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Review Article

REVIEW ARTICLE

Gut biofilms: *Bacteroides* as model symbionts to study biofilm formation by intestinal anaerobes

Nathalie Béchon[1,2,†] and Jean-Marc Ghigo[1,*,‡]

[1]Institut Pasteur, Université de Paris, UMR CNRS2001, Genetics of Biofilms laboratory, 25-28 rue du docteur Roux, 75015 Paris, France and [2]Université de Paris, Bio Sorbonne Paris Cité (BioSPC), Cellule Pasteur, France

*Corresponding author:Institut Pasteur, Université de Paris, UMR CNRS2001, Genetics of Biofilms laboratory, 25-28 rue du docteur Roux, 75015 Paris, France. Tel: + 33 (0)1 40 61 34 18; E-mail: jmghigo@pasteur.fr

One sentence summary: *Bacteroides* are abundant anaerobic mutualists and facultative pathogens of the gut microbiota that are relevant model organisms to study the impact of adhesion and biofilm formation on gut microbiota stability and function.

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†Nathalie Béchon, https://orcid.org/0000-0002-0838-7946
‡Jean-Marc Ghigo, https://orcid.org/0000-0001-6528-118X

ABSTRACT

Bacterial biofilms are communities of adhering bacteria that express distinct properties compared to their free-living counterparts, including increased antibiotic tolerance and original metabolic capabilities. Despite the potential impact of the biofilm lifestyle on the stability and function of the dense community of micro-organisms constituting the mammalian gut microbiota, the overwhelming majority of studies performed on biofilm formation by gut bacteria focused either on minor and often aerobic members of the community or on pathogenic bacteria. In this review, we discuss the reported evidence for biofilm-like structures formed by gut bacteria, the importance of considering the anaerobic nature of gut biofilms and we present the most recent advances on biofilm formation by *Bacteroides*, one of the most abundant genera of the human gut microbiota. *Bacteroides* species can be found attached to food particles and colonizing the mucus layer and we propose that *Bacteroides* symbionts are relevant models to probe the physiology of gut microbiota biofilms.

Keywords: gut microbiota; biofilms; Bacteroides; anaerobic bacteria; adhesion; mucosal community

INTRODUCTION

The mammalian gastro-intestinal (GI) tract is home to a dense community of micro-organisms, composed of bacteria, viruses, archaea and eukaryotes, collectively referred to as the gut microbiota (Eckburg *et al.* 2005). The colon, where retention time is highest, hosts 10^{13} bacteria and is the major intestinal site for many processes key to human health (Sender, Fuchs and Milo 2016). This dense microbiota is involved in food digestion and nutrient intake; gut epithelium, immune and nervous system maturation; colonization resistance against pathogens; cross-talk with the nervous systems and so on. (Savage *et al.* 1981; Wostmann 1981; Bäckhed *et al.* 2004; Sudo *et al.* 2004; Mazmanian *et al.* 2005; Rooks and Garrett 2016; Kim *et al.* 2018). Consistently, community imbalances, known as dysbiosis, are known to have strong pathological implications and a lot of attention was given to factors impacting gut microbiota composition, function and stability (Xu and Gordon 2003; Bäckhed *et al.* 2005; de Vos and De Vos 2012; Laukens *et al.* 2016; Sarkar *et al.* 2016; Adak and Khan 2019).

In particular, bacterial adhesion to host surfaces has been proposed to mediate bacterial strain–host specificity, to prevent excessive shedding of bacterial cells, therefore, increasing their

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retention time in the gut, and to allow persistence of disadvantaged bacterial population during fasting periods (Sonnenburg, Angenent and Gordon 2004; Johansson, Holmén Larsson and Hansson 2011; Frese *et al.* 2013; McLoughlin *et al.* 2016). Moreover, bacterial adhesion to host surfaces often leads to biofilm formation. Biofilms are communities of micro-organisms adhering to each other or a substrate and encased in an extracellular matrix (ECM), which express lifestyle-specific physiological adaptations (Hall-Stoodley, Costerton and Stoodley 2004; Flemming and Wuertz 2019). For example, biofilm formation by gut commensals can increase complex polysaccharide degradation efficiency and increase tolerance to bile-mediated killing and to other stresses (Hung *et al.* 2006; Macfarlane and Macfarlane 2006; Dubois *et al.* 2019). Therefore, increasing attention has been given to the impact of biofilm formation on gut microbiota stability and function, and several devices have been proposed to grow gut microbiota samples as biofilms (Crowther *et al.* 2014a; Fehlbaum *et al.* 2015; Motta *et al.* 2018; Shin *et al.* 2019). However, studies on model organisms, in pure or mixed culture, still mostly focus on enterobacterial facultative anaerobes that are minor members of the community, or pathogenic bacteria such as *Clostridioides difficile*, rather than on major members of the healthy gut microbiota.

The *Bacteroides* are Gram-negative, rod-shaped, bile-resistant and non-spore forming bacteria that represent *circa* 25% of the fecal microbiota. The most represented species are *Bacteroides vulgatus, Bacteroides thetaiotaomicron* and *Bacteroides distasonis* (now reclassified *Parabacteroides distasonis*). *Bacteroides fragilis, Bacteroides ovatus, Bacteroides eggerthii* and *Bacteroides uniformis* are also abundant, but less frequently found (Salyers 1984). *Bacteroides* maintain a well-documented beneficial relationship with the host, although they can sometimes behave as pathogenic species (Xu and Gordon 2003; Wexler 2007; Wexler and Goodman 2017). *Bacteroides* are oxygen tolerant, so they can be manipulated at the bench, and they have a relatively fast generation time, making them convenient models for the study of anaerobic bacteria. The most studied *Bacteroides* species are *B. thetaiotaomicron* and *B. fragilis*.

B. thetaiotaomicron represents 6% of all feces microbiota, and 12% of all gut Bacteroidetes (Eckburg *et al.* 2005). It was the first *Bacteroides* species to be sequenced (Xu *et al.* 2003) and, since then, it has been established as a model symbiont organism studied for its ability to degrade complex sugars (Hooper, Midtvedt and Gordon 2002; Xu and Gordon 2003; Rakoff-Nahoum, Coyne and Comstock 2014). *B. thetaiotaomicron* has also been shown to provide various benefits to the host, such as immune system maturation, defense against pathogens (López-Boado *et al.* 2000; Scharek *et al.* 2000; Hooper *et al.* 2003; Kelly *et al.* 2004; De Sablet *et al.* 2009; Kamada *et al.* 2012; Delday *et al.* 2019) and gut epithelium maturation (Hooper *et al.* 2001; Stappenbeck, Hooper and Gordon 2002; Wrzosek *et al.* 2013). On the other hand, *B. fragilis*, although 10–100 times less abundant than *B. thetaiotaomicron* in the fecal microbiota, is recognized as the most virulent *Bacteroides* species as it is commonly isolated in bloodstream infections and abdominal abscesses (Wexler 2007; Sears 2009). In particular, enterotoxigenic *B. fragilis*, carrying the fragilysin metalloprotease toxin (Moncrief *et al.* 1995), was shown to cause diarrhea and has been associated with colorectal cancer (Sears 2009; Wu *et al.* 2009; Boleij *et al.* 2015; Pierce and Bernstein 2016). However, *B. fragilis* is also an important symbiont, shown to be critical for gut immune system maturation (Mazmanian *et al.* 2005; Troy and Kasper 2010). *B. thetaiotaomicron* and *B. fragilis* have been reported in the mucus layer and attached to food particles (Sonnenburg *et al.* 2005; Macfarlane and Macfarlane 2006; Lee *et al.* 2013; Yasuda *et al.* 2015; Mark Welch *et al.* 2017; Donaldson *et al.* 2020), suggesting that biofilm formation could be an important part of their lifestyle.

In this review, we will briefly present biofilms, then discuss to what extent the gut microbiota can be considered a biofilm-like structure, followed by the importance of oxygen, considering the anaerobic nature of this community, and present the most recent advances on our understanding of biofilm formation by one of the most abundant genera of the human gut microbiota, the *Bacteroides*.

HOW TO DEFINE THE DIVERSITY OF SYSTEMS CALLED BIOFILMS?

The international union of pure and applied chemistry (IUPAC) broadly defines biofilms as 'aggregates of microorganisms in which cells are frequently embedded in a self-produced matrix of extracellular polymeric substances (EPS) that are adherent to each other and/or a surface' (Vert *et al.* 2012), a generic definition that covers diverse *in vitro* and *in vivo* biofilm-like structures formed by all types of micro-organisms, including bacteria, archaea, eukaryotes and even viruses (Harding *et al.* 2009; Thoulouze and Alcover 2011; Orell, Fröls and Albers 2013). Bacterial biofilms have been extensively reviewed previously and we will only briefly describe the hallmarks of biofilm formation required to support the focus of this review on *Bacteroides* biofilms. Bacterial biofilm formation schematically involves three steps (Fig. 1): bacteria initially adhere to a surface or to other cells. Adhesion is mediated by diverse structures, such as extracellular polysaccharides, or proteinaceous adhesins such as curli, pili or autotransporters (Korea, Ghigo and Beloin 2011; Berne *et al.* 2015; Meuskens *et al.* 2019). After initial adhesion, the biofilm matures, from aggregates of a few dozen to a few thousand cells called microcolonies, to a complex 3D structure encased in the ECM known as a biofilm. The ECM is a highly hydrated structure, estimated to comprise 73–98% of the biofilm mass (Lawrence *et al.* 1991) that is composed of polysaccharides, lipids, proteins, nucleic acids, ions and so on (Fig. 1; Flemming 2016; Karygianni *et al.* 2020). The ECM contributes to the adhesive properties of the biofilm and its structure (Lawrence *et al.* 1991; Flemming 2016; Schlafer and Meyer 2017; Karygianni *et al.* 2020), prevents desiccation (Lawrence *et al.* 1991; Flemming 2016; Karygianni *et al.* 2020), and allows the formation of gradients of waste and nutrients that drive bacterial heterogeneity, leading to the emergence of biofilm-specific properties (Rani *et al.* 2007; Stewart and Franklin 2008). For instance, biofilms commonly harbor an increased tolerance to multiple stresses such as antibiotic exposure, osmotic and oxidative stress, host immune defenses and grazing protozoa compared to their free-living counterparts, in part due to the presence of the ECM itself and to the heterogeneity of bacterial growth rates within the biofilm (Flemming 2016; Karygianni *et al.* 2020). Moreover, the high cell density promotes a range of social interactions, including communication (quorum sensing and electrical communication; Parsek and Greenberg 2005; Prindle *et al.* 2015; Passos da Silva *et al.* 2017; Manna *et al.* 2020), horizontal gene transfer (Ghigo 2001; Nesse and Simm 2018), cooperation (sharing of public goods or trophic networks; Liu *et al.* 2015; Dragoš *et al.* 2018; Sivadon *et al.* 2019) and competition in multi-species biofilms (killing of other members and cheating; Rendueles and Ghigo 2015; Nadell, Drescher and Foster 2016), all of which could have very strong implications in the context of the gut microbiota. The biofilm eventually disperses to colonize other niches.

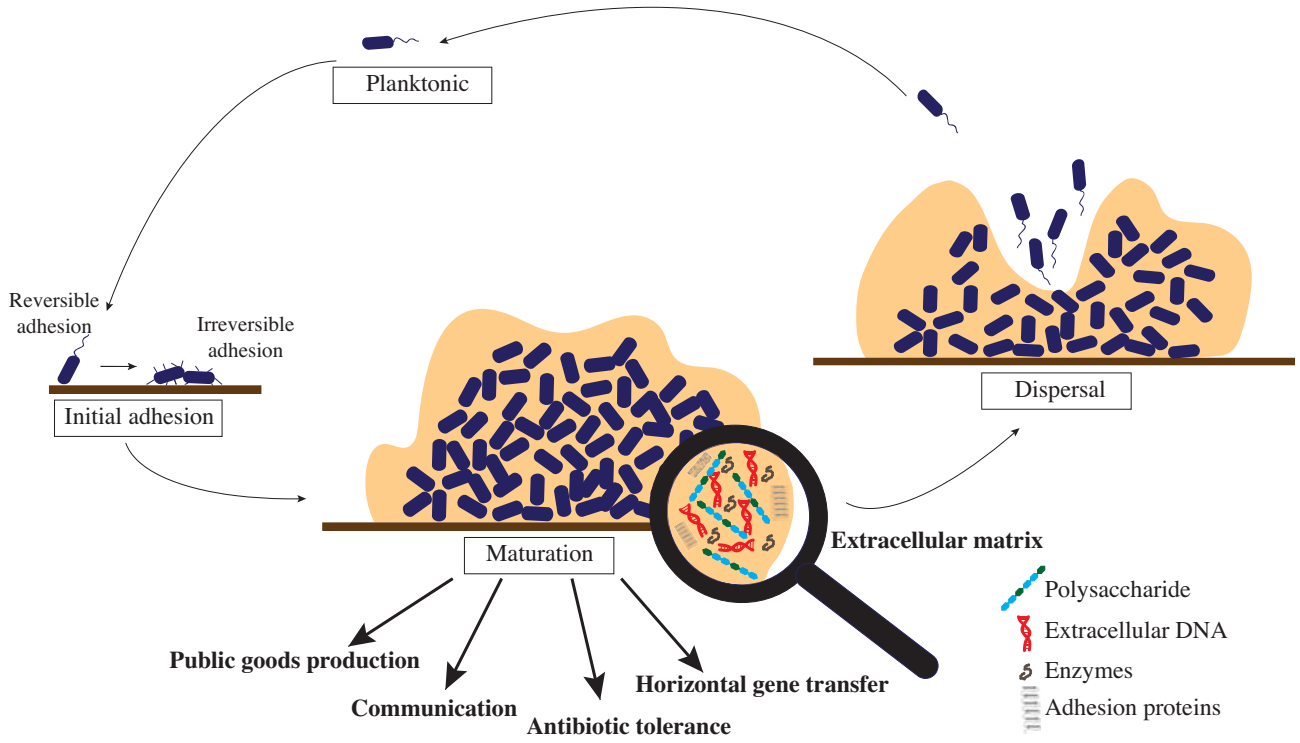

Figure 1. *In vitro* biofilm formation. *In vitro,* free-floating (planktonic) bacteria initially come in contact with a surface. After irreversibly adhering to the surface, the biofilm then develops and matures in a 3D structure encased in extracellular matrix composed of polysaccharides, proteins, nucleic acids, ions, water and so on. This organization drives cell heterogeneity and cell–cell interactions and leads to biofilm-specific properties. Eventually, cells can disperse from the biofilm and released aggregates and individual planktonic bacteria can colonize new surfaces.

ARE SOME PARTS OF THE GUT MICROBIOTA ORGANIZED AS AN ANAEROBIC BIOFILM?

Common gut molecules induce biofilm formation by gut bacteria

Several environmental conditions can impact gut bacteria biofilm formation, such as pH, oxygen concentration, nutrient availability and so on, which could determine their preferred colonization niche (Lebeer *et al.* 2007; Houot *et al.* 2010; De Weirdt and Van De Wiele 2015; Mashruwala, van de Guchte and Boyd 2017; Chiang *et al.* 2020). Moreover, as will be described in the following section, several relevant gut molecules have been shown to induce biofilm formation, suggesting biofilms might be an important lifestyle for gut bacteria.

Bile is a complex mix of proteins, such as biliverdin, and numerous cholesterol-derived bile acids (Urdaneta and Casadesús 2017). It is secreted in the GI tract upon food intake to favor the emulsification of dietary lipids, and has antimicrobials properties (Urdaneta and Casadesús 2017). Bile has been shown to induce biofilm formation in a wide range of gut bacteria including symbionts such as *B. thetaiotaomicron, Lactobacillus* and *Bifidobacterium* and the pathogens *C. difficile, B. fragilis, Listeria monocytogenes, Klebsiella pneumoniae* and *Vibrio cholera* (Hung *et al.* 2006; Pumbwe *et al.* 2007; Begley, Kerr and Hill 2009; Ambalam *et al.* 2012, 2014; Chen *et al.* 2014; Dubois *et al.* 2019; Bechon *et al.* 2021). However, when pure bile acids were tested, rather than bile extract, different bacteria reacted to different bile acids (Ambalam *et al.* 2012; Dubois *et al.* 2019; Kelly *et al.* 2020) and some authors even reported that taurine-conjugated bile acids disperse biofilms of *V. cholerae* and *Pseudomonas aeruginosa* (Hay and Zhu 2015; Sanchez *et al.* 2016), showing that bile could differentially impact biofilm formation depending on its composition. Moreover, bile promoted biofilm formation in different ways, including increased production of adhesins, extracellular polysaccharide (Crawford *et al.* 2008; Chen *et al.* 2014; Nickerson *et al.* 2017) or proteinacceous curli and autotransporters (González *et al.* 2019; Köseoglu *et al.* 2019)), increased production of the secondary messenger cyclic-di-GMP (Koestler and Waters 2014), a well-known regulator of biofilm formation, and increased production of extracellular DNA, an important component of the ECM (Béchon *et al.* 2021). Bile-dependent biofilm formation was shown to increase bacterial tolerance to bile and to antibiotics of the pathogenic gut bacteria *C. difficile, B. fragilis* and *V. cholerae* (Hung *et al.* 2006; Pumbwe *et al.* 2007; Dubois *et al.* 2019), to increase the probiotic potential of *Lactobacillus* strains (Aoudia *et al.* 2016), and impairment of *K. pneumoniae* poly-N-acetylglucosamine production reduced both *in vitro* bile-dependent biofilm formation and colonization of the mouse gut (Chen *et al.* 2014) demonstrating that bile-mediated biofilm formation could have a strong impact on gut microbiota stability and function.

In the colon, bacteria colonize the mucus layer covering the epithelium (Sicard *et al.* 2017). This gel-like structure made of glycoproteins, known as mucins, lubricates the intestine and protects the epithelium. It is composed of: (1) a dense inner layer attached to the epithelium that physically prevents contact between the luminal content and the epithelium, and (2) a looser outer layer densely colonized by micro-organisms (Johansson *et al.* 2008). Like bile, the mucus layer has complex interactions with the gut microbiota: bacteria can use it as a food source and a colonization niche, but it also physically protects the gut

epithelium from gut micro-organisms (Sicard *et al*. 2017). Several gut bacteria have been shown to be able to adhere to the O-glycan of mucins *in vitro,* including *Escherichia coli*, *Lactobacillus, B. fragilis*, *Bifidobacteria* and several pathogenic bacteria. This interaction is mediated by different structures including pili, flagellum, polysaccharides and different sugar-binding proteins (Sicard *et al*. 2017). Adhesion to mucins has been proposed to be key factor for gut colonization, allowing the colonization of the mucus outer layer in order to prevent bacterial wash out with the luminal content (Sonnenburg, Angenent and Gordon 2004). Moreover, agarose gels can be used to mimic biofilm formation within mucus, suggesting the gel-like properties of mucus are enough to induce biofilm formation, regardless of their chemical structure (Jouenne, Tresse and Junter 1994; Pabst *et al*. 2016). Conversely, it has been proposed that mucins could prevent biofilm formation of some bacterial species, by acting as a chemoattractant and favoring cell motility of *Campylobacter jejuni* or by impacting expression of biofilm-related genes in the fungus *Candida albicans* (Hugdahl, Beery and Doyle 1988; Kavanaugh *et al*. 2014; Wang, Wu and Ribbeck 2021).

Secreted IgA (sIgA) are part of the immune defense preventing contact between the gut epithelium and bacteria. sIgA bind bacteria and trap them in aggregates, preventing breaching of epithelium, a process known as immune exclusion. However, this binding was also shown to facilitate biofilm formation by *E. coli* through type 1 pilus/sIgA interactions and *B. fragilis* colonization of the mucus layer (Randal Bollinger *et al*. 2003; Bollinger *et al*. 2006; Donaldson *et al*. 2018).

Other important gut molecules can modulate biofilm formation, such as H_2S (Motta *et al*. 2015), short chain fatty acids (Chen *et al*. 2015), dietary fibers (Mirande *et al*. 2010; Rajasekharan *et al*. 2020) and so on. They have not, however, been studied as extensively as bile or mucins were in the context of biofilm formation.

Microscopic and phenotypic arguments in favor of a biofilm-like organization of the colonic microbiota *in vivo*

Observations by Palestrant and colleagues of imaging of healthy animals and human intestines suggested that the colonic microbiota of mammals is commonly organized in dense and structured communities that are phenotypically heterogeneous, with layers of cells of different size, oriented in the direction of the flow and encased in porous material reminiscent of an ECM, which most likely corresponded to the mucus layer (Palestrant *et al*. 2004). Electronic microscopy and fluorescent light microscopy also showed that in the human GI tract, bacteria often occur as aggregates or microcolonies in different microhabitats: associated to the mucus, the epithelium or to food particles (Macfarlane, Hopkins and Macfarlane 2000). Finally, fluorescent *in situ* hybridization (FISH) of a defined 15-member consortia of bacteria in a gnotobiotic mouse model showed that this community was not randomly distributed in the gut, but rather spatially structured, a process which might be mediated by adhesion to mucus, epithelium or between bacteria (Mark Welch *et al*. 2017). More generally, the observation that a part of the gut microbiota colonized the mucus layer, forming a dense community encased in a hydrated, gel-like structure, has led several authors to propose that the mucosal community was an anoxic biofilm, with consequences on host health and disease (Sonnenburg, Angenent and Gordon 2004; Macfarlane and Dillon 2007; Sproule-Willoughby *et al*. 2010), as was recently reviewed in (Motta *et al*. 2021). Moreover, bacteria from the mucus layer are phenotypically distinct from their luminal counterparts. They were shown to have an altered metabolism, and a plasmid transfer rate that was closer to that observed in *in vitro* biofilms, rather than planktonic cultures (Licht *et al*. 1999; Macfarlane, Woodmansey and Macfarlane 2005).

The controversy

There is, however, still some debate about whether these complex gut structures are proper biofilms (Tytgat *et al*. 2019; Motta *et al*. 2021). First, some laboratories failed to replicate the observations of biofilm-like structure by electronic microscopy (Swidsinski *et al*. 2007a). This might be due to protocol variations leading to biofilm destruction during sample preparation, as mucosal communities were shown to be extremely sensitive to sample preparation (Palestrant *et al*. 2004; Bollinger *et al*. 2007). Moreover, in the strictest sense, a structure can only be called a biofilm if it is encased in a self-produced ECM; something that is extremely difficult to show from *in vivo* gut samples due to the confusing presence of host-produced gel-like matrix, the mucus, and the fragility of polysaccharidic structures. Mucosal communities were shown to form biofilm *ex vivo* and to secrete ECM, but whether or not this occurs *in vivo* remains to be addressed (Buret *et al*. 2019). *Lactobacillus reuteri* was shown to form biofilms in the forestomach of rats, where no mucus layer is present, and the bacteria directly adhered to the epithelium and produced ECM. However, no such structure exists in humans, as the GI tract is continuously protected by mucus. Interestingly, only *L. reuteri* isolates from rats adhered to the rat epithelium. Strains from humans did not, but they showed adhesion to mucus, hinting that adhesion and biofilm formation could be common between strains of different hosts, but mediated by different binding abilities (Frese *et al*. 2013). The self-production of an ECM, a hallmark of biofilm formation *in vitro,* might not be so relevant in a context where host-produced mucus can display exactly the same properties as classical bacteria-produced ECM. This situation is often encountered in cystic fibrosis patients, in which aberrant host mucus production in the lungs creates a niche of biofilm formation even for the non-mucoid strains of the opportunistic pathogen *P. aeruginosa*, which produce low levels of the alginate exopolysaccharide. Yet, this model is one of the gold standard of biofilm formation *in vivo* (Moreau-Marquis, Stanton and O'Toole 2008). Finally, some authors argue that the relatively short retention time in the gut and the fast shedding of mucus makes it impossible for a biofilm to form. However, other theoretical work suggests that, on the contrary, biofilm formation is required to escape luminal content shedding and to allow maintenance in the gut (Sonnenburg, Angenent and Gordon 2004). Indeed, bacteria unable to colonize the mucus layer were shown to be deficient for gut colonization (Sonnenburg, Angenent and Gordon 2004). Moreover, high-molecular weight dietary fibers led to compression of the mucus layer in mice, and it was hypothesized that this could increase bacterial retention time in the mucus layer, allowing biofilm formation (Datta, Steinberga and Ismagilova 2016; Arias and Brito 2021).

Ultimately, the recurring problem is the perceived lack of a consistent gut biofilm definition, impeding meaningful comparisons between studies. Some authors concluded to biofilm formation so long as they observed aggregates of cells, while others strictly differentiated between aggregates, microcolonies and biofilms (Tytgat *et al*. 2019). Some authors only considered communities as biofilms if they reached a specific width, or only if they could observe direct contact with a surface, the epithelium.

The continuum between adhesion, aggregation and biofilm is easily distinguishable *in vitro*, and also in some model systems that are the tools of the trade to study *in vitro* biofilm. This transition is much more difficult to monitor in an environment such as the human gut. Perhaps the complex, dense 3D structures readily observed *in vitro* are lacking in the gut, where adhering bacteria would occur mostly as small aggregates of cells or microcolonies, similar to what has been observed for chronic infection biofilms (Bjarnsholt *et al.* 2013). Yet, considering the observed density and spatial structure of bacteria within the gut microbiota, their known adhesive properties to mucus or food particles, the presence of an extracellular gel-like structure (produced, or not, by bacteria themselves) and their original phenotypes compared to freely diffusing non-adherent bacteria; we propose that parts of the gut microbiota develop as a biofilm-like structure even in healthy condition at least in the colon. *In vitro* studies on gut bacteria should consider their adhesion properties and biofilm-specific phenotypes, as they would, in fact, be much better predictors of *in vivo* phenotypes than planktonic bacteria. In the next part, we will distinguish three niches of biofilm-like structures described to date in the colon: food particles, the outer mucus layer, and the gut epithelium surface (Fig. 2).

IN VIVO NICHES OF BIOFILM FORMATION IN THE COLON

Food-particle associated biofilm formation

Food particles transiting through the GI tract are rapidly colonized by bacteria in the cecum and the colon and 5% of the luminal bacteria were found to be strongly attached to food particles, mainly *Bacteroides* and *Bifidobacteria*, forming microcolonies (Macfarlane and Macfarlane 2006). The composition of the bacterial community recovered from the soluble feces material, a proxy for non-adhering bacteria, was found to differ from the composition of the feces insoluble material, corresponding to undigested fiber-adhering bacteria (Walker *et al.* 2008). In particular, the Bacteroidetes/Firmicutes ratio was lower in the fiber-associated community and *Ruminococcus* was found to be strongly associated to undigested fibers, demonstrating that specific communities associate with food-particles (Walker *et al.* 2008). Different insoluble plant polysaccharides were colonized by distinct communities of bacteria in continuous-flow fermenters inoculated with fecal microbiota, and the diversity of each community was lower than that of the inoculum, suggesting that colonization of food particles involves a specific set of bacteria. The initial inoculum composition, which is very variable between individuals, also had a strong impact on the food-particles community composition, showing that the same substrate can be colonized by different communities in different individuals (Leitch *et al.* 2007). Moreover, the food particle-associated community showed altered metabolic activity that digested polysaccharides such as arabinogalactan and xylan more efficiently and produced mainly acetate. By contrast, their planktonic counterparts digested oligosaccharides faster and produced more butyrate (Macfarlane, McBain and Macfarlane 1997; Macfarlane and Macfarlane 2006). These studies suggest that food particles are colonized by a specific subset of bacteria in the gut, and that this adhesion induces metabolic changes, increasing the efficiency of complex polysaccharide degradation.

The mucosal biofilm

Several studies showed that the mucus-associated microbiota, also referred to as the mucosal community, differed in composition from the fecal community (Zoetendal *et al.* 2002; Eckburg *et al.* 2005; Motta *et al.* 2021). Abundant mucosal genus include *Bacteroides, Faecalibacterium, Roseburia, Blautia* and lactic acid bacteria (De Weirdt and Van De Wiele 2015). Moreover, use of laser microdissection of mice colon allowed the comparison of mucosal and luminal communities. Mucosal communities had a lower Bacteroidetes/Firmicutes ratio, and were in particular enriched in Lachnospiraceae and Ruminococcaceae compared to luminal communities (Nava, Friedrichsen and Stappenbeck 2011). Several mucus characteristics might contribute to its colonization (De Weirdt and Van De Wiele 2015), including bacterial adhesion to the O-glycan of mucins described above (Sicard *et al.* 2017). In some cases, this correlates with mucin-bound oligosaccharides degradation, such as that performed by *Akkermansia muciniphila, B. thetaiotaomicron* or *Ruminoccocus*, although *in vivo* mucin degradation seems to implicate a consortium of bacteria rather than a single species (Sonnenburg, Angenent and Gordon 2004; Sicard *et al.* 2017). Interestingly, the composition of the mucin O-glycan varies between species, suggesting a possible mechanism to select host-specific colonizing bacterial strains (Johansson, Holmén Larsson and Hansson 2011; Wang, Wu and Ribbeck 2021). Moreover, as mentioned before, the gel-like properties of the mucus layer could also favor biofilm formation (Jouenne, Tresse and Junter 1994; Pabst *et al.* 2016). Indeed, bacterial shape and motility was shown to determine bacterial penetration in gels of different viscosity, and to correlate with spatial organization of bacteria within the mucus layer. This suggests that local viscosity of the mucus could select for specific bacterial shapes, rather than species (Swidsinski *et al.* 2007b). A recent review examined in detail the biophysical properties of mucus that could impact biofilm formation. Interaction between bacteria, bacteria-secreted extracellular polymers, host-produced mucins and environmental parameters can drive aggregation through osmotic pressure or electrostatic interactions, independently of classical biofilm determinants such as adhesins (Arias and Brito 2021). Thus, the intrinsic biochemical properties of the mucus layer could favor initial bacterial aggregation and lead to biofilm formation.

Interactions between bacteria and host factors, such as the immune system or oxygen diffusion through the epithelium, are also key factors to allow colonization of the mucus layer (Van den Abbeele *et al.* 2011). *In vitro* studies mimicking the gut environment enabled the functional characterization of mucus-associated versus luminal communities. A fecal microbiota rapidly colonized an *in vitro* mucus gel in a fermenter, and this mucus-colonizing community differed from a planktonic community in both taxa representation and sugar metabolism. However, only one fermenter was followed for each tested condition, preventing definitive conclusions from being drawn (Macfarlane, Woodmansey and Macfarlane 2005). Using an *in vitro* chemostat system mimicking the lumen and mucosal compartments of the gut, McDonald *et al.* (2015) also showed that the planktonic and mucus-associated biofilm communities differed in composition and in reaction to antibiotic perturbations. These studies highlight the importance of taking into account, when studying the stress metabolism and stress responses of symbionts, where bacteria are localized in the gut, not just which bacteria are present, and whether they are in a biofilm or planktonic state.

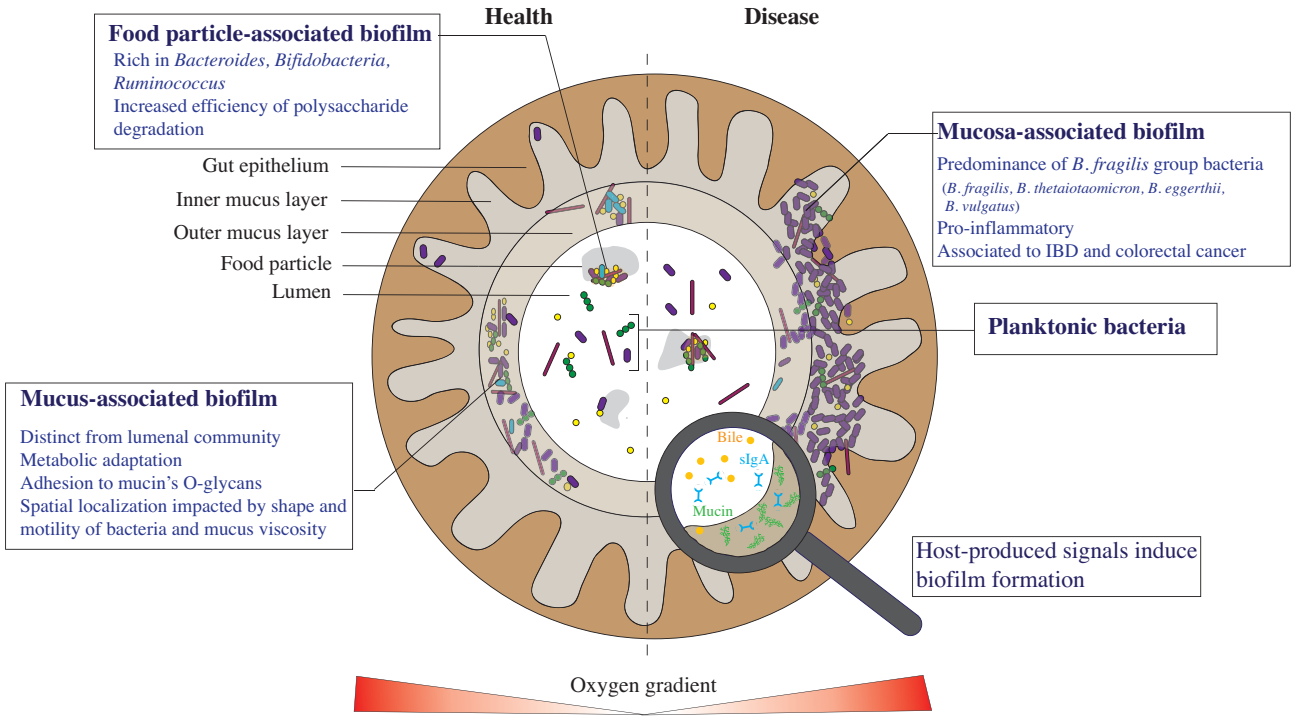

Figure 2. Biofilm formation of the colonic microbiota. Biofilm-like structures of the gut microbiota in the colon can be described in three main niches: on food particles, in the outer mucus layer or in contact with the epithelium in disease conditions. Each niche is associated with a specific gut microbiota composition and physiology.

Mucus-invasive biofilm formation

In healthy conditions, very few bacteria are found in the inner mucus layer or in contact with the gut epithelium. By contrast, mucus-invasive biofilms have been described in disease states. Swidsinski *et al.* (2005) strictly defined mucus-invasive biofilms as a lawn of more than 10^9 bacterial/mL, spanning a linear distance of at least 50 μm and observed within 1 μm of the epithelium. Only 35% of the gut of healthy individuals were shown to contain mucus-invasive biofilms, whereas more than 90% of subjects suffering inflammatory bowel disease (IBD) or self-limiting colitis contained these biofilms. The *B. fragilis* group bacteria (comprised of *B. fragilis, B. thetaiotaomicron, B. eggerthii* and *B. vulgatus*) accounted for more than 60% of the biofilm mass of IBD patients (Swidsinski *et al.* 2005). *In vitro* biofilm cultivation of mucosal communities in anaerobic conditions showed that IBD patients-mucosal communities formed thicker biofilms than healthy patients-mucosal communities. Bacteria dispersed from biofilms from IBD patients were more invasive into Caco-2 cells, and translocated across epithelia more often, than bacteria dispersed from the healthy control. Moreover, bacteria dispersed from IBD-biofilms led to an increased inflammatory response in Caco-2 cells and *Caenorhabditis elegans,* showing that these biofilms were pro-inflammatory (Motta *et al.* 2018). Whereas correlation between increased biofilm formation and IBD does not imply causation, the demonstration that communities from IBD patients are pro-inflammatory seems to suggest that the development of a mucus-invasive biofilm might be important in IBD onset.

In another study, mucus-invasive biofilms were defined similarly as a lawn of more than 10^9 bacteria/mL in the mucus layer, spanning a linear distance of at least 200 μm of the epithelial surface. Biofilms were detected in 50% and 67% of biopsies from patients suffering from colorectal cancer and adenomas respectively, but only in 15% of healthy individuals. This correlation varied depending on the colon site: biofilms were detected in 87% of biopsies of ascending colon containing tumors, but only in 13% of biopsies of transverse and descending colon containing tumors. Mucus-invasive biofilms also correlated with known pro-oncogenesis marker apparition (Dejea *et al.* 2014). Inoculation of human mucus-invasive biofilm biopsies into three different mouse colon tumor models led to biofilm formation, inflammation and carcinogenesis, regardless of whether the samples came from healthy or tumor-positive individuals. However, inoculation of biofilm-negative samples from healthy humans never induced tumor apparition nor biofilm formation, suggesting mucus-invasive biofilms are carcinogenic, even in healthy individuals (Tomkovich *et al.* 2019). Hence, biofilm formation in the gut can be associated to both health and disease. The density of bacteria and their distance to the gut epithelium seem key to distinguish between healthy biofilms and disease-associated biofilms. Therefore, in *vivo* studies should, when possible, take the gut microbiota spatial organization into account.

What of planktonic bacteria?

Ex vivo studies in gut-mimicking systems have allowed the comparison of the sessile and planktonic populations of the microbiota and showed that they differed in terms of composition, metabolism, stress tolerance and pathogenicity (Crowther *et al.* 2014b; Hay and Zhu 2015; Bircher *et al.* 2020). The sessile community can act as a reservoir of cells for healthy members of the microbiota, stabilizing the community over long periods of

time and allowing recovery of bacteria after antibiotic treatment (Cinquin *et al.* 2004; Crowther *et al.* 2014b; Bircher *et al.* 2020). However, this can also have strong implications in disease onset. Indeed, *C. difficile* was shown to germinate and produce cytotoxin mostly in the planktonic phase, whereas the sessile community served as a spore reservoir that could seed the planktonic fraction after antibiotic treatment, leading to recurring *C. difficile* growth in the system (Crowther *et al.* 2014b). Moreover, bacteria dispersed from gut biofilms can become pathobionts, meaning that members of the healthy gut microbiota can become pathogenic, as was recently reviewed (Buret *et al.* 2019). For instance, bacteria dispersed from the biofilms of IBD patients were pro-inflammatory and could translocate epithelial barriers (Motta *et al.* 2018; Buret *et al.* 2019).

OXYGEN—OR LACK THEREOF—IN BIOFILMS

As discussed above, adhesion and biofilm formation contribute to gut microbiota stability and function. Considering the difficulty of studying these processes *in vivo*, biofilm formation by gut bacteria has been mostly studied *in vitro* using only a limited number of bacterial species that are often either minor members of the community or pathogenic bacteria (Beloin, Roux and Ghigo 2008; Yildiz and Visick 2009; Pantaléon *et al.* 2014; Salas-Jara *et al.* 2016). Though the colon is a mostly anoxic environment, a rapid PubMed search showed that close to 99% of these studies were performed on facultative anaerobes and studied almost exclusively in the presence of oxygen. In this section, we will focus on the impact of oxygen on bacterial physiology within biofilms.

Biofilms formed in aerobic conditions are exposed to complex oxygen gradients that drive phenotypic heterogeneity

Measurements of oxygen concentration in aerobic *in vitro* biofilms consistently revealed the presence of a gradient of oxygen. Although this gradient is not linear, schematically, oxygen concentration is high at the interface with liquid and low or even null in the deeper parts of the biofilm (de Beer *et al.* 1994; Rani *et al.* 2007; Stewart and Franklin 2008; Klementiev, Jin and Whiteley 2020). This is due to the lowered oxygen diffusion rate within biofilms, that is estimated to be approximately 60% of the rate measured in water based on observation for similar compounds, and the rapid consumption of oxygen by bacteria close to the surface (Stewart and Franklin 2008). This gradient of oxygen drives phenotypic heterogeneity in the biofilms, which gives rise to biofilm-specific phenotypes (Fig. 3).

Oxygen concentration can lead to differential gene expression. Biofilm gene expression shows similarity to both aerobic and anaerobic gene expression, consistent with an oxygen gradient (Beloin *et al.* 2004; Teal *et al.* 2006). Moreover, oxygen concentration was shown to impact the expression of different adhesion factors in *P. aeruginosa* and uropathogenic *E. coli*, driving differential expression of adhesins within the biofilm (Bayer *et al.* 1990; Floyd *et al.* 2015), and of a major biofilm regulator in *Salmonella typhimurium* (Gerstel and Römling 2001). Oxygen also impacts the 3D structure of biofilms: wrinkle formation of *P. aeruginosa* biofilms was proposed to be a way to maximize oxygen availability (Dietrich *et al.* 2013) and in *E. coli*, the differential expression of quinol oxidases along the oxygen gradient was shown to be correlated with increased ECM production in the well-aerated parts of the biofilm, driving biofilm architecture (Beebout *et al.* 2019).

The anoxic conditions in the deep layers of the biofilm induce metabolic changes. In particular, *E. coli* biofilms were shown to produce propanol, an industrial-relevant chemical, exclusively in biofilm condition to regulate the bacterial redox balance in anoxic conditions (Létoffé *et al.* 2017) and to express specific redox protein modifications, such as S-nitrosylation (Barraud *et al.* 2021). These metabolic changes can be used as signals to promote biofilm dispersal (Karatan and Watnick 2009). Reactive nitrogen intermediates such as nitric oxide formed by anaerobic metabolism were shown to induce biofilm dispersal of *P. aeruginosa*. These reactive nitrogen species were detected in mature biofilms, and mutants defective for nitric oxide production failed to disperse (Barraud *et al.* 2006). Moreover, in *Shewanella oneidensis*, the sudden drop of oxygen concentration upon arrest of the medium flow is enough to induce biofilm dispersal (Thormann *et al.* 2005).

The distribution of oxygen in a biofilm correlates with bacterial metabolic activity: it is higher in oxygen-rich regions of the biofilm where aerobic respiration can take place, whereas in the anoxic and nutrient-depleted parts of the biofilm it is low, and some cells even enter into dormancy (Xu *et al.* 1998; Rani *et al.* 2007; Stewart and Franklin 2008). Several studies showed that reduced metabolic activity in the deeper parts of the biofilm lead to an increased tolerance to multiple antibiotics, compared to early biofilms in which the gradient of oxygen has not yet formed. The anoxic or hypoxic conditions could contribute to up to 70% of the observed antimicrobial tolerance in mature biofilms (Walters *et al.* 2003; Borriello *et al.* 2004). In addition to lowering the metabolic activity of cells, hypoxia was also shown to modify multidrug efflux pump expression, increasing *P. aeruginosa* tolerance to antimicrobials (Schaible, Taylor and Schaffer 2012). Oxygen was also found to impact conjugation efficiency, restricting plasmid transfer to the metabolically active, outer-layers of the biofilm (Król *et al.* 2011).

Facultative anaerobes form different biofilms in aerobic and anaerobic conditions

Several studies compared biofilms formation by facultative anaerobes in presence or in absence of oxygen, showing that different bacterial species reacted differently to anaerobiosis. Absence of oxygen was found to increase biofilm formation of *Streptococcus mutans* in an autolysin A (AtlA)-dependent manner (Ahn and Burne 2007). Similarly, anaerobic conditions were associated with higher production of polysaccharidic adhesins (Cramton *et al.* 2001) and higher cell lysis of *S. aureus*, increasing extracellular DNA production and ultimately leading to increased biofilm formation (Mashruwala, van de Guchte and Boyd 2017). However, *E. coli* formed patchier and thinner biofilms in anaerobic conditions compared to aerobic conditions (Bayramoglu, Toubiana and Gillor 2017). *Escherichia coli* transcriptome profiling showed that many genes were repressed in anaerobic compared to aerobic biofilms, with a global reduction of metabolic activity in anaerobic biofilms. Only three *E. coli* genes were upregulated in anaerobic biofilms: *safA*, encoding a two-component system connector, and two genes of unknown function. However, surprisingly the composition of the ECM and the expression of genes regulating ECM production were quite similar in anaerobic and aerobic biofilms, showing that while metabolism was reduced in absence of oxygen, the structure

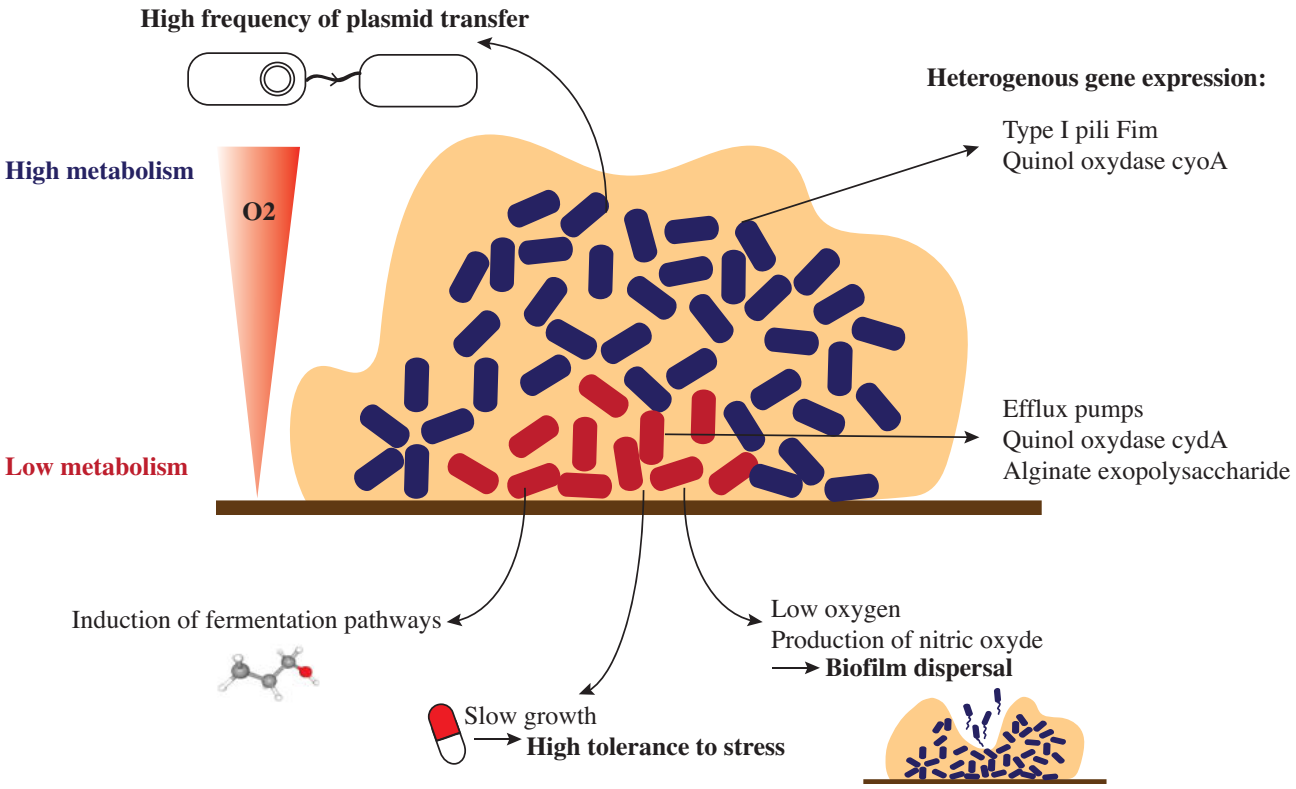

Figure 3. Oxygen in *in vitro* biofilm formation. An oxygen gradient develops in biofilms grown aerobically. In the outer biofilm layer, in contact with media nutrients and oxygen, bacteria are metabolically active. In the deeper layers of the biofilm, bacteria are nutrient limited and exposed to anaerobic conditions, which drives metabolic adaptations that can lead to antibiotic tolerance.

of the biofilm might be quite similar in both conditions (Bayramoglu, Toubiana and Gillor 2017).

Biofilms formed in the mucus layer of cystic fibrosis patients' lungs were shown to be mostly anaerobic (Worlitzsch *et al.* 2002). Therefore, increasing attention has been given to biofilms formed in anaerobic conditions by *P. aeruginosa*, one of the major pathogens responsible for infections in cystic fibrosis patients. Contrary to *E. coli*, *P. aeruginosa* was found to form stronger biofilms in anaerobic conditions. A flagellar mutant formed poor biofilms regardless of oxygenation, whereas a mutant unable to synthetize a type IV pilus showed a severe reduction specifically in anaerobic biofilm formation, showing that both flagellum and type IV pilus are important for adhesion in anaerobic conditions (Yoon *et al.* 2002). Expression of factors preventing accumulation of nitric oxide was also necessary to preserve cell viability within anaerobic biofilms, showing that anaerobic metabolism was associated with specific stress conditions. A proteomic analysis revealed that several proteins were enriched in anaerobic biofilms compared to aerobic biofilms, including two proteins that were present exclusively in anaerobic biofilms: the 50S ribosomal protein L9 and the channel-forming porin OprF (Yoon *et al.* 2002).

Oxygen gradients, therefore, drive many biofilm properties in aerobic conditions *in vitro*. *In vivo*, although there is a gradient of oxygen both longitudinally and laterally in the mammalian gut microbiota (oxygen is higher next to the epithelium and in the small intestine, whereas it is minimal in the lumen and in the colon), the colon is a mostly anaerobic environment (Pereira and Berry 2017). Although oxygen concentration in the gut was shown to impact gut microbiota composition and localization (Albenberg *et al.* 2014; Rivera-Chávez, Lopez and Bäumler 2017; Friedman *et al.* 2018), strict anaerobic bacteria outnumber the aerobes by 100–1000 times in the colon (Eckburg *et al.* 2005; Adak and Khan 2019), highlighting the importance of increasing our understanding of biofilm formed by anaerobes, rather than facultative aerobes, in the context of the gut microbiota.

BACTEROIDES AS MODELS OF MUTUALIST AND PATHOGENIC ANAEROBIC BIOFILM FORMATION IN THE GUT

Despite variations in species and strains composition, the colon microbiota is always dominated by Firmicutes (genera *Clostridium*, *Eubacterium* and *Ruminococcus*) and Bacteroidetes (genera *Bacteroides* and *Prevotella*), which make up more than 90% of the community. Proteobacteria, Actinobacteria, Fusobacteria, Verrucomicrobia and Cyanobacteria are also represented, but not dominant (Bäckhed *et al.* 2005). In this section, we will focus on what is known about biofilm formation by the strict anaerobic *Bacteroides*, one of the most abundant genera of gut symbionts, known to provide a wide range of benefits to the host, but that can also become pathogenic (Xu and Gordon 2003; Wexler 2007; Fig. 4). *Bacteroides* have relatively fast generation times, a high oxygen tolerance that allows easy manipulation at the bench, and they are genetically amenable, therefore they are convenient model organism for the study of the gut microbiota. Moreover, *Bacteroides* were shown to rapidly adhere to food particles, and to colonize the mucus layer in both health and disease conditions, suggesting adhesion and biofilm formation could be important colonization factors (Sonnenburg *et al.* 2005; Macfarlane and Macfarlane 2006; Lee *et al.* 2013; Yasuda *et al.* 2015; Mark Welch *et al.* 2017; Donaldson *et al.* 2020). However, *Bacteroides* do

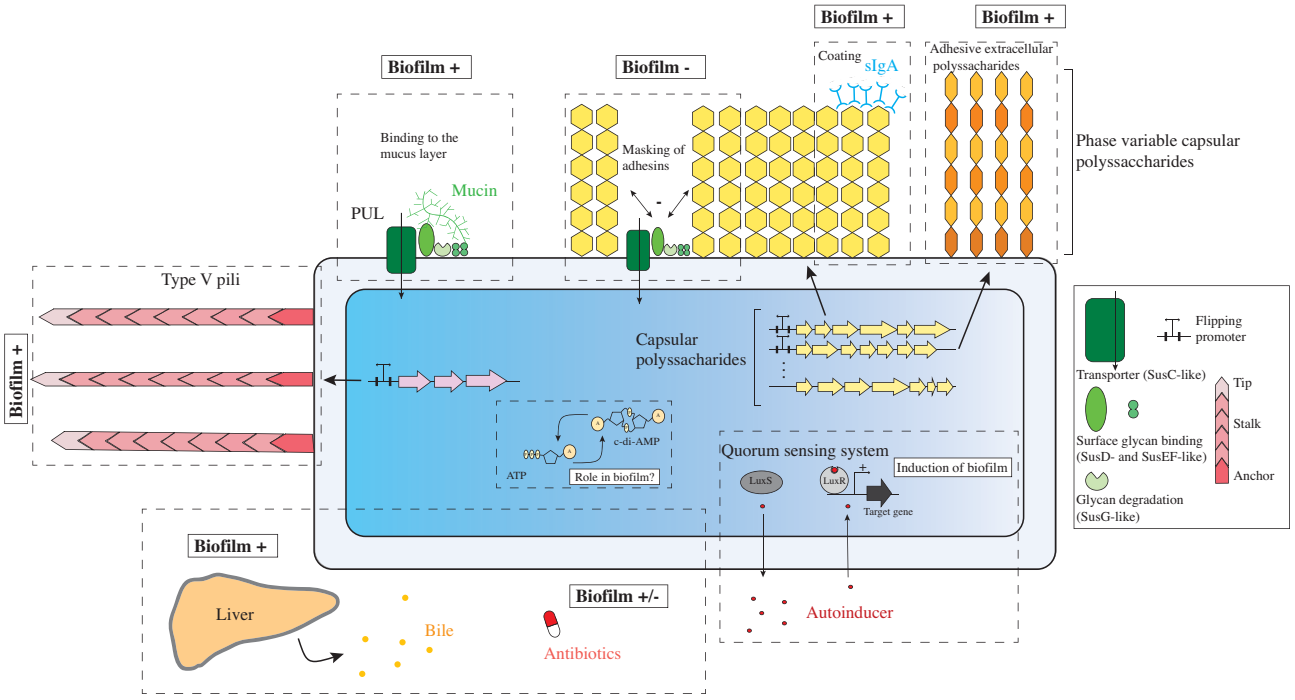

Figure 4. *Bacteroides* biofilm formation. *Bacteroides* biofilm formation involves adhesive structures such as type V pilus, polysaccharidic utilization locus (PUL) that can bind host-derived sugars such as mucin O-glycosylation, and capsular polysaccharides. Capsular polysaccharides can also be coated with host-secreted immunoglobulin A (sIgA), leading to aggregation. However, some capsular polysaccharide inhibits biofilm formation by masking short adhesins. *Bacteroides* biofilm formation is modulated by environmental cues: bile and sub-inhibitory concentration of antibiotic can either induce (bile, enrofloxacin) or inhibit biofilm formation (imipenem, metronidazole). Moreover, *Bacteroides* encode *luxS–luxR* homologs and induce biofilm formation upon sensing of the autoinducer homoserine lactone. *Bacteroides* also produce cyclic di-AMP (c-di-AMP), a known regulator of biofilm formation in *Firmicutes*, although its contribution to *Bacteroides* biofilm formation has not been demonstrated.

not encode classical fimbriae, pili or autotransporter adhesins (Xu *et al.* 2003), and must, therefore, produce original adhesins (Fig. 4).

Biofilm formation by *B. fragilis* and *B. thetaiotaomicron* strains

Bacteroides fragilis and *B. thetaiotaomicron* reference strains (*B. fragilis* NCTC 9343 and 638R, *B. thetaiotaomicron* VPI-5482) are poor biofilm formers *in vitro*, which *de facto* limited their use as models of strict anaerobe biofilm formation (TerAvest *et al.* 2014; Pierce and Bernstein 2016; Mihajlovic *et al.* 2019). However, several clinical and natural isolates of *B. thetaiotaomicron* and *B. fragilis* were shown to form biofilms *in vitro*, indicative of a widespread biofilm ability in the *Bacteroides* genus (Donelli *et al.* 2012; Reis *et al.* 2014; Pierce and Bernstein 2016; Mihajlovic *et al.* 2019; Jasemi *et al.* 2020). In particular, enterotoxigenic *B. fragilis* strains were shown to be better biofilm-formers than non-toxigenic *B. fragilis*, suggesting that *in vitro* adhesion capacity could correlate with virulence (Pierce and Bernstein 2016; Jasemi *et al.* 2020).

The regulation of *Bacteroides* biofilm formation is poorly understood. *Bacteroides* do not encode diguanylate cyclase enzymes (Xu *et al.* 2003), responsible for the synthesis of the secondary messenger cyclic di-GMP (c-diGMP), a well-known regulator of biofilm formation in many Gamma-Proteobacteria species including *E. coli, P. aeruginosa, Salmonella* and *V. cholerae* (Karatan and Watnick 2009; Jenal, Reinders and Lori 2017; Hengge 2020). They do, however, produce cyclic di-AMP, a secondary messenger that is increasingly recognized as a regulator of biofilm formation in Firmicutes, although whether this molecule mediates biofilm formation in *Bacteroides* is not known (Peng *et al.* 2016; Townsley *et al.* 2018; Stülke and Krüger 2020). Quorum sensing, a mechanism by which bacteria sense surrounding cell density by reacting to small molecules called autoinducers, has been shown to regulate adhesion, maturation and dispersal of biofilm in different species (Parsek and Greenberg 2005; Passos da Silva *et al.* 2017; Saxena *et al.* 2019). Quorum sensing has early been proposed to operate in biofilms, which are characterized by a high density of cells and a low diffusion rate favoring accumulation of autoinducers. *B. fragilis* encodes a quorum sensing system homologous to *luxS–luxR*. *B. fragilis* biofilm formation was shown to increase in the presence of the autoinducer N-hexanoyl-L-Homoserine lactone (C6-HSL) and in presence of culture supernatant of different autoinducer-producing bacteria, suggesting that quorum sensing is an important regulator of biofilm formation in this species (Pumbwe, Skilbeck and Wexler 2008; Peixoto *et al.* 2014; Fig. 4).

Non-endogenous molecules can also impact *Bacteroides* biofilm formation. For example, sub-inhibitory concentrations of imipenem and metronidazole antibiotics inhibit *B. fragilis* clinical isolates biofilm formation whereas enrofloxacin induced it (Silva *et al.* 2014). Moreover, bile was shown to induce biofilm formation in *B. fragilis, B. thetaiotaomicron* and several other Bacteroidales species (Pumbwe *et al.* 2007; Béchon *et al.* 2021). In presence of bile, *B. fragilis* also showed increased co-aggregation and adhesion to human cells, and an increased tolerance to bile cytotoxic effects, and antimicrobials (Pumbwe *et al.* 2007), but the mechanism of bile-dependent biofilm formation was not solved. As for *B. thetaiotaomicron*, it was proposed that bile could increase the release of extracellular DNA (eDNA), a known

scaffolding component of the ECM that promotes biofilm formation in many bacteria. However, *B. thetaiotaomicron* was surprisingly shown to require the production of an extracellular DNase for maximum bile-induced biofilms, suggesting that biofilm formation in this genus could involve previously unrecognized factors and that the interplay between eDNA and biofilm formation might be complex (Béchon *et al.* 2021). Bile is a complex mixture of diverse bile acids, and its composition can be impacted by members of the gut microbiota. The two most common modifications are deconjugation, in which a bile salt hydrolase (BSH) removes the taurine or glycine residues of conjugated bile acids, and dehydroxylation, that transforms primary bile acids into secondary bile acids. Gut microbiota-mediated bile acids modifications can impact biofilm formation of other members of the community (Dubois *et al.* 2019), although this has not yet been demonstrated for *Bacteroides*. Interestingly, a mixture of cholic and deoxycholic (non-conjugated bile acids) acid was shown to be a better inducer of *B. fragilis* biofilm formation *in vitro* than porcine bile extract rich in conjugated bile acids, suggesting that the activity of a BSH, commonly produced by gut bacteria including *Bacteroides* (Yao *et al.* 2018), could impact *Bacteroides* biofilm formation. Moreover, in one study that compared the transcriptomes of *B. thetaiotaomicron* biofilm and planktonic culture in chemostats, the gene encoding BSH was found to be slightly overexpressed (1.7-fold) in biofilms, although whether this occurs in *in vivo* biofilms is unknown.

Interactions with other bacteria can also impact *Bacteroides* biofilm formation. *Bacteroides fragilis* BfBs12 strain, isolated from a biliary stent, grew as aggregates but did not produce a lot of ECM on its own. When it was co-cultivated with *Finegoldia magna* FmBs12 strain, isolated from the same biliary stent, it grew within the dense ECM produced by its partner and formed dense mixed-species biofilm (Donelli *et al.* 2012). A mix of *Bifidobacterium bifidum, Bifidobacterium longum* subsp. *infantis, P. distasonis* and *B. ovatus* was also shown to form more biofilm together than the sum of each individual capacity to form a biofilm, showing a strong synergistic interaction. A dual biofilm of *B. longum* and *B. ovatus* was enough to observe a 4-fold increase in biofilm formation compared to the sum of individual biofilm formation. The relative amount of each bacteria within mixed biofilms differed from their abundance in mixed planktonic cultures, suggesting mixed-species biofilm formation could impact community composition in the gut (Sadiq *et al.* 2021).

Biofilm formation has been identified as a *B. fragilis* virulence factor (Jasemi *et al.* 2020), and biofilms are known to be refractory to antimicrobial treatments. Therefore, several groups have attempted to identify *Bacteroides* biofilm-inhibiting molecules. The phytochemicals α-humulene and zerumbone both inhibited *B. fragilis* biofilm formation and reduced efflux pump expression, suggesting that these molecules could be good therapeutic targets (Kim, Rhee and Eom 2019; Jang, Rhee and Eom 2020). Moreover, the gut epithelium-secreted antimicrobial peptide lactoferrin did not affect planktonic bacterial growth but reduced biofilm formation of *B. thetaiotaomicron* and *B. fragilis,* and reduced *B. fragilis* binding to laminin (de Sá Almeida *et al.* 2020). Likewise, culture supernatant of *Clostridium butyricum* was shown to inhibit biofilm formation, disperse pre-formed biofilms and inhibit efflux pump expression of *B. fragilis,* suggesting this strain had potential as a probiotic (Shin, Rhee and Eom 2020).

Bacteroides adhesion factors

Microscopy imaging revealed the presence of fimbria-like structures around *B. fragilis* cells in presence of bile, but these structures were not characterized (Pumbwe *et al.* 2007). Although pili have been described at the surface of *B. fragilis* cells (van Doorn *et al.* 1987; van Doom, Oudega and MacLaren 1992), there are relatively few studies on gut Bacteroidales pili synthesis, and most of the literature on Bacteroidales pili focuses on the oral pathogen *Porphyromonas gingivalis,* in which pili are known to be important virulence factors, mediating adhesion to host cells and other bacteria (Enersen, Nakano and Amano 2013). Pili are cell-surface appendages composed of polymerized subunits called pilin. A structural analysis of FimA homologs, which are putative pilin subunits found in different Bacteroidales, led to the identification of a novel type of pilus synthesis pathway, the type V, which relies on protease-mediated polymerization (Xu *et al.* 2016; Fig. 4). The pilus assembles from tip to stalk. Tip and major stalk subunits are synthetized as lipidated protein at the cell surface where they are cleaved by a protease to remove both the lipid moiety and part of the N-terminal region of the protein, thereby uncovering the N-terminal groove involved in polymerization. Anchor pilin are also lipidated proteins, but they do not undergo cleavage and thus stay attached to the outer membrane (Coyne and Comstock 2016; Xu *et al.* 2016; Shibata *et al.* 2020).

Bacteroidales pilin share little sequence homology with pilin of other bacteria. Recently, a search for homologs of these newly described type V FimA homologs in Bacteroidales genomes led to the identification of multiple pilin genes among gut Bacteroidales, including *B. thetaiotaomicron* and *B. fragilis* (Xu *et al.* 2016). Interestingly, several of these pilin-encoding loci were under the control of an invertible promoter and were previously shown to be regulated by the global invertase Mpi, or the tyrosine site-specific recombinase that regulates production of phase variable surface structures in *B. fragilis* (Weinacht *et al.* 2004; Coyne and Comstock 2016). In particular, deletion of a *B. fragilis* tyrosine site-specific recombinase led to the constitutive expression of a locus encoding a putative type V pilus, the *aap* (aggregative adherent phenotype) locus, increasing co-aggregation and biofilm formation, although pilus formation was not confirmed (Weinacht *et al.* 2004; Xu *et al.* 2016). Moreover, deletion of the last nine amino acids of a FimA homolog led to polymerization of a pilus and biofilm formation in *B. thetaiotaomicron,* providing the first characterization of a putative adhesin in *B. thetaiotaomicron* (Mihajlovic *et al.* 2019).

Apart from proteinaceous adhesins, extracellular polysaccharides can also contribute to *Bacteroides* biofilm formation. Capsular polysaccharides (CPS) are tightly packed polysaccharidic structures that surround the cell with a protective layer. Gut Bacteroidales encode multiple CPS, whereas other Bacteroidales do not, suggesting multiple CPS production is an important gut colonization factor (Coyne and Comstock 2008). *B. fragilis* NCTC9343 and *B. thetaiotaomicron* VPI-5482 encode 8 CPS operons, named respectively *PSA-H* and *cps1-8* (Krinos *et al.* 2001; Xu *et al.* 2003; Coyne and Comstock 2008). Mutants unable to synthetize any capsules were shown to have a strong autoaggregation phenotype and increased biofilm formation in both *B. thetaiotaomicron* and *B. fragilis* (Coyne *et al.* 2008; Béchon *et al.* 2020), showing that CPS could be masking adhesion factors at the cell surface. Additionally, CPS1, 3, 4 and 6 were downregulated in *B. thetaiotaomicron* biofilms grown in chemostat compared to planktonic cultures, and CPS8 and CPS7 were upregu-

lated in biofilms (TerAvest *et al.* 2014). Consistently, constitutive expression of CPS8 was sufficient to increase *B. thetaiotaomicron* biofilm formation (Béchon *et al.* 2020). Interestingly, CPS8 is the only *B. thetaiotaomicron* CPS encoding FimA homologs (Xu *et al.* 2016), although, pilus synthesis was not confirmed in this study. Alternatively, CPS8 could be an extracellular polysaccharidic adhesin. *Bacteroides* capsule regulation involves transcriptional regulators, flipping promoters and ECF sigma/anti sigma factors (Krinos *et al.* 2001; Patrick *et al.* 2003; Xu *et al.* 2003; Chatzidaki-Livanis, Coyne and Comstock 2009; Martens *et al.* 2009). This complex regulation ensures that, whilst each cell expresses a single capsule, the population always expresses multiple ones. This phenotypic heterogeneity could have a strong impact on the adhesion properties of each cell within the population.

In vivo biofilm formation

Interestingly, the transcriptome of *B. thetaiotaomicron in vitro* biofilms was closer to that of bacteria colonizing the gnotobiotic mouse gut than to planktonic cells, suggesting that *in vitro* biofilm formation might be a better proxy for *in vivo* phenotypes than planktonic cultures (TerAvest *et al.* 2014). In gnotobiotic mouse guts, *B. thetaiotaomicron* was shown to be part of dense communities of bacteria that were not randomly distributed but rather spatially organized (Mark Welch *et al.* 2017). In general, *Bacteroides* are abundant mucus-associated bacteria (Sonnenburg *et al.* 2005; Huang, Lee and Mazmanian 2011; Lee *et al.* 2013; De Weirdt and Van De Wiele 2015) capable of forming microcolonies on the rectal mucosa (Macfarlane, Hopkins and Ma 2000). Members of the *B. fragilis* group (*B. fragilis, B. thetaiotaomicron, B. eggerthii* and *B. vulgatus*) were shown to rapidly adhere to and colonize mucus gels *in vitro* (Macfarlane, Woodmansey and Macfarlane 2005; Huang, Lee and Mazmanian 2011). Interestingly, only a small subpopulation of *B. fragilis* cells bound mucins, which suggests that phase-variable surface adhesion structures present in *Bacteroides* might be involved (Huang, Lee and Mazmanian 2011). Several studies showed that *Bacteroides* can degrade mucin-bound sugars as a carbon source (Roberton and Stanley 1982; Macfarlane 1991; Tsai *et al.* 1992; Martens, Chiang and Gordon 2008; Koropatkin *et al.* 2009; Martens *et al.* 2011). In *Bacteroides,* polysaccharide degradation is performed by polysaccharide utilization loci (PUL) that are defined *a minima* as a pair of a TonB-dependent transporter (TBDT) and a lipidated cell-surface glycan-binding protein (SGBP; Martens, Chiang and Gordon 2008; Grondin *et al.* 2017; Fig. 4). SGBP have been suggested to be able to mediate the adhesion of *Bacteroides* to mucins, suggesting they are involved in mucus colonization. Consistently, *B. fragilis* and *B. thetaiotaomicron* sampled from the mucosal community were shown to overexpress genes involved in mucin degradation compared to *Bacteroides* sampled from the lumen, and deletion of these genes in *B. fragilis* led to impaired mucus colonization (Li *et al.* 2015; Donaldson *et al.* 2020). Moreover, transcriptomic comparison between *B. thetaiotaomicron* biofilms grown in chemostats and planktonic cultures, or between cultures grown in presence or absence of bile, an inducer of biofilm formation, showed that PULs involved in host-derived mucin O-glycan degradation were upregulated in biofilm conditions and in presence of bile (TerAvest *et al.* 2014; Béchon *et al.* 2021). A unique class of *Bacteroides* PULs, the commensal colonization factors (ccf), was shown to mediate species-specific *Bacteroides* colonization resistance, in which colonization by a *Bacteroides* prevented colonization by other *Bacteroides* (Lee *et al.* 2013). *B. fragilis* lacking the *ccf* locus lost their ability to both colonize the mucus layer and form aggregates. Furthermore, the *ccf* regulator ccfA was shown to induce *B. fragilis* capsule PSC production and PSC deletion led to a loss of aggregation and mucus colonization capacity. PSC production allowed the coating of *B. fragilis* cells by gut produced IgA, surprisingly without impacting bacterial viability but rather increasing bacterial adhesion to epithelial cells and mucus, showing that *B. fragilis* developed a way to attract, rather than evade, the host immune system in order to increase its colonization capacity (Donaldson *et al.* 2018). Finally, members of the *B. fragilis* group were shown to make up to 60% of mucus-invasive biofilms associated to IBD, and to be the main feature of IBD, showing mucus colonization and biofilm formation could have consequences on host health (Swidsinski *et al.* 2005).

CONCLUSION

Biofilm formation by pathogenic bacteria has been extensively studied as a virulence factor, impacting clinical outcomes and leading to therapeutic failures and chronic infections. Indeed, the National institute for health (NIH) estimates that 65% of microbial infections and 80% chronic infections involve a biofilm (Jamal *et al.* 2018) and these infections often prove challenging to treat because of their high resistance to antibiotics (Lebeaux *et al.* 2014). However, new studies have shown that in the context of the gut, we should consider biofilm formation capacity not only as a virulence factor but also as a potentially beneficial lifestyle for the host. Indeed, biofilm formation by gut bacteria leads to a better degradation of complex sugars, allows selection of specific bacterial strains and increases tolerance to stresses such as antibiotics, which could contribute to prevention of antibiotic-induced dysbiosis or facilitate the recovery of the microbiota after dramatic procedures such as colonoscopy. Therefore, the understanding of bacterial adhesion and biofilm formation in the healthy gut microbiota could allow the development of new therapeutic tools. The use of *Bacteroides* as anaerobic gut symbionts models for the study of biofilm formation can pave the way to our increased understanding of the gut microbiota/host interactions. Moreover, as they are phylogenetically distant from classical Proteobacteria and Firmicutes models of biofilm formation, we may uncover unique *Bacteroides* mechanisms of biofilm formation, regulation and physiology.

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