Microbiome-mediated colonization resistance: defense against enteropathogens and multi-drug resistant organisms

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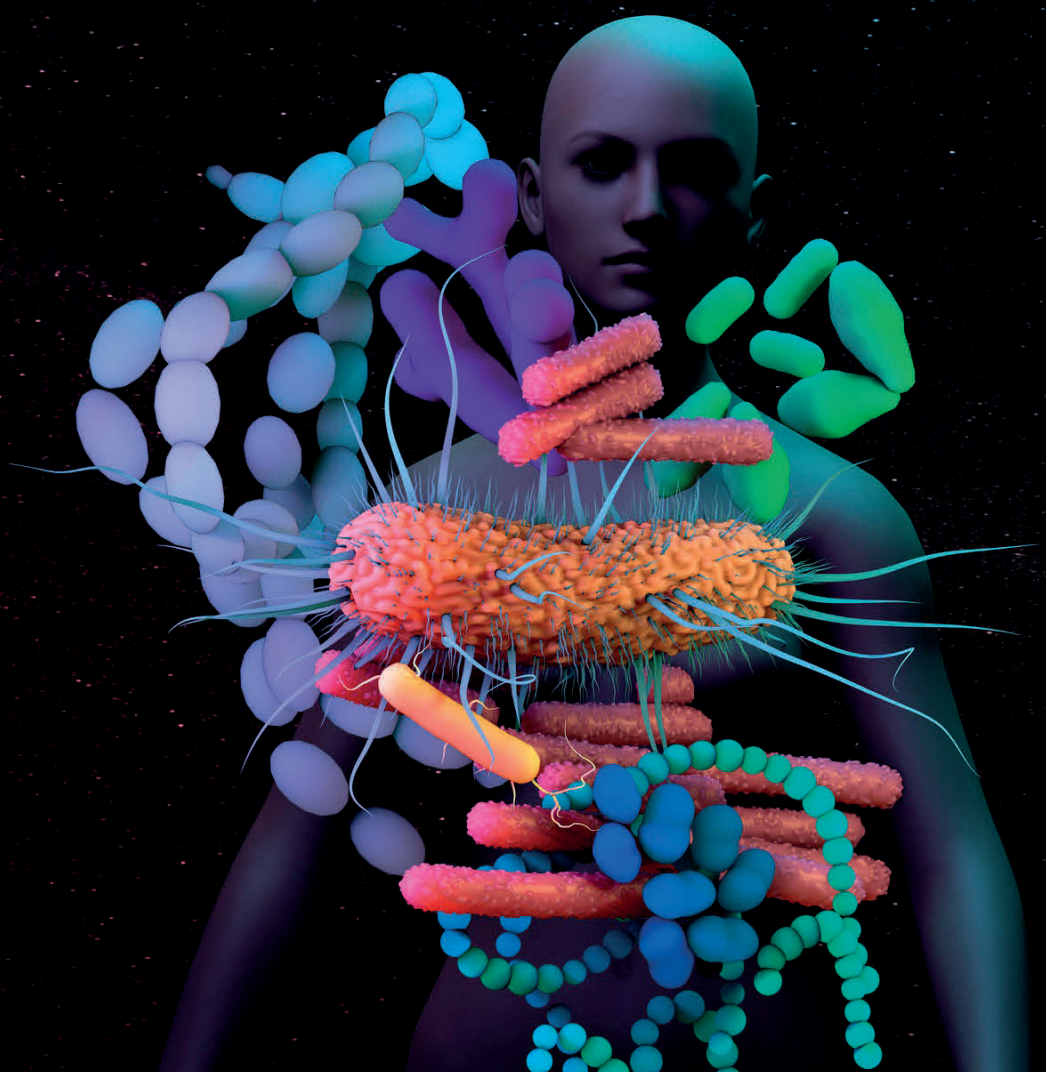
Part I

Mechanisms of microbiome-mediated colonization resistance and how to develop microbiome-based therapies



Chapter 2

Gut microbiota and colonization resistance against bacterial enteric infection

Gut microbiota, colonization resistance and infection
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Summary

The gut microbiome is critical in providing resistance against colonization by exogenous microorganisms. The mechanisms via which the gut microbiota provides colonization resistance (CR) have not been fully elucidated, but include secretion of antimicrobial products, nutrient competition, support of gut barrier integrity and bacteriophage deployment. However, bacterial enteric infections are an important cause of disease globally, indicating that microbiota-mediated CR can be disturbed, and become ineffective. Changes in microbiota composition, and potential subsequent disruption of CR, can be caused by various drugs, such as antibiotics, proton pump inhibitors, antidiabetics and antipsychotics, thereby providing opportunities for exogenous pathogens to colonize the gut and ultimately cause infection. In addition, the most prevalent bacterial enteropathogens, including *Clostridioides difficile*, *Salmonella enterica* serovar Typhimurium, enterohemorrhagic *Escherichia coli*, *Shigella flexneri*, *Campylobacter jejuni, Vibrio cholerae*, *Yersinia enterocolitica* and *Listeria monocytogenes*, can employ a wide array of mechanisms to overcome colonization resistance. This review aims to summarize current knowledge on how the gut microbiota can mediate colonization resistance against bacterial enteric infection, and on how bacterial enteropathogens can overcome this resistance.

Introduction

The human gastrointestinal tract is colonized by an enormous number of microbes, collectively termed gut microbiota, including bacteria, viruses, fungi, archaea and protozoa. Bacteria achieve the highest cell density, estimated to be approximately 10^{11} bacteria/ml in the colon [1]. Research has long focused on pathogenicity of microbes and not on their potential beneficial roles for human health. Beneficial roles include aiding in immune system maturation, production of short-chain fatty acids (SCFAs), vitamin synthesis and providing a barrier against colonization with potential pathogens [2]. Additionally, the gut microbiota has extensive interactions with our immune system and it has been associated with many immune-mediated diseases both in and outside of the gut [3-5]. Over the last ten years, there has been an increased interest in elucidating the bidirectional relationship between gut microbiota and human health and disease. This has been partly propelled by improved sequencing technologies, allowing the profiling of entire microbial communities at high efficiency and low costs [6].

Hundreds of different bacterial species inhabiting the healthy human gut have been identified [7, 8]. Initial studies seeking to elucidate the relationship between human microbiota and health and disease were largely observational; gut microbiota composition would be compared between diseased and healthy groups and subsequently associated with clinical markers [9]. Currently, the field is moving towards more functional and mechanistic studies by including other –omics techniques.

In healthy individuals, the gut microbiota provides protection against infection by deploying multiple mechanisms including secretion of antimicrobial products, nutrient competition, support of epithelial barrier integrity, bacteriophage deployment, and immune activation. Together, these mechanisms contribute to resistance against colonization of exogenous microorganisms (colonization resistance, CR) [10]. However, also in absence of a fully functional immune system, the gut microbiota can provide a crucial and nonredundant protection against a potentially lethal pathogen [11]. This review will discuss the mechanisms used by gut microbiota to provide CR, the impact of various drugs on gut microbiota and thereby CR, and the strategies of specific bacterial pathogens to overcome CR and ultimately cause enteric infection.

Mechanisms providing colonization resistance

The gut microbiota produces various products with antimicrobial effects, including SCFAs, secondary bile acids and bacteriocins. Each of these contribute to CR in a product-specific manner. The following section describes their general mechanisms of

action. The contribution of the immune system in conferring CR has been extensively reviewed elsewhere and is outside the scope of this review [12, 13].

Short-chain fatty acids

SCFAs are mainly produced by bacteria through fermentation of non-digestible carbohydrates (Fig. 1) [14]. The three main SCFAs are acetate, propionate and butyrate, constituting 90-95% of the total SCFA pool [15]. During homeostatic conditions, butyrate is the main nutrient for enterocytes and is metabolized through β-oxidation. Hereby, an anaerobic milieu inside the gut can be maintained [16]. SCFAs can impair bacterial growth by affecting intracellular pH and metabolic functioning. SCFA concentrations have been shown to inversely relate to pH throughout different regions of the gut [17]. At lower pH, SCFAs are more prevalent in their non-ionized form and these non-ionized acids can diffuse across the bacterial membrane into the cytoplasm. Within the cytoplasm they will dissociate, resulting in a build-up of anions and protons leading to a lower intracellular pH [18].

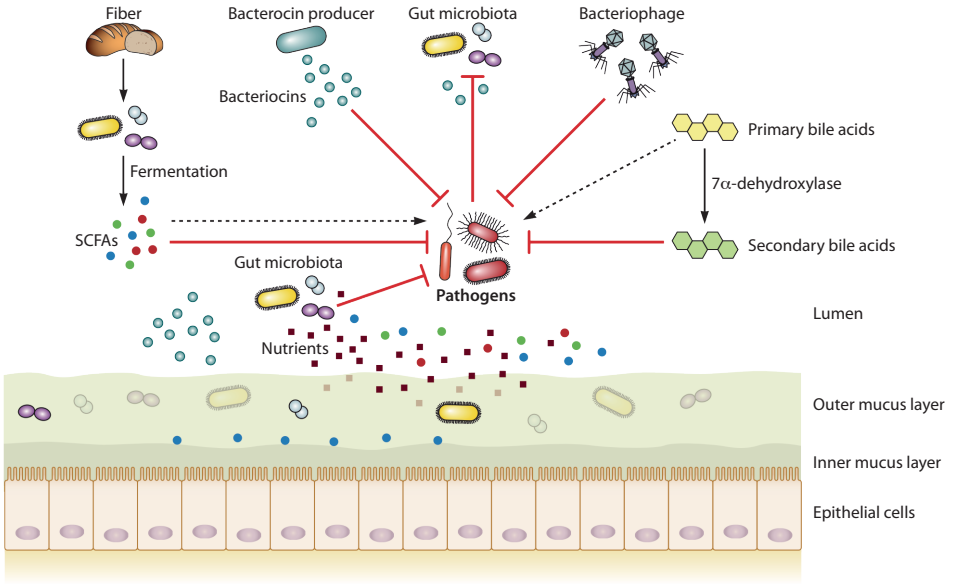

Figure 1: Outline of gut microbiota-mediated colonization resistance mechanisms. Fiber obtained from the diet is fermented by gut microbiota into short-chain fatty acids (SCFAs). Bacteriocin producers produce bacteriocins capable of targeting a specific pathogen. Primary bile acids can be converted by a very select group of gut microbiota into secondary bile acids, which generally have antagonistic properties against pathogens. Nutrient competition of native microbiota can limit access to nutrients for a pathogen. Specific organisms can use SCFAs, bacteriocins and primary bile acids to increase their virulence, as will be discussed in later sections.

In presence of acetate, metabolic functioning of *Escherichia coli* could be impaired by preventing biosynthesis of methionine, leading to accumulation of toxic homocysteine and growth inhibition. Growth inhibition was partly relieved by supplementing the growth medium with methionine, showing that this metabolic dysfunction is one of the factors by which SCFAs impair bacterial growth [19].

Bile acids

Bile acids, possessing antimicrobial properties, are produced by the liver and excreted in the intestinal tract to aid in the digestion of dietary lipids. After production of primary bile acids in the liver, they are subsequently conjugated with glycine or taurine, to increase solubility [20]. These are then stored in the gallbladder, and upon food intake, are released into the duodenum to increase solubilization of ingested lipids. A large part of conjugated primary bile acids is reabsorbed in the distal ileum (50-90%), while the remainder can be subjected to bacterial metabolism in the colon [20]. Here, conjugated bile acids can be deconjugated by bile salt hydrolases (BSH), which are abundantly present in the gut microbiome [21]. Deconjugated primary bile acids can subsequently be converted into the two main secondary bile acids, deoxycholic acid and lithocholic acid, by few bacteria, mostly *Clostridium* species, via 7α-dehydroxylation through a complex biochemical pathway [21-23] (Fig. 1). A crucial step during the conversion is encoded by the *baiCD* gene, which is found in several *Clostridium* strains, including *Clostridium scindens* [24]. Deoxycholic acid is bactericidal to many bacteria, including *Staphylococcus aureus, Bacteroides thetaiotaomicron*, *Clostridioides difficile*, bifidobacteria and lactobacilli by membrane disruption and subsequent leakage of cellular content [25-28].

The importance of bacteria for conversion of primary bile acids was demonstrated by investigating bile acid profiles in germ-free mice, where no secondary bile acids could be measured [29]. Very few colonic bacteria, less than 0.025% of total gut microbiota, are capable of performing 7α-dehydroxylation [23, 30]. One of these bacteria, *C. scindens*, is associated with colonization resistance against *C. difficile* through secondary bile acid production [22, 31]. A follow-up *in vivo* study demonstrated that *C. scindens* provided CR in the first day post infection (p.i), but protection and secondary bile acid production was lost at 72 p.i [32]. *C. scindens* on its own was also not sufficient to inhibit *C. difficile* outgrowth in humans [33]. Together, these studies suggest that *C. scindens* either requires cooperation with other secondary-bile acid producing bacteria or that other mechanisms were involved in providing CR. The secondary bile acid lithocholic acid may exert its antimicrobial effects, and potentially its effects on CR, in an indirect manner. Lithocholic acid has been shown to enhance transcription for the antimicrobial peptide LL-37, in gut epithelium using a HT-29 cell line [34]. However, no increased mRNA transcription nor protein translation of LL-37 was observed in another study using a Caco2 cell line [35].

Bacteriocins

Bacteriocins are short, toxic peptides produced by specific bacterial species that can inhibit colonization and growth of other species [36] (Fig. 1). Their mechanisms of action are multifold and include disturbing RNA and DNA metabolism, and killing cells through pore formation in the cell membrane [37-40]. Bacteriocins can be divided into those produced by Gram-positive bacteria, and those produced by Gram-negative bacteria. Further classification of bacteriocins has been extensively discussed elsewhere [41, 42]. Bacteriocins produced by Gram-positive bacteria are mostly produced by lactic acid bacteria (e.g. *Lactococcus* and *Lactobacillus*) and some *Streptococcus* species, and are further subdivided into three major classes on the basis of the molecular weight of the bacteriocins and the presence of post-translational modifications [42]. Bacteriocins produced by Gram-negative bacteria, mostly by *Enterobacteriaceae*, can be broadly divided into high molecular weight proteins (colicins) and lower molecular weight peptides (microcins) [41].

The lantibiotic nisin is the best studied bacteriocin and is produced by *Lactococcus lactis* strains. It has potent activity against many Gram-positive bacteria but has much less intrinsic activity against Gram-negative organisms [43-45]. By itself, nisin does not induce growth inhibition of Gram-negative bacteria, since binding to lipid II – the main target – is prevented by the outer bacterial membrane [46]. Therefore, studies have used different methods to overcome this problem by combining nisin with chelating agents like EDTA, antibiotics and engineered nisin peptides [47-52]. These compounds can destabilize the outer membrane, allowing nisin to exert its damaging effect [53, 54].

Several *in vivo* models have confirmed the potency of bacteriocins in providing CR. *Lactobacillus salivarius* UCC 118, which produces the bacteriocin Abp118, was able to significantly protect mice from infection by direct killing of *Listeria monocytogenes*, while an UCC 118 mutant could not, confirming the protective role of Abp118 against this food-borne pathogen [55].

Another example is *Bacillus thuringiensis* DPC 6431, which produces the bacteriocin thuricin [36]. Thuricin targets several *C. difficile* strains, including the highly virulent PCR ribotype 027. *In vitro*, its activity was more potent than metronidazole, the common treatment for *C. difficile* infection [56]. In a colon model system, metronidazole, vancomycin and thuricin all effectively reduced *C. difficile* levels. However, thuricin has the advantage of conserving gut microbiota composition. This is highly relevant, as a disturbed microbiota is associated with increased susceptibility to infection [57, 58].

Enterobacteriaceae members can produce specific bacteriocins called colicins and one example, colicin F_Y, is encoded by the *Yersinia frederiksenii* Y27601 plasmid.

Recombinant *E. coli* strains, capable of producing colicin $F_{Y,}$ were shown to be highly effective against *Yersinia enterocolitica in vitro* [59]. *In vivo* experiments were performed by first administering the recombinant *E. coli* strains, after which mice were infected with *Y. enterocolitica*. In mice with a normal gut microbiota the recombinant strains did not inhibit *Y. enterocolitica* infection, while infection was effectively reduced in mice pre-treated with streptomycin [59]. This was most probably the result of increased colonization capacity of recombinant *E. coli* in the inflamed gut, while the normal gut microbiota provided sufficient CR to prevent *E. coli* colonization [59].

Microcins are also produced by *Enterobacteriaceae*, but differ from colicins in several ways [60]. For example, microcins are of much smaller size (<10 kDa) and microcin production is not lethal to the producing bacterium, in contrast to colicin production [60]. *E. coli* Nissle 1917, capable of producing microcin M and microcin H47, could significantly inhibit *Salmonella enterica* serovar Typhimurium *in vitro* and *in vivo* [61]. This inhibition was however only seen during intestinal inflammation, during which *S.* Typhimurium expresses siderophores to scavenge iron from an iron-depleted environment. As microcins are able to conjugate to siderophores and *S.* Typhimurium takes up the siderophore during iron scavenging, microcins are introduced into the bacterial cell in a Trojan-horse like manner [62].

In silico identification of bacteriocin gene clusters shows that much remains to be discovered in this area, as 74 clusters were identified in the gut microbiota [63]. Not all of these clusters may be active *in vivo*, but it illustrates the potential relevance of bacteriocin production by the gut microbiota to provide colonization resistance.

Nutrient competition

Bacteria have to compete for nutrients present in the gut. This is especially relevant for bacterial strains belonging to the same species, as they will often require similar nutrients. The importance of nutrient competition in providing CR has been shown in multiple studies using multiple *E. coli* strains [64-67]. Indigenous *E. coli* strains compete with pathogenic *E. coli* O157:H7 for the amino acid proline [64]. In fecal suspensions, depletion of the proline pool by high-proline-utilizing *E. coli* strains inhibited growth of pathogenic *E. coli*. This inhibition could be reversed by adding proline to the medium, thereby confirming nutrient competition between the strains [64]. In addition to amino acids, different *E. coli* strains use distinct sugars present in the intestinal mucus [65]. When two commensal *E. coli* strains were present in the mouse gut that together utilize the same sugars as *E. coli* O157:H7, *E. coli* O157:H7 was unable to colonize after it was administered to these mice. However, *E. coli* O157:H7 successfully colonized when only one of these commensals was present. This indicated that the two commensals complement each other to sufficiently deplete all sugars used by this pathogenic *E.*

coli strain [66]. Nutrient competition is not limited to macronutrients, but can extend to micronutrients such as iron. *S.* Typhimurium is known to take up large amounts of iron from the inflamed gut during infection [67]. Upon a single administration of the probiotic *E. coli* Nissle 1917, which was proposed to scavenge iron very efficiently, *S.* Typhimurium levels were reduced more than two log-fold during infection via the limitation of iron availability. Administration of *E. coli* Nissle 1917 prior to infection with *S.* Typhimurium led to a 445-fold lower colonization [67].

Finally, genome-scale metabolic models have been used to reconstruct microbiome-wide metabolic networks, which could partly predict which species utilize specific compounds from their environment [68]. These models have been used to study nutrient utilization by *C. difficile*, which will be described in the section on this organism below. Together, these studies show that colonization resistance by nutrient competition is most effective when microbiota take up key nutrients that are required by the pathogen (Fig. 1). Future strategies could therefore aim at administrating probiotic strains that are able to outcompete pathogens for specific nutrients. This is especially relevant at times of gut microbiota disturbances, e.g. during and following an antibiotic treatment, as this is the time window where it is easiest for exogenous bacteria to colonize the GI tract.

Mucus layers

The gut barrier consists of the inner and outer mucus layer, the epithelial barrier and its related immune barrier. It is out of the scope of this review to discuss the full immunological characteristics of the epithelial barrier, the highly complex host-microbe interactions occurring at the mucus layer and host-associated genetic polymorphisms associated with mucus layer composition, as these have been extensively described elsewhere [12, 13, 69, 70]. Instead, a general description with various examples of how the mucus layer provides CR will be given.

The inner mucus layer is impenetrable and firmly attached to the epithelium, forming a physical barrier for bacteria thereby preventing direct interaction with the epithelial layer and a potential inflammatory response [71, 72]. Commensal gut microbes reside and metabolize nutrients in the nonattached outer mucus layer. Thinning of the mucus layer leads to an increased susceptibility for pathogen colonization, which can result from a Western-style diet deficient in microbiota-accessible-carbohydrates (MACs) [58]. When MACs were scarce, mucus-degrading bacteria (*Akkermansia muciniphila* and *Bacteroides caccae*) fed on the outer mucus layer in a gnotobiotic mouse model, resulting in closer proximity of bacteria to the epithelial layer [58]. The host adapts by increasing *muc2* expression, the main producer of intestinal mucin glycans, but fails to sufficiently do so. Inner mucus layer damage could however be reversed by administration of *Bifidobacterium longum*, perhaps due to stimulation of mucus generation [73].

The composition of the microbiota is thus a contributing factor to the integrity of the mucus barrier. Genetically identical mice housed in different rooms at the same facility showed a distinct microbiota composition, with one group of mice showing a more penetrable barrier [74]. When fecal-microbiota transplant (FMT) was performed on germ-free mice, they displayed the same barrier function as their respective donor. No specific microbes were identified to be responsible for the change in observed barrier function [74].

In conclusion, the mucus layers provide a first barrier of defense against colonization of exogenous microorganisms. Diet has been shown to be an important factor for proper functioning of this layer, suggesting that dietary intervention, or specific pro- and prebiotics, may be a future therapeutic option.

Bacteriophages

Bacteriophages are the most abundant microorganisms on our planet and are also highly present in the human gut [75, 76]. Bacteriophages have been proposed as potential alternatives to antibiotics, as they are highly specific, only targeting a single or a few bacterial strains thereby minimizing the impact on commensal members of the microbiota [75, 77] (Fig. 1). Their complex interactions in the intestine with both host immunity and bacterial inhabitants are starting to be explored, but much remains to be elucidated [76]. Here, we will focus on their relationship with bacterial enteropathogens.

Vibrio cholerae infection could be controlled using a prophylactic phage cocktail in mice and rabbits [78]. This prophylactic cocktail killed *V. cholerae in vitro*, reduced colonization of *V. cholerae* in the mouse gut and prevented cholera-like diarrhea in rabbits. Importantly, the authors suggest that the concentration of phages in the gut is an important criterion for successful prevention of infection, as timing between phage cocktail administration and *V. cholerae* inoculation was associated with treatment outcome [78]. Similar findings have been demonstrated for *Campylobacter jejuni* colonization in chickens, where a phage cocktail reduced *C. jejuni* levels several orders of magnitude [79].

Bacteriophages can also confer a competitive advantage for commensals. *Enterococcus faecalis* V583 harbors phages that infect and kill other *E. faecalis* strains, thereby creating a niche for *E. faecalis* V583 [80].

Phages play an important role in excluding specific gut bacteria and can thereby contribute to CR. Therapeutic use in humans is not yet performed at a wide scale in the Western world, as sufficient evidence for their safety and efficacy is still lacking [81]. However, recent case reports indicate that bacteriophage treatment has definite future potential for treating multi-drug resistant bacteria [82, 83].

Effects of various non-antibiotic drugs on gut colonization resistance

Antibiotics are long known for their deleterious effect on gut microbiota. Recently, various other drugs have come to attention for their impact on our microbial ecosystem. As effects of antibiotics have been extensively reviewed elsewhere [84, 85], the focus in the current review will be on non-antibiotic drugs, namely proton-pump inhibitors (PPIs), antidiabetics and antipsychotics.

Proton-pump inhibitors

PPIs inhibit gastric acid production and are among the most prescribed drugs in Western countries [86]. A significant association between long-term use of PPIs and the risk on several bacterial enteric infections has been demonstrated in multiple systematic reviews [87-90].

Several studies have associated PPI use with microbiota alterations that may specifically predispose to *C. difficile* infection and to small intestinal bacterial outgrowth [91-95]. Especially taxa prevalent in oral microbiota (e.g. *Streptococcus*) were associated with PPI use, likely resulting from increased gastric pH and thereby allowing for colonization of these bacteria further down the gastrointestinal tract [91-94]. Administering PPIs to twelve healthy volunteers for four weeks did not result in changes in diversity or changes in overall microbiota composition. However, abundance of specific taxa associated with *C. difficile* infection and gastrointestinal bacterial overgrowth increased, thereby potentially lowering colonization resistance against *C. difficile* [91].

Results of two mouse studies suggest that the reduced bactericidal effect, due to increased stomach pH, may be the most important factor for increased enteric infection risk. Mice received PPIs seven days prior to infection with the murine pathogen *Citrobacter rodentium*, which resulted in increased numbers of *C. rodentium* in the cecum one hour post inoculation as compared to control mice [96]. Similar results were observed in another study where treatment of mice with PPIs led to increased colonization of vancomycin-resistant enterococci and *Klebsiella pneumoniae* [97]. In spite of its general acceptance as a model for gut disturbances, it is important to note that mice were pre-treated with clindamycin, which may limit generalizability [97]. This is an important issue when studying effects of PPIs, as the combined use of medication in the human population complicates the study of the effects of PPIs on microbiota and CR. Even though large-scale studies have adjusted for cofounders to filter out the effect of PPIs on the gut microbiota, this does not represent a mechanistic study where only PPIs would be administered [92, 98].

Therefore, more mechanistic studies investigating how PPIs increase the risk for enteric infection are required. These studies should then exclusively administer PPIs to healthy human volunteers or animals.

Antidiabetics

Metformin is the primary prescribed drug for treatment of type II diabetes mellitus (T2DM) and mainly acts by reducing hepatic glucose production, thereby lowering blood glucose levels [99]. The current increase in the number of T2DM patients is unprecedented and it is therefore crucial to evaluate metformin's effect on gut microbiota and colonization resistance [100].

The microbiota of T2DM patients is, amongst other changes, characterized by a depletion in butyrate-producing bacteria [101, 102]. Metformin administration increased both the abundance of butyrate and other SCFA-producing bacteria, as well as fecal SCFA levels and may thus contribute to colonization resistance. The underlying mechanisms remain unknown [101, 103].

Another effect of metformin has been studied in an *in vitro* model, where it was found to reduce tight junction dysfunction of the gut barrier by preventing TNF-α induced damage to tight junctions [104]. Similar findings for improvement of tight junction dysfunction were demonstrated using two *in vivo* models, one using interleukin-10 deficient mice and one using a colitis mouse model [105, 106]. As tight junctions are a critical part of epithelial barrier integrity, alleviating their impaired functioning likely improves CR.
In conclusion, metformin may have beneficial effects on CR, as its ability to raise SCFA concentrations and improved tight junction function suggests. The effects of metformin on gut microbiota and CR in healthy organisms needs further evaluation.

Antipsychotics

The interest in whether antipsychotics affect gut microbiota composition and colonization resistance may surge after a recent publication demonstrating that antipsychotics target microbes based on their structural composition [107]. This led to the suggestion that antibacterial activity may not simply be a side effect of antipsychotics, but can be part of their mechanism of action [107]. Various antipsychotics have been investigated for their antibacterial effects, of which several will be highlighted here.

In an *in vitro* model, olanzapine has been demonstrated to completely inhibit growth of two potentially pathogenic bacteria, *E. coli* and *E. faecalis* [108]. Pimozide has been shown to inhibit internalization of several bacteria, including *L. monocytogenes* [109]. An *in vitro* screening test evaluated effects of fluphenazine on 482 bacterial strains, belonging to ten different genera. Growth inhibition was demonstrated in multiple

species, including five out of six *Bacillus spp.,* 95 out of 164 staphylococci, 138 out of 153 *V. cholerae* strains and *Salmonella* serovars Typhi and Typhimurium. Significant protection by administering fluphenazine was shown in a mouse model infected with *S*. Typhimurium, as viable cells in several organs was lower and overall survival was higher as compared to controls [110].

Antipsychotics can also be used in combination with antibiotics, to exert a synergistic antibacterial effect. Flupenthixol dihydrochloride (FD) was demonstrated to have antibacterial activity, both *in vitro* and *in vivo* [111]. Co-administration of FD and penicillin yielded extra protection against *S.* Typhimurium as compared to singular administration of either drug[111]. As antipsychotics have only recently been recognized for their potential antimicrobial effects, studies have only looked at the effects on pathogens. It is likely that gut commensals are also affected by these drugs, but future studies will have to confirm this hypothesis.

Apart from their potential antibacterial effects, several antipsychotics were shown to increase intestinal permeability in the distal ileum in rats, and therefore showing a possibly detrimental effect on CR [112]. Curiously enough, use of antidepressants was associated with increased risk of *C. difficile* infection development, although no underlying mechanism has been elucidated yet [113].

In conclusion, antipsychotics have definite antibacterial effects, but, to our knowledge, no studies have yet been performed regarding their effects on colonization resistance and bacterial enteric infection *in vivo*.

Colonization resistance towards specific bacterial enteric pathogens

Other than antibiotic resistance acquisition, enteric pathogens possess multiple virulence factors to overcome CR and cause infection. Some of these factors are common and apply to many bacterial species, others are organism-specific. Mechanisms implicated in antibiotic resistance development include horizontal gene transfer, mutational resistance and altering structure and thereby efficacy of the antibiotic molecule. Full reviews describing these mechanisms in depth can be found elsewhere [114, 115]. Here, the main focus will be on how several of the most prevalent and dangerous bacterial enteropathogens overcome the mechanisms providing CR as described herein, namely secretion of antimicrobial products, nutrient competition, mucus barrier integrity and bacteriophage deployment. As insufficient knowledge is available on how each specific enteropathogen overcomes CR by rendering bacteriophages ineffective, apart from the

well-known and conserved CRISPR-Cas, an overview of the currently known bacterial defense mechanisms will be given at the end of this review.

C. difficile

C. difficile-associated diarrhea is the most common hospital-acquired infection, causing more than 450.000 diarrheal cases per year in the United States alone [116]. Clinical symptoms can range from self-limiting diarrhea to bloody diarrhea, pseudomembranous colitis and ultimately death [117]. However, also in healthy individuals CR is not always successful against this opportunistic pathogen, resulting in asymptomatic colonization in 2-15% of the healthy population [118]. The reason why some asymptomatically colonized patients do not develop infection, while others do, may well be found in the gut microbiome, although no mechanisms have yet been elucidated. *C. difficile* contains a pathogenicity locus with the information to produce its two major toxins, TcdA and TcdB. The significance of a third toxin, called binary toxin, is less clear. Toxin production in the colon is facilitated by disruption of the native gut microbiota, for instance through antibiotic use [119].

Effects of SCFAs on *C. difficile* throughout its life cycle are currently unclear [120-122]. In an antibiotic-treated mouse model, decreased SCFA levels were associated with impaired CR against *C. difficile* [120]. CR was subsequently restored six weeks after ending antibiotic treatment with a concomitant increase in SCFAs, probably resulting from restoration of the fermentative activity of the microbiota [120]. Restoration of SCFA levels is also seen as an effect after fecal microbiota transplantations in humans [122]. However, SCFA supplementation could not induce a significant decrease in *C. difficile* shedding levels up to six weeks post infection [121]. No study has yet investigated whether *C. difficile* possesses any mechanisms by which it becomes resistant against the effects of SCFAs, which warrants further research.

Compared to the effects of SCFAs, there is more clarity on the effects of bile acids on *C. difficile*. Secondary bile acids are toxic to both *C. difficile* spores and vegetative cells, while primary bile acids generally stimulate growth and spore germination [123-125]. During antibiotic treatment, conversion of primary into secondary bile acids is suppressed and the reduction of secondary bile acids leads to a more favorable environment for *C. difficile* [120]. In addition, *C. difficile* isolates causing most severe disease in mice were also the isolates that showed highest resistance against lithocholic acid *in vitro* [126]. A relationship between disease score and deoxycholic acid could not be shown [126]. Secondary bile acid resistance may be strain-dependent, but further research is warranted to draw this conclusion with certainty.

Intrinsic anti-bacteriocin properties have been described for *C. difficile* [127, 128]. Nisin can

inhibit growth of vegetative cells and prevent spore germination of *C. difficile in vitro* [44]. However, this does not hold for all *C. difficile* strains, as the mutant strain MC119 had normal growth in sub-lethal concentrations. It was demonstrated that this resistance was at least partly due to export of nisin by an ABC-transporter [127]. Another identified mechanism was a net positive charge on the bacterial cell surface resulting in lower efficacy of nisin, since nisin is attracted to a low negative charge on the cell surface [128].

Using genome-scale metabolic models in antibiotic-treated mice, it was demonstrated that *C. difficile* does not necessarily compete for specific nutrients against specialized bacteria, but that it adapts to utilize a wide array of nutrients. This allows for colonization of diverse microbiomes, wherein *C. difficile* is not limited to a specific nutrient niche [129]. A follow-up study, also using a multi-omics approach, showed that *C. difficile* alters transcriptional activity of especially low abundant taxa. The main genes showing decreased transcription in these low abundant taxa during infection, as compared to mock infected mice, were carbohydrate-acquisition and utilization genes. A possible reason for this could be that *C. difficile* attempts to create its own nutrient niche to facilitate colonization [130].

However, others have found specific nutrients that may be important for *C. difficile* colonization and/or outgrowth. Three highly virulent ribotypes (RT), RT017, RT027 and RT078, have recently been demonstrated to utilize trehalose as a nutrient source [131, 132]. This was confirmed in a mouse model, where mice were challenged with spores of either RT027 or a non-trehalose metabolizing ribotype. After trehalose administration, RT027 mice showed higher mortality in a dose-dependent manner [131].

C. difficile post-antibiotic outgrowth depends partly on the production of succinate and sialic acid by commensals. *B. thetaiotaomicron* is capable of metabolizing polysaccharides and thereby produces sialic acid. Upon inoculation with *C. difficile,* monocolonized *B. thetaiotaomicron* mice had approximately a five times higher density of *C. difficile* in feces as compared to germ-free mice [133]. Expression levels of genes involved in sialic acid metabolism were increased in the *B. thetaiotaomicron* model, and, as expected, a sialidase-deficient *B. thetaiotaomicron* mutant led to highly reduced production of sialic acid and *C. difficile* density was lower [133]. Density of *C. difficile* was higher in *B. thetaiotaomicron* mice fed a polysaccharide-rich diet as compared to a chow diet [134]. The succinate to butyrate pathway was crucial for *C. difficile* expansion in *B. thetaiotaomicron* mice, as WT *C. difficile* was more effective in establishing infection than a succinate-transporter deficient *C. difficile* [134].

Micronutrient availability can affect virulence of *C. difficile*. High zinc levels have been demonstrated to exacerbate *C. difficile* infection in mouse models [135]. Mice fed a high-

zinc diet had higher toxin levels, higher pro-inflammatory cytokines levels and increased loss of barrier function. Furthermore, it was shown that calprotectin, a zinc-binding protein, was important for limiting zinc availability to *C. difficile* during infection [135].

Together, these studies demonstrate the importance of specific nutrients used by *C. difficile* to establish colonization and infection.

Efficient colonization of the epithelial barrier is made possible by flagella and pili [136, 137]. When mice were inoculated with flagellated or non-flagellated *C. difficile* strains, higher levels of flagellated C. *difficile* were found in mouse cecum [136]. The exact destination of non-flagellated *C. difficile* remained unknown, as levels were not measured in feces or in sections of the small intestine. Regarding pili, it has been shown that type IV pili were not playing a role in initial colonization, but were crucial for epithelial adherence and long-lasting infection [137].

_S._ Typhimurium

S. Typhimurium is a nontyphoidal S*almonella* and an important cause of gastroenteritis in humans. It was estimated that globally 3.4 million invasive nontyphoidal *Salmonella* infections occur each year, of which 65.2% are attributable to serovar Typhimurium [138]. It mostly causes self-limiting, non-bloody diarrhea in otherwise healthy individuals. However, it can lead to bloodstream infections and metastatic spread with eventually death in especially infants and immunocompromised individuals [138, 139]. *S.* Typhimurium contains two pathogenicity islands, SPI1 and SPI2. SPI1 mostly contains information for causing intestinal disease and cell invasion, while SPI2 is necessary for intracellular survival [140].

Effects of SCFAs on *S.* Typhimurium are not yet well defined. Butyrate and propionate have been demonstrated to reduce expression of invasion genes, while acetate increased their expression in *S.* Typhimurium [141, 142]. However, conflicting results exist. A *S.* Typhimurium knockout mutant, unable to metabolize butyrate, caused less inflammation than a WT *S.* Typhimurium, suggesting that butyrate is crucial for *S.* Typhimurium virulence [143]. Furthermore, this study demonstrated that butyrate was necessary for expression of invasion genes in mouse models. In contrast, propionate inhibited *S.* Typhimurium in a dose-dependent manner *in vitro*, probably due to disturbance of intracellular pH [144]. In an *in vivo* setting, it was demonstrated that a cocktail of propionate-producing *Bacteroides* species was sufficient to mediate CR against *S.* Typhimurium [144].

S. Typhimurium has developed mechanisms to overcome bile acids encountered in the gut. When exposed to individual bile acids at sub-lethal levels *in vitro*, it can become

resistant to originally lethal levels by changing gene and protein expression of several virulence regulators [145, 146]. In addition, it has been demonstrated that a mixture of cholate and deoxycholate confers a synergistic inhibition on invasion gene expression in *S.* Typhimurium [147].

Innate resistance of *S.* Typhimurium against bacteriocins produced by Gram-positive bacteria is naturally conferred through its Gram-negative outer membrane [148].

Usage of nutrients produced by gut microbiota is believed to facilitate *S.* Typhimurium outgrowth. By causing inflammation and thereby altering microbiota composition, *S.* Typhimurium provides itself with a competitive advantage [149, 150].

Metabolic profiling in mice showed increased luminal lactate levels in the inflamed gut during *S.* Typhimurium infection, which could result from a depletion in butyrate-producing bacteria [149]. When butyrate is scarce, enterocytes switch to glycolysis with lactate as end product. Lactate is an important nutrient for *S.* Typhimurium, as indicated by decreased colonization of cecal and colonic lumen by a *S.* Typhimurium mutant lacking two lactate dehydrogenases [149]. As explained in the introduction, an anaerobic milieu is maintained in the gut during homeostatic conditions. However, diffusion of oxygen from the tissue to the lumen is enabled by inflammation caused by *S.* Typhimurium, which alters enterocyte metabolism [151]. Oxygen can then be used by *S.* Typhimurium to ferment several carbohydrates through respiration [152-155]. In conclusion, these findings suggest that *S.* Typhimurium creates its own niche in the gut by causing inflammation, subsequently shifting microbiota composition and thereby nutrient availability, so that it can optimally colonize and expand.

An intact and well-functioning mucus layer is crucial for protection against *S.* Typhimurium infection. WT mice infected with the attenuated Δ*aroA* strain, which causes severe colitis, showed increased *muc2* gene expression and MUC2 production [156]. Mortality and morbidity was high in Δ*muc2* mice and higher numbers of the pathogen were found in their liver, ceca and close to the epithelial layer [156].

S. Typhimurium may profit from mucin-degrading commensal microbiota. In a gnotobiotic mouse model, complementation with mucin degrading *A. muciniphila* during *S.* Typhimurium infection allowed *S.* Typhimurium to dominate the bacterial community five days p.i [157]. This was not caused by an absolute increase in cell number, but by a decrease in other microbiota members. In addition, the complementation with *A. muciniphila* led to increased inflammation, as indicated by increased histopathology scores and protein and mRNA levels of pro-inflammatory cytokines. Although generally considered a beneficial bacterium, *A. muciniphilia* exacerbated *S.* Typhimurium

infection by thinning the mucus layer, thereby promoting translocation of the pathogen to the epithelial layer [157].

Enterohemorrhagic *E. coli*

Shiga-toxin producing *E. coli* (STEC) comprises a group of *E. coli* strains capable of producing Shiga-toxins. Enterohemorrhagic *E. coli* (EHEC) is a subgroup of STEC causing more severe disease, often with complications. Each year, approximately 100,000 people are infected by the most common EHEC serotype, O157:H7 [158]. Clinical presentation includes abdominal pain and bloody diarrhea which can progress into toxin-mediated hemolytic uremic syndrome [159]. Virulence of EHEC strains is mostly encoded by Shiga toxin genes, *stx1* and *stx2*, and by locus of enterocyte effacement (*lee*) genes, which are imperative for initial attachment to epithelial cells [160].

At present, outcomes regarding the effects of SCFAs on EHEC are mixed [161-165]. LEE protein and gene expression was already enhanced at 1.25mM of butyrate, while for acetate and propionate, only minor changes were detected at 20mM, with acetate giving a repressive effect. In a separate growth experiment, acetate was more efficient in inhibiting growth of EHEC as compared to butyrate and propionate [162]. Acetate was observed to have small repressive effects on EHEC in the study by Nakanishi *et al*., and this was also found by Fukuda *et al.* [162, 165]. Mice fed acetylated starch prior to infection showed higher fecal acetate levels and improved survival rate compared to starch-fed mice [165]. Acetate also prevented gut barrier dysfunction as measured by transepithelial electrical resistance and prevented translocation of the Shiga toxin to the basolateral side of the epithelial cells [165]. In Caco2 cells, EHEC epithelial adherence was 10-fold higher when grown on butyrate than on acetate or propionate [162]. These results indicate that butyrate may be less effective in inhibiting EHEC growth and potentially colonization as compared to acetate and propionate, for which the exact pathways and genes involved have been elucidated [162, 163]. In contrast, butyrate was found to be effective against EHEC in a pig model [161]. Piglets given sodium butyrate two days prior to being infected with EHEC showed no symptoms 24 hours p.i, while the control group developed multiple signs of disease, e.g. histopathological signs of kidney damage. The sodium butyrate group did not show any signs of inflammation and shed less viable cells compared to the control group within 48h [161]. *In vitro* assays demonstrated that butyrate enhanced bacterial clearance, ultimately making the authors suggest that butyrate can be developed as a new drug to treat EHEC [161].

EHEC has multiple traits to fight against the potentially deleterious effects of bile acids. Bile acid mixtures upregulated gene expression of the *A*crAB efflux pump and downregulated *ompF*, a gene encoding for an outer membrane porin [166]. In addition, other genes responsible for limiting penetration of bile acids through the membrane (*basR* and

basS), were upregulated, and this effect was concentration-dependent. Interestingly, the bile acid mixtures did slightly downregulate *stx2* subunit genes, encoding for Shiga toxin production [166].

EHEC possesses natural resistance against bacteriocins, especially nisin, through its Gram-negative outer membrane, as described in the chapter on bacteriocins. Three EHEC strains were screened for, amongst others, potential resistance against several colicinogenic *E. coli* strains [167]. *In vitro*, resistance against *E. coli* strains producing a single colicin was observed, but resistance was rarely observed against multiple colicins and could never be linked to acquiring a specific plasmid [167].

Nutrient competition for proline and several sugars between EHEC and commensal *E. coli* strains is described in the introductory section. In addition, ethanolamine (EA), a source of carbon, nitrogen and energy for EHEC, has been investigated. It was demonstrated that EA could diffuse across the bacterial membrane and that the *eut* genes were crucial for metabolizing EA. *Eut* sequences were absent in native bacterial genomes in the bovine gut, apart from commensal *E.coli*, indicating that EA provides a nutrient niche for *E. coli*. When the *eutB* gene was knocked out in EDL933, it was outcompeted by commensal *E. coli* due to its inability of utilizing EA, indicating its critical importance for colonization [168]. During further transcriptomic investigations of EA utilization, it was noticed that genes involved in gluconeogenesis were upregulated if no glucose was supplemented. A knockout of two genes within the gluconeogenesis pathway led to a growth defect in a coculture with the wildtype [169]. This is in line with a previous finding that optimal usage of gluconeogenic substrates by EDL933 is important for colonization [170]. Since this effect was seen in a medium consisting of bovine small intestinal contents, the relevance for the human gut remains unclear [169].

Co-culturing of EHEC with *B. thetaiotaomicron* led to an upregulation of genes involved in nutrient competition in EHEC as compared to culturing EHEC alone [171]. In addition, presence of *B. thetaiotaomicron* resulted in upregulation of multiple virulence genes including *lee*, likely due to regulation of a transcription factor involved in sensing carbon metabolite concentrations in the environment [171]. Using a combination of *in vitro* and *in vivo* methods, Pacheco *et al.* showed that fucose cleaved from mucins by *B. thetaiotaomicron* could be an important nutrient for upregulating virulence and intestinal colonization of EHEC [172]. Interestingly, fucose sensing and subsequent regulation of virulence genes was more important for successful colonization than utilization of fucose for energy. This example indicates that nutrients cannot only be utilized for energy, but that they can be important environmental signals for properly regulating timing of virulence [172].

Human colonoid monolayers were used to study initial colonization mechanisms of EHEC [173]. This study showed that EHEC disturbs the tight junctions, preferentially attaches to mucus producing cells and subsequently impairs the mucus layer [173]. In addition, by using various *in vitro* models, it was demonstrated that the metalloprotease StcE, produced by EHEC, enables degradation of MUC2 in the inner mucus layer which may pave the way to the epithelial surface [174].

S. flexneri

Shigella infections mostly occur in developing countries, with *S. flexneri* as the most frequently found species [175]. Annually, an estimated 164,000 people die of shigellosis worldwide [176]. Clinical presentation includes a wide variety of symptoms, including severe diarrhea, possibly containing blood and mucus, and abdominal pain [160]. *S. flexneri* contains a virulence plasmid (pINV) which is necessary for invasion of epithelial cells and intracellular survival [160].

No studies seem to have investigated resistance mechanisms of *S. flexneri* against SCFAs yet. Butyrate has been investigated as a potential therapeutic agent as it counteracts a putative virulence mechanism of *S. flexneri*, namely decreasing LL-37 expression in the gut [177, 178]. By suppressing LL-37 expression *S. flexneri* is able to colonize deeper into intestinal crypts [178]. Butyrate was able to increase rectal LL-37 expression in a subgroup of patients, which was associated with lower inflammation in rectal mucosa and lower levels of pro-inflammatory cytokines [177]. However, butyrate treatment did not seem to impact clinical recovery [177].

The type three secretion system (T3SS) which is able to directly inject bacterial protein into host cells and cause infection, is considered a key virulence factor. *S. flexneri* T3SS can sense and bind secondary bile acid deoxycholate, which leads to co-localization of protein translocators at the needle tip [179, 180]. In *S. flexneri* mutants lacking the needle structure, the deoxycholate-associated adhesion and invasion of *S. flexneri* to host epithelial cells was diminished [181]. At physiological levels of bile salts, *S. flexneri* is able to grow normally *in vitro*, but at increased concentrations growth is significantly reduced [182]. Transcriptomics showed that during exposure to physiological bile salt levels, genes involved in drug resistance and virulence were upregulated, which was subsequently confirmed using reverse transcription-quantitative PCR (RT-qPCR). Deletion of a multidrug efflux pump led to sensitivity to bile salts and growth inability, confirming the importance of this pump in bile salt resistance [182].

Bacteriocin resistance has not been well studied in *S. flexneri*, but downregulating antimicrobial peptide production in the gut is suggested to be an important virulence mechanism [183]. The downregulation of LL-37 early in infection was demonstrated both

in gut biopsies of patients and in cell lines [183]. Since protein and gene expression were not downregulated to the same degree, the authors speculated that there is an interference mechanism during active transcription of LL-37. Transcription of other antimicrobial peptides was also downregulated, especially in the human β-defensin hBD family [178, 183]. It was demonstrated that *S. flexneri* shows high sensitivity to LL-37 and hBD-3 peptides *in vitro* [178]. This suggests that by downregulating expression of antimicrobial peptides, *S. flexneri* creates an environment in which it can survive and ultimately cause severe disease.

It is unknown how *S. flexneri* competes and utilizes nutrients in the luminal side of the gut. Therefore, a short description will be given on how the bacterium rewires host cell metabolism for supporting its survival after entering the host cells. These findings might be translatable, and can at least provide insight in potential nutrient usage of *S. flexneri* in the lumen. Using a combination of metabolomics and proteomics it was demonstrated that *S. flexneri* does not alter host cell metabolism in HeLa cells, but that it captures the majority of the pyruvate output [184]. Pyruvate was demonstrated to be a crucial carbon source for *S. flexneri* cultured on a HeLa derivative, using metabolomics, transcriptomics and bacterial mutants [185]. *S. flexneri* converts pyruvate into acetate via a very quick, but energy-inefficient pathway, allowing for rapid expansion of the bacterium intracellularly without rapid destruction of the host cell [184].

S. flexneri possess special systems to alter mucus composition. Human colonoid monolayers infected with *S. flexneri* showed increased extracellular release of mucins [186]. The increased extracellular mucins were trapped at the cell surface which surprisingly favored access of *S. flexneri* to the apical surface, subsequently promoting cell invasion and cell-to-cell spread [186]. Furthermore, expression of several genes encoding for production of mucins and mucin glycosylation patterns were altered [186]. Together, these results suggest that *S. flexneri* can alter the mucus environment such that it can promote its own virulence.

C. jejuni

C. jejuni is associated with food-borne gastroenteritis and is estimated to cause more than 800,000 infections annually in the USA alone [187]. Major clinical symptoms include diarrhea (both with and without blood), fever and abdominal cramping [160]. In rare cases, it can give rise to the Guillain-Barré syndrome and reactive arthritis [187]. It is a commensal bacterium in avian species and it is not yet well understood why it causes disease in humans [188].

There is a distinct lack of research on the resistance mechanisms of *C. jejuni* against SCFAs, but one study found that SCFAs are important for colonization in chickens [189].

Acetinogenesis, the conversion of pyruvate to acetate, is a crucial metabolic pathway for optimal colonization of *C. jejuni*. Mutants unable to use this pathway show impaired colonization and decreased expression of acetinogenesis genes. Upon encountering a mixture of SCFAs at physiological levels, this mutant was surprisingly able to restore acetinogenesis gene expression to WT levels. Therefore, it was investigated whether expression of acetinogenic genes differs throughout the intestinal tract, as SCFAs are most abundant in distal parts of the intestine. It was observed that both gene expression and *C. jejuni* levels were highest in the cecum. The authors suggested that *C. jejuni* can monitor SCFA levels in the gut, so that in response it can express colonization factors [189]. As this is the only study suggesting this hypothesis, further research is required for validation.

Results regarding bile acid resistance in *C. jejuni* are mixed, which may stem from using different animal models or bile acids. A specific multidrug efflux pump, CmeABC, was important for bile resistance in chickens [190]. Δ*cmeABC* mutants showed impaired growth *in vitro* and unsuccessful colonization in chicken upon cholate administration, while cholate did not affect growth and colonization of the WT [190]. This suggests that the efflux pump is critical for proper colonization of *C. jejuni* by mediating bile-acid resistance. Another study elucidated the effects of secondary bile acids on *C. jejuni* [191]. Upon administration of deoxycholate prior to, and during, infection, mice showed decreased colitis. Unexpectedly, *C. jejuni* luminal colonization levels were not affected [191]. In conclusion, *C. jejuni* colonization seems not to be affected by bile acids, but may be important in limiting disease progression.

Bacteriocin resistance is not common in *C. jejuni*. Multiple *C. jejuni* (n=137) isolates were screened for resistance against two anti-*Campylobacter* bacteriocins, OR-7 and E-760, produced by the gut inhabitants *L. salivarius* and *Enterococcus faecium*. However, no isolates were found to harbor resistance [192]. In a follow-up study, chickens were successfully colonized with a *C. jejuni* strain prior to bacteriocin treatment, with the aim of studying bacteriocin resistance. Resistance developed in most chickens, but was lost upon ending bacteriocin administration, suggesting resistance instability *in vivo* [193].

In contrast to most other enteric pathogens, *C. jejuni* does not metabolize carbohydrates as its main energy source. It is unable to oxidize glucose, fructose, galactose and several disaccharides, including lactose, maltose and trehalose, resulting from the absence of 6-phosphofructokinase [194-197]. Fucose could be metabolized by some *C. jejuni* strains, due to the occurrence of an extra genomic island [197]. Main energy sources for *C. jejuni* are organic acids, including acetate, and a limited number of amino acids [198-200]. It is currently unclear what these metabolic adaptations mean for its colonization potential,

but it is possible that *C. jejuni* occupies a unique macronutrient niche.
Iron regulation systems are critical for colonization and persistence of *C. jejuni*. In presence of sufficient iron, transporter and acquisition genes are downregulated [201]. Mutants lacking genes involved in either iron acquisition or transport were severely impaired in colonizing the chick gut [201]. Free iron concentrations are extremely low in the gut, which forces *C. jejuni* to utilize other iron sources. It was demonstrated that lactoferrin and transferrin can also be used for this purpose and molecular pathways have been described [202]. In short, transferrin-bound iron can only be utilized if it is in close proximity to the bacterial cell surface. Thereafter, it is most likely that iron is freed from the bacterial cell surface proteins, transported across the outer membrane and subsequently internalized by an ABC-transporter [202]. Additionally, both in an *in vitro* setting and in a controlled human infection model with *C. jejuni* the most upregulated genes were involved in iron acquisition [188, 203]. These results suggest that iron regulation is maintained extremely well, and that *C. jejuni* can obtain sufficient iron even in a harsh environment as the gut.

C. jejuni resides in the mucus layer prior to invading the epithelial cell. It can cross and reside here because of its powerful flagellum, which can change in conformation or rotation upon being challenged by higher viscosity [204, 205]. *C. jejuni* can hereby cross the mucus layer at speeds which cannot be met by other enteric pathogens, and the flagellum can subsequently be used as an adhesin [205, 206].

Another important characteristic for *C. jejuni* 's success in crossing the mucus layer is its helix-shape. In a mouse model, a WT strain or either of two rod shaped *C. jejuni* bacteria, Δ*pgp1* or Δ*pgp2*, were administered to cause infection [207]. Rod-shaped mutants were demonstrated to be mostly non-pathogenic, whereas the WT strain caused severe inflammation. Mutants were to some extent able to colonize the mucus layer, but could not cross it, explaining their non-pathogenicity [207].

V. cholerae

V. cholerae is one of the first bacterial pathogens where the microbiota has been considered to play an important role against infection [208]. It is mainly prevalent in contaminated brackish or salt water and can cause outbreaks, particularly during wars and after natural disasters. In the first two years following the earthquake in Haiti, 2010, more than 600,000 people were infected with *V. cholerae* serogroup O1, biotype Ogawa, resulting in more than 7,000 deaths [209]. The clinical course is characterized by watery diarrhea, which can be so severe that it can result in dehydration, hypovolemic shock and death [210]. *V. cholerae* colonizes the small intestine by employing the toxin-coregulated pilus, after which it can cause severe infection and clinical symptoms through cholera enterotoxin production [210].

V. cholerae is able to utilize its acetate switch, the shift from elimination to assimilation of acetate, to increase its own virulence [211]. In a *Drosophila* model, it was demonstrated that *crbRS* controlled the acetate switch, while *acs1* was required for acetate assimilation [211]. When either of these genes were knocked-out, mortality decreased. Competition experiments demonstrated that WT *V. cholerae* had a growth advantage over strain when the Δ*crbS* strain and WT *V. cholerae* strains were administered together in a 9:1 ratio. This led the authors to suggest that acetate utilization may be important early in infection, when low levels of *V. cholerae* cells are present [211]. Furthermore, acetate consumption led to dysregulation of host insulin signaling pathways, ultimately leading to intestinal steatosis and increased mortality. Dysregulation of host insulin signaling was not observed in Δ*crbS* or Δ*acs1*, further confirming the role of acetate in *V. cholerae* virulence [211].

V. cholerae has a master regulator, *toxT*, which can directly activate several virulence factors including toxin production. Cholera toxin production was reduced by 97% when *V. cholerae* was grown in presence of bile, which could be reversed after growing the same cells in bile-free medium for a few hours [212]. C*tx* and *tcpA*, encoding for cholera toxin and the major structural unit of the toxin-coregulated pilus and regulated by *toxT*, were highly repressed during bile exposure [212]. Additionally, motility was increased approximately 1.6-fold in presence of bile [212]. To elucidate which exact components of bile acids were responsible for the repression of these virulence genes, bile was fractionated. It was found that several unsaturated fatty acids strongly repressed *ctx* and *tcpA* and that they upregulated expression of *flrA*, leading to increased motility [213]. The reason for upregulation of *flrA* and downregulation of *tcpA* could be that the flagellum increases the speed of passing through the mucus layer, while the pilus would only slow it down. When lower concentrations of bile at the epithelial surface are encountered, expression can be reversed [214].

Two outer membrane porins, OmpU and OmpT, are directly regulated by the master regulator *toxR*. Upon encountering bile acids, *ompU* and *ompT* are regulated in such a way that bile acid entrance is prevented [215, 216]. Furthermore, Δ*toxR* mutants are more sensitive to bile acids due to changed outer membrane composition [215]. Recently, it was shown that *toxR* also regulates *leuO* [217]. *LeuO* was demonstrated to confer bile resistance independent of the two porins, although its exact resistance mechanism is not yet elucidated [217].

Bacteriocin resistance in *V. cholerae* has, to our knowledge, not been studied and future studies will have to reveal whether any resistance is present.

An important nutrient through which *V. cholerae* gains a competitive advantage is

sialic acid, a component of the mucus layer. Using streptomycin pre-treated mice who were given a mutant strain defective in sialic acid transport (Δ*siaM)*, it was shown that sialic acid is not required for initial colonization, but that it is important for persistent colonization [218]. Competition assays of the two mutant strains in mouse intestine (small intestine, cecum and large intestine) showed that Δ*siaM* was less fit to compete in each environment, further indicating the necessity of sialic acid utilization for niche expansion of *V. cholerae* [218].

The El Tor strain may have a competitive advantage over 'classical' strains due to its differential carbohydrate metabolism [219]. When grown in a glucose-rich medium, classical strains display a growth defect as compared to El Tor. It was observed that this was due to production of organic acids through glucose metabolism, leading to acidification of the medium. El Tor biotypes were found to produce acetoin, a neutral compound, and decrease organic acid production. This prevented acidification of the medium, leading to better growth. El Tor strains were also more successful in colonizing mice, especially when extra glucose was administered. The classical types were shown to be able to produce acetoin, but glucose only led to a minor increase in transcription of genes necessary for acetoin production [219]. These studies have shown that specific metabolic pathways are used by *V. cholerae* to successfully colonize the gut.

One of the first studies on how the mucus layer can potentially be crossed by *V. cholerae* was reported almost 50 years ago [220]. Here, motile and non-motile strains were compared for pathogenicity after administration to mice. It was observed that motile strains were almost always deadly 36 hours p.i, while most non-motile strains had a mortality of under 35% [220]. One hypothesis offered by the authors was that together with mucinase, the flagellum could effectively pass the mucus barrier [220]. Specific mucin degradation mechanisms employed *by V. cholerae* have been identified since, with hemagglutinin/ protease (Hap), and TagA being the major ones [221-225]. Presence of mucins, limitation of carbon sources and bile acids maximized production of Hap, while glucose could partly reverse this effect [221]. This may indicate that during conditions as encountered in the gut, *V. cholerae* quickly aims to cross the mucus layer and be in close contact with the epithelial cells. TagA, which is similar to StcE as described for EHEC, is also capable of degrading mucin [222]. In conclusion, *V. cholerae* has developed a way of sensing environmental conditions, and in response to these, is able to upregulate virulence factors which can degrade mucins. A simplified overview of *V. cholerae* virulence factors opposing CR can be found in Fig. 2.

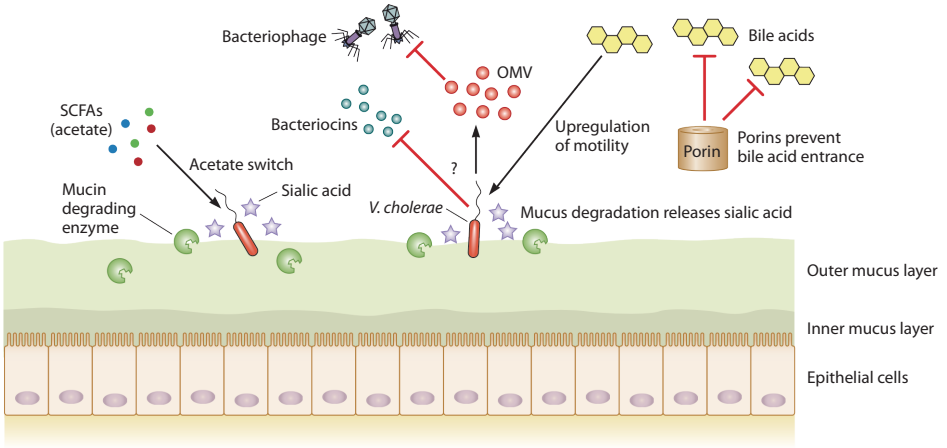

Figure 2: *Vibrio cholerae* uses a wide array of mechanisms to overcome CR. First, it employs its acetate switch to use acetate for upregulating its own virulence. Nothing about potential bacteriocin resistance is presently known, and this subject remains to be studied. To protect itself from bacteriophages, *V. cholerae* produces outer membrane vesicles (OMVs) which act as a decoy binding site for the attacking phages (see section: Bacterial defense mechanisms against bacteriophages). Regulation of outer membrane porins is such that they prevent entry of bile acids when they are encountered. By employing specific mucin-degrading enzymes, *V. cholerae* releases sialic acid and subsequently metabolizes it.

Y. enterocolitica

Yersiniosis is mostly contracted through contaminated food or water with *Y. enterocolitica*, and its prevalence is much higher in developing countries than in high-income nations [160, 226]. It is characterized by mild gastroenteritis, abdominal pain and is usually self-limiting, though pseudo-appendicitis illnesses can occur [160]. Virulence is mostly conferred through presence of a 64-75 kb plasmid on which several virulence genes are present, including *yadA*, which is crucial for epithelial adherence [227].

Resistance of *Y. enterocolitica* against antibacterial compounds has not been much studied. One study investigated effects of SCFAs, including acetic acid and propionic acid, on *Y. enterocolitica* at 4°C. *Y. enterocolitica* was less sensitive to acetic acid when cultured anaerobically than under aerobic culturing. Propionic acid was similarly effective in inhibiting growth with both culture methods [228]. Even though conditions like 4°C are not representative for the intestinal environment, this study might provide some initial clues on the effects of SCFAs on *Y. enterocolitica*. It is clear that more research is required to further elucidate potential resistance mechanisms.

ompR, a transcriptional regulator in *Y. enterocolitica*, is probably able to upregulate expression of the AcrAB-TolC efflux pump, which, in turn, is regulated by two components of the efflux pump, *acrR* and *acrAB* [229]. A mixture of bile acids, but not

the secondary bile acid deoxycholate, was found to be the strongest inducer of *acR* and *acrAB* [229]. Whether the upregulation of these efflux pump components contributes to bile acid resistance, remains to be elucidated.

Bacteriocin resistance is so far mostly unknown in *Y. enterocolitica*. WA-314 and 8081 are both 1B:O8 strains that are highly infective in murine models [230]. WA-314 possesses a putative colicin cluster for colicin production, but no expression was observed in a spot-on-lawn assay with 8081 and the colicin-sensitive *E. coli* K12 [230]. It is likely that no specific resistance against colicin is present, as colicin has been shown to effectively inhibit *Y. enterocolitica* infections *in vivo* [59].

Like most other enteric pathogens, *Y. enterocolitica* has sophisticated systems to acquire sufficient iron. Using these systems, *Y. enterocolitica* may be more efficient at scavenging iron than commensal members, thereby providing itself with a competitive advantage. *Y. enterocolitica* expresses yersiniabactin, *ybt*, a highly efficient siderophore and a crucial component for lethality in mouse models [231, 232]. The exact mechanisms for iron uptake and transport have been extensively reviewed elsewhere [233]. Proteomics analysis revealed that *Y. enterocolitica* serovar 1A, whose pathogenic role is unclear, uses different proteins to successfully scavenge iron, as it lacks the Ybt protein [234].

Y. enterocolitica is the only pathogenic Yersinia species which can metabolize sucrose, cellobiose, indole, sorbose and inositol [235]. Additionally, it can degrade EA and 1,2-PD by using tetrathionate as a terminal electron acceptor [235].

Mucus layer invasion and adherence of *Y. enterocolitica* have been elucidated in great detail several decades ago [236-240]. The YadA protein is used for initial attachment to the mucus [240]. The preferential binding side on mucins is their carbohydrate moiety, but binding to mucin proteins is also possible under specific conditions [238]. *Y. enterocolitica* uses a plasmid, pYV, with mucin-degradation enzymes to thin the mucus layer, facilitating crossing of the mucus layer [237, 240]. *Y. enterocolitica* containing the pYV plasmid is not only able to successfully invade and degrade the mucus layer, but is also highly efficient in multiplying in this environment [240]. After interacting with the mucus layer, its bacterial cell surface was altered so that *Y. enterocolitica* became less efficient in colonizing the brush border [240]. This may be a host response mechanism to prevent *Y. enterocolitica* invasion in deeper tissues. In a rabbit infection model, persistent goblet cell hyperplasia and increased mucin secretion was observed throughout the small intestine over 14 days [236]. The extent of hyperplasia was associated with severity of mucosal damage, indicating a compensatory mechanism. Mucin composition changed in infected rabbits, with a decrease in sialic acid and an increase in sulfate [236].

L. monocytogenes

L. monocytogenes causes listeriosis, a food-borne disease. Listeriosis is not highly prevalent, with an estimated 23,150 people infected in 2010 worldwide, but has a high mortality rate of 20-30% [241]. The most common syndrome is febrile gastroenteritis, but complications can develop, such as bacterial sepsis and meningitis [241]. This is especially relevant for vulnerable patient groups, such as immunocompromised individuals, neonates and fetuses [242]. Virulence genes are present on an 8.2-kb pathogenicity island, which includes internalin genes necessary for invading host cells [243].

Culturing *L. monocytogenes* in presence of high levels of butyrate leads to incorporation of more straight-chain fatty acids in the membrane [244, 245]. This is not a natural state for *L. monocytogenes*, as normally its membrane consists for a very high percentage of branched-chain fatty acids. When subsequently exposed to LL-37, it displays a survival defect as compared to bacteria not grown in presence of butyrate [244]. It was not elucidated whether this survival defect was due to increased stress, altered membrane composition or differentially regulated virulence factors. Effects of propionate on *L. monocytogenes* growth, metabolism and virulence factor expression are dependent on temperature, oxygen availability and pH [246]. Therefore, it is not possible to ascribe a general function to propionate in relation to *L. monocytogenes*.

L. monocytogenes possesses several bile acid resistance mechanisms, and *in vitro* transcriptome and proteome analyses have provided insight into these. Transcriptomics analysis revealed that in response to cholic acid, amongst others, two efflux pumps were upregulated, *mdrM* and *mdrT* [247]. BrtA was shown to regulate expression of the efflux pumps, and to be able to sense bile acid levels. Bacterial abundance was determined in multiple organs of mice infected with knockout strains of either efflux pump, but not in the intestine [247]. Proteomic analyses found many changes in response to bile salts and included proteins associated with efflux pumps, metabolism and DNA repair [248].

Bile salt hydrolases (BSH) are another way of combatting encountered bile acids. It was demonstrated that all *Listeria* species which infect mammals showed BSH enzyme activity. BSH was crucial during infection of guinea pigs, demonstrated by the decreased ability of Δ*bsh* to cause a persistent infection [249]. At decreased pH levels, e.g. in the duodenum, bile salts are more acidic and show higher toxicity [250]. However, this toxicity seems to be strain-dependent [251]. The strain responsible for a 2011 outbreak even displayed higher bile resistance at pH 5.5 than at 7.0, further indicating that bile susceptibility may be strain-dependent [251].

As discussed in the introductory section on bacteriocins, the Abp118 bacteriocin produced by *L. salivarius*, protected mice from *L. monocytogenes* infection [55].

However, several bacteriocins have been shown ineffective against *L. monocytogenes* and responsible mechanisms have been partly elucidated. Innate nisin resistance has been associated with multiple loci [252]. One crucial gene was *anrB*, encoding for a permease in an ABC transporter. Loss of this gene resulted in high sensitivity, not only to nisin, but also to several other bacteriocins [252]. The mannose phosphotransferase system (Man-PTS), encoded by *mptACD*, is a main sugar uptake system and two of its outer membrane proteins, IIC and IID, can serve as a class II bacteriocin receptor [253]. In natural resistant and spontaneous resistant strains, a reduced expression of *mptC* and *mptD* was observed, although this could not be linked to receptor mutations [254]. The *mpt* operon is partly regulated by *manR*, and a *manR* mutant did not show any activation of the *mpt* operon [255]. Development of bacteriocin resistance was to some extent dependent on available carbohydrates [256]. Several sugar sources impaired growth of *L. monocytogenes* when exposed to bacteriocin leucocin A. Increased sensitivity to leucocin A was hypothesized to relate to sugar uptake by Man-PTS. When specific sugars are present, cells may not downregulate this system even in presence of bacteriocins, which possibly allows leucocin A to use the Man-PTS as a docking molecule [256]. Not only does *L. monocytogenes* display bacteriocin resistance, it also produces a bacteriocin, Lysteriolysin S, which modifies the gut microbiota such that intestinal colonization is promoted [257]. *Allobaculum* and *Alloprevotella*, genera known to contain SCFA-producing strains, were significantly decreased in mice treated with Lysteriolysin S. *L. monocytogenes* strains unable to produce Lysteriolysin S were impaired in competing with native gut microbiota and colonized less efficiently [257].

Most reports about metabolic adaptations of *L. monocytogenes* have logically described intracytosolic adaptations, as *L. monocytogenes* replicates intracellularly [258]. Limited information is available on nutrient competition of *L. monocytogenes* inside the lumen. Comparison of genome sequences between colonizing *Listeria* and non-colonizing *Listeria* led to identification of, amongst others, a vitamin B12-dependent 1,2-propanediol (1,2-PD) degradation pathway in colonizing *Listeria*, dependent on the *pduD* gene [259]. Mice were co-infected with a Δ*pduD* strain and a WT strain. Within 3 hours after feeding, a large amount of the Δ*pduD* was shed in feces and 21 hours later the number of viable cells decreased significantly. At ten days p.i, the Δ*pduD* strain was completely cleared, while the WT strain shed for up to four more days. This indicates that the ability to degrade 1,2-PD offers *L. monocytogenes* a distinct competitive advantage [259].

Multiple adhesins and internalins have been characterized which facilitate *L. monocytogenes* retention in the mucus layer [260-263]. InlB, InlC, InlL and InlJ were demonstrated to bind to MUC2, but not to epithelial cell surface MUC1 [262, 263]. Histopathological analysis of a listeriosis rat model revealed that *L. monocytogenes* was present in the mucus layer after less than 3 hours p.i [261]. At this time point, very few *L. monocytogenes* were present on the epithelial cells [261].

Bacterial defense mechanisms against bacteriophages

As research investigating how each enteric pathogen overcomes CR by rendering bacteriophages ineffective is still in its infancy, this general section will describe the most employed resistance mechanisms. The bacteriophage infectious cycle involves a lytic and a lysogenic cycle. Phages have to bind to a receptor on the bacterial surface to be able to insert their genomic material, usually DNA, into the bacterial cytoplasm and subsequently circularize their DNA [264]. Here, lysogenic and lytic bacteriophages' mechanisms start to branch (Fig. 3). Lytic phages start DNA replication, assemble their proteins and pack their DNA into the typical bacteriophage shape with a capsid head and tail. After sufficient replication, phages use lytic enzymes to form holes in the bacterial cell membrane, eventually leading to lysis of the cell and phage spreading. Lysogenic phages integrate their DNA in the bacterial chromosome and become prophages. Reproduction is then ensured through vertical transmission, and upon induction, prophages can also enter the lytic cycle [265] (Fig. 3). In general, factors that induce the lytic phase are compounds or conditions with bactericidal effects, e.g. a DNA damaging-agent [266].

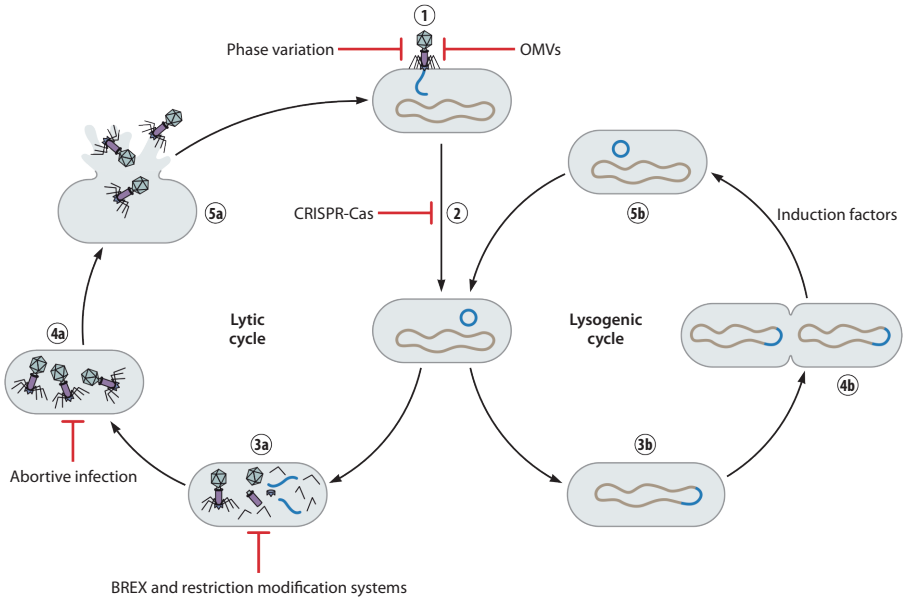

Figure 3: Lytic and lysogenic bacteriophage infection cycle with bacterial defense mechanisms. The first two steps (1 and 2) of infection are identical for the lytic and lysogenic cycle, namely phage binding followed by DNA insertion and DNA circularization. The lysogenic cycle then branches off by integrating its DNA into the bacterial chromosome and becoming prophage, thereby ensuring its replication (3b). Only upon encountering induction factors will the prophage leave the bacterial chromosome, after which it can enter the lytic cycle (4b and 5b). In the lytic cycle, phage DNA and protein is replicated and subsequently assembled into full phages (3a and 4a). The phages then lyse the bacterial cell, are released and can infect other bacteria (5a).

Bacteria possess multiple mechanisms to prevent killing by bacteriophages, starting with blocking attachment. This can be achieved through phase variation or production of OMVs. After phage DNA entry, CRISPR-Cas can recognize this foreign DNA and degrade it. Phage DNA and protein replication can be prevented by BREX and restriction modification systems, while full phage assembly can be prevented by abortive infection.

The first step for preventing bacteriophage infection is to prevent surface receptor recognition. Outer membrane vesicles are produced by Gram-negative bacteria and have several functions, including interbacterial communication [267]. They have highly similar surface composition as the bacterium and may thereby serve as decoys for attacking phages [268] (Fig. 3). Indeed, *V. cholerae* outer membrane vesicles were shown to neutralize a *V. cholerae* specific phage in a dose-dependent manner (Fig. 2) [268]. This effect was only seen when the O1 antigen, the bacteriophage target on *V. cholerae*, was included in the outer membrane vesicle structure [268].

V. cholerae possesses another mechanism to prevent O1 phage receptor recognition [269] (Fig. 3). Two genes necessary for O1 biosynthesis were shown to use phase variation to induce variation in the O1 antigen composition [269]. Mutants using phase variation were resistant to the O1 antigen phage, but displayed impaired colonization in a mouse model [269]. As the O1 antigen is an important virulence factor, e.g. for immune evasion, this demonstrates that enteric pathogens constantly have to deal with multiple CR mechanisms [269].

The second step in phage infection is injection of its DNA, and this can be prevented by superinfection exclusion systems which are mostly coded by prophages (Fig. 3). The *E. coli* prophage HK97 encodes for gp15, a probable inner transmembrane protein [270]. Remarkably, HK97 gp15 has putative homologues resembling the YebO protein family in many *Enterobacteriaceae* [270]. GP15 prevented DNA injection into the bacterial cytoplasm by preventing proper formation of a complex consisting of an inner membrane glucose transporter and part of the tape measure protein [270, 271]. This example illustrates how bacteria can incorporate phage DNA to prevent itself against future phage attacks.

DNA replication can be prevented by restriction-modification systems (Fig. 3). These systems consist of a methyltransferase and a restriction endonuclease. Exogenous DNA is not tagged by this methyltransferase, while ‘self’ DNA does get tagged [272, 273]. Subsequently, non-tagged DNA can be cleaved. This system is viewed as a primitive innate bacterial defense system. However, it was found that this system is not perfect, as these restriction-modification systems can also attack self-DNA [274].

Currently, many groups are actively investigating the adaptive bacterial immune system

CRISPR-Cas and this has been extensively reviewed elsewhere [275, 276]. CRISPR-Cas is present in about 45% of sequenced bacterial genomes, although it is unknown if its prevalence is similar in gut bacteria [277, 278]. In short, it consists of CRISPR arrays, sets of short repetitive DNA elements with variable DNA sequences (spacers) separating the repetitive DNA sets, and of an operon of CRISPR associated genes (Cas). Spacers are pieces of foreign DNA, derived from bacteriophage DNA or other mobile genetic elements such as plasmids. The defense mechanism consists of adaptation followed by expression and interference. During adaptation, Cas proteins can recognize foreign phage DNA and integrate a piece of this DNA as a new spacer into the CRISPR array. This allows the bacterium to build an immunological memory of all phages it previously encountered. The expression response entails transcription of the CRISPR array, followed by processing into smaller RNA pieces (crRNAs). CrRNAs consist of two outer parts of repeated DNA sequences, with a spacer in between. To form the eventual Cas-crRNA complex, crRNAs are combined with at least one Cas protein. This complex then travels through the bacterial cell and when it identifies a complementary DNA sequence, representative for the previously encountered bacteriophage, it cleaves and degrades this foreign DNA.

In 2015, a novel phage resistance system was discovered, called bacteriophage exclusion (BREX) [279]. BREX is able to block DNA replication, but does not prevent bacteriophage attachment to the bacterium (Fig. 3). It also uses methylation as guidance to identify self and exogenous DNA, but is different from restriction-modification systems as it does not cleave exogenous DNA [279]. Almost 10% of all bacterial genomes sequenced were found to have this BREX, suggesting that it is quite a conserved defense mechanism against bacteriophages [279]. In spite of this promising defense mechanism, no further papers have been released regarding BREX functioning in e.g. pathogenic bacteria.

Bacterial cells can perform an apoptosis-like action called abortive infection, resulting in death of the infected cell and hereby protecting surrounding bacterial cells [280] (Fig. 3). These systems have not been much elucidated for enteric pathogens at a molecular level, though, relevance of this system has been shown for the gut bacteria *S. dysenteriae* and *E. coli* [281, 282]. The abortive infection systems are best studied in *L. lactis*, a bacterium widely used in production of fermented foods [283].

Concluding remarks

Currently, bacterial enteric infections still cause a heavy disease burden worldwide. For many bacterial pathogens, the virulence factors involved in infection are understood, but less is known concerning the failure of gut microbiota to provide colonization resistance

against these enteropathogens. A more comprehensive understanding of why the microbiota fail to confer sufficient CR could lead to development of specific therapies aiming to restore CR. It is likely that not a single bacterium will be used as the 'holy grail' to restore CR, but that bacterial consortia with complementary functions will be used instead. This would be preferable over the currently often used FMT, where it is not well known what exact components are transferred to the patient. One could imagine that these consortia could not only be used to treat existing infections, but that they could also be administered prophylactically in susceptible patient groups. In addition, more attention has recently been given to several drugs that were previously not linked to gut health for their potentially disturbing effect on gut microbiota and perhaps CR. In conclusion, we reviewed many of the latest insights in the rapidly evolving fields of gut microbiota, colonization resistance and bacterial enteric infection. We are looking forward to the coming years, where undoubtedly more knowledge will be gained on gut microbiota and CR, ultimately leading to more microbiota-based therapies.

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