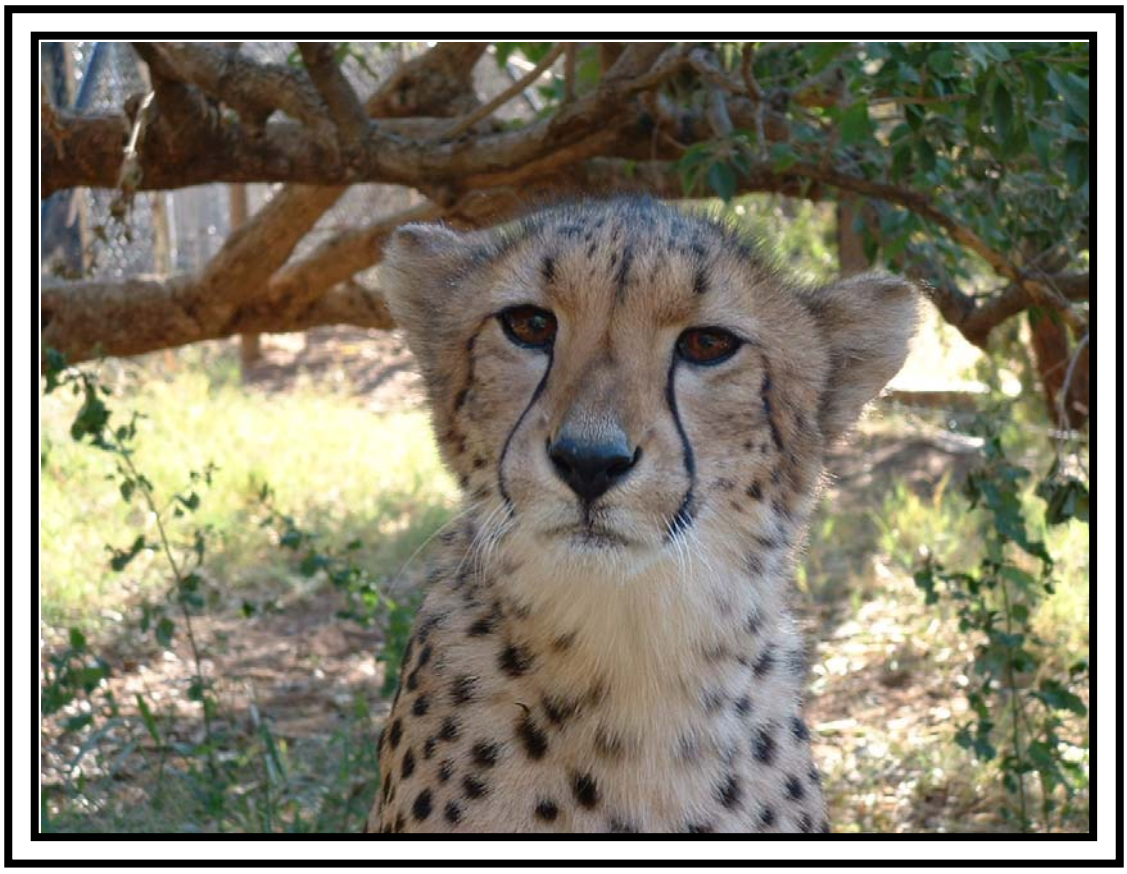
THE USE OF A PROBIOTIC IN CAPTIVE CHEETAHS (*Acinonyx jubatus*)

by

Katja Natalie Koeppel



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Abstract

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Supervisor : Professor Moritz van Vuuren
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Submitted in partial fulfilment of the requirements for the degree

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The project was undertaken to establish the normal intestinal flora of healthy cheetahs and to produce a species-specific probiotic for use in juvenile cheetahs in captivity to improve weight gain and reduce diarrhoea.

The normal intestinal flora of healthy cheetahs was established using non-selective and selective media. High numbers of anaerobic bacteria and aerobic bacteria were isolated from the faeces of cheetahs in this study. Eight percent of isolates were *Enterococcus* spp. Both *Enterococcus faecium* and *Lactobacillus* Group 1 were selected for use in the probiotic.

Twenty-seven juvenile cheetahs between eight and thirteen months of age were included in the probiotic trial (Median: 12 months). The probiotic was fed for 28 days to the Probiotic Group. Both the Probiotic and Control groups were monitored for 70 days prior to the administration of the probiotic and 14 days after administration.

The feeding of the cheetah-specific probiotic resulted in an increase of weight in the treatment group ($p=0.026$, ANOVA, $p<0.05$) in comparison to the Control Group. There was a relative improvement in the faecal quality in the Probiotic Group in comparison to the Control Group. This was accompanied by an absence of blood and mucus in the faeces, which had been present prior to the start of the 28-day administration of the probiotic.

The feeding of a cheetah-specific probiotic resulted in an improved weight gain and food conversion in the Probiotic Group in comparison to the Control Group as well as in a reduction of diarrhoea in the Probiotic Group. More research is needed on the effect of the probiotic on different age groups and animals suffering from specific diseases such as liver disease and gastritis.

Opsomming

DIE GEBRUIK VAN 'n PROBIOTIKUM IN JAGLUIPERDS (*Acinonyx jubatus)* IN GEVANGENESKAP

Deur

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Die projek was onderneem om die normale dermflora van gesonde jagluiperds te bepaal en ‘n species-spesifieke probiotikum to produseer vir gebruik in jong jagluipers in gevangeneskap om gewigstoename te verbeter en diarree te verminder.

Die normale dermflora van gesonde jagluiperds was bepaal deur die gebruik van nie-selektiewe and selektiewe groei media. Hoë getalle anaerobe en aerobe bakterieë was geisoleer vanuit die faeces van jagluiperds gedurende hierdie studie. Agt persent van die isolate was *Enterococcus* spp.. Beide *Enterococcus faecium* en *Lactobacillus* Groep 1 was geselekteer vir gebruik in die probiotikum.

Sewe en twintig jong jagluiperds tussen die ouderdomme van agt en dertien maande was ingesluit in die probiotikum proef (Gemiddeld: 12 maande). Die probiotikum was gevoer vir 28 dae aan die probiotikum groep. Beide die probiotikum en kontrole groepe was waargeneem vir 70 dae voor toediening van die probiotikum en 14 dae daarna.

Die inname van die jagluiperd-spesifieke probiotikum het ‘n toename in gewig teweeggebring in die behandelde groep ($p=0.026$, ANOVA, $p<0.05$) in vergelyking met die kontrole groep. Daar was ‘n relatiewe verbetering in die kwaliteit van faeces in die probiotikum groep in vergelyking met die kontrole groep. Dit het gepaard gegaan met ‘n afwesigheid van bloed en slym in die faeces wat wel teenwoordig was voor die 28 dae toedieningsperiode van die probiotikum.

Die inname van ‘n jagluiperd-spesifieke probiotikum het gewigstoename en verbeterde voeromset teweeggebring in die probiotikum groep asook ‘n vermindering van diarree, in vergelyking met die kontrole groep. Meer navorsing word benodig om die effek waar te neem van die probiotikum op verskillende ouderdomsgroepe van jagluiperds wat lei aan spesifieke siektes soos lewerversaking en gastritis.

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Abbreviations

BHI	brain heart infusion
BilT	total bilirubin
CO_2	carbon dioxide gas
CFU	colony forming unit
CG	Control Group
CPV	canine parvovirus
°C	degrees Celsius
EDTA	ethylenediaminetetraacetic acid
FCoV	feline enteric coronavirus
FeLV	feline leukaemia virus
FIP	feline infectious peritonitis
FIV	feline immunodeficiency virus
FPLV	feline panleukopenia virus
g	gram
GI	gastrointestinal
Hb	haemoglobin
Ht	haematocrit
KNP	Kruger National Park
l	litre
L	lactulose
MCHC	mean cell haemoglobin concentration
MCV	mean cell volume
min	minutes
ml	millilitre
mg	milligram
MRS	De Man, Rogosa and Sharpe
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	Probiotic Group
R	rhamnose
RBCC	red blood cell count

RLB	reverse line blot
RNA	ribonucleic acid
SD	standard deviation
SIBO	small intestinal bacterial overgrowth
Spp.	species
ThrC	thrombocyte count
TSP	total serum protein
WG	weight gain
XLD	xylose lysine deoxychocolate
µg	microgram
µl	microlitre

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CHAPTER 1

1.1 INTRODUCTION

Probiotics have been used in humans since the use of fermented milk, but their association with health benefits dates from the turn of the century. Metchnikoff (1908) drew attention to the adverse effects of the gut microflora on the host. Probiotics have been used in the treatment of various intestinal problems in humans and animals, and in production animals to increase weight gain and improve feed conversion (Apgar *et al.,* 1993; Fox, 1988). Probiotics have also been used in companion animals and humans to treat viral and bacterial enteritis and antibiotic-induced enteritis (Fuller, 1989). A wide range of commercial probiotics is available for the management of intestinal disorders in humans and domestic animals (Fuller, 1989, 1991). Commercial probiotics are also used in the treatment of intestinal problems in wildlife, in particular cheetahs, but those have been formulated especially for companion and production animals, not cheetahs. Clinical trials have shown that probiotics work best in the species from which they have been derived, therefore a cheetah-specific probiotic would more desirable for the treatment of intestinal diseases in cheetahs.

The cheetah has until recently been regarded as an endangered species as their natural environment is continuously being decreased due to human settlement. Breeding in captivity is important for the survival of the species. The intestinal tract of captive cheetahs is very susceptible to bacterial infection, as the cheetahs' intestinal tract is not adapted to deal with spoilt and contaminated food (Meltzer, 1993). Free-ranging cheetahs will normally not return to a carcass (Skinner and Smithers, 1990).

Enteritis is associated with high mortality in cheetahs in zoos and breeding facilities worldwide, particularly in cubs and juvenile animals (Schaller, 1991; Munson *et al.,* 1999). Commercial probiotics have been used to reduce intestinal problems in cheetahs. A species-specific probiotic was selected in this trial as it is more likely to improve microbial flora of cheetahs than a commercial probiotic (Fuller, 1989). An effective probiotic could reduce mortalities, increase weight gain and reduce the amounts of antimicrobial drugs needed to treat the animals. The quantity of antibiotics used in cheetahs, particularly juvenile cheetahs,

to combat intestinal disturbances is high (H. Bertschinger, University of Pretoria, personal communication, 2003). A probiotic, if used long-term could also prevent or reduce *Helicobacter* spp. infection in cheetahs. *Helicobacter* spp. infections in cheetahs result in gastritis. Gastritis results in vomiting, hypersalivation, weight loss, and partial or complete anorexia (Wack, 1999). *Helicobacter* spp. infections are very important in cheetahs in captivity with up to 100 % of cheetahs in zoos in the United States being infected. Most cheetahs suffering from *Helicobacter* spp infection, present with a chronic gastritis (Lobetti *et al.,* 1999b*)*. Gastritis can cause serious debilitation with chronic vomiting, weight loss and can result in death of the animal (Munson, 1999). For the selection of a species-specific probiotic the normal intestinal flora of cheetahs was first established. Once the normal flora had been identified, selective media were used to select bacteria suitable for a probiotic.

Twenty-seven juvenile cheetahs with a history of chronic diarrhoea were selected to evaluate the effectiveness of a species-specific probiotic. The animals were split into two groups, namely a Probiotic and a Control group. The probiotic was fed over a 28-day period and the faecal quality, body mass index and intestinal permeability of the cheetahs were compared.

The aims of this project were:

- To determine the normal intestinal bacterial flora in a population of captive cheetahs
- To select and culture bacteria for use as probiotic in cheetahs
- To test the effects of the selected probiotic in a population of juvenile cheetahs

1.2 LITERATURE REVIEW

1.2.1 Global status of cheetahs

In the past, cheetahs (*Acinonyx jubatus*) were widely distributed throughout Africa and Asia. This is not so today as the free-ranging cheetah population has declined by over 50 % in the past 25 years, from 30,000 animals, to less then 15,000. Cheetahs have become extinct in at least 13 countries over the past 50 years. The remaining strongholds of the cheetah in Africa are Kenya, Tanzania, Namibia, Botswana and South Africa. The conservation of cheetahs in protected areas is often complicated by competition with abundant lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*) (Marker, *et al.,* 2003b).

Their survival in the wild depends on reduction in hunting by humans, and conservation of their habitat and prey species. Many farmers regard them as vermin due to their predation upon domestic livestock and game and have poisoned and shot cheetahs (Marker, *et al.*, 2003a). The greatest threat to the survival of cheetahs in the Serengeti in Kenya and Tanzania is predation by larger carnivores, particularly lions (Laurenson *et al.*,1995). Captive breeding and education of farmers have contributed to the conservation of the species. In fact, cheetahs were taken off the South African endangered species list in 1989 due to conservation, success of captive breeding programmes and reintroduction into game reserves from Namibia (Marker, 1998). The De Wildt Cheetah and Wildlife Centre, near Pretoria, South Africa and founded by Ann van Dyk in 1971 in collaboration with the National Zoological Gardens of South Africa, is one of the largest and most successful breeding centres in the world (Meltzer and van Dyk, 1998). It usually manages a population of 80 to 120 cheetahs.

1.2.2 Diseases of captive and free-ranging cheetahs

Cheetahs are diurnal animals. They feed on smaller antelope, small mammals (insectivora, lagomorpha), birds, rodents and reptiles. Unlike other large felids, they do not return to a carcass after feeding and do not scavenge meat (Skinner and Smithers, 1990). This makes them particularly susceptible to spoilt or contaminated food, as their gastrointestinal tract is not accustomed to it (Meltzer, 1993).

Captive cheetah cubs are particularly vulnerable to enteric bacterial infection. Neonatal mortalities of up to 87 % and poor survival rates in cheetahs are problems encountered in breeding facilities and zoos (Kriek, *et al.*, 1998; Wack, *et al.*, 1991; Meltzer and van Dyk, 1998; Schaller 1991). Kriek (1998) showed that supplementing the diet with calcium, magnesium, phosphate and vitamins and reducing the level of faecal contamination of meat in the diet reduced mortalities. Enteritis is a problem in both juveniles and adults (Burroughs, 1998; Munson, *et al.,* 1998). In the Allwetter Zoo, Muenster, Germany, three out of ten deaths of cheetahs younger than seven months of age were associated with enteritis and infection with pathogenic bacteria such as *Escherichia coli* and *Salmonella* species. In animals older then seven months one in four died due to *Salmonella* infection (Schaller, 1991). Munson (1999) reported enteritis in a proportion (50 %) of cheetahs that died in South Africa between 1975-1995. The enteritis was often associated with some degree of gastritis and was characterised by chronic plasmacytic infiltrates with villous atrophy, necrosis of crypts and mild neutrophilic infiltrates. Lymphocytic-plasmacytic colitis has been associated with loose faeces with fresh blood and mucus in captive cheetahs (Gillespie and Fowler, 1984).

Outbreaks of salmonellosis in cheetahs are usually associated with contaminated food and usually presents with severe enteritis and occasionally septicaemia (Burroughs, 1998). It is a particular problem in cubs that results in severe haemorrhagic diarrhoea and death (Meltzer, 1993 and 1999). At De Wildt and Hoedspruit Cheetah Centres cubs died after the ingestion of *Salmonella* infected meat (Meltzer and van Dyk, 1998). *Salmonella* Typhimurium and *Salmonella* Muenchen were the most frequent isolates in faeces of cheetahs in a breeding establishment in South Africa (Venter *et al.,* 2003). Salmonellosis was identified as a secondary factor affecting cub mortality (Kriek *et al.*; Venter *et al.,* 2003). Improvement of meat hygiene for cheetah rations and nutrition decreased cub mortality in this study. Cub survival increased from 43-64 % to 93-100 % after changes in the food processing and improvement of nutrition (Kriek *et al.*, 1998; Venter *et al.,* 2003).

Fifty per cent of deaths of cheetahs in captivity in South Africa and the USA have been associated with gastritis, glomerulosclerosis and veno-occlusive disease. The prevalence of gastritis in cheetahs in South Africa and the USA is nearly 100 %, but a higher proportion of cheetahs in South Africa presented with a moderate to severe gastritis (Munson *et al.*, 1999). Chronic gastritis in cheetahs is characterised by infiltration of lymphocytes, plasma cells or neutrophils in the lamina propria and ulceration of the mucosa (Munson, 1993). Epithelial

erosions and spiral-shaped bacteria are seen in the stomach (Lobetti *et al.*, 1999a, 1999b). The two species of bacteria that were isolated from gastric biopsies in cheetahs are *Helicobacter acinonychis* (formerly *Helicobacter acinonyx*) and *Helicobacter heilmannii* (Wack, 1999). *Helicobacter acinonychis* is most commonly associated with chronic active gastritis, but stress might play a role in the severity of clinical signs (Lobetti *et al.*, 1999b). Anti-gastric antibodies are proposed to play a role in the pathogenesis of gastritis in cheetahs since the disease progresses in spite of the eradication of the bacteria (Terio *et al.,* 1998). The aetiology of gastritis in cheetahs is multifactorial (Lobetti *et al.*, 1999b). Gastritis in cheetahs in the USA seems to be aggravated by stressful conditions including large numbers of cheetahs in small enclosures and confinement of adult males in adjacent enclosures (Wack, 1997).

Renal disease is a major problem in captive cheetahs (Burroughs, 1998). Histopathologically these cheetahs show glomerulonephritis and glomerulosclerosis (Bolton and Munson, 1999). Eighty-two % of captive cheetahs in this study showed some degree of glomerulosclerosis with 30 % of cheetahs showing moderate to severe sclerosis (Bolton and Munson, 1999). Cheetahs often develop systemic amyloidosis in response to inflammation (Papendick *et al.,* 1997). Chronic gastritis has been associated with systemic amyloidosis (Munson *et al.,* 1998). Cheetahs are predisposed to develop systemic amyloidosis (Papendick *et al.,* 1997). Amyloid deposits occur primarily in the kidney and liver. The amyloid deposits in the kidney obstruct the normal blood circulation and result in papillary necrosis or cortical atrophy, eventually leading to kidney failure (Papendick *et al.,* 1997). Veno-occlusive disease and hepatic necrosis have been mainly associated with cheetahs in zoos, suggesting environmental factors being important in the pathogenesis of these diseases (Munson *et al*., 1999).

Feline panleukopaenia virus (FPLV) and canine parvovirus (CPV-2a and CPV-2b) are closely related viruses that belong to the feline parvovirus subgroup. FPLV causes a syndrome described as feline infectious enteritis, malignant panleukopenia, feline distemper or spontaneous agranulocytosis in domestic cats (Steinel *et al.*, 2001). The syndrome has been reported in both captive and free-ranging cheetahs (Steinel *et al.,* 1999 and 2000). CPV-2b is the predominant antigenic type circulating in cheetahs in southern Africa and North America, but FPLV has also been isolated from cheetahs (Steinel *et al.,* 1999; Van Vuuren *et al.,* 2000).

Serological evidence indicates that coronavirus infection occurs in captive and free-ranging populations of cheetahs (Heeney *et al.,* 1990). The viruses are antigenically distinct from

coronaviruses in domestic cats (Kennedy *et al.*, 2000). FCoV infection has been associated with fatal systemic disease, feline infectious peritonitis (FIP), necrotizing enterocolitis and chronic diarrhoea in cheetahs (Kennedy *et al.*, 2000, 2001 and 2003). Cheetahs are particularly susceptible to FCoV induced disease (Evermann, *et al.*, 1993; Brown *et al.,* 1993). Kennedy *et al.* (2003) suggested that clearance and re-infection, as well as continuous shedding of virus follow infection. Stress might induce viral shedding or predispose cheetahs to infection (Kennedy *et al.* 2001).

A lentivirus antigenically closely related to feline immunodeficiency virus (FIV) of domestic cats is wide spread in wild felids and felids kept in European zoos (Lutz *et al.;* 1992). In certain natural cheetah populations FIV is more endemic. Twenty-six percent of cheetahs from the Serengeti National Park, Tanzania, were positive for FIV antibodies (Brown *et al.,* 1993). Feline immunodeficiency virus and feline leukaemia virus have never been a problem in captive cheetahs in South Africa (Burroughs, 1998). FeLV has not been detected in free-ranging cheetahs (Munson *et al.*, 1998). FIV has not been associated with immunological or pathological impairment in non-domestic felines (Brown *et al.,* 1993).

Feline herpesvirus type 1 has been associated with upper respiratory disease in cheetahs, and chronic progressive skin disease (Munson *et al.*, 1998). Infection persists for life and the virus is periodically shed. Infected epithelial cells show a marked inflammatory response (Munson *et al.*, 1998).

Cryptococcus neoformans gattii infection has been associated with nervous signs, retinal infections and skin tumours in captive cheetahs (Burroughs, 1998).

1.2.3 The gastrointestinal tract and its interaction with the microflora

The gastrointestinal tract of mammals is a complex ecosystem. Folding of epithelium and formation of microvilli results in an increased surface area for the digestive processes and microbial interactions (Holzapfel and Schillinger, 2002). The diversity of the intestinal microbial flora varies from segment to segment and is also determined by factors such as diet, genetic background and physiological state of the host (Holzapfel and Schillinger, 2002). The species composition of the microflora varies between different hosts. The microbial species composition within the gastrointestinal tract is more stable than bacterial strains within the population. This means that the species are stable but the strains of bacteria change frequently

as a result of changes in diet and environment. Diarrhoea is the most consistent manifestation of intestinal disease or upset (Guilford and Strombeck, 1996). The colon has a waste buffering capacity. If the colonic buffering capacity is overwhelmed it results in acidification, which damages the epithelium leading to an increased permeability (Argenzio, 1978). This will result in diarrhoea. Bacterial enterotoxins and endotoxins from pathogns such as, *Clostridium perfringens*, *E. coli*, *Salmonella* spp. and *Yersinia enterocolitica* result in a secretory diarrhoea (Ettinger and Feldman, 2000b). Enteropathogenic *E. coli* adhere to the mucosal cells of the small and large intestine, causing loss of microvilli ("attaching and effacing lesions") and formation of filamentous actin pestrals or cuplike structures under the organism (Greene, 1998). Enterotoxigenic *E. coli* adhere to the small intestine and produce symptoms by elaborating toxins, therefore there are no histological changes to the mucosal tissue to which the bacteria are attached (Greene, 1998) In cats *E. coli* infection resulted in diffuse atrophy and focal fusion of the villi with elongation and dilation of the crypts in the ileum (Pospischil *et al.,* 1987). Salmonellosis results in active secretion of the gut but does not change the mucosal permeability. This is caused by an increased release of prostaglandins from the inflamed intestinal mucosa (Argenzio, 1978). *Salmonella, Klebsiella* and *Pseudomonas* spp. are associated with neonatal septicaemia and death in cheetah cubs, particularly in association with vitamin E and selenium deficiency (Kriek *et al.,* 1998).

Feline panleukopenia virus (FPLV) was the most important primary enteric virus in cats, outbreaks are now less common because of routine vaccination (Ettinger and Feldman, 2000b). FPLV is associated with damage to the germinal intestinal gland epithelium and results in degeneration of the gland and collapse (Greene, 1998). Enteric coronavirus, toravirus, reovirus, rotavirus and astrovirus have also been associated with diarrhoea in felines, including cheetahs (Ettinger and Feldman, 2000a). Other viruses such as calicivirus, reovirus type III and non-cultivable enteric picornaviridae-like virus have been identified from feline faeces but their importance in causing intestinal disease is uncertain (Ettinger, 1989). The intestinal tract might also be involved with a generalized viral infection such as feline leukaemia virus (FeLV), feline immunodeficiency virus (FIV) infection or feline infectious peritonitis (FIP) (Ettinger and Feldman, 2000b). FIV and FeLV could not be isolated from free-ranging cheetahs by Lutz *et al.* (1992). Munson *et al.* (1998) stated that only captive cheetahs have tested positive for FeLV and FIV infection. Two out of 31 (6.45 %) cheetahs in North American zoos died of FIP (Munson, 1993).

FPLV has a predilection for rapidly dividing cells particularly of the crypt epithelium resulting in acute, severe enteritis, for haemopoietic tissue (panleukopenia) and for lympoid tissue (lympoid depletion) (Ettinger and Feldman, 2000a). FPLV has been isolated from cheetahs with acute enteritis (Steinel *et al.*, 2001). Histopathologically, FPLV has been associated with focal necrosis of the intestinal mucosa, collapse of villi, as well as necrosis of the gut-associated lympoid tissue (Van Vuuren *et al.*, 2000). The occurrence of coronavirus in mammals is widespread. FCoV is an important contagious pathogen of captive cheetahs (Kennedy *et al.*, 2001). Infection results in histological lesions of villous atrophy (Williams and Barker, 2001). Rotavirus has been isolated from normal and diarrhoeic faeces and its enteropathogenic significance is unclear in cats, experimental infection results in inapparent disease or mild self-limiting diarrhoea (Ettinger and Feldman, 2000a).

Ancylostoma spp., *Toxocara* spp., *Trichinella* spp., *Taenia* spp., *Ollulanus tricuspis* and *Spirocerca lupi* have been isolated from cheetah faeces (Penzhorn *et al.*, 1989). The most consistent findings of intestinal parasitism are diarrhoea and weight loss. Young growing animals are more frequently and severely affected. *Toxocara* spp. only causes clinical signs in severe infection. *Toxocara* can also cause damage by larval migrans through liver-lung, wall of the GI tract and somatic tissue migration (Ettinger and Feldman, 2000b). *Ancylostoma* spp. cause intestinal blood loss due to their bloodsucking activity. *Trichinella* have been noted to cause transient haemorrhagic enteritis in cats (Ettinger, 1989). *Trichinella nelsoni* has been isolated from faeces of cheetah in Tanzania (Pozio *et al.*, 1997). Other nematodes rarely cause clinical disease unless high numbers are present within the intestinal tract. Stress and an impaired immune system usually result in high numbers of helminths, which can result in clinical signs such as stunted growth, dull haircoat, unthriftyness and diarrhoea (Ettinger and Feldman, 2000b).

The gastrointestinal immune system is important in the prevention of diarrhoea. M cells are specialised epithelial cells present in the gut-associated lymphoid tissue (Guilford and Strombeck, 1991b). Some pathogens such as salmonellae, chlamydophila, reoviruses, retroviruses and coronaviruses can utilise M cells to access the body. M cells also play a role in colonisation of the intestine by some bacteria, e.g. *E. coli* adheres to M cells prior to the adherence to absorptive enterocytes. (Strombeck and Guilford, 1991b).

The normal microbial flora acts as a host defensive barrier by making the epithelium unavailable to the pathogens or by creating an environment detrimental to pathogens. A healthy intestinal epithelium, in association with an optimal intestinal flora, provides a vital barrier against the invasion or uptake of pathogenic microorganisms, antigens and harmful compounds from the intestinal lumen (Holzapfel and Schillinger, 2002).

1.2.4 Properties of probiotics

Lilly and Stillwell first used the term probiotics in 1965 in reference to substances produced by protozoa, that stimulated the growth of other organisms (Kaur, *et al.,* 2002). Probiotics have been defined as products containing viable organisms, which have a beneficial effect on the host animal in the prevention and treatment of specific pathological conditions. Fuller (1989) defined them as "live microbial feed supplements, which beneficially affects the host animal by improving its intestinal microbial balance". They consist of lactic acid-producing bacteria such as lactobacilli, certain streptococci, bifidobacteria and yeasts (Chow, 2002; Hall, 1996). To survive in the intestinal tract a probiotic must be able to withstand the chemical and physical conditions of the intestines such as the constant flushing of bacteria by peristalsis. To avoid flushing out by peristalsis with the food, the bacteria either have to grow at a rate faster than their removal or attach themselves to the gut wall. They can either adhere to structures on the surface or colonize secretions such as mucin overlying the epithelial layer (Fox, 1988; Fuller, 1989). The microbial flora is down-regulated by the antibacterial properties of gastric acid, bile and pancreatic juices. Mucus provides a physicochemical barrier, which entraps bacteria and facilitates phagocytosis by the local immune system (Batt, *et al.,* 1996). The normal gastrointestinal microbial flora has a symbiotic relationship with the host. The normal microbial flora has the ability to adhere to the epithelial cells and thereby exclude or reduce adherence by pathogens. They also produce nutrients such as short chain fatty acids and vitamins required by the host as well as antibacterial substances.

Several requirements have been identified for an "effective" probiotic:
The ability to:

- Adhere to cells in the intestinal tract
- Exclude or reduce adherence by pathogens
- Persist and multiply
- Produce acids, hydrogen peroxide, and bacteriocins antagonistic to pathogen growth

- Be safe, non-invasive, non carcinogenic and non-pathogenic
- Co-aggregated to form a normal balanced flora

1.2.5 Action of probiotics

Probiotics beneficially affect the host by improving its intestinal microbial balance. They are thought to function in several ways to reduce pathogens in the gastrointestinal tract.

1. Antibiotic production:

   Primary metabolites derived from probiotics, such as organic acids and hydrogen peroxide are known to be effective *in vitro* against pathogenic bacteria (Fuller, 1989). *Lactobacillus* spp. have been reported to produce acidophilin, lactocidin, and acidolin and lactolin (Fox, 1988). Volatile fatty acids, derived from probiotics, prevent colonisation of the intestine by *Salmonella* Sonnei and enteropathogenic *E. coli.*

2. Competitive antagonism:

   The normal microbial flora acts as a host defensive barrier by making the epithelium unavailable or by creating an environment detrimental to pathogens and competing for nutrients. *Lactobacillus rhamnose* strain GG, reduced S fimbriae-mediated adhesion of *Salmonella* Typhimurium *in vitro* (Tuomola *et al.,* 1999).

3. Immunostimulation:

   The attachment of probiotic bacteria to cell surface receptors of enterocytes initiates signalling events resulting in the synthesis of cytokines. They balance the control of pro-inflammatory and anti-inflammatory cytokines and thereby provide an innovative tool to alleviate intestinal inflammation, normalise gut mucosa dysfunction and down-regulate hypersensitivity (Holzapfel and Schillinger, 2002). The enzymatic and phagocytic activity of macrophages can also be stimulated by lactic acid producing bacteria.

4. Regulation of colonocyte gene expression:

   Probiotics can result in the expression of mucin genes preventing attachment of pathogenic *E. coli* (Tuohy *et al.,* 2003).

5. Production of toxic metabolites and increased turnover of enterocytes:

   The most important metabolite is hydrogen peroxide. It has a bactericidal effect on most pathogens. Production of short chain fatty acids reduces luminal pH, which directly inhibits certain pathogens (Tuohy *et al.,* 2003). Chow (2002) believes that probiotics inhibit potential pathogens by reducing blood ammonia levels, but the work of Zentek *et al.* (1998) with *Enterococcus faecium in vitro* showed that the ammonia concentration was only minimally affected by the probiotics. There was an increase in lactate production. Feeding *E. faecium* to domestic dogs resulted in an increase in the enterococcal concentration in their faeces.

6. Neutralisation of dietary carcinogens:

   Probiotic bacteria such as bifidobacteria and lactobacilli have been shown to reduce enzyme activity that has been associated with colonorectal cancer in humans (Tuohy *et al.,* 2003).

7. Restoration of normal gut flora after antibiotic therapy:

   Diarrhoea occurs in approximately 20 % of human patients receiving antibiotics (Tuohy *et al.,* 2003). Several probiotic strains e.g. *Bifidobacterium longum*, *Lactobacillus* spp., *Enterococcus faecium* and *Streptococcus boulardi* have been shown to reduce the incidence and duration of antibiotic-associated diarrhoea (Tuohy *et al.,* 2003).

1.2.6 Specific action of *Lactobacillus* strains

Lactobacilli are characterised as Gram-positive, non-spore forming, non-motile rods or coccobacilli (Charteris *et al.*, 1997). They are distributed throughout the gastrointestinal and genital tracts and are an important part of the normal microbial flora of animals and humans (Charteris *et al.*, 1997).

Most of the work relating to the use of probiotics has been carried out in production and laboratory animals.

The normal microflora colonizing the gut is very host-specific (Barrows and Deam, 1985). Experimentally, *Lactobacillus* strains adhered in a host-specific fashion to the keratinised epithelial cells of rats (De Waard *et al.,* 2002).

Lactobacilli attach to the surface epithelium in the chicken crop and squamous epithelial cells of the pig's stomach (Fuller 1989; Fox, 1988). The microbial strains are slightly different in different animals and receptors required for attachment to epithelial cells are host species-specific (Fuller, 1989). Therefore artificially cultured probiotics may work well only when used in the species from which the strain was isolated. Bacterial strains in the gastrointestinal tract depend not only on the animal species but also on the environment in which the animal is kept. Comparison of indigenous lactobacilli strains in mice showed that the environmental background of the animal rather then the hosts' genetics determines the indigenous *Lactobacillus* species strains found (De Waard *et al.,* 2002). Animal feed is an important factor that influences the composition of the intestinal microflora (De Waard *et al.,* 2002). Therefore it is important to collect faecal samples for microbial culture from different enclosures and animals fed different diets.

Lactobacilli have been reported to produce various types of antibiotics such as acidophilin, lactocidin, lactobacillin and lactolin. They inhibit growth of potential pathogens such as *E. coli, Salmonella, Shigella, Pseudomonas, Bacillus* and *Vibrio* species. *Lactobacillus rhamnose* strain *GG* modulates the intestinal immunity in humans by increasing the number of immunoglobin A and stimulating the local release of interferon (Tuomola *et al.,* 1999).

Lactobacillus gasseri has been effective in suppressing *Heliobacter pylori* and reducing gastric mucosal inflammation in humans (Kaur *et al.*, 2002). *Lactobacillus johnsonii* La1 restricted the size of the population of *H. pylori*, suggesting an interference with the colonisation of *H. pylori* (Cruchet *et al.,* 2003). Regular ingestion of lactobacilli could be effective in modulating *H. pylori* infection (Cruchet *et al.,* 2003).

Lactobacillus spp. have also been shown to be effective in reducing the severity of acute pancreatitis (Bonn, 2002) and acute gastroenteritis in children, in particular rotavirus-induced diarrhoea (Sullivan and Nord, 2003). In infants probiotics are most important treatment of virus-associated diarrhoea e.g. rotavirus (Kaur, *et al.,* 2002).

Clinical trials in humans affected with chronic liver disease and clinical signs of hepatic encephalopathy have shown that probiotics, in particular *Lactobacillus acidophilus* and *Enterococcus faecium* could be effective in reducing the severity of clinical signs associated

with liver disease (Solga, 2003). Efficacy of probiotics in the treatment of hepatic encephalopathy is thought to be associated with the decrease of ammonia in the portal blood by decreasing bacterial urease activity, decreasing ammonia absorption, decreasing intestinal permeability and improving the nutritional status of the intestinal epithelium (Solga, 2003).

1.2.7 Specific action of *Bifidobacterium* strains

The genus *Bifidobacterium* was first isolated from the faeces of human infants (Jones and Collins, 1986). They are generally characterised as Gram-positive, non-spore forming, non-motile, catalase-negative anaerobes. At present there are 29 recognised species (Charteris *et al.*, 1997).

Scharek *et al*. (2002) showed that *Bifidobacterium adolescentis* and *B. thetaiomicron* are able to colonise the intestinal tract of rats effectively. Oral administration of bifidobacteria has been shown to balance the intestinal flora and control the bacterial metabolism in the gastrointestinal tract of animals (Suzuki *et al.,* 1997).

Strains of bifidobacteria have been shown to be antagonistic against *Salmonella* spp. *in vitro.* The antagonism between *Bifidobacterium* and *Salmonella* spp. is strain dependent. All strains of *Bifidobacterium* tested by Bielecka *et al.* (1998) reduced or eliminated the *Salmonella* populations. Fifteen strains of *Bifidobacterium* were tested against six *Salmonella* strains *in vitro* and the degree of inhibition ranged from 44 to 100 % (Bielecka *et al.*, 1998). The antagonistic effect have not only been associated with acid production but also with the competition for nutrients, the modification of oxidation-reduction potential and bacteriocin-like inhibitory substances produced by some strains of *Bifidobacterium* spp. and other lactic acid-producing bacteria (Bielecka *et al.* 1998)

Administration of *Bifidobacterium longum* to germ-free (gnobiotic) mice challenged with *E. coli* C25 lowered the numbers of *E. coli* translocating to the mesenteric lymph nodes (Suzuki *et al.,* 1997). Administration of *Bifidobacterium lactis* HN019 reduced the severity of diarrhoea in piglets challenged with *E. coli* and rotavirus (Shu *et al.,* 2001). The animals also showed a higher feed conversion suggesting an improvement in overall health in the probiotic group compared to the control group (Shu *et al.,* 2001). Apgar *et al.* (1993) also noted an increase in weight gain in pigs receiving *Bifidobacterium* in their food.

Consumption of a diet containing *Bifidobacerium longum* has been associated with a decrease of beta-glucuronidase activity and ammonium concentration, both of which have been associated with carcinogenesis of the colon in rats (Kaur, *et al.*, 2002).

1.2.8 Specific action of *Enterococcus* strains

Enterococci can be found in soil, food and water, and they make up a significant portion of the normal intestinal flora of humans (10^5-10^7/g of stool) and animals (Kayser, 2003). *Enterococcus faecium* and *E. faecalis* are residents of the normal intestinal flora of humans and animals. They are usually Gram-positive oval or spherical cells arranged in pairs or chains. They are aerobic or facultative anaerobes. *Enterococcus* spp, belonging to the normal intestinal flora have been documented to produce bacteriocins against *Listeria* spp. (Sullivan and Nord, 2002). They have been documented to reduce antibiotic-associated diarrhoea in humans (Tuohy *et al*., 2003) and gastroenteritis in adults (Holzapfel and Schillinger, 2002).

Enterococci are increasingly involved in nosocomial infections and readily transfer antibiotic resistance (Sullivan and Nord, 2002). Most pathogenic isolates in humans are *E. faecalis*, which account for 80 - 90 % of clinical isolates. *E. faecium* represented 5 – 10 % of clinical isolates (Kayser, 2003). *Enterococcus faecium* can transmit vancomycin resistance (Weese, 2002). Many clinical strains of *E. faecalis* produce a cytolysin (haemolysin) that causes tissue damage (Kayser, 2003). Many of the clinical isolates also possess aggregation substances on the surface and an extracellular surface protein. These contribute to their ability to adhere to eukaryotic cells. *E. faecium* has been shown to favour the adhesion and colonization of *Clostridium jejuni* in the dog's intestine (Rinkinen *et al.*, 2003). Even though not all strains of enterococci are considered a health risk, the use of enterococci as probiotics is controversial. Kayser (2003) proposes a two stage process in the establishment of pathogenic enterococci: firstly colonisation of the gastrointestinal tract by enterococcal strains possessing virulence traits, followed by a subsequent tissue invasion associated with elimination or disturbance of the normal microbial flora particularly in immunocompromised humans (Kayser, 2003). Pathogenic strains of *Enterococcus* spp. should be avoided when selecting stains for probiotics.

1.2.9 Probiotics and antibiotics

A number of trials have shown that probiotics can offer the same benefits in animals as low-dose antibiotics when used as growth promoters (Fox, 1988). They increase the feed conversion particularly in animals with a disturbed microbial flora (Fuller, 1989).

Antibiotic therapy can cause fungal and yeast overgrowth in the intestines and thereby increase susceptibility to infection by pathogens and interfere with nutrient uptake. This is due to antibiotics removing producers of volatile fatty acids, which normally control the growth of yeasts and fungi. *Saccharomyces cerevisiae* has been reported to cause episodic diarrhoea in conjunction with prolonged antibiotic therapy (Milner *et al.*, 1997). *Candida* spp. infection is often a consequence of antibiotic therapy (Fuller, 1989, 1991). Administration of antibiotics causes a decrease of the total bacterial numbers in the large intestine particularly anaerobes and an increase in the number of coliforms present, thus allowing pathogenic opportunists such as salmonellae to colonize the gut. Antibiotics suppress the indigenous microbial population for prolonged periods of time (Strombeck and Guilford, 1991a). Bacteria in the small intestine are able to synthesise folate and bind cobalamin (vitamin B12). Increased serum concentrations of folate and decreased serum concentrations of cobalamin have been associated with small intestinal bacterial overgrowth (SIBO). A disturbance of the gastrointestinal flora of up to nine months and also alterations in cobalamin and albumin were noted after the administration of an antibiotic (metronidazole) *per os* to cats (Johnston *et al.,* 2000). Diarrhoea is one of the most frequent side effects of antimicrobial therapy in humans (Sullivan and Nord, 2002). The disease pseudomembranous colitis in humans is almost always associated with administration of antibiotics *per os* (Fuller, 1989).

Probiotics can be used on their own in uncomplicated diarrhoea, i.e. no fever, depression or degenerative left-shift leukograms. Antibiotics would only be required if the bacteria has invaded the intestinal mucosa causing bacteraemia or septicaemia. The use of a probiotic together with antibiotic therapy allows the beneficial microbial flora to re-establish itself and reduces the risk of antibiotic-induced diarrhoea. Probiotics have been effectively used in reducing side effects of antibiotics (rabeprazole, clarithromycin and tinidazole) used to eradicate *H. pylori* infection in humans (Tuohy *et al.,* 2003).

1.2.10 Examples of how probiotics have benefited the health of animals

The addition of a probiotic might reduce mortalities particularly in those animals with a disturbed microbial gut flora. This is shown by the observation that germ-free animals are more susceptible to disease than are the corresponding conventional animals with a complete intestinal flora (Maia *et al.,* 2001; Scharek *et al.,* 2002). Gnotobiotic pigs fed *Enterococcus faecium* had less diarrhoea and no mortality when challenged with *E. coli*, than pigs only given *E. coli* (Underdahl, 1982). The use of probiotics is well documented in suppressing neonatal scours and improving the growth of young and stressed animals. Stress can be nutritional, environmental, or emotional. For example, Barrows and Deam (1985) used a product made from the spores of a strain of *Bacillus subtilis*, which was routinely fed to all hospitalised dogs and cats. Less digestive disturbances and improved appetites were observed in the animals receiving the additive.

Shu *et al.* (2001) reduced the severity of weaning diarrhoea in piglets and maintained greater feed conversion efficiency by adding *Bifidobacterium lactis* to the diet. The beneficial effect was through enhancement of the immune-mediated protection against rotavirus and *Escherichia coli*. Underdahl *et al.* (1982) indicated that the presence of lactic acid-producing bacteria can lower the pH of the intestine and reduce the number of pathogenic *E. coli* adhering to the microvilli of the lymphoepithelial cells, clinically preventing severe diarrhoea and death.

In mice the addition of *L. rhamnosus* HN 001 resulted in lower morbidity following infection with *E. coli* O157:H7 in comparison to the control group (Shu and Gill, 2002). The probiotic group also showed a lower incidence of *E. coli* translocation into extra-intestinal tissue. This was thought to be due to an increase of levels of intestinal IgA antibodies and a greater proportion of blood leukocytes exhibiting phagocytic activity in the probiotic group compared to control group (Shu and Gill, 2002). Thus feeding of *L. rhamnosus* HN 001 resulted in enhanced acquired and innate immunity in mice.

Maia *et al.* (2000) fed mice with Vitacanis^{R}, a probiotic containing *Lactobacillus acidophilus*, *E. faecium* and *Saccharomyces cerevisiae*. They then challenged them with *Salmonella* Typhimurium. A higher survival rate (82 %) was observed in mice given only *E. faecium.* All the animals in the groups receiving *L. acidophilus* or a combination of the three bacteria died after being challenged with *S.* Typhimurium but the survival time was increased. No

significant increase in survival rate was noted in the animals receiving only *S. cerevisiae* (Maia *et al.* 2000). This further underlines the importance of analysis of the microbial flora in probiotic studies.

1.2.11 Bacterial flora of cheetahs

The bacterial numbers of the flora in the proximal small intestine in felids is higher than in canids. The total bacterial counts in undiluted juices from the proximal small intestine ranged from 2.2 x 10^5 to 1.6 x 10^8 colony-forming units per ml in clinically healthy domestic cats (Johnston *et al.,* 1993). These numbers would be consistent with small intestinal bacterial overgrowth in humans and dogs (Johnston *et al.,* 1993).

Samples from duodenal fluid from healthy cats contained between 10^4-10^8 cfu/ml anaerobes, most commonly *Bacteroides* and *Clostridium* spp. Total bacterial numbers in cats with chronic intestinal disease, with a history of chronic diarrhoea, weight loss or vomiting, were comparable to healthy cats, but there was a difference between individual species present (Johnston *et al.,* 2001). *Pasteurella, Bacteroides* and *Lactobacillus* spp. in the duodenal fluid of cats with chronic intestinal disease were lower (Johnston *et al.,* 2001). In cats the individual species of bacteria rather than the total number of bacteria seems to be important in gastrointestinal disease. Gram-positive bacteria, including streptococci, staphylococci and lactobacilli are found in the proximal intestine of healthy dogs (Batt, 1996). The numbers of lactobacilli present in the faeces is decreased or even diminished in diarrhoea.

Rectal swabs from domestic cats showed both Gram-negative (43 %) and Gram-positive (57 %) bacteria. Beta-haemolytic *E. coli* was the most common isolate. In the same study 98 % of isolates from cheetahs were Gram-negative. *E. coli* and *Proteus* spp. were the most common isolates from cheetahs (Howard, *et al.,* 1993).

1.2.12 Selection of bacteria suitable as probiotics

Lactobacillus, Bifidobacterium and *Enterococcus* spp. are the three bacteria most often used in probiotics in monogastric animals. The species often used in commercial probiotic preparations are *Enterococcus faecium, E. faecalis, Lactobacillus rhamnosus, L. casei, L. acidophilus, L. farciminis, L. bulgaricus, Bifidobacterium bifidum* and *B. longum* (Fox, 1988; Kaur, 2002; Reuter, 2001; Yuan-Kun, 1999). Other organisms not belonging to the lactic acid

bacteria such as *Aspergillus*, *Saccharomyces*, *Bacillus subtilis and B. toyoi* have also been used (Fox, 1988; Holzapfel and Schillinger, 2002). The concentration of bacteria used in probiotics varied between 10^2 to 10^{10} (Holzapfel and Schillinger, 2002) but the best clinical results have been reported with concentrations between 10^8 to 10^9 CFU per day (Yuan-Kun *et al.*, 1999).

1.2.13 Intestinal permeability in gastrointestinal disease

The determination of intestinal permeability has been established as a non-invasive approach to the assessment of intestinal damage (Sørensen, 1993). Intestinal abnormalities might not result in clinical disease and are therefore unlikely to be detected on routine biochemical and haematological analysis of blood (Batt, *et al.,* 1992). Intestinal function can be assessed by measuring the rise in blood concentration or renal excretion of a selected test substance following the oral administration of a standard dose (Menzies, 1993). Hollander (1992) proposed a permeability model to explain the different rate at which compounds of different size penetrate the intestinal barrier. An increase in permeability to larger compounds is not necessarily associated with a concomitant increase in permeability in all smaller compounds. He explains this by the difference in tight junctions between the villous epithelium and crypt epithelium (Figure 1).

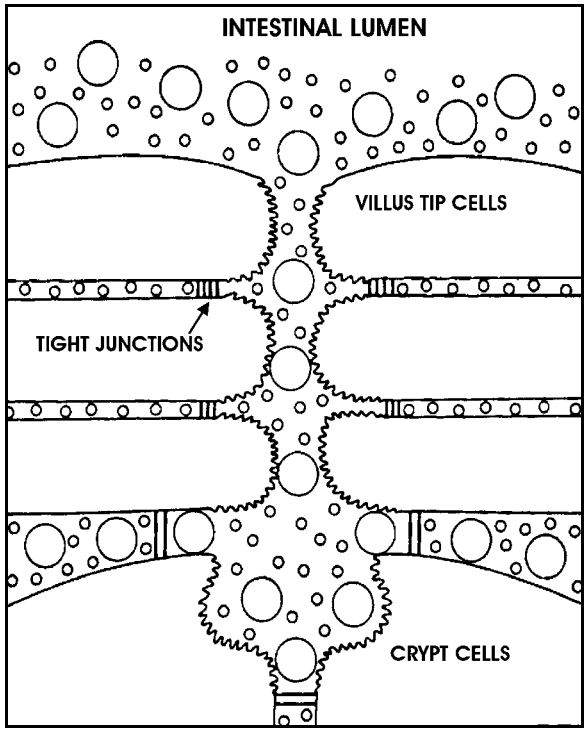


Figure 1: Model of tight junction difference between intestinal villi and crypts (Hollander, 1992)

The tight junctions of the crypt epithelium have a higher mean linear density and lower strand counts. This results in a difference of permeability of the two regions to probe molecules of different sizes. Smaller compounds can penetrate smaller and more resistant tight junctions at the tips of the villi; whereas larger compounds can only penetrate the more difficult accessible crypts (Hollander, 1992).

Sugars of different molecular size have been used to test intestinal function. It has been proposed that monosaccharides are absorbed transcellulary and disaccharides are absorbed paracellulary through gaps in tight junctions. Disaccharides are unable to penetrate healthy enterocytes (Papasouliotis *et al.*, 1993). Diseases that are characterised by decreased surface area or villous atrophy result in decreased absorption of monosaccharides. Disruption of mucosal integrity causes increased absorption of disaccharides (Randell *et al.*, 2001). As both sugars will be affected equally by non-mucosal factors, comparison of the ratio of the two is more accurate in determining intestinal disease (Menzies, 1993). Non-mucosal factors affecting sugar absorption are delayed gastric emptying, intestinal dilution, intestinal transit time, impaired renal excretion, renal function and incomplete urinary recovery (Quigg *et al.*, 1993; Papasouliotis *et al.*, 1993).

Cr-labeled EDTA has been used as a sensitive indicator of intestinal damage in dogs and humans. It is able to detect sub-clinical abnormalities, with only minor or no histological changes in the mucosa. An increase in the permeability is usually related to an increase in urinary recovery of Cr-labelled EDTA. Beagles with small intestinal bacterial overgrowth (SIBO) have a higher intestinal permeability than Beagles with no overgrowth (Batt *et al.,* 1992). Urinary recovery of Cr-labelled EDTA was 30.5 to 37.6 % compared to 11.1 to 17.3 % in normal beagles. The increase in intestinal permeability was directly related to the numbers of bacteria in the duodenal fluid (Batt *et al.,* 1992). Disadvantages of Cr-EDTA are the requirement for a 24-hour urinary collection, a gamma counter and the possibility of colonic absorption. The major limitation to the use of a single marker is the effect of non-mucosal factors. The advantage of blood collection is that the sample is not influenced by poor renal clearance (Menzies, 1993).

Intestinal permeability is better assessed by determining the ratio of urinary recoveries of two sugars with different molecular sizes, such as lactulose/rhamnose or cellobiose/mannitol (Hall and Batt, 1991; Garden *et al.*, 1997). As cellobiose is susceptible to intestinal

betagalactosidase activity and there is endogenous production of mannitol, lactulose and rhamnose are considered to be the more appropriate probes in assessing intestinal permeability (Quigg *et al.,* 1993). Mannitol absorption is dependent on the efficiency of the countercurrent multiplier in the intestinal villi to induce water absorption in cats and humans (Bijlsma *et al.,* 2002). Therefore impaired function of the countercurrent mechanism will affect the recovery of mannitol. D-xylose/3-O-methylglucose (X/G) ratio reflects intestinal absorptive function while lactulose/rhamnose (L/R) ratios reflects permeability. In SIBO the X/G ratio is less sensitive in identifying affected dogs than L/R ratios (Rutgers *et al.*, 1996).

The amount of disaccharide excreted in the urine in humans is similar to the quantity permeating across the intestinal wall and entering the blood stream (Menzies, 1993). The estimations of the concentrations of rhamnose, 3-O-methylglucose, xylose and lactulose in plasma are accurately comparable to their urine concentrations (Sørensen *et al.*, 1997). The plasma ratio of D-xylose to 3-O-methyl-D-glucose 60 minutes after oral administration gave a reproducible normal range in humans (Menzies, 1993). In healthy cats the maximum plasma concentration after the administration of xylose were reached after 60 minutes and remained elevated for 90 minutes before starting to decrease (Hawkins *et al*., 1986). Lactulose/rhamnose ratios in plasma and urine were compared in healthy Labrador puppies and the correlation was best for plasma collection after 120 minutes (Sørensen *et al.,* 1997). Sørensen *et al.* (1997) also showed that although there is variation in increase or decrease of the concentration of the individual sugars, the lactulose/rhamnose ratio remains relatively stable between 90 to 180 minutes post administration in dogs.

The lactulose to rhamnose ratio was increased by greater than 0.12 in dogs with SIBO compared to normal dogs (Rutgers *et al.,* 1996). The urinary recovery of lactulose was increased two to four fold and the urinary rhamnose recovery was two to four fold in dogs suffering from parvoviral enteritis (Möhr, 2002). Cellobiose to mannitol ratio was higher in dogs suffering from gluten-sensitive enteropathy. Thus intestinal permeability is increased in diseases causing a disruption of mucosal integrity.

There is little difference in the permeability of the gastrointestinal tract to rhamnose between different species. The permeability to lactulose in cats has been reported to be four times higher than in dogs and a difference greater than 20 has been seen between cats and humans. Differences in the resistance and number of tight junctions between species have been

associated with the increased permeability to larger molecules in cats (Johnston *et al.*, 2001). Metabolism of different sugars might also be responsible for the differences between urinary and plasma recovery. Lactulose is not metabolised in dogs and humans but metabolism of 10 –30 % has been reported in cats. Metabolism of approximately 25 % of rhamnose has been reported in dogs and humans (Hall and Batt, 1996). The lactulose to rhamnose urinary excretion test showed that gut permeability was higher in cats than in dogs (Randell *et al.*, 2001). This has been associated with the higher number of small intestinal bacteria and a shorter intestine in cats resulting in a decreased surface area, which will alter intestinal permeability (Johnston *et al.*, 1993, 2001).

CHAPTER 2 MATERIALS AND METHODS

2.1 ANIMALS

All animals used in the study were housed in the De Wildt Cheetah and Wildlife Centre. The adult animals are housed separately in one-hectare enclosures. The males are only allowed access to the females during breeding.

The cubs are reared by their mother, or if this is not possible they are hand-reared. Once weaned, litters are housed together and fed a commercial IAMS kitten and junior diet (IAMS Co, USA) mixed with thawed, minced horsemeat with the addition of a commercial mineral mix. The adult animals either receive IAMS adult cat diet or a meat-based diet of horsemeat and whole chicken carcasses. Adults are fed once daily and juveniles are fed twice daily. Fresh water is available ad lib.

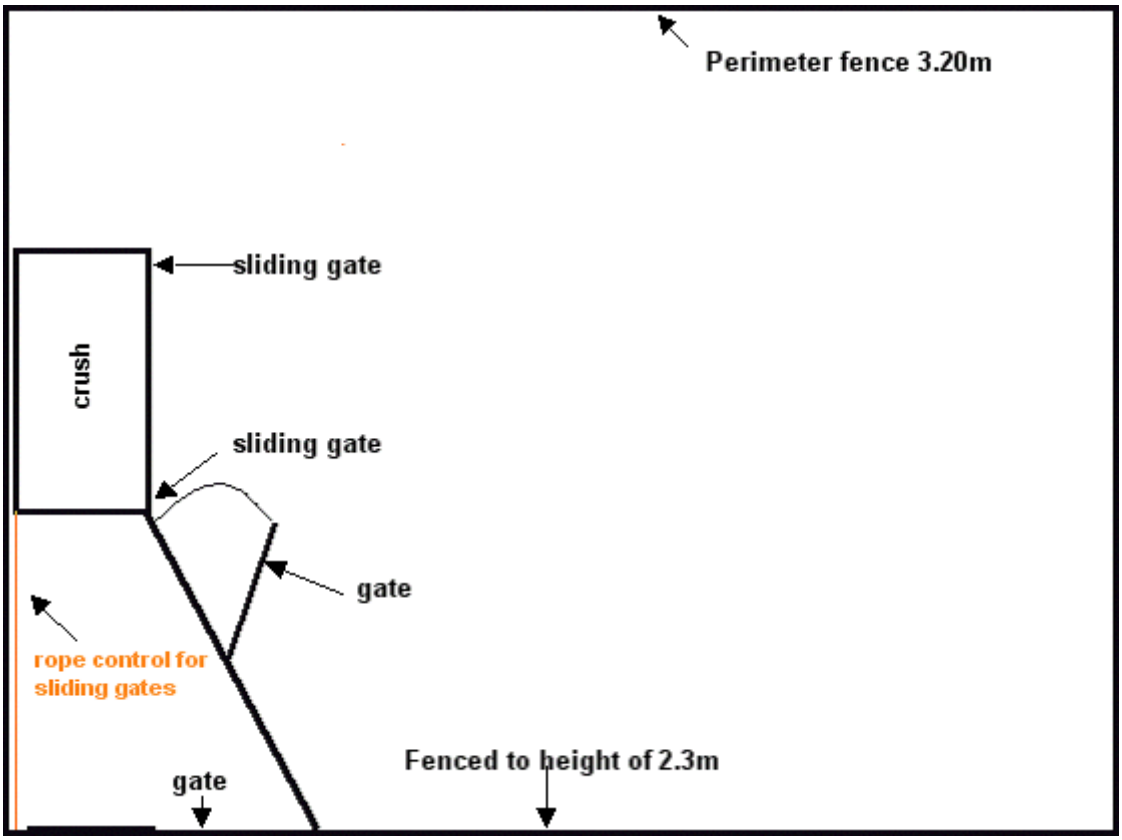


Figure 2: Cheetah camp layout (Meltzer, 1999)

All enclosures were designed to allow easy handling of the cheetahs (see Figure 2). A fence separates the camp and the animals get accustomed to walk through the crush to get access to water and food. If required, the gates of the crush can be lowered from the outside to confine the cheetah in the crush. Once confined in the crush, blood can be collected from the cheetah without immobilisation. All enclosures are cleaned every third day.

2.2 ESTABLISHMENT OF NORMAL INTESTINAL FLORA

For the establishment of the normal intestinal flora, faeces from eight adult healthy cheetahs were collected. The faeces were collected from animals F309 and Q46, both fed on a commercial IAMS cat diet. Faeces were also collected from animals F283, F282, F318, F331, F362 and F327 fed on a meat-based diet (see Table 5). All animals were clinically healthy at the time of collection and did not have a history of gastritis or other chronic medical conditions. All animals were born in the De Wildt Centre except F283, which came to De Wildt from the Kalahari in 1999. The faeces were collected into sterile containers and stored under anaerobic conditions (Gas Pack, Oxoid) until processed in the laboratory.

Duodenal samples were collected from animals M286 and F352. The female was fed on IAMS cat food and the male was fed on a meat-based diet. Both had undergone gastroscopy in 2000 and 2001 as part of a study on the prevalence of gastritis in cheetahs and had no macroscopic or histological abnormalities in the stomach. The animals were starved for 24 hours prior to the procedure and were anaesthetised with a combination of 2 mg medetomidine (Domitor, Novartis Animal Health, SA) and 100 mg ketamine (Anaket-V, Centaur) (Rogers, 1998) in the crush using a pole syringe. Endoscopies were performed in left lateral recumbency with the aid of a mouth gag. A 7.9 mm diameter, 1.3 m length flexible fibre-optic scope (Olympus Optical, Tokyo, Japan) was advanced into the stomach and proximal duodenum (R. Lobetti, Bryanston Animal Hospital, personal communication, 2003). The endoscope was advanced as far as possible along the proximal duodenum and a duodenal wash was obtained by flushing with 10 ml of sterile saline solution. Care was taken to avoid gastric acid contamination of the sample but it could not be completely avoided as the endoscope had to pass through the stomach to advance into the proximal duodenum. The medetomidine antidote, atipamezole hydrochloride (Antisedan, Novartis Animal Health, SA)

was given intramuscularly at a dosage rate of 10 mg per animal 45 minutes after initial immobilisation before releasing the animals back into the camps.

The fresh faeces and duodenal juice samples were cultured in the laboratory to obtain a general idea of the bacteria present. To identify and quantify the amount of bacteria present in faeces and duodenal samples respectively, dilutions of the samples were made. Two grams of faeces and 0,1 ml of duodenal fluid were diluted 10-fold in normal saline up to a dilution of 10^{-12} for the faeces and 10^{-6} for the duodenal samples.

One tenth of a ml of the dilutions 10^{-1}, 10^{-3}, 10^{-9} and 10^{-12} were plated out on Columbia blood agar (Oxoid, Basingstoke, UK) containing 7 % horse blood and MacConkey agar without crystal violet (Oxoid Basingstoke, UK) for growth of members of the enterobactericeae, and kanamycin-aesculin medium (bile aesculin agar (Oxoid, Basingstoke, UK) with the addition of kanamycin sulfate, 0.02 g/l) for the growth of enterococci. The blood agar and kanamycin agar were then incubated at 37°C for 24 hours in an atmosphere of air with 5 % CO_2. MacConkey agar was incubated in an aerobic atmosphere at 37°C. Pre-reduced Columbia blood agar with 7 % horse blood was also incubated anaerobically at 37°C for 48 hours in an anaerobic cabinet.

The isolates from the different dilutions were counted. The bacterial counts were expressed as colony-forming units (CFU) per gram of faeces and CFU per ml of duodenal fluid. The bacteria were grouped by colony morphology, Gram's stain, catalase and oxidase reactions, growth on MacConkey agar, and oxidation-fermentation and motility tests (Picard, 2003). The identification of the bacteria to species level was done by using published biochemical tests (Balows, 1991, Picard, 2003, Quinn *et al*, 1994).

2.3 SELECTION OF BACTERIA SUITABLE FOR USE IN A PROBIOTIC

The faeces were diluted ten-fold and plated onto different media for the selection of suitable bacteria. One tenth of a ml of the duodenal sample was inoculated onto different media. Kanamycin aesculin medium was used for the selection of enterococci, clostridia agar (Sigma,

USA) and SL medium were used for the selection of lactobacilli (Yuan-Kun *et al.*, 1999). Kanamycin aesculin medium was made up using commercial bile aesculin agar (Oxoid, Basingstoke, UK) with the addition of kanamycin sulfate, 0.02 g/l. De Man, Rogosa and Sharpe (MRS) medium (Oxoid, Basingstoke, UK), TPY medium (Yuan-Kun *et al.*, 1999) and Beehrens medium (Beehrens, 1990; Hartemink and Rombouts, 1999) were used for the selection of bifidobacteria. Media for bifidobacteria and lactobacilli were incubated anaerobically for 48 hours whereas those for enterococci were incubated in 5 % CO_2 for 24 hours. SL, TPY and Beehrens medium were not commercially available. For their compositions and references see Appendix B.

Bacteria that on primary identification tests (i.e. colony morphology, microscopic morphology, catalase, OF, oxidase and motility) were positive for lactobacilli were inoculated into the following sugars and secondary tests: bile-aesculin, lactose, galactose, maltose, mannitol, melibiose, salicin, sorbitol, sucrose, trehalose, and xylose (Kandler and Weiss, 1986). The species of enterococci were identified by Lancefield cell wall antigen grouping (Streptococcal Grouping Kit (Oxoid) and whether they fermented lactose, arabinose, sorbitol, mannitol and grew in 6.5 % salt broth (Schleifer, 1986). The *Enterococcus faecium* isolates were further tested for sensitivity to a wide range of antibiotics to see if different strains of *Enterococcus faecium* were present. *Bifidobacterium* species were identified by their ability to ferment the sugars, arabinose, sorbitol, starch, meleziotose, cellobiose, raffinose; sucrose, ribose and lactose. Instead of using a peptone broth base for the sugars to identify the lactobacilli and enterococci, Viande-Levure (VL) (Oxoid, Basingstoke, UK) broth was used (Murray *et al.,* 1995; Picard, 2003).

2.4 FEEDING OF PROBIOTIC BACTERIA

Once bacteria, suitable as probiotics, were identified, they were cultured in brain-heart infusion (BHI) (Merck, Germany) broth. A non-selective enrichment nutrient broth; MRS broth was used for the non-selective isolation of *Bifidobacterium* and *Lactobacillus* spp. (Yuan-Kun, 1999; Starr 1981) for 24 to 48 hours. The broth was then decanted into cryotubes and stored at -86°C until required. Just before needed they were thawed; a loop full was placed in BHI broth and MRS broth and incubated at 37°C for 24 to 48 hours. *Enterococcus faecium* was grown in BHI and *Lactobacillus* was grown in MRS broth. The cloudiness of the

solution was measured at a wavelength of 560nm. The density was standardised with either MRS or BHI broth depending on the bacteria measured. The density was then compared to that of a known standard. This was determined measuring the optical densities of 10-fold dilutions of a pure culture of *enterococci* and correlating them with the CFU obtained for each corresponding dilution on a blood agar plate. The solution was then diluted in BHI broth so that 10^9 to 10^{10} CFU/ ml were obtained.

Twenty-seven juvenile cheetahs between the ages of eight and thirteen months of age were used in the probiotic trial (Median: 12 months old, standard deviation: 1.32). The animals were randomly split into two groups depending on their camps, since different feeding schedules of animals in one camp was not possible due to logistical reasons. The Probiotic Group (PG) consisted of camps 5, 54, 55 and 57 and the Control Group (CG) of camps 6, 53 and 56 (Figure 3). The animals ID, camps and dates of birth and approximate age at the start of treatment are presented in Table 21.

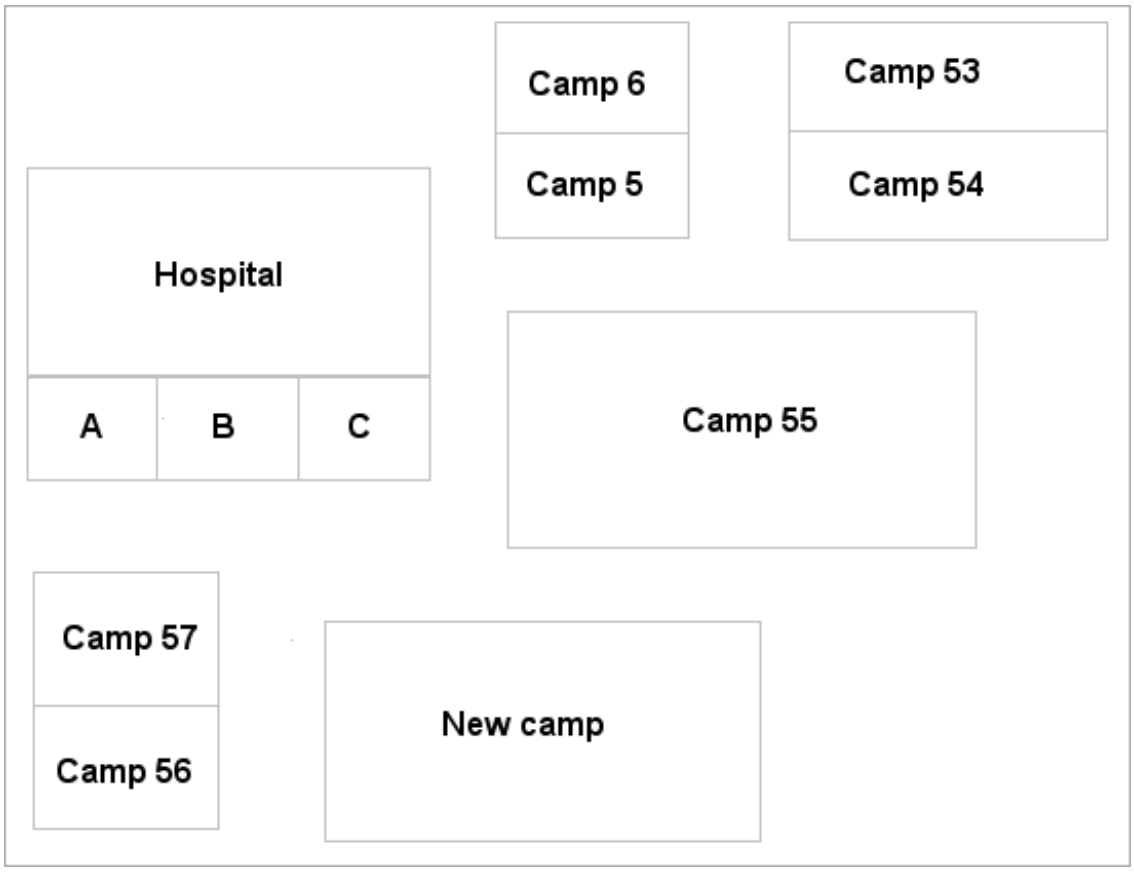


Figure 3: Camp layout for juvenile cheetahs at the De Wildt Centre

The probiotic was mixed into the normal food and each animal in the PG received between 10^9 to 10^{10} CFU (equal mix of *Enterococcus* and *Lactobacillus*) once daily. These numbers of bacteria have given the best results in clinical trials associated with the prevention of diarrhoea in animals (Underdahl, 1982; Maia, 2001; Shu, 2001). The CG received the same volume of BHI and MRS broth as the PG but without any bacteria.

The samples were made up once a week for all animals and stored in the fridge until used. The samples were clearly labelled with the camp numbers. The staff at the De Wildt Centre was not aware of which camps received the probiotic and which camps were used as controls.

2.5 EXPERIMENTAL DESIGN

The trial was split into three monitoring periods, pre-treatment, treatment and post-treatment. The two groups PG and CG were treated as indicated in Table 1.

Table 1: Experimental design and treatments of probiotic trial

Period	Days	Treatments	
		Probiotic Group	Control Group
pre treatment	-70 to -1	no treatment	no treatment
treatment	0 to 28	probiotic bacteria	sterile broth
post treatment	29 to 42	no treatment	no treatment

2.6 FAECAL SCORING AND OBSERVATIONS

Once a week all faeces in the camps were recorded in the morning and scored under the following criteria (see Table 2).

Table 2: Example of faecal score data collection table

Camp No	Faeces normal	Faeces soft, pasty	Faeces watery, some solids	Faeces watery, no solids	Faeces mucoid/bloody

Faeces that were well formed with a little bit of pasty, faecal material surrounding it were considered normal. Faeces, which did not have a well-formed part but just consisted of pasty material were considered soft and pasty. The percentage of diarrhoea was calculated from the third and fourth columns (seeTable 2), as the first two are considered normal. Soft and pasty faeces are associated with the diet and more normal faeces are seen when the percentage of meat in the diet is increased (H. Bertschinger, University of Pretoria, personal communication, 2003). The presence of any mucus or blood was recorded in the last column.

A cross tabulation report was used to analyze the faecal scores. Chi-square statistics for non-continues variables were used. A comparison between Probiotic and Control groups during the different treatment periods was made. The effect of the probiotic was compared in the pre-treatment, treatment and post-treatment periods within groups. The level of statistical significance was set at $P<0.05$.

Faecal scoring was not possible on the 23rd of April (day 0) and the 21st of May (day 28) as all animals in the trial had been starved for 24 hours previously to evaluate their intestinal function and had not produced faeces.

2.7 ANALYSIS OF FAECAL WATER

The percentage water in the faeces was recorded by collecting two fresh faecal samples from each camp once a week. The faeces were weighed before and after drying in an oven at 100ºC for at least 5 days and the percentage water in the sample was recorded.

2.8 DIARRHOEIC FAECES

Ten percent of diarrhoeic faeces were collected and cultured for the presence of pathogenic *E. coli* and *Salmonella* species. To improve the sensitivity of the isolation of salmonellae a swab of faeces was placed in peptone water (Oxoid) and incubated for 2 days followed by selective enrichment in selenite broth (Oxoid) at 37°C for 1 day. Aliquots of selenite broth (Oxoid) were inoculated onto Salmonella selective xylose lysine deoxycholate (XLD) media (Oxoid) and incubated at 37°C. Black colonies were subcultured on blood and MacConkey agar and identified with biochemical tests (Picard, 2003).

Samples were cultured on blood and MacConkey agars for the isolation of *E. coli*. The colony morphology was used to differentiate between rough and smooth colonies.

Faecal flotations for the presence of worm eggs were done on faeces collected from all camps during an outbreak of diarrhoea on the 14th of May and 28th of May. The animals were treated on the 26th of March with anthelmintics: Antezole tablets (Kyron) containing praziquantel 20 mg and pyrantel pamoate 230 mg. They were treated on the 12th to the 14th of May with Panacur (Intervet) containing fenbendazole for 5 days.

2.9 CHANGES IN BODY MASS

All animals were weighed at the beginning (day 0) and end (day 28) of the probiotic treatment period. The animals were caught in the crush and transferred to a transportation crate and weighed on a flatbed scale accurate to 0.1 kg. The weight of the crate was subtracted to obtain the weight of the individual cheetahs. The increase in weight over the four week period was expressed as a percentage weight gain to account for the variation in weights and ages between the animals at the start of the trial.

2.10 CLINICAL PATHOLOGY

Blood samples for haematology and biochemistry were collected from all 27 juvenile cheetahs at the start of the probiotic treatment period on the 23rd of April (day 0) and at the end of the trial on the 21st of May (day 28). Animals were caught in the crush and pinned down within the crush (Meltzer, 1999). Blood was collected from the femoral vein into serum and EDTA (Ethylenediaminetetraacetic acid) blood tubes.

General hematology and the total serum protein, albumin, globulin, albumin to globulin ratio, total bilirubin, cholesterol and urea and creatinine were recorded to eliminate any other systemic disease which could be responsible for diarrhoea (Bechert, 2002). Repeated measures of ANOVA were used to compare values between PG and CG and between the groups and time of collection.

2.11 POLYMERASE CHAIN REACTION (PCR)

The faecal samples were analyzed by means of the PCR for the presence of feline enteric coronavirus (FCoV). Faecal samples were suspended in phosphate buffered saline (PBS). The supernatant fraction was diluted (0.2 g in 10 ml PBS). After dilution, the supernatant was added to the carrier ribonucleic acid (RNA). It was then incubated and centrifuged and the sample added to premix 1. After spinning and incubation the second premix was added. A positive field sample, negative field sample and water were used as controls. The controls with a 100base pair marker (Promega) were analysed on 2 % agarose gel. The result was captured on an EDAC documentation system (Laboratory Specialist Services) (Herrewegh *et al,* 1995; Anne-Marie Bosman, University of Pretoria, personal communication, 2003).

For the detection of blood parasites the Reverse Line Blot (RLB) hybridisation assay, described by Gubbels *et. al.* (1999), a recently developed diagnostic technique, was used. It assisted in the characterization of the blood parasites present in the cheetahs.

After the RNA sequence had been isolated and amplified, a 2 % agarose gel was used to analyse the PCR. The positive PCR amplicons has a size of about 500 base pairs. The results

were read from the X-ray film after development of the film. Spots occurred at the sites where species-specific oligonucleotides and PCR products hybridised. The results were compared to known *Theileria* and *Babesia* species (Gubbels *et al.,* 1999; Anne-Marie Bosman, University of Pretoria, personal communication, 2003).

2.12 EVALUATION OF INTESTINAL PERMEABILITY

Intestinal permeability was tested at the beginning (day 0) and the end (day 28) of the four-week treatment period using isomolar solutions of lactulose and rhamnose. The cheetahs were fasted for 24 hours and water was withheld overnight. An isomolar solution of the two sugars rhamnose (R) and lactulose (L) was given orally. The cheetahs were placed into the crate to insure individual intake. The solution was offered to the cheetahs in the crate with a little bit of minced meat to facilitate voluntary intake. Each animal received 20 ml of the solution containing 102,67 gm of lactulose and 61,55 gm of rhamnose per litre. If the solution was spilt a further 20 ml were offered. This resulted in a solution with an osmolality of 300 mmol/L. The serum concentration in felines is approximately 300 mmol/L depending on concentrations of sodium, glucose and urea in the blood (Carlson, 1997). Hypertonic solutions have shown to cause transient diarrhoea in dogs and humans (Menzies, 1993; Steiner, 2002).

The amount of rhamnose and lactulose in the blood was tested in sera after one hour. The exact time of the blood sample was recorded. To take the blood sample the cheetahs were gently restrained in the crate and a blood sample taken from the femoral vein. Centrifuging for 5 min separated the serum from the rest of the sample. The serum was placed into a clean sample tube and frozen at -86ºC until analysed by HPLC method (Sørensen *et al.*, 1993 and 1997) at the GIT Laboratory, Texas A & M University, USA.

Analysis of variance was used to compare L and R values and L/R ratios between Control Group and Probiotic Group. Repeated measures of ANOVA were used to compare the effects of the timing of the blood collection before and after one hour and before and after one and-a-half hour to the value of sugars present in the blood.

CHAPTER 3 RESULTS

3.1 NORMAL INTESTINAL FLORA

Table 3 shows the aerobic and anaerobic bacteria and enterococci grown per ml of duodenal fluid, obtained by endoscopy. No aerobic bacteria were isolated. The anaerobic bacteria in the sample are obligate anaerobes. *Enterococcus* species was the only member of the facultative anaerobes isolated.

Table 3: Bacteria isolated from duodenal fluid (CFU)

	Cheetah ID			
	F 352	M 286	Mean	Std Dev
Diet	IAMS	Meat		
Strict Aerobes	0[a]	0[a]		
Anaerobic	60	110	85	35.36
Enterococci	300	100	200	141.42

[a] no growth occurred after 48 hours of incubation

Table 4 shows the number of aerobic and anaerobic bacteria and enterococci isolated from 1 g of faeces from 8 healthy adult cheetahs on different diets.

Table 4: Bacterial counts (CFU) of cheetah faecal samples

	Cheetah ID							
	F309	**F283**	**Q46**	**F282**	**F318**	**F331**	**F362**	**F327**
Aerobic	$4.67x10^6$	$1.56x10^9$	$6.40x10^5$	$3.50x10^7$	$1.86x10^6$	$2.34x10^8$	$4.77x10^8$	$1.44x10^6$
Median				$1.99x10^7$				
Mean				$2.89x10^8$				
Std Dev				$5.41x10^8$				
Min				$6.40x10^5$				
Max				$1.56x10^9$				
Anaerobic	$4.66x10^7$	$6.12x10^8$	$1.94x10^6$	0[a]	$2.54x10^6$	$1.32x10^6$	$3.06x10^9$	$1.30x10^6$
Median				$2.54x10^6$				
Mean				$5.32x10^8$				
Std Dev				$1.14x10^9$				
Min				$1.30x10^6$				
Max				$3.06x10^9$				
Enterococci	$1.00x10^5$	$5.00x10^8$	$1.40x10^5$	$6.60x10^3$	$1.88x10^4$	$2.20x10^3$	$4.60x10^5$	$7.00x10^3$
Median				$5.94x10^4$				
Mean				$6.26x10^7$				
Std Dev				$1.77x10^8$				
Min				$2.20Ex10^3$				
Max				$5.00x10^8$				

[a] no growth occurred after 48 hours of incubation

A high proportion of bacteria isolated were *Enterococcus* species. The average number of anaerobic bacteria from the faecal samples was higher than the aerobic isolate numbers. The mean number of aerobic and anaerobic bacteria, and enterococccci isolated from the eight samples was $2.89x10^8$ (SD $5.41x10^8$), $5.32x10^8$ ($1.14x10^9$) and $6.26x10^7$ ($1.77x10^8$), respectively.

The mean comparative number of bacteria isolated from the eight faecal samples is shown in Figure 4.

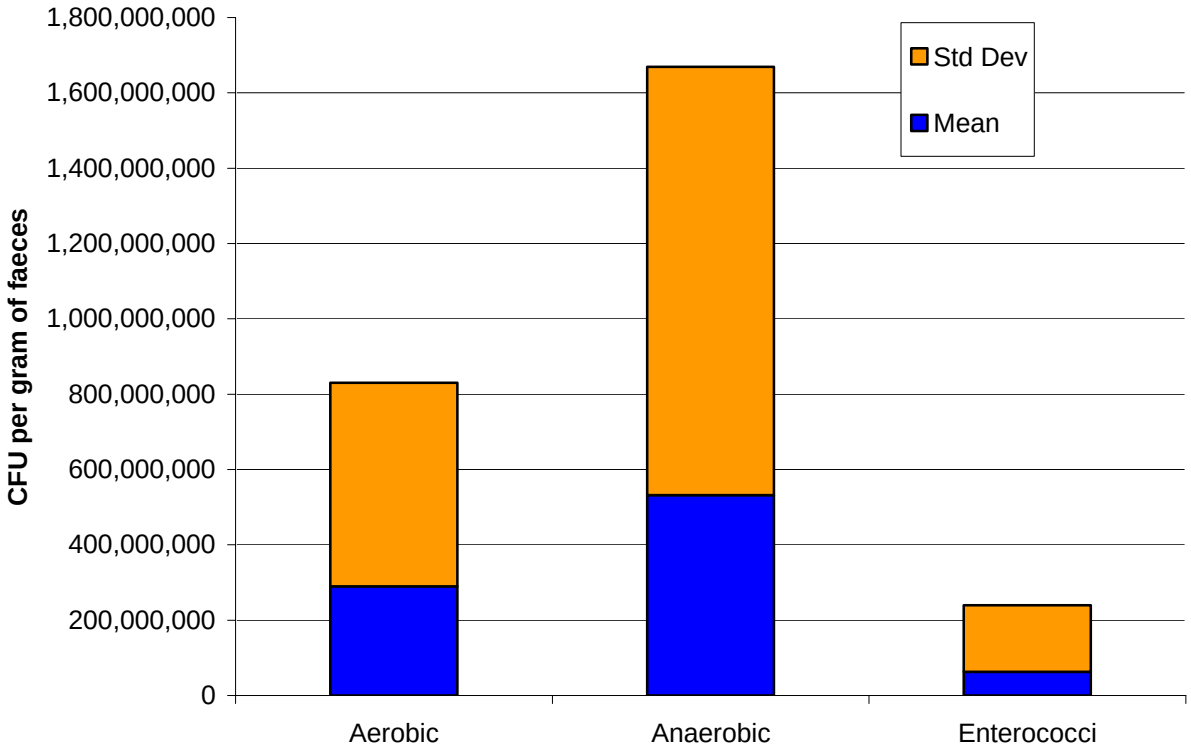


Figure 4: Bacterial numbers (CFU) isolated from cheetah faecal samples

Table 5 shows the effect of diet (IAMS adult cat or meat based) on the numbers of bacteria in the faeces of adult cheetahs.

Table 5: Bacterial counts (CFU) of faeces and diets of adult cheetahs on two diets

Diet	Aerobic	Anaerobic	Enterococci
Mean IAMS	$2.70x10^{6}$	$2.43x10^{7}$	$1.20x10^{5}$
Median IAMS	$2.70x10^{6}$	$2.43x10^{7}$	$1.20x10^{5}$
Std Dev	$2.91x10^{6}$	$3.16x10^{7}$	$2.83x10^{4}$
Mean Meat	$3.85x10^{8}$	$7.35x10^{8}$	$8.34x10^{7}$
Median Meat	$1.35x10^{8}$	$2.54x10^{6}$	$1.29x10^{4}$
Std Dev	$6.05x10^{8}$	$1.33x10^{9}$	$2.04x10^{8}$

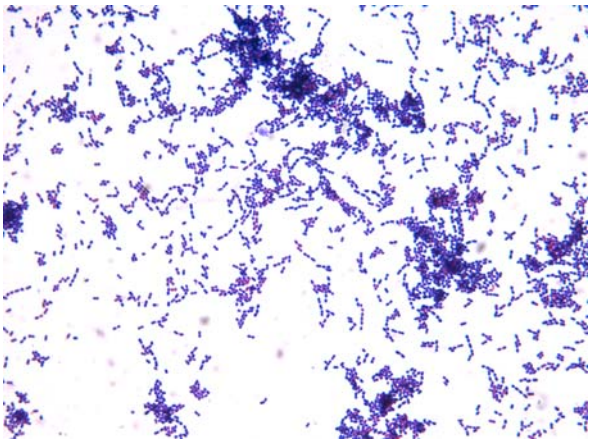
Table 6 shows the different bacteria and yeasts isolated from the faeces and duodenal samples of the cheetahs. Not all genera were identified to species level.

Table 6: Bacteria and yeasts isolated from cheetah faeces

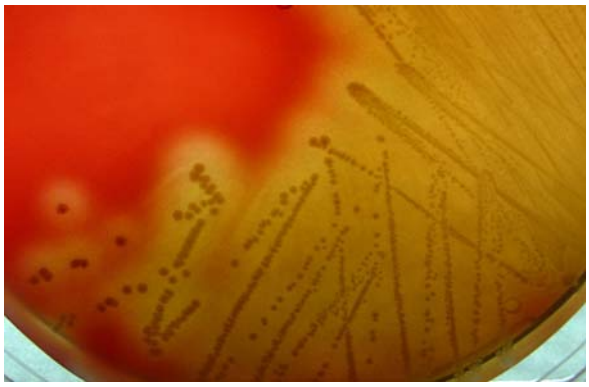
Bacteria isolated	
Genus	Species
Acinetobacter spp.	*A. wolfii, A. calcoaceticus*
Bacillus spp	
Clostridium spp.	*C. perfringens*
Corynebacterium spp.	
Edwardsiella spp.	*E. hoshinae*
Escherichia spp.	*E. coli*
Enterobacter spp.	*E. agglomerans*
Enterococcus spp.	*E. durans, E. agglomerans, E. faecium*
Lactobacillus spp.	Group 1, Group 2
Moraxella spp.	
Pasteurella spp.	
Proteus spp.	
Pseudmonas spp	
Staphylococcus spp.	*S. epidermalis*
Vibrio spp.	*V. alginolyticus, V. cholera*
Yeasts isolated	
Cryptococcus spp.	

3.2 BACTERIA FOR THE PROBIOTIC

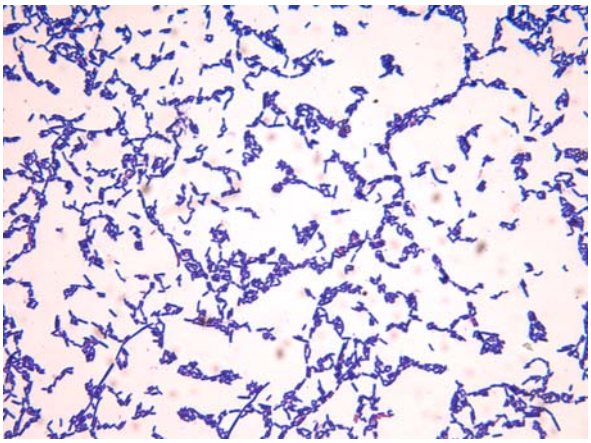
Enterococcus faecium and *Lactobacillus* group 1 were isolated from cheetah faeces to be included in the probiotic. The bifidobacterial isolates were only present in very low numbers. The isolates of *Bifidobacterium* ssp stored did not grow in the BHI or MRS broth and could therefore not be used in the trial.



Entercoccus faecium

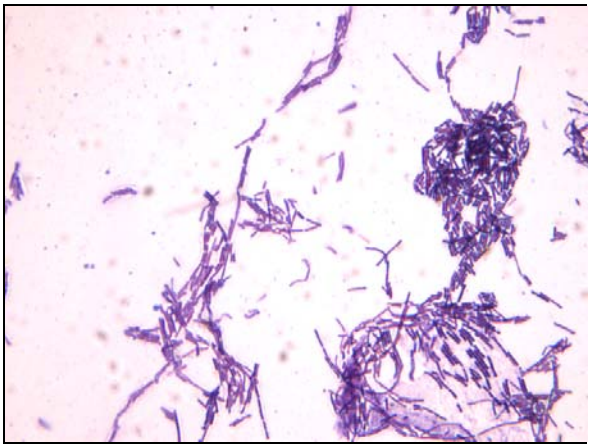


Enterococcus culture

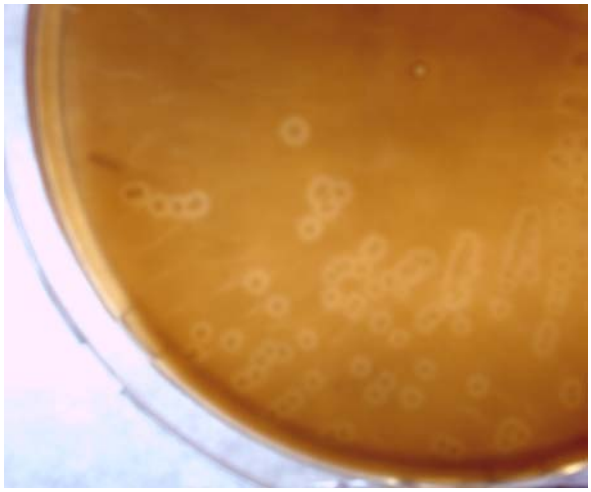


Lactobacillus species

Lactobacillus culture



Bifidobacterium species



Bifidobacterium culture

Figure 5: Smears and culture of bacteria used as probiotic

3.3 HEALTH OF JUVENILE CHEETAHS

All animals were healthy at the beginning of the trial on day –70. There was episodic diarrhoea present in all camps, rarely accompanied with a depressed or altered appetite. No animal showed signs of systemic disease before, during or after the treatment period (day 0 to day 28), except diarrhoea. F 457 was treated with 2 ml Synulox (140 mg amoxycillin and 35 mg clavulanic acid per ml; Pfitzer) and 2 ml Duplocillin (procaine penicillin 150 000 IU per ml; Intervet) on day –56 due to a bite wound on the shoulder. Synulox treatment was continued for 5 consecutive days.

An outbreak of severe diarrhoea in camp 55 on day –35 was treated with Biosol (neomycin sulphate 200 mg, methscopolamine bromide 2 mg; Pharmacia and Upjohn). Each animal received 1.5 ml orally. Antibiotic therapy was continued for three days with Enteritis Tablets (sulphathiazole 75 mg, phthalylsulphathiazole 175 mg, neomycin sulphate 15 mg, streptomycin sulphate 10 mg, aminopentamide sulphate 0.025 mg, kaolin 200 mg, pectin 2 mg; Bayer Animal Health). The dosage was three tablets per animal twice daily.

There was a significant difference between the ages of the Probiotic and Control groups (two sampled t-test, $P<0.0069$).

3.4 FAECAL SCORING

The faeces were analysed from day –70 to day 42. Days –70 to –56 showed significant difference ($P=0.0137$) in the Probiotic Group in comparison to days –42 to –7. This is thought to be associated with subjective differences in scoring faeces, as a different person scored the first three weeks (days –70 to –56). For statistical analysis the first three weeks were excluded from the statistics in both groups.

Table 7: Comparison of diarrhoeic scores of Probiotic and Control groups in the pre-treatment period

Probiotic Group				
Camps	5	54	55	57
Diarrhoea Score	35.71%	26.32%	42.31%	39.39%
Control Group				
Camps	6	53	56	
Diarrhoea Score	27.54%	26.67%	11.11%	

Table 7 compares the differences in percentage diarrhoea between different camps in the PG and CG. The percentage diarrhoea was lower in camp 56 in the pre-treatment period but it was not significant. There were no significant differences in the percentage diarrhoea between camps in the PG and CG in the pre-treatment period (days –42 to –7).

Table 8: Percentage diarrhoea in Probiotic and Control groups during probiotic trial.

	Percentage diarrhoea		
Period	Probiotic Group	Control Group	Total
Pre-treatment	46.85%	24.68%	37.77%
Treatment	30.77%	31.37%	31.03%
Post-treatment	75.00%	36.00%	53.33%

Table 8 compares the percentage diarrhoea between PG and CG during the trial. There was a significant difference (P=0.0021) in the percentage diarrhoea between the PG and CG in the pre-treatment period. There was no statistical significant difference between PG and CG during the treatment period, but a significant difference was noted in the post-treatment period (P=0.0092).

Comparing the diarrhoeic scores between the pre-treatment, treatment and post-treatment periods in the control group, there was no statistical significant difference in percentage diarrhoea during different periods. However, there was a statistical significant difference between the pre-treatment and treatment period in the PG (P=0.0363) and the treatment and post-treatment period (P=0.0004) (see Table 8 and Figure 6).

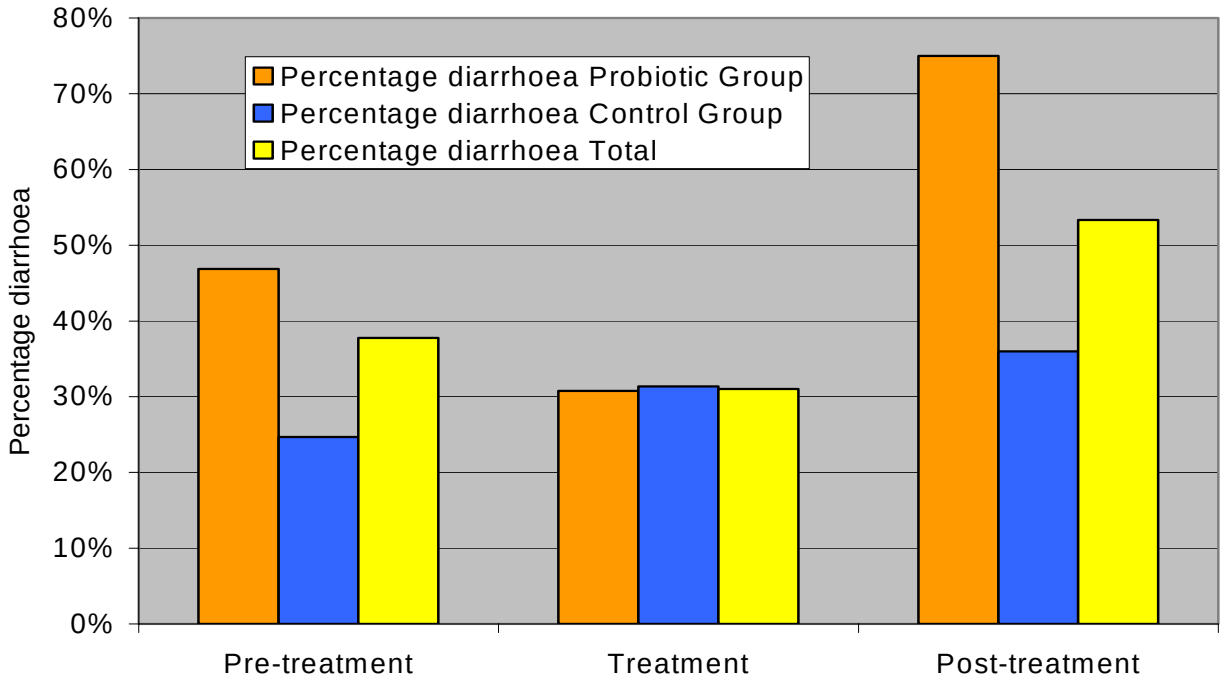


Figure 6: Comparison of percentage diarrhoea in Probiotic and Control groups during trial

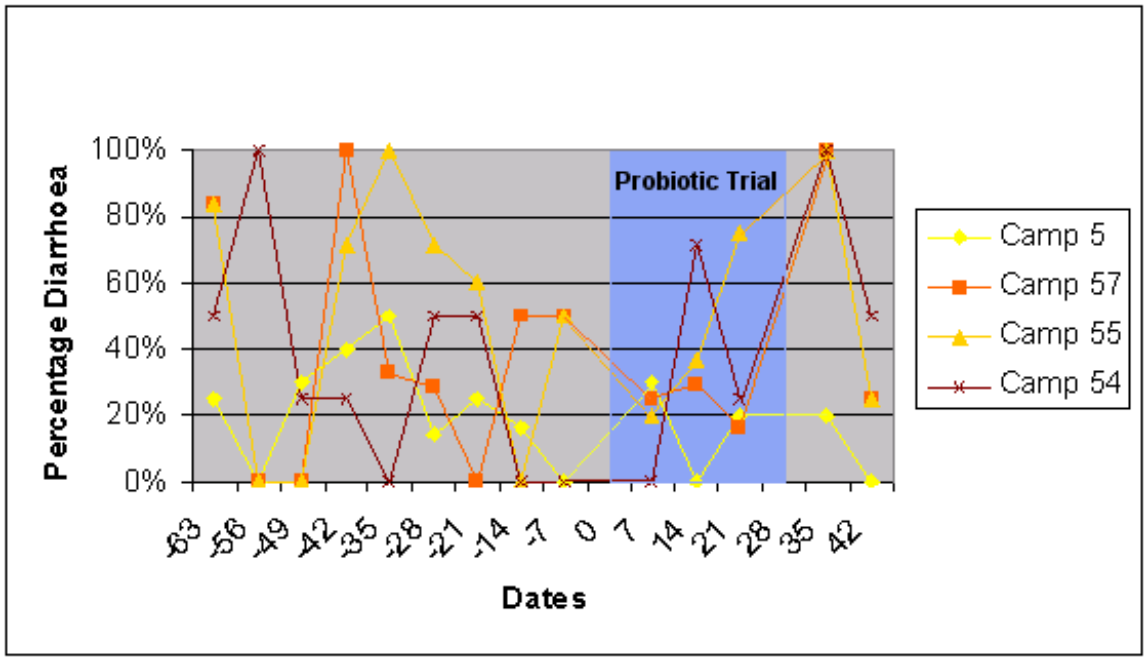


Figure 7: Percentage diarrhoea in the Probiotic Group during the trial

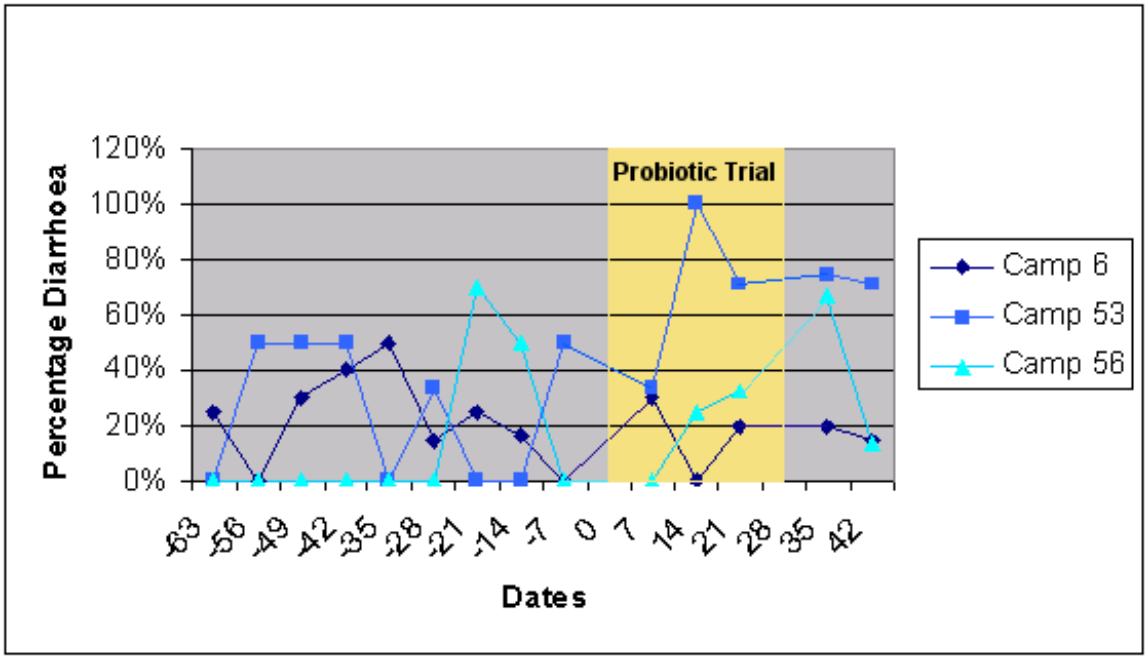


Figure 8: Percentage diarrhoea of the Control Group during the trial

Figure 7 and Figure 8 show the percentage diarrhoea of the PG and CG over time from day –63 to day 42, on weekly intervals. The probiotic was fed from the Day 0 until Day 28.

The prevalence of bloody or mucoid faecal samples in the PG decreased to nil during the treatment period (Figure 9and Table 24)

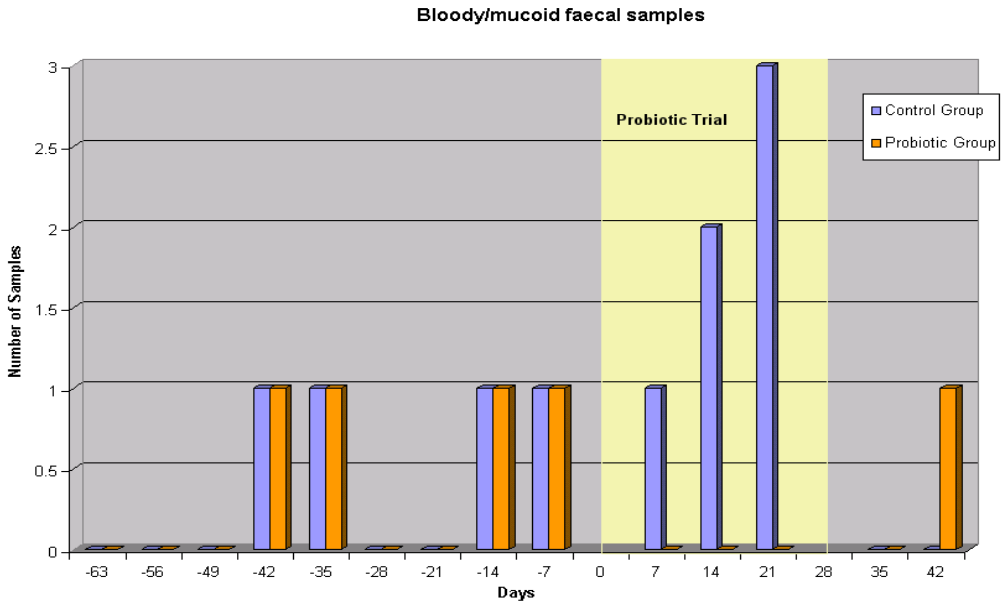


Figure 9: Prevalence of bloody/mucoid faecal samples in Probiotic Group and Control Group during trial

The lightly shaded area in Figure 9 shows the time during which the probiotic bacteria were fed to the Probiotic Group. The orange markers represent the PG and the CG is represented by the blue colour.

3.5 FAECAL WATER CONTENT

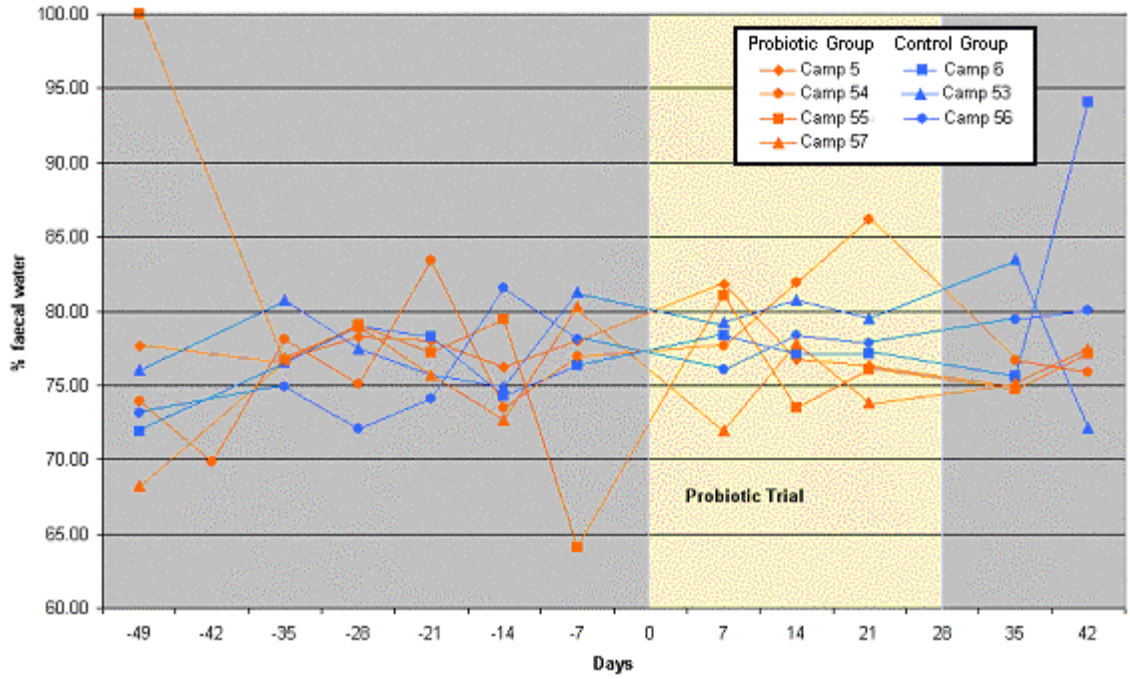


Figure 10: Percentage of faecal water in Probiotic Group and Control Group during trial

Figure 10 shows the maximal percentage of water in faeces in weekly samples collected in different camps during the trial.

There was no statistical difference in faecal water between different camps or different dates. The maximal faecal water was not affected by the administration of the probiotic bacteria.

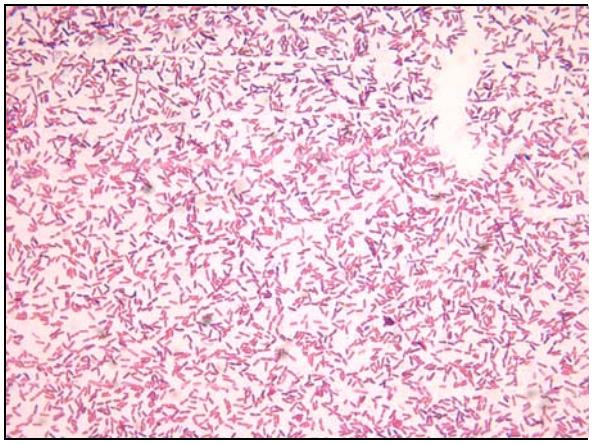
3.6 PATHOGENIC BACTERIA IN FAECES

Four diarrhoeic samples, collected in different camps, were cultured for pathogenic bacteria. No *Salmonella* spp. and no smooth *E. coli* were isolated (see Table 9).

Table 9: Bacteria isolated from diarrhoeic faeces

Day sampled	Camp	Bacteria isolated
Day -7	57	Rough *E. coli*
Day 14	53	Rough *E. coli*
Day 42	57	Rough *E. coli* and *Proteus* species
Day 42	55	Rough *E. coli*

In summary, no smooth *E.coli*, *Salmonella* or *Yersinia* were isolated from the diarrhoeic faeces during the entire trial.



Escherichia coli

Figure 11: *E. coli* smear- Gram's stain

Figure 11 shows Gram-negative slender bacilli typical for *E.coli.*

3.7 BODY MASS

Table 10 and Table 11 show the weight of each cheetah at Day 0 and Day 28, after the four-week treatment period. Only one weight was available for F460 as the cheetah was sold before day 28. F459 was also sold earlier, however, it was weighed on day 13. The percentage weight increase of each individual cheetah is also provided. Animals F460, F459 and F444 were excluded from the statically analysis. F444 was excluded as a result of an incorrect reading (see Table 11).

Table 10: Body mass changes of cheetah in Probiotic Group

Camp	Animal	D.o.B.	Age months	Weight 23.04	2nd Weight	Date	% increase	Comment
5	460	01.06.2002	11	20.7				Sold
	465	Unknown	11 [a]	17.7	19.7	21.05.04	10.15 %	
54	50	17.04.2002	13	25.5	27.7	21.05.05	7.94 %	
	433	17.04.2002	13	29.1	30.2	21.05.06	3.64 %	
	434	17.04.2002	13	27.8	31.3	21.05.07	11.18 %	
	440	28.04.2002	13	23.7	25.9	21.05.08	8.49 %	
	441	28.04.2002	13	22.3	24.4	21.05.09	8.61 %	
55	438	28.04.2002	13	19.8	21.1	21.05.10	6.16 %	
	450	30.05.2002	12	20	22.1	21.05.11	9.50 %	
	455	05.06.2002	11	23.2	24.9	21.05.12	6.83 %	
	437	21.04.2002	13	17.2	17.8	21.05.13	3.37 %	
	446	18.05.2002	12	16.7	18.6	21.05.14	10.22 %	
	459	02.06.2002	11	19.1	20.8	06.05.03	8.17 %	
	461	01.06.2002	11	17	17.9	21.05.03	5.03 %	
57	458	01.08.2002	9	16.8	17.8	21.05.04	5.62 %	
	457	01.08.2002	9	13.9	15.54	21.05.05	10.55 %	

[a] approximated age of cheetah

Table 11: Body mass changes of cheetah in Control Group

Camp	Animal	D.o.B.	Age month	Weight 23.04	2nd Weight	Date	% increase	
6	430	16.04.2002	13	22.1	23.2	21.05.03	4.74 %	
	431	16.04.2002	13	18.6	19.8	21.05.04	6.06 %	
	432	16.04.2002	13	21.5	23.1	21.05.05	6.93 %	
	435	20.04.2002	13	27.4	28.2	21.05.06	2.84 %	
	436	20.04.2002	13	25.7	28.3	21.05.07	9.19 %	
	447	20.04.2002	13	21	22.7	21.05.08	7.49 %	
53	426	22.03.2002	14	30.6	32.6	21.05.09	6.13 %	
	427	22.03.2002	14	33.2	36.3	21.05.10	8.54 %	
	428	22.03.2002	14	30.6	32.2	21.05.11	4.97 %	
56	443	12.05.2002	12	26.5	27.1	21.05.12	2.21 %	
	444	12.05.2002	12	26	22.9	21.05.13	-13.54 %	Incorrect reading

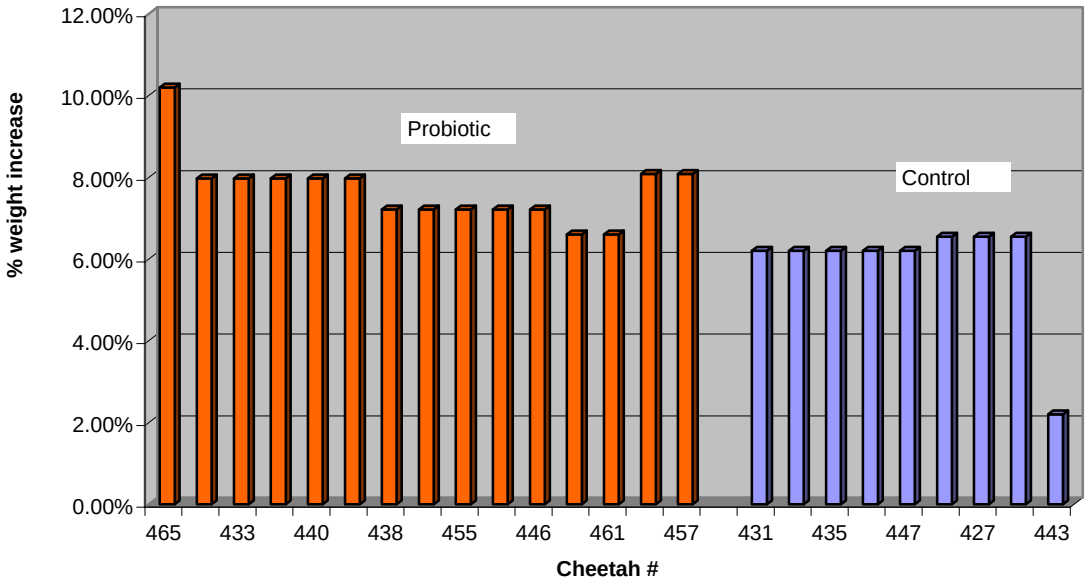


Figure 12: Comparison of percentage body mass increase in Probiotic Group and Control Group

There was no difference in weight increase between the PG and CG if actual weight change was considered, however when considered as a percentage, the PG gained considerably more weight than the CG ($p = 0.026$, ANOVA, $p < 0.05$).

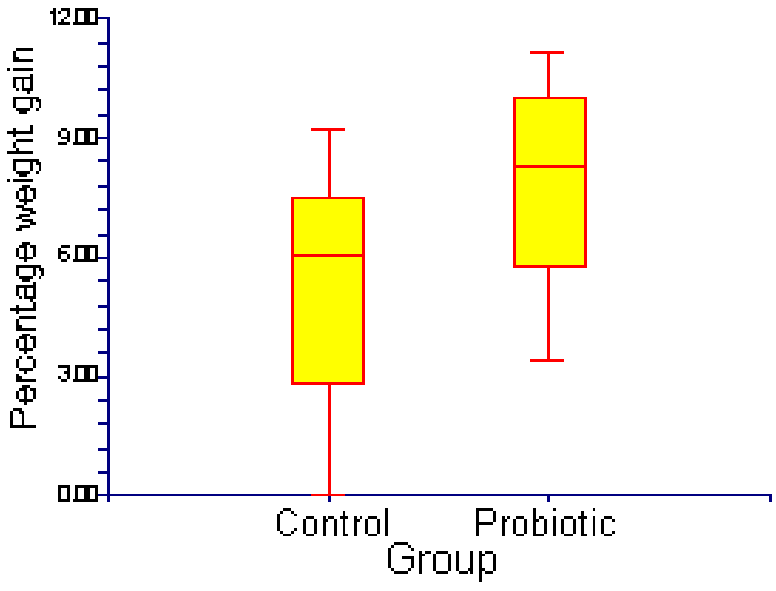


Figure 13: Box plot comparison of percentage weight gain of Probiotic Group and Control Group

The mean increase in weight of the PG was 7.754 % (SE=0.645) and of the CG 5.372 % (SE=0.778) (Tukey-Kramer test, $p < 0.05$). The mean and standard deviation are presented in Figure 13.

3.8 SERUM BIOCHEMISTRY AND HAEMATOLOGY

Except for the eosinophil count of cheetah F446, the leukocyte values of the cheetahs were all within the reference range, published by the International Species Information System (1999).

The mean haemoglobin (Hb) at the beginning of the treatment period was higher in the PG than the CG and lower at the end, however, the difference was not statistically significant. The red cell count (RCC) showed a significant decrease in the CG over time (P=0.023), but none of the animals fell outside the published reference range for RCC. There was no significant difference in mean haematocrit (Ht) in both groups over time. There was a significant difference in the mean cell volume (MCV) over time in both groups (P=0.002). The initial MCV in the CG was higher. There was a significant increase in mean cell haemoglobin concentration (MCHC) in both groups over time (P=0.000002). The MCHC was higher in the PG at the start. There was a significant decrease in white blood cell count (WBCC) in both groups over time (P=0.0014). There was a decreasing trend in segmented neutrophils but this was not significant. The mean number of banded neutrophils did not change significantly over time but the PG had lower counts at all times. There was a lot of individual variation in neutrophil count over time. The mean number of lymphocytes in both groups decreased insignificantly over time. The number of monocytes at the end of the treatment period decreased non-significantly in the PG. There was little variation in eosinophil count between groups and over time. F446 had the highest eosinophil count on Day 0 (4.23 $x10^3/\mu l$) and on Day 28 it had increased to 7.92 x $10^3/\mu l$. The highest physiological reference recorded is 5.84 $x10^3/\mu l$ (International Species Information System, 1999).

Table 12: Biochemistry and haematology values of Probiotic and Control groups at the start (day 0) and end of treatment (day 28).

		Control Group day 0		Control Group day 28		Probiotic Group day 0		Probiotic Group day 28		Reference Range	
Variables	Units	Mean	St dev.	Mean	St dev.	Mean	St Dev.	Mean	St Dev.	Min	Max
TSP	g/l	62.35	4.87	62.05	2.26	63.24	2.18	62.52	2.69	51.00	88.00
Alb	g/l	33.85	2.58	34.37	1.51	35.18	1.70	35.03	1.37	23.00	51.00
Globulin	g/l	28.51	2.69	27.65	1.37	28.04	1.61	27.49	1.98	17.00	55.00
A/G		1.19	0.08	1.25	0.07	1.26	0.10	1.28	0.08	0.60	1.20
Bil-T	ymol/l	2.45	0.46	2.68	0.49	3.08	0.76	2.67	0.50	0.00	29.00
Cholesterol	mmol/l	3.24	0.72	3.68	0.50	3.05	0.46	3.49	0.38	2.07	13.68
Urea	mmol/l	12.93	1.30	10.55	0.68	12.18	2.05	11.65	1.85	5.36	29.63
Creatine	ymol/l	207.73	15.75	210.82	27.91	195.50	31.89	198.93	24.81	53.04	716.04
Haemoglobin	g/l	138.27	6.33	134.18	13.39	133.50	5.18	137.21	5.48	6.90	20.20
RCC	1000/yl	8.18	0.40	7.77	0.69	7.89	0.42	7.93	0.38	4.18	11.10
Haematocrit	l/l	0.47	0.02	0.43	0.04	25.92	0.02	0.45	0.03	0.20	0.58
MCV	fL	57.62	1.87	56.13	1.96	56.86	2.25	55.95	1.53	34.20	86.10
MCHC	g/dl	29.36	0.36	30.87	0.92	29.80	0.98	30.99	0.40	20.50	48.80
WBCC	1000/yl	11.55	2.41	9.81	1.09	11.76	2.88	10.50	2.56	3.70	25.20
Seg. neutrophils	1000/yl	6.88	1.57	6.16	0.96	6.81	2.09	6.02	1.23	1.34	20.90
Neutrophils (bands)	1000/yl	0.05	0.11	0.05	0.08	0.01	0.05	0.01	0.03	0.00	6.30
Lymphocytes	1000/yl	3.35	1.58	2.51	0.49	3.64	1.36	3.03	0.78	0.14	8.26
Monocytes	1000/yl	0.61	0.38	0.54	0.33	0.60	0.25	0.45	0.21	0.00	2.59
Eosinophils	1000/yl	0.66	0.42	0.50	0.28	0.64	0.98	0.96	2.02	0.00	5.80
Basophils	1000/yl	0.02	0.05	0.04	0.07	0.03	0.06	0.03	0.04	0.00	0.26
ThrC	1000000/yl	364.09	185.04	379.36	146.32	358.78	187.70	403.57	181.39	96.00	842.00

There was no significant difference in the mean number of basophils. There was no significance difference in the level of thrombocytes (ThrC), total serum protein (TSP), albumin or globulin. There was, however, a significant increase in albumin/globulin ratios (A/G) in the CG from Day 0 to Day 28 (P = 0.044). Although, total bilirubin (BilT) decreased in the PG, the difference was not significant. Cholesterol increased significantly in both groups over time (Epsilon probability level = 0.000014). Urea decreased significantly in the CG in relation to the PG and time (P = 0.0054), but there was no significant change in creatinine over time or between groups. The mean values of the PG and CG at the start (day0) and end (day 28) of the feeding of the probiotic are represented inTable 12.

3.9 PATHOGEN IDENTIFICATION

PCR tests for presence of feline coronavirus in diarrhoeic faecal samples were negative, as no nucleic acid could be detected. Blood smears from M427, M440 and F446 were positive for *Babesia* species on repeated blood sampling (seeTable 25, Table 26, Table 27 and Table 28). Blood samples were further analysed by PCR and reverse line blot to identify the species of *Babesia*, as described by Penzhorn *et al.* (2001). The parasite did not match any known isolates for *Theileria/Babesia* species (Anna-Marie Bosman, University of Pretoria, personal communication, 2003).

Table 13: Faecal flotation of faeces (no of eggs/gram of faeces)

Group	Camp	Dates	
		14.05.03 Day 21	28.05.03 Day 35
Probiotic	5	63	0
	54	226	0
	55	3	0
	57	35	0
Control	6	0	0
	53	110	0
	56	33	0

Results of the faecal flotations from faeces collected on day 21 and day 35 are presented in Table 13. Eggs showing the characteristics of *Toxocara leonina* and other *Toxocara* species were identified in the faeces. Adult helminths belonging to *Toxocara* spp. were identified in the faeces.

3.10 INTESTINAL PERMEABILITY

The intestinal permeability of the Probiotic and Control groups was measured at the start (day 0) and the end (day 28) of the treatment period. The timing between the administration of the sugars and the collection of blood is shown in Table 29 and Table 30. The median for day 0 for all animals was 82 minutes (range 40 to 170 minutes) and for day 28 was 70 minutes

(range 44 to 112 minutes). The delayed blood collection after administration of the sugars lactulose (L) and rhamnose (R) occurred mainly in the CG. Comparing L and R-values and L/R ratios, there was no significant difference between sugar concentration in the blood before and after one hour and one and-a-half hour.

The concentration of L and R was measured in the serum and the ratios of the two sugars were calculated for each animal. Table 14 and Table 16 show the sugar concentrations for the CG and PG at the start of the trial. Table 15 and Table 17 show the sugar concentrations of the PG and CG at the end of the trial.

Table 14: Sugar concentration of Control Group at the start of treatment period

Rhamnose (R) conc.		Lactulose (L) conc.		L/R ratio	
Min	0.00	Min	0.00	Min	0.00
Max	6.20	Max	1.10	Max	0.79
Mean	2.84	Mean	0.56	Mean	0.20
Median	2.20	Median	0.64	Median	0.19
SD	2.07	SD	0.47	SD	0.21

Table 15: Sugar concentration of Control Group at the end of treatment period

Rhamnose (R) conc.		Lactulose (L) conc.		L/R ratio	
Min	1.40	Min	0.02	Min	0.00
Max	5.20	Max	2.50	Max	0.48
Mean	3.22	Mean	0.40	Mean	0.09
Median	3.20	Median	0.20	Median	0.05
SD	1.33	SD	0.71	SD	0.14

Table 16: Sugar concentration of Probiotic Group at the start of treatment period

Rhamnose (R) conc.		Lactulose (L) conc.		L/R ratio	
Min	0.30	Min	0.00	Min	0.00
Max	5.40	Max	2.50	Max	0.92
Mean	1.89	Mean	0.55	Mean	0.27
Median	1.60	Median	0.40	Median	0.14
SD	1.30	SD	0.67	SD	0.29

Table 17: Sugar concentration Probiotic group at the end of treatment period

Rhamnose (R) conc.		Lactulose (L) conc.		L/R ratio	
Min	0.200	Min	0.010	Min	0.003
Max	7.200	Max	2.100	Max	0.913
Mean	1.993	Mean	0.382	Mean	0.264
Median	1.450	Median	0.130	Median	0.100
SD	1.927	SD	0.565	SD	0.308

Table 19 shows the changes in the L/R ratios of the individual animals in the Control and Probiotic groups, respectively. Figure 14 shows a comparison of the mean lactulose/rhamnose ratios of the CG and PG at the beginning and end of the probiotic trial.

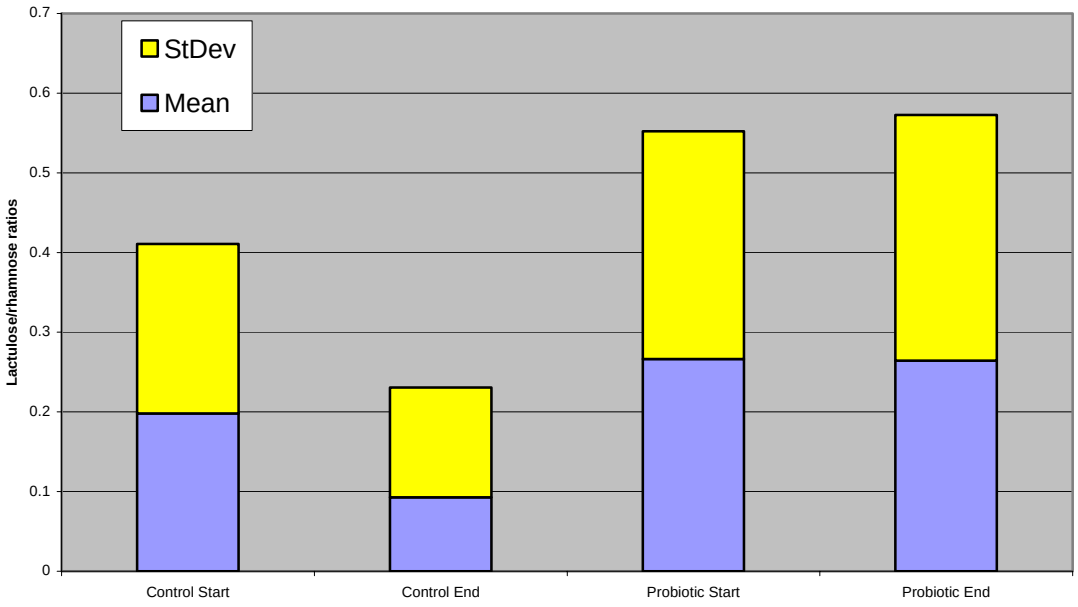


Figure 14: Comparison of lactulose/rhamnose ratios in PG and CG at the start and end of treatment period

Table 18: Differences in rhamnose (R) and lactulose (L) ratios in Control Group

Cheetah ID	Start L/R	End L/R	Difference
430	0.214	0.045	-0.169
431	0.045	0.010	-0.036
432	0.006	0.014	0.008
435	0.140	0.061	-0.080
436	0.190	0.128	-0.062
447	0.224	0.008	-0.217
426	0.188	0.154	-0.034
427	0.000	0.000	0.000
428	0.150	0.073	-0.077
443	0.786	0.481	-0.305
444	0.233	0.045	-0.187

Table 19: Differences in rhamnose (R) and lactulose (L) ratios in Probiotic Group

Cheetah ID	Start L/R	End L/R	Difference
465	0.500	0.733	0.233
438	0.000	0.100	0.100
450	0.076	0.132	0.056
455	0.917	0.338	-0.578
437	0.353	0.056	-0.297
446	0.100	0.003	-0.097
461	0.067	0.017	-0.050
50	0.045	0.008	-0.037
433	0.029	0.050	0.021
434	0.040	0.088	0.048
440	0.714	0.600	-0.114
441	0.338	0.913	0.575
458	0.072	0.10	0.03
457	0.653	0.56	-0.09
459	0.173	[a]	
460	0.181	[a]	

[a] no second reading available since cheetah was sold

There was a significant difference in rhamnose concentration between the CG and PG on Day 0 of the treatment period (P=0.034, ANOVA, significance α=0.05). The mean rhamnose

concentration of CG on Day 0 was 2.836 (SE 0.466) and the PG was 1.894 (SE 0.386). The mean concentration of rhamnose at the end in CG was 3.218 (SE: 0.466) and in PG was 1.993 (SE 0.413). There was no significant difference between lactulose concentrations between the two groups on Day 0. Both groups had a non-significant decrease in the mean lactulose concentration on Day 28. There was a significant difference in the lactulose to rhamnose ratio (L/R) between the Probiotic and the Control groups at the end of the treatment period, with the Control Group showing a decrease in the L/R ratio ($P=0.044$, ANOVA, significance $\alpha=0.05$).

CHAPTER 4 DISCUSSION

4.1 THE INTESTINAL FLORA OF HEALTHY CHEETAHS

There were no aerobic bacteria isolated from the proximal duodenum of the two cheetahs and the number of anaerobic bacteria and *Enterococcus* spp. varied. Many of the Enterobacteriaceae and *Enterococcus* spp. are facultative anaerobes. They will grow in the presence or absence of oxygen.

Only two animals were available for duodenal sampling. One can therefore only deduce that there is a difference in bacterial numbers in the proximal duodenum between cheetahs. The difference might be associated with diet. The two animals were fed on a different diet, namely meat-based or adult cat food (IAMS). Vulfson *et al.* (2003) proposed a difference in bacterial counts associated with diet in mink. He also showed an effect on the different bacteria where beta-haemolytic staphylococci decreased after birth and *E. coli* increased gradually from birth to weaning.

High numbers of aerobic bacteria were cultured from the proximal intestine in cats (Johnston, *et al.*, 1993). The reason that no growth of aerobic bacteria occurred from the duodenal samples of the two cheetahs might be associated with the proximity of sampling to the stomach. Even so Johnston, *et al.* (1999), proposed no differences in bacterial counts in either qualitative or quantitative microbiological flora when duodenal juice is collected by endoscopy compared with direct needle aspiration during laparotomy.

Low numbers of anaerobes and high numbers of enterococci were found in the duodenum of the cheetahs in this study. An increase in fermentable fibre has been associated with a trend of lower total bacterial counts in the duodenum of cats (Johnston *et al.*, 1999). The IAMS diet contains more fermentable fibre than the meat-based diet. Therefore the difference in fibre or fundamental difference in the diet composition could be associated with the difference in bacterial counts found in the cheetahs.

High numbers of anaerobic bacteria and aerobic bacteria were isolated in the faeces of cheetahs in this study. The bacterial counts found by Howard *et al.* (1993) differed. The predominant bacterial isolates in cheetahs were Gram-negative (98 %) in comparison to 43 % of Gram-negative bacteria in cats.

There were higher numbers of bacteria, both aerobic and anaerobic, isolated from faeces of cheetahs fed a meat-based diet in this study. The higher bacterial counts could be related to their exclusive meat-based diet. Johnston *et al.,* (1993), proposed the higher number of bacteria in the gastrointestinal tract of cats to be associated with an exclusive meat-based diet. Higher numbers of bacteria should also be expected in cheetahs on a meat diet.

The most common bacterium found in cheetahs and cats in the study of Howard *et al.* (1993) was *E. coli*, but only 1.4 % of their isolates were *Enterococcus spp.* In contrast, 8 % of isolates were *Enterococcus spp* in this study. Medium to high numbers of *E. coli* were found in this study but no colony counts were performed making it difficult to compare the two studies.

Howard *et al.* (1993) isolated the following Gram-negative bacteria from rectal swabs of cheetahs: *E. coli, Campylobacter, Klebsiella* and *Proteus*. *Enterococcus* spp. were the only Gram-positive bacteria present. This is in contrast with the results of this study where a variety of bacteria and yeasts were isolated (see Table 6). The variety of isolates was similar to the composition of isolates found in the domestic cat (Howard *et al.* 1993). All cheetahs in their study were housed in zoos and received a commercial carnivore diet with the addition of various meat supplements. The differences in housing and diet may also be responsible for the differences in bacterial isolates. The use of selective media in our study may also have enhanced the recovery of those bacteria only present in small numbers. No selective media were used by Howard *et al.* (1993). To get a true representation of the microflora of cheetahs, more animals would need to be studied to evaluate the effects of age, diet and housing. The influence of the diet on the susceptibility of cheetahs to intestinal upsets and diarrhoea also needs to be studied more.

Ideally faeces of free-ranging cheetahs should also have been analysed. The normal flora of free-ranging cheetah may be different from the flora of captive cheetah fed on IAMS adult cat

pellets, horsemeat, or chicken. Different diets fed to animals in captivity may also change the normal flora. Unfortunatly no faecal specimens from free-ranging cheetahs were available. One of the female cheetahs (F331) used for faecal collection was wild-caught (see Table 20), but not recently enough to qualify as free-ranging. The faecal cultures of F331 were not significantly different from those of captive-bred animals.

4.2 COMPOSITION OF THE CHEETAH PROBIOTIC

Lactobacillus Group 1 was selected as one of the bacteria to be included in a probiotic. *Lactobacillus* Group 1 contains several species of lactobacilli, namely: *L. delbrueckii, L. acidophilus, L. amylophilus, L. amylovorus, L. animalis, L. crispatus, L. farciminis, L. gasseri, L. helveticus, L. jensenii, L. ruminis, L. salivarius, L. sharpeae, L. vitulinus* and *L. omanashiensis* (Kandler and Weiss, 1986). The species of lactobacilli are closely linked when comparing rRNA sequences (de Waard *et al.*, 2002). This makes analysis of individual species difficult using biochemical tests. Several species of *Lactobacillus* Group I are currently used as probiotics (Fuller, 1998; Fox, 1988) and none of them are considered to be animal pathogens. Their use is thus considered to be safe. No further analyses of specific species were performed. Supplementing the diet with a probiotic containing lactobacilli increases the numbers of lactobacilli in the gut. Supplementing the diet of diarrhoeic cheetahs with a probiotic could increase survival rate as well as reduce the severity of clinical signs of diarrhoea caused by infectious diseases, stress and antibiotic use. The probiotic must be able to survive an acid and bile environment, adhere to the intestinal wall, colonize the intestinal tract and inhibit pathogens (Weese, 2002). A species-specific probiotic produced from healthy cheetahs was presumed to be more effective in supporting the intestinal flora of cheetahs than non-specific probiotics.

Enterococcus faecium was the most common *Enterococcus* spp. isolated from the cheetahs in this clinical trial. *Enterococcus faecium* has been used in commercial probiotics (Fox, 1988). It has been used to increase weight gain in pigs and feed conversion in calves (Fox, 1988). In calves the addition of *E. faecium* to the feed also lowered the requirement for medical treatment (Fox, 1988) and lowered the severity of *E. coli*-induced diarrhoea (Underdahl, 1982). *E. faecium* could reduce the severity of *Salmonella* infection in captive cheetahs. It has been found to reduce the severity and increase survival time of mice challenged with

Salmonella (Maia *et al.*, 2001). Lloyd *et al.*, (1977) also reduced the severity of *Salmonella* infection in chickens by feeding intestinal content of adult birds. *E. faecium* was thus selected as the second bacterium to be included in a probiotic for cheetahs.

Lactobacillus Group 1 and *E. faecium* have for the above-mentioned benefits on animal health, been selected to be used in a cheetah-specific probiotic in this trial.

4.3 EFFECT OF THE PROBIOTIC ON FAECAL QUALITIY

If the difference in percentage diarrhoea is compared before and after the treatment period with the treatment period in the PG group, it can be concluded that there was a significant decrease in the percentage of diarrhoea in the PG during the feeding of the probiotic. The CG had a lower percentage of diarrhoea throughout the trial. This was incidental.

The reduction in diarrhoea during the feeding of the probiotic in the PG was accompanied by an absence of blood and mucus in the faeces, which had been present prior to the start of the 28-day administration of the probiotic (see Figure 9). In pigs treated with probiotic, Underdahl *et al.* (1982) were able to reduce the severity and duration of diarrhoea brought about by challenging them with different strains of *E. coli.* However, in this study the effects of the probiotic was only short term; mucoid/bloody faeces reappeared on day 42, 14 days post treatment (see Figure 9). Antibiotics were administered on two occasions to camp 57 and camp 55 on day –56 and day –35 respectively. These will also have affected the faecal qualities in these camps.

The percentage of faecal water increases when any intestinal problem causes an increase in secretions or a decrease in the absorption of fluids. A water content of 60 to 80 % is considered normal in dogs and cats. Water content of 70 to 90 % are found in unformed to watery faeces in domestic cats (Guilford and Strombeck, 1996). Small changes in faecal water content are normally responsible for the transformation of semi-solid faeces to liquid faeces.

The faecal water content did not change over time and there was no difference between the Probiotic and Control groups. The faecal water is highly dependent on the food provided. Water content increased as the proportion of IAMS adult cat diet increased. The quality of

food during the entire trial varied on a day-to-day basis and depended on the availability of horsemeat. In camp 55, the diet was changed to IAMS intestinal diet on Day 7 for approximately 14 days in an attempt to reduce the loose faeces in the group. In addition the group was moved to a new camp on Day 19 as a result of a high helminth burden found in faecal samples (see Figure 3). Animals in Camps 5, 6, 55 and 57 had to be moved to different camps or groups for managemental reasons during the trial. Possible stress associated with movement may also have affected faecal water content. All these factors and the two outbreaks of *Toxocara* spp. infestation probably contributed to the variation in diarrhoeic score and faecal water before and during the probiotic treatment period.

Faecal water may also be affected by the environment. The water content of the soil, drainage and weather conditions influence the water content of the faeces collected. Even though only fresh faeces were collected, some water would have already drained into the surrounding soil. To improve the reliability of the faecal water content animals should be housed on a concrete floor. Due to practical considerations this was not possible in this study.

4.4 EFFECT OF PROBIOTIC TREATMENT ON WEIGHT GAIN

The weight gains of each animal over the 28-day treatment period are shown in Figure 12. The Probiotic Group had a mean increase of 7.70 % and the Control Group 5.91 %. Cheetah M444 was excluded because his weight had decreased by 13.54 %. This would have been a substantial reduction in weight and would thus have been noticeable. Because it was in perfect condition the difference was put down to error and the reading excluded later when the data was evaluated (see Table 11). The animals were of different ages and sexes at the start of treatment. There was a difference between the ages of the Probiotic and Control groups. This was a constraint of the trial, which was determined by management at the De Wildt Centre. The animals were only available in those groups as this was determined by litters of siblings staying together, some of which were mixed with others long before the trial started. Later mixing to match or stratify animals in groups according to ages, weight and sex was not possible, as the animals would have fought causing severe stress.

Sex does not influence weight increase in neonatal cubs (Wack *et al.,* 1991), therefore it is unlikely that the sex of the juvenile cheetahs influenced their individual weight gains. Growth rate in neonatal cubs up to 40 days of life is linear (Beekman *et al.,* 1999). Birth weight of cubs influences individual growth rate rather then sex (Beekman *et al.,* 1999). Therefore the weight gain as a percentage of individual weight at the start of the probiotic feeding (day 0) was used as a measure of increase in weight. It is unlikely that the difference in ages between the Probiotic and Control groups influenced the weight gain of the animals in this study.

The difference in percentage weight gain between the Probiotic and Control groups of 1.79 % is significant particularly considering the short duration of the trial. Dilworth *et al.* (1978) reported increased growth rates in chickens fed *Lactobacillus* spp. over an eight-week period. The finding was similar to that of Bernardeau *et al.* (2002) who reported a weight increase of up to 31.7 % in mice fed different *Lactobacillus* spp. in comparison with controls over a 17-day period. The greater response seen in the mice compared to the juvenile cheetahs in this experiment was probably due to the shorter generation interval and smaller body mass of mice.

4.5 ROLE OF INFECTIOUS AGENTS AND PARASITES ON WEIGHT GAIN AND DIARRHOEA

Reduced weight gain and diarrhoea have also been associated with coronavirus infection and feline infectious peritonitis (FIP). The mortality due to FIP in cheetahs and reported in the literature is low but coronavirus can cause significant morbidity in a population (Munson *et al*, 1998). It is thus important to evaluate diarrhoeic samples of the juvenile cheetahs for the presence of coronavirus, particularly if they are showing depression and reduced appetite alongside diarrhoea. Coronavirus could not be isolated from faeces and therefore was unlikely to have influenced results.

Diarrhoea may occur with *Babesia felis* infection in cats (Taboada, 1998). Three animals, M427, M440 and F446, were found to be positive for *Babesia* spp. in two consecutive blood smears. None of the animals however, showed severe diarrhoea, lethargy, anaemia, anorexia or icterus that are associated with *B. felis* infection in cats under the age of 2 years (Taboada, 1998; Schoeman *et al.,* 2001). Macrocytic, hypochromic, regenerative anaemia was present in

57 % of the young cats infected with *B. felis* (Schoeman *et al.,* 2001). No other haematological abnormalities were detected in the three cheetahs (Table 25, Table 26, Table 27 and Table 28).

Theileria-like piroplasms have been reported in lions (Averbeck *et al.,* 1990) in Serengeti National Park and Ngorongoro Crater, Tanzania and the Kruger National Park (KNP) South Africa. The piroplasms in lions in the KNP have been described as a distinct species: *Babesia leo,* based on the phylogenetic analysis of 18S rRNA gene (Penzhorn *et al.*, 2001). *Theileria*-like piroplasms have been described in the blood of all the cheetahs tested in the Serengeti National Park and Ngorongoro Crater, Tanzania (Averbeck *et al.,* 1990). None of these cheetahs showed clinical signs of disease. A non-pathogenic *Theileria*-like piroplasm has been previously identified at the De Wildt Center (D. Meltzer, University of Pretoria, DGA, personal communication, 2003). The *Theileria*-like piroplasm identified from the cheetah in this study were analysed by PCR and reverse line blot but did not match any known *Theileria*-like piroplasm. It is likely that the piroplasm identified in the cheetah is a new, non-pathogenic species specific to cheetah but further research is needed to evaluate this.

4.6 SERUM BIOCHEMISTRY AND HAEMATOLOGY

Serum biochemistry and haematology were monitored in this trial as an indicator of systemic disease. The results showed that none of the cheetahs suffered from systemic illnesses. Only results that deviated from the expected values were therefore considered.

The high eosinophil count found in F446 could have been associated with the *Theileria*-like piroplasm (Ettinger and Feldman, 2000b). F446 was one of the three cheetahs infected with the *Theileria*-like piroplasm. The two other cheetahs, M440 and M427, found to have positive smears, did not show increased eosinophil counts. It is unlikely therefore that the eosinophilia was associated with the *Theileria*-like piroplasm unless there were two different species with different pathogenicities present. In addition, Schoeman *et al.* (2001) did not report an eosinophilia in cats affected with *B. felis*.

4.7 PATHOGEN ISOLATION

No pathogenic bacteria (smooth *E.coli* or *Salmonella* spp.) were isolated from the cheetahs during the study. The pathogenicity of *E. coli* is *inter alia* associated with the morphological appearance of the colonies. Smooth colonies are round, shiny and domed and rough colonies are irregular, flat and have a pitted surface. This effect is best noted after two to three days of incubation. Rough colonies are usually non-pathogenic. The disturbances of the intestinal tract were more associated with a disturbance of the normal flora rather than growth of pathogenic bacteria or osmotic diarrhoea as a result of the diet.

Faecal samples from all camps had evidence of helminth infestations, but no individual faecal egg counts were carried out. Ettinger and Feldman (2000b) reported eosinophilia in association with parasitism, particularly where nematodes such as *Toxocara* were undergoing tissue migration. Faecal egg counts represents only the presence of adult worms in the intestinal tract. The higher eosinophil counts could have been associated with a higher helminth burden in that particular animal (F446). *Toxocara* spp. have been reported as a problem in various zoos around the world (Penzhorn *et al.,* 1998). This is probably due too high concentrations of animals in a confined area.

4.8 EFFECT OF PROBIOTIC TREATMENT ON INTESTINAL PERMEABILITY AND TRANSIT TIME

Intestinal permeability varies greatly between different species and different breeds (Garden *et al.*, 1997). The level of exercise also has an effect on intestinal permeability, a lower permeability has been found in racing greyhounds (Randell *et al.,* 2001). The recovery of rhamnose and lactulose also varies between species (Delahunty and Hollander, 1987). Higher ratios of lactulose to rhamnose have been recorded in clinically healthy cats (Randell *et al.,* 2001). An increase in lactulose uptake has been associated with an increase in intestinal permeability, an increase in the perviousness of tight junctions and increased accessibility of molecules to the crypts. A reduction in the rhamnose blood concentration indicates a decrease in gut transit time or a reduced surface area. Normal cats have a shorter intestine and therefore a decreased surface area for absorption resulting in a higher intestinal permeability, when comparing sugar absorption, in relation to dogs (Randell *et al.,* 2001). The higher bacterial

counts in cat intestines has also been associated with an increased permeability (Johnston, 1999; Johnston *et al.,* 2001). Higher inherent permeability has also been recorded in juvenile animals (Garden *et al.*, 1997). All cheetahs in this study were juvenile therefore a higher intestinal permeability would have been expected.

The reduction in the L/R ratios, reflecting a lower permeability in the Control Group could have been caused by several factors. There might be a variation in permeability between different animals. There are different publications of mean values in healthy dogs. L/R ratios of 0.19 (± 0.07) has been reported by Randell *et al.,* (2001), but lower mean values of intestinal permeability in urine (0.08) and in plasma (0.09) have been reported by Sørensen *et al.* (1993, 1997). High L/R ratios of up to 0.40 +/- 0.20 have been recorded in clinically healthy cats (Johnston *et al.,* 2001). Randell *et al.,* (2001) reported even higher values (0.52+/- 0.19 with a range of 0.3-0.98) as normal in clinically healthy cats. The L/R ratios in this study in cheetahs varied from 0.00 to 0.79, which is within the range of ratios reported by Randell *et al.* (2001) for domestic cats.

Another factor affecting the recovery of the sugars and thus intestinal permeability is the timing of the blood collection after the administration of the sugars. The timing will affect the recovery of the sugars in plasma. The percentage recovery of the sugars in plasma of cats is quite consistent between 60 and 90 minutes after oral administration of the sugars (Hawkins *et al.*, 1986). A sharp decline of the sugar concentration in plasma was noted at 120 minutes (Hawkins *et al.*, 1986). No reference for the ideal timing of collection of plasma after the administration of the sugar was available and therefore plasma was collected as close to 60 minutes after administration as possible. However there was a variation in the collection time of the plasma between cheetahs from 40 min to 170 min (median 74 min). There was no significant difference in the amount of R and L recovered after one hour and after one and-a-half hour between the CG and PG. The differences in timing between administration and blood collection were therefore unlikely to affect the results.

Johnston *et al.,* (2001) looked at the effect of diet and antibiotic therapy (metronidazole) on the intestinal permeability in healthy cats. Higher intestinal permeability has been associated with antibiotic therapy. Higher permeability has also been associated with canned cat food in relation to dry cat food (Johnston *et al.,* 2001). This could be associated with differences in bacterial numbers. More animals in the PG were treated with antibiotics than the CP during

the probiotic treatment period, which could have resulted in higher intestinal permeability in the PG. Food was also not kept as a constant during the trial and varied from day to day, and between camps, which would have had an effect on the intestinal permeability.

There was high variability in the collection time of the plasma for the analysis of sugar concentration as well as other factors affecting the intestinal permeability as discussed above. This in conjunction with no reference range for intestinal permeability in healthy cheetahs makes it difficult to analyse the results. Therefore it is difficult to discuss the significance of the different L/R ratios obtained in this study. The establishment of a reference range in cheetahs would be necessary to evaluate the results of this study further.

CHAPTER 5 CONCLUSIONS

The intestinal flora of cheetahs was shown to be similar to those of the domestic cat, although differences in bacterial species composition were seen (see section 4.1). More samples need to be taken from healthy cheetahs housed in different environments in order to establish a reference range of normal intestinal flora in the cheetah. Cheetahs tend to have higher numbers of faecal anaerobic bacteria and *Enterococci*, particularly if they are fed a meat-based diet.

The isolation of selected bacteria from healthy adult cheetahs produced a probiotic that showed promising results in this study. The juvenile cheetahs treated with the probiotic over a 28-day period gained relatively more weight than the control animals. There was a significant improvement in faecal quality in the Probiotic Group during the feeding of the probiotic. There was also a reduction in the number of faecal samples containing mucus and blood in the Probiotic Group. Faecal water, however, did not seem to be affected by treatment as discussed in section 4.3. As only juvenile cheetahs were tested, the study should be repeated on a wider range of animals of different ages, because the effects of the probiotic will differ in different age groups.

Unfortunately there were several confounding factors affecting some of the tests used to quantify the effect of the probiotic. The animals in the PG were slightly younger then the CG. High helminth burdens in the juvenile cheetahs and movement of animals into different camps affected the faecal consistency and the percentage diarrhoea in the camps. There were also changes in diet due to managemental reasons. Standardising the diet and routinely performing faecal flotation for helminths would have been beneficial. Potentially stressful situations such as movement of animals in and between camps should also be avoided. It would have been beneficial to standardise procedures particularly for faecal water and intestinal permeability by providing a stable environment and diet (see section 4.3). When dealing with wildlife and in particular valuable and endangered species such as the cheetah, this is not always possible.

APPENDIX I TABLES

Table 20: Year of birth, sex, place of birth and diets of adult cheetahs used for faecal culturing

Animal Number	Year of birth	Sex	Place of birth	Diet
F 282	1996	F	De Wildt	Meat
F 283	1998	M	De Wildt	Meat
F 331	approx. 1998	F	Kalahari	Meat
F 318	1998	F	De Wildt	Meat
F 327	1998	F	De Wildt	Meat
F 362	2000	F	De Wildt	Meat
F 309	1996	F	De Wildt	IAMS
Q 46	1998	F	De Wildt	IAMS
M286	1996	M	De Wildt	Meat
F 352	1999	F	De Wildt	IAMS

Table 21: Ages, sex and camps of cheetahs in Probiotic Group

Camp No	Sex/ Animal #	Date of birth	Age at start of probiotic trial in month
5	F 460	01.06.2002	10
5	F 465	unknown	10[a]
55	M 438	28.04.2002	12
55	M 450	30.05.2002	11
55	M 455	05.06.2002	10
55	M 437	21.04.2002	12
55	F 446	18.05.2002	11
55	F 459	02.06.2002	10
55	F 461	01.06.2002	10
54	K 50[b]	17.04.2002	12
54	M 433	17.04.2002	12
54	M 434	17.04.2002	12
54	M 440	28.04.2002	12
54	M 441	28.04.2002	12
57	M 458	01.08.2002	8
57	F 457	01.08.2002	8

[a] approximated age of cheetah, [b] King cheetah- male

Table 22: Ages, sex and camps of cheetahs in Control Group

Camp No	Sex/ Animal #	Date of birth	Age at start of probiotic trial in months
6	M 430	16/04/2002	12
6	M 431	16.04.2002	12
6	M 432	16.04.2002	12
6	M 435	20.04.2002	12
6	M 436	20.04.2002	12
6	F 447	20.04.2002	12
53	M 426	22.03.2002	13
53	M 427	22.03.2002	13
53	M 428	22.03.2002	13
56	M 443	12.05.2002	11
56	M 444	12.05.2002	11

Table 23: Bacteria (CFU) isolated per gram of faeces

Aerobic	Anaerobic	Enterococci
$4.67x10^6$	$4.66x10^7$	$1.00x10^5$
$1.56x10^9$	$6.12x10^8$	$5.00x10^8$
$6.40x10^5$	$1.94x10^6$	$1.40x10^5$
$3.50x10^7$	0[a]	$6.60x10^3$
$1.86x10^6$	$2.54x10^6$	$1.88x10^4$
$2.34x10^8$	$1.32x10^6$	$2.20x10^3$
$4.77x10^8$	$3.06x10^9$	$4.60x10^5$
$1.44x10^6$	$1.30x10^6$	$7.00x10^3$

[a] no growth after 48 hours of incubation

Table 24: Number of bloody/mucoid faecal samples in individual camps

Camp	Control Group			Probiotic Group				Total	
Date/ Day	Camp 6	Camp 53	Camp 56	Camp 5	Camp 54	Camp 55	Camp 57	Control	Probiotic
19.02.03 Day -70	0	0	0	0	0	0	0	0	0
26.02.03 Day -63	0	0	0	0	0	0	0	0	0
05.03.03 Day -56	0	0	0	0	0	0	0	0	0
12.03.03 Day -42	1	0	0	1	0	0	0	1	1
19.03.03 Day -35	1	0	0	0	0	1	0	1	1
26.03.03 Day- 28	0	0	0	0	0	0	0	0	0
02.04.03 Day -21	0	0	0	0	0	0	0	0	0
09.04.03 Day -14	0	0	1	0	0	1	0	1	1
16.04.03 Day -7	0	0	1	0	0	0	1	1	1
23.04.03 Day 0	No data-start of probiotic trial								
30.04.03 Day 7	1	0	0	0	0	0	0	1	0
07.05.03 Day 14	0	1	1	0	0	0	0	2	0
14.05.03 Day 21	0	1	2	0	0	0	0	3	0
21.05.03 Day 28	No data-end of probiotic trial								
28.05.03 Day 35	0	0	0	0	0	0	0	0	0
04.06.03 Day 42	0	0	0	0	0	0	1	0	1

Table 25: Serum biochemistry and haematology results Control Group on day 0

Variables	Units	M 430	M 431	M 432	M 435	M 436	F 447	M 426	M 427	M 428	M 443	M 444	Median	St dev.	Mean	Min	Max
TSP	g/l	61.6	64.6	59.8	51.7	60.9	63.9	62.6	60.2	63.5	72	65.1	62.6	4.87	62.35	51.7	72
Alb	g/l	32.2	34.6	32.5	27.8	33.2	35.3	34.1	33.2	36.7	37.2	35.5	34.1	2.58	33.85	27.8	37.2
Globulin	g/l	29.4	30	27.3	23.9	27.7	28.6	28.5	27	26.8	34.8	29.6	28.5	2.69	28.51	23.9	34.8
A/G		1.1	1.15	1.19	1.16	1.2	1.23	1.2	1.23	1.37	1.07	1.2	1.2	0.08	1.19	1.07	1.37
Bil-T	ymol/l	1.8	1.9	2.2	1.9	2.5	2.8	2.6	3	2.5	2.6	3.2	2.5	0.46	2.45	1.8	3.2
Cholesterol	mmol/l	2.74	2.38	2.98	3.03	3.53	3.59	2.72	3.2	2.58	4.82	4.03	3.03	0.72	3.24	2.38	4.82
Urea	mmol/l	12.4	11.5	14	14	11.6	14.1	11.7	11.9	12.2	13.5	15.3	12.4	1.30	12.93	11.5	15.3
Creatine	ymol/l	198	212	217	177	205	206	201	191	225	226	227	206	15.75	207.73	177	227
Haemoglobin	g/l	134	130	137	136	142	130	146	135	147	137	147	137	6.33	138.27	130	147
RCC	1000/yl	7.58	7.54	7.94	8.27	8.35	8.1	8.89	8.17	8.21	8.28	8.67	8.21	0.40	8.18	7.54	8.89
Haematocrit	l/l	0.46	0.442	0.473	0.465	0.474	0.443	0.499	0.462	0.488	0.469	0.505	0.469	0.02	0.47	0.442	0.505
MCV	fL	60.8	58.7	59.6	56.2	56.8	54.7	56.1	56.5	59.5	56.6	58.3	56.8	1.87	57.62	54.7	60.8
MCHC	g/dl	29.2	29.3	28.9	29.4	30	29.3	29.2	29.2	30.1	29.2	29.2	29.2	0.36	29.36	28.9	30.1
RDW		22.3	22.3	22.3	22.1	23.6	24.3	22.1	23.3	21	22.4	20	22.3	1.17	22.34	20	24.3
WBCC	1000/yl	13.7	13.3	7.8	15	12.6	13.3	7.7	11.5	12.2	9.7	10.3	12.2	2.41	11.55	7.7	15
seg. Neutrophils	1000/yl	8.22	9.14	4.76	7.2	8.08	6.25	5.08	7.21	8.7	4.85	6.18	7.2	1.57	6.88	4.76	9.14
Neutrophils bands	1000/yl	0	0	0	0.3	0.25	0	0	0	0	0	0	0	0.11	0.05	0	0.3
Lymphocytes	1000/yl	5.21	2.27	2.18	6.3	2.12	5.45	2	3.05	1.83	3.3	3.09	3.05	1.58	3.35	1.83	6.3
Monocytes	1000/yl	0.27	1.08	0.31	0.6	0.86	0.13	0	0.87	0.96	0.97	0.62	0.62	0.38	0.61	0	1.08
Eosinophils	1000/yl	0	0.82	0.53	0.6	1.3	1.46	0.62	0.21	0.7	0.58	0.41	0.6	0.42	0.66	0	1.46
Basophils	1000/yl	0	0	0.02	0	0	0	0	0.17	0	0	0	0	0.05	0.02	0	0.17
ThrC	1000000/yl	739	140	236	400	437	212	264	141	454	543	439	400	185.04	364.09	140	739
MPV	fl					40.6	28.6			27.3		30.2	29.4	6.07	31.68	27.3	40.6
PDW	%					24.9	21.5			22.6		22.5	22.55	1.44	22.88	21.5	24.9
Aniso		2+	3+	2+	2+	2+	2+	1+	2+	2+	1+	1+					
H.J.B.		1+	1+	2+	1+	1+	1+			1+	1+	1+					
L Bl		1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+					
Norm	% of NCC																
Para									B.felis+								
Comment																	

Table 26: Serum biochemistry and haematology values Control Group on day 28

Variables	Units	M430	M431	M432	M435	M436	F447	M426	M427	M428	M443	M444	Median	St dev.	Mean	Min	Max
TSP	g/l	57.7	64.6	62.8	60.8	62.2	61	64.7	61.5	65	59.6	62.6	62.2	2.26	62.05	57.7	65
Alb	g/l	32.1	33.8	34.1	32.7	34.7	34.1	36.4	33.9	37.4	33.8	35.1	34.1	1.51	34.37	32.1	37.4
Globulin	g/l	25.6	30.6	28.7	28.1	27.5	26.9	28.3	27.6	27.6	25.8	27.5	27.6	1.37	27.65	25.6	30.6
A/G		1.25	1.1	1.19	1.16	1.26	1.27	1.29	1.23	1.36	1.31	1.28	1.26	0.07	1.25	1.1	1.36
Bil-T	ymol/l	2.5	2.6	3.1	2.7	2.2	2.5	3.3	3.6	2.7	1.9	2.4	2.6	0.49	2.68	1.9	3.6
Cholesterol	mmol/l	2.84	3.17	3.57	3.86	4.58	3.95	3.48	3.62	3.21	4.17	4.02	3.62	0.50	3.68	2.84	4.58
Urea	mmol/l	9.5	9.8	11.3	10.6	10.4	11.8	9.8	10.6	10.5	10.8	10.9	10.6	0.68	10.55	9.5	11.8
Creatine	ymol/l	195	219	221	226	225	204	182	173	179	226	269	219	27.91	210.82	173	269
Haemoglobin	g/l	128	129	133	137	158	125	153	128	147	114	124	129	13.39	134.18	114	158
RCC	1000/yl	7.02	7.44	7.94	7.94	8.93	7.64	9	7.44	7.94	6.83	7.32	7.64	0.69	7.77	6.83	9
Haematocrit	l/l	0.4	0.418	0.429	0.473	0.505	0.397	0.49	0.423	0.458	0.375	0.412	0.423	0.04	0.43	0.375	0.505
MCV	fL	57	56.1	56	59.6	56.6	51.9	54.4	56.9	57.7	54.9	56.3	56.3	1.96	56.13	51.9	59.6
MCHC	g/dl	32	30.8	31	28.9	31.2	31.6	31.1	30.3	32.1	30.4	30.2	31	0.92	30.87	28.9	32.1
RDW		22.7	21.8	22.2	22.3	23.3	24.3	21.9	22.6	20.4	23.8	22.9	22.6	1.05	22.56	20.4	24.3
WBCC	1000/yl	10.1	11	8.5	7.8	9.9	10.4	9.4	10.3	9.9	11.6	9	9.9	1.09	9.81	7.8	11.6
seg. Neutrophils	1000/yl	5.98	6.71	5.27	4.76	6.1	6.05	5.8	6.02	7.13	8.35	5.62	6.02	0.96	6.16	4.76	8.35
Neutrophils bands	1000/yl	0	0.22	0.09	0	0	0	0.09	0	0.2	0	0	0	0.08	0.05	0	0.22
Lymphocytes	1000/yl	2.5	3.41	2.38	2.18	2.37	2.82	2.38	3.09	2.18	1.62	2.73	2.38	0.49	2.51	1.62	3.41
Monocytes	1000/yl	0.72	0.22	0.34	0.31	0.47	0.62	0.77	0.91	0	1.16	0.43	0.47	0.33	0.54	0	1.16
Eosinophils	1000/yl	0.88	0.44	0.43	0.53	0.95	0.9	0.3	0.26	0.4	0.23	0.23	0.43	0.28	0.50	0.23	0.95
Basophils	1000/yl	0.02	0	0	0.02	0.01	0.01	0.06	0.03	0	0.23	0.01	0.01	0.07	0.04	0	0.23
ThrC	1000000/yl	554	239	320	236	511	286	511	111	470	486	449	449	146.32	379.36	111	554
MPV	fl	14.6		26.8						19.3	23.1	35.1	23.1	7.78	23.78	14.6	35.1
PDW	%	17.7		20.7						20.4	21.2	23.3	20.7	2.01	20.66	17.7	23.3
Aniso		2+	2+	2+	2+	2+	3+	3+	2+	2+	2+	2+					
H.J.B.		1+		1+	2+	1+	1+	1+	1+	1+		1+					
L Bl		1+	1+	1+	1+	1+	1+	1+	1+	1+	1+	1+					
M Bl									1+								
Acanth												few					
Poly												1+					
Para									B.felis2+								
Comment												Blister cells					

Table 27: Serum biochemistry and haematology values Probiotic Group on day 0

Variables	Units	K50	M433	M434	M440	M441	M458	F457	F465	M438	M455	F437	M450	F446	F461	F459	F460	Mean	Median	St Dev.	Max	Min
TSP	g/l	63.3	60.2	61.8	61	62.6	61.2	59.6	63.2	66.7	65.3	66	63.2	62.2	65.70	64.40	65.60	63.24	63.20	2.18	66.7	59.6
Alb	g/l	34.2	32.5	33	33.8	33.3	34.8	33.3	35.8	35	36.3	37	37.9	36.1	37.80	36.30	35.80	35.18	35.40	1.70	37.9	32.5
Globulin	g/l	29.1	27.7	28.8	27.2	29.3	26.4	26.3	27.4	31.7	28.6	29	25.3	26.1	27.90	28.10	29.80	28.04	28.00	1.61	31.7	25.3
A/G		1.18	1.17	1.15	1.24	1.14	1.32	1.27	1.31	1.1	1.28	1.3	1.5	1.38	1.35	1.29	1.20	1.26	1.28	0.10	1.5	1.1
Bil-T	ymol/l	3.3	3	3.8	3.2	3.4	2.4	2.6	2.3	2.5	2.9	4.2	2.2	1.9	4.00	4.50	3.00	3.08	3.00	0.76	4.5	1.9
Cholesterol	mmol/l	2.8	2.78	2.77	2.59	2.31	3.46	3.47	3.55	3.28	4.13	2.5	3.28	2.95	2.78	3.06	3.07	3.05	3.01	0.46	4.13	2.31
Urea	mmol/l	11.9	13.3	13.8	10.6	11.9	8.6	11.1	10.1	13.2	11.3	12	11.5	12.1	15.80	16.80	11.00	12.18	11.90	2.05	16.8	8.6
Creatine	ymol/l	257	261	213	205	207	167	154	182	216	169	185	174	215	158.00	175.00	190.00	195.50	187.50	31.89	261	154
Hemoglobin	g/l	140	141	129	134	131	134	129	133	136	138	137	137	121	137.00	128.00	131.00	133.50	134.00	5.18	141	121
RCC	1000/yl	8.55	8.16	7.8	7.89	7.57	8.03	7.42	7.54	7.98	8.24	8.2	8.44	6.89	7.92	7.60	7.96	7.89	7.94	0.42	8.55	6.89
Hematocrit	%	0.47	0.47	0.44	0.46	0.45	0.46	0.42	0.46	0.46	0.46	0.5	0.465	0.408	0.47	0.39	0.44	0.45	0.46	0.02	0.469	0.387
MCV	fL	54.7	57.7	55.8	58.4	59.5	57.2	56.4	60.4	57.3	56.3	57	55.1	58.4	59.00	50.90	55.80	56.86	57.00	2.25	60.4	50.9
MCHC	g/dl	29.9	30	29.8	29	29.2	29.3	30.8	29.1	29.7	29.7	29	29.4	29.6	29.30	33.10	29.50	29.80	29.55	0.98	33.1	29
RDW		23.8	22.3	21	22.9	22.4	22.8	23.9	22.1	23.4	21.2	23	21.5	23.8	24.20	21.80	22.40	22.63	22.45	0.99	24.2	21
WBCC	1000/yl	16.9	12.4	17.7	8.8	13.4	11.6	9.7	9.5	11.4	10.2	9.8	11.8	14.5	13.70	7.80	8.90	11.76	11.50	2.88	17.7	7.8
seg. N-ophils	1000/yl	10.1	6.94	12.5	4.6	7.5	5.34	5.57	6.73	8.11	6.79	5.5	5.11	6.32	7.67	5.15	5.06	6.81	6.53	2.09	12.48	4.6
N-phils bands	1000/yl	0	0	0	0	0	0	0.19	0	0	0	0	0	0	0.00	0.00	0.00	0.01	0.00	0.05	0.19	0
Lymphocytes	1000/yl	5.07	4.15	3.49	3.06	5.09	5.8	2.98	1.67	2.28	2.26	3.7	5.63	3.13	5.21	2.11	2.60	3.64	3.31	1.36	5.8	1.67
Monocytes	1000/yl	0.68	0.76	1.13	0.7	0.8	0.23	0.51	0.67	0.66	0.81	0.6	0.63	0.61	0.27	0.08	0.52	0.60	0.65	0.25	1.13	0.08
Eosinophils	1000/yl	0.68	0.55	0.62	0.29	0	0.23	0.46	0.44	0.31	0.34	0	0.3	4.23	0.55	0.46	0.74	0.64	0.45	0.98	4.23	0
Basophils	1000/yl	0	0	0	0.15	0	0	0	0	0.03	0	0	0.14	0.19	0.00	0.00	0.00	0.03	0.00	0.06	0.19	0
ThrC	10^6/yl	434	45.5	155	282	128	660	589	276	634	585	370	333	258	469.00	307.00	215.00	358.78	320.00	187.70	660	45.5
MPV	fl	16.3					17.4	18.9		16.3	17	21			38.50	18.00		20.40	17.70	7.46	38.5	16.3
PDW	%	19.5					9.5	19.2		19.3	20.5	19			23.80	18.20		18.61	19.25	4.06	23.8	9.5
Aniso		2+	2+	1+	2+	1+	2+	3+	2+	2+	2+	2+	2+	2+	2+	2+	2+					
H.J.B.		1+	1+	1+	1+		1+		1+		1+	1+		1+	1+	1+	1+					
L Bl		1+	1+	1+	1+		1+	1+	1+		1+	1+	1+	1+	1+	1+	1+					
M act						1+				1+												
Norm	% NCC								2 %													
Parasites					B.felis 2+									B.felis 2+								
Comment																						

Table 28: Serum biochemistry and haematology values Probiotic Group on day 28

Variables	Units	K50	M433	M434	M440	M441	M458	F457	F465	M438	M455	F437	M450	F446	F461	Mean	Median	St Dev.	Max	Min
TSP	g/l	63.9	61.9	61	57.9	60.5	59.7	61.9	66	63.5	64.3	61	60.70	67.30	65.80	62.52	61.90	2.69	67.3	57.9
Alb	g/l	37.3	33.8	34.5	32.5	33.5	33.9	35	36.8	36.2	36.7	35	34.90	34.90	35.40	35.03	34.95	1.37	37.3	32.5
Globulin	g/l	26.6	28.1	26.5	25.4	27	25.8	26.9	29.2	27.3	27.6	26	25.80	32.40	30.40	27.49	26.95	1.98	32.4	25.4
A/G		1.4	1.2	1.3	1.28	1.24	1.31	1.3	1.26	1.33	1.33	1.4	1.35	1.08	1.16	1.28	1.30	0.08	1.4	1.08
Bil-T	ymol/l	2.2	2.4	2.9	3.6	2.2	2.3	2.5	2.4	2.4	3	2.1	2.60	3.50	3.30	2.67	2.45	0.50	3.6	2.1
Cholesterol	mmol/l	3.3	3.59	3.4	3.05	3.17	3.35	3.37	3.99	3.65	4.16	3.2	3.90	3.83	2.90	3.49	3.39	0.38	4.16	2.9
Urea	mmol/l	11	12.2	9.6	10.2	10.9	9.7	12.8	10.3	12.6	10.4	15	10.10	14.90	13.30	11.65	10.95	1.85	15.1	9.6
Creatine	ymol/l	214	239	196	181	201	185	167	185	194	202	219	166.00	251.00	185.00	198.93	195.00	24.81	251	166
Hemoglobin	g/l	145	144	138	125	138	144	139	139	137	140	133	134	132.00	133.00	137.21	138.00	5.48	145	125
RCC	1000/yl	8.74	8.32	8.15	7.3	7.54	8.35	7.76	7.72	7.88	8.08	7.8	7.99	7.47	7.89	7.93	7.89	0.38	8.74	7.3
Hematocrit	%	0.48	0.47	0.45	0.41	0.45	0.47	0.45	0.45	0.43	0.44	0.4	0.53	0.42	0.43	0.45	0.45	0.03	0.533	0.408
MCV	fL	54.6	56.3	54.8	56	59	55.7	57.9	58.1	54.9	54.7	56	54.20	56.50	54.20	55.95	55.85	1.53	59	54.2
MCHC	g/dl	30.5	30.7	30.9	30.7	31	31	31	30.9	31.6	31.8	30	30.90	31.40	31.10	30.99	30.95	0.40	31.8	30.3
RDW		23.1	23.1	25.1	22.7	22.9	22.1	22.5	20.4	23	23.3	24	20.90	22.20	22.00	22.67	22.80	1.18	25.1	20.4
WBCC	1000/yl	13.1	9.5	9.9	7.1	10.4	10.2	11.8	8.2	8.3	8.9	11	10.10	17.60	11.30	10.50	10.15	2.56	17.6	7.1
seg. N-phils	1000/yl	8.65	5.86	4.95	4.37	5.06	6.12	7.15	5.41	5.14	6.05	7.6	4.78	5.81	7.24	6.02	5.84	1.23	8.65	4.37
N-phils bands	1000/yl	0.13	0	0	0	0	0	0	0	0	0	0	0.00	0.00	0.00	0.01	0.00	0.03	0.13	0
Lymphocytes	1000/yl	3.16	2.79	2.97	2.13	4.51	3.47	3.59	2.38	2.12	2.31	2.4	4.37	3.52	2.69	3.03	2.88	0.78	4.51	2.12
Monocytes	1000/yl	0.39	0.45	0.79	0.43	0.52	0.41	0.59	0.16	0.7	0.18	0.1	0.62	0.35	0.63	0.45	0.44	0.21	0.79	0.11
Eosinophils	1000/yl	0.77	0.4	1.19	0.16	0.24	0.2	0.44	0.25	0.29	0.36	0.3	0.28	7.92	0.67	0.96	0.34	2.02	7.92	0.16
Basophils	1000/yl	0	0	0	0.02	0.06	0	0.04	0	0.05	0	0.1	0.05	0.00	0.07	0.03	0.01	0.04	0.11	0
ThrC	10^6/yl	463	269	271	315	297	784	580	172	650	591	337	311	229	381	403.57	326.00	181.39	784	172
MPV	fl	27.3	17.5	26.8			14.7	21.3	19.5	19.5	17					20.45	19.50	4.53	27.3	14.7
PDW	%	22.5	19	24.8			19.4	21.7	19.8	21.4	20.3					21.11	20.85	1.92	24.8	19
Aniso		2+	2+	2+	2+	2+	1+	2+	2+	2+	1+	2+	2+	2+	2+					
H.J.B.		1+		1+	1+	1+		1+		1+		1+			1+					
L Bl		1+	1+	1+			1+	2+	1+	1+	1+	1+	1+	1+						
M Bl									1+		1+									
Acanth						few														
Schizo										few					few					
Parasites					B.felis 2+									B.felis 2+						
Comment																				

Table 29: Timing of blood collection for intestinal permeability- day 0

Camp	Animal #	Sex	Time of admin.	Time of collection	Time between
6	430	M	09:31	10:35	01:04
	431	M	09:35	11:24	01:49
	432	M	09:40	11:02	01:22
	435	M	09:35	11:20	01:45
	436	M	09:31	10:35	01:04
	447	F	09:40	10:20	00:40
5	460	F	07:32	08:25	00:53
	465	F	07:40	08:30	00:50
55	438	M	11:15	12:25	01:10
	450	M	11:15	12:38	01:23
	455	M	11:15	12:35	01:20
	437	F	11:15	12:20	01:05
	446	F	11:15	12:40	01:25
54	50	K (M)	12:50	14:40	01:50
	433	M	12:50	14:44	01:54
	434	M	12:50	14:40	01:50
	440	M	12:50	14:30	01:40
	441	M	12:50	14:37	01:47
53	426	M	12:00	13:05	01:05
	427	M	12:00	12:55	00:55
	428	M	12:00	13:14	01:14
57	458	M	12:20	15:10	02:50
	457	F	12:20	15:05	02:45
55	459	F	07:45	08:53	01:08
	461	F	07:45	09:00	01:15
56	443	M	07:50	09:15	01:25
	444	M	07:50	09:25	01:35

Table 30: Timing of blood collection for intestinal permeability- day 28

Camp	Animal #	Sex	Time of admin.	Time of collection	Time between
6	430	M	7:15	8:39	1:24
	431	M	7:15	8:45	1:30
	432	M	7:15	8:26	1:11
	435	M	7:15	8:29	1:14
	436	M	7:15	8:55	1:40
	447	F	7:15	8:20	1:05
55	465	F	7:00	7:55	0:55
	438	M	10:35	11:58	1:23
	450	M	10:35	11:45	1:10
	455	M	10:35	12:05	1:30
	437	F	10:35	12:10	1:35
	446	F	10:35	12:27	1:52
	461	F	10:35	11:40	1:05
54	50	K (M)	9:45	10:40	0:55
	433	M	9:45	10:52	1:07
	434	M	9:45	10:40	0:55
	440	M	9:45	10:47	1:02
	441	M	9:45	10:29	0:44
53	426	M	9:00	10:04	1:04
	427	M	9:00	10:00	1:00
	428	M	9:00	10:10	1:10
57	458	M	12:00	13:20	1:20
	457	F	12:00	13:40	1:40
56	443	M	11:30	12:34	1:04
	444	M	11:30	12:40	1:10

Table 31: Sugar concentrations on day 0 of the Control Group

ID/sex		Rhamnose (R) conc. mg/l	Lactulose (L) conc. mg/l	L/R ratio
430	M	1.4	0.3	0.214
431	M	2.2	0.1	0.045
432	M	1.6	0.01	0.006
435	M	6.2	0.87	0.140
436	M	5.2	0.99	0.190
447	F	4.9	1.1	0.224
426	M	3.4	0.64	0.188
427	M	0	0	0.000
428	M	0.6	0.09	0.150
443	M	1.4	1.1	0.786
444	M	4.3	1	0.233

Table 32: Sugar concentrations on day 0 of the Probiotic Group

ID/sex		Rhamnose (R) conc. mg/l	Lactulose (L) conc. mg/l	L/R ratio
465	F	2	1	0.500
438	M	0.6	0	0.000
450	M	5.4	0.41	0.076
455	M	1.2	1.1	0.917
437	F	1.7	0.6	0.353
446	F	0.3	0.03	0.100
461	F	0.6	0.04	0.067
50	K	1.1	0.05	0.045
433	M	1.4	0.04	0.029
434	M	2	0.08	0.040
440	M	3.5	2.5	0.714
441	M	3.4	1.15	0.338
458	M	1.1	0.08	0.073
457	F	1.5	0.98	0.653
459	F	2.3	0.4	0.174
460	F	2.2	0.4	0.182

Table 33: Sugar concentrations on day 28 of the Control Group

ID/sex		Rhamnose (R) conc. mg/l	Lactulose (L) conc. mg/l	L/R ratio
430	M	2.2	0.1	0.045
431	M	2.1	0.02	0.010
432	M	1.4	0.02	0.014
435	M	3.3	0.2	0.061
436	M	3.2	0.41	0.128
447	F	5.1	0.04	0.008
426	M	2.6	0.4	0.154
427	M	1.8	0.2	0.000
428	M	4.1	0.3	0.073
443	M	5.2	2.5	0.481
444	M	4.4	0.2	0.045

Table 34: Sugar concentrations on day 28 of the Probiotic Group

ID/sex		Rhamnose (R) conc. mg/l	Lactulose (L) conc. mg/l	L/R ratio
465	F	0.6	0.44	0.733
438	M	1.1	0.11	0.100
450	M	5.3	0.7	0.132
455	M	1.3	0.44	0.338
437	F	1.6	0.09	0.056
446	F	7.2	0.02	0.003
461	F	0.6	0.01	0.017
50	K	1.2	0.01	0.008
433	M	1.2	0.06	0.050
434	M	1.6	0.14	0.088
440	M	0.2	0.12	0.600
441	M	2.3	2.1	0.913
458	M	2.1	0.21	0.100
457	F	1.6	0.9	0.563

APPENDIX II CULTURE MEDIA

SL Medium

(Starr *et al.*, 1981)

Ingredients: for 1 litre

- Trypticase (BBL), 10 g
- Yeast extract, 5 g
- KH_2PO_4, 6 g
- Diammonium citrate, 2 g
- $MgSO_4$ $7H_2O$, 0.58 g
- $MnSO_4$ $4H_2O$, 0.28 g
- Glucose, 10 g
- Arabinose, 5 g
- Sucrose, 5 g
- Tween 80, 1 g
- Na acetate $3H_2O$, 2.5 g
- Glacial acetic acid (to titrate medium to pH 5.4), 99.5%
- Agar, 15 g (DIFCO Laboratories)

Dissolve the agar separately by steaming in 500 ml distilled water. Dissolve all the other ingredients except acetate and acetic acid in 300 ml distilled water without heating, then add this to the melted agar and steam for a further 5 min; excessive heating at this stage must be avoided. Dissolve the Na acetate in about 15 ml distilled water, without heating, and add acetic acid as a 10% vol/vol aqueous solution to pH5.4. Make up volume to 20 ml. Add this buffer mixture to the hot basal medium and mix well. Cool a small portion and check with a glass electrode that the pH is 5.4. If too high, adjust with further acetic acid. While hot, distribute the medium in convenient amounts in sterile screw-capped bottles; no further sterilization is done. The medium should be a clear, light straw colour, giving a firm gel. For use, dissolve in free-flowing steam. Avoid repeated melting and cooling. The addition of arabinose and sucrose as well as glucose allows the growth of strains that preferentially ferment these sugars.

TPY Medium

(Starr *et al.*, 1981)

Ingredients: for 1 litre

- Trypticase (BBL), 10 g
- Phytone (BBL), 5 g
- Glucose, 5 g
- Yeast extract (Difco), 2.5 g
- Tween 80, 1 ml
- Cysteine hydrocloride, 0.5 g
- K_2HPO_4, 2 g
- $MgCl_2$ $6H_2O$, 0.5 g
- $ZnSO_4$ $7H_2O$, 0.25 g
- $CaCl_2$, 0.15 g
- $FeCl_3$, a trace
- Agar, 15 g (DIFCO Laboratories)
- Distilled water to 1000 ml

Dissolve all ingredients and sterilise. The final pH is about 6.5 after autoclaving at 121^{o}C for 25 min; dilutions can be made with the same liquid medium.

Beehrens Media

(Beehrens, 1990)

Ingredients: for 1 litre

- Columbia agar base (42 g per l)(Merck)
- Glucose 5 g
- Cysteine hydrochloride 0.5 g
- Agar 5 g (Final agar concentration 15 g/l) (DIFCO Laboratories)
- Propionic acid 5 ml

The mixture is boiled to dissolve all the ingredients (except propionic acid). The medium is then cooled to 70ºC. Propionic acid (5 ml) is added to 1000 ml of the medium and the pH adjusted to 5.0 with NaOH. The media is not sterilised.

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