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Commensal and Pathogenic *Escherichia coli* Metabolism in the Gut

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ABSTRACT *E. coli* is a ubiquitous member of the intestinal microbiome. This organism resides in a biofilm comprised of a complex microbial community within the mucus layer where it must compete for the limiting nutrients that it needs to grow fast enough to stably colonize. In this article we discuss the nutritional basis of intestinal colonization. Beginning with basic ecological principles we describe what is known about the metabolism that makes *E. coli* such a remarkably successful member of the intestinal microbiota. To obtain the simple sugars and amino acids that it requires, *E. coli* depends on degradation of complex glycoproteins by strict anaerobes. Despite having essentially the same core genome and hence the same metabolism when grown in the laboratory, different *E. coli* strains display considerable catabolic diversity when colonized in mice. To explain why some *E. coli* mutants do not grow as well on mucus in vitro as their wild type parents yet are better colonizers, we postulate that each one resides in a distinct "Restaurant" where it is served different nutrients because it interacts physically and metabolically with different species of anaerobes. Since enteric pathogens that fail to compete successfully for nutrients cannot colonize, a basic understanding of the nutritional basis of intestinal colonization will inform efforts to develop prebiotics and probiotics to combat infection.

INTRODUCTION

Every mammal on the planet is colonized with *E. coli* (1), as well as cold-blooded animals (e.g., fish) at an appropriately warm temperature (2). We estimate there are 10^{21} *E. coli* cells in the human population alone. *E. coli* is frequently the first bacterium to colonize human infants and is a lifelong colonizer of adults (3). *E. coli* is arguably the best understood of all model organisms (4). Yet the essence of how *E. coli* colonizes and/or causes disease is still not completely understood. Certainly, innate immunity, adaptive immunity, and bacterial cell-to-cell communication play important roles in modulating the populations of the 500–1000 different commensal species in the intestine (5–11); however, these topics will not be a focus of this chapter. We have reviewed the mucus layer as habitat for *E. coli* to colonize the intestine, aspects of *E. coli* physiology that enable its success, and the model systems employed for colonization research (12–14). Here, we focus on *E. coli* metabolism in the intestinal mucus layer. We discuss evidence that *E. coli* must obtain nutrients in the mucus layer to colonize, that it resides in the mucus layer as a member of mixed biofilms, and that each *E. coli* strain displays a unique nutritional program in the intestine. We also discuss evidence supporting the "Restaurant" hypothesis for commensal *E. coli* strains, i.e., that they colonize the intestine as sessile members of mixed biofilms obtaining the nutrients they need for growth locally, but compete for nutrients with invading *E. coli* pathogens planktonically.

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FROM INGESTION TO COLONIZATION

When *E. coli* is eliminated by a host animal, it is not growing because it cannot grow in the luminal contents of the intestine (15). *E. coli* persists in the environment until its next host consumes viable bacteria in contaminated water or adulterated food. Following ingestion, a stressor faced by *E. coli* is acidity in the stomach, which it survives because stationary phase bacteria induce protective acid-resistance systems (16). Extreme acid tolerance makes *E. coli* transmissible by as few as ten bacterial cells (17). Upon reaching the colon, *E. coli* must find the nutrients it needs to exit lag phase and grow from low to high numbers. Failure to transition from lag phase to logarithmic phase will lead to elimination of the invading *E. coli* bacteria (18). Successful colonization of the colon by *E. coli* depends upon competition for nutrients with a dense and diverse microbiota (18), penetration of the mucus layer (19) (but not motility [20]), avoid host defenses (21, 22), and grow rapidly, exceeding the turnover rate of the mucus layer (23). *E. coli* resides in mucus until being sloughed into the lumen of the intestine (24, 25), from whence some cells are eliminated in the host feces and the cycle begins again. This circle of colonization and extra-intestinal survival is the reality for commensal and pathogenic *E. coli* alike.

BASIC PRINCIPLES OF COLONIZATION

Colonization is defined as the indefinite persistence of a particular bacterial population without reintroduction of that bacterium. We agree with Rolf Freter, a true pioneer in the field of intestinal colonization, who concluded that although several factors could theoretically contribute to an organism's ability to colonize, competition for nutrients is paramount for success in the intestinal ecosystem (26). According to Freter's nutrient-niche hypothesis, the mammalian intestine is analogous to a chemostat in which several hundreds of species of bacteria are in equilibrium. To co-colonize, each species must use at least one limiting nutrient better than all the other species (18, 27, 28). The nutrient-niche hypothesis further predicts that invading species will have difficulty colonizing a stable ecosystem, such as the healthy intestine. The ability of the microbiota to resist invasion is termed colonization resistance (29), an example of which being that when human volunteers were fed *E. coli* strains isolated from their own feces, those *E. coli* failed to colonize (30). Yet, despite colonization resistance, humans are colonized on average with five different *E. coli* strains and there is a continuous succession of strains in individuals (30). This suggests that diversity exists among commensal *E. coli* strains and that different strains may possess different strategies for utilizing growth-limiting nutrients.

If diversity amongst *E. coli* commensal strains plays a role in colonization resistance, then mice pre-colonized with a human *E. coli* commensal strain would resist colonization by the same strain (isogenic challenge strain) because bacteria that consume the nutrients it needs to colonize already occupy its preferred niche. However, if mice pre-colonized with one human *E. coli* commensal strain were subsequently fed a different *E. coli* strain (non-isogenic challenge strain) then, if the second strain could occupy a distinct niche in the intestine, it would co-colonize with the first strain. The results of such experiments showed that each of several pre-colonized *E. coli* strains nearly eliminated its isogenic challenge strain from the intestine, confirming that colonization resistance can be modeled in mice, but non-isogenic challenge *E. coli* strains grew to higher numbers in the presence of different pre-colonized strains, suggesting that the newly introduced non-isogenic challenge strain either grows faster than the pre-colonized strain on one or more nutrients or uses nutrient(s) not being used by the pre-colonized strain (31).

How might an invading enteric pathogen subvert colonization resistance? According to the nutrient-niche hypothesis, upon reaching the intestine the pathogen would first have to outcompete the resident microbiota for at least one nutrient, allowing it initially to colonize the intestine. However, colonization would not in itself result in pathogenesis if the pathogen must reach the epithelium and either bind to epithelial cells or invade the epithelium. In such instances, the pathogen must presumably penetrate the mucus layer. In a series of groundbreaking studies (32–34), Stecher, Hardt, and colleagues showed that when *Salmonella enterica* serovar Typhimurium induces inflammation in a mouse colitis model, the composition of the microbiota is changed and its growth is suppressed while serovar Typhimurium growth is enhanced. The authors also showed that serovar Typhimurium is attracted by chemotaxis to galactose-containing nutrients on the mucosal surface (e.g., galactose-containing glycoconjugates and mucin) and, as expected, flagella and motility were required (32). Thus, to quote the authors (34), "Triggering the host's immune defense can shift the balance between the protective microbiota and the pathogen in favor of the pathogen."

In streptomycin-treated mice, nutrient consumption by colonized *E. coli* strains can prevent invading *E. coli*

strains from colonizing (35). By examining the sugars used by various human commensal *E. coli* strains to colonize, we identified a pair of strains (*E. coli* HS and *E. coli* Nissle 1917) that together use the five sugars previously found to be most important for colonization by the enterohemorrhagic *E. coli* (EHEC) strain EDL933 (O157:H7) (36). When mice were precolonized with *E. coli* HS and *E. coli* Nissle 1917, invading *E. coli* EDL933 was eliminated from the intestine (35). Clearly, one therapeutic strategy to prevent pathogenesis would be to outcompete the pathogen for nutrients normally present in the intestine and eliminate it before it can colonize and subsequently cause inflammation (5, 6, 37).

Implicit in the nutrient-niche hypothesis is the idea that different species compete for preferred nutrients from a mixture that is equally available to all species. However, there is growing evidence that, at least under some circumstances, *E. coli* receives the nutrients it needs through direct interactions with neighboring microbes in the intestinal community. Thus, we take a renewed look at the metabolism of and nutrient flow between members of the intestinal microbiota.

CENTRAL METABOLISM AND INTESTINAL COLONIZATION

E. coli is a Gram-negative, prototrophic, facultative anaerobe with the ability to respire oxygen, use alternative anaerobic electron acceptors, or ferment, depending on electron-acceptor availability. Central metabolism in *E. coli* consists of the Embden-Meyerhof-Parnas glycolytic pathway (EMP), the pentose phosphate pathway (PP), the Entner-Doudoroff pathway (ED), the TCA cycle, and diverse fermentation pathways. *E. coli* grows best on sugars, including a wide range of mono- and disaccharides, but it cannot grow on complex polysaccharides because it lacks the necessary hydrolase enzymes (36). *E. coli* also can grow on amino acids and dicarboxylates that feed into the TCA cycle; the metabolism of these nutrients requires gluconeogenesis, the biosynthesis of glucose phosphate to be used as precursors of macromolecules such as LPS and peptidoglycan. Central metabolic pathways in *E. coli* are highly conserved, constituting a significant part of the core *E. coli* genome (38). The role of central metabolism during intestinal colonization has been studied in *E. coli*. The results of these experiments are summarized below (Table 1).

Mutants blocked in glycolysis or the ED pathway, but not the PP pathway, have major colonization defects

TABLE 1 Central metabolism mutants tested for colonization defects in the mouse intestine

Pathway	Gene Defect	MG1655	EDL933
Glyoxylate bypass	*aceA*	No	Yes
ED	*edd*	Yes	Yes
glycolysis	*pgi*	Yes	ND
PPP	*gnd*	No	ND
gluconeogenesis	*ppsA pckA*	No	No
TCA cycle	*frdA*	Yes	Yes
TCA cycle	*sdhAB*	No	Yes
TCA cycle	*frdA sdhAB*	Yes	Yes

Results show the difference in population sizes of wild-type verses mutant strains at Day 9.
Yes indicates the difference exceeds a 0.8 $\log_{10}$ colonization advantage and students *t* test value $P < 0.05$
ND indicates not determined

in competition with their wild type parents (39). Given its role in hexose metabolism, it is expected that glycolysis is important for colonization. Indeed, a *pgi* mutant lacking the key enzyme, phosphoglucose isomerase, of the EMP glycolytic pathway has a substantial colonization defect when competed against its wild-type *E. coli* K-12 parent (Table 1). The role of the EMP pathway goes beyond colonization by *E. coli*. For example, glucose catabolism and glycolysis are known to play a role in intracellular growth of serovar Typhimurium within macrophage vacuoles (40), and proper regulation of glucose catabolism and glycolysis are coupled to virulence-factor expression in EHEC (41). A recent study of *Shigella flexneri* revealed similar usage of these central metabolic pathways to support replication within host cells (42). We conclude that glycolysis is important for *E. coli* colonization and other aspects of enteric pathogenesis.

Gluconate was the first nutrient that was shown to be used by *E. coli* to colonize the streptomycin-treated mouse intestine (43). Since gluconate and other sugar acids are primarily catabolized via the ED pathway, it is reasonable to expect that mutants lacking the pathway will be defective in colonization (44). The ED pathway is encoded by the *edd-eda* operon (45). The promoter-proximal *edd* gene encodes 6-phosphogluconate dehydratase, which converts 6-phosphogluconate to 2-keto-3-deoxy-6-phosphogluconate. The *eda* gene encodes 2-keto-3-deoxy-6-phosphogluconate aldolase, which converts 2-keto-3-deoxy-6-phosphogluconate to glyceraldehye-3-phosphate and pyruvate. *E. coli edd* mutants lacking the ED pathway, but retaining the pentose phosphate (PP) pathway, are poor colonizers of the mouse intestine, suggesting that *E. coli* utilizes the ED pathway for growth in the intestine (43). Other enteric bacteria require the

ED pathway. For example, intracellular serovar Typhimurium induces genes of the ED pathway and gluconate catabolism during growth in macrophages (46). Moreover, the ED pathway is induced by *Vibrio cholerae in vivo* and an *edd* mutant failed to colonize the mouse intestine (47).

In contrast to the importance of the ED pathway, an *E. coli gnd* mutant, missing 6-phosphogluconate dehydrogenase and therefore deficient in the oxidative branch of the PP pathway, was as good a mouse-intestine colonizer as the wild-type (39). It should be noted that *gnd* mutants retain the non-oxidative PP pathway; therefore, they retain the ability to make essential precursor metabolites (e.g., ribose-5-phosphate) (48). We conclude that *E. coli* has alternative mechanisms for generating reducing power (nicotinamide adenine dinucleotide phosphate; NADPH) other than the oxidative PP pathway, but that the ED pathway for sugar acid catabolism is required to colonize efficiently (Table 1).

The role of the TCA cycle in commensal *E. coli* colonization of the intestine and in *E. coli* pathogenesis is poorly studied. It has been reported that an *sdhB* mutant lacking succinate dehydrogenase colonized as well as its wild-type parent (39). However, *E. coli* has a second isoform of succinate dehydrogenase: fumarate reductase, which provides redundant enzyme function under some circumstances (49). Indeed, an *E. coli sdhAB frdA* double mutant has a significant colonization defect (Table 1). The role of the TCA cycle in colonization and pathogenesis by other Enterobacteriaceae is better understood, as described immediately below.

A fully functional TCA cycle is required for virulence of *Salmonella enterica* serovar Typhimurium via oral infection of BALB/c mice, i.e., a *sucCD* mutant, which prevents the conversion of succinyl coenzyme A to succinate, was attenuated. Also, an *sdhCDA* mutant, which blocks the conversion of succinate to fumarate, was attenuated, whereas both an *aspA* mutant and an *frdABC* mutant, deficient in the ability to run the reductive branch of the TCA cycle, were fully virulent (50). Moreover, although it appears that serovar Typhimurium replenishes TCA cycle intermediates from substrates present in mouse tissues, fatty acid degradation and the glyoxylate bypass are not required, since a *fadD*, *fadF*, and *aceA* mutants were all fully virulent during acute infection (50–52). Interestingly, it appears that the TCA cycle is required for virulence of *Edwardsiella ictaluri* in catfish fingerlings (53) and that the glyoxylate bypass is required for serovar Typhimurium persistent infection of mice (51).

The fact that *E. coli* depends on the TCA cycle for colonization implies that gluconeogenesis also is important. Using mutants that are unable to synthesize glucose from fatty acids, acetate, and TCA cycle intermediates because they are blocked in converting pyruvate to phosphoenolpyruvate (*ppsA pckA*), a critical step in gluconeogenesis, it was shown that neither the commensal *E. coli* K-12 strain MG1655 nor EHEC use gluconeogenesis for growth in the streptomycin-treated mouse intestine when each is the only *E. coli* strain fed to mice (54). However, *E. coli* Nissle 1917, the probiotic strain, does use gluconeogenesis to colonize (55). In addition, while *E. coli* EDL933 did not use gluconeogenic nutrients when it was the only *E. coli* strain in the mouse intestine, it used metabolic flexibility to switch to gluconeogenic nutrients when in competition in the intestine with either *E. coli* MG1655 (54) or *E. coli* Nissle 1917 (55). These findings are of extreme interest in view of a recent report showing that *E. coli* EDL933 activates expression of virulence factor genes only under gluconeogenic conditions (41).

CATABOLIC PATHWAY DIVERSITY IN *E. COLI*

The substrate range of *E. coli* is limited to monosaccharides, disaccharides, a small number of larger sugars, some polyols, and sugar acids (56). Amino acids and carboxylates also are consumed (56, 57). The corresponding catabolic pathways feed these substrates into central metabolism. While the genes encoding central metabolism in *E. coli* fall within the highly conserved core genome (38), there is predicted to be some variation between strains with respect to the catabolic pathways that feed various substrates into central metabolism, as indicated by genome-based metabolic modeling (58). For example, pathogenic *E. coli* strains are predicted to grow on sucrose while commensals are not. In contrast, commensals are predicted to grow on galactonate while pathogens are not. However, most of the substrates predicted by modeling to be used differentially by different *E. coli* strains are not known to be present in the intestine (58). *E. coli* EDL933, the prototypical EHEC strain, is able to grow on sucrose, whereas most commensal strains do not because they lack the *sac* genes, and some strains are missing genes within the N-acetylgalactosamine operon and are thus unable to grow on this substrate (36). Despite the modest differences between strains regarding their substrate range, in laboratory cultures containing a mixture of 13 different sugars known to be present in mucus polysaccharides, *E. coli* EDL933 and *E. coli* MG1655

each use the sugars in the same order (36). However, although *E. coli* strains have nearly identical catabolic potential, they vary significantly in the sugars that support their colonization (35, 36, 39, 43, 54, 59, 60).

NUTRIENT AVAILABILITY IN THE INTESTINE

Fluorescent *in situ* hybridization (FISH) microscopy of thin sections of the cecum of streptomycin-treated mice shows that colonized *E. coli* are surrounded by other members of the microbiota within the mucus layer (19, 54, 61). Indeed, *E. coli* grows well *in vitro* on cecal mucus, but fails to grow in intestinal contents (15, 19). When a transposon insertion-mutant library was screened for poor growth on mucus agar plates, a *waaQ* mutant of *E. coli* K-12 was isolated that also was defective in lipopolysaccharide biosynthesis, sensitive to detergents, clumped *in vitro* in broth culture, and failed to colonize streptomycin-treated mice (19). While the *waaQ* strain initially (during the first 24 h) grew from low to high numbers in the intestine, it rapidly declined in fecal plate counts and was undetectable by day 7 of the experiment. FISH showed that that the *waaQ* mutant formed clumps in the cecal mucus layer of streptomycin-treated mice at 24 h post-feeding, leading to the conclusion that failure to penetrate mucus and grow as dispersed cells within the mucus layer prevented it from colonizing (19).

The sources of nutrients that support intestinal colonization by *E. coli* are shed epithelial cells, dietary fiber, and mucosal polysaccharides (12–14). Most of the amino acids are available in the cecum, as growth of *E. coli* in mucus results in repression of the majority of genes involved in amino acid biosynthesis (39). In rat, mouse, and human, colonic mucus is organized by Muc2, the major glycoprotein, which is a high molecular weight gel-forming glycoprotein containing L-fucose, D-galactose, D-mannose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, and N-acetylneuraminic acid (62). Mucin is 80% polysaccharide and 20% protein and is highly viscous (63). In addition to mucin, the mucus layer contains a number of smaller glycoproteins, proteins, glycolipids, and lipids (62–65). There are two mucus layers, a loosely adherent suction-removable layer closest to the lumen of the intestine and an adherent layer firmly attached to the mucosa (62, 66, 67). In the rat colon, the thickness of the adherent layer is about 100 μm and that of the loose layer about 700 μm (62). In the mouse colon, the thickness of the adherent layer is about 50 μm and that of the loose layer about 100 μm (67). The mucus layer itself is in a dynamic state, constantly being synthesized and secreted by the mucin-secreting, specialized goblet cells and degraded to a large extent by the indigenous intestinal microbes (68, 69). Degraded mucus components are shed into the intestinal lumen forming a part of the luminal contents that is excreted in the feces (68).

The loosely adherent mucus layer contains large numbers of bacteria in the mouse, but the inner adherent mucus layer is largely devoid of bacteria (70) and is not penetrated by beads the size of bacteria (71), suggesting that the inner mucus layer protects the colonic epithelium from the commensal microbiota. Commensal *E. coli* strains do not attach to intestinal epithelial cells and growth takes place predominantly in the mucus layer (19, 24). The mucus layer of the conventional mouse large intestine turns over about every 2 hours (23). Hence, to maintain a stable population, the bacterial growth rate in mucus must keep pace with the turnover rate of the mucus layer. For example, *E. coli* BJ4 has a generation time of 40–80 minutes in the streptomycin-treated mouse cecum, which is more than fast enough to maintain its population (23).

E. coli cannot degrade oligosaccharides or polysaccharides, except dextrin (36, 72). In the intestine, this is the job of anaerobes. To obtain the mono- and di-saccharides it needs for growth, *E. coli* relies on hydrolysis of complex polysaccharides by members of the intestinal community such as *Bacteroides thetaiotaomicron*, a Gram-negative obligate anaerobe and a major member of the human intestinal microbiota (73–78). This symbiotic relationship is illustrated in Fig 1. Complex polysaccharides derived from epithelial cell debris, dietary fiber, or mucus are degraded by extracellular polysaccharide hydrolases secreted by anaerobes. The anaerobes preferentially take up the resultant oligosaccharides, which are further degraded intracellularly to monosaccharides that enter central metabolism. The mono- and di-saccharides that are released by polysaccharide hydrolysis are discarded by the anaerobes and thereby made available to *E. coli* and organisms with similar metabolism.

Evidence for the model shown in Fig 1 is mounting. In response to competition from *Eubacterium rectale*, *B. thetaiotaomicron* upregulates a number of polysaccharide-utilization loci that encode a variety of glycosyl hydrolases (79). In contrast, *E. rectale* responds to *B. thetaiotaomicron* by down-regulating expression of loci encoding glycan-degrading enzymes and up-regulating expression of a number of sugar transporters, suggesting that *E. rectale* uses metabolic flexibility to take advantage of the superior ability of *B. thetaiotaomicron* to degrade polysaccharides (79). In a *tour de*

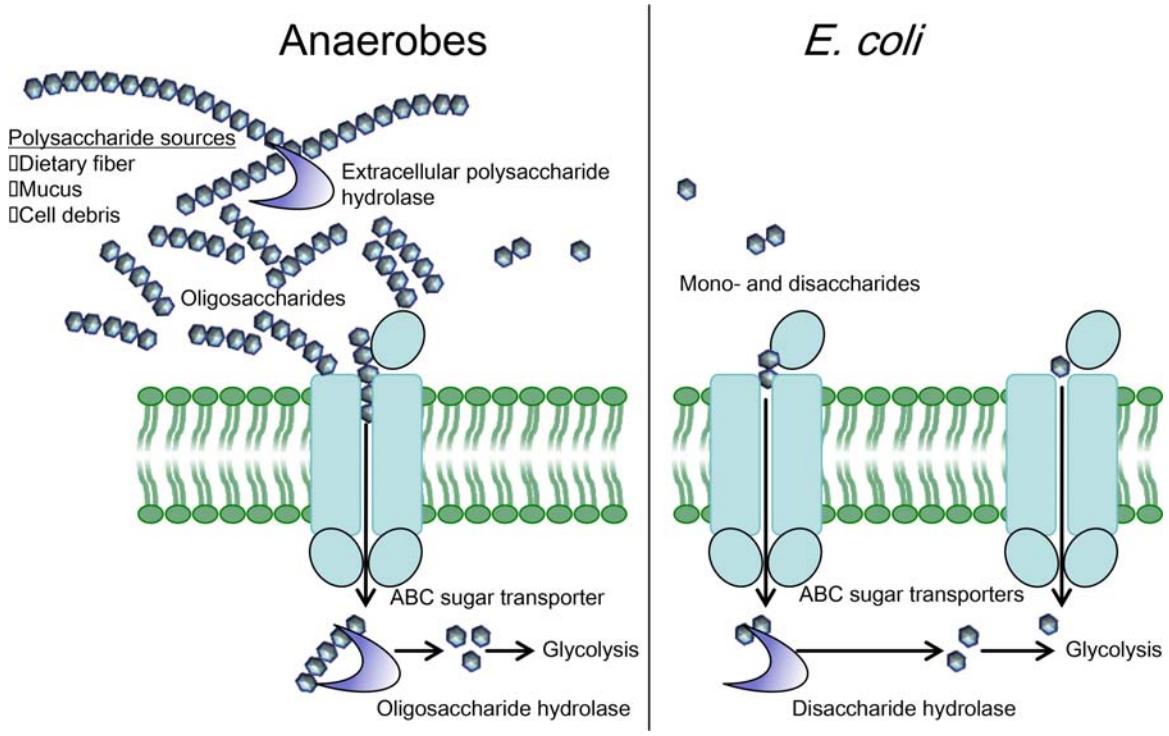

FIGURE 1 Nutrient flow in the intestine. The primary sources of carbohydrates in the large intestine are mucus, dietary fiber, and epithelial cell debris. Mucus and dietary fiber consist of complex polysaccharides. *E. coli* typically cannot degrade complex polysaccharides; that is the job of anaerobes. Hence, degradation of polysaccharides by anaerobes releases oligosaccharides, which are preferred by anaerobes, as well as mono- and disaccharides, which are preferred by *E. coli*. doi:10.1128/microbiolspec.MBP-0006-2014.f1

force series of experiments from the Sonnenberg laboratory, it was recently shown in gnotobiotic mice associated with *B. thetaiotaomicron* and *S.* Typhimurium that fucose and sialic acid catabolic-gene systems are up-regulated in the latter organism and its growth is stimulated (77). Importantly, *Salmonella* mutants lacking the capacity to catabolize either fucose or sialic acid showed substantial fitness defects in streptomycin-treated mice (77). A *B. thetaiotaomicron* mutant lacking a predicted surface-associated sialidase failed to release free sialic acid in mice, whereas the wild-type *B. thetaiotaomicron* released free sialic acid and stimulated intestinal growth of *Clostridium difficile* (77). Free sialic acid was very low in conventional mice and greatly elevated in mice that were treated with streptomycin, which is consistent with the idea that the microbiota consumed sialic acid in the conventional animals, but streptomycin treatment removed those members of the microbiota (77). These experiments prove that polysaccharide hydrolysis by anaerobes in the intestine can provide monosaccharides to members of the microbiota that can use them.

NUTRIENT LIMITATION IN THE INTESTINE

Competition for limiting resources drives ecosystems (80). In microbial ecosystems, such as the intestine, the microbial community competes for carbon and energy sources and terminal electron acceptors (27). It appears that *E. coli* uses strategies for maximizing its population in a stiffly competitive environment. *E. coli* can simultaneously utilize a mixture of six sugars under nutrient-limiting conditions in chemostats (81). When presented with a mixture of 13 sugars *in vitro*, *E. coli* uses up to nine of them at a time (36). Bacteria expand their transcriptome to induce a number of gene systems for carbon-source transport and catabolism when growing slowly (82) or when they are nutrient-deprived (83). While there is a fine line between "hunger" and "starvation", in fact, the two are distinct. Hunger is a state that is defined by physiological and genetic changes that expand the metabolic capacity of the cell (84). To better understand the role of the hunger lifestyle *in vivo*, the importance of carbon stores for colonization fitness was examined (60). It is well known that stored glycogen promotes survival during times of carbon limitation.

It was found that mutants of commensal and pathogenic *E. coli* that cannot synthesize or degrade glycogen have significant intestinal colonization defects in mice (60). Furthermore, in support of the hypothesis that *E. coli* uses glycogen to withstand hunger, when a constant supply of a readily metabolized carbon source was supplied in the drinking water of the mice, these colonization defects were rescued (60).

E. coli Nissle 1917 is a non-pathogenic strain that has been used since the early 1920's as a probiotic agent to treat gastrointestinal infections in humans, sold under the name "Mutaflor" (85). Despite having the *E. coli* Nissle 1917 genome sequence to search for clues as to its success as a probiotic agent (86, 87), little attention has been paid to the possibility that it may out-compete pathogens for essential nutrients. When *E. coli* Nissle 1917 is the only *E. coli* strain in the streptomycin-treated mouse intestine, it appears to use arabinose, fucose, galactose, gluconate, mannose, N-acetylgalactosamine, and sialic acid to colonize (Table 2 and [59]). In contrast, *E. coli* Nissle 1917 does not appear to use ribose to colonize (Table 2). Identifying the nutrients used by *E. coli* Nissle 1917 when competing with *E. coli* pathotypes might lead to new approaches to prevent *E. coli* infections.

Metabolic flexibility is also exhibited by uropathogenic *E. coli* (UPEC) strain CFT073, a human urinary-tract pathogen, when its nutritional program in the mouse intestine is compared to that in the mouse urinary tract. *E. coli* CFT073 utilizes several sugars simultaneously to colonize the intestine (Table 2 and [35]), but not to infect the urinary tract. Instead, in the urinary tract, transport of peptides and gluconeogenesis are required for maximum *E. coli* CFT073 growth (88). When *E. coli* EDL933 is the only *E. coli* strain in the mouse intestine, it does not use gluconeogenic substrates to colonize, but it switches to gluconeogenic nutrients when competing in the intestine with *E. coli* MG1655 (55). As an additional example of metabolic flexibility *in vivo*, neither *E. coli* MG1655 nor *E. coli* Nissle 1917 use ribose for growth in the intestine, unless fuculose-1-phosphate accumulates in mutants unable to catabolize it further (59). Moreover, it was shown that fucose at a concentration too low to support growth, stimulated the utilization of ribose by the wild-type *E. coli* strains *in vitro*, suggesting that fuculose-1-phosphate plays a role in regulating the use of ribose as a carbon source by *E. coli* MG1655 and *E. coli* Nissle 1917 in the mouse intestine (59). In summary, to colonize successfully, *E. coli* must compete for limiting nutrients, so it uses several sugars at a time, is flexible in its nutrient preference, and relies on glycogen-carbon stores in the intestine.

COMPETITION FOR NUTRIENTS IN THE INTESTINE

E. coli competes for nutrients in the intestine in (at least) three ways. First, it can use nutrients that are available because no other community member has used it (Table 2). Second, it can outcompete other strains for the nutrients it prefers by growing faster on them (89). Third, it can enter into a symbiotic association with the anaerobe(s) that releases its preferred sugar(s) (61, 90). Evidence for each of these mechanisms comes from

TABLE 2 Sugar utilization in the intestine by *E. coli* strains

Sugar defect	Mutation	MG1655	Nissle	HS	EDL933	UPEC	EPEC
Arabinose	*araBAD*	Yes	Yes	Yes	Yes	Yes	Yes
Fucose	*fucK*	No	Yes	No	No	No	No
Galactose	*galK*	No	Yes	Yes	Yes	Yes	Yes
Gluconate	*gntK*/Δ*idnK*	Yes	Yes	Yes	No	ND	ND
Hexuronates	*uxaC*	No	No	No	Yes	ND	ND
Lactose	*lacZ*	No	No	Yes	No	ND	ND
Mannose	*manA*	No	Yes	No	Yes	Yes	Yes
N-acetylglucosamine	*nagE*	Yes	No	Yes	Yes	Yes	Yes
N-acetylgalactosamine	*agaWEFA*	NA	Yes	ND	No	ND	ND
N-acetylneuraminate	*nanAT*	Yes	Yes	Yes	No	ND	ND
Ribose	*rbsK*	No	No	Yes	Yes	Yes	Yes
Sucrose	*sacH*	NA	NA	NA	Yes	NA	ND

Results show the difference in population sizes of wild-type verses mutant strains at Day 9.
Yes indicates the difference exceeds a 0.8 $\log_{10}$ colonization advantage and students *t* test value $P < 0.05$
NA indicates that the pathway is not intact in this genetic background
ND indicates not determined

competitive-fitness studies in streptomycin-treated mice. Streptomycin treatment (5 g/l in drinking water) perturbs the microbiota by selectively removing the facultative anaerobes with which *E. coli* competes, making nutrients available and allowing experimentally introduced strains to overcome colonization resistance (27, 91, 92). Colonization resistance in animal models can also be overcome in other ways, e.g., by gnotobiotic mice in which there is no native microbiota (93), by mice that have a contrived microbiota (94), or by mice with intestinal inflammation, which generates nitrate that is respired by *E. coli* (95).

The three ways that *E. coli* can compete with the microbiota for nutrients are described in detail here. First, different *E. coli* strains use different nutrients *in vivo*, despite using the same nutrients in the same order *in vitro* (Table 2). Mutants with deletions in genes corresponding to metabolic pathways induced in mucus were constructed by allelic replacement (29) and were tested for their ability to compete with their wild-type parent strain when simultaneously fed to mice in low numbers (10^5 CFU/mouse). The data obtained from these studies showed that the human commensals *E. coli* HS, *E. coli* Nissle 1917, *E. coli* MG1655, and the pathogen *E. coli* EDL933 each occupy unique nutritional niches in the mouse intestine (Table 2 and [35, 36]). Of the 12 sugars available in the mucus layer, *E. coli* HS utilizes six for colonization: arabinose, galactose, gluconate, N-acetylglucosamine, lactose, and ribose (Table 2). *E. coli* Nissle 1917 uses a different list of seven carbon sources to support colonization, including arabinose, fucose, galactose, gluconate, N-acetylglucosamine, and N-acetylneuraminate, and mannose (Table 2) and *E. coli* MG1655 utilizes five sugars for colonization: arabinose, fucose, gluconate, N-acetylglucosamine, and N-acetylneuraminate (Table 2). Each of these commensals is capable of colonizing mice that are pre-colonized with any one of the others (31), and each strain is capable of utilizing at least one sugar not used by the others *in vivo*, which suggests that differences in their *in vivo* sugar preferences allows them to occupy distinct nutrient-defined niches in the intestine. Furthermore, the intestinal niche occupied by pathogenic *E. coli* EDL933 is also unique and is defined by utilization of seven sugars: arabinose, galactose, hexuronates, mannose, N-acetylglucosamine, ribose, and sucrose (Table 2).

That *E. coli* MG1655 and *E. coli* EDL933 display different nutritional programs in the mouse intestine, e.g., *E. coli* MG1655 uses N-acetylneuraminate but not mannose whereas *E. coli* EDL933 uses mannose but not N-acetylneuraminate (Table 2), was surprising in view of the fact that *E. coli* MG1655 and *E. coli* EDL933 utilize them equally well *in vitro* and display identical nutritional preferences *in vitro*, i.e., they use sugars in the same order *in vitro* as follows: N-acetylglucosamine, gluconate, ribose, sialic acid, mannose, arabinose, maltose, and fucose (36). These findings will be discussed in the context of the "Restaurant" hypothesis below.

Since different commensal *E. coli* strains use different sugars to colonize the intestine, it seemed reasonable that a potential strategy for preventing colonization by the enterohemorrhagic *E. coli* EDL933 would be to pre-colonize mice with a combination of commensal strains that would fill the sugar-defined nutritional niches normally available to the invading pathogen. When mice were pre-colonized for 10 days with either the commensal *E. coli* MG1655, *E. coli* HS, or *E. coli* Nissle 1917 and then fed 10^5 CFU of the pathogenic *E. coli* EDL933, *E. coli* Nissle 1917 limited growth of *E. coli* EDL933 in the intestine (10^3 to 10^4 CFU/gram of feces), whereas *E. coli* MG1655 and *E. coli* HS allowed growth to higher numbers (10^6 to 10^7 CFU/gram of feces). However, when *E. coli* EDL933 was fed to mice previously pre-colonized with the three commensal *E. coli* strains (MG1655, HS, and Nissle 1917), each of which displays a different nutritional program *in vivo* (Table 2), *E. coli* EDL933 was eliminated from the intestine (31). Therefore, a combination of as few as three commensal *E. coli* strains provided a barrier to *E. coli* EDL933 infection.

If the basis for exclusion of *E. coli* EDL933 was because the three commensal *E. coli* strains utilize the nutrients needed by *E. coli* EDL933 to compete and colonize, then any *E. coli* commensal strain or combination of strains that effectively catabolizes the sugars used by *E. coli* EDL933 would prevent its colonization. Indeed, when the ability of *E. coli* EDL933 to colonize mice that were pre-colonized with *E. coli* HS and *E. coli* Nissle 1917 was tested, which the data in Table 2 indicate should be equally effective without *E. coli* MG1655 present, *E. coli* EDL933, was indeed eliminated 5 days following association (35). However, the same two commensal *E. coli* strains could not prevent colonization of *E. coli* CFT073, an uropathogenic strain, and *E. coli* E2348/69, an enteropathogenic strain (96). Therefore, it is unlikely that any particular commensal strain(s) of *E. coli* will be generally effective as a probiotic to prevent colonization by enteric pathogens. Nevertheless, the data support the hypothesis that nutrient consumption by commensal *E. coli* can limit nutrient availability to pathogens, which in turn points to the potential of probiotics for preventing disease.

A second way of competing for nutrients in the intestine is illustrated by what happens when *E. coli* MG1655 adapts to the mouse intestine. When mice were fed *E. coli* MG1655, non-motile *flhDC* deletion mutants appeared in the feces 3 days post-feeding and reached approximately 90% of the total population by day 15 of the experiment (97). These mutants had a striking colonization advantage over the wild-type *E. coli* MG1655 parent strain. The deletions were of varying length and began immediately downstream of the IS*1* element in the *flhDC* promoter region. One such mutant, designated MG1655*, was a better colonizer than its parent, grew in cecal mucus faster than its parent *in vitro* (90 ± 2 min generation time vs 105 ± 2 min, $P < 0.001$), and grew 15–30% faster than its parent on a number of sugars present in the mouse intestine (98). The *E. coli flhDC* operon encodes the $FlhD_4C_2$ regulatory complex, which is the master positive regulator of the more than 40 gene flagella regulon (99). The $FlhD_4C_2$ complex has also been reported to negatively regulate *E. coli* K-12 genes involved in galactose transport, the ED pathway, and the TCA cycle and positively regulate genes involved in ribose transport (89, 100). *E. coli* MG1655 *flhDC* deletion mutants have also been reported to be selected in the intestines of ex-germfree mono-associated mice (101).

Several high-throughput genomic approaches were taken to further characterize *E. coli* MG1655*. Whole-genome pyrosequencing did not reveal any changes on its genome, aside from the deletion at the *flhDC* locus, that could explain the colonization advantage of *E. coli* MG1655* (89). Microarray analysis revealed modest yet significant induction of catabolic gene systems across the genome in both *E. coli* MG1655* and an isogenic *flhD* mutant constructed in the laboratory (89). Catabolome analysis with Biolog GN2 microplates revealed an enhanced ability of both *E. coli* MG1655* and the isogenic *flhD* mutant to oxidize a variety of carbon sources (89). Collectively, the results showed that intestine-adapted *E. coli* MG1655* is more fit than the wild-type for intestinal colonization, because loss of FlhD results in elevated expression of genes involved in carbon and energy metabolism, allowing the mutants to outcompete their wild-type parent for the same nutrients. Hence, a second strategy for gaining a colonization advantage is to outcompete other members of the microbiota for their preferred nutrients.

There is a third way that *E. coli* competes for nutrients in the intestine. The intestine selects for mutants that gain a colonization advantage by promoting occupation of a distinct niche. The selection of non-motile *E. coli* MG1655 *flhDC* mutants by the streptomycin-treated mouse intestine is easily explained by the nutrient-niche hypothesis, i.e., the mutants grow 15% faster *in vitro* in mouse cecal mucus and 15%–30% faster on several sugars present in cecal mucus than *E. coli* MG1655 (97). In addition to *flhDC* mutants, *E. coli* MG1655 mutants with reduced motility also were selected by adaptation in the streptomycin-treated mouse intestine and these turned out to be *E. coli* MG1655 *envZ* missense mutants (61). *E. coli* MG1655 *envZ* missense mutants have also been reported to be selected in the intestines of ex-germfree mono-associated mice (102). The *envZ* gene encodes a histidine kinase that is a member of the *envZ/ompR* two-component signal-transduction system that modulates gene expression in response to osmolarity. The genes regulated include *flhDC*, the porin genes *ompC* and *ompF*, and several other genes encoding outer-membrane proteins (103, 104). These *E. coli* MG1655 *envZ* missense mutants produced more phosphorylated OmpR than both *E. coli* MG1655 and the *E. coli* MG1655 *flhDC* deletion mutants and produced more of the outer-membrane porin OmpC and less of the outer-membrane porin OmpF (90). As a result, the *E. coli* MG1655 *envZ* missense mutants were more resistant to bile salts and colicin V than *E. coli* MG1655 and the *E. coli* MG1655 *flhDC* deletion mutants (61). One of the *E. coli* MG1655 *envZ* missense mutants, which was studied further, contained the $envZ_{P41L}$ missense mutation and grew about 15% slower *in vitro* in mouse cecal mucus and on several sugars present in mucus compared to the *flhDC* deletion mutants, yet was as good an intestinal colonizer as the *flhDC* deletion mutants and far better than *E. coli* MG1655 (61). Moreover, *E. coli* MG1655 $envZ_{P41L}$ and the *E. coli* MG1655 *flhDC* deletion mutants appeared to colonize equally well in one major intestinal niche, but *E. coli* MG1655 $envZ_{P41L}$ appeared to use galactose to colonize a second intestinal niche either not colonized or colonized poorly by the *E. coli* MG1655 *flhDC* deletion mutants. These data are not consistent with the nutrient-niche hypothesis, but they are consistent with what we call the "Restaurant" hypothesis, which will be discussed below.

Since *E. coli* MG1655 $envZ_{P41L}$ was a far better intestinal colonizer than wild-type *E. coli* MG1655, the $envZ_{P41L}$ gene was transferred for further study into *E. coli* Nissle 1917, the human probiotic strain used to treat gastrointestinal infections. Like *E. coli* MG1655 $envZ_{P41L}$, *E. coli* Nissle 1917 $envZ_{P41L}$ produced more phosphorylated OmpR than its parent and produced more of the outer membrane porin OmpC and less of the outer membrane porin OmpF (90). It also became more resistant to bile salts and colicin V, grew 50% slower

in vitro in mucus and 15%-30% slower on several sugars present in mucus, yet was a 10-fold better colonizer than *E. coli* Nissle 1917 (90). Furthermore, like *E. coli* MG1655 *envZ*$_{P41L}$, *E. coli* Nissle 1917 envZ$_{P41L}$ appeared to use galactose to colonize a second intestinal niche either not colonized or colonized poorly by wild-type *E. coli* Nissle 1917, despite not growing as well as *E. coli* Nissle 1917 on galactose as a sole carbon source (90). Moreover, despite being a better colonizer, *E. coli* Nissle 1917 *envZ*$_{P41L}$ was not better than its parent at preventing colonization by enterohemorrhagic *E. coli* EDL933 and, in fact, appeared to be worse (90). The data can be explained according to our "Restaurant" hypothesis for commensal *E. coli* strains, i.e., that they colonize the intestine as sessile members of mixed biofilms obtaining the sugars they need for growth locally, but compete for sugars with invading *E. coli* pathogens planktonically as described below.

BIOFILMS IN THE INTESTINE

Much attention has been given to the role played by biofilms in bacterial colonization of many environments, but until recently surprisingly little information was available regarding biofilms in the intestine. On the one hand, the transit time of intestinal contents is short compared to the timescale of biofilm development (105), so it was hard to imagine how a stable biofilm might be maintained in the intestine, yet the kinetics of plasmid transfer between *E. coli* strains in the streptomycin-treated mouse intestine suggested that *E. coli* resides in biofilms *in vivo* (106). Moreover, the mucus layer itself has many of the characteristics of a secreted biofilm matrix. Add to this the concept of bacterial binding to mucus components and it became reasonable to consider the possibility of bacterial biofilms in the gastrointestinal tract. Indeed, it's been shown that biofilms form in the mucus layers of the large intestines of healthy humans, rats, baboons, and mice (107–110) and that mixed biofilms consisting of *Bacteroides*, *Enterobacter*, and *Clostridia* species form rapidly on strands of mucin in mucus introduced into a growing human microbiota contained in a continuous-flow culture system constructed to mimic the human intestine (111). Furthermore, it appears that human colonic-mucosal biofilms and bacterial communities in feces differ greatly in composition (112) and dysbiosis in the community structure of mucosal biofilms may play an important role in contributing to chronic inflammatory-bowel diseases such as ulcerative colitis and Crohn's disease (107, 108). In *Vibrio cholerae*, biofilm formation is important for pathogenesis (113). Intestinal biofilms would provide the habitat for microbe-microbe interactions such as those that are thought to occur between *E. coli* and the polysaccharide-degrading anaerobes.

THE "RESTAURANT" HYPOTHESIS

As discussed above, commensal strains of *E. coli* appear to reside in mixed biofilms in the large intestines of mice (61, 90). Moreover, commensal and pathogenic strains of *E. coli* use mono- and disaccharides for growth in the intestine (36). However, these sugars are absorbed in the small intestine, whereas dietary fiber reaches the large intestine intact. In contrast to the anaerobes, most *E. coli* strains do not secrete extracellular polysaccharide hydrolases (72, 114) and therefore cannot degrade dietary fiber-derived and mucin-derived oligo- and polysaccharides. Since commensal and pathogenic *E. coli* strains colonize the mouse large intestine by growing in intestinal mucus (43, 115–119), it appears likely that *E. coli* depends on the anaerobes present in mucus that can degrade oligo- and polysaccharides to provide them with the mono-and disaccharides and maltodextrins they need for growth. Indeed, *Salmonella enterica* serovar Typhimurium, which is in the same family as *E. coli* and has very similar metabolism, catabolizes fucose and sialic acid liberated from mucosal polysaccharides by *Bacteroides thetaiotaomicron* (77). It is therefore possible that anaerobes in the mixed biofilms provide *E. coli* with the sugars it needs for growth locally, rather than from a perfectly mixed pool available to all species, which is an assumption of the nutrient-niche hypothesis. We call the mixed biofilms that feed the *E. coli* strains "Restaurants" and we hypothesize that different commensal *E. coli* strains reside in different "Restaurants" interacting physically and metabolically with different anaerobes. Each restaurant might serve different nutrients, i.e., each commensal *E. coli* strain could be exposed to a different menu, which explains why different *E. coli* strains display different nutritional programs in the mouse intestine despite displaying identical nutritional programs *in vitro* (35, 36). The restaurant hypothesis can also explain how *E. coli* strains that grow more slowly in mucus and on several sugars found in mucus are better colonizers than their parents as long as they have a higher affinity for biofilm-binding sites than their parents. Indeed, the outer membranes of both *E. coli* MG1655 *envZ*$_{P41L}$ and *E. coli* Nissle 1917 envZ$_{P41L}$ are very different from those of their parents (61, 90), which could result in increased affinities for mixed biofilms.

The "Restaurant" hypothesis can also explain why, despite being a better colonizer than *E. coli* Nissle 1917, *E. coli* Nissle 1917 *envZ*$_{P41L}$ is not better at limiting enterohemorrhagic *E. coli* EDL933 colonization than *E. coli* Nissle 1917 and, in fact, may be worse (90). It is possible when *E. coli* EDL933 invades the mouse intestine it initially grows planktonically in mucus and not in mixed biofilms. If we are correct that *E. coli* Nissle1917 and *E. coli* Nissle1917 *envZ*$_{P41L}$ colonize the mouse intestine by being served specific sugars by the anaerobes in the mixed biofilms they inhabit, then small amounts of these sugars that escape the mixed biofilms might be available to invading *E. coli* EDL933 as well as to the small numbers of planktonic *E. coli* Nissle1917 or *E. coli* Nissle1917 *envZ*$_{P41L}$ that leave the mixed biofilms. Therefore, it may be that both planktonic *E. coli* Nissle 1917 *envZ*$_{P41L}$ and *E. coli* Nissle 1917 compete directly with planktonic *E. coli* EDL933 for the sugars that escape the biofilms or that are produced by small numbers of planktonic members of the microbiota that leave the biofilms. This scenario would allow planktonic *E. coli* EDL933 to grow to the extent allowed by the available concentrations of those sugars in competition with planktonic *E. coli* Nissle 1917 *envZ*$_{P41L}$ or *E. coli* Nissle 1917, which could explain why *E. coli* Nissle 1917, the faster grower in perfectly mixed bacteria-free mucus *in vitro*, appears to limit *E. coli* EDL933 growth in the intestine to a greater extent than does *E. coli* Nissle 1917 *envZ*$_{P41L}$ (90).

We stress that the granularity of mixed intestinal biofilms and nutrient flow between the microbes that reside within them is not known. These interactions could be so finite as to allow two different *E. coli* strains to interact with the same anaerobe cell and each grow on a different preferred nutrient. If so, it will not be possible to find zones within the biofilm (i.e., restaurants) that contain only a single population of *E. coli* and interacting anaerobe partner.

CONCLUSIONS

It is becoming increasingly clear that once *E. coli* strains reach the large intestine, in order to colonize, they must enter the mucus layer and utilize nutrients there for growth. It is also clear that different strains of *E. coli* display different nutritional programs in the intestine. However, it is not known whether a specific *E. coli* commensal strain utilizes the same nutrients when it is the only *E. coli* strain in the intestine compared to a situation in which it colonizes along with several different commensal *E. coli* strains with which it must compete for nutrients. Metabolic flexibility could be a key requirement for successful colonization of the intestine by several *E. coli* strains simultaneously. However, the "Restaurant" hypothesis explains long-term colonization by several established commensal *E. coli* strains without invoking metabolic flexibility, i.e., each commensal *E. coli* strain resides as a sessile member of a mixed biofilm in the intestine and obtains nutrients locally rather than from a perfectly mixed pot of nutrients. However, as described above, it seems likely that when mice colonized long-term with one *E. coli* strain are fed a pathogenic *E. coli* strain, planktonic members of the pre-colonized strain that escape the mixed biofilm compete directly with the invading pathogen for nutrients from the same perfectly mixed pot, according to the Freter nutrient-niche hypothesis. Since it appears likely that a pathogen must be able to grow in the intestine in order to initiate the pathogenic process, we hope that future research will provide a nutritional framework for colonizing humans with a combination of commensal *E. coli* strains or with one commensal *E. coli* strain that has been engineered to be as effective as several strains and can serve as an effective first line of defense against pathogenic *E. coli* intestinal infections.

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