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ARTICLE

Integrated Nutraceutical – Nutritional Approaches to Address Equine Leaky Gut Syndrome

Michael I. Lindinger*

The Nutraceutical Alliance, Torrevieja, Alicante, 03185, Spain

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ABSTRACT

Many of the nutrients beneficial for intestinal health are present in normal foods, but their normal daily intake may be too low to exert optimum effects on intestinal barrier function and immune status. Evidence from laboratory and farm production animals strongly supports dietary supplementation with additional nutrients and nutraceuticals, however research in horses remains scarce and inconclusive. Careful consideration of the outcome desired for horses in care, together with the types of nutraceuticals available, is needed to develop effective strategies for maintenance of healthy intestinal barrier function and for treatment of various leaky gut syndromes in horses. This review presents these issues in the context of what is known about the effects of nutraceutical-type nutrients on the mammalian (including equine) g.i. tract and intestinal microbiome with the aim of providing suggestions for the equine situation.

1. Introduction

Leaky gut syndrome denotes a range of conditions whereby the barrier functions of the intestinal system have been compromised, thereby making the intestinal wall permeable to molecules and substances that should not freely enter into the interior of the horse. There are numerous, intimate relationships between the gastrointestinal tract (GIT), immune system and the microbiota within the GIT. The purpose of this review is to highlight a number of nutrients and nutraceuticals that are of importance to GIT health, particularly in maintaining healthy GIT barrier function and in repairing barrier function [1].

Barrier function refers to the fact that the GIT keeps ingested matter outside of the interior of the animal [2-4]. The healthy GIT is supposed to act like a true physical barrier because much of what is ingested, and much of the digesta and microbiome within the GIT, are in fact harmful if they enter the body. As with any structure, its integrity is a reflection of how well it is maintained, and failure to maintain barrier function of the GIT results in a leaky gut, an increased permeability of the barrier formed by intestinal epithelial cells (Figure 1).

**Corresponding Author:*
Michael I. Lindinger,
The Nutraceutical Alliance, Torrevieja, Alicante, 03185, Spain;
Email: mi.lindinger@gmail.com

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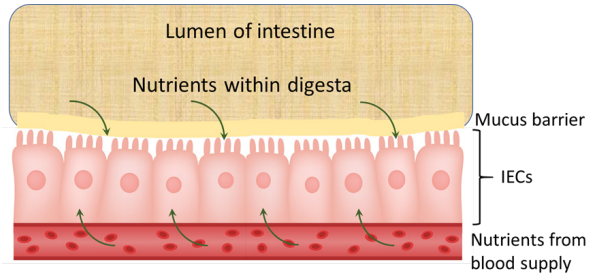

Figure 1a. Schematic representation of a portion of intestinal wall showing the physical barrier formed by intestinal epithelial cells (IECs) and the mucous barrier that overlies the IECs in intestinal lumen. Tight junctions, adherens junctions, and zonula occludens junctions normally hold IECs tightly together

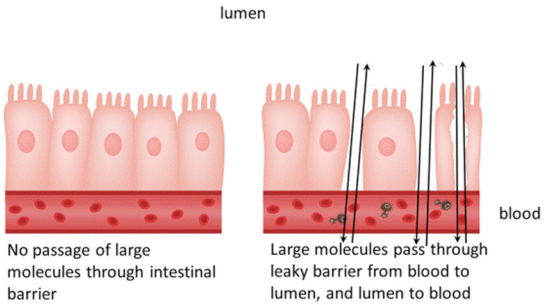

Figure 1b. Left panel: Healthy IEC layer with no leaks. Right panel: Intestinal pathogens attack IEC structures, weakening the physical barrier, which can result in inflammation and leak through the weakened barriers. Leak can occur between cells (paracellular pathway) or through severely damaged cells

For the purposes of this review we have considered nutrients to be the molecules arising from the digestion of traditional feedstuffs such as forages and grains; these include amino acids, peptides, mono- and disaccharides, free fatty acids, electrolytes, minerals, water and some vitamins. Nutraceuticals, in contrast, are ingredients that can be added to feeds, to the feed as a supplement, or on its own that impart to the horse benefits beyond those normally attributed to nutritional molecules. Nutraceutical ingredients are derived mainly from plants, animals, fungi and bacteria and a nutraceutical product may contain a blend of ingredients derived from one or more of these main sources. A nutraceutical may also be a product of digestion, butyrate for example which is produced by some gut microbiota but can also be supplemented to the normal diet specifically for the purpose of improving GIT barrier function. Probiotics and prebiotics may also be used as nutraceuticals. Probiotics are living microorganisms, typically bacteria and yeasts, that must survive the acid environment of the stomach and when in the intestine contribute beneficial effects to one or more of the GIT microbiome, to the mucous layer, to immune system cells and to intestinal epithelial cells (IECs). Examples includes some bacterial strains of lactobacillus, acidophilus and yeast strains of *Saccharomyces cerevisiae* . Prebiotics are compounds that provide nutritional substrate for beneficial microbiota (commensal bacteria) which thereby result in increased growth, proliferation and metabolism of beneficial microbiota. The products of beneficial microbiota contribute to GIT health by a number of mechanisms including antibiotic effects on pathogenic microbiota, augmenting the molecular defenses of the mucous barrier, production of molecules that signal other commensal bacteria, dendritic cells of the innate immune system as well as the intestinal epithelial cells themselves. Examples of prebiotics includes plant, yeast or bacterial cell wall material – an example are beta-glucans derived from oats or from fungi.

2. Nutrients and Nutraceuticals with Demonstrated Benefits

Suggested approaches that may be used when feeding supplements to horses for the purposes of correcting a leaky gut, or better still to prevent a leaky gut from occurring, include:

(1) Provide nutrients and nutraceuticals that are specific to the needs of the IECs

(2) Provide nutrients and nutraceuticals that are specific to the needs for maintaining / repairing the mucosal barrier that lines the luminal (interior) surface of IECs;

(3) Provide beneficial microbiota (probiotics) to augment or repopulate the commensal microbiome within the small intestine, cecum and lower GIT -- these can include microbes that destroy undesirable GIT microbes;

(4) Provide nutrients and nutraceuticals that are specific to the health and proliferation of the commensal microbiome (prebiotics).

An effective strategy for correction of leaky gut will employ these four approaches, while effective strategies for maintaining healthy GIT barrier function in stressed horses will use at least three of these four approaches. A balance of these, and the absence of nutritional gaps, is important, when designing effective strategies.

There are two main ways in which the GIT can be nourished: (1) from within the lumen (exterior to the body); and (2) from the arterial blood supply in the basal lamina (interior of the body) side of the barrier. This article focuses on providing nutrients on the luminal side (feedstuffs) and that will have effects on the GIT without

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having to be absorbed into the body by the IECs. Supplements such as vitamins, amino acids, nucleic acids, carbohydrates and fatty acids of the correct types and in the right balance may have beneficial effects on GIT mucous layer and IEC growth and proliferation [5]. Some of these are included within complete feed rations and premium feeds, but there often remain nutritional gaps that result in inadequate defense against factors contributing to leaky gut.

Luminal nutrients and nutraceuticals serve a number of important functions including (1) providing fuel to (L-glutamine) to IECs; (2) stimulating the growth and proliferation of new IECs by their interactions with existing cells (galactose and 3-O-methyl-d-glucose); (3) stimulating the release of gut hormones from the distal small intestine, cecum and colon; (4) molecular signaling functions to increase or decrease nutrient transport systems, i.e. for glucose or amino acids; and (5) stimulation of intestinal mucus production [1,6-8].

Some of the main sites of action of various nutrient and nutraceutical classes are presented in List 1. The disaccharides sucrose, maltose and lactose are more potent than monosaccharides such as glucose, galactose and fructose for stimulating growth and proliferation (trophic effect) of IECs, and this trophic effect requires the hydrolysis (breakdown) of disaccharides to the monosaccharides [9]. This trophic effect is pronounced within the small intestine, the primary site for absorption of nutrient molecules coming from dietary sources of carbohydrates, fats and protein. Different amino acids such as ornithine, L-glutamine, histidine, valine, glycine appear to stimulate growth and proliferation by different mechanisms from each other and from carbohydrates. The IECs use 20% of the extracted amino acids for mucosal protein synthesis (the intestinal mucous is rich in proteins) and the remainder for many other metabolic processes including providing fuel for oxidative energy production with the IECs. Some long-chain triglycerides (fats) enhance adaptive responses in the small intestine and the effect is more pronounced in the presence of some long-chain free fatty acids [10]. One of the smallest molecules, the short chain fatty acid butyrate, is produced by beneficial GIT microbes and it can also be supplemented in the diet. Butyrate plays a crucial role in maintaining the tight junctions between IECs throughout the length of the GIT, is involved in mucosal barrier integrity [11-13]. Dietary water- and fat-soluble vitamins (mainly vitamins A, C, D and riboflavin) are also required for intestinal epithelial cell growth and proliferation [7,14-16].

L-arginine – IEC growth, barrier function, immunostimulant

Beta-glucans – anti-parasitic, anti-bacterial, anti-oxidant, anti-inflammatory

Butyrate – IEC tight junctions, mucosal barrier, energy source, immune system, nervous system

Disaccharides – energy source for transport systems and trophic effects

Fatty acids – oleic acid, linoleic acid, palmitic acid are closely associated with immunological function of the intestinal mucosa

L-glutamine - IECs energy source, protein synthesis, growth, proliferation, repair, barrier function, immunostimulant

L-threonine – mucosal barrier

List 1. Key sites of action of specific GIT-beneficial nutrients and nutraceuticals

2.1 Amino Acids

Amino acids can be provided in the form of proteins sources from the diet, or as supplements of specific amino acids. Providing amino acids solely from dietary protein sources can result in an oversupply of some amino acids and inadequate provision of other, GIT-important amino acids and is not recommended in cases of suspected leaky gut syndrome. Three amino acids worthy of consideration for maintenance gut health and barrier integrity and for inclusion into a strategy for treatment of leaky gut syndrome are L-glutamine, L-arginine and L-threonine.

2.1.1 L-Glutamine

L-glutamine is a highly digestible amino acid that has many important nutritional, immune function, performance and general health benefits in healthy and unwell mammals. These include regulation of cellular gene expression, neuronal excitability, protein turnover, cellular metabolism, immunity and acid-base balance. For the GIT, L-glutamine can be considered an "essential" amino acid [17-19] and barrier function is dependent on dietary L-glutamine availability [20].

The proteolysis or breakdown of dietary protein and peptide sources provides about 87% of L-glutamine within the body, while the remaining 13% arises from synthesis within the body [21]. However most (>90%) of the L-glutamine absorbed from the lumen of the small intestine does not enter the portal circulation and is used by IECs. Most of the uptake occurs in the small intestine [22-23] but dietary L-glutamine is transported into IECs along the entire length of the GIT. Up to two-thirds being is used to provide energy within these cells along the entire length of the GIT[19]. Among the various types of IECs, the absorptive columnar epithelial cells of the small intestine is the major site of L-glutamine extraction and oxidative

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energy (ATP) production [25].

Numerous researchers have shown that dietary L-glutamine supplementation is important to maintain a normal intestinal barrier against pathogens and preserve mucosal integrity [20,27-30]. Within the GIT L-glutamine is involved in the regulation of cell growth and numerous cellular functions including cell / tissue regeneration. L-glutamine is one of the most important amino acids for IECs as an important energy source, for its ability to build protein within the cells, for its regulatory roles in the metabolic pathways of other amino acids such as ornithine, citrulline, L-arginine, and proline [31-33]. Removal of L-glutamine by starvation of cultured intestinal mucosal cells prevents cell growth and proliferation, and results in a breakdown of tight- and adherens junctions with loss of barrier function, leading to a leaky gut. L-Glutamine supplementation decreases intestinal permeability and preserves gut mucosa integrity in an experimental mouse model [28]. Dietary L-glutamine is necessary for normal intestinal mucosal growth and for maintenance of the intestinal mucosal integrity [7].

Inadequate L-glutamine supply is associated with impaired function of TIT-associated immune function, and L-glutamine been shown to be essential for lymphocytes (which are unable to synthesize L-L-glutamine) and other rapidly dividing cells, such as gut mucosa and bone marrow stem cells [27,32]. High rates of extraction and utilization of L-glutamine by leukocytes, and by lymphocytes in particular, has led to the classification of L-glutamine as an immunostimulant [33].

Table 1. Benefits of dietary sources of L-glutamine to support growth and health (from Ruth and Field [29])

• serves as a precursor and energy substrate for immune and epithelial cells;
• is important for intestinal development and function and for maintaining the integrity of the gut barrier, the structure of the intestinal mucosa, and redox homeostasis;
• supports proliferative rates and reduces enterocyte apoptosis;
• protects against pathogenic bacterial damage to intestinal structure and barrier function;
• lowers inflammatory response and increases immunoregulatory cytokine production; and
• improves the proliferative responses and numbers of intestinal immune cells.

2.1.2 L-arginine

The amino acid L-arginine is also highly digestible (85 – 92%) within the small intestine and is taken up and metabolized within IECs, in addition to active absorption into the blood. When dietary L-arginine is low (less than 1% of diet) supplementary L-arginine (up to 2% of diet) may stimulate growth of intestinal epithelial cells [34]. Compared to diets low or absent of L-L-arginine, 7 days of consuming a diet having 2% L-arginine resulted in preservation of intestinal barrier function within mice when bolus *E. coli* was introduced into the stomach [36]. This confirms an earlier study in rats receiving 300 and 600 mg L-arginine per day, where an effective barrier to *E. coli* was maintained in the presence of an induced lower small intestine (illeal) obstruction [37]. This beneficial effect appears to be due to a reduction in / modulation of inflammatory signaling molecules within the GIT including proinflammatory cytokines, and with stimulation of immunoglobulin A production [36].

Performance horses are subjected to periods of training, transport and competition, of which heat and exercise stresses contributed to intestinal dysfunction. Exercise heat stress results in loss of small intestine barrier function and compromised immune responses [38]. L-arginine supplementation (2% of diet) to mice subjected to exercise heat stress prevented the increases in intestinal permeability and bacterial translocation caused by exertional hyperthermia. The authors concluded that "dietary l-L-arginine supplementation preserves the integrity of the intestinal epithelium during exercise under heat stress". In the large intestine the provision of L-arginine is essential for maintaining the integrity of the epithelial barrier. L-arginine is transported into epithelial cells lining the large intestine by the cationic amino acid transporter resulting in the production of polyamines that are required for maintaining barrier function and for repair of impaired barrier function within the large intestine. Within the intestinal immune systems, L-arginine also stimulates T cell proliferation and activity thus combating inflammation [40,41]. One of the beneficial effects of L-arginine is by inducing the immune system to stimulate T cell proliferation and activity in conditions such as peritonitis and sepsis [41,42].

2.1.3 L-Threonine

The amino acid L-threonine is also highly digestible (84 – 93%) within the small intestine and is taken up and metabolized within intestinal epithelial cells, in addition to active absorption in the blood. Within the small and large intestine, L-threonine is oxidized by epithelial cells and specifically used for mucin production. L-Threonine is one of the nine indispensable amino acids that cannot be synthesized to meet body needs in animals and therefore must be provided in the diet. Dietary L-threonine imbalance reduced the growth of the small intestine, liver and skeletal muscle in young animals, and reduced protein synthesis and mucin production in the jejunum of growing pigs [43]. This translates to an optimum dietary L-threonine

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of about 1% of digestible protein.

In neonates especially, the gastrointestinal tract extracts the majority of dietary L-threonine on the first pass to maintain synthesis of L-threonine-rich mucins in mucus. As dietary L-threonine becomes limiting, this extraction must limit protein synthesis in extra-intestinal tissues at the expense of maintaining protein synthesis in mucin-producing tissues [44]. These authors concluded that "If dietary L-threonine intake is deficient, then muscle growth and the functions of other tissues are likely compromised at the expense of maintenance of the mucus layer in mucin-producing tissues".

L-threonine is required by IECs of both the small and large intestine to produce the mucin [45] that is such an essential component of barrier function and intestinal immunity. Mucin, and the L-threonine-requiring cells that produce it, form an essential and important part of the (enteric) intestinal immune system involved in protection from physical and chemical insult [46,47]. Ileal losses of L-threonine through mucin contribute to increased L-threonine usage in humans [48] and intestinal mucin production is considered a major metabolic fate for L-threonine [49]. In addition to its use for mucin and muscle protein synthesis, other major functions of L-threonine include immune function, protein phosphorylation, and glycine synthesis, as reviewed by [49].

Horses and other animals are routinely challenged by pathogenic bacteria that are ingested with foods or accidently. Provision of supplementary L-threonine resulted in improved growth performance, health, immunity and gastrointestinal function of weaning pigs challenged with *E. coli* [50]. Even small (0.5 g / kg feed) increases in dietary L-threonine improved feed intake, overall feed efficiency, intestinal IgA secretion and beneficially regulated the population of gut microbiota in growing pigs. In poultry it was similarly concluded that L-threonine supplementation can improve immunity, antioxidant capacity and intestinal health [51], building on the earlier work of many researchers including Azzam et al. [52] who suggested that L-threonine functions as a nutrient immunomodulator in maintaining intestinal barrier function.

2.2 Short Chain Fatty Acids

Short chain fatty acids (SFCAs) such as butyrate, proprionate and acetate are produced by many types of commensal microbes within the distal small intestine, cecum and large intestine. All of these can be used as fuel for oxidative metabolism by all cells of the body and some, i.e. butyrate, exerts direct effects within the GIT. Microbial fermentation is most commonly associated with the hindgut of horses, however, foregut fermentation in horses also occurs such that starch fermentation in the foregut contributes to the overall response [53]. Fermentation of dietary starch in the foregut, cecum and hindgut results in the production of lactate, which is used by beneficial GIT microbes to produce butyrate. Butyrate has immunomodulatory properties and reduces intestinal [54] and systemic inflammation when fed to geriatric horses [55]. Butyrate can also be rapidly transported by IECs into the blood from which it can be used as an energy source by numerous cells and tissues of the body [56].

Butyrate is a very important molecule within the GIT because it has direct inputs into intestinal, immune [7] and nervous system physiology and a role in gut-brain communication [57]. An increasing number of studies indicate a primary role of butyrate in reinforcing epithelial barrier function through signaling within IECs to maintain / repair tight junctions [13] and by stimulating increases in mucus production [11,58]. Butyrate also contributes to the energetic balance of IECs, is involved in the regulation of oxidative stress and inflammatory status of cells. [2,12]. Changes in diet affects the population of gut microbiota [8] and this in turn modulates the peripheral nervous system and brain function via what has been termed a microbiota gut-brain axis [56]. The effects of intestinal microbiota on the nervous system cannot be disassociated from effects on the immune system since both systems are in constant bidirectional communication. Alterations to the microbial population in the GIT may affect the production neurotransmitter molecules such as gamma amino butyric acid, and the products of fermentation (SCFAs such as butyrate, propionate, and acetate).

Butyrate is produced by ileal, cecal and colonic microbial fermentation of dietary fibers (complex carbohydrates) present within forage and other feedstuffs [57]. While not as important, proprionate and acetate are also pleomorphic and positively influence IEC [2] and whole body [59] glucose and energy homeostasis. A high abundance of intestinal Bifidobacterium, Lactobacillus and *Clostridium leptum* results in healthy production of butyrate and other SCFAs. Sodium butyrate supplementation is also shown to enhance the GI mucosal growth and high carbohydrate improve gastrointestinal functions in piglets after weaning [57]. However, when the abundance of SCFA-producing microorganisms is low, the result is often poor epithelial barrier and tight-junction integrity, a reduced ability to repair of epithelial lesions, and a reduced ability to combat exercise-associated GI barrier perturbations. Overfeeding of grain is common, and this results in elevated cecal and hindgut production of lactate, which lowers luminal pH to favour the proliferation of non-beneficial microbes and reduces the populations of commensal

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bacteria, therefore lowering the production of butyrate [60].

Because of butyrate's recognized importance in many aspects of healthy GIT function, research has been undertaken to find effective ways of increasing cecal and hindgut butyrate concentrations through dietary supplementation of butyrate products. Because butyrate is so rapidly taken up by many cells, it is necessary to encapsulate the butyrate to allow it to travel with digesta into the cecum and hindgut. Here, the capsule is degraded, releasing butyrate which raises luminal concentrations to desired levels where beneficial effects can occur. One such product is ButiPearl Z EQ (Kemin Industries) which, after ingestion, results in a sustained release of butyrate and provision of zinc which is a beneficial cofactor for commensal gut microbes. This product acts to promote intestinal health and barrier function through provision of energy, maintenance of tight junctions and mucous production. Studies have shown that butyrate supplementation enhances the GI mucosal growth and improves several indicators of GIT function [3,11-13].

2.3 Beta-glucans

Beta-glucans are polysaccharide cell wall components of cereals such as oats, fungi, some yeasts and some bacteria. Beta-glucans present in mushrooms and yeasts exhibit β-l,3- and β-l,6-linkages. In barley and oats, the β-l,3- and β-l,4-linked water-soluble beta-glucans are predominant and account for about 75% of the cell wall dry matter [61]. The beta-glucan content of oat bran is about 9% which is three times greater than that of oat flour [61]. Beta-glucans are a form of dietary fiber that are not degraded in the stomach and the small intestine, therefore are delivered to the cecum and large intestine where they provide a source of non-starch polysaccharides to microbiota [62].

Animal studies using oat beta-glucan have shown uptake or interaction with cells of the gastrointestinal tract, with benefits including protection against intestinal parasites and bacterial infection, anti-oxidant, anti-inflammatory. In vitro studies reported effects on cytokine secretion, phagocytic activity and cytotoxicity of isolated immune cells, and activation of the complement system [63]. Reported effects in animal studies include a protective effect against an intestinal parasite, protection against bacterial infection, and a synergistic effect in antibody-dependent cellular cytotoxicity [64]. Dietary oat beta-glucans have been associated with anti-inflammatory, immune-stimulating, and gut microbiota-modulating activities, as well as the ability to beneficially modify microbial SCFA production [63, 65].

Dietary supplementation with yeasts or mushrooms rich in beta-glucans exhibit immune stimulating effects in humans [66-68] and other animals [69]. In animals, dietary yeast beta-glucans have reduced the incidence of bacterial infections and levels of stress-induced cytokines, and enhanced antineoplastic effects of cytotoxic agents. Protective effects toward drug intoxication and ischemia/reperfusion injury have also been reported [70]. Toxicity studies performed on laboratory animals have shown that beta-glucans are safe at high dietary inclusion levels (>2 g / kg body mass / day) [71,72].

2.4 Triglycerides (TGs), Free Fatty Acids (FFAs) and Polyunsaturated Free Fatty Acids (PUFAs)

TGs, FFAs and PUFAs are dietary fats or lipids and some of these beneficially the functions and structures of cells and tissues, including those of the GIT. TGs can be comprised of both FFAs and PUFAs on a glycerol backbone, and TGs are easily broken down into its four molecular parts. Intestinal barrier function is directly modified by cell membrane lipid content, and therefore providing beneficial dietary lipids is important. Rapid increases in dietary fats should be avoided as enzyme systems needed for lipolysis and transport need to be gradually upregulated, and sudden changes can result in increased intestinal permeability.

IEC barrier permeability is directly modified by cell membrane lipid content and dietary lipids appear to exert rapid effects on IEC membrane composition and function. This highlights the importance of providing beneficial dietary fats. As such, omega-3 polyunsaturated fatty acids have been proposed as an adjuvant therapy in animals with leaky gut [73,74]. Part of the rationale behind this approach is that phosphatidylcholine and other phospholipids serve as major components of the intestinal mucus layer and are integral in establishing the gut mucosal barrier. Kunisawa et al. [75] showed that dietary palmitic acid and its metabolites enhance intestinal IgA responses including increased numbers of IgA-producing plasma cells in the large intestine. Thus, omega-3 PUFAs have been proposed as a nutritional therapy in leaky gut conditions in humans. Randomized and controlled clinical trials showed that the administration of dietary PUFAs reduces the GIT inflammatory activity in ulcerative colitis patients by serving as major components of the intestinal mucus layer, generating and maintaining the protective layer overlying IECs and thus helping to re-establish an effective mucosal barrier [76].

The quality and quantity of dietary fat intake is also closely associated with immunological function of the intestinal mucosa mainly through induction of gut-associated lymphoid tissue [77]. Oat oil, sunflour oil, borage oil and fish oil are excellent sources of beneficial fatty acids,

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including palmitic acid, omega-3 and omega-6 polyunsaturated fatty acids. Some oils are high in polyphenol and tocopherol antioxidant activities and exert beneficial effects on cells and tissues [78]. The unsaturated fatty acids oleic acid, linoleic acid, as well as palmitic acid, confer beneficial effects on maintaining and restoring intestinal health and immunity after various challenges including gliadin-induced depletion of intestinal defenses [79], Chron's disease in humans [77], nutritional depletion of intestinal defenses [80] and restitution after small bowel resection [81]. These fatty acids are highly digestible in the small intestine, and also exert direct and indirect effects on IEC function and on modulating the intestinal microbiota. Studies using rats have shown that dietary oleic acid supplements contributed to maintenance of immunological function of the intestinal mucosa [80]. They also exert anti-inflammatory activity and may exert trophic effects such as cellular proliferation, increased mucosal mass and increased mucosal IgA activity [82]. High levels of IgA within the intestine protect against pathogenic microorganisms by preventing their passage through the mucosal barrier and attachment to IECs, as well as by neutralizing their toxins. Their beneficial effects are particularly evident in vitro studies when used prophylactically in the face of disease-causing agents [77].

2.5 Probiotics and Prebiotics

Effective nutritional strategies for maintaining a healthy GIT rely on more than one approach. This is mainly due to the fact that the GIT tract is a very complex physiological system that integrates the physiology and metabolism of intestinal epithelial cells (IECs), immune system cells, and both beneficial (commensal) and pathogenic microbiota dwelling in the GIT. Prebiotics, probiotics, antimicrobials and fecal microbial transfaunation continue to be explored to manipulate GIT microbiota composition and, by doing so, achieve a healthy GIT.

Gut microbiota dysbiosis, i.e. unfavorable alterations in microbiota populations as a whole, are associated with acute colitis [83], equine grass sickness [84], laminitis [88] and a wide range of other diseases [8]. The microbiota refers to the microbes living within the GIT, and these living organisms include bacteria, yeasts and fungi and archaea (Figure 2). Probiotics and prebiotics work to help restore a balanced, favourable gut microbiota. The microbiota is unique for each horse, but in healthy horses the phylum Firmicutes is predominant (46 – 70%) in feces. Bacteroidetes, Proteobacteria, Verrucomicrobia, Actinobacteria, and Spirochaetes contribute up to 15% each [86-87]. Microbiota dysbiosis is characterized by substantial shifts in the phyla as observed in a range of equine gastrointestinal disease. Healthy horses are abundant in Actinobacteria, Spirochetes, and order Clostridiales while many GIT diseases are characterized by increased abundance of Fusobacteria [8]. There appears to be little or no difference in the abundance of Lactobacillales (majority of lactic acid-producing bacteria) between healthy and diseased horses.

In a healthy horse microbiotal populations are in balance with respect to one another and with respect to the IECs and the immune cells. In a healthy GIT the populations of beneficial microbiota are high keep the populations of pathogenic microbiota in check. The pathogenic microbiota are so called because some of the products of their metabolism is toxic to IECs, immune cells and to beneficial microbiota. High starch diets, sudden changes in diet [88], medications, excessive stress and ingested pathogens can all result in increased populations of pathogenic microbiota [89]. One nutritional strategy, therefore, is to ensure that the GIT is regularly provided with probiotics that are capable of supporting the populations of beneficial microbiota while suppressing the population of pathogenic microbiota. Balance is key – it is not desirable that all pathogenic microbiota are destroyed.

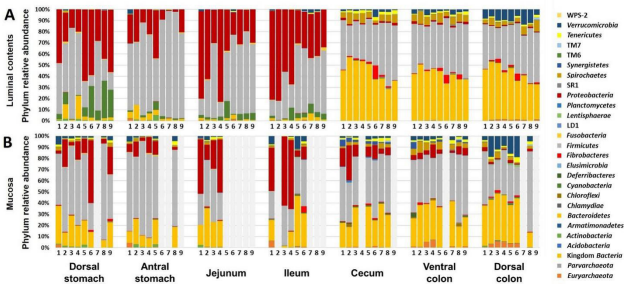

Figure 2. A microbiome map of the phylla resident in the healthy horse GIT. The equine GIT contains more than 150 different species of microbiota from 27 different phyla, of which about 25 species predominate. [87]

Note: From Ericsson et al. (2016) PLoS ONE 11(11): e0166523

Probiotics are simply living biological organisms, mainly bacteria and yeasts, that are good for GIT health. The 'pro' means good or beneficial, the 'biotic' means alive. A probiotic is not to be confused with a prebiotic, which is a compound that is not alive but that serves to provide beneficial nutrients to beneficial microbiota in the gut. Inactivated yeasts and bacteria as well as products from these organisms may be probiotics. There are numerous ways in which probiotics can be beneficial, including:

(1) Production of molecules that inhibit growth of pathogenic microbiota (antibiotic effect)

(2) Production of molecules that provide nutrition for IECs

(3) Production of molecules that provide nutrition for and / or modulate cells of the innate and acquired immune

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systems

(4) Production of molecules that provide nutrition for other beneficial microbiota

(5) Production of molecules that directly contribute to barrier functions of the mucosal layer and of the IEC tight junction barrier

(6) Production of molecules that are used as nutrition (fuel) by other cells of the body once absorbed by the GIT, for example the volatile fatty acids butyrate, propionate and acetate

(7) Inhibition or inactivation of pathogenic toxins

(8) Competitive exclusion of pathogenic microbiota

Some of the commonly available and effective probiotics for horses include both bacteria and yeasts when used as supplements or as microbial feed additives. The most commonly used genera for probiotics, Lactobacillus, Bifidobacterium and Enterococci are normally in low abundance in the equine GIT. It is also not required that probiotics colonize the GIT in order to obtain beneficial effects. While colonization may be considered superior to mere survival due to a prolongation of the beneficial activity, even transient probiotics may act beyond the period of administration. However, in many animals studied to date the ability of probiotics to colonize the GIT is not host-specific, therefore strains are typically selected on the basis of their probiotic properties, and not their species of origin. It may also be beneficial that probiotic bacterial strains are also antibiotic resistant, particularly when antibiotics are needed for post-surgery and injury situations [90].

3. Bacterial Products

Probiotic species commonly used commercially are Bifidobacterium and Lactobacillus and examples of each has been briefly described. This will be followed by brief consideration of *Bacillus subtilis* and *E.coli*. The reader is referred to other reviews for more detailed treatments of this topic [91,92]. While there is evidence-based research supporting positive effects on intestinal barrier function and health in laboratory and production animals, results obtain to date in healthy and diseased horses remain inconclusive.

Different species of Bifidobacterium have had positive effects in animal models of intestinal infection and inflammation. For example, mice that received *Bifidobacterium longum* showed an increased number of IgA-producing cells in the intestine, and significantly improved survival, against *Salmonella typhimurium* infection [93]. Similar results have been obtained in response to *C. difficile* challenge [94]. *Bifidobacterium bifidum* S17 exerted beneficial effects on intestinal histology, chemokine, cytokine, and inflammatory tissue marker profiles in a murine model of colitis [95].

Lactobacillus, of various species, had beneficial effects murine models of colitis. *L. brevis* G-101 induced the expression of IL-10 in peritoneal macrophages and significantly inhibited the expression of inflammatory cytokines which was associated with improved intestinal barrier function and cell morphology [96]. Heat-killed *Lactobacillus brevis* SBC8803 (a prebiotic) resulted in improved intestinal barrier, attenuated intestinal injury and decreased mRNA expression of the proinflammatory cytokines TNF-a, IL-1-b, and IL-12 [97]. Application of a medium containing secretagogues from a combined probiotic (*L. plantarum*, *L. acidophilus*, and *B. infantis*) reduced necrotizing enterocolitis-like intestinal injury and improved the inflammatory profile [98]. The commercial probiotic mixture VSL#3 (*Streptococcus thermophilus, B. longum, B. breve, B. infantis, L. acidophilus, L. plantarum, L. casei,* and *L. bulgaricus*) reduced inflammation and prevented increases in colonic epithelial permeability that were associated with maintained (as compared to disrupted) expression and distribution of junctional proteins [99].

Bacillus subtilis is a naturally occurring species of bacteria commonly found in soil, but also present in the GIT of many animals including horses. In horses, the Bacillus species represent less than 1% of the microbiome, but they may play a role that is larger than what their numbers indicate. There are many different strains of *B. subtilis* that are used as probiotics in a variety of animals including humans and horses. Each different strain will act in unique ways, so it is incorrect to state that one strain is better than another for every situation.

The PB6 strain of *B. subtilis* that was identified in the GIT of stressed poultry nearly 20 years ago when found to be associated with increased survival of the GIT disease necrotic enteritis. This strain has been developed and extensively tested for function and safety. The mechanism of action of strain PB6 appears to be through its ability to produce and secrete an active molecule into the GIT that retards the proliferation of Clostridium species as well as other pathogenic species [100,101].

In poultry with induced necrotic enteritis (using *Eimeria sp*. and *C. perfringens*) *B. subtilis* PB6 reduced feed conversion ratio, and this was associated with reduced intestinal *C. perfringens* counts, improved villi length and increased villi length to crypt depth ratio [102,103]. In neonatal pigs receiving formula supplemented with PB6 for 21 days, compared to controls, treatment decreased the feed conversion ratio due to increased villous height and intestinal activities of maltase and sucrase. This was associated with upregulation of mRNA and protein abundances of zonula occludens-1 and claudin-1 in the ileum evidence

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of Bacillus proliferation in colonic digesta [104]. Using a rat model of induced in inflammatory bowel disease, PB6 appeared to secrete surfactins (cyclic lipopeptides) with anti-bacterial potential that inhibited PLA2, a rate-limiting enzyme involved in the arachidonic acid associated inflammatory pathway [101]. Ten days of oral PB6 administration suppressed the colitis as measured by mortality, changes in weight gain, colon morphology and reduced levels of plasma proinflammatory cytokines.[101]

In many animals, Clostridium species of bacteria are associated with gastrointestinal distress, and in horses *C. difficile* is unfortunately all too prevalent and one of the most important causes of diarrhea and enterocolitis in foals and adult horses [105]. The Clostridium species produce toxins that breakdown the structural integrity of the mucosal barrier and IECs, resulting in a leaky gut. Infection is typically caused by ingestion of spores from animal (including equine) feces, contaminated soil from the animal hospital environment. Hospitalization and antibiotic treatment are the two major risk factors for the development of *C. difficile* associated disease. The intestinal lesions caused by *C. difficile* produced toxin A and toxin B are not distinguishable from the lesions caused by other pathogenic bacteria. So detection of fecal toxin A and B are diagnostic for *C. difficile* 105. The strategy of providing the PB6 strain of *B. subtilis* routinely as part of the horse's diet may help prevent the occurrence of leaky gut or reduce the severity of leaky gut. In an in vitro study of five common equine intestinal and respiratory pathogenic bacteria (*C. difficile*, *C. perfringens, R. equi, S. equi, Salmonella typhimurium*) application of PB6 to plated media or broth resulted in inhibition of growth of all pathogenic species [106].

The probiotic product ColiCure contains a strain of *E. coli* approved for use in Europe to improve fecal consistency in adult horses [107]. Results presented in the EFSA report indicate a more rapid improvement in fecal consistency in diarrheic horses treated with Colicure (1 X 10 [11] CFU daily for three days) than with control diarrheic horses.

4. Yeast Products

The primary probiotic yeasts used are the nonpathogenic *Saccharomyces cerevisiae* and *S. boulardii* of various strains. They are typically used live (probiotics) or heat inactivated or dead (prebiotic). Some inactivated yeast products also used as prebiotics include those that are rich in mannan oligosaccharides and / or beta glucans – both of which are associated with nutraceutical benefits in animals [108].

Research under controlled conditions using laboratory animals provide good evidence for efficacy of probiotics and prebiotics. Supplementary feeding with Saccharomyces strains (*S. boulardii* and *S. cerevisiae* UFMG 905) have been associated with preservation of intestinal barrier integrity and reduce BT in animals [109-111]. In a murine model of intestinal obstruction, both viable and heat-killed *S. boulardii* and *S. cerevisiae* 905 were associated with improvement in intestinal morphology, reduced intestinal damage, stimulation of intestinal IgA production, and increased cytokine IL-10 after intestinal obstruction [109-110]. Daily treatment of mice challenged *S. typhimurium* with *S. boulardii* prevented weight loss, enhanced survival, protected against liver damage and inhibited inflammatory signal transduction pathway activation in mice after challenge [111].

Positive results on intestinal barrier function in various laboratory and production animals led to research investigating the efficacy of probiotics and prebiotics in healthy horses and in horses with gastrointestinal disease. In a randomized blinded placebo-controlled clinical trial the efficacy of *S. boulardii* in treating the diarrhea associated with acute colitis in horses was assessed [112]. Acute colitis can be caused by several pathogens, making identification of a specific cause difficult. Horses receiving *S. boulardii*had a shorter duration of diarrhea and watery diarrhea compared to controls, but the duration of loose feces was similar in both groups.

When *S. boulardii* was assessed in horses affected with antimicrobial-associated no differences were observed between groups (12 horses per group) with respect to fecal consistency or cessation of watery diarrhea [113]. Also similar between groups were: days to improvement in attitude, resolution of leukopenia, return of: appetite, normal heart rate, normal respiratory rate, normal temperature. The lack of efficacy was attributed to difficulty in standardization of treatment, and a possible lack of colonization by *S. boulardii* because the fecal samples of some horses were negative for *S. boulardii*. In the study by Desrocher et al. [112], administration of 10 X 10 [9] CFU with the feed twice daily showed viable fecal *S. boulardii* at 5 days, but not at 20 days. Therefore *S. boulardii* may have beneficial effects but does not appear to colonize the ceca and colons of horses [114,115]. Any beneficial effect of these yeast probiotics may only persist during the period of administration, and therefore consideration needs to be given to long-term feeding.

The safety of commercially available probiotics and prebiotics appears to be high, and large amounts and repeated dosing do not appear to have harmful effects [8,116]. Many commercially available products either have successful GRAS and / or EFSA notifications and are

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thus considered safe by regulatory authorities when used as intended in the target species. In animals, including humans, there are reports of extra-intestinal infections associated with the use of some products, this may reflect translocation of pathogenic material across a leaky gut with resulting infection and inflammation [117]. There have been no such published reports in horses, even when up to three times the manufacturers recommended serving amount was used in horses with colic [118]. While research in horses remains inconclusive regarding efficacy, researchers and clinicians generally consider probiotics and prebiotics as safe for use in healthy and diseased adult horses.

5. Summary and Conclusions

This review has highlighted a number of nutraceutical and nutritional ingredients that can be supplemented to the normal diet of horses with the specific goal of better maintaining and / or repairing barrier functions of the GIT. These ingredients include specific amino acids, free fatty acids, butyrate, probiotics and prebiotics. Definitive *in vivo* information regarding efficacy of probiotics and prebiotics is often lacking in horses, and usage is indicated from studies using animal models of intestinal disease. There is a clear need for both descriptive and mechanistic studies on all aspects of nutraceutical treatments for equine leaky gut syndrome.

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