MOLECULAR CHARACTERIZATION OF THE FIBRE-DIGESTING BACTERIA ISOLATED FROM THE OSTRICH (*Struthio camelus* var. *domesticus*) HIINDGUT

by

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ABSTRACT

Eleven bacterial strains that have been isolated from the ostrich hindgut, have successfully been identified with the aid of PCR amplification of the 16S rDNA genes and sequence analysis using the BLASTN search of the GenBank database. The nucleotide sequences were determined either through direct sequencing of the PCR products or after cloning the products into the plasmid vector pBluescript (SK). The results obtained from the 16S rDNA sequence homologies strongly suggests that the newly identified bacteria should be assigned to three major bacterial genera, namely, *Ruminococcus*, *Butyrivibrio* and *Bacteroides*. Four highly cellulolytic strains were identified as *ruminococci* while two weakly cellulolytic strains, showed highest homology to members of the genus *Butyrivibrio*. Another weakly cellulolytic strain showed closest identity to the sulfur reducing bacterial genus *Desulfovibrio*. Four non-cellulolytic strains showed highest homology to the genus *Bacteroides*.

A comprehensive comparative sequence analysis was then performed to determine the phylogenetic positions of the newly identified bacterial strains. The phylogenetic analyses based on 16S rDNA gene sequence data supported the 16S rDNA sequence results. The *Ruminococcus* strains were shown to be phylogenetically related to cluster IV of the *Clostridium* subphylum of the Gram-positive bacteria, while the two *Butyrivibrio*-like strains were related to cluster XIVa of the *Clostridium* subphylum. The

Bacteroides strains were shown to be members of the *Bacteroides* subgroup within the *Bacteroides* cluster of the *Cytophaga-Flavobacter-Bacteroides* phylum.

Repetitive extragenic palindromic PCR (REP-PCR), a PCR-based typing method was employed to show that there was considerable variability between strains of the same species.

Since feces remain the most difficult specimens for DNA amplification, two extraction protocols were employed to isolate genomic DNA from domestic and wild ostrich fecal samples and to overcome problems related to the presence of substances that inhibit PCR amplification. Both methods yielded PCR-quality DNA although one method proved to be more rapid and less hazardous. In order to detect the presence of *Bifidobacterium* as well as *Bacteroides* and *Prevotella*, PCR with 16S rRNA-targeted primers specific for these genera, were applied to the DNA samples. *Bifidobacterium* was not detected in any of the fecal samples, however, *Bacteroides* and *Prevotella* were detected in the domestic fecal samples at 0.5 ng, 1 ng and 10 ng using the genomic DNA as a template. In contrast, when the 16S rDNA was employed as the template, *Bacteroides* and *Prevotella* was also detected at the lowest fecal DNA concentration, 0.1 ng. No PCR signals were obtained at any of the higher fecal concentrations employed, namely 100 ng and 200 ng. Similarly, no PCR signals were observed in any of the wild fecal samples using both the genomic DNA and the 16S rDNA.

After transferring the 16S rDNA amplicon of the fecal samples to membranes by southern blotting, the universal eubacterial primer, FD1, or rBacPre, the primer specific for *Bacteroides* and *Prevotella* were end-labelled and employed as probes. The percentage of *Bacteroides* in total eubacterial 16S rDNA was calculated from the relative binding of these probes to amplified material from the gut samples and from the control sample, *B. fragilis* ATCC9343. The percentage of *Bacteroides* in the total eubacterial 16S rDNA was estimated to account for between 33% and 89% for the various samples tested.

ABBREVIATIONS

λ	lambda
γ	gamma
×	times
%	percent
°C	degrees celcius
±	approximately
A	adenine
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
A_{260}	absorbance at 260 nm
BHI	brain heart infusion media
bp	base pair
C	cytosine
cm	centimetre
cm^2	centimetre squared
cpm	counts per minute
CTAB	hexadecyltrimethylammonium bromide
dATP	deoxy-adenine 5'-triphosphate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleoside triphosphates (dATP, dCTP, dGTP and dTTP)
EDTA	ethylenediaminetetraacetic acid (disodium salt)
g	standard gravitational acceleration (9.81 m/s^2)
g	gram
G	guanine
hr	hour
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase
kcal/kg	kilocalories per kilogram
kg	kilogram
kJ	kilojoule
km hr^{-1}	kilometre per hour

l	litre
LB	Luria broth
lb	pounds
M	molar
mA	milli-Amperes
mg	milligrams
ml	millilitre
mm	millimetre
mM	millimolar
mmol/l	milllimoles per litre
mol/day	moles per day
MRS	Mann Rogosa Sharpe medium
ng	nanogram
nm	nanometre
OD_{600}	optical density at 600 nm
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pmol	picomole
^{32}P	phosphorous-32
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulphate (sodium lauryl sulphate)
SSC	sodium chloride trisodium citrate buffer
STE	sodium chloride tris-EDTA buffer
T	thymine
TBE	Tris-boric-EDTA buffer
TE	Tris-EDTA buffer
TEMED	N,N,N',N'-tetramethylethylene-diamine
TENS buffer	Tris-EDTA-NaOH-SDS buffer
Tris	Tris (hydroxymethyl) aminomethane
µg	microgram
µl	microlitre
µM	micromolar
µm	micrometre
UV	ultraviolet

V	volts
VFA	volatile fatty acid
W	watt
(w/v)	weight per volume
YT	Yeast Tryptone

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

CONTENTS

INTRODUCTION

Ostriches (*Struthio camelus* var. *domesticus*) are the largest flightless avian herbivores, weighing approximately 90-120 kg adult body mass, and belonging to the subclass Ratitae of the Family Struthionidae (Oelofsen *et al.*, 1991; Swart *et al.*, 1987; Swart *et al.*, 1993b). They are related to the rheas of South America, the emu and cassawaries of Australia and the kiwis of New Zealand (Swart *et al.*, 1987; Swart *et al.*, 1993b). Ostriches have no keel on the sternum, no stiff contour feathers and no oil glands, so when exposed to rain they get wet. They have long, powerful legs which enable them to run at speeds of up to 70 km hr^{-1}, which helps in their defense and escape (Swart *et al.*, 1987). The male ostrich can be distinguished from the female bird by its black plumage except for the white wing and tail feathers while the female bird is brown with a pale edging to the feathers.

Unlike some other birds, they effectively digest plant fibre such as cellulose and hemicellulose (Swart *et al.*, 1993a; Swart *et al.*, 1993b). However, all herbivorous vertebrates as well as many invertebrates depend on the gut microflora for digestion of cellulose and hemicellulose since the digestive enzymes secreted by these animals are unable to digest these biopolymers (Angel *et al.*, 1995; Atlas and Bartha, 1993; Hobson, 1966; Latham, 1979; Swart *et al.*,1987; Swart *et al.*, 1993a).

1. 1 Ostrich Farming

About 200 years ago, this species extended over much of Africa, Syria and Arabia in desert and bush regions but are now extinct or very rare over most of these areas and are only found in settled areas where domesticated animals have gone feral. Wild birds can still be found in parts of Southern and East Africa, for example Kenya (Swart *et al.*, 1987).

Domestication of the African ostrich for commercial farming purposes dates back to 1864-1875 (Swart *et al.*, 1993b). During this time, ostrich feathers were the only commercial product of that activity and held fourth place on South Africa's export list,

after gold, diamonds and wool. The ostrich feathers satisfied the tastes of fashion in America and Europe, and the demands of music hall and cabaret. The South African ostrich population swelled until by 1913, nine million birds were being bred exclusively for their plumes. However, during the world depression of 1914-1945, the demand for ostrich feathers vanished and the market collapsed (Nixon, 1999; Swart *et al.*, 1987).

Currently, commercial farming with domestic ostriches is flourishing in South Africa's Klein Karoo and Southern Cape. Although it is relatively small in the general stock-farming scenario, it provides a source of income for approximately 400 farmers who own about 120 000 ostriches (Swart *et al.*, 1987).

During December 1999, it was reported that Karoo ostrich feathers from an ostrich co-operation in the little Karoo town of Oudtshoorn was to add exotic flair to millennium parties around the world. Normally, the Klein Karoo Feather and Duster company exports about six tons of feathers annually. However, in 1999 more than 12 tons of plumes were exported to places as far as the US, Germany, Spain, Canary Islands, Portugal and Brazil. It has also been stated that the wing feathers could fetch up to R1 200 a kg. The company's biggest customer was Rio de Janiero in Brazil, home to the famous street carnival (Govender, 1999).

Besides ostrich feathers, leather, eggs and meat have all become important export products accounting for foreign exchange earnings of more than 30 million rand. In terms of monetary value, the ostrich industry has grown by more than 300% over the past decade and by almost 100% in the last five years. The steep growth rate is mainly due to the stable developing slaughter bird industry where meat, leather and feathers make up more than 80% of the industry's turnover (Swart *et al.*, 1987). With the increasing worldwide trend towards health concerns, ostrich meat has recently become increasingly popular since it is extremely lean. A 100 g portion of ostrich meat contains 590 kJ and only 2 mg cholesterol. An equivalent amount of mutton contains 799 kJ and 89 mg cholesterol. Ostrich eggs are eaten on a relatively small scale and are primarily used for

hatching purposes. One ostrich egg is roughly equivalent to 24 hen's eggs (Clasen *et al.*, 1997).

The ostrich industry experiences major productive losses due to the high rate of chick mortality during the first 3 months of life (Table 1.1). These losses are the result of a number of factors including disease such diarrhea which is greatest in young farm animals and they suffer the highest incidences of morbidity and mortality (Buddington and Weiher, 1999; Van Niekerk, 1996). Other factors include faulty hatchery practice, the environment, poor management, and malnutrition of parents as well as chicks (Van Niekerk, 1996). Part of the reason could also be the absence of the required bacteria during early development since intense modern methods of animal rearing often restrict the access that the newborn bird has to the mother and prevents it obtaining the full complement of characteristic microbes (Fuller *et al.*, 1989).

Table 1.1. Average rates of chick mortality

Age	Rate of mortality (%)
Day old to 90 days	50 ± 30
2 to 6 months	10 ± 8
6 to 14 months	3 ± 2

In the wild state, ie. under natural rearing conditions, newly hatched ostrich chicks will consume their parents fresh feces and because this is such a strong instinct, it must surely have an evolutionary basis. Since ostrich manure contains almost no protein, the only benefit they are likely to derive from practising coprophagy is obtaining the characteristic gut flora and gaining access to microbially manufactured B-vitamins (Van Niekerk, 1996).

Coprophagy is defined as the feeding or eating of dung or excrement. It is normal behaviour among many insects, birds and other animals. Ingestion of feces can only

benefit the animal if it contains more nutrients than the food they displace. This is overcome in many small mammalian herbivores by practising cecotrophy. The term cecotrophy is preferred by some nutritionists when referring to rabbits and small herbivores because these animals obtain nutrition through the selective ingestion of highly nutritious excreta formed in the cecum (Bergman, 1990; Soave and Brand, 1991; Stevens and Hume, 1998). When an animal ingests its own excreta it is called autocoprophagy. However, if the animal ingests the feces of other animals of the same species it is referred to as allocoprophagy (Soave and Brand, 1991).

Coprophagy was first observed in the rabbit in 1882 but the practice is also known to occur in mice, guinea pigs, rats, mountain beavers, dogs, foals, swine and non-human primates. It is of nutritional significance to many animals providing a source of vitamins, minerals, amino acids, bacteria and other nutrients which are excreted with the feces and not effectively utilised in the gastrointestinal tract that contribute to growth, development and maturation (Soave and Brand, 1991; Stevens and Hume, 1998). The rate of coprophagy seems to be highest during the first few weeks of life. A study conducted on foals showed that the rate of coprophagy was highest during the first 8 weeks of life (Crowell-Davis and Houpt, 1985).

During the day domesticated rabbits excrete dry hard fecal pellets and soft, moist pellets at night. The hard pellets are the result of a colonic separation mechanism (CSM), which allows the more rapid passage of larger particles into the feces. Interruption of CSM results in the formation of cecal contents into soft pellets and their passage through the colon. The soft feces contains high levels of volatile fatty acids and microbial protein with a high content of essential amino acids (Savage, 1986; Stevens and Hume, 1998).

Sufficient evidence exists showing that prevention of coprophagy has a vast number of implications in coprophagous animals. It has a marked effect on the composition of gut bacteria and the consequent performance of the intestinal microflora. A study showed that prevention of coprophagy reduced the levels of *lactobacilli* and increased the

coliforms in rat feces. The efficiency of food conversion was also shown to be reduced (Latham, 1979).

In rabbits and guinea pigs, prevention of coprophagy reduced digestibility of crude protein (CP), organic matter, dry matter, amino acids and the ether of the acid detergent fiber (ADF) of alfalfa meal, indicating that coprophagy is involved with protein digestibility (Soave and Brand, 1991; Takahashi and Sakaguchi, 1998). Results obtained from estimating the contribution of coprophagy to protein intake in captive adult and young nutrias, suggested that the ingestion of feces is quite an effective manner for nutrias to ingest extra protein. Contribution of coprophagy to CP intake in adult nutrias was estimated to be 16%, superior to that for rabbits on a diet with a similar ADF concentration (Takahashi and Sakaguchi, 1998).

A rat consumes a variable amount of its feces, from 5 to 50% of its total fecal output. Prevention of coprophagy may depress the growth rate in young rats by as much as 15 to 20%. The decreased growth rate is partly due to a deficiency of cysteine which results from the prevention of coprophagy. If rats are not permitted to eat their feces, vitamin K must be added to the diet since it is produced in the intestinal tract of the rat but is not absorbed. Instead it is passed out with the feces and thus only obtained through coprophagy. Coprophagy also enables guinea-pigs to utilise the B-complex vitamins and amino acids in the feces that are synthesised by the microflora of the cecum (Soave and Brand, 1991).

Generally an iron deficiency may result if coprophagy is prevented in coprophagous animals since iron is eliminated in the feces. For example, adult swine feces are rich in iron and piglets will ingest about 20 g of their mother's feces daily (Soave and Brand, 1991). It is hypothesised that foals and rats may consume feces in response to a maternal pheromone, which signals the presence of deoxycholic acid or other acids that these animals may be deficient in, and which they may require for gut immunocompetence and myelination of the nervous system (Crowell-Davis and Houpt, 1985; Soave and Brand, 1991).

Much of the nutritional benefit from microbial cells in the hindgut flora can be derived through coprophagy by monogastric mammals. For example, rabbits have efficient cecal fermentation and are able to recover some of the microbial cells synthesised in the cecum by practising coprophagy (Latham, 1979). However, non-coprophagic animals may derive little benefit unless molecules produced by or released from lysing microbial cells are absorbed into the blood in the cecum and colon (Savage, 1986).

Whether or not the hatchlings should be allowed access to their parents' feces remains controversial. However the belief that coprophagy will result in internal parasite infection is unfounded as the parasites must first complete their life-cycle before they become infective which requires at least five days under favourable conditions (Van Niekerk, 1996).

1.2 Diet of Wild and Domestic Ostriches

The ostrich in its wild or domesticated state is well known for the unusual and often surprising variety of items composing its diet. On this basis they could be classified as herbivorous, granivorous, frugivorous and possibly even omnivorous (Oelofsen *et al.*, 1991).

Little was known about the feeding behaviour and food composition of wild ostriches until very recently. Ostriches consume all parts of herbs and grass but being highly selective, prefer dicotyledonous plants. Besides a variety of vegetation, they were also shown to consume locusts and other life forms. They feed on fallen figs, flowers, pods of acacias and seed pods of species such as aloes which are unpalatable to other animals. Even though recent studies confirm that adult birds normally survive well purely on vegetation, there is no doubt that ostriches will sometimes eat animal matter. This could depend on whether or not the food available to them is deficient in proteins. For example, captive birds at a farm in southern Africa were seen stripping off all the skin and flesh from an adult bird that died in their extensive enclosure (Jarvis, 1995).

There seems to be a lack of research conducted on food consumed by wild ostriches. Studies of their nutrition could serve as a basis for understanding the food requirements of captive birds. Recent findings show that ostriches, including those in deserts, feed almost exclusively on plant material such as annual grasses, roots, leaves, flowers and fruits. Total analysis of the diet showed energy densities ranging between 8 and 9 mg per kg dry mass and a protein content of 12%. The birds consumed between 5 and 8 kg of green matter daily which represents an average of about 2 kg dry matter (Jarvis, 1995).

Ostriches also ingest hard objects such as pebbles or coarse sand that help in the grinding and crushing of food in the gizzard (Jarvis, 1995; Swart *et al.*, 1987; Swart *et al.*, 1993b). The type of stone used for grinding food in the stomach is another point of interest. Mainly hard stones like quartz, with sizes ranging from 3 to 15 mm in diameter are found in adult birds (Jarvis, 1995; Van Nierkerk, 1996). Obviously the stone size for chicks is much smaller. Newborn birds only select stones of up to 3 mm in diameter. As the chicks age they consume bigger stones but what is notable is that the weight of stones will never exceed 1% of the chick's total body weight. The overall weight of stones represents an average of 0.83% body weight.

Many commercial feeds available have been developed through observing ostriches in their natural habitats. However, the exact nutritional requirements of ostriches are not known due to a lack of research in this area. Most feed ingredients are based on what the ostrich would normally eat in its natural wild diet in order to maintain daily nutritional requirements (Ultra Bio-Logics inc, Acidola OER).

Normally the diet has a high fibre content which is expected since ostriches are one of the only birds capable of digesting up to 60% of the fibre in the diet. Ostrich rations can be formulated without any animal proteins since the chicks can be reared healthily on pure vegetable diets, with vegetable proteins. In the wild there is some evidence that chicks will eat insects if they get the chance. It has also been demonstrated that captive birds will sometimes eat live termites placed in their feed (Jarvis, 1995).

In South Africa the most common feed ingredients are fish meal, soyabean meal, ostrich meat and bonemeal, full-fat soya, sunflower meal, and maize gluten 60. Lucerne is the most generally used forage source, with wheaten bran as an alternative fibre source (Van Niekerk, 1996). These feed ingredients help to prevent deficiencies commonly associated with the use of modern, highly processed feeds, which are not found in the ratites' natural environment. They also help to maintain a positive microflora, improving feed conversion and stamina, and the overall health of the bird. In addition, feed preparations usually contain a source of live, beneficial, naturally occurring bacteria, vitamins, electrolytes and trace elements. The presence of *Lactobacillus acidophilus* ensures the decreased growth rate of *Pseudomonas*, *Bacillus* and *Proteus* because *Lactobacillus* produces hydrogen peroxide that is detrimental to these pathogens (Ultra Bio - Logics inc, Acidola OER).

1. 3 Important Factors in Feed Formulation

A. Protein Content

Proteins of body tissues contain as many as 22 amino acids and about 50% of these amino acids must be supplied because the ostrich cannot synthesize them rapidly enough to meet requirements. Deficiencies of dietary protein and amino acids result in reduced growth of chicks, decreased reproductive performance of mature animals, impaired feed efficiency, poor feather development, lethargy, and reduced resistance to disease (Angel *et al.*, 1995).

The amino acid requirements of young poultry are relatively high during early growth and as birds mature physically, dietary requirements for amino acids decrease because of changes in growth rate and metabolic needs in relation to feed intake. Ostrich feeds are usually formulated to contain prescribed percentages of protein and amino acids. Therefore any factor that affects feed intake will influence the amounts of protein and amino acids consumed. To maintain adequate, but not excessive protein and amino acid intake, adjustments may be required in protein and amino acid percentages in the feed to compensate for changes in feed intake (Angel *et al.*, 1995).

Studies were performed using 8 to 10 day old ostrich chicks to evaluate the effect of an iso-caloric diet and 4 levels of protein (14, 16, 18 and 20%) on weight gain and feed conversion efficiency in ostrich chicks. The best rate of weight gain was recorded on the highest level of protein (20%) with a lysine level of 0.99% but efficiency of weight gain was best on the 18% protein diet containing only 0.90% lysine. Other authors achieved excellent weight gain and efficiencies of feed conversion using a 12.97 MJ ME turkey starter diet fed for 35 days, followed by a 12.55 MJ ME turkey grower diet fed for the rest of the experiment. Previous studies also compared 14.5 and 17.0% protein diets for the age group 0 to 56 days and found superior weight gains on the 17.0% protein level, although the low protein levels used in this comparison do not allow meaningful comparisons (Van Niekerk, 1996).

B. Energy Value

The energy value of feed represents the capacity of dietary carbohydrates, fats and amino acids to provide energy to support all life processes through metabolic oxidation. The most common energy term used to quantitate energy in ostrich diets is apparent metabolisable energy (ME), defined as the gross energy of the feed minus the gross energy of the feces, urine and gaseous products of digestion (Angel *et al.*, 1995).

In poultry, the capacity to ferment fibre is very low, therefore fibre contributes very little to the metabolisable energy of an ingredient. The apparent fibre digestion (neutral detergent fibre) (NDF) in adult chickens varies between 6 and 9%, while in ostriches the digestibility ranges between 42 and 63%. The capacity of the ostrich to ferment fibre will have a considerable effect on the amount of metabolisable energy it can obtain from ingredients containing varying levels of fibre. For example, the metabolisable energy of alfalfa for poultry is 1200 kcal/kg while the determined metabolisable energy of alfalfa for ostriches is 2127 kcal/kg.

Other studies show that ostriches are able to utilize energy locked up in forages far better than poultry. From these studies it became apparent that using poultry ME values for formulating ostrich feeds results in an underestimation of the actual energy value of the

diet, especially when the diet contains high fibre levels (Angel *et al.*, 1995;Van Niekerk, 1996). It has also been shown that the ostrich chick makes relatively poor use of the fibre in its feed and that it takes as long as ± 17 weeks to reach mature status in this respect (Van Niekerk, 1996).

It is important to note that fibre not only affects the energetic value of feeds, but may profoundly alter the utilization of the other components of the diet such as proteins, amino acids and minerals (Dierick *et al.*, 1989).

C. Fibre

The natural feed of ruminants is generally referred to as crude fibre and the nutritive value of these compounds is often estimated from apparent digestibility. Studies showed that organic matter (OM) digestibility of untreated fibrous roughage is about 0.50 with hemicellulose digestibility being somewhat lower than cellulose digestibility. The reason for hemicellulose digestibility falling below the rate of cellulose digestibility may be due to the covalent links between hemicellulose and lignin. It was also shown that the proportion digested beyond the rumen seems to increase with decreasing overall digestibility, due for example to grinding or pelleting or increasing maturity of the forage (Demeyer, 1981).

The capacity to digest fibre changes with age. The apparent NDF digestibility in ostriches was found to be 6.5, 27.9, 51.2, 58.0 and 61.6% at 3, 6, 10, 17 weeks and 30 months of age. Chicks up to the age of 10 to 17 weeks were shown to have a limited ability to digest fibre (Angel *et al.*, 1995).

Fibrous feeds serve to dilute the energy content of the diet and also to make it bulky. Unless fibrous feeds are finely milled and /or pelleted, they can lead to impaction of the proventriculus or gizzard. Therefore it is advised to enforce maximum restriction on fibre in the early-starter diet. Some authorities believe that there is no need for a minimum fibre specification and the reasons for this include the need to stimulate microbial fermentation in the hindgut and the prevention of abnormal behaviour such as feather

pecking and pica. There is also the belief that low-fibre, high-energy diets lead to excessively fast growth that precipitates leg problems (Van Niekerk, 1996).

D. Other nutrients

Restricting water, sometimes advised by ostrich farmers, serves no purpose but to add to the many stress factors which domestic chicks are subjected to. A deficiency of water results in a marked reduction in growth, poor feed efficiency, and impaired reproduction. Young ostriches (4 to 6 months of age), deprived of water for 24 hours reduced their feed intake by 45% (Angel *et al.*, 1995).

Feed-grade fats are useful for increasing the energy concentration of diets as they contribute about 2.25 times as much energy to animal metabolism per unit weight as carbohydrate or proteins. In addition to contributing energy to diets, supplemental fats (fish oil added at a level of ± 2%) seems to improve absorption of fat-soluble nutrients, improve palatability, contributes essential fatty acids to the ration and also reduces dustiness of the feed (Angel *et* al., 1995; Van Niekerk, 1996).

Although mature ostriches digest fat as well as poultry, chicks do not digest lipids well until they are 10 to 17 weeks of age (Van Nierkerk, 1996). It was shown that the digestibility of fat increased up to 17 weeks of age (85.7, 91.1, and 92.9% at 10 and 17 weeks and 30 months of age, respectively). Chickens and turkeys have been shown to have difficulty digesting fat up to 1 and 3 weeks of age respectively (Angel *et al.*, 1995).

Most vitamins required by ostriches are not adequately supplied through cereals and forages typically found in ostrich rations and must therefore be supplemented. Growing captive ostrich chicks may have little or no access to green grazing and thereby lack the source of β-carotene which wild ostrich chicks obtain in their environment. Therefore, it is recommended to supplement vitamin A in the diet of young chicks since it is required for reproduction, vision and immunological functions. Too much vitamin A is harmful to animal production and will compete with the absorption of vitamin D, leading to reduced plasma vitamin E levels (Angel *et al.*, 1995; Van Niekerk, 1996). Vitamin D must be

supplemented in bird diets in the D_3 form and thereafter it is hydroxylated in the liver and kidneys into the active form (Angel *et al.*, 1995).

There is reason to believe that vitamin E is one of the most crucial vitamins in ostrich nutrition. It has been reported that degenerative myopathy in ratites have been linked to vitamin E/selenium deficiencies. There is also some evidence that the efficiency of vitamin E absorption in the gastrointestinal tract of the ostrich chick is limited and the embryonic stores are often used up before the ostrich becomes efficient at using dietary vitamin E (Angel *et al.*, 1995; Van Niekerk, 1996).

There is no research evidence to prove that growing ostriches need a supplemental source of B-vitamins and no report of B-vitamin deficiencies. One reason for this lack of B-vitamin deficiency reports can probably be attributed to the fact that the ostrich, as a hindgut fermenter, produces sufficient amounts of these vitamins to satisfy the birds needs through direct absorption and through the practice of coprophagy (Angel *et al.*, 1995; Van Niekerk, 1996).

Vitamin C is not known to be required in bird rations, however, there is an indication of some benefits from vitamin C supplementation during stressful periods, and for decreased leg abnormalities in domestic poultry (Angel *et al.*, 1995).

Ostriches also have general requirements for macro-minerals (calcium, phosphorus, potassium, sodium and chloride) and micro-minerals (magnesium, zinc, iron, copper, iodine, selenium, cobalt, and others). Deficiencies of these minerals can result in various implications, therefore it is essential to maintain adequate but not excessive inclusions of these minerals (Angel *et al.*, 1995).

The Rumen and Gastrointestinal Systems of Animals

The rumen and the gastrointestinal tract (GIT) harbour a very complex collection of mostly anaerobic microorganisms, which form an integral and biologically essential component of the body. Within these systems exist many interrelationships between the

different microorganisms and between the microorganisms and the host. Not all bacteria are capable of surviving and growing in the gut (Fuller, 1989; Fuller and Gibson, 1997). The established microflora which is a collection of about 10^{14} microorganisms consisting of 400 different types of bacteria, has to withstand the effects of antimicrobial chemicals present in the gut and avoid the effects of peristalsis which tends to flush bacteria out with the food. Bacterial survival can be achieved by either growing at a rate faster than the rate of removal by peristalsis or by attaching itself to the gut wall (Fuller, 1989). Together the established microflora carry out a process known as fermentation, where dietary and endogenous substrates are metabolised to release a variety of end products and these metabolites may have varying consequences for host health (Fuller and Gibson, 1997).

The rumen is a specialised chamber present in all ruminants such as deer, moose, antelope, giraffe, cow, sheep and goat that contains large populations of bacteria and protozoa that contribute to food digestion. It provides a stable optimal environment that is anaerobic, 30-34^{0}C, and has a pH range of 5.5 to 7 (Atlas and Bartha, 1993). The rumen is located strategically before the small intestine where the majority of essential nutrients such as amino acids, lipids, minerals and vitamins are absorbed (Scheideler, 1996). Essentially the rumen is a continuous culture of long turnover time, about a day, in which microorganisms are mixed with incoming food by contraction and expansion of the rumen wall and by rumination. Rumination physically grinds the plant material and provides an increased surface area for microbial attack (Hobson, 1966; Latham, 1979).

The rumen microflora consists of non-sporeforming anaerobic bacteria, anaerobic ciliate protozoa and other organisms such as flagellated fungal zoospores and viruses (Atlas and Bartha, 1993; Latham, 1979). Although protozoa comprise about 50% of the total biomass in the rumen, the metabolic and growth rates of the bacteria are much higher than those of the protozoa. Therefore the contribution of bacteria to the total metabolic activity in the rumen is substantially greater (Latham, 1979). The bacterial species of the rumen are divided into functional groups based on the substrates they ferment in vivo, and includes cellulose digestors, starch digestors, hemicellulose digestors, sugar

fermenters, fatty acid utilisers, methanogenic bacteria, proteolytic bacteria and lipolytic bacteria (Atlas and Bartha, 1993; Latham, 1979).

The organisms responsible for the initial degradation of plant materials are the cellulolytic *Ruminococcus flavefaciens*, *Ruminococcus albus* and *Fibrobacter succinogens* (Demeyer, 1981; Latham, 1979) (Table 1.2). The most metabolically versatile genus *Bacteroides*, contains species which are able to degrade cellulose, hemicellulose, pectin, starch and proteins. Bacteria such as the *Butyrivibrio* sp. and *Selenomonas* also show metabolic variability. Also found are the more specialised lipolytic vibrios *Anaerovibrio lipolytica* and the highly specialised methanogens, *Methanobacterium ruminantium* and *M. mobilis*. These bacteria are strict anaerobes except for *Streptococcus bovis*, the only facultative anaerobic species present in the majority flora. *Megasphaera elsdenii* and *Veillonella alcalescens*, two lactate fermenting species are also found in the majority flora and are able to ferment lactate as a sole energy source which is a rare property (Latham, 1979). This varied bacterial community possesses the broad enzymatic capabilities that are required for digesting various plant materials ingested by ruminants.

Table 1.2. Fermentation products and energy sources of some rumen bacteria

Organism	Energy Sources[1]	Major Fermentation products[2]
Fibrobacter succinogens	C, S, G	A, S,
Ruminococcus flavefaciens	C, X, G	A, S, F, H
Ruminococcus albus	C, X, G	A, E, F, H
Butyrivibrio fibrisolvens	C, S, X, G	B, F, H
Selenomonas ruminantium	S, G, L, Y	A, P, L
Veillonella alcalescens	L	A, P, H
Streptococcus bovis	S, G	L
Methanobacterium ruminantium	$H_2 + CO_2$, F	M

[1]Energy Sources: C = cellulose; S = starch; X = xylan; G = glucose; L = lactate; Y = glycerol; F = formate.

[2]Fermentation Products: A = acetate; F = formate; S = succinate; H = hydrogen; E = ethanol; B = butyrate; P = propionate; L = lactate; M = methane; many also produce CO_2.

Similarly, GIT is populated by hundreds of species of anaerobic bacteria, protozoa, and fungi, each species occupying a particular niche (Bergman, 1990; Stevens and Hume, 1998). It provides an extensive surface area where direct contact takes place between the host and a variety of dietary substances, microorganisms, and exogenous toxins (Gaskins, 1996). Temperatures in the GIT, where the fermentation rate may be lower, do not deviate significantly from body temperature (Latham, 1979). The bacterial species from numerous genera that can be recovered from the GIT of most mammals include both resident species and those that are transient (Buddington and Weiher, 1999). The dominant bacterial species within the GIT are either oligately anaerobic (eg. *Bacteroides*; *Veillonella*; *Clostridium* and the *Peptococcaceae*) or facultatively anaerobic (eg. *streptococci*; *lactobacilli* and the *Enterobacteriaceae*) (Latham, 1979).

1. 4 Digestive Systems of Ruminant and Non-Ruminant Mammals

Since herbivores digest cellulose, hemicellulose or pectin, they have evolved adaptations to their GIT which provide large fermentation chambers in which proliferation of microorganisms and digestion can take place.

In mammals two types of structural adaptations are found: enlargement of the oesophagus or diverticulae of the stomach to form a rumen or rumen-like stomach respectively, and enlargement of the cecum or colon (Hobson, 1966; Latham, 1979). In monogastric animals food passes down the oesophagus directly to the stomach, where it undergoes proteolytic attack by the acidic gastric secretions. However, in ruminants a pregastric microbial fermentation occurs in the rumen, a site anterior to the true stomach, which is physically separated from the acidic secretions of the stomach (Latham, 1979). Numerous differences in the anatomy of the GIT can be found throughout the animal kingdom and many can be related to the type of diet on which the animal lives. In addition, each

segment of the gut provides different environmental conditions for microbial growth, that frequently change in some way as the animal matures (Latham, 1979; Orpin and Anderson, 1988; Vanbelle *et al.*, 1990).

Unlike poultry, ostriches are devoid of a crop that serves as a storage organ. Instead the large size of the gizzard in ostriches makes it seem that this organ may play a storage role. The ostrich gizzard is thick-walled and has well developed muscles which is partly due to the presence of hard materials such as grit inside the gizzard that promotes muscle contractions, resulting in greater muscle development. The small intestine of ostriches constitutes 36% of the total length of the intestinal portion of the GIT (Table 3) (Angel *et al.*, 1995).

Table 1.3. Comparative intestinal lengths of ratites

Length	Ostrich [a]		Emu [b]		Rhea [c]		Chicken [d]	
	cm	% [e]	cm	%	cm	%	cm	%
Small intestine	512	36	315	88.5	132	63.2	61	90
Cecum	94	7	12	3.3	46	22.0	5	7
Colon	800	57	29	8.2	31	14.8	2	3

[a] adult 122kg (268 lb) female ostrich

[b] emu adult 38.3kg (84.3lb)

[c] Cho *et al.* (1984)

[d] Adult chicken

[e] % of total length of the intestinal tract (from duodenum to the end of the colon).

Ostriches have modifications in the hindgut, such as well-developed paired, sacculated ceca and a capacious, haustrated colon. These modifications are suitable for retention and degradation of plant fibre (Swart *et* al., 1987; Swart *et al.*, 1993a; Swart *et al.*, 1993b). When compared to other ratite species, the ostrich has the longest colon (Angel *et al.*, 1995; Stevens and Hume, 1998). The ostrich digestive tract is more similar to a chicken than the cow. Their anatomy and location of forage digestion is also quite different from

that of bovines. It has a relatively small stomach that serves primarily as a storage organ while ruminants such as cattle have a four-compartment forestomach, namely, the rumen, reticulum, omasum and the secretory compartment, the abomasum (Scheideler, 1996; Stevens and Hume, 1998). The rumen and the reticulum serve as the major fermentation organs while the omasum serves mainly for the transfer of digesta from the reticulum into the abomasum (Stevens and Hume, 1998).

Besides the ostrich, other small mammals, birds, reptiles and possibly some fish, also have anatomical adaptations in their large intestine. Insects, most notably the lower termites and wood-roaches have highly developed hindgut structures in which microbial fermentation can take place. Animals such as horses and zebras are the most abundant of the large terrestrial non-ruminant herbivores and are dependant to a large extent on microbial fermentation in their large intestine for their source of energy (Latham, 1979).

Generally, the cecum is pouch-shaped but may be elongated into a tubular form in some animals. In small herbivores, such as herbivorous rodents, the large cecum serves as the primary site for microbial fermentation, while an enlarged colon is the principal fermentation site in many of the larger herbivorous mammals (Stevens and Hume, 1998). For example, horses have large cecums and even larger colons where fermentation and absorption can continue (Latham, 1979; Orpin and Anderson, 1988).

1. 5 Influence of Diet on the Gut Microflora and GIT Structure

The protective flora which establishes itself in the gut is very stable, but is influenced by dietary and environmental factors (Fuller, 1989). Diet can indirectly influence the bacteria by modulating GIT structure and associated functions. The resulting changes in the physical and chemical characteristics of the environments in the different GIT regions can be expected to change the densities, relative proportions and metabolic characteristics of the resident bacteria (Buddington and Weiher, 1999).

In the rumen, the relative proportions of microbial populations shift according to the nature of the plant materials ingested. Sudden changes in diet, such as the change from

hay in winter to grass in spring, can disrupt the rumen fermentation system, resulting in excessive production of methane that can distend the rumen, sometimes to the extent that it compresses the lungs, suffocating the animal (Angel *et al.*, 1995; Atlas and Bartha, 1993). Similarly, the rumen of wild animals experience large fluctuations in bacterial counts that can be related to seasonal variations in the quality of their food. Generally, counts decrease in winter when there is less succulent vegetation and the material consumed is fibrous, highly lignified and frequently low in nitrogen (Latham, 1979). The composition of the rumen microbial population is also affected by the rate of passage since organisms with generation times greater than the turnover time are eliminated (Orpin and Anderson, 1988).

When animals are fed roughage rations, the cellulolytic bacteria are at the apex of a food chain involving interactions with many species which begins with structural plant cell wall carbohydrates and ends with the production of volatile fatty acids and methane. Changing the physical form of roughage by grinding, increases the surface area available for digestion and may also cause changes in the microbial population. On the other hand, rations high in cereal starch promote the growth of the amylolytic, lactic acid and propionate producing species (Angel *et al.*, 1995; Latham, 1979). When large amounts of cereals are fed to domestic cattle previously given roughage, an almost immediate proliferation of lactic acid-producing species ensues causing a severe, and often fatal, alimentary disorder known as acidosis. Under these circumstances the rate of lactic acid production far exceeds the capacity of other rumen bacteria to use it, the pH falls and many of the strictly anaerobic bacteria, such as the cellulolytic species, become much reduced in numbers. Artificial diets or diets lacking various nutrients can cause loss of certain species from the microflora within the GIT (Latham, 1979).

Carbohydrate-based diets support the growth of *Lactobacillus*, *Streptococcus* and *Bifidobacterium* in the stomach, small intestine and to a lesser extent the large intestine of homothermal animals (Latham, 1979). In addition, some feeding methods, principally those where large amounts of starch material are fed, may produce rumen pH values below 6, causing the bacteria to be of the more aciduric types (Hobson, 1966).

When pigs were fed high protein diets, the number of proteolytic bacteria in the colon contents increased. These results suggest that when more protein substrate is entering the colon of the pigs, it favours the growth of proteolytic bacteria. Similar responses were seen with the number of cellulolytic bacteria when the pigs were fed high fibre diets. However, it took 34 days to see the greatest number of cellulolytic bacteria as opposed to 17 days for the proteolytic bacteria. This could mean that it may take a longer time for fibre-digesting bacteria to establish and stabilise in the pig intestinal tract, as compared to the proteolytic bacteria. In addition, when pigs are fed with a high fibre diet, the number of cellulolytic bacteria and relative weight of the stomach were greatest. Therefore relative stomach weight was more directly related to the numbers of cellulolytic bacteria and appeared to be inversely related to numbers of anaerobes, proteolytic bacteria and *Campylobacter* sp. (Anugwa *et al.*, 1989).

It is also well known that an increase in fibre in the diet results not only in increase in the weight gain and volume of the gut, but also in an increase in the empty weight of the gut and the supporting tissues. Fibre components such as hemicellulose also have a propulsive effect on nutrients, shifting their digestion from the small intestine to the hindgut. This "digestive shift" can be explained by the effect of fibre on transit time in the small intestine so that there is less time for further processes of digestion and absorption (Dierick *et al.*, 1989).

The effects of poorly digested dietary components such as fibre and certain sugars on the flora arise from their resistance to degradation by mammalian intestinal enzymes, therefore allowing the passage of unchanged carbohydrate into the large intestine. The provision of additional fermentable energy by such materials may bring about changes in the concentration or relative species diversity of bacteria in the large intestine (Mallett *et al.*, 1988).

Studies investigating the influence of dietary levels of fibre and starch on the composition of cecal contents of the rabbit, showed that an increase of dietary crude fibre increased the crude fibre level in cecal contents from 11.58% to 26.53%. However, a relatively

lower proportion of fibrous materials was found in the cecal contents when rabbits were fed more fibrous diets. These results suggest that dietary fibre had a direct influence on the particle separation efficiency in the digestive tract. When dietary crude fibre increased, the volatile fatty acids and crude protein concentrations of cecal contents decreased from 47.8 to 36.7 and from 30.14% to 19.45% mmol/l, respectively. This may be due to the availability of energy to the microflora of the ceca (Carabaño *et al.*, 1988).

Fermentable fibres were shown to increase the densities of beneficial bacteria and stimulate growth and functions of the healthy intestine. Following acute diarrhea, the use of an oral electrolyte solution with oligofructose accelerated the recovery of beneficial bacteria, reduced the relative abundance of detrimental bacteria, stimulated mucosal growth and enhanced digestive and immune functions. Similarly, fermentable fibres, such as oligofructose and inulin, selectively increased the abundance of lactic acid bacteria while decreasing the percentages of potential pathogens and putrefactive bacteria in several species. These fibres also influenced the metabolic activities of the bacteria (Buddington and Weiher, 1999).

When the diet of dogs was supplemented with oligofructose and beet pulp, which include fermentable components, it resulted in longer intestines with a greater surface area and greater mucosal mass compared with the intestines of dogs fed a diet with cellulose, which is poorly fermented. Similar responses occurred in mice fed diets with oligofructose and inulin compared with those fed a diet with cellulose. Since mammals are unable to digest fermentable fibres, the increases in intestinal dimensions and functional capacities provide evidence for interactions among the diet, the resident bacteria and GIT characteristics. Recent findings also indicate that bacterial fermentation of fibre triggers the release of glucagon-like peptides 1 and 2, gastric inhibitory peptide and perhaps other enteric hormones. These stimulate mucosal growth and upregulation of transport processes in the proximal intestine. Thus, the GIT bacteria, like many organisms in other ecosystems are able to modify their environment (Buddington and Weiher, 1999).

1.6 Characteristics and Distribution of Indigenous Gut Microbes

Due to the anatomical differences between animal species, the location and the number of organisms along the GIT varies. Three interacting populations of microorganisms may be found in each region of the gut: those free in the aqueous phase of the gut lumen, those attached to food particles, and those attached to the epithelium lining the tract (Orpin and Anderson, 1988; Savage, 1986).

The bacterial assemblages found in the mammalian GIT are highly variable and species specific (Buddington and Weiher, 1999). Detailed analysis of the fecal flora of man demonstrated that while there are many similarities in composition between individuals, each person nevertheless has its own characteristic microflora (Latham, 1979).

In man and rabbits, few if any organisms occur in the stomach or anterior small intestine, whereas in the rat and mouse counts are high in the region of non-secreting tissue (Table 4) (Latham, 1979).

Table 1.4. Numbers ($\log_{10}$) of bacteria in the GIT and feces of different animals

Site	Man	Rabbit	Rodent
Stomach	0-5	0-6	7-9
Small Intestine (A)	0-5	0-5	6-8
Small Intestine (P)	6-7	6-7	7-8
Large Intestine	7-10	8-9	8-9
Feces	10-11	9-10	9-10

A, Anterior. P, Posterior

The pH and the time that digesta are retained in a given GIT segment can determine the number and type of indigenous bacteria and the degree of microbial fermentation. The low pH of gastric contents and rapid transit of digesta through the small intestine of most

species tend to inhibit the growth of many microbes. However, the long retention time and relatively neutral pH of digesta in the hindgut of most terrestrial vertebrates and the foregut of a few of these species result in large numbers and greater diversification (Stevens and Hume, 1998).

During the first few weeks after birth, the forestomach becomes colonised with *Escherichia coli aerogens* and lactic streptococci, which are joined by lactobacilli in the suckling animal. Weaning is followed by development of the extremely complex microbiota that are characteristic of adult animals (Orpin and Anderson, 1988; Stevens and Hume, 1998). The succession of organisms in the feces of unweaned animals such as man, ruminants and pigs are remarkably similar to the early microflora of streptococci and coliforms being replaced with *Lactobacillus* and *Bacteroides* (Latham, 1979; Vanbelle *et al.*, 1990). Changes in the fecal flora occur soon after weaning as the diet changes towards one typical of the adult animal. The established fecal population of various adult animal species often show marked dissimilarities which can be related to the different diets of the host (Latham, 1979).

Generally the more oxygen tolerant anaerobic bacteria such as lactobacilli and the *Streptococcus* sp. are among the first species to appear in the stomach with *Lactobacillus* frequently being the dominant organisms. The secreting regions of the stomach do not support much microbial growth due to the hydrochloric acid (HCl) that has a marked bactericidal activity. In the absence of HCl secretion, the stomach contents usually have a high count of bacteria. Therefore gastric acid undoubtedly controls the passage of bacteria into the small intestine and is probably important in preventing the invasion of pathogenic bacteria into lower reaches of the gut. The numbers of microorganisms increase distal to the stomach as the digesta becomes less acidic (Latham, 1979).

The small intestine harbours a more complex flora of lactobacilli and streptococci; coliforms and *Bacteroides* becoming more predominant towards the most favourable GIT region, the large intestine. If the organisms in the lumen of the small intestine do not multiply very rapidly their existence will only be transient due to the continuous passage

of digesta down the gut. Organisms adhering to epithelial cells, however, have a much more permanent existence and are only displaced when the epithelial cells are shed into the lumen (Gaskins, 1996; Latham, 1979). Growth in the small intestine is limited by the following mechanical factors; gastric motility which continuously moves organisms down the intestine in the digesta, the mucous covering of the epithelium which acts as a barrier and limits access of intestinal organisms to the epithelial cells, and the ileocaecal valve which restricts any back-flow of organisms from the large intestine (Latham, 1979).

Due to a neutral pH, prolonged retention time, anaerobiosis and diminished motility, the largest bacterial populations are found in the hindgut or large intestine of mammals and in the foregut of a few mammals (Gaskins, 1996; Stevens and Hume, 1998). These hindgut bacteria produce substantial levels of VFA (volatile fatty acids) by fermenting starches that escape digestion in the upper digestive tract and the endogenous carbohydrates contained in mucus and sloughed epithelial cells. They also produce ammonia and microbial protein from digestion of amino acids that escape absorption in the midgut, digestive enzymes, mucus, and sloughed cells released into the gut (Low, 1989; Stevens and Hume, 1998).

The cecum and colon harbour the more extremely complex flora of strictly anaerobic species while coliforms and streptococci often comprise the minority (Latham, 1979; Savage, 1986).

It is becoming increasingly apparent that the ability of an organism to adhere to surfaces is a major ecological determinant in the mammalian gut. Some organisms, particularly lactobacilli show a remarkable degree of specificity to the animal host, region of the gut and type of epithelial cell to which they will adhere. For example, the cellulolytic rumen bacterium, *R. flavefaciens* show a great propensity to attach to plant cell wall material and the intense competition for nutrients in the rumen makes it seem likely that adhesion may well be essential for the organism to obtain the maximum nutritional benefit from the material it is degrading (Latham, 1979).

Generally, the bacterial numbers in the mammalian midgut or small intestine are much lower than in the rumen. Researchers also found that anaerobic bacterial counts in grass fed horses ranged from 10^6 /g in the duodenum to 10^8 /g in the ileum. Bacterial counts of 10^7 to 10^{12} /g have been reported in mammalian hindguts. In addition, bacterial populations associated with the epithelial surface and lumen contents of the hindgut can differ from one another, from those in the feces and can also vary between segments of the hindgut (Stevens and Hume, 1998).

It is recognised that the bacteria associated with the mucosa are likely to have greater potential to influence the host than those present in the lumen since the bacterial populations associated with the mucosa differ from those of the digesta (Buddington and Weiher, 1999).

1.7 Fibre Digestion and Nutrient Absorption

For fermentation to occur, a viable microbial population including bacteria, protozoa and yeasts need to live and reproduce within the intestinal lumen. For these organisms to thrive long term in the GIT, there has to be a location within the gut where fibrous materials have a relatively long residence time. Therefore location of forage fermentation and passage rate is important (Angel *et al.*, 1995).

The degree of plant fibre degradation depends on the composition of the gut microflora, its retention in the fermentative parts of the gut and the nature of the plant cell wall (Swart *et al.*, 1987)

Anything that alters the microflora such as diet changes, stress and antimicrobial medications, alters the bird's capacity to digest fibre. When a high fibre diet is being fed, the microbial populations that digest fibre will be high. When the diet is suddenly changed from a high-fibre to a low-fibre-high grain diet, the substrate for fibre-digesting microbes will no longer be abundant, and the populations will fall with an increase in starch-digesting bacteria (Angel *et al.*, 1995).

The ostrich is able to utilise as much as 60% of the neutral detergent fibre (NDF) fraction of its feed, resulting in excellent digestion of fibre sources such as alfalfa (Jarvis, 1995; Scheideler, 1996). Similarly, the ruminant is also known for its excellent use of fibre, however, the digestive mechanisms for fibre digestion in the ostrich compared to the ruminant, is very different. These differences call for quite different feeding programs to meet the respective animal's nutrient requirements.

Fermentation of fibre in the ostrich occurs in the proventriculus, gizzard, ceca and colon although it occurs primarily in the large intestine and cecum, where the microbial populations are substantial and after most of the absorption of amino acids, vitamins and minerals has occurred in the small intestine (Angel *et al.*, 1995; Scheideler, 1996). Fibre digestion provides the ostrich with a rich energy source of VFA (Angel *et al.*, 1995; Scheideler, 1996). The large intestine and cecum of the ostrich is capable of absorbing these primary energy sources, which provide as much as 60% of the birds energy. Volatile fatty acids can either be utilised as energy sources by the bird, utilised for muscle deposition, or deposited as fat if their diet is not balanced for amino acids (Scheideler, 1996). VFA were found to be high in the gizzard and proventriculus, decreased in the small intestine and was very high in the hindgut (Angel *et al.*, 1995).

Protein that passes protected from microbial breakdown through the rumen to the small intestine, where it is absorbed and utilised by the animal and not the microbes, is referred to as "by- pass protein". Little or no protein or amino acid utilization can occur during microbial forage breakdown because the site of fibre digestion is after the small intestine. Absorption of these nutrients only occurs in the small intestine before fibre digestion occurs in the large intestine of the ostrich. Unlike ruminants, by-pass protein is not beneficial to the ostrich since there is a very limited microbial population in the ostrich stomach to by-pass. It is hypothesised that forage is not an essential requirement in the ostrich diet, however, it is known to be a key factor for a healthy gut flora, optimum rate of passage, and efficiency of nutrient digestion (Scheideler, 1996).

In cattle, the rumen is the primary site for microbial digestion of fibre. Microbes leaving the rumen provide a protein source to the host. VFA produced are absorbed through the ruminal wall for energy utilization and fat synthesis by the bovine. This system of rumen fibre digestion allows the bovine maximum utilization of the energy and protein stores in forages mainly because the location of forage digestion in the rumen is before the small intestine (Scheideler, 1996).

In other non-ruminant mammals such as horses, pigs, and rabbits, microbial cellulose digestion occurs in an enlarged cecum with the production of volatile fatty acids (Atlas and Bartha, 1993; Dierick *et al.*, 1989). These acids are absorbed through the intestinal lining and enter the bloodstream where they are ultimately oxidised within the animals cells, producing $C0_2$ and H_20 (Atlas and Bartha, 1993).

Fibre digestion is influenced not only by structure and capacity of the GIT, but also by the rate of passage of digesta. Studies conducted to determine mean retention time for ostriches with different live-weights showed that retention time for the particulate phase varied, ranging between 21 to 76 hours (Swart *et al.*, 1987; Swart *et al.*, 1993b). Young birds with a mean live-weight of about 6,8 kg had a mean retention time averaging 39 hours compared to older birds with a live-weight of 45,8 kg, which averaged 48 hours. These results obtained show significant retention of feed in the ostrich gut and that passage rate or retention time varies with age (Angel *et al.*, 1995; Swart *et al.*, 1987). The selective or prolonged retention time of fibrous feeds in the GIT ensures that fibre is exposed to microbial digestion for extended times, allowing efficient fermentation of fibre. This is supported by the high digestibility of cell walls (NDF) (47%), cellulose (38%) and especially hemicellulose (66%) (Swart *et al.*, 1987; Swart *et al.*, 1993a; Swart *et al.*, 1993b).

1. 8 Fermentation of Carbohydrates

The major end products of microbial fermentation of cellulose, hemicellulose, pectins, starch, dextrans and sugars are the volatile fatty acids (VFA), mainly acetate, propionate and butyrate plus CO_2, methane (CH_4), hydrogen gas and available energy as ATP

(Bergman, 1990; Demeyer, 1981; Savage, 1986). Dietary and endogenous nitrogenous compounds are also converted into ammonia and microbial proteins, and synthesis of B-vitamins (Stevens, *et al.*, 1998). Mucus, sloughed cells and endogenous secretions also provide fermentable substances, especially proteins and polysaccharides. The low molecular weight acids are absorbed through the surrounding epithelial tissue into the animal's bloodstream where they are oxidised to release the most important single source of carbon and energy available to ruminants and monogastric animals (Atlas and Bartha, 1993; Savage, 1986). Removal of these acids from the rumen or cecum and/or colon by absorption allows continued prolific growth of the microbial population since an accumulation of these acids would be toxic to the microbes (Atlas and Bartha, 1993; Swart *et al.*, 1987).

The continuous production of VFA is counteracted by the buffers secreted by the gut epithelium and in the rumen by the saliva which helps to maintain a pH above 5.6, a critical level for cellulolysis (Demeyer, 1981; Latham, 1979; Swart *et al.*, 1987).

Primary products of food fermentation are lactic, formic and succinic acid, but these are rapidly utilised in secondary fermentations and converted to CH_4, acetic and propionic acid. Any hydrogen formed as a primary fermentation product is utilised in secondary reactions, principally in the formation of CH_4 (Hobson, 1966). The soluble sugars released from the degradation of carbohydrates are the major energy substrates for most rumen bacteria and are fermented mainly to VFA, CH_4 and CO_2 (Latham, 1979). Fermentation gases such as CH_4 and $C0_2$, that collects above the rumen digesta are vented by means of a reflex belching action (Demeyer, 1981; Latham, 1979; Orpin and Anderson, 1988). In all mammals examined, acetic acid is the main VFA produced and is present in higher concentrations than all other organic acids combined. Propionate and butyrate are also present in large concentrations but their amounts can vary considerably with the diet (Bergman, 1990).

Two main features distinguish hindgut fermentation from rumen-type fermentation systems. Firstly, in non-ruminants, the fermentation substrates have already been exposed

to the digestive enzymes of the animal before fermentation, and, secondly, the mass of microbial cells synthesised in the hindgut or large intestine cannot be directly utilised by the animal and are voided in the feces (Orpin and Anderson, 1988).

The overall fermentation that occurs within the rumen can be described by the equation (Bergman, 1990; Latham, 1979):

$57.5\ (C_6H_{12}O_6) \longrightarrow 65$ acetate + 20 propionate + 15 butyrate + 60 CO_2 + 35 CH_4 + 25 H_2O

Generally, herbivores depending on cecal fermentation have higher dietary requirements for preformed amino acids and vitamins than ruminants, since the latter obtain these substances by digesting the continuous supply of rumen microorganisms which flow from the rumen to the small intestine (Latham, 1979; Orpin and Anderson, 1988).

In the ostrich, nutrients first undergo gastric digestion and intestinal absorption before microbial fermentation in the cecum and/or colon. The principal site for microbial fermentation in the ostrich is the colon (Stevens and Hume, 1998). There is some evidence for B-vitamin absorption from the hindgut although most microbial biosynthetic products are not utilised by the animal and are voided in the feces. Therefore many hindgut fermenters resort to coprophagy and cecotrophy (Stevens and Hume, 1998).

The high levels of VFA in the proventriculus, 158.8 mM, and gizzard, 139.3 mM, suggest that there may be some foregut fermentative digestion in ostriches (Angel *et al.*, 1995; Swart *et al.*, 1987). VFA concentrations decreased in the small intestine ranging between 65-78 mM and increased in the hingut. The concentration in the ceca was 140,7 mM and increased further from the proximal colon to the distal colon. Quantitatively, acetate is the most important VFA and in the ostrich gizzard, proventriculus and stomach, it was the only VFA. The high molar proportions of acetate and low propionate proportions, are indicative of fibre fermentation or material high in plant cell walls (Swart *et al.*, 1987).

Since the production of VFA in the ostrich GIT does not necessarily mean that it is able to absorb and metabolise the acids, studies were conducted to assess the contribution of microbial digestion to the energy economy of these birds. The theoretical energy contribution of VFA could be as high as 76% of the metabolisable energy intake and the absorption and oxidative metabolism of end products from cellulose fermentation, was shown to contribute to the metabolisable energy requirements of the growing ostrich (Swart *et al.*, 1993a).

Diet influences the quantity and nature of the fermentation end products because it can change the metabolic activities of the microflora by providing new or different substrates (Bergman, 1990; Demeyer, 1981). The amount of VFA produced is affected by the amount of fibre in the diet, therefore the contribution of VFA to the energy needs of the body could become considerably greater as dietary fibre increases (Bergman, 1990). In ruminants, an increase in starch or soluble carbohydrate, such as cereal grain, and a decrease in roughage or a diet that is rapidly fermented, results in a decrease in the proportion of acetic acid and an increase in the proportion of propionic and butyric acid (Bergman, 1990; Demeyer, 1981; Latham, 1979). VFA concentrations in pigs increased as the fibre proportion reaching the hindgut increased. A high-fibre diet in rats has also been shown to increase VFA fecal production or absorption into the bloodstream. The amount of soluble carbohydrate as well as fibre reaching the large intestine thus obviously varies with diet as well with the animal species. Similarly, although not as highly variable as in the rumen, carbohydrates and sugars tend to result in a high propionate-to-acetate ratio and a decrease in the pH of the large intestinal contents (Bergman, 1990).

The rate of VFA production in the rumen also depends on the diet and the amount of feed consumed. As an example, for a 55 kg sheep on a maintenance dried-grass diet, rumen production rates obtained for acetate, propionate, and butyrate were 3.7, 1.0, and 0.7 mol/day, respectively, and these had energy equivalents of 773, 386, and 357 kcal/day, respectively. Therefore acetate provided about one-half while propionate and butyrate

each provided about one-fourth of the caloric energy contained in the three VFA (Bergman, 1990).

Even though VFA production in the rumen is highly variable, the total amount present is usually between 60 and 150 mM (Bergman, 1990). In cattle, daily VFA production has been calculated to be 3.7 kg of acetic acid, 1.1 kg of propionic acid and 0.6 kg of n-butyric acid, which yield 399 g of bacterial cells and 315 g of protozoan cells (dry weight) (Orpin and Anderson, 1988). VFA production in the rumen is dependent on both the composition of diet and the time after feeding. When animals graze on fresh grass or when fed starch-rich diets, exceptionally high values rising to 200 mM can occur. Maximal concentrations usually occur two to four hours after feeding. Total concentrations of VFA in the cecum, colon, rectum, and feces of all animals have been measured in the range of 30-240 mM but more commonly averaging 70-120 mM (Bergman, 1990).

When comparing total VFA concentrations at different GIT sites of mammalian species, ruminants had the highest VFA concentrations in the rumen while values in the small intestine were one-fifth less, followed by a peak in the cecum and colon. In non-ruminants and pigs, the highest VFA concentration was in the cecum, followed by the colon. Concentrations in the stomach and small intestine of horses and pigs was slightly higher than in rats and rabbits, suggesting slight gastric fermentation in the first two species. These findings of slight gastric fermentation have been confirmed in pigs and horses and have been extended to other non-ruminant herbivores or omnivores (Bergman, 1990).

In all species studied, each of the VFA is readily absorbed into the blood from all segments of the lower digestive tract as in the rumen (Bergman, 1990). VFA absorption is independent of the location of the fermentation chamber and appears to be mostly passive and increases linearly with corresponding decreases in pH or increases in concentration (Bergman, 1990; von Engelhardt *et al.*, 1989). Unlike in the rumen, individual VFA in the GIT are probably absorbed at comparable rates rather than in the

ascending order of acetate<propionate<butyrate (Bergman, 1990). Absorption of these acids also plays an important role in the absorption of Na and water (Stevens and Hume, 1998).

Most small herbivorous birds and mammals compensate for a limited gut capacity and higher rate of metabolism by selective retention of fluid and small particles in their cecum and more rapid excretion of larger digesta particles. Some species can adapt to a reduction in the digestibility of dietary fibre by increasing the cecal retention time of fluid, bacteria, and, to varying degrees, the small digesta particles. However, increased cecal retention time limits their ability to increase food intake and recover soluble nutrients on poorly digestible high fibre diets. However, greater gut capacities allow larger herbivores to adapt to less digestible diets by either increasing digesta passage, food intake, and soluble nutrient recovery, as seen in colon fermenters such as equids and wombat, or increasing digesta retention and fermentation time in a forestomach. In addition, foregut fermenters and coprophagic fermenters also have the advantage of utilising both their midgut and hindgut for recovery of the large secretions of fluid and electrolytes required for microbial fermentation, for digestion of microbial protein, absorption of amino acids and B-vitamins synthesised by bacteria (Stevens and Hume, 1998).

Other than providing energy and nutrition to the body, VFA have various physiological functions (Bergman, 1990; von Engelhardt *et al.*, 1989). They may indirectly influence cholesterol synthesis and even help regulate insulin or glucagon secretion (Bergman, 1990). In vivo, they stimulate cell proliferation in the intestinal epithelium, inhibit cell proliferation in vitro but are potent enhancers of gene expression in cultured cells (von Engelhardt *et al.*, 1989). VFA production and absorption have a very significant effect on epithelial cell growth, blood flow, and the normal secretory and absorptive functions of the large intestine, cecum, and rumen (Bergman, 1990). At physiological concentrations, VFA also stimulate epithelial cell division in both ruminant and non-ruminant animals (Dierick *et al.*, 1989).

1. 9 Protection by Gut Microflora

The role of the gut flora is multifunctional ranging from fermentation of feed compounds, induction of anatomical and physiological changes in the intestinal cell wall structure and increasing host resistance against enteropathogenic bacteria (Vanbelle *et al.,* 1990).

The property whereby the gut flora helps the animal to resist infections, particularly in the GIT has been referred to as bacterial antagonism; bacterial interference; barrier effect; colonization resistance and competitive exclusion (Fuller, 1989). Maybe the most important contribution of the gut microbiota is their ability to inhibit the colonization of pathogenic bacteria in the intestine. Mechanisms by which these bacteria contribute to colonization resistance include the establishment of an unfavourable pH, competition for nutrients, competition for attachment sites on the intestinal epithelium, and the local production of antibiotics known as bacteriocins (Gaskins, 1996).

The protective effect of the gut flora stems from the observation that germ-free animals, were more susceptible to disease than conventional animals with a complete intestinal microflora (Fuller, 1989; Savage, 1986). Studies conducted showed that it took ten cells of *Salmonella enteritidis* to kill a germ-free mouse but to kill a conventional mouse, 10^6 cells were required. This could be due to conventional animals having increased phagocytic activity and immunoglobulin levels compared with germ-free animals (Fuller, 1989). The indigenous flora is also known to influence the transit time in the tracts of conventional animals whereby digesta passes more rapidly through the tracts of conventional animals than those of germ-free animals. It has also been observed that some germ-free rats require vitamin K in their diet while conventional animals do not. In addition, some germ-free animal species require certain B vitamins in higher concentrations than their conventional counterparts (Savage, 1986).

Sufficient evidence exists to show that the indigenous gut bacteria also stimulates the host immune system to respond more quickly to pathogen invasion through bacterial antagonism and also stimulates the development and effectiveness of immunologic mechanims at the mucosal and systemic level (Berg, 1996; Gaskins, 1996). For example,

germ-free animals without normal gut bacteria have vastly underdeveloped immunologically relevant organs, including the intestines and when pathogenic gut bacteria was introduced, normal immunologic structure and function was swiftly restored in these germ-free animals (Gaskins, 1996). Studies have also shown that pretreatment of chicks with adult cecal cultures immediately prevents the growth of *Salmonella* in the lumen, therefore preventing the bird from shedding large numbers of salmonellas into the rearing environment (Impey and Mead, 1989).

1.10 Aims of this study

Despite its importance to the overall development and health of the GIT, relatively little is known about the gut microbiota of the ostrich. However, recent studies have shown that the ostrich hindgut harbour high concentrations of cellulolytic and hemicellulolytic bacteria (Van Gylswyk *et al.*, in press). These studies also showed that the anaerobic, intestinal bacteria play a role in the digestion of plant fibre. Therefore this study aims to determine the identity of the fibre-digesting bacteria isolated from the ostrich hindgut and also to estimate the relative abundance of some of these bacteria in the feces of adult domestic and wild ostriches. Domestic and wild fecal samples were analysed since the diets of these birds differ substantially and therefore the gut microflora may also differ. Knowledge of the fibre-digesting bacteria found in wild ostriches may allow the development of improved feed formulations for farm-reared birds.

CHAPTER 2

IDENTIFICATION AND CHARACTERISATION OF THE FIBRE-DIGESTING BACTERIA ISOLATED FROM THE OSTRICH HINDGUT

2.1 SUMMARY

With the aid of PCR amplification of the 16S rDNA genes and sequence analysis using the BLASTN search of the GenBank database, 11 bacterial strains isolated from the ostrich hind-gut were identified. Four of the cellulolytic strains, C1a, X18, X25 and TC30, were identified as *Ruminococcus* while two weakly cellulolytic strains, X13 and TC26, showed highest homology to the genus *Butyrivibrio*. Another weakly cellulolytic strain, B2 was identified as a member of the sulfur reducing bacteria, *Desulfovibrio*. Sequence analysis of four non-cellulolytic strains, X5, TC5, TC14 and TC23, showed closest identity to the genus *Bacteroides*. Sequence analysis was usually performed directly on the PCR product, however, those PCR products that did not yield a unique product were cloned into the plasmid vector pBluescript (SK) prior to sequencing. Results of the treeing analyses, using the neighbor-joining method, clearly indicated that the newly identified strains should be assigned to the genera *Ruminococcus*, *Butyrivibrio* or *Bacteroides*. The four *Ruminococcus* strains were shown to be phylogenetically related to cluster IV of the *Clostridium* subphylum. The phylogenetic analysis also revealed that the two *Butyrivibrio*-like isolates that were examined were phylogenetically related to cluster XIVa of the *Clostridium* subphylum of the Gram-positive bacteria. The non-cellulolytic strains were shown to be members of the *Bacteroides* subgroup within the *Bacteroides* cluster of the *Cytophaga-Flavobacter-Bacteroides* phylum. Analysis by the PCR-based typing method, repetitive extragenic palindromic PCR (REP-PCR), showed that there was considerable variability between strains of the same species.

2.2 INTRODUCTION

With the development of strictly anaerobic techniques and habit-simulating media, a variety of numerically important strictly anaerobic gut bacteria have been isolated (Hobson, 1966; Krause and Russell, 1996; Latham, 1979). However, analysing the microbial ecology of natural communities using traditional culture methods requires a great deal of time and effort, and has major shortcomings, in particular lack of accuracy (O'Sullivan, 1999). In addition, it has been recognised that a significant fraction of the microflora cannot be cultivated by standard culture techniques (Marchesi *et al.*, 1998; Sghir *et al.*, 2000; Suau *et al.*, 1999; Ward *et al.*, 1990). Despite these limitations, the combination of both culture and molecular based non-culture techniques are necessary to study such complex ecosystems (O'Sullivan, 1999).

Culture-dependant techniques are used to isolate culturable bacteria from fecal or intestinal samples using selective or non-selective media. Examples of non-selective media include rumen fluid-glucose-cellobiose agar (RGCA) (Hobson, 1966) and brain heart infusion (BHI) (Kaufmann *et al.*, 1997). Enumeration of specific bacterial genera is generally achieved with selective media such as YN-6 (Resnick and Lewin, 1981), a medium specifically used for the isolation of *Bifidobacterium* and acidified Man Rogosa Sharpe (MRS; Difco) (Clara *et al.*, 1992) media for the isolation of *Lactobacillus*. Following isolation, a variety of morphological and biochemical tests are required to confirm genus identity, however, the confidence level for species identification only increases as more tests are performed. Culture-independent techniques such as direct microscopic analysis and monitoring specific enzymes or metabolites have proven useful in giving valuable insight into the real numbers of microflora *in situ* in fecal samples. However, these tools are very limited in their ability to give any in depth characterisation of specific organisms (O'Sullivan, 1999).

The emergence of molecular approaches has greatly expanded the ability to reliably identify isolates, in some cases without prior cultivation of community members, provide

data on culturable and non-culturable bacteria and also to calculate the evolutionary relatedness between strains (Marchesi, 1998; O'Sullivan, 1999).

Currently sequence analysis of 16S ribosomal rDNA (rDNA) is being employed for the accurate typing of unknown isolates. This sequence-based typing method for classifying organisms and evaluating their evolutionary relatedness was first described by Woese (1987). Detailed studies involving the phylogenetic positions of unknown isolates has been made possible due to the available databases of rDNA sequences being extensive. Molecular phylogeny has not only revolutionised the field of microbial ecology but has also allowed meaningful phylogenetic relationships between microbes in natural communities to be discerned (Olsen *et al.*, 1994). This is feasible with the polymerase chain reaction (PCR) which can be used to directly amplify the 16S rRNA gene from the isolates using primers that are targeted at universally conserved regions at both ends of the gene. The entire PCR amplicon, which is approximately 1.5 kb, can then be directly sequenced and compared to the rRNA database (O'Sullivan, 1999). Molecular phylogeny has also greatly shaped our understanding of the phylogenetic relationships of the major genera found in the rumen and gastrointestinal tract of humans and animals. Three major genera, *Ruminococcus*, *Butyrivibrio* and *Bacteroides* are very heterogenous and the employment of 16S rDNA sequence analysis has contributed enormously to their phylogeny (Forster *et al.*, 1996; Rainey and Jansen, 1995; Shah and Collins, 1990).

Two other sequence-based typing methods include the internal transcribed spacer (ITS), the sequence between the 16S and 23S rRNA genes, and an internal portion of the *recA* gene. Comparing the three sequence-based typing and phylogenetic approaches, O'Sullivan stated that the 16S rRNA molecule was the only one capable of evaluating phylogenetic relationships between multiple genera while ITS and the short segment of the *recA* gene were only useful for intrageneric characterization. However, the phylogenetic relationship obtained by sequence analysis of the short segment of the *recA* gene, compared favorably with the analysis from the complete rRNA gene while the ITS molecule cannot be relied upon for meaningful phylogenetic analysis due to its high heterogeneity and low sequence conservation (O'Sullivan, 1999).

Other molecular typing tools available include phenotypic and genotypic fingerprint analysis. These methods are less sensitive than the sequence-based approaches. However, they can be used to divide the isolates into broader groups prior to the sequence-based typing protocols. These rapid techniques also provide information on the relatedness of different strains. Examples of phenotypic fingerprints include polyacrylamide gel electrophoresis of soluble proteins, fatty acid analysis, bacteriophage typing and serotyping. Serotyping has proven to be the most rapid and useful procedure as colonies can directly be typed, without subculturing, by colony hybridization with a monoclonal antibody specific for a particular genus, species or strain (O'Sullivan, 1999). This methodology has been applied to the analysis of two *Bacteroides* species in different human intestines (Corthier *et al.*, 1996).

Shortcomings of phenotypically based typing methods, particularly changes in the fingerprint which may not necessary mean a different organism but rather a change in expression of a particular phenotypic trait, has led to the development of typing methods based on the microbial genotype or DNA sequence (Olive and Bean, 1999; O'Sullivan, 1999). DNA-based typing methods not only minimize problems with typeability and reproducibility, but in some cases enables the establishment of large databases of characterized organisms (Olive and Bean, 1999). A few examples of DNA-based typing methods include pulsed-field gel electrophoresis (PFGE), ribotyping, random amplified polymorphic DNA (RAPD) assays and colony hybridization with nucleic acid probes, particularly targeted at 16S rRNA (Olive and Bean, 1999; O'Sullivan, 1999).

PCR-based fingerprinting techniques include multiplex PCR, arbitrary primed (AP) PCR, and triplet arbitrary primed (TAP) PCR (O'Sullivan, 1999). However, repetitive extragenic palindromic (REP) PCR is fast becoming the most widely used method of DNA typing (Olive and Bean, 1999). Repeated sequences are ubiquitous in the genomes of prokaryotes and although the precise functions of these repetitive elements are still obscure, their presence can be exploited for several applications and molecular genetic manipulations. Several authors have employed PCR with primers based on these highly conserved, defined interspersed repetitive elements for the amplification of regions

between neighboring repetitive elements (REP-PCR, ERIC-PCR and BOX-PCR) and this has been collectively designated as REP-PCR (Moraes *et al.*, 2000; Versalovic *et al.*, 1991).

PCR-based DNA typing methods such as repetitive sequence-based PCR (REP-PCR), have been cited as techniques that are universally applicable, rapid and with considerable discriminatory power (Lupski and Weinstock, 1992; Moraes *et al.*, 2000; Olive and Bean, 1999; Versalovic *et al.*, 1991). This PCR method employing the REP and ERIC sequences as PCR primers have successfully been used to differentiate strains of various pathogenic and enteric bacteria and to define the genetic relatedness among *Bacteroides fragilis* stains (Olive and Bean, 199; Moraes *et al*, 2000).

In this chapter, eleven bacterial strains isolated from the ostrich hindgut, were identified with the aid of PCR amplification of the 16S rDNA genes and sequence analysis. A comprehensive comparative sequence analysis was then performed to determine the phylogenetic positions of the newly identified strains.

2.3 MATERIALS AND METHODS

2.3.1 Bacterial isolates and plasmids

The bacteria were isolated from the ostrich hindgut in a previous study (van Gylswyk *et al.*, in press). Strains C1a, X18, X25 and TC30 were highly cellulolytic, while strains TC26, B2 and X13 were only weakly cellulolytic. The remaining strains, X5, TC5, TC14 and TC23 were non-cellulolytic. Strains X18, X25, X13 and X5 were isolated from xylan agar media while strains C1a ,TC30 and B2 were isolated from cellobiose liquid medium and GSCX media (0.1% each of glucose, starch, cellobiose and xylan) respectively. The remaining strains, TC5, TC14, TC23 and TC26 were isolated from non-specific media. The plasmid SK Bluescript (Appendix D) was used for cloning some of the 16S rDNA PCR products of the above mentioned isolates.

2.3.2 Culture media and growth conditions

All media were prepared under strict anaerobic conditions with the gas phase being O_2-free $C0_2$. The reducing solution (Appendix A) contained cysteine and sulfide in concentrated O_2-free alkaline solution and was added to the media after dispelling O_2 by heating to boiling point. The cellulolytic isolates were grown anaerobically at 37°C in cellobiose medium (medium A) (Appendix A) supplemented with 0.5% yeast extract and 1% cellobiose. The cellobiose was replaced with 1% glucose when growing the non-cellulolytic bacteria.

2.3.3 DNA extraction

Chromosomal DNA was isolated from 37°C overnight cultures grown in cellobiose medium (medium A) according to the methods of Demuyter *et al.* (1988) and Avgustin *et al.* (1994). A 100 ml of culture was harvested by centrifugation at 10 000 rpm for 10 minutes at 4°C and resupended in 5 ml TE buffer (Appendix A). A 2.5 ml volume of lysozyme solution (Appendix A) was then added and held at room temperature for 3 hours. A 0.5 ml portion of 10% SDS and 50 µl Proteinase K (20 mg/ml) was added and the mixture was incubated for 1 hour at 37°C. After the addition of 1.5 ml of CTAB (hexadecyltrimethylammonium bromide) and 1.8 ml of NaCl (5 M) the mixture was incubated for 20 minutes at 65°C. Thereafter the DNA was extracted with equal volumes of phenol and chloroform. This step was repeated several times. An equal volume of chloroform-isoamyl alcohol (24:1) was used to rid the mixture of any residual phenol. The nucleic acids were recovered from the aqueous phase by precipitating with 2.5 volumes of 100% ice cold ethanol and one-tenth volume 3 M NaAc (pH 5.2). The suspension was then centrifuged at 14 000 rpm for 10 minutes and the supernatant discarded. The pellet was washed with 1 ml 70% ethanol and centrifuged at 14 000 rpm for 5 minutes. The supernatant was removed, the pellet dried, resuspended in TE buffer (Appendix A) and treated with RNase (10 mg/ml) for 1 hour at 37°C. The DNA concentration was then quantified spectrophometrically by measuring the absorbance at 260 nm (A_{260} =1 is equivalent to 50 µg DNA).

2.3.4 Determination of the 16S rDNA gene sequences

A forward primer sequence tail (17-24 bases), homologous to the M13 forward sequencing primer, was attached to the PCR primer. Therefore a sequencing priming site was introduced into the PCR product (Figure 2.1). The 16S rDNA PCR primers were homologous to conserved regions of the 16S rDNA (represented by the bold bases in Figure 2.1). Fluoresecent end-labelled primers were used for direct PCR product sequencing (Figure 2.2) (Appendix B). The 16S rDNA was sequenced using a modified PCR strategy described by Weisburg *et al.* (1991) (Figure 2.3). The PCR primers utilized were homologous to the 16S rDNA gene at positions: F1 (8-27 bp), F3 (517-536 bp), R5 (1492-1512 bp) and R3 (1053-1074 bp). A DNA fragment encompassing the complete 16S rDNA gene (F1R5) and two overlapping DNA fragments (F1R3 and F3R5) encompassing most of the 16S rDNA gene were amplified using the GeneAmp PCR System 9700, Perkin Elmer thermal cycler. The PCR primers (F1, R1, F3, R3, F5, R5, Appendix B), with average sizes of 46 bases, were designed so that the 20-22 bases on the 3′ end were complimentary to one of the six highly conserved sequences in the bacterial 16S rDNA gene (see underlined sequences in Appendix B).

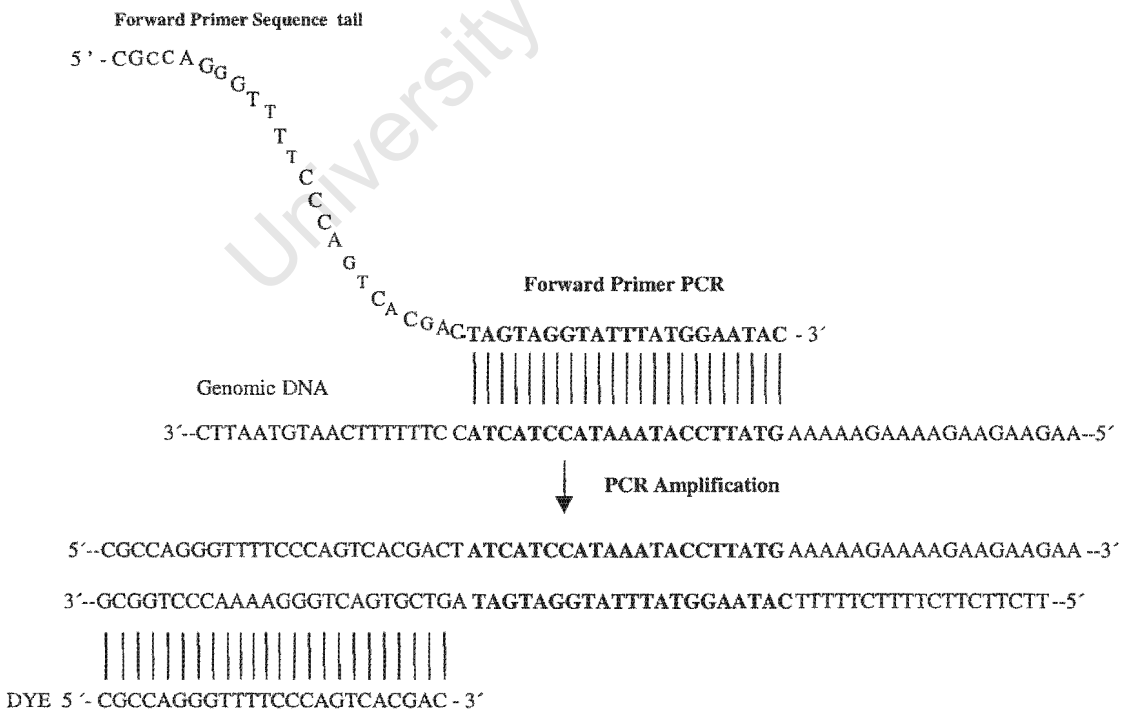


Figure 2.1. Strategy used for amplification of 16S rDNA sequences

Forward

PCR Primer

5′ CGC CAG GGT TTT CCC AGT CAC GAC _ _ _ _ _ _ _ _ _ _ 3′

Reverse

5′ CAG GAA ACA GCT ATG AC _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ _ 3′

Figure 2.2. Forward and reverse sequences of the fluorescent end-labelled primers.

Three primer pairs were used in the amplification reactions using 2.5 µl of 10 µM of each of the following combinations of forward (F) and reverse (R) primers: reaction 1 – F1R5; reaction 2 – F1R3 and reaction 3 – F3R5. Fifty nanograms of genomic DNA was amplified using cycle profile 1 (Appendix B). Fifty microlitre reactions were carried out using 5 units of SuperTherm Taq polymerase (Amersham), 10 µl 10 x Taq polymerase buffer (Amersham), equimolar amounts of all four dNTPS (6.25 mM each, Boehringer Mannheim) and 6 µl (25 mM) $MgCl_2$ (Boehringer Mannheim). The PCR products (20 µl) were analysed by electrophoresis (Sambrook *et al.*, 1989) on a 0.8% agarose gel (Appendix C) using a lambda (λ) *Pst*I digest to verify their sizes. The amplified products were subsequently purified using a HighPure PCR purification kit (Boehringer Mannheim). The DNA concentrations were then quantified spectrophometrically by measuring the absorbance at 260 nm.

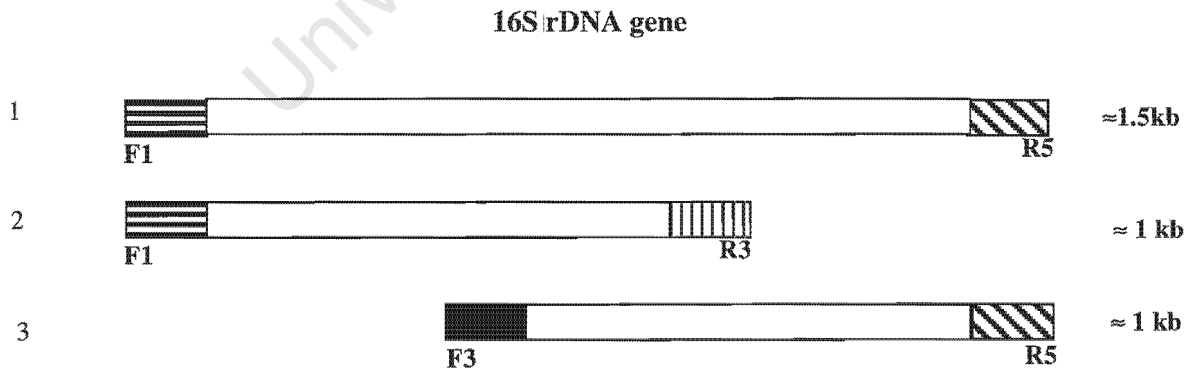


Figure 2.3. PCR strategy for amplifying the 16S rDNA gene using three different pairs of primers. Sizes of the amplified products are shown in kb.

2.3.5 Direct sequencing of 16S rDNA PCR products

The amplified 16S rDNA PCR products were sequenced using a Thermosequenase fluorescent- labeled primer cycle-sequencing kit (Amersham) and an ALFexpress™ DNA sequencer (Amersham Pharmacia Biotech using the ALFWin Version 1.10 software). The method is described in greater detail in Appendix C. The PCR products that did not yield a unique product were cloned and sequenced.

2.3.6 Cloning of 16S rDNA PCR products and restriction endonuclease digestion of cloned DNA

The plasmid vector pBluescript (SK) was cut with the restriction endonuclease *Eco*RV and dephosphorylated using the enzyme calf intestinal phosphatase (CIP) according to the methods of Sambrook *et al.* (1989). The ends of the PCR products were filled in using 1 unit of T4 polymerase (Boehringer Mannheim), 5 units of polynucleotide kinase (Boehringer Mannheim), 1 µl of 1 mM ATP, 5 µl of restriction buffer A (Boehringer Mannheim) and 6 µl of equimolar amounts of all four dNTPS (6.25 mM each, Boehringer Mannheim) in a final volume of 50 µl. The reaction tubes were incubated at 25°C for 30 minutes before heat inactivating the reaction at 70°C for 15 minutes.

After the end-repair reaction, the PCR products were ligated into the SK vector at a vector:insert ratio of 1 pmol:4 pmol using the method described by Sambrook *et al.* (1989). The ligated PCR products were then transformed into *E. coli* JM109 cells (Yannisch-Perron *et al.*, 1985) made competent with $CaCl_2$ using the method described by Dagert and Ehrlich (1979; Appendix C). One hundred microlitre amounts of transformed cells were plated on Mackonkey agar (Appendix A) supplemented with 100 µg/ml ampicillin (Sigma) to select for transformants. Pale pink colonies representing transformants were selected, inoculated into 5 ml sterile Luria broth (LB; Appendix A) containing 100 µg/ml ampicillin and incubated overnight at 37°C with agitation. Plasmid DNA was extracted from pelleted cells using the quick mini-prep method described by Zhou *et al.* (1990; Appendix C). The presence of cloned DNA was verified by digesting

the DNA according to the method described by Sambrook *et al.* (1989) with the restriction endonuclease *Pvu*II and resolving the fragments on a 0.8% agarose gel together with pBluescript (SK) restricted with *Pvu*II. The plasmid DNA containing the insert was quantified spectrophometrically.

2.3.7 Computer analysis of DNA sequences

In order to generate complete 16S rDNA gene sequences, the 16S rDNA fragment sequences obtained were aligned with each other using the Winconsin Package, version 9.1 (Genetics Computer Group, Madison Wis.) and DNAMan (Version 4.13, Lynnon Biosoft). Homology searches were performed using the program BLASTN V.2.0.4 (Altschul *et al.*, 1997) from the National Centre for Biotechnology Information (NCBI) via the Internet (www.ncbi.nlm.nih.gov).

2.3.8 Phylogenetic analysis

The 16S rDNA gene sequences were aligned against a number of sequences obtained from the Blast search results and the GenBank database (Appendix F) using DNAMan (Version 4.13, Lynnon Biosoft). Using DNAMan, a final region of 1269 nucleotides (positions 103 to 1398; *Escherichia coli* numbering, Accession J01859) was aligned for the *Ruminococcus* tree analysis, while the final region for the *Butyrivibrio* tree analysis was 1251 nucleotides (positions 103 to 1375). For the *Bacteroides* phylogenetic tree analysis, the final region was 1292 nucleotides (positions 110 to 1407). The stability of relationships was assessed using the programs SEQBOOT, DNADIST (Kimura's method option), NEIGHBOR, and CONSENSE of the PHYLIP package (Felsenstein, 1993). The unrooted phylogenetic trees were viewed using TreeView (Page, 1998). The resultant trees were evaluated in bootstrap analyses (Felsenstein, 1985), using the neighbour-joining method based on 1000 resamplings.

2.3.9 Repetitive extragenic palindromic PCR (REP-PCR)

To investigate the genetic relationship among the newly identified anaerobic bacteria, the profile of each sample was determined by REP-PCR according to the methods of Redkar *et al.* (1996) in the laboratory of Dr. Vito Delvecchio, Scranton, USA. The primer employed, RW3A, was derived from a *Mycoplasma pneumoniae* repetitive sequence (Wenzel and Herman, 1988).

2.4 RESULTS

2.4.1 PCR amplification of the 16S rDNA genes and restriction digestion of the cloned DNA

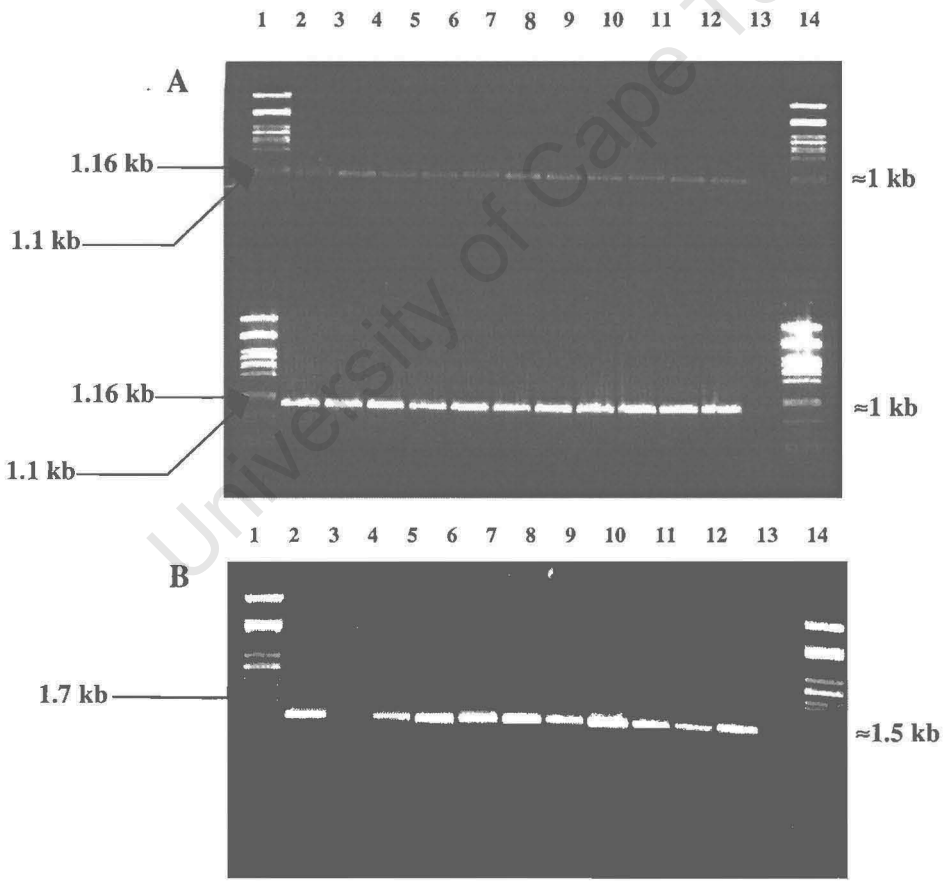


Figure 2.4. PCR amplification of the 16S rDNA genes using primer pairs (A). F1R3 (top) and F3R5 (bottom) (B). F1R5. Lane 1, λ-*Pst*I digest; lane 2, C1a; lane 3, X18; lane 4, X25; lane 5, TC30; lane 6, TC26; lane 7, X13; lane 8, X5; lane 9, TC5; lane 10, TC14; lane 11, TC23; lane 12, B2; lane 13, sterile distilled water and lane 14, λ-*Pst*I digest. Sizes of the amplified products are shown in kb.

With the aid of the universal eubacterial 16S rDNA primers F1R3, F3R5 and F1R5, the 16S rDNA gene sequences of all the isolates were successfully amplified. Amplification with the F1R3 and F3R5 primer pairs yielded PCR fragments that were about 1 kilobase pair (kb) in size (Figure 2.4A). The F1R5 primer pair yielded a fragment of about 1.5 kb which represents the complete 16S rDNA gene sequence (Figure 2.4B).

Following amplification, the PCR products were either directly sequenced or cloned into SK (Figure 2.5). Digestion of recombinant plasmids with the restriction endonuclease *Pvu*II would generate two DNA fragments of 2.9 kb and 1.45 kb in size, the latter representing the insert (lanes 2, 8 and 9).

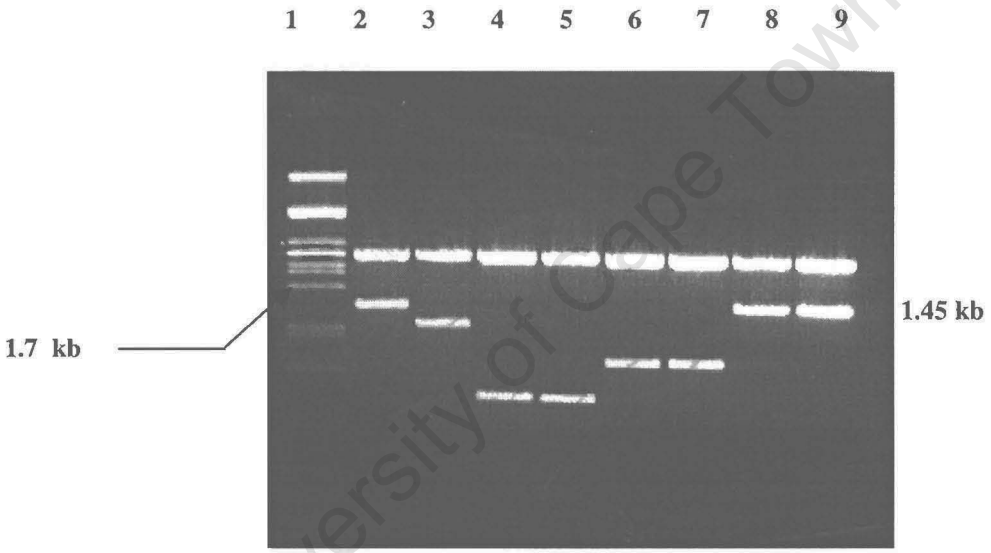


Figure 2.5. Restriction digestion of the 16S rDNA PCR products cloned into SK pBluescript and digested with *Pvu*II. Lane 1, λ- *Pst*I digest; lanes 2, 8 and 9 represent the recombinant plasmids containing the 1.45 kb insert. Sizes of the digested plasmids are shown in kb.

2.4.2 Sequence analysis of the 16S rDNA gene sequences

The 16S rDNA gene sequences ranged in size from 1368 to 1527 nucleotides (Appendix E). To determine whether they were homologous to any known sequences, a homology search of the GenBank database was performed using BLAST (Table 2.1). This indicated that the cellulolytic isolates C1a, X18, X25 and TC30 were members of the genus *Ruminococcus*. Strains C1a, X18 and TC30 were highly homologous to the species *Ruminococcus flavefaciens*, showing 97%, 96% and 95% sequence identity respectively,

while strain X25 exhibited 93% sequence identity to *Ruminococcus callidus*. The weakly cellulolytic strains TC26 and X13 showed closest identity to the genus *Butyrivibrio* with TC26 having 95% sequence identity to an unidentified rumen bacterium RF22 while X13 showed 94% sequence identity to an uncultured bacterium A11. The isolate RF22 has been shown to have 95% sequence identity to *Butyrivibrio fibrisolvens* H10b while strain A11 has been shown to have 91% sequence identity to *Butyrivibrio fibrisolvens* NCDO2397. Sequence analysis of the non-cellulolytic strains, namely TC5, TC14, TC23 and X5 showed highest identity to the *Bacteroides* genus. Strains TC5 and TC14 showed 92% and 89% sequence identity to *Bacteroides* sp. AR29 respectively while TC23 had 97% sequence identity to *Bacteroides ovatus* ATCC8484T. Strain X5 showed closest identity (94%) to *Bacteroides eggerthi* NCTC11185 while strain B2 had a 95% sequence identity to *Desulfovibrio desulfuricans*.

Table 2.1. Percentage sequence similarity of the 16S rDNA sequences of the isolates to the 16S rDNA sequences available in the GenBank database.

Strain	Species	% Sequence Similarity	GenBank Accession number
X25	*Ruminococcus callidus*	93%	X85100
TC30	*Ruminococcus flavefaciens* LP C14-Adx	95%	AF104834
X18	*Ruminococcus flavefaciens NJ*	96%	AF030446
C1a	*Ruminococcus flavefaciens*	97%	X83430
TC26	Unidentified rumen bacterium RF22	95%	AF001754
X13	Uncultured rumen bacterium A11	94%	AF052412
TC14	*Bacteroides* sp. AR29	89%	AF139525
TC5	*Bacteroides* sp. AR29	92%	AF139525
TC23	*Bacteroides ovatus* ATCC8483T	97%	X83952
X5	*Bacteroides eggerthii* NCTC11185	94%	L16485
B2	*Desulfovibrio desulfuricans*	95%	M34113

2.4.4 Phylogenetic analysis of the identified isolates

The phylogenetic dendrograms (Figures 2.6 and 2.7), show the positions of the newly identified strains C1a, X18, X25, TC30, TC26 and X13 within the *Clostridium* subphylum of the Gram-positive bacteria. Figure 2.6 shows that strains C1a, X25, X18 and TC30 are highly related to members of the genus *Ruminococcus* and fall within cluster IV of the *Clostridium* subphylum as defined by Collins *et al.* (1994). Similarly, from the unrooted phylogenetic tree in Figure 2.7, it is clear that strains TC26 and X13 are members of the *Butyrivibrio* genus and fall within cluster XIVa of the *Clostridium* subphylum. In contrast, Figure 2.8 shows that strains TC5, TC14, TC23 and X5 are members of the *Bacteroides* subgroup and fall within the *Bacteroides* cluster of the *Cytophaga-Flavobacter-Bacteroides* (CFB) phylum (Paster *et al.*, 1994).

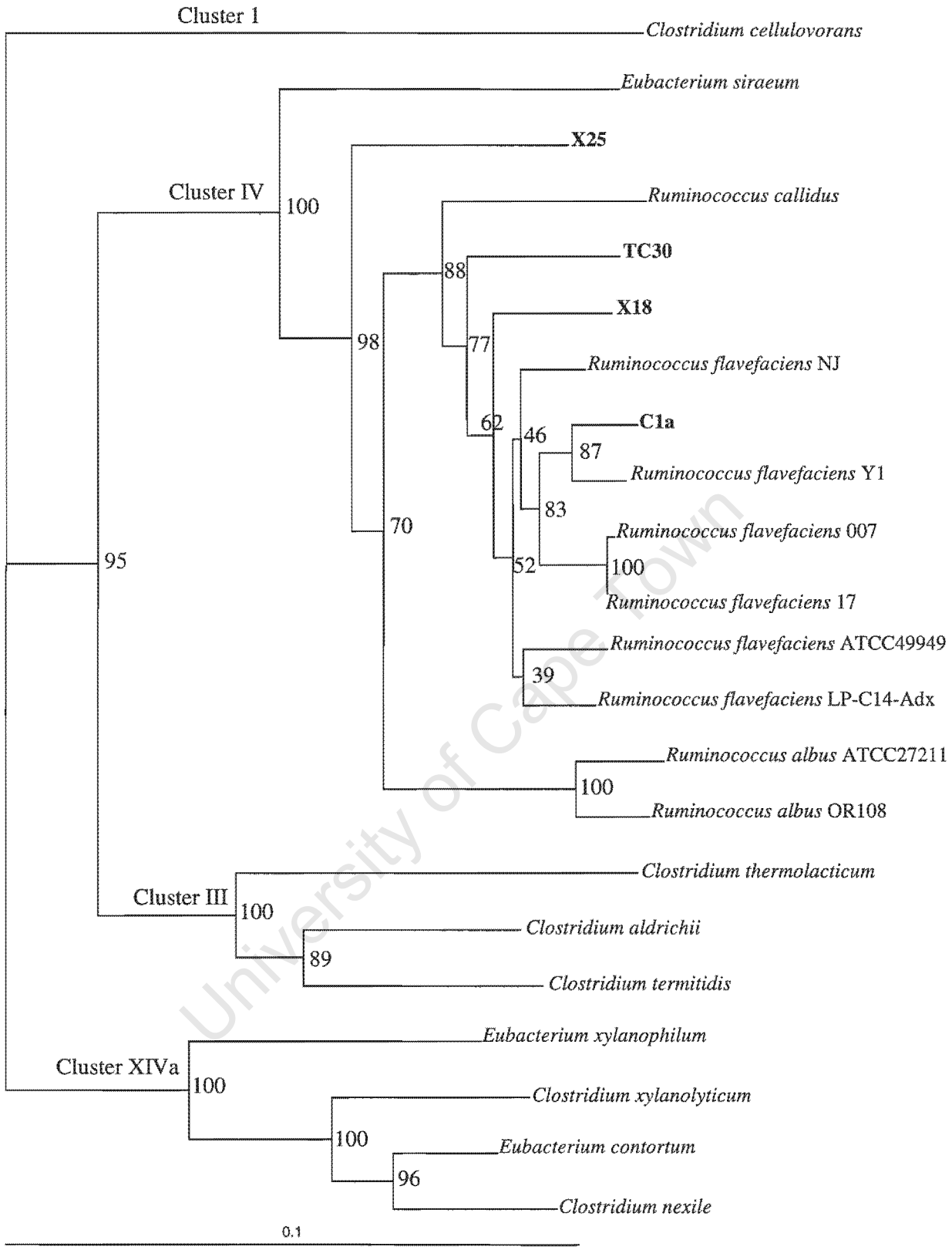


Figure 2.6. Unrooted phylogenetic tree obtained from performing a neighbor-joining analysis of a distance matrix from a multiple sequence alignment, showing the positions of *Ruminococcus* strains, C1a, X18, X25 and TC30 within cluster IV of the *Clostridium* subphylum of the gram-positive bacteria (Collins *et al.*, 1994). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. The scale bar represents 0.1 nucleotide substitutions per site.

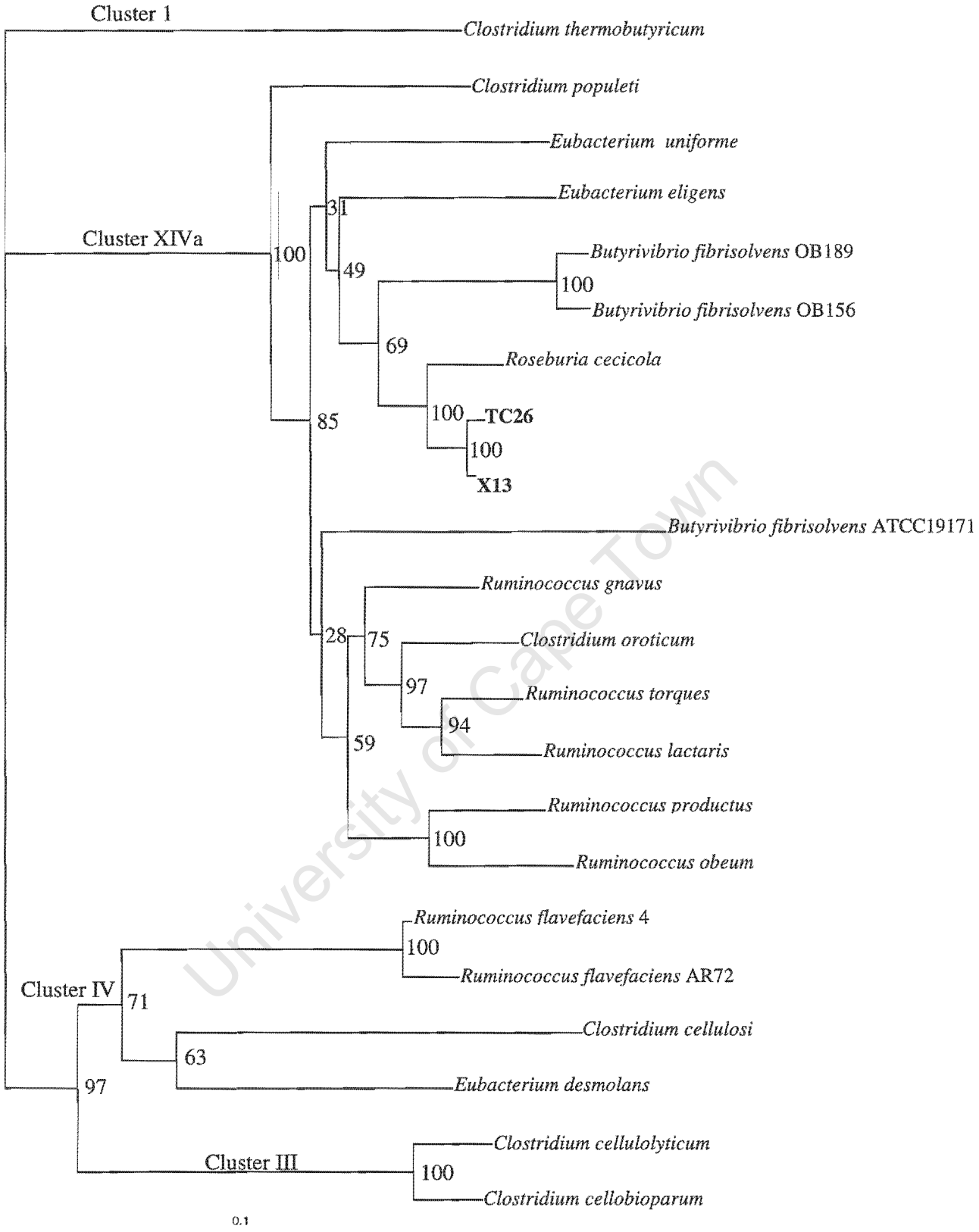


Figure 2.7. Unrooted phylogenetic tree obtained from performing a neighbor-joining analysis of a distance matrix from a multiple sequence alignment, showing the positions of *Butyrivibrio* strains, TC26 and X13, within cluster XIVa of the *Clostridium* subphylum of the gram-positive bacteria (Collins *et al.*, 1994). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. The scale bar represents 0.1 nucleotide substitutions per site.

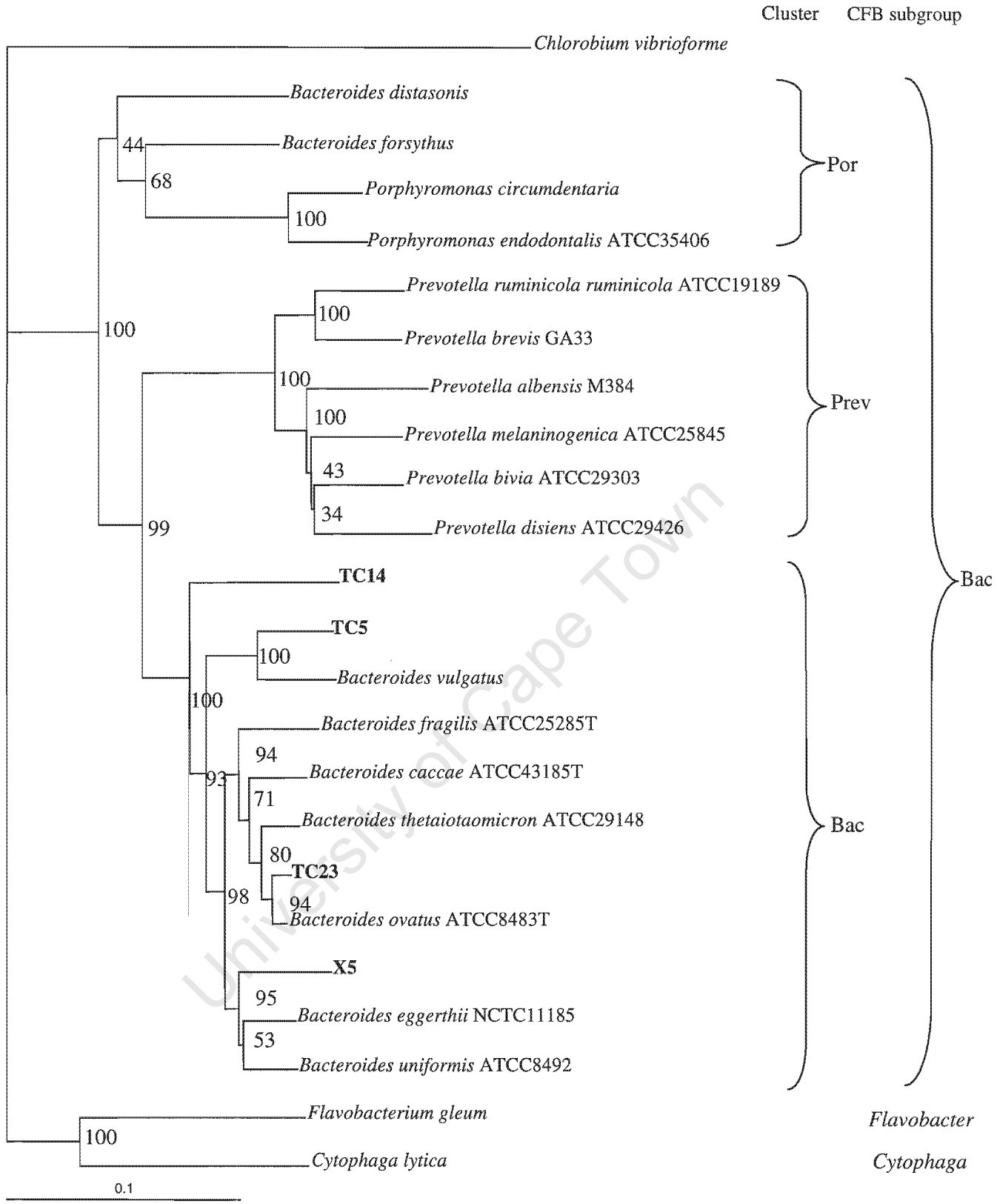


Figure 2.8. Unrooted phylogenetic tree obtained from performing a neighbor-joining analysis of a distance matrix from a multiple sequence analysis, showing the positions of *Bacteroides* strains, TC5, TC14, TC23 and X5 in the *Bacteroides* (Bac) subgroup within the *Bacteroides* cluster of the CFB phylum (Paster *et al.*, 1994). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. The abbreviations Por and Prev represent the *Porphyromonas* and *Prevotella* clusters respectively. The scale bar represents 0.1 nucleotide substitutions per site.

2.4.5 Repetitive extragenic palindromic PCR (REP-PCR)

The rep-PCR genomic fingerprint (Figure 2.9) showed that all of the newly identified bacteria had distinct patterns. This method was effective in showing that strains C1a (lane 2), TC30 (lane 3) and X18 (lane 4) which were all identified as members of the type species *R. flavefaciens*, are indeed different strains. Similarly, it also showed that strains TC5 (lane 10) and TC14 (lane 9) are distinct strains, even though they were had 92% and 89% sequence identity to *Bacteroides* sp. AR29 respectively.

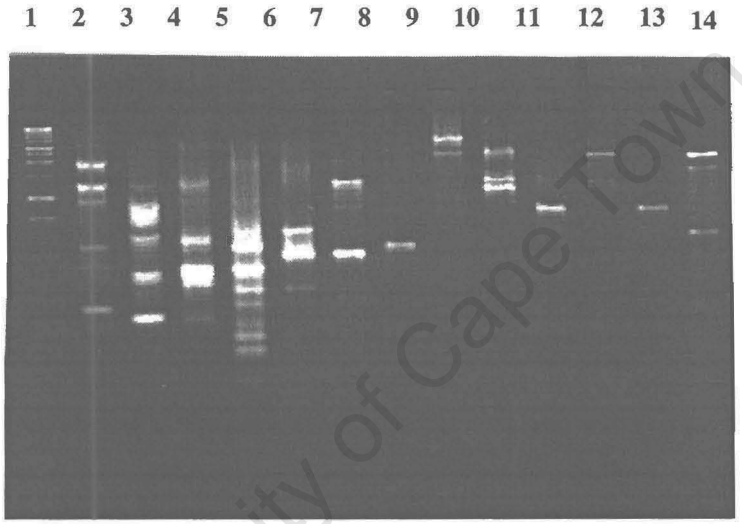


Figure 2.9. Rep-PCR fingerprints of the ostrich hindgut bacteria. Lane 1, 1-kb ladder; lane 2, C1a; lane 3, TC30; lane 4, X18; lane 5, X25; lane 6, TC26; lane 7, X13; lane 8, TC23; lane 9, TC14; lane 10, TC5; lane 11, X5; lane 12, B2; lane 13, M2 and lane 14, 100-bp marker.

2.5 DISCUSSION

The 16S rDNA gene sequences of 11 bacterial strains were amplified using PCR and their nucleotide sequences were determined either through direct sequencing of the PCR products or after cloning the PCR products into SK. The sequence strategy employed resulted in the derivation of approximately 90% of their complete 16S rDNA primary structures (> 1300 nucleotides per gene). The derived 16S DNA sequences have not been deposited in any of the available data libraries.

The high 16S rDNA sequence homologies (89%-97%) (Table 2.1) strongly suggests that the newly identified bacteria should be assigned to three major bacterial genera, namely, *Ruminococcus*, *Butyrivibrio* and *Bacteroides*. One other bacterial strain, B2, was identified as a member of the sulfur reducing genus *Desulfovibrio*. The sequence results also confirm the phenotypic and biochemical characterisation tests which have been performed on these bacteria in a previous study conducted by van Gylswyk *et al.* (in press). It is important to note that the bacterial isolates have only been identified to the genus level. When classifying strains to the species level using 16S rDNA sequences, there may be a small amount of intraspecific variation within and between 16S rDNA sequences of strains which have been deposited in the GenBank database (Clayton *et al.*, 1995). In addition, when distinguishing between species, one should not rely solely on 16S rDNA information. Based on different biochemical characteristics, Fox *et al.* (1992) argued that *Bacillus globisporus* W25 and *B. psychrophilus* W16A are distinct species despite sharing 99.5% 16S rDNA sequence similarity.

In order to determine the phylogenetic positions of the newly identified strains, a comprehensive phylogenetic analysis was performed which included a number of sequences obtained from the Blast search results and from the GenBank database. These sequences included representatives of some of the clusters of the *Clostridium* subphylum. For example, representatives of cluster I included *C. cellulovorans* and *C. thermobutyricum*, and these were used as outgroups in the *Ruminococcus* and the *Butyrivibrio* tree analyses respectively. The other sequences used in the analysis were representatives from clusters III, IV and XIVa. For the *Bacteroides* tree analysis, representatives from three of the five major phylogenetic subgroups of the CFB phylum were included, namely *Cytophaga*, *Bacteroides*, and *Flavobacter* (Paster *et al.*, 1994). The outgroup used in the *Bacteroides* tree was *Chlorobium vibrioforme*. The phylogenetic analyses based on 16S rDNA gene sequence data confirm the 16S rDNA sequence results that were obtained. High bootstrap values give a strong indication of the relatedness of each of the new strains to strains used in the phylogenetic tree analysis. Of the eleven new strains identified, four (C1a, X25, X18 and TC30) fell into the genus *Ruminococcus* within the *Clostridium* cluster IV (Collins *et al.*, 1994) which hosts the

type species *R. flavefaciens* as well as *R. albus* and *R. callidus*. Strains C1a, X18 and TC30 fell within the same branch along with the type strains *R. flavefaciens* ATCC49949 and *R. albus* ATCC27211. Therefore these strains may be more closely related to each other than X25 which forms part of a different branch but still shows strong relatedness to members of cluster IV. The treeing analyses also revealed that TC26 and X13 are closely related to members of the *Butyrivibrio* genus within the *Clostridium* cluster XIVa (Collins *et al.*, 1994). This cluster has been shown to encompass a broad spectrum of species belonging to various genera. For example, *Eubacteria*, *Roseburia*, *Clostridia* and *Ruminococcus* species such as *R. torques*, *R. obeum*, *R. productus*, *R. lactaris* and *R. gnavus*. The *Butyrivbrio* tree analysis also reveals that TC26 and X13 exhibit a closer affinity with *Roseburia cecicola* and the plasmid-bearing strains OB156 and OB189 than to the type strain D1 (ATCC19171) (Forster *et al.*, 1997). This explains the negative signals obtained when probe D1 was used as a PCR primer in conjunction with the universal eubacterial primer R5. Perhaps TC26 and X13 are similar to the strains of *Butyrivibrio*-like bacteria isolated from the rumen of deer (Forster *et al.*, 1997). Phylogenetic analysis of the non-cellulolytic strains clearly indicated that these strains should be assigned to the *Bacteroides* subgroup within the *Bacteroides* cluster (Paster *et al.*, 1994) which hosts *B. fragilis*, the type species of the genus *Bacteroides* (Paster *et al.*, 19994; Shah and Collins, 1990).

While the 16S rDNA genes have been used to identify new species of organisms, they show limited variability between strains the same species (Olsen and Woese, 1993; Woese, 1987). For this reason, the PCR-based typing method, REP-PCR was employed. This study demonstrated that repetitive extragenic sequences such as REP are present in the genomes of *Ruminococcus*, *Butyrivibrio* and the *Bacteroides* strains that were examined. Therefore it confirms the suggestion made by other authors that these sequences are ubiquitous in prokaryotic genomes (Lupski and Weinstock, 1992; Versalovic *et al.*, 1991). Since 16S rDNA is not a reliable method for identifying bacteria to the species or strain level, PCR-based typing methods such as REP-PCR is particularly useful in showing variability among strains of the same species. For example, from the sequence analyses (Table 2.1), isolates TC5 and TC14 were shown to have 92% and 89%

sequence identity respectively, to *Bacteroides* sp. AR29. However, the REP-PCR method (Figure 9) clearly showed that these two bacterial strains have completely different DNA fingerprints. Therefore these two isolates must be two distinct strains, possibly belonging to different species.

Previous reports suggest that the bacteria isolated from the ostrich hind-gut, and identified as members of the genera *Ruminococcus*, *Butyrivibrio* or *Bacteroides*, are also found in other hind-gut fermenters as well as ruminants. According to Hespell *et al.* (1997) and Krause *et al.* (1999), *R. flavefaciens* and *R. albus* have commonly been isolated as major cellulolytic species in the rumen contents of cattle and sheep. These species were also found in the rumens of wild ruminants such as musk oxen, the cecum and colon of swine, and the gut of guinea pigs (Hespell *et al.*, 1997). *R. flavefaciens* has also been shown to be the predominant cellulolytic bacterial species within the equine ceca (Julliand *et al.*, 1999). Other cellulolytic bacteria such as *R. albus* were also present but in lesser numbers. *R. flavefaciens* was also isolated from pigs, however, to date *R. albus* does not appear to have been isolated from the pig gut (Stewart, 1997). *Ruminococcus* sp. such as *R. flavefaciens*, *R. albus* and *R. callidus* have also been isolated from humans (Wang *et al.*, 1997; Wedekind *et al.*, 1988). Unlike the results obtained in this study, Krause *et al.* (1999) stated that *R. flavefaciens* and *R. albus* were usually the predominant species in the rumen, while *R. callidus*, *R. bromii*, *R. torques*, *R. gnavus*, *R. lactaris* and *R. obeum* were usually isolated from the hind-gut of non-ruminants.

The hemicellulolytic bacterium, *B. fibrisolvens* has also been one of the most common bacteria isolated from the rumen of domestic and wild animals. Forster *et al.* (1997) stated that *B. fibrisolvens* has been isolated from cattle fed hay, hay grain mixtures, or alfalfa sileage or from animals on pasture. They were also found in the gastrointestinal tract of a variety of animals (Hespell *et al.*, 1997). Hemicellulolytic bacterial species found in humans included the genera *Butyrivibrio*, *Bacteroides* and *Clostridia* (Wedekind *et al.*, 1988). *Bacteroides* sp. were also isolated from pigs (Stewart, 1997). *Desulfovibrio* species were shown to be the most abundant Gram-negative sulfur reducing bacteria

present in the rumen and the gastrointestinal tract of nearly all animals (Hespell *et al.*, 1997; Lin *et al.*, 1997).

When comparing the ostrich hindgut bacteria to those found in other birds, many other genera have been identified in these other birds. For example, the predominant cecal bacteria found in chickens were Gram-positive anaerobic cocci such as *Coprococcus*, *Peptostreptococcus*, and several types of *Streptococcus*, particularly *Streptococcus pleomorphus* (Barnes, 1979; Mead, 1989). *S. pleomorphus* has also been found in turkeys and ducks (Mead, 1989).

Other major bacterial types were Gram-negative, non-sporing rods belonging to *Bacteroidaceae* and Gram-positive, non-sporing rods that were identified as *Eubacterium* sp (Barnes, 1986; Mead, 1989). Strains of *Bacteroidaceae* from chickens include *Bacteroides hypermegas* which was reclassified by Shah and Collins (1982) within the genus *Megamonas* (Barnes, 1979; Mead, 1989). *M. hypermegasi* has also been found in game birds such as turkey and ducks (Mead, 1989). Another species of *Bacteroides*, *B. vulgatus* is also normally found in the ceca of chicks (Barnes, 1979). *Bacteroides* sp. was also shown to be one of the main gut genera present in captive willow grouse but was not reported to be found in wild willow grouse. Instead, spirochetes, flagellates and amoebae were found in wild willow grouse but not in the captive grouse (Hanssen, 1979). Other major microflora present in chickens include *Clostridium* sp., *Bifidobacterium* sp. and budding bacteria such as *Gemmiger formicilis* (Barnes, 1979; Barnes, 1986; Mead, 1989).

CHAPTER 3

DETECTION OF *BACTEROIDES* AND *PREVOTELLA* AND THEIR CONTRIBUTION TO TOTAL EUBACTERIAL 16S rDNA IN ADULT DOMESTIC AND WILD OSTRICH BIRDS

3.1 SUMMARY

In an attempt to overcome problems related to the presence of endogenous PCR inhibitors in fecal extracts, two extraction methods were employed to isolate genomic DNA from domestic and wild ostrich fecal samples. One of these methods, method A, combined cell lysis (chemical and mechanical) with protein denaturation followed by purification of the DNA with phenol-chloroform and phase lock gel (PLG) columns (5 Prime). Method B (QIAamp method) was similar to method A with a notable exception: DNA purification and recovery was achieved using a silica-gel membrane immobilised on a small filter in a 1.5 ml centrifuge tube. However, both methods yielded PCR-quality DNA although the QIAamp method proved to be more rapid and less hazardous compared to method A. PCR was applied to the DNA samples to detect the presence of *Bifidobacterium* as well as *Bacteroides* and *Prevotella*. This was achieved by employing 16S rRNA-targeted primers specific for these genera. *Bifidobacterium* was not detected in any of the fecal samples tested. However, *Bacteroides* and *Prevotella* were detected in the domestic fecal samples at 0.5 ng, 1 ng and 10 ng using the genomic DNA as templates. In contrast, when the 16S rDNA were employed as templates, *Bacteroides* and *Prevotella* were also detected at the lowest fecal concentration employed, 0.1 ng. No PCR results were observed at the higher fecal concentrations of 100 ng and 200 ng. Similarly, no PCR signals, using the *Bacteroides/Prevotella* specific primers, were observed for any of the wild fecal samples using the genomic DNA or the 16S rDNA, irrespective of the method from which they were obtained. In order to estimate the approximate proportion of *Bacteroides* 16S rDNA to total eubacterial 16S rDNA, FD1, a universal eubacterial primer, or rBacPre, a primer specific for *Bacteroides* and *Prevotella*, were end-labelled and applied as probes to the 16S rDNA amplicon from the fecal samples. *Bacteroides* was estimated to account for 40%, 89% and 25% for domestic samples 1, 2 and 3 respectively, when employing the DNA amplified from method A genomic DNA. However, when method B DNA was utilised, the percentages ranged from 33% to 87% for domestic samples 1 and 3 respectively. The data obtained for domestic sample 2, when employing method B DNA resulted in an overestimation which could possibly be linked to errors in the methodology employed.

3.2 INTRODUCTION

It has long been recognised that the gut microflora play significant roles in human and animal health. Therefore their composition and activities are of paramount importance to human and animal immunology, nutrition and pathogenesis and hence to the health of the host (Kaufmann *et al.*, 1997; Matsuki *et al.*, 1999; Wang *et al.*, 1996). Traditional methods for identifying fecal bacteria are time-consuming and sometimes cannot distinguish the bacteria on the species level (Wang *et al.*, 1996).

In contrast, the polymerase chain reaction (PCR) is an extremely powerful and rapid procedure for detecting a wide variety of microorganisms in complex biological samples (Monteiro *et al.*, 1997; Wang *et al.*, 1996; Wang *et al.*, 1997). Several authors have employed PCR with genus, species or strain-specific primers based on the 16S rDNA genes to examine the gastrointestinal communities of humans and various animals (Kok *et al.*, 1996; Matsuki *et al.*, 1999; Wang *et al.*, 1996; Wang *et al.*, 1997, Wood *et al.*, 1998). This approach has led to the detection of a variety of strictly anaerobic bacteria including species of *Bifidobacterium*, *Ruminococcus*, *Lactobacillus*, *Eubacteria* and *Bacteroides*.

Another innovative application of PCR has been the cataloging of bacterial abundance in the environment. This method involves the extraction of nucleic acids from an environmental sample and PCR amplification of the 16S rDNA genes with universal, degenerate primers (Polz and Cavanaugh, 1998). Following isolation of the 16S rDNA product, three strategies are currently being employed for the analysis of the intestinal microflora: cloning and sequencing of individual rDNA genes, separation of individual rDNA products by denaturing gradient gel electrophoresis (DGGE) and checkerboard hybridization with specific probes (O'Sullivan, 1999; Polz and Cavanaugh, 1998). These approaches have revealed the existence of many novel microorganisms because it circumvents bias introduced by traditional culture-based methods which typically detect only a fraction (<1%) of the total bacteria present in an environment (Polz and Cavanaugh, 1998).

When the amplified 16S rDNA product from a fecal sample is cloned into a standard sequencing vector, a bank of individual rRNA gene clones can result which can then be sequenced and phylogenetically analysed (O'Sullivan, 1999). This PCR cloning system has been successfully applied to a variety of ecosystems including the rumen (Whitford *et al.*, 1998), the human colon (Suau *et al.*, 1999; Wilson and Blitchington, 1996) and the gut of termites (Paster *et al.*, 1996).

On the other hand, DGGE can separate individual rRNA genes from the universally amplified product, and although the individual rRNA species within the amplicon are of the same length, electrophoresis through a linearly increasing gradient of denaturants can separate the products of different sequence (O'Sullivan, 1999). This procedure is based on the melting of rRNA genes at specific denaturing points based on their sequence. Therefore each individual sequence will begin to melt at a characteristic denaturing point. The melting changes the conformation of the DNA molecule, slowing its migration through the gel. Instead of denaturing, temperature can also be used, thus creating a temperature gradient gel electrophoresis (TGGE).

In contrast, the checkerboard hybridization approach has the ability to rapidly detect certain microbes in a mixed population (O'Sullivan, 1999). Kok *et al.* (1996) used this approach to detect a probiotic *Bifidobacterium* strain and amplify its DNA directly from fecal samples using species-specific primers targeted at the 16S rDNA gene. A more sensitive approach entails the use of universal rDNA primers to amplify the rDNA amplicon from feces and subsequently probe the amplicon with genus or species-specific oligonucleotide probes. This approach can also be adapted to the analysis of multiple samples with multiple probes at once using checkerboard hybridization (O'Sullivan, 1999).

Other non-PCR approaches used to identify bacterial species from natural communities include the use of genus or species-specific oligonucleotide probes targeted at the 16S rRNA gene. Employment of 16S rRNA-targeted probes has led to the discovery of *Ruminococcus* sp., such as *R. albus* and *R. flavefaciens* (Krause and Russell, 1996;

Odenyo *et al.*, 1994); *Bacteroides* (*Fibrobacter*) *succinogens* (Odenyo *et al.*, 1994; Lin and Stahl, 1995; Stahl *et al.*, 1988); *Bifidobacterium* sp., such as *B. bifidum*, *B. breve*, *B. infantis*, *B. longum* and *B. adolescentis* (Yamamoto *et al.*, 1992) and *Butyrivibrio fibrisolvens* (Forster *et al.*, 1997). The *in situ* application of 16S rRNA-targeted probes has also been described for the genus *Bifidobacterium* (Langendijk *et al.*, 1995). Some authors have also used 16S rRNA-targeted probes to quantify bacterial groups such as *Bacteroides*, *Bifidobacterium* and *Lactobacillus* (Sghir *et al.*, 2000) and species of *Bifidobacterium* (Kaufmann *et al.*, 1997). Oligonucleotide probes targeting other regions of the ribosomal RNA has also been described and these have been used to quantify several phylogenetically-defined groups of methanogens and sulfur reducing bacteria in the gastrointestinal tracts of a variety of domestic animals (Lin *et al.*, 1997). A short region of the *recA* gene has also been employed to detect *Bifidobacterium* in the human intestine (Kullen *et al.*, 1997).

Fecal samples are a convenient means of studying the microbial communities within the gastrointestinal tract, however, feces remain the most difficult specimens for DNA extraction and amplification (Clement and Kitts, 2000; Monteiro *et al.*, 1997; Morell, 1995). The major difficulty in employing PCR for detection of microorganisms from fecal extracts is the presence of substances that inhibit PCR or reduce the amplification efficiency (Al-Soud and Rådström, 1998; Clement and Kitts, 2000; Monnier *et al.*, 1996). These inhibitors may act through one or more of the following mechanisms: (a) interference with the cell lysis step, (b) degradation or capture of the nucleic acids, or (c) inactivation of the thermostable DNA polymerase (Al-Soud and Rådström, 1998).

The most common PCR inhibitors present in feces are the complex polysaccharides, particularly the aciduric types such as dextran sulfate and gum ghatti, possibly originating from plant material in the diet (Al-Soud and Rådström, 1998; Demeke and Adams, 1992; Monteiro *et al.*, 1997). Studies conducted by Demeke and Adams (1992) showed that the inhibitory effects of some aciduric polysaccharides could be reversed by the addition of certain buffer additives, such as Tween 20, DMSO and polyethylene glycol at certain concentrations. Other studies also showed that various aciduric polysaccharides had

inhibitory effects on the activity of certain DNA restriction endonucleases such as *Hind*III and *Eco*RI, even at low concentrations. These authors also found that Elutip-d columns (Schleicher and Schuell) were effective in removing polysaccharides from DNA except for certain aciduric polysaccharides (Do and Adams, 1991).

Different techniques such as boiling, dilution, filtration, enrichment media, aqueous two phase-systems, density gradient centrifugation and immunological techniques are currently being employed to facilitate PCR and to reduce the effect of PCR inhibitors (Al-Soud and Rådström, 1998). Other researchers have employed methods that include separating cells and fecal debris by dilution and centrifugation (Wang *et al.*, 1996). According to Clement and Kitts (2000), these procedures may eliminate cells attached to the debris and therefore render the assay biased.

Al-Soud and Rådström (1998) showed that inhibition of *Taq* DNA polymerase amplification by PCR inhibitors present in biological samples can be reduced or eliminated by using an appropriate thermostable DNA polymerase. These authors also showed that decreasing the fecal concentration resulted in a marked difference in the activity of the polymerases, such that the lowest fecal homogenate, 0.04%, was found not to be inhibitory on most of the polymerases they tested. Their studies also showed that the polymerase *Pwo*, from *Pyrococcus woesei* was the only enzyme capable of effectively amplifying at a fecal concentration of 2%.

Much effort has also been devoted to the extraction of DNA from feces since the commonly used methods are long, complex and time-consuming. Examples of these include phenol-chloroform extractions; treatment with CTAB, a detergent that has been reported to effectively remove PCR inhibitors from feces, followed by a phenol-chloroform extraction; and purification of nucleic acids with silica particles in the presence of guanidium thiocyanate (Monteiro *et al.*, 1997). Less hazardous and laborious methods include those described by Monnier *et al.* (1996) and Monteiro *et al.* (1997). However, several authors such as Höss and Pääbo (1993) and Boom *et al.* (1990) have also successfully proposed reliable protocols for extracting DNA from complex sources.

Their methods are based on the lysing and nuclease-inactivating properties of the chaotropic agent guanidium thiocyanate together with the nucleic acid-binding properties of silica particles or diatoms.

With the increasing worldwide trend towards health concerns, the field of probiotics has developed rapidly. Recent advances in probiotic research have confirmed the health benefits of some successful probiotic bacterial strains (Lee and Salminen, 1995). One such strain, *Bifidobacterium bifidum*, has been reported to be successful in the treatment of rotavirus and viral diarrhea as well as promoting a balanced intestinal microflora (Lee and Salminen, 1995; Salminen and Saxelin, 1996). However, since 1906, much research has been conducted on *Bifidobacteriumin* in an attempt to elucidate its mechanism of action in the gut of healthy individuals. This focus on *Bifidiobacterium* stemmed from the fact that this species was shown to be the predominant flora in breast-fed infants and it was speculated by Tissier that infant diarrhea could be treated by administering large doses of *Bifidobacterium* orally (Symons, 1997). Several other authors have also suggested that *Bifidobacterium* species are important in monitoring general health and have also been reported to be responsible for many beneficial effects such as contributing to a beneficial intestinal microflora and an increased production of secretory IgA (Matsuki *et al.*, 1999; Symons, 1997).

In this chapter, PCR was employed to detect *Bifidobacterium*, *Bacteroides* and *Prevotella* in adult domestic and wild ostrich fecal samples using primers specific for these genera.

3.3 MATERIALS AND METHODS

3.3.1 Animals and sampling

Three fecal samples D1, D2 and D3 were obtained from domesticated ostriches on a farm in Malmesbury about 60 km from the laboratory in Cape Town. Samples W1, W2 and W3 were taken from wild ostriches at the Cape Point Nature Reserve, situated about 60 km south of the laboratory. The captive birds were fed a balanced, pelleted commercial

feed while the wild ostriches fed on the natural, indigenous vegetation. The wild birds tended to frequent places where perennial grass had been introduced in earlier times and their intake could have included such grass. The sampling procedure entailed watching the birds carefully until it was noticed that a bird defecated. The feces were immediately sampled by filling a screw-capped bottle and placing it on ice. The samples were transported to the laboratory and processed within one hour of collection.

3.3.2 Bacterial strains and growth conditions

All the bacterial strains were grown under strict anaerobic conditions at 37°C overnight except for *Bacteroides thetaiotaomicron* 5482 that was incubated for 48 hours. The three Bifidobacterial strains (*B. breve* 2257, *B. bifidum* 2203 and *B. longum* 2259) were obtained from the National Collections of Industrial and Marine Bacteria NCIMB (Aberdeen) and were grown in anaerobic brain heart infusion (BHI) media (Appendix A). Genomic DNA was extracted according to the methods of of Demuyter *et al.* (1988) and Avgustin *et al.* (1994). The *Prevotella* type culture ATCC23 was received from Harry Flint and genomic DNA was extracted in M2GSC media (Appendix A) according to the method of Miyazaki *et al.* (1997). *Bacteroides thetaiotaomicron* 5482 and *Bacteroides fragilis* ATCC9343 were obtained from Dr Val Abratt (UCT) and were grown in BHI supplemented with hemin-menadione (Appendix A). Genomic DNA was extracted according to the method of Dachs (1992). The National Institute for Dairy Research (Irene) supplied us with the *Butyrivibrio fibrisolvens* ATCC19171 that was extracted in cellobiose media (Appendix A) according to the methods of Demuyter *et al.* (1988) and Avgustin *et al.* (1994). The Microbiology Department at the University of Stellenbosch supplied the *Lactobacillus acidophilus* (lab strain) and genomic DNA was extracted in Mann Rogosa Sharpe (MRS media) (Appendix A) according to the methods of Clara *et al.* (1992) and Avgustin *et al.* (1994).

3.3.3 Two methods of genomic DNA extraction from feces

Method A

Genomic fecal DNA was extracted according to the methods of Avgustin *et al.* (1994) and Wood *et al.* (1998). A sterile 2-ml screw cap Eppendorf was filled with 0.6 g sterile zirconium beads (0.1 mm in diameter). A 0.1 g (wet weight) portion of fecal material and 900 µl sterile phosphate-buffered saline (PBS; 0.05 M, pH 7.4) (Appendix A) were added to the tube and homogenised using a Mini-Bead Beater (Biospec Products, Bartlesville, Oklahoma) at a speed of 3800 for 30 seconds. The mixture was homogenised 5 times and chilled on ice for at least 1 minute during each interval. The homogenate was then centrifuged at a low speed of 2000 rpm for 5 minutes and the upper phase was removed and transferred to a clean tube. Then 20 µl of 25% SDS and 20 µl proteinase K (20 mg/ml) were added to the mixture and incubated for 1 hour at 55°C. Thereafter 15 µl of CTAB and 18 µl of 5 M NaCl were added and mixed thoroughly. This mixture was incubated for 1 hour at 65°C. At this stage an equal volume of lysate and phenol/chloroform/isoamyl (25:24:1) were applied to the phase lock gel columns (5 Prime) and gently mixed. Before any mixture was added to the spin columns, the wax was spun down at 14 0000 rpm for 30 seconds. The tubes were then centrifuged at 10 000 rpm for 3 minutes. The top layer was gently removed and the procedure was repeated as many times as necessary. The DNA was precipitated with 2.5 x 100% ice cold ethanol and one-tenth 3 M NaAc (pH 5.2), and centrifuged at 14 000 rpm for 10 minutes. The supernatant was poured off and the pellet was washed with 70% cold ethanol. After this, the DNA was centrifuged again for 2 minutes at 14 000 rpm and the supernatant removed. The pellet was air-dried and the DNA was resuspended in sterile distilled water. After treating with RNase (10 mg/ml) for 1 hour at 37°C, the DNA concentrations were determined spectrophometrically by measuring the absorbance at 260 nm (A_{260} =1 is equivalent to 50 µg DNA/ml).

Method B

Genomic fecal DNA was extracted according to the methods of the Tissue Protocol (Qiagen QIAamp Blood and Tissue Kit) and Monteiro *et al.* (1997) with a few modifications. A 25 mg portion of feces was added to 180 µl lysis buffer provided and homogenised using a Mini-Bead Beater 2 times with a 30 second interval on ice between each beat. Then 20 µl proteinase K (20 mg/ml) and 20 µl of 25% SDS were added to the homogenate and incubated at 55°C for 1 hour. A 200 µl volume of buffer AL was added to the sample and mixed thoroughly without vortexing. The lysate was incubated at 70°C for 10 minute. Then 210 µl of 96-100% ethanol was added and mixed thoroughly without vortexing. This mixture was centrifuged at 14000 rpm for 10 seconds. The supernatant was added to the spin column and centrifuged at 6000 rpm for 1 minute. The spin column was placed in a 2 ml collection microfuge tube and the tube containing the filtrate was discarded. Column material was washed 5 times (200 µl each) with the washing buffer provided in the kit. Finally the DNA was eluted with 200 µl of sterile distilled water preheated to 70°C. After treating the DNA with RNase (10 mg/ml) for 1 hour at 37°C, the DNA concentrations were quantified spectrophometrically by measuring the absorbance at 260 nm.

3.3.4 Total 16S rDNA amplification of the genomic fecal DNA

A 2.5 µl volume of 10 µl of each primer F1 and R5 (Appendix B) were used to amplify the complete 16S rDNA genes from the domestic and wild ostrich birds. Fifty nanograms of genomic DNA from each fecal sample was amplified according to section 2.3.4 using cycle profile 1 (Appendix B).

3.3.5 *Bifidobacterium* detection in fecal samples using *Bifidobacterium*-specific primers

Primers Bif164 and Bif662, used for the detection of *Bifidobacterium* in the domestic and the wild genomic and total 16S rDNA fecal samples, are listed in Appendix B. A positive signal with this primer pair will yield a 523 bp fragment according to Kok *et al.* (1996). A 2.5 µl volume of 10 µM of each of the primers were used in the amplification

reactions. Various concentrations (0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng) of both the genomic fecal DNA and the total 16S rDNA fecal samples were amplified using the GeneAmp PCR System 9700, Perkin Elmer thermal cycler and cycle profile 2 (Appendix B). Fifty µl reactions were carried out using 5 units of SuperTherm *Taq* polymerase (Amersham), 10 µl 10 x *Taq* polymerase buffer (Amersham), equimolar amounts of all four dNTPS (6.25 mM each, Boehringer Mannheim) and 6 µl (25 mM) $MgCl_2$ (Boehringer Mannheim). The PCR products (20 µl) were analysed by electrophoresis (Sambrook *et al.*, 1989) on a 0.8% agarose gel (Appendix C) using a λ-*Pst*I digest to verify their sizes.

Before screening the fecal samples with the *Bifidobacterium* primers, the specificity of the primers were checked. Fifty nanograms of genomic DNA from *B. breve* 2257 or *B. longum* 2259 were used as a positive control. The negative controls included 50 ng of genomic DNA from *L. acidophilus*, 50 ng of genomic DNA from *B. fibrisolvens* ATCC19171 and sterile distilled water.

In a separate experiment, 100 ng of the genomic fecal DNA samples and the total 16S rDNA fecal samples were spiked with 5 ng of genomic DNA from *B. breve* 2257. The positive control included 10 ng of genomic DNA from *B. bifidum* 2203 and the negative control was sterile distilled water. None of the control samples were spiked. This experiment was conducted to exclude the possibility of PCR inhibitors commonly found in fecal samples.

3.3.6 *Bacteroides* and *Prevotella* detection in fecal samples using *Bacteroides/Prevotella*-specific primers

Primers, FD1 and rBacPre, used to detect the presence of *Bacteroides/Prevotella* in the fecal samples, are listed in Appendix B. The forward FD1 primer is a universal Eubacterial primer while the reverse rBacPre primer is specific for *Bacteroides* and *Prevotella*. A positive signal with this primer pair will yield a fragment of about 900 bp according to Wood *et al.* (1998). A 2.5 µl volume of 10 µM of each of the primers were

used in the amplification reaction. Various concentrations (0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng) of both the genomic fecal DNA and the total 16S rDNA fecal samples were amplified using the GeneAmp PCR System 9700, Perkin Elmer thermal cycler and cycle profile 3 (Appendix B). Fifty microlitre reactions were carried out using 5 units of SuperTherm *Taq* polymerase (Amersham), 10 µl 10 x *Taq* polymerase buffer (Amersham), equimolar amounts of all four dNTPS (6.25 mM each, Boehringer Mannheim) and 6 µl (25 mM) $MgCl_2$ (Boehringer Mannheim). The PCR products (20 µl) were analysed by electrophoresis (Sambrook *et al.*, 1989) on a 0.8% agarose gel (Appendix C) using a λ-*Pst*I digest to verify their sizes.

The specificity of these primers was checked before screening the fecal samples. Fifty nanograms of *B. fragilis* ATCC9343 and *Prevotella ruminicola* ATCC23 were used as positive controls in the amplification reactions. The negative controls included 50 ng of *B. breve* 2257, 50 ng of *B. fibrisolvens* ATCC19171, 50 ng of *L. acidophilus* and sterile distilled water.

3.3.7 Procedures employed for estimating the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA in fecal samples

The method utilized to estimate the contribution of *Bacteroides* and *Prevotella* 16S rDNA total eubacterial 16S rDNA in the ostrich fecal samples was adapted from the approach described by Wood *et al.* (1998). Southern transfer of DNA was followed according to a modified method from Reed and Mann (1985). The method is described in greater detail in Appendix C. Three hundred nanograms of the total 16S rDNA from the domestic and wild fecal samples were transferred to nylon membranes. *B. fragilis* ATCC9343 and *P. ruminicola* ATCC23 were amplified using the *Bacteroides/Prevotella*-specific primers, FD1 and rBacPre. This amplification gave the expected 900-bp fragment and these amplified PCR products at a total concentration of approximately 300 ng were used as positive controls. The negative controls included 300 ng of unlabelled FD1 or rBacPre primer, sterile distilled water and 300 ng of amplified DNA from *B. fibrisolvens* ATCC19171. Following the transfer of DNA, the membranes were

hybridized with radioactively labelled primers FD1 or rBacPre according to a modified method from Church and Gilbert (1984). The method is described in greater detail in Appendix C. The procedure described in Figure 3.1 was also performed using the total 16S rDNA product amplified from Method B extracted genomic DNA. After exposure, the band intensities on the X-ray film were measured using a Transmission Densitometer TD-901 (Macbeth) in the Electron Microscope Unit, University of Cape Town. This experiment was done in duplicate for both the domestic and the wild fecal samples (Figure 3.1) and the average of the two densitometer readings obtained were used for further calculations in Table 3.2.

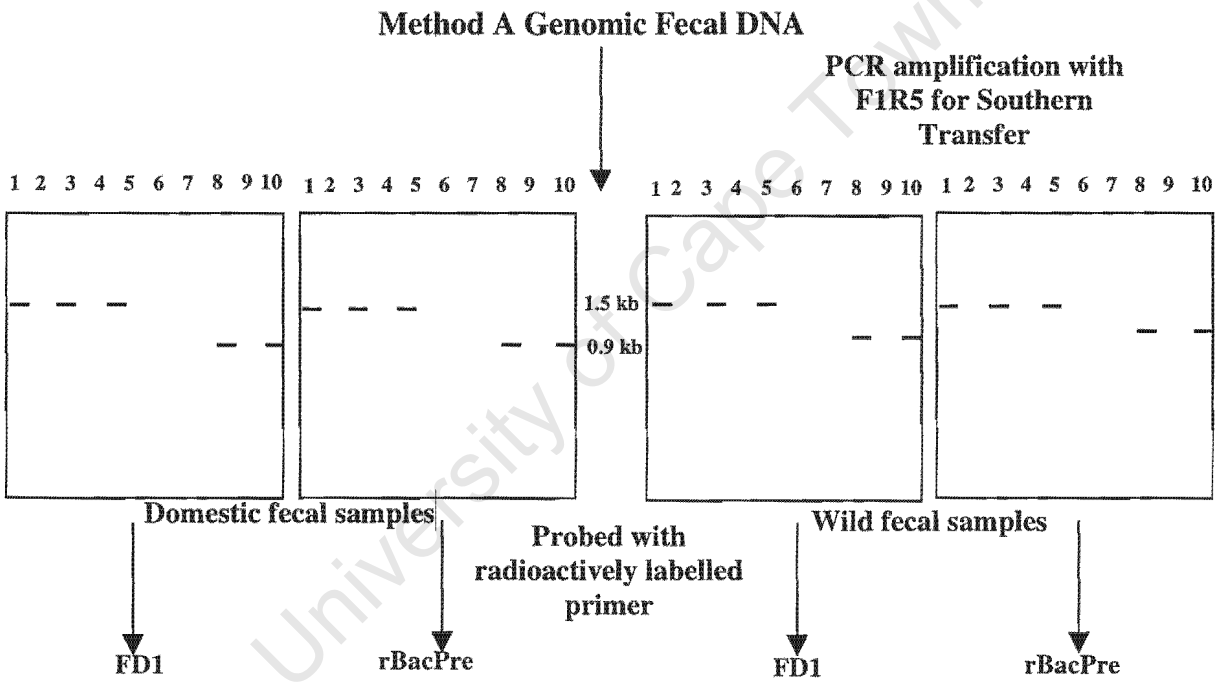


Figure 3.1. Schematic representation of the procedure for transferred DNA probed with radioactively labelled primers FD1 and rBacPre. Lanes 1, 3 and 5 of gel A had the domestic samples D1, D2 and D3 respectively. Lanes 6, 7, 8, 9 and 10 had the unlabelled FD1 primer, amplified DNA from *B. fibrisolvens* ATCC19171, amplified DNA from *P. ruminicola* ATCC23, sterile distilled water and amplified DNA from *B. fragilis* ATCC9343 respectively. Gel B had the same controls and order as Gel A except it had the unlabelled rBacPre primer in lane 6 instead of the unlabelled FD1 primer. Gels C and D also had the same controls and order as in Gels A and B respectively but had the wild fecal samples instead of the domestic samples. Sizes of the products are shown in kb.

3.4 RESULTS

3.4.1 Yield of genomic DNA from two methods of DNA extraction from feces and total 16S rDNA amplification of genomic fecal DNA

The genomic DNA obtained using the two methods of fecal DNA extraction is depicted in Figures 3.2A and 3.2B. Methods A and B gave very similar yields of DNA. For example, using extraction method A (100 mg feces), domestic sample 1 yielded 414 ng/µl of DNA while Method B (100 mg feces) yielded 432 ng/µl of DNA. However, when comparing Figures 3.2A and 3.2B, it appears as though Method B yielded more intact DNA with less DNA degradation. Prior to amplification, the genomic fecal DNA and total 16S rDNA was diluted to the following concentrations, 0.1, 0.5, 1, 10, 100 and 200 ng.

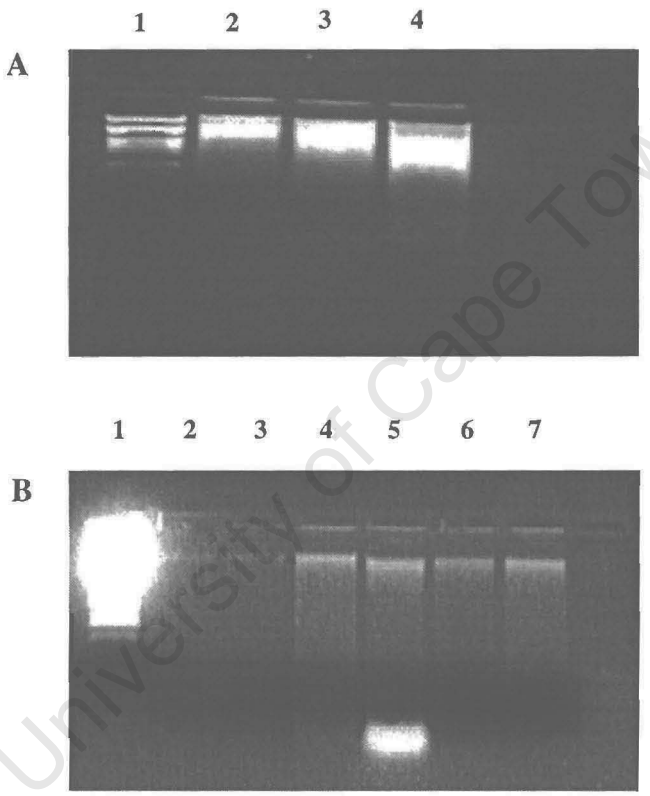


Figure 3.2. Yields of DNA obtained from two methods of fecal DNA extraction. (A) Method A fecal DNA from the domestic samples. Lane 1, λ-*Pst*I digest; lanes 2, 3, and 4, domestic samples 1, 2 and 3 respectively. (B) Method B fecal DNA from the domestic and wild fecal samples. Lane 1, λ-*Pst*I digest; lanes 2, 3, and 4, domestic samples 1, 2, and 3 respectively; lane 5, 6, and 7, wild samples 1, 2, and 3 respectively.

As previously described in Chapter 2, the primer pair F1R5 yields a 1.5-kb PCR fragment which encompasses the entire 16S rDNA of all bacterial species. Therefore the PCR products depicted in Figure 3.3 represent the total 16S rDNA genes of most, if not all of the bacteria present in the domestic and wild fecal samples.

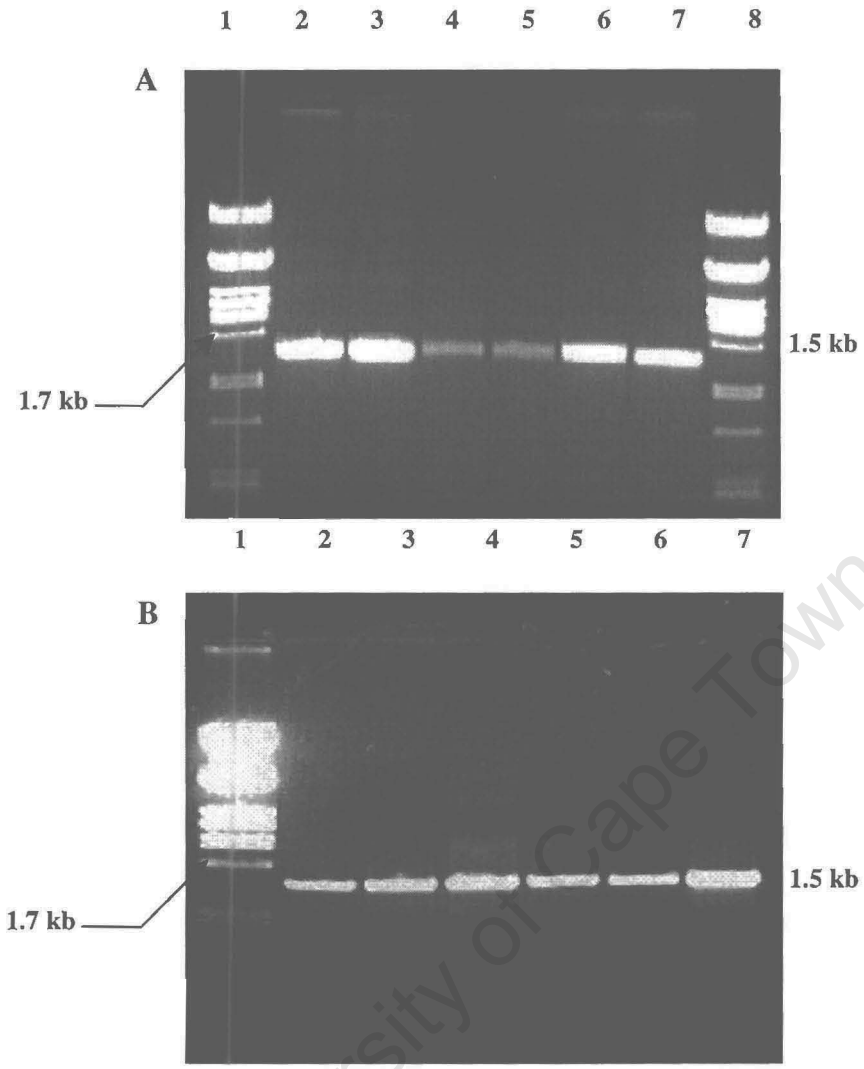


Figure 3.3. PCR amplification of the total 16S rDNA amplified from 50 ng of genomic fecal DNA obtained from Methods A (A) and B (B) with the primer pair F1R5. Lane 1, λ-*Pst*I digest; lane 2, 3 and 4, domestic samples 1, 2 and 3 respectively; lane 5, 6 and 7, wild samples 1, 2and 3 respectively and lane 8, λ-*Pst*I digest (A). Sizes of the amplified products are shown in kb.

3.4.2 *Bifidobacterium* detection in fecal samples using *Bifidobacterium*-specific primers

Primers Bif164 and Bif662 were proven to be specific for *Bifidobacterium* since positive signals were only obtained for *B. breve* 2257 and *B. longum* 2259. No signals were obtained for the negative controls employed, *L. acidophilus*, *B. fibrisolvens* ATCC19171 and sterile distilled water (data not shown). Figures 3.4A and 3.4B clearly show that after the fecal samples were spiked with genomic DNA from *B. breve* 2257, the amplification reactions were not inhibited by any substances since positive signals were obtained in all

the fecal samples. This is evident from the 523 bp fragment generated. This experiment also shows the specificity of the *Bifidobacterium* primers (lanes 15 and 16).

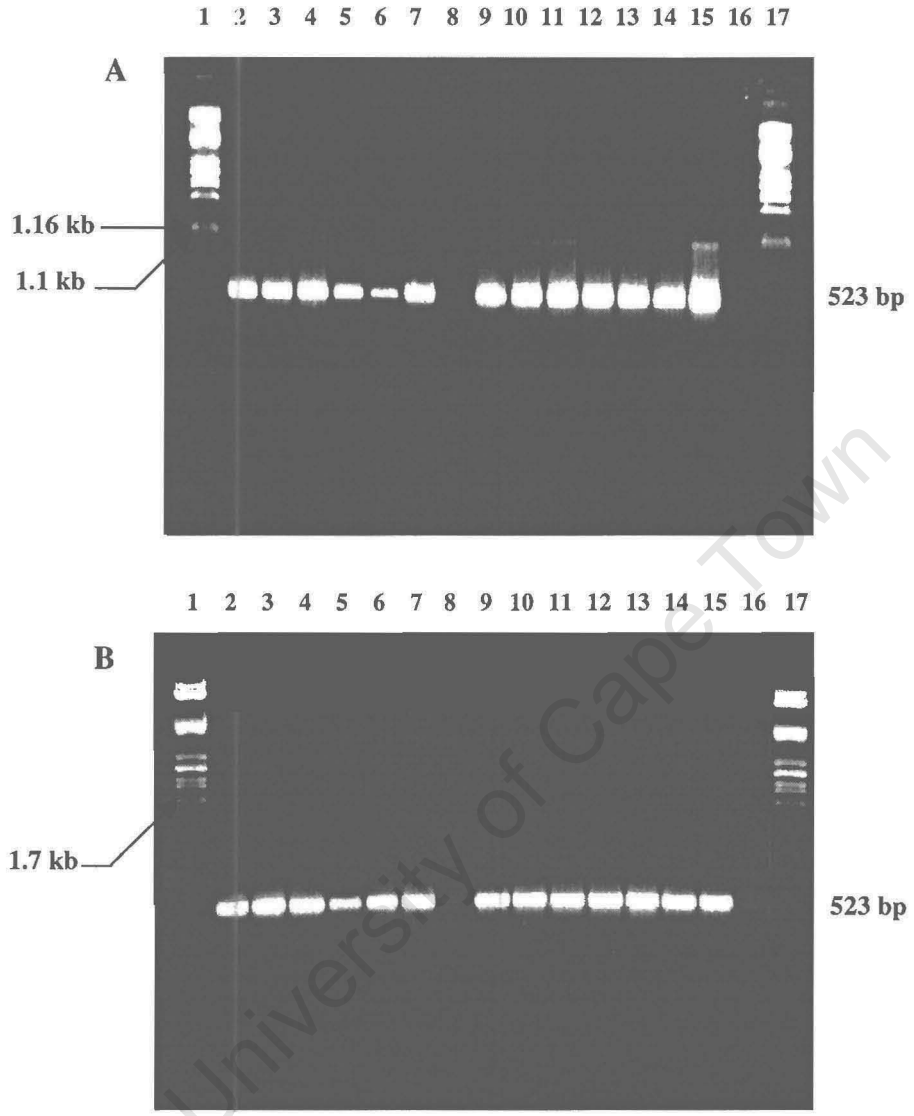


Figure 3.4. Genomic DNA and total 16S rDNA (100 ng) from Method A (A) and Method B (B) spiked with 5 ng of genomic DNA from *B. breve* 2257. Lane 1, λ-*Pst*I digest; lanes 2, 3 and 4, genomic DNA from domestic samples 1, 2 and 3 respectively; lanes 5, 6 and 7, genomic DNA from wild samples 1, 2 and 3 respectively; lane 8, sterile distilled water; lanes 9, 10 and 11, total 16S rDNA from domestic samples 1, 2 and 3 respectively; lanes 12, 13 and 14, total 16S rDNA from wild samples 1, 2 and 3 respectively; lane 15, 10 ng of amplified DNA from *B. bifidum* 2203; lane 16, 10 ng of amplified DNA from *B. fibrisolvens* ATCC19171 and lane 17, λ-*Pst*I digest. Sizes of the amplified products are shown in kb and bp.

Bifidobacterium was not detected in the domestic and the wild fecal samples using the genomic DNA and the total 16S rDNA obtained from methods A and B. Various concentrations including 0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng of the fecal DNA were used as a template for screening.

3.4.3 *Bacteroides* and *Prevotella* detection in fecal samples using *Bacteroides/Prevotella*-specific primers

Positive signals were only obtained from the controls *B. fragilis* ATCC9343 and *P. ruminicola* ATCC23. No signals were obtained for the negative controls *B. breve* 2257, *B. fibrisolvens* ATCC19171, *L. acidophilus* and sterile distilled water (data not shown). Therefore primers FD1 and rBacPre were specific for *Bacteroides* and *Prevotella*.

Since positive signals were obtained when the genomic DNA samples were spiked with genomic DNA from *Bifidobacterium* (Figure 3.4), it was not necessary to spike the fecal samples with *Bacteroides*. When the genomic DNA obtained from extraction method A was employed as a template, *Bacteroides* and *Prevotella* were detected at fecal concentrations of 0.5 ng, 1 ng and 10 ng. This is evident from the 900-bp fragments generated in Figure 3.5A.

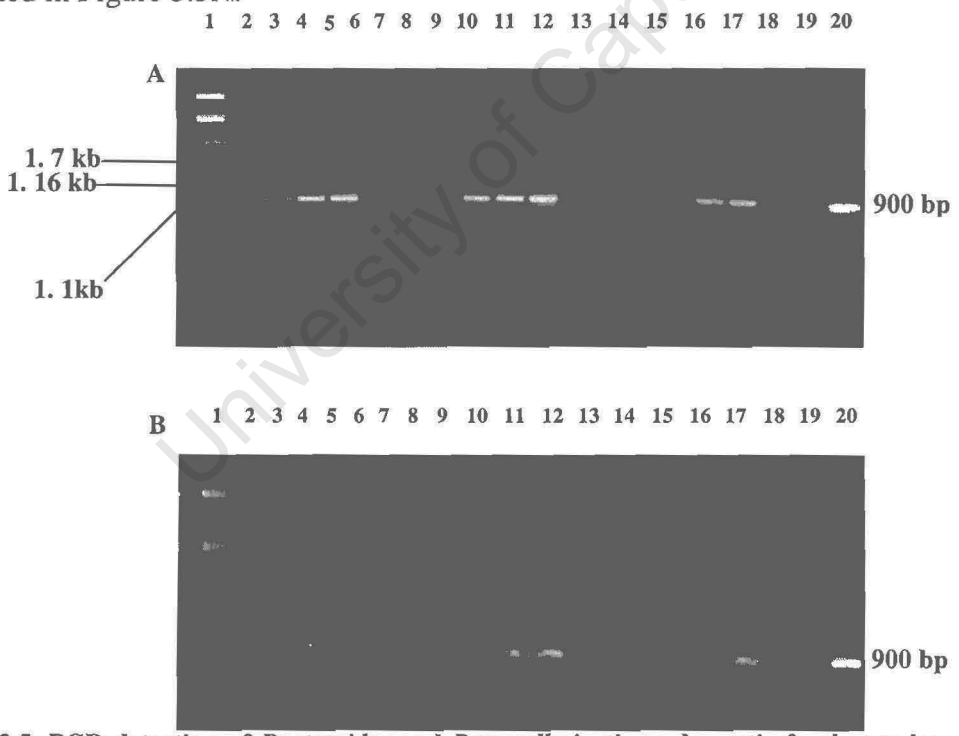


Figure 3.5. PCR detection of *Bacteroides* and *Prevotella* in three domestic fecal samples employing the genomic DNA obtained from method A (A) or the total 16S rDNA product amplified from method A genomic DNA (B). Lane 1, λ-PstI digest; lanes 2, 3, 4, 5, 6 and 7, D1 at 0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng respectively; lanes 8, 9, 10, 11, 12 and 13, D2 at 0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng respectively; lanes 14, 15, 16, 17, 18 and 19, D3 at 0.1 ng, 0.5 ng, 1 ng, 10 ng, 100 ng and 200 ng respectively and lane 20, genomic DNA from *B. fragilis* ATCC9343.

Similar results were observed when the genomic DNA obtained from method B was employed as a template (data not shown). However, when the 16S rDNA products, amplified from the genomic DNA obtained from method A were used as templates, *Bacteroides* and *Prevotella* were also detected at the lowest fecal concentration of 0.1 ng (Figure 3.5B). The same observations were seen with the 16S rDNA products amplified from genomic DNA obtained from method B (data not shown). At the higher fecal concentrations of 100 ng and 200 ng, negative PCR results were observed when employing the genomic DNA and the 16S rDNA, irrespective of the method used. No positive signals were detected in the wild fecal samples using the genomic DNA or the amplified total 16S rDNA obtained from both methods of extraction (data not shown).

3.4.4 Estimation of the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA in domestic fecal samples

To estimate the amount of *Bacteroides* and *Prevotella* 16S rDNA relative to total eubacterial 16S rDNA, two universal eubacterial primers FD1 and R5 were employed to amplify most of the 16S rRNA gene. The amplified material was then transferred to membranes by Southern blotting and probed with FD1 or the *Bacteroides* and *Prevotella*-specific oligonucleotide rBacPre (Figure 3.1). The percentage of *Bacteroides* in total eubacterial 16S rDNA (Table 3.2) was calculated from the relative binding of probes FD1 and rBacPre to material amplified from the gut samples and from the control sample *B. fragilis* ATCC9343 (Figure 3.6). This was achieved by estimating the radioactivity for each band using a transmission densitometer. The approximate proportion of eubacterial 16S rDNA sequences due to *Bacteroides* was estimated as $(a_e/a_b)x\ (b_b/b_e)$ where a_e and b_e are the counts obtained for the control and fecal samples respectively, with the universal eubacterial probe (FD1), and a_b and b_b are the corresponding counts obtained with the rBacPre probe. Only the *B. fragilis* ATCC9343 control was used in the percentage calculations in Table 3.2. *Bacteroides* sequences were estimated to account for between 25 and 89% of the total eubacterial 16S rDNA in the domestic ostrich samples. Figure 3.7 depicts a graphical representation of the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA of the domestic samples.

Table 3.1. The averaged data obtained from the transmission densitometer for the domestic fecal samples and the control samples probed with the universal eubacterial probe, FD1 or the rBacPre probe.

Labelled probe	Fecal samples			Control samples	
				Bacteroides	*Prevotella*
	D1	D2	D3		
Method A: FD1	0.27	0.06	1.08	1.53	0.43
rBacPre	0.04	0.02	0.10	0.57	0.10
Method B: FD1	0.75	0.49	0.48	3.48	3.43
rBacPre	0.23	0.97	0.39	3.27	2.58

Table 3.2. Approximate proportion of *Bacteroides* 16S rDNA in the total eubacterial 16S rDNA from domestic samples 1, 2 and 3.

Extraction method	Fecal samples	% of *Bacteroides* in total eubacterial 16S rDNA
Method A	D1	40
	D2	89
	D3	25
Method B	D1	33
	D2	211
	D3	87

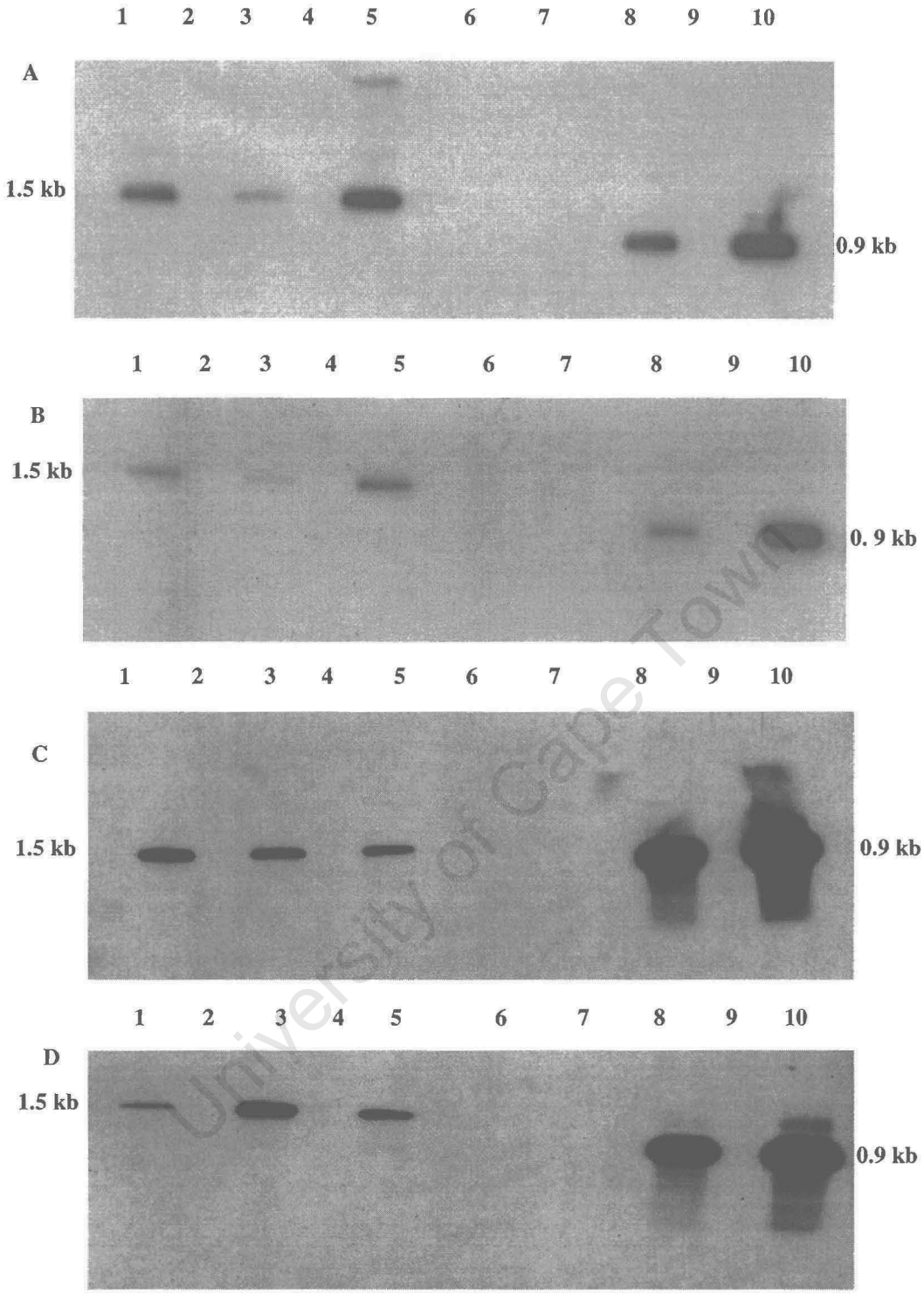


Figure 3.6. Estimation of the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA sequences. Figures A and B, 16S rDNA amplified from genomic DNA obtained from method A and figures C and D, 16S rDNA amplified from genomic DNA obtained from method B. Amplified sequences were transferred onto a nylon membrane by Southern blotting and probed with either the universal 16S eubacterial probe FD1 (Figures A and C) or rBacPre probe (Figures B and D). Lanes 1, 3 and 5, total 16S rDNA from domestic fecal samples 1, 2 and 3; lane 6, unlabelled FD1 primer (Figures A and C) and unlabelled rBacPre primer (Figures B and D); lane 7, amplified DNA from *B. fibrisolvens* ATCC19171 control; lane 8, amplified DNA from *P. ruminicola* ATCC23 control; lane 9, sterile distilled water and lane 10, amplified DNA from *B. fragilis* ATCC9343 control. Sizes of the products are shown in kb.

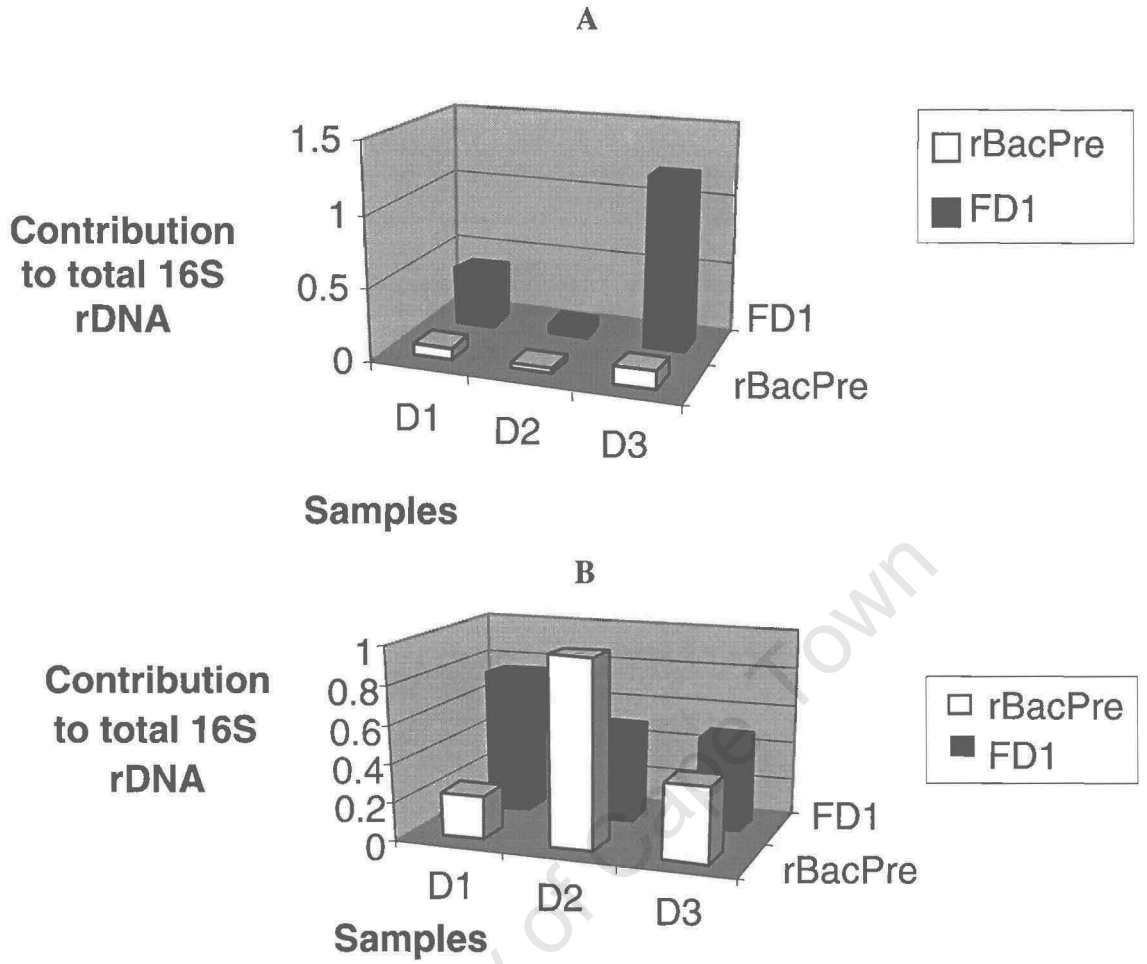


Figure 3.7. Estimation of the contribution of *Bacteroides* and *Prevotella* to the total eubacterial 16S rDNA of the domestic samples D1, D2 and D3 amplified from method A (A) and method B (B) genomic DNA (derived from data in Table 3.1, excluding the control sample data).

3.5 DISCUSSION

The two extraction methods employed produced similar yields of genomic DNA although method B seems to have yielded DNA with less degradation. DNA obtained from method B was subjected to less chemical and mechanical procedures than DNA obtained from method A. In addition, method B was far more rapid than method A and therefore the DNA was less likely to be degraded by DNA-degrading enzymes. Method B which is based on a silica-gel membrane, for purification of DNA, was less hazardous than method A since it is devoid of phenol-chloroform extraction procedures and the use of CTAB, a detergent which has been reported to effectively remove PCR inhibitors from

fecal material (Monteiro *et al.*, 1997). It has been reported that the use of phase lock gel (PLG) not only offers increased protection from exposure to hazardous compounds such as phenol and/or chloroform but also produces an increased yield of nucleic acids. The increased yield is the result of the tight seal that PLG forms between the phases of an aqueous/organic extraction, thereby the entire aqueous phase can be collected. Both methods did, however, produce DNA suitable for PCR directly from feces.

When amplifying DNA extracted from complex sources such as feces, various parameters can be manipulated so as to reduce or even eliminate the effect of PCR inhibitors. For example, dilutions of fecal DNA have been shown to increase the performance of PCR in such a way that negative results were obtained without prior dilution of the fecal DNA samples (Monnier *et al.*, 1996). Monteiro *et al.* (1997) also stated that in fecal DNA samples where inhibitors persisted, diluting the fecal DNA samples was sufficient to remove the effects and render the PCR positive. Therefore it is imperative to dilute fecal DNA samples prior to the amplification reactions. In this study, the fecal DNA obtained from the two extraction methods were diluted to 0.1, 0.5, 1, 10, 100 and 200 nanograms before any PCR reactions were conducted.

When the genomic DNA obtained from extraction methods A and B were employed as templates, *Bacteroides* and *Prevotella* were only detected at 0.5 ng, 1 ng and 10 ng. However, when the 16S rDNA products were utilised as templates, *Bacteroides* and *Prevotella* were also detected at 0.1 ng, the lowest fecal concentration employed in this study. In contrast, negative PCR results were obtained at the higher fecal concentrations of 100 ng and 200 ng when utilising both the genomic and the 16S rDNA as templates. As previously mentioned, Monteiro *et al.* (1997) stated that when fecal DNA samples where inhibited by substances, diluting the fecal DNA samples was sufficient to remove the effects of the inhibitors and render the PCR positive. The dilutions 100 ng and 200 ng employed in this particular study, are apparently not high enough since by further increasing the dilutions of the fecal DNA samples, positive PCR signals resulted. This observation is most probably due to the PCR-inhibiting substances also being diluted and therefore their effects on the PCR amplification are reduced. A possible reason for the

detection of *Bacteroides* and *Prevotella* at the lowest fecal DNA concentration employed, 0.1 ng, when using the 16S rDNA amplicon as a template, could be due to the possibility that this approach is more sensitive than using the genomic DNA, directly extracted from fecal samples, as a template. A similar idea has been advanced by O'Sullivan (1999). No PCR signals were detected in the wild fecal samples.

Bacteroides has been reported to be the most prevalent genus in the human gut followed by *Bifidobacterium* and *Clostridium* (Langendijk *et al.*, 1995; Wang *et al.*, 1996). A variety of *Bacteroides* species including *B. thetaiotaomicron*, *B. vulgatus* and *B. distasonis* have been detected in human and animal fecal samples employing PCR hybridization assays (Kreader, 1995). Hanssen also reported *Bacteroides* sp. to be one of the main gut and cecal microorganisms in captive grouse while spirochetes, flagellates and amoebae, which were absent in captive grouse, were the dominant species in wild willow grouse. He also stated that wild willow grouse fed on a low-quality, high fibre rich diet while captive grouse were fed a diet very similar to commercial chicken food and thus promoting a gut microflora very similar to that of the domestic fowl, both with regards to types, numbers and distribution. According to Hanssen, these findings combined with the extensive differences in cecal morphology of captive and wild willow grouse observed in a previous study, explains why captive grouse digest natural food, especially cellulose and lignin, only half as efficiently as wild grouse, and cannot survive on it.

A possible reason for *Bacteroides* being detected in the domestic ostrich fecal samples and not in the wild fecal samples could also be linked to diet. It has been stated that roughage rations would promote the growth of cellulolytic species whereas rations high in cereal starch stimulates the growth of the amylolytic, lactic acid and propionate producing species (Latham, 1979). The rations fed to the captive ostrich birds were low in fibrous material and high in cereal grain, thereby promoting the growth of species such as *Bacteroides*. In comparison, the ostrich birds in the wild were probably exposed to feed which was high in fibrous components and this could result in higher populations of fibre-digesting bacteria. In addition, it is highly probable that the increased intake of

fibrous materials could also be responsible for the negative PCR signals obtained in the wild fecal samples. This idea stems from the fact that most of the common PCR inhibitors in feces are complex polysaccharides, possibly originating from plant material in the diet (Al-Soud and Rådström , 1998; Demeke and Adams, 1992; Monteiro *et al.*, 1997). Therefore the extraction protocols employed in this study were perhaps only sufficient to remove the inhibitors present in the domestic fecal samples, but failed to rid the wild fecal samples of more resistant inhibitors present due to the high-fibre diet of the wild birds.

Another important factor that has been shown to reduce or eliminate the effect of PCR inhibitors is the use of an appropriate thermostable DNA polymerase (Al-Soud and Rådström , 1998). In this study, a comparison was made between the effectiveness of polymerases *Taq* and *Pwo*. The enzyme *Pwo* was shown to be equally effective at half the concentration of the *Taq* DNA polymerase employed (data not shown). *Pwo* was also shown to be the only enzyme capable of effectively amplifying at a fecal concentration of 2% (Al-Soud and Rådström , 1998). Since the amplification reactions employing *Taq* polymerase were not affected negatively by PCR inhibitors, this DNA polymerase proved effective when amplifying the fecal DNA obtained from the two extraction methods employed in this study.

The potential use of *Bifidobacterium* species in the treatment and prevention of gastrointestinal disorders has raised the demand for an accurate and rapid method for their detection and enumeration. In this study, PCR was applied to domestic and wild ostrich fecal samples employing 16S rRNA-targeted primers specific for the genus *Bifidobacterium*. No signal was obtained in any of the fecal samples. However, these primers have successfully been used to detect *Bifidobacterium* in infant feces (Kok *et al.*, 1996).

Like most intestinal microorganisms, the populations of *Bifidobacterium* are influenced by factors such as diet, drugs, climate and stress (Hoover, 1993; Symons, 1997). In addition, advancing age of the host has also been reported to cause a decline in the

Bifidobacterium populations (Mitsuoka and Hayakawa, 1972). Perhaps one of these factors is responsible for *Bifidobacterium* not being detected in the ostrich fecal samples. In addition, the negative PCR signals obtained do not necessarily imply that *Bifidobacterium* is not found in ostriches. Rather, it could mean that this species was not present in the ostrich fecal samples that were examined in this particular study.

The approach used in this study to estimate the contribution of *Bacteroides* and *Prevotella* 16S rDNA to total eubacterial 16S rDNA in ostrich fecal samples was adapted from that described by Wood *et al.* (1998). When the DNA obtained from extraction method A was utilised as a template, the percentage of *Bacteroides* in total eubacterial 16S rDNA was estimated to account for 40%, 89% and 25% for domestic samples 1, 2 and 3 respectively. However, when method B DNA was employed, the percentages ranged from 33% to 87% for domestic samples 1 and 3 respectively. The data and the percentage value obtained for the domestic sample 2 (Table 3.1 and 3.2), using method B DNA, could possibly be due to errors in the methodology employed. This idea comes from the observation that the control samples in Figure 3.6 should essentially have the same band intensities since *B. fragilis* ATCC9343 and *P. ruminicola* ATCC23 were both employed at a final concentration of approximately 300 ng. However, it is evident that the intensities vary and therefore could cause discrepancies in the results obtained in this kind of experiment.

CHAPTER 4

GENERAL CONCLUSIONS

Even though the molecular approaches used in this study are more reliable for classifying bacteria than previously used phenotypic and biochemical tests, it is imperative to employ 16S rDNA sequencing in conjunction with the latter and not as the sole method for identifying bacteria. The phenotypic and biochemical approaches, however, can be troublesome since many gut anaerobes are either difficult to cultivate or are unculturable. Determination of the phylogenetic positions of the strains also increases the confidence level of the identification.

Results of the phylogenetic analyses of the newly identified strains in this study, confirm the findings of Willems and Collins (1995) and Rainey and Jansen (1995) in showing that the genera *Ruminococcus* and *Butyrivibrio* are not monophyletic groups but are phylogenetically heterogenous. This study is also in full support of the suggestion that cluster XIVa of the *Clostridium* subphylum requires extensive taxonomic revision since many of the species are currently classified in the genera *Clostridium*, *Eubacterium* and *Ruminococcus,* although they are in fact phylogenetically only remotely related to the type species of their respective genera.

Since *Bifidobacterium* was not detected in any of the fecal samples, there is a need to examine more domestic and wild fecal samples, and perhaps healthy and sick ostrich chicks, to determine whether these species are indeed not found in ostrich birds. By examining a wider variety of ostrich fecal samples, one could also determine whether the genera, *Bacteroides* and *Prevotella* are only found in domestic ostrich birds.

In addition, the error rates associated with quantitation studies are high, since all the bacteria in a mixed population may not necessarily lyse under the same extraction conditions employed and some gut anaerobes are known to be intractable to lysing

Therefore this kind of experiment requires more accurate controls which would give increased confidence in the results obtained.

APPENDICES

APPENDIX A

MEDIA, BUFFERS AND SOLUTIONS

CONTENTS

APPENDIX A

MEDIA, BUFFERS AND SOLUTIONS

All media were autoclaved at 121°C for 20 minutes prior to use, unless otherwise specified. Sterile (autoclaved) distilled water was used for making solutions, media and diluting buffers. Ultrapure water was obtained by further purification of the above water using a Milli-Q Plus (Millipore) water purification system.

A.1 MEDIA

A.1.1 BHI

BHI media (Difco)	37 g
Yeast extract	5 g
Cysteine	0.5 g
Resazurin (see A.3.29)	2 ml
Agar	15 g
Distilled water	1 l

Steam for 20 minutes. Then add resazurin and gas before autoclaving.

A.1.2 BHI for *Bacteroides*

BHI media (Difco)	37 g
Yeast extract (Difco)	5 g
Cysteine	0.5 g
Na_2CO_3	4 g
Resazurin	2 ml
Hemin-Menadione stock (see A.3.2)	10 ml
Agar (Biolab)	15 g
Distilled water	1 l

Hemin-Menadione and resazurin are only added after steaming for 20 minutes.

Gas with CO_2 and H_2 before autoclaving.

A.1.3 Mackonkey agar

Mackonkey agar base	40 g
Lactose	10 g
Distilled water	1 l

Cool to 50°C and add 1 ml ampicillin (100 mg/ml)

A.1.4 Cellobiose (medium A) medium

Cellobiose	1 g
Mineral solution 1 (see A.3.8)	7.5 ml
Mineral solution 2 (see A.3.9)	7.5 ml
Indigocarmine solution (see A.3.7)	1 ml
Yeast extract	0.5 g
Rumen fluid (clarified)	40 ml
Distilled water	42 ml

After gassing with CO_2 and H_2, add 2 ml of the reducing agent (see A.3.26).

A.1.5 MRS medium

Peptone from casein	10 g
Meat extract	8 g
Yeast extract	4 g
D-glucose	20 g
K_2HPO_4	2 g
Tween 80	1 g
$(NH_4)_2H$ citrate	2 g
NaAc	5 g
$MgSO_4$	0.2 g
$MnSO_4$	0.04 g
Agar	15 g

Adjust pH to 5.7 before making up volume to 1 l with distilled water.

A.1.6 M2GSC medium

Casitone	1 g
Yeast extract	0.25 g
$NaHCO_3$	0.4 g
Glucose	0.2 g
Cellobiose	0.2 g
Soluble starch	0.2 g
Rumen fluid	30 ml
Cysteine	0.1 g
K_2HPO_4	0.045 g
KH_2PO_4	0.045 g
$(NH_4)_2SO_4$	0.09 g
NaCl	0.09 g
$MgSO_4.7H_20$	0.009 g
$CaCl_2$	0.009 g
Distilled water	100 ml

Add 0.1 mg resazurin.

A.1.7 LB

Tryptone	4 g
Yeast extract	2g
NaCl	2 g
Distilled water	400 ml

A.1.8 YT broth (2 x)

Yeast extract	10 g
Tryptone	16 g
NaCl	5 g
Distilled water	1 l

A.2 BUFFERS

A.2.1 TENS buffer for quick preparation of plasmid DNA

25% SDS	500 µl
10 N NaOH	250 µl
1 M Tris-Cl (pH 8)	250 µl
0.5 M EDTA	50 µl

Add sterile (autoclaved) distilled water to 25 ml.

A.2.2 PBS (0.05 M)

NaCl	8 g
KCl	0.2 g
Na_2HPO4	1.44 g
KH_2PO4	0.24 g
Distilled water	800 ml

Adjust to pH 7.4 with HCl and make up volume to 1 l with distilled water before Autoclaving.

A.2.3 Buffers for Southern Transfer of DNA

0.25 M HCl

21.35 ml in 1 l distilled water.

0.4 M NaOH/1 M NaCl

NaOH	16 g
NaCl	58.44 g
Distilled water	1 l

SSC buffer (20 x)

NaCl	17.50 g
$Na_3C_6H_5O_7.2H_2O$ (Saarchem)	8.82 g
Distilled water	80 ml

Adjust the pH with 10 N NaOH to 7.4 before making up the volume to 100 ml with

sterile distilled water. Dilutions were made from the stock solution when required.

A.2.4 Buffers for DNA-DNA Hybridisation

0.1 M EDTA

Dissolve 3.7 g of EDTA in 100 ml distilled water.

1 M Phosphate buffer stock (PB)

$Na_2HPO_4.7H_2O$	134 g
85% H_3PO_4	4 ml
Distilled water	1 l

Church pre-hybridisation buffer (CPHB)

Non-fat dry milk (Carnation)	0.5 g
0.5 M EDTA	0.2 ml
25% SDS	28 ml
1 M PB stock	50 ml
Distilled water	21.5 ml

Church hybridisation buffer (CHB)

Same as CPHB excluding the non-fat dry milk powder.

Wash buffer A (WBA)

25% SDS	100 ml
1 M PB	20 ml
0.1 M EDTA	5 ml
Distilled water	375 ml

Wash buffer B (WBB)

25% SDS	40 ml
1 M PB	40 ml
0.1 M EDTA	10 ml

Distilled water	910 ml

A.2.5 TBE buffer (10 x stock)

Tris base	108 g
Boric acid	55 g
0.5 M EDTA (pH 8)	20 ml
Distilled water	1 l

This solution was diluted in distilled water to a 1 x concentration for use.

A.2.6 TE buffer (pH 8)

1 M Tris-HCl (pH 8)	1 ml = (10 mM)
0.5 M EDTA (pH8)	200 µl ≒ (1 mM)
Distilled water	100 ml

A.2.7 STE buffer

1 M Tris-HCl (pH 8)	1 ml
1 M NaCl	1 ml
0.5 M EDTA (pH 8)	200 µl
Distilled water	100 ml

A.3 SOLUTIONS

A.3.1 DNA electrophoresis loading buffer

Bromophenol blue	0.25 g
Sucrose	40 g
0.5 M EDTA (pH 8)	4 ml
Distilled water	100 ml

A.3.2 Hemin-Menadione stock

Menadione solution	1 ml
Hemin stock	100 ml

Store at 4°C.

A.3.3 Vitamin K (Menadione) stock solution (5 mg/ml)

Menadione	100 mg
96% ethanol	20 ml

Filter sterilise and store at 4°C.

A.3.4 Hemin stock solution (5 mg/ml)

Hemin	50 mg
1M NaOH	1 ml
Distilled water	100 ml

Store at 4°C.

A.3.5 Sephadex G-25 for separation of radiolabelled DNA from unincorporated nucleotides

Sephadex G-25 (fine; Pharmacia)	30 g
STE buffer	250 ml

The Sephadex beads were slowly added to the STE buffer in a 500 ml beaker before autoclaving. The mixture was stored at 4° C.

A.3.6 Tracking dye for separation of radiolabelled DNA from unincorporated nucleotides

Dextran blue 2000 (Pharmacia)	0.3 g
50 mM NaCl	10 ml

The dextran blue was dissolved in the NaCl before adding Orange G (BDH) to a final concentration of 1%.

A.3.7 Indigocarmine solution (50 mg/ml)

Dissolve 0.05 g in 1 ml distilled water and store at 4°C. Make up fresh each month.

A.3.8 Mineral solution 1

K_2HPO_4	3 g

Make volume up to 500 ml with distilled water. Store at 4°C after autoclaving.

A.3.9 Mineral solution 2

NaCl	6 g
$(NH_4)_2SO_4$	6 g
KH_2PO_4	3 g
$MgSO_4.7H_2O$	1.2 g
$CaCl_2$ (anhydrous) or dihydrate	0.6 g or 0.67 g

Make volume up to 500 ml with distilled water. Store at 4°C after autoclaving.

A.3.10 Lysozyme solution

Sucrose (25%)	125 g
NaCl (0.1 M)	2.9 g
Tris	3.05 g

Adjust the pH to 8 then make volume up to 500 ml with distilled water. Add lysozyme at 1mg/ml.

A.3.11 SDS

Make 100 ml of a 25% (w/v) solution. Heat to about 80°C to dissolve but do not autoclave. Dilutions were made from the stock solution when required.

A.3.12 Proteinase K (20 mg/ml)

Dissolve 0.02 g of Proteinase K in 1 ml of sterile distilled water. Store at –20°C.

A.3.13 NaCl (5 M)

Dissolve 29.22 g of sodium chloride to 100 ml distilled water.

A.3.14 NaAc (3 M)

Dissolve 204.05 g of sodium acetate in 400 ml distilled water. Adjust pH to 5.2 with glacial acetic acid, and then make up to 500 ml with distilled water.

A.3.15 Chloroform – isoamylalcohol (CI)

Mix at a ratio of 24 : 1

A.3.16 CTAB/NaCl solution

Dissolve 4.1 g NaCl in 80 ml distilled water. Slowly add 10 g of CTAB (hexadecyltrimethyl ammonium bromide) while heating and stirring. If necessary, heat to 65°C to dissolve. Adjust final volume to 100 ml with distilled water.

A.3.17 Phenol (P)

Use 500 g commercial crystallised phenol. Add 0.6 g 8 – hydroxyquinoline, 7.5 ml NaOH (2 M), 130 ml water, and 6 ml Tris-HCl (1 M, pH 7.6). Leave overnight to liquefy (or at 40°C until the solution is clear). Store at –20°C.

A.3.18 RNase (10 mg/ml)

Dissolved pancreatic RNase A at a concentration of 10 mg/ml in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl. Heat to 100°C for 15 minutes and allow to cool slowly at room temperature. Store aliqouts at – 20°C.

A.3.19 Denaturing gel for automated sequencing (ALFexpress, Pharmacia)

Urea (Pharmacia)	19 g
1.5 x TBE buffer (see A.2.5.)	7.5 ml
50% Long Ranger gel solution (Pharmacia)	5.0 ml
ultrapure water	50 ml

The above solution was gently stirred in a 100 ml Erlenmyer flask covered with parafilm for 30 minutes on a magnetic stirrer. When all the urea had dissolved, the solution was filter-sterilised through a 0.45 µm vinyl filter (Millex, Millipore) to

rid the solution of any contaminating crystals. Before pouring the gel, the following was added:

10% (w/v) ammonium persulphate (Pharmacia)	250 µl
N,N,N',N'-tetramethyletylene-diamine (TEMED; Sigma)	25 µl

This mixture was mixed gently in a fumehood without aerating too much and syringed between two glass plates using two 25 ml syringes. The acrylamide was left to polymerise for 2.5 hours.

A.3.20 Ampicillin (100 mg/ml)

0.1 g of ampicillin (Sigma) was dissolved in 1 ml of distilled water. The solution was filtered sterilised and stored at –20°C. Add 1 µl/ml for a final concentration of 100 µg/ml. Only add when media has cooled down to ± 45°C.

A.3.21 ATP (0.1 M)

Dissolve 60 mg ATP in 800 µl sterile distilled water. Adjust pH to 7 with 0.1 M NaOH. Freeze at –70°C. Dilutions of this stock was made when required.

A.3.22 $CaCl_2$ (0.1 M)

Dissolve 1.47 g of calcium chloride in 100 ml of distilled water.

A.3.23 $MgCl_2$ (0.1 M)

Dissolve 2.03 g of magnesium chloride in 100 ml of distilled water.

A.3.24 EtBr (10 mg/ml)

Dissolve 0.01 g of ethidium bromide in 1 ml distilled water.

A.3.25 NaOH (10 N)

Dissolve 40 g of sodium hydroxide in 100 ml distilled water. Dilutions were made from the stock solution when required.

A.3.26 Reducing solution

Place 100 ml of 0.2 N NaOH in a conical flask and bring to the boil for 1 minute. Then add 1.25 g cysteine.HCl.H_20 and 1.25 g $Na_2S.H_20$. Cover the solution and gas with N_2 for 10 to 15 minutes. This solution was stored by dispensing into N_2 purged tubes using syringe needles. Add 2 ml of the reducing agent to 100 ml cellobiose medium.

A.3.27 1 M Tris-HCl

Dissolve 12.1 g in 80 ml distilled water. Adjust the pH to 8 and make volume up to 100 ml with distilled water.

A.3.28 EDTA (0.5 M)

Dissolve 93.05 g of EDTA in 400 ml distilled water while adjusting the pH to 8. Make volume up to 500 ml with distilled water before autoclaving. Dilutions were made from the stock solution when required.

A.3.29 Resazurin (25 mg/ml)

Dissolve 0.025 g of resazurin in 1 ml distilled water.

APPENDIX B

DNA PRIMER SEQUENCES AND PCR CYCLE PROFILE

APPENDIX B

DNA PRIMER SEQUENCES AND PCR CYCLE PROFILES

B.1 DNA PRIMER SEQUENCES

All synthetic oligonucleotide sequences were supplied by the Oligonucleotide Synthesising Service of the Biochemistry Department, University of Cape Town, Cape Town, South Africa.

B.1.1 Primers used for 16S rDNA amplification

F1 5´CGC CAG GGT TTT CCC AGT CAC GAC AGA GTT TGA TCC TGG CTC AG 3´

R1 5´CAG GAA ACA GCT ATG AC GTA TTA CCG CGG CTG CTG GCA C 3´

F3 5´CGC CAG GGT TTT CCC AGT CAC GCC AGC AGC CC GGT AAT AC GAC 3´

R3 5´CAG GAA ACA GCT ATG AC CAC GAG CTG ACG ACA ICC ATG 3´

F5 5´CGC CAG GGT TTT CCC AGT CAC GAC GCA TGG ITG TCG GCA GCT CGT G 3´

R5 5´CAG GAA ACA GCT ATG AC ACG GIT ACC TTG TTA CGA CTT 3´

I = A, C, T, or G

Underlined sequence represents PCR primers that were homologous to highly conserved regions in the bacterial 16S rDNA gene.

Non-underlined sequence represents sequence homologous to the primers used for cycle-sequencing (see B.1.4).

B.1.2 Primers used for amplification of *Bifidobacterium*

Bif164 (Forward) primer	5´CGC CAG GGT TTT CCC AGT CAC GAC GGG TGG TAA TGC CGG ATG 3´
Bif662 (Reverse) primer	5´CAG GAA ACA GCT ATG ACC CAC CGT TAC ACC GGG AA 3´

B.1.3 Primers used for amplification of *Bacteroides/Prevotella*

FD1 (F1) primer	5´AGA GTT TGA TCC TGG CTC AG 3´
rBacPre primer	5´TCA CCG TTG CCG GCG TAC TC 3´

B.1.4 Primers for cycle-sequencing protocol (end-labelled with Cy5 fluorescent dye)

Forward primer	5´CGC CAG GGT TTT CCC AGT CAC GAC 3´
Reverse primer	5´CAG GAA ACA GCT ATG AC 3´

B.2 PCR CYCLE PROFILES

B.2.1 PCR cycle profile 1 (used for amplification of the 16S rDNA)

Denaturation:	96°C for 5 minutes	1 cycle
Denaturation: Annealing: Extension:	96°C for 45 seconds 55°C for 30 seconds 72°C for 90 seconds	25 cylces
Extension:	72°C for 5 minutes 4°C for 5 minutes	1 cycle

B.2.2 PCR cycle profile 2 (used for amplification of *Bifidobacterium*)

Denaturation:	96°C for 5 minutes	1 cycle

Denaturation:	96°C for 1 minute	} 25 cycles
Annealing:	66°C for 45 seconds	
Extension:	72°C for 1 minute	
Extension:	72°C for 5 minutes	} 1 cycle
	4°C for 5 minutes	

B.2.3 PCR cycle profile 3 (used for amplification of *Bacteroides/Prevotella*)

Denaturation:	96°C for 5 minutes	1 cycle
Denaturation:	96°C for 1 minute	} 25 cycles
Annealing:	71°C for 45 seconds	
Extension:	72°C for 1 minute	
Extension:	72°C for 5 minutes	} 1 cycle
	4°C for 5 minutes	

B.2.4 PCR cycle profile 4 (used for cycle-sequencing)

Denaturation:	96°C for 3 minutes	1 cycle
Denaturation:	96°C for 30 seconds	} 30 cycles
Annealing:	55°C for 30 seconds	
Extension:	70°C for 1 minute	
Denaturation:	96°C for 30 seconds	} 1 cycle
Annealing:	55°C for 30 seconds	
Extension:	70°C for 5 minute	

APPENDIX C

STANDARD PROCEDURES

APPENDIX C

STANDARD PROCEDURES

C.1 AGAROSE GEL ELECTROPHORESIS

Agarose gel electrophoresis of DNA was carried out according to Sambrook *et al.*, 1989 in 1 x TBE running buffer (Appendix A). Agarose (D-1 LE, Whitehead Scientific) was dissolved in TBE buffer (Appendix A) and 5 µl ethidium bromide (10 mg/ml) added per 100 ml agarose solution. A sixth the final volume of DNA electrophoresis loading buffer (Appendix A) was added to the DNA samples before loading the DNA into the wells. DNA was visualised on a 264 nm transilluminator (UVP).

C.2 PREPARATION OF COMPETENT *E.COLI* JM 109 CELLS

A 5 ml culture of *E. coli* JM 109 (Yannisch-Perron *et al.*, 1985) was incubated overnight at 37°C with agitation in 2 x yeast tryptone (YT) broth (Appendix A). A one in two hundred dilution of this culture was made in a volume of 25 ml of 2 x YT and incubated for 3 hours at 37°C with agitation until the OD_{600} reached 0.3 (early log phase). The cells were collected at 4°C by centrifugation at 5000 x g for 5 minutes and the supernatant was discarded. The cells were always kept cold on ice. The cells were resuspended in one culture volume (25 ml) of ice cold 0.1 M $MgCl_2$ and kept on ice for 1 minute. The cells were pelleted as before, resuspended in half the original volume (12.5 ml) of 0.1 M $CaCl_2$ and maintained on ice for 1.5 hours. Then the cells were pelleted as before and gently resuspended in one tenth the original culture volume (2.5 ml) of 0.1 M $CaCl_2$. The cells were kept on ice and 100 µl amounts were used for transformation of ligated DNA.

C.3 QUICK MINI-PREP METHOD FOR PLASMID DNA EXTRACTION

Two millilitres of a 6-hour *E. coli* JM 109 culture (containing plasmid) incubated in 5 ml LB (Appendix A) at 37°C, was centrifuged for 30 seconds at 15 800 x g. The supernatant

was discarded and the pellet was resuspended in 300 µl of TENS buffer (Appendix A) for no longer than 10 minutes. One hundred and fifty microlitres 3 M sodium acetate (pH 5.4) was added and the contents briefly vortexed and then centrifuged for 5 minutes at 15 800 x g. The supernatant was transferred to a clean microfuge tube, and re-centrifuged for 5 minutes at the same speed. After transferring the supernatant to a clean tube again, 900 µl ice-cold 100% ethanol was added and mixed thoroughly. The tubes were then centrifuged for 5 minutes at 15 800 x g to pellet the DNA, which was then washed with 70% ethanol, air-dried and re-dissolved in 30 µl TE (Appendix A) containing RNase A (Appendix A)

C.4 THERMOSEQUENASE CYCLE-SEQUENCING OF 16S rDNA

The procedure for the Thermosequenase Cycle-Sequencing Kit (Amersham Life Sciences) was followed according to the manufacturers instructions. Two microlitres of A, C, G, or T termination mix was added to 200 µl sterile PCR tubes. In separate microfuge tubes, 1 µg of DNA was diluted to 22 µl using sterile distilled water containing 2.1 pmol of cycle-sequencing primers (Appendix B) which were end-labelled with Cy5-Far Red fluorescein (Amersham Pharmacia Biotech). The DNA mix (5.1 µl) was aliquotted into each termination mix tube, mixed and placed in a thermal cycler (Perkin Elmer PCR System 9700) with a heated lid. The DNA products were amplified using cycle profile 4 (Appendix B).

Immediately after amplification, the tubes were placed on ice and 4 µl of Stop solution was added to each tube before sequencing the DNA using an automated sequencer (ALFexpress™; Amersham Pharmacia Biotech). Prior to sequencing, the DNA samples were heat-denatured at 96°C for 5 minutes and 4 µl of each sample was loaded into the wells. The gel was run in 0.6% TBE buffer (Appendix A). The ALFexpress™ was run with the following settings: 1000 V, 60 mA, 25 W, 55°C, 2 seconds sample time and 720 minutes running time. The data was processed using the Pharmacia software package ALFWin Version 1.10, which controlled and evaluated the sequence data generated by the Pharmacia ALFexpress™ automated sequencer.

C.5 SOUTHERN TRANFSER OF DNA ONTO NYLON MEMBRANES

The DNA gel was soaked in 2 x volume of 0.25 M HCl (Appendix A) for 5 minutes with agitation at room temperature or until the stop buffer turns yellow. The gel was then rinsed in distilled water. Approximately 10 sheets of Whatman 3MM chromotography paper, cut to size of gel was placed on an inverted gel tray soaked with 0.4 M NaOH (Appendix A) and placed in a container such that the overhanging ends acted as a wick when the container was filled with 0.4 M NaOH/1 M NaCl (Appendix A). The inverted the gel was placed on top of saturated Whatman paper making sure that no air bubbles remain trapped. A Hybond N^+ nylon membrane (cut to the size of gel) which had been wet with distilled water and pre-soaked for 5 minutes in 0.4 M NaOH/1M NaCl was placed onto the gel. This was then covered with 3 sheets of Whatman 3MM chromatography paper, the first sheet was wet with distilled water and the other two were placed on dry to blot off excess fluid. This was then covered with an 8 centimetre stack of dry absorbant paper towel. A glass plate and approximately 0.3 kilograms weight was placed on top of this and the DNA was allowed to transfer from the gel onto the membrane overnight. The gel was subsequently removed and the well positions were marked on the membrane before it was rinsed in 2 x SSC (Appendix A) for 2-5 minutes and air-dried. The DNA was crosslinked using the Ultraviolet Crosslinker (Amersham) at 70 000 microjoules/cm^2 and stored in a plastic bag until probing with the radioactively labelled DNA.

C.6 T4 POLYNUCLEOTIDE KINASE 5' END LABELLING REACTION

The FD1 and rBacPre primers were labelled in a total volume of 25 µl. One hundred nanograms of either FD1 or rBacPre were added to 10 units of T4 polynucleotide kinase and 2.5 µl of a 10 x polynucleotide buffer. Before making the volume up to 25 µl with sterile distilled water, fifty microcuries of [γ ^{32}P] dATP was added to the reaction mix. After mixing well, the reaction tubes was incubated at 37°C for 1.5 hours followed by another incubation at 94°C for 3 minutes. The labelled reaction mix was then subjected to the spin column procedure for separation of unincorporated nucleotides from the radiolabelled DNA. All reaction steps were performed on ice.

C.7 SPIN COLUMN PROCEDURE FOR SEPARATION OF UNINCORPORATED NUCLEOTIDES FROM RADIOLABLLED DNA

A column of Sephadex G-25 equilibrated in STE buffer (Appendix A) was made up by pipetting the mix into a 1 ml syringe barrel and centrifuging the column in a test tube at 1600 x g for 4 minutes. This step was repeated until the column bed volume measured about 1 ml. One hundred microlitres of STE buffer was added to the column that was then re-centrifuged at 1600 x g for 4 minutes. The radiolabelled DNA sample was prepared by adding 10 µl Dextran blue tracking dye (Appendix A) and the volume was then made up to 100 µl with STE buffer. The sample was loaded onto the column and centrifuged at 1600 x g for 4 minutes. The sample was collected in a microfuge tube at the base of the column and the specific activity of the labelled DNA was determined by counting 1 µl of DNA probe in 2 ml scintillation fluid (Packard Scintillator 299™) in a Beckman scintillation counter.

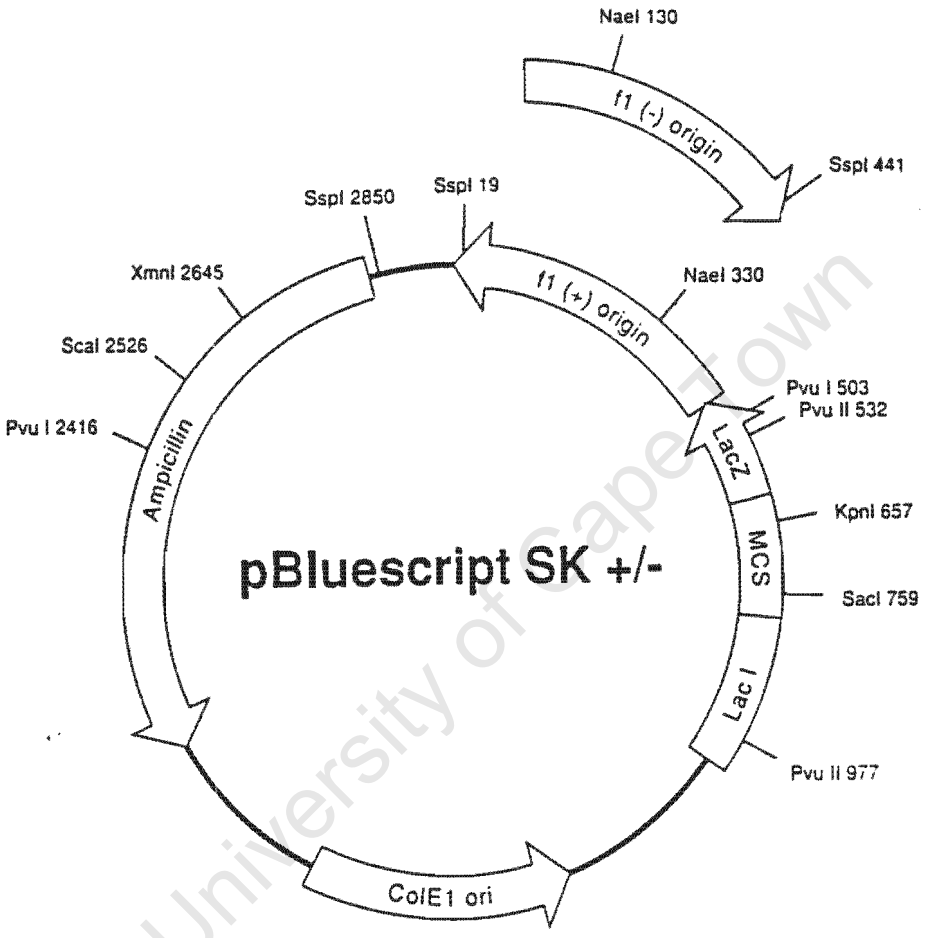
C.8 DNA-DNA HYBRIDISATION

The nylon membrane was wet with sufficient (CPHB) (Appendix A) i.e. a 0.2 ml CPHB/cm^2 membrane and sealed in a plastic bag. The bag was incubated at 65°C for 2 to 3 hours with agitation. The CPHB was removed and CHB (Appendix A) was added to the bag (50 µl CHB/cm^2 membrane). The radiolabelled DNA probe was denatured at 100°C for 5 minutes in a heating block (Techne) and sufficient probe was added to the plastic bag to satisfy the following: 1 x 10^6 cpm/ml CHB. The bag was then re-sealed and incubated overnight at 65°C with agitation. The membrane was then removed and washed two times for 5 minutes at 65°C in pre-warmed WBA (Appendix A) with agitation. After discarding the WBA, the membrane was then washed 2 to 4 times for 5 minutes at 65°C in pre-warmed WBB (Appendix A) with agitation. The radioactive counts were monitored between washes using the Geiger counter and washes were stopped if most of the membrane begins to count around between 200-500 cpm. The membrane was then washed briefly in distilled water to remove SDS and then sealed in plastic without

allowing to dry, and exposed to X-ray film in an X-ray cassette overnight or for a longer period.

APPENDIX D

Restriction enzyme site map of the vector pBluescript (SK) (Stratagene), that was used for cloning 16S rDNA PCR products (section 2.3.6.).



APPENDIX E

16S rDNA GENE SEQUENCES OF THE IDENTIFIED ISOLATES

1 TATCTTAGTG GCGGACGGGT GAGTAACACG TGAGCAACCT GCCTTTGAGA
GAGGGATAGC

61 TTCTGGAAAC GGATGGTAAT ACCTCATAAC GTATTGAGAC CGCATGATCT
TGATACCAAA

121 GATTTATCAC TCAAAGATGG GCTCGCGTCT GATTAGCTAG ATGGTGAGGT
AACGGCTCAC

181 CATGGCGACG ATCAGTAGCC GGACTGAGAG GTTGAACGGC CACATTGGGA
CTGAGACACG

241 GCCCAGACTC CTACGGGAGG CAGCAGTGGG GAATATTGCA CAATGGGGGA
AACCCTGATG

301 CAGCGATGCC GCGTGGAGGA AGAAGGTTTT CGGATTGTAA ACTCCTGTCT
TAWAGGACGA

361 TAATGACGGT ACTTTAGGAG GAAGCTCCGG CTAACTACGT GCCAGCAGCC
GCGGTAATAC

421 GTAGGGAGCG AGCGTTGTCC GGAATTACTG GGTGTAAAGG GAGCGTAGGC
GGGAGTGCAA

481 GTCAGATGTG AAATACATGG GCTCAACCCA TGGGCTGCAT TTGAAACTGC
ATTTCTTGAG

541 TGAAGTAGAG GTAAGCGGAA TTCCTGGTGT AGCGGTGAAA TGCGTAGATA
TCAGGAGGAA

601 CACCGGTGGC GAAGGCGGCT TACTGGGCTT TTACTGACGC TGAGGCTCGA
AAGCGTGGGG

661 AGCAAACAGG ATTAGATACC CTGGTAGTCC ACGCTGTAAA CGATGATTAC
TAGGTGTGGG

721 GGGACTGACC CCTTCCGTGC CGCAGTTAAC ACAATAAGTA ATCCACCTGG
GGAGTACGGC

781 CGCAAGGTTG AAACTCAAAG GAATTGACGG GGGCCCGCAC AAGCAGTGGA
GTATGTGGTT

841 TAATTCGAAG CAACGCGAAG AACCTTACCA GGTCTTGACA TCGTATGCAT AGTCTAGAGA

901 TAGATGAAAT TCCTTCGGGG ACATATAGAC AGGTGGTGCA TGGCTGTCGT CAGCTCGTGT

961 CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT TACCTTTTAG TTGTACGCAA

1021 GAGCACTCTA AAGGGACTGC CGTTGACAAA ACGGAGGAAG GTGGGGATGA CGTCAAATCA

1081 TCATGCCCCT TATGACCTGG GCTACACACG TACTACAATG GCAATTAACA AAGAGAAGCG

1141 AGACGGTGAC GTGGAGCGAA TCTCAAAAAA TTGTCCCAGT TCAGATTGCA GGCTGCAACT

1201 CGCCTGCATG AAGTCGGAAT TGCTAGTAAT CGCGGATCAG CATGCCGCGG TGAATACGTT

1261 CCCGGGCCTT GTACACACCG CCCGTCACAC CATGGGAGTC GGTAACACCC GAAGTCGGTA

1321 GTCTAACAGC AATGAGGACG CCGCCGAAGG TGGGATTGAT GACTGGGGTG AAGTCGTAAC

1381 AAGGTAA

Figure A.1. Nucleotide sequence (1387 bp) of 16S rDNA gene (5 ´- 3 ´) of strain C1a. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 GTTTTGAGAG CTTGCTCTTA AAACTTAGTG GCGGACGGGT GAGTAACACG
TGAGTAACCT
61 GCCTTTCAGA GAGGGATAGC TTCTGGAAAC GGATGGTAAT ACCTCATAAC
ATATCATTAC
121 GGCATCGTGA AGATATCAAA GATTTATCGC TGAGAGATGG GCTCGCGTCT
GATTAGCTGG
181 TTGGTGAGGT AACGGCTCAC CAAGGCAACG ATCAGTAGCC GGACTGAGAG
GTTGAACGGC
241 CACATTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTGGG
GAATATTGCA
301 CAATGGCGGC AACCTTGATG CAGCGATGCC GCGTGAGGGA AGAAGGTTTT
CGGATTGTAA
361 ACCTCTGTCA TCAGGGACGA AAATGACGGT ACCTGAGGAG GAAGCTCCGG
CTAACTACGT
421 GCCAGCAGCC GCGGTAATAC GTAGGGAGCA AGCGTTATCC GGAATTACTG
GGTGTAAAGG
481 GAGTGTAGGC GGGACTGTAA GTCAGATGTG AAATATGCCG GCTCAACTGG
CAGACTGCAT
541 TTGAAACTGC GGTTCTTGAG TGAAGTAGAG GTAAGCGGAA TTCCTAGTGT
AGCGGTGAAA
601 TGCGTAGATA TTAGGAGGAA CATCAGTGGC GAAGGCGGCT TACTGGGCTT
TAACTGACGC
661 TGAGGCTCGA AAGCGTGGGG AGCAAACAGG ATTAGATACC CTGGTAGTCC
ACGCTGTAAA
721 CGATGATTAC TAGGTGTGGG GGGACTGACC CCTTCCGTGC CGGAGCAAAC
GCAATAAGTA
781 ATCCACCTGG GGAGTACGGC CGCAAGGTTG AAACTCAAAG GAATTGACGG
GGGCCCGCAC
841 AAGCAGTGGA GTATGTGGTT TAATTCGAAG CAACGCGAAG AACCTTACCA
GGTCTTGACA
901 TCGAGTGAAG GACTAAGAGA TTAGTTTCGC CTTCGGGCAC AAAGACAGGT
GGTGCATGGT
961 TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG CAACGAGCGC
AACCCTTACC

1021 ATTAGTTGCT ACGCAAGAGC ACTCTAATGG GACTGCCGTT GACAAAACGG AGGAAGGTGG

1081 GGATGACGTC AAATCATCAT GCCCCTTATG ACCTGGGCTA CACACGTACT ACAATGGCAA

1141 TAAAACAGAG GGAAGCAATA CAGCGATGTA AAGCAAAACC CGAAAAATTG TCTCAGTTCG

1201 GATTGCAGGC TGCAACTCGC CTGCATGAAG TCGGAATTGC TAGTAATCGC AGATCAGCAT

1261 GCTGCGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT GGGAGTCGGT

1321 AACACCCGAA GCCAGTAGTC TAACCGCAAG GAGGACGCTG TCGAAGGTGG GATTGATGAC

1381 TGGGGTGAAG TCGTAACAAG GTATCCGTGT C

Figure A.2. Nucleotide sequence (1411 bp) of 16S rDNA gene (5 ´- 3 ´) of strain X25. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 ATTTATTCAG AATTCTTAGT GGCGGACGGG TGAGTAACAC GTGAGCAACC TGCCTTTGAG

61 AGAGGGATAG CTTCGGGAAA CTGATGGTAA TACCTCATAA CGTATATTTA AGGCATCTTA

121 GATATACCAA AGATTTATCA CTCAAAGATG GGCTCGCGTC TGATTAGCTA GTTGGTGAGG

181 TAACGGCTCA CCAAGGCGAC GATCAGTAGC CGGACTGAGA GGTTGAACGG CCACATTGGG

241 ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGGG

301 AAACCCTGAT GCAGCGATGC CGCGTGGAGG AAGAAGGTTT TCGGATTGTA AACTCCTGTC

361 TTAAAGGACG ATAATGACGG TACTTTAGGA GGAAGCTCCG GCTAACTACG TGCCAGCAGC

421 CCCGGTAATA CGTAGGGAGC GAACGTTGTC CGGAATTACT GGGTGTAAAG GGAGCGTAGG

481 CGGGACGGCA AGTCAGATGT GAAAACCATG GGCTCAACCC ATAGACTGCA TTTGAAACTG

541 TTGTTCTTGA GTGAAGTAGA GGTAAGCGGA ATTCCTGGTG TAGCGGTGAA ATGCGTAGAT

601 ATCAGGAGGA ACACCGGTGG CGAAGGCGGC TTACTGGGCT TTAACTGACG CTGAGGCTCG

661 AAAGCGTGGG TAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCTGTAA ACGATGATTA

721 CTAGGTGTGG GGGGACTGAC CCCTTCCGTG CCGCAGTTAA CACAATAAGT AATCCACCTG

781 GGGAGTACGG CCGCAAGGTT GAAACTCAAA GGAATTGACG GGGGCCCGCA CAAGCAGTGG

841 AGTATGTGGT TTAATTCGAA GCAACGCGAA GAACCTTACC AGGTCTTGAC ATCGTATGCA

901 TACTAAGAGA TTAGAGAAAT CCCTTCGGGG ACATATAGAC AGGTGGTGCA TGGTTGTCGT

961 CAGCTCGTGT CGTGAGAGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTT ACCATTAGTT

1021 GCTACGCAAG AGCACTCTAA TGGGACTGCC GTTGACAAAA CGGAGGAAGG TGGGGATGAC

1081 GTCAAATCAT CATGCCCCTT ATGACCTGGG CTACACACGT ACTACAATGG CAATTAACAA

1141 AGAGCAGCGA AGCGGTGACG TGGAGCGAAT CTCCAAAAAT TGTCCCAGTT CGGATTGCAG

1201 GCTGCAACTC GCCTGCATGA AGTCGGAATT GCTAGTAATC GCGGATCAGC ACGCCACGGT

1261 GAATACGTTC CCGGGCCTTG TACACACCGC CCGTCACACC ATGGGAGTTG GTTTTACCTG

1321 AAGACGGTGC GCTAACCGAA AAGGGGGCAG CCGGCCACGG TAGGGTCAGC GACTGGGGTG

1381 AAGTCGTAAC AAGGTA

Figure A.3. Nucleotide sequence (1396 bp) of 16S rDNA gene (5 ´- 3 ´) of strain X18. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 GAGTTTGATC TTGGCTCAGG ACGAACGCTG GCGGCACCTA AACACAAGCA AGTCGAACGG

61 AGATAATTGA GTATACTTAG TTATCTTAGT GGCGGACGGG TGAGTAACAC GTGAGCAACC

121 TGCCTCTGAG AGAGGGATAG CTTCTGGAAA CGGATGGTAA TACCTCATGA CATATACTTA

181 AGGCATCTTA GGTATATCAA AGATTTATCA CTCAGAGATG GGCTCGCGTC TGATTAGCTA

241 GATGGTGAGG TAACGGCTCA CCATGGCGAC GATCAGTAGC CGGACTGAGA GGTTGAACGG

301 CCACATTGGG ACTGAGACAC GGCCCAGACT CCTACGGGAG GCAGCAGTGG GGAATATTGC

361 ACAATGGGGG AAACCCTGAT GCAGCGATGC CGCGTGGAGG AAGAAGGTTT TCGGATTGTA

421 AACTCCTGTC TTAAAGGACG ATAATGACGG TACTTTAGGA GGAAGCTCCG GCTAACTACG

481 TGCCAGCAGC CGCGGTAATA CGTAGGGAGC GAGCGTTGTC CGGAATTACT GGGTGTAAAG

541 GGAGCGTAGG CGGGATGGTA AGTTAGATGT GAAATGCAGG GGCTCAACCC CTGAGCTGCA

601 TTTAAAACTG CTGTTCTTGA GTGAAGTAGA GGTAAGCGGA ATTCCTGGTG TAGCGGTGAA

661 ATGCGTAGAT ATCAGGAGGA ACATCGGTGG CGAAGGCGGC TTACTGGGCT TTTACTGACG

721 CTGAGGCTCG AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCTGTAA

781 ACGATGATTA CTAGGTGTGG GGGGACTGAC CCCTTCCGTG CCGCAGTTAA CACAATAAGT

841 AATCCACCTG GGGAGTACGG CCGCAAGGTT GAAACTCAAA GGAATTGACG GGGGCCCGCA

901 CAAGCAGTGG AGTATGTGGT TTAATTCGAA GCAACGCGAA GAACCTTACC AGGTCTTGAC

961 ATCGAGTGAC GAACTAAGAG ATTAGTTTTT CTTCGGGACA CGAAGACAGG TGGTGCATGG

1021 TTGTCGTCAG CTCGTGTCGT GAGATGTTGG GTTAAGTCCC GCAACGAGCG
CAACCCTTAC
1081 CTTTAGTTGC TACGCAAGAG CACTCTAAAG GGACTGCCGT TGACAAAACG
GAGGAAGGTG
1141 GGGATGACGT CAAATCATCA TGCCCCTTAT GACCTGGGCT ACACACGTAC
TACAATGGCA
1201 ATTAACAAAG AGCAGCAAAG CGGTGACGCA GAGCGAATCT CCAAAAATTG
TCCCAGTTCG
1261 GATTGCAGGC TGCAACTCGC CTGCATGAAG TCGGAATTGC TAGTAATCGC
AGATCAGAAT
1321 GCTGCGGTGA ATACGTTCCC GGGCCTTGTA CACACCGCCC GTCACACCAT
GGGAGTCGGT
1381 AACACCCGAA GCCTGTAGTC TAACGAAAGA GGACGCAGTC GAAGGTGGGA
TTGATGACTG
1441 GGGTGAAGTC GTAACAAGGT ATCCGT

Figure A.4. Nucleotide sequence (1466 bp) of 16S rDNA gene (5 ´- 3 ´) of strain TC30. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 TCGCCAGGGT TTTCCAGTCA CGACGAGTTT GATCCTGGCT CAGGATGAAC GCTGGCGGCG

61 TGCTTAACAC ATGCAAGTCG AACGAAGCAC TTTACTTGAT TTCTTCGGAA TGATTGTTCT

121 GTGACTGAGT GGCGGACGGG TGAGTAACGC GTGGGTAACC TGCCTCATAC AGGGGGATAA

181 CAGTTGGAAA CGACTGCTAA TACCGCATAA GCGCACAGTA CTGCATGGTA CAGTGTGAAA

241 AACTCCGGTG GTATGAGATG GACCCGCGTC TGATTAGCTA GTTGGTGAGG TAGCGGCCCA

301 CCAAGGCGAC GATCAGTAGC CGACCTGAGA GGGTGACCGG CCACATTGGG ACTGAGACAC

361 GGCCCAAACT CCTACGGGAG GCAGCAGTGG GGAATATTGC ACAATGGGGG AAACCCTGAT

421 GCAGCGACGC CGCGTGAGCA AGAAGTATTT CGGTATGTAA AGCTCTATCA GCAGGGAAGA

481 AAAATGACGG TACCTGACTA AGAAGCACCG GCTAAATACG TGCCAGCAGC CGCGGTAATA

541 CGTATGGTGC AAGCGTTATC CGGATTTACT GGGTGTAAAG GGAGCGCAGG CGGTACGGCA

601 AGTCTGATGT GAAAGCCCGG GGCTCAACCC CGGTACTGCA TTGGAAACTG TCGAACTAGA

661 GTGTCGGAGG GGTAAGTGGA ATTCCTAGTG TAGCGGTGAA ATGCGTAGAT ATTAGGAGGA

721 ACACCAGTGG CGAAGGCGGC TTACTGGACG ATAACTGACG CTGAGGCTCG AAAGCGTGGG

781 GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGAATA CTAGGTGTCG

841 GGGAGCAAAG CTTCTCGGTG CCGCAGCAAA CGCAATAAGT ATTCCACCTG GGGAGTACGT

901 TCGCAAGAAT GAAACTCAAA GGAATTGACG GGGACCCGCA CAAGCGGTGG AGCATGTGGT

961 TTAATTCGAA GCAACGCRAA GAACCTTACC AAGTCTTGAC ATCCCGATGA CCAGCTATGT

1021 AATGTAGCCT CTCTTCGGAG CATCGGTGAC AGGTGGTGCA TGGTTGTCGT
CAGCTCGTGT
1081 CGTGAGATGT TGGGTTAAGT CCCGCAACGA GCGCAACCCT TATCCTTAGT
AGCCAGCGGT
1141 TCGGTCGGGC ACTCTAGGGA GACTGCCAGG GATAACCTGG AGGAAGGTGG
GGATGACGTC
1201 AAATCATCAT GCCCCTTATG ACTTGGGCTA CACACGTGCT ACAATGGCGT
AAACAAAGGG
1261 AAGCAAAACC GTGAGGTCGA GCAAATCTCA AAAATAACGT CTCAGTTCGG
ATTGTAGTCT
1321 GCAACTCGAC TACATGAAGC TGGAATCGCT AGTAATCGCG AATCAGAATG
TCGCGGTGAA
1381 TACGTTCCCG GGTCTTGTAC ACACCGCCCG TCACACCATG GGAGTTGGTA
ATGCCCGAAG
1441 TCAGTGACCC AACCGCAAGG AGGGAGCTGC CGAAGGCAGG ATCGATAACT
GGGGTGAAGT
1501 CGT

Figure A.5. Nucleotide sequence (1503 bp) of 16S rDNA gene (5 ´- 3 ´) of strain TC26. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 GAGTTTGATC ATGGCTCAGG ATGAACGCTG GCGGCGTGCT TAACACATGC AAGTCGAACG

61 AAGCACTTTA CTTGATTTCT TCGGAATGAT TGTTCTGTGA CTGAGTGGCG GACGGGTGAG

121 TAACGCGTGG GTAACCTGCC TCATACAGGG GGATAACAGT TGGAAACGAC TGCTAATACC

181 GCATAAGCGC ACAGTACTGC ATGGTACAGT GTGAAAAACT CCGGTGGTAT GAGATGGACC

241 CGCGTCTGAT TAGCTAGTTG GTGAGGTAAC GGCTCACCAA GGCGACGATC AGTAGCCGAC

301 CTGAGAGGGT GACCGGCCAC ATTGGGACTG AGACACGGCC CAAACTCCTA CGGGAGGCAG

361 CAGTGGGGAA TATTGCACAA TGGGGGAAAC CCTGATGCAG CGACGCCGCG TGAGCGAAGA

421 AGTATTTCGG TATGTAAAGC TCTATCAGCA GGGAAGAAAA AGACGGTACC TGACTTAAGA

481 AGCACCGGCT AAATASGTGC CAGCAGCCGC GGTAATACGT ATGGTGCAAG CGTTATCCGG

541 ATTTACTGGG TGTAAAGGGA GCGCAGGCGG TACGGCAAGT CTGATGTGAA AGCCCGGGGC

601 TCAACCCCGG TACTGCATTG GAAACTGTCG AACTAGAGTG TCGGAGGGGT AAGTGGAATT

661 CCTAGTGTAG CGGTGAAATG CGTAGATATT AGGAGGAACA CCAGTGGCGA AGGCGGCTTA

721 CTGGACGATA ACTGACGCTG AGGCTCGAAA GCGTGGGGAG CAAACAGGAT TAGATACCCT

781 GGTAGTCCAC GCCGTAAACG ATGAATACTA GGTGTCGGGG AGCATAGCTT CTCGGTGCCG

841 CAGCAAACGC AATAAGTATT CCACCCGGGG AGTACGTTCG CAAGAATGAA ACTCAAAGGA

901 ATTGACGGGG ACCGCACAAG CGGTGGAGCA TGTGGTTTAA TTCGAAGCAA CGCGAAGAAC

961 CTTACCAAGT CTTGACATCC CGATGACCAG CTATGTAATG TAGCCTCTCT TCGGAGCATC

1021 GGTGACAGGT GGTGCATGGT TGTCGTCAGC TCGTGTCGTG AGATGTTGGG TTAAGTCCCG

1081 CAACGAGCGC AACCCTTATC CTTAGTAGCC AGCGGTTCGG CCGGGCACTC TAGGGAGACT

1141 GCCAGGGATA ACCTGGAGGA AGGTGGGGAT GACGTCAAAT CATCATGCCC CTTATGACTT

1201 GGGCTACACA CGTGCTACAA TGGCGTAAAC AAAGGGAAGC AAAACCGTGA GGTCGAGCAA

1261 ATCTCAAAAA TAACGTCTCA GTTCGGATTG TAGTCTGCAA CTCGACTACA TGAAGCTGGA

1321 ATCGCTAGTA ACCGCGAACC AGAATGTCGC GGTGAATACG TTCCCGGGTC TTGTACACAC

1381 CGCCCGTCAC ACCATGGGAG TTGGTAATGC CCGAAGTCAG TGACCCAACC GCAAGGAGGG

1441 AGCTGCCGAA GGCAGGATCG ATAACTGGGG TGAAGTCGTA ACAAGGTATC CGTATC

Figure A.6. Nucleotide sequence (1496 bp) of 16S rDNA gene (5 ´- 3 ´) of strain X13. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 CGGCGCACGG GTGAGTAACA CGTATCCAAC CTGCCTTATA CTCCCGGACA GCCTTCTGAA

61 AGGAAGATTA ATACGGGATG GTATCACGAG CCGGCATTTT CTCGTGATTA AAGATACTTC

121 GGTATAAGAT GGGGATGCGT TCCATTAGAT AGTAGGCGGG GTAACGGCCC ACCTAGTCTA

181 CGATGGATAG GGGTTCTGAC AGGAAGGTCC CCCACATTGG AACTGAGACA CGGTCCAAAC

241 TCCTACGGGA GGCAGCAGTG AGGAATATTG GTCAATGGGC GCGAGCTGAA GCAGCCAAGT

301 AGCGTGAAGG ATGACTGCCC TACGGGTTGT AAACTTCTTT TATAAAGGAA TAAAGTGAGG

361 CACGTGTGCC TTTTTGTATG TACTTTATGA ATAAGGATCG GCTAACTCCG TGCCAGCAGC

421 CGCGGTAATA CGGAGGATCC GAGCGTTATC CGGATTTATT GGGTTTAAAG GGAGCGTAGA

481 TGGGTTGTTA AGTCAGTTGT GAAAGTTTGC GGCTCAACCG TAAAATTGCA ATTGATACTG

541 GCAGTCTTGA GTACAGTTGA GGTAGGCGGA ATTCGTGGTG TAGCGGTGAA ATGCTTAGAT

601 ATCACGAAGA ACTCCGATTG CGAAGGCAGC CTACTAAACT GCCACTGACA TTGATGCTCG

661 AAAGTGTGGG TATCAAACAG GATTAGATAC CCTGGTAGTC CACACGGTAA ACGATGAATA

721 CTCGCTGTTT GCGATATACT GCAAGCGGCC AAGCGAAAGC GTTAAGTATT CCACCTGGGG

781 AGTACGCCGG CAACGGTGAA ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGAGGAAC

841 ATGTGGTTTA ATTCGATGAT ACGCGAGGAA CCTTACCCGG GCTTGAATTG CACTCGAATA

901 TAGCGGAAAC GTTATAGCTA GCAATAGCGA GTGTGAAGGT GCTGCATGGT TGTCGTCAGC

961 TCGTGCCGTG AGGTGTCGGC TTAAGTGCCA TAACGAGCGC AACCCTTGTC TATAGTTACT

1021 AACAGGTCAT GCTGAGGACT CTATGGAGAC TGCCATCGTA AGATGTGAGG AAGGTGGGGA

1081 TGACGTCAAA TCAGCACGGC CCTTACGTCC GGGGCCACAC ACGTGTTACA ATGGGGGGTA

1141 CAGAGGGCCG CTACCACGCA AGTGGATGCC AATCCCGAAA GCCTCTCTCA GTTCGGACTG

1201 GAGTCTGCAA CCCGACTCCA CGAAGCTGGA TTCGCTAGTA ATCGCGCATC AGCCATGGCG

1261 CGGTGAATAC GTTCCCGGGC CTTGTACACA CCGCCCGTCA AGCCATGGGA GCCGGGGGTA

1321 CCTGAAGTGC GTAACCGCAA GGAGCGCCCT AGGGTAAAAC TGGTGACTGG GGCTAAGTCG

1381 TA

Figure A.7. Nucleotide sequence (1382 bp) of 16S rDNA gene (5 ´- 3 ´) of strain TC5. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 TCGCAGGGTT TCCAGTCACG ACGAGTTTGA TCTTGGCTCA GGATGAACGC TAGCTACAGG

61 CTTAACACAT GCAAGTCGAG GGGCAGCGGG ATTGAAGCTT GCTTCAATTG CCGGCGACCG

121 GCGCACGGGT GAGTAACGCG TATCCAACCT TCCGTTTACT CAGGGATAGC CTTTCGAAAG

181 AAAGATTAAT ACCTGATAGT ATGGTAAGAT TGCATGATAA TACCATTAAA GATTTATCGG

241 TAAACGATGG GGATGCGTTC CATTAGGTAG TAGGCGGGGT AACGGCCCAC CTAGCCGACG

301 ATGGATAGGG GTTCTGAGAG GAAGGTCCCC CACATTGGAA CTGAGACACG GTCCAAACTC

361 CTACGGGAGG CAGCAGTGAG GAATATTGGT CAATGGGCGA GAGCCTGAAC CAGCCAAGTA

421 GCGTGAAGGA TGAAGGTTCT ATGGATTGTA AACTTCTTTT ATAAGGGGAA TAACCGACAC

481 CACGTGTGGT GTTCTGCATG TACCTTATGA ATAAGCATCG GCTAACTCCG TGCCAGCAGC

541 CGCGGTAATA CGGAGGATGC GAGCGTTATC CGGATTTATT GGGTTTAAAG GGAGCGTAGA

601 CGGGATGTTA AGTCAGCTGT GAAAGTTTGG GGCTCACCCT TAAAATTGCA GTTGAAACTG

661 GCGTTCTTGA GTGCGGTAGA GGCAGGCGGA ATTCGTGGTG TAGCGGTGAA ATGCTTAGAT

721 ATCACGAAGA ACTCCAATTG CGAAGGCAGC TTGCTGGAGC GTAACTGACG TTGATGCTCG

781 AAAGTGTGGG TATCAAACAG GATTAGATAC CCTGGTAGTC CACACGGTAA ACGATGAATA

841 CTCGCTGTTG GCGATATACG GTCAGCGGCC AAGCGAAAGC ATTAAGTATT CCACCTGGGG

901 AGTACGCCGG CAACGGTGAA ACTCAAAGGA ATTGACGGGG GCCCGCACAA GCGGAGGAAC

961 ATGTGGTTTA ATTCGATGAT ACGCGAGGAC CTTACCCGGG CTTAAATTAC GCATGAATCA

1021 TTTGGAGACA GATGAGCCGC AAGGCCATGT ATGAGGGTGC TGCATGGTTG TCGTCAGCAC

1081 GTGCCGTGAG GTGTCGGCTT AAGTGCCATA ACGAGCGCAA CCCTTTCTGC CAGTTACTAA

1141 CAGGTTGAGC TGAGGACTCT GGCGGTACTG CCATCGTAAG ATGTGAGGAG GGTGGGGATG

1201 ACGTCAAATC AGCACGGCCC TTACGTCCTT TTCTACACAC GTGTTACAAT GGGGGGTACA

1261 GAAGGCAGCT TGCCGGCGAC GGTTGGCCAA TCCCAAAAGC CTCTCTCAGT TCGGACTGGA

1321 GTCTGCAACC CGACTCCACG AAGCTGGATT CGCTAGTAAT CGCGCATCAG CCACGGCGCG

1381 GTGAACACGT TCCCGGGCCT TGTACACACC GCCCGTCAAG CCATGAAAGC CGGGAGTACC

1441 TGAAGTGCGT AACCGCAAGG AGCGCCCTAG GGTCCTGGTA ATTGGGGCTA AGTCGTAACA

1501 AGGTAGCCGT GTCATAGCTG TTTCCTG

Figure A.8. Nucleotide sequence (1527 bp) of 16S rDNA gene (5 ´- 3 ´) of strain TC14. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 TTGCTTGCAA ACTGAAGATG GCGACCGGCG CACGGGTGAG TAACACGTAT
CCAACCTGCC
61 GATAACTCGG GGATAGCCTT TCGAAAGAAA GATTAATATC CGATAGTATT
AAAACCGCAT
121 GGTTTTACTA TTAAAGAATT TCGGTTATCG ATGGGGATGC GTTCCATTAG
TTTGTTGGCG
181 GGGTAACGGC CCACCAAGAC TACGATGGAT AGGGGTTCTG AGAGGAAGGT
CCCCCACATT
241 GGAACTGAGA CACGGTCCAA ACTCCTACGG GAGGCAGCAG TGAGGAATAT
TGGTCAATGG
301 GCGAGAGCCT GAACCAGCCA AGTAGCGTGA AGGATGACTG CCCTATGGGT
TGTAAACTTC
361 TTTTATATGG GGAATAAAGT ATTCCACGTG TGGGATTTTG TATGTACCAT
ATGAATAAGG
421 ATCGGCTAAC TCCGTGCCAG CAGCCGCGGT AATACGGAGG ATCCGAGCGT
TATCCGGATT
481 TATTGGGTTT AAAGGGAGCG TAGGTGGATT GTTAAGTCAG TTGTGAAAGT
TTGCGGCTCA
541 ACCGTAAAAT TGCAGTTGAA ACTGGCAGTC TTGAGTACAG TAGAGGTGGG
CGGAATTCGT
601 GGTGTAGCGG TGAAATGCTT AGATATCACG AAGAACTCCG ATTGCGAAGG
CAGCTCACTA
661 GACTGCAACT GACACTGATG CTCGAAAGTG TGGGTATCAA ACAGGATTAG
ATACCCTGGT
721 AGTCCACACA GTAAACGATG AATACTCGCT GTTTGCGATA TACAGTAAGC
GGCCAAGCGA
781 AAGCATTAAG TATTCCACCT GGGGAGTACG CCGGCAACGG TGAAACTCAA
AGGAATTGAC
841 GGGGGCCCGC ACAAGCGGAG GAACATGTGG TTTAATTCGA TGATACGCGA
GGAACCTTAC
901 CCGGGCTTAA ATTGCAAATG AATAATCTGG AAACAGGTTA GCCGCAAGGC
TGTTGTGAAG
961 GTGCTGCATG GTTGTCGTCA GCTCGTGCCG TGAGGTGTCG GCTTAAGTGC
CATAACGAGC

1021 GCAACCCTTA TCTTTAGTTA CTAACAGGTC ATGCTGAGGG ACTCTAGAGA
GACTGCCGTC
1081 GTAAGATTTG AGGAAGGTGG GGATGACGTC AAATCAGCAC GGCCCTTACG
TCCGGGGCTA
1141 CACACGTGTT ACAATGGGGG GTACAGAAGG CAGCTACCTG GCGACAGGAT
GCTAATCCCA
1201 AAAACCTCTC TCAGTTCGGA TCGAAGTCTG CAACCCGACT TCGTGAAGCT
GGATTCGCTA
1261 GTAATCGCGC ATCAGCCATG GCGCGGTGAA TACGTTCCCG GGCCTTGTAC
ACACCGCCCG
1321 TCAAGCCATG AAAGCCGGGG GTACCTGAAG TACGTAACCG CAAGGAGC

Figure A.9. Nucleotide sequence (1368 bp) of 16S rDNA gene (5 ´- 3 ´) of strain TC23. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 ATGACGCTAG CTACAGGCTT AACACATGCA AGTGGAGGGG CAGCAGGGAT GTAGCAATAC

61 ATTGCTGGCG ACCGGCGCAC GGGTGAGTAA CACGTATCCA ACCTGCCGAT TATTCCGGGA

121 TAGCCTTTCG AAAGAAAGAT TAATACTGGA TAGTATAACG AGAAGGCATC TTCTCGTTAT

181 TAAAGAATTT CGATAATTGA TGGGGATGCG TTTCATTAGT TTGTTGGCGG GGTAACGGCC

241 CACCAAGACA TCGATGGATA GGGGTTCTGA GAGGAAGGTC CCCCACATTG GGACTGAGAC

301 ACGGTCCAAA CTCCTACGGG AGGCAGCAGT GAGGAATATT GGTCAATGGA CGGGAGTCTG

361 AACCAGCCAA GTATCGTGAA GGATGCTGCC CTATGGGTTG TAAACTTCTT TTATATGGGA

421 ATAAAGTGCA GTATGTATAC TGTTTTGTAT GTACCATATG AATAAGGATC GGCTAACTCC

481 GTGCCAGCAG CCGCGGTAAT ACGGAGGATC CGAGCGTTAT CCGGATTTAT TGGGTTTAAA

541 GGGAGCGTAG GCGGAAGCTT AAGTCAGTTG TGAAAGTTTG CGGCTCAACC GTAAAATTGC

601 AGTTGATACT GGGTTTCTTG AGTGCAGTAG AGGTAGGCGG AATTCGTGGT GTAGCGGTGA

661 AATGCTTAGA TATCACGAAG AACTCCGATT GCGAAGGCAG CTTACTGGAC TGTAACTGAC

721 GCTGATGCTC GAAAGTGTGG GTATCAAACA GGATTAGATA CCCTGGTAGT CCACACAGTA

781 AACGATGAAT ACTCGCTGTT TGCGATATAC AGCAAGCGGC CAAGCGAAAG CATTAAGTAT

841 TCCACCTGGG GAGTACGCCG GCAACGGTGA AACTCAAAGG AATTGACGGG GGCCCGCACA

901 AGCGGAGGAA CATGTGGTTT AATTCGATGA TACGCGAGGA ACCTTACCCG GGCTTAAATT

961 GCAACTGACG TAGGTGGAAA CATCTATTCC TTCGGGCAGT TGTGAAGGTG CTGCATGGCT

1021 GTCGTCAGCT CGTGGTCATA GCTGTTTCCT TAAGTGCCAT AACGAGCGCA ACCCTTATCT

1081 TTAGTTACTA ACAGGTTATG CTGAGGACTC TGAGAGAGAC TGCCGTCGTA AGATGTGAGG

1141 AAGGTGGGGA TGACGTCAAA TCAGCACGGC CCTTACGTCC GGGGCTACAC ACGTTGTTAC

1201 AATGGGGGGT ACAGAAGGCA GCTACACGGC GACGTGATGC TAATCCCCAA AACCTCTCTC

1261 AGTTCGGATT GGAGTCTGCA ACCCGGACTC CATGAAGCTG GATTCGCTAG TAATCGCGCA

1321 TCAGCCACGG CGCGGTGAAT ACGTTCCCGG GCCTTGTACA CACCGCCCGT CAAGCCATGA

1381 AAGCCGGGGG TA

Figure A.10. Nucleotide sequence (1392 bp) of 16S rDNA gene (5 ´- 3 ´) of strain X5. The underlined sequence represents the region that was aligned with 16S rDNA gene sequences obtained from the BLAST search results and from the GenBank database (Appendix F).

1 CGAGTTGATC ATGGCTCAGA TTGAACGCTG GCGGCGTGC TTAACACATG
CAAGTCGTAC
61 GCGAAAGGGA CTTCGGTCCC GAGTAAAGTG GCGCACGGGT GAGTAACACG
TGGATAATCT
121 GCCTCTATGA TGGGGATAAC AGTTGGAAAC GACTGCTAAT ACCGAATAGG
CTCATGATGA
181 ACTTTGTGAG GAAAGGTGGC CTCTGCTTGC AAGCTATCGC ATAGAGATGA
GTCCGCGTCC
241 CATTAGCTAG TTGGTGGGGT AACGGCCTAC CAAGGCAACG ATGGGTAGCC
GATCTGAGAG
301 GATGATCGGC CACACTGGAA CTGAAACACG GTCCAGACTC CTACGGGAGG
CAGCAGTGGG
361 GAATATTGCG CAATGGGCGA AAGCCTGACG CAGCGACGCC GCGTGAGGGA
TGAAGGTCTT
421 CGGATCGTAA ACCTCTGTCA GAAGGGAAGA AACTAGGGTG CTCTAATCAT
CATCCTACTG
481 ACGGTACCTT CAAAGGAAGC ACCGGCTAAC TCCGGCCAGC AGCCGCGGTA
ATACGGAGGG
541 TGCAAGCGTT AATCGGAATC ACTGGGCGTA AAGCGCACGT AGGCTGTTAT
GTAAGTCAGG
601 GGTGAAATCC CACGGCTCAA CCGTGGAACT GCCCTTGATA CTGCACGACT
TGAATCCGGG
661 AGAGGGTGGC GGAATTCCAG GTGTAGGAGT GAAATCCGTA GATATCTGGA
GGAACATCAG
721 TGGCGAAGGC GGCCACCTGG ACCGGTATTG ACGCTGAGGT GCGAAACGTG
GGGAGCAAAC
781 AGGATTAGAT ACCCTGGTAG TCCACGCCGT AAACGATGGA TGCTAGATGT
CGGGATGTAT
841 GTCTCGGTGT CGTAGTTAAC GCGTTAAGCA TCCCGCCTGG GGAGTACGGT
CCCAAGGCTG
901 AAACTCAAAG AAATTGACGG GGGCCCGCAC AAGCGTKGGA GTATGTGGTT
TAATTCGATG
961 CAACGCGAAG AACCTTACCT AGGTTTGACA TCTGGGGAAC CCTCCCGAAA
AGGAGGGGTG

1021 CCTTCGGGGA GCCCCAAGAC AGGTGCTGCA TGGCTGTCGT CAGCTCGTGT
CGTGAGATGT
1081 TGGGTTAAGT CCCGCAACGA GCGCAACCCC TATGCATAGT TGCCAGCAGG
TAAAGCTGGG
1141 CACTCTATGC AGACTGCCCG GGTTAACCGG GAGGAAGGTG GGGACGACGT
CAAGTCATCA
1201 TGGCCCTTAC ACCTAGGGCT ACACACGTAC TACAATGGCA CGCACAAAGG
GCAGCGATAC
1261 CGTGAGGTGG AGCCAATCCC AAAAAACGTG TCCCAGTCCG GATTGCAGTC
TGCAACTCGA
1321 CTGCATGAAG TCGGAATCGC TAGTAATTCG AGGTCAGCAT ACTCGGGTGA
ATGCGTTCCC
1381 GGGCCTTGTA CACACCGCCC GTCACACCAC GAAAGTCGGT TTTACCCGAA
GCCGGTGAGC
1441 CAACTAGCAA TAGAGGCAGC CGTCTACGGT AGGGCCGATG ATTGGGGTGA
AGTCGTAACA
1501 AGGTAACCGT GTCATAGCTG TTTC

Figure A.11. Nucleotide sequence (1524 bp) of 16S rDNA gene (5 ´- 3 ´) of strain B2.

APPENDIX F

List of GenBank Accession numbers of nucleotide sequences used in the multiple sequence alignments and tree construction.

Partial and Complete 16S rDNA gene sequences

Organism	GenBank Accession Number
Ruminococcus flavefaciens NJ	AF030446
Ruminococcus flavefaciens 007	AF030447
Ruminococcus flavefaciens 4	AF030448
Ruminococcus flavefaciens ATCC49949	AF030449
Ruminococcus flavefaciens 17	AF030450
Ruminococcus albus 0R108	AF030452
Ruminococcus albus ATCC27211	AF030451
Ruminococcus flavefaciens LP-C14-Adx	AF104834
Ruminococcus flavefaciens AR72	AF104841
Ruminococcus flavefaciens Y1	AF104846
Ruminococcus productus	L76595
Ruminococcus lactaris	L76602
Ruminococcus torques	L76604
Ruminococcus gnavus	X94967
Ruminococcus callidus	X85100
Ruminococcus obeum	X85101
Eubacteria uniforme	L34626
Eubacteria siraeum	L34625
Eubacteria contortum	L34615

Partial and Complete 16S rDNA gene sequences

Organism	GenBank Accession Number
Eubacteria eligens	L34420
Eubacteria xylanophilum	L34628
Eubacteria desmolans	L34618
Escherichia coli	J01859
Butyrivibrio fibrisolvens OB189	U41169
Butyrivibrio fibrisolvens OB156	U41168
Butyrivibrio fibrisolvens ATCC19171	U41172
Clostridium cellulovorans	X73438
Clostridium termitidis	X71854
Clostridium thermolacticum	X72870
Clostridium aldrichii	X71846
Clostridium oroticum	M59109
Clostridium nexile	X73443
Clostridium xylanolyticum	X71855
Clostridium thermobutyricum	X72868
Clostridium populeti	X71853
Clostridium cellulosi	L09177
Clostridium cellulolyticum	X71847
Clostridium cellobioparum	X71856
Chlorobium vibrioforme	M62791
Roseburia cecicola	L14676
Bacteroides vulgatus	M58762
Bacteroides uniformis ATCC8492	L16486
Bacteroides thetaiotaomicron ATCC29148	16489
Bacteroides ovatus ATCC8483T	X83952

Partial and Complete 16S rDNA gene sequences

Bacteroides forsythus	AB035460
Bacteroides fragilis ATCC25285T	X83935
Bacteroides eggerthii NCTC11185	L16485
Bacteroides distasonis	M86695
Bacteroides caccae ATCC43185T	X83951
Prevotella ruminicola rum. ATCC19189	L16482
Prevotella brevis GA33	AJ011682
Prevotella bivia ATCC29303	L16475
Prevotella disiens ATCC29426	L16483
Prevotella albensis M384	AJ011683
Prevotella melaninogenica ATCC25845	L16469
Cytophaga lytica	M62796
Porphyromonas circumdentaria	L26102
Porphyromonas endodontalis ATCC35406	L16491
Flavobacterium gleum	M58772

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