Fecal Microflora and Dysbosis; Contribution to Metabolic Syndrome, Inflammation and Leaky Gut Syndrome

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**Introduction**

**Intestinal bacterial overgrowth has been a recognized condition in humans, often correlated with systemic health conditions ranging from allergies to cancer, and is a common connection with obesity. Intestinal hyperpermeability or leaky gut syndrome, is a primary problem that has been also related to various health conditions, including diabetes, cardiovascular disease, metabolic syndrome, multi-organ failure, kidney disease, liver ailments and a common consequence to radiation or chemotherapy.  The connection between the two is that through the process of increased permeability, bacteria may gain access to the systemic circulation, contributing to organ infection and immune dysfunction. (1,5,6,14)**

The microbial balance within the gastrointestinal tract is an important contributor to overall health, digestion and nutrient assimilation.  The balance is easily upset due to changes within the diet and secondary to various internal and external stressors, including training, competition and transportation. As the balance is altered, certain bacterial strains can become more dominant, inhibiting more normal predominant strains, creating potentially harmful byproducts that impact health and even impact digestion and/or nutrient absorption.

The concept of intestinal hyperpermeability, leaky gut, has been explored for quite some time in research in both human and animal models.  The intestinal tract is designed to be a restricted, somewhat closed system, in which nutrients and fluid are absorbed, but other potentially harmful products including bacteria are contained and eliminated.  Permeability can be impacted by numerous factors but is often related to localized inflammation within the gastrointestinal tract, which can be secondary to numerous events.  As permeability is affected, tight junctions between normal cells in the gastrointestinal lining become compromised, leading to increased absorption of potentially noxious substances and bacteria.  As these substances are absorbed, they can contribute to systemic infection, immune dysfunction, inflammation, organ failure and a variety of other health conditions.

One example in human literature is the connection between *Streptococcus bovis* overgrowth in the bowel, valvular dysfunction and colon cancer.  The connection is so strong that in the human medical field, an individual undergoing a cardiac valve replacement that cultures positive for *Streptococcus bovis*, is recommended to have a colonoscopy performed to rule out concurrent colonic neoplasia. The exact connection can only be theorized at this time, with speculation as to underlying gastrointestinal overgrowth which then contributes to increased intestinal permeability with subsequent translocation of the bacteria. (7,9,11,13)

In the equine industry, it has been noted that in cases of metabolic syndrome and even colitis in horses, that a bacterial overgrowth or shift is present.  In most papers, it has been identified that the shift is in favor of an overgrowth of lactic acid producers as in the case of carbohydrate overload induced laminitis. (3,10)

In starch and oligofructose models of laminitis, bacterial shifts in population have been noted, mostly in favor of overgrowth of*Lactobacillus* and *Streptococcus* species with concurrent decrease in luminal pH.(8) In other studies, evaluating equine patients with active colitis compared to normal horses, there were dramatic variations in the microbial biome which resulted in the conclusion that colitis was more likely a result of gut dysbiosis rather than overgrowth on one particular organism.(2)  This further reinforces the importance of balance within the intestinal microbiome in the horse and how imbalance may contribute to disease and even culminating in death.(2)

In theory, based on human data regarding intestinal hyperpermeability, it may be possible that this shift in bacteria could be directly responsible for the ‘leakiness’ of the intestinal mucosa, secondary to local environmental changes, immune response with cytokine release and subsequent inflammation.  If this is the case, it would then be possible that the local inflammatory response could be transferred to a systemic level with possible translocation of bacteria, furthering an immune response and subsequent inflammation. In some studies, the by products produced as a result of overgrowth of certain bacterial populations have also been implicated directly with inflammation, including amines and fructose utilization. (3,10)

Chronic inflammation is a main focus of human and veterinary literature, making connections with a host of diseases in horses ranging from allergies to joint dysfunction to metabolic conditions and even laminitis.  As a whole, we have known the inflammatory response is primarily responsible for the progression of these diseases, but to date, the question has always been as to the source of the response.  In horses, one primary condition of focus and connection with gastrointestinal health is laminitis.  The potential connection with poor GI health has been shown in numerous studies, but has yet to be thoroughly explored as a means of intervention and treatment.

Through the exploration of feces from normal and clinically affected equine patients, we hope to provide a small piece of what may be a larger puzzle to consider.

**Purpose of the Trial:**

The overall goal with this research trial was to evaluate differences in fecal microflora between clinically perceived normal and abnormal horse populations.  The intention was to confirm prior data that indicated an overgrowth of lactic acid producing bacteria in certain populations and if present, potentially make a connection with ongoing health and lameness concerns.

**Methodology:**

Fifty three (53) horse were selected voluntarily at random from owners with the request for fresh fecal sample submission.  The request for was for a group of perceived normal horses and metabolic horses, with no specifications on breed, age, health or lameness conditions.

All fecal samples were submitted and processed within 12 hours of submission by the owner with full history including diet, supplementation and health related concerns.

Samples were recorded for color, moisture and odor, then further processing was performed for data accumulation.

Initially, 1 gram of fecal material was combined with 5 ml of 0.9% saline for purposes of wet mount evaluation, pH assessment and gram stain.  After dilution was performed, the suspension was mixed and then strained using sterile gauze to as to remove obvious debris.  A 1 ml syringe was filled with 1 drop placed onto a slide with cover slip for wet mount observation and another drop was added to a slide, then smeared out for gram stain evaluation.  The pH was performed by using a standardized pH strip with range of 5.0-8.5 with increments of 0.3 based on color chart.

The wet mount was performed under 100x magnification, evaluating several fields for motile bacteria with focus on obvious motile bacilli.  The motile bacilli were generally graded as present or not present.

A Sudan stain was also performed by directly compressing feces into a slide, making a moist impression, then removing obvious debris.  Sudan stain was then applied to the impression region by adding 2 drops, then performing an initial review under 10x magnification, focusing on the margins of the impression.  After initial review, Vinegar was added at the rate of 2 drops, then gentle heat applied to the slide for 5 seconds.  The slide was then reviewed again under 10X magnification, assessing for changes in coloration intensity or retention of Sudan stain by fat globules.  The rate of Sudan uptake was graded on a scale of 0-4 with 0 representing no uptake and a 4 indicating marked uptake.

The Gram Stain evaluation was performed under oil microscopy, gathering data from 10 different fields of view, then averaging.  The initial goal was to determine relative presence of gram positive and gram negative bacteria, based on staining.  Then the fields were examined for the presence of obvious gram positive bacilli and gram positive streptococcus, with numbers averaged per high power field.

The next step were fecal cultures using four different agar media, including CNA, MacConkey, and Enterococcus (Bile Esculin) agar.

Fecal cultures were performed through the use of 0.9% saline and 1 gram of feces, diluting out to a 1:10,000 dilution.  Plating was performed using 0.1 ml of the final diluent per agar plate, which was then streaked out accordingly, then incubated at 37°C for 24 hours, at which time were read, colonies noted, counted and recorded.

Distinct and consistent colony formations were further cultured on TSA/Blood Agar plates, cultured and submitted to the Ohio State University College of Veterinary Medicine for further identification through MALDI methodology.

**Patient Information:**

The trial enrolled a total of 53 horse of a wide variety of breeds and genders.  In the group, there were 19 reported normal horses with no clinical health problems and 34 horses with clinical problems ranging from anxiety, ulcers to allergies and active laminitis.

**Breed Demographics:**

Normal Horses

|  |  |
| --- | --- |
| Thoroughbred | 7 |
| Arabian | 4 |
| Mini | 1 |
| Quarter | 1 |
| Warmblood | 5 |
| Pony | 1 |

Abnormal/Clinical Horses

|  |  |  |  |
| --- | --- | --- | --- |
| Mini | 2 | Morgan | 2 |
| Appaloosa | 2 | Welsh | 3 |
| Quarter | 13 | Paint | 2 |
| Arabian | 1 | Pony | 1 |
| Friesan | 1 | Hafflinger | 1 |
| Thoroughbred | 5 | Warmblood | 1 |

**Patient Clinical Problems (abnormal horse group):**

|  |  |
| --- | --- |
| Laminitis | 13 |
| Allergies | 2 |
| Diarrhea | 1 |
| Lyme | 2 |
| Joint lameness | 2 |
| Weight loss | 1 |
| COPD | 2 |
| Foot Concerns | 3 |
| Anxiety/Ulcers | 5 |
| Navicular | 3 |

**Patient Categories & Data Determination:**

Patients were broken down into two general categories; normal or abnormal (implying clinical problems).  They were then broken down within those groups and subcategorized as easy keeper or non-easy keeper body condition.  An easy keeper horse was defined as one that was perceived as more ‘fleshy’ or one that was prone to gaining weight easily.

Patient results were determined for several categories:

1. pH
2. Sudan Stain
3. Gram positive Bacilli count under gram stain per high power field
4. Lactose positive colony growth on MacConkey agar
5. Enterococcus agar growth

Sudan stain is a laboratory technique used in human and companion animal veterinary practice in order to assist in the detection of malabsorption/maldigestion syndrome.  Sudan stain is applied to fresh fecal smear on a glass slide, then microscopically evaluated for baseline evaluation.  Vinegar is then applied to slide with gentle heating and re-evaluated in the same fashion, assessing for bright orange uptake in fat globules within the smear. The presence of high Sudan stain uptake is considered to be related to malabsorptive concerns.

The bacilli evident in some patients under Gram Stain has been isolated via culture and laboratory methodology to being predominantly *Lactobacillus* species, based on morphology and growth on MRS agar under CO2 conditions.  In some isolated cases there was also suspected concurrent *Clostridial* species overgrowth based on gram stain evaluation.

MacConkey agar is a selective and differential culture medium used to isolate gram negative bacteria and further differentiate them based on lactose utilization.  Lactose positive bacteria include *Escherichia coli*, *Enterobacter* and *Klebsiella*.  On MacConkey agar, lactose positive gram negative bacteria exhibit pink colonies due to fermentation of lactose and lowering of agar pH.  The specific colonies formed were consistent between patients and determined to be Gram negative rods on Gram Stain, while confirmed to be lactose positive *E. coli* on follow up MALDI identification methodology.

Enterococcus or bile esculin agar is a selective media utilized to isolate *Enterococcus*and group D *Streptococcus* bacteria which are normally present within the gastrointestinal tract.  One of the benefits of this agar is that its use helps to eliminate overgrowth of other gram positive organisms, including other non-D groups of *Streptococcus*. Growth on enterococcus media was confirmed gram positive and also reactive to Strep D antisera, which aids in confirming identification. On further isolation and identification through MALDI methodology, the predominant colonies were identified as strains of *Enterococcus*.

**Patient Data:**

**Normal Horse Group:**

The normal horse group did not include any that classified as ‘easy keepers’ by their owners on submission reports.

All values reported as colony forming units (cfu)/ml at a 1:10,000 dilution

Average values of normal horses as a group: (Total 19 horses)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 7.0 | 18 | 230 | 880 |

Average values of normal horses that were Sudan negative: (6 horses)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 7.0 | 7 | 20 | 350 |

Average value of normal horses that were Sudan positive: (13 horses)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus |
| 7.0 | 24 | 330 | 1130 |

Dietary Regimen of groups on average:

|  |  |
| --- | --- |
| Sudan Negative | Hays + Whole Grain +1 supplement |
| Sudan Positive | Hays, predominant commercial grains +1 synthetic supplement |

**Abnormal or Clinical Horse Group:**

The abnormal horse group consisted of horses that demonstrated clinical health or lameness problems by their owners.  This group contained both ‘easy’ and ‘non-easy’ keeper body conditions as determined by their owners.  All values reported as colony forming units (cfu)/ml at a 1:10,000 dilution.

Average values of clinical horses as a group: (Total of 34)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 6.7 | 16 | 510 | 1020 |

Average values of non-easy keeper Sudan Negative clinical horses:  (Total of 2)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 6.6 | 4 | 290 | 900 |

Average values of easy keeper Sudan Negative clinical horses:  (Total 4)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 6.7 | 25 | 890 | 1370 |

Average values of non-easy keeper Sudan Positive clinical horses: (Total 8)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 6.7 | 13 | 270 | 770 |

Average values of easy keeper Sudan Positive clinical horses: (Total 20)

|  |  |  |  |
| --- | --- | --- | --- |
| pH | Bacilli Gram Stain # | Lactose + | Enterococcus # |
| 6.8 | 17 | 560 | 1070 |

Dietary regimens of groups on average:

|  |  |
| --- | --- |
| Sudan Negative/Non-easy keeper | Hay + whole grains or no grain |
| Sudan Negative / Easy keeper | Hay + Commercial Grain + 1 synthetic supplement |
| Sudan Positive / Non-easy keeper | Hay + Commercial Grain +2 or 3 synthetic supplements |
| Sudan Positive/ Easy keeper | Hay + Commercial Grains or hay pellets + 3 or more synthetic supplements |

**Discussion:**

The equine intestinal tract contains trillions of different species of bacteria, which are often susceptible to changes in diet and environmental stressors, contributing to fluctuations in specific levels. Prior research has documented changes in bacterial flora levels within the intestinal tract and feces of horses potentially contributing to various health conditions ranging from metabolic syndrome, laminits to cases of diarrhea involving *Clostridial* species. (2,15)  Even stress from transportation has been noted to result in fluctuations in intestinal bacterial growth, impacting equine health on various levels. (4)  In many aspects, the shift in microbial levels is documented but overall, we are unsure as to the connection; cause or effect.

In this research model, the normal group of non-clinical horses that demonstrate no Sudan stain uptake exhibit a bacterial population that is much lower at a 1:10,000 dilution as compared to the other groups. The bacterial populations evaluated in this study, including lactose positive *E. coli*, *Lactobacillus* and *Enterococcus* species are considered normal parts of the equine intestinal flora, but due to overgrowth and shifting of populations, the levels are much higher in some clinical populations, thus demonstrating growth at higher dilutions as compared to the normal, Sudan negative group.

In looking at the two groups, normal compared to abnormal or clinical, we do note that the colony counts for lactose + gram negative bacteria, *E. coli*, and Enterococcus populations tend to increase as we move from normal to clinically affected horses.  In actuality, as we move into higher counts, we also move from a Sudan negative to a Sudan Positive in most respects.

In the normal, non-clinical, Sudan negative equine patient, the average number of gram positive Bacilli under Gram stain was 7/hpf, lactose positive gram negative colonies averaged 20 cfu/ml, and Enterococcus counts averaged 350 cfu/ml at 1:10,000 dilution.  In comparison, our clinical, non-easy keeper, Sudan negative equine patients averaged 4/hpf, 290 cfu/ml and 900 cfu/ml respectively.

In the normal, non-clinical group, we did note some horses that demonstrated a positive Sudan stain uptake, however, they also demonstrated higher bacterial levels than the normal, Sudan negative group, averaging up to 3 times the levels of Gram positive bacilli, 12 times the levels of lactose positive gram negative bacteria and 4 times the levels of *Enterococcus* species. This opens the possibility for an association between increasing bacterial counts, intestinal inflammation, localized damage to the intestinal barrier and malabsorption concerns.

Visual comparison of Gram stain and agar growth evaluations for normal horses compared to clinical or abnormal horses can be seen in figure 1.

The level of gram positive bacilli (*Lactobacillus*species), lactose positive *E. coli* and *Enterococcus* species between the non-clinical Sudan positive and clinical equine groups did not vary significantly.  Given the difference in one group demonstrating clinical signs and the other group being clinically normal, one has to raise the question, what is the variable?  What would make one group with a markedly elevated bacterial profile demonstrate clinical signs over another?

Based on our results and patient analysis, the one key determinant appears to be dietary related.  In the clinical equine patients, Sudan negative or positive, there is a distinct difference in dietary regimens as compared to the normal group.

In the normal Sudan negative equine population with no clinical signs, we noted that the horses were primarily fed hay, whole grains with one or less supplements.  In the normal population, Sudan positive, we note that there was a higher incidence of commercial grain use as opposed to whole cereal grains along with one or less supplements.

As we assess our clinical group, we note that there is an increasing trend towards higher use of commercial grain products and increased use of supplements, correlating with Sudan status and even easy keeper status of the horse.  The Sudan positive, easy keeper group tended to exhibit the highest use of commercial grains with an average of 3 or more supplements.

In regards to specifics on supplements, the main supplements utilized included joint, hoof and coat conditioning products.  When evaluating labels supplied by the owners, the majority of these supplements utilized synthetic based ingredients rather than whole foods or herbs, with some demonstrating high use of flavoring and sweetening enhancers for palatability reasons.

Evaluating this information, we have to determine if there is a correlation between diet, intestinal bacterial overgrowth, intestinal permeability and chronic inflammatory conditions.  In some human studies there has been a correlation made between food processing and intestinal permeability, with the concern over advanced glycation end products (AGEs) and glycated lipids (ALEs), associated with high temperatures utilized in food processing, flavoring and increased use of sugars.  AGEs, once formed in the diet or body, are strongly associated with inflammation.(12). It does appear, based on our data, as the diet increases in commercial or processed feeds, the bacterial counts of lactic acid producing strains tends to also increase.  This is also noted with the higher use of synthetic supplements that may contain added sugars or flavoring.

High intake of sugars and various fermentable carbohydrates is also known to increase the tendency towards overgrowth of lactic acid producing bacteria, including *Lactobacillus*and *Streptococcus* species.  In some studies, the metabolism of fructose at high levels by bacteria within the intestinal tract may contribute to intracellular ATP depletion and predispose to the genetic phenotype of metabolic syndrome.  Fructose metabolism, when in excess, has also been shown to lead to local inflammation, increased intestinal permeability and lactic acidosis. Chronic high ingestion of fructans in the diet will lead to an altered gut flora, favoring fructanase positive bacteria including *Lactobacillus* and *Streptococcus* species.(10).  The concern for excessive intake of fructans is not only present with pasture and hay access, but also of concern in many supplements due to added flavors and sugars that may be present.

In one study regarding acute equine laminitis, it was noted that in pasture associated laminitis, there was overgrowth of Gram positive bacteria, *Lactobacillis* and *Streptococcus*, within the hindgut.  The associated laminitis was thought to be contributed to toxic compounds produced by the carbohydrate fermenting bacteria or from the death of other bacteria, which were then released from the intestine into the circulation.  It was theorized that these compounds were then directly or indirectly responsible for the trigger of active laminitis. These trigger factors from the gastrointestinal tract could directly activate an inflammatory response with subsequent up-regulation of matrix metalloproteinase (MMP) activity, which is a key feature of laminitis and laminar deterioration. In other theories, the overgrowth of specific gram negative bacteria, including *E. coli*, may contribute to the systemic inflammatory response through endotoxin (LPS) release.  (3)

In cases of laminitis, disruption of normal blood circulation to the sensitive lamina is known to occur with some research indicating that ischaemia reperfusion injury is one of the key components to the condition.  Monoamines produced within the gastrointestinal tract of the horse, secondary to high fermentable carbohydrate intake, have the potential to mimick the effects of Serotonin and norepinephrine, resulting in vasoconstriction with marked selectivity to digital vasculature, once absorbed into the systemic circulation.  *Lactobacillus* species and Group D *Streptococcus* have been identified as some of the main bacteria capable of monamine production. (3)

**Conclusion:**

Gut dysbiosis has been long implicated in connection with many human and equine health conditions ranging from allergies, metabolic syndrome, cancer and laminitis.  In actuality, many health conditions are directly associated with chronic inflammation, which may actually be localized in the gastrointestinal tract related to microbial overgrowth.

Many studies have made the connection between overgrowth of lactic acid producing bacteria in the clinical metabolic and laminitic patient, but none have really evaluated a perceived normal horse that may be predisposed to health concerns.  Based on the results of our evaluation, we have noted several perceived clinically normal horses that demonstrated overgrowth of these key bacterial types, which may predispose them to future ailments, especially upon exposure to lush Spring pastures or even stress. It may be possible that this particular group of horses with demonstrable overgrowth may also be potentially exacerbated through the concurrent use of processed foods and added sugars, furthering overgrowth and gastrointestinal inflammation.  Another consideration is the concurrent use of probiotics, especially Lactobacillus strains, which are commonly present not only in gastrointestinal support supplements, but also found in many commercial diets and other supplements.  It may be possible that these probiotics may be adding to the current overgrowth problem, contributing to clinical predisposition to various health ailments.

In this study, we open the door to possibilities not only for evaluation of the perceived clinically normal patient, but also raise concerns regarding possible implications of processed feeds and added sugars to the equine diet with influence on health and soundness.  Through earlier diagnostics and fecal examination, it may be possible to detect problems earlier, which may allow for intervention on a dietary level to improve outcome.

**Disclosures:**

Tom Schell, D.V.M is a private practicing veterinarian and head of product research and development for Nouvelle Research, Inc.  This research trial was funded in part by Nouvelle Research, Inc.

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