Pathogens, microbiome and the host: emergence of the ecological Koch's postulates

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Abstract

Even though tremendous progress has been made in the last decades to elucidate the mechanisms of intestinal homeostasis, dysbiosis and disease, we are only at the beginning of understanding the complexity of the gut ecosystem and the underlying interaction networks. We are also only starting to unravel the mechanisms that pathogens have evolved to overcome the barriers imposed by the microbiota and host to exploit the system to their own benefit. Recent work in these domains clearly indicates that the "traditional Koch's postulate", which state that a given pathogen leads to a distinct disease, are not valid for all "infectious" diseases, but that a more complete and complex interpretation of the Koch's postulate is needed in order to understand and explain them.

This review summarizes the current understanding of what defines a healthy gut ecosystem and highlights recent progress in uncovering the interplay between the host, its microbiota and invading intestinal pathogens. Based on these recent findings, we propose a new interpretation of the Koch's postulate, that we term "ecological Koch's postulate".

One sentence summary

This review summarizes the current understanding of what defines a healthy gut ecosystem, highlights recent progress in uncovering the interplay between the host, its microbiota and invading intestinal pathogens and, based on the most recent findings in this field, proposes a new interpretation of the Koch's postulate, the "ecological Koch's postulates".

Introduction

Traditionally, pathogens have been viewed as armed warriors, fighting against the host (Sansonetti 2004). However, it is well established that not everyone that has ingested a typical infectious dose of a given pathogen, for example *Salmonella* Typhimurium, *Helicobacter pylori* or *Campylobacter jejuni*, will develop disease. There also is increasing evidence for asymptomatic carriage of enteric pathogens (Kotloff *et al.* 2013; Breurec *et al.* 2016; Randremanana *et al.* 2016). In addition, it is also known that antibiotic use leads to an increased susceptibility to infection by enteropathogens (BOHNHOFF, DRAKE and MILLER 1954; MILLER, BOHNHOFF and DRAKE 1954; Barthel *et al.* 2003; Sekirov *et al.* 2008; Van der Waaij, Berghuis-de Vries and Lekkerkerk-van der Wees 2009). These observations led to an extended concept that acknowledged the role of the microbiota in protecting the host against pathogens, referred to first as "microbial barrier" and later as "colonization resistance" (Ducluzeau and Railbaud 1989).. Bacteria can have an inhibitory effect on phylogenetically unrelated species/groups of bacteria ("interspecies barrier effect"). This was demonstrated as early as the late 1970s through work showing that pre-colonization of axenic mice with *Escherichia coli* could inhibit the colonization of *Shigella flexneri* (Ducluzeau *et al.* 1977). Further, bacteria from the same species/group can have an inhibitory effect on the installation of their peers ("intra-species barrier effect"). This concept was shown in the 1980s in studies using closely related *Clostridium* species (Corthier and Muller 1988). Several studies have also assessed the role of the prokaryotic microbiota on

the susceptibility to viral infections. Indeed, susceptibility to rota- and norovirus seems to depend, at least in part, on the composition of a host's prokaryotic microbiota (Rodríguez-Díaz *et al.* 2017), as do sexually transmitted diseases, such as for example Human Immunodeficiency Virus (HIV, (Nunn *et al.* 2015)), Cervical Human Papillomavirus (HPV, (Shannon *et al.* 2017b)) or Herpes Simplex Virus (Shannon *et al.* 2017a), in the context of a changed vaginal microbiota. For many years, the exact mechanisms underlying the phenomenon of colonization resistance remained unclear. However, work over the last decades has demonstrated that the microbiota forms a complex ecosystem and interacts with the host and invading pathogens in a dynamic manner.

The intestinal microbiota is composed of trillions of organisms belonging to hundreds of different species (Eckburg *et al.* 2005; Yatsunenko *et al.* 2012), reviewed in (Cho and Blaser 2012). While the majority belongs to the prokaryota (bacteria and *archae*), it also comprises viruses (including phages) and different eukaryotes, especially yeasts and protists (Parfrey, Walters and Knight 2011; Ursell *et al.* 2012; Clemente *et al.* 2012; Parfrey *et al.* 2014; Hamad, Raoult and Bittar 2016). Members of the gram-negative *Bacteroidetes* and the gram-positive *Firmicutes* dominate the bacterial community residing in the gut. Less abundant groups include members of the *Proteobacteria*, the *Verrucomicrobia*, the *Tenericutes*, the *Defferibacteres* and the *Fusobacteria* (Eckburg *et al.* 2005; Yatsunenko *et al.* 2012). In areas of the world without developed water supplies, the intestinal microbiota can also include multicellular organisms, for example helminths (Giacomin, Agha and Loukas 2016).

Several different ecosystems formed by microbial communities in and on the body (e.g. the skin microbiota, the gastric microbiota, the vaginal microbiota), engage in a constant crosstalk with the human host (Costello *et al.* 2009). The microbes belonging to each of these communities are often specific and adapted to living in their particular environment (e.g. anaerobic bacteria in the gut). This review will focus on the interactions found in the intestine, relying mainly on data gathered in laboratory mice. Even though similar mechanisms are at work in all ecosystems found in and on the human host, a complete description would go beyond the scope of this review. In this review, we will summarize the current understanding of what defines a healthy gut ecosystem and highlight recent progress in uncovering the interplay between the host, its microbiota and invading intestinal pathogens.

The gut ecosystem- species abundance, chance, and the environment

It has been hypothesized that at least some of the gut microbes have co-evolved and/or co-speciated with mammals (Groussin *et al.* 2017). However, co-occurrence of microbes and their host, even if they affect each others fitness, does not necessarily mean a shared evolutionary history but can also be forged by numerous other mechanisms, including unidirectional selection (Moran and Sloan 2015). Regardless of their evolutionary origin, most mammals have a distinct assembly of microorganisms organized into a complex social network, which is remarkably robust and resilient to aggressions from, for example, allogenic/pathogenic intruders.

Two major competing ecological theories have been proposed to explain how microbial communities are organized and maintained, i.e. how communities are assembled. The first, termed niche theory (Chase and Leibold 2004), is based on deterministic processes and assumes that each species occupies a given realized niche (i.e. a particular position in the abiotic and biotic space) due to its species-specific properties that define its fundamental niche. The grounding of this theory for microbes lies in the work of Martinus Willem Beijerinck (1851–1931) and in the famous statement: "Everything is everywhere, but the environment selects" (O'Malley 2007).

On the other hand, the "neutral theory" is based only on stochastic processes and was proposed at the beginning of the century by Hubbell (Hubbell [2001. The Unified Neutral Theory of Biodiversity and Biogeography, Princeton University Press, Princeton, NJ]). The theory suggests that local communities are assembled independently of

species fitness differences, hence assuming that all individual have the same fitness. Further, the neutral theory claims that the fitness differences between species are not larger than the fitness differences within a given species. Competition is therefore not the driving factor of the observed community structure ("all species are equivalent; they have the same chance of immigration, extinction and speciation"). Of course, these two theories are not mutually exclusive and can act in parallel to drive microbial community assembly at (i) different spatial scales (e.g. at different locations in the intestine) or (ii) in different environments e.g. in different location on or in our body.

Testing the fit of both theories on intestinal microbial communities has been hampered by the inability to culture many of the present species and hence define the composition of the microbial communities. With the rise of advanced sequencing methodologies and the decreasing associated costs, large datasets have been put together that now allow testing of these theories on highly complex communities, for example the intestinal microbiota. In studies conducted in the past few years, evidence is emerging that the intestine is not only an ecosystem based on Hubbell's "neutral theory" where each member has the same fitness (Costello *et al.* 2012). Indeed the Human Microbiome Project, which screened several hundred stool samples from individuals residing in the United States, revealed that the microbiota of only one individual showed a composition structure consistent primarily with the "neutral theory" (Li and Ma 2016). Instead, modelling confirmed that the microbiota is governed mainly by deterministic processes, including environmental factors (Li and Ma 2016). This, however, does not exclude that both processes are shaping the communities at the same time and clearly more work is needed to elucidate these questions.

The microbiome is shaped by different deterministic forces, including maternal transmission at birth (Dogra *et al.* 2015; Frese and Mills 2015; Rutayisire *et al.* 2016; Stokholm *et al.* 2016; Edwards 2017), nutrition (Turnbaugh *et al.* 2006; De Filippo *et al.* 2010; Muegge *et al.* 2011; David *et al.* 2014; Kashtanova *et al.* 2016; Donovan and Comstock 2016; Edwards 2017; Donovan 2017; Araújo *et al.* 2017), host genetics (Leamy *et al.* 2014; Camarinha-Silva *et al.* 2017), the use of different food additives or drugs (Dethlefsen *et al.* 2008; Modi, Collins and Relman 2014; Chassaing *et al.* 2015; 2017; Namasivayam *et al.* 2017; Pourabedin *et al.* 2017; Uebanso *et al.* 2017) and infection (Hoffmann *et al.* 2009; Hill *et al.* 2010; Braun *et al.* 2017). The immune system's dynamic IgA host response to the microbiota (Suzuki *et al.* 2004; Peterson *et al.* 2007; Slack *et al.* 2012; Macpherson *et al.* 2012; Pabst, Cerovic and Hornef 2016; Moor *et al.* 2017) and its action to hamper bacteria from interacting with the gut tissue ("immune exclusion") and to grow ("enchained growth", (Moor *et al.* 2017)), as well as other innate and adaptive immune mechanisms, also have an important role in controlling the microbiota and shaping the community structure (Slack *et al.* 2009; Ivanov *et al.* 2009; Macpherson, Geuking and McCoy 2012; Schnupf, Gaboriau-Routhiau and Cerf-Bensussan 2013; Spasova and Surh 2014; Rescigno 2014; Kato *et al.* 2014; Bang *et al.* 2014; Goto *et al.* 2014; Dowds, Blumberg and Zeissig 2015; Atarashi *et al.* 2015; Furusawa, Obata and Hase 2015; Ivanov 2017).

The different environments that individuals and their gut ecosystem experience therefore select for a particular set of strains within the microbiota. However, a core bacterial genetic pool can be defined that is common to all individuals (Ley *et al.* 2006; Qin *et al.* 2010). Scarce resources, such as nutrients and access to a given niche are limiting factors for growth in this confined ecosystem (reviewed in (Stecher and Hardt 2011)). These thoughts were already, in part, formulated earlier in the "nutrient niche theory", a refined niche theory established by Rolf Freter in 1983 (Freter *et al.* 1983a; 1983b; 1983c). The "nutrient niche theory" or "niche co-existence theory" stresses that ecological niches are defined by the available nutrients. Further, Freter asserted that a given species can only establish itself if it is using at least one of the limiting nutrients in the most efficient way. He also hypothesized that only a few nutrients are responsible for shaping the whole community as they limit the growth potential of the whole

ecological niche (Freter *et al.* 1983a; 1983b; 1983c). Although Freter's classic "nutrient niche theory" is useful to understand some of the mechanisms occurring in the gut ecosystem, it is not reflective of the entire complexity observed. Indeed, several cases of mixed-substrate utilization and metabolic flexibility have been described. For example, *E. coli* and *Salmonella spp.*, can thrive on tetrathionate, nitrate, succinate, 1,2-propanediol, or ethanolamines among others, and therefore rapidly adapt to a changing environment in the intestine during inflammation (Thiennimitr *et al.* 2011; Winter *et al.* 2013; Rivera-Chávez *et al.* 2016a; Faber *et al.* 2017; Spiga *et al.* 2017). There are also a few examples of nutritional cooperation between gut microbes described (Rakoff-Nahoum, Foster and Comstock 2016).

To date, the complexity of the gut ecosystem and the underlying interaction networks are only beginning to be understood. We are also only starting to unravel the mechanisms that pathogens have evolved to overcome the barriers imposed by the microbiota and host to exploit the system to their own benefit.

Hallmarks of homeostasis

Intestinal pathogens are mainly ingested through consumption of contaminated food or water. During their travel to their preferred "niche", they encounter several obstacles and barriers imposed by the members of the ecosystem, which impede colonization and invasion by intruders. Most pathogens must replicate in the gut lumen in order to elicit disease (Ackermann *et al.* 2008). Below, we discuss the primary mechanisms in a healthy host that hamper pathogens from overcoming these obstacles and cause disease (see Figure 1).

Stomach acidity and bile acids

After ingestion and resisting salivary enzymes, the first major barrier to infection is the low pH environment of the stomach. Indeed, pharmacological perturbation of stomach acidity through the use of proton-pump inhibitors leads to an increased pH and greater susceptibility to enteric infections (reviewed in (Eusebi *et al.* 2017)). After surviving the stomach, the pathogen then enters the duodenum, where it is exposed to the massive influx of bile acids, that are produced by the liver and released by the gall bladder. The primary bile acids are involved in lipid absorption from food. They also show toxicity towards given groups of bacteria. For example, in rats treated with cholic acid, phylum-level alterations in the composition of the gut microbiota were observed with an increase in *Firmicutes* and a concomitant decrease in *Bacteroidetes*. Cholic acid feeding also led to a less complex composition of the microbiota with overrepresentation of members of the classes *Clostridia* and *Erysipelotrichi* (Islam *et al.* 2011). Therefore, bile acids shape the resident bacterial community by promoting the growth of bile acid-metabolizing bacteria and by inhibiting in turn, the growth of bile-sensitive bacteria. Several studies in patients suffering from biliary obstruction, who display blocked bile flow into the small intestine, have shown an association with bacterial overgrowth and translocation of bacteria in the small intestine (Clements *et al.* 1996). It was also shown that this phenotype can be reversed by the administration of bile acids (Lorenzo-Zúñiga *et al.* 2003). Bile acids thus play an important role in regulating the microbiota in the small intestine.

Over the length of the intestinal tract, these primary bile acids are metabolized by the microbiota to over 50 different secondary bile acids. One of the possible transformations is deconjugation of bile acids through extracellular bile salt hydrolases (BSHs). BSHs are encoded by different members of the microbiota, especially in members of the *Firmicutes*, *Bacteroidetes* and some *Actinobacteria*. The deconjugated secondary bile acids show less toxicity towards the microbiota than the primary bile acids. Bile acids can also be oxidized and epimerized (transformation in between two stereoisomers) at specific hydroxyl-groups through transformation by 3-α, 7-α, or 12-α hydroxysteroid dehydrogenases. The secondary bile acids have an important role in gut homeostasis

by inhibiting inflammation (reviewed in (Ridlon *et al.* 2014; Winston and Theriot 2016; Wahlström *et al.* 2016)). A recent study has shown that disuccinimidyl suberate (DSS)-mediated colitis was ameliorated in the presence of ursodeoxycholic acid or its taurine- or glycine-conjugated derivatives. Even though daily administration of these bile acids did not restore the full diversity of the microbiota to pre-DSS levels, specific species, such as *Akkermansia muciniphila* and the *Clostridium* cluster XIVa, were less depleted upon DSS treatment and the ratio of *Firmicutes* to *Bacteroidetes* remained normal (Van den Bossche *et al.* 2017). Secondary bile acids have also been implicated in colony resistance, for example resistance against infection by *Clostridium difficile* (Winston and Theriot 2016; Van den Bossche *et al.* 2017). Reducing the pool of secondary bile acids through antibiotic treatment relieves colonization resistance towards *C. difficile* and enhances spore germination. A later study demonstrated that the colonization resistance was mediated by a close relative, *Clostridium scindens,* through the production of the 7-α-dehydroxylated bile acids lithocholic acid and deoxycholic acid (Studer *et al.* 2016).

Box 1- Colonization resistance

Colonization resistance describes a phenomenon, observed in the human gut as well as in many other ecosystems, that invading pathogens (and other organisms) face resistance against establishing themselves in this densely populated space. This "colonization resistance" is due to several factors, namely competition for nutrients or direct inhibition by chemical compounds or "molecular weapons", for example Type 6 secretion systems and translocated toxins or other toxins. Colonization resistance can be breached by interfering with the equilibrium of the ecosystem, for example by the administration of antibiotics or by infection.

Short-chain fatty acids and other mechanisms of colony resistance through direct inhibition

In the colon, complex carbohydrates present in the food or eaten in the form of prebiotics are metabolized by the resident microbiota into short chain fatty acids (SCFA), the three most abundant being acetate, propionate and butyrate. Acetate and propionate are produced mainly by members of the *Lactobacilli* and *Bifidobacteriae.* Butyrate is produced by bacteria of the phylum *Firmicutes*, for example *Roseburia* spp. and *Faecalibacterium prausnitzii*, or different members of the genus *Clostridium* (Ramirez-Farias *et al.* 2009; Rivière *et al.* 2016). Acetate triggers anti-inflammatory and anti-apoptotic responses in host epithelial cells, which leads to protection in the gut against colonization with pathogenic bacteria like *Enterobacteriaceae* and *Clostridiae* (Fukuda *et al.* 2011). Recently, butyrate production by *Clostridia* species has been implicated in colonization resistance against *S.* Typhimurium (Rivera-Chávez *et al.* 2016b). The authors show that either through antibiotic treatment, or to a lesser extent, through *Salmonella* infection and the action of the *Salmonella* Type Three secretion system (T3SS) and associated virulence factors, a depletion of butyrate-producing *Clostridia* species occurs in the intestine of affected mice. This then leads to an increased epithelial oxygenation and subsequently aerobic expansion of *S.* Typhimurium. The colonization resistance against *Salmonella*, alongside an anaerobic epithelial environment, could be restored through gavage of the mice with tributyrin, a butyrate metabolic precursor, clearly demonstrating the inhibitory role of butyrate on *Salmonella* invasion.

Colonization *resistance and maintenance of the ecosystem through competition and cooperation*

In addition to direct inhibition, invading bacteria must also contend with limited available nutrient resources. Nutrient availability in the gut varies with food intake and time of day. Therefore, the microbiota faces a constantly changing environment. In a healthy, fully established intestine, all nutrient niches are occupied and incoming species must utilize methods to displace resident species from their established niches in order to create their own niche. A recent study analysing the gene expression of co-occurring human gut microbes showed that for 41% of all co-occurring species the presence of one of the organisms was associated with an altered transcriptional profile in the other, the most affected genes being involved in nutrient uptake and anaerobic respiration (Plichta *et al.* 2016).

This suggests that nutrient niche partitioning is prevalent within the gut ecosystem.

Furthermore, early in development or after a destabilization of the equilibrium, e.g. by an infection or antibiotic treatment, the microbiota has to re-establish itself. In this context, a so called "priority effect" is observed, whereby the first re-colonizing species establish a large colony which hampers the subsequent re-establishment of otherwise fully adapted species to the other's niche (Fukami and Nakajima 2011) (reviewed in (Pereira and Berry 2017)). This leads to fierce competition for nutrients within the microbiota and by invading pathogens. It also suggests that most of the interactions in the gut are based on competition. To date, cooperation is a type of interaction only very rarely described and does not seem to be as successful as competition in a crowded and competitive environment such as the intestine (Foster and Bell 2012). In one study of cooperation within the gut microbiota, *Bacteroides ovatus* was shown to produce two outer surface glycoside hydrolases, which digest complex carbohydrates, for example inulin. However, these two hydrolases are not necessary for *B. ovatus* to grow on inulin. Rather, other members of the microbiota, such as *Bacteroides vulgatus*, grow on the inulin breakdown products produced by *B. ovatus*. *B. ovatus* then uses products produced by *B. vulgatus* and both species flourish (Rakoff-Nahoum, Foster and Comstock 2016).

Despite the rare documented cases of cooperation, most microbial interactions in the intestine are indeed competitive.

The metabolic landscape is shaped by a few so-called "keystone species" or "keystone taxa", which have a large impact on the rest of the community by degrading initial substrates and making these accessible to many other species. Keystone species may only be detectable in very low relative abundance, however their outsized effect on the global microbial composition makes them a "keystone" of the microbiota (reviewed in (Pereira and Berry 2017). One example of "keystone species" is *Akkermansia muciniphila*, which degrades secreted host mucus into products that are then accessible to other bacteria, such as *B. vulgatus* (Png *et al.* 2010). Hydrogen and sulfate/sulfite consuming species represent an example of a "keystone taxa" due to their regulatory effect on the fermentative activity of other species (Carbonero *et al.* 2012; Rey *et al.* 2013). Yet, some of these taxa, for example *Ruminococcus*, have evolved to degrade mucin without giving other species access to the degradation products. The intramolecular transsialidase produced by *R. gnavus* releases 2,7-anhydro- Neu5Ac instead of sialic acid from mucin and other glycoproteins, a product that cannot by utilized by other species (Tailford *et al.* 2015b) (reviewed in (Tailford *et al.* 2015a)).

This nutritional limitation represents a strong barrier for the niche establishment of invading species.

Box 2- The concept of the nutritional/dietary niche

A nutritional niche describes the nutrient sources which are available to, and usable by, a given set of organisms at a given time and space. The nutritional niche therefore defines if an organism is capable of establishing itself in a given place or not. The concept of the nutritional niche is a sub-definition of the ecological niche concept. The concept has been first proposed by Rolf Freter (Freter's nutritional niche theory) and has subsequently been adapted to take into account the instable flux of nutrients in given ecosystems, for example the intestine, and to take into account the co-existence of other microorganisms utilizing the same nutrients either at different geographic locations in the intestine or at different time points. The theory also has been expanded to take into account the metabolic flexibility and mixed-substrate utilization that most microorganisms exhibit.

The process by which a given organism changes its ecological niche is known as "niche construction". (See (Pereira and Berry 2017) for an extended review of the topic.)

Mucus layer

The mucus layer forms a physical barrier to the microbiota, preventing direct interaction with the epithelium (reviewed in (McGuckin *et al.* 2011; Pelaseyed *et al.* 2014)). In the gut, the goblet cells are responsible for the secretion of the mucin MUC2, which forms a disulfide cross-linked network. This network is comprised of an inner layer, which is tightly attached to the epithelium and mainly impenetrable to bacteria, as well as a looser, outer layer. This outer layer harbours a specific community of bacteria, feeding on the mucus (Li *et al.* 2015) and

attaching to its o-glycosylated side-chains (Johansson, Larsson and Hansson 2011). Mucus production is dynamic and depends on the presence of bacterial stimuli, especially LPS (lipopolysaccharide) and PGN (peptidoglycan) as reported in an elegant study using germ-free mice (Petersson *et al.* 2011). It has also been known for a long time that SCFAs are involved in mucus secretion from goblet cells into the gut lumen (Shimotoyodome *et al.* 2000; Willemsen *et al.* 2003). Homeostasis of mucus production is regulated by two complementary bacteria, *Bacteroides thetaiotaomicron* (stimulating mucus production through increased goblet cell differentiation) and *F. prausnitzii* (inhibiting goblet cell proliferation and mucus glycosylation) (Wrzosek *et al.* 2013). A preponderance of evidence shows that mucus composition and structure directly depends on the interplay between resident microbiota and epithelial tissues (Jakobsson *et al.* 2015). Several studies have also linked reduced or aberrant *O*-glycosylation of mucin to the development of intestinal inflammation (Fu *et al.* 2011; Larsson *et al.* 2011; Sommer *et al.* 2014). Other studies have shown penetration of commensal bacteria into the inner mucus layer in the context of colitis (Johansson *et al.* 2014). The mucus layer thickness is also related to nutrition, especially dietary intake, as it has been shown recently that low-fibre diets increase mucus-eroding bacteria communities, leading to greater access for pathogens at the epithelial surface and subsequently increased susceptibility to infection (Desai *et al.* 2016). Furthermore, the attachment of the mucus layer to the epithelium is dependent on the microbiota. Meprin β, a host-derived zinc-dependent metalloprotease induced by the microbiota, is needed to detach the mucus in the small intestine and to subsequently release it into the intestinal lumen (Schütte *et al.* 2014).

A healthy mucus layer is therefore essential to protect the underlying epithelium from the dense bacterial population and to physically separate this population from immune cells in the underlying tissue in order to prevent exaggerated immune activation.

Mucosal immune system

Prokaryotic microbiota and the immune system

It is now well established that the immune system relies on the microbiota for proper maturation (reviewed in (Schnupf, Gaboriau-Routhiau and Cerf-Bensussan 2013; Spasova and Surh 2014; Turfkruyer and Verhasselt 2015; Donovan and Comstock 2016; Torow and Hornef 2017)). Different bacterial species guide the development of specific cell subsets; for example, Segmented filamentous Bacteria (SFB) induce the development of T_H17 cells (Ivanov *et al.* 2009; Gaboriau-Routhiau *et al.* 2009; Goto *et al.* 2014)(reviewed in (Schnupf *et al.* 2017; Ivanov 2017), and *Bacteroides fragilis* has been shown to induce T_{reg} proliferation and to act on the T(H)1/T(H)2 balance (Mazmanian *et al.* 2005; Round and Mazmanian 2010). *F. prausnitzii* increases antigen-specific T cells and decreases the number of IFN-γ(+) T cells (Rossi *et al.* 2016) and other *Clostridia* species induce different T_{reg} subsets (Atarashi *et al.* 2011). Additionally, short chained fatty acids derived from bacteria regulate T_{reg} numbers in the intestine (Geuking *et al.* 2011; Smith *et al.* 2013; Furusawa, Obata and Hase 2015) and have a direct impact on overall IgA levels (Kim *et al.* 2016) (reviewed in (Velasquez-Manoff 2015)).

Mucosal IgA plays a crucial role in gut homeostasis (Peterson *et al.* 2007; Macpherson and Slack 2007; Pabst, Cerovic and Hornef 2016). It is known to protect the gut mucosa from access of bacteria ("immune exclusion") and to inhibit bacterial replication in the gut lumen ("enchained growth" (Moor *et al.* 2017)). Through these mechanisms, IgA regulates the composition and dynamics of the gut microbiota. IgA and the microbiota therefore regulate each other, leading to a delicate homeostatic balance between stimulation and control (reviewed in (Peterson *et al.* 2007; Macpherson and Slack 2007; Slack *et al.* 2012; Macpherson *et al.* 2012; Pabst, Cerovic and Hornef 2016)).

The microbiota's effect on the mucosal immune system is not limited to regulatory T cells and IgA secretion. It also plays a major role in inducing production of antimicrobial peptides (AMPs), which concentrate at the interphase between the thick, tightly formed, mucus layer and the more dispersed outer layer (reviewed in (Lehrer,

Lichtenstein and Ganz 1993; Salzman *et al.* 2010))(Meyer-Hoffert *et al.* 2008; Vaishnava *et al.* 2011). Antimicrobial peptides include the defensins, the Reg-protein family and several other proteins, which act directly on the bacteria by targeting the bacterial cell wall. In addition, other host factors with antimicrobial activity include lipocalin2/NGAL, which chelates bacterial siderophores involved in iron acquisition, as well as calprotectin, which leads to the chelation of two other essential trace elements, zinc and manganese. .

Eukaryotic microbiota and the immune system

The intestinal microbiota is not only composed of bacteria, but also harbours a whole array of *archae*, viruses and eukaryotes. It has been shown that the presence of helminths, through their effect on the host immune system (Walsh *et al.* 2009; Finlay, Walsh and Mills 2014; Finlay *et al.* 2016; Ramanan *et al.* 2016), can have profound effects on the microbiota composition in the intestine (Walk *et al.* 2010; Broadhurst *et al.* 2012; Giacomin *et al.* 2015; McKenney *et al.* 2015; Ramanan *et al.* 2016; Li *et al.* 2016; Guernier *et al.* 2017) , reviewed in (Gause and Maizels 2016)) as well as on disease susceptibility within the intestine (Reynolds *et al.* 2017) and at distant sites, e.g. the respiratory system (McFarlane *et al.* 2017). However, alterations in the microbiota composition due to the presence of helminths are not generalisable, as changes were observed in a first study of 51 persons infected with *Trichuris trichiuria* in Malaysia (Lee *et al.* 2014), but no changes were found in a second study in 97 children from Ecuador (Cooper *et al.* 2013) nor in 8 persons living in Australia infected experimentally by *Necator americanus* (Cantacessi *et al.* 2014). Helminths have also been shown to attenuate the effect of intestinal bowel disease by restoring the number of goblet cells and preventing outgrowth of *B. vulgatus* in the context of *Nod2*$^{-/-}$ mice and associated intestinal inflammation (Ramanan *et al.* 2016). For some of the observed immune changes, the helminth-induced changes seem to be mediated through the prokaryotic microbiota (Zaiss *et al.* 2015). For other effects, they seem to be independent from the microbiota (Osborne *et al.* 2014), highlighting the complex interplay found within the gut ecosystem. Very recently, a member of the eukaryome, *Tritrichomonas musculis*, was shown to activate the epithelial inflammasome to induce protection against bacterial mucosal infection (Chudnovskiy *et al.* 2016). Other protists, such as *Giardia* (Barash *et al.* 2017) and possibly *Blastocystis* (Audebert *et al.* 2016; Siegwald *et al.* 2017) have also been shown to change the resident prokaryotic microbiota. However, data remains conflicting, pointing towards the fact that shifts in the microbiota through given eukaryotes might be strain-specific. Due to incomplete databases, it is currently difficult to determine the exact strain of a eukaryotic organism by comparison against the database. More work in curating these databases is therefore needed to better appreciate the influence that specific eukaryotes might have on the prokaryotic microbiota. More work is also needed to unravel the triad of eukaryotes, prokaryotes, and the immune system to better understand the mutual interactions that take place.

Taken together, the microbiota and the development and proper function of the mucosal immune system are exquisitely intertwined. Thus, perturbations on either side of this ecosystem can deregulate the balance and leave the host open to infection or inflammatory diseases (Clarke 2014).

Friend or foe: pathogens facing the microbiota and the host

As discussed, the host and its microbiota have set-up a tightly regulated network of mutual control. Invading pathogens therefore have several barriers to overcome in order to establish themselves and cause disease (see Figure 2).

Within the last decade, much progress has been made in understanding the “ménage à trois” of the microbiota, the host, and pathogens. In this section we will discuss the mechanisms that have evolved in pathogens to overcome

the protective environment arising from homeostasis and summarize the complex interactions that take place between pathogens, the microbrobiota and their host.

Combatting the resident microbiota by direct killing or by inhibition

Small antibacterial toxins

A number of small, mainly plasmid-encoded, antibacterial peptides termed bacteriocins or microcins, are produced and secreted by a broad range of bacteria, including the *Bifidobacteria* (reviewed in (Martinez *et al.* 2013; Alvarez-Sieiro *et al.* 2016)), *Lactobacilli* (Collins *et al.* 2017), *Enterococci* (Kommineni *et al.* 2015) and many more. It has been demonstrated that bacteriocin production leads to a niche advantage for the bacteria expressing them (Riley and Wertz 2002; Kommineni *et al.* 2015). Some of the bacteriocins and microcins have been shown to act against pathogenic strains (Kommineni *et al.* 2015; Sassone-Corsi *et al.* 2016b), thereby augmenting colonization resistance and mediating resistance to invading pathogens. One bacteriocin subclass produced by *Enterobacteriaceae* and termed colicins are encoded by several pathogenic strains including *S.* Typhimurium (Nedialkova *et al.* 2014) and *Shigella sonnei* (Anderson *et al.* 2017; Calcuttawala *et al.* 2017). It has been shown recently that *S.* Typhimurium expresses colicin Ib (ColIB), giving it a fitness advantage over the closely related *E. coli,* which blooms simultaneously in the gut upon *Salmonella*-induced intestinal inflammation. ColIb is regulated through the SOS-response and iron-limitation and upregulated in the context of inflammation (Nedialkova *et al.* 2014). Therefore, the carriage and induced upregulation of colicins seems to be an evolutionary adaption of enteropathogens in order to have a selective advantage in the ecological niche they share with commensal *E. coli* in the inflamed gut.

Type 6 secretion system (T6SS)

T6SS are encoded by a substantial number of gram-negative pathogens, as diverse as *Helicobacter hepaticus* (Chow and Mazmanian 2010), *S.* Typhimurium (Sana *et al.* 2016), *S. sonnei* (Anderson *et al.* 2017), *Pseudomonas aeruginosa* (Mougous *et al.* 2006)*, enteroaggregative E. coli* (Dudley *et al.* 2006)*, Vibrio cholera* (Pukatzki *et al.* 2006) and *B. fragilis* (Hecht *et al.* 2016; Chatzidaki-Livanis, Geva-Zatorsky and Comstock 2016) to name a few. Indeed, T6SS homologous have been described in up to 25% of all sequenced gram-negative genomes. While some bacteria use their T6SS to interfere with host processes (Brodmann *et al.* 2017), alter the immune response upon infection (Chow and Mazmanian 2010; Hachani, Wood and Filloux 2016; Aubert *et al.* 2016; Chen *et al.* 2017), or modulate virulence (Bladergroen, Badelt and Spaink 2003; Parsons and Heffron 2005; Pukatzki *et al.* 2006), growing evidence suggests that T6SS are also used to attack the resident microbiota and to confer the bacteria expressing them with a competitive advantage ((Russell *et al.* 2014; Unterweger *et al.* 2014; Sana *et al.* 2016; Chatzidaki-Livanis, Geva-Zatorsky and Comstock 2016; Anderson *et al.* 2017; Bernal *et al.* 2017; Tian *et al.* 2017; Kim *et al.* 2017), reviewed in (Sana, Lugo and Monack 2017)). Indeed, *Shigella*, which encodes a T6SS had a selective advantage in colonization of the mouse gut compared to *S. flexneri* or *E. coli*, a phenomenon which was shown to be largely due to the T6SS (Anderson *et al.* 2017). In *B. fragilis* this could also be observed at the strain level, where symbiotic, non-toxic *B. fragilis* was shown to outcompete a pathogenic strain in a T6SS-dependent manner (Hecht *et al.* 2016; Chatzidaki-Livanis, Geva-Zatorsky and Comstock 2016).

The T6SS is also implicated in other important tasks that confer the bacterium harbouring it a selective advantage over the resident microbiota. These include iron acquisition (Lin *et al.* 2017) as well as different mechanisms to handle oxidative stress induced by the host (Wang *et al.* 2015; Si *et al.* 2017; Wan *et al.* 2017). It has been shown recently in marine bacteria that T6SS are horizontally shared between different species (Salomon *et al.* 2015). Furthermore, in *Vibrio cholera*, the T6SS was shown to be able to foster horizontal gene transfer (Borgeaud *et al.*

2015). Clearly, the last few years have seen strong advancements in the understanding of T6SS contributions to microbial life cycles (Filloux 2013), while more work is needed in order to fully understand the full scope of T6SS functions.

Exploiting nutrients to gain a selective advantage over the resident microbiota

The most limiting nutrients in the gut are micronutrients, especially iron, as well as general energy sources, such as carbohydrates. Bacterial pathogens have evolved a number of strategies to selectively acquire nutrients over the resident microbiota in the race for these resources.

Iron scavenging and use of specific siderophores

The host has evolved sophisticated strategies termed "nutritional immunity" to limit the amount of available iron that the microbiota has access to, (reviewed in (Cassat and Skaar 2013) and (Kortman *et al.* 2014)). Bacteria have responded by maximizing their ability to uptake iron through the secretion of iron-scavenging molecules, termed siderophores, which give them a selective advantage over strains lacking the scavenging capability (Niehus *et al.* 2017). Lipocalin2 (NGAL in humans) is a potent antimicrobial factor secreted by the host whose function is to sequester iron bound siderophores (Flo *et al.* 2004). Bacteria do not only have to compete with the host for iron, but also with the resident members of the microbiota. Indeed, many microbes encode species-specific siderophores that require specific re-uptake machinery to bind and import the iron-siderophore complex (reviewed in (Miethke and Marahiel 2007)). Many enteropathogens have also evolved specific strategies to more efficiently scavenge any available iron. *S.* Typhimurium for example secretes Salmochelin, which is not recognized by lipocalin2/NGAL (Raffatellu *et al.* 2009). Using Salmochelin, *S. typhimurium is* able to obtain enough iron to overcome iron-restriction, leading to a selective growth advantage over neighbouring bacteria. This proves especially important in the context of the inflamed intestine where *Salmonella* thrives amidst large quantities of Lipocalin2 secreted mainly by recruited neutrophils.

Use of alternative energy sources

S. Typhimurium is a useful model pathogen to illustrate the basic mechanisms that pathogens employ to bypass the metabolic environment established by the microbiota. Initial replication of *Salmonella* in the yet undisturbed gut depends on hydrogen gas, an important intermediate in microbiota metabolism (Maier *et al.* 2013). Once *Salmonella* has invaded the gut mucosa and induced inflammation, other energy sources become available and are exploited by the pathogen. One of these is the aerobic and anaerobic respiration of 1, 2-propanediol generated by the resident microbiota (demonstrated in mono-associated mice using *B. fragilis* and *B. thetaiotaomicron*) through fermentation of fucose or rhamnose in the inflamed intestine (Faber *et al.* 2017). *Salmonella* can also thrive by oxidative respiration on succinate, which is released by the resident microbiota (Spiga *et al.* 2017). As an alternative energy source, *Salmonella* is also capable of using galactarate and glucarate, generated by the microbiota after antibiotic treatment. This metabolic versatility, especially in an oxidative environment as is found in the inflamed intestine, is likely the basis of the long known condition of antibiotic-mediated *Salmonella* expansion (Faber *et al.* 2016). Another nutrient source is siacylic acid, liberated from the breakdown of sialyated mucins by *B. thetaiotaomicron* and other microbiota members. *B. thetaiotaomicron* secretes a sialidase but lacks the ability to use the freed siacylic acid itself. In turn, this acid is metabolized by *C. difficile* and *S.* Typhimurium as an alternative nutrient source giving them a selective nutritional advantage over neighbouring bacteria (Ng *et al.* 2013; Huang *et al.* 2015). Differential energy utilization has also been recently shown in some *E. coli* species, which use microbiota-derived formate as an alternative energy source to increase their advantage over other resident species

(Hughes *et al.* 2017).

Several enteropathogens, including *Citrobacter rodentium*, *Campylobacter jejuni* and *S. Typhimurium*, induce acute intestinal inflammation through their virulence factors (reviewed in (Winter *et al.* 2010a)). The inflammation provides these pathogens with an advantage over the resident microbiota by transforming the intestine into an aerobic environment. Niche creation can be exemplified by the widely studied enteropathogen *S.* Typhimurium (reviewed in (Winter, Lopez and Bäumler 2013; Winter and Bäumler 2014a)). It is likely that similar mechanisms that have evolved in *Salmonella* have also evolved in other enteropathogens, for example *E. coli* and *Shigella* spp. and together contribute to the "enterobacterial bloom" that is observed in the inflamed gut ((Lupp *et al.* 2007; Stecher *et al.* 2010) reviewed in (Winter and Bäumler 2014a)). Inflammation leads to the production of respiratory electron acceptors, for example nitrogen species and reactive oxygen species. These products are converted in the intestine to nitrate. Nitrate can be used by *Salmonella spp.*, *E. coli* and potentially other facultative anaerobe members of the *Enterobacteriaceae* as an alternative electron acceptor, giving them a selective advantage over other resident bacteria, which rely mainly on anaerobic fermentation of carbohydrates (Lopez *et al.* 2012; Spees *et al.* 2013; Winter *et al.* 2013). *Salmonella* has also been shown to use other electron acceptors, for example S-oxides, ethanolamine or tetrathionate, which are present in larger amounts in the inflamed intestine (Winter *et al.* 2010b; Thiennimitr *et al.* 2011). In a recent study, it could be shown that *Salmonella* T3SS activation leads to a depletion of *Clostridium* species, which in turn leads to a decrease in butyrate levels. Butyrate is the most important energy source of colonocytes and butyrate oxidation to carbon dioxide leads to the consumption of local oxygen and the generation of an anaerobic environment. The lack of butyrate therefore increases tissue oxygenation, generating a favourable niche for *Salmonella*'s aerobic expansion in the gut lumen (Rivera-Chávez *et al.* 2016b).

Under physiological conditions, the microbiota induces expression of PPARγ, resulting in an increase in β-oxidation of the colonocytes and hence an anoxic environment. In the context of antibiotic treatment, PPARγ-induction is inhibited and the colonocytes switch to anaerobic glucose oxidation. This then leads to increased availability of nitrate and allows for aerobic expansion of *Enterobacteriaceae* (Byndloss *et al.* 2017). Inflammation also leads to outgrowth of other bacterial species, for example *B. vulgatus* (Huang *et al.* 2015). Through its sialidase activity, *B. vulgatus* releases sialic acid from the intestinal tissue, supporting the growth of *E. coli*. *B. vulgatus* thereby contributes to the occurrence of the "enterobacterial blooms" observed during inflammation. Conversely, enterobacterial blooms during infection are abrogated when sialidase inhibitors are administered (Huang *et al.* 2015). Gut inflammation can also boost horizontal gene transfer, either between pathogenic and commensal enteropathogens or between dense populations of enteropathogens in the context of the so-called "enterobacterial booms" (see text above). This has been shown for the transfer of a colicin-carrying plasmid p2 (Stecher *et al.* 2012) as well as of temperate phages (Diard *et al.* 2017). Enterobacterial blooms can therefore contribute to pathogen evolution of some species.

Virulence genes

Once in contact with the host, pathogens have developed different strategies to influence the host and exploit it to their own benefit. This is mainly achieved through so called "virulence factors". First and foremost, pathogens express "classical" virulence factors, for example toxins and the T3SS and their associated effector proteins. The function and specific role of these virulence factors have been discussed in detail elsewhere ((Puhar and Sansonetti 2014; Qiu and Luo 2017) and many others for specific pathogens). The expression of these virulence factors can be constitutive. However, with the immense fitness cost virulence gene expression imparts on the pathogen, virulence gene expression is often regulated by environmental cues. This means that the

bacterium only expresses the virulence genes once it is in close contact with the host and at the right location along the gastrointestinal tract.

Induction of pathogen virulence genes by cues from the host

Different host cues allow invading pathogens to pinpoint their position within their host and to regulate virulence genes only once the appropriate location has been reached. Host cues for virulence regulation include bile acids (Antunes *et al.* 2012; Brotcke Zumsteg *et al.* 2014; Eade *et al.* 2016), pH (Behari, Stagon and Calderwood 2001), temperature (Elhadad *et al.* 2015; Nuss *et al.* 2015; Fraser and Brown 2017), nutrient availability (reviewed in (Porcheron, Schouler and Dozois 2016)), and oxygen levels ((Marteyn *et al.* 2010; Fraser and Brown 2017) reviewed in (Marteyn *et al.* 2011; Marteyn, Gazi and Sansonetti 2012)). Oxygen levels are dynamic within the intestine and even within the anoxic colon an oxygen tension gradient is present in close proximity to the epithelial surface. *Shigella flexneri* has adapted to this gradient by repressing its T3SS in response to reduced oxygen levels encountered in the lumen of the intestine. The key outcome is to conserve metabolic resources in this energy and nutrient depleted environment. This regulation has the added benefit of more closely aligning the expression of virulence factors to the site of infection at the epithelial layer, where they are actually used. The suppression of the T3SS system is mediated by the oxygen sensitive regulator gene *fnr.* When oxygen pressure increases near the epithelium, the anaerobic block on the master regulators of the T3SS, *spa32* and *spa33,* is released and the genes of the T3SS can be expressed (Marteyn *et al.* 2010).

Another example of regulated virulence is the bile salt-mediated activation of virulence in *Vibrio cholerae*. It was recently shown through a series of elegant in vitro experiments in a tissue model of infection that virulence genes of *V. cholerae* are induced by the bile salt taurocholate, glyocholate and cholate, but not the deconjugated deoxycholate or chenodeoxycholate. This virulence activation is mediated through dimerization of the transcription factor TcpP by disulfide bond formation. Consequently, a *V. cholerae* strain mutated in the respective cysteine is unable to respond to the bile salts, leading to a competitive disadvantage compared to the wildtype strain in an infant mouse model of colonization. This colonization difference was abolished when a bile-salt sequestering resine, cholestyramine, was co-administered, confirming the crucial role of bile acids in the observed colonization defect (Yang *et al.* 2013).

A third example is the regulation of virulence genes in *Enterotoxigenic E. coli* (ETEC) and its close relative, the mouse pathogen *C. rodentium* in the intestine. A recent study has shown that the two neurotransmitters epinephrine and norepinephrine, that are produced by the endocrine cells localized in the intestine are needed for the full expression of virulence/colonization genes (Moreira *et al.* 2016).

Together, these observations show that pathogens have evolved varied and complex mechanisms to tightly regulate their virulence attributes. This helps pathogens avoid unnecessary energetic costs that may lead to a loss of fitness in the highly competitive gut environment (see (Diard and Hardt 2017) for a recent review on the evolution of bacterial virulence).

Induction of pathogens's virulence genes by members of the microbiota

We previously discussed the cues from the host that lead to the induction of virulence genes expression in the invading pathogens. Beside this host-mediated induction, some virulence genes are also controlled by sensing metabolites derived from the microbiota, for example SCFAs (butyrate, actetate, lactate and propionate). A recent study has shown that microbiota-derived SCFAs modulate the expression of virulence genes in *Campylobacter jejuni*. The authors discovered that the gradient of SCFAs butyrate, acetate, and lactate along the intestinal tract guide expression of genes involved in virulence and commensalism of *C. jejuni*. Lactate, which is abundant in the

upper intestinal tract, suppresses production of *C. jejuni* virulence genes, while acetate and butyrate, two SCFA's that are mostly produced in the lower intestinal tract, activate virulence pathways (Luethy *et al.* 2017).

Expression of the *Salmonella* pathogenicity island 1 (SPI-1) is inhibited in the presence of propionate, a SCFA that is produced mainly by *Lactobacilli* and *Bifidobacteria* and is more abundant in the upper gastrointestinal tract. Propionate acts through posttranslational modification on HilD, the master regulator of the *Salmonella* SPI-1 (Hung *et al.* 2013). This inhibition ensures that the coordinated expression of SPI-1 genes starts only in the distal small intestine, the main site of *Salmonella* infection. Another example of microbiota-mediated virulence gene expression is the enhancement of enterohemorraghic *E. coli (EHEC)* T3SS by *B. thetaiotaomicron*. B. thetaiotaomicron is an abundant member of the gut microbiota metabolizing complex polysaccharides into monosaccharides that can be further processed by a number of other bacteria. The presence of *B. thetaiotaomicron* leads to a local increase in the levels of succinate, which is sensed by the transcription factor Cra of EHEC. Cra activation leads to an increase in the expression of the genes encoding EHEC's T3SS while leaving the general growth of the pathogen unaffected (Curtis *et al.* 2014).

Induction of AMP's by pathogens to inhibit microbiota competition

Some pathogens have evolved mechanisms to exploit the host's own antibacterial defenses. By developing countermeasures against host AMPs, pathogens can rely on the host response to combat the resident microbiota and gain a selective advantage. *Salmonella* Typhimurium for example uses an alternative siderophore (Salmochelin, described above), which is resistant to chelation by Lipocalin2. *Salmonella* has also evolved strategies to resist against calprotectin-induced sequestration of zinc and manganese (Liu *et al.* 2012), giving *Salmonella* a selective advantage over the majority of microbiota members that do not have the necessary tools to survive in the inflamed gut.

Salmonella infection induces the cytokine IL-22, which in turn activates AMP's (Behnsen *et al.* 2014). One of the proteins induced is the antimicrobial peptide Reg3beta. Previously, Stelter and colleagues showed that induction of this antimicrobial peptide inhibits the competing microbiota (Miki, Holst and Hardt 2012), while having no effect on the resistant *S*. Typhimurium (Stelter *et al.* 2011). In a new study, Miki and collaborators could show that Reg3beta extends gut colonization by *S*. Typhimurium through the prolonged induction of a pro-inflammatory environment and changes to the microbiota, especially an inhibition of *Bacteroides* species. The alteration of the microbiota also leads to profound changes in the metabolic landscape with metabolites of Vitamin B6 being the most affected. The authors could show that re-insertion of *Bacteroides* species or supplementation of Vitamin B6 alone was able to accelerate clearance of Salmonella from infected mice (Miki *et al.* 2017).

A recent study in *Ixodes scacpularis* ticks showed a similar mechanism whereby *Anaplasma phagocytophilum* infection induced *Ixodes* anti-freeze glycoprotein (Iafgp), which alters biofilm formation in the *Ixodes* gut to destabilize the resident microbiota and facilitate niche construction of *A. phagocytophilum*. It is likely that many pathogens have evolved similar mechanisms to take advantage of host defence mechanisms to facilitate niche construction and induce a host-derived selective advantage over the resident microbiota.

Evolutionary adaptions of pathogens to overcome homeostasis: penetrating the mucus layer

As described previously, the mucus layer constitutes a thick, protective layer between the gut lumen and the epithelium. To get access to the epithelium, pathogens have evolved strategies to penetrate the mucus layer and enter the underlying epithelium. *Porphyromonas gingivalis*, a pathogen found mostly in the oral cavity, secretes a cysteine protease (RgpB), which leads to Muc2 cleavage (van der Post *et al.* 2013). Mucinases also play an important role in the colonization and fitness of pathogenic *E. coli* (Valeri *et al.* 2015), an *E. coli* strain associated

with Crohn's disease (Gibold *et al.* 2016), and also eukaryotic pathogens such as *Candida albicans* (Colina *et al.* 1996) or *Entamoeba histolytica* (Lidell *et al.* 2006). It is likely that other enteropathogens also express mucinases to gain access to the underlying epithelium although more research is needed to fully address the scope of mucinase secretion and usage by pathogens.

Mechanisms established by the microbiota to clear off invading pathogen after infection

The microbiota not only plays an important role in preventing the colonization of pathogens, but also in the pathogen clearing from the gut upon resolution of the inflammation. The mechanisms underlying this process differ from those governing colonization resistance as the mucosa and the microbiota have both been affected and therefore need to return to homeostasis (Endt *et al.* 2010). In the case of *S.* Typhimurium infection it was shown that recovery is mainly mediated by the microbiota and was largely independent of the IgA pool (Endt *et al.* 2010). Mechanistic insights on the underlying causes of clearance were elucidated in a study on *Vibrio cholera* infection, showing an increase in *Ruminococcus obeum* upon infection of mice with the pathogen. The same was also observed in a cohort of infected humans from Bangladesh recovering from the disease. In elegant mouse studies, the authors showed that *Ruminococcus obeum*, through the expression of the *luxS* gene (autoinducer-2 synthase), promotes quorum sensing-mediated restriction of virulence gene expression in *V. cholerae*, leading to a decrease in host symptoms (Hsiao *et al.* 2014)

Dysbiosis: Towards a new interpretation of Koch's postulate

It is now common knowledge that the gut is a complex ecosystem with different interacting entities and that infections must be understood in this context rather than isolated as a pathogen and a host. In consequence, for these complex diseases, neither Koch's postulate ("a pathogen, a disease") nor the molecular Koch's postulate as proposed by Stanley Falkow ("a virulence gene, a disease", (Falkow 1988)) are sufficient. In a very recent review by Neville and collaborators, a third interpretation of the Koch's postulates, the commensal Koch's postulate, was proposed ("a beneficial microbe", "an ameliorated disease state"). The authors infer a new framework for establishing causation in microbiome studies were they use the commensal Koch's postulate to test if a given microorganism is able to ameliorate a disease state in a reproducible manner (Neville, Forster and Lawley 2017). As in the original Koch's postulate, they propose that for inferring causation, the "beneficial" commensals need to be isolated in pure cultures before they are re-introduced and tested in a host for their capacity to mitigate disease.

We propose yet another interpretation of Koch' postulate, which we have termed "ecological Koch's postulate ("a gut ecosystem state, a disease"). Underlying these postulates is the fact that the gut harbours a full ecosystem, rather than an isolated bacterium or pathogen. This means that rather than an isolated microorganism or group of microorganisms, a whole ecosystem, including the microbiota, the genetic make-up of the host as well as nutrition, age etc. form an entity, which can ultimately lead to disease (see Figure 3 and Box 3).

The ecological Koch's postulates are based on two major observations, both pointing towards the fact that the clear distinction between a pathogen and a commensal are probably too simple of a model to explain complex disease states.

Box 3- Postulates for defining a disease-promoting ecosystem (dysbiosis)

Ecological Koch's postulates

1 The dysbiotic microbiota is found in similar composition/ with similar characteristics in all affected individuals

2 The dysbiotic microbiota can be retrieved from the affected host

3 Gavaging of germ-free hosts with this retrieved microbiota leads, in combination with a similar environment (ex. genetic make-up of the host, nutrition, age), to similar symptoms as in the affected individual

4 The dysbiotic microbiota composition remains fairly stable in the newly affected host

Original Koch's postulates

1 The microorganism must be present in all diseased individuals

2 The microorganism must be isolated from the diseased host and be grown in a pure culture

3 The re-inoculation of a naïve host with this pure culture must lead to the same disease as in the original host

4 The microorganism must be recovered from the newly diseased host

(I) Not every person infected with a "pathogen" will manifest disease. Therefore, host susceptibility, from a genetic point of view, but also from the resident microbiota, the nutrition, earlier infections, or other insults to the microbiota, play as much of a role in the manifestation of disease as does the presence of given virulence factors in an aggressing pathogen. The pathogens not only need to be present and harbour the virulence genes, but they also need to express these genes and be able to establish a niche for themselves within the competitive environment of the already established microbial community in order to replicate and then cause disease.

(II) In the last years, several gastrointestinal diseases have emerged, which are not associated with an overt pathogen, but where the microbial community as a whole seems to mediate the disease. Examples are intestinal bowel disease, colorectal cancer, obesity, and different states of malnutrition. Indeed, depending on the microbial community pathogens find themselves in, bacteria, which normally do behave as commensals may become invasive and cause disease. For all of these diseases, a decrease in the composition complexity of the microbiota leads to dysbiosis and an oxidation of the gut environment as well as an increase in aeortolerant species such as *Enterobacteriaceae* (Rivera-Chávez, Lopez and Bäumler 2017). These disturbances in the ecosystem lead to a lowered resilience and increased susceptibility to pathogens and other, normally commensal, bacteria with potential harmful properties (often called "pathobionts"). Indeed, these diseases are characterized by the fact that the wrong bacteria are in wrong proportions, in wrong "company" (Huang *et al.* 2015) or in the wrong "place" (Brown *et al.* 2015; Tomas *et al.* 2016). The presence of commensal bacteria near the epithelial surface has been put in relation with the breakdown of gut homeostasis and emergence of pathological states in the context of environmental enteropathy (Brown *et al.* 2015) or in a pre-diabetic state (Tomas *et al.* 2016).

In the ecological Koch's postulate, a dysbiotic community, including or not "classical pathogens" or pathobionts therefore represents a disease. The entity of transmission is the complete dysbiotic microbiota rather than a pathogen (Koch's postulate), a virulence gene (molecular Koch's postulate) or a commensal (commensal Koch's postulate).

In accordance to the other Koch's postulate, the ecological Koch's postulate are proven through the fact that a given entity (here the dysbiotic microbiota) from a diseased individual can provoke disease in a formerly healthy individual. To prove this hypothesis, one hence has to transmit the microbiota from a diseased individual into a germfree individual/ mouse and this transplanted individual subsequently has to develop the signs of the disease. This transfer can either be performed in (I) "standard" conditions, using a "wild type, normally fed" germ-free host (showing a direct effect of the microbiota on disease), or, else, in (II) a "pre-fragilised" host, as an example in a mouse exhibiting a mutation in a given gene or eating a specific chow. In syndromes, which do need a pre-fragilised host, the dysbiotic microbiota contributes to disease, without however being the only cause for it.

As stated earlier, several inflammatory, gastrointestinal diseases can be explained through the ecological Koch's postulate, including obesity. Indeed, if a microbiota from obese mice is transplanted into lean mice, the transplanted mice showed increased fat deposition (Turnbaugh *et al.* 2006; Ridaura *et al.* 2013). The same could also be proven for kwashiorkor, the oedematous form of acute undernutrition (Smith *et al.* 2013) as well as for environmental enteropathy (Brown *et al.* 2015). On the other hand, it is well known that mutations in *nod2* facilitate and support the onset of IBD (Cho 2001). Therefore, to prove the contribution of the microbiota in IBD, a susceptible host, mutated for *nod2*, should be transplanted with the dysbiotic microbiota and this transplanted microbiota should worsen the disease state.

An instructive example of a "pathological dysbiosis" and hence a disease following the ecological Koch's postulates is chronic and acute malnutrition coupled to associated environmental enteropathy. Several reports have shown evidence that children suffering from one of these two syndromes have an altered colonic microbiota (Smith *et al.* 2013; Subramanian *et al.* 2014; Gough *et al.* 2015; Blanton *et al.* 2016a; 2016b) and increased abundance of asymptomatic pathogen carriage, including enteroaggregative *E. coli* (Havt *et al.* 2017), *Campylobacter spp*. and *Giardia spp.* (Platts-Mills *et al.* 2017). Further, a recent study sequencing cultured microbes from two Bangladeshi children suffering or not of undernutrition showed that the *B. fragilis* strain found in the undernourished child is enterotoxic, while the strains found within the normally nourished child were not. When transplanting the native community into germfree mice and infecting with the enterotoxigenic *B. fragilis* strain, the authors could show that the enterotoxigenic strain causally led to malnutrition and associated pathophysiological disturbances only in its native community, but not when administered to mice harboring the microbiota of the healthy child (Wagner *et al.* 2016). These observations put forward the hypotheses that (I) even subclinical infections with enteropathogens can have negative effects on gut health and that (II) pathogens or pathobionts, depending on the community they dwell in, might have negative effects on host homeostasis or not. This indeed supports the concept of the "ecological Koch's postulates", stating that the whole ecosystem, rather than an isolated element contributes to morbidity.

Overall, we are only beginning to understand the complex relationships and interactions within the gut ecosystem and more research is needed in order to elucidate the origin and pathophysiological effect of the different dysbiotic communities and to understand the crosstalk they have between each other as well as with the host.

Conclusion

Infection biology has been moving in the last decades from the original Koch's postulate looking at pathogens, to molecular Koch's postulate looking at virulence factors, to the newly proposed ecological Koch's postulate looking at dysbiosis. Indeed, infection biology has shifted towards an integrated approach of systems biology, ecology, and evolution. This increasing complexity makes it more and more difficult to untangle the causative effects of disease states and asks for imaginative and sophisticated designs of experiments to explain the underlying pathophysiological mechanisms. Particularly, experiments need to take into account the physiological and pathophysiological state of the infected host, for example the microbiota and the exact nutrition the model animals are receiving. In recent years, several initiatives have been launched to standardize the microbiota of model animals (Brugiroux *et al.* 2016) or at least to meticulously report, not only the exact strain of pathogen used and the genetics of the mouse model, but also the microbiota composition, as well as the food composition of the animal models used (Ma *et al.* 2012; Macpherson and McCoy 2015). This will prove indispensable in the future in order to compare different studies and explain the pathophysiological mechanisms underlying the complex interplay between pathogens, the microbiota, and their host. To date, we are only at the beginning of understanding the

interactions within these ecosystems, the perturbations, which can be induced, and their effect on pathogen susceptibility and disease. The widely available techniques of sequencing, especially of metagenomics, metatranscriptomics and metabolomics, will prove essential in this endeavour. Special attention should also be paid to not forget that the microbiota harbours other organisms than prokaryotes, first and foremost viruses (including phages and prophages), and eukaryotes. Only an integrated view of the gut ecosystem, including the host, the pro- and eukaryome and virome as well as the pathogens will allow us to move forward in our understanding of which mechanisms are governing infection.

The scientific community is on the verge of experiencing another revolution in understanding the complex network of gut interactions. This will surely open the way for more targeted and personalized interventions to infectious diseases based on interference or corrections to the misbalances in the gut ecosystem and restoration of gut homeostasis. This could include siderophore-based immunization strategies (Mike *et al.* 2016; Sassone-Corsi *et al.* 2016a), probiotic bacteria (e.g. *E. coli* strain Nissle) using similar iron-scavenging mechanisms than the invading pathogen (Deriu *et al.* 2013), probiotic strains consuming H_2 and hence restricting the use of this energy source for invading pathogens (Maier *et al.* 2013), the development of probiotic strains expressing bacteriocins or microcins targeting the pathogen (Kommineni *et al.* 2015; Hegarty *et al.* 2016; Sassone-Corsi *et al.* 2016b), expressing iron-sequestering mechanisms to inhibit invading pathogens (Vazquez-Gutierrez *et al.* 2016), siacylidase inhibitors (Huang *et al.* 2015) or inhibitors of anaerobic respiration (Winter and Bäumler 2014b) (see Table 1). There are certainly many other possible intervention strategies yet to be discovered. The generated knowledge will therefore prove very important in paving the way to propose other intervention strategies, which do not rely on antibiotics. In a world where antibiotic resistance is on a constant rise this aspect will be of utmost importance.

Figure 1: Homeostatic gut environment (A) versus a dysbiotic gut environment (B). Scheme of an intestine in homeostasis (left) and in dysbiosis upon invasion of a pathogen (depicted in red). Upon dysbiosis, the mucus layer is thinner, larger amounts of antimicrobial peptides are secreted (e.g. C-type lectins as Reg3γ) to prevent bacteria breach the barrier and get access to the underlying tissue. This leads to villous blunting, influx of inflammatory cells into the lamina propria and depletion of members of the microbiota, leading to a changed composition and lower diversity of the resident microbiota.

Figure 2: Mechanisms evolved by pathogen to combat the resident microbiota and the host

Pathogens have evolved several mechanisms to overcome the barrier imposed by the resident microbiota and the host. These include direct (bacteriocins, microcins, T6SS) and indirect (nutrient restriction) inhibition of members of the microbiota, the use of alternative energy sources as well as different mechanisms to overcome the barriers imposed by the hosts (mucinases, T3SS and other virulence factors acting on the host).

Figure 3: The evolution of the Koch's postulate

In recent years, the original Koch's postulate ("a pathogen, a disease") have been extended to the molecular Koch's postulate (Stanley Falkow, "a virulence genes, a disease") and here to the ecological Koch's postulate ("a dysbiosis, a disease")

Table 1: Possible new intervention strategies intervening with the gut ecosystem to target diseases related to dysbioses-induced enteropathogenic blooms

Intervention strategy	Evidence for intervention	References
Siderophore-based immunization strategies	Mice immunized mice with siderophores conjugated to an immunogenic carrier protein were able to elicit a potent immune response and to protect against urinary tract infections. Mice immunized with a cholera toxin β-siderophore conjugate show a potent immune response and are able to protect against infection with *Salmonella* Typhimurium.	(Mike *et al.* 2016; Sassone-Corsi *et al.* 2016a)
Probiotic strains with similar or more efficient iron-sequestering mechanisms inhibiting invading pathogens	Oral gavage with *Escherichia coli* strain Nissle 1917 reduces *S*. Typhimurium colonization in mouse models of acute colitis or chronic persistent infection. The observed probiotic activity depends on the iron-sequestering mechanisms of *E. coli* Nissle, which is highly similar to the one found in *Salmonella* Typhimurium. The two bifidobacterial strains *Bifidobacterium pseudolongum* PV8-2 (Bp PV8-2) and *Bifidobacterium kashiwanohense* PV20-2 (Bk PV20-2) are able to inhibit growth of *S.* Typhimurium and *E. coli* O157:H45 (EHEC) in *in vitro* co-culture experiments and are able to displace the pathogens on mucus-producing HT29-MTX cell lines.	(Deriu *et al.* 2013) (Vazquez-Gutierrez *et al.* 2016),
Probiotic strains consuming H_2 to prevent initial ecosystem invasion	In a non-inflamed intestine, *S.* Typhimurium relys on H_2 metabolisms for invasion. Introducing H_2-consuming bacteria into the microbiota reduces *hyb*-dependent *S*. Typhimurium growth.	(Maier *et al.* 2013)
Probiotic strains producing butyrate	Oral gavage of mice with tributyrin reduces growth of *S*. Typhimurium in the inflamed intestine.	(Rivera-Chávez *et al.* 2016b)
Probiotic strains expressing bacteriocins or microcins targeting the pathogen	Colonization of mice with a bacteriocin-carrying *E. faecalis* strain defective for conjugation leads to clearance of vancomycin resistant enterococci. Microcin-producing *E. coli* Nissle is able to limit the growth of commensal *E. coli*, adherent–invasive *E. coli* and *Salmonella enterica* in the inflamed intestine.	(Kommineni *et al.* 2015; Hegarty *et al.* 2016; Sassone-Corsi *et al.* 2016b)
Siacylidase inhibitors	Oral administration of sialidase inhibitors decreases outgrowth of *E. coli* as well as the severity of colitis in a mouse model.	(Huang *et al.* 2015)
Inhibitors of aerobic respiration	Aerobic respiration is used by *Salmonella* spp and other *Enterobacteriaceae* to thrive in the inflamed intestine.	(Winter and Bäumler 2014b)
Sustaining PPAR-γ signaling	PPAR-γ signaling in the homeostatic intestine leads to β-oxidation in the colonocytes and hence an anoxic environment limiting nitrate availability and outgrowth of *Enterobacteriaceae*	(Byndloss *et al.* 2017)

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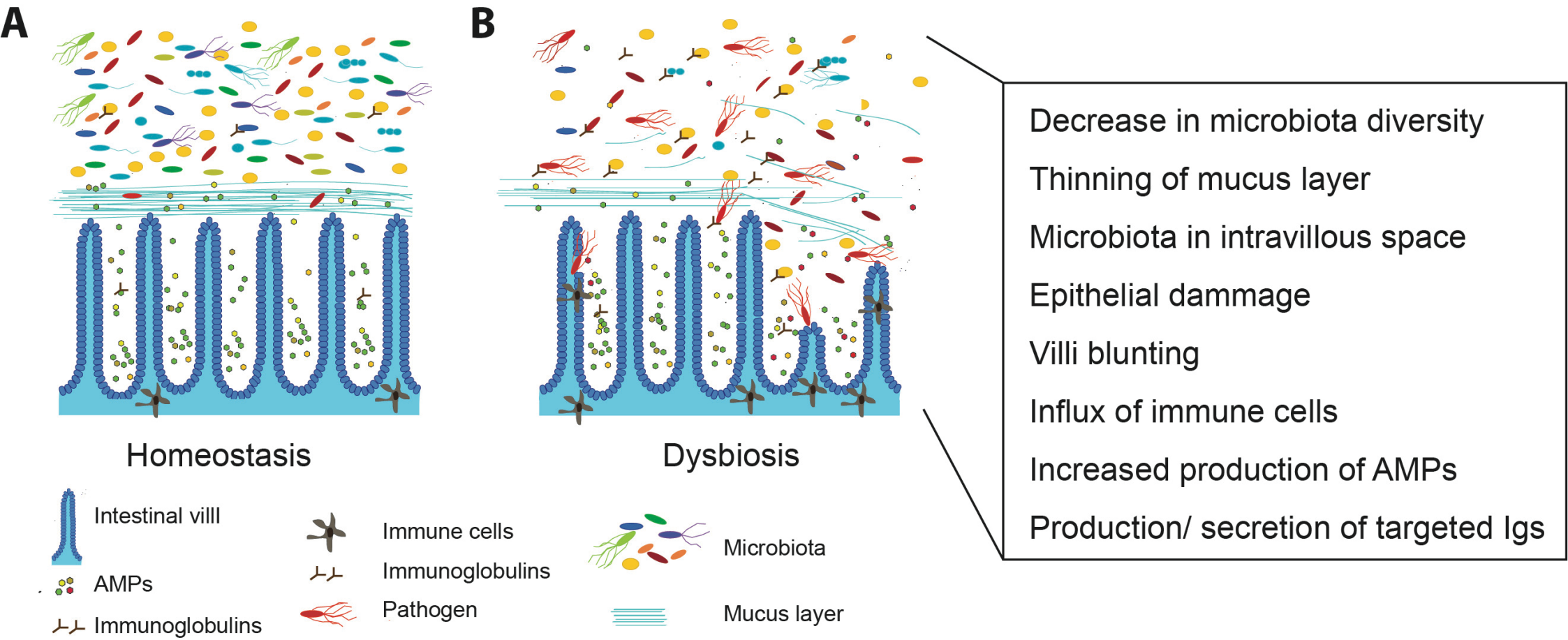
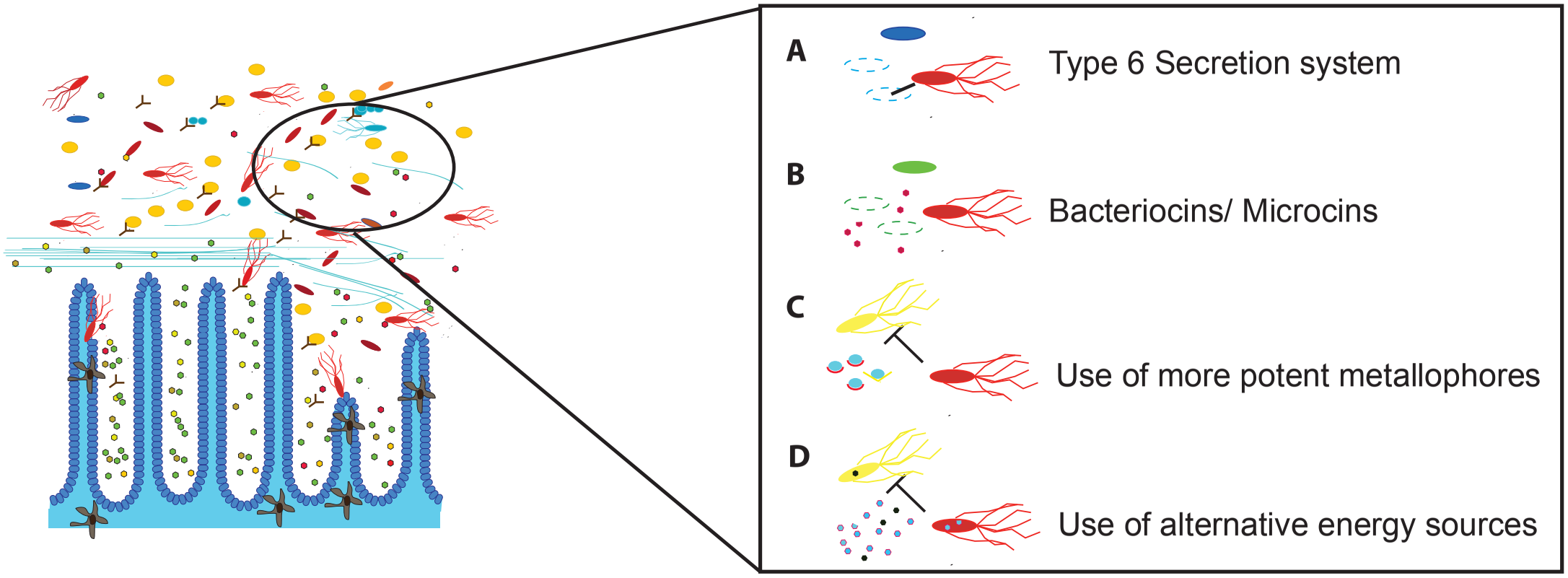
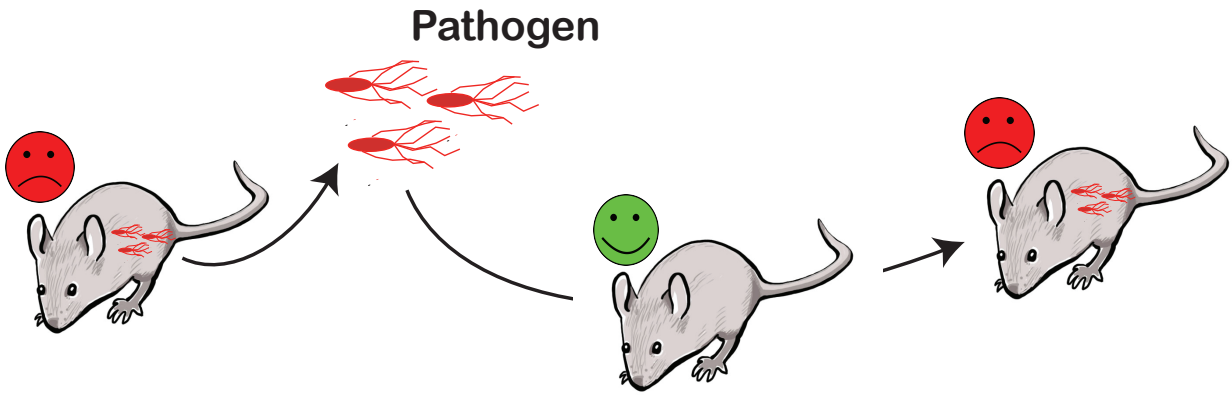

A
B
Homeostasis
Dysbiosis
Intestinal villl
AMPs
Immunoglobulins
Immune cells
Immunoglobulins
Pathogen
Microbiota
Mucus layer
Decrease in microbiota diversity
Thinning of mucus layer
Microbiota in intravillous space
Epithelial dammage
Villi blunting
Influx of immune cells
Increased production of AMPs
Production/ secretion of targeted Igs

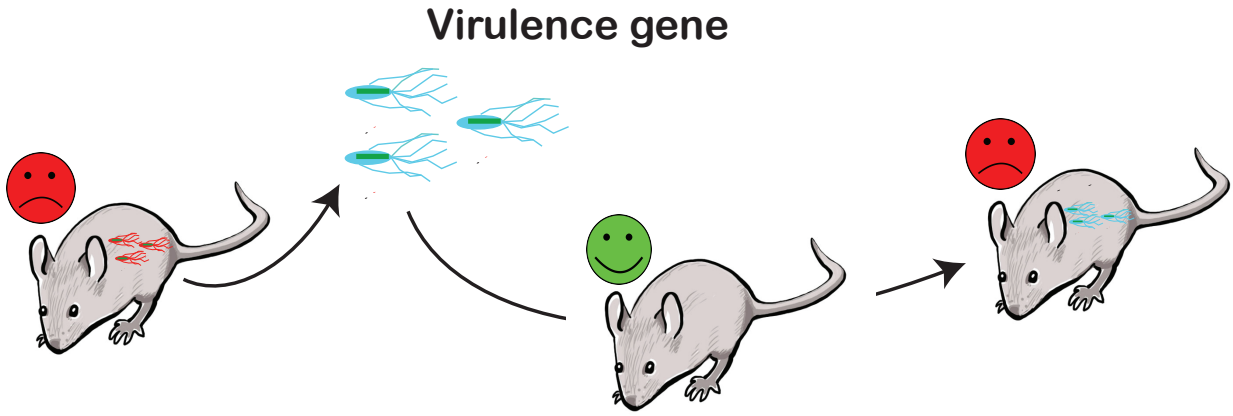

A
Type 6 Secretion system
B
Bacteriocins/ Microcins
C
Use of more potent metallophores
D
Use of alternative energy sources

Koch’s postulate



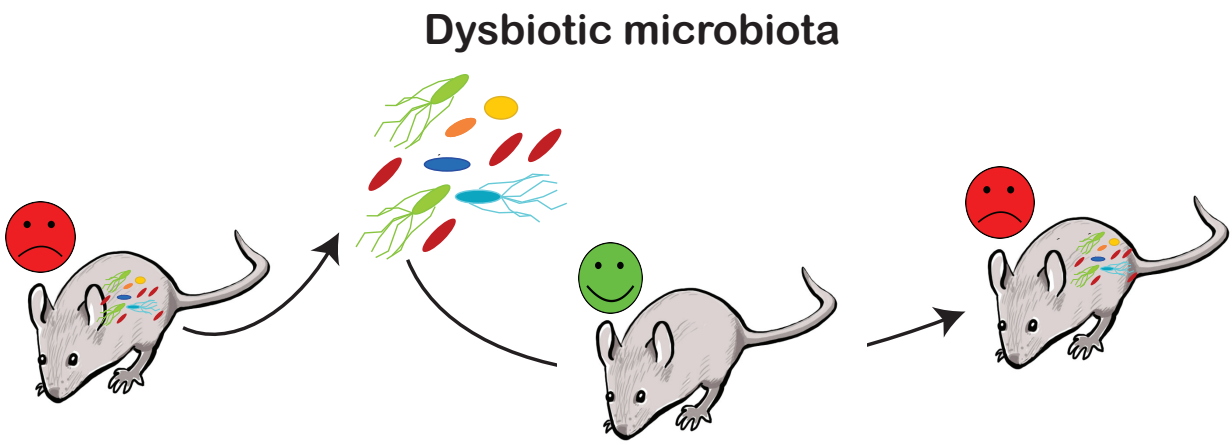
«A pathogen, a disease»

Molecular Koch’s postulate



«A virulence gene, a disease»

Ecological Koch’s postulate



«A dysbiosis, a disease»