ABSTRACT

The mucus layer of the intestinal tract plays an important role of forming the front line of innate host defense. Recent studies have suggested that the involvement of feeding natural additives on protection/promotion of mucus production in the intestinal environment is beneficial. The goblet cells continually produce mucins for the retention of the mucus barrier under physiological conditions, but different factors (e.g. microorganisms, microbial toxins, viruses, cytokines, and enzymes) can have profound effects on the integrity of the intestinal epithelium covered by a protective mucus. The intestinal mucus forms enterocytes covered by transmembrane mucins and goblet cells produce by the secreted gel-forming mucins (MUC2). The mucus is organized in a single unattached mucus layer in the small intestine and in two mucus layers (inner, outer) in the colon. The main part of the review evaluates the effects of natural additives/substances supplementation to stimulate increased expression of MUC2 mucin in the intestine of animals.

Key words: additives; intestinum; layer, mucin; mucus; protection

INTRODUCTION

The important role of the intestine is: digestion, absorption as well as the elimination of ingested/undigested food, microorganisms and their microbial products and luminal contents. The intestine is the major line of bacterial colonization and the system of dynamic balanced interactions between microbiota, intestinal epithelial cells, mucus layers as well as host immune defense to maintain the intestinal mucosal homeostasis [26]. The mucosal tissues in the gastrointestinal tract are exposed to a large number of exogenous, water or food born microbiota and their products (e.g. bacteria, parasites, viruses, enzymes and toxins). The epithelium of the intestinal tract is covered by a layer of mucus composed predominantly of mucin glycoproteins that are synthesized and secreted by the goblet cells [41]. The mucus layer acts as a medium for: protection, lubrication, transport, a physical barrier and a trap for microbes.
as well as a positive environment for the beneficial endogenous microbiota to adapted to symbiotic living [12].

Passage through the small intestine is relatively fast, which gives limited time for bacteria to increase in number. This is in contrast to the colon, where bacteria reside for a much longer time. Mucus is important for the protection of the gastrointestinal tract [24]. The mucus function is to separate the luminal content (especially bacteria) from direct contact with the epithelial cells [25].

**CHARACTERIZATION OF MUCUS IN THE INTESTINAL EPITHELIUM**

The intestinal epithelium is covered by a protective mucus gel composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells [12]. The intestinal mucosal epithelium consists of four principal cells: absorptive enterocytes, enteroendocrine cells, Paneth cells and goblet cells [26].

The intestinal enterocytes have their apical surfaces covered by transmembrane mucins and the whole intestinal surface is further covered by mucus, built around the gel-forming mucin MUC2 [23]. Goblet cells synthesize secretory mucin glycoproteins (MUC2; secreted gel-forming mucin) and bioactive molecules such as: epithelial membrane-bound mucins (MUC1, MUC3, MUC17), trefoil factor peptides (TFF), resistin-like molecule β (RELMβ) and Fc-γ binding protein (Fcgbp) in the intestine [26]. The mucus of the small intestine has only one layer, whereas the large intestine has a two-layered mucus where the inner, attached layer has a protective function for the intestine, as it is impermeable to the luminal bacteria. Goblet cells function can be disrupted by certain factors (e.g. microbes, microbial toxins and cytokines) that can affect the integrity of the mucus barrier (e.g. inhibit mucin production/secretion, alter the chemical composition of mucins, and degrade the mucus layer [8]). Goblet cells and their main secretory product, mucus/mucus system differs substantially between the small and large intestine, although it is built around MUC2 mucin polymers in both. The surface colonic goblet cells secrete continuously to maintain the inner mucus layer, whereas goblet cells of the colonic and small intestinal crypts secrete upon stimulation [6]. The epithelial cells as well as the enterocytes provide the best separation of the luminal material from the lamina propria. Of special importance is the enterocyte apical glycocalyx that is built by transmembrane mucins and the tight junctions that firmly anchors the cells to each other [25].

**INTESTINAL MUCUS LAYER**

The intestinal mucus gel layer is an integral structural component of the intestine used for protection, lubrication, and transport between the luminal contents and the epithelial cells [12]. For protection, the gastrointestinal epithelium is covered by mucus in which the main constituent is the secreted gel-forming mucins (in the intestine MUC2) [16]. The net-like mucins forming the intestinal mucus have different properties in the small and large intestine. The regulation of mucus secretion is controlled by the neural, hormonal and paracrine system and also by the immune system [6].

The small intestine has a single unattached mucus layer and the colon is composed of two mucus layers [25]. In the small intestine the large pore sizes allowing bacteria or bacterial particles/products/biofilms to penetrate the mucus. The mucus fills the space between the villi and covers the villi tips, but bacteria are typically not found in contact with the epithelium except at the villus tip. The carbohydrate-rich polymeric mucin binds water that limits and slows down diffusion. The antibacterial peptides and proteins secreted from the crypt of Paneth cells and enterocytes into the mucus are of major importance for keeping bacteria at a distance. This penetrability of the small intestinal mucus may be the reason why pathogenic bacteria mostly infect this region of the gut. The small intestinal mucus is normally non-attached [6]. The thickness of the inner mucus layer in the distal colon has been estimated to be approximately 50 μm in the mice and 100 μm in the rat. The mucus in the colon is organized in two layers (Figure 1): an inner, stratified mucus layer that is firmly adherent to the epithelial cells and approximately 50 μm thick; and an outer, non-attached layer that is usually approximately 100 μm thick as measured in the mouse [24].

The inner mucus layer is converted into the outer layer, which is the habitat of the commensal flora. The outer mucus layer has an expanded volume due to proteolytic activities provided by the host but probably also caused by commensal bacterial proteases and glycosidases. The numerous O-glycans on the MUC2 mucin not only serve as nutrients...
for the bacteria but also as attachment sites and, as such, probably contribute to the selection of the species-specific colon microbiota. This is in contrast to the small intestine, where the mucus is discontinuous and is secreted at the top of the crypts and then moves upward between the villi [23, 24].

THE MAJOR COMPONENT OF INTESTINAL MUCUS

The viscoelastic, polymer-like properties of mucus are derived from the major gel-forming glycoprotein components called mucins [5]. For protection, the gastrointestinal epithelium is covered by mucus in which the main constituent is the secreted gel-forming mucins: in the stomach MUC5AC and in the intestine MUC2, which are also the two most similar of the secreted gel-forming mucins [16]. Other components of intestinal mucus are Fcgbp protein, Clca3, Zg16, Agr2, immunoglobulins, and many more proteins. The mucus also contains cellular proteins because cells are continuously shed out into the lumen and trapped in the mucus. The Fcgbp protein or Fc Ig binding protein was originally suggested to bind IgG [24]. Mucins refers to high molecular weight, appear as long filaments with a wide range of lengths ranging from 200 to more than 1000 nm (corresponding to $0.5 \times 10^6$ to $25 \times 10^6$ Da) [29], polydisperse, highly glycosylated molecules consist of a peptide backbone containing alternating glycosylated and nonglycosylated domains, the carbohydrate content of mucins (makes up to 60—90 % of their molecular mass) with O-linked glycosylated (O-glycans) regions comprising 70—80 % of the polymer. N-Acetylgalactosamine, N-acetylglucosamine, fucose and galactose are the 4 primary mucin oligosaccharides. Secretory mucins are secreted from the apical surface of specialized columnar epithelial cells...
(goblet cells) by 2 distinct processes; baseline secretion and compound exocytosis [12].

MUC2 mucin — the major mucus component is stored in a condensed way in the goblet cell mucin granules. When the granules are released, MUC2 mucin expands in volume. The formation of MUC2 organized sheets due by the net-like structure of the MUC2 polymer could be responsible for the lamellar stratified appearance of the inner mucus layer [24]. The primary sequence of the MUC2 mucin encodes approximately 5200 amino acids [3]. The amino acids act as attachment sites for the O-glycans [45], that are attached to the proline, threonine and serine in the PTS (proline-threonine-serine) domains [3]. The PTS domains are often highly repetitive. The O-glycans make the mucin domains highly protease resistant. Once the mucin apoprotein reaches the Golgi apparatus, it is densely decorated by consecutive additions of monosaccharides, a modification which turns these domains into long, stiff bottle brush-like rods where the glycans make up to more than 80% of the mass [3]. The high density of these often branched oligosaccharides gives the mucin domains their extended structure and will bind water molecules to give the mucins their viscous properties [3] and give mucins their high water-binding capacity [18].

INTESTINUM AND BACTERIA

Animals assemble and maintain a diverse but host-specific gut microbial community. In addition to characteristic microbial compositions along the longitudinal axis of the intestines, discrete bacterial communities form in microhabitats, such as the gut lumen, colonic mucus layers and colonic crypts [14].

The distal small intestine and the large intestine are the reservoirs for an enormous and complex community of microorganisms (about 1000 species belonging to the phyla Bacteroides and Firmicutes; in the number of $10^{12}$ colony forming units per gram of faeces in the distal colon) [22]. The mucus in the small intestine fills up the space between the villi and covers these, but is not attached to the epithelium and has a structure that can allow particles as large as bacteria to penetrate. The mucus protection acts as a diffusion barrier with a high concentration of antibacterial products close to the epithelium and few bacteria reaching near the cell surface. The higher bacterial load in the colon and the slow transit time requires a different protective strategy [22]. The commensal bacteria in the colon live and thrive in the outer loose mucus layer. This is possible after the MUC2 mucin network has expanded in volume, such that it allows the bacteria to penetrate into the mucus network. Once inside the mucus gel, the commensal bacteria can use its large number of glycan-degrading enzymes that release one monosaccharide at a time from the mucin glycans—a very important energy source for commensal bacteria [18].

In this way, it will take some time for the bacterial enzymes to reach and expose the mucin protein core for proteolysis that will degrade the mucin protein core. The mucin polymeric network of the loose mucus is maintained for some time to give a relatively thick outer mucus layer. The volume expansion of the mucus network of the outer loose mucus layer is a process that involves endogenous proteases of the host that degrade MUC2 in such a way that the polymeric network remains largely intact [24]. The outer colon mucus layer has an expanded volume due to proteolytic activities provided by the host but probably also caused by proteases and glycosidases of the commensal bacterial. The numerous O-glycans on the MUC2 mucin not only serve as nutrients for the bacteria but also as attachment sites and, as such, probably contribute to the selection of the specific colon flora [23].

The inner colon mucus layer is rapidly renewed and converted into the outer mucus layer by host controlled endogenous proteolytic processing. MUC2 mucin forms an enormously large net-like structure that builds the laminated inner mucus layer that largely acts as a size exclusion filter excluding bacteria. In the absence of MUC2 mucin, there is no inner mucus layer and bacteria reach the epithelial cell surface, penetrate the crypts and are also found inside epithelial cells, something that leads to severe inflammation [25].

Enzymatic digestion of the mucus coat provides access to readily available sources of carbon and energy and enables bacteria to reach the epithelial surface. Mucin degradation is a multistep process that begins with proteolysis of the nonglycosylated "naked" regions of the mucin glycoproteins by host and microbial proteases. This initial step markedly reduces mucin gelation and viscosity. Mucin glycopeptides are then degraded by various bacterial enzymes [16].

The maintenance of gut health is complex and relies on a delicate balance between the diet, the commensal
microflora and the mucosa, including the digestive epithelium and the overlying mucus layer. Superimposed on this balance is the frequent presence of enteric bacteria with pathogenic potential, the proliferation and metabolic activity of which may perturb digestive function, and lead to diarrhoea, poor growth rates and even death. Such enteric infections with pathogenic bacteria are common especially during the weaning period in young animals [21, 28, 38, 42].

Bacterial species present in the mucus show differential proliferation and resource utilization compared with the same species in the intestinal lumen. Functional competition for existence in this intimate layer is a major determinant of microbiota composition in the host [27]. Adherence of bacteria to the surface layer cells/epithelial cells or colonize cellular secretions (mainly mucin) of the host enables commensal bacteria and potential pathogens to overcome flushing mechanisms which cleanse mucus membranes. Although adhesion is essential for maintaining members of the normal microflora in their host, it is also the crucial first stage in any infectious disease [47]. Diet as well as dietary components (e.g. dietary fibre, natural additives/substances) have an important influence on gut health, including effects on the proliferation of pathogenic bacteria, and it can provide either beneficial or harmful input [38]. One of the protective factors of the beneficial bacteria against microbial pathogens is the formation of biofilms representing an initial barrier delaying penetration of the antimicrobial agents including physical/chemical diffusion barriers to make resistance of the transport of antimicrobial agents [32]. The bacterial species can attach to an intestinal surface in the form of a biofilm. Microbial biofilms (single/multiple bacterial species) are ubiquitous self-produced polymeric exopolysacharide matrix or glocalyx expressed properties distinct from planctonic cells and play an important role in the host digestive processes, gut physiology and metabolism [31].

NATURAL SUBSTANCES AND MUC2 MUCIN

Natural substances belong to a large group of feed additives. Feed additives are products used in animal nutrition for purposes of improving the quality of feed and the quality of food from animal origin, or to improve the animals’ performance and health, e.g. providing enhanced digestibility of the feed materials. Feed additives may not be put on the market unless authorisation has been given following a scientific evaluation demonstrating that the additive has no harmful effects, on human and animal health and on the environment [15].

The selected supplemented natural substances/additives such as probiotics as well as plant extracts/plant essential oils play the important role to protection/prevention of intestinal mucus layers and their compounds from colonization/invasion by the pathogens as well as have a stimulating effect on MUC2 gene expression (MUC2 mucin is forming part of mucous barrier to protect the intestinal epithelium). Major probiotic mechanisms of action include: enhancement of the epithelial barrier, increasing adhesion to intestinal mucosa and inhibition of pathogen adhesion, competitive exclusion of pathogenic microorganisms, production of anti-microorganism substances such as bacteriocins and modulation of the immune system [4].

Probiotics or/and bacteria with probiotic properties may promote mucous secretion as one mechanism to improve barrier function and the exclusion of pathogens [7]. The addition of Lactobacillus casei GG to the enterocyte monolayer surface resulted in significantly increased MUC2 expression compared to the untreated monolayers; in addition, both mucin and the probiotic strain Lactobacillus casei GG have an inhibitory effect on bacterial translocation in both an in vitro Caco-2 cell model and a neonatal rabbit model [35]. Exposure to both gram-positive (Staphylococcus aureus, Staphylococcus epidermis, and Streptococcus pyogenes) and gram-negative (Pseudomonas aeruginosa and Escherichia coli) bacteria increase MUC2 and MUC5AC gene expression in mucin-producing NCIH292 epithelial cells as well as the probiotic strains Lactobacillus plantarum 299v and Lactobacillus rhamnosus GG increase expression of both MUC2 and MUC3 genes in HT29 colon cell cultures [13, 36].

Lactobacillus rhamnosus GG-derived soluble protein — p40, has been shown to transactivate the EGF receptor by inhibition of apoptosis and preservation of barrier function in intestinal colonic epithelial cells, thereby ameliorating intestinal injury and colitis. The results suggest that p40-stimulated activation of epidermal growth factor receptor (EGF receptor) contribute up-regulation of mucin production to protect the mouse colonic and small intestinal epithelial cells from injury [46].

O h et al. [39] evaluated the effect of mulberry leaf extract fermented with Lactobacillus acidophilus A4 on in-
intestinal mucositis induced by 5-fluorouracil in rats. These treatments stimulated MUC2 and MUC5AC gene expression and mucin production and showed protective as well as synergistic therapeutic benefit effects on 5-fluorouracil-induced mucositis in a rat model.

Aliakbarpour et al. [2] quantified the intestinal MUC2 gene expression and/or intestinal morphology after probiotic strains supplementation in chickens. The relative expression of MUC2 mRNA was significantly greater in the jejunum of the mono-strain (*Bacillus subtilis*) probiotic diet fed chicks compared with the control group, but no significant differences were found in relative higher intestinal MUC2 gene expression between broilers fed with mono-strain diet and multi-strain lactic acid bacteria (LAB) probiotics (*Lactobacillus casei*, *Lactobacillus acidophilus*, *Bifidobacterium thermophilum* and *Enterococcus faecium*) supplemented diets. However, inclusion of lactic acid bacteria strain diets (multi-strain feeding) significantly increased goblet cell number and villus length. The higher synthesis of the mucin gene after probiotic administration may positively affect bacterial interactions in the intestinal digestive tract, intestinal mucosal cell proliferation and consequently efficient nutrient absorption. The average MUC2 expression as well as villus length, and crypt depth increased in a linear fashion after the administration of *Bacillus licheniformis* to a probiotic diet in turkeys [30].

The positive impact of applied probiotic strain *Enterococcus faecium* EF55 (the isolate from chicken origin producing bacteriocin enterocin Ent 55; [43]) on mucus dynamics, intestinal morphometry as well as the increased proliferative activity of epithelial intestinal cells in the jejunum of chickens was demonstrated after infection with *Salmonella enterica* serovar *Enteritidis* phage type 4 [17, 34, 44]. The prebiotic properties of β-glucan as the principal structural components of the cellular walls of grains, yeasts, algae and some bacteria were confirmed by β-glucan supplementation to the chicken diet by the significantly increased thickness of mucus in the caecum [11].

The mucus layer plays an important role in the gut protection against digestive enzymes, chyme and pathogens as well as it acts as a lubricant and facilitator of nutrient compounds transport [18, 40]. In addition, some phytochemical compounds seem to show properties to promote intestinal mucus production [19]. The diet addition of plant extracts and/or essential oils obtained from Labiatae family herbs (e. g. *Thymus vulgaris* L., *Salvia officinalis* L., *Origanum vul- gare*) caused the increased quantity of acid mucins in the duodenum/ileum of chickens [9, 10]. The beneficial effect of oregano components on jejunal mucin quantity and its turnover in relation to oregano and coccidia was found in ROSS 308 hybrid broilers infected with *Eimeria acervulina* [33]. The increased MUC2 gene expression was observed in the small intestine of broiler chickens by the diet supplementation of turmeric, thyme and cinnamon [40]. Also the gene expression of mucosal barrier proteins MUC2, MUC3 and villin were up-regulated as well as a decreased colonic damage score was showed by administration of an ethanolic extract of the stem bark of *Terminalia catappa* L. to trinitrobenzenesulfonic acid-treatment colitic rats [1].

Also the supplementation of carbohydrates or specific amino acids of proteins such as threonine to a diet demonstrated the alteration of intestinal mucin secretion by increasing of MUC2 expression in broiler chickens [37, 41]. The increased villus height in the ileum, the ratio of villus height to crypt depth in jejunum and ileum, goblet cells density in the jejunum and ileum was observed by the threonine treatment in chickens [20].

The mucus layer provides homeostasis in the intestine by affecting several aspects of the intestinal biology (physical/chemical protection, immunomodulation and growth). An intestinal part modulating the communication between the luminal contents including microbial bacteria and the mucosa is the mucus layer and its secretion, which plays the important role on the influence of pathogen’s behaviour in the intestinal ecosystem. Several different studies and results have demonstrated the protective and beneficial effects of natural substances, first probiotics, on maintaining the physiological intestinal environment function.

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