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17   Running Head: 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

99   immune activation. Together, these mechanisms contribute to resistance against

100 colonization of exogenous microorganisms (colonization resistance, CR) (10).

101 However, also in absence of a fully functional immune system, the gut microbiota

102 can provide a crucial and nonredundant protection against a potentially lethal

103 pathogen (11). This review will discuss the mechanisms used by gut microbiota to

104 provide CR, the impact of various drugs on gut microbiota and thereby CR, and the

105 strategies of specific bacterial pathogens to overcome CR and ultimately cause

106 enteric infection.

107

108 **MECHANISMS PROVIDING COLONIZATION RESISTANCE**

109 The gut microbiota produces various products with antimicrobial effects, including

110 SCFAs, secondary bile acids and bacteriocins. Each of these contribute to CR in a

111 product-specific manner. The following section describes their general mechanisms

112 of action. The contribution of the immune system in conferring CR has been

113 extensively reviewed elsewhere and is outside the scope of this review (12, 13).

114

115 **Short-chain fatty acids**

116 SCFAs are mainly produced by bacteria through fermentation of non-digestible

117 carbohydrates (Fig. 1) (14). The three main SCFAs are acetate, propionate and

118 butyrate, constituting 90-95% of the total SCFA pool (15). During homeostatic

119 conditions, butyrate is the main nutrient for enterocytes and is metabolized through

120 β-oxidation. Hereby, an anaerobic milieu inside the gut can be maintained (16).

121 SCFAs can impair bacterial growth by affecting intracellular pH and metabolic

122 functioning. SCFA concentrations have been shown to inversely relate to pH

123 throughout different regions of the gut (17). At lower pH, SCFAs are more prevalent

124 in their non-ionized form and these non-ionized acids can diffuse across the bacterial

125 membrane into the cytoplasm. Within the cytoplasm they will dissociate, resulting in

126 a build-up of anions and protons leading to a lower intracellular pH (18).

127 In presence of acetate, metabolic functioning of *Escherichia coli* could be impaired

128 by preventing biosynthesis of methionine, leading to accumulation of toxic

129 homocysteine and growth inhibition. Growth inhibition was partly relieved by

130 supplementing the growth medium with methionine, showing that this metabolic

131 dysfunction is one of the factors by which SCFAs impair bacterial growth (19).

132

133 **Bile acids**

134 Bile acids, possessing antimicrobial properties, are produced by the liver and

135 excreted in the intestinal tract to aid in the digestion of dietary lipids. After production

136 of primary bile acids in the liver, they are subsequently conjugated with glycine or

137 taurine, to increase solubility (20). These are then stored in the gallbladder, and

138 upon food intake, are released into the duodenum to increase solubilization of

139 ingested lipids. A large part of conjugated primary bile acids is reabsorbed in the

140 distal ileum (50-90%), while the remainder can be subjected to bacterial metabolism

141 in the colon (20). Here, conjugated bile acids can be deconjugated by bile salt

142 hydrolases (BSH), which are abundantly present in the gut microbiome (21).

143 Deconjugated primary bile acids can subsequently be converted into the two main

144 secondary bile acids, deoxycholic acid and lithocholic acid, by few bacteria, mostly

145 *Clostridium* species, via 7α-dehydroxylation through a complex biochemical pathway

146 (21-23) (Fig. 1). A crucial step during the conversion is encoded by the *baiCD* gene,

147 which is found in several *Clostridium* strains, including *Clostridium scindens* (24).

148 Deoxycholic acid is bactericidal to many bacteria, including *Staphylococcus aureus,*

149 *Bacteroides thetaiotaomicron*, *Clostridioides difficile*, bifidobacteria and lactobacilli by

150 membrane disruption and subsequent leakage of cellular content (25-28).

151 The importance of bacteria for conversion of primary bile acids was demonstrated by

152 investigating bile acid profiles in germ-free mice, where no secondary bile acids

153 could be measured (29). Very few colonic bacteria, less than 0.025% of total gut

154 microbiota, are capable of performing 7α-dehydroxylation (23, 30). One of these

155 bacteria, *C. scindens*, is associated with colonization resistance against *C. difficile*

156 through secondary bile acid production (22, 31). A follow-up *in vivo* study

157 demonstrated that *C. scindens* provided CR in the first day post infection (p.i), but

158 protection and secondary bile acid production was lost at 72 p.i (32). *C. scindens* on

159 its own was also not sufficient to inhibit *C. difficile* outgrowth in humans (33).

160 Together, these studies suggest that *C. scindens* either requires cooperation with

161 other secondary-bile acid producing bacteria or that other mechanisms were

162 involved in providing CR. The secondary bile acid lithocholic acid may exert its

163 antimicrobial effects, and potentially its effects on CR, in an indirect manner.

164 Lithocholic acid has been shown to enhance transcription for the antimicrobial

165 peptide LL-37, in gut epithelium using a HT-29 cell line (34). However, no increased

166 mRNA transcription nor protein translation of LL-37 was observed in another study

167 using a Caco2 cell line (35).

168

169 **Bacteriocins**

170 Bacteriocins are short, toxic peptides produced by specific bacterial species that can

171 inhibit colonization and growth of other species (36) (Fig. 1). Their mechanisms of

172 action are multifold and include disturbing RNA and DNA metabolism, and killing

173 cells through pore formation in the cell membrane (37-40). Bacteriocins can be

174 divided into those produced by Gram-positive bacteria, and those produced by

175 Gram-negative bacteria. Further classification of bacteriocins has been extensively

176 discussed elsewhere, (41, 42). Bacteriocins produced by Gram-positive bacteria are

177 mostly produced by lactic acid bacteria (e.g. *Lactococcus* and *Lactobacillus*) and

178 some *Streptococcus* species, and are further subdivided into three major classes on

179 the basis of the molecular weight of the bacteriocins and the presence of post-

180 translational modifications (42). Bacteriocins produced by Gram-negative bacteria,

181 mostly by *Enterobacteriaceae*, can be broadly divided into high molecular weight

182 proteins (colicins) and lower molecular weight peptides (microcins) (41).

183 The lantibiotic nisin is the best studied bacteriocin and is produced by *Lactococcus*

184 *lactis* strains. It has potent activity against many Gram-positive bacteria but has

185 much less intrinsic activity against Gram-negative organisms (43-45). By itself, nisin

186 does not induce growth inhibition of Gram-negative bacteria, since binding to lipid II

187 – the main target – is prevented by the outer bacterial membrane (46). Therefore,

188 studies have used different methods to overcome this problem by combining nisin

189 with chelating agents like EDTA, antibiotics and engineered nisin peptides (47-52).

190 These compounds can destabilize the outer membrane, allowing nisin to exert its

191 damaging effect (53, 54).

192 Several *in vivo* models have confirmed the potency of bacteriocins in providing CR.

193 *Lactobacillus salivarius* UCC 118, which produces the bacteriocin Abp118, was able

194 to significantly protect mice from infection by direct killing of *Listeria monocytogenes*,

195 while an UCC 118 mutant could not, confirming the protective role of Abp118 against

196 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,

222 during which *S.* Typhimurium expresses siderophores to scavenge iron from an iron-

223 depleted environment. As microcins are able to conjugate to siderophores and *S.*

224 Typhimurium takes up the siderophore during iron scavenging, microcins are

225 introduced into the bacterial cell in a Trojan-horse like manner (62).

226 *In silico* identification of bacteriocin gene clusters shows that much remains to be

227 discovered in this area, as 74 clusters were identified in the gut microbiota (63). Not

228 all of these clusters may be active *in vivo*, but it illustrates the potential relevance of

229 bacteriocin production by the gut microbiota to provide colonization resistance.

230

Nutrient competition

231 **Nutrient competition**

232 Bacteria have to compete for nutrients present in the gut. This is especially relevant

233 for bacterial strains belonging to the same species, as they will often require similar

234 nutrients. The importance of nutrient competition in providing CR has been shown in

235 multiple studies using multiple *E. coli* strains (64-67). Indigenous *E. coli* strains

236 compete with pathogenic *E. coli* O157:H7 for the amino acid proline (64). In fecal

237 suspensions, depletion of the proline pool by high-proline-utilizing *E. coli* strains

238 inhibited growth of pathogenic *E. coli*. This inhibition could be reversed by adding

239 proline to the medium, thereby confirming nutrient competition between the strains

240 (64). In addition to amino acids, different *E. coli* strains use distinct sugars present in

241 the intestinal mucus (65). When two commensal *E. coli* strains were present in the

242 mouse gut that together utilize the same sugars as *E. coli* O157:H7, *E. coli* O157:H7

243 was unable to colonize after it was administered to these mice. However, *E. coli*

244 O157:H7 successfully colonized when only one of these commensals was present.

245 This indicated that the two commensals complement each other to sufficiently

246 deplete all sugars used by this pathogenic *E. coli* strain (66). Nutrient competition is

247   not limited to macronutrients, but can extend to micronutrients such as iron. *S.*

248   Typhimurium is known to take up large amounts of iron from the inflamed gut during

249   infection (67). Upon a single administration of the probiotic *E. coli* Nissle 1917, which

250   was proposed to scavenge iron very efficiently, *S.* Typhimurium levels were reduced

251   more than two log-fold during infection via the limitation of iron availability.

252   Administration of *E. coli* Nissle 1917 prior to infection with *S.* Typhimurium led to a

253   445-fold lower colonization (67).

254   Finally, genome-scale metabolic models have been used to reconstruct microbiome-

255   wide metabolic networks, which could partly predict which species utilize specific

256   compounds from their environment (68). These models have been used to study

257   nutrient utilization by *C. difficile*, which will be described in the section on this

258   organism below.

259   Together, these studies show that colonization resistance by nutrient competition is

260   most effective when microbiota take up key nutrients that are required by the

261   pathogen (Fig. 1). Future strategies could therefore aim at administrating probiotic

262   strains that are able to outcompete pathogens for specific nutrients. This is

263   especially relevant at times of gut microbiota disturbances, e.g. during and following

264   an antibiotic treatment, as this is the time window where it is easiest for exogenous

265   bacteria to colonize the GI tract.

266

267   **Mucus layers**

268   The gut barrier consists of the inner and outer mucus layer, the epithelial barrier and

269   its related immune barrier. It is out of the scope of this review to discuss the full

270   immunological characteristics of the epithelial barrier, the highly complex host-

271   microbe interactions occurring at the mucus layer and host-associated genetic

272 polymorphisms associated with mucus layer composition, as these have been

273 extensively described elsewhere (12, 13, 69, 70). Instead, a general description with

274 various examples of how the mucus layer provides CR will be given.

275 The inner mucus layer is impenetrable and firmly attached to the epithelium, forming

276 a physical barrier for bacteria thereby preventing direct interaction with the epithelial

277 layer and a potential inflammatory response (71, 72). Commensal gut microbes

278 reside and metabolize nutrients in the nonattached outer mucus layer. Thinning of

279 the mucus layer leads to an increased susceptibility for pathogen colonization, which

280 can result from a Western-style diet deficient in microbiota-accessible-carbohydrates

281 (MACs) (58). When MACs were scarce, mucus-degrading bacteria (*Akkermansia*

282 *muciniphila* and *Bacteroides caccae*) fed on the outer mucus layer in a gnotobiotic

283 mouse model, resulting in closer proximity of bacteria to the epithelial layer (58). The

284 host adapts by increasing *muc2* expression, the main producer of intestinal mucin

285 glycans, but fails to sufficiently do so. Inner mucus layer damage could however be

286 reversed by administration of *Bifidobacterium longum*, perhaps due to stimulation of

287 mucus generation (73).

288 The composition of the microbiota is thus a contributing factor to the integrity of the

289 mucus barrier. Genetically identical mice housed in different rooms at the same

290 facility showed a distinct microbiota composition, with one group of mice showing a

291 more penetrable barrier (74). When FMT was performed on germ-free mice, they

292 displayed the same barrier function as their respective donor. No specific microbes

293 were identified to be responsible for the change in observed barrier function (74).

294 In conclusion, the mucus layers provide a first barrier of defense against colonization

295 of exogenous microorganisms. Diet has been shown to be an important factor for

12

296 proper functioning of this layer, suggesting that dietary intervention, or specific pro-

297 and prebiotics, may be a future therapeutic option.

298

299 **Bacteriophages**

300 Bacteriophages are the most abundant microorganisms on our planet and are also

301 highly present in the human gut (75, 76). Bacteriophages have been proposed as

302 potential alternatives to antibiotics, as they are highly specific, only targeting a single

303 or a few bacterial strains thereby minimizing the impact on commensal members of

304 the microbiota (75, 77) (Fig. 1). Their complex interactions in the intestine with both

305 host immunity and bacterial inhabitants are starting to be explored, but much

306 remains to be elucidated (76). Here, we will focus on their relationship with bacterial

307 enteropathogens.

308 *Vibrio cholerae* infection could be controlled using a prophylactic phage cocktail in

309 mice and rabbits (78). This prophylactic cocktail killed *V. cholerae in vitro*, reduced

310 colonization of *V. cholerae* in the mouse gut and prevented cholera-like diarrhea in

311 rabbits. Importantly, the authors suggest that the concentration of phages in the gut

312 is an important criterion for successful prevention of infection, as timing between

313 phage cocktail administration and *V. cholerae* inoculation was associated with

314 treatment outcome (78). Similar findings have been demonstrated for *Campylobacter*

315 *jejuni* colonization in chickens, where a phage cocktail reduced *C. jejuni* levels

316 several orders of magnitude (79).

317 Bacteriophages can also confer a competitive advantage for commensals.

318 *Enterococcus faecalis* V583 harbors phages that infect and kill other *E. faecalis*

319 strains, thereby creating a niche for *E. faecalis* V583 (80).

320 Phages play an important role in excluding specific gut bacteria and can thereby

321 contribute to CR. Therapeutic use in humans is not yet performed at a wide scale in

322 the Western world, as sufficient evidence for their safety and efficacy is still lacking

323 (81). However, recent case reports indicate that bacteriophage treatment has definite

324 future potential for treating multi-drug resistant bacteria (82, 83).

325

EFFECTS OF VARIOUS NON-ANTIBIOTIC DRUGS ON GUT COLONIZATION

RESISTANCE

328 Antibiotics are long known for their deleterious effect on gut microbiota. Recently,

329 various other drugs have come to attention for their impact on our microbial

330 ecosystem. As effects of antibiotics have been extensively reviewed elsewhere (84,

331 85), the focus in the current review will be on non-antibiotic drugs, namely proton-

332 pump inhibitors (PPIs), antidiabetics and antipsychotics.

333

Proton-pump inhibitors

335 PPIs inhibit gastric acid production and are among the most prescribed drugs in

336 Western countries (86). A significant association between long-term use of PPIs and

337 the risk on several bacterial enteric infections has been demonstrated in multiple

338 systematic reviews (87-90).

339 Several studies have associated PPI use with microbiota alterations that may

340 specifically predispose to *C. difficile* infection and to small intestinal bacterial

341 outgrowth (91-95). Especially taxa prevalent in oral microbiota (e.g. *Streptococcus*)

342 were associated with PPI use, likely resulting from increased gastric pH and thereby

343 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 several *Salmonella* species. 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.

466

467   Compared to the effects of SCFAs, there is more clarity on the effects of bile acids

468   on *C. difficile*. Secondary bile acids are toxic to both *C. difficile* spores and vegetative

469   cells, while primary bile acids generally stimulate growth and spore germination

470   (123-125). During antibiotic treatment, conversion of primary into secondary bile

471   acids is suppressed and the reduction of secondary bile acids leads to a more

472   favorable environment for *C. difficile* (120).In addition, *C. difficile* isolates causing

473   most severe disease in mice were also the isolates that showed highest resistance

474   against lithocholic acid *in vitro* (126). A relationship between disease score and

475   deoxycholic acid could not be shown (126). Secondary bile acid resistance may be

476   strain-dependent, but further research is warranted to draw this conclusion with

477   certainty.

478

479   Intrinsic anti-bacteriocin properties have been described for *C. difficile* (127, 128).

480   Nisin can inhibit growth of vegetative cells and prevent spore germination of *C.*

481   *difficile in vitro* (44). However, this does not hold for all *C. difficile* strains, as the

482   mutant strain MC119 had normal growth in sub-lethal concentrations. It was

483   demonstrated that this resistance was at least partly due to export of nisin by an

484   ABC-transporter (127). Another identified mechanism was a net positive charge on

485   the bacterial cell surface resulting in lower efficacy of nisin, since nisin is attracted to

486   a low negative charge on the cell surface (128).

487

488   Using genome-scale metabolic models in antibiotic-treated mice, it was

489   demonstrated that *C. difficile* does not necessarily compete for specific nutrients

490   against specialized bacteria, but that it adapts to utilize a wide array of nutrients. This

491 allows for colonization of diverse microbiomes, wherein *C. difficile* is not limited to a

492 specific nutrient niche (129). A follow-up study, also using a multi-omics approach,

493 showed that *C. difficile* alters transcriptional activity of especially low abundant taxa.

494 The main genes showing decreased transcription in these low abundant taxa during

495 infection, as compared to mock infected mice, were carbohydrate-acquisition and

496 utilization genes. A possible reason for this could be that *C. difficile* attempts to

497 create its own nutrient niche to facilitate colonization (130).

498 However, others have found specific nutrients that may be important for *C. difficile*

499 colonization and/or outgrowth. Three highly virulent ribotypes (RT), RT017, RT027

500 and RT078, have recently been demonstrated to utilize trehalose as a nutrient

501 source (131, 132). This was confirmed in a mouse model, where mice were

502 challenged with spores of either RT027 or a non-trehalose metabolizing ribotype.

503 After trehalose administration, RT027 mice showed higher mortality in a dose-

504 dependent manner (131).

505 *C. difficile* post-antibiotic outgrowth depends partly on the production of succinate

506 and sialic acid by commensals. *B. thetaiotaomicron* is capable of metabolizing

507 polysaccharides and thereby produces sialic acid. Upon inoculation with *C. difficile,*

508 monocolonized *B. thetaiotaomicron* mice had approximately a five times higher

509 density of *C. difficile* in feces as compared to germ-free mice (133). Expression

510 levels of genes involved in sialic acid metabolism were increased in the *B.*

511 *thetaiotaomicron* model, and, as expected, a sialidase-deficient *B. thetaiotaomicron*

512 mutant led to highly reduced production of sialic acid and *C. difficile* density was

513 lower (133).

514 Density of *C. difficile* was higher in *B. thetaiotaomicron* mice fed a polysaccharide-

515 rich diet as compared to a chow diet (134). The succinate to butyrate pathway was

516   crucial for *C. difficile* expansion in *B. thetaiotaomicron* mice, as WT *C. difficile* was

517   more effective in establishing infection than a succinate-transporter deficient *C.*

518   *difficile* (134).

519   Micronutrient availability can affect virulence of *C. difficile*. High zinc levels have

520   been demonstrated to exacerbate *C. difficile* infection in mouse models (135). Mice

521   fed a high-zinc diet had higher toxin levels, higher pro-inflammatory cytokines levels

522   and increased loss of barrier function. Furthermore, it was shown that calprotectin, a

523   zinc-binding protein, was important for limiting zinc availability to *C. difficile* during

524   infection (135).

525   Together, these studies demonstrate the importance of specific nutrients used by *C.*

526   *difficile* to establish colonization and infection.

527

528   Efficient colonization of the epithelial barrier is made possible by flagella and pili

529   (136, 137). When mice were inoculated with flagellated or non-flagellated *C. difficile*

530   strains, higher levels of flagellated C. *difficile* were found in mouse cecum (136). The

531   exact destination of non-flagellated *C. difficile* remained unknown, as levels were not

532   measured in feces or in sections of the small intestine. Regarding pili, it has been

533   shown that type IV pili were not playing a role in initial colonization, but were crucial

534   for epithelial adherence and long-lasting infection (137).

535

536   **S. Typhimurium**

537   *S.* Typhimurium is a nontyphoidal S*almonella* and an important cause of

538   gastroenteritis in humans. It was estimated that globally 3.4 million invasive

539   nontyphoidal *Salmonella* infections occur each year, of which 65.2% are attributable

540   to serovar Typhimurium (138). It mostly causes self-limiting, non-bloody diarrhea in

541 otherwise healthy individuals. However, it can lead to bloodstream infections and

542 metastatic spread with eventually death in especially infants and

543 immunocompromised individuals (138, 139). *S.* Typhimurium contains two

544 pathogenicity islands, SPI1 and SPI2. SPI1 mostly contains information for causing

545 intestinal disease and cell invasion, while SPI2 is necessary for intracellular survival

546 (140).

547

548 Effects of SCFAs on *S.* Typhimurium are not yet well defined. Butyrate and

549 propionate have been demonstrated to reduce expression of invasion genes, while

550 acetate increased their expression in *S.* Typhimurium (141, 142). However,

551 conflicting results exist. A *S.* Typhimurium knockout mutant, unable to metabolize

552 butyrate, caused less inflammation than a WT *S.* Typhimurium, suggesting that

553 butyrate is crucial for *S.* Typhimurium virulence (143). Furthermore, this study

554 demonstrated that butyrate was necessary for expression of invasion genes in

555 mouse models. In contrast, propionate inhibited *S.* Typhimurium in a dose-

556 dependent manner *in vitro*, probably due to disturbance of intracellular pH (144). In

557 an *in vivo* setting, it was demonstrated that a cocktail of propionate-producing

558 *Bacteroides* species was sufficient to mediate CR against *S.* Typhimurium (144).

559

560 *S.* Typhimurium has developed mechanisms to overcome bile acids encountered in

561 the gut. When exposed to individual bile acids at sub-lethal levels *in vitro*, it can

562 become resistant to originally lethal levels by changing gene and protein expression

563 of several virulence regulators (145, 146). In addition, it has been demonstrated that

564 a mixture of cholate and deoxycholate confers a synergistic inhibition on invasion

565 gene expression in *S.* Typhimurium (147).

566

567 Innate resistance of *S.* Typhimurium against bacteriocins produced by Gram-positive

568 bacteria is naturally conferred through its Gram-negative outer membrane (148).

569

570 Usage of nutrients produced by gut microbiota is believed to facilitate *S.*

571 Typhimurium outgrowth. By causing inflammation and thereby altering microbiota

572 composition, *S.* Typhimurium provides itself with a competitive advantage (149, 150).

573 Metabolic profiling in mice showed increased luminal lactate levels in the inflamed

574 gut during *S.* Typhimurium infection, which could result from a depletion in butyrate-

575 producing bacteria (149). When butyrate is scarce, enterocytes switch to glycolysis

576 with lactate as end product. Lactate is an important nutrient for *S.* Typhimurium, as

577 indicated by decreased colonization of cecal and colonic lumen by a *S.* Typhimurium

578 mutant lacking two lactate dehydrogenases (149).

579 As explained in the introduction, an anaerobic milieu is maintained in the gut during

580 homeostatic conditions. However, diffusion of oxygen from the tissue to the lumen is

581 enabled by inflammation caused by *S.* Typhimurium, which alters enterocyte

582 metabolism (151). Oxygen can then be used by *S.* Typhimurium to ferment several

583 carbohydrates through respiration (152-155).

584 In conclusion, these findings suggest that *S.* Typhimurium creates its own niche in

585 the gut by causing inflammation, subsequently shifting microbiota composition and

586 thereby nutrient availability, so that it can optimally colonize and expand.

587

588 An intact and well-functioning mucus layer is crucial for protection against *S.*

589 Typhimurium infection. WT mice infected with the attenuated Δ*aroA* strain, which

590 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).

616   At present, outcomes regarding the effects of SCFAs on EHEC are mixed (161-165).

617   LEE protein and gene expression was already enhanced at 1.25mM of butyrate,

618   while for acetate and propionate, only minor changes were detected at 20mM, with

619   acetate giving a repressive effect. In a separate growth experiment, acetate was

620   more efficient in inhibiting growth of EHEC as compared to butyrate and propionate

621   (162). Acetate was observed to have small repressive effects on EHEC in the study

622   by Nakanishi *et al.*, and this was also found by Fukuda *et al.* (162, 165). Mice fed

623   acetylated starch prior to infection showed higher fecal acetate levels and improved

624   survival rate compared to starch-fed mice (165). Acetate also prevented gut barrier

625   dysfunction as measured by transepithelial electrical resistance and prevented

626   translocation of the Shiga toxin to the basolateral side of the epithelial cells (165).

627   In Caco2 cells, EHEC epithelial adherence was 10-fold higher when grown on

628   butyrate than on acetate or propionate (162). These results indicate that butyrate

629   may be less effective in inhibiting EHEC growth and potentially colonization as

630   compared to acetate and propionate, for which the exact pathways and genes

631   involved have been elucidated (162, 163).

632   In contrast, butyrate was found to be effective against EHEC in a pig model (161).

633   Piglets given sodium butyrate two days prior to being infected with EHEC showed no

634   symptoms 24 hours p.i, while the control group developed multiple signs of disease,

635   e.g. histopathological signs of kidney damage. The sodium butyrate group did not

636   show any signs of inflammation and shed less viable cells compared to the control

637   group within 48h (161). *In vitro* assays demonstrated that butyrate enhanced

638   bacterial clearance, ultimately making the authors suggest that butyrate can be

639   developed as a new drug to treat EHEC (161).

640

641 EHEC has multiple traits to fight against the potentially deleterious effects of bile

642 acids. Bile acid mixtures upregulated gene expression of the *A*crAB efflux pump and

643 downregulated *ompF*, a gene encoding for an outer membrane porin (166). In

644 addition, other genes responsible for limiting penetration of bile acids through the

645 membrane (*basR* and *basS*), were upregulated, and this effect was concentration-

646 dependent. Interestingly, the bile acid mixtures did slightly downregulate *stx2* subunit

647 genes, encoding for Shiga toxin production (166).

648

649 EHEC possesses natural resistance against bacteriocins, especially nisin, through its

650 Gram-negative outer membrane, as described in the chapter on bacteriocins. Three

651 EHEC strains were screened for, amongst others, potential resistance against

652 several colicinogenic *E. coli* strains (167). *In vitro*, resistance against *E. coli* strains

653 producing a single colicin was observed, but resistance was rarely observed against

654 multiple colicins and could never be linked to acquiring a specific plasmid (167).

655

656 Nutrient competition for proline and several sugars between EHEC and commensal

657 *E. coli* strains is described in the introductory section. In addition, ethanolamine (EA),

658 a source of carbon, nitrogen and energy for EHEC, has been investigated. It was

659 demonstrated that EA could diffuse across the bacterial membrane and that the *eut*

660 genes were crucial for metabolizing EA. *Eut* sequences were absent in native

661 bacterial genomes in the bovine gut, apart from commensal *E.coli*, indicating that EA

662 provides a nutrient niche for *E. coli*. When the *eutB* gene was knocked out in

663 EDL933, it was outcompeted by commensal *E. coli* due to its inability of utilizing EA,

664 indicating its critical importance for colonization (168).

665   During further transcriptomic investigations of EA utilization, it was noticed that

666   genes involved in gluconeogenesis were upregulated if no glucose was

667   supplemented. A knockout of two genes within the gluconeogenesis pathway led to a

668   growth defect in a coculture with the wildtype (169). This is in line with a previous

669   finding that optimal usage of gluconeogenic substrates by EDL933 is important for

670   colonization (170). Since this effect was seen in a medium consisting of bovine small

671   intestinal contents, the relevance for the human gut remains unclear (169).

672   Co-culturing of EHEC with *B. thetaiotaomicron* led to an upregulation of genes

673   involved in nutrient competition in EHEC as compared to culturing EHEC alone

674   (171). In addition, presence of *B. thetaiotaomicron* resulted in upregulation of

675   multiple virulence genes including *lee*, likely due to regulation of a transcription factor

676   involved in sensing carbon metabolite concentrations in the environment (171).

677   Using a combination of *in vitro* and *in vivo* methods, Pacheco *et al.* showed that

678   fucose cleaved from mucins by *B. thetaiotaomicron* could be an important nutrient for

679   upregulating virulence and intestinal colonization of EHEC (172). Interestingly,

680   fucose sensing and subsequent regulation of virulence genes was more important

681   for successful colonization than utilization of fucose for energy. This example

682   indicates that nutrients cannot only be utilized for energy, but that they can be

683   important environmental signals for properly regulating timing of virulence (172).

684

685   Human colonoid monolayers were used to study initial colonization mechanisms of

686   EHEC (173). This study showed that EHEC disturbs the tight junctions, preferentially

687   attaches to mucus producing cells and subsequently impairs the mucus layer (173).

688   In addition, by using various *in vitro* models, it was demonstrated that the

689   metalloprotease StcE, produced by EHEC, enables degradation of MUC2 in the

690   inner mucus layer which may pave the way to the epithelial surface (174).

691

692   ***S. flexneri***

693   *Shigella* infections mostly occur in developing countries, with *S. flexneri* as the most

694   frequently found species (175). Annually, an estimated 164,000 people die of

695   shigellosis worldwide (176). Clinical presentation includes a wide variety of

696   symptoms, including severe diarrhea, possibly containing blood and mucus, and

697   abdominal pain (160). *S. flexneri* contains a virulence plasmid (pINV) which is

698   necessary for invasion of epithelial cells and intracellular survival (160).

699

700   No studies seem to have investigated resistance mechanisms of *S. flexneri* against

701   SCFAs yet. Butyrate has been investigated as a potential therapeutic agent as it

702   counteracts a putative virulence mechanism of *S. flexneri*, namely decreasing LL-37

703   expression in the gut (177, 178). By suppressing LL-37 expression *S. flexneri* is able

704   to colonize deeper into intestinal crypts (178). Butyrate was able to increase rectal

705   LL-37 expression in a subgroup of patients, which was associated with lower

706   inflammation in rectal mucosa and lower levels of pro-inflammatory cytokines (177).

707   However, butyrate treatment did not seem to impact clinical recovery (177).

708

709   The type three secretion system (T3SS) which is able to directly inject bacterial

710   protein into host cells and cause infection, is considered a key virulence factor. *S.*

711   *flexneri* T3SS can sense and bind secondary bile acid deoxycholate, which leads to

712   co-localization of protein translocators at the needle tip (179, 180). In *S. flexneri*

713   mutants lacking the needle structure, the deoxycholate-associated adhesion and

714 invasion of *S. flexneri* to host epithelial cells was diminished (181). At physiological

715 levels of bile salts, *S. flexneri* is able to grow normally *in vitro*, but at increased

716 concentrations growth is significantly reduced (182). Transcriptomics showed that

717 during exposure to physiological bile salt levels, genes involved in drug resistance

718 and virulence were upregulated, which was subsequently confirmed using RT-qPCR.

719 Deletion of a multidrug efflux pump led to sensitivity to bile salts and growth inability,

720 confirming the importance of this pump in bile salt resistance (182).

721

722 Bacteriocin resistance has not been well studied in *S. flexneri*, but downregulating

723 antimicrobial peptide production in the gut is suggested to be an important virulence

724 mechanism (183). The downregulation of LL-37 early in infection was demonstrated

725 both in gut biopsies of patients and in cell lines (183). Since protein and gene

726 expression were not downregulated to the same degree, the authors speculated that

727 there is an interference mechanism during active transcription of LL-37. Transcription

728 of other antimicrobial peptides was also downregulated, especially in the human β-

729 defensin hBD family (178, 183). It was demonstrated that *S. flexneri* shows high

730 sensitivity to LL-37 and hBD-3 peptides *in vitro* (178). This suggests that by

731 downregulating expression of antimicrobial peptides, *S. flexneri* creates an

732 environment in which it can survive and ultimately cause severe disease.

733 It is unknown how *S. flexneri* competes and utilizes nutrients in the luminal side of

734 the gut. Therefore, a short description will be given on how the bacterium rewires

735 host cell metabolism for supporting its survival after entering the host cells. These

736 findings might be translatable, and can at least provide insight in potential nutrient

737 usage of *S. flexneri* in the lumen. Using a combination of metabolomics and

738   proteomics it was demonstrated that *S. flexneri* does not alter host cell metabolism in

739   HeLa cells, but that it captures the majority of the pyruvate output (184). Pyruvate

740   was demonstrated to be a crucial carbon source for *S. flexneri* cultured on a HeLa

741   derivative, using metabolomics, transcriptomics and bacterial mutants (185). *S.*

742   *flexneri* converts pyruvate into acetate via a very quick, but energy-inefficient

743   pathway, allowing for rapid expansion of the bacterium intracellularly without rapid

744   destruction of the host cell (184).

745

746   *S. flexneri* possess special systems to alter mucus composition. Human colonoid

747   monolayers infected with *S. flexneri* showed increased extracellular release of

748   mucins (186). The increased extracellular mucins were trapped at the cell surface

749   which surprisingly favored access of *S. flexneri* to the apical surface, subsequently

750   promoting cell invasion and cell-to-cell spread (186). Furthermore, expression of

751   several genes encoding for production of mucins and mucin glycosylation patterns

752   were altered (186). Together, these results suggest that *S. flexneri* can alter the

753   mucus environment such that it can promote its own virulence.

754   **C. jejuni**

755   *C. jejuni* is associated with food-borne gastroenteritis and is estimated to cause more

756   than 800,000 infections annually in the USA alone (187). Major clinical symptoms

757   include diarrhea (both with and without blood), fever and abdominal cramping (160).

758   In rare cases, it can give rise to the Guillain-Barré syndrome and reactive arthritis

759   (187). It is a commensal bacterium in avian species and it is not yet well understood

760   why it causes disease in humans (188).

761

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

786   affected (191). In conclusion, *C. jejuni* colonization seems not to be affected by bile

787   acids, but may be important in limiting disease progression.

788

789   Bacteriocin resistance is not common in *C. jejuni*. Multiple *C. jejuni* (n=137) isolates

790   were screened for resistance against two anti-*Campylobacter* bacteriocins, OR-7

791   and E-760, produced by the gut inhabitants *L. salivarius* and *Enterococcus faecium.*

792   However, no isolates were found to harbor resistance (192). In a follow-up study,

793   chickens were successfully colonized with a *C. jejuni* strain prior to bacteriocin

794   treatment, with the aim of studying bacteriocin resistance. Resistance developed in

795   most chickens, but was lost upon ending bacteriocin administration, suggesting

796   resistance instability *in vivo* (193).

797

798   In contrast to most other enteric pathogens, *C. jejuni* does not metabolize

799   carbohydrates as its main energy source. It is unable to oxidize glucose, fructose,

800   galactose and several disaccharides, including lactose, maltose and trehalose,

801   resulting from the absence of 6-phosphofructokinase (194-197). Fucose could be

802   metabolized by some *C. jejuni* strains, due to the occurrence of an extra genomic

803   island (197). Main energy sources for *C. jejuni* are organic acids, including acetate,

804   and a limited number of amino acids (198-200). It is currently unclear what these

805   metabolic adaptations mean for its colonization potential, but it is possible that *C.*

806   *jejuni* occupies a unique macronutrient niche.

807   Iron regulation systems are critical for colonization and persistence of *C. jejuni*. In

808   presence of sufficient iron, transporter and acquisition genes are downregulated

809   (201). Mutants lacking genes involved in either iron acquisition or transport were

810   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).

834   *V. cholerae*

835   *V. cholerae* is one of the first bacterial pathogens where the microbiota has been

836   considered to play an important role against infection (208). It is mainly prevalent in

837   contaminated brackish or salt water and can cause outbreaks, particularly during

838   wars and after natural disasters. In the first two years following the earthquake in

839   Haiti, 2010, more than 600,000 people were infected with *V. cholerae* serogroup O1,

840   biotype Ogawa, resulting in more than 7,000 deaths (209). The clinical course is

841   characterized by watery diarrhea, which can be so severe that it can result in

842   dehydration, hypovolemic shock and death (210). *V. cholerae* colonizes the small

843   intestine by employing the toxin-coregulated pilus, after which it can cause severe

844   infection and clinical symptoms through cholera enterotoxin production (210).

845

846   *V. cholerae* is able to utilize its acetate switch, the shift from elimination to

847   assimilation of acetate, to increase its own virulence (211). In a *Drosophila* model, it

848   was demonstrated that *crbRS* controlled the acetate switch, while *acs1* was required

849   for acetate assimilation (211). When either of these genes were knocked-out,

850   mortality decreased. Competition experiments demonstrated that WT *V. cholerae*

851   had a growth advantage over Δ*crbS* when WT *V. cholerae* was administered in

852   minority. This led the authors to suggest that acetate utilization may be important

853   early in infection, when low levels of *V. cholerae* cells are present (211).

854   Furthermore, acetate consumption led to dysregulation of host insulin signaling

855   pathways, ultimately leading to intestinal steatosis and increased mortality.

856   Dysregulation of host insulin signaling was not observed in Δ*crbS* or Δ*acs1*, further

857   confirming the role of acetate in *V. cholerae* virulence (211).

858

859   *V. cholerae* has a master regulator, *toxT*, which can directly activate several

860   virulence factors including toxin production. Cholera toxin production was reduced by

861   97% when *V. cholerae* was grown in presence of bile, which could be reversed after

862   growing the same cells in bile-free medium for a few hours (212). C*tx* and *tcpA*,

863   encoding for cholera toxin and the major structural unit of the toxin-coregulated pilus

864   and regulated by *toxT*, were highly repressed during bile exposure (212).

865   Additionally, motility was increased approximately 1.6-fold in presence of bile (212).

866   To elucidate which exact components of bile acids were responsible for the

867   repression of these virulence genes, bile was fractionated. It was found that several

868   unsaturated fatty acids strongly repressed *ctx* and *tcpA* and that they upregulated

869   expression of *flrA*, leading to increased motility (213). The reason for upregulation of

870   *flrA* and downregulation of *tcpA* could be that the flagellum increases the speed of

871   passing through the mucus layer, while the pilus would only slow it down. When

872   lower concentrations of bile at the epithelial surface are encountered, expression can

873   be reversed (214).

874   Two outer membrane porins, OmpU and OmpT, are directly regulated by the master

875   regulator *toxR*. Upon encountering bile acids, *ompU* and *ompT* are regulated in such

876   a way that bile acid entrance is prevented (215, 216). Furthermore, Δ*toxR* mutants

877   are more sensitive to bile acids due to changed outer membrane composition (215).

878   Recently, it was shown that *toxR* also regulates *leuO* (217). *LeuO* was demonstrated

879   to confer bile resistance independent of the two porins, although its exact resistance

880   mechanism is not yet elucidated (217).

881

882   Bacteriocin resistance in *V. cholerae* has, to our knowledge, not been studied and

883   future studies will have to reveal whether any resistance is present.

884

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

886 sialic acid, a component of the mucus layer. Using streptomycin pre-treated mice

887 who were given a mutant strain defective in sialic acid transport (Δ*siaM*) , it was

888 shown that sialic acid is not required for initial colonization, but that it is important for

889 persistent colonization (218). Competition assays of the two mutant strains in mouse

890 intestine (small intestine, cecum and large intestine) showed that Δ*siaM* was less fit

891 to compete in each environment, further indicating the necessity of sialic acid

892 utilization for niche expansion of *V. cholerae* (218).

893 The El Tor strain may have a competitive advantage over 'classical' strains due to its

894 differential carbohydrate metabolism (219). When grown in a glucose-rich medium,

895 classical strains display a growth defect as compared to El Tor. It was observed that

896 this was due to production of organic acids through glucose metabolism, leading to

897 acidification of the medium. El Tor biotypes were found to produce acetoin, a neutral

898 compound, and decrease organic acid production. This prevented acidification of the

899 medium, leading to better growth. El Tor strains were also more successful in

900 colonizing mice, especially when extra glucose was administered. The classical

901 types were shown to be able to produce acetoin, but glucose only led to a minor

902 increase in transcription of genes necessary for acetoin production (219). These

903 studies have shown that specific metabolic pathways are used by *V. cholerae* to

904 successfully colonize the gut.

905

906 One of the first studies on how the mucus layer can potentially be crossed by *V.*

907 *cholerae* was reported almost 50 years ago (220). Here, motile and non-motile

908 strains were compared for pathogenicity after administration to mice. It was observed

909   that motile strains were almost always deadly 36 hours p.i, while most non-motile

910   strains had a mortality of under 35% (220). One hypothesis offered by the authors

911   was that together with mucinase, the flagellum could effectively pass the mucus

912   barrier (220). Specific mucin degradation mechanisms employed *by V. cholerae*

913   have been identified since, with hemagglutinin/protease (Hap), and TagA being the

914   major ones (221-225). Presence of mucins, limitation of carbon sources and bile

915   acids maximized production of Hap, while glucose could partly reverse this effect

916   (221). This may indicate that during conditions as encountered in the gut, *V.*

917   *cholerae* quickly aims to cross the mucus layer and be in close contact with the

918   epithelial cells. TagA, which is similar to StcE as described for EHEC, is also capable

919   of degrading mucin (222). In conclusion, *V. cholerae* has developed a way of

920   sensing environmental conditions, and in response to these, is able to upregulate

921   virulence factors which can degrade mucins. A simplified overview of *V. cholerae*

922   virulence factors opposing CR can be found in Fig. 2.

923

924   ***Y. enterocolitica***

925   Yersiniosis is mostly contracted through contaminated food or water with *Y.*

926   *enterocolitica*, and its prevalence is much higher in developing countries than in

927   high-income nations (160, 226). It is characterized by mild gastroenteritis, abdominal

928   pain and is usually self-limiting, though pseudo-appendicitis illnesses can occur

929   (160). Virulence is mostly conferred through presence of a 64-75 kb plasmid on

930   which several virulence genes are present, including *yadA*, which is crucial for

931   epithelial adherence (227).

932

933   Resistance of *Y. enterocolitica* against antibacterial compounds has not been much

934   studied. One study investigated effects of SCFAs on *Y. enterocolitica* at 4°C,

935   including acetic acid and propionic acid. *Y. enterocolitica* was less sensitive to acetic

936   acid when cultured anaerobically as compared to anaerobic culturing. Propionic acid

937   was similarly effective in inhibiting growth with both culture methods (228). Even

938   though conditions like 4°C are not representative for the intestinal environment, this

939   study might provide some initial clues on the effects of SCFAs on *Y. enterocolitica*. It

940   is clear that more research is required to further elucidate potential resistance

941   mechanisms.

942

943   *OmpR*, a transcriptional regulator in *Y. enterocolitica*, is probably able to upregulate

944   expression of the AcrAB-TolC efflux pump, which, in turn, is regulated by two

945   components of the efflux pump, *acrR* and *acrAB* (229). A mixture of bile acids, but

946   not the secondary bile acid deoxycholate, was found to be the strongest inducer of

947   *acR* and *acrAB* (229). Whether the upregulation of these efflux pump components

948   contributes to bile acid resistance, remains to be elucidated.

949

950   Bacteriocin resistance is so far mostly unknown in *Y. enterocolitica*. WA-314 and

951   8081 are both 1B:O8 strains that are highly infective in murine models (230). WA-

952   314 possesses a putative colicin cluster for colicin production, but no expression was

953   observed in a spot-on-lawn assay with 8081 and the colicin-sensitive *E. coli* K12

954   (230). It is likely that no specific resistance against colicin is present, as colicin has

955   been shown to effectively inhibit *Y. enterocolitica* infections *in vivo* (59).

956

957 Like most other enteric pathogens, *Y. enterocolitica* has sophisticated systems to

958 acquire sufficient iron. Using these systems, *Y. enterocolitica* may be more efficient

959 at scavenging iron than commensal members, thereby providing itself with a

960 competitive advantage. *Y. enterocolitica* expresses yersiniabactin, *ybt*, a highly

961 efficient siderophore and a crucial component for lethality in mouse models (231,

962 232). The exact mechanisms for iron uptake and transport have been extensively

963 reviewed elsewhere (233). Proteomics analysis revealed that *Y. enterocolitica*

964 serovar 1A, whose pathogenic role is unclear, uses different proteins to successfully

965 scavenge iron, as it lacks the Ybt protein (234).

966 *Y. enterocolitica* is the only pathogenic Yersinia species which can metabolize

967 sucrose, cellobiose, indole, sorbose and inositol (235). Additionally, it can degrade

968 EA and 1,2-PD by using tetrathionate as a terminal electron acceptor (235).

969

970 Mucus layer invasion and adherence of *Y. enterocolitica* have been elucidated in

971 great detail several decades ago (236-240). The YadA protein is used for initial

972 attachment to the mucus (240). The preferential binding side on mucins is their

973 carbohydrate moiety, but binding to mucin proteins is also possible under specific

974 conditions (238). *Y. enterocolitica* uses a plasmid, pYV, with mucin-degradation

975 enzymes to thin the mucus layer, facilitating crossing of the mucus layer (237, 240).

976 *Y. enterocolitica* containing the pYV plasmid is not only able to successfully invade

977 and degrade the mucus layer, but is also highly efficient in multiplying in this

978 environment (240). After interacting with the mucus layer, its bacterial cell surface

979 was altered so that *Y. enterocolitica* became less efficient in colonizing the brush

980 border (240). This may be a host response mechanism to prevent *Y. enterocolitica*

981 invasion in deeper tissues. In a rabbit infection model, persistent goblet cell

982 hyperplasia and increased mucin secretion was observed throughout the small

983 intestine over 14 days (236). The extent of hyperplasia was associated with severity

984 of mucosal damage, indicating a compensatory mechanism. Mucin composition

985 changed in infected rabbits, with a decrease in sialic acid and an increase in sulfate

986 (236).

987

988 ***L. monocytogenes***

989 *L. monocytogenes* causes listeriosis, a food-borne disease. Listeriosis is not highly

990 prevalent, with an estimated 23,150 people infected in 2010 worldwide, but has a

991 high mortality rate of 20-30% (241). The most common syndrome is febrile

992 gastroenteritis, but complications can develop, such as bacterial sepsis and

993 meningitis (241). This is especially relevant for vulnerable patient groups, such as

994 immunocompromised individuals, neonates and fetuses (242). Virulence genes are

995 present on an 8.2-kb pathogenicity island, which includes internalin genes necessary

996 for invading host cells (243).

997

998 Culturing *L. monocytogenes* in presence of high levels of butyrate leads to

999 incorporation of more straight-chain fatty acids in the membrane (244, 245). This is

1000 not a natural state for *L. monocytogenes*, as normally its membrane consists for a

1001 very high percentage of branched-chain fatty acids. When subsequently exposed to

1002 LL-37, it displays a survival defect as compared to bacteria not grown in presence of

1003 butyrate (244). It was not elucidated whether this survival defect was due to

1004 increased stress, altered membrane composition or differentially regulated virulence

1005 factors. Effects of propionate on *L. monocytogenes* growth, metabolism and

1006 virulence factor expression are dependent on temperature, oxygen availability and

1007 pH (246). Therefore, it is not possible to ascribe a general function to propionate in

1008 relation to *L. monocytogenes*.

1009

1010 *L. monocytogenes* possesses several bile acid resistance mechanisms, and *in vitro*

1011 transcriptome and proteome analyses have provided insight into these.

1012 Transcriptomics analysis revealed that in response to cholic acid, amongst others,

1013 two efflux pumps were upregulated, *mdrM* and *mdrT* (247). BrtA was shown to

1014 regulate expression of the efflux pumps, and to be able to sense bile acid levels.

1015 Bacterial abundance was determined in multiple organs of mice infected with

1016 knockout strains of either efflux pump, but not in the intestine (247). Proteomic

1017 analyses found many changes in response to bile salts and included proteins

1018 associated with efflux pumps, metabolism and DNA repair (248).

1019 Bile salt hydrolases (BSH) are another way of combatting encountered bile acids. It

1020 was demonstrated that all *Listeria* species which infect mammals showed BSH

1021 enzyme activity. BSH was crucial during infection of guinea pigs, demonstrated by

1022 the decreased ability of Δ*bsh* to cause a persistent infection (249).

1023 At decreased pH levels, e.g. in the duodenum, bile salts are more acidic and show

1024 higher toxicity (250). However, this toxicity seems to be strain-dependent (251). The

1025 strain responsible for a 2011 outbreak even displayed higher bile resistance at pH

1026 5.5 than at 7.0, further indicating that bile susceptibility may be strain-dependent

1027 (251).

1028 As discussed in the introductory section on bacteriocins, the Abp118 bacteriocin

1029 produced by *L. salivarius*, protected mice from *L. monocytogenes* infection (55).

1030 However, several bacteriocins have been shown ineffective against *L.*

1031 *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

1057 (258). Limited information is available on nutrient competition of *L. monocytogenes*

1058 inside the lumen. Comparison of genome sequences between colonizing *Listeria* and

1059 non-colonizing *Listeria* led to identification of, amongst others, a vitamin B12-

1060 dependent 1,2-propanediol (1,2-PD) degradation pathway in colonizing *Listeria*,

1061 dependent on the *pduD* gene (259). Mice were co-infected with a Δ*pduD* strain and a

1062 WT strain. Within 3 hours after feeding, a large amount of the Δ*pduD* was shed in

1063 feces and 21 hours later the number of viable cells decreased significantly. At ten

1064 days p.i, the Δ*pduD* strain was completely cleared, while the WT strain shed for up to

1065 four more days. This indicates that the ability to degrade 1,2-PD offers *L.*

1066 *monocytogenes* a distinct competitive advantage (259).

1067

1068 Multiple adhesins and internalins have been characterized which facilitate *L.*

1069 *monocytogenes* retention in the mucus layer (260-263). InlB, InlC, InlL and InlJ were

1070 demonstrated to bind to MUC2, but not to epithelial cell surface MUC1 (262, 263).

1071 Histopathological analysis of a listeriosis rat model revealed that *L. monocytogenes*

1072 was present in the mucus layer after less than 3 hours p.i (261). At this time point,

1073 very few *L. monocytogenes* were present on the epithelial cells (261).

1074

1075 **Bacterial defense mechanisms against bacteriophages**

1076 As research investigating how each enteric pathogen overcomes CR by rendering

1077 bacteriophages ineffective is still in its infancy, this general section will describe the

1078 most employed resistance mechanisms. The bacteriophage infectious cycle involves

1079 a lytic and a lysogenic cycle. Phages have to bind to a receptor on the bacterial

1080 surface to be able to insert their genomic material, usually DNA, into the bacterial

1081 cytoplasm and subsequently circularize their DNA (264). Here, lysogenic and lytic

1082 bacteriophages' mechanisms start to branch (Fig. 3). Lytic phages start DNA

1083 replication, assemble their proteins and pack their DNA into the typical

1084 bacteriophage shape with a capsid head and tail. After sufficient replication, phages

1085 use lytic enzymes to form holes in the bacterial cell membrane, eventually leading to

1086 lysis of the cell and phage spreading. Lysogenic phages integrate their DNA in the

1087 bacterial chromosome and become prophages. Reproduction is then ensured

1088 through vertical transmission, and upon induction, prophages can also enter the lytic

1089 cycle (265) (Fig. 3). In general, factors that induce the lytic phase are compounds or

1090 conditions with bactericidal effects, e.g. a DNA damaging-agent (266).

1091

1092 The first step for preventing bacteriophage infection is to prevent surface receptor

1093 recognition. Outer membrane vesicles are produced by Gram-negative bacteria and

1094 have several functions, including interbacterial communication (267). They have

1095 highly similar surface composition as the bacterium and may thereby serve as

1096 decoys for attacking phages (268) (Fig. 3). Indeed, *V. cholerae* outer membrane

1097 vesicles were shown to neutralize a *V. cholerae* specific phage in a dose-dependent

1098 manner (Fig. 2) (268). This effect was only seen when the O1 antigen, the

1099 bacteriophage target on *V. cholerae*, was included in the outer membrane vesicle

1100 structure (268).

1101 *V. cholerae* possesses another mechanism to prevent O1 phage receptor

1102 recognition (269) (Fig. 3). Two genes necessary for O1 biosynthesis were shown to

1103 use phase variation to induce variation in the O1 antigen composition (269). Mutants

1104 using phase variation were resistant to the O1 antigen phage, but displayed impaired

1105 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.

1156   Bacterial cells can perform an apoptosis-like action called abortive infection, resulting

1157   in death of the infected cell and hereby protecting surrounding bacterial cells (280)

1158   (Fig. 3). These systems have not been much elucidated for enteric pathogens at a

1159   molecular level, though, relevance of this system has been shown for the gut

1160   bacteria *S. dysenteriae* and *E. coli* (281, 282). The abortive infection systems are

1161   best studied in *L. lactis*, a bacterium widely used in production of fermented foods

1162   (283).

1163

1164   **CONCLUDING REMARKS**

1165   Currently, bacterial enteric infections still cause a heavy disease burden worldwide.

1166   For many bacterial pathogens, the virulence factors involved in infection are

1167   understood, but less is known concerning the failure of gut microbiota to provide

1168   colonization resistance against these enteropathogens. A more comprehensive

1169   understanding of why the microbiota fail to confer sufficient CR could lead to

1170   development of specific therapies aiming to restore CR. It is likely that not a single

1171   bacterium will be used as the 'holy grail' to restore CR, but that bacterial consortia

1172   with complementary functions will be used instead. This would be preferable over the

1173   currently often used FMT, where it is not well known what exact components are

1174   transferred to the patient. One could imagine that these consortia could not only be

1175   used to treat existing infections, but that they could also be administered

1176   prophylactically in susceptible patient groups. In addition, more attention has recently

1177   been given to several drugs that were previously not linked to gut health for their

1178   potentially disturbing effect on gut microbiota and perhaps CR. In conclusion, we

1179   reviewed many of the latest insights in the rapidly evolving fields of gut microbiota,

1180 colonization resistance and bacterial enteric infection. We are looking forward to the

1181 coming years, where undoubtedly more knowledge will be gained on gut microbiota

1182 and CR, ultimately leading to more microbiota-based therapies.

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1195

1196

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1979

FIGURE LEGENDS

FIG 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.

Fig 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. Bacteriocin resistance remains to be mostly elucidated. 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.

Fig 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).

2005 Only upon encountering induction factors will the prophage leave the bacterial

2006 chromosome, after which it can enter the lytic cycle (4b and 5b). In the lytic cycle,

2007 phage DNA and protein is replicated and subsequently assembled into full phages

2008 (3a and 4a). The phages then lyse the bacterial cell, are released and can infect

2009 other bacteria (5a). Bacteria possess multiple mechanisms to prevent killing by

2010 bacteriophages, starting with blocking attachment. This can be achieved through

2011 phase variation or production of OMVs. After phage DNA entry, CRISPR-Cas can

2012 recognize this foreign DNA and degrade it. Phage DNA and protein replication can

2013 be prevented by BREX and restriction modification systems, while full phage

2014 assembly can be prevented by abortive infection.

2015

Fiber

Bacteriocin producer

Gut microbiota

Bacteriophages

Bacteriocins

Primary bile acids

Fermentation

7α-dehydroxylase

SCFAs

Secondary bile acids

Gut microbiota

Pathogens

Lumen

Nutrients

Outer mucus layer

Inner mucus layer

Epithelial cells

Bacteriophages

SCFAs (acetate)

Bacteriocins

OMVs

Bile acids

Acetate switch

Upregulation of motility

Porin

Porins prevent bile acid entrance

Mucin degrading enzyme

Sialic acid

?

V. cholerae

Mucus degradation releases sialic acid

Outer mucus layer

Inner mucus layer

Epithelial cells

① Phase variation OMVs

CRISPR-Cas ②

Lytic cycle

Lysogenic cycle

Induction factors

⑤b

⑤a

④a

④b

Abortive infection

③a

③b

BREX and restriction modification systems