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Role of trefoil factors in maintaining gut health in food animals

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It is imperative to preserve the integrity of the gastrointestinal system in spite of the persistent existence of harmful chemicals and microbial flora in the gut. This is made possible by essential healing initiators called Trefoil factors which helps in mucosal reconstitution and tissue development on the gastrointestinal surface. The trefoil factors are a class of abundant secreted proteins that are essential for epithelial continuity (TFFs). Trefoil factor family (TFF) proteins are biologically active peptides that play significant role in safeguarding, restoring and continuity of the gastrointestinal tract (GIT) epithelium, through collaborative modulations with mucins in the mucosal layer. These peptides are readily produced in reaction to epithelial damage in the digestive tract, thereby contributing to the healing and restituting of the epithelial layers of the intestine. In addition, considerable evidence indicated that TFF peptides trigger proliferation, migration and angiogenesis, all which are crucial processes for wound healing. There is also increasing evidence that TFF peptides modulate the mucosal immune system. These protective properties, suggest that dietary manipulation strategies targeted at enhancing the expression and synthesis of TFF peptides at optimal levels in the GIT epithelium, may constitute a plausible alternative strategy to the use of in-feed antibiotic growth promoters to maintain epithelial integrity and promote resistance to enteric pathogens. This review describes TFF peptides, with importance to their biological functions and involvement in gastrointestinal mucosal protection and repair in food animals.

KEYWORDS

trefoil factor family, peptides, gastrointestinal mucosal injury, mucins, epithelial repair

1 Introduction

The gastrointestinal tract is a complex environment that assembles myriad of components such as peptides, cellular matters, pathogens, microorganisms and nutritional biomaterials. The presence and interaction of these components determines the outcome put up against threat or challenge to the system (1). The GIT harbors immune cells and molecules that grants immunity and biological responses to pathogens and toxins made possible to protect the lubricated epithelial surface of the GIT. Consistent with these functions, it's interesting to note that TFFs are mostly expressed in the healthy gastrointestinal (GI) tract (2), thus it makes sense that they would either positively or negatively correlate with stomach disorders. The peptides known as the trefoil factor family (TFF1, TFF2, and TFF3) are essential for the upkeep, preservation, and repair of the gastrointestinal system.

Intestinal trefoil factor (ITF) is an important member of TFF domain peptides with pivotal roles in the protection of the intestinal epithelium (1, 3, 4). There are three secretory proteins (12–22KD) that make up TFF, and are often produced in adequate quantity in the tissues containing cells that secrete mucus (3). TFF proteins are readily produced in reaction to epithelial damage in the digestive tract (1, 4) thereby contributing to the healing of the gastrointestinal epithelium (5). When an injury occurs to the epithelium, TFF are produced close to the injury site and migrate to the site by a process that causes a rapid change in the shape and volume of the cells, thereby allowing free flow of water in and out of the cells (6). There are three known mammalian Trefoil Factors namely TFF1 or the pS2 (7), pancreatic spasmodic polypeptide TFF2 (8), and TFF3 (or ITF) (1, 9). The distribution of TFF cut across numerous tissues such as Liver, Pancreas, Kidney, brain salivary gland as well as the respiratory tract (10). The isomers of TFF are found in the gastrointestinal tracts with variation in distributions (10). The TFF1 is a 9-KDa mucin-associated secretory protein primarily expressed in the gastrointestinal (GI) tract mucosa, and is characterized by a 38- to 39-amino-acid trefoil domain which contains six cysteine residues that form a cloverleaf disulfide structure (11). This structure renders TFF1 resistant to both acid and enzymatic breakdown, and thus perhaps responsible for the ability of TFF1 to inhibit tumor growth in the gastrointestinal tract (GIT) (12). TFF1 exerts its protective action by stimulating the restitution of the tissues destroyed as a result of inflammation and ulceration (12). Pancreatic spasmodic polypeptide TFF2 is secreted by the antral and pyloric region of the stomach, and the Brunner's glands of the duodenum (13). In a healthy animal, there is an interaction between TFF2 and mucins that often lead to development and maintenance of the mucus barrier in the gastric and duodenal sites (14). At the site of an injury, TFF2 can be rapidly expressed in the epithelia of the entire GIT and critically contribute to epithelial "restitution" and regenerative processes (9). Furthermore, TFF could modulate immune responses, cell chemotaxis, and cytokine release (15). The gastrointestinal mucosa is the primary site for TFF3 secretion (16). From this site, TFF3 is excreted from the granules with mucins onto the surface of the epithelium, thereby forming a protective mucosal layer. The mechanism of action of TFF3 is such that it stimulates the activation of extracellular signal-regulated kinase/mitogen-activated protein kinase and activates serine phosphorylation of Akt, a kinase associated with apoptotic pathways (17, 18), thereby modulating the E-cadherin/catenin cell adhesion complex in various ways. For instance, TFF3 peptide caused reduction in the level of E-cadherin, β -catenin, α -catenin and the adenomatous polyposis coli (APC) protein in HT-29 cells in, consequently resulting in significant alterations in cell

aggregation, detachment from the substratum, and translocation of APC from the cytoplasm to nucleus (19).

Since TFFs are mostly expressed in the healthy gastrointestinal (GI) tract, there exist correlation, either positive or negative, between them and stomach disorders (20). This review aims to examine the significance and therapeutic potentials of TFF in the gastrointestinal tract health, provide current knowledge on the target factors and steer future research toward their perceived mechanisms and clinical applications in gastrointestinal disease management.

1.1 Trefoil factor (ITF): types, structure, and functions

The process of breaking down and absorbing consumed food and liquids is carried out by the gastrointestinal (GI) system. The intricacy of the GI tracts and complex nature of biological processes that occur with the system, preserving the gastrointestinal tract's integrity and homeostasis is crucial even in the face of constant exposure to microbial flora and other detrimental agents. The gut tissues and epithelium are designed to express polypeptide growth factors capable of stimulating cellular growth, proliferation, or differentiation after attachment to surface membrane receptors (21). These factors include epidermal growth factor (EGF), transforming growth factor beta (TGF- β), insulin-like growth factor (IGF), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), trefoil factor, wingless (Wnt) family, and Hedgehog (Hh) family proteins (22). The latter three has been identified over the past few decades and discovered to play mitogenic roles in the development and adult function of the GI tract in animals. TFF peptide is discovered in both serum and luminal fluid of the GIT. TFF has been reported to be expressed in a cell-specific manner on the mucosal surfaces of normal gastrointestinal tissues (21). ITF proteins have been demonstrated to have important functions after mucosal damage by improving superficial cell resistance, lowering epithelial cell permeability, strengthening the cell-to-cell connection in intestinal mucosal injury, suppressing apoptosis and inflammatory signaling (2).

Additionally, some research has shown that ITF reduces the inflammatory response by preventing the digestive tract's production of pro-inflammatory factors (23, 24). These small molecular peptides factors called TFF are produced by the goblet cells scattered across the absorptive cells onto the gastrointestinal tract's surface. It is believed that mechanism of action of TFF is via luminal secretion of the peptide from the epithelial cells lining the GI tract, suggestive that systemic TFF peptides can be secreted into the gastric lumen in tandem with mucus secretion from mucous cells (10). The concept of growth factor started in the 80's with the identification of spasmodic protein SP (25) and pS2 (7). The advancement in knowledge and efforts over subsequent decades in the identification of some cell division activities, proteins classified trefoil (TFF) and subsequent of a third ITF in mammals (9). The pS2, spasmodic peptide, and intestinal trefoil factor which share a distinct motif of six cysteine residues defined as "trefoil" domain, were renamed with the current nomenclature of TFF1, TFF2 and TFF3, respectively, in 1997 at the Philippe Laudat Conference

Abbreviations: TFF, Trefoil factor family; GIT, gastrointestinal tract; ITF, Intestinal trefoil factor; PI3K/Akt, phosphatidylinositol 3'-kinase; TGF- α , transforming growth factor-alpha; GKN2, gastrokine; TFF1, Trefoil Factor 1; TFF2, Trefoil Factor 2; TFF3, Trefoil factor 3; COX2, cyclooxygenase; IL-6, Interleukin-6; TNF- α , tumor necrosis factor- α ; NF- κ B, Nuclear factor-kappaB; IFABP, intestinal fatty acid binding protein; LFABP, liver fatty acid binding protein; IAP, Intestinal alkaline phosphatase.

(26, 27). The protein data bank (PDB) structure of TFF1, TFF2, and TFF3 is shown in [Figure 1](#).

The TFFs are small secretory peptides with three loop structures that contain a highly conserved motif of cysteine disulfide bonds that maintain the functional stability of the protein. Depending on anatomical location, TFF proteins are both constitutively and inducible expressed (28). The trefoil, or P domain, is a conserved 38–39 amino acid motif containing six cysteine residues held together by three pairs of disulfide bonds configured 1–5, 2–4, and 3–6 (28, 29). It is a collection of secreted peptides with a C-terminal dimerization domain and a trefoil domain (s). The TFF2 has two trefoil motifs as a result of chromosomal duplication, whereas TFF1 and TFF3 only have a single trefoil domain (9, 30, 31).

TFF1 is a monomer that consists of a single trefoil domain, which includes about 40 amino acids forming a stable three-loop structure. This domain is characterized by six conserved cysteine residues that form three disulfide bonds, creating a compact and stable structure (26, 29). TFF1 is glycosylated and this influences its protective function in mucosal tissues. TFF2 is a dimer, composed of two trefoil domains, making it structurally different from TFF1 ([Figure 1](#)). These domains are also stabilized by three disulfide bonds in each domain and can form dimers through non-covalent interactions, and this dimerization is critical for its biological activity (26) and it is also similarly glycosylated to TFF1. TFF3 is a dimer, much like TFF2, but can also exist in a monomeric form. It contains a single trefoil domain, similar to TFF1, but typically forms dimers through disulfide bonds between two TFF3 monomers (26). The dimerization of TFF3 enhances its mucosal protective properties and like TFF1 and TFF2, TFF3 is also glycosylated.

The cell-specific patterns of occurrence of TFF expression result in a regional colocalization of specific TFF proteins and secretory mucins, providing a potential molecular basis for the functional mucosa specificity. Trefoil factor family peptides are typically co-secreted together with mucins and interact with mucins in the lumen to enhance protective barriers through mucosal innate immune defense, mucosal repair, and prevention of the infiltration of microorganisms and toxin insults (32, 33). TFF proteins and secretory mucins colocalize in certain regions as a result of cell-specific TFF expression patterns, which may provide a mechanistic explanation for the functional mucosa specificity. It is significant to note that expression patterns of TFF proteins vary greatly among infection specific, species and age-related. Early in the development of the embryo, TFF expression takes place, with the gastrointestinal tract expressing all three TFF proteins in unison with individual expressions becoming more restricted resulting in cell-specific patterns in adult (34, 35).

The expression of TFF is modulated by different factors. Expression of TFFs in the gastrointestinal tract is abundant and is second, in weight of protein, only to the mucins. TFF1 is mostly restricted to the pit cells of the stomach, TFF2 to mucous neck cells of the gastric gland, and TFF3 to goblet cells of the small and large intestine (22).

1.1.1 Interactions and regulations with other immune cells

The immune system of the mucosal of the GIT is crucial because it keeps pathogens from gaining entrance into the body

system while simultaneously facilitating the transfer of nutrients from the intestinal lumen to the systemic circulation for proper function (1). TFF peptides are major secretory products of mucous epithelia and play a multifunctional role in cytoprotection, apoptosis, and immune response. TFF influence the activity of immune cells and cytokines, helping to regulate inflammation and maintain immune homeostasis in the gut ([Figure 2](#)). Trefoil factors play significant role in the homeostasis of the gastrointestinal tract and are expressed rapidly in response to injury and are up-regulated in inflammation and ulceration (3, 21, 22). This perceived protective is achieved through regulations by pro-inflammatory and anti-inflammatory cytokine expressions (32). Cytokines such as IL-1 β and IL-6 down-regulate TFF genes expression by transcriptional repression in GI cells; IL-6 represses TFF1, IL-1 β and IL-6 inhibit TFF2 and TFF3 transcription, TNF- α (acting via NF κ B transcription factor) decreases trefoil expression during inflammation thereby delaying mucosal restoration, and cytoprotection (36) while IL-4 and IL-13 induce TFF2 (37).

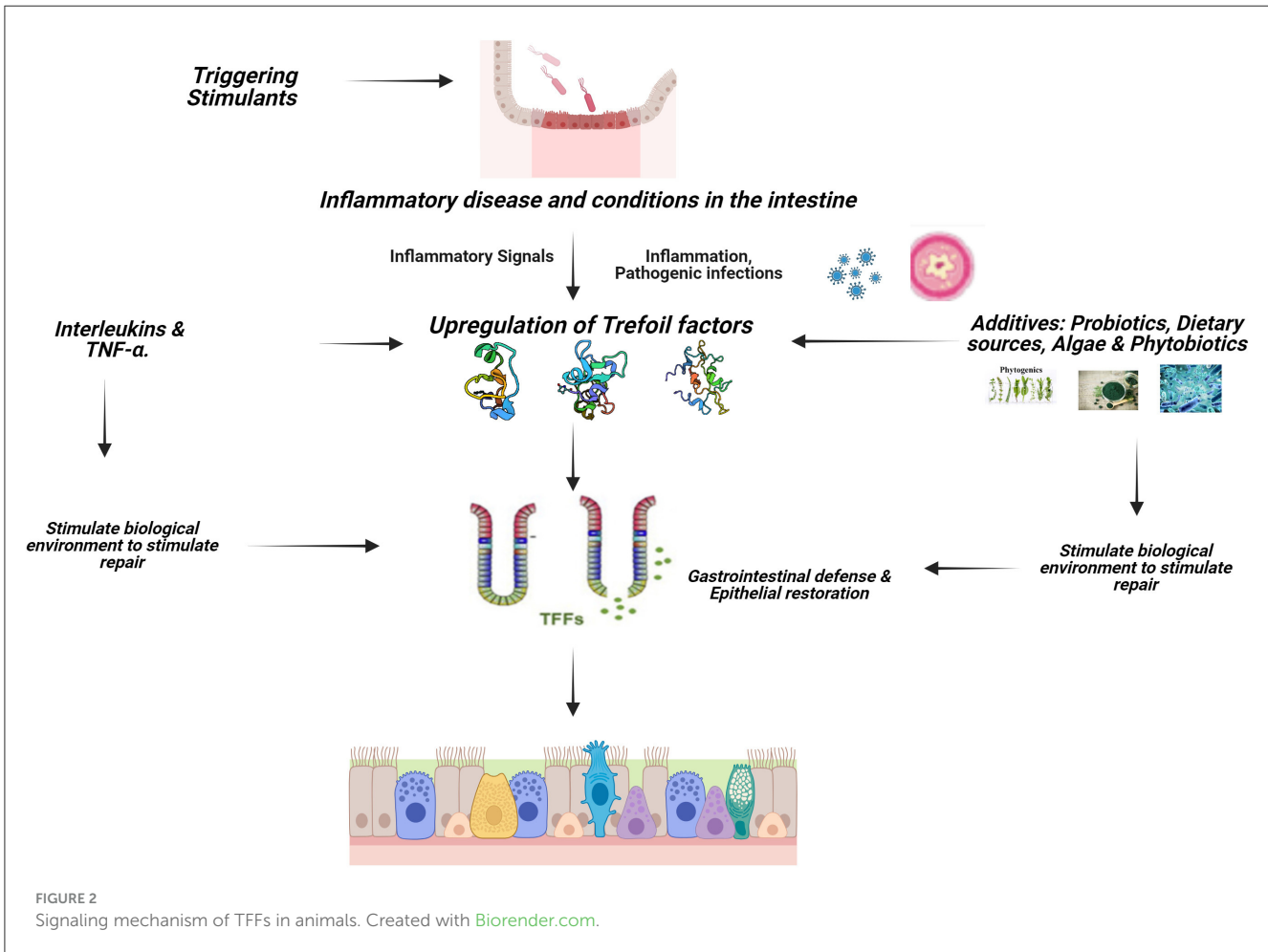
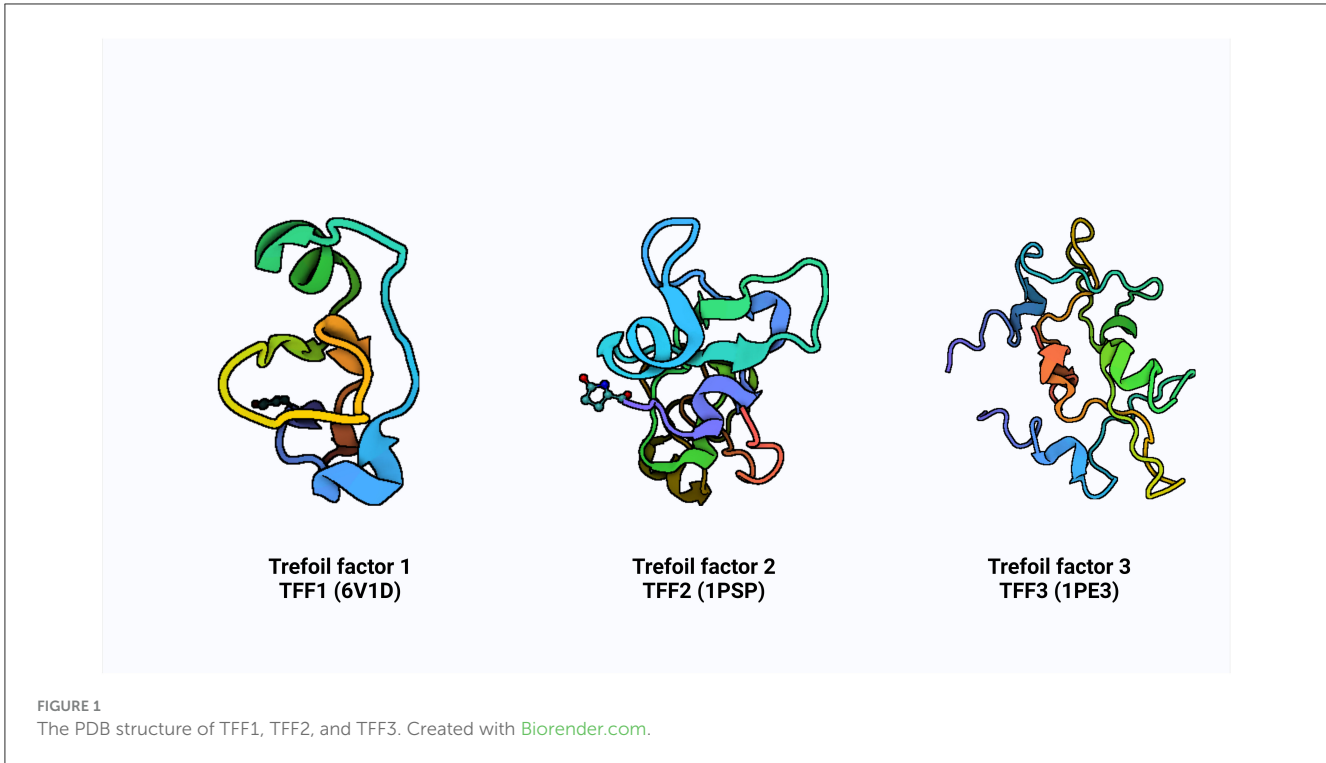
Pattern recognition Toll-like receptors (TLRs) is a key player in the initiation of an inflammatory process, TLR2 regulates TFF3 expression in the gut to control terminal GC differentiation, which protects the intestinal mucosa against apoptosis (38). The gut epithelial activating transcription factor 4 (ATF4) plays an important role, as its deficiency results in decreased peptide trefoil factor 3 levels (39). The identification of TFF-interacting proteins presents a potential regulatory mechanism for TFF function in the GI tract.

1.2 Trefoil factor family peptides (TFF1, TFF2, and TFF3)

1.2.1 Trefoil factor 1

TFF1 is mainly expressed in the fundus and antrum of gastric mucous cells but with low expressions in ileum, colon, salivary glands, pancreas and respiratory tract (2). The major site of expression of TFF1 is primarily in the gastric foveolar cells and surface epithelial cells across the entire stomach, and it is also present in the upper ducts of brunner's glands localized in the proximal duodenum, salivary glands and gastric juice (10). TFF1 plays an important role in the number of differentiated cells in the epithelium of the stomach. TFF1 is induced by hypoxia-inducible factor-1 (HIF-1 α) pathway resulting in the inhibition of activation of IL6-STAT3 pro-inflammatory signaling axis that stimulate gastric lesions (40). The Tff1 and the gastrokine genes (Gkn1, Gkn2, and Gkn3) are expressed selectively in the duodenum and but at much lower levels when compared with the stomach (41). The subcutaneous administration of TFF1 has been recorded to suppress tumor growth *in vivo*, affirming that TFF1 enters the bloodstream to reach tumor cells, where it functions as a tumor suppressor.

TFF1 help cells to counteract bacteria colonization and the development of a chronic inflammation. TFF 1 induces the following function with respective interactions; MUC2/MUC5AC (protection of the mucosal) (42), Fragment of IgG Binding Protein (FCGBP) (binding of microorganisms) (43) and Gastrokine 2 (GKN2) (antiproliferative and pro-apoptotic and homeostasis) (44).



1.2.2 Trefoil factor 2

The spatial localization of TFF2 in the gastrointestinal tract varies among different species and this offers highlights on species-specific expression patterns and the potential functional diversity of TFF genes across species (13). TFF2 peptides expressions have been found in acinar cells of the pancreas, gastric, pyloric and Brunner's glands (Humans and rodents), mucous cells of stomach and throughout the intestine (13, 18). The functional diversity can be increased by the interactions of each trefoil loops with different substrates such as the amino acids. Expressions shift suggests a spatial transition of expression abundance from upper intestine to lower intestine as the gut matures as illustrated in the study in chickens (45). It has been particularly observed that TFF2, when added to a mucin solution, greatly improves the viscosity and flexibility of the mucus, indicating that TFF2 plays a vital role in repair responses to maintain mucosal surface integrity during pathological processes (46). Trefoil factor-2 is a stable secretory protein expressed in gastrointestinal mucosa responsible for protecting the epithelial layer from insults, stabilizing the mucus layer and promoting healing of the epithelium. Two trefoil motifs identified and present in TFF2 is believed to be essential for the protein's proper function as *in vitro* recombinants with a single trefoil domain was discovered to lose its anti-apoptotic properties but continue to stimulate cell migration (45). TFF2 and Fragment of IgG Binding Protein (FCGBP) interaction has been proven to participate in intestinal immunity (mucosal protection). TFF2 binds to the mucin MUC6 thereby stabilizing the inner insoluble gastric mucus barrier layer (47). The interaction of TFF2 and MUC6 also alters the viscoelastic properties of gastric mucous gels thereby contributing to the gastric mucosal innate immune defense.

1.2.3 Trefoil factor 3

Trefoil factor family protein 3 (Tff3) is a small peptide (59 amino acids; 7 kDa) that is a member of the trefoil factor family proteins (Tffs) (48), and it is specifically distributed in the surface of the intestinal mucosa (49). It is known as intestinal trefoil factor and was identified from a rat cDNA sequence in 1991 (50, 51). It is a stable secretory protein expressed in gastrointestinal mucosa, selectively dispersed on the intestinal mucosa's surface (49) and has been discovered to be predominantly expressed by goblet cells of the large and small intestines (50, 52), and also interact with mucins on the apical cell surface (10). TFF3 plays a significant role in mucosal regeneration and repair in intestinal goblet cells, where it is primarily co-secreted or co-expressed alongside MUC2, while its expression is termed "intestinal". By controlling tight junctions, TFF3 improves the function of the intestinal barrier by reducing the intestinal epithelium's paracellular permeability via regulation of tight junctions. This finding provide insight into the protective functions of TFF3 in epithelial cells and demonstrate its potential for treatment of inflammatory diseases in the GIT (10).

TFF3 suppresses the proliferation and differentiation of activated T cell subsets early during the process of CD4+ T cell differentiation. In recent times TFF3 has been recognized to affect liver metabolism and possible involvement in metabolic pathways through its action in improvement of glucose tolerance in diet-induced obesity model (53). Intestinal trefoil factor 3 (TFF3)

protects and repairs the gastrointestinal mucosa and restores normal intestinal permeability, which is dependent on the integrity of the tight junction (TJ) barrier and the TJ associated proteins claudin-1, zona occludens-1 (ZO-1) and occludin (54). Mucus is essential to the gastrointestinal tract's integrity and for protecting epithelial cells from outside stimuli, infections, and mechanical damage (55). TFF3-FCGBP and MUC2 are the major components of intestinal mucus, and they both colocalize in the gut and play a key role in the innate immune defense of mucous epithelia (22). The combination of TFFs and mucin was effective in protecting the barrier function of epithelial cells. TFF3 promotes immunity and wound healing by de-repressing inhibitory LINGO2-EGFR complexes through interactions with LINGO2. TFF3 binds leucine rich repeat receptor and nogo-interacting protein 2 (LINGO2) to de-repress and enhance epidermal growth factor receptor protein (EGFR) signaling that drives wound healing and immunity against helminths (56).

2 Role of trefoil factors in protecting the gastrointestinal epithelium

Trefoil factors represent group of proteins with a crucial involvement in mucosal protection and repair processes. The complex interplay within the mucosal environment resulted in keen interest in understanding their functions in advancing treatments in gastrointestinal disease (57). This section unravels their biological significance and potential therapeutic implications in various animal species with a summary of actions in Table 1.

2.1 Trefoil factors on the gut health of pigs/piglets

Intestinal epithelial cells form the basic unit of the gastrointestinal tract, and therefore play a unique role of maintaining the integrity of the mucosal barrier, nutrient absorption, and disease prevention (57). In the GIT of pigs, TFFs functions in the establishment of mucosal protection and repair. Although TFF1 is mainly expressed in the pyloric gland and the neck cells of the stomach, all TFFs are abundantly produced in the goblet cells—a mucin-producing epithelial cell in the small and large intestines (2). In general, the intestinal epithelial cells in the gut of pigs contribute to maintaining the defense and integrity of the mucosal layer (58). In general, TFF peptides maintain intestinal epithelial integrity in various organisms through restitution, wound healing, apoptosis, cell motility, and as well as establishing the protective effects of the intestinal barrier (59, 60). During mucosal repair, both pro and anti-inflammatory cytokines regulate the actions of TFFs (61). For instance, during intestinal development in pigs, transforming growth factor-alpha (TGF- α , a cytokine) modulates TFF2 and TFF3 to exhibit different regulation patterns (36, 62). Accordingly, TFF2 expression was significantly upregulated after weaning phase, and this in-turn enhanced mucosal integrity. During the pre-weaning phase in piglets, TFFs such as TFF3 may facilitate intestinal repair of injury induced by inflammation, thereby enhancing intestinal

TABLE 1 Summary of expressions of intestinal trefoil factors in animals.

Species	Treatment	Pre-condition	Action	References
Rat	Wheat peptides (0.1, 0.2, 0.4 g/kgbw) and omeprazole (20 mg/kgbw)	–	Increasing trefoil factor 1 (TFF1) levels	(84)
Rat	Low dose administration of mature silkworm powder	Ethanol-induced gastric damage	Promoting genes related gastric mucosal protection and biosynthesis including mucin 5AC and trefoil factors. Induces gastric mucosal defense factors in ethanol-induced gastric injury rat model	(85)
Rodents	Astragalus polysaccharides and matrine treatment	Ulcerative colitis (UC)	Upregulating trefoil factor 3 expression	(49)
Rodents	Oral administration of TFF3	Hemotherapy- and radiotherapy-induced mucositis	Decreased the intestinal damage	(86)
Rodents	¹ Lingo2 mice	¹ 200 <i>Trichuris muris</i> eggs/mouse by oral gavage	ITF(TFF3) restrains T _H 1 cell proliferation, promotes Type 2 immunity against infection	(87)
Rodents	Oxyresveratrol, an active ingredient of <i>Artocarpus lakoocha</i> administered to five treatment groups	Induction of gastric ulcer in mice	Elevated expression levels of cytoprotective TFF-2 levels, and attenuated expression levels of IL-6, TNF- α , NF- κ B, and COX-2	(88)
Pigs	Control diet (supplemented 25 mg/kg quinocetone and 11.25 mg/kg aureomycin in the basal diet), three treatment diets supplemented with 200, 400, or 600 mg/kg bacteriophage in the basal diet	Healthy weaned piglets	ITF, and tumor growth factor-alpha (TGF- α) was enhanced	(89)
Pigs	Weaned piglets (28 D old)	Diarrhea occurrence (Enterotoxigenic <i>E. coli</i>) Five levels of Wheat bran fiber or pea fiber.	Increased colonic peptide trefoil factors in piglets on the WBF and PF diets	(90)
Pigs	Weaned Piglets	Post-weaning (7 and 14 days) Dietary treatment [0, 200, or 400 μ g/kg epidermal growth factor (EGF) supplementation].	Enhanced abundance of mucin 2 and intestinal trefoil factor 3 at 200 μ g/kg EGF	(91)
Calves	Calves with coccidiosis received a single dose of toltrazuril (15 mg/kg) and supportive care	Control group (healthy calves) vs. Treatment group (calves with coccidiosis at 0th and 72nd hours infection)	Calves with coccidiosis had higher TFF-3 levels and ACTG2 after treatment (72nd hours) compared to the control group.	(91)
Cattle	Control group (negative controls) Positive control Orally infected with 10,000 <i>Ostertagia ostertagi</i> L3s (third-stage larva) and killed at 6, 9, and 24 days post infection (dpi)	Infection with nematode <i>Ostertagia ostertagi</i>	TFF1 and TFF3, co-expressed with mucins in the GI tract was upregulated in infected animals.	(46)
Cattle	Healthy cow, right displacement of the abomasum (RDA) and left displacement of the abomasum (LDA)	Displacement of abomasum	TFF-3 concentrations lower in RDA. Negative correlation between lactate and TFF-3	(92)
New-born Calves	Normal (NG) and test group (TG): <i>E. coli</i> O1(2.5 \times 10 ¹¹ CFU/mL, 100 mL)	Early pathogenic <i>Escherichia coli</i> infection	Lower ITF levels in the colons of TG calves than NG. Concentration of ITF in both the TG and NG decreased over experimental time	(23)
Sheep	¹ Group One (Chiswick Avermectin Resistant CAVRS) and Group two (ivermectin-sensitive McMaster)	<i>Haemonchus contortus</i> infection (two isolates: 500 McMaster L3 and 500 CAVR L3)	TFF3 was the second most highly upregulated gene after Intelectin 2 (IL-2) in the comparisons of day 22 vs. 3.	(93)
	Genetically Resistant group and Susceptible group	10,000 <i>H. contortus</i> L3s	TFF 2 was higher in resistant sheep	(94)
Sheep	Control group two (nfected with 30,000 <i>T. colubriformis</i> larvae L3) Group three: (infected with 10,000 <i>H. contortus</i> L3)	Sheep infected with <i>Haemonchus contortus</i> and <i>Trichostrongylus columbriformis</i>	TFF3 was also found to be significantly upregulated in the small intestine (proximal jejunum), TFF2 was expressed in the abomasum.	(95)
Poultry	Arbor acre broilers	Supplementing paraformic acid (PFA)	Elevated intestinal mucosal factors (mucin 2, trefoil factor family, and zonula occludens-1) concentrations	(33)

(Continued)

TABLE 1 (Continued)

Species	Treatment	Pre-condition	Action	References
	Arbor Acre broilers: basal diet [control (CON) group] and Tannic Acid (TA) group	Tannic acid extracted from <i>Galla chinensis</i> (300 mg/Kg TA in basal diet)	Intestinal mucosal ZO-1 and TFF expression was similar in both groups.	(96)
	Control (PBS-treated), AKK (orally administered 1×10^6 CFU Akkermansia muciniphila for 10 days), SAL (orally administered 1×10^9 CFU Salmonella pullorum-treated on the 5 th day) & AKK+SAL (orally administered 1×10^6 CFU Akkermansia muciniphila for 10 days and orally administered 1×10^9 CFU Salmonella pullorum-treated on the 5 th day).	<i>S. pullorum</i> (1×10^9 CFU) and <i>Akkermansia muciniphila</i> (1×10^6 CFU) gavaging of birds.	Increased number of goblet cells in the intestine and up-regulation of Mucin 2 and trefoil factor 2 (TFF2)	(97)
	Male Cobb 500	live <i>Eimeria</i> vaccination or salinomycin and combinations of in-feed inclusion of gelatin and vitamin E	TFF2, cytokines, and MUC2 was increased by <i>Eimeria</i> vaccination.	(98)
	SPF chickens (control group and infection group)	Avian Influenza Virus Subtype H9N2 (i inoculated with 3 doses of A/Chicken/Henan/SH01/2015 at 10^6 TCID ₅₀ /0.1 mL through the respiratory tract	TFF2, MUC2, ZO-1, and caudin-3 expressions was downregulated by H9N2 AIV infection	(99)
	d-1 Cherry Valley ducks	0, 1, 2, 4, 8, and 16 mg/kg iodine (dietary ethylenediamine dihydroiodide) in diet	Similar expression level of TFF2 in treatment groups	(100)
	Arbor acre broilers CON (basal diet group), Group two (AB) Group three (BP)	Basal diet supplemented with 50 mg/kg aureomycin (AB group) and basal diet supplemented with 40 mg/kg Bopu powder (BP group)	Dietary Bopu powder supplementation increased concentration of trefoil factor family member and mRNA expressions of superoxide dismutase	(101)
	Fertile Cobb 500 eggs	Eggs Injected in the amnion with 100 μ L of either sterile water (sham), 1×10^5 , 1×10^6 , or 1×10^7 (P1, P2, and P3) probiotic product (Primalac W/S containing <i>Lactobacillus acidophilus</i> , <i>Lactobacillus casei</i> , <i>Enterococcus faecium</i> , and <i>Bifidobacterium bifidum</i>)	In ovo probiotic supplementation was associated with downregulated expression of Toll-like receptors-2 and-4, inducible nitric oxide synthase, trefoil factor 2, mucin-2, interferon- γ , and interleukins-4 and-13 in both the ileum and cecal tonsils	(102)

development and growth performance (50). The ability of TFFs to rebuild (or repair) the intestinal epithelium has been attributed to their anti-apoptotic mechanisms that drive the migration of epithelial cells over the damaged section of the intestinal mucosa, where the apoptosis is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix (52) in pigs. In addition, TFF3 has also been reported to suppress factors that downregulate tight junction proteins, thereby decreasing mucosal permeability, promoting the integrity of tight junctions, and consequently enhancing intestinal barrier function (63).

2.2 Trefoil factors on the gut health in chicken

Although the molecular mechanisms underlying the functions of TFFs has been well-explored in mammalian species like rodents, humans and other amphibians, information is scanty regarding avian species (64). It has been established that the protective effect of TFFs result from their interactions with mucins to generate signaling mechanisms that result in their overexpression during mucosal damage, consequently culminating in intestinal restitution of the mucosa and enhanced barrier function (65). A similar mechanism of action has been reported in the chicken,

and this was characterized by migration of the epithelial cells to the epithelial layers of the intestinal villi during injury (50). TFF2 bearing one single trefoil domain promote cell migration (45), since a damaged intestinal mucosa encourages the migration of pathogens into the body thereby causing systemic infection, trefoil factors mediate as a host defense mechanism by keeping the gastrointestinal mucosal layer impermissible to pathogenic attack (66). Accordingly, it can be proposed that the activities of TFFs inhibit pathogenic infection in concert with the actions of other antimicrobial peptides. This notion is corroborated by the finding that chickens infected with avian influenza (H9N2) showed significant decrease in the expression of TFFs and other antimicrobial peptides, and consequently, this resulted in infection with *E. coli* bacteria (67).

2.3 Trefoil factors on the gut health in rodents

The fact that most of the studies investigating the role of TFFs in gastrointestinal health were done in rodents warrant the exploration of literature in this regard in mice. Several studies have demonstrated the protective and healing effects of TFFs following mucosal damage in rodents (68). Conversely, anomalies

TABLE 2 Feed additives and their impact on trefoil factor (TFF) activation in animal models.

Additive	Treatment	Action	References
Lactic acid bacteria (LAB)	LAB-fermented formula milk supplementation given to weaned piglets	Increased the average daily gain, UP-regulated TFF2, selective enrichment of lactate-producing and short-chain fatty acid (SCFA)-producing bacteria in the ileum	(103)
<i>Lactobacillus rhamnosus</i>	Newborn piglets were orally administered with Wild-type (WT) and mutant (Δ luxS) <i>Lactobacillus rhamnosus</i> , Control (CON)	ITF levels were increased in the WT and Δ luxS group than in the CON group	(104)
Epidermal growth factor	Dietary treatment (0, 200, or 400 μ g/kg EGF supplementation)	Dietary supplementation with 200 μ g/kg enhanced abundances of mucin 2 and intestinal TFF3 compared with the control	(91)
Dietary Fiber	Control diet without fiber source (CT), and diets: maize was replaced by 10 % maize fiber (MF), 10 % soyabean fiber (SF), 10 % wheat bran fiber (WBF) or 10 % pea fiber (PF).	WB and PF resulted in increased expression of transforming growth factor-alpha (TGF- α), trefoil factors.	(90)
Dietary crude protein	Weanling pigs fed Three (3) diets containing 22, 19, or 16% CP	A reduction in occludin, trefoil factor-2, trefoil factor-3, and mucin 2 was observed at 16% CP	(105)
Zearalenone (ZEA)	Diet supplemented with ZEA at 0, 0.15, 1.5, and 3.0 mg/kg fed to weaned piglets	ZEA at 0.15 upregulated TFF3	(60)
Probiotic mixture	Probiotic mixture VSL containing four species of <i>Lactobacilli</i> : three species of <i>Bifidobacteria</i> and <i>Streptococcus salivarius</i> subsp. <i>thermophilus</i> sp.	Increased production of mucus, TFF1 and TFF2	(106)
Taurine	Lipopolysaccharide (LPS) and dietary taurine	Taurine increased the expression of TFF-3 mRNA. LPS-challenged animals lowered expressions of trefoil factor-3, transforming growth factor β -1 expression and number of goblet cells	(107)
Palygorskite	Basal diet supplemented with 0, 5, and 10 g/kg palygorskite for 21 days in chicks	Supplementation of 5 g/kg palygorskite increased jejunal trefoil factor 2 (TFF2) mRNA abundance	(108)
<i>Lactiplantibacillus plantarum</i>	Negative control (NC): basal diet only; OTC: basal diet + 0.01% oxytetracycline; RG11: basal diet + 0.1% Postbiotic RG11; RI11: basal diet + 0.1% postbiotics RI11 and RS5: basal diet + 0.1% postbiotic RS5	The concentrations of acidic mucin, sulfated mucin, and intestinal trefoil factor were higher in the birds fed with RI11 and RS5.	(109)
Paraformic acid	Basal diet (CON) or a basal diet supplemented with 1,000 mg/kg PFA	PFA supplementation elevated intestinal mucosal factors (mucin 2, trefoil factor family, and zonula occludens-1)	(33)
Yeast nucleotides	Control (CON = basal diet), groups 2–4 were fed the basal diet supplemented with 0.1, 0.3, and 0.5% yeast nucleotides	0.1 and 0.3% yeast nucleotides exhibited higher expression of Mucin 2 (MUC2) and trefoil factor 2 (TFF2) gene	(110)
Probiotics (Primalac W/S)	Probiotics (Primalac: 1×10^5 , 1×10^6 , or 1×10^7 (P1, P2, and P3 probiotic bacteria.) and Sham <i>in ovo</i> injection of Cobb500 eggs	Probiotic supplementation downregulated expression of Toll-like receptors-2 and-4, inducible nitric oxide synthase, TFF2, mucin-2, interferon- γ , and interleukins-4 and-13 in both the ileum and cecal tonsils.	(102)
<i>Macleaya Cordata</i> (Bopu powder)	Basal diet (CON group), basal diet supplemented with 50 mg/kg aureomycin (AB group), or a basal diet supplemented with 40 mg/kg Bopu powder (BP group)	Dietary Bopu powder supplementation significantly increased the concentration of trefoil factor family member	(101)
Algae	Seaweed extract (SWE; 10.0 g/d) and fish oil (FO; 100 g/d) inclusion	SWE supplementation increase colonic TFF3 mRNA expression	(111)
<i>Bifidobacterium bifidum</i>	Milk formula containing 5×10^6 CFU per day of <i>Bifidobacterium bifidum</i> OLB6378 and dam-fed littermates fed by surrogate mothers as a baseline control	<i>B. bifidum</i> treatment of neonatal necrotizing enterocolitis (NEC) markedly reduced number of Tff3-positive cells to values seen in normal healthy controls.	(112)
Cinnamaldehyde	Treatment with cinnamaldehyde (100 or 200 mg/kg bodyweight/day)	Cinnamaldehyde upregulated the expression of TFF3	(113)

develop in mucosal layer when there is deficiency of TFFs in mice. Studies have shown that TFF-2 deficient mice showed high susceptibility to gastric injury, and TFF-3 deficient mice showed a reduced resistance to colonic injury (69, 70). Mode of action

of TFFs involve the formation of mucous defense and barriers, modulation of the mucosal immune response as well as enhancing a rapid mucosal repair via cell migration; a process known as restitution (71). The restitution process is a pivotal mechanism

following a mucosal damage as it helps in mucosal repair by migrating neighboring cells to the injury sites (71). This is evident in previous research reporting that TFF3 restitutes epithelial cells by stimulating the migration of cells surrounding the injured area to the damaged site (73). In addition, TFFs also exhibit antiapoptotic effects by rapidly preventing cell death through maintaining cell migration and cell survival (71). A research study conducted by Sturm and Dignass (72) showed that TFF3 promotes migration of epithelial cells by influencing localization and expression of catenin in epithelial cells, and by stimulating phosphorylation of catenin. The expression of TFF2 and TFF3 have also been observed to simultaneously induce the migration of monocytes in the bone marrow, lymph nodes, thymus and other lymphoid tissues (71). It has been established that the inflammatory responses of immune systems of mice is dysfunctional when TFF2 is mostly deficient (15), thus confirming that TFF2 is closely associated with immune response in addition to its involvement in gastric repair of epithelial cells. The phosphatidylinositol 3 γ -kinase (PI3K/Akt) signaling pathway which is activated by ITF, serves as a regulatory intracellular avenue that restores the epithelial mucosal integrity following an injury (74).

Many studies have confirmed that the PI3K/Akt signaling pathway actively participates in key physiological and pathological processes such as regulating cell proliferation, migration and apoptosis, as well as exhibiting inflammatory responses in the epithelial mucosa (75, 76). For instance, it was observed that higher concentration of ITF increased the proliferation and migration of gut esterase-1 (GES-1) cells (74), thereby corroborating the findings of previous studies (77, 78). In the large intestine, the absence of TFF3 expression was found to increase sensitivity to colonic injury by stabilizing the mucosal layer and inducing repair at injury sites, and rapidly upregulating and enhancing the restorative process (6). This protective effect may be attributed to the collaborative action of TFF3 and Muc2 in the intestinal mucosa as the first line of defense against epithelial injury (79). It has also been reported that recombinant human TFF3 (rhTFF3) can stimulate the expression of tight junction proteins which are directly connected with intestinal barrier functions, thereby reducing intestinal mucosal permeability (74). This was evident in an rhTFF3 expression study on intestinal wall injury in the rat (80). In this study, Wang et al. (80) observed a significant reduction in damage to ileal mucosa, and a reduced intestinal permeability.

2.4 Trefoil factors on the gut health in calves

The intestinal mucosa is crucial for regulating the interaction between bacteria and host cells, as well as affecting nutrient absorption. Calves are mostly susceptible to numerous mechanical obstructions as a result of abnormalities in the bowel lumen, intestinal wall, or outside the intestinal tract (81). Calves, like other young animals, experience gastrointestinal injuries or insults due to factors such as weaning stress, infections, or dietary imbalances. Trefoil factors, particularly TFF2 and TFF3 has been established to play a significant role in maintaining and repairing the intestinal mucosa in calves (23). Studies have shown that

trefoil factors like TFF3 can help prevent intestinal epithelial damage and promote repair of the intestinal mucosa. In calves, this protection is particularly crucial during the early stages of life when gastrointestinal system is still developing and vulnerable to various stressors, including dietary changes and pathogens. The immunological barrier function mediated by intestinal microbes is destroyed by changes in the makeup of the intestinal flora in calves, making the intestinal tract more vulnerable to harmful germs and putting the animals' health at risk (82). The abomasal infection by nematodes in calves such as *Ostertagia ostertagi* larvae causes substantial tissue injury to the abomasal mucosa, and these injuries include acute epithelial cytolysis, hyperplasia of gastric glands, and reduction in acid-producing cells, all culminating in increased gastric pH concentration and inhibition of protein metabolism (83). In another study, the mucin glucosaminyl (N-acetyl) transferase 3 which catalyzes one of the key rate-limiting steps in mucin biosynthesis was up-regulated in the bovine abomasum during nematode infections (46). Together, these results suggest TFF-induced enhanced tissue repair and mucin secretion may contribute directly to mucosal protective immunity. It can be suggested that TFFs enhance mucosal defense by inducing tissue repair mechanisms in the GIT through stimulating cell movement in a manner that reestablishes a healthy mucosa and inhibit apoptosis (84). Intestinal biomarkers have been found to be elevated in inflammatory bowel damage and acute intestinal ischemia (blood flow to the intestine), and intestinal mucosal injury has been linked to elevated expression levels of IFABP, LFABP, IAP, and TFF3 (81).

3 Feed additives: TFFs activation

TFFs play critical roles in maintaining the integrity and repair of mucosal barriers, particularly in the gastrointestinal tract (1). Certain feed additives and nutrients have been studied for their ability to stimulate or enhance the expression of TFFs, potentially improving gut health and promoting healing. Some feed additives that can stimulate or activate trefoil factors are listed in Table 2.

4 Conclusion

The GIT tracts of animals is often exposed to injuries, proliferation of pathogenic bacterial, stress caused by chemicals, physical stress and also adverse effects related to side effects of medical drugs used as growth promoters, consequently necessitating the need for tissue repairs. Various studies have indicated that TFFs enhanced protection and restitution of the mucosal surfaces of various vital organs, including the GIT. The beneficial effects of TFFs are achieved through molecular interactions with mucins to improve cellular migration. The findings from this literature exploration confirms that TFF peptides play significant beneficial roles in intestinal epithelial healing and amalgamation of the mucus layer. Such protection of the intestinal epithelial barrier is prone to culminate in improved animal health, growth performance, and production. Accordingly, future research

should be conducted to identify biogenic feed additives that have potential to induce optimum synthesis of TFFs in the GIT of food animals.

Author contributions

YF: Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing. TO: Conceptualization, Investigation, Supervision, Writing – original draft, Writing – review & editing. DE: Investigation, Writing – original draft, Writing – review & editing, Conceptualization, Supervision. GD: Investigation, Writing – original draft, Writing – review & editing. JR: Investigation, Writing – original draft, Writing – review & editing. OA: Investigation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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