Dietary Factors in Prevention of Pediatric Escherichia coli Infection: A Model Using Domestic Piglets

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Abstract

Enterotoxigenic Escherichia coli (ETEC) is the major etiological agent causing acute watery diarrhea that is most frequently seen in young children in lower-income countries. The duration of diarrheal symptom may be shortened by antibiotic treatment, but ETEC is relative refractory to common antibiotics. Burgeoning evidence suggests bioactive components that naturally occur in human milk (e.g., lysozyme and oligosaccharides) and plants (e.g., nondigestible carbohydrates and phytochemicals) contain antimicrobial functions are promising preventive measures to control ETEC infection. Although the exact protective mechanisms may vary for each compound and are still not completely understood, they generally act to (1) competitively inhibit the binding of pathogenic bacteria and toxins to gut epithelium; (2) directly kill pathogens; and (3) stimulate and/or enhance host mucosal and systemic immune defense against pathogenic microorganisms. An appropriate ETEC-challenge animal model is critical to evaluate the effect and unveil the mechanism of bioactive compounds in prevention of enteric infection. Despite wide application in biomedical research, rodents do not usually manifest typical clinical signs of enteric infections. The remarkable differences in digestive physiology, immune response, and gut microbiota between rodents and human beings necessitate the use of alternative animal models. Pigs are closely related to humans in terms of genomes, physiology, anatomy of gastrointestinal tracts, digestive enzymes, components of immune system, and gut microbiota. Like human infants and young children, nursing and nursery piglets are more susceptible to ETEC infection and reproduce the clinical signs as observed in humans. Hence, the ETEC-challenge piglet represents a valuable translational model to study pathogenesis and evaluate dietary factors (e.g., milk bioactive compounds, nondigestible carbohydrates, and phytochemicals) as preventive measures for ETEC infection in pediatrics.

Key words: bioactive compounds; diarrhea; enterotoxigenic Escherichia coli; pig

Introduction

Infectious diarrhea disease has long been one of the leading causes of morbidity and mortality in children living in developing countries. Based on WHO UNICEF 2017 data, children in low-income countries experience on average 3 episodes of diarrhea each year. Even though the mortality has halved since 2000, diarrhea-caused under-5 deaths still account for 477,293 deaths in 2016. One of the most common infectious agents causing diarrhea is Escherichia coli (E. coli), which includes 6 different categories: enteropathogenic E. coli, enterotoxigenic E. coli (ETEC), enteroinvasive E. coli, enterohemorrhagic E. coli, diffusely adherent E. coli, and enteroaggregative E. coli. In a systemic review of pathogens involved in diarrhea, enteropathogenic E. coli, ETEC, and enteroaggregative E. coli are responsible for 30 to 40% of all prolonged infections in children who live in low- and middle-income countries. In another study conducted in Bangladesh, ETEC accounted for 19.5% cases of diarrhea in children under 2 years old, representing the most common pathogen followed by rotavirus. Young children who live in areas with limited public healthcare and poor sanitation are the population most susceptible to ETEC infection. ETEC strains colonize the small intestines and produce enterotoxins...
that produce diarrhea in both humans and animals.\textsuperscript{8} ETEC is a noninvasive bacterium, and the pathogenesis mainly relies on 2 major virulent factors: colonization factors that are fimbrial structures on the bacterial surface, and the secretion of enterotoxins. Fimbriae help bacteria adhere to the small intestinal epithelial cells. Then the colonized ETEC produce one or more enterotoxins, such as heat-labile (LT), heat-stable (ST), or shiga toxins. These internalized enterotoxins in epithelial cells acti-

Advantages and Limitations of using Domestic Piglet as a Model for Human Enteric Infectious Disease

Domestic pig (Sus scrofa) is a promising animal model that provides a number of translational advantages in the study of human nutrition and gastrointestinal pathophysiology.\textsuperscript{24,34,35} They are readily available and very comparable to humans in genomics,\textsuperscript{34} digestive physiology,\textsuperscript{36,37} and immune system.\textsuperscript{38} Use of pigs for biomedical research is also ethically more acceptable compared with nonhuman primates.\textsuperscript{28,39} Pigs, like humans, are omnivorous and have a glandular stomach lined with cardiac, gastric, and pyloric mucosa.\textsuperscript{40} In comparison, the majority portion of stomach in rats is nonglandular.\textsuperscript{31} The intestinal epithelial structure (the crypt-villus axis) and functions are very comparable between pigs and human beings, including absorption of nutrients and identification of self and non-self antigens.\textsuperscript{45} As the major fermentation site, the colon of both pigs and humans is sacculated in comparison with a nonsacculated structure in rodents.\textsuperscript{37} Our understanding about the modulatory role of gut microbiota on host metabolism has been significantly improved in the past several years. The intestinal microflora are broadly similar between pigs and human beings, whereas the majority (~85%) of gut microbiota of the mouse was not present in humans.\textsuperscript{32} Furthermore, because pigs are omnivorous and precocious, they could be rapidly adapted to artificial feeding shortly after birth. It is feasible to manipulate the composition and feeding regimen of their liquid and solid diet at neonatal age for nutritional studies. Collectively, the domestic pig is an excellent model for translational research of pediatric nutrition.\textsuperscript{41,42}

Pigs are also equipped with the whole innate and adaptive immune system, of which most effector clusters could match with their human counterparts. Additionally, the relevant features of the intestinal mucosal immunity between pigs and humans are very similar, including the distribution of Peyer’s patches in the distal ileum and the pattern of intra-epithelial lymphocytes in the mucosal surfaces.\textsuperscript{28} It has been implicated that the pig immune response resembles that of humans for 80% of analyzed parameters, whereas mice are similar in less than 10%.\textsuperscript{45,46} There was remarkable improvement in genomic database and functional annotation of porcine immune-related homologenes compared with those identified in humans and

Exclusive breastfeeding during infancy has been identified as the most effective intervention against infectious diarrheal disease in early childhood.\textsuperscript{19-21} The protective effect of mater-
nal milk is presumably attributed to an array of bioactive com-
ponents such as secretory antibodies, bactericidal enzyme (e.g., lysozyme), oligosaccharides, and lactoferrin. Meanwhile, a growing number of phytochemicals derived from food plants were reported for their bactericidal and immune-stimulating functions and have been increasingly used as natural alternatives to antibiotics in animal feeds to prevent enteric infections.\textsuperscript{22} Their application in pediatric nutrition is largely hampered by the concern or lack of understanding on the potential adverse effects. Nevertheless, due to the risk and ethi-
cal concern, it is extremely difficult or impossible to test the effectiveness of milk- or plant-derived bioactive compounds as prevention of infectious diarrhea in pathogen-challenge clinical trials with human subjects, especially young children.

A translatable pathogen-challenge animal model may serve as an invaluable approach to study the impact of pediatric nutrition on host resilience to enteric infections.\textsuperscript{23,24} The animal model should not only resemble humans in terms of anatomy, gastrointestinal development, and digestive physiology, but also display similar symptoms and pathogenicity after infections and respond with comparable immune defense as young children. Rodent models are an important tool in biomedical research. Particularly, genetically modified mice are more readily available than any other model animals and are therefore extremely valuable and still going to be the predominant ani-

minal models in basic research.\textsuperscript{25,26} Nevertheless, the remarkable differences between mice and human beings should also be

mentioned, including their genomes, organ size, phases of development, lifestyle, behavior, and so forth.\textsuperscript{27,28} The range of differences in the immune system between mice and humans is also very extensive and has been completely reviewed by other research groups.\textsuperscript{29,30} A few researchers have applied small ruminants as comparative models for studies with mucosa immune function, but the model is highly restricted to research in the upper gastrointestinal tract.\textsuperscript{31} Nonhuman pri-
mates are currently considered to be the most representative animal model for human research because of their irresistible similarities to human; however, the ethical consideration, expensive raising and maintenance costs, and the high level of biosecurity requirement restricts the use of primates as research objects.\textsuperscript{32,33} The comparisons of different animal mod-
els have been fully summarized by Jiminez et al.\textsuperscript{28} The focus of this review is to highlight the suitability and significance of the pig model to explore the mechanisms of nutritional supple-
ments on gut health with the emphasis on resistance to enteric ETEC infections.
Hence, findings in pig immune response under various pathological states are translatable to humans and rodents. Pigs are naturally susceptible to many pathogens that are either identical or closely related to those infecting humans, such as Rotavirus, influenza, E. coli, and Salmonella. As we discussed above, ETEC-caused diarrhea is a huge disease burden in young children who live in areas with poor sanitary infrastructures. In swine production, ETEC also impose immense threat to nursing and nursery piglets. However, rodents (mice and rats) are not normally susceptible to ETEC, and experimental challenges with the pathogen hardly reproduce clinical signs (e.g., diarrhea and dehydration) associated with ETEC, which is, at least partially, due to insufficient pathogen attachment to the mucosa of small intestine. It was reported that strains of human ETEC and porcine K88 (F4+) strains cross-bound to isolated small intestinal cells from either humans or pigs, whereas the ETEC strain (K99+) that prevalently infects calves and lambs failed to bind both human and pig small intestinal cells under the same conditions. Consistently, gnotobiotic piglets challenged with the ETEC strain with the expression of porcine enterotoxins (pLT or pSta) displayed equivalent colonization of pathogens and identical signs of disease as piglets inoculated with the isogenic ETEC strain constructed with human enterotoxins (hLT or hSta). Domestic piglets thus may be a superior model to study pediatric ETEC infection.

Despite the aforementioned advantages of pigs as a translational model, it is also important to recognize the differences between the 2 species in anatomy of gastrointestinal tracts, immunity, and gut microbiota. Compared with humans, pigs have bigger gastric capacity, longer absolute length of intestine, and a greater proportion of the large intestine. Additionally, the cecum, the ascending and transverse colon, and the proximal section of descending colon are arranged in spiral coils, which structurally differs from that of humans. As the pig placenta has 6 layers, the embryo is separated from the sow’s blood supply during the intrauterine period. Piglets lack transplacental transfer of immunoglobins; therefore, colostal immunoglobulin intake is essential for their efficient immune function. Gut closure for the macromolecule uptake occurs within 24 to 48 hours after birth. During this period, colostal immunoglobulins are transported into piglets via enterocytes. In addition to this major difference in passive immunity, other differences include the inversion of lymph nodes, types of Peyer’s patches, and cluster of differentiation. Cell-surface proteins that allow the identification and characterization of various immune cells vary across species (human vs pig) have been thoroughly described by Brandtzæg, Mair et al., Rothkötter, and Summerfeld and McCullough. Although the majority of human intestinal microbiota are present in pigs, there were marked differences in relative abundance of gut bacteria at the phyla level between neonatal piglets and human infants. In piglets, the predominant phyla are Bacteroidetes and Firmicutes (rather than Bifidobacteria), which represents more than 50% of 16S rRNA sequence in human neonates. Given the important role of gut microbiota in modulation of host immune response, such innate differences between the neonates of the 2 species impose challenges using piglets for the study of pediatric enteric infections. Cautious explanation is warranted when translating results from piglets to human infants.

**ETEC Challenge Piglet Model**

Pigs are naturally susceptible to postweaning diarrhea, which is another reason that the pig is a very suitable model to study diarrhea or other environmentally acquired enteric infectious diseases. Postweaning E. coli diarrhea in pigs, characterized as anorexia, depression, rapid dehydration, decreased growth performance, and increased mortality, remains a major cause of loss for the pig industry. E. coli that express F18 and F4 (K88) fimbriae are the predominant strains that cause diarrhea in postweaning and preweaning pigs. Several published studies thoroughly investigated the influences of F18 and F4 E. coli on gut morphology and immunity and systemic immunity of newly weaned pigs. Other literature also reviewed the role of gut microbiota in modulation of host immune response, and differences with the pathogenesis of F18 E. coli. Both strains have been explored to use for E. coli challenge studies in pigs. As we mentioned above, 2 important virulence factors are involved in E. coli infection: fimbriae and toxins. The F4 and F18 receptors are located on different porcine chromosomes and are genetically determined by autosomal dominate genes. It has been observed that the colonization of F4 E. coli in the small intestine may be quicker than that of F18 E. coli. The peak excretion of F4 E. coli from feces was observed 2 days post-infection, whereas the peak excretion of F18 E. coli from feces was 1 to 3 days later than F4 E. coli infection. Many factors could influence their adhesion, including the amount of fimbriae expressed by E. coli, the strength of their binding, and environmental factors in intestinal lumen that could impact the interaction of bacteria and their receptors. F18 E. coli infection is more commonly observed in postweaning pigs (3 to 6 weeks of age) and may be the result of lack of expression of receptors in the small intestinal enterocytes of piglets for F18 fimbriae. Different immune responses were also observed in F4+ and F18+ E. coli, depending on the toxins they expressed.

In this review section, we will focus only on the description of F18 E. coli infection. In this disease challenge model, the F18 E. coli that was used for inoculation was derived from a field disease outbreak by the University of Illinois Veterinary Diagnostic Laboratory (isolate no.: U.I.L-VDL #05-27242) and expressed LT, STb, and Shiga-like toxin 2. The inoculums were provided at 1010 cfu per dose per day in phosphate buffer saline for 3 consecutive days. It has been observed that F18 E. coli inoculation with the dose described above consistently induced moderate diarrhea (brown to yellow scours), as the peak of diarrhea was around 5 to 6 days postinoculation and most pigs recovered around 11 to 12 days after the first inoculation. The feecal culture results were in agreement with the observations of diarrhea trend, as indicated that the majority of E. coli in feces were F18 E. coli on day 5 or 6 postinoculation, then the percentage of F18 E. coli in total culfsoms gradually decreased after day 5 or 6. Toxins (LT and STb) produced by E. coli are able to induce partial villus atrophy in young pigs. In this disease challenge model, it was also observed that F18 E. coli infection reduced villus height in jejunum and ileum. The villus volume is highly related to the nutrient absorptive capacity of the small intestine. Therefore, the villus atrophy could induce decreased nutrient absorption, reduced feed intake, and finally reduced growth performance, which was also confirmed in this disease challenge model. The clinical signs observed in the E. coli challenge model with pigs are pretty similar to young children, including loss of appetite, watery diarrhea, severe dehydration, or potential growth retardation.

In the F18 E. coli disease challenge model, it was also observed that F18 E. coli infection induced systemic inflammation, as indicated by the gradually increased total white blood cells, neutrophils, and lymphocytes postinoculation. Consistent with this, several proinflammatory cytokines...
(i.e., TNF-α) and acute phase proteins (C-reactive protein and haptoglobin) in pig serum were also elevated by E. coli infection. Lymphocytes and neutrophils are the most abundant circulating immune cells in humans and play a fundamental role in the immune response. There is limited information about the nature of immune responses of ETEC in general, with the exception that the antibody responses against different virulence factors were determined in children or adult patients who were infected with ETEC.27–29 Examination of white blood cells and neutrophils is particularly prevalent in patients with bacterial infection, and a similar trend is also observed in infected patients or clinical trials.80 Therefore, data reflect the systemic immunity confirmed that the E. coli disease challenge model with newly weaned pigs could provide a valuable tool to examine the immune responses of bacterial infection in young children.

As the first line of defense, the mucosal layer of the intestine is in direct contact with luminal contents; thus, mucosal immunity is very important for the immune defense against pathogens.81 In the F18 E. coli challenge model, we also observed that E. coli infection enhanced specific local inflammation, as indicated by the increased neutrophil and macrophage recruitment in the distal ileum of weaned pigs.22 During inflammatory responses, neutrophils are the first cells to migrate into infected tissues and then secrete monocyte chemotactants, which will contribute to the recruitment of other immune cells, such as macrophages.82 The recruited neutrophils and macrophages in the infected sites will phagocytose bacteria and their particles, release large amounts of inflammatory mediators, and facilitate the resolution of inflammation. To characterize the effects of F18 E. coli infection on the expression of immune-related genes in ileal mucosa of weaned pigs, a porcine genome array was performed for the ileal mucosa samples collected from E. coli-infected pigs at day 5 postinoculation.83 In summary, E. coli infection altered the expression of 418 of 5168 genes in the ileal mucosa. Within this, E. coli infection altered the expression level of genes related to LPS activation, cytokine and chemokine production, complement cascades, receptors and co-stimulators, heat stress, antigen presentation, cell apoptosis, and endoplasmic reticulum stress. Although the effects of E. coli infection on mucosa immunity have been well evaluated with mice or rat models, the results from this pig model provide more valuable information on the regulation of mucosal immune response against bacterial infection due to the intestinal similarities of pigs and humans.

### Milk and Plant-derived Bioactive Compounds on Enteric ETEC Infection

Accumulating evidence has confirmed the importance of nutritional interventions, including modified feeding strategies and nutrient supplements, in the control of diarrheal diseases and prevention of enteric infection (Table 1).84–87 For example, probiotics are probably the most popular supplements recommended to be used to treat or prevent infant diarrhea.86–89 Supplementation with micronutrients, such as zinc, also showed a protective effect in both well-nourished and malnourished children with diarrhea.90 To explore the novel nutritional strategies and decipher the underlying mechanisms, animal models are highly preferred prior to clinical trials with humans. In the F4 or F18 E. coli challenge pig model described above, the effects of different dietary factors or nutrient supplements on diarrhea, disease resistance, physiology, and immunity of newly weaned pigs could be assessed. Many nutritional strategies and/or feed additives have been applied to improve health and maximize the production of weaned pigs.91–93 Those strategies target different aims: (1) improvement of nutrient digestion and absorption, (2) regulation of gut microbiota to more favorable bacterial species, and (3) immune modulation to enhance disease resistance of weaned pigs. In this review, we will focus only on a few milk- and plant-derived bioactive compounds, such as phytochemicals, oligosaccharides, and lysosomes, as examples to introduce novel interventions on enteric infection of young children using pigs as a model.

#### Phytochemicals

Phytochemicals are secondary plant metabolites and can be obtained naturally from plant materials. Phytochemicals can be used in solid powder form or as crude or concentrated extracts.

<table>
<thead>
<tr>
<th>Pathogens</th>
<th>Dietary Supplements</th>
<th>Outcome</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETEC, K88</td>
<td>Milk from human lysozyme transgenic goats</td>
<td>Reduced diarrhea, reduced bacterial translocation in mesenteric lymph nodes</td>
<td>172,182,183</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Chito-oligosaccharide</td>
<td>Reduced diarrhea</td>
<td>184</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Combination of raw potato starch and probiotic E. coli strains</td>
<td>Reduced diarrhea, enhanced gut microbial diversity</td>
<td>185</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Probiotics: Pediococcus acidilactici, Sacharomyces cerevisiae boulardii</td>
<td>Reduced ETEC attachment to ileal mucosa, upregulated inflammatory responses in gut</td>
<td>186</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Sacharomyces cerevisiae fermented products</td>
<td>Enhanced appetite and ileal digesta bacteria richness, reduced ETEC adhering to the mucosa and colonic ammonia</td>
<td>187,188</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Probiotics: Lactobacillus plantarum CJLP243</td>
<td>Enhanced growth performance, reduced diarrhea, reduced gut inflammation, enhanced gut barrier function</td>
<td>189,190</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Phytochemicals</td>
<td>Enhanced growth performance</td>
<td>191</td>
</tr>
<tr>
<td>ETEC, K88</td>
<td>Nucleotides</td>
<td>Enhanced growth performance and nutrient digestibility, reduced diarrhea</td>
<td>192</td>
</tr>
<tr>
<td>ETEC, F18</td>
<td>Clays (smectite, zeolite, kaolinite)</td>
<td>Reduced diarrhea, enhanced gut integrity</td>
<td>61,63</td>
</tr>
<tr>
<td>ETEC, F18</td>
<td>Phytochemicals (capsicum oleoresin, garlic botanical, turmeric, oleoresin)</td>
<td>Reduced diarrhea, enhanced gut morphology, decreased systemic and gut mucosal inflammation</td>
<td>22,83</td>
</tr>
<tr>
<td>ETEC, F18</td>
<td>β-Glucan</td>
<td>Enhanced gut barrier function, reduced systemic inflammation</td>
<td>62</td>
</tr>
</tbody>
</table>

*ETEC = enterotoxigenic Escherichia coli.*
Depending on the process used to derive the active ingredients, the extracts can be classified as essential oils that are volatile lipophilic substances obtained by cold extraction or distillation and oleoresins that are derived by nonaqueous solvents. The major bioactive compounds in phytochemicals are polyphenols, terpenoids, alkaloids, and sulfur-containing compounds. The composition and concentration of bioactive compounds vary according to the plants, parts of the plant, geographical origins, harvesting season, environmental factors, storage conditions, and processing techniques. Phytochemicals have been largely applied for human nutrition and improvement of human health due to their potential biological functions, such as, antiviral, antimicrobial, antioxidant, and antiinflammatory effects (Table 2). It has been reported that various phytochemicals exhibit a wide spectrum of antibacterial activities against gram-negative and gram-positive bacteria with several general modes of action. First, owing to the lipophilic nature, many essential oils exert their antibacterial effect through increasing permeability and fluidity of plasma membranes that cause leakage of intracellular materials (e.g., ions, proton). Second, phytochemicals contain a high percentage of phenolic compounds, which possess strong antibacterial properties. Third, the active components in phytochemicals could interfere with the enzyme system of bacteria, then block the microbe’s virulence. Fourth, certain bioactive components in phytochemicals may prevent the development of virulent structures in bacteria, such as flagella that is critical for bacterial adhesion. Fifth, certain plant polyphenols could inhibit ETEC adhesion and toxin binding in vitro. A low dose of phytochemicals has been recommended to serve as a potential natural antimicrobial in reconstituted infant rice cereal.

The antiinflammatory effects of phytochemicals have been widely reported with in vitro cell culture models. Essential oils from clove, tea, garlic, cinnamon, and others have potential antiinflammatory activities because they are able to suppress the production of TNF-α, IL-1β, and nitric oxide from LPS-induced mouse and porcine macrophages. In addition, Lang et al. reported that garlic extract also can inhibit intestinal epithelial cell secretion of several chemokines, including IL-8, IP-10, and MIG, which mediate the inflammatory response by recruitment of various circulating leukocytes into the inflamed tissue. The modes of action for the antiinflammatory activities of phytochemicals are not clear, but evidence suggests that these effects are partially mediated by blocking the NF-κB activation pathway. For example, curcumin can block cytokine-induced NF-κB DNA binding activity, RelA nuclear translocation, IκB degradation, IκB serine 32 phosphorylation, and IκB kinase activity.

In an ETEC challenge model with weaned pigs, it has been observed that dietary supplementation of 10 mg/kg of capsicum oleoresin, 20 mg/kg of garlic botanical, or 10 mg/kg of turmeric oleoresin alleviated signs of diarrhea in ETEC-infected pigs. Capsicum and turmeric are extracted from oleoresins, which were standardized to 6% capsaicin and dihydrocapsaicin and 98% curcuminoids, respectively. Garlic botanical is standardized to 40% propyl thiosulfonates. Although the supplementation of those phytochemicals reduced diarrhea of ETEC-infected pigs, the proportions of β-hemolytic coliforms in feces were not affected, indicating that the dose of phytochemicals was probably too low to have an antimicrobial effect. Thus, the reduction of diarrhea may be due to other potential mechanisms instead of antimicrobial effects. The analysis of gene expression patterns by microarray showed

Table 2 Several Commonly Used Phytochemicals and Their Main Components Exhibiting Different Biological Activities

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Scientific Name</th>
<th>Main Components</th>
<th>General Modes of Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamon</td>
<td>Cinnamomum verum J. Presl</td>
<td>Cinnamaldehyde</td>
<td>1. Antimicrobial effect¹⁰⁵,¹⁹³–¹⁹⁴</td>
</tr>
<tr>
<td></td>
<td>Cinnamomum osmophloeum</td>
<td></td>
<td>Increase permeability and depolarize cytoplasmic membrane</td>
</tr>
<tr>
<td>Clove</td>
<td>Eugenia Caryophyllus Spreng.</td>
<td>Eugenol</td>
<td>2. Effect on host cells</td>
</tr>
<tr>
<td></td>
<td>Eugenia caryophylla Thunb Syzygium aromaticum (L.)</td>
<td></td>
<td>Antioxidant</td>
</tr>
<tr>
<td>Fennel</td>
<td>Eugenia Caryophylla</td>
<td>Anethol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Foeniculum vulgare</td>
<td>Eugenol</td>
<td></td>
</tr>
<tr>
<td>Garlic</td>
<td>Allium sativum</td>
<td>Allicin</td>
<td></td>
</tr>
<tr>
<td>Ginger</td>
<td>Zingiber officinale</td>
<td>Curcumin</td>
<td></td>
</tr>
<tr>
<td>Oregano Thyme</td>
<td>Origanum vulgare spp.</td>
<td>Gingerol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Origanum onites</td>
<td>Carvacrol</td>
<td></td>
</tr>
<tr>
<td>Pepper</td>
<td>Capsicum</td>
<td>Capsaicin</td>
<td></td>
</tr>
<tr>
<td>Pomegranate</td>
<td>Punica granatum</td>
<td>Ellagic acid</td>
<td></td>
</tr>
<tr>
<td>Rutaceae</td>
<td>Zanthoxylum schinifolium</td>
<td>Citronellal</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Antinflammatory (suppress NF-κB expression/signaling pathway; inhibit TNF-α, IL-1β, IL-6, IP-10, MIG, and PGE2 production; suppress iNOS and COX-2 expression)</td>
</tr>
<tr>
<td>Thyme</td>
<td>Thymus vulgaris L.</td>
<td>Thymol</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thymbra spicata</td>
<td>Carvacrol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Terpinene</td>
<td></td>
</tr>
</tbody>
</table>

Modified from Liu 2011.²⁴ COX-2 = cyclooxygenase-2; IL = interleukin; iNOS = inducible nitric oxide synthase; IP-10 = interferon gamma-induce protein 10; NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells; MIG = monokine induced by gamma interferon; PGE2 = prostaglandin E2; TNF-α = tumor necrosis factor. ²⁴The general modes of action were listed in the table, because many studies tested on essential oil containing a number of compounds rather than pure compound. The exact mode of action of each compound is not completely clear.
that dietary phytochemicals affected the expression of genes related to mucin, membrane structure, and function in ileal mucosa of weaned pigs, indicating consumption of phytochemicals may enhance gut mucosal health of E. coli-infected pigs.

Moreover, feeding those phytochemicals also reduced neutrophil and macrophage recruitment in the ileum of E. coli-infected pigs compared with pigs fed the control diet. During the inflammatory response, neutrophils are the first cells to migrate into the infected gut as part of the host defense system. The recruitment of other immune cells, such as macrophages, was activated by the secretion of monocyte chemoattractants from neutrophils in the infected tissues. Both neutrophils and macrophages can facilitate resolution of inflammation by phagocytizing bacteria and their particles and release large amounts of mediators. But excessive recruitment of those activated immune cells in the infected area will induce the excessive production of inflammatory mediators and then exacerbate gut inflammation.

The reduced recruitment of immune cells suggests that weaned pigs supplemented with those phytochemicals actually had less gut inflammation compared with infected control. The microarray analysis also confirmed the reduced gut inflammation by feeding those phytochemicals to weaned pigs. Compared with the ETEC-infected control pigs, feeding capscium oleoresin, garlic botanical, or turmeric oleoresin altered the expression of 52 genes (18 up and 34 down), 117 genes (34 up and 83 down), or 84 genes (16 up and 68 down), respectively, often counteracting E. coli infection. The overall findings from this ETEC challenge study indicate that supplementation of low-dose phytochemicals could enhance disease resistance and stimulate the recovery of young pigs from ETEC infection by modulating gut immunity and barrier functions.

Previous studies also demonstrated that perfusion of F4 E. coli-infected jejunal segments with black or green tea extract reduced net fluid and electrolyte losses, suggesting the antidepressant activity of those tea extracts. Supplementation of 1 g/L of cranberry extract in drinking water remarkably reduced diarrhea of F18 E. coli-challenged piglets. The use of herbal medicinal products and supplements has grown rapidly across the world over the past decades. There are more than 80% of people worldwide, representing the majority in the developing countries, relying on herbal medicines as primary healthcare. A wide variety of herbal extracts are employed to treat diarrhea, especially in the developing world. Although many promising potential benefits were observed in a good number of herbal products, many of them remain untested and their modes of action and potential side effects are not clear. A valuable animal model (i.e., pigs) will absolutely help us overcome the wide range of challenges of utilization of phytochemicals as medicine or nutritional therapy for fighting diarrhea in young children.

**Prebiotics and Nondigestible Functional Carbohydrates**

Prebiotics are a category of nutritional compounds that may not share similar structures but have the ability to improve the growth of beneficial microorganism in the gastrointestinal tract. It is important that prebiotics are resistant to hydrolysis by mammalian enzymes in humans and animals in the small intestine and preferentially utilized by Lactobacilli and Bifidobacteria in the large intestine, which confers benefit to gut health through competitive inhibition of pathogenic bacterial species. Gibson et al. offered a definition of prebiotics, which contains 3 key aspects: resistance to digestion, fermentation by the large intestinal microbiota, and a selective effect on the microbiota associated with health-promoting effects.

Many dietary fibers exhibit some prebiotic activity, but other nonfiber dietary components may be classified as prebiotics if they meet the requisite functional criteria. The number of potential prebiotic substances has grown beyond those that are naturally occurring, such as inulin found in chicory products, to include a large number of chemically/enzymatically manufactured prebiotics, the most notable of which is galacto-oligosaccharides (GOS), produced from lactose by β-galactosidase. The most well-characterized prebiotics are nondigestible oligosaccharides, such as inulin, fructo-oligosaccharides (FOS), GOS, lactulose, polydextrose, xylo-oligosaccharides, transgalactooligosaccharides, pyrodextrins, and isomalto-oligosaccharides. Inulin, oligofructose, and FOS are considered inulin-type prebiotics, which have been commonly used in the pig industry and human foods. GOS also have attracted interest, mainly because these are the compounds in human milk that have been associated with the improved colonic health of breast-fed infants. Owing to the beneficial effect of human milk oligosaccharides, the use of prebiotics is encouraged in infant formula with the intention to stimulate the effects of human milk oligosaccharides. A few other nondigestible carbohydrates not categorized as prebiotics, however, manifest health-promoting functions. For example, β-glucan is linear and branched polysaccharides that are produced by bacteria and are also found in cereals, algae, and fungi. The use of β-glucan has drawn growing interest in the food industry due to its immunomodulatory effects as demonstrated in animal and human. In vitro study supports a prebiotic effect of nondairy bacterial origin β-glucan on 3 strains from Lactobacillus genus. Oat β-glucan has been allowed to use to fortify cereals for young children (ages 1–3 years old) in the European Union. Clinical research on fermentation characters of β-glucan is still in scant.

The most notable effect of prebiotics is their modification of the balance of the microbiota, both in the lumen and at the mucosal surface. They can specifically stimulate growth of a limited number of beneficial microorganisms, generally Bifidobacteria and Lactobacilli, which suppress the growth of potentially pathogenic microorganisms such as E. coli by various means described below and therefore reduce the adverse effects caused by bacterial infection. For example, the desired bacteria produce short-chain fatty acids and lactic acid, which may indirectly and specifically kill or inhibit the growth of pathogens. The reduction of the pH of the intestinal environment through production of acids creates an environment unsupportive of the growth of several pathogens. The desired bacteria may produce antimicrobial compounds such as bacteriocins or antibiotics, although regulatory agencies try to avoid production of antibiotics. The desired bacteria compete for the available nutrients against pathogens.

More potential mechanisms are involved in the benefits of prebiotic supplements. For instance, the beneficial bacteria induced in the gastrointestinal tract by prebiotics could also inhibit the attachment of pathogens to the intestine by competing for binding sites on the intestinal wall, by producing acids that may reduce pathogen binding, by stimulating mucus production, or by strengthening gut barrier functions. Some prebiotics may contact with mucus to directly compete for intestinal binding sites. In addition, some prebiotics and their subsequent increase in short-chain fatty acids appear to have direct immunomodulatory properties. The most common studies in prebiotics and nondigestible carbohydrates and their potential modes of action are briefly summarized in Table 3.
Table 3 Prebiotics and Nondigestible Carbohydrates and Their Potential Mechanisms of Action

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<td>- Stimulate mucin production, strengthen tight junctions, and enhance gut barrier function</td>
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|                                      |                                      | Lysozyme is an antimicrobial enzyme naturally present in body fluids (e.g., tears, saliva, and milk) of all mammalian species. Its muramidase activity catalyzes the hydrolysis of the peptidoglycan layer of the bacterial cell wall that leads to cell lysis. Gram-positive bacteria is thus susceptible to the enzymatic degradation of lysozyme. However, lysozyme also displayed bactericidal activity against a variety of Gram-positive and Gram-negative species through the mechanism that is independent of its enzymatic function. Particularly, lysozyme has been found to act synergistically with lactoferrin in killing gram-negative bacteria. It has also been reported for its antiinflammatory property that was mediated through inhibiting neutrophil migration. Early research reported that human milk contains an average of 390 mg/L lysozyme. Based on data from 4 studies, Lönnertal et al. recently reported that the median concentration of lysozyme was 320 mg/L in human colostrum, peaked at 1100 mg/L in the second month of lactation, and decreased to 850 mg/L in the following month, whereas data of lysozyme concentration after 90 days in lactation were unavailable. The lysozyme concentration is remarkably low in milk of most livestock species (cow, sow, and goat). For instance, the lysozyme content of cow milk ranged from 0.18 to 0.45 mg/L across 5 dairy breeds, whereas the lysozyme concentration is approximately 0.25 mg/L in goat milk and 0.065 mg/L in sow milk. Deficiency in antimicrobial proteins in cow milk presumably contributes to the difference in gut microbiota profiles observed between breast-fed and formula-fed infants. For instance, breast-fed infants harbor fecal microbiota of more uniformity that is predominated by bifdobacteria. In contrast, the microbiota of formula-fed newborns demonstrated greater diversity and higher prevalence of clostridia, and transgalactooligosaccharides due to their relatively high cost. In a K88 ETEC challenge model with weaned pigs, it has been observed that supplementation of 8% inulin reduced the incidence and severity of postweaning diarrhea, probably by increasing short-chain fatty acid production in the cecum and proximal colon. It has been also reported that the addition of FOS could prevent mortality and morbidity of weaned pigs infected with K88 ETEC. Supplementation of β-glucan originated from different sources (yeast or algae) could enhance the resistance of pigs against ETEC infection. The likely reasons may include enhanced gut integrity and health and reduced paracellular permeability, reduced colonization of the small intestine with ETEC, and boosted host immune response against ETEC infection. Both dendritic-1 and CR3 expressed on several immune cells (i.e., macrophages, neutrophils) are highly involved in the immuno-modulatory effects of β-glucan, which need to be further elucidated.

Multiple studies have also evaluated the different combinations of oligosaccharides in pediatric research, suggesting that the preventive use of prebiotics could reduce the rate of acute infectious diseases requiring antibiotic therapy in infants and children younger than 2 years old. Supplementation of prebiotic oligosaccharides to infant formula has also been shown to modify gut microrganisms of formula-fed infants closer to the flora in breast-fed infants. Although a large amount of studies have been published to explore the potential benefits of prebiotics on human health and modes of action, the majority of research was done with in vitro cell culture models or laboratory animal models. The use of the pig model with ETEC challenge will provide more supportive data to validate the efficacy of different combinations of prebiotic carbohydrates against intestinal infection in young children and to help explore the potential mechanisms they may have.

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In a clinical trial, children hospitalized with acute diarrhea had faster recovery and a lower relapse rate by receiving an oral rehydration solution supplemented with human lysozyme and human lactoferrin. It has not been evaluated whether dietary supplementation of lysozyme per se could modulate microbiota and enhance host resistance to enteric infections in young children. The question has been addressed by a research group at the University of California, Davis (Drs. Elizabeth Maga and James Murray) and others through a translational model using milk from transgenic goats expressing human lysozyme at 68% of the level found in human milk, and young pigs as feeding subject. Six-week-old crossbred domestic pigs were artificially reared and fed milk from either nontransgenic control goat or human lysozyme transgenic goat for 14 days. Consumption of lysozyme-rich milk significantly increased the proportion of Bacteroidetes and decreased the proportion of Firmicutes (Clostridia) in fecal microbiota. Within phyla, there was an enrichment in the abundance of Bifidobacteriaceae and Lactobacillaceae, families known for their health-promoting function in lower GI tract, whereas the abundance of bacteria (Mycobacteriaceae, Streptococcaceae, Campylobacterales) associated with diseases were underrepresented in response to consumption of lysozyme milk. In another trial, after 14 days of feeding lysozyme milk, pigs were orally inoculated with porcine-specific ETEC (O149:F4 strain) at 2 × 10⁵ total CFU for 4 times at 12-hour intervals. Fecal score decreased from 24 to 96 hours post-ETEC inoculation, suggesting successful induction of diarrhea. The lowest score was observed at 24 to 48 hours postinoculation. In comparison with pigs that consumed control goat milk, feeding lysozyme-rich milk alleviated the severity of diarrhea and reduced total bacteria translocation into the mesenteric lymph nodes by 83%, which corresponded to a tendency of reduced fecal Enterobacteriaceae in pigs fed lysozyme-rich milk. Because many prevalent enteric pathogens such as E. coli and Salmonella belong to the family of Enterobacteriaceae, this possibly explained the dampened signs of diarrhea. A line of transgenic pigs that expresses high levels of recombinant human lysozyme (approximately 1300 mg/L) in their milk was also generated at China Agricultural University by Dr. Li’s group. Consumption of human lysozyme-rich milk reduced diarrhea, increased survival rate, and facilitated the recovery of neonatal pigs from F4 E. coli infection. The observed benefits are likely due to the increase in the abundance of intestinal Lactobacillus as well as enhanced intestinal integrity and mucosa immunity of neonatal pigs consumed lysozyme.

Further Applications and Conclusions
The pediatric population is especially vulnerable to ETEC infection. The tremendous infectious disease burden requires continued and extensive studies aimed at exploring more therapeutic/preventive interventions to improve young children’s health/survival and to alleviate bacterial infection. Young pigs have demonstrated their potential as a new animal model for pediatric research. The translational features of the piglet model in terms of anatomy of the gastrointestinal tract, digestive physiology, components of the immune system, dynamics of neurodevelopment, and morphological structure of CNS foretells its broad applications for mechanistic research in human nutrition, immunity, and neurodevelopment in early life. Considering that enteric infections are the leading causes of morbidity and mortality in early childhood, a well-characterized pig model incorporating enteric pathogen challenges presented by our group (bacterial infection) and others (viral infection) is promising in preclinical studies to uncover the mechanism of pathogenesis and evaluate the effects of nutraceutical interventions in youth. The underlying mechanisms will be further explored by combining both functional measurements (i.e., gut permeability, feed efficiency, nutrient digestibility, etc.) and descriptive analysis (i.e., gut morphology and gut barrier function, etc.). The important roles of the gut microbiota in host resistance against invading pathogens in the small intestine should not be negligible. The protective effects of gut microbiota against pathogenic bacterial infection could be deeply approached with this pig challenge model by investigating metagenomics and the changes of bacterial metabolites. Last but not least, this translational model could also be expanded to different pathogens, for instance, different strains of E. coli, Salmonella, other infectious agents, or combinations.

It is also important to keep in mind that each animal species shows some similarity to the physiology of humans and therefore provides valuable insights from different angles on the research of nutritional intervention in pediatric enteric infection. The purpose of this review is not to compare different animal models with their pros and cons. The overall objective is to highlight another potential model that could serve as a powerful tool for pediatric research.

References


