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Benefits of bacteria-derived exopolysaccharides on gastrointestinal microbiota, immunity and health

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ABSTRACT

The gastrointestinal tract (GIT) is colonized by a broad spectrum of microorganism, of which bacteria are especially recognized for the health effects they exert. However insight in the effector molecules on the bacteria responsible are still largely lacking. Recently, bacterial exopolysaccharides (EPS), which are glycan structures on the bacterial cell wall, have gained considerable attention for their effects on human health. EPS are found to act as polymers that directly influence bacterial interactions with their host, such as bacterial adhesion to the GIT epithelium, their influence on immune responses, and the intestinal microbiota. These effects are highly dependent on the type of EPS structures and might be related to specific monosaccharides or linkages in EPS. Here, current knowledge on structure-specific EPS functions is reviewed in view of possible use of EPS to stimulate intestinal health and the formulation of dietary EPS mixtures or the possible creation of new EPS structures.

1. Introduction

The human gastrointestinal tract (GIT) is densely colonized by a broad spectrum of microorganisms that live in symbiosis with their host (Berg, 1996; Proctor et al., 2019). It is estimated that the gut microbiome is composed of>1000 different species of bacteria, fungi, archaea, protozoa and viruses (Marchesi & Ravel, 2015). The majority of these species are bacterial, which are known to exert various beneficial effects to the host (Sánchez et al., 2017)). This includes a role in food digestion processes, protection against pathogenic invasions and regulation of intestinal immunity (Hardy, Harris, Lyon, Beal, & Foey, 2013; Shi, Li, Duan, & Niu, 2017). In healthy adults, Firmicutes and Bacteroidetes are the most abundant bacterial species. Also present, although less abundant, are the actinobacteria, proteobacteria, bifidobacteria and lactobacilli (Thursby & Juge, 2017).

Dysbiosis or alterations in the composition of this complex community of bacteria have been linked or associated with over 100 diseases or disorders demonstrating the importance of a healthy microbiota for the host (Rojo et al., 2017). For example, it has been shown that symptoms in patients suffering from irritable bowel syndrome (IBS) or inflammatory bowel disease (IBD) can be reduced by modifying the gut microbiome by e.g. a microbiota transfer from a healthy individual or by addition of specific beneficial bacteria into the diet (Staudacher et al., 2017). Especially the presence of *Lactobacillus* and *Bifidobacterium* within the intestinal microbial environment has been proposed to contribute to a better healthy state of the host. *Lactobacillus* is particularly found in the small intestine, while *Bifidobacterium* is a common member of the colon (Arumugam et al., 2011; Milani et al., 2017; Stearns et al., 2011; Xiao et al., 2015).

Changes in the abundance of *Lactobacillus* and *Bifidobacterium,* as

Abbreviations: GIT, gastrointestinal tract; EPS, exopolysaccharides; IBS, irritable bowel syndrome; IBD, inflammatory bowel disease; SCFAs, short-chain fatty acids; UC, ulcerative colitis; HoPS, homopolysaccharides; HePS, heteropolysaccharides; GlcNAc, N-acetyl-glucosamine; GalNAc, N-acetyl-galactosamine; GH, glycosyl hydrolase; GT, glucosyltransferase; TEM, Transmission Electron Microscopy; TEER, *trans*-epithelial electrical resistance; PRRs, pattern-recognition receptors; TLRs, Toll-like receptors; CLRs, C-type lectin receptors; NODs, nucleotide oligomerization domain-like receptors; PAMPs, pathogen-associated molecular patterns; PSA, Polysaccharide A; FMDV, food-and-mouth disease vaccine; PP, Peyer's patch; GRAS, generally regarded as safe.

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well as other bacteria, are linked to specific health issues. However, this should not be interpreted as a suggestion that these bacteria are the only bacteria responsible for health benefits. Gut microbiota are living in a specific community and ecology, and together are responsible for production of essential molecules such as short-chain fatty acids (SCFAs) (Morrison & Preston, 2016) and indole derivates (Agus, Planchais, & Sokol, 2018), but also bacterial cell wall products that are impacting human health. Nonetheless, simple supplementation of beneficial bacterial strains in the diet has been shown to alleviate disease symptoms by restoring shortage of specific microorganisms in the gut. This was for example demonstrated in IBS, in which a reduction in both bifidobacteria and lactobacilli levels is associated with worsening of the disease symptoms (Liu et al., 2017). Similar observation were done in patients suffering from ulcerative colitis (UC), in which a decreased abundance of *Bifidobacterium bifidum*, among other bifidobacteria species, is a characteristic of disease (Duranti et al., 2016). Restoring the dysbiosis by e.g. administration of bifidobacteria and lactobacilli as probiotic contributes to lowering of UC symptoms, probably by impacting the ecology of the gut microbiota as a whole (Staudacher et al., 2017).

Another strategy that has been shown to improve health by restoring microbiota communities is by providing prebiotic fibers. These prebiotic fibers are usually carbohydrate polymers resistant to digestion in the upper intestinal tract and reach the colon intact, where they can serve as energy source for the gut microbiota and stimulate growth of specific species (Holscher, 2017). Up to now, health benefits of intervention in gut microbiota are mainly attributed to measures such as the enhancement of specific species, prevention of dysbiosis or keeping diversity of species high (Dal Bello, Walter, Hertel, & Hammes, 2001; Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, & De Los Reyes-Gavilán, 2008; Salazar et al., 2009). Although as outlined above this seems to correlate with improved health, insight in the effector molecules on the bacteria which are responsible for these health effects is still largely lacking. Some reports have proposed enhanced expression or production of cell-wall components such as polysaccharide-A (Surana & Kasper, 2012) and lipoteichoic acid (J. Y. Kim et al., 2013; Lightfoot & Mohamadzadeh, 2013; Smelt et al., 2013), as molecules responsible for health benefits.

During recent years, glycans which form part of the microbial envelope, have been proposed as important effector molecules in promoting intestinal health (Hidalgo-Cantabrana et al., 2012; Jones, Paynich, Kearns, & Knight, 2014; Verma, Lee, & Jeun, 2019; Zhou et al., 2017). Two types of glycans can be distinguished: (1) capsular polysaccharides that are tightly linked to the cells surface, and (2) exopolysaccharides (EPS), which are loosely attached to the extracellular surface or secreted into the surrounding environment. There has been a growing interest in EPS due to the role they play in the human host (Poole, Day, von Itzstein, Paton, & Jennings, 2018). It is also hypothesized that the health benefits of probiotics are linked to specific EPS structures that probiotics produce which makes studies on composition and health benefits of EPS timely and relevant (Rossi et al., 2015; Round et al., 2011).

Many bacteria produce EPS, and it appears that they exert different functions such as changing microbiota compositions, interacting with the intestinal epithelial barrier and the intestinal immune system (Castro-Bravo, Wells, Margolles, & Ruas-Madiedo, 2018; Hidalgo-Cantabrana et al., 2012). In this review, currently known EPS functions will be reviewed, as more insight in working mechanisms by which EPS exert their beneficial effects to the host might contribute to the formulation of tailored dietary EPS mixtures or the possible creation of new modified EPS structures to support specific health benefits.

2. Exopolysaccharides (EPS)

EPS can be isolated from bacteria. Some EPS molecules are already commercially available such as levans and xanthan (Mortensen et al., 2017). They hold the advantage over for example probiotics that specific bacterial products can be administered instead of whole, often living bacteria, which makes application more cost-effective, effects more reproducible and transport and shelf-life less complicated. EPS, however, are a heterogenic family of molecules and much research is still necessary to gain insight in the chemical structures responsible for health benefits. Insight in the different EPS structures and synthesis pathways involved in the production of EPS by bacteria can contribute to the formulation of tailored or modified EPS structures to promote specific health benefits. Therefore, in this section we review different molecular EPS structures and current knowledge available on the genetic background of the synthesis of EPS.

2.1. EPS structures

EPS can be divided into two main categories, the homopolysaccharides (HoPS) and heteropolysaccharides (HePS). HoPS are composed of a single type of monosaccharide, which is mostly D-glucose or D-fructose. HoPS composed of glucose can be connected through α-glycosidic linkages or with β-glycosidic linkages, resulting in α-glucans or β-glucans, respectively. Polysaccharides composed of fructose are generally constructed through β-glycosidic linkages, as in the β-fructans (Anwar, Kralj, Van Der Maarel, & Dijkhuizen, 2008; Dertli, Colquhoun, Côté, Le Gall, & Narbad, 2018; Kralj et al., 2004; Meng, Pijning, et al., 2016; Nácher-Vázquez et al., 2017; Sims et al., 2011). Apart from the glycosidic linkage of glucose or fructose, the linkage position is also used to determine the type of glucan or fructan.

In contrast, heteropolysaccharides (HePS) are comprised of two or more types of monosaccharides, with D-glucose, D-galactose and L-rhamnose being the major components in the EPS of lactobacilli and bifidobacteria. N-acetylated monosaccharides, including N-acetyl-glucosamine (GlcNAc) and N-acetyl-galactosamine (GalNAc), and L-fucose, D-glucuronic acid, D-mannose, and glycerol, are occasionally present in HePS (Badel, Bernardi, & Michaud, 2011; Hidalgo-Cantabrana, Sánchez, et al., 2014). The variety in monosaccharide composition, linkage position, branching patterns, and modifications with pyruvate and phosphate, create a large diversity in HePS structures, that generally require careful characterization.

2.2. Genetics and EPS synthesis

There is a wealth of information available about the large number of species that are able to produce HoPS and HePS (Badel et al., 2011; Hidalgo-Cantabrana et al., 2012; Lynch, Zannini, Coffey, & Arendt, 2018; Mozzi et al., 2006; Oleksy & Klewicka, 2018; Ruijssenaars, Stingele, & Hartmans, 2000; Ryan, Ross, Fitzgerald, Caplice, & Stanton, 2015; Salazar, Gueimonde, de los Reyes-Gavilán, & Ruas-Madiedo, 2016; Živković et al., 2015). In Fig. 1, a variety of examples of EPS structures that will be discussed in this review are illustrated. The biosynthesis of HoPS and HePS occur via different enzymatic pathways. In the case of HoPS, EPS is synthesized extracellularly, from either sucrose or activated UDP-glucose as donor building blocks. The synthesis of α-glucans and β-fructans is well-established, and starts off with sucrose as the donor substrate for the glycosyl hydrolase (GH) enzymes glucansucrases and fructansucrases, which catalyze the synthesis of α-glucans or β-fructans, respectively (Gangoiti, Pijning, & Dijkhuizen, 2018; Meng, Gangoiti, et al., 2016; van Hijum, Kralj, Ozimek, Dijkhuizen, & van Geel-Schutten, 2006; M. Yan et al., 2018). In contrast, the synthesis of β-glucans occurs via glucosyltransferases (GT) that use activated UDP-glucose as donor substrate (Dols-Lafargue et al., 2008; Fraunhofer et al., 2018; Werning et al., 2006). Clusters of genes can be found in HoPS-producing bacteria encoding for glycosyltransferases (GT), which are involved in the biosynthesis of repeating units, proteins related to polymerization and export of these units, as well as many other genes with unknown functions (Chapot-Chartier et al., 2010; Dols-Lafargue et al., 2008; Lebeer, Claes, Verhoeven, Vanderleyden, & De Keersmaecker, 2010).

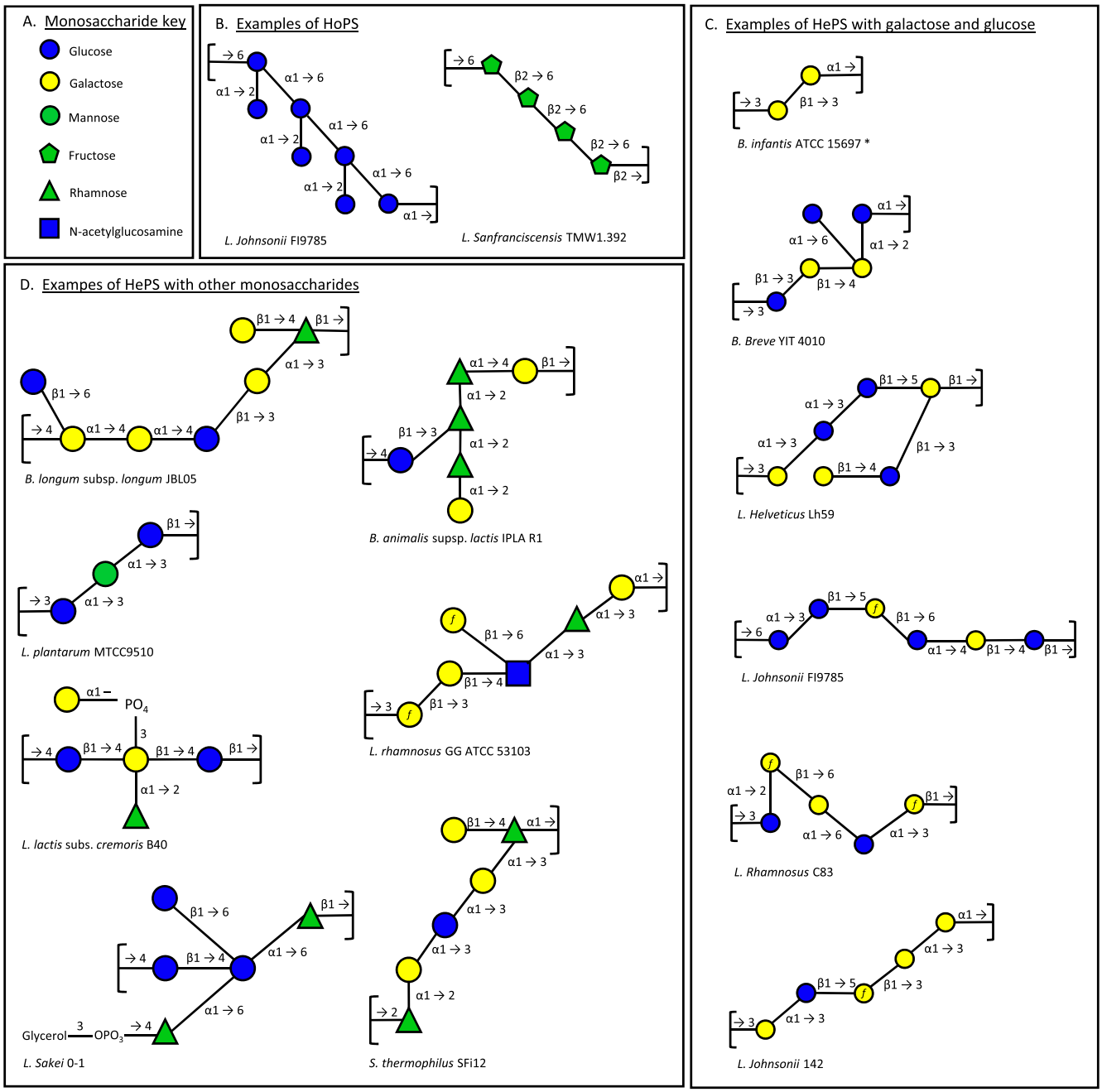

Fig. 1. Examples of HoPS and HePS structures. (A) Monosaccharides that compose HoPS and HePS. (B) Examples of HoPS structures (Dal Bello et al., 2001; Dertli et al., 2013). (C) Examples of HePS composed of galactose and glucose (Dertli et al., 2013; Górska et al., 2010; Habu, Nagaoka, Yokokura, & Azuma, 1987; Ruijssenaars et al., 2000; Tone-Shimokawa, Toida, & Kawashima, 1996; Vanhaverbeke et al., 1998) and (D) examples of HePS comprised of other monosaccharides (Hidalgo-Cantabrana, Nikolic, et al., 2014; Ismail & Nampoothiri, 2010; Landersjö, Yang, Huttunen, & Widmalm, 2002; Ruijssenaars et al., 2000). * backbone is partially substituted with β-D-Glucose in the carbon C-6 of both monosaccharides.

As HePS are structurally more complex than HoPS, it naturally follows that the mechanisms of biosynthesis are also of higher complexity. (Hidalgo-Cantabrana, Sánchez, et al., 2014). The biosynthesis of HePS is very complex and many details still remain to be elucidated. Overall, the biosynthesis is hypothesized to occur in four steps (Hidalgo-Cantabrana, Sánchez, et al., 2014; Oleksy & Klewicka, 2018). Firstly, an intracellular build-up of individual building blocks such as monosaccharides occurs. The first building block is then linked to a lipid carrier located in the cytoplasmic membrane by the priming-GT. During the third step, other building blocks are added through different GTs to create the repeating units. The fourth step is the extracellular secretion of the units whereupon the repeating units polymerize into EPS. The secretion has been hypothesized to occur either via ABC-transporters or the "flippase"-polymerase complex. The ABC-transporter genes have only been found in bifidobacteria (Ferrario et al., 2016). Even though it is hypothesized that this system could participate in heteropolymer formation, the exact function of ABC-transporters within this system is still not well understood. The flippase-polymerase complex can be found in both bifidobacteria and lactobacilli, and consists of Wzx (flippase), Wzy (polymerase) and Wzz (a tyrosine kinase). The latter determines the final length of the HePS, as demonstrated in both lactobacilli (Hidalgo-Cantabrana, Sánchez, et al., 2014; Horn et al., 2013) and bifidobacteria (Castro-Bravo et al., 2017).

Within the lactobacilli, genes involved in the production of HePS are

organized in *eps* clusters and have a functional-structural organization. In the case of bifidobacteria, Ferrario et al (Ferrario et al., 2016), conducted a wide search for *eps* clusters in *Bifidobacterium,* and identified at least one *eps* cluster in each *Bifidobacterium* genome, with the exception of *B. bifidum*. The similarity in organization however, as has been described in lactobacilli, was lacking in the *Bifidobacterium* genome (Salazar, Gueimonde, de los Reyes-Gavilán, & Ruas-Madiedo, 2016). Additionally, interspecies variability in terms of cluster length and the number of genes within the *eps* loci was detected. Ferrario et al. identified nine relatively conserved *eps* regions, as well as 44 unique gene cassettes, that were shown to be exclusively present in a single bifidobacterial genome (Ferrario et al., 2016). This wide variety of genes within bifidobacteria contributes to the hypothesis, that although many bifidobacteria produce HePS, the structure and length of these polymers can differ tremendously, resulting in diverse biological functions in the host.

3. Beneficial effects of EPS in the gastrointestinal tract.

Over the past years a lot of knowledge has become available on the various beneficial effects bacterial EPS exert within the gastrointestinal tract of the host (see Fig. 2). These effects are highly dependent on the molecular structures of the EPS, as there are studies showing that slight modifications of EPS structures can change or even completely abolish their biological effects (Salazar et al., 2008). In the following sections we will discuss the effects of specific EPS structures on digestion and fermentability by intestinal microbiota by acting as a prebiotic carbohydrate, the effects of EPS on the intestinal epithelial barrier and the interactions of EPS with the mucosal immune system.

3.1. Fermentation of EPS by the intestinal microbiota and the formation of SCFAs

To consider the use of EPS as prebiotic food supplements, according to Gibson et al., they should act as "substrates that are selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). To meet this, EPS should exert two important functions. First of all, they should not be digested or absorbed in the upper gastrointestinal tract and arrive in the colon. For fructans and β-glucans it has been shown that there are no human enzymes that can degrade them, and therefore HoPS can reach the colon in an undigested form. The indigestibility of EPS was tested, for example, for EPS from *Lactobacillus sanfranciscensis*, a β-2,6 linked fructan, which was shown to be stable at low pH and after heat treatment. (Korakli, Gänzle, & Vogel, 2002). Due to the complex nature of HePS, an even higher resistance of digestion has been postulated compared to HoPS, however this has not yet been demonstrated *in vitro* or *in vivo* (Castro-Bravo et al., 2018).

The second function that EPS should exert to be considered as prebiotic food supplements is that they should be utilized by specific members of the gut microbiota when reaching the colon. The degradation of EPS structures by microbiota-derived enzymes into products beneficial for the host has been described for several HoPS (Korakli et al., 2002). For example, in a study by Korakli et al. (Korakli et al., 2002) fermentability of high molecular weight HoPS produced by

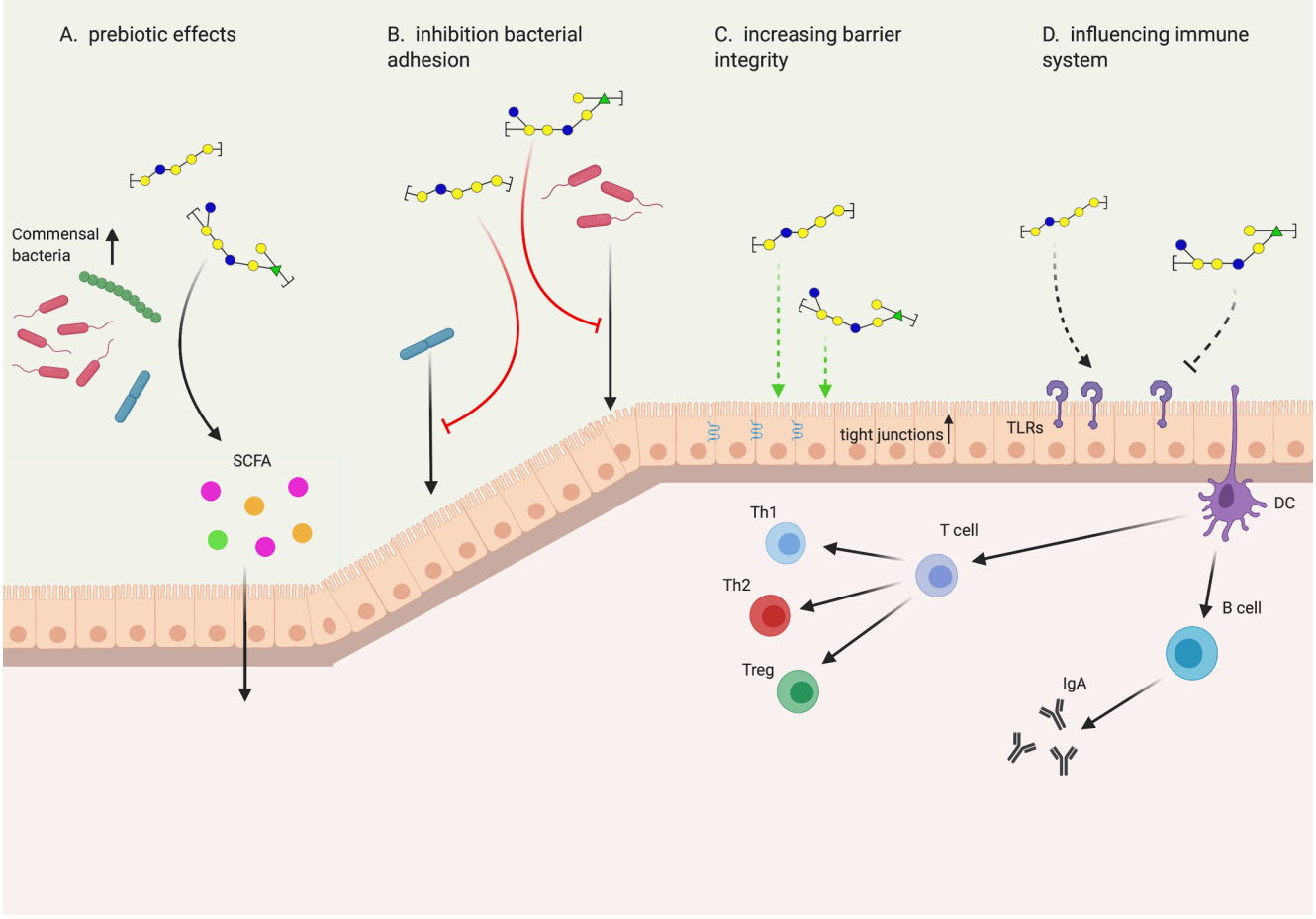

Fig. 2. Overview of the beneficial effects EPS exert within the gastrointestinal tract. (A) EPS can stimulate the growth of beneficial bacteria, (B) inhibits the bacterial adhesion to the intestinal epithelium and (C) increase the intestinal barrier integrity by upregulation of tight junctions. EPS can also (D) influence the immune system by direct and indirect interactions with Toll-like receptors.

Lactobacillus sanfranciscensis by bifidobacteria and lactobacilli was studied. The HoPS used in this study consisted of 99.3% fructose and 0.7% glucose. Fermentation capability was dependent on the type of bacteria as bifidobacteria, including *B. adolescentis, B. bifidum, B. breve* and *B. infantis* were able to ferment the EPS of *Lactobacillus sanfranciscensis* and produced acetate, lactate and formate, whereas lactobacilli failed to ferment this EPS

Ruijssenaars et al. (Ruijssenaars et al., 2000) investigated fermentability of HePS produced by streptococci, lactobacilli and lactococci (Fig. 1). They found that HePS from *Streptococcus thermophilus* SFi12, constituted of rhamnose, galactose and glucose, could be readily degraded by adult human microbiota. However, EPS isolated from *Lactococcus lactis* ssp. *cremoris*, constituted of rhamnose, galactose and glucose, as well as EPS isolated from *Lactobacillus sakei*, containing rhamnose, glucose, phosphate and glycerol, were found to be less accessible to degrading enzymes due to the composition (Ruijssenaars et al., 2000). Both nondegradable EPS contained two side chains, for *Lactococcus lactis* ssp. *cremori, one side chain* was composed of rhamnose and the second side chain contained galactose and phosphate. For *Lactobacillus sakei* one side chain is composed of glucose, whereas the second side chain consists of rhamnose, phosphate and glycerol. These side chains are uncharged and charged, whereas the degradable EPS from *Streptococcus thermophilus* SFi12 only had a single beta-galactosyl residue as a side chain, illustrating the effect of EPS structure on the degradation capacity of the microbiota (Ruijssenaars et al., 2000).

In another *in vitro* study, HePS isolated from bifidobacteria, *i.e. Bifidobacterium animalis, Bifidobacterium longum,* were evaluated for their potential to be fermented by human fecal slurry obtained from three healthy adult donors, aged 31 to 40 years (Salazar et al., 2008). These bifidobacteria HePS were mainly composed of glucose and galactose, but can also contain rhamnose and differ in specific composition, however, these structural differences were not further elucidated. It was concluded that HePS of bifidobacteria are readily fermentable substrates for the microorganisms of the human gut environment, as fermentations with these HePS resulted in increased levels of short chain fatty acids (SCFAs) (Salazar et al., 2008; Salazar et al., 2009). Furthermore, externally added EPS were found to influence microbiota compositions. It was illustrated that the EPS exerted a moderate bifidobacteria enhancing effect (Salazar et al., 2008).

The enhancing effects of different EPS structures on bifidobacteria-populations were also shown by Dal Bello et al (Dal Bello et al., 2001). In their study, levan-type EPS from *Lactobacillus sanfranciscensis*, which were composed of β-2,6 linked fructose moieties, were fermented in batch cultures inoculated with human fecal samples of healthy adult subjects. The enrichment of *Bifidobacterium* spp. upon fermentation of the EPS was accompanied by growth of *Eubacterium biforme* and *Clostridium perfingens* (Dal Bello et al., 2001). Salazar et al. (Salazar et al., 2008) determined the microbial modulation by HePS of *B. animalis, B. longum and B. pseudocatenulatum*. In this study all HePS included were found to increase the levels of bifidobacterial species in batch cultures using adult human fecal microbiota. In addition, increases of microbial levels were observed for *Anaerostipes caccae* and *Clostridium polysaccharolyticum* (Salazar et al., 2008). This illustrates the fermentability of EPS in the colon by several microorganisms known for their health benefits.

Even though bifidobacteria species have been most studied for their EPS fermenting capacity, there are also other species known that can degrade EPS structures. For example, Rios-Covian and colleagues (Rios-Covian et al., 2016) showed that *Bacteroides fragilis* DSMZ 2151 was able to grow on purified EPS from *B. animalis* ssp. *lactis,* consisting of glucose, galactose, rhamnose and phosphate, and *B. longum* E44, consisting only of glucose and galactose, when added as the sole carbon source in a minimal medium. Bacterial counts increased upon fermentation and a concomitant release of SCFA and organic acids was measured.

As mentioned before, fermentation of EPS by specific bacteria may lead to the formation of SCFAs, which have various beneficial effects in the host, such as providing energy for colonocytes (Hamer et al., 2008), maintenance of the intestinal barrier (de la Cuesta-Zuluaga et al., 2019) and modulating host metabolism (Delzenne, Neyrinck, & Cani, 2011) and immune responses (Vieira et al., 2015). In a recent study, it was found that fermentation of the EPS from *B. breve* in an infant colon model with feces from healthy, full-term breast-fed infants increased the formation of the SCFAs acetate, propionate and butyrate, which might contribute to the promotion of infant health (Püngel et al., 2020). Another study, by Salazar et al., showed that SCFA formation can be directly influenced by specific EPS structures (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, De Los Reyes-Gavilán, et al., 2008). They found that the SCFA production upon the fermentation of HePS from different bifidobacteria strains resulted in the production of different acetic acid-to-propionic acid ratios, indicating that the SCFA production was dependent on the specific EPS composition rather than on the EPS producing species, as different bifidobacteria strains all produce different HePS structures. In addition, this study also found structure dependent differences in the production of butyric acid (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, De Los Reyes-Gavilán, et al., 2008). This indicates that it might be possible to use specific EPS structures to promote the formation of specific metabolites, and thereby stimulate certain health benefits.

EPS might also stimulate bacterial cross-feeding, where the fermentation products of one bacterial strain can provide substrates for other bacterial populations and thereby promote the growth of bacteria that cannot degrade EPS themselves (Belenguer, Duncan, & Calder, a G., Holtrop, G., Louis, P., Lobley, G. E., & Flint, H. J. , 2006; Duncan, Louis, & Flint, 2004; Falony, Vlachou, Verbrugghe, & De Vuyst, 2006). For example, the fermentation of β2,1-fructans increased the number of bifidobacteria and increased the butyrate production in the colon (Falony et al., 2006). Bifidobacteria are able to produce several different SCFAs, however, has never been reported to produce butyrate. Furthermore, direct fermentation of β2,1-fructans by butyrate-producing bacteria is scarce (Duncan et al., 2003). Falony et al. (Falony et al., 2006) described a cross-feeding mechanism between bifidobacteria and butyrate-producing bacteria, where the free fructose and acetate produced by bifidobacteria supported the growth of butyrate-producing bacteria, leading to an increase of bifidobacteria as well as a higher butyrate production. This illustrates the complex role of EPS in changing in microbiota compositions and subsequent production of bacterial metabolites.

4. Interactions between EPS and the intestinal epithelial barrier

In addition to the effects on the intestinal microbiota compositions, EPS can also interact with processes related to the intestinal epithelial barrier, including influencing bacterial adhesion of both pathogenic and beneficial strains to the epithelial cell layer. There are also studies available on the direct effects EPS on epithelial cell barrier integrity (P. Ruas-Madiedo et al., 2010; Zhou et al., 2018). In the next sections the effect of EPS on the intestinal epithelial barrier will be discussed.

4.1. Effect of EPS on bacterial adhesion to the intestinal epithelium

Different techniques can be used to quantify the adhesion of EPS-producing bacteria *in vitro*, including: (1) labeling the bacteria either using ^{3}H nucleotides or fluorescent probes, (2) expressing fluorescent proteins on the bacterial surface or (3) using conventional culturing techniques and count the number of adhered bacteria (Castro-Bravo et al., 2017; Patricia Ruas-Madiedo, Gueimonde, Margolles, de los Reyes-Gavilán, & Salminen, 2006; Živković et al., 2015). EPS-negative mutants were created in order to compare the adherence to gut epithelial cells. All these techniques have been applied to demonstrate that EPS can contribute to reduction of adherence of pathogens to intestinal cells, possibly due to shielding of the surface macromolecules acting as adhesins (Castro-Bravo et al., 2018). The effects of EPS on

adhesion by beneficial bacteria and pathogens have been investigated, and will be described in the next sections.

4.1.1. EPS prevents bacterial adhesion to the intestinal epithelium

As EPS is surrounding the bacterial cell wall and possibly shields surface proteins involved in adhesion, is was hypothesized that EPS has a negative effect on the bacterial adhesion to the intestinal epithelium. Although shielding surface proteins might not result in efficient bacterial adhesion, by shielding other surface proteins normally targeted by the immune system, EPS is required for evasion of the host immune system and therefor essential to the survival of the bacteria in the gut (Aslam et al., 2008; Chapot-Chartier et al., 2010; Lebeer et al., 2010; Murofushi et al., 2015). There are various studies available showing that EPS decreases adhesion to the gut epithelium.

In an *in vitro* study by Lee et al. (Lee et al., 2016), mutated *L. plantarum* Lp90 bacteria, that were unable to produce EPS, demonstrated that EPS prevents commensal bacteria to adhere to epithelial cells. A similar observation was done with *B. longum* in which deletion of *cpsD*, the gene encoding the priming glycosyltransferase, resulted in no EPS production and therefor a lower density of the capsular layer in *B. longum*, as determined with Transmission Electron Microscopy (TEM). This gene deletion increased the adherence to Caco-2 cells compared to the wildtype, demonstrating that EPS prevents adhesion to the epithelium (Tahoun et al., 2017). A different bacteria, *Lactobacillus johnsonii* FI9785, has been shown to produce two types of EPS, the HoPS α-glucan and a HePS composed of galactose and glucose (Dertli et al., 2013). Deletion of *epsE*, which is responsible for the priming glycosyltransferase of the HePS, resulted in the production of only α-glucan, and increased the adherence ability to chicken gut explants compared to the wildtype. A different *Lactobacillus johnsonii* FI9785 mutant epsCD88N, which has an accumulation of both EPS types, showed lower adherence than the wildtype (Dertli, Mayer, & Narbad, 2015). Lastly, Lebeer et al. (Lebeer et al., 2010) showed with their *in vitro* work that an EPS non producing mutant of *L. rhamnosus GG* had improved adhesion to Caco-2 cells. This indicates that EPS negatively influences the bacterial adhesion to the epithelial cells.

In vivo studies confirm this phenomenon that EPS prevents bacterial adhesion to the intestinal epithelium as EPS of *B. breve* UCC2003, with its composition still unknown, did not increase the colonization in mice when compared with the non-EPS-producing mutant strain, which is in line with the *in vitro* studies mentioned above (Fanning et al., 2012). Other *in vivo* studies confirm the importance of adhesion-preventing effects of EPS to gut epithelium (Denou et al., 2008; Dertli et al., 2015), and simultaneously illustrate a complex role of EPS for survival of bacteria in the host. As will be discussed later on, EPS is required for evasion of the host immune system and therefore essential to the survival of the bacteria in the gut.

4.1.2. EPS-dependent effects on pathogen adhesion to the intestinal epithelial

The role of EPS in epithelial adhesion of commensal bacteria makes EPS of particular interest for preventing adhesion of pathogenic bacteria. This is supported by several reports. For example, Živković et al. (Živković et al., 2016) demonstrated that EPS produced by *Lactobacillus paracasei* subsp. *Paracasei* BGSJ2 plays an essential role in the prevention of adhesion of *E. coli* to Caco-2 cells. This strain, that produces two different HePS with still unknown composition, one with a low molecular weight (13,600 Da) and one with a higher molecular weight (41,200 Da), was able to reduce the adherence of *E. coli* to Caco-2 cells. However, a mutant *Lactobacillus paracasei* subsp. *Paracasei* EPS7, that was not able to produce the low molecular weight HePS, was not able to inhibit *E.coli* adherence, indicating that only one of the EPS of *Lactobacillus paracasei* subsp. *Paracasei BGSJ2* was responsible for the observed anti-adhesion effects (Živković et al., 2016).

There are also *in vivo* studies showing a decrease of pathogen adhesion induced by EPS. In poultry, the administration of *L. johnsonii* FI9785, a poultry-derived isolate that produces the HoPS α-glucan and a HePS constituted from galactose and glucose, suppressed the colonization and persistence of *Clostridium perfringens* and reduced the colonization of *E. coli* in twenty day old chicks (Dertli et al., 2013; La Ragione, Narbad, Gasson, & Woodward, 2004). In a different study, mice were treated with *B. breve* UCC2003 and the non-EPS-producing isogenic mutant *B.breve* UCC2003-EPSdel, followed by infection with *C. rodentium* (Fanning et al., 2012). *B. breve* UCC2003 was able to protect the mice against the pathogen, and at the end of the study (day 14) *C. rodentium* was absent in the colon or cecum. *B.breve* UCC2003-EPSdel did not protect against *C. rodentium* (Fanning et al., 2012). Taken all together, this data indicates that EPS can play an important role in the prevention of adherence of pathogenic bacteria to the host intestinal epithelium.

4.1.3. Decoy effect of EPS

Administration of EPS can lead to binding of adhesion molecules on pathogenic bacteria in the gut and thereby serve as decoy receptor (Lynch et al., 2018). It has been believed that this leads to decreased accessibility and binding of the pathogen to the intestinal epithelium (Aslim, Onal, & Beyatli, 2007). The decoy ability of EPS to pathogens is frequently studied by the use *in vitro* models. Early studies focused on of the ability of probiotic bacteria to bind to pathogens, as illustrated by García-Cayuela et al. In this study, 126 strains of *Lactobacillus plantarum* were isolated from raw milk cheeses and the binding capability to *Staphylococcus aureus, Listeria monocytogenes* and *Escherichia coli* O157:H7 was determined. Different levels of binding were found between *Lactobacillus plantarum* strains as well as differences in pathogen specificity. *L. plantarum IFPL156* showed a higher binding affinity towards *L. monocytogenes*, whereas *L. plantarum IFPL162* bound *S. aureus* to a higher extend. However not specifically described, it can be speculated that these effects might be dependent on the differences in EPS structures that the different *Lactobacillus plantarum* strains produce. In another study, it was shown that the supernatant of *Lactobacillus acidophilus* A4, which includes EPS of unknown composition, was able to reduce the adhesion of *E. coli* O157:H7 to HT-29 cells (Y. Kim, Kim, Whang, Kim, & Oh, 2008). Inhibition was only found during co-treatment, since pre-treating the cells with supernatant did not result in a reduction in adhesion. This illustrates the effect on the pathogenic bacteria specifically, possible due to a decoy effect which prevents the bacteria from binding to epithelial cell layers (Y. Kim et al., 2008).

4.2. Effects of EPS on epithelial barrier integrity

The integrity of the host epithelial cell layer can be influenced by EPS of various bacteria. Epithelial cells are sealed together by tight junctions which compromise the gut barrier. These tight junctions consist of complex lipoprotein structures that link with proteins from the adjacent cell. The primary proteins identified as tight junction transmembrane proteins are claudins and occludin (Van Itallie & Anderson, 2006). Many different claudins are known and several have shown to decrease permeability of the intestinal barrier (claudins 1, 4, 5, 7, 8, and 14), whereas others appear to increase permeability (claudins 2 and 16) (Van Itallie & Anderson, 2006).

HePS isolated from *Lactobacillus rhamnosus GG, Bifidobacterium animalis* and *Bifidobacterium longum* were studied for enhancing the barrier integrity. All HePS contained glucose, galactose and rhamnose, but differed in monosaccharide ratios. In addition, HePS from *B. animalis* also contained phosphate, which was not detected in the HePS from *B. longum* and *L. rhamnosus* (P. Ruas-Madiedo et al., 2010). HePS from all three strains were shown to mitigate the cytotoxic effects of *Bacillus cereus* toxins on barrier integrity in a model using Caco-2 cells (P. Ruas-Madiedo et al., 2010). In a different study, HePS produced by *Streptococcus thermophilus* MN-BM-A01, consisting of rhamnose, glucose, galactose and mannose, was able to rescue the barrier integrity of Caco-2 cells upon lipopolysaccharide (LPS) stimulation (Chen, Zhang, & Ren,

2019). In this study, LPS decreased the *trans*-epithelial electrical resistance (TEER) and increased the permeability as indicated by higher dextran-FITC flow through the epithelial cell-layer. Pretreatment of the Caco-2 cells with HePS dampened both indicators of barrier integrity illustrating EPS induced protection for barrier disruption. On gene expression levels, LPS decreased the expression of several tight junction genes, and treatment with the *S. thermophilus* HePS prevented these disturbances in the barrier integrity *in vivo* (Chen et al., 2019).

EPS isolated from *Lactobacillus plantarum* NCU116 was shown to increase TEER and the expression of tight junction genes in Caco-2 cells. The HePS contained mannose, glucose, glucuronic acid, glucosamine and galactosamine (Zhou et al., 2017). When Caco-2 cells were treated with this HePS the expression of occludin and ZO-1 was increased, and a higher TEER value was observed compared to the control condition (Zhou et al., 2018). In another study, conditioned medium containing, among other compounds, EPS derived from *Bifidobacterium infantis* increased the TEER of human intestinal cell line T84 by upregulation of claudin-4, ZO-1 and occludin expression and downregulation of claudin-2. Upon stimulation with IFNγ and TNFα, claudin-1 and occludin was redistributed intracellularly and TEER was decreased. Pre-treatment with *B. infantis* conditioned medium protected against intracellular redistribution and attenuated the drop in epithelial resistance (Ewaschuk et al., 2008).

There are also some *in vivo* experiments available that demonstrated the gut barrier protecting effects of EPS. An *in vivo* study by Chen et al. (Chen et al., 2019) investigated the effect of HePS originating from *Streptococcus thermophilus* MN-BM-A01 in a barrier disrupting mice-DSS model. This EPS is composed of rhamnose, glucose, galactose and rhamnose in a molar ratio of 12.9:26.0:60.9:0.25, with a molecular weight of 4.23×10^5 Da. It was tested in a DSS reduced gut barrier function models. DSS reduces the colonic epithelial tight junction expression of claudin-1, occludin, and E-cadherin. Administration of the HePS of *Streptococcus thermophilus* MN-BM-A01 was able to counteract this effect and prevent disease progression of colitis in mice (Chen et al., 2019). A different *in vivo* study using the DSS mouse model, the effect of three *Bifidobacteria longum* subsp. *longum* strains with a different EPS status were investigated. In this study, DSS treatment decreased the expression of tight junction genes as well as *MUC2*, a gene involved in mucus production (S. Yan et al., 2019). Treatment with HAN4-25, a *Bifidobacteria longum* subsp. *longum* strain that does not produce EPS, did not rescue the expression. The EPS-producing strain C11A10B, not further characterized, could increase both claudin-1 and MUC2. This, however, did not result in a fully recovered mucosal barrier. Treatment with the YS108R strain, a spontaneous mutant of C11A10B which produces a not further characterized EPS with a ropy texture, indicating a higher molecular weight, was able to maintain or even increase the expression of *claudin-1, ZO-1 and MUC-2*, and resulted in alleviated colonic damage induced by DSS. Even though the exact composition of the EPS produced by these strains was not determined, the results of this study highlight the diverse functions of different EPS.

The HePS from *Lactobacillus plantarum* NCU116 was also tested in a DSS mouse model. Treatment with this HePS, consisting of mannose, glucose, glucuronic acid, glucosamine and galactosamine, prevented the intestinal barrier disruption as indicated by reduced dextran-FITC levels in the serum. The expression of claudin-1, occludin and ZO-1 was increased distinctly, whereas claudin-2 was decreased (Zhou et al., 2018). The role of *Bifidobacterium infantis* was also investigated in IL-10 deficient mice, whom develop spontaneous colitis at 6–8 weeks of age. Administration of *B. infantis* conditioned medium decreased the barrier permeability disruption and improved the clinical signs of colitis (Ewaschuk et al., 2008). In a double-blind, placebo-controlled human study, healthy volunteers where given *Streptococcus thermophilus* ST10, which produces a HePS consisting of mainly galactose and rhamnose. Supplementation with this HePS-producing bacteria significantly improved the intestinal functional barrier. The barrier function of the small intestine was enhanced as shown by a decrease in lactitol/mannitol ratio. Urinary excretion of sucralose was used as indicator of colonic permeability and was decreased upon HePS supplementation (Del Piano et al., 2014). All these studies highlight the strengthening of barrier integrity with the use of specific EPS.

5. The effects of EPS on mucosal immune responses

EPS have also been shown to influence different cell-types of both the innate and adaptive immune system. As the mucosal immune system of the gastrointestinal tract is known to be one of the largest immune sites of the body, containing almost 80% of the body's immune cells (Lewis & Blutt, 2019), interactions with luminal content, including bacterial components like EPS, are considered to be of great influence on overall human health (Shi et al., 2017). Understanding how EPS interacts with the different receptors and immune cells of the mucosal immune system might contribute to the specified use of EPS to treat disease and to promote health.

5.1. Recognition of EPS structures and interactions with the innate immune system

Microorganisms can be recognized by the innate immune system through several classes of pattern-recognition receptors (PRRs) present on a wide variety of immune cells, that play a crucial role in the regulation and initiation of immune responses (Hill & Diehl, 2018). Examples of PRRs that are able to recognize microorganisms are Toll-like receptors (TLRs), lectin receptors, including C-type lectin receptors (CLRs) and nucleotide oligomerization domain-like receptors (NODs). Nowadays there are various studies available on EPS recognition and interactions with PRRs (Fig. 3a). In the next sections, the interactions between EPS and CLRs and TLRs will be discussed.

5.1.1. Interactions of EPS with Toll-like receptors

TLRs are known to recognize a broad spectrum of pathogen-associated molecular patterns (PAMPs) (Janeway & Medzhitov, 2002). In the past decade, there have been various *in vitro* studies that investigated both the direct and indirect immune responses of activating and inhibiting nature by various EPS through their interactions with TLRs. As example of indirect immune responses, Murofushi *et al.* (Murofushi et al., 2015) found that the acidic fraction of EPS isolated from the biofilm of *Lactobacillus plantarum* using ion-exchange chromatography, could decrease the production of the pro-inflammatory cytokines IL-6, IL-8 and MCP-1 by increasing the negative regulators of TLR4 in a porcine intestinal epithelial cell line during a challenge with an enterotoxigenic *Escherichia coli* strain. The exact composition of the EPS used in this study was not determined (Murofushi et al., 2015). In a similar study, EPS from of lactobacilli and bifidobacteria were also shown to be effective in attenuating the pro-inflammatory response of enterotoxigenic *E. coli* on porcine intestinal enterocytes (Wachi et al., 2014). Capsular polysaccharide from *Salmonella typhi* was also shown to be able to reduce IL-8 production in human intestinal epithelial cells by reducing the activity of both TLR5 and TLR4 (Raffatellu et al., 2006). Although these studies showed a TLR-dependent effect, there was no direct interaction between the polysaccharide and the TLRs reported in these studies. An additional study by Aslam et al. concluded that EPS from various plants and plant pathogens are able to indirectly influence PRR signal transduction pathways by binding to calcium ions, which are a secondary messenger in multiple PRR signal-transduction pathways (Aslam et al., 2008).

Next to the studies showing the inhibitory effects of EPS by an indirect interaction with TLR receptors, there are also studies available indicating that other EPS-types might contain structural features that can be directly recognized by TLRs and thereby induce a stimulatory immune response. Lin et al. (M. H. Lin et al., 2011) demonstrated that an exopolysaccharide isolated from the biofilm of *Thermus aquaticus* YT-1 exerted an immunostimulatory effect in macrophages through the

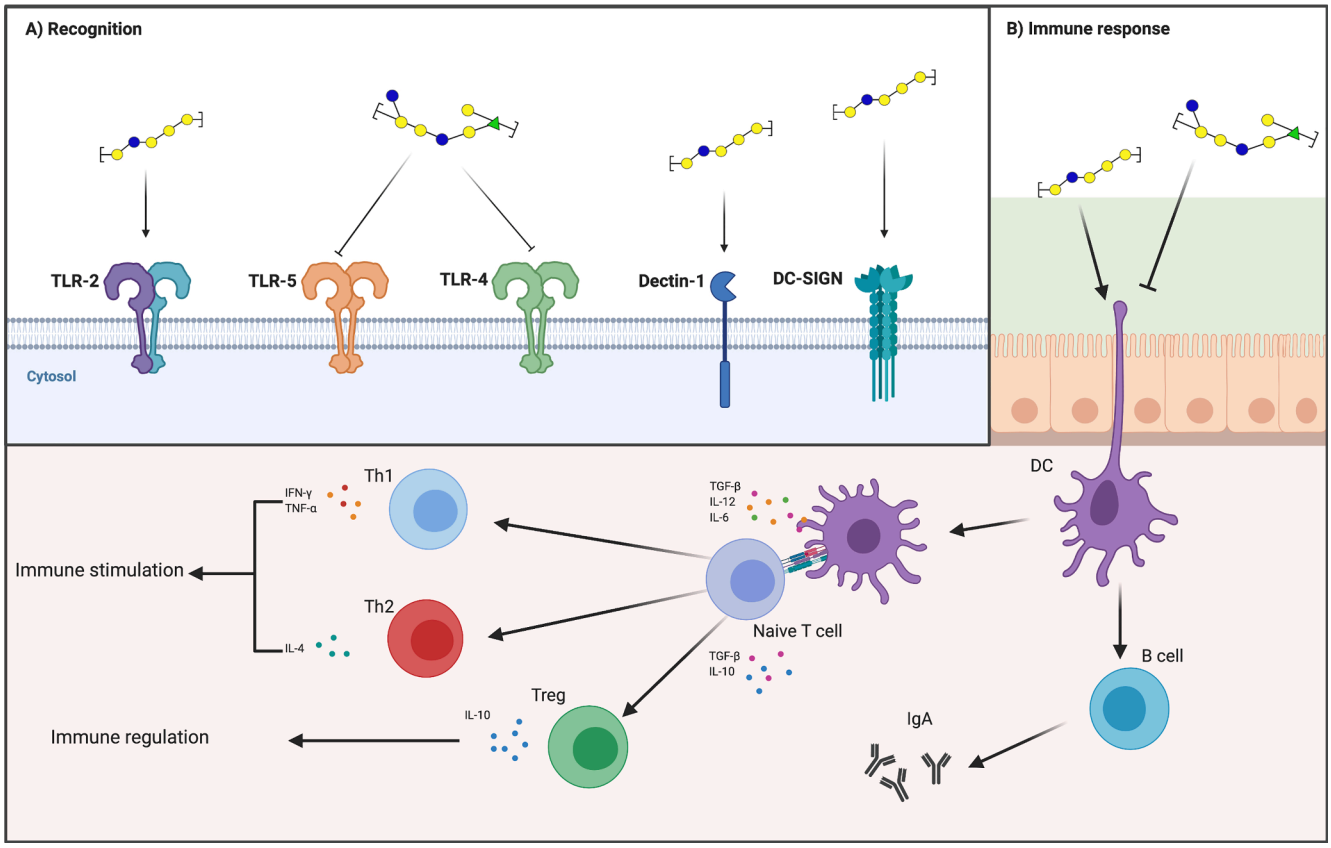

Fig. 3. Overview of the effects of EPS on the immune system. (A) EPS can interact by different PRRs, like TLRs, Dectin-1 and DC-SIGN. (B) EPS can also influence immune responses by interacting with dendritic cells (DCs) and thereby influence subsequent immune responses.

activation of the TLR2 receptor. The respective polysaccharide TA-1, a HePS composed of a tetrasaccharide repeating unit containing galactofuranose, galactopyranose, and N-acetylgalactosamine (ratio 1:1:2, respectively), induced production of the pro-inflammatory cytokines TNF-α and IL-6 in macrophages (M. H. Lin et al., 2011). Graveline et al. (Graveline, Segura, Radzioch, & Gottschalk, 2007) also demonstrated that capsular polysaccharides produced by *Streptococcus suis* were recognized by human monocyte TLR2s, triggering the release of pro-inflammatory cytokines IL-1, IL-6, IL-8 and MCP-1 *in vitro*.

Taken together, these studies show that there are different ways in which EPS interact with TLRs and TLR pathways. Although the described interactions between EPS and TLRs can contribute to the pathogenicity of certain bacteria by reducing their TLR recognition and attenuation of induced immune responses against them, modulation of TLR activity is also a strategy by which beneficial bacteria exert their immune modulatory responses. This was further illustrated by an *in vivo* study performed by Jones et al. in which administration of a single dose of EPS-producing *Bacillus subtilis* could prevent disease and inflammation induced by the enteric mouse pathogen *Citrobacter rodentium*. The EPS of *B. subtilis* was composed of mannose (88%) and glucose (11.9%). These protective effects were not observed when an EPS-deficient mutant of *B. subtilis* was used. The effects were found to be TLR4-dependent as EPS-treated TLR4 knockout mice developed disease (Jones et al., 2014).

5.1.2. Interactions of EPS and C-type lectin receptors

A class of receptors that recently gained considerable attention for their possible ability to recognize and interact with bacterial EPS are the CLRs. CLRs are a vastly heterogenous pool of carbohydrate-binding proteins (lectins), and as a result they have the ability to recognize a large variety of carbohydrates (Castro-Bravo et al., 2018). Secreted soluble CLRs, such as SP-A, SP-D and mannose- binding lectins, are involved in the innate immunity. Bound to microbes, they promote direct and indirect killing, through phagocytic uptake and activation of the lectin pathway of the complement (Castro-Bravo et al., 2018). CLRs can also be expressed in the transmembrane of immune cells from the myeloid lineage, including dendritic cells, monocytes and macrophages (McGreal, Miller, & Gordon, 2005). These CLRs can trigger signaling pathways involved in antimicrobial immunity. The ligand and binding mode have only been characterized for a few CLRs, with Dectin-1, Dectin-2 and DC-SIGN being the most examined.

Dectin-1 is expressed in primary human enterocytes and is primarily known to recognize β-1,3 glucans found in fungal cell walls (Cohen-Kedar et al., 2014). However, there are also reports of Dectin-1 activation by lipopeptide (Mae et al., 2007), trehalose-6,6-dibehenate (Dzharullaeva et al., 2018), mannoprotein (Dzharullaeva et al., 2018), and bacterial cell walls of *Lactobacillus casei* and *Mycobacterium tuberculosis* (I. C. Lin et al., 2013; Rothfuchs et al., 2007). Whether the EPS on the bacterial cell walls in these studies are responsible for the Dectin-1 activation remains unclear. Recently it was also demonstrated that different fractions of surface polysaccharides of *Bifidobacterium bifidum* strain *PRI1* induce structure specific immune responses in dendritic cells. The first fraction, a phospho-*glycero*-β-galactofuranan, PGβG, enhanced pro-inflammatory responses in DCs by increasing IFN-γ, whereas the second fraction, a mixture composed of four neutral polysaccharides named as (CSGG), composed of β-(1 → 6)-glucan, β-(1 → 4)-galactan, β-(1 → 6)-galactan and β-galactofuranan, exerted an immunosuppressive action on DCs (Speciale et al., 2019). From these results, it may be postulated that bacterial β-glucans also activate Dectin-1 receptors and thereby exert immunomodulatory effects. However, to date, this is not fully understood and more research is needed to confirm this suggestion.

Other CLRs include Dectin-2 and DC-SIGN. Dectin-2 recognizes α-mannan in fungal cell walls, mannose-capped lipoarabinomannan

from *Mycobacterium tuberculosis* and mannosylated O-antigens of Gram-negative bacterial LPS. DC-SIGN receptors are mainly expressed by DCs and bind high mannose structures and fucose-terminated glycan structures. Pathogens that are known to bind to DC-SIGN include *M. tuberculosis* and *H. pylori* (Gringhuis, den Dunnen, Litjens, van der Vlist, & Geijtenbeek, 2009). Singh et al. also found that DC-SIGN can bind the high-molecular weight fraction (>132,000 kDa) of carbohydrates purified from *Pseudomonas aeruginosa* biofilms which contained 74.9–80% mannose (Singh, Almuhanna, Alshahrani, Lowman, Rice, Gell, & Martinez-Pomares, 2020). Signaling of EPS via DC-SIGN regulates DC maturation and T cell polarization, which will be described in the next section.

As it remains undetermined how EPS interacts with transmembrane CLRs, further studies are needed to elucidate the role of these receptors in the immunomodulatory effects of EPS. The recent development of CLR-Fc fusion protein might be instrumental to study these interactions (Mayer et al., 2018), as well as the use of specific reporter cell lines expressing CLRs (Sahasrabudhe, Schols, Faas, & de Vos, 2016).

5.2. EPS effects on mucosal immune responses

As described before, EPS modulates immune responses through interaction with immune receptors. In addition, there are various studies reporting the effect of EPS on specific immune cell types and responses (Fig. 3b). Polysaccharide A (PSA) is one of 8 polysaccharides produced by *B. fragilis*. It is comprised of an unusual repeating tetrasaccharide moiety ([→3)α-D-2-acetamido-4-amino-2,4,6-trideoxygalactosep(1 → 4)β-D-Galp(1 → 3])α-D-GalpNAc(1 → 3)β-D-Galp(1 →]) and has free carboxyl, phosphate, and amino groups that contribute to the zwitterionic nature of PSA, which is important for its function (Baumann, Tzianabos, Brisson, Kasper, & Jennings, 1992). In several studies, the effect of PSA on dendritic and T cell responses has been investigated. *B. fragilis* secretes outer membrane vesicles that contain PSA. These vesicles can be taken up by DCs and induce development of plasmacytoid DCs, which produce IL-12, TNF-α and IFN-γ in a TLR2 dependent manner and thereby are able to influence the cytokine milieu *in vivo* (Round et al., 2011; Wang et al., 2006).

There are also studies showing that PSA can be processed by DCs via the endocytic pathway and present deaminated PSA fragments to CD4 + T cells carrying αβ-T-cell receptors via MHCII (Cobb, Wang, Tzianabos, & Kasper, 2004). In a study performed by Mazmanian et al. (Mazmanian, Cui, Tzianabos, & Kasper, 2005), administration of either PSA or PSA-producing *B. fragilis* altered T-cell populations in germfree mice. In this study an expansion of splenic CD4 + T cells was observed, indicating that the single species *B. fragilis* could restore part of the stunted systemic immune system in germfree mice. PSA can also influence T-cell skewing in conventional mice by inducing anti-inflammatory IL-10 producing Foxp3 + T-regulatory cells that allow the persistence of *B. fragilis* in the gastrointestinal tract (Kalka-Moll et al., 2002). Another EPS that was shown to exert anti-inflammatory effects is the EPS produced by the HTF-F strain of *F. prausnitzii* (Rossi et al., 2015). Even though the EPS itself did not activate DCs or induce cytokine production, it did reduce secretion of the pro-inflammatory cytokine IL-12p70 and upon stimulation with *L. plantarum* it increased the production of IL-10 in a DSS mouse model (Rossi et al., 2015).

As described before, Jones et al. found that EPS of *B. subtilis* could prevent disease in mice infected with *Citrobacter rodentium* (Jones et al., 2014). The same group found that EPS purified from *B. subtilis*, comprised of the 3 sugars mannose (88%), glucose (11.9%) and N-acetyl-glucosamine (<0.1%), could bind to F4/80$^+$CD11b$^+$ peritoneal macrophages after intraperitoneal administration. When EPS-treated macrophage-rich peritoneal cells of treated mice were transferred to naïve mice it could still prevent *C. rodentium* induced colitis in the recipient mice, indicating that macrophages play an important role in EPS-mediated protection against *C. rodentium* (Jones et al., 2014). EPS isolated from *L. casei* WXD030 was also found to modulate immune responses in RAW264.7 cells, a macrophage cell line. This EPS was found to be mainly composed of glucose, glucosamine and mannose in an approximate molar ratio of 1.4:1.1:1 and had a molecular weight of around 38 kDa. Incubation with this EPS induced production of nitric oxide, TNF-α, IL-1β and IL-6. In addition, it could induce maturation in bone marrow derived cells, increase production of OVA-specific antibodies and enhanced T-cell proliferation *in vitro*. When used as an *in vivo* adjuvant with the food-and-mouth disease vaccine (FMDV) in mice, the EPS enhanced FMDV-specific antibody production (Xiu et al., 2018).

There are also several other studies available reporting the effects of EPS from beneficial bacteria on antibody production of B cells, thereby serving as a mucosal adjuvant. Vinderola et al. administered kefiran to mice in their drinking water and observed an increased IgA$^+$ production in B cells in both the small and large intestine 2 days after the treatment. Kefiran was isolated from *L. kefiranofaciens*, a bacteria strain found in the fermented milk product kefir. This EPS is comprised of equal amounts of glucose and galactose and had a molecular weight of $> 10^7$ Da (Vinderola, Perdigón, Duarte, Farnworth, & Matar, 2006). EPS produced by *Leuconostoc mesenteroides* strain NTM048 was also shown to exert IgA inducing abilities in an *in vitro* and *in vivo* in a murine model. *In vitro*, the NTM048 EPS, with a size ranging from 10 to 40 kDa and mainly composed of glucose and fructose, induced total and antigen-specific IgA in Peyer's patch (PP) cells and induced Th1 and Th2 cell-mediated responses in splenocytes. *In vivo*, oral administration of the EPS induced a dose-dependent fecal IgA production and upregulation of retinoic acid synthase and TGF-β genes in PP cells. This indicates that NTM048 EPS enhances mucosal immune responses (Matsuzaki et al., 2015).

5.3. EPS to protect commensal, beneficial bacteria against immune responses

In addition to the studies investigating the interactions between EPS and the different cell types of the gastrointestinal immune system, there are also a number of studies available investigating the role of EPS of different beneficial bacteria on shielding them from innate and adaptive immune responses. For example, Lebeer et al. (Lebeer et al., 2010) also found that a long galactose-rich EPS from *Lactobacillus rhamnosus* GG could provide a protective shield against innate defense molecules, such as LL-37 antimicrobial peptide and complement factors when compared to the isogenic EPS mutant CMPG5351, increasing their persistence in the murine gastrointestinal tract. A study by Chapot-Chartier et al. (Chapot-Chartier et al., 2010) showed that the bacterial cell wall polysaccharide pellicle on the surface of *Lactococcus lactis* formed a protective barrier against host phagocytosis by murine macrophages.

Fanning et al. (Fanning et al., 2012) showed that the EPS-producing *B. breve* UCC2003 could specifically evade adaptive B-cell responses as the EPS, composed of a mixture of EPS associated with the pellicle layer and surface EPS, played a role in shielding the bacteria from phagocytosis, immune activation and antigen presentation. In this study, the EPS producing *B. breve* UCC2003 induced a lower production of IgA and IgG and thereby failed to elicit a strong immune response that would clear the microorganism from its intestinal niche, compared with EPS-deficient variants (Fanning et al., 2012). From other studies it is also known that polysaccharide capsules are associated with reduced opsonization as a result of their sterically shielding capacity (Meijerink et al., 2012). This shield also protects the bacteria against antimicrobial peptides and modulates the immune responses, possibly by interactions with immune receptor as described above. A similar effect can be envisioned for EPS.

6. Concluding remarks and future perspectives

Nowadays, some EPS are already applied in the food industry as a thickening agent (Nwodo, Green, & Okoh, 2012). Commercially available EPS, for example xanthan, are authorized as a food additive in the European Union and is approved by the US FDA for many years. EPS

produced by lactic acid bacteria are already considered to be safe immunomodifiers for inducing the mucosal immune system due to their 'generally regarded as safe' (GRAS) status (Ahmed et al. 2017). However, EPS are not yet applied for their immunomodulatory and other beneficial functions. More insight in the specific structure–function relationships of EPS of lactobacilli as well as from other strains might contribute to the formulation of specific EPS structures and promote their application as novel food additives or as therapeutics to promote intestinal health, as current research shows that the beneficial effects of EPS present on the bacterial cell walls are highly dependent on their molecular structures.

For example, it was shown that small differences in EPS structures can directly influence the growth of bifidobacterial strains and their fermentation kinetics (Ruijssenaars et al., 2000) such as kinetics of SCFA formation (Salazar, Gueimonde, Hernández-Barranco, Ruas-Madiedo, De Los Reyes-Gavilán, et al., 2008). In addition, specific EPS structures have been shown to reduce pathogen adhesion (García-Cayuela, Korany, Bustos, Gómez de Cadiñanos, & L., Requena, T., Peláez, C., & Martínez-Cuesta, M. C. , 2014; Živković et al., 2016), and to induce health benefits by interacting with certain immune receptors or cell types (Castro-Bravo et al., 2018; Matsuzaki et al., 2015; Wachi et al., 2014). The stimulation of specific bacterial strains by the application of dietary EPS might be of interest for groups of patients that have illnesses that can be directly related to a low abundance of certain bacterial strains. For example, it was found that a low abundance of *Bifidobacteria* in infants is related to the development of atopic diseases in later life (Penders et al., 2006). The effects of EPS on the host epithelium can be of particular interest as barrier disruption is associated to a variety of pathophysiologies (Chelakkot, Ghim, & Ryu, 2018; Cheru, Saylor, & Lo, 2019; Lechuga & Ivanov, 2017; Yang et al., 2019). The strengthening of the epithelial barrier by EPS could be of great benefit for patients suffering for 'leaky gut' diseases such as inflammatory bowel disease.

However, application of dietary EPS for specific patients with different health statuses comes with some challenges. Even though many studies shed light on the effects of differences in EPS structures on health benefits, there are still large gaps in our understanding of EPS structures. More research efforts on chemical structures responsible for specific health benefits are urgently needed. One of the restrictions currently faced by scientists is the limited sample size available EPS. The small amounts of EPS isolated during extractions, resulted in the need for other methods of obtaining EPS. Synthesizing EPS could lead to a higher availability of EPS. Modification of EPS structures or chemically creating EPS could also be of great value, to create single monosaccharide changes or specific side chains, in order to elucidate or enhance specific functions of EPS.

While various beneficial effects of extracted EPS have been illustrated, it is also important to identify the specific composition of the EPS in future research. As is illustrated in our review, EPS structures can differ significantly between bacterial strains and result in different outcomes in biological effect. Details into the specific EPS structures used in research are often lacking or insufficient to determine the exact structure–function relationships. When reporting findings on biological functions of EPS, special care should be taken in providing detailed information on the used bacterial strain or specific EPS structure. Additionally, the growth conditions of the EPS-producing bacteria can result in different EPS structures, as shown by (Ua-Arak, Jakob, & Vogel, 2017). Moreover, care should be taken with extracting and purifying EPS from bacteria, to prevent the presence of any impurities that might impact results, such as lipopolysaccharides, or prevent degrading the EPS during the extracting procedure. To arrive at accurate conclusions about structure–activity relationships, information on the chemical composition, linkage analysis, types of EPS modifications, molecular weight, and sample purity are essential.

In order to introduce EPS into the health sector for the promotion of specific health benefits, more insights into the positive effects of EPS on the host is required. Influences of EPS on PRRs still requires additional research, as well as a more *in vivo* safety and efficacy studies. These results will create a better understanding of the immune modulatory functions that EPS might possess. The effects of EPS on the host epithelium can be of particular interest for the promotion of health benefits, as barrier disruption is associated to a variety of pathophysiology's (Chelakkot et al., 2018; Cheru et al., 2019; Lechuga & Ivanov, 2017; Yang et al., 2019). Together with strengthening the epithelial barrier, prevention of pathogenic adhesion to the intestinal epithelium by EPS could be of great benefit for consumers.

Understanding which specific EPS structures are responsible for specific health effects will contribute to further understanding of the structure–function relation of EPS and it might also provide new ways to be explored as therapeutics. For example, with the rise of antibiotic resistance in bacteria, a desire to combat bacteria-related infections is rising. Additionally, the use of antibiotics also has its downsides, such as lowering activity and diversity of gut microbiota and side effects including diarrhea (Ladirat et al., 2013; Scribano & Prantera, 2013; Shaw, 1990). By reducing the adhesion of pathogenic bacteria to the host intestinal epithelium and enhancing the host immune response and intestinal barrier function, EPS could be used to reduce infection and support the recovery of specific pathological conditions like microbial dysbiosis after antibiotic treatment, and thereby contribute to mitigate the antibiotic burden.

Ethics statement

This work did not include any human subjects or animal experiments.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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