

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 than 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 than 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 panleukopaemia 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 pathogens 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 pedestals 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 lymphoid tissue (lymphoid 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 lymphoid 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, unthriftiness 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, chlamydia, 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 than 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 immunoglobulin A and stimulating the local release of interferon (Tuomola *et al.*, 1999).

Lactobacillus gasseri has been effective in suppressing *Helicobacter 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. thetaiomicon* 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 *Bifidobacterium 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 2.2×10^5 to 1.6×10^8 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).

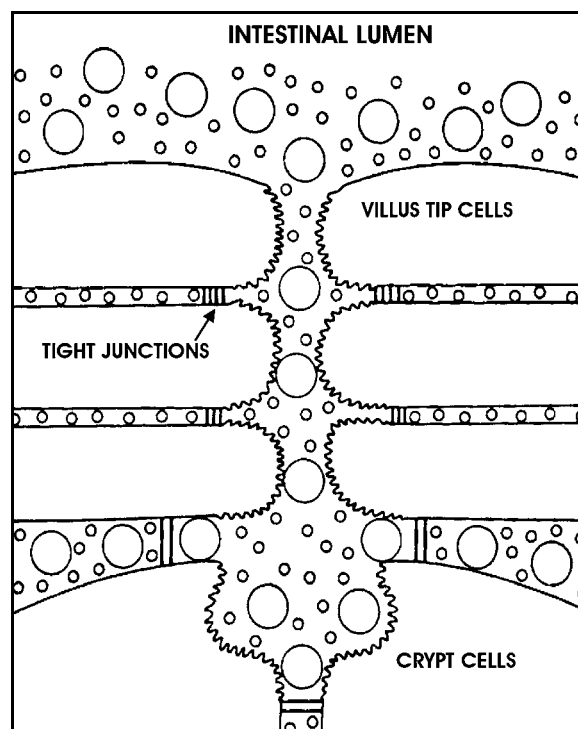


Figure 1: Model of tight junction difference between intestinal villi and crypts (Hollander, 1992)

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 transcellularly and disaccharides are absorbed paracellularly 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).