Prevention of enterotoxigenic *Escherichia coli* infections in pigs by dairy-based nutrition

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Abstract

Postweaning diarrhoea (PWD) is a significant enteric disease causing considerable economic losses for the pig industry. Among several aetiological risk factors, enterotoxigenic *Escherichia coli* (ETEC) is considered to be a major cause. After being routinely used for several decades to control bacterial disease outbreaks in piglets, the *use* of antibiotics at subtherapeutic concentrations has been banned in the European Union because of the increasing prevalence of resistance to antibiotics in pigs. The removal of in-feed antibiotics from piglet diets has negative economic consequences as it dramatically increases the rate of morbidity and mortality due to ETEC as well as the use of antibiotics for therapeutic purposes. Other than subtherapeutic antibiotics, zinc oxide (ZnO) has been reported to ameliorate and/or prevent the development of PWD in piglets, but its excretion may have negative impacts on the environment. Thus, other alternatives that can control ETEC infections in piglets postweaning will be of great advantage. A number of nutritional strategies have been proposed as alternative means of preventing ETEC infections, of which feeding dairy-based products to piglets could be one of such strategies. It is apparent that colostrum, milk and milk fractions such as whey and casein contain several biologically active compounds with anti-microbial and immunomodulatory properties. Recently, these dairy products and their isolated compounds such as lactoferrin and oligosaccharides have been employed as anti-infective agents against ETEC infections in piglets postweaning. The dairy by-products (e.g. whey or whey permeate) may also be fermented to further improve the anti-infective potential of the products. Overall, the anti-infective activities of dairy-based products against ETEC could be attributed to the improvement of the intestinal barrier functions, microbial ecosystem and immunity of the piglets.

Keywords: Dairy-based product, Anti-microbial, Immunomodulation, Anti-infective, Enterotoxigenic *Escherichia coli*, Postweaning diarrhoea, Piglet

Review Methodology: We conducted a literature search with focus on ETEC-induced PWD and the preventive effect of dairy-based nutrition using the following criteria: (1) peer-reviewed journal articles in English were included; (2) articles or chapters in an edited book were selectively included; (3) grey literature such as Ph.D. theses was selectively included; (4) published reports from the official organization such as The Danish Integrated Anti-microbial Resistance Monitoring and Research Programme (DANMAP) and European Food Safety Authority (EFSA) were selectively included; (5) *in vitro* and *in vivo* investigations on pigs were included; (6) *in vitro* and *in vivo* studies on humans and rodents were selectively included, provided that the aims were to investigate the cell/host–pathogen interactions and/or enteric infections; (7) *in vivo* studies with the aim to investigate the gastric infections were excluded.

Introduction

Postweaning diarrhoea (PWD) has been reported to be the most common enteric disease with a great economic impact on the pig industry [1]. Bacteria, viruses and parasites are among the infectious agents associated with diarrhoea [2]. In the pig industry, much attention has been focused on enterotoxigenic *Escherichia coli* (ETEC), as it is

estimated that half of piglet mortality due to diarrhoea is attributable to ETEC [3]. A subtherapeutic use of antibiotics has been routinely practiced in swine industry for decades to deal with ETEC infections postweaning. However, this practice has been banned by the European Union since 1 January 2006 because of the risk of development of anti-microbial resistance and transference of antibiotic resistance genes from animal to human microbiota [1]. Because of this ban, a number of alternatives based on the nutritional practices have been proposed to control the increased rate of mortality and morbidity [4].

Apart from inorganic zinc that may contaminate the environment [1], some feeds or feed ingredients have been suggested to possess immunomodulatory and anti-microbial properties, and thus can help to protect the piglets against ETEC infections [5]. Of these ingredients, dairy-based products have gained increasing interest as a potential nutritional tool to prevent piglets from developing diarrhoea due to ETEC [6–9]. Freeze-dried (defatted) bovine colostrum (BC), liquid whey, spray dried whey isolate (whey meal), whey protein concentrate, whey permeate and spray dried calcium caseinate are among the milk-derived products that have been studied to improve the intestinal health of pigs. Likewise, several biologically active compounds such as lactoferrin, lysozyme, lactoperoxidase, immunoglobulin (Ig), phospholipid, oligosaccharides and glycoconjugates have been isolated from milk or milk fractions, and these components may have therapeutic properties in prevention and inhibition of ETEC infections [10–16]. The purpose of this review is to update the current knowledge pertaining to the potential preventive effects of dairy-based products on ETEC infections in piglets postweaning.

ETEC Infections in Piglets

Escherichia coli are part of the normal microbiota in the gastrointestinal tract and are found in both healthy and diseased pigs. Although most of *E. coli* are not pathogenic, some of them encode genes that can impart virulence [17]. To date, eight *E. coli* pathovars have been characterized, and each uses several virulence factors to subvert host cellular functions and to potentiate its virulence. Six out of the eight pathovars have been classified as diarrhoeagenic, including enteropathogenic *E. coli*, enterohaemorrhagic *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli*, enteroaggregative *E. coli* and diffusely adherent *E. coli* [2]. Among these diarrhoeagenic *E. coli*, ETEC is the most common cause of diarrhoea in newborn and weaned piglets [4, 17].

ETEC strain expressing F4 fimbriae are highly prevalent in pigs. Currently, three antigenic variants of the F4 fimbriae have been identified in porcine ETEC strains, i.e. F4ab, F4ac and F4ad [18]. Of these variants, F4ac are the most important variants of ETEC F4 [4, 18]. ETEC F18 is another common strain of ETEC isolated from piglets suffering from diarrhoea. So far, two antigenic variants of F18 can be characterized, i.e. F18ab and F18ac. To elicit diarrhoea in piglets, ETEC F4 or F18 must first adhere to specific porcine enterocyte receptors for the F4 or F18 fimbriae, respectively [1, 4]. This adhesion facilitates colonization of the distal jejunum and proximal ileum mucosa by ETEC and enables them to transmit enterotoxins leading to diarrhoea [17]. The detailed pathogenesis of ETEC infections and the mechanisms by which enterotoxins (produced by ETEC) induce diarrhoea in piglets have been extensively reviewed elsewhere and are not discussed further here.

Antibiotics and Zinc to Control ETEC infections in Piglets

In the past, antibiotics were routinely used to control enteric bacterial infections, such as diarrhoea, in piglets after weaning. The risk of developing anti-microbial resistance and transference of antibiotic resistance genes from pig to human microbiota has led the European Union to ban the application of subtherapeutic use of antibiotics in the swine industry since 1 January 2006. Consequences of this ban include a dramatic increase in the prevalence of PWD, an increase in the postweaning mortality rate and an increased use of therapeutic antibiotics [19]. In Denmark, it was reported that therapeutic use of antibiotics in pigs increased by 25% compared with the pre-ban levels [20], and during the period 2001–2009 the anti-microbial consumption in the pig production increased gradually by 36% [21].

Apart from the long-term effect of antibiotics on the development of antibiotic resistance, the subtherapeutic use of antibiotics has been reported to have a short-term impact on the composition of microbiota in the intestine [22, 23]. Antibiotics may, concomitant with the reduction in pathogens, decrease the number of *Lactobacillus*, species which are generally considered to be beneficial to the host [24]. Both the reduced species diversity and the total number of bacteria in the intestine of pigs have also been attributed to the treatment with antibiotics [24, 25]. It has been widely accepted that an altered composition and diversity of the intestinal microbiota may affect the intestinal immune system and intestinal epithelial barrier functions of the host. In this regard, the use of antibiotics may thereby affect host immune defence mechanisms [23]. For example, Hill *et al.* [26] reported that antibiotic treatment reduced (10-fold) the amount of intestinal bacteria and changed the intestinal microbial communities in the gut of murine model. Concurrently, this antibiotic administration caused significant alterations in the expression of proinflammatory cytokines by $CD4^+$ T lymphocytes in the gut-associated lymphoid tissue. Overall, it should be noted that both oral and intravenously administered antibiotics may

have implications on the intestinal microbiota composition and the immune functions of the host [23]. In addition, the alterations in the intestinal microbiota persist for a long period after termination of the antibiotics administration [22].

Feeding supplemental zinc (zinc oxide, ZnO) to weaned pigs at pharmacological levels up to 2500 mg/kg, which is allowed in Denmark and Belgium, have been reported to ameliorate and/or prevent the development of PWD [4, 27], and hence could be a substitute for in-feed antibiotics. The mechanism by which zinc exert the PWD-reducing effect has not yet been fully elucidated, but it may be due to the anti-microbial and immune-enhancing properties of zinc (Table 1). According to Højberg *et al.* [27] the effect of zinc resembles the working mechanism suggested for some in-feed antibiotics, i.e. the suppression of gram positive commensals (lactic acid bacteria; LAB) rather than potentially pathogenic Gram-negative organisms (coliforms). This condition may therefore have long-term negative consequences on the immune functions of piglets [26]. Janczyk *et al.* [28] reported that feeding supplemental zinc (2500 mg/kg) immediately after weaning could positively affect the immune responses of piglets infected with *Salmonella* typhimurium, but for a short period only. After 2 weeks, all positive effects disappeared and rather negative effects, such as lower T-cell frequencies, occurred. Apart from the potential protection of zinc against ETEC in pigs, environmental considerations should be taken into account when using zinc at high levels, as this leads to heavy metal contamination of the soil [4]. Taken together, zinc is not feasible alternatives to in-feed antibiotics in piglets.

Dairy Products to Control ETEC infections in Piglets

The compromised immune functions and subsequent increased colonization of the porcine small intestine by ETEC during the weaning transition period have been proposed to contribute to the outbreaks of PWD [1]. Considering that nutrition and diet play substantial roles in the composition of intestinal microbiota and immune status of the host [23], various nutritional interventions (Table 1) have been investigated as possible tools to prevent the disruption of gut microbial ecosystem and impairment of the immune system, hence preventing the development of PWD. Dietary intervention studies have especially been intensified following the ban of sub-therapeutic use of antibiotics in pig productions, because of the need for alternative strategies. A number of dietary components and/or feeding regimes have been suggested to provide factors that beneficially influence the intestinal microbiota, the immune system as well as the epithelial barrier function, and thus the antigen exposure [5, 29]. In this review, some basic relationships between nutritional interventions and intestinal health of piglets postweaning will be highlighted, giving dairy-based products the major focus.

Mammalian milk including bovine milk contains a wide variety of factors that contribute to the protection of the newborn with immature immune defence system against infections [30]. In addition to the anti-microbial and immune components, milk contains several growth factors (e.g. beta cellulin, epidermal growth factor, insulin-like growth factor, transforming growth factor [TGF], etc.) which are essential for the general development of the young mammals [13, 30]. There is now a lot of evidence suggesting that bioactive components of bovine milk can act as anti-microbial and immunomodulatory agents in ruminant as well as in non-ruminant species [31]. Therefore, the use of dairy-based products may be expected to preserve a healthy intestine and prevent ETEC-induced PWD in piglets.

The detailed mechanisms through which the dairy-based products protect pigs from ETEC infections are largely unknown, but in general these mechanisms may lead to an improvement of the intestinal microbial ecosystem and immune functions of the pigs [7, 32–34]. In addition, the dairy products may preserve the intestinal integrity and prevent the intestinal barrier (mucosa) from disruption by controlling the excessive expression of proinflammatory mediators in response to invading pathogens [34–37].

Preventive potential of bovine colostrum on ETEC infections in piglets

Colostrum is milk secreted by mammary glands during the first few days postpartum. In the case of the cow, colostrum is defined as the milk secreted for the first 4 days postpartum [38]. In addition to its value as a complete nutrient source for the newborn calf, BC has gained increasing interest as a source of nutraceuticals for other mammals including pigs. Colostrum contains various anti-microbial components, such as Igs, lactoferrin, lysozyme and lactoperoxidase, which could exert both bacteriostatic and bactericidal activity [13, 35]. It also contains several immune components which play important roles in host defence against infections, including Igs, cytokines and lactoferrin. Of these immune components, Igs appear to be the most abundant immune components as they represent 70–80% of the total protein content in colostrum. Three isotypes of Igs are present in colostrum, i.e. IgG, IgA and IgM. The most abundant Ig in colostrum is IgG of which 95% belong to the subclass IgG_1 and 5% to the IgG_2 [13]. It is known that the content of Igs in colostrum varies among animal species [39]. Compared with sow colostrum, BC has lower proportions of IgA and higher IgM, but the proportion of IgG does not significantly differ between both species (Figure 1). The major bioactive components in BC are presented in Table 2. It should be noted that the mentioned-bioactive

Table 1 Examples of feeds or feed components that could potentially prevent and/or reduce ETEC-induced diarrhoea in piglets

Feeds or feed components	Results/diarrhoea incidence	Possible mechanisms through which feed components prevent/reduce diarrhoea	References
Chicken egg-yolk antibodies	Reduced ETEC F18-induced PWD by 4× compared with piglets fed egg-yolk powder (contain no antibodies) [72]	Prevent gut from ETEC adhesion and colonization	[4, 72]
Spray-dried plasma	Reduced ETEC F18-induced PWD by 4× compared with piglets fed egg-yolk powder [72]	Prevents gut from ETEC adhesion and colonization Inhibits ETEC growth in the gut (anti-bacterial effect) Prevents disruption of intestinal mucosal integrity Improves host immune competence Reduces cytokine-induced inflammation	[73–75]
Zinc oxide	Reduced ETEC F4-induced PWD in piglets fed high protein diet (4.8 versus 13.1%) compared with piglets fed high protein diet without zinc oxide during the first week postweaning [76]	Inhibits the growth of ETEC in the gut Reduces ETEC adhesion and invasivity (of Caco-2 cells) Maintains the stability and diversity of the intestinal microbiota particularly with respect to coliforms Prevents disruption of intestinal mucosal integrity Improves host immune competence Reduces excessive inflammatory responses Reduces electrolyte secretion from enterocytes	[1, 77–80]
Dietary oligosaccharides	Reduced ETEC F4-induced PWD incidence by ±8% compared with piglets fed control basal diet [15]	Prevent gut from ETEC adhesion and colonization Reduce the sensitivity of fimbrial receptors on the small intestine Block the fimbriae of ETEC Maintain a balanced intestinal microbiota Improve host immune functions Reduce cytokine-induced inflammation	[4, 10, 15, 81–83]
Probiotics	Reduced incidence of ETEC F4-induced PWD [8], and reduced incidence of diarrhoea in suckling piglets due to rotavirus and ETEC by 25% [84]	Hinder ETEC adhesion and colonization to gut Produce substances and/or organic acids (especially lactic acids) possessing an anti-bacterial effect Lower the pH of the stomach resulting in less enterobacteria reaching the small intestine Preserve a balanced intestinal microflora Improve the host immune system Reduce the bacterial translocation to mesenteric lymph nodes Modulate the establishment of lymphocyte populations and IgA secretion in the gut Enhance the intestinal epithelial barrier function	[8, 84–87]
β-glucan derived from *Saccharomyces cerevisiae* (unpurified form)	Shortened the duration of ETEC F4-induced PWD (3.4 versus 4.3 days) compared with piglets received control basal diet [88]	Protects the small intestine from adhesion and colonization by ETEC Improves host immune system Modulates the immune response of porcine neutrophils *in vitro*	[88–90]
β-glucan derived from *Saccharomyces cerevisiae* (purified form)	Enhanced the immune functions of weaned piglets upon challenge with *E. coli* [91]	Increases plasma IL-6, TNF-α and IL-10 after *E. coli* challenge Enhances the cellular and humoral immune functions of weaned piglets via decreased prostaglandins E_2 (PGE_2)	[91, 92]
Mannan oligosaccharides (unpurified form)	Reduced diarrhoea score in weaned piglets (1.70 versus 1.83; diarrhoea score 1 = well-formed faeces, 2 = sloppy faeces, and 3 = diarrhoea) compared with piglets received basal diet [93]	Prevents ETEC from binding to the gut wall and causing damage Improves the immune system of piglets both systemically and enterically Reduces cytokine-induced inflammation	[93, 94]

Hydrolysed whole yeast cell derivate (β-glucans and mannan are the main active compounds)	Reduced the incidence of ETEC F4-induced PWD compared with piglets received no yeast (detailed ratio of piglets with PWD over time postweaning can be seen in Jensen *et al*. [95])	Block the fimbriae of ETEC Improve the balanced intestinal microbiota Improve immune competence of piglets	[95, 96]
Short- and medium chain fatty acids	Lowered the mortality of piglets [97]	Stabilize the intestinal microbiota postweaning Reduce cytokine-induced inflammation Prevent disruption of intestinal mucosal integrity	[86, 97–99]
Organic acids	Alleviated the incidence of ETEC F4-induced PWD compared with piglets offered feed without organic acids (detailed diarrhoea score among the treatments can be seen in Tsiloyiannis *et al*. [100])	Lower the pH of the stomach (below 6) which prevents the entry of exogenous ETEC to the small intestine Exhibit bactericidal and bacteriostatic effects which may prevent outgrowth of pathogenic bacteria (ETEC) in the small intestine Stabilize gut microbiota	[85, 100, 101]
Freeze-dried defatted BC	Reduced PWD incidence (piglets had 9% less soft faeces and 4% less liquid faeces compared with piglets received no BC) [7]	Controls pathogenic bacteria overgrowth in the small intestine of piglets Protects pig intestinal IPEC-1 cells against the increased membrane permeability caused by ETEC Enhances total serum IgA and stimulates ileal Peyer's patches in weaned piglets	[7, 33, 43]
Milk products	Improved intestinal health	Maintain a balanced intestinal microflora Preserve intestinal epithelial barrier function	[102] [13]
Fermented products	Improved intestinal health	Reduce the number of ETEC entering the intestine by lowering the pH of the stomach Maintain a balanced intestinal microflora Control the growth and colonization of intestine by pathogenic enterobacteria Improve the systemic and mucosal immune responses of pigs Control the excessive inflammatory reaction upon infection	[29, 103, 104]
Lactoferrin	Reduced the incidence of PWD by 66.2% compared with piglets received control basal diet [12]	Reduces the total viable counts of *E. coli* and *Salmonella* in the small intestine Increases the number of *Lactobacillus* and *Bifidobacterium* in the intestine Improves the morphology of small intestine (increased villus height and lowered crypt depth)	[12]
Prebiotics		Preserve the intestinal ecosystem Supports the growth of specific lactobacilli in the ileum and colon of weaned piglets	[105]
Herbs (e.g. allicin isolated from garlic)	Reduced the incidence of PWD (0.87% versus 2.91%) compared with piglets fed basal diet supplemented with antibiotics [106]	Exhibit anti-microbial activities against human enteric bacteria *in vitro* Protect host cells against the increased membrane permeability caused by ETEC *in vitro*	[106–108]
Conjugated linoleic acid		Improves host immune system Reduces cytokine-induced inflammation	[99, 109]
Phospholipids		Improve the immune system of the host Prevent disruption of intestinal mucosal integrity	[110]
Essential oil		Inhibits the growth of ETEC in the intestine	[5]

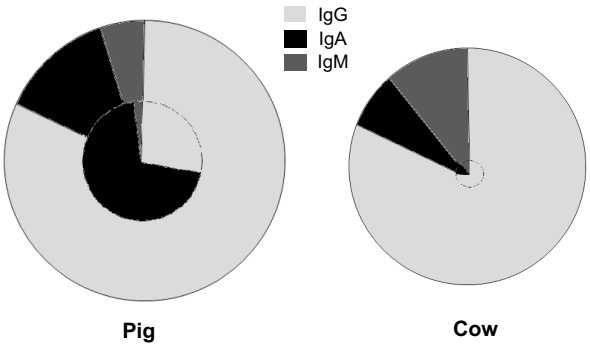

Figure 1 Relative distribution of Igs in colostrum (outer circle) and in milk (inner circle) of the sow and the cow (adopted from [39]). The overall concentration of total Igs is higher in colostrum than that in milk, and is relatively higher in the sow milk than that in the bovine milk (based on the relative size of the circles).

components in colostrum are also present in milk, but in much lower concentrations (e.g. typically 1 : 100 to 1 : 1000 of what is found in colostrum) [13].

In addition to the content of anti-microbial and immune factors, BC is a good source of a range of bioactive peptides which act as regulatory compounds and support the defence system of the host [40]. Colostrum also contains oligosaccharides and glycoconjugates, which may provide the host a protection against bacterial pathogens [10]. Several potentially proinflammatory components in BC, including tumour necrosis factor-α (TNF-α) and interferon- γ (IFN-γ) [13, 30], makes BC capable of augmenting the immune responses leading to rapid clearance and/or less colonization of the intestine by pathogens. However, it should be noted that excessive expression of these proinflammatory cytokines in the gut may induce inflammation resulting in diarrhoea in piglets. Fortunately, BC is also rich in TGF-β, which is able to control inappropriate inflammatory responses and preserve the epithelial barrier function after infections [13]. BC is also claimed to contain anti-oxidative factors, which are beneficial against the oxidative damage (stress) caused by infections [38, 41]. The anti-oxidative factors include enzymatic as well as non-enzymatic anti-oxidants (Table 2).

BC is one of the commercially available co-products of the dairy industry that has been shown to control the outbreaks of PWD in piglets [7, 42]. The definite mechanism through which BC shortens PWD episodes remains unknown, but a combined action of anti-microbial and immunomodulatory factors of BC are likely to be involved [33, 43]. In addition, the potential of BC to lower the gastric pH and to increase the duodenal lactobacilli: coliform ratio [32] may balance the intestinal ecosystem, which in turn may prevent PWD. The potential of BC to preserve the intestinal integrity by maintaining the villi height and crypt depth after weaning [13] seems also to contribute to the reduced PWD. In continuation of the earlier works, Sugiharto *et al.* [42] showed that feeding BC reduced the adherence of ETEC F18 to porcine intestinal tissue *ex vivo*, and hence may reduce the intestinal colonization by ETEC and prevent the development of ETEC-induced diarrhoea. Apart from the preventing potential of BC on the development of PWD, the dose of BC included in the diet of pigs is another concern that should be taken into account. This is most likely ascribed by the high price of BC in the current market. In their review, Boudry *et al.* [13] noticed that most of the cited authors incorporated 5–10% of BC in the diet of piglets during their studies. Hence, further studies with less proportion of BC included in the diet of piglets are of importance in order to make BC applicable for the commercial pork production systems.

Preventive potential of whey and whey protein on ETEC infections in piglets

Whey is a byproduct of cheese-making, which was traditionally regarded as a waste with little or no commercial value [44]. Whey is the second most abundant source of protein in the milk (approximately 20% of the total milk proteins) after casein. From the nutritional point of view, whey protein has been regarded to be superior to casein, in that whey protein is absorbed faster than casein in humans [45]. Moreover, whey proteins have proportionately more sulphur-containing amino acids (cysteine and methionine) than casein, contributing to the higher protein efficiency ratio of whey proteins than of casein [44]. Beyond the nutritional aspect, whey proteins and their derived-peptides are known to modulate a variety of immune functions including lymphocyte activation and proliferation, cytokine secretion, antibody production, phagocytic activity and granulocyte and NK cell activity [31, 46]. Besides being responsible for the nutritional quality of whey proteins, the sulphur-containing amino acids appear important to the ability of whey proteins to enhance immune functions via modulation of the sulphur-containing tripeptide glutathione [44]. Likewise, some individual proteins derived from whey, such as β-lactoglobulin, α-lactalbumin, Igs, lactoferrin and lactoperoxidase may contribute to the immunomodulatory and/or anti-microbial activities of whey or whey proteins [31, 44, 46]. The major individual proteins derived from whey and their potential immunomodulatory and/or anti-microbial activities are summarized in Table 3. Recently, there has also been a considerable interest in the non-protein components of whey that may possess anti-microbial and immunomodulatory properties, including phospholipid (phosphatidylethanolamine, phosphatidylcholine and sphingomyelin are the predominant fractions), oligosaccharides and glycoconjugates (Table 3).

Most of the studies on anti-microbial and immunomodulatory activities of whey-derived bioactive compounds have been conducted *in vitro* with focus on specific isolated whey components (see references in Table 3).

Table 2 Bioactive components in bovine colostrum

Bioactive compounds	Biological activities	References
Anti-microbial functions:		
- Lactoferrin	Binds the iron released from transferrin which prevents its further use for bacterial growth	[39, 111]
	Damages the outer membrane of gram-negative bacteria	[13, 111]
	Attacks gram-negative bacteria directly by destabilizing its coat membranes	[41]
- Lysozyme	Degrades or lyses bacterial cell walls through the enzymatic activity of lysozyme and its cationic and hydrophobic properties	[111]
	Partly activates lactoperoxidase by forming a complex with it	[112]
	Works in synergy with lactoferrin and Igs against pathogenic bacteria	[113]
- Lactoperoxidase	In the presence of H_2O_2, catalyzes the oxidation of thiocyanate (SCN^-) and produces intermediate products that inhibit bacterial metabolism (via the oxidation of essential sulphydryl groups in microbial enzymes and other protein)	[13, 40]
	Exhibits an additive anti-bacterial effect with lactoferrin	[111]
- Igs	Bind and agglutinate the invading organisms in the intestine	[13, 41]
	Prevent bacteria from binding to the intestinal surface (mucosal epithelial cells)	[39, 41]
	Activate complement-mediated bacteriolytic reactions	[13]
	Increase the recognition and phagocytosis of bacteria by leukocytes	[13]
	Limit ETEC-induced damage in the IPEC-1 cell membrane	[43]
Immunological functions:		
- Cytokines (IL-1, IL-2, IL-6, TNF-α and INF-γ)	Mediate the regulation of the immune and inflammatory responses	[114]
	Enhance B and T cell maturation and increase the level of endogenous antibody production	[41]
	Regulate intestinal inflammation and epithelial restitution following mucosal damage	[13]
- Lactoferrin	Activates the phagocytes and immune response to antigens	[13, 37]
	Regulates macrophage activity and proliferation of lymphocytes	[35]
	Binds to receptors in the intestine and acts as a signal to stimulate the immune system	[41]
- Igs	Provide passive immune protection for the neonate and function in antigens binding	[13, 111]
	Perform an immunosuppressive mechanism in the intestine that inhibits excessive proinflammatory responses to oral antigens	[39]
	Are involved in part of the oral tolerance mechanisms in the intestine	[39]
Anti-oxidant functions:		
- Enzymatic anti-oxidants (lactoperoxidase, catalase, superoxide dismutase, glutathione peroxidase)	Are involved in the natural host defence system against invading microorganisms and protection of cells against peroxidative effects	[41, 115]
- Non-enzymatic anti-oxidants (lactoferrin, vitamin E, A, C, selenium, copper, zinc, cysteine)	Promote a proper activity of anti-oxidant enzymes, decrease the intensity of peroxidative processes and protect the immune cells against free radicals	[13, 37, 38, 41]

Table 3 Bioactive components in whey

Bioactive compounds	Biological activities	References
Whey protein:		
- β-lactoglobulin	Mediates cellular activation in both human and murine immune cells	[116]
	Enhances the human neutrophil responses to antigen stimulation	[117]
	Increases cell proliferation and antibody production in murine spleen cell culture	[118]
	Exhibits potential anti-oxidative effects	[37]
- α-lactalbumin	Inhibits the association of the pathogens (ETEC, *S.* typhimurium, and *S.* flexneri) with intestinal cells	[119]
	Activates human neutrophils in response to antigen stimulation	[117]
	Increases the humoral responses of mice and splenic mitogen responses	[119]
	Enhances IL-1β production	[49]
- Serum albumin	Increases antibody production in chickens	[120]
	Exhibits potential anti-oxidative activity	[121]
- Igs	Exhibits anti-microbial activity	[122]
	Provides passive immunity	[37, 119]
- Lactoferrin	Kills or slows bacterial growth synergistically with other factors present in mucosal secretion (Igs, complement, lysozime).	[123]
	Exerts anti-microbial activity through iron deprivation or damages the outer membrane of bacteria	[119, 122, 124]
	Decreases the binding of cholera toxin (CT) and CT's B subunit to GM1-ganglioside on the surface of intestinal cells of mice.	[47]
	Stimulates murine lymphocyte proliferation *in vitro* and Igs production	[125]
	Potentiates the gut immune system, and enhances and reconstitutes the peripheral immune system	[119]
	Attenuates cytokine production of Th1, but not Th2 cell line	[119]
	Exhibits potential anti-oxidative effects	[37]
- Lactoperoxidase	Exhibits anti-microbial functions	[37]
	Inhibits proliferation and IFN-γ production of ovine blood lymphocytes in response to mitogenic stimulation	[49]
- Phospholipid	Exhibits anti-microbial and anti-viral activity *in vitro*	[35]
	Inhibits the excessive expression of proinflammatory mediator expression	[36]
	Prevents gastric mucosal damage	[35, 110]
Oligosaccharides	Stimulate the growth and/or activity of favourable bacteria in the intestine	[10]
	Inhibit the proliferation of pathogenic gram-negative bacteria in the intestine	[37]
	Provide a means to inhibit pathogen adhesion to the intestinal epithelium	[10]
	Modulate (induce) the inflammatory immune responses	[37]
Glycoconjugates	Act as receptor analogues of epithelial cell-surface carbohydrate and can therefore compete with pathogenic bacteria for attachment sites	[10, 16, 124]

However, differences between the results of *in vitro* and *in vivo* studies have often been seen. For example, Sarelli *et al.* [11] showed that lactoferrin (purified from cheese whey) inhibited the growth of *E. coli* O147 *in vitro*, but had no effect on the faecal haemolytic *E. coli* counts and PWD in piglets. Therefore, further *in vivo* studies are needed to confirm the anti-microbial and immunomodulatory activities of the bioactive compounds derived from whey at the whole-animal level. Rivera *et al.* [47] showed that lactoferrin inhibited ETEC H10407 growth in a dose-dependent manner and reduced secretory diarrhoea caused by cholera toxin in mice. Likewise, Wang *et al.* [12] reported that feeding lactoferrin reduced total viable counts of *E. coli* and *Salmonella*, increased the number of *Lactobacillus* and *Bifidobacterium* in the small intestine and improved the morphology of the small intestine (increased villus height and lowered crypt depth) in piglets. This study also revealed that treatment with lactoferrin was able to reduce the incidence of PWD in piglets by 66.2% from piglets received control basal diet. Based on the anti-microbial and immunomodulatory potential of each single isolated whey components, there is a potential in combining these components and feeding them to piglets in order to improve health. This strategy, however, may not give the expected synergistic effects as factors such as lactoferrin and lactoperoxidase exhibit the immunomodulating actions only in their isolated form [48] and the effects of both compounds are diminished when they are combined with other milk factors [48, 49]. However, Kobayashi *et al.* [6] observed that feeding liquid whey to piglets resulted in larger populations of lactobacilli and bifidobacteria and less population of *Clostridium disporicum* in the intestine and they suggested that whey is useful for promoting the intestinal health of piglets and preventing PWD. Concomitant with this, a study in Wistar albino rats by Anbarasu *et al.* [50] demonstrated

Table 4 Bioactive components in casein

Bioactive compounds	Biological activities	References
Anti-microbial peptides:		
- α_{s1}-casein	Isracidin from α_{s1}-casein exhibits antibiotic-type activity *in vivo* against *S. aureus* and *Candida albicans*	[55, 126]
	Isracidin protects mice against *Candida albicans* through stimulation of both phagocytosis and immune responses	[126]
- α_{s2}-casein	Casocidin-I from α_{s2}-casein has anti-bacterial properties against *E. coli*, *Staphylococcus* spp., *Sarcina* spp., *Bacillus subtilis*, *Diplococcus pneumoniae* and *Streptococcus pyogenes in vitro*	[55, 126, 127]
- β-casein	Peptides from bovine β-casein (f193–209) enhance the anti-microbial activity of mouse macrophages	[55]
- κ-casein	GMPs from κ-casein reduce the adhesion of ETEC F4 to the intestinal mucosa, increase the lactobacilli population in the intestine and reduce the overgrowth of enterobacteria in the digestive tract of piglets after an ETEC F4 challenge	[59]
Immunomodulating peptides:		
- α_{s1}-casein	Acts as a humoral immunostimulator in cell culture	[128]
	Phosphoserine-rich residues 59–79 from bovine α_{s1}-casein exhibit a mitogenic activity and a stimulatory activity on Igs production	[48]
	Peptides isolated from bovine α_{s1}-casein (f194–199 and f1–23) increase the phagocytotic activity of human and murine macrophages *in vitro*	[129]
- α_{s2}-casein	Peptides isolated from bovine α_{s2}-casein (f1–32) enhance the proliferative response induced by lipopolysaccharide (LPS), phytohaemagglutinin (PHA) and concanavalin A (ConA) stimulation and the Igs production in mouse spleen cell cultures	[55, 128]
- β-casein	Peptides isolated from bovine β-casein (f63–68, f191–193, and f193–209) increase the phagocytotic activity of human and murine macrophages *in vitro*	[55]
	Peptides (f1–28) enhance the proliferative response induced by LPS, PHA and ConA stimulation and the Igs production in mouse spleen cell cultures	[128]
- κ-casein	Increases proliferation of human peripheral blood lymphocytes *in vivo*	[127]
	GMPs derived from κ-casein inhibit the proliferation of mouse splenocytes *in vitro*	[48, 130]
	GMPs derived from κ-casein have immunomodulatory and bacterial toxin-binding effects, and exert intestinal anti-inflammatory activity in the trinitrobenzenesulfonic acid-induced model of colitis in rats	[56]

anti-diarrhoeal action of whey powder against diarrhoea induced by castor oil. Overall, the potential of whey and whey components to improve the intestinal ecosystem and immune functions of the host seems to prevent ETEC infections in piglets especially during the postweaning period.

Preventive potential of whey permeate on ETEC infections in piglets

Whey permeate is produced by removing protein and other solids from whey resulting in a product with a high content of lactose (~80% lactose). Because of its lactose content, whey permeate has been used as a pure-lactose replacer for weaned piglets to increase their feed intake and growth performance during the first 2 weeks post-weaning [51]. It has been reported that provision of lactose was associated with an increase in population of probiotic species (*Lactobacillus johnsonii* and *Lactobacillus reuteri*) in the intestine of nursery pigs, as lactose may act as a specific substrate for lactobacilli [52]. Taking these facts into consideration, the use of whey permeate as a substitute for the pure-lactose in piglet diet is likely to improve the intestinal ecosystem and health of the piglets as well. In the study of Manurung [8], it was shown that feeding whey permeate at 60 or 120 g/kg reduced the coliform counts in caecum and colon of piglets after weaning although the balance of the colonic microbiota was not affected. As *E. coli* can utilize lactose as an energy and carbon source [53], fermentation of whey permeate to minimize the content of lactose before feeding to piglets may reduce the amount of substrate for pathogenic *E. coli* in the intestine. Consistent with this, we previously showed that feeding fermented whey permeate resulted in a lowered population of coliform bacteria in the gastrointestinal digesta of piglets postweaning challenged with ETEC F4 [34] and Manurung [8] reported that feeding whey permeate fermented with *Weissella viridescens* reduced ETEC F4-induced PWD in piglets.

Beyond the source of lactose, whey permeate contains oligosaccharides with compositions similar to those present in human milk [14]. These oligosaccharides have been shown to exhibit prebiotic activity, and are therefore essential for supporting the growth of LAB in the gut of the host. In addition, oligosaccharides may act as competitive inhibitors for the binding sites of pathogenic *E. coli* on the epithelial surfaces of the intestine [10] and they enhance the inflammatory immune responses against invading pathogens [37]. These properties of oligosaccharides appear to be essential for the prevention of ETEC colonization in the gut and consequently for the development of ETEC-induced diarrhoea in piglets.

Preventive potential of casein on ETEC infections in piglets

Casein is the major source of protein in bovine milk, approximately 80% of the total milk protein fraction [54]. The physical properties of casein in the gut are quite distinct from the properties of whey protein [44]. Because of the low pH condition, micellar casein forms clots within the stomach, which slows gastric emptying and their exit from the stomach. This is in part responsible for the previously mentioned lower absorption rate of casein in its native micellar form compared with whey [44, 45]. Casein is formed by α-, β- and κ-casein subunits, which have been shown to possess several immunomodulatory properties [31, 54]. Unlike whey, no specific anti-microbial or immunomodulatory properties have been proposed for the individual fractions of casein, but various peptides are hidden in an active state inside the polypeptide chain of casein [54, 55]. The polypeptide chains of casein are broken down by enzymatic proteolysis during *in vitro* as well as *in vivo* digestion, resulting in biologically active peptides with anti-microbial and immunomodulatory traits [40, 54]. Table 4 shows the anti-microbial and immunomodulating peptides derived from casein. Apart from the immunopotentiating effects of casein-derived bioactive peptides, several immunosuppressive properties of κ-casein may be of benefit by regulating intestinal inflammation of the host and counteracting excessive production of proinflammatory cytokines [31]. López-Posadas *et al*. [56] reported that κ-casein-derived bovine glycomacropeptides (GMPs) exert intestinal anti-inflammatory activity in the trinitrobenzenesulfonic acid-induced model of colitis in rats, which may be primarily related to actions on Th1 and Th17 lymphocytes and macrophages. In addition, GMPs appeared to preserve the intestinal epithelium of rats [56].

It has been apparent that amino acids are important for the development and/or regulation of the host immune responses. Compared with whey protein, casein contains higher proportions of histidine, phenylalanine, valine, arginine, glutamic acid, proline, serine and tyrosine [45]. In addition, casein and whey differ in the nature of peptides produced by *in vitro* and *in vivo* enzymatic proteolysis [45, 54]. As certain amino acids and peptides may exert specific immunomodulatory effects [45], treatments with milk proteins have been speculated to affect the immune regulation of the host. In our previous study, feeding piglets with casein compared with whey resulted in a higher concentration of total IgA in the intestinal mucosa and a higher percentage of $CD3^+$ T cells in the blood [9]. Because of its potential anti-microbial and immunomodulatory capacities, interest has arisen in the use of casein or biologically active peptides of casein as a means to prevent diarrhoea. For example, β-casomorphins derived from β-casein have been reported to modulate water and electrolyte absorption and exert an anti-diarrhoeal action in animals and in humans [57]. Accordingly, κ-casein-derived GMPs protect the mice against diarrhoea caused by cholera toxins [58]. However, Hermes *et al*. [59] reported that feeding casein-derived GMPs did not reduce the incidence of PWD in piglets challenged with ETEC F4, although the treatment reduced the adhesion of ETEC F4 to the intestinal mucosa. With regard to the whole casein, de Mattos *et al*. [60] reported that a casein-based diet did not interfere with the improvement and/or recovery of patients with persistent diarrhoea. In a recent pig study, inclusion of casein in the diet had no significant impact on the development of PWD in piglets after challenged with ETEC F4 [9]. So far, the data regarding the effect of casein or casein-derived bioactive peptides on the development of PWD in piglets is limited.

Preventive potential of fermented milk and related products on ETEC infections in piglets

Fermented milk products have long been considered as an important component in the human nutrition [61]. Yogurt and kefir are examples of the fermented milk products that have been consumed by human for centuries. Fermented milk is prepared by fermenting the milk with LAB (most commonly used), which is naturally found in milk or which may be added to the milk. This fermentation makes the products totally digestible and results in the release of peptides and amino acids that are more easily utilized by the host. In addition, the enzymatic activity of LAB in fermentation may produce a variety of milk-derived biologically active peptides with anti-bacterial and immunomodulatory properties [30, 62]. It should be noted that the specific activity and amount of bioactive peptides in fermented dairy products may depend on many factors, including the chemical composition of the raw milk, the type of bacterial starters used, the processing of the product and the time of fermentation [30]. In addition to a decreased content of potentially harmful bacteria, fermentation generates a product with high content of favourable bacteria such as LAB. This bacterial line plays, in addition to the bioactive peptides released from milk protein during fermentation, a significant role in

determining the positive health effects of fermented milks and related products [61].

In general, the beneficial effects of fermented dairy products have been attributed to their ability to control the growth of microbial pathogens in the gut as well as to improve the intestinal immune functions of the host [61, 63]. There have been several studies reporting the benefits of fermented milk or related products on the gastrointestinal health of humans and/or animals, as indicated by the number of a specific bacterial population in the gut. For example, fermented milk, prepared with a starter culture *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* and supplemented with the probiotic strains *Lactobacillus acidophilus* NCFM and *Bifidobacterium lactis* Bi-07 and the prebiotic isomalto-oligosaccharide, resulted in an increased number of faecal populations of lactobacilli and bifidobacteria and a decreased number of enterobacilli and *Clostridium perfringens* in the human subjects [63]. These authors also reported that the numbers of lactobacilli and bifidobacteria were significantly increased, whereas enterobacilli were decreased in the mice administered fermented milk when compared with the control group. In another study by de LeBlanc *et al*. [64], the consumption of probiotic-fermented milk either by the mother during nursing or by the offspring after weaning increased the number of bifidobacteria in the large intestine of mice, and this was accompanied by a decrease of the enterobacteria population. In pigs, feeding fermented milk prepared with *Lactobacillus casei* strain Shirota increased the numbers of indigenous lactobacilli and bifidobacteria in the intestine [65]. In addition, feeding fermented milk increased faecal organic acid concentration and reduced the faecal pH. In another study, Ohashi *et al*. [66] reported that feeding yogurt, prepared with *Lactobacillus delbrueckii* subsp. *bulgaricus* strain 2038, increased the relative abundance of lactobacilli in the intestine of pigs.

The impact of fermented milk on the intestinal microbiota of the host has been regarded to have a positive consequence on the modulation of the gut immune responses [64]. This condition, in concert with the activities of the immunomodulatory peptides (produced during the fermentation), may therefore improve host immunity. In the study of Wang *et al*. [63], administration of fermented milk prepared with *B. lactis* Bi-07 and *L. acidophilus* NCFM influenced the cellular and humoral immunity of mice, apparently by increasing delayed-type hypersensitivity (cell-mediated response), plaque-forming cells and half-haemolysis values (closely associated with humoral immunity). In addition, de LeBlanc *et al*. [64] reported that treatment with fermented milk stimulated the IgA^+ cells, macrophages and dendritic cells in the small intestine of mice, and thus improved the intestinal mucosal immunity of the mice. In another study, oral administration of milk fermentation products of *Lactobacillus helveticus* R389 to mice increased total secretory IgA in the intestinal lumen and enhanced the number of IgA and various cytokine producing cells as well as the secretion of interleukin (IL)-6 by small intestinal epithelial cells [67]. The latter authors also revealed that the fermented milk improved the intestinal mucosal immunity by improving the mechanisms that reinforce epithelial and non-specific barriers and the gut functioning at sites of infection. More recent *in vitro* study by Zagato *et al*. [68] reported that *Lactobacillus paracasei* CBA L74-fermented milk had a strong anti-inflammatory activity on dendritic cells in response to the inflammatory enteric pathogen, *Salmonella* typhimurium, and hence may prevent inflammation and tissue destruction.

Owing to the intestinal health benefits of fermented milks, interest has arisen in the application of these products as means to prevent and/or treat the enteric infections [61]. In the study of Guérin-Danan *et al*. [69], provision of milk fermented with *Lactobacillus casei* DN-114 001 significantly decreased the clinical signs of rotavirus-associated diarrhoea, prevented rotavirus infection in all sections of the intestine and reinforced mucosa integrity of suckling rats. In another study, treatment with milk fermented with *L. helveticus* R389 was effective in prevention of *Salmonella* typhimurium infection in mice [67]. Moreover, provision of *L. paracasei* CBA L74-fermented milk protected infants against colitis or enteric pathogens *in vivo* [68]. Unlike for human consumption, fermented milk and related products for pigs are most commonly prepared from the inexpensive by-products of dairy industry such as whey or whey permeate. In general, the application of these products to control the development of ETEC-induced diarrhoea in piglets is still scarce, and the results appear to be variable. As mentioned above, Manurung [8] showed benefits of fermented whey permeate for reducing ETEC F4-PWD incidence. However, Amezcua *et al*. [70] did not observe significant positive effect of feeding fermented liquid whey (plus dextrose) prepared with *Lactobacillus plantarum* on the occurrence of ETEC F4-PWD, although less occurrences of diarrhoea were actually observed in the piglets fed fermented liquid whey than those of fed non-fermented liquid whey.

In addition to the characteristics (e.g. chemical composition) of the raw milk or the milk by-product, studies have revealed that the impacts of LAB on the health of humans and/or animals are species or strain-specific [71]. The difference in species or strain of LAB used as a starter inoculum during fermentation may in part be responsible for the preventing potential of specific fermented milk products or milk by-product on ETEC infections in pigs.

Conclusions

The removal of in-feed antibiotics from swine diets has encouraged researchers to find antibiotic alternatives that can control ETEC infections in piglets postweaning. A number of nutritional strategies have been suggested to

prevent ETEC infections in piglets. BC, milk and milk fractions such as whey and casein contain several bioactive components (e.g. whey protein, casein-derived peptides, lactoferrin, lysozyme, lactoperoxidase, oligosaccharides, phospholipid and glycoconjugates) with antimicrobial and immunomodulatory properties, and thus possessing preventive effects on ETEC infections in piglets postweaning. Likewise, fermented dairy by-products (e.g. whey or whey permeate) appear to exert potentially anti-infective properties against ETEC. The preventing potential of dairy-based products on ETEC infections is attributable to their capacity to improve the intestinal barrier functions, the microbial ecology and the immunity of piglets. To date, the application of dairy-based products as anti-infective agents against ETEC infections in piglets is still limited, and thus further research is needed to elucidate the effectiveness of the products in preventing ETEC infections in piglets. Hence, collaboration between the dairy and the pig production industry is suggested to utilize the preventing potentials of dairy-based nutrition to increase the resilience of pigs toward pathogenic infections and diseases.

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