antimicrobial soaps, smaller family size, less social crowding, more cesarean births, and less breastfeeding, may have had unintended effects on the composition of the intestinal microbiota, and therefore human physiology and disease risks (Blaser & Falkow, 2009).

The microbiome is not inherited fully formed; it is acquired following birth via vertical or horizontal transfer (Bocci, 1992; Cho & Blaser, 2012; Proctor, 2011). If born by vaginal delivery, the founding microbial population reflects the vaginal microbiome of the mother, being primarily *Lactobacillus* , *Prevotella* , or *Sneathia* species; if born by cesarean section, it is similar to the skin microbiome of the mother, with primarily *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* species (Dominguez-Bello et al., 2010). Lactobacilli (Cho & Blaser, 2012)

and Bifidobacteria (Houghteling & Walker, 2015) are also contributed from mother's milk. Regardless of delivery mode, the initial bacterial colonies are undifferentiated in the newborn, with homogeneous distribution across the skin, nasopharyngeal, oral, and intestinal habitats (Dominguez-Bello et al., 2010). The initial colonization of the intestinal microbiota by pioneer colonizers prepares the baby's GI tract for subsequent additional bacterial population (Cho & Blaser, 2012; Dominguez-Bello et al., 2010). Bacterial diversity increases with age, achieving a configuration similar to adults by about 3 years of age (Yatsunenko et al., 2012).

Several studies have documented that birth method influences fecal microbiota populations in infants (Dominguez-Bello et al., 2010; Grönlund, Lehtonen, Eerola, & Kero, 1999; Yatsunenko et al., 2012). One study comparing babies born by cesarean and vaginal delivery found that intestinal colonization was delayed and remained disturbed for 6 months or longer in those born by cesarean (Grönlund et al., 1999). *Lactobacillus* and *Bifidobacterium* colonization normalized at 10 and 30 days, respectively, but only 36% of cesarean-delivered infants were colonized by *Bacteroides fragilis* at 6 months, versus 76% of vaginally delivered infants ($p = .009$); colonization by *Clostridium perfringens* at 30 days was 57% in babies delivered by cesarean, versus 17% in vaginally delivered infants ($p = .003$) (Grönlund et al., 1999).

Colonization by *B. fragilis* may never normalize, due to interbacterial inhibition by one or more ecological competitors established in its absence (Grönlund et al., 1999). Reduced colonization may have unintended consequences, both because *B. fragilis* prevents colitis by supporting differentiation of T-cells that express interleukin (IL)-10, which reduces inflammatory responses in the intestine, and because a capsular component of *B. fragilis* , polysaccharide A, plays a regulatory role in autoimmune processes in the central nervous system (CNS) (Jarchum & Pamer, 2011). In contrast, with vaginal birth, the primary means of intestinal colonization appears to be vertical transmission from the mother, with the founding microbial community of the baby principally composed of Lactobacilli from the vagina and mother's milk (Cho & Blaser, 2012).

The *disappearing microbiota hypothesis* postulates that loss of indigenous beneficial bacteria over time (for whatever reason) has contributed to the recent rapid increases in conditions such as Type 2 diabetes mellitus (T2DM), obesity, and metabolic syndrome (Blaser & Falkow, 2009). The loss of keystone species can result in the extinction of secondary species as well, with a cascading downstream effect on the host and on co-colonizing species; functional redundancy can mask these effects in the short-term, but over time, the microbiota's ability to adapt and respond appropriately is compromised (Cho & Blaser, 2012). The loss of *Helicobacter pylori* in particular is related to the increased incidence of allergies, asthma, and esophageal reflux, with concurrent decreases in rates of peptic ulcers and certain gastric cancers (Blaser & Falkow, 2009).

A related concept, known as the *hygiene hypothesis* , suggests that reduced exposure to microbes and infectious diseases in childhood is directly linked to the increasing incidence in recent years of IBDs and chronic immune-related disorders (Biedermann & Rogler, 2015). Exposure to foreign microorganisms in early childhood influences microbial composition and stimulates correct development of the immune system (Biedermann & Rogler, 2015; Rescigno, 2013). The use of antibiotics, especially in early infancy, may therefore be linked to the rising incidence of

food and airborne allergies, other immune-related disorders, metabolic disorders, and autoimmune diseases (Rescigno, 2013).

4 THE INTESTINAL BARRIER

Several mechanisms work together in the intestinal lumen to protect host integrity and prevent bacterial invasion. These include the mechanical barrier of the epithelium; tight junctions where cell–cell contact occurs; expression of mucins to form the mucus layer; secretion of chloride; production of defensins and cytokines; the protective presence of beneficial bacteria; and gut motility (Biedermann & Rogler, 2015). The epithelium is a single layer of cells, composed of enterocytes, goblet cells, and enteroendocrine cells (Wells, Rossi, Meijerink, & van Baarlen, 2011), which supports the largest mucosal surface of the human body (Artis, 2008).

The primary function of the epithelium lining the GI tract is to maintain health and homeostasis (Artis, 2008). It does this by regulating digestion, absorption, and transport of vital nutrients and macromolecules, while simultaneously tolerating food antigens, avoiding overreaction and potentially destructive inflammatory responses to beneficial bacteria, and preventing invasion by pathogenic organisms (Artis, 2008; Rescigno, 2013; Wells et al., 2011). Maintaining homeostatic balance between tolerance and immunity is a regulatory challenge that involves delicate choreography, orchestrated by the epithelial cells, between the signals and responses of the adaptive and innate immune systems. Antigens activate both of these systems; any molecule that can be recognized by the immune system serves as an antigen (Delves, 2017a); however, every antigen evokes a unique type and degree of response.

The innate immune system does not require prior exposure to specific antigens, but instead identifies broadly distributed antigens as "self," distinguishes potentially harmful or foreign molecules and cells as "non-self," and mounts an immediate defense against them (Alonso et al., 2014; Delves, 2017a). The primary components of innate immune response include antimicrobial peptides and pattern recognition receptors (PRRs) (Alonso et al., 2014). Other components of the innate immune system include phagocytes, which ingest foreign antigens; natural killer cells, which kill virus-infected cells and some tumor cells; and leukocytes, which release inflammatory mediators (Alonso et al., 2014; Delves, 2017a).

Defensins are peptides that are expressed in mucosal epithelial cells and phagocytes, and are released into the intestinal lumen (Alonso et al., 2014). In addition to acting as effector and regulatory molecules of the innate immune response, they induce the release of inflammatory mediators, regulate the complement system, and increase antigen-specific immune response by interacting with dendritic cells and T-cells (Alonso et al., 2014). Cytokines, complement, and acute-phase proteins are molecular components that participate in both innate and adaptive immunity (Delves, 2017a). Cytokines are not antigen-specific, but they influence the magnitude of inflammatory or immune responses and can act sequentially, synergistically, or antagonistically (Delves, 2017b).

Although the acquired or adaptive immune system takes longer to respond after initial exposure to an antigen, after it acquires antigen-specific memory, subsequent response to the same antigen is rapid (Delves, 2017a, 2017c). Cell-mediated responses are derived from activation of certain T

cell receptors, while humoral responses are derived from B cells, which secrete soluble antigen-specific antibodies (Alonso et al., 2014; Delves, 2017a). The innate and adaptive components of the immune system also coordinate their efforts; for example, most microorganisms are killed after they are phagocytosed, but when necessary, phagocytes can be stimulated to produce more lytic enzymes and microbicidal products by T-cell-derived cytokines such as interferon-γ (IFN-γ) (Delves, 2017a).

PRRs are a class of proteins that respond to small molecular sequences that are consistently found on pathogens and other microbes, such as flagella, cilia, Gram-positive peptidoglycans, Gram-negative lipopolysaccharides (LPSs), oligosaccharides, and nucleic acids (Alonso et al., 2014; Artis, 2008; Delves, 2017a; Guzman et al., 2013; Proctor, 2011; Wells et al., 2011). Upon detection of these antigens or other bacterial ligands, toxins, bacterial DNA, or viral double-stranded RNA, PRRs on epithelial cells trigger signaling cascades that tailor the immunological memory of the adaptive immune system to recognize and respond to these molecular patterns in specific ways (Alonso et al., 2014; Artis, 2008; Delves, 2017a; Guzman et al., 2013; Proctor, 2011; Wells et al., 2011). Activation of some signaling pathways leads to initiation of the adaptive immune response, including induction of T-cells and maturation of dendritic cells, which confer a homeostatic response and tolerance to future contact, whereas activation of other pathways elicits a defensive response, involving activation of the innate immune system and release of antimicrobial peptides, inflammatory cytokines, chemokines, and macrophages (Alonso et al., 2014; Rescigno, 2011, 2013; Wells et al., 2011).

The synergistic interaction between the microbiota, the epithelial barrier, and the immune system is complicated by the fact that all three respond to and are affected by dietary influences; this has been called the *diet-microbiota-immune axis* (Kau, Ahern, Griffin, Goodman, & Gordon, 2011). Metabolites from epithelial cells directly regulate the functions of antigen-presenting cells (APCs) and lymphocytes (Artis, 2008); metabolites in the diet indirectly influence the function of the mucosal barrier (Guzman et al., 2013); and the intestinal microbiota produce most of the metabolites found in blood plasma (Cho & Blaser, 2012). Metabolic activities of the microbiota are monitored by the immune system, which is in turn modified by microbial signaling and mucosal absorption of metabolites and other dietary compounds (Kau et al., 2011).

4.1 Establishing and preserving homeostasis

The preservation of homeostasis requires microbial sensing by epithelial cells; this in turn depends on the adequate presence and correct functioning of these receptors, which are critical for preventing the development of chronic intestinal inflammation, infectious colitis, and even cell apoptosis (Rescigno, 2011). These conditions can lead to increased permeability of the intestinal barrier and translocation of the microbiota across the epithelium (Rescigno, 2011). However, this damage is mitigated by intraepithelial lymphocytes, located between the epithelial cells in the intestinal barrier (Alonso et al., 2014), which promote repair of injured epithelia and release antimicrobial peptides that prevent entrance of enteric bacteria (Rescigno, 2013).

The tissue directly beneath the epithelium (on the basolateral side) is known as the *lamina propria* ; it contains a variety of plasma cells, lymphocytes, stromal cells, and APCs (Alonso et al., 2014; Wells et al., 2011). It also produces both secretory IgA (sIgA), which is constantly

transported across the intestinal epithelium into the gut lumen (Benckert et al., 2011), and polymeric IgA, which promotes excretion of antigens present in the lamina propria and neutralizes pathogens intracellularly during transepithelial transport (Mantis, Rol, & Corthesy, 2011). The lamina propria is a key component of the innate immune system (Pagnini et al., 2010), which quickly responds to defend, support, or restore mucosal barrier integrity and function, and prevent uptake of pathogenic organisms and toxins (Guzman et al., 2013).

The apical side of the epithelial cells is protected by the glycocalyx, which consists of a high-density mixture of mucins (glycoproteins), glycolipids, and proteoglycans attached directly to the epithelial cell membrane (Etzold et al., 2014; Ouwerkerk et al., 2013), as well as immunoglobulins, antimicrobial peptides, and electrolytes (Etzold et al., 2014). The glycocalyx is negatively charged and repels most microorganisms that are also negatively charged (Rescigno, 2013). On the apical side of the glycocalyx, the negatively charged net-like polymer of mucins secreted by the enterocytes and goblet cells of the epithelium is heavily glycosylated and forms two layers of viscous and relatively impermeable extracellular mucus; the glycans in the inner layer are more densely packed than those in the outer layer, and their smaller pore size physically prevents bacterial penetration (Artis, 2008; Ouwerkerk et al., 2013).

Mucins are composed of a variety of both membrane-bound mucins (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, and MUC17) and gel-forming mucins (MUC2, MUC5AC, MUC5B, and MUC6); the majority of the mucus in the GI tract from duodenum to colon is composed of the gel-forming mucin MUC2 (Etzold et al., 2014; Varum & Basit, 2014). MUC6 is also secreted by Brunner's glands in the duodenum, while MUC5AC and MUC6 are secreted in the stomach (Varum & Basit, 2014). Membrane-bound mucins of the inner layer influence cell growth, metastasis of tumors, and immune system recognition, and may play a role in cell differentiation, but are primarily involved in cell signaling (Moncada & Chadee, 2002). Gel-forming secreted mucins of the outer layer provide lubrication as well as protection from digestive enzymes, gastric pH, free radicals, toxins, and carcinogens (Varum & Basit, 2014). This unregulated, baseline constitutive mucus secretion can be quickly augmented by receptor-mediated secretion, when stimulated by alcohol, mustard, other chemical irritants, prostaglandins, secretions from monocytes and macrophages, or bacterial toxins (Moncada & Chadee, 2002; Varum & Basit, 2014).

The mucus layers are the only point where there is direct contact between the host and the intestinal microbiota. Mucus keeps epithelial cells hydrated; provides a chemical, physical, and immunological barrier that protects the underlying epithelium against luminal microbes; and has hydrophobic qualities that also help fight enteric bacteria and regulate gut permeability (Alonso et al., 2014; Varum & Basit, 2014). Thus, defects that affect the function or differentiation of goblet cells and the production of mucus result in gut inflammatory disorders, dysfunction, and impaired resistance to infection (Alonso et al., 2014; Rescigno, 2011).

Bacterial invasion is prevented by antimicrobial peptides that are produced in large quantities throughout the GI tract, and by antibodies and defensins that are usually bound to the mucins; despite attachment of segmented filamentous bacteria (SFB) to the epithelium, in a healthy individual the inner mucus layer is virtually sterile (Biedermann & Rogler, 2015; Jarchum & Pamer, 2011; Ouwerkerk et al., 2013). SFB are host-friendly bacteria that penetrate the mucus

layers and adhere tightly to the epithelium, which induces development of the adaptive immune system, including strong IgA responses, as well as differentiation and activation of T-cells (Jarchum & Pamer, 2011; Ohnmacht et al., 2011; Rescigno, 2011). SIgA coats beneficial microorganisms and anchors them to the mucus layer, which allows their colonization (Rescigno, 2013). SIgA antibodies are also believed to be involved in the progressive, controlled establishment of the newborn's microbiota (Mantis et al., 2011).

Antigen-specific sIgA antibodies attached to the mucus layer block specific binding sites on the bacterial cell wall, prevent direct contact with the epithelium by binding with pathogenic bacteria and toxins, and interfere with their adherence to epithelial receptors by stearic hindrance, all of which result in decreased colonization and faster removal through a process known as *immune exclusion* (Benckert et al., 2011; Jarchum & Pamer, 2011; Mantis et al., 2011; Rescigno, 2011; Walker, 1978). Immune exclusion, which involves agglutination, entrapment in mucus, and/or clearance through peristalsis (Mantis et al., 2011), may be the main and most important function of intestinal antibodies, because their ability to selectively interfere with specific bacterial adherence determines the composition of the resident microbiota (Walker, 1978).

The term *mucosal barrier* refers to the lamina propria, epithelium, and glycocalyx, the mucus layers of the intestine, and the membrane-bound microbiota; the release of various chemical mediators and regulatory molecules drives multidirectional communication between the components of this local regulatory network (Alonso et al., 2014). Homeostasis is possible—and is maintained—because barrier integrity is maintained while the intestinal epithelial cells closely regulate the activity of dendritic cells and the responses of macrophages and lymphocytes to prevent overreaction to resident microbiota (Wells et al., 2011).

The relative percentage of mucus-secreting goblet cells increases from 4% in the duodenum to 16% in the descending colon; perhaps not surprisingly, the thickness of the mucus layers varies in each part of the GI tract (Goto & Kiyono, 2012), and is thickest in the highly colonized colonic segment of the distal gut (Alonso et al., 2014; Ouwerkerk et al., 2013). There is very little data on the thickness of human mucus layers, and much disagreement as to which methodology gives the most accurate results, but the murine GI tract can be used as a representative model (Varum & Basit, 2014), although the layout of the intestinal tract is different.

An in vivo study in rats found that the firmly adherent, continuous inner mucus layer of the colon was 115 ± 51 µm, whereas the inner mucus layer of the small intestine was extremely thin (ranging from 15 ± 2 µm in the duodenum and jejunum to 29 ± 8 µm in the ileum), and was patchy and discontinuous (Atuma, Strugala, Allen, & Holm, 2001). Similarly, the less dense outer mucus layer of the colon was 714 ± 109 µm, whereas the outer mucus layer of the duodenum, jejunum, and ileum was 154 ± 39 µm, 108 ± 5 µm, and 447 ± 47 µm, respectively (Atuma et al., 2001). Extrapolating from the available data, it appears that human mucus layers should have similar relative proportions (Varum & Basit, 2014).

The thinner outer mucus layer of the duodenum and jejunum, and the extremely thin, discontinuous inner layer are necessary for enterocytes to absorb nutrients, but this leaves the epithelium much more vulnerable to bacteria (Atuma et al., 2001). In humans, direct bacterial

contact is minimized because transit time through the small intestine is rapid, the mucus layers are continuously replenished, and antimicrobial peptides and lysozymes are expressed in much higher quantities that are concentrated closer to the epithelial cells in the small intestine than elsewhere in the GI tract (Ouwerkerk et al., 2013). In addition to their absorptive and transport functions, enterocytes also develop immunological activity in self-defense; they not only express innate immune receptors and secrete several chemokines and cytokines, but also function as nonprofessional APCs (Alonso et al., 2014). Additional protection is conferred by antibodies present in the glycocalyx that bind with antigens, as well as by pancreatic enzymes, which adsorb to the surface of the enterocytes and facilitate the breakdown of these antigen–antibody complexes; similarly, secretory antitoxins protect against the effects of toxic bacterial by-products (Walker, 1978).

Although these defense strategies can limit exposure of the epithelium to gut microbes and prevent hypersensitive immune responses, controlled and regulated bacterial contact and direct sampling throughout the GI tract are required to maintain optimum gut immune functions (Alonso et al., 2014; Kelly & Mulder, 2012). Specialized regions of organized lymphoid tissues such as microfold (M) cells, Peyer's patches, and mesenteric lymph nodes play a critical role in direct antigen sampling of the gut; they are considered the inductive sites of the immune system that both induce and regulate immune responses (Alonso et al., 2014; Kelly & Mulder, 2012). Dendritic cells, which are found throughout the lamina propria and lymphoid tissue of the mucosa, also produce finger-like membrane extensions called dendrites that are extruded between epithelial cells to obtain direct antigen samples from the bacteria in the lumen (Swiatczak & Rescigno, 2012; Wells et al., 2011).

Dendritic cells are the most important of the APCs in the innate immune system, and express up to 100 times more major histocompatibility complex (MHC) molecules than other APCs; those in the lymphoid tissue drive differentiation of naïve T-cells and production of sIgA, whereas those in the lamina propria kill invading microorganisms and induce inflammatory responses to defensive signaling (Swiatczak & Rescigno, 2012; Wells et al., 2011). Among other factors, the type of microorganism encountered, the type and number of PRRs activated, and the local cytokine/chemokine environment influence the type of immune response (Kelly & Mulder, 2012; Swiatczak & Rescigno, 2012).

5 HOST–MICROBIAL INTERACTIONS

Antigen binding to mature naïve B cells stimulates their differentiation into memory cells, with slow and limited production of antibodies; subsequent binding by the same antigen induces rapid proliferation and maturation of memory cells into plasma cells with immediate production of large amounts of antigen-specific antibodies (Delves, 2017c). Although B cells can present antigens to T-cells and secrete cytokines, their primary function is to develop into plasma cells that manufacture and secrete antibodies (Delves, 2017c).

T-cell-dependent adaptive responses are typically triggered by MHC molecules expressed by APCs (Toivanen, Vaahtovuo, & Eerola, 2001). Class I MHC molecules, which are expressed by all nucleated cells (Toivanen et al., 2001), present intracellular antigens such as those found on viruses to CD8 cytotoxic T-cells (Delves, 2017c). Class II MHC molecules, which are

constitutively expressed by B cells, monocytes, macrophages, and dendritic cells, complex with peptides derived from extracellular antigens, which are then presented to CD4 helper T (T_H) cells (Delves, 2017c; Swiatczak & Rescigno, 2012).

Microbial composition is an important factor determining the intricate balance between different T-cell subsets in the GI tract; beneficial microbiota have been shown to drive local expansion of T_H cell populations (Kelly & Mulder, 2012). There are three types of T-cells: cytotoxic T-cells, regulatory T-cells (T_{REG}), and T_H cells (Delves, 2017c). Cytotoxic T-cells can secrete cytokines and are critical for eliminating intracellular pathogens, especially viruses, usually by inducing apoptosis of infected cells, whereas T_{REG} cells either suppress the immune response by direct cell-to-cell contact or by secreting cytokines with immunosuppressive properties (Delves, 2017c).

T_H cells are differentiated into three types, each of which secretes several cytokines (Delves, 2017c). T_H1 cells promote cell-mediated immunity against intracellular pathogens (e.g., viruses) by facilitating cytotoxic T-cell and macrophage responses, whereas T_H2 cells stimulate antibody production by B cells and direct responses to extracellular pathogens (e.g., parasites, bacteria) (Delves, 2017c). In contrast, T_H17 cells promote tissue inflammation (Delves, 2017c), and are also important mediators of intestinal immune defenses that protect against intestinal pathogens (Jarchum & Pamer, 2011). T_H17 cells are induced by the intestinal microbiota via SFB colonization of the epithelium, by phagocytosis of infected apoptotic cells by dendritic cells, and by direct flagellin-mediated stimulation of lamina propria dendritic cells, but are negatively regulated by inhibition of T-cell proliferation following microbial recognition by intestinal epithelial cells (Jarchum & Pamer, 2011).

In addition, natural killer T (NKT) cells are a distinct subset of T-cells in the adaptive immune system; when activated, NKT cells secrete IL-4 and IFN-γ, and are thought to regulate immune responses (Delves, 2017c). NKT cells are discrete from natural killer cells, which are cytotoxic leukocytes in the innate immune system (Delves, 2017c). Natural killer cells secrete various cytokines, influence the adaptive immune system by stimulating and inhibiting differentiation of T_H1 cells and T_H2 cells, respectively (Delves, 2017c), and some subtypes appear to have mucosal barrier-protective effects (Rescigno, 2013).

Communication between the mucosal layer and the microbiota is bidirectional. *Cross-talk* is the term used to describe this ability to sense and appropriately respond to the microbial environment within the lumen; the release of cytokines, chemokines, and other signaling molecules, and the response by leukocytes and other immune cells, enables the body to mount a defensive response only when necessary (Servin, 2004). This dynamic interplay between the intestinal microbiota, epithelial cells, and gut-associated immune cells of the intestinal barrier maintains the crucial balance between immunity and inflammation in the gut (Servin, 2004).

Different responses by the immune system to beneficial and pathogenic bacteria are evoked by the recognition of their different signature molecules; inflammatory and defensive responses occur in response to pathogen-associated molecular patterns, but these responses are not triggered by the microbe-associated molecular patterns of commensal bacteria (Servin, 2004). The adequate presence and correct functioning of PRRs and signaling mechanisms in epithelial

and monocytic cells are critical to prevent chronic stimulation by beneficial microorganisms of inappropriate and potentially destructive inflammatory responses, while simultaneously maintaining robust defensive responses against pathogenic organisms (Kelly & Mulder, 2012; Servin, 2004; Wells et al., 2011).

These very different responses can initiate via the same pathway or receptor, depending on what activates it and where it is located, because the apical side of the epithelial cell interacts with the microbiota via the mucin layer, but the basolateral side interacts with immune cells in the lamina propria (Alonso et al., 2014; Rescigno, 2011). For example, if TLR-9 receptors on the apical side of epithelial cells are triggered, a tolerogenic response is induced, but when TLR-9 receptors on the basolateral side are triggered, the innate immune system is activated and expresses proinflammatory cytokines and chemokines (Alonso et al., 2014; Wells et al., 2011). Similarly, intrinsic signaling by nuclear factor kappa-B (NF-κB) prevents apoptosis of epithelial cells (Goto & Kiyono, 2012), yet when the NF-κB pathway is triggered by defensive signaling, it initiates a proinflammatory response, and if triggered by homeostatic signaling, it confers tolerance of resident bacteria, regulates T_H cell responses, and prevents inflammation (Alonso et al., 2014; Jarchum & Pamer, 2011; Kelly & Mulder, 2012; Wells et al., 2011).

Surface-associated proteins, such as pili, flagella, and fimbria, not only provide motility for bacteria but also allow adhesion to glycosylated mucins or fibronectins in the extracellular matrix (Etzold et al., 2014; Ruiz, Hevia, Bernardo, Margolles, & Sánchez, 2014). Once shed in the lumen, their monomeric subunits interact directly with epithelial and immune cells to induce β-defensins, tumor necrosis factor α (TNF-α), and IL-8 cytokines (Ruiz et al., 2014). Bacteria can also highjack certain signaling pathways and actively inhibit others (Artis, 2008). For example, both pathogenic and beneficial bacteria have evolved mechanisms to suppress or induce NF-κB activation, and inhibit or modulate its signaling pathway; recent evidence suggests that bacteria-derived secreted factors may be involved (Kelly & Mulder, 2012; Ruiz et al., 2014; Sánchez, Urdaci, & Margolles, 2010). The microbiome produces numerous such compounds that regulate the expression of antimicrobial peptides (Rescigno, 2013) and modulate intestinal barrier function (Guzman et al., 2013), so alterations to the composition of the intestinal microbiota can affect mucosal barrier integrity and susceptibility to intestinal pathogens (Guzman et al., 2013; Jarchum & Pamer, 2011; Servin, 2004).

5.1 Microbial mechanisms and effects

Commensal bacteria help to determine the composition of the resident microbiota by inhibiting pathogen colonization, and can thus have an anti-inflammatory effect, but they can also help to trigger inflammatory diseases and other gut disorders (Round & Mazmanian, 2009; Ruiz et al., 2014). Likewise, pathogenic bacteria can have beneficial effects. For example, the polysaccharide chain and lipid tail of LPS are components of the bacterial outer cell membrane that identify it as "non-self" and there is evidence that trace amounts of LPSs bound to Gram-negative bacteria act beneficially to stimulate or "prime" the innate immune system without initiating a full-blown response (Bocci, 1992). Free LPS in large quantities in body fluids (such as during sepsis, gastroenteritis, or altered bowel permeability) induces synthesis of prostaglandins and production of cytokines (IFNs, IL-1, IL-6, and TNF-α), activates complements, and stimulates macrophages and leukocytes (Bocci, 1992).

In contrast, when trace amounts of LPS are bound to very low-density or low-density lipoproteins, chylomicrons, bile salts, serum albumin, Ig, or LPS-binding protein, the LPS remains biologically active and has a physiological effect rather than a pathological effect (Bocci, 1992). Locally, it induces IgA and cytokine production in the gut-associated lymphoid tissue (GALT) and stimulates enterocyte differentiation, whereas systemically it results in highly restricted, controlled immunological responses in the reticuloendothelial cells of organs such as bone marrow, spleen, liver, and lungs (Bocci, 1992). The beneficial effect of this physiological stimulation is that the immune system is maintained in a state of alertness, primed for an immediate response, but without toxic effects from high circulating levels of cytokines and without developing a tolerance for LPS (Bocci, 1992).

Most probiotics are derivatives of the intestinal bacteria and have beneficial effects on the host and most of the resident microbiota, with either inflammatory or anti-inflammatory capacity, which stimulate immune and nonimmune cells, respectively (Rescigno, 2011; Ruiz et al., 2014). Probiotics can preserve or restore intestinal epithelial integrity and homeostasis, contribute to increased production of antimicrobial substances, and compete with pathogenic bacteria for adhesion sites (Pagnini et al., 2010; Sánchez et al., 2010; Servin, 2004). Additional proposed mechanisms include downmodulating the immune response; stimulating innate immunity that protects against chronic inflammation; stimulating production of intestinal mucins and regeneration of epithelial cells; reducing permeability; and improving barrier tightness (Alonso et al., 2014; González-Rodríguez, Ruiz, Gueimonde, Margolles, & Sánchez, 2013; Pagnini et al., 2010; Rescigno, 2011; Sánchez et al., 2010; Servin, 2004). Specific strains can: prevent reduction in transepithelial resistance induced by hydrogen peroxide; downregulate nitric oxide production by macrophages; promote cytoprotective responses; reduce bacterial translocation to the mesenteric lymph nodes from the gut; increase mucus production; maintain colonic mucosal barrier integrity; and regulate apoptosis (Alonso et al., 2014; Sánchez et al., 2010). One novel mechanism that has been observed in some intestinal bacteria is the production of antibacterial substances that, when cleaved by the pancreatic enzyme trypsin, are activated against Gram-positive pathogenic bacteria (Servin, 2004).

Recent advances in technology and greater understanding of functional genomics have enabled researchers to better understand some of the processes by which bacterial colonization and survival occur, and the mechanisms by which cross-talk occurs between bacteria and host mucosal cells (Etzold et al., 2014; González-Rodríguez et al., 2013; Ruiz et al., 2014; Sánchez et al., 2010; Servin, 2004). Through technological advances, these very properties have been exploited for a variety of uses, from cosmetics, toiletries, paints, printer ink, and pesticides, to the medical field (including drug delivery, anticoagulants, plasma substitutes, wound dressings, tissue reengineering and replacement, and tumor inhibition) as well as functional foods such as probiotics (Nwodo, Green, & Okoh, 2012). Digestive enzymes and low pH of the stomach are natural barriers to many bacteria, and the small intestine harbors an equally harsh environment that includes pancreatic enzymes, bile salts, and antimicrobial peptides such as defensins and cathelicidins (González-Rodríguez et al., 2013). Probiotics help to prevent infection from surviving pathogens by stimulating production of these peptides and overcompeting for colonization sites in the gut (González-Rodríguez et al., 2013; Servin, 2004).

Bacteria have developed acid-induced survival mechanisms, such as strain-specific changes in sulfur amino acid metabolism; increased production of ATP synthases to reduce cytoplasmic H^+ accumulation; increased production of ammonia and branched-chain amino acids, which capture excess H^+; as well as changes in surface properties and fermentation capabilities that increase acid tolerance (González-Rodríguez et al., 2013). Exposure to bile, which has a detergent-like effect on the lipid layer of the bacterial cell membrane, has resulted in strain-specific adaptive responses that include increased production of glycolytic enzymes and enzymes that synthesize fatty acids; expression of bile salt hydrolase to deconjugate bile salts; and increased activity of efflux pumps to reduce cytoplasmic accumulation of bile acids (González-Rodríguez et al., 2013). Other adaptive responses to bile exposure include overexpression of chaperones and proteases to repair misfolding and prevent aggregation; induction of SOS response proteins to repair DNA damage; and production of exopolysaccharides (González-Rodríguez et al., 2013). Exopolysaccharides will be discussed in more detail in section 8.

In addition to exopolysaccharides, some bacteria produce H_2O_2, some secrete bactericidins, and some secrete lactic acid and acetic acid, which lower the pH in the environment; these secretions inhibit growth and colonization of some pathogenic bacteria, whereas secretion of teichoic acid and lipoteichoic acid increases adherence by certain strains of Gram-positive bacteria (Didierlaurent, Sirard, Kraehenbuhl, & Neutra, 2002; Servin, 2004). Secreted molecules interact with dendritic cells to modulate their regulatory mechanisms and maintain immune tolerance for the bacteria (Ruiz et al., 2014). The cell wall-associated proteins p40 and p75 also modulate dendritic cell response and serve as adhesion molecules, but in addition, they stimulate phosphorylation of epidermal growth factor receptors, and prevent epithelial injury and cytokine-induced apoptosis of epithelial cells (Ruiz et al., 2014; Sánchez et al., 2010). In response to signals by probiotic and commensal bacteria, IgA antibody production and phagocytic activity are higher, and translocation of pathogens across the mucosal barrier is lower (Servin, 2004).

Intestinal barrier dysfunction, which can include both increased intestinal permeability and inflammation, often involves dysfunction of tight junctions between epithelial cells (Alonso et al., 2014). Pathogenic bacteria use flagella for motility, or produce mucus-degrading enzymes such as glycosidases to break down mucin oligosaccharides so they can digest mucus barriers to reach the host epithelial layer (Etzold et al., 2014; Goto & Kiyono, 2012). They have also developed strategies to gain access to the lamina propria, such as activating the inflammatory cascade, directly disrupting paracellular tight junctions, and inducing fluid and electrolyte secretions in response to toxin production (Alonso et al., 2014). *Vibrio cholera* disrupts tight junctions through its cytotoxin hemagglutinin protease; *Clostridium difficile* produces two distinct exotoxins that cause increased paracellular permeability; *Clostridium perfringens* enterotoxin triggers massive permeability changes; and enteropathic *Escherichia coli* directly disrupts tight junctions and enhances intestinal permeability through selective dephosphorylation and phosphorylation (Alonso et al., 2014).

Bacterial survival strategies can make it difficult for the body to mount an effective immune defense and also increase the possibility of autoimmune issues (Nwodo et al., 2012). Some pathogenic bacteria (e.g., *E. coli*) produce capsular polysaccharides known as glycosaminoglycans for molecular camouflage, and some of the most challenging pathogenic bacteria are difficult to treat because they form biofilms (e.g., *Streptococcus pyogenes* , which

causes necrotizing fasciitis), whereas polysaccharides on some bacteria are very similar to those on human cells (e.g., *Neisseria meningitidis*) (Nwodo et al., 2012).

The immunogenic LPSs produced by *E. coli* suppress macrophage growth and promote secretion of TNF-α, whereas commensal exopolysaccharides have the opposite effect, inhibiting secretion of TNF-α and promoting macrophage growth (Ruiz et al., 2014). Similarly, commensal and probiotic exopolysaccharides have been correlated with stimulation of Peyer's patch cells in the intestinal mucosa and increased natural killer cell activity (Ruiz et al., 2014). They have also demonstrated an ability to inhibit biofilm formation, attenuate infection-related cytotoxic effects on enterocytes through their antioxidant and free-radical scavenging activity, and improve recovery time after viral infection (Ruiz et al., 2014).

6 ANTIGENS, ADHESINS, AND RECEPTORS

In the human body, the immune system uses cell-surface molecules known as *antigens* to distinguish "self" from "not self." Infection by pathogens occurs when they are able to evade or resist the body's immune response, express sufficient amounts of the appropriate adhesion molecules to bind with the host's receptors, establish residency in the tissue of choice, and produce toxins or other virulence factors in large enough amounts to cause destruction or disruption of normal cellular function (Didierlaurent et al., 2002; Klemm & Schembri, 2000). Phagocytes have learned to recognize and respond to certain bacterial antigens, with phagocytosis being assisted by the actions of Toll-like receptors (TLRs) and scavenger receptors: TLRs can recognize and bind with LPS and lipopeptides, which upregulate scavenger receptors, whereas both TLRs and scavenger receptors bind with bacterial polysaccharides (Kline, Fälker, Dahlberg, Normark, & Henriques-Normark, 2009).

In their turn, pathogenic bacteria have evolved several ways to avoid detection and elimination by the immune system: the IgA-binding proteins that are found in hemolytic *Streptococcus* species and *S. aureus* (specifically M-proteins, β-antigen, and SSL), some of which also bind complement proteins, prevent phagocytosis and block the respiratory burst of neutrophils (Kazeeva & Shevelev, 2009). The filamentous hemagglutinin (FHA) of *Bordatella pertussis* upregulates complement receptor 3 (CR3) by binding with the phagocytic integrin signal transduction complex, and when it then binds with CR3, it is safely internalized within the macrophage; similarly, the FimH adhesin of *E. coli* binds to the CD48 receptor of macrophages, which results in internalization and survival within the phagocyte (Kline et al., 2009).

After surviving macrophage engulfment, *Salmonella enterica* serovar Typhi and serovar Paratyphi are then spread through the blood and lymph systems of their human hosts (Kato & Ishiwa, 2015). *E. coli* , *N. meningitidis* , and some *Streptococcus* species are among the pathogens that use molecular mimicry to evade detection by the host's immune system and protect themselves from phagocytosis; negatively charged polysaccharides such as polysialic acid, heparosan, chondroitin, and hyaluronan not only repel phagocytes but also allow the bacteria to masquerade as "self," which increases its virulence (Cress et al., 2014).

Superantigens (SAgs) are an extremely potent bacterial defense against the immune system, which cause fever, shock, and often death; they stimulate mitogenesis of T-cells by first binding

with MHC Class II molecules and then binding to the variable β-chain region of T-cell receptors, leading to uncontrolled T-cell activation followed by systemic release of massive amounts of TNFα, IL-2, and other proinflammatory cytokines (Proft & Fraser, 2003). The first bacterial SAgs identified were toxins from Gram-positive *S. aureus* and *S. pyogenes* , and there are now more than 40 known SAgs that have been identified with genomic sequencing, with some resulting from horizontal gene transfer between subgroups of streptococcal and staphylococcal species (Proft & Fraser, 2003). SAgs have also been identified in *Mycoplasma arthritidis* and *Yersinia pseudotuberculosis* , which are Gram-negative bacteria (Proft & Fraser, 2003). SAgs cause staphylococcal food poisoning and endotoxic shock, staphylococcal toxic shock syndrome, streptococcal toxic shock syndrome (which is lethal in up to 50% of cases), acute rheumatic fever, and Kawasaki disease; some studies have also found a possible SAg role in autoimmune diseases, such as multiple sclerosis (MS) and Type 1 diabetes mellitus (T1DM) (Proft & Fraser, 2003).

6.1 Histo-blood group antigens

Histo-blood group antigens (HBGAs) are specific surface-associated structures that have been classified into more than 30 blood group systems by researchers investigating the cause of life-threatening reactions to blood transfusions and rejection of tissue or organ transplants (Daniels, 2013). The most well-known HBGAs are the ABO and Rh "blood type" antigens found on red blood cells (RBCs) and platelets, but HBGAs are found on the surface of most endothelial and epithelial cells, and in the body fluids and secretions of about 80% of those of European descent (Daniels, 2013; Lloyd, 1987; Ravn & Dabelsteen, 2000). Because blood group antigens are found throughout the body, they are considered histocompatibility antigens (Daniels, 2013). To reflect the fact that these antigens are not just found on RBCs, the term "histo-blood group antigens" is used to specifically indicate that antigens from these blood group systems are also found on histological tissues (Daniels, 2013; Ravn & Dabelsteen, 2000). Interested readers are referred to an extensive review of the associations between blood type biochemistry and human disease, which has been previously published (Ewald & Sumner, 2016).

To better understand the microbiome, we need to consider the blood type antigens we were born with and their influence on the microbiota. These surface-associated markers not only serve as antigens but also as a food source for intestinal bacteria (Stowell et al., 2010; Weng & Walker, 2013). In the intestine, the glycocalyx is constantly renewed, with an average turnover time of 6–12 hr, while the epithelial cells of the colon have a 24-hr turnover rate (Ouwerkerk et al., 2013). Terminally differentiated goblet cells, enteroendocrine cells, and enterocytes reach the tip of the villi within 3–5 days, undergo apoptosis, and are extruded into the intestinal lumen (Alonso et al., 2014; Goto & Kiyono, 2012). Mucosal bacteria degrade the glycoproteins found in mucus, including the glycans on antigens (Makivuokko et al., 2012). A small but intriguing study in Finland found a statistically significant correlation between the composition of the intestinal mucosal microbiota and the ABO blood type of the host (Makivuokko et al., 2012). The overall mucosal microbial profile as well as the relative proportion of major bacterial groups in the mucosal layer were significantly different in the presence of the B antigen (from B and AB blood types) (Makivuokko et al., 2012).

The HBGAs found on cell surfaces and in body fluids and secretions are not identical. There are two glycoside "precursor" chains or acceptor substrates with slightly different structures, known as Type 1 and Type 2 chains. Type 1 chains and their terminal antigens, shown in Figure 1, are detectable in sweat, breast milk, saliva, mucous, and other body fluids of individuals who are known as "secretors," but these antigens are missing in the body fluids of about 20% of people of European descent, who are known as "nonsecretors" (Ewald & Sumner, 2016; Watkins, 2001). Type 2 chains, depicted in Figure 2, are the basis for the addition of the ABO "blood type" antigens that serve as receptors on the surface of almost every cell and tissue in the body, including the endothelial cells of the GI tract (Ewald & Sumner, 2016; Watkins, 2001).
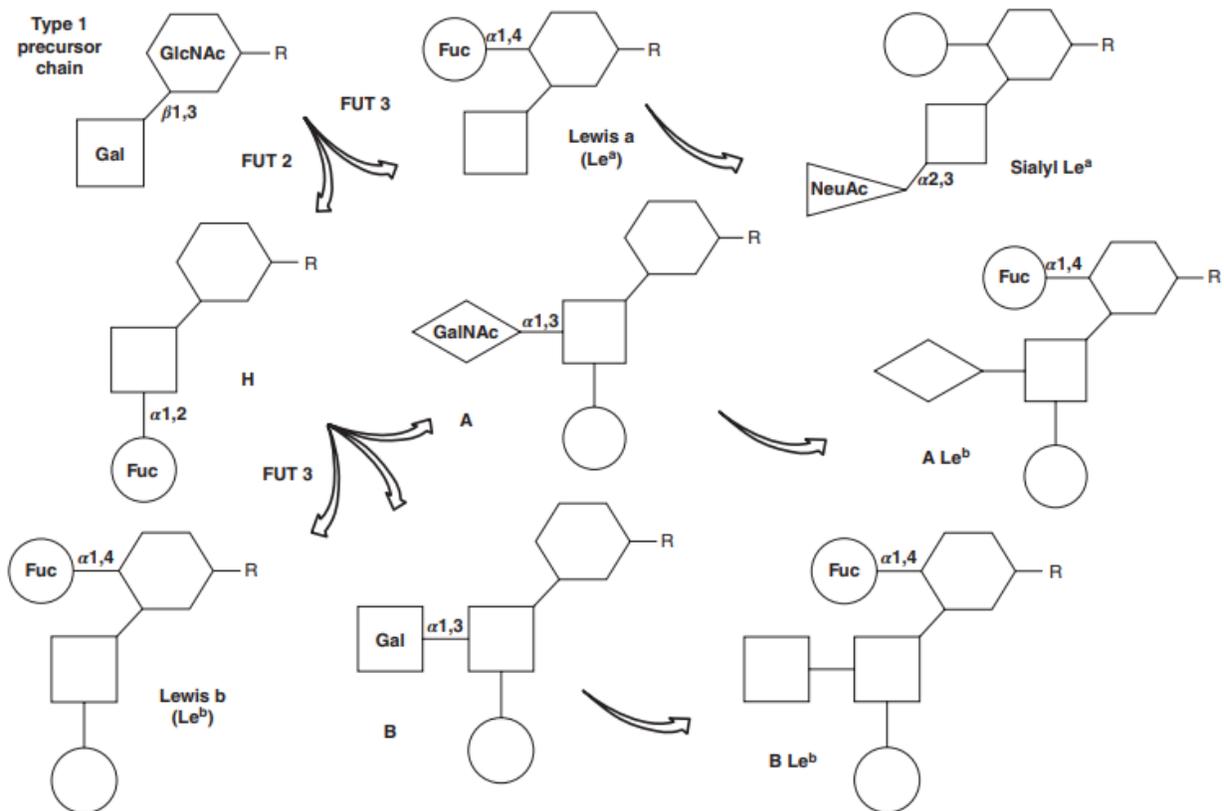

Figure 1. Type 1 precursor chain and associated antigen structures; the Lewis[b] antigen is found in the body fluids of about 80% of "secretors." Fuc = fucose; FUT2 = α1,2-fucosyltransferase enzymes expressed by *Se* genes; FUT3 = α1,3/4-fucosyltransferase enzymes expressed by *Le* genes; Gal = galactose; GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; NeuAc = *N*-acetylneuraminic (sialic) acid

Each person's "blood type" is determined by the combined expression of the HH gene and the ABO gene, which results in six possible allelic combinations and four phenotypes (Daniels, 2013). Those with the *H/H* or *H/h* alleles have the fucosyltransferase enzyme FUT1 required for the acceptor substrate, but if they lack the glycosyltransferase enzymes which are required to attach either of the final glycosides, these individuals have OO antigens and type O blood (Daniels, 2013; Ewald & Sumner, 2016; Watkins, 2001). People with Type A blood have either AO or AA antigens; those with Type B blood have either BO or BB antigens; whereas those with Type AB blood have alleles that express both enzymes and therefore have both the A

and B antigens (Ewald & Sumner, 2016; Franchini & Bonfanti, 2015; Watkins, 2001). Those with the so-called Bombay phenotype have the *h/h* allele, and because the FUT1 enzyme is nonfunctional, they do not carry the O, A, or B antigens (Watkins, 2001).
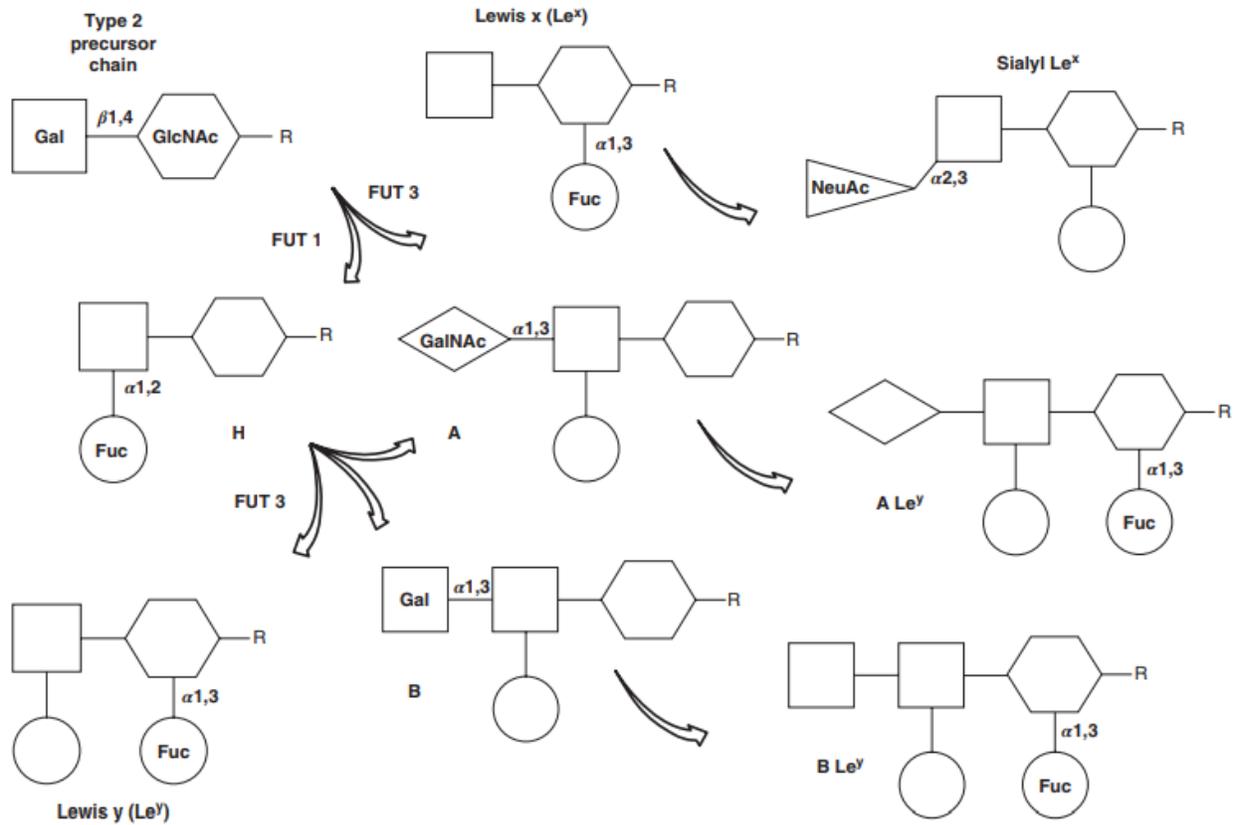

Figure 2. Type 2 precursor chain and associated antigen structures, found on endothelial, epithelial, and red blood cells throughout the body. The H antigen is the acceptor molecule needed for attachment of the A, B, or AB terminal glycosides. Fuc = fucose; FUT1 = α1,2-fucosyltransferase enzymes expressed by *HH* genes; FUT3 = α1,3/4-fucosyltransferase enzymes expressed by *Le* genes; Gal = galactose; GalNAc = *N*-acetylgalactosamine; GlcNAc = *N*-acetylglucosamine; NeuAc = *N*-acetylneuraminic (sialic) acid

The Lewis (*Le*) and Secretor (*Se*) genes function independently from the HH and ABO loci, but play a significant role in antigen expression; they both express fucosyltransferase enzymes that attach a terminal fucose in specific ways to Type 1 and Type 2 precursor chains and their derivative structures, as shown in Figures 1 and 2 (Ewald & Sumner, 2016). Sialylation or sulfuration occurs when *N*-acetylneuraminic (sialic) acid is attached in α2–3 linkage to a β-d - galactose residue of an existing chain (Daniels, 2013). Fucosyltransferase enzymes expressed by *Le* genes (FUT3) synthesize and release Lewis antigens from exocrine epithelial cells; those with *Le/Le* or *Le/le* alleles express Lewis antigens, while those with *le/le* do not (Ewald & Sumner, 2016; Henry, 1996; Henry, Oriol, & Samuelsson, 1995). These antigens can be glycolipids or glycoproteins, found as membrane-bound antigens on the surface of cells and tissues or as oligosaccharides that freely circulate in blood and body fluids, and can be taken up by RBCs, platelets, and lymphocytes (Ewald & Sumner, 2016; Henry, 1996; Henry et al., 1995).

The fucosyltransferase enzymes expressed by *Se* genes (FUT2) primarily interact with Type 1 precursor chains; about 80% of Caucasians are "secretors" with *Se/Se* or *Se/se* alleles, while those known as "nonsecretors" have two nonfunctional copies (*se/se*) and the ABO antigens are not found in their saliva or other body fluids (Ewald & Sumner, 2016; Henry, 1996; Henry et al., 1995). By adulthood, the distal colon and rectum only express Le^a and Le^x antigens, regardless of secretor status, but the cells and secretions of the esophagus, stomach, and small intestine continue to strongly express the same HBGAs found in the fetal GI tract, with levels of HBGAs 10 to 12 times higher in the GI tract of secretors than in nonsecretors (Lloyd, 1987).

HBGAs are found on tissues derived from ectodermal, mesodermal, and endodermal origins, as well as RBCs and platelets; although ABO(H) and Lewis antigens may be detected on lymphocytes, they are acquired or adsorbed from the plasma (Daniels, 2013). Regardless of secretor status, lymphocytes, granulocytes, and monocytes do not express ABO(H) and Lewis antigens that require FUT2 activity, however, they do have FUT3 activity and express Le^x and sialyl-Le^x antigens, which serve as ligands for selectins, a type of cell adhesion molecule found on endothelium, leucocytes, and platelets (Daniels, 2013).

Because HBGAs are "self" antigens, the adaptive immune system must be trained not to mount a response to these antigens. Central tolerance mechanisms include negative selection, in which MHC Class I and Class II molecules present endogenously expressed antigens from peripheral tissues in medullary thymic epithelial cells, whereupon most but not all self-reactive T-cells are eliminated (Lee et al., 2007). Peripheral tolerance involves the presentation of self-peptide–MHC complexes by CD8$^+$ dendritic cells to circulating T-cells in the lymph nodes, which subdues or eliminates the remaining self-reactive T-cells (Lee et al., 2007). Antigen proteins from parenchymal tissues can also be transported to draining lymph nodes, where MHC Class I and Class II molecules present them to circulating naïve T-cells (Lee et al., 2007).

However, APCs are not required for induction of tolerance to antigens from intestinal epithelial cells (Lee et al., 2007). Instead, CD8$^+$ dendritic cells present naïve T-cells with self-antigens from the intestinal mucosa (both epithelial cells and mucus) in the secondary GALT, and cortical stromal cells (which constitutively express many peripheral tissue antigens) present intestinal mucosa self-antigens in a similar manner in lymph nodes throughout the body (Lee et al., 2007). By activating and inducing deletional tolerance in T-cells, lymph node stromal cells function like medullary thymic epithelial cells to directly eliminate self-reactive T-cells (Lee et al., 2007).

In a similar manner, MHC Class II molecules present microbiota-derived antigens from intestinal commensal bacteria to CD4$^+$ T-cells in Group 3 innate lymphoid cells (ILC3), thus inducing negative selection and deletion of activated commensal bacteria-specific T-cells (Hepworth et al., 2015). This process is very similar to negative selection of self-reactive T-cells by the medullary thymic epithelial cells and stromal cells, and results in control of adaptive immune responses that maintain intestinal homeostasis (Hepworth et al., 2015). Dysregulation of this process may be a factor in development of IBD. In tissue biopsies from pediatric Crohn's disease patients, ILC3 MHC Class II levels were inversely correlated with colonic T_H17 cell frequencies and commensal-bacteria-specific serum IgG titers, indicating proinflammatory responses to commensal intestinal bacteria by the adaptive immune system (Hepworth et al., 2015).

Dysregulation of mucosal immune function is thought to occur as a result of inadequate or abnormal initial colonization of the GI tract (Weng & Walker, 2013). The small intestine of the fetus has a thin, immature epithelium, with slow mucosal cell turnover and a limited amount of GALT cells in the lamina propria, whereas in the infant, the mature epithelium is thicker, with fast mucosal cell turnover and robust, diverse, and abundant GALT (Weng & Walker, 2013). Although the fetal intestinal mucosa is permeable, which allows exchange between fetal serum and amniotic fluid, intestinal membrane closure occurs during the first week after birth; intestinal epithelial growth and maturation is stimulated by human milk hormones and growth factors (Anderson et al., 2012).

The intestinal mucosal barrier continues to grow and develop during the first year of life, undergoing significant tissue remodeling and profound modifications of absorptive and digestive functions (Anderson et al., 2012; Gritz & Bhandari, 2015). Immunological and morphological maturation of the mucosal barrier requires the presence of intestinal microbiota (Anderson et al., 2012; Gritz & Bhandari, 2015; Houghteling & Walker, 2015). For example, they induce expression of microbicidal proteins from Paneth cells as well as development of blood vessels in the villi (Anderson et al., 2012). Maturation of the mucosal barrier also depends on crosstalk between the colonizing bacteria and the immature mucosal barrier of the newborn, which produces the initial functional innate and adaptive immune phenotype and establishes homeostasis in the GI tract (Weng & Walker, 2013). The maturation of the mucosal barrier is further strengthened with the introduction of oral feedings and solid foods (Gritz & Bhandari, 2015; Weng & Walker, 2013).

The newborn is not entirely defenseless, but the function of all components of the innate immune system is weaker at birth than compared with later in life, and the adaptive immune system actively promotes self-tolerance (Simon, Hollander, & McMichael, 2015). Macrophages and monocytes are immature, natural killer cells are hyporesponsive, and serum concentrations of complement are lower than in adults, with less biological activity (Simon et al., 2015). In the fetal intestine, T-cells and macrophages appear as early as the 11th week of gestation, and by the 16th week, Peyer's patches form; the mucin gene is fully expressed between the 23rd and 27th weeks of gestation; dendritic cells are seen throughout the gut; by about the 19th week, CD4 T-cells begin to predominate in Peyer's patches, but CD8 T-cells do not appear until around the time of birth (Battersby & Gibbons, 2013).

During pregnancy, the maternal immune system is biased toward T_H2 responses, allowing tolerance of the fetus; it is thought that fetal T-cells are from a separate hematopoietic lineage that is predisposed to a tolerogenic response to antigens encountered before birth (Battersby & Gibbons, 2013). Intraepithelial lymphocytes are found which differ from naïve systemic lymphocytes and respond at a lower threshold (Battersby & Gibbons, 2013; Simon et al., 2015). The fetus receives passive immunity from transplacental maternal IgG, and at birth, as a result of sensitization to maternally derived antigens, the newborn displays immune competence with T-cells that are capable of antigen-specific responses; by the second week, the newborn produces cells containing IgA and IgM (Battersby & Gibbons, 2013). In breast-fed babies, mother's milk contains sIgA, which binds with microbial antigens and is thought to protect the baby's developing immune system from its own intestinal microbiota (Gritz & Bhandari, 2015).

The tolerogenic bias in the newborn's immune system allows for the colonization of the intestinal microbiome without an overwhelming inflammatory response (Battersby & Gibbons, 2013). In maturing epithelial cells of the intestine, surface TLR expression and downstream signaling is reduced and inhibitory factor kappa B is increased, which suppresses NF-κB activation (Battersby & Gibbons, 2013). In addition, commensal bacteria downregulate expression of IL-17 and upregulate expression of IL-25 and IL-10, which results in anti-inflammatory effects; they also promote sealing of tight junctions and apical tightening, which maintains barrier integrity (Battersby & Gibbons, 2013). This tolerogenic bias benefits the newborn in another way: although ABO(H) and Lewis antigens are expressed throughout the fetal GI tract (Daniels, 2013; Ravn & Dabelsteen, 2000), in the first months of life, babies do not produce ABO antibodies (Daniels, 2013). Fortunately, loss of self-reactive T-cells and adaptation to commensal bacteria and HBGAs occurs during the period before the baby's immune response loses the tolerogenic bias.

Because ABO(H) blood group antigens result in negative selection and deletion of self-reactive T-cells, the adaptive immune system does not produce antibodies to pathogenic bacteria that exhibit blood group antigens (Stowell et al., 2010). Fortunately, the innate immune system contains various glycan-binding proteins, such as C-type lectins and galectins, which recognize a variety of cell-surface carbohydrate moieties and participate in innate immune responses to pathogens bearing blood group antigens (Stowell et al., 2010). The galectins Gal-4 and Gal-8 are specifically reactive to blood group A and B antigens on pathogens, regardless of the individual's blood group antigen profile, but do not react to blood group A and B antigens if present on the individual's RBCs (Stowell et al., 2010).

Unlike other innate immune lectins, Gal-4 and Gal-8 do not require or activate complement, but directly kill bacteria by drastically disrupting membrane integrity and altering bacterial motility (Stowell et al., 2010). Galectins are highly expressed in the intestinal mucosa, where the adaptive immune system has been trained to tolerate "self" antigens, thus providing direct protection via the innate immune system against bacteria masquerading as "self" (Stowell et al., 2010). Galectins are able to bind with and kill bacteria expressing either α-Gal or β-Gal epitopes, indicating that their protection is not limited to bacteria expressing HBGAs; indeed, they function as pathogen recognition proteins for multiple populations of intestinal bacteria, and may play a previously unrecognized role in modulating the intestinal microbiome (Stowell et al., 2010).

Some pathogens mimic HBGAs as a way of evading detection by the immune system. For example, *E. coli* O86 mimics the blood group B antigen and thus does not generate an immune response in blood group B people, but blood group A or O people generate high titers of blood group B antibodies when exposed to the bacteria (Stowell et al., 2010). *H. pylori* expresses both Le[a] and Le[b] HBGAs on its cell surface (Kato & Ishiwa, 2015), and the Le[b] antigen mediates attachment of *H. pylori* to gastric epithelial cells (Toivanen et al., 2001). Secretor status influences susceptibility to pathogens such as *V. cholera* , *Campylobacter jejuni* , *H. pylori* , Norwalk, and certain respiratory viruses; nonsecretors are known to be more susceptible to Crohn's disease, primary sclerosing cholangitis, and other chronic inflammatory diseases (Kashyap et al., 2013). Although negatively charged surface-associated carbohydrate antigens, such as heparan sulfate and sialic acid, are recognized as receptors by most viruses, bacteria, and

protozoans, some pathogens recognize and bind to HBGAs, which do not carry a charge (Kato & Ishiwa, 2015).

6.2 Adhesins and receptors

Bacterial adhesion is a critical mechanism in the human gut, for without it, colonization and survival are unlikely, and bacterial virulence is impaired (Etzold et al., 2014; Servin, 2004). Exopolysaccharides serve a variety of functions for bacterial species, including slime and biofilm formation, establishment of colonies, as well as migration, adherence, and protection of individual microorganisms (Nwodo et al., 2012; Ruiz et al., 2014). Exopolysaccharides can be made of homogeneous repeating monomers or heterogeneous polysaccharides, which affect the chemical nature of the polymers and the type of linkage bonds that are formed (Nwodo et al., 2012). The monomeric units found in homopolysaccharides include fructans, polygalactans, and α- and β-d -glucans, while the heteropolysaccharide units include l -rhamnose, d -glucose, d - galactose, and sometimes glucuronic acid (GlcA), *N*-acetylglucosamine (GlcNAc), and *N*- acetylgalactosamine (GalNAc) (Nwodo et al., 2012).

Exopolysaccharides that remain attached to the surface of the bacteria are known as *capsules* ; these capsular polysaccharides are the antigenic basis for bacterial serotyping as well as antibody development leading to successful vaccines, and account for the diversity of diseases caused by bacteria of the same species (Nwodo et al., 2012). Exopolysaccharides can also be made in the external environment by bacterial enzymes attached to the external cell wall, or made within the bacteria and then secreted through the cell wall; some of these extracellular proteins are known as *adhesins* (González-Rodríguez et al., 2013; Nwodo et al., 2012; Sánchez et al., 2010).

In addition to polysaccharides, bacteria secrete extracellular proteins and small peptides that bind with receptors on host mucosal cells in the lumen, activate signaling pathways, and stimulate or suppress gene expression, with resulting physiological changes in the host cells (Gerlach & Hensel, 2007; Ruiz et al., 2014; Sánchez et al., 2010; Servin, 2004). As shown in Table 1, quite a few secretion systems have been identified, each with unique functional features and structural mechanisms. These secretion systems are used to transport extracellular proteins, peptides, toxins, and assembled adhesins across bacterial membranes, with different systems used by Gram-positive and Gram-negative bacteria; it is quite common for a bacterial species to use multiple systems (Abdallah et al., 2007; Amor et al., 2005; Campos, Cisneros, Nivaskumar, & Francetic, 2013; Chatzidaki-Livanis, Geva-Zatorsky, & Comstock, 2016; Christie, Atmakuri, Krishnamoorthy, Jakubowski, & Cascales, 2005; Costa et al., 2015; Coulthurst, 2013; Evans & Chapman, 2014; Freudl, 2013; Gerlach & Hensel, 2007; Kato & Ishiwa, 2015; Kazeeva & Shevelev, 2009; Kline et al., 2009; Korea, Ghigo, & Beloin, 2011; Natale, Bruser, & Driessen, 2008; Nivaskumar & Francetic, 2014; Nuccio & Bäumler, 2007; Pizarro-Cerdá & Cossart, 2006; Rennoll-Bankert et al., 2015; Russell, Peterson, & Mougous, 2014; Schneewind & Missiakas, 2014).

Table 1. Known secretion systems of Gram-negative and Gram-positive bacteria

Gram	Secretion system	Membrane(s) spanned	Starting molecule	Target environment	Some of the known bacteria using each system	Secreted molecule(s)	Examples	Ref
Neg, Pos	General secretion pathway	Bacterial inner	Preproteins containing signal peptide	Periplasm	Most	Unfolded proteins; lipoproteins	Sortase-assembled pili	(Abdallah et al., 2007; Freudl, 2013; Gerlach & Hensel, 2007; Kline et al., 2009; Natale et al., 2008; Schneewind & Missiakas, 2014)
Pos	Accessory secretion pathway	Bacterial inner	Large serine-rich glycoproteins	Periplasm	*Bacillus* spp.; *Streptococcus* spp; *Staphylococcus* spp.; *Listeria monocytogenes*; *Mycobacterium tuberculosis*; *Clostridium difficile*; *Corynebacterium glutamicum*	Virulence factors	FbpA; SodA; MnSOD; SRRPs; catalase-peroxidase; autolysin p60	(Freudl, 2013; Kline et al., 2009)
Neg, Pos	Twin arginine translocase	Bacterial inner	Folded proteins with signal peptide	Periplasm	*Escherichia coli*; *Bacillus subtilis*; *Streptomyces* spp.	Folded proteins	Peroxidase; phosphodiesterase	(Freudl, 2013; Natale et al., 2008)
Neg	Curli biogenesis system	Bacterial outer	Unfolded soluble peptide	Extracellular space	*Salmonella* spp.; enteric *E. coli*	Insoluble amyloid fiber	Curli	(Evans & Chapman, 2014; Kline et al., 2009)
Neg	Chaperone/usher	Bacterial outer	Unfolded proteins	Extracellular space	*E. coli* spp.; *Burkholderia* spp.; *Yersinia* spp.; *Salmonella enterica*; *Pseudomonas* spp.; *Acinetobacter* spp.; *Bordatella* spp.; *Haemophilus* spp.; *Klebsiella* spp.; *Proteus mirabilis*	B-, γ-, κ-, and π-Fimbrial adhesins; nonfimbrial surface structures; Dr[a]-blood group antigens	Type I pili; P pili; Afa/Dr	(Abdallah et al., 2007; Gerlach & Hensel, 2007; Kato & Ishiwa, 2015; Korea et al., 2011; Nuccio & Bäumler, 2007)
Neg	Alternate chaperone/usher	Bacterial outer	Unfolded proteins	Extracellular space	Human enterotoxigenic *E. coli*; *Yersinia pestis*; *Aeromonas hydrophila*; *Salmonella enterica* serovar Typhi; *Burkholderia* spp.	α-Fimbrial adhesins	Coli surface antigen 1	(Korea et al., 2011; Nuccio & Bäumler, 2007)
Neg	Type 2	Bacterial inner and outer	Unfolded or folded proteins	Extracellular space	*Klebsiella oxytoca*; *Klebsiella pneumoniae*; *Pseudomonas aeruginosa*; *Vibrio cholera*; *Neisseria gonorrhoeae*; *Neisseria meningitidis*; *E. coli* spp.; *Legionella pneumophila*; *Yersinia* spp.; *S. enterica* serovar Typhi; *Shewanella* spp.; *Chlamydia* spp.	Hydrolytic enzymes; slime proteins; toxins; adhesins; cytochromes	Type IV pili; Pullulanase; pseudolysin; cholera toxin	(Abdallah et al., 2007; Campos et al., 2013; Costa et al., 2015; Gerlach & Hensel, 2007; Kato & Ishiwa, 2015; Nivaskumar & Francetic, 2014;

Gram	Secretion system	Membrane(s) spanned	Starting molecule	Target environment	Some of the known bacteria using each system	Secreted molecule(s)	Examples	Ref
Neg	Type 5	Bacterial outer	Unfolded proteins	Extracellular space	*Neisseria gonorrhoeae; Neisseria meningitidis; Yersinia* spp.; *Bartonella henselae; S. enterica* serovar Typhimurium; *Helicobacter pylori; Bordatella pertussis*	Virulence factors; adhesins; proteases	OMPs; Intimin; ATs; TAAs; TPS/FHA; IgA-binding proteins	Pizarro-Cerdá & Cossart, 2006) (Abdallah et al., 2007; Gerlach & Hensel, 2007; Kazeeva & Shevelev, 2009; Pizarro-Cerdá & Cossart, 2006)
Neg	Type 1	Bacterial inner and outer	Preproteins containing signal peptide	Extracellular space	Uropathogenic *E. coli; S. enterica*	Large partially unfolded proteins	α-Hemolysin; BAP family; SiiE	(Abdallah et al., 2007; Gerlach & Hensel, 2007)
Neg	Type 3	Bacterial inner and outer, and target cell wall	Preproteins containing signal peptide	Target cell cytoplasm	*S. enterica; Yersinia* spp; *Pseudomonas* spp.; *Shigella* spp.; enteropathogenic *Escherichia* spp.	Partially unfolded proteins; virulence factors; effector molecules	Tir	(Abdallah et al., 2007; Costa et al., 2015; Gerlach & Hensel, 2007)
Neg, Pos	Type 4	Bacterial inner and outer, and target cell wall	Proteins containing signal peptide	Extracellular space; target cell cytoplasm	*B. pertussis; Neisseria gonorrhea; Helicobacter pylori; Brucella* spp.; *B. henselae; Campylobacter jejuni; Ricksettia typhi; Ricksettia felis; Ricksettia bellii; Legionella pneumophila; Enterococcus faecalis*	Proteins; virulence factors; or complexes of single-stranded DNA and proteins	Pertussis toxin; CagA; RalF	(Abdallah et al., 2007; Amor et al., 2005; Christie et al., 2005; Costa et al., 2015; Gerlach & Hensel, 2007; Rennoll-Bankert et al., 2015)
Neg	Type 6	Bacterial inner and outer, and target cell wall	Proteins without signal peptides	Target cell cytoplasm or periplasm	*Vibrio cholera; Aeromonas hydrophila; Pseudomonas aeruginosa; Burkholderia* spp.	Effector proteins; antibacterial toxins	VrgG-1; Hcp; Tse1–3; Tle1–5	(Chatzidaki-Livanis et al., 2016; Coulthurst, 2013; Russell et al., 2014)
Pos	Type 7	Bacterial inner, peptidoglycan layer (and mycomembrane), cell capsule	Protein complexes containing C-terminal signal sequences	Extracellular space	*M. tuberculosis; Mycobacterium leprae; L. monocytogenes; Corynebacterium diphtheriae; Norcardia* spp.; *Streptomyces* spp.; *Clostridium* spp.; *Staphylococcus aureus; Streptococcus agalactiae; B. subtilis; Bacillus anthracis*	Protein complexes; virulence factors	ESAT-6; (EsxA) CFP-10 (EsxB); WXG100; PE/PPE proteins; Rv3881c (MTB48)	(Abdallah et al., 2007; Freudl, 2013)

Note. Each secretion system transports different types of molecules across the bacterial membrane(s), and some also enable translocation of effector proteins, single-stranded DNA, or toxins through the membrane of target cells.

7 SECRETION SYSTEMS

The general secretion pathway (Sec) is used by both Gram-negative and Gram-positive bacteria; in Gram-negative bacteria, the Sec pathway is a mechanism composed of multisubunit complexes, including an ATPase subunit, located in the bacterial inner membrane to transport unfolded proteins and lipoproteins across the inner membrane into the periplasm (Freudl, 2013; Schneewind & Missiakas, 2014). There are two separate processes, known as co-translational and posttranslational, which result in proteins being translocated by this mechanism (Gerlach & Hensel, 2007). In the co-translational process, a cytoplasmic signal recognition particle (SRP) binds to the signal peptide as it emerges from the ribosome, which temporarily suspends translation; the SRP guides the ribosome to the Sec mechanism and docks with it, which causes translation to resume and the protein emerges directly into the channel of the mechanism (Gerlach & Hensel, 2007; Natale et al., 2008; Schneewind & Missiakas, 2014).

In the posttranslational pathway, cytoplasmic chaperone proteins prevent premature folding as they guide unfolded preproteins to the protein-conducting channel of the mechanism for translocation, whereupon periplasmic signal peptidases cleave the N-terminal signal peptide from them to produce mature proteins (Gerlach & Hensel, 2007; Natale et al., 2008; Schneewind & Missiakas, 2014). Lipoprotein precursor proteins are modified by the addition of diacylglycerol prior to translocation, which enables recognition and cleavage of their N-terminal signal peptide by a second type of periplasmic signal peptidases (Freudl, 2013).

In Gram-positive bacteria, the Sec pathway also transports preproteins across the cell membrane, where the N-terminal signal peptide is cleaved and the mature protein is released, unless the presence of a C-terminal sorting signal causes it to be retained (Kline et al., 2009; Schneewind & Missiakas, 2014). Different sorting signals result in the retention of surface proteins that can either impede detection by the immune system, enable tissue adhesion, promote heme-iron uptake, or polymerize into pili (Kline et al., 2009; Schneewind & Missiakas, 2014). The sorting signals are cleaved by membrane-anchored transpeptidases known as sortase enzymes; the *housekeeping sortase* of individual surface proteins catalyzes the transfer of the protein to pentaglycine cross-bridges that connect peptidoglycan strands in the cell wall, and in the case of pilus assembly, sequential transpeptidation reactions by *pilin-specific sortases* result in polymerization of pilin subunits until the completed pilus is transferred to the cell wall and anchored there by the housekeeping sortase (Schneewind & Missiakas, 2014).

The accessory secretion pathway (Sec2) is a second independent Sec pathway found in primarily pathogenic Gram-positive bacteria; Sec2 exports virulence factors that are not translocated by Sec because they are highly glycosylated (Freudl, 2013; Schneewind & Missiakas, 2014). These large proteins are also anchored in the bacterial cell wall and facilitate pathogenic activities as diverse as platelet aggregation, endocarditis, and biofilm formation (Kline et al., 2009; Schneewind & Missiakas, 2014). The twin arginine translocase (Tat) is a multisubunit complex in the inner or cell membrane of Gram-negative and Gram-positive bacteria, respectively, that uses proton motive force to translocate certain precursor proteins when it recognizes a highly conserved twin arginine consensus motif in signal peptides attached to them (Freudl, 2013; Natale et al., 2008). These are usually proteins that contain a cofactor and therefore must fold

prior to translocation, but some rapidly folding proteins without cofactors are also translocated through this pathway (Freudl, 2013; Natale et al., 2008).

The Curli biogenesis system is a multisubunit, outer membrane Sec-dependent mechanism that generates thin fimbriae that are unstructured soluble peptides until they get to the outer surface, where interaction with a nucleator subunit in the mechanism results in a conformational change that produces insoluble amyloid fibers (Evans & Chapman, 2014; Kline et al., 2009). Two unique features of this system are that it uses an entropy gradient that is diffusion-based and therefore does not require ATP for translocation of the subunit proteins (Costa et al., 2015), and that the amyloids it secretes self-polymerize when they contact the nucleator subunit (Evans & Chapman, 2014). Similar to other systems, it uses periplasmic chaperones to prevent premature amyloid formation and to direct the peptide subunit to the secretion mechanism (Costa et al., 2015; Evans & Chapman, 2014). Of interest, closely related bacteria (e.g., *E. coli* and *S. enterica*) can "cross-seed" with their own unstructured soluble peptides to accelerate the other's amyloid polymerization (Evans & Chapman, 2014).

Gram-positive bacteria use the Type 4 secretion system (T4SS) or the Type 7 secretion system (T7SS) to export mature proteins (Christie et al., 2005; Freudl, 2013). In Gram-negative bacteria, mature proteins are secreted through the outer membrane via one of three additional systems: the chaperone/usher secretion systems, which transport fimbrial adhesins and pili; the Type 2 secretion system (T2SS), which secretes most bacterial enzymes and toxins; and the Type 5 secretion system (T5SS), which is the mechanism used by bacterial autotransporter proteins (Abdallah et al., 2007; Gerlach & Hensel, 2007). The Sec and T2SS pathways are ATP-dependent, but the T5SS and chaperone/usher mechanisms are not (Abdallah et al., 2007; Gerlach & Hensel, 2007). Regardless of the secretion system used, translocated proteins must fold properly and quickly into their native, active conformation to avoid degradation by proteases in the periplasmic or extracellular environment; the close proximity of metal ions such as calcium was shown to increase the speed with which protein folding occurred, and the release of folded proteins was significantly decreased when calcium was removed (van Wely, Swaving, Freudl, & Driessen, 2001).

The classical and alternate chaperone/usher secretion systems are located in the outer membrane and are mechanisms by which discrete fimbrial adhesin proteins are secreted (Gerlach & Hensel, 2007;Korea et al., 2011 ; Nuccio & Bäumler, 2007). These fimbriae enable biofilm formation, host cell recognition, and cell-to-cell attachment, and contribute to pathogenicity (Costa et al., 2015). In both mechanisms, periplasmic proteins (the "chaperones") bind with pilins to ensure proper folding, prevent premature assembly of the pilus in the periplasm, and guide the pilins to an integral protein (the "usher") in the outer membrane, which translocates folded proteins and serves as a platform for pilus assembly (Gerlach & Hensel, 2007; Korea et al., 2011), via noncovalent polymerization of pilin subunits (Korea et al., 2011).

However, the chaperone and usher proteins involved in the classical mechanism as well as the pilins that are assembled in the pilus itself are genetically distinct from those in the alternate mechanism (Korea et al., 2011; Nuccio & Bäumler, 2007). A classification system (Nuccio & Bäumler, 2007) has recently been proposed based on the genetic distinctions of the usher proteins and their associated chaperones and substrates (see Nuccio & Bäumler, 2007 for a

review of this system). In both secretion systems, the pilus has a rigid rod-like component anchored to the usher platform and a flexible structure at the tip; both components contribute to binding specificity (Costa et al., 2015; Gerlach & Hensel, 2007).

The T2SS consists of a large multisubunit ring-shaped complex in the outer bacterial membrane (the "secretin pore") that is attached to an inner membrane secreton also consisting of multiple subunits, some of which extend into the cytoplasm of the bacteria (Campos et al., 2013; Gerlach & Hensel, 2007). A periplasmic pseudopilus participates in loading periplasmic proteins into the mechanism, and then pushes the proteins through the secretin pore channel and across the outer membrane; it is not clear whether the pseudopilus extends in a piston-like manner or turns in a manner reminiscent of Archimedes' screw (Campos et al., 2013; Costa et al., 2015).

Many components that are similar to those of the T2SS are also found in the Type IV pilus assembly mechanism, in bacterial competence systems, and in the systems that construct archaeal pili and flagella (Campos et al., 2013; Nivaskumar & Francetic, 2014). Important distinctions are that the pili generated by Type IV assembly mechanisms are long and stabilized by covalent disulfide bonds which require energy from a retraction ATPase for their disassembly and retraction, whereas the pseudopilus of the T2SS is short and unstable, relying on electrostatic and hydrogen bonds, and does not retract (Campos et al., 2013).

The T5SS is only found in the outer membrane and consists of a variety of autotransporter proteins, such as surface-attached coiled-coil oligomeric autotransporters, classical single-polypeptide autotransporters, and the two-partner system (TPS) of autotransporters, consisting of two proteins that are translated separately with each containing one of the components needed for protein secretion (Abdallah et al., 2007; Gerlach & Hensel, 2007). *Autotransporters* have a translocation domain or unit and a passenger domain or unit (Costa et al., 2015; Gerlach & Hensel, 2007). The translocated proteins are primarily virulence factors, but adhesins are also involved in biofilm formation and cell-to-cell adhesion (Costa et al., 2015).

With TPS and classical autotransporters, the translocation component is a single protein that forms a β-barrel in the outer membrane and the passenger component is secreted through this pore; once in the extracellular space, it may remain attached or it may be cleaved and released (Abdallah et al., 2007; Costa et al., 2015; Gerlach & Hensel, 2007). With oligomeric autotransporters, the β-barrel is formed by the coiled coiling of the C-terminal translocation units of a number of proteins which form an oligomeric complex in the outer membrane; this complex anchors the nonfimbrial adhesin proteins that are secreted through this pore (Gerlach & Hensel, 2007). TPSs have been shown to bind to flagella on some enterohemorrhagic and enterotoxigenic *E. coli* ; however, the exact manner is unknown (Kline et al., 2009).

The Type 1 secretion system (T1SS), Type 3 secretion system (T3SS), and T4SS are complexes that act as protein-translocation channels but, unlike the Sec-dependent pathways, they do not secrete periplasmic proteins (Abdallah et al., 2007; Gerlach & Hensel, 2007), with the exception of *B. pertussis* , which uses a T4SS to translocate the pertussis toxin from the periplasm (Christie et al., 2005). The T1SS is a heterotrimeric complex anchored in the inner membrane that forms a pore in the outer membrane, allowing secretion directly from the bacterial cytoplasm to the extracellular space, whereas the T3SS and T4SS are multisubunit complexes that actually create

a mechanism that spans both the bacterial membranes as well as the target cell membrane, so that secretion is directly into the cytoplasm of the target cell (Abdallah et al., 2007; Gerlach & Hensel, 2007). All three use ATP to provide the energy for translocation, but the T3SS transports proteins with N-terminal signal peptides (Abdallah et al., 2007; Gerlach & Hensel, 2007), whereas the T1SS and T4SS recognize C-terminal signal peptides attached to a variety of nonproteinaceous substrates, proteins, or protein–DNA complexes (Abdallah et al., 2007; Christie et al., 2005). In the T1SS, this signal peptide is recognized by the ATP-binding cassette subunit anchored in its inner membrane (Christie et al., 2005; Gerlach & Hensel, 2007).

The T3SS has evolved from and is similar in structure to flagellar systems, yet has the ability to deliver its translocator and effector proteins directly into the target cell; this capacity and the shape of the multisubunit complex, which has a long cylinder with a needle-like extension at the tip, has resulted in the T3SS being called an "injectosome" or a "molecular needle" (Abdallah et al., 2007; Gerlach & Hensel, 2007). The inner membrane, multiunit ring complex found at the base makes use of cognate chaperones to prevent proteins from folding before they are loaded into the cylinder (Costa et al., 2015; Gerlach & Hensel, 2007). It is thought that while the cylinder is being constructed, the loaded proteins are subunits and needle components needed to complete its length, and when its polymerization is complete, the same chaperones begin to load effector proteins for export (Costa et al., 2015; Gerlach & Hensel, 2007).

The T4SS has similar DNA sequences that appear to have evolved from bacterial conjugation systems, and not surprisingly, has the ability to translocate single-stranded DNA–protein complexes as well as single proteins (Abdallah et al., 2007; Christie et al., 2005; Costa et al., 2015; Gerlach & Hensel, 2007). This process appears to involve the use of a surface protein structure, such as a filament, pilus, adhesin, or fibrous mesh (Christie et al., 2005; Costa et al., 2015), and is used by both Gram-positive and Gram-negative bacteria (Costa et al., 2015). This secretion system allows bacteria to perform three very different functions: cell-to-cell DNA conjugation; export of DNA to the extracellular environment; and translocation of proteins from cell to cell (Gerlach & Hensel, 2007; Christie et al., 2005). The translocation process has been further subdivided into Type 4A and Type 4B: Type 4A has been shown to secrete effector proteins that induce inflammation and alter the target cell's cytoskeleton; Type 4B (more recently discovered) has been associated with secretion of the protein RalF, a guanosine nucleotide exchange factor protein that upregulates the target cell's ADP-ribosylation factor (Arf) (Gerlach & Hensel, 2007). To date, translocation of RalF has only been found in *Legionella pneumophila* and some *Rickettsia* infections, but in both cases, it alters normal cellular function (Amor et al., 2005; Rennoll-Bankert et al., 2015).

After phagocytosis of *L. pneumophila* , membrane fusion between the phagosome and the lysosome is prevented, and the phagosome is positioned at the endoplasmic reticulum; the translocation of RalF recruits Arf1 to the surface of the phagosome and activates it, whereupon it assists in the formation of an intracellular vacuole that the bacteria use for replication and survival (Amor et al., 2005; Rennoll-Bankert et al., 2015). RalF is also translocated by *Ricksettia typhi* , *R. felis* , and *R. bellii* , but with slight differences: *Rickettsia* RalF recruits Arf6 to the plasma membrane and increases the concentration of phosphatidylinositol bisphosphate, causing actin remodeling that induces phagocytosis and facilitates cellular invasion, following which Rickettsia species lyse the phagosome and replicate in the cytoplasm (Rennoll-Bankert et

al., 2015). *R. typhi* and *R. felis* remain co-located with the plasma membrane, but *R. bellii* co-locates with the perinuclear membrane (Rennoll-Bankert et al., 2015).

The Type 6 secretion system (T6SS) has a complex multisubunit two-part mechanism consisting of a membrane-spanning component, with proteins that are similar to the Type 4B secretion system, and another cytoplasmic component that is perpendicular to the inner cell membrane (Costa et al., 2015; Coulthurst, 2013; Russell et al., 2014). Reminiscent of the bacteriophage sheath, tube, and tail-spike assembly, the T6SS forms a needle-like cell-puncturing device and the extended tube of this sheath contracts as if it were spring-loaded to penetrate and eject its toxic contents through the outer membrane or cell wall of the target cell (Costa et al., 2015; Coulthurst, 2013; Russell et al., 2014). In contrast to some of the other secretion systems, direct cell-to-cell contact is the trigger for the T6SS (Chatzidaki-Livanis et al., 2016; Russell et al., 2014), which is used by some of the most pathogenic bacteria.

Antibacterial effectors translocated through this system include phospholipases that destroy cell membranes, glycohydrolase toxin that degrades $NAD(P)^+$, and toxins that form pores in the target cell membrane (Chatzidaki-Livanis et al., 2016), as well as nucleases that degrade plasmid and chromosomal DNA, and amidases that degrade the peptidoglycan cell wall (Chatzidaki-Livanis et al., 2016; Coulthurst, 2013; Russell et al., 2014). Following phagocytosis or endocytosis, pathogenic bacteria translocate an effector protein that results in actin cross-linking, which protects the remaining pathogenic bacteria from being engulfed (Coulthurst, 2013). These effectors and toxins do not appear to have an amino-terminal signal sequence, and it is not clear how they are directed into the secretion mechanism (Abdallah et al., 2007; Costa et al., 2015).

Among Gram-negative bacteria, this system appears to function as an interbacterial defense system or to confer competitive advantage during bacterial invasion (Chatzidaki-Livanis et al., 2016; Coulthurst, 2013; Russell et al., 2014). Without exception, the genes for every cytotoxic effector protein are found adjacent to genes for an immunity protein that binds and inactivates the effector, thus preventing self-intoxication (Russell et al., 2014). These immunity proteins are also found in bacteria from other species that do not produce the corresponding effector proteins, indicating that they are needed to defend against attack (Chatzidaki-Livanis et al., 2016; Coulthurst, 2013; Russell et al., 2014). It is possible that the T6SS is used to distinguish between friend and foe in areas with high bacterial density, or to remove defective or infected bacteria to protect the colony (Chatzidaki-Livanis et al., 2016; Russell et al., 2014). Recent research has revealed that some probiotic bacteria also use the T6SS to create or protect their environmental niche; in these bacteria, the Type 6 loci are highly conserved with three distinct genetic signatures; two are found in most Bacteroidales species, but a third structure is only found in *B. fragilis* (Chatzidaki-Livanis et al., 2016).

The T7SS found in Gram-positive bacteria was first identified in *Mycobacterium tuberculosis* ; mycobacteria have a thick hydrophobic layer of mycolic acids, called a *mycomembrane* , made of hydroxylated branched-chain fatty acids covalently bound to the peptidoglycan layer (Abdallah et al., 2007). The T7SS translocates proteins with a C-terminal signal sequence, and appears to be similar in structure to the Type 1 through Type 4 secretion systems, with a multisubunit mechanism that is thought to span the inner cell membrane, the peptidoglycan layer (and in mycobacteria, the mycomembrane), as well as the capsule or outer layer of

polysaccharides, although a two-step mechanism has also been proposed (Abdallah et al., 2007; Freudl, 2013).

Mycobacteria use five homologous so-called ESX secretion systems that appear to function independently of each other; in addition to mycobacteria, which belong to the Actinobacteria phylum, the T7SS has been identified in other high G+C Gram-positive species that do not have a mycomembrane, such as Streptomyces, as well as in members of the Firmicutes phylum, including *Listeria monocytogenes* , *Streptococcus agalactiae* , *Staphylococcus aureus* , and other Bacillus and Clostridium species (Abdallah et al., 2007; Freudl, 2013). Based on genomic analysis, some researchers have proposed that bacteria using the T7SS should be segregated into subtypes, with those in the Actinobacteria phylum designated as Type 7A and those in Firmicutes designated as Type 7B (Abdallah et al., 2007), whereas other researchers think that the T7SS is unique to the high G+C species with a mycomembrane and that the similar secretion system used by those without a mycomembrane should be classified as a separate WXG100 system (Freudl, 2013).

8 ADHESINS AND RECEPTORS

Adhesins and receptors are the mechanisms by which bacteria and their hosts interact; as with immunoglobins and enzymes, adhesins bind with their specific "substrate," which is their receptor molecule (Klemm & Schembri, 2000; Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006). Fimbrial adhesins that bind with carbohydrate receptor molecules are referred to as *lectins* (Klemm & Schembri, 2000). Soluble molecules in serum, interacting with proteins on the surface of bacteria, and bacterial adhesins interacting with cell surface molecules can initiate signaling events that result in activation of the innate immune system, generating either proinflammatory or anti-inflammatory responses (Kline et al., 2009). They can also mediate invasion of or uptake by the host cell, production of antibacterial peptides, and coagulation events (Kline et al., 2009). Cell surface structures on the intestinal mucosal layer, including mucins and glycans, act as receptors for bacterial ligands and provide adhesin binding sites (Etzold et al., 2014). C-type lectin receptors, found on dendritic cells and macrophages, and TLRs are among the known intestinal receptors that recognize glycolipids, lipoproteins, glycoproteins, peptidoglycans, and other extracellular structures that may be present, such as flagella, pili, and fimbria (Didierlaurent et al., 2002; Etzold et al., 2014; Sánchez et al., 2010).

The bacterial surface structures produced by many of these secretion systems are organelles that have traditionally been known as pili; while the terms pili and fimbria are often used interchangeably, they actually designate functions (Pizarro-Cerdá & Cossart, 2006). To distinguish those that transfer bacterial DNA during conjugation, still known as *pili* , those used for adhesion to surfaces for any other purpose are now more commonly called *fimbria* (Pizarro-Cerdá & Cossart, 2006); despite this, the traditional terms *Type IV pili* , *Type I pili* , and *P pili* have been retained by researchers.

While adhesins can be categorized by the secretion system that transports them across the inner and outer bacterial cell membranes, they are perhaps better classified into three broad categories based on their structure and adhesion system. Polymeric fimbria have different subunits in the shaft and the adhesion protein at its tip, and are generally assembled through the chaperone/usher

system; in contrast, monomeric/oligomeric filaments, and secreted soluble molecules that enable adhesion are primarily generated through T1SS and T5SS (Gerlach & Hensel, 2007; Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006). Fimbria can be further classified by their structural subunits, which distinguish their method of attachment to the mucosal or epithelial cell surface and their binding domains (Gerlach & Hensel, 2007; González-Rodríguez et al., 2013; Sánchez et al., 2010). While it is impossible to include every type of molecule secreted by bacteria, examples of some adhesins and their receptors are shown in Table 2 (Gerlach & Hensel, 2007; Kato & Ishiwa, 2015; Klemm & Schembri, 2000; Kline et al., 2009; Korea et al., 2011; Pizarro-Cerdá & Cossart, 2006; Rendón et al., 2007; Thomas, Holland, & Schmitt, 2014; van Wely et al., 2001).

Table 2. Examples of known classes of adhesion molecules of Gram-negative and Gram-positive bacteria

Adhesion system	Adhesin produced	Secretion system	Host tissue	Receptor	Type of receptor	Ref
Fimbrial	Type P pili	Chaperone/usher	Upper urinary tract	α-d -Galactopyranosyl-(1-4)-β-d -galactopyranoside; Toll-like receptor 4	Glycolipids; proteins	(Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006)
	Type I pili	Chaperone/usher	Urinary tract, intestinal tract	Monomannose; trimannose; fibronectin; plasminogen; laminin	Glycoproteins; ECM proteins	(Kato & Ishiwa, 2015; Klemm & Schembri, 2000; Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006)
	Coli surface antigen I	Alternate chaperone/usher	Epithelial cells	Unknown	Unknown	(Gerlach & Hensel, 2007)
	Afa/Dr adhesins	Chaperone/usher	Various	Drª-blood group antigen of CD55 (DAF); CEACAMs; Type IV collagen; α5β1 integrins	Proteins	(Gerlach & Hensel, 2007; Pizarro-Cerdá & Cossart, 2006)
	Type IV pili	T2SS	Mucosal epithelial cells	Possibly integrins; CD46	Glycolipids	(Gerlach & Hensel, 2007; Kato & Ishiwa, 2015; Kline et al., 2009)
	Flagella	T3SS	Intestinal tract	Toll-like receptor 5	Proteins	(Kline et al., 2009)
	Curli	Curli biogenesis system	Intestinal tract	Human contact phase proteins; fibronectin; plasminogen	ECM proteins	(Evans & Chapman, 2014; Klemm & Schembri, 2000; Kline et al., 2009)
	SRRPs	Sec2	Epithelial cells	Sialylated glycoconjugates	Glycoconjugates	(Kline et al., 2009)
	Sortase-assembled pili	Sec	Epithelial cells; brain endothelial cells	Proline-rich proteins; Gal-β(1-3)-GalNAc; Gal-β(1-3)-Gal; fibronectin; collagen	Proteins; saccharides; ECM proteins	(Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006)
Filamentous OMPs						

Adhesion system	Adhesin produced	Secretion system	Host tissue	Receptor	Type of receptor	Ref
	OmpA	T5SS	Brain endothelial cells	Ecgp	Glycoprotein	(Gerlach & Hensel, 2007)
	OpcA	T5SS	Endothelial and epithelial cells	Heparan sulfates; pyranose saccharides; sialic acids	Glycoproteins; proteoglycans; saccharides	(Gerlach & Hensel, 2007)
	Invasin	T5SS	M-cells	β1 Integrins	Integrins	(Gerlach & Hensel, 2007)
	Intimin/Tir	T5SS/T3SS	Intestinal tract	Tir; integrin; nucleolin	Proteins	(Gerlach & Hensel, 2007; Rendón et al., 2007)
	TAA	T5SS	Epithelial cells	Collagen; laminin; fibronectin	ECM proteins	(Gerlach & Hensel, 2007; Kline et al., 2009)
	Classical AT	T5SS	Epithelial cells	Fibronectin; Lewis blood group antigens	ECM protein; saccharides	(Gerlach & Hensel, 2007; Pizarro-Cerdá & Cossart, 2006)
	TPS	T5SS	Mucosal epithelial cells	Various	Various	(Gerlach & Hensel, 2007)
Soluble	TPS	T5SS	Epithelial cells; dendritic cells	Various	Various	(Gerlach & Hensel, 2007)
	BAP family	T1SS	Epithelial cells	Calcium ions	Calcium ions	(Gerlach & Hensel, 2007)
	α-hemolysin	T1SS	RBCs; WBCs; fibroblasts; endothelial and epithelial cells	Glycophorin; β-2 integrins	Glycoprotein; integrins	(Gerlach & Hensel, 2007; Thomas et al., 2014)
	SiiE	T1SS	Epithelial cells	Various	Various	(Gerlach & Hensel, 2007)
	GbpA	T2SS	Epithelial cells	*N*-Acetylglucosamine residues	Saccharides	(Gerlach & Hensel, 2007)

Note . Adhesion molecules are secreted by both pathogenic and commensal bacteria. Their polymeric fimbrial structures are primarily assembled via the chaperone/usher secretion system, while monomeric or oligomeric filamentous structures are associated with the Type 5 secretion system and many soluble adhesion molecules are secreted via the Type 1 secretion system. Most receptors, whether found on host tissues or other bacterial species, are surface-bound molecules that have a conformation which is compatible with a given adhesion molecule. AT = autotransporter; BAP = biofilm-associated protein; CD55 (DAF) = complement delay-accelerating factor; CEACAMS = carcinoembryonic antigen-related cell adhesion molecule; ECM = extracellular matrix; OMPs = outer membrane proteins; RBCs = red blood cells; SRRPs = serine-rich repeat proteins; TAA = trimeric autotransporter adhesins; TPS = two-partner-secreted filamentous adhesins; WBCs = white blood cells.

Adhesins determine survival as well as virulence, and many bacteria express more than one type of adhesin in response to the tissue or environment in which they find themselves; however, each adhesin is specific to its ligand on a given receptor, known as *tissue tropism* , and bacteria selectively and sequentially express a series of adhesion molecules during infection (Gerlach & Hensel, 2007; Klemm & Schembri, 2000; Korea et al., 2011). Microbial adhesion mechanisms

include nonspecific factors such as hydrophobic and/or electrostatic forces, steric hindrance, and secretion of lipoteichoic acids, as well as specific structures found in cell wall properties, and secretion of adhesins or lectin-like proteins (González-Rodríguez et al., 2013; Servin, 2004); flagellar-mediated adhesion has also recently been recognized (Kline et al., 2009). The most-studied adhesins are proteins found at the distal end of polymeric fimbria anchored in the outer membrane of Gram-negative bacteria; polymeric fimbria are now known to also exist in the cell wall of some Gram-positive bacteria (Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006). These hair-like extensions are composed of hundreds or even thousands of secreted subunits that are assembled into polymeric structures as they are translocated into the extracellular environment (Gerlach & Hensel, 2007; Kline et al., 2009).

The β, γ, κ, and π fimbriae that are classified as Type I and P pili are generated through the chaperon/usher system, and are anchored in the outer membrane of the bacteria; these fimbriae are particularly abundant in *E. coli* , but although they have different adhesin proteins and bind to different receptors, they result in agglutination of human RBCs (Gerlach & Hensel, 2007; Kline et al., 2009). Class III adhesins of P pili bind specifically to the terminal gal-α(1–4)-βgal unit, found on the globo-A antigen of the P-blood group or the globopentosylceramide antigen of the Forssman blood group (Gerlach & Hensel, 2007; Klemm & Schembri, 2000; Kline et al., 2009), whereas Class I and Class II adhesins of P pili bind to globotriaosylceramides or globosides, respectively (Klemm & Schembri, 2000).

Adhesins on Type I pili of pathogenic *E. coli* strains recognize mannose monomers, which are abundant glycoproteins found on the cells of the urinary tract; however, those on nonpathogenic strains bind with receptors containing trimannose structures (Gerlach & Hensel, 2007). In addition, adaptations have occurred among bacterial species; while Type I pili are found in *Salmonella enterica* , genome sequencing of just serovar Typhimurium alone revealed 13 homologous fimbrial gene sequences and at least 11 serovar-specific fimbria with phase variations of some operons (Gerlach & Hensel, 2007).

The α-fimbriae classified as Coli surface antigen pili are generated through the alternate chaperon/usher system, and like those of the Type I and P pili, are anchored in the outer membrane of the bacteria; however, although these pili have very similar functions, the Coli surface antigen pili are genetically discrete (Gerlach & Hensel, 2007). Further, these α-fimbriae are only found in enterotoxigenic *E. coli* and other bacteria which bind to intestinal epithelial cells, but to date the host-cell receptor molecule has not been determined (Gerlach & Hensel, 2007).

The family of diffuse adherence fibrillar adhesin/Dr blood group antigen adhesins (Afa/Dr adhesins) are generated through the chaperon/usher system and form a variety of outer-membrane structures, which differ depending on the genetic sequence of the adhesin itself, resulting in nonfimbrial, afimbrial, as well as fimbrial structures (Gerlach & Hensel, 2007; Pizarro-Cerdá & Cossart, 2006). Afa/Dr adhesins are thought to interact with α5β1 integrins and with various carcinoembryonic antigen-related cell adhesion molecule (CEACAM) surface molecules (Gerlach & Hensel, 2007; Pizarro-Cerdá & Cossart, 2006). Dr adhesins primarily form fimbrial adhesins which bind with the Dr[a]-blood group antigen of complement delay-accelerating factor CD55 (DAF), a complement regulatory molecule, and with collagen IV,

while Afa adhesins form afimbrial sheaths (Gerlach & Hensel, 2007; Pizarro-Cerdá & Cossart, 2006). The host signaling cascade that follows Afa/Dr adhesin interaction with surface receptors on epithelial cells may have a zipper-like effect that enables cell invasion (Gerlach & Hensel, 2007).

The Type IV pili are generated by a multisubunit mechanism that is similar to yet distinct from the T2SS; unlike that system's pseudopilus, which is anchored in the inner membrane but only extends across the periplasm, the Type IV pilus extends across the outer membrane (Ayers, Howell, & Burrows, 2010; Costa et al., 2015; Gerlach & Hensel, 2007). The Type IV pili enable cell motility, adhesion, and signaling (Nivaskumar & Francetic, 2014), aggregation and biofilm formation (Campos et al., 2013), and DNA uptake from the external environment (termed "competence") (Ayers et al., 2010; Campos et al., 2013).

Bundle-forming pili occur when Type IV pili aggregate laterally in some bacterial species like *V. cholera* and enteropathogenic *E. coli* (Pizarro-Cerdá & Cossart, 2006). The Type IV pilus should not be confused with the T4SS; the Type IV pilus does not secrete anything and it does not penetrate the host or target cell wall, unlike the pili of the T4SS, which are used for DNA conjugation and release, and transmembrane protein translocation (Costa et al., 2015; Freudl, 2013; Gerlach & Hensel, 2007). Adherence by Type IV pili induces changes in the host-cell cytoskeleton that result in formation of filopodium that protect the bacteria and allow formation and survival of microcolonies (Kline et al., 2009).

Two subclasses of Type IV pili are distinguished by their functions, their assembly systems, and their pilin subunit sequences and lengths: in addition to competence, Type IVa pili provide both adhesion and motility functions, while Type IVb pili are more associated with adherence and aggregation of bacterial cells (Ayers et al., 2010) and have only been found in bacteria that colonize the intestinal tract in humans (Gerlach & Hensel, 2007). Once the Type IVa pilus is attached to a receptor or cell surface, it can be forcefully retracted, released, and then extended and reattached, which results in a distinctive type of movement termed "twitching motility" (Ayers et al., 2010; Gerlach & Hensel, 2007). It is not clear whether bacteria with the Type IVb pilus have this capacity (Ayers et al., 2010). The cystic fibrosis transmembrane conductance regulator (CFTCR) is a Type IVb epithelial apical membrane receptor whose concentrations are thought to be increased when activated by binding with *S. Typhi* , which results in greater adhesion and increased uptake of the bacteria (Gerlach & Hensel, 2007).

Curli functional amyloids are nonbranching fibers secreted by many enteric bacteria through extracellular nucleation, which are important for surface adhesion, surface colonization of host tissues (Evans & Chapman, 2014), and host immune system interactions (Costa et al., 2015). Curli amyloids are extremely sticky aggregative adhesins that do not require a receptor and do not bind to specific ligands; they play a major role in stability of the bacterial biofilm and establishment of enteric populations (Evans & Chapman, 2014; Kline et al., 2009). Curli amyloid production within a biofilm is regulated by temperature, oxygen, osmolarity, oxidative stress, and other environmental signals (Evans & Chapman, 2014). In humans, curli are known to interact with plasminogen, laminin, fibronectin, MHC Class I molecules, and contact phase proteins (Gerlach & Hensel, 2007), and may interact with cellulose (Kline et al., 2009). Curli induce cytokine production leading to an inflammatory response and, in addition to releasing

bradykinin, curliated *E. coli* and *Salmonella* species can also induce expression of nitric oxide synthase 2, resulting in serious hypotension (Kline et al., 2009).

After translocation through the Sec system, some periplasmic proteins form integral outer membrane proteins (Omp) that are anchored with a β-barrel, and some of these Omps are important pathogenic adhesins (Gerlach & Hensel, 2007). Meningitis in newborns is a result of the adhesin OmpA recognizing the receptor Ecgp on microvascular endothelial cells in the human brain, allowing *E. coli* to cross the blood–brain barrier, whereas the adhesin OpcA, the Omp found in *N. meningitidis* , recognizes integrins, proteoglycans, and saccharides, which enables invasion of both endothelial and epithelial cells (Gerlach & Hensel, 2007). Invasin is an Omp found in *Yersinia pseudotuberculosis* , which has a lectin adhesin that recognizes β1-integrin receptors on the host cell; adhesion to the host cell results in a signaling cascade that produces pseudopods which enable the bacteria to invade M cells (Gerlach & Hensel, 2007).

Although the intimin Omp is genetically and structurally similar to the invasin Omp, the Intimin/Tir system is unusual in that it consists of an Omp adhesin (intimin) and a cognate receptor (Gerlach & Hensel, 2007). The translocated intimin receptor (Tir) is actually provided to the host cell by the bacteria using its T3SS; after the effector molecule is injected into the host cell, it is phosphorylated and inserted in the cell membrane, whereupon the intimin lectin adhesin binds to it (Gerlach & Hensel, 2007). This type of system is used by enteropathogenic and enterohemorrhagic *E. coli* , and by *Citrobacter* species (Gerlach & Hensel, 2007).

The trimeric autotransporter adhesins (TAAs) have varying lengths, but all share the same general structure: a β-barrel in the outer membrane anchors a complex of proteins that are secreted through this pore, consisting of a filamentous trimer made up of repeating coiled coils, with an adhesin molecule at the distal end, similar to a balloon on a string (Gerlach & Hensel, 2007). TAAs bind to complement proteins, enable binding to extracellular membrane proteins and host cells, and can also result in target cell invasion (Gerlach & Hensel, 2007). TAAs are highly stable and have been found in bacteria ranging from *Bartonella henselae* (BadA) to *N. meninigitidis* , enteropathogenic *Yersinia* species (YadA), and *Haemophilus influenza* (Gerlach & Hensel, 2007; Kline et al., 2009). YadA is the primary adhesin in *Yersina* species and the most studied; however, it is known that there are significant differences in the length of Yad filaments among various *Yersinia* serotypes; further, while YadA filaments extend about 28 nm from the outer membrane, BadA filaments extend 100–300 nm from the bacterial surface (Gerlach & Hensel, 2007).

Classical autotransporters (ATs) consist of a translocation component and a passenger component translated in the same protein, and have an adhesin at the distal end of a monomeric filament that remains attached to the β-barrel in the outer membrane (Gerlach & Hensel, 2007). The adhesin MisL, found in *Salmonella enterica* serovar Typhimurium, and ShdA, an AT also found in *Salmonella* species, appear to be encoded in two different pathogenicity islands, namely, SPI3 and SC54; ShdA was found to bind with fibronectin and to play a role in both adhesion and virulence (Gerlach & Hensel, 2007).

Two-partner system autotransporters (TPS) differ from ATs in two significant ways. First, the translocation component (β-barrel) and the passenger component (monomeric filament) are

translated as two separate proteins which recognize each other in the periplasmic space (Gerlach & Hensel, 2007). Second, once translocated, the monomeric filament can either remain attached or be cleaved and released to function as a secreted protein molecule (Gerlach & Hensel, 2007). One example of a TPS is FHA, which in *B. pertussis* is found in both a membrane bound and a secreted form (Gerlach & Hensel, 2007). A significant portion of the translocated monomeric filaments are cleaved and released into the extracellular space and only a small portion remains bound to the bacterial outer membrane; while surface-bound FHA may enable binding to several ligands and play more than one role in adhesion, it is thought that secreted FHA may act to influence immune response by dendritic cells and modulate cytokine release (Gerlach & Hensel, 2007).

Some soluble proteins secreted by the T1SS are members of the biofilm-associated protein (BAP) family, involved primarily in biofilm formation but also in adhesion (Gerlach & Hensel, 2007). The common factors in these large proteins are that they contain a signal sequence that results in extracellular secretion, they have a glycine-rich motif that is usually a repeated sequence at the C-terminal, and they bind calcium ions (Costa et al., 2015; Gerlach & Hensel, 2007). BAP family proteins are found in Gram-positive as well as Gram-negative bacteria; those found in *S. enterica*, *S. aureus*, *Staphylococcus epidermis*, *Burkholderia cepacia*, *Enterococcus faecalis*, and *Vibrio parahaemolyticus* enable biofilm formation, whereas those found in *E. coli*, *Pseudomonas putida*, *S. pyogenes*, *Lactobacillus reuteri*, and *Enterococcus faecium* enable adhesion (Gerlach & Hensel, 2007).

The soluble adhesin molecule α-hemolysin, which is secreted through the T1SS of certain uropathogenic *E. coli* bacteria, acts as a toxin by forming a pore in the membrane of the host cell that causes cell death as a result of Ca^{2+} influx (Thomas et al., 2014). Because α-hemolysin undergoes acylation at two lysine residues before it is secreted, it is thought that these lipid chains allow it to penetrate the lipid membrane of the host cell; however, some research has shown that β2-integrin receptors or endocytosis of outer membrane vesicles containing α-hemolysin also play a role (Thomas et al., 2014). Also secreted by the T1SS, SiiE is an adhesin produced by *S. enterica* that is not used for biofilm formation but is needed for adhesion to epithelial cells during bacterial invasion; secretion of SiiE is co-regulated with expression of the T3SS of *S. enterica* (Gerlach & Hensel, 2007). Although most soluble adhesion molecules are secreted through the T1SS, GbpA is an adhesion molecule secreted by the T2SS of *V. cholera* that binds to epithelial cells through surface-associated *N*-acetylglucosamine residues (Gerlach & Hensel, 2007).

Bacteria do not use the translocation structures of T3SS, T4SS, and T6SS to secrete adhesins, because the translocated effector molecules are delivered directly into the host cell, but they do use other secretion systems in their outer membrane to facilitate adhesion to the target cell, via adhesins such as intimin, curli, and TAAs (Gerlach & Hensel, 2007; Kline et al., 2009). For example, the T4SS used by *H. pylori* appears to be capable of coordinated adhesion as well as secretion of effector molecules: its CagL protein binds with α5β1 integrins on the surface of gastric epithelial cells to ensure delivery of its CagA effector molecule, which upregulates glycosyltransferases that synthesize Lewis antigens, whereas its autotransporter BabA and SabA adhesins recognize and bind with nonsialylated and sialylated Lewis blood group antigens, respectively (Kline et al., 2009; Pizarro-Cerdá & Cossart, 2006).

Type IV pili can also be encoded on plasmids, which can then be transferred via the T4SS; plasmid-encoded fimbria adhere specifically to the terminal Gal-β(1–4)-Fuc-α(1–3)-GlcNAc moiety of the Lewis[x] HBGA, which is found primarily on the crypt cells of the GI tract (Kato & Ishiwa, 2015). These plasmid-encoded fimbria are known to be expressed by *S. enterica* serovar Typhimurium, which causes numerous crypt abscesses in those who are infected by this pathogen (Kato & Ishiwa, 2015). There are additional adhesive pili that appear to provide additional ways for both pathogenic and commensal bacterial to establish microcolonies and biofilms, such as the bundle-forming pili of the T2SS, and the *E. coli* common pili, which have been described but not classified into one of the secretion systems (Kline et al., 2009; Rendón et al., 2007).

Serine-rich repeat proteins (SRRPs) are a family of large glycosylated proteins exported by the Sec2 pathway in pathogenic Gram-positive bacteria which remain anchored to the cell wall; although not technically containing an adhesin molecule, SRRPs act as adhesins because the N-terminal is quite basic and binds to sialylated glycoconjugates on the host cell surface (Kline et al., 2009). SRRPs contribute to the invasiveness and persistence of *Streptococcus pneumoniae* in the lungs, and are one of the reasons for the virulence and comorbidities of other *Streptococcus* and *Staphylococcus* species (Kline et al., 2009).

Sortase-assembled pili are also anchored to the peptidoglycan cell wall of pathogenic Gram-positive bacteria, and consist of covalently linked pilin subunits forming the main pilus, with minor pilin subunits that function as adhesin molecules (Kline et al., 2009). Sortase-assembled pili are important for adhesion, aggregation, biofilm formation, and invasion of host cells by pathogens as varied as *Actinomyces naeslundii* (dental caries and gingivitis); *E. faecalis* (urinary tract infections, endocarditis); *Corynebacterium diphtheriae* and *S. pneumoniae* (severe respiratory infections); *S. agalactiae* (meningitis, sepsis, and pneumonia in newborns) (Kline et al., 2009); *S. pyogenes* (necrotizing fasciitis); and *S. aureus* (toxic shock syndrome, endocarditis, mastitis, septic arthritis, and hospital-acquired pneumonia) (Henderson, Nair, Pallas, & Williams, 2011; Pizarro-Cerdá & Cossart, 2006).

Fibronectin-binding proteins (FnBPs) are adhesins secreted by bacteria, which bind to fibronectin glycoproteins found on epithelial cells, in the ECM, and in body fluids of the host; in Gram-negative bacteria, many FnBPs are autotransporters, whereas in Gram-positive bacteria, FnBPs are sortase-assembled structures (Henderson et al., 2011). Plasma fibronectin, which is produced by hepatocytes, is found in blood, saliva, and other body fluids and is an important component of blood clotting and wound healing (Henderson et al., 2011). Cellular fibronectin, a dimer secreted by most cells, acts as a ligand that binds with β-1 integrins to facilitate adhesion to the ECM and is also known to bind with DNA, tissue transglutaminase, fibrin, glycosaminoglycans such as heparin, and various collagens (Henderson et al., 2011). Fibronectin also plays a role in inflammatory responses and macrophage activation, and mediates leukocyte function (Henderson et al., 2011). FnBPs enable pathogen-specific binding to the ECM, laminin, collagen, fibroblasts, osteoblasts, RBCs, endothelial cells, or epithelial cells of the host, followed by invasion of the host cell (Henderson et al., 2011). Although FnBPs contribute to the virulence of the pathogen, if the bacteria happen to bind to the fibronectin receptor on macrophages, it is ingested (Henderson et al., 2011).

Moonlighting proteins, which are not adhesins, are also found on the surface of bacteria, and although their normal function may be as housekeeping, ribosomal, or glycolytic proteins, once they are exposed to the external environment, they can assume other functions, similar to adhesins (González-Rodríguez et al., 2013; Sánchez et al., 2010). Some are involved in attachment to mucus or mucosal components, or have an affinity for plasminogen, fibronectin, or other extracellular matrices (González-Rodríguez et al., 2013); however, not all strains have the same adhesive capacity. For example, Lactobacilli have evolved mucus-binding adhesin proteins consisting of long fibrils that have Ig and mucin-binding domains repeated in tandem along the length, which hold them securely in the mucosal layer and, like most commensal bacteria, do not penetrate to the epithelium (Etzold et al., 2014). In contrast, some pathogenic Gram-positive bacteria appear to have only one or two mucus-binding repeats at the end of their adhesin proteins, but have evolved the ability to penetrate the mucosal layer and adhere to the epithelial surface with lectins or lectin domains (Etzold et al., 2014).

9 ENTEROTYPES

Secretors have an abundance of bacteria with glycan-degrading enzymes such as α-l -fucosidase and β-galactosidase that enable them to degrade the HBGAs found in the intestinal mucosa, thus taking advantage of a readily available food source as well as making the resulting monosaccharides available to co-occurring species that do not have these enzymes (Wacklin et al., 2014). In contrast, bacteria that are known to be abundant in nonsecretors have also been associated with irritable bowel syndrome (IBS) and autoimmune conditions such as IBD, celiac disease, and T1DM (Wacklin et al., 2014), as well as chronic inflammatory diseases like primary sclerosing cholangitis and Crohn's disease (Kashyap et al., 2013). These associations might be due to the fact that nonsecretors also have fewer commensal bacteria such as Bifidobacteria, which generally play a protective role in the GI tract by preventing pathogens from achieving colonization (Wacklin et al., 2011).

A recent study used reference genomes and metagenomic techniques to map known species by their phylogenetic profiles; the data were sorted and analyzed, and the resulting three clusters were designated as "enterotypes" composed of groups of species that were found to contribute specific functions to what could be considered a microbial community (Arumugam et al., 2011). The main contributors to each enterotype were the *Bacteroides*, *Prevotella*, and *Ruminococcus* genera, with each community composed of a network of co-occurring groups of species (Arumugam et al., 2011).

Although HBGAs were not identified as host properties in this study, degradation of mucosal glycoproteins played a fundamental role (Arumugam et al., 2011). Further analysis showed that each enterotype derived energy from specific fermentable substrates; one degraded carbohydrates and proteins, while the other two degraded glycoproteins found in the mucosal lining of the intestine (Arumugam et al., 2011). It is possible that these enterotypes reflect the method of hydrogen disposal utilized during fermentation; enterotype 1 (*Prevotella*) is enriched with a sulfate reducer and enterotype 3 (*Bacteroides*) is enriched with a methanogen, and these just happen to be two of the ways by which hydrogen is removed (Arumugam et al., 2011).

Also of interest are the findings that secretors were more likely to have enterotype 3, and have a higher risk of *C. difficile* or *Salmonella* infection following antibiotic treatment, whereas nonsecretors were more likely to have enterotype 1, and have a higher risk of urinary tract infections, vaginal candidiasis, T1DM, or necrotizing enterocolitis (Wacklin et al., 2014). Secretor status also determines susceptibility to a variety of other pathogens: nonsecretors have a lower risk of Norovirus infection and a greater risk of *H. pylori* , *V. cholera* , and *C. jejuni* infection (Kashyap et al., 2013). These differences in risk are thought to be due to differences in the gut microbial composition as a result of availability of the mucosal carbohydrate antigens in the intestinal lumen of secretors and nonsecretors (Ewald & Sumner, 2016; Kashyap et al., 2013; Wacklin et al., 2014).

Carbohydrates are the primary energy source for the microbiota, and aside from diet, one of the main sources of glycosides are the oligosaccharides which make up 50–80% of mucins by weight; in secretors, fucose residues are a major component of the mucosal glycans but these residues are not present in nonsecretors (Kashyap et al., 2013). *B. fragilis* is known to incorporate fucose residues into its polysaccharide layer, and *Bacteroides thetaiotaomicron* , a prominent Gram-negative commensal bacteria, not only metabolizes fucose residues but also actually induces fucosylation of intestinal mucins (Kashyap et al., 2013). Although the greatest influence appears to be secretor/nonsecretor status as a result of FUT2 allelic expression, in addition to HBGAs, long-term diet is known to influence the microbial profile for each enterotype (Wacklin et al., 2011, 2014).

9.1 Dietary and genetic factors

In a human study that assessed the effect of long-term diet on microbiota composition (Wu et al., 2011), enterotype 1 (*Prevotella*) was strongly associated with a high-carbohydrate and low animal protein diet, such as that found in agrarian populations. However, enterotype 3 (*Bacteroides*) was strongly associated with a diet high in animal fat and animal proteins, typical of the Western diet. While short-term dietary changes had a significant effect within 24 hr on microbiome composition of the subjects, the effect was not strong enough to change their enterotype classification (Wu et al., 2011).

Another study found that variation in bacterial diversity was distributed along a *Prevotella* /*Bacteroides* gradient that was strongly affected by the presence of Bifidobacteria (Yatsunenko et al., 2012). Studies with humanized gnotobiotic mice have shown that nonsecretors have greater abundance of Bacteroidetes and decreased diversity within their bacterial microbiota, simply because of the lack of fucosylation of intestinal mucosa (Kashyap et al., 2013). However, substituting a diet deficient in plant polysaccharides but rich in glucose resulted in microbial diversity similar to secretors; these findings indicate that although the microbiota adapt to the environment dictated by the host genotype, diet has a greater impact than genotype (Kashyap et al., 2013).

Microbial ability to rapidly adjust to dietary changes gave selective advantage to ancestral populations whose diet depended on success in foraging and hunting, and was further affected by season, climate, and temperature (David et al., 2014). A short-term dietary intervention in humans found that significant changes in the structure of the gut microbiota were observed

within 24 hr of introducing an animal-based diet, which reverted to the baseline profile within 2 days of the end of the intervention (David et al., 2014). In this study, the high-fat diet resulted in increased microbial production of the secondary bile acid, deoxycholic acid (DCA), which is known to inhibit Bacteroidetes and Firmicutes growth and may explain the change in the microbiota profile (David et al., 2014).

A high-fat diet is also associated with *Bilophila wadsworthia* , a pathogen whose growth is stimulated if secretion of bile acids occurs while consuming a diet high in saturated milk fats (David et al., 2014). *B. wadsworthia* is in the Desulfovibrionaceae family that reduces sulfite to hydrogen sulfide, a genotoxic and cytotoxic gas that can cause impaired barrier function, endotoxemia, and IBD, and contribute to the development of cancer (Shen, Gaskins, & McIntosh, 2014). Hydrogen sulfide reduces beneficial butyrate oxidation, causing altered redox status and increased MAP kinase activation, and is extremely toxic to colonocytes, resulting in increased colonocyte turnover, thereby increasing intestinal permeability and reducing integrity of the epithelial barrier (Shen et al., 2014). Proinflammatory microbial products from a high-fat diet can also result in metabolic changes in the host leading to increased energy harvest, obesity, and insulin resistance (Shen et al., 2014).

Diets high in plant polyphenols and polysaccharides such as dietary fiber, which are naturally resistant to digestion and absorption in the small intestine, provide important substrates for colonic bacteria, which produce small phenolic compounds and short-chain fatty acids (SCFAs) (Tuohy, Conterno, Gasperotti, & Viola, 2012). Once absorbed, SCFAs provide energy for colonocytes as well as heart, brain, and muscle cells; regulate immune function; and play important roles in lipid metabolism, thermogenesis, cell differentiation, proliferation, and apoptosis (Tuohy et al., 2012). Plant polyphenols are antioxidants and free radical scavengers, while small phenolic acids are known to participate in biological processes that include antibacterial, anti-inflammatory, and phytoestrogenic activities; anti-AGE formation; and stimulation of enzymes needed for detoxification and degrading xenobiotics (Tuohy et al., 2012).

Prebiotic dietary fiber is the primary energy source that supports the composition and metabolic activity of the colonic microbiota and maintains human health by protecting against obesity, T2DM, metabolic syndrome, IBD, and colon cancer (Tuohy et al., 2012). Although consumption of dietary fiber ranges between 70 and 120 g per day in populations with a more traditional plant-based diet, in populations with a Western diet, intake averages just 20 g per day (Tuohy et al., 2012). When the availability of fermentable polysaccharide substrate is inadequate, colonic bacteria substitute amino acid fermentation, which generates potentially harmful metabolites that can be cytotoxic, genotoxic, or carcinogenic (Tuohy et al., 2012). Bacteroidetes have developed an ecological network that enables them to maximize polysaccharide substrate availability: strains and species that can utilize one type of polysaccharide structure, such as inulin, provide breakdown products that support others that need a different type of polysaccharide structure, such as fructose (Rakoff-Nahoum, Coyne, & Comstock, 2014).

Tissues that are not in direct contact with intestinal microorganisms can be profoundly affected by changes in the composition and density of bacterial species in the gut which alter immune responses; this should be considered when inflammatory processes are being investigated, including chronic inflammatory responses and metabolic syndrome (Jarchum & Pamer, 2011).

Commensal bacteria prevent liver inflammation and fibrosis as a result of translocation of pathogenic bacteria and their products across the intestinal barrier (Mazagova et al., 2015). There is evidence that both beneficial and pathogenic bacteria may respond directly to stress-related catecholamines, which can affect their growth, motility, and virulence (Konturek, Brzozowski, & Konturek, 2011). Exposure to stress not only modifies the gut microbiota but also enhances bacterial mucosal adherence, reduces sIgA secretions, and increases paracellular permeability of the epithelium, which may seriously influence the outcome of infections (Alonso et al., 2014; Foster & McVey Neufeld, 2013; Konturek et al., 2011).

In addition, changes in the intestinal microbiota directly correlate with the appearance of stress-related disorders (Konturek et al., 2011), including affective states such as anxiety and depression (Foster & McVey Neufeld, 2013). Physiological responses to inflammation and stress also affect a variety of GI functions as diverse as visceral sensitivity, mucosal blood flow, gut motility, acid secretion, and release of various hormones and neuropeptides, which can result in dyspepsia, abdominal pain, diarrhea, and other unpleasant symptoms (Alonso et al., 2014; Konturek et al., 2011). Thus, effective treatment of inflammatory, stress-related, and functional disorders of the GI tract requires a thorough understanding of how the physiological responses of the gut are regulated.

10 THE ENTERIC NERVOUS SYSTEM

The uniqueness of the enteric nervous system (ENS) and the central role it plays in the proper physiological functioning of the GI tract cannot be fully described without a brief review of the distinctions between the CNS, the peripheral nervous system (PNS), and the ENS. The CNS consists of the brain and the nerves of the spinal cord, while the PNS consists of all other nerves in the body that communicate with the CNS (Gershon, 1998). Thus the efferent nerves of the PNS carry all nerve signals from the CNS to muscles, tissues, and glands, which evokes their response, and information gathered by sensory receptors in the body is carried by the afferent nerves of the PNS back to the CNS, where it is processed (Gershon, 1998).

The nerves of the skeletal motor system carry signals from the CNS directly to the skeletal muscles, while the autonomic nervous system (ANS) carries the nerve signals that control the behavior of the heart, blood vessels, glands, and visceral muscles (Gershon, 1998). However, unlike the skeletal motor nerves, the nerves of the ANS are characterized by ganglionic synapses, thus the signals they carry can be amplified, weakened, or otherwise modified by processes that occur at those synapses, which allows for instant adaptation to changing circumstances (Gershon, 1998).

The efferent nerves of the ANS are further classified as either sympathetic or parasympathetic, based on anatomical characteristics of the nerve fibers and the proximity of their ganglia to their target organ or tissue (Gershon, 1998). The parasympathetic division consists of the cranial and sacral nerves, with their ganglia located just outside of or within the organ or tissue they innervate, whereas the sympathetic division is made up of the thoracic and lumbar nerves, with their ganglia located immediately adjacent to the spinal column (Gershon, 1998). Parasympathetic responses are usually faster and more precisely targeted, and sympathetic

responses are usually slower and more diffuse, but these are tendencies and not absolute distinctions (Gershon, 1998).

The GI tract is innervated with efferent nerves from both the sympathetic and parasympathetic divisions of the PNS: it is supplied with a set of nerves from the sympathetic ganglia associated with the thoracic and abdominal nerves, while the vagus nerves (the large cranial nerves that connect the brain to the gut) innervate it from the esophagus to the middle of the colon and the sacral nerves innervate the distal colon (Gershon, 1998). As with other organs, the efferent nerves of the PNS deliver catecholamines to the GI tract, and the afferent nerves of the PNS carry sensory information, such as pain and pressure, from the GI tract back to the CNS (Gershon, 1998). However, the minute-to-minute control, coordination, and regulation of the myriad physiological functions of the GI tract fall to the ENS.

Auerbach discovered the existence of the ENS in the 1860s, which is now known to include not only Auerbach's (myenteric) nerve plexus but also Meissner's (submucosal) nerve plexus; these are complex networks of nerve cells and intrinsic nerve fibers (Gershon, 1998) which contain sensory neurons, interneurons, and motor neurons (Alonso et al., 2014). The interneurons are serotonin-secreting nerve cells that enable communication between other nerve cells; interneurons are only found in the CNS and ENS, and add layers of complexity and sophistication to neural communication that allow the brain to modulate and process the information it receives (Gershon, 1998).

However, the number of motor nerve fibers connecting the CNS to the GI tract is incredibly small in comparison to the number of ENS nerve cells in the gut; for example, there are only about 2,000 nerve fibers in the vagus nerves at the point where they enter the abdomen but there are over 100 million ENS nerve cells in the human small intestine alone (Gershon, 1998). Given this overwhelming disparity, it seems highly unlikely that such a small number of motor nerve fibers can innervate so many ENS nerve cells, along with the many hundreds of millions of intrinsic nerve fibers that these cells put out to communicate with each other—and indeed, this is true (Gershon, 1998).

In a simple yet elegant experiment (Gershon, 1998), an isolated section of intestine suspended in an organ bath detected and responded to pressure-inducing stimuli with a coordinated descending oral-to-anal wave of contraction and relaxation. This reproducible result was especially startling because the brain, spinal cord, and sensory ganglia had been discarded, leaving only the segment of gut. Such groundbreaking research by Trendelenburg in the early 1900s, confirmed by Langley and others in subsequent investigations, established that—unlike any other organ—the gut could manifest reflex activity independently of input from the CNS, and that the ENS did not require commands from the CNS in order to function. Trendelenburg introduced the term "peristaltic reflex" to describe the pressure-induced, unidirectional propulsive activity of the gut (Gershon, 1998).

Thus, while the nerves of the PNS do communicate between the CNS and the GI tract itself, the nerves of the PNS are virtually irrelevant to many of the behaviors of the gut because the ENS is not affected when these motor nerves are severed; the smooth muscle and glands of the gut are not supplied by them but by complex intrinsic enteric neural circuits that may involve many

nerve cells (Gershon, 1998). The nerve cells of the ENS are not just postganglionic links in the parasympathetic nerve pathway; the ENS is actually an integrated system of neural communication and processing that is anatomically and functionally independent from the CNS and therefore can be considered neither sympathetic nor parasympathetic (Gershon, 1998).

The sensory receptor nerves of the ENS independently detect, process, and act on data from the gut to activate a set of effector nerves that it alone controls, which means that all of the necessary elements of this neural apparatus are intrinsic components of the wall of the gut, and that the intrinsic nervous system of the gut has properties that are like those of the brain (Gershon, 1998). In fact, over 95% of the body's serotonin is made in the nerve cells of the ENS, and more importantly, every one of the classes of neurotransmitters found in the brain is represented in the ENS (Gershon, 1998). The proper understanding of the ENS as a separate but equal division of the ANS, and as a "second brain" that is independent of the CNS, holds great potential for clinical medicine and may hold the key to understanding some of our most challenging conditions.

11 THE BRAIN-GUT-MICROBIOTA AXIS

The term *brain-gut microbiota axis* refers to the multiple parallel pathways by which the brain and the gut communicate, which include the splanchnic and vagus pathways of the ANS, the hormonal pathways of the hypothalamic–pituitary–adrenal (HPA) axis, and the various components of the immune system (Konturek et al., 2011). Because this is a bidirectional axis, the term also refers to the various mechanisms by which the gut microbiota communicates with the mucosal cells, immune cells, and neural endings of the intestines, which in turn modulate permeability, visceral sensitivity, and motility of the GI tract (Konturek et al., 2011). Through these mechanisms, changes in the bacterial status of the intestinal lumen can also activate signaling pathways between the ANS (either the ENS or the vagus nerves, or both) and the brain, thus this axis plays an important role in modulating the stress response of the gut (Foster & McVey Neufeld, 2013). It is also possible that psychosocial or life stressors which induce changes in the CNS and alter brain chemistry may in turn result in disruptions to the gut microbiota, and that these changes could then modulate host responses and behaviors (Forsythe, Kunze, & Bienenstock, 2016).

The enteroendocrine cells of the epithelium are also an important part of the brain-gut-microbiota axis, and they too communicate through multiple parallel pathways: their primary influence on the axis is through secretion of serotonin, which activates neural pathways in the ENS, PNS, and CNS (Alonso et al., 2014). Serotonin plays a role in regulating cognition and mood in the brain, but peripherally it is involved in perception of pain, gut motility (contraction and relaxation of smooth muscles), and regulating secretions in the GI tract (Foster, Rinaman, & Cryan, 2017). Of note, certain bacteria can promote intestinal synthesis of serotonin (Forsythe et al., 2016; Foster et al., 2017). The enteroendocrine cells of the epithelium also act as highly specialized chemoreceptors, which sense changes in the pH, osmolarity, and nutrient composition of the lumen (Alonso et al., 2014). As the largest endocrine organ of the human body, they secrete hormones such as ghrelin, somatostatin, cholecystokinin, glucagon-like peptide, gastric inhibitory peptide, and peptide YY, which affect both the brain and the gut (Alonso et al., 2014).

The role of the intestinal microbiota in the development of metabolic diseases (discussed later) and neuropsychiatric disorders has become an area of intense research interest since 2010 (Khanna & Tosh, 2014). CNS receptors for cytokines and other immune system molecules suggest the importance of the immune system in normal brain function; the discovery of anti-brain antibodies in conditions as diverse as autism spectrum disorder (ASD), obsessive–compulsive disorder, and schizophrenia indicates that autoimmunity may be a factor in the pathogenesis of some neuropsychiatric conditions (Hornig, 2013). In addition to possibly contributing to neurodevelopmental disorders such as ASD, intestinal microbiota may be involved in the pathophysiology of depression, anxiety, and other mood disorders (Alonso et al., 2014; Biedermann & Rogler, 2015; Blank et al., 2016; Bradstreet, Ruggiero, & Pacini, 2015; Herz & Kipnis, 2016).

The brain-gut-microbiota axis is also thought to be involved in functional GI disorders, which have been associated with psychiatric comorbidities (Alonso et al., 2014). Major depressive disorder (MDD) may be associated with epithelial barrier dysfunction and greater permeability, known as "leaky gut," which increases the probability of intestinal inflammation, followed by activation of the immune system and upregulation of IL-6 and IFN-γ production (Foster et al., 2017). MDD patients have significantly higher serum concentrations of IgA and IgM antibodies against LPS from enterobacteria, suggestive of leaky gut as a possible contributor to MDD development (Foster et al., 2017).

11.1 Fecal transfers

Historic records document that fecal transfers have occasionally been done but there have been no fecal transfer studies in humans that specifically focused on neuropsychiatric diseases (Evrensel & Ceylan, 2016). Fecal transfer studies with germ-free mice are of limited use, due to known alterations in physiology and immune development (Forsythe et al., 2016). A few fecal transplant studies with wild-type mice have investigated the associations, if any, between diet, intestinal permeability, inflammatory markers, and behavior (Foster et al., 2017). In one study, chow-fed mice, treated with antibiotics to deplete their intestinal bacteria, received microbiota from donor mice fed a high-fat diet; the recipient mice were subsequently found to have increased gut permeability and circulating inflammatory markers, and exhibited selective yet significant disturbances in cognitive, exploratory, and stereotypical behaviors (Forsythe et al., 2016; Foster et al., 2017). Experimental fecal transfers in humans to treat IBS, chronic fatigue syndrome, MS, ASD, and Parkinson's disease have had limited success in some cases; however, much more research is needed to obtain reliable evidence of the efficacy of fecal transplants for treatment of neuropsychiatric disorders (Evrensel & Ceylan, 2016).

11.2 Probiotic effects

There is significant evidence that probiotics can prevent intestinal barrier damage from inflammatory conditions such as colitis or IBD, and can lessen stress-induced intestinal damage, by minimizing gut hypersensitivity and mucosal barrier disruption, thereby preventing the cascade of hyperpermeability and endotoxemia, HPA axis response, and neuroinflammation (Alonso et al., 2014). In addition, several studies have found that direct manipulation of the

intestinal microbiota, including with probiotic supplementation, can modulate behavior (Biedermann & Rogler, 2015; Foster & McVey Neufeld, 2013; Kinross et al., 2011; Lepage et al., 2013). Thus, addressing functional GI disorders with probiotics may better regulate intestinal and brain barrier function, which may in turn alleviate psychiatric comorbidities (Alonso et al., 2014).

Depressive behavior, social avoidance, inactivity, and impaired appetite are frequently associated with systemic viral infections, and conversely, treatment of hepatitis C, MS, and metastatic cancer with INF-α and IL-2 often results in development of MDD (Blank et al., 2016; Herz & Kipnis, 2016). It is thought that proinflammatory cytokines induce secretion of CXCL10 by the epithelial and endothelial cells of the meninges in the brain, which results in activation of CXCR3 signaling followed by a subsequent decrease in synaptic activity (Blank et al., 2016; Herz & Kipnis, 2016). Recently, research has established that lymphatic vessels exist in the meninges of the brain, which appear to play a role in drainage of cerebral spinal fluid and may be responsible for immune surveillance of the CNS as well as removal of waste products from the brain parenchyma (Louveau et al., 2015; Raper, Louveau, & Kipnis, 2016). Impaired function of these lymph vessels may be associated with neurological disorders such as cerebral amyloid angiopathy, neuromyelitis optica, Alzheimer's disease, or MS (Louveau et al., 2015; Ueno et al., 2016).

In a mouse model of MS, concurrent treatment with several *Lactobacillus* strains decreased proinflammatory responses and reduced clinical symptoms, and treatment with *B. fragilis* induced protection against demyelination (Ochoa-Reparaz, Mielcarz, Begum-Haque, & Kasper, 2011). In a mouse model of ASD, treatment with *B. fragilis* has been found to change microbial composition; restore gut permeability; decrease repetitive, anxiety-like, sensorimotor behaviors; and improve social behaviors (Alonso et al., 2014). These findings suggest that ASD behaviors may be a result of viral infection during pregnancy, at least some of which may be reversible with *B. fragilis* administration (Forsythe et al., 2016). Recall that *B. fragilis* colonization was seriously impaired and remained depressed in babies following cesarean birth when compared to babies delivered vaginally (Grönlund et al., 1999). Stool samples from children with ASD have a significantly greater prevalence of *Clostridium* species than samples from children without ASD (Ochoa-Reparaz et al., 2011). Further studies are clearly warranted to assess whether there is any correlation between intestinal dysbiosis and ASD or MS, but targeted probiotics appear to have some prophylactic value.

Most Gram-positive commensal or probiotic bacteria have the same secretion systems and adhesins as Gram-positive pathogenic bacteria, and recognize the same receptor molecules (González-Rodríguez et al., 2013; Sánchez et al., 2010). Overcompetition and competitive exclusion by such probiotic bacteria can thus prevent infection by both Gram-negative and Gram-positive pathogenic bacteria by ensuring that adhesion and colonization cannot occur (González-Rodríguez et al., 2013; Sánchez et al., 2010). Extracellular proteins and peptides produced by probiotic bacteria, such as Lactobacilli and Bifidobacteria, are important for maintaining intestinal health and homeostasis (González-Rodríguez et al., 2013; Sánchez et al., 2010). Bifidobacteria are known to secrete a serine protease inhibitor (serpin) that prevents inflammation by inhibiting secretion of inflammatory cytokines and pancreatic and neutrophil elastases (Sánchez et al., 2010). Various species of Bifidobacteria and Lactobacilli secrete other

extracellular proteins that regulate cell proliferation and apoptosis; modulate immune function and activity; promote dendritic cell maturation and survival; induce production of defensins; strengthen the mucosal barrier; increase production of tight-junction proteins; reduce intestinal permeability; and regulate transcription of proto-oncogenes (Sánchez et al., 2010).

Of note, the Gram-positive Lactobacilli strain, *L. rhamnosus GG* (LGG), has pili which form strong attachments to the intestinal mucus layer and the epithelial cells beneath, as well as between bacteria within the colony, enabling it to form biofilms and resist intestinal shear from peristalsis (Segers & Lebeer, 2014). The pili can form a zipper-like mechanism that brings the bacteria into closer interaction with host cells, enabling attachment of surface adhesins and immune modulation by effector molecules (Lebeer et al., 2012; Segers & Lebeer, 2014). Although lipoteichoic acid molecules from the Gram-positive bacterial surface can stimulate proinflammatory IL-8 cytokines and induce NF-κB activation, the LGG pili directly induce anti-inflammatory responses and suppress NF-κB activity, and indirectly bring about the secretion of anti-inflammatory proteins p40 and p75, which are known to have an antiapoptotic effect and reduce cell damage (Lebeer et al., 2012; Segers & Lebeer, 2014). As a probiotic, LGG has been shown to be safe, was effective in clearing vancomycin-resistant Enterococcus infections, and showed promising results in treating *C. difficile* infections, preventing *Candida* colonization, and reducing intensity and frequency of pain in patients with IBS (Segers & Lebeer, 2014).

Perhaps not surprisingly, there is growing interest in the targeted use of prebiotics and probiotics as therapeutic approaches to the prevention or treatment of a variety of diseases (Dominguez-Bello & Blaser, 2008; Kootte et al., 2012), and to offset the deleterious effects of stress on the intestinal microbiota which contribute to dysregulation of the brain-gut-microbiota axis (Konturek et al., 2011). This approach may be of particular interest to those exploring a possible treatment for the high prevalence of *Clostridium bolteae* found in some children with ASD (Kinross et al., 2011). Although extensive research with mice has shown that probiotics have a consistent antiobesity effect (Arora, Singh, & Sharma, 2013), further trials with humans are now needed. When mouse and human bacterial diversity were compared using distal gut rRNA sequences, a high degree of taxonomic similarity was seen at the division level but a full 85% of the mouse sequences belonged to genera that have not been found in humans (Ley, Backhed, & Turnbaugh, 2005). Despite the fact that there are large differences in the composition of their gut microbiota at lower levels, because mice and humans are genetically similar host organisms with highly conserved metagenomic core functions and similar microbiotic composition at higher taxonomic levels (Cho & Blaser, 2012), the use of germ-free or humanized gnotobiotic mice is an important research tool.

It should also be noted that, although the dynamics of the microbiota and the immune system have been studied, the inter-kingdom signaling pathways between intestinal bacteria, fungi, parasites, and viruses are not well understood (Kinross et al., 2011). One virome study with monozygotic twins and their mothers did not find the expected viral-microbial dynamic typical of other ecosystems (Kinross et al., 2011). The gut virome of the participants was not influenced by their genetic relatedness, but was found to be unique to each individual, and the diversity of each person's virome remained virtually unchanged over the research period (95% of virotypes were retained) (Kinross et al., 2011).

12 DISEASE MECHANISMS

Although there are still significant gaps in our knowledge, completion of the HMP has resulted in much greater understanding of the mechanisms by which the microbiota influence health and disease, some of which are reviewed here. Certain diseases associated with microbiome characteristics are also shown in Table 3. The human microbiota influences our mood, alters our behavior, and affects our physiology in a variety of ways, including modulating predisposition to obesity, diabetes, colitis, cancer, skin and mucosal disorders, and infection (Foster & McVey Neufeld, 2013; Kinross et al., 2011; Lepage et al., 2013; Ohnmacht et al., 2011). Tight junction dysfunction has been associated with conditions as diverse as autoimmune thyroiditis, IgA nephropathy, chronic kidney disease, T1DM, primary biliary cirrhosis, primary sclerosing cholangitis, alcoholic liver disease, and cirrhosis of the liver (Alonso et al., 2014).

Table 3. Association of diseases with characteristics of the microbiome

Microbiome	Disease or condition	Relevant characteristics
Cutaneous	Psoriasis	Significantly increased ratio of Firmicutes to Actinobacteria in lesions (Cho & Blaser, 2012)
	Acne	*Propionibacterium acnes* infection of pilosebaceous units (Cho & Blaser, 2012)
	Chronic skin ulcers	Increased Pseudomonadaceae following antibiotic treatment (Cho & Blaser, 2012)
	Diabetic skin ulcers	Increased Streptococcaceae (Cho & Blaser, 2012)
Gastric	*Increased* risk of peptic ulcer disease, gastric mucosa-associated lymphoid tissue tumors, and noncardia gastric adenocarcinomas. *Decreased* risk of reflux esophagitis and childhood-onset asthma	Presence of *Helicobacter pylori* in gastric microbiota (Cho & Blaser, 2012)
	Increased risk for childhood-onset asthma, reflux esophagitis, Gastroesophageal reflux disease, Barrett's esophagus, and esophageal and gastric cardia adenocarcinomas	Absence of *H. pylori* in gastric microbiota (Blaser & Falkow, 2009)
	Reflux esophagitis	Esophageal microbiota dominated by Gram-negative anaerobes; gastric microbiota has low or absent *H. pylori* (Cho & Blaser, 2012)
	Childhood-onset asthma	Absence of *H. pylori* in gastric microbiota (Cho & Blaser, 2012)
	Age-related gastric atrophy	Enhanced by presence of *H. pylori* in gastric microbiota (Lepage et al., 2013)
Upper gastrointestinal	Obesity	Reduced ratio of Bacteroidetes to Firmicutes; enrichment of genes related to lipid and carbohydrate metabolism; risk significantly increased with antibiotic use prior to 6 months of age (Cho & Blaser, 2012). Successful weight loss associated with higher levels of *Bacteroides fragilis* , *Lactobacilli* , and *Bifidobacteria* (Biedermann & Rogler, 2015)
	Cardiovascular disease	Gut-microbiota-dependent metabolism of phosphatidylcholine (Cho & Blaser, 2012)

Microbiome	Disease or condition	Relevant characteristics
	Diseases of the liver: nonalcoholic fatty liver disease, alcoholic steatosis, hepatocellular carcinoma	Exposure to metabolic products of microbiome: acetaldehyde, phenols, ammonia (Cho & Blaser, 2012)
	Cirrhosis	Substantially altered microbiome, with enrichment of Proteobacteria and Fusobacteria phyla, and Enterobacteriaceae, Veillonellaceae, and Streptococcaceae families (Cho & Blaser, 2012)
	ASD	Significantly increased presence of *Clostridium bolteae* (Kinross et al., 2011)
	Small intestine bacterial overgrowth	Associated with recurrent antibiotics, gastric acid inhibitors, Crohn's disease, cirrhosis, chronic pancreatitis, end-stage renal disease (Wang & Yang, 2013)
Colonic	Inflammatory bowel disease	Host polymorphisms in bacterial sensor genes (NOD2, CARD15, TLR4); symptoms may improve with antibiotic treatment (Cho & Blaser, 2012). Bacterial sensor genes (NOD2, TLR4, CARD8, CARD9, NLRP3) and autophagy genes (ATG16L1, IRGM, LRRK2) (Biedermann & Rogler, 2015). Reduced butyrate, acetate, methylamine, trimethylamine; elevated amino acids; metabolites from arachidonic acids and bile acids were most affected (Lepage et al., 2013). Impaired production of α-defensin (Goto & Kiyono, 2012)
	Functional bowel diseases	Larger populations of *Veillonella* and *Lactobacillus* (Cho & Blaser, 2012)
	Ulcerative colitis	Large populations of Enterobacteriaceae, increased proportions of Actinobacteria and Proteobacteria (Cho & Blaser, 2012). Increased taurine and cadaverine (Lepage et al., 2013)
	Crohn's disease	Increased risk with early childhood exposure to antibiotics; significantly diminished microbial diversity; large populations of *Enterococcus faecium* and several Proteobacteria (Cho & Blaser, 2012). Genes involved in epithelial barrier integrity (IBD5, DLG5, PDGER4, DMBT1, XBPI); decreased *Faecalibacterium prausnitzii* and *Roseburia hominis* (Biedermann & Rogler, 2015). Linked to disruption of duodenal microbiota (Wang & Yang, 2013). Higher amounts of bacteriophages (Lepage et al., 2013)
	Colorectal cancer	Larger populations of *Fusobacterium* spp.; significantly lower *Desulfovibrio* spp. (Cho & Blaser, 2012; Biedermann & Rogler, 2015; Kinross et al., 2011). Invasive CRC associated with *Escherichia coli NC101* (Biedermann & Rogler, 2015). Lower butyrate and acetate, higher proline and cysteine (Lepage et al., 2013)
	Irritable bowel syndrome	Distinctive alterations in *Coprococcus* , *Collinsella* , and *Coprobacillus* genera (Biedermann & Rogler, 2015). Higher levels of *Pseudomonas aeruginosa* and lower levels of *Bifidobacterium catenulatum* in upper GI (Wang & Yang, 2013). Higher bile acid concentration, lower levels of branched-chain fatty acids (Lepage et al., 2013). May follow infection by *Campylobacter enteritis* , *Shigella* , or *Salmonella* (Goto & Kiyono, 2012)

Note . The presence or absence of certain human diseases is associated with and influenced by genetic and bacterial conditions of the relevant microbiome.

Life-threatening conditions such as multiple severe injuries, hemorrhagic shock, splanchnic ischemia–reperfusion injury, and severe burns can result in increased intestinal permeability, gut inflammation, loss of intestinal barrier function, and translocation of endotoxins and/or bacteria from the GI tract (Alonso et al., 2014). Inflammatory cytokines such as IFN-γ and TNF-α can upregulate expression of channel-forming proteins found in tight junctions, whereas butyrate, glutamine, and estrogens protect the intestinal barrier by increasing mucus production and enhancing expression of tight junction proteins that act to reduce permeability (Alonso et al., 2014).

12.1 Autoimmune diseases

Dysregulation of the intestinal immune system and activation of T_H1 cells have been associated with T1DM, Crohn's disease, MS, and other autoimmune diseases, whereas activated T_H2 cells are associated with allergic disorders, asthma, and ulcerative colitis (Delves, 2017c; Ochoa-Reparaz et al., 2011). T_H17 cells may contribute to autoimmune disorders such as psoriasis and rheumatoid arthritis (Delves, 2017c). In autoimmune diseases such as celiac disease and T1DM, the protein zonulin plays a critical role in tight junction regulation, but wheat gluten is the dietary trigger that induces increased intestinal permeability and inflammation in those who are genetically predisposed to celiac disease (Alonso et al., 2014). Recall that the hygiene hypothesis suggests that some allergies and autoimmune disorders could be avoided with more exposure to bacteria in early life, which is less common in our modern Western society than in more rural, bacteria-filled lifestyles (Biedermann & Rogler, 2015). Lack of exposure during development of the immune system may result in alterations that lead to loss of self-tolerance, which may be irreversible (Khanna & Tosh, 2014).

It should be noted that the pathophysiology of food allergies can involve enhanced intestinal permeability even in the absence of food allergens (Alonso et al., 2014), and antibodies are known to exist against some proteins secreted by commensal bacterial in the gut (Sánchez et al., 2010). Drugs and toxins can also induce intestinal barrier dysfunction: for example, tacrolimus treatment can result in new-onset food allergies by increasing intestinal permeability; NSAIDs can increase intestinal permeability, inhibit epithelial repair, and increase intestinal nitric oxide synthesis; and ethanol is oxidized by intestinal bacteria to acetaldehyde, which is a toxic product that increases intestinal permeability (Alonso et al., 2014).

In individuals who are genetically susceptible to celiac disease, the transferrin receptor CD71 is expressed at high levels on the apical surface of intestinal epithelial cells (Mantis et al., 2011). When antigen-specific sIgA antibodies bind with gluten-derived deamidated gliadin peptides in the lumen, this receptor recognizes the complex, which is then transcytosed and delivered intact to the basolateral side, where these highly reactive peptides activate CD4 T cells and stimulate inflammatory processes (Mantis et al., 2011). Some bacteria secrete proteases that degrade the gliadin protein in the duodenum, and may therefore contribute to the severity of symptoms (Ruiz et al., 2014). However, there is a subset of celiac disease patients whose symptoms do not resolve with a gluten-free diet, indicating that microbial dysbiosis may be an independent risk factor (Cenit, Olivares, Codoñer-Franch, & Sanz, 2015). If commensal bacteria from the lumen penetrate the mucosal barrier, LPS from the Gram-negative bacterial cell wall would be

recognized by the CD14–TLR-4 complex and would activate the innate immune system, resulting in release of proinflammatory cytokines (Cenit et al., 2015).

T1DM is not classified as a metabolic disorder, but instead is considered an autoimmune disease; it appears to be a consequence of disruption of the mucosal microbiota and the intestinal epithelial cells, which results in intestinal immune activation and eventually leads to destruction of the pancreatic beta cells by the autoimmune system (Vaarala, 2013). A large genetic study has established that nonsecretors are more susceptible to T1DM (OR = 1.29, 95% CI 1.20–1.37; $p = 7.3 \times 10^{-14}$) (Smyth et al., 2011; Yang, Li, & Wang, 2011). It is thought that the associated decrease in diversity and stability of intestinal microbiota results in decreased mucus production and increased gut permeability followed by translocation of bacteria, with corresponding extra-intestinal inflammation and upregulation of T-cell response (Giongo et al., 2011; Vaarala, 2013).

12.2 Chronic pancreatitis

Unlike acute pancreatitis, no serum tests exist for diagnosis of chronic pancreatitis, because elevated serum lipase activity, which also occurs with a number of critical illnesses, typically does not meet the threshold of three times the upper normal limit and may even be within normal limits (Weiss et al., 2014). A recent GWAS study conducted on chronic pancreatitis patients and volunteer blood donors (Weiss et al., 2014) reported that chronic pancreatitis was significantly associated with nonsecretor status (OR = 1.53; $p = 8.56 \times 10^{-4}$) and ABO blood type B (OR = 1.69; $p = 1.0 \times 10^{-4}$). Surprisingly, asymptomatic elevated serum lipase in volunteer blood donors was also significantly associated with nonsecretor status (OR = 1.49; $p = .012$) and ABO blood type B (OR = 2.48; $p = 7.29 \times 10^{-8}$). Those with ABO blood type O had a significantly reduced risk of chronic pancreatitis (OR = 0.62; $p = 1.22 \times 10^{-05}$) and elevated serum lipase activity (OR = 0.59; $p = 8.14 \times 10^{-05}$) (Weiss et al., 2014).

Nonsecretors with ABO blood type B have a genetic predisposition to chronic pancreatitis; asymptomatic patients with elevated serum lipase who have this phenotype may have subclinical pancreatic injury, even when serum lipase levels are within the normal range, and also have an increased risk of developing chronic pancreatitis (Weiss et al., 2014). Although nonsecretors make up about 20% of most ethnic populations, because ABO blood type frequencies vary by population (ABO blood type B ranges from 8% in Norwegians to 12–15% in Western populations and 32% in Kashmiris) and genetic background varies with ethnicity, future genetic and environmental studies will need to adjust for these confounding factors in each particular cohort (Weiss et al., 2014).

12.3 Irritable bowel syndrome

Anxiety, depression, posttraumatic stress disorder, sexual abuse, and other life stressors are psychosocial risk factors for the development, worsening, and persistence of functional GI disorders such as IBS and functional dyspepsia (Alonso et al., 2014). However, it is now known that these disorders involve both intestinal barrier dysfunction and dysregulation of the brain-gut-microbiota axis (Alonso et al., 2014). Psychological and physical stress are associated with increased small intestine motility (in IBS) and enhanced visceral sensitivity, but clinical

manifestations of these functional disorders also include mucosal inflammation and increased intestinal permeability (Alonso et al., 2014).

Further, it should be noted that low-grade mucosal inflammation causes increased infiltration of mast cells, which affect intestinal permeability and activate visceral afferent nerves that signal abdominal pain (Alonso et al., 2014). Indeed, new imaging techniques have shown significant overlap between regions of the brain which modulate gut motility and visceral pain, and regions involved in processing emotion, with greater activation of the latter in response to GI distress than in healthy subjects (Alonso et al., 2014). Enteric mast cells are able to communicate bidirectionally with the ENS, ANS, and CNS, and the presence of inflammation increases contacts between mast cells and enteric nerve fibers, which has been positively associated with frequency and severity of abdominal pain (Alonso et al., 2014).

12.4 Inflammatory bowel disease

Ulcerative colitis and Crohn's disease are the two most common forms of IBD, and are distinguished by being both chronic and relapsing (Biedermann & Rogler, 2015). Such inflammatory processes may be due to functional interactions of an entire subset of the microbial community, rather than a single microorganism; known as 'community as a pathogen,' this is an emerging concept in the study of microbial pathogenesis (Blaser & Falkow, 2009; Swiatczak & Rescigno, 2012). Alternatively, a host-specific genetic effect on the composition of the microbiota could explain some of the dysbiosis that is associated with these conditions (Cho & Blaser, 2012). Autophagy, an important component of physiological processes involved in cell growth and homeostasis, is required for bacterial degradation; mutations in several genes in the autophagy pathway have been linked with susceptibility to Crohn's disease (Swiatczak & Rescigno, 2012).

At least 160 single nucleotide polymorphisms are associated with increased risk of developing one or both forms of IBD; however, the genes involved are not disease specific but are instead genes associated with PRRs, defensins, and maintenance of epithelial barrier integrity (Biedermann & Rogler, 2015). Increased antigen permeability and uptake of hepatotoxins, whether due to IgA deficiency or altered intestinal mucosa, can result in damage to hepatocytes and the chronic active (nonviral) hepatitis associated with IBD (Walker, 1978). Removal of inflamed bowel tissue, which concordantly reduces uptake of toxins, has resulted in improvement in the liver condition (Walker, 1978).

Receptor polymorphisms are associated with deregulation of resident microbial tolerance by the adaptive immune system, reduced expression of antimicrobial peptides, impaired innate immunity, and severe intestinal inflammation (Artis, 2008; Wells et al., 2011). Not surprisingly, such polymorphisms are associated with increased susceptibility to IBD, and symptoms sometimes improve following antibiotic treatment; however, it should be noted that exposure to antibiotics in early childhood has been significantly associated with increased risk for Crohn's disease (Cho & Blaser, 2012). Nonsecretor status has been associated with Crohn's disease but not ulcerative colitis (Goto & Kiyono, 2012; Weiss et al., 2014), and would account for the reduced microbial diversity found in Crohn's disease patients (Biedermann & Rogler, 2015), which has been associated with increased intestinal permeability (Box 1).

BOX 1. LESION TOPOLOGY AND SECRETOR STATUS IN CROHN'S DISEASE

When Japanese researchers (Miyoshi et al., 2011) stratified inflammatory lesions by intestinal region, secretor status was associated with colonic Crohn's disease (CD) lesions, but not with ileal or ileocolonic lesions or with ulcerative colitis (UC). The nonsense mutation (G428A) in the *FUT2* gene of nonsecretors, found in about 20% of those with Northern European heritage, is not found in the Japanese population, which instead has a different nonsense mutation (A385T), two missense mutations (C571T and C628T), and a fusion gene, with a nonsecretor prevalence of about 25%. All study subjects had the A385T mutation and ABO blood type A; whereas 80% of healthy controls and patients with UC and ileocolonic CD were secretors, only 67% of ileal CD patients were secretors, but all colonic CD patients were secretors ($p = .036$). There is normally almost no expression of ABO blood group antigens in the rectum, yet staining of rectal tissue specimens found abnormally high expression of blood group A antigen in some colonic CD patients. Colonic tissue from experimental IBD mice used in the study was stained for blood group H1 antigens and was strongly positive in symptomatic 10-week-old IL-$10^{-/-}$ mice. Of note, similar staining in asymptomatic 8-week-old IL-$10^{-/-}$ mice was also strong for H1 antibodies, although inflammation was not present. Although colonic CD is less prevalent, abnormal colonic expression of HBGAs in secretors may be a diagnostic indicator. Further studies on populations with different *FUT2* mutations and ABO blood group antigens, stratified by topology, would be highly instructive.

12.5 Metabolic diseases

A metagenomic study found a strong correlation between gut microbial communities, certain associated enzyme profiles, and obesity and IBD phenotypes in the hosts (Greenblum, Turnbaugh, & Borenstein, 2012). A large proportion of these host-state associated enzymes are involved in either the phosphotransferase system, which regulates carbohydrate uptake and has been noted as a biomarker for IBD, or the nitrate reductase pathway, which converts nitrate to nitrite, nitric oxide, and other nitrogenous end-products (Greenblum et al., 2012). Increased nitric oxide levels are carcinogenic, have inflammatory effects, and have been linked with both insulin resistance in the obese and IBD (Greenblum et al., 2012). A study with monozygotic twins found that although the proportion of microbes in fecal samples were not significantly affected by probiotics and prebiotics, RNA sequencing showed altered expression of microbial genes that control carbohydrate metabolism (McNulty et al., 2011). Metabolomic analysis of the less diverse microbiota has confirmed lower levels of butyrate, increased potential for production of hydrogen sulfide, and less capability to manage oxidative stress (Ursell et al., 2014).

Other studies with lean and obese twins have found an association between obesity and decreased numbers and diversity of bacterial species, and have identified increased energy-harvesting abilities of the bacteria that function within the obese person's microbiome (Greenblum et al., 2012; Kau et al., 2011; Kinross et al., 2011; Turnbaugh et al., 2006, 2009). There appears to be a direct correlation between the amount of weight loss and the initial bacterial makeup of the gut microbiota before dieting (Santacruz et al., 2009), and people with plentiful *B. fragilis*, *Lactobacilli*, and *Bifidobacteria* were more successful in losing weight (Biedermann & Rogler, 2015). Weight gain after a smoking cessation intervention was associated with a decrease in Proteobacteria and Bacteroidetes and a significant increase in

Firmicutes and Actinobacteria, even though caloric intake was stable or even lower than when subjects smoked (Biedermann & Rogler, 2015).

In a small study comparing bacterial diversity in normal-weight, obese, and postgastric bypass patients (Kinross et al., 2011), phylogenetic analysis found that obese patients had enhanced levels of Prevotellaceae, gastric bypass patients had higher levels of Gammaproteobacteria, whereas Firmicutes were significantly decreased in the gastric bypass patients but were dominant in obese and normal-weight patients. In addition, obese patients had significantly higher levels of hydrogen-oxidizing methanogens, mainly Methanobacteriales, resulting in increased energy uptake in the colon (Kinross et al., 2011).

Gastric bypass surgery permanently alters the gut microbiota; this change may be the driver behind complete resolution of T2DM reported in these patients before surgery-associated weight loss has occurred (Kinross et al., 2011). Recent studies with mice found that alterations in the innate immune system of the gut mucosa correlate with hyperphagia and development of T2DM and metabolic syndrome, and these metabolic changes are associated with modifications in gut microbial composition (Kinross et al., 2011). If such dysregulation of the intestinal microbiota is a central mechanism in the development of obesity and T2DM, it may be possible to reverse these conditions by changing the composition of the gut microbiota without resorting to surgery (Kinross et al., 2011).

Researchers are using fecal transplant studies with mice to investigate possible factors involved in metabolic dysfunction, ranging from insulin resistance, glucose intolerance, and cholesterol metabolism, to obesity, T2DM, and metabolic syndrome (Kinross et al., 2011). These last three conditions are all associated with low-grade inflammation, indicating a possible activation of the innate immune system in response to the intestinal microbiota (Kau et al., 2011; Tilg & Kaser, 2011). Chronic low-grade inflammation, which is associated with both obesity and metabolic syndrome, increases expression of TNF-α, which inactivates insulin receptor substrate-1, leading to insulin resistance, hyperinsulinemia, T2DM, fatty liver, and excess adiposity (Kallus & Brandt, 2012). Transplantation of the microbiota has resulted in transfer of the donor's altered phenotypic expression for cholesterol metabolism, colitis, and obesity (Kinross et al., 2011; Turnbaugh et al., 2006).

Two small but intriguing studies looked at the difference in microbial gene richness among obese and overweight people. They found that people with low gene count (LGC) were more likely to have more pronounced low-grade inflammation, more disturbed metabolism, and more overall body fat (adiposity), and were more likely to gain more body weight than people with high gene count (HGC) (Cotillard et al., 2013; Le Chatelier et al., 2013). Gene richness represents the abundance and diversity of bacterial species, or the complexity of the microbiota (Cotillard et al., 2013; Fang & Evans, 2013; Le Chatelier et al., 2013). At baseline in one study, 40% of participants were LGC, having only two-thirds of the gene richness of HGC subjects; after a 6-week calorie-restriction intervention, LGC subjects had a significant increase in gene richness that was not seen in HGC participants, accompanied by a significant decrease in adiposity and serum cholesterol and a less-robust decrease in inflammation (Cotillard et al., 2013; Fang & Evans, 2013). In the other study, although only 23% of the participants were LGC, this group had a significantly larger proportion of obese people who had gained significantly more weight

during the previous 9 years than participants in the HGC group, and their most abundant microbial species were associated with inflammation (Le Chatelier et al., 2013). Stratification of obese and overweight people by LGC and HGC may be a way to define subsets with different metabolic risk profiles and severity of inflammation-associated comorbidities (Le Chatelier et al., 2013).

Human fecal transplants have also shown promise as a possible treatment for people with conditions such as antibiotic-induced diarrhea or *C. difficile* infection (Evrensel & Ceylan, 2016; Kinross et al., 2011), and fecal transplants from lean donors to recipients with metabolic syndrome resulted in statistically significant increases in insulin sensitivity after just 6 weeks (Kootte et al., 2012). Building on these studies, fecal transplants could become a mainstream treatment following intensive or long-term antibiotic therapy, to restore the microbiota to healthy proportions and to prevent or correct dysregulation of the epithelial membrane. It is also possible that the structure and metabolic function of the "obese" microbiota could be influenced through targeted antibiotic treatment (Cho & Blaser, 2012).

Cardiovascular disease is also associated with the intestinal microbiome. Gut microbes play an obligate role in the process of converting dietary phospholipids to trimethylamine N-oxide (TMAO), which leads to formation of atherosclerotic plaques (Wang et al., 2011). The current medical approach is to take drugs and change the diet, but it may be possible to prevent or treat cardiovascular disease by changing the gut microbiome instead. One study reported statistically significant reductions of plasma lipoproteins and liver concentrations of di- and trimethylamine (TMAO precursors) following dietary supplementation with *Lactobacillus paracasei* when compared with controls (Martin et al., 2008), which suggests that targeted probiotic consumption can induce modulation of bacterial metabolism with consequent changes in host metabolic phenotypes and health status.

12.6 Effect of vaccines and antibiotics

There is no doubt that vaccinations and antibiotics have saved countless lives, and there is also no doubt that they have had unintended consequences. Loss of one pathogenic species may give opportunity to an ecological competitor, which may be more virulent and more easily transmissible (Blaser & Falkow, 2009). For example, following successful vaccination programs targeting *S. pneumoniae* , replacement with *S. aureus* has occurred, concurrent with an unprecedented surge in community-acquired methicillin-resistant *S. aureus* infections (Blaser & Falkow, 2009). And while it is not yet known whether low bacterial diversity associated with some diseases is a side effect of the specific disorder or a causative factor (Ursell et al., 2014), it is known that antibiotic usage affects the diversity of the microbiota.

Ciprofloxacin is a commonly used, broad-spectrum antibiotic that does not have some of the more unpleasant side effects of clindamycin or amoxicillin-clavulanic acid and was thought to be less disturbing to gut microbial diversity (Dethlefsen, Huse, Sogin, & Relman, 2008). However, pyrosequencing of the distal gut bacteria prior to and following a short course of treatment revealed that it rapidly and profoundly affected the abundance of 30% of the bacterial taxa, with additional individualized effects in each participant, and was pervasive in its effects, decreasing richness, diversity, and evenness (Dethlefsen et al., 2008). Although no functional disturbances

were reported, and the composition at 4 weeks posttreatment was similar to its pretreatment state, some taxa still had not recovered 6 months posttreatment (Dethlefsen et al., 2008). Despite functional redundancy, continuity of metabolic activity does not inform about more specialized functions that may have been affected, such as immune modulation, pathogen resistance, or bile transformation (Dethlefsen et al., 2008).

Two courses of ciprofloxacin separated by 6 months were each followed by a return to a state approximating the pretreatment state; however, recovery was incomplete and although the composition was stable, it was different from the original pretreatment composition (Dethlefsen & Relman, 2011). The long-term effects of a persistent altered state are unknown (Dethlefsen & Relman, 2011), but it is known that the effects of one course of antibiotics can persist for years, that some effects may not be apparent right away (Dethlefsen et al., 2008), and that antibiotics are associated with both acute and chronic health conditions (Dethlefsen & Relman, 2011). One of the consequences that may have the most long-term ramifications is acquired antibiotic resistance in the intestinal microbiota; another is replacement of a mutualist that provides a specialized function with a commensal species or strain that does not (Dethlefsen & Relman, 2011).

Since the 1950s, one of the primary sources of antibiotic intake in humans has been the U.S. food supply; subtherapeutic antibiotic treatment (STAT) of mammals and birds used for food production has reduced infection and disease rates and increased weight gain in farm animals, but there may have been unintended long-term effects on human consumers and their microbiota (Cho et al., 2012). In a murine model, significant alterations in the gut microbiome due to early-life STAT exposure resulted in increased production of hormones that regulate metabolism; changes in regulation of lipids and cholesterol in the liver; changes in genes involved in metabolism of carbohydrates to SCFAs and increased SCFAs in the colon; and not surprisingly, increased adiposity (Cho et al., 2012). It is not unreasonable to think that repeated daily STAT intake through our food supply could have similar effects in humans (Box 2).

BOX 2. ANTIBIOTICS AND TYPE I DIABETES MELLITUS

In nonobese diabetic (NOD) mice, autoimmune diabetes spontaneously develops as a result of immune-mediated destruction of pancreatic β-cells, similar to Type 1 diabetes mellitus (T1DM) in humans (Markle et al., 2013). In postpubertal, 6-week-old NOD mice, the incidence of T1DM is 2:1 female-to-male, which is thought to be due to protective effects of testosterone (Markle et al., 2013). The 6-week-old male NOD mice exposed to pulsed therapeutic-dose antibiotics (PAT) in early life (Livanos et al., 2016) had higher T1DM incidence compared to male controls (53 vs. 26%, respectively, $p < .05$). They also had reduced T_{REG} and T_H17 populations in the small intestinal lamina propria; upregulated gene expression for cholesterol biosynthesis and for steroid, sterol, lipid, and cholesterol metabolism; and downregulated serum amyloid A (SAA) gene expression (Livanos et al., 2016). In addition, they had reduced intestinal microbiota diversity with a distinct community structure, nearly complete loss of Bacteroidetes and Actinobacteria but increased Proteobacteria and *Akkermansia mucinophila* , and accelerated development of T1DM and insulitis (Livanos et al., 2016). Reduced SAA gene expression may be associated with impaired intestinal barrier function, translocation of gut bacteria, and inflammation (Livanos et al., 2016). In humans, increased risk for T1DM is associated with barrier function loss and

prior antibiotic exposure, and the PAT model was chosen because its dosing and pharmacokinetics are representative of the type and frequency of antibiotic treatments received annually by U.S. children (Livanos et al., 2016). Of note, although incidence of T1DM in humans is not sex-biased, women have a higher incidence of autoimmune diseases; the female-to-male ratio is inversely associated with age of onset for rheumatoid arthritis and MS, which is consistent with declining testosterone levels in men (Markle et al., 2013).

A new vaccine for *N. meningitidis* serogroup B is a result of completion of the HMP, which has given drug developers the ability to reverse-engineer vaccines by analyzing whole genome sequences, selecting one or more bacterial surface-exposed proteins, and developing a vaccine that includes these bacterial antigens (Gebhardt, Hutchins, Comella, & Aho, 2014). Use of such a targeted vaccine will result in production of bactericidal antibodies that recognize those proteins and generate an immune response (Gebhardt et al., 2014). However, due to *antibody cross-reactivity* , any microorganisms with antigen sequences that are highly similar to the selected proteins will also be targeted by the immune response, even if they are beneficial and protect against pathogens (Gebhardt et al., 2014).

Indeed, about 5% of the total human microbiota are nonpathogenic *Neisseria* species that share some of these very same sequences; found in the mouth and airway, some protect against dental caries, and some are thought to promote natural immunity to *N. meningitidis* (Gebhardt et al., 2014). Given existing concern about the effects of vaccines, vaccine developers will need to be careful that new reverse-engineered vaccines do not have unintended consequences for our beneficial, health-promoting bacteria.

Although no credible evidence has been found that establishes an association between childhood vaccinations and development of ASD (Mnookin, 2011), other vaccines have occasionally been associated with serious adverse events. The live virus smallpox vaccine given to active duty U.S. military service members sometimes results in myopericarditis, which is inflammation of the pericardium and/or the myocardium (McClenathan et al., 2017). Among the millions of U.S. military service members who received the vaccine since 2002, occurrence of myopericarditis and subclinical myopericarditis is estimated at 0.46 and 2.87%, respectively (McClenathan et al., 2017). Preliminary metabolomic analysis distinguished unique prevaccine and postvaccine profiles in those who experienced adverse effects (McClenathan et al., 2017). With the refinement of case definitions for this and other vaccines, metabolomics may be able to identify a metabotype or metabolic signature that could predict who might experience adverse events from their prevaccine profile alone (McClenathan et al., 2017).

12.7 Metabolomics and the microbiome

Metabolomics is used to assess the presence and quantity of thousands of metabolites simultaneously, identify biomarkers of disease, and explore genomic variations that affect function (Ursell et al., 2014). Unlike genomic analysis, which delineates potential for metabolism, metabolomic analysis provides information about actual metabolism and elucidates possible mechanisms; metabolic outcomes in the host can be directly compared with the metabolism of the intestinal microbiota (Ursell et al., 2014). The emerging field of metabolomics provides an invaluable tool to explore how disease may be associated with perturbations in either

the metabotype or gut microbial populations; how bacterial metabolites can contribute to inflammation or disease processes if the human host is unable to adequately metabolize them; and how nutritional and metabolic imbalances influence health and disease in previously unknown ways.

In a metabolomics study comparing germ-free and conventional mice (Wikoff et al., 2009), metabolism of certain dietary compounds by enteric bacteria resulted in higher serum concentrations in the conventional mice. When provided with dietary tryptophan, mice with enteric bacteria that expressed the enzyme tryptophanase had decreased serum tryptophan concentrations and increased concentrations of indole, ammonia, and pyruvate. In metabolomics studies of human patients with chronic kidney failure, elevated indole was transformed by the liver into indoxyl sulfate, which is a nephrotoxin (Wikoff et al., 2009).

Conversely, in mice colonized by different subsets of enteric bacteria, indole was transformed into indole-3-propionic acid, which is a powerful antioxidant. The tyrosine metabolites phenyl and *p*-cresol sulfate were found to be 1.4-fold higher, which was thought to be due to activation of the sulfation mechanism used by the body during phase II drug metabolism (Wikoff et al., 2009). However, glycine conjugation, another phase II drug metabolism mechanism, resulted in elevated serum concentrations of hippuric acid without reducing serum phenylalanine (Wikoff et al., 2009). These findings demonstrate a significant effect on the host's health from microbial metabolic products, and indicate the complexity of the interaction between the host and the microbiota.

A metabolomics study of progressive knee osteoarthritis in overweight and obese people (Loeser et al., 2016) identified distinct metabolomics profiles at baseline and at 18 months. Different metabolite patterns distinguished between progressors and nonprogressors at baseline for glycolate, Hippurate, and trigonelline, and at 18 months for alanine, *N,N*-dimethylglycine, glycolate, Hippurate, histidine, and trigonelline, despite BMI being similar in subjects at baseline, at 18 months, and when comparing baseline and 18 months (Loeser et al., 2016). In addition, while the mean plasma level of IL-6 was similar at baseline, it decreased by 18 months in nonprogressors but increased in progressors (Loeser et al., 2016). Hippurate and trigonelline are believed to be metabolites produced by enteric bacteria (Loeser et al., 2016), indicating that the makeup of the microbiome plays an important role in metabolic differences associated with progression of inflammation and degenerative diseases.

13 FUTURE RESEARCH

Completion of the HMP has resulted in a comprehensive catalog of reference data, ranging from taxonomically characterized discrete microbial communities to the overall function and metabolism of the healthy human microbiome (Methé et al., 2012). Not only are these data an invaluable resource that can be used to correlate and compare with data from future studies, but the protocols and methods developed and refined during this project can serve as a model for future research efforts (Methé et al., 2012). A factor that appears to have relevance for future studies is the effect of racial and ethnic heritage on the microbial communities within the human microbiota. In reporting on the progress of the HMP, areas that required further exploration included host ancestry and genetics, non-European heritage, effects of environment, and

microbiome interactions with the host (Proctor, 2011). In a follow-up HMP analysis, microbial metabolic and functional pathways were found to correlate with clinical metadata and host phenotypes, and were strongly associated with racial and ethnic heritage (Huttenhower et al., 2012).

There are still significant challenges and limitations associated with identifying the intestinal microbiota that play a significant role in metabolism of nutrients in the small intestine and research in this area should provide much-needed understanding of some of our most difficult and hard-to-treat diseases. Although the microbiota found in stool samples is generally considered to be representative of the lower intestinal microbiome, it does not accurately represent the flora found in the upper GI tract, and further, in the distal GI, the composition of the fecal microbiota differs substantially from mucosa-associated microbiota (Ouwerkerk et al., 2013). Despite recent advances in sequencing technology and metagenomic analysis of the microbiota of the large intestine, research into the small intestinal microbiota continues to be hampered (Wang & Yang, 2013).

Owing to the discrete functions and pH levels of the esophagus, stomach, and small intestine, different microorganisms are associated with each of these environments; however, relatively little research has been done into upper GI conditions, because each region must be sampled separately and there is no easy method to obtain uncontaminated specimens of these mucosal microbes (Wang & Yang, 2013). Studies have reported that the most common genera in the normal esophagus are *Streptococcus* , *Prevotella* , and *Veillonella* , with *Streptococcus viridans* being the most common microorganism (Wang & Yang, 2013). However, Gram-negative anaerobes dominated in Barrett's esophagus and esophagitis, and aerobic Gram-positive and anaerobic bacteria were most common with mega-esophageal disease (Wang & Yang, 2013). Unless *H. pylori* is present, many of these esophageal bacteria are also found in the stomach (Cho & Blaser, 2012).

Conventional testing has included cultures or biochemical assays, tissue histology, and host serological responses, which may or may not reveal the presence of a particular organism, and polymerase chain reaction-based techniques, which can detect low levels of microbes belonging to minor populations (Blaser & Falkow, 2009). It must then be determined whether the organism is part of the resident population of that region, or transient from another proximal region of the GI tract (Blaser & Falkow, 2009). For example, one study of 17 patients receiving small intestine transplants found that the normal ileal population of strict anaerobic *Bacteroides* and *Clostridium* species was temporarily replaced by facultative anaerobic species of *Lactobacillus* and Enterobacteriacae until their ileostomies were closed, whereupon the normal community structure was restored (Kinross et al., 2011).

The influence of diet on the microbiome is another emerging area of research. A well-designed study correlated changes in the diet with changes in the intestinal microbiota (Wu et al., 2011); although there were immediate effects on the microbiota (within 24 hr of dietary changes), short-term dietary changes did not correlate with reclassifications from one enterotype to another. However, this study (Wu et al., 2011) found a very strong association between long-term dietary patterns and proposed enterotype classifications (Arumugam et al., 2011). If future research shows similar strong associations between these enterotypes and certain disease states, then long-

term dietary changes may become the intervention of choice for both preventing and treating these diseases.

In addition to dietary changes or enteric infections, antibiotics induce functional changes in the microbiota, which are mediated by changes in population structure, or by introduction or extinction of specific microbial groups (Cho & Blaser, 2012). Thus, the effect of long-term subtherapeutic antibiotic levels in the diet and the evolution of antibiotic-resistant bacteria are both of increasing concern. As we have seen, low-dose antibiotic administration modulates the gut microbiota as well as the host metabolism and phenotype (Cho et al., 2012). Recent research has also documented the destabilizing effect on the microbiota of repeated or extensive antibiotic use (Dethlefsen et al., 2008; Dethlefsen & Relman, 2011) as well as the increase in adiposity associated with Fermicutes dominance (Cho & Blaser, 2012; Dethlefsen & Relman, 2011). In Italy, probiotics such as *Bifidobacterium bifidum* and *Lactobacillus acidophilus* are routinely prescribed following antibiotic treatment (Bocci, 1992).

With further delineation of the various bacterial secretion systems and greater understanding of the role of adhesins in colonization and survival of pathogenic and beneficial bacteria, anti-virulence compounds have been developed that target key proteins needed for pilus biosynthesis and curli biogenesis, or impair dimerization needed for T3SS needle production or assembly of the T4SS (Costa et al., 2015). The use of anti-adhesion vaccines that are carefully targeted to specific adhesion molecules used by specific pathogens, and which do not impair the protective functioning of probiotic or commensal bacteria, have been shown to be effective in both cattle and pigs; development of similar vaccines for human use is a research area that would have potentially life-saving benefits (Klemm & Schembri, 2000). Future research could also focus on the development of targeted drugs that block signal peptidases or impair translocase function along with the selective use of probiotics to compete with pathogens and rebalance the intestinal population (Freudl, 2013).

The growing number of children with ASD is another area of intense research. Various metabolomics studies of people with ASD have found changes in the enteric microbiota; decreased glutamate and increased taurine levels in urine; decreased plasma levels of polyunsaturated fatty acids and increased saturated fatty acids; abnormal amino acid metabolism; greater oxidative stress; and changes in plasma metabolites associated with mitochondrial dysfunction (Wang et al., 2016). A recent metabolomics study using independent discovery and validation case–control cohorts found 17 serum metabolites associated with ASD, validated 11 metabolites with univariate analysis, and in a logistic regression model, identified 2 metabolites, docosahexaenoic acid (DHA) and sphingosine 1-phosphate, that were significant predictors of ASD (Wang et al., 2016). Polyunsaturated fatty acids (PUFAs) such as DHA are necessary for the structural and functional integrity of the lipid membranes of the CNS, while sphingomyelin metabolism is associated with cerebral white matter, and abnormal white matter has been associated with ASD (Wang et al., 2016).

Among the metabolites associated with ASD, this study (Wang et al., 2016) found significant differences between cases and controls in serum l -acetylcarnitine and pregnanetriol levels, and abnormal lysophosphatide metabolism in cases. It was speculated that the abnormal fatty acid metabolism associated with ASD may be related to disturbed metabolism of estrogen; reduced l -

acetylcarnitine and decanoylcarnitine metabolism may be associated with mitochondrial dysfunction; and abnormal lysophosphatide metabolism may be associated with abnormalities in metabolism of sphingomyelin and PUFAs (Wang et al., 2016). Future metabolomics research investigating the role of the enteric microbiota and their metabolites may reveal additional associations with ASD, and could also provide a mechanism for evaluating the effectiveness of dietary, probiotic, or other therapeutic treatment of ASD.

Perhaps the most pressing concern that could be addressed by future research is the obesity epidemic, because of its associated health implications. The common wisdom has been that obesity is no more than the result of not exercising enough to burn off the calories that are consumed, and that the solution is simply to eat less and exercise more. However, millions of frustrated dieters can attest that it is not that simple. And they are correct. As this review has made clear, many factors contribute to obesity, and the unique microbiome and genetic makeup of each person must be taken into account. Here again, metabolomics analysis, as a data-driven diagnostic and predictive tool that can identify metabolic and pathway biomarkers as well as potential pathophysiologic mechanisms and phenotypes, would add a dimension to obesity research that complements, clarifies, and strengthens our ability to understand the still-emerging dynamics of host–microbiome symbiosis and may aid in changing the common belief that weight loss is simply a matter of lack of willpower.

Metabolomics research will also help us better understand functional GI disorders, unravel the mysteries of the brain-gut-microbiome axis, and explore the metabolites associated with depression, anxiety, and stress-related disorders. A small study used metabolomics to evaluate an immersion treatment program for weight loss in obese adolescents that included psychosocial inventories of depression and self-esteem (Pathmasiri et al., 2012). The urinary metabolites Hippurate, leucine, and 2-oxoisocaproate differentiated responders and nonresponders to weight loss efforts, as measured by change in BMI (Pathmasiri et al., 2012). Participants with healthy and impaired depression and self-esteem classifications on the psychosocial inventories could also be distinguished by the score plots of certain metabolites at baseline and conclusion of the study (Pathmasiri et al., 2012). The ability to integrate metabolomic signatures, psychosocial inventory classifications, and metabolic responses to dietary changes and exercise is an invaluable contribution to developing successful weight loss interventions and personalizing weight management (Pathmasiri et al., 2012). The identification of obesity-associated metabotypes consisting of specific metabolites may also help researchers understand why certain people are nonresponders and then develop weight-loss strategies that overcome those obstacles to weight loss.

14 CONCLUSION

The human intestinal microbiota plays such an important role in the healthy functioning of our bodies that it is hard to imagine how much our approach to illness and disease will change as the results of current and future research are translated into mainstream medicine and education. The current medical model and standard treatments use a "one size fits all" approach for most conditions, but as genomic and metagenomic research unravels the mystery of each of us, personalized medicine becomes more and more likely, necessary, and realistic. This review has explored how the enteric microbiota modulates mucosal barrier function and immune response,

influences the host metabolic functions, and stimulates host gene expression. We have reviewed the mechanisms used by bacteria for communication, colonization, and pathogenesis, as well as the ways the human intestine establishes and maintains homeostasis, and the role the ENS plays. We have seen how delicate the balance is between health and disease, and discussed how complex and challenging it can be to regain homeostasis and health once it is lost. The HMP project and the advances in genetic and genomic sequencing technologies have created exciting opportunities for research using newer technologies like metabolomics.

One focus for future research that may not be as obvious yet could be groundbreaking is the influence of the host genotype on the composition, function, and metabolic activity of the mucosal microbiome. Although the presence or absence of HBGAs has not been determined in most microbiome or metabolome research, they play such an important role in human health and disease that they should be controlled for as confounders or modifiers. Further, analysis of results by including HBGAs as a variable may reveal associations with statistical significance that have been missed. There is a perception that the intestinal microbiota is a controlling factor in human health; however, the HBGAs predate the microbiome. These antigens are present at birth, before the microbiome has been established and before any nutrients have passed the lips of the newborn. The glycans that form these antigens are receptors for the adhesion molecules of the assembling bacterial community, and ultimately determine which bacterial species find a supporting environment, and which do not. You are what you eat, but only because you are who you are!

ACKNOWLEDGMENTS

CONFLICT OF INTEREST

The authors have declared no conflicts of interest for this article.

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