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.

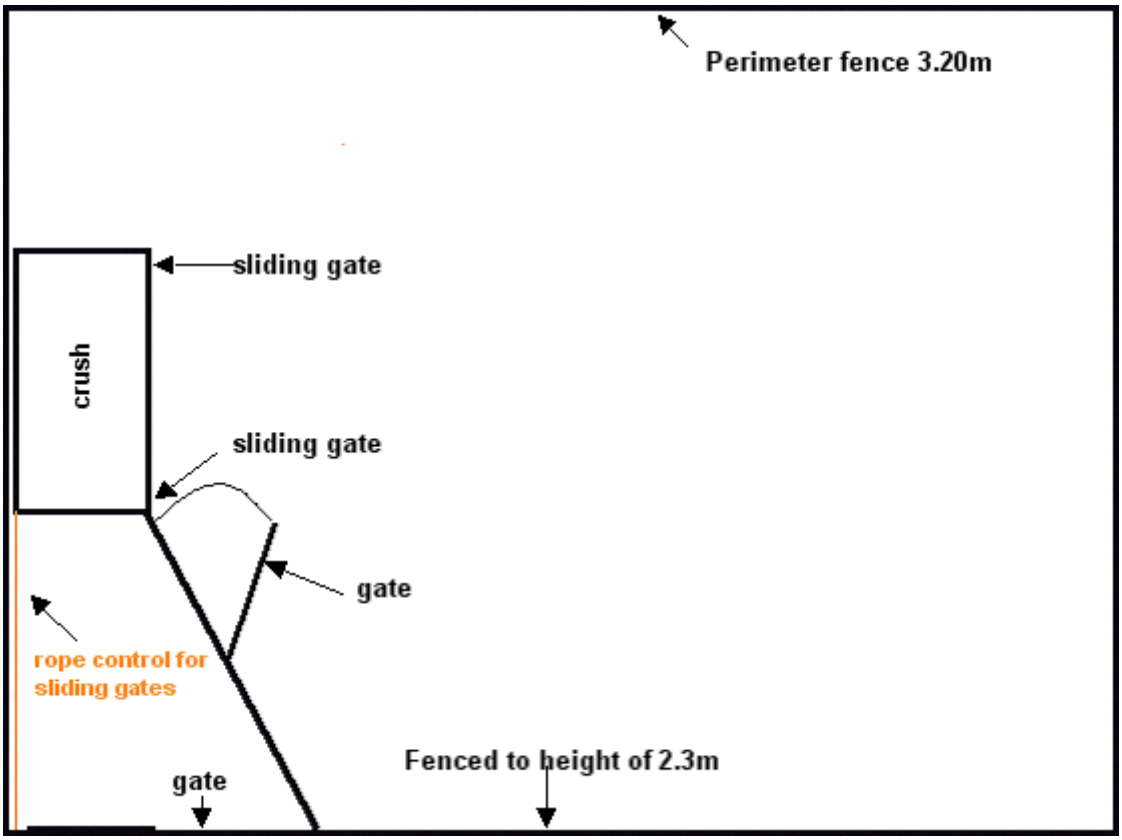

Figure 2: Cheetah camp layout (Meltzer, 1999)

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.

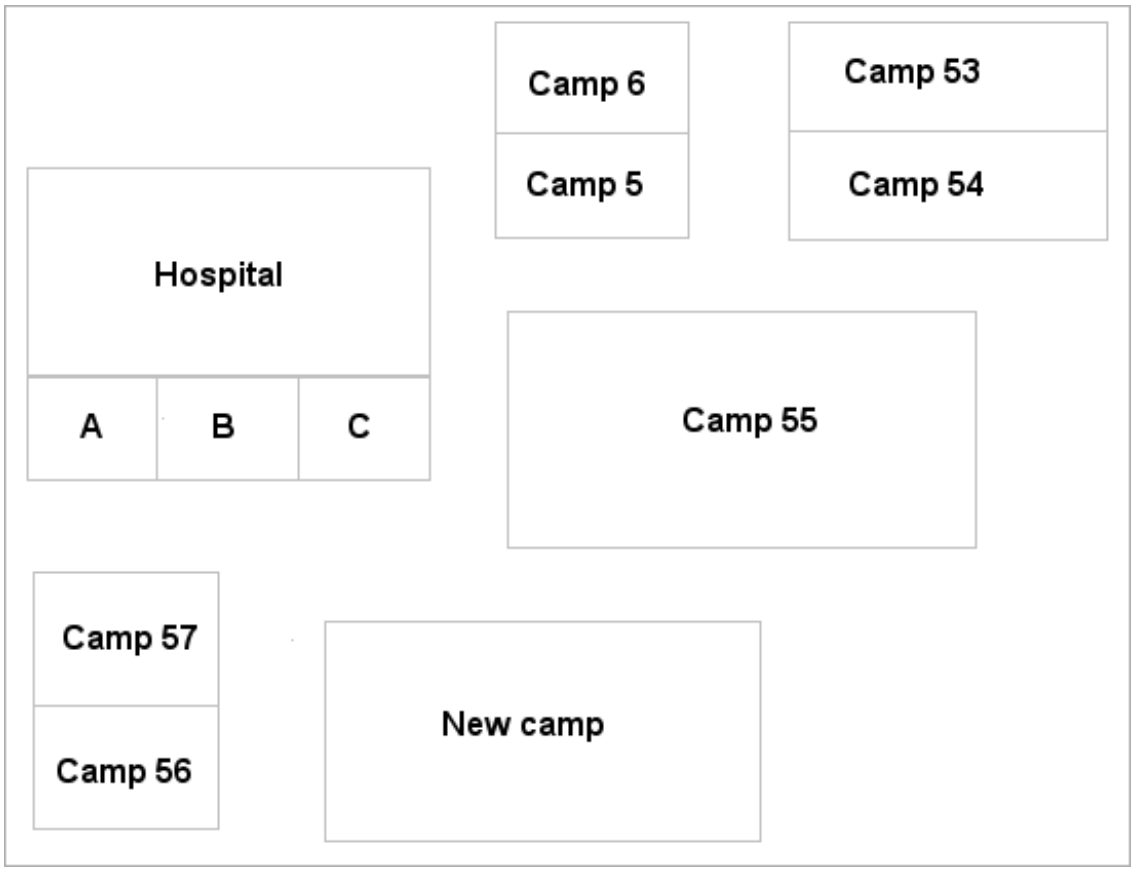

Figure 3: Camp layout for juvenile cheetahs at the De Wildt Centre

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.