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ARTICLE

Molecular Analysis of Pathogenicity Differences of Avian Paramyxovirus 1 Genotypes VI and VII in Chickens

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ABSTRACT

Background: Genotypes VI and VII of (APMV-1) have different host range and pathogenicity in pigeons and chickens. However, the molecular determinants of these differences are still unclear. Methods: Here, we aligned the DNA sequences of 56 genotype VI and 33 genotype VII APMV-1 strains. Sequence alignment results revealed that there are 17 amino acids sites differed between APMV-1 strains of these two genotypes. We then constructed a plasmid based on the full-length genome of rSG10 APMV-1 strain, which belongs to genotype VII but was mutated with these 17 VI-genotype-specific amino acids, and rescued as rSG10-17 strain. The restriction digestion and ligation and overlapping PCR methods were used in the construction of plasmids with amino acids mutation. This virus was evaluated for its virulence and growth characteristics. Results and conclusion: The results indicated that the virulence and the growth characteristics have no obvious difference between the rSG10-17 virus and its parental strain rSG10. The simultaneous mutation of 17 genotype-specific amino acids did not affect the virulence of APMV-1 in chickens. Further analysis of these amino acids is required by taking into consideration of the functions of encoded proteins.

1. Introduction

Newcastle disease (ND) is a highly infectious viral disease in the poultry industry caused by Avian avulavirus-1 (APMV-1), which is taxonomically classified in the genus *Orthoavulavirus* in the family of *Paramyxoviridae*.[1] APMV-1 is an enveloped virus with a non-segmented, negative-sense, single-stranded RNA genome of approximately 15 kb. The viral RNA encodes six structural proteins, including nucleoprotein (N), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin–neuraminidase (HN), and large protein (L), and two nonstructural proteins, V and W. [2]

Since it was first discovered in Newcastle-on-Tyne in 1926, there has been 18 genotypes and caused four panzootics. [3, 4] Pigeon paramyxovirus type 1 (PPMV-1), which is belonged to genotype VI, was responsible for the third panzootics. [5] PPMV-1 isolates first appeared in the late 1970s in the Middle East, and mainly infects birds in the family of Columbidae, which contains species of domestic and wild doves and pigeons. [6, 7] This panzootic peaked during the early 1980s. Although the reported cases have decreased since the panzoonosis, there is still occurrence in China and other countries. [8, 9] In general, the disease

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symptoms include a series of nervous disorders. Pathogenicity tests and monoclonal antibody binding studies have shown that PPMV-1 is a variant form of classical APMV-1. [10] Even though strains of PPMV-1 cause morbidity in pigeons, most of them are considered mesogenic or lentogenic according to the intracerebral pathogenicity index (ICPI) in chicken and they are unable to cause disease even for chicks. [11, 12]

The genotype VII strains were responsible for the fourth panzootics. [13] And genotype VII APMV-1 has become the predominant strains circulating in the world since it was first isolated in 2000s. [14, 15] Viruses of genotype VII have a wide range of host, and most birds are reportedly infected by the virus. [16] Almost all genotype VII APMV-1 strains are velogenic and result in more mortality in poultry. [17,18] Many studies also showed that strains of genotype VII APMV-1 induced more innate immune response and cell death in lymphoid tissues compared with virulent strains of other genotypes. [19-21]

The complete genome of genotype VI and genotype VII strains is 15,192 nt, which could encode six structural proteins. Although the F protein of those viruses is associated with virulent APMV-1 strains, the pathogenicity in chickens is different obviously. However, the molecular determinants of pathogenicity differences are still unclear. In this study, we wanted to find amino acids which could influence the pathogenicity of genotype VI and genotype VII strains in chickens. 17 specific amino acids were identified between genotype VI viruses group and genotype VII viruses group. Then we used reverse genetics to study the functions of these amino acids, to identify the molecular determinant(s) of the different pathogenicities of these two APMV-1 genotypes in chickens.

2. Methods

2.1 Cells, Virus and Animals

BSR T7/5 cells expressing the T7 RNA polymerase were cultured in Dulbecco's modified eagle medium (DMEM; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS; Gibco) and G418 (1 mg/ml) (Invitrogen, CA, USA). The recombinant strain rSG10 was generated in our laboratory, which belongs to genotype VII, with an MDT of 45 h and ICPI value of 1.79. [22] The SPF chicken embryos and chickens used for the pathogenicity test were from Beijing Merial Vital Laboratory Animal Technology Co., Ltd, China. All of the birds used in this experiment were cared for in accordance with established guidelines, and the protocols were performed with the approval of the Animal Welfare and Ethical Censor Committee of China Agricultural University (CAU approval number 1901–01).

2.2 Phylogenetic and Sequence Analyses

The complete APMV-1 genomic sequences were obtained from NCBI, including 58 strains of genotype VI and 33 strains of genotype VII, and other reference strains known as APMV-1 genotype. A phylogenetic tree was constructed by the MEGA 4.0 (Molecular Evolutionary Genetics Analysis) with the neighbor-joining method (1000 replicates for bootstrapping). Nucleotide sequence editing, analysis, deduction of amino acids, and alignments were performed in the MegAlign program (v4.00) with the Clustal W multiple alignment algorithm in the Lasergene package.

2.3 Reverse Genetic Constructs

The construction of rSG10 has been described previously. [22] To investigate the function of the 17 genotype-specific amino acids in viral pathogenicity, the pOK–rSG10 full-length cDNA was mutated with overlapping PCR, and designated pOK–rSG10-17. The restriction digestion and ligation were used in the construction of plasmids and the enzymes sites were as same as rSG10. Because the APMV-1 rSG10 strain full-length antigenomic cDNA is made up of six fragments, we mutated each identified amino acid belonged genotype VII into genotype VI in the relevant fragment and then cloned those fragments into the pOK12 vector at seven restriction sites. The mutations were confirmed with DNA sequencing.

The method of rescue infectious APMV-1 was described previously. [23] Briefly, BSR T7/5 cells were cultured in six-well plates of 90% confluence and then transfected with 10 μg of DNA, containing a mixture of pOK–rSG10, pCI–NP, pCI–P and pCI–L, using Lipofectamine 2000 (Invitrogen). At 6 h post-transfection, the cells were washed with phosphate-buffered saline (PBS) and maintained in 2% (v/v) FBS DMEM. 72 hours later, the cells were harvested and SPF chicken embryos were used to recover the viruses. After incubation for 96 hours, the allantoic fluid was harvested and the rescued virus named rSG10-17 was collected with an HA test. The mutated amino acids were confirmed with DNA sequencing.

2.4 MDT and ICPI of the Virus

The pathogenicity of rSG10-17 was determined with an Mean Death Time (MDT) assay in 9-11 days old SPF chicken embryos and an Intracerebral Pathogenicity Index (ICPI) assay in day-old SPF chicks as described previously. [24] Briefly, for the MDT assay, the allantoic cavities of five 9 days old embryos were inoculated with serial 10-fold dilutions of allantoic fluid. The time at which each embryo was first observed dead was recorded. MDT was

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calculated as the mean time (hours) required for the minimum lethal dose of the virus that killed all the inoculated embryos. For the ICPI assay, ten of day-old SPF chicks were inoculated with 50μl of a 1:10 dilution of fresh allantoic fluid via the intracerebral route. The clinical symptoms and mortality of the birds were monitored for every 12 h for 8 days. At each observation, the birds were scored as follows: 0 for normal, 1 for sick, and 2 for dead. The ICPI was the mean of the scores per bird per observation point over the observation period.

2.5 Viral Growth Kinetics

The growth kinetics of rSG10 and rSG10-17 were determined under multicycle growth conditions in DF-1 cells. The cells in duplicate wells of six-well plates were infected at a multiplicity of infection (MOI) of 0.01. After 1 h adsorption, the cells were washed once with pure DMEM and then incubated in 5% (v/v) CO_2 incubator at 37 °C. The supernatants were harvested at 12-h intervals for 72 h, and the viral titers were determined by $TCID_{50}$.

3. Results

3.1 Phylogenetic and Sequence Analyses

To reconfirm the genotypes of the selected viruses, a phylogenetic tree was constructed based on the nucleotide sequences of the F genes of the 91 viruses and other reference strains (Figure 1). The phylogenetic tree showed that 58 strains clustered in the genotype VI group and 33 strains clustered in the genotype VII group. Therefore, the selected viruses could be used for further study. We set the 58 genotype VI viruses as one group and the 33 genotype VII viruses as another group, and analyzed the similarities of the complete genomes and the coding sequences of each gene between the two groups. The results showed that the complete genomes of the two genotypes shared 86.4%–91.7% identity (Table 1). For the contractual proteins, the NP and L genes were relatively conserved between two genotypes, with amino acid identities of 91.7%–98% and 94.2%–97.2%, respectively. The P gene was the most variable, with amino acid identities of 78.8%–89.9% among this two groups. Therefore, from an evolutionary perspective, the NP and L proteins are relatively well conserved and the P protein is the least conserved.

3.2 Genotype-specific Amino Acid Analysis

To analyze the molecular determinant(s) of the difference in pathogenicity of APMV-1 genotypes VI and VII, we aligned the amino acid sequences of every structural protein of the 91 APMV-1 strains. We identified 17 consensus amino acids that are conserved among the genotype VI viruses, which were also completely conserved among the genotype VII strains but differed between the two genotypes (Table 2). Therefore, these genotype-specific amino acids might be the common characters shared by the viruses of a single genotype that contribute to the differences between the two genotypes.

Of the identified 17 genotype-specific amino acids, one site located in each M, F, and HN proteins coding region, while the NP and P proteins each contain two sites. Surprisingly, the L protein contains 10 sites of these amino acids. To exclude the high number of conserved sites in L protein was due to its relatively large scale, we divided the number of genotype-specific amino acids in each protein by the total number of amino acids of that protein. The ratio was represented as the proportion of genotype-specific amino acids in each protein (Table 3). We found that the viral replication complex contains more genotype-specific amino acids than the M, HN, and F proteins, although the NP and L proteins are relatively conserved between the two viral genotypes. The L protein also has a high ratio of conserved genotype-specific amino acids, which indicates it is not attributed to the length of the protein.

3.3 Recovery of Strain rSG10-17

To study the role of the 17 genotype-specific amino acids, the cDNA clone pOK-rSG10-17 encoding the antigenome of strain SG10, with mutations at the 17 genotype-specific amino acid sites was constructed with reverse transcription PCR from the genomic RNA. The cDNA sequence analysis confirmed the intended amino acid mutations. According to the methods, the strain rSG10-17 was rescued and detected with a hemagglutination (HA) test. Then strain rSG10-17 was amplified by RT-PCR and confirmed the correct mutation sites and a lack of adventitious mutations.

3.4 Mean Death Time (MDT) and ICPI Test of rSG10-17

To determine the contributions of the genotype-specific amino acids to the pathogenicity of APMV-1 in chickens, the virulence of the rSG10-17 viral strain was evaluated by determining its MDT and its ICPI. The MDT value for rSG10-17 was 48 h, and its ICPI was 1.92. The results indicated that the virulence and the growth characteristics have no obvious difference between the rSG10-17 virus and its parental strain rSG10.

3.5 Growth Characteristics of rSG10-17 and rSG10

To further compare the properties of strains rSG10-17

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and its parental rSG10, the kinetics and final viral titers under multistep growth conditions in DF-1 cells were analyzed. The kinetics and replication of rSG10-17 were very similar to those of rSG10, which meant that rSG10-17 retained the replicate characteristics of the parental virus (Figure 2).

4. Discussion

Researchers have reported that genotype VI and VII of APMV-1 have different pathogenicities in chickens.[2,12,13] However, the molecular determinants of this difference are still unclear. In this study, we aligned 91 APMV-1 strains and identified 17 genotype-specific amino acids. Then we used a reverse genetics method to investigate the functions of these amino acids. The results indicated that these mutations do not influence the virulence of the virus. However, these amino acids located in different functional proteins regions, thus we need to further analyze of the effects of these individual amino acids by taking the protein function into consideration.

While all APMV-1viruses belong to a single serotype, the virulence among different strains is varied. The virulence of a virus is determined by its tissue tropism, efficacy of replication and ability to deal with the host immune response. The cleavage sites of the F protein were known as a primary determinant and some other genes have also been shown to contribute to the virulence of APMV-1. [25, 26]

As is well known, the velogenic and mesogenic APMV-1 viruses contain a polybasic amino acid motif [112] (K/R)–R–(Q/K)–(R/K)–R [116] and a phenylalanine at the position 117 of F protein, which could be cleaved by furin-like proteases, resulting in systemic infections. However, the lentogenic viruses can only replicate in the respiratory and intestinal tracts. [27] The F protein of genotypes VI and VII viruses are associated with virulent APMV-1 strains, but their pathogenicity in chickens is very different. It is reported that besides the proteolytic cleavage site, other regions of the F protein were also influence pathogenicity. [28] The F protein is a class I transmembrane protein that is synthesized as a precursor protein F0 and functional domain of the F protein are signal peptide, fusion peptide, three heptad repeats, transmembrane domain. [29] Only one of the selected amino acids residues at position 52 (I52 of genotypes VI and V52 of VII viruses) of the F protein, but not in any already known functional domain of the F protein.

There is a correlation between virulence and the efficiency of viral replication. It has been reported for APMV-1 that the replication complex is related with the virulence. [30] TheAPMV-1 replication complex is made up of the NP protein, P protein, and L protein. The NP protein encapsidates the RNA genome, which acts as the template for viral transcription and replication, forming the nucleocapsid. The P protein and L protein make up the viral polymerase, which transcribes the viral genomic RNA. [31] Several studies have demonstrated an increased virulence after the passage of some PPMV-1 isolates in chicken eggs, and they identified the amino acids changed in the L protein during the process. [32] Dortmans JC et al. have rescued recombinant viruses, including the viral replication complex of one PPMV-1 virus, using reverse genetics. The researchers' conclusion was that the viral replication complex is related with the virulence of APMV-1. [33] In this study, although the NP and L proteins were relatively conserved between genotypes VI and VII viruses, they have 12 genotype-specifically conserved amino acids. And this meant the NP and L proteins may associated with the virulence of PPMV-1 virus as other studies reported. From nucleotide and amino acid sequence similarities, we know that P is the least conserved protein of APMV-1. But the ratio that represented the proportion of selected amino acids of P protein is high. Therefore, we infer that these genotype-specifically conserved amino acids in the replication complex may influence the pathogenicity of the virus, even though, the growth characteristics of rSG10-17 were very similar to those of rSG10 in DF-1 Cells. It is possible that some of these 17 amino acid sites positively affect the pathogenicity of APMV-1 and others may have a negative effect.

HN and M protein also contain genotype-specific amino acids. The HN proteins are multifunctional molecules with three distinct activities: binding activity, neuraminidase activity and fusion-promotion activity. [34, 35] It is reported that HN protein also affect virulence. [36] The genotype-specific amino acid residues at position 390 of HN protein, belonging to the globular head domain. Maybe this genotype-specific amino acids influence the binding activity and neuraminidase activity. The matrix protein (M), plays a crucial role in the viral replication. The genotype-specific amino acid residues at position 193 of M protein. A previous study demonstrated that arginine 36 (R36) of the M protein is a key factor that evolved in the adaptation of PPMV-1 to pigeons. [37]

In this study, we only focus on the influence of six structural proteins on the different pathogenicity. We did not study the unique amino acid in V proteins in the manuscript. However, several studies have shown that the nonstructural V protein is also closely associated with the pathogenicity and host range restriction of APMV-1. [38, 39] So the role of V protein in the different pathogenicity between these two viral genotypes will be evaluated in the future study.

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In conclusion, to analyze the molecular basis reasons of the differences in chicken pathogenicity between genotypes VI and VII of APMV-1, we simultaneously mutated all the genotype-specific amino acids in APMV-1 to investigate their influence on the pathogenicity of the virus. Although the ICPI and MDT values of strain rSG10-17 did not differ from those of the parental strain, the effects of each genotype-specific amino acid should be investigated individually in the further study, the function of the relevant protein should also be taken into consideration at the same time.

Acknowledgments

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Conflicts of Interest

The authors declare no conflicts of interest relevant to this study.

SupplementsFigures

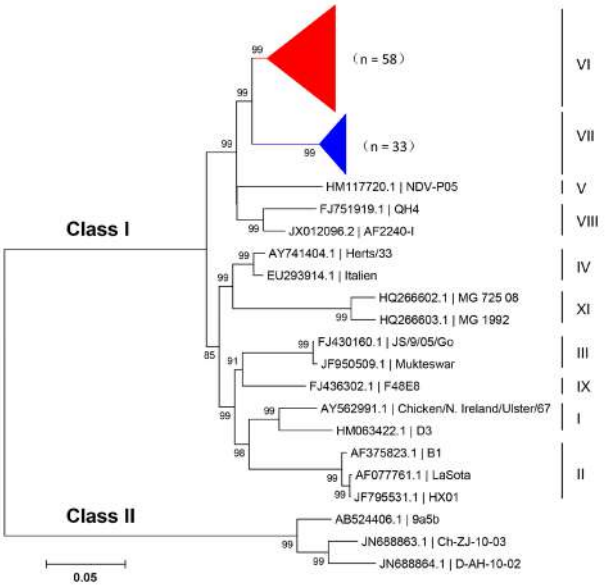

Figure 1. The phylogenetic tree based on the nucleotide sequences of the fusion (F) gene of NDV

Note: This phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replicates. The red and the blue branches represent viruses of genotypes VI and VII, respectively.

Table 2. Seventeen consensus amino acid sites that differ between 58 genotype VI and 33 genotype VII APMV-1 strains

Genotype	Virus	NP^{450}	NP^{464}	P^{162}	P^{243}	M^{193}	F^{52}	HN^{390}	L^{329}	L^{423}	L^{1096}	L^{1241}	L^{1334}	L^{1495}	L^{1542}	L^{1840}	L^{2017}	L^{2107}
		L	S	E	Y	A	I	V	D	K	V	K	K	S	I	F	R	T
VI	01. AB853926.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	02. AB853928.2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	03. AY562989.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	……	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	56. KM374059.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	57. KM374060.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	58. KM374061.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		F	P	G	H	V	V	I	N	N	I	N	R	N	V	Y	K	F
VII	01. AF431744.3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	02. DQ485229.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	03. DQ485230.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	……	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	31. KC542914.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	32. KC853019.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
	33. KM885167.1	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.

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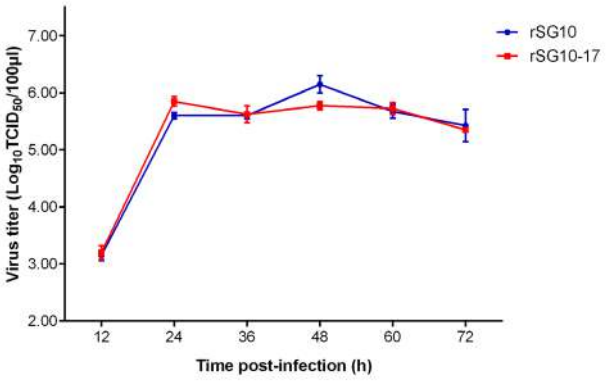

Figure 2. Multistep growth curves for rSG10 and aSG10-17 in DF-1 cells

Note: The cells were infected with the indicated APMV-1 viruses at 0.01 MOI. Samples were harvested at 12h intervals for 72 h, and the viral titers were determined with the $TCID_{50}$ method in DF-1 cells.

Table 1. Nucleotide (nt) and amino acid (aa) sequence identities (%) between 58 genotype VI and 33 genotype VII APMV-1 strains

	Nucleotide sequence identity (%)	Amino acid sequence identity (%)
Genome	86.4-91.7	
NP	87.3-92.7	91.7-98.0
P	82.5-90.2	78.8-89.9
M	86.8-93.6	90.7-97.0
F	86.6-93.2	90.6-96.9
HN	85.6-92.0	89.2-95.3
L	88.6-93.3	94.2-97.2

Table 3. Ratios of the number of genotype-specific amino acids to the total amino acids in each APMV-1 protein

Protein	Total amino acids	Selected amino acid sites	No. selected amino acid sites / No. total amino acids (%)
NP	490	2	0.41
P	396	2	0.51
M	365	1	0.27
F	554	1	0.18
HN	571	1	0.18
L	2205	10	0.45

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ARTICLE

Integrated Nutraceutical – Nutritional Approaches to Address Equine Leaky Gut Syndrome

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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).

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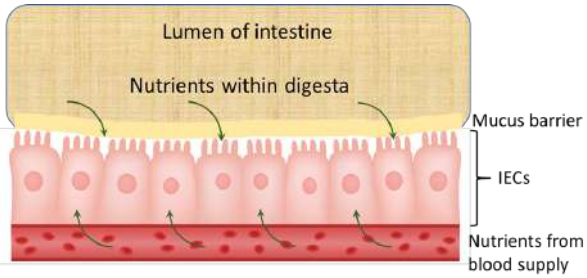

Figure 1a. Schematic representation of a portion of intestinal wall showing the physical barrier formed by intestinal epithelial cells (IECs) and the mucous barrier that overlies the IECs in intestinal lumen. Tight junctions, adherens junctions, and zonula ocludens junctions normally hold IECs tightly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

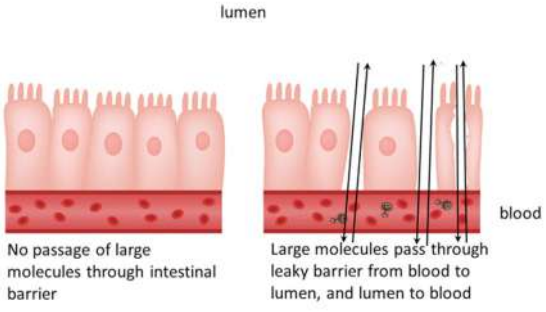

Figure 1b. Left panel: Healthy IEC layer with no leaks. Right panel: Intestinal pathogens attack IEC structures, weakening the physical barrier, which can result in inflammation and leak through the weakened barriers. Leak can occur between cells (paracellular pathway) or through severely damaged cells

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 ar-

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ticle focuses on providing nutrients on the luminal side (feedstuffs) and that will have effects on the GIT without 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

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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 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

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pigs [43]. This translates to an optimum dietary L-threonine 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-bene-

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ficial microbes and reduces the populations of commensal 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.

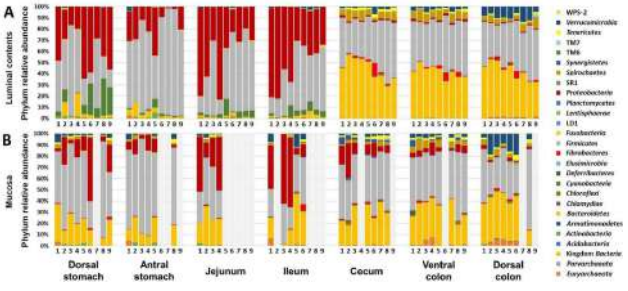

Figure 2. A microbiome map of the phylla resident in the healthy horse GIT. The equine GIT contains more than 150 different species of microbiota from 27 different phyla, of which about 25 species predominate. [87]

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 diarrhea 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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ARTICLE

Comparison of Secnidazole and Fenbendazole for the Treatment of Asymptomatic Giardia Infection in Dogs

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ABSTRACT

The objetive of this study was to compare a single dose of secnidazole versus multiple doses of fenbendazole for the treatment of dogs with asymptomatic *Giardia* infection. Materials and methods: Twenty-four asymptomatic dogs with a positive test result for *Giardia* spp were randomized in two equal groups to receive a single dose of secnidazole at 30 mg/ kg PO, or fenbendazole at 50 mg/ kg PO q24h for 3 days. Hematological parameters were evaluated before and 8 days after treatment, and feces were re-examined at days 8, 15, and 30 post-treatment by fecal flotation and antigen test. Results: The number of positive dogs in the fenbendazole group was: 1 (day 8) and 3 (days 15 and 30). In the secnidazole group, the number of positive cases were: 4 (day 8), 3 (day 15), and 1 (day 30). Conclusion: Treatment with secnidazole or fenbendazole, were effective between 75% and 92% to eliminate the excretion of *Giardia* cysts in canines together with hygienic measures to control, like disinfection with quaternary ammonium of patients and their environment. Further studies that include more animals and multiple fecal exams on consecutive days would be necessary to confirm its efficacy in dogs.

1. Introduction

Giardia spp are flagellated protozoan parasites found in the intestinal tract of mammals, birds, and reptiles worldwide. *Giardia duodenalis (syn. G. intestinalis* or *G. lamblia*) inhabits the small intestine of humans, dogs, and cats, and is considered as a potential zoonotic risk [1]. In developing countries with a high prevalence and incidence of infection, some studies suggest that chronic *Giardia*sis causes delayed growth in children [2,3,4].

Although *Giardia* infection may be common, clinical signs of diarrhea are not always present and many dogs and cats are subclinical carriers [5,6,7]. The prevalence in dogs has been reported to vary from 1% to nearly 28%, with some predisposing factors being age and lack of hygienic conditions [8,9]. Although *G. intestinalis* is a species complex with a wide mammalian host range, including people, the role of pets as a source of human *Giardia*sis remains unclear and there are no current recommendations to test and treat healthy pets for *Giardia* spp infection [1,2,10].

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However, animals housed under conditions of stress or overcrowding may have high prevalences of *Giardia*, for this reason it is necessary to treat the infected animals in all cases.

Treatment of *Giardia*sis with fenbendazole at 50 mg/kg orally every 24 hours for 3–5 days has been reported to effectively eliminate infections in 86%–100% of dogs and cats [11,12]. An alternative treatment is metronidazole at 50 mg/kg q24h for 5 days, but it is only about 67% effective in eliminating *Giardia* spp from infected dogs and may be associated with adverse side effects such as development of anorexia and vomiting, which may progress to neurotoxicosis [13,14]. In addition, treatment for 5 days is impractical for some animals that are difficult to handle or for treating large populations (e.g., kennels, catteries). In these situations, the search for alternative therapeutic options, including single-dose treatments, could be quite valuable.

Secnidazole is an antiparasitic used in humans that it is administered as a single dose for the control and treatment of *Giardia*sis [15,16]. When a single oral dose of 30 mg/kg was used extralabel in 18 naturally infected cats it achieved a 100% efficacy to eliminate cyst shedding in the feces [17]. The aim of this study was to evaluate the use of a similar single oral dose of secnidazole for treating naturally acquired *Giardia*sis in dogs.

2. Materials and Methods

2.1 Ethics Committee

This study was approved by the Ethics Committee for Animal Experimentation of the University of Antioquia, Colombia, record No. 72.

2.2 Type of Study

A prospective positive-controlled study with dogs randomized to receive fenbendazole or secnidazole.

2.3 Study Population and Treatment Protocols

This is a field study the owners maintained the dogs. Dogs eligible for inclusion in the study had owner consent as well as a positive test for *Giardia* cysts by fecal flotation with zinc sulfate solution on the day of examination. A total of 34 out of 250 clinically healthy dogs from the metropolitan area of Medellin met these criteria. The following hematological and biochemical parameters were analyzed on the day 0 of examination and at day 8 after treatment: complete blood count, BUN, creatinine, ALT, alkaline phosphatase, albumin, and direct and indirect bilirubin. Ten animals were excluded from the study due to concurrent illness, death, or being lost to follow-up. The study involved 24 dogs that were divided in 2 groups: secnidazole at 30 mg/kg PO single dose or fenbendazole at 50 mg/kg PO daily for 3 days. The secnidazole group was comprised of 4 females and 8 males with a mean age of 33 months (range 12–48 months). The fenbendazole group included 7 females and 5 males, with a mean age of 28 months (range 10–48 months).

2.4 Diagnostic Tests

Blood samples were collected on the day of examination (day 0) and at day 8 post-treatment. Stool samples were collected on days 0, 8, 15, and 30 post-treatment. The day of treatment (day 1), the perineal area was thoroughly bathed with a quaternary ammonium solution 0.05% to remove cysts from the hair coat and prevent reinfection from grooming. The owners were advised and trained to disinfect every day the premises where the dogs lived and to promptly remove feces to limit environmental contamination. They were also instructed to notify for any signs of vomiting, diarrhea, anorexia, or other abnormalities.

The diagnostic tests for *Giardia* included a direct smear examination for cysts following centrifugal fecal flotation with zinc sulfate, and multiple ELISA for the detection of *Giardia* antigens in feces (SNAP *Giardia* Test, IDEXX Laboratories) [18]. The sample was considered infected when resulted positive at least in one test. Statistical analysis was performed using SAS software with chi square analysis for qualitative variables (positive or negative infection) and Student's t for quantitative variables, and the level of statistical significance was set at $P<0.05$.

3. Results

A total of 250 stool samples from a random population of dogs from Medellin were processed by fecal flotation with zinc sulfate to detect *Giardia* cysts. There were 34 positive samples, giving a prevalence of 13.6% of the studied population. Some of these animals had to be removed from the study because they have clinical signs and a more complete treatment that included hydration, antibiotics and analgesics was required, leaving 24 suitable dogs that were equally divided in 2 groups to receive fenbendazole or secnidazole. This study did not include an untreated infected group, and effectiveness of treatment was determined by comparing the different evaluation days with day 0.

The number of positive and negative animals following treatment is shown in Table 1. In the group of animals that received secnidazole, there were 4, 3, and 1 dogs positive on days 8, 15, and 30, respectively, being always the

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same positive animals. In the fenbendazole group, only 1 of 12 animals had *Giardia* cysts on day 8; however, 3 dogs were positive at 15 days and the same 3 dogs were positive at 30 days after treatment. No statistical differences were observed in outcomes between the treatment groups ($p>0.05$).

With regard to the hematological and biochemical parameters analyzed, all values were within the normal range in every animal before treatment and on day 8 after treatment (Tables 2, 3, and 4). In addition, the owners did not report diarrhea or signs suggestive of adverse drug effects throughout the study period.

4. Discussion

Prevalence rates for *Giardia* infection in dogs have been reported to vary from 1% in fecal samples from well-managed pets to 28% in shelter dogs [5,8,9,19,20]. Typically, younger animals may show signs of infection, with adults being subclinical carriers [5,19,21]. In this study, 34 out of 250 (13.6%) dogs were positive by fecal flotation technique.

The classical treatment used in dogs and cats against *Giardia* has been fenbendazole (50 mg/kg/day for 3–5 days), and experimental infestations have shown its efficacy close to or at 100% [11,12]. Those studies are in accordance with the present results where only 1 of 12 animals was positive on day 8 following a 3-day treatment protocol with fenbendazole. The fact that a total of 3 animals in the fenbendazole group tested positive on days 15 and 30 suggests that re-infection occurred in 3 dogs.

Other studies have used alternative drugs with more variable results, including metronidazole at 30–50 mg/kg q12h for 15 days with or without silimarin [14], and albendazole at 25 mg/kg q12h for 2 days [11]. Similar to the results of this study, these products have good effects on the control of *Giardia* in dogs, but none over 100% of treated canines at all assessment times.

Earlier studies in cats showed that a single oral dose of 30 mg/kg secnidazol reached 100% efficacy at days 6, 7, and 8 post-treatment [17]. In this study, only 8 of 12 dogs were negative on day 8 post-treatment, even though all animals, except for one, turned negative by day 30. It is possible that because of the intermittent nature of *Giardia* shedding (from undetectable to large concentrations in feces), the negative test results on day 30 coincided with a time of no shedding. Therefore, infection cannot be definitively ruled out in spite of the lack of cysts in the fecal samples.

The pharmacological treatment of *Giardia* infection in dogs and cats is very effective; however, there may appear cases of therapeutic failure, that are very likely due to re-infection phenomena through the ingestion of cysts from the environment [22,23]. Reinfection is a phenomenon that in this study probably occurred since the animals were all the time in their homes with their owners and the disinfection was in charge of these, with possible flaws in this process. In addition, this study did not have information on the coexistence of other animals or humans that could be able to favor reinfection.

Thus, besides the antiprotozoal treatment, accompanying measures such as post-treatment bath and sanitation of the environment have been recommended before considering resistance to treatment [23]. Therefore, in this study we used the combination of treatment with secnidazol or fenbendazol together with the disinfection of the quarters with quaternary ammonium and washing of the perineum of the study subjects, thus reducing the risk of recontamination with the cysts excreted by the parasite. The high resistance and ubiquity of the *Giardia* cysts also play an important role in the recontamination. Nevertheless, our results indicate that treatment with secnidazol or fenbendazol, together with hygienic measures like disinfection with quaternary ammonium of patients and their environment, may be effective to eliminate the excretion of *Giardia* cysts in canines.

The administration of nitroimidazoles in animals may cause adverse reactions such as anorexia, nausea, and diarrhea [23] and a study reported that in felines secnidazol may cause increase of liver enzymes [17]. In the present study, none of the animals treated, that were asymptomatic, showed alterations or changes in their laboratory parameters. Nonetheless, more studies are suggested to help confirm drug´s safety mainly, if second doses of treatment are employed to counter reinfections.

Supplement

Table 1. Number of dogs shedding *Giardia intestinalis* cysts before and after treatment with secnidazole or fenbendazole

	N° of positive animals/Total (%)*			
Day	**0**	**8**	**15**	**30**
Secnidazole 30 mg/kg PO once	12/12 (100%)[a]	4/12 (33.3%)[b]	3/12 (25%)[b]	1/12 (8.3%)[b]
Fenbendazole 50 mg/kg PO for 3 days	12/12 (100%)[a]	1/12 (8.3%)[b]	3/12 (25%)[b]	2/12 (25%[b])

Note: *A positive animal was diagnosed based on detection of cysts by fecal centrifugation-flotation technique using zinc sulfate, and/or a positive ELISA result (SNAP *Giardia* Test, IDEXX Lab).

[a,b] Numbers followed by different letters in each row are statistically different from each other ($P<0.05$). No differences were observed between groups at any given time.

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Table 2. Mean (±SD) of hematological parameters in dogs infected with *Giardia intestinalis*, before and after treatment with secnidazole and fenbendazole

Parameter*	Before treatment	After treatment
Erythrocyte (x10^6/ul)	Reference range (5.5-8.5)	
Secnidazole	6.87 ± 1.06	6.91 ± 0.95
Fenbendazole	6.63 ± 0.81	6.68 ± 0.79
Hematocrit (%)	Reference range (37-55)	
Secnidazole	45.02 ± 7.22	45.50 ± 8.11
Fenbendazole	43.50 ± 5.93	42.11 ± 5.99
Hemoglobin (g/dl)	Reference range (12-18)	
Secnidazole	14.84 ± 2.32	14.93 ± 2.36
Fenbendazole	14.23 ± 2.28	13.72 ± 2.09
MCV (fl)	Reference range (60-77)	
Secnidazole	63.17 ± 2.08	62.33 ± 3.06
Fenbendazole	64.25 ± 3.41	63.83 ± 4.04
MCH (Pg)	Reference range (21-27)	
Secnidazole	22.14 ± 0.74	22.09 ± 0.94
Fenbendazole	21.68 ± 1.29	21.01 ± 1.17
MCHC (g/dl)	Reference range (32-37)	
Secnidazole	35.14 ± 0.99	34.99 ± 2.69
Fenbendazole	33.67 ± 1.38	32.81 ± 1.22
RDW (%)	Reference range (12-16)	
Secnidazole	14.85 ± 0.64	15.03 ± 0.94
Fenbendazole	15.32 ± 1.26	15.45 ± 1.51
Platelets (x10^3/ul)	Reference range (190-500)	
Secnidazole	195.33 ± 56.70	220.08 ± 87.35
Fenbendazole	252.58 ± 77.75	248.08 ± 72.33

Note: MCV: Mean Corpuscular Volume.

MCH: Mean Corpuscular Hemoglobin.

MCHC: Mean Corpuscular Hemoglobin Concentration.

RDW: Red blood cell Distribution Width.

*No statistical differences were observed among groups or before and after treatment.

Table 3. Mean (±SD) of white blood cells in dogs infected with *Giardia intestinalis*, before and after treatment with secnidazole and fenbendazole

Variable*	Before treatment	After treatment
Total leukocyte count (/ul)	Reference range (100-1700)	
Secnidazole	11385.83 ± 3513.78	11764.17 ± 3165.25
Fenbendazole	18095.00 ± 5569.51	14962.50 ± 5660.67
Eosinophils (/ul)	Reference range (100-1700)	
Secnidazole	1624.61 ± 1551.33	1582.78 ± 1400.74
Fenbendazole	1669.04 ± 935.04	1599.68 ± 1775.98
Neutrophils (/ul)	Reference range (3300-12000)	
Secnidazole	6113.69 ± 2834.44	6952.72 ± 2805.93
Fenbendazole	11982.08 ± 5245.06	9444.13 ± 3895.04
Bands (/ul)	Reference range (0-300)	
Secnidazole	224.05 ± 340.88	84.87 ± 186.14
Fenbendazole	184.73 ± 220.34	32.14 ± 82.22
Lymphocytes (/ul)	Reference range (1000-4500)	
Secnidazole	3070.91 ± 971.06	2848.98 ± 1232.76
Fenbendazole	3856.25 ± 1563.57	3474.21 ± 1020.68
Monocytes (/ul)	Reference range (100-700)	
Secnidazole	314.91 ± 296.98	278.91 ± 211.43
Fenbendazole	402.91 ± 296.87	412.38 ± 245.09

Note: * No statistical differences were observed among groups or before and after treatment.

Table 4. Mean (±SD) of some clinical biochemistry in dogs infected with *Giardia intestinalis*, before and after treatment with secnidazole and fenbendazole

Parameter*	Before treatment	After treatment
ALT(UI/l)	Reference range (21-102)	
Secnidazole	47.20 ± 34.91	36.45 ± 11.29
Fenbendazole	43.42 ± 31.11	36.25 ± 9.09
Alkaline phosphatase (UI/l)	Reference range (10-73)	
Secnidazole	40.00 ± 17.26	27.75 ± 15.74
Fenbendazole	61.25 ± 37.77	58.09 ± 33.32
BUN (mg/dl)	Reference range (10-28)	
Secnidazole	25.60 ± 4.41	29.13 ± 4.76
Fenbendazole	26.26 ±5 .20	24.16 ± 7.25
Creatinine (mg/dl)	Reference range (0.5-1.5)	
Secnidazole	1.03 ± 0.14	1.05 ± 0.18
Fenbendazole	0.87 ± 0.13	0.92 ± 0.17
Total protein (g/l)	Reference range (54-71)	
Secnidazole	77.50 ± 12.48	79.50 ± 8.27
Fenbendazole	69.83 ± 5.56	69.33 ± 5.93
Albumin (g/l)	Reference range (26-33)	
Secnidazole	28.10 ± 4.56	28.08 ± 4.56
Fenbendazole	30.00 ± 3.77	31.58 ± 5.66
Total bilirubin (mg/dl)	Reference range (0.1-0.9)	
Secnidazole	0.72 ± 0.35	0.74 ± 0.55
Fenbendazole	0.94 ± 0.44	0.86 ± 0.71

Note: * No statistical differences were observed among groups or before and after treatment.

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ARTICLE

A Pilot Study on Behavioural Responses of Shelter Dogs to Olfactory Enrichment

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ABSTRACT

The influence of essential oils (EOs) on emotions has been widely described among humans and animals. Several studies have investigated the effects and the actions of EOs on behaviour, mood and perception. In this study, shelter dogs (n=23) were exposed to olfactory stimulation through diffusion of 9 anxiolytic essential oils in one blend (olfactory enrichment) for 8 weeks in order to check long-term effects on behaviour. First, dog's postures have been evaluated in both groups before and after exposure. Secondly, in order to collect the preliminary results on the distance necessary to obtain an effect of EOs, dogs were divided in 2 groups according to the distance from the diffuser.
Our results indicate that olfactory enrichment with *this blend of EOs* is related to less time spent by dogs in high posture. More research is needed to investigate a potential gradual effect of distance and concentration of EOs on dog's welfare.

1. Introduction

Essential oils (EOs), which are obtained through distillation from aromatic plants, have been widely used for bactericidal, viricidal, fungicidal, antiparasitic, insecticidal, medicinal and cosmetic applications [1].

The influence of EOs on emotions has extensively been described among humans [2-6]. Several studies have investigated the effects and the physiological pathways of EOs on behaviour, emotions and perception [7]. In the last few years, scientific research has focused on very specific effects of different EOs among humans (e.g., the anti-anxiety effects of *Lavandula angustifolia* or the improved alertness effect of *Mentha piperita*).

Also, in animals, the use of aromatherapy as environmental enrichment has been studied in e.g. zoo animals [8-10], kenneled dogs [11-13], kenneled cats [14], and horses [15-16].

Shelter dogs often live in stressful situations. In some of these cases, specific anxiolytic essential oils may be helpful for these dogs in order to decrease their stress.

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Graham and colleagues [11] exposed 55 dogs of mixed breed housed in an animal rescue shelter to four types of olfactory stimulation: the diffused essential oils of lavender, chamomile, rosemary and peppermint. Animals were also studied in their normal kennel environment minus the introduction of any artificial odours (control). The dogs received each condition of stimulation for 4 hours a day for 5 days, with an intervening period of 2 days between conditions. Certain aspects of the dogs' behaviour were found to be influenced by the odours. Specifically, dogs spent significantly more time resting and less time moving upon exposure to lavender and chamomile than any of the other olfactory stimuli. These odours also encouraged less vocalization than other types of scent. The diffusion of rosemary and peppermint into the dogs' environment encouraged significantly more standing, moving and vocalizing than other types of odour.

The Cognitive Bias Test is another approach that can be used to understand the effect of Eos. Olfactory enrichment with a blend of 9 anxiolytic essential oils (*Cananga odorata, Cistus ladaniferus, Citrus aurantium, Cupressus sempervirens, Juniperus communis var. montana, Lavandula angustifolia, Laurus nobilis, Litsea citrata, Pelargonium graveolens*) resulted in a reduced latency to the ambiguous cue (cognitive bias test), indicating a more optimistic bias [13]. A cognitive bias test in this context refers to the propensity of a subject to show behaviour indicating the anticipation of either relatively positive or relatively negative outcomes in response to affectively ambiguous stimuli [17]. Changes in cognitive bias reflect an individual's experience of positive and negative events and thus its affective valence and welfare [18]. This recent and innovative approach utilizes the influence of affective states on the interpretation of current experience. The resulting affect-induced cognitive biases can be measured [17] through cognitive bias tests as indicators of the animal's psychological well-being [17, 19].

Observation of dog's posture is often used as a valid instrument to interpret emotions. In a study where researchers checked potential welfare effects of two different housing conditions through behavioural and physiological parameters, shelter dog's postures were evaluated as behavioural parameters [20]. In the SAB Test (Socially Acceptable Behaviour Test) most dogs that threaten or bite other dogs will have a high posture or a behavioural state of high arousal [21].

We tested the same blend of Eos applied in [13]. The aim of this study was to evaluate dog's postures before and after EOs diffusion. Dogs have been exposed to the blend at two different distances in order to explore the distance to induce an effect (diffuser in the same corridor and diffuser at 10 m distance from the corridor). We hypothesized that the blend could reduce the dogs' reactivity demonstrated by a reduction in time spent in high posture due to the anxiolytic effect of the EOs-blend. However, the group that was directly exposed to the diffusion is expected to display bigger changes in posture then the group at a 10 m distance of the diffuser.

2. Material and Methods

2.1 Subjects and housing

This study was carried out during a period of 8 weeks at a rescue shelter (Het Blauwe Kruis, Zinnialaan 2, Oostende, Belgium). A total of 23 shelter dogs participated: Group 1 (G1) included 7 males and 4 females, Group 2 (G2) included 10 males and 2 females. All dogs were declared to be in good health at the onset of the study.

The dogs were between 1 and 9 years old and arrived at the shelter within 1 month up to 5 years of the start of the study (mean for G1 and G2: 1.1 year). All dogs were spayed.

The sample of dogs was comprised of several breeds (G1: 1 Belgium shepherd, 2 Staffordshire terriers, 1 Bull terrier, 2 Labradors, 2 crossed Rottweilers, 1 Red nose pitbull, 1 Husky, 1 Mixed breed; G2: 1 Belgium shepherd, 2 Staffordshire terriers, 1 Shar-Pei, 1 Pincher, 1 Teckel, 2 Jack Russell terriers, 1 Yorkshire terrier, 1 Akita Inu, 1 French bulldog, and 1 Cross poodle).

The dogs of G1 and G2 were individually housed in typical indoor (G1: 2 x 2 x 2.5 m; G2: 1.5 x 2 x 2.5 m) – outdoor (G1 and G2: 2 x 4 x 2.5 m) pens (Figure 1). The diffuser was located in the G1 corridor, meanwhile the G2 was at 10 meters far from the diffuser. All the dogs remained in their own shelters throughout the study.

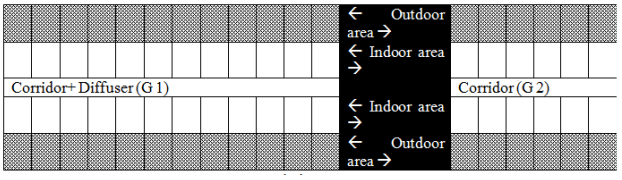

Figure 1. Shelter

2.2 Experimental Design of the Olfactory Enrichment Procedure

The dogs were exposed to olfactory enrichment through a blend of essential oils (*Litsea citrata, Cupressus sempervirens, Citrus aurantium, Pelargonium graveolens, Lavandula angustifolia, Cananga odorata, Juniperus communis var. Montana, Cistus ladaniferus and Laurus nobilis*). The

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blend was diffused by a specific instrument (diffuser) manufactured by Voith©, able to diffuse up to 300m². This diffuser was placed in the central corridor of G1 and was activated from day 3 until day 56 from 5PM until 2PM the following day (21 hours per day). A concentration of 3 ml was gradually diffused each day over 21 hours. This concentration was chosen according to preliminary data collected by authors. The diffuser was silent and there was no unfamiliar auditory stimulation during the experiment.

The dogs of G2 were not directly exposed to this olfactory enrichment, though, but were expected to perceive these odours. The dogs of G1 and G2 were used to the normal odors from the shelter, and those odors were considered to be the neutral control odors. Dogs of G1 and G2 were all studied at the same time within the same kennel environment.

A camera filmed each dog during a session of 20 minutes each day: (1) throughout the control period (day 1 until day 3; no diffusion of EOs), (2) during week 1 (day 4 until day 10) and (3) again during week 8 (day 57 until day 63) to check long-term effect. Filming occurred after feeding the dogs at 9AM. According to [22], the first 5 minutes and the last 5 minutes were not used to avoid human interference due to installing and removing the camera. However, dogs ignored the camera in all occasion. Additionally, no human disturbance was allowed during testing.

The postures of the dogs were recorded on videotape using a surveillance camera (Digital Video Camera Recorder, DCR-TRV27E, Sony®). The camera was placed in front of the cage. Videotapes were scored by one of the authors, and independently by one student. This student received training by the authors to score the behaviours. Intra-observer reliability exceeded 95%. Postures were evaluated according to Table 1.

Table 1. Postures description according to [22] and [23]

Postures	
High	The breed specific posture as shown by dogs under neutral conditions, but in addition the tail is positioned higher or the position of the head is elevated, and the ears are pointed forwards, or the animal is standing extremely erect
Neutral	The breed posture shown by dogs under neutral conditions
Half low	Two or more of the following three features are displayed: a lowered position of the tail (compared to the neutral posture), a backward position of the ears and bent legs
Low	The position of the tail is lowered, the ears are positioned backwards, and the legs are bent
Very low	Low posture, but now the tail is curled forward between the hind legs
Not seen	Unable to determine the behaviour of the dog owing to darkness or the position of the dog

3. Data analysis

The effect of period (control, week 1, week 8) on the percentage of high posture was modelled. However, since the observations belonging to the same dog are correlated, as well as the observations taken on the same day, two random effects were included in the model to capture these correlations. This implies the estimation of the following mixed linear model:

$$Y_{ij}=\beta_0+\beta_1 x_{ij}+\delta_i+\gamma_j+\varepsilon_{ij}$$

Where Y_{ij} is the measured percentage of high posture of dog_i on day_j, x_{ij} represents the period (control, week 1, week 8) for dog_i on day_j, δ_i is the random effect of dog_i , γ_j is the random effect of day_j , and ε_{ij} is the random error of the individual observation. If hypothesis testing showed that there was a significant effect of "period" on the percentage of high posture, multiple comparison testing with Tukey correction was performed to identify the significant differences between the three periods considered.

The statistical analysis was performed using JMP pro 13. The significance level was set at 0.05.

4. Results

4.1 Results for Group 1 (G1)

The average percentages of high posture suggest a decrease over experimental period in G1 (Table 2). The standard deviations are however quite high.

Table 2. Average percentage of high posture for each period as well as the standard deviation for G1

Period	Mean	Std dev
Control	86.75	23.12
Week 1	84.31	28.96
Week 8	57.53	36.74

The estimation of the mixed linear model reveals that G1 shows a significant effect of "period" on the percentage of high posture (p-value<0.0001, Table 3).

Table 3. Results fixed effects test of mixed linear model

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Period	2	2	161.4	21.507886	<.0001*

This significant effect is the result of a significant difference in average percentage of high posture between control period and week 8 (p-value <0.0001), and between week 1 and week 8 (p-value <0.0001). There is no significant difference between control period and week 1 for G1 (Table 4).

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Table 4. Subsequent pairwise comparison testing for G1

Period	-period	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Control	week_1	0.90607	6.033051	0.15	0.9876	-13.3656	15.17774
Control	week_8	27.65306	6.053850	4.57	<.0001*	13.3322	41.97394
week_1	week_8	26.74699	4.413390	6.06	<.0001*	16.3068	37.18723

4.2 Results for Group 2 (G2)

G2 shows similar results than G1. The results for G2 show a decline in average percentage of high posture over the experimental period, suggesting that period has an effect on the percentage of high posture. Also here, the standard deviations are quite high (Table 5).

Table 5. Average percentage of high posture for each period as well as the standard deviation for G2

Period	Mean	Std dev
Control	87.91	25.89
Week 1	77.45	34.41
Week 8	56.59	37.98

The estimation of the mixed linear model reveals that G2 shows a significant effect of "period" on the percentage of high posture (Table 6).

Table 6. Results fixed effects test of mixed linear model

Source	Nparm	DFNum	DFDen	F Ratio	Prob > F
Period	2	2	143.4	7.2587544	0.0010*

Subsequent pairwise comparison testing shows that there is a significant difference in average percentage of high posture between the control period and week 8 (p-value = 0.0007), as well as between week 1 and week 8 (p-value = 0.0368) for G2. There is no significant difference between control period and week 1 (p-value = 0.1403) for G2 (Table 7).

Table 7. Subsequent pairwise comparison testing for G2

Period	-period	Difference	Std Error	t Ratio	Prob>|t|	Lower 95%	Upper 95%
Control	week_1	11.05006	5.793235	1.91	0.1403	-2.67027	24.77040
Control	week_8	24.34497	6.463416	3.77	0.0007*	9.03742	39.65252
week_1	week_8	13.29490	5.338127	2.49	0.0368*	0.65242	25.93739

5. Discussion

The results of the present study indicate that olfactory enrichment with this blend may be helpful for dogs in shelter in order to spend less time in high posture. When dogs are decreasing their high posture, they become more relaxed [23]. In a daily routine at the shelter, when no stimuli are present, dogs are supposed to stay in a neutral posture that could be considered as an energy-saving posture.

The diffusion of the EOs blend can be helpful in relaxing dogs during long-term confinement in shelter. Effects have been reported both in G1 and G2: dogs spend significantly less time in high postures at the end of the study (week 8) than during the control period. Although we still do not define precisely the maximum distance that still has an effect, we observed that time spent in "high posture" decreased after exposure to EOs blend, even in dogs at 10m distance from the diffuser. Canine high posture has been categorized among agonistic behaviours [20-21]. In dogs' encounters with other conspecifics, body size and body posture are the first visual signals perceived, providing the very first information about other individuals' intentions. Dogs can communicate confidence, but also arousal, alertness, or threat by increasing their body size, pulling themselves up to their full height, and increasing the tension of the body muscles [24].

Living in a shelter has been correlated with some behavioural and welfare problems: abnormal social behaviour, enhanced or abnormal reactions in threatening situations, and retarded development of independence [25]. Abnormal reactions are quite common in routine shelter life. Vigilance and alertness without any apparent stimulus are often reported. According to [11], the use of EOs blend as olfactory enrichment provides new possibilities to enhance animal welfare and decrease behavioural problems due to stress (e.g., barking, whining, and high activity) among shelter dogs. Moreover, a combination of EOs seems more effective than the application of the EOs separately [13]. These results also build further on [26], who found that olfactory enrichment provides a sense of safety for animals.

Our results can be useful in order to understand how exactly EOs can be applied. However, this study shows that there are still several unsolved research questions. The minimal concentration and the minimal distance to get an effect has not been tested yet. The bibliography mentions various methods for analyzing the effects of EOs on behaviour in the lab and under field conditions. There is however a lack of consistency between these methods, and the authors of this study favor a standardized method for EO olfactory exposure, exposure time, observations (filming) and exposure to external stimuli like e.g. shelter stimuli.

The authors are convinced that olfactory enrichment can become part of routine management not only for animal shelters but also for veterinarians who are seeking to prevent the development of behavioural problems and to stimulate a positive relationship between owners and their animals.

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6. Conclusion

Our results indicate that olfactory enrichment with this blend of EOs is related to less time spent by dogs in high posture. More research is needed to investigate a potential gradual effect of distance and concentration of EOs on dog's welfare.

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Authorship

The idea for the paper was conceived by Dr. Anouck Haverbeke. The experiments were designed by Dr. Anouck Haverbeke and Dr. Stefania Uccheddu. The experiments were performed by Dr. Anouck Haverbeke, Dr. Stefania Uccheddu and Mathilde Debel. The data were analyzed by Dr. Heidi Arnouts Arnouts and Dr. Adinda Sannen. The paper was written by all the authors.

Conflicts of Interest

There is no conflict of interest.

Approval of the ethical treatment of animals, including the identification of the institutional committee that approved the experiments, was not required in the present study.

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REVIEW

Ground Flaxseed – How Safe is it for Companion Animals and for us?

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ABSTRACT

EFSA released the 89-page Scientific Opinion "Evaluation of the health risks related to the presence of cyanogenic glycosides in foods other than raw apricot kernels". This opinion, and the ensuring media coverage, has left uncertainty in the minds of consumers, feed and supplement manufacturers and flaxseed producers of how much ground flaxseed can safely be consumed without crossing the threshold of cyanide toxicity. This editorial updates the science and tries to bring clarity to the question "how much flaxseed can I safely feed my dog, cat, horse on a daily basis?" and "how much can I safely eat?" The great majority of ground flaxseed products have a cyanogenic glycoside content of less than 200 mg / kg seed. For people, consuming 30 grams of such flaxseed the average peak blood cyanide concentration will be about 5 µmole / L, much less than the toxic threshold value of 20 to 40 µmole / L favoured by EFSA. Thus, as much as 120 grams of crushed / ground flaxseed can be consumed by a 70 kg adult person before a toxic threshold of 40 µmole / L is reached (up to 1.7 grams ground flaxseed / kg body weight). The toxic threshold of cyanide for dogs is 2 to 4-fold greater than for humans, and unknown for cats and horses. The daily serving amounts for dogs and cats are about 0.23 grams / kg body mass per day, which will result in blood cyanide well below the toxic threshold. The highest recommended daily serving amount for horses is 454 grams per day, or 0.8 to 2 grams per kg / body mass depending on mass of the horse. This amount for horses should not be exceeded.

1. Introduction

The feed ingredient and food supplement industries for both humans and animals are reacting to the release of the 89-page EFSA "Scientific Opinion" entitled **Evaluation of the health risks related to the presence of cyanogenic glycosides in foods other than raw apricot kernels** https://www.efsa.europa.eu/sites/default/files/consultation/consultation/181127-ag.pdf. Statements at the heart of the issue include:

"Estimated acute {and chronic} exposures to cyanide originating from foods containing CNGs [cyanogenic glycosides] across 43 different dietary surveys and all age groups ranged from 0.0 to 13.5 µg/kg bw per day"

and

"At the 95th percentile, the ARfD was exceeded by up to about 2.5-fold in some consumption surveys for 'Infants', 'Toddlers', 'Other children' and the adolescent age groups."

Note: ARfD is the acute reference dose, above which

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cyanide is more likely to have toxic effects; the ARfD for humans is 20 µg cyanide / kg bw per day for chronic consumption, and 80 µg CN / kg bw per day for acute consumption. Read below for how much ground flaxseed this equates to.

The reporting of this document by the press, in particularly that in Britain (https://www.dailymail.co.uk/sciencetech/article-7367955/Scientists-warn-superfood-porridge-topping-flaxseed-cause-cyanide-poisoning.html), is generating justifiable concerns by both manufacturers and consumers of flaxseed products. The basis for the concerns arises from the following two statements:

(1) "Depending on the body weight, consumption of 1.3 g – 14.7 g ground linseed containing a high concentration of 407 mg CN/kg could reach the ARfD".

(2) "The highest acute and chronic exposures were estimated for 'Infants', 'Toddlers', and 'Other children'".

Granted, the EFSA Scientific Opinion admits that this is a worst-case scenario if consuming a product with a very high content of cyanogenic precursors.

Most people are aware that cyanide is a poison, and they would be correct in saying that there is really no safe amount of cyanide, i.e. there is no known concentration of cyanide in the body that exerts a beneficial effect in normal, healthy individuals. The concern, therefore, is that ingestion of flaxseed in foods and supplements will result in the production of cyanide in the body and produce toxic effects. The threshold for cyanide toxicity in the human body is a daily ingestion in the range 0.5 to 3.5 µg / kg bw, so concentrations lower than 0.5 µg / kg bw should not result in any observable toxic effect in over 97% of the population. Some people (~3% of the population) may be more sensitive.

Directive 2002/32/EC16 provides a maximum content of hydrocyanic acid (essentially cyanide) in feed materials and complete feeding stuffs of 50 mg/kg of food (relative to a moisture content of 12%). Exceptions are linseed, linseed cakes, and manioc products/almond cakes for which maximum contents are 250, 350 and 100 mg of cyanide / kg, respectively and complete feeding stuffs for chicks which can contain a maximum of only 10 mg/kg (EFSA, 2018).

But before getting into how much is safe of us or our companion animals to eat, we need to have a better picture of what type of flaxseed (or linseed) product is being consumed. The main concern is with fresh, crushed or ground flaxseed. Not whole flaxseed from which little cyanide is formed and released when consumed, nor flax oil which is extracted from the seed and does not contain appreciable amounts of cyanogenic glycosides or the enzymes needed to produce cyanide from cyanogenic glycosides. It is also not with some types of treated ground flaxseed.

Scientists have developed ways of treating flaxseed to prevent cyanide production, with the ability to conserve all of the nutritive and nutraceutical benefits. Using fresh, crushed flaxseed Yamashita et al. [24] developed a commercial-scale method to enzymatically release cyanide and effectively remove the cyanide by steam-evaporation. Steam-evaporation was more effective than heating or lyophilisation to evaporate the cyanide. This method lowered the residual cyanide content below the detection limit without affecting the protein, fat, fibre and lignan content of the linseed. More recently, Wu et al. [23] developed a fermentation technique that can be performed on a commercial scale with the benefit of lower energy consumption and no environmental pollution compared to steam evaporation. Importantly, "the detoxified flaxseed retained the beneficial nutrients, lignans and fatty acids at the same level as untreated flaxseed".

Other forms of heat-treatment to detoxify crushed flaxseed (boiling, roasting, autoclaving, microwave) may not result in appreciable cyanide production in the body because the heat destroys the enzymes responsible for cyanide production. Some people may not want buy such heat-treated products because they are of lower nutrient value, particularly for the beneficial unsaturated fatty acids and because removal of cynanogenic precursors is incomplete [2,7]. Such heat-treated products would, however, certainly be safer as far as the cyanide issue is concerned.

2. Fresh, Crushed or Ground and Untreated Flaxseed – How Much is Safe to Eat?

So how much fresh crushed or ground flaxseed can one safely eat? Particularly in view of the fact that many product labels and websites are advising people to consume 30 grams / day and sometimes more. How much is safe depends on the content of the cyanogenic precursors present in the product.

Table 23 of the EFSA Scientific Opinion provides for how much ground flaxseed can be consumed without exceeding the ARfD. For toddlers (infants) the amount should not exceed 1.7 grams per eating occasion, and this value becomes 10.9 grams for adults. Therefore a daily maximum amount for adults with 3 eating occasions would be 32.7 grams.

In flaxseed the two main cyanogenic precursors are the glycosides linustatin and neolinustatin [4] and there may be small amounts of linamarin [3]. Enzymes (ß-glycosidase enzymes) naturally present in the cell walls of flaxseed hulls catalyze the conversion of the glycoside precursors to cyanide. Cyanide binds to proteins in the mitochondria

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of all cells and this results in the inhibition of oxidative phosphorylation, the primary process by which cells generate ATP (cellular energy). Oxidative phosphorylation is required for life.

The EFSA Scientific Opinion provides the following information:

"The acute lethal oral dose of cyanide in humans is between 0.5 and 3.5 mg/kg body mass [9]. The toxic threshold value for cyanide in blood is considered to be between 0.5 (~20 μM) and 1.0 mg/L (~40 μM), the lethal threshold value ranges between 2.5 (~100 μM) and 3.0 mg/L (~120 μM). Signs of acute cyanide poisoning in humans include headache, vertigo, agitation, respiratory depression, metabolic acidosis, confusion, coma, convulsions, and death. Poisoning cases, some fatal, have resulted from ingestion of amygdalin preparations, bitter almonds and cassava. Several neurological disorders and other diseases have been associated with chronic exposure to cyanide in populations where cassava constitutes the main source of calories."

Based on the limited data available, the EFSA recommends an acute reference dose (ARfD) of 0.020 mg CN / kg body mass, also established by FAO/WHO [6] for chronic consumption. For acute consumption an ARfD of 0.080 mg CN / kg is used. In an Australian assessment [8] it was determined that a "high consumption of linseed containing bread led to exposure estimates of up to 511 μg HCN/kg bw per day thereby exceeding the ARfD of 80 μg HCN/kg bw per day (EFSA, 2018).

With respect to flaxseed, there have been only two human studies that have examined the pharmacokinetic aspects of cyanide after ingestion of linseed (flaxseed) [1,25]. Also, the Schultz et al. study appears to have used whole flaxseed, not crushed or ground flaxseed, so therefore the bioavailability of cyanide will be much lower than if a ground product had been used [1].

Schultz et al. [25] reported 3 main trials:

In the acute trial, 20 normal subjects and 5 patients consumed 30 grams of flaxseed (capable of producing ~9 mg of cyanide in the body). The dosage of cyanide, for a 70 kg person, is thus 0.13 mg / kg body mass, which is 1.6 times higher than the EFSA-recommended acute ARfD of 0.080 mg / kg body mass. Now one can appreciate the concern. A peak increasein plasma cyanide occurred at 15 – 30 minutes and concentration returned to baseline by 2 hours. No adverse effects were reported.

In the second chronic trial, 25 normal subjects consumed 15 grams of flaxseed, three times daily, for up to 5 weeks. The daily dose of cyanide equivalents was 13.5 mg, or 0.19 mg / kg body mass, which is 10 times higher than EFSA-recommended chronic ARfD of 0.020 mg / kg body mass. There were increases in thiocyanate (cyanide breakdown metabolite) in plasma and urine throughout the trials.

One subject consumed 100 grams of flaxseed in a single dose, and for this subject plasma cyanide did not increase above baseline. In contrast, when this subject ingested 3, 6 or 12 mg of potassium cyanide the concentration of plasma cyanide peaked 20 to 30 minutes later and returned to baseline by 3 hours.

Abraham et al. [1] used fresh, ground flaxseed with a serving amount of 30.9 grams and a cyanide-equivalent dose of 6.8 mg (0.08 to 0.1 mg / kg body mass), consumed by 12 healthy subjects. This was approximately the EFSA chronic ARfD of 0.08 mg / kg body mass). It is also known that the concentration of cyanide inside red blood cells is about 10-fold greater than in plasma [9], and Abraham et al. [1] measured total blood (not just plasma) concentration of cyanide. Peak blood cyanide occurred 30 – 60 minutes after ingestion, and varied widely from 1.7 to 13.9 μmoles / L with a mean (± SD) of 6.4 ± 3.3 μmoles / L. In one subject that consumed 100 grams, a sustained peak cyanide of 42 μmoles / L occurred between 2 and 3 hours after ingestion and rapidly returned towards baseline over the next 3 hours. No adverse events were reported.

Abraham et al. [1] also reported that there are "no reports on cyanide poisoning after consumption of linseed [were] found in the literature". And I also could find no reports.

The highest daily dose reported in the literature is 80 g of ground linseed given as "fiber shock" in a private spa setting [20]. The typical high dose recommendation by health practitioners is 15 grams three times daily (EMA 2006) with Abraham et al. [1] stating this "this dose is safe with respect to possible acute toxicity of cyanide".

The normal content of cyanogenic precursors present in flaxseed from many sources in different continents ranges from less than 80 up to about 300 mg cyanide-equivalents / kg seed [1]. Using data from Abraham et al. [1] one can arrive at the following generalizations for fresh, ground flaxseed: Consuming 30 grams of flaxseed with a cyanogenic precursor content of 200 mg / kg seed will result in an average peak blood cyanide concentration of 5 μmole / L. This is less than the toxic threshold value of 20 to 40 μmole / L favoured by EFSA. Using these scientific data as a guide, as much as 120 grams of crushed / ground flaxseed can be consumed before a toxic threshold of 40 μmole / L is reached. For the 'average' 70 kg person this equates to 1.7 grams ground flaxseed / kg body mass.

3. Translating This for Our Dogs, Cats, Horses

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Ground flaxseed is widely used in pet foods, as well as in veterinary health products and various types of supplements. Evidence-based research in the target species is very low, with the majority of studies supporting product claims coming from studies performed on mice and rats. Research performed using dogs suggests that it can be added to the diet to support g.i. health [12,21] blood lipid profile [5], skin health [14,15,19] and reproductive health [13]. Studies using cats also support a role for reproductive health [13] and modulation of immune health and inflammatory responses [17]. While flaxseed, as well as flaxseed oil, is widely used to supplement the diet of horses there are few published research studies. Research using flaxseed (not the oil) in horses supports an improved blood and tissue lipid profile and hematology [10,18,22], immune function [22] and skin health and immune response [16].

In dogs and rats, LD50s (lethal dose for 50% of the animals) were equivalent to 2.13 and 4.0–6.03 mg CN / kg bw, respectively. The lowest lethal dose identified in humans was 0.56 mg cyanide/kg body mass. These values have not been determined for cats and horses, and it will be assumed that the LD50 would be in the range of 1 mg CN / kg body mass.

(For a typical ground flaxseed product containing 200 mg cyanide-equivalents / kg, a mammal would need to consume 10 grams of flaxseed per kg body mass to reach the lowest LD50 of 0.56 mg cyanide / kg body mass. A safe limit would be 10-fold lower, or 1 gram of flaxseed / kg body mass, which is about 2-fold greater than for humans, as described above).

The serving amounts for dogs and cats are typically provided using teaspoons and tablespoons. One teaspoon of ground flaxseed weighs about 2 grams, and a tablespoon weighs about 7 grams. The body mass range for adult cats is from 2 to 10 kg, and serving amounts are scaled to body mass. The lowest serving amounts for very small dogs (~2 kg body mass) is 1/8 teaspoon / day, thus the daily serving dosage works out to 0.125 grams / kg body mass per day. In the largest breeds (~60 kg body mass) a recommendation is to start with up to 2 tablespoons (14 grams), thus the daily serving dosage is 0.23 grams / kg body mass per day. These dosages are 5 to 10 times lower than what could be considered a safe maximum limit. It is likely that all known commercial dog and cat foods or supplements containing ground flaxseed should not be of any concern to the pet owner or veterinarian.

For horses, a recent recommendation in a trade blog is that up to 1 pound (454 grams)of fresh, ground flaxseed can be fed to horses per day ([11] https://thehorse.com/148473/5-facts-about-flax/). No research has been cited to support the statements. Therefore the 1 pound per day recommendation would be 'safe' as the cyanide dose would be about 10-fold lower than the assumed LD50. Most recommendations are in the 100 gram per day range, with some short-term recommendations approaching 800 grams per day for adult horses. Short-term use of fresh, ground flaxseed at more than 500 grams per day should be used under close veterinary supervision because, based on rodent studies, signs of toxicity may occur in sensitive animals at this dose.

4. What do Consumers Need to do?

Consumers are right to ask questions. They should ask questions like: Is this product safe, and how much can I safely eat? What is the level of cyanogenic precursors present in these products? How do I know if this product has been processed to destroy the enzymes responsible for producing cyanide? Are you able to show me something on paper that certifies these levels for this product?

Consumers should not take just the word of their retailer. Retailers are not scientiststhey do not fully understand the science or health concerns and may not be able to properly understand product specification sheets. If you can, obtain the information and take it to your physician or pharmacist.

5. What do Manufacturers Need to do?

Manufacturers of ingredients, foods and supplements are for the most part very responsible and want to produce safe products that work the way they are supposed to.

Manufacturers need to ask their flaxseed suppliers for product specification sheets that detail the amount of cyanogenic precursor, state if the product is processed to destroy the cyanide-producing enzymes, among many other things. If their supplier is not willing to provide this information, then they should not purchase that product.

Manufacturers should be proactive in allaying the concerns of companies and people purchasing their products, and freely supply supporting documentation and information.

6. What do Flaxseed Producers Need to do?

Aside from the consumer, the flaxseed producer remains in a high-risk situation with the classification of flaxseed as having a high potential for cyanide production. The current media coverage may translate to a moderate potential for lost revenue resulting from decreased sales. Now, we have known for decades that crushed / ground flaxseed results in cyanide production, but it has not really been an issue because the amounts produced do not cause

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toxic levels of cyanide in people or animals when consumed in the amounts that most people and animals are having.

Producers need to know the levels of cyanogenic precursors in their products. This means that for each field of flaxseed that is harvested, they need to obtain a triplicate analysis of a number of representative samples of flaxseed for the cyanogenic glycosides. The average and range of these values needs to be reported on the specification sheet of each 'batch' of flaxseed or flaxseed product, and this information provided to their customers.

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This document provides some guidelines to authors for submission in order to work towards a seamless submission process. While complete adherence to the following guidelines is not enforced, authors should note that following through with the guidelines will be helpful in expediting the copyediting and proofreading processes, and allow for improved readability during the review process.

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All articles should include a cover letter as a separate document.

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Declaration

v Conflict of Interest

Examples of conflicts of interest include (but are not limited to):

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- Employment or consultation
- Project sponsors
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- Informed Consent

This section confirms that written consent was obtained from all participants prior to the study.

- Ethical Approval

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The role(s) that each author undertook should be reflected in this section. This section affirms that each credited author has had a significant contribution to the article.

1. Main Manuscript

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In this section, the methods used to obtain the results in the paper should be clearly elucidated. This allows readers to be able to replicate the study in the future. Authors should ensure that any references made to other research or experiments should be clearly cited.

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