THE POTENTIAL OF NEOKESTOSE AS A PREBIOTIC FOR BROILER CHICKENS

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“The germ is nothing; the terrain is everything”

Louis Pasteur (1822-1895)

I declare that the dissertation hereby submitted for the degree of Magister Scientiae (Microbiology) at the University of the Free State, Bloemfontein, is my own independent work and that I have not previously submitted this work for a qualification at/ in another university/ faculty. All sources of information used have been indicated and acknowledged by means of complete references.

Signature:

Date: 6 May 2008

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List of Abbreviations

%G+C	–	percentage guasine plus cytosine
1-FFT	–	1F-fructan-fructan fructosyltransferase
1-SST	–	sucrose:sucrose 1-fructosyltransgerase
6-FFT	–	6F-fructan-fructan fructosyltransferase
6G-FFT	–	6G-fructan-fructan fructosyltransferase
ADT	–	Agri Data Trust
AGP	–	antimicrobial growth promoter
ATCC	–	American Type Culure Collection
BHI	–	Brain heart infusion medium
CaEDTA	–	calcium
CE	–	competitive exclusion
CFU	–	colony forming unit
DGGE	–	denaturing gel electrophoresis
DNA	–	deoxynucleic acid
DP	–	degree of polimerisation
DSMZ	–	Deutsche Sammlung von Mikroorganismen un Zellkulturen
EtOH	–	ethanol
F6PPK	–	fructose-6-phosphate phosphoketolase
FCR	–	feed conversion ratio
FEH	–	Fructan exohydrolases
FOS	–	fructo-oligosaccharide
g	–	gram
GF	–	glucofructoside
GI	–	Gastro intestine
glc	–	glucose
HPLC	–	high performance liquid chromatography
Inu	–	inulin
kg	–	kilogram
l	–	liter
LAMVAB	–	MRS supplemented with vancomycin acetic acid and bromocresol green.
LiCl	–	Lithium Chloride
MCE	–	modified competitive exclusion
MDCP	–	mono-and di-calcium phosphate
MIC	–	minimum inhibitory concentration
Min	–	minute
ml	–	millilitre
MRS	–	De Man Regosa and Sharpe medium
MTPY	–	modified trypticase phytone yeast extract
MW	–	modified Wilkins-Chalgren
NCFB	–	National Collection of Food Bacteria
ND	–	not determined
neo	–	Neokestose mixture
OVI	–	Onderstepoort Veterinary Institute
PBS	–	phosphate buffered saline
PCR	–	Polimerase chain reaction
ppm	–	parts per million

PROP	–	propionate agar
PY	–	peptone yeast extract medium
RB	–	Raffinose Bifidobacterium medium
RCA	–	Reinforced Clostridial Medium base
rDNA	–	ribosomal deoxynucleic acid
SCFA	–	short chain fatty acid
T-cell	–	thymus cell
TOS-A	–	Trans-oligosaccharide propionate agar supplemented with acetic acid
VFA	–	volatile fatty acid
XLD	–	Xylose Lysine Deoxycholate medium
YM	–	Yeast extract malt medium

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Antibiotics have been used in animal feed as growth promotants since the mid-seventies. This has been, since its inception, very popular and even regarded as a necessary practice in the increasingly competitive agricultural economic environment. Antibiotics in animal feed have been shown to eliminate or inhibit intestinal microbial populations (Butaye ***et al.***, 2003)

Throughout the developed world there is public and governmental concern about the increasing prevalence of resistance to antibiotics in disease-causing bacteria. There are concerns that many antibiotics currently available to treat human diseases will in future no longer be effective (Casewell ***et al.***, 2003). There is a parallel concern that the development of resistance among bacteria is outstripping the ability of the pharmaceutical industry to develop new antibacterial agents.

Replacing in-feed antibiotics with non-antibiotic alternatives is, therefore, an ever increasing necessity. However, the withdrawal of all growth promotants is not a simple matter since this will not only affect feed efficiency but will also increase the mortality and morbidity of animals (Huyghebaert, 2003). It is therefore important to research alternative feed additives that may be used to alleviate the problems associated with the withdrawal of antibiotics from animal feeds.

Prebiotics are one class of feed additives that has shown promise in replacing or reducing antibiotics used as prophylactics or growth promoters. These are food ingredients which beneficially affect the host by stimulating the fraction of the intestinal microbiota which is regarded as essential for a healthy intestinal environment.

One such class of prebiotics is the fructo-oligosaccharides, to which neokestose, a trisaccharide, belongs. Neokestose consists of a sucrose (glucose and fructose) molecule to which a fructosyl is β-(2-6) linked to the carbon 6 atom of the glucose moiety on the sucrose molecule. There has been much work done on the prebiotic effects or the inulin type fructans, whereas little information is available on the inulin neoseries type, to which neokestose belongs.

Poultry is one of the major meat protein sources produced for the consumer market. The highly competitive nature and small profit margins of the market have led to extremely intensive rearing practices found in this industry. With such enormous numbers of animals cooped up in small spaces, it is important for pre-emptive approaches when dealing with various types of diseases. The use of in-feed antibiotics for chickens is one such approach which has become unpopular, leaving a gap for alternatives.

The aim of this project was to produce neokestose and then to study its effect on the intestinal microbial population of the chicken intestine ***in vitro***.

1.2. Literature Review

1.2.1. Antibiotics in the animal industry

1.2.1.1. Administration

Antibiotics are principally used in the animal industry for the treatment of disease, the prophylactic prevention of disease, and growth promotion. Antibiotics that are used therapeutically are administered in high dosages for a limited period to an individual or groups of animals showing signs of disease. For poultry the commercial value of the animal dictates that therapeutic antibiotics, which in most cases have to be prescribed by a veterinarian, are usually administered in the water. Prophylactic use involves the administration of antibiotics for a limited period to a group of healthy animals which are deemed to be at risk of disease caused by pathogens susceptible to the drugs. Growth promotant use of antibiotics generally refers to orally administered (in-feed, in-water) antibiotics which, when given to healthy animals for long periods at concentrations below the minimum inhibitory concentration (MIC) for most pathogens, will increase the rate and efficiency of growth. Prophylactic and growth-enhancing antibiotics are administered at sub MIC levels and at this dosage these are referred to as sub-therapeutics.

1.2.1.2. Antibiotic Growth Promoters and Prophylactics

1.2.1.2.1. General

Sub-therapeutic in-feed levels of antibiotics in chicken rearing, especially for broilers, are used routinely all over the world. Prophylactic and growth-enhancing effects have been explained by the inhibition of bacteria in the intestine (Bedford, 2000a). Muramatsu ***et al.*** (1994) stated that antimicrobial growth promoters (AGP's) seem to have no direct interaction with the physiology of the animal, but rather improve performance by influencing the microbial activity in the host. This performance is measured by the feed conversion ratio (FCR) which is calculated by the weight gained (kg) per unit of food (kg) over a given time period. Reduction in low-level infection and elimination of naturally resident bacteria increases the FCR.

1.2.1.2.2. Effects of sub therapeutic antibiotics

Over the years a number of benefits have been attributed to the use of antibiotics sub therapeutically when administered in feed and these have led to the reluctance in abandoning their use in animal rearing.

One of the main reasons has to do with the increase in growth rate and feed efficiency, which has been observed with feeding antibiotics (Rosen, 1995; Thomke & Elwinger, 1998a:1998b; Page, 2003). This growth promotion with the administration of antibiotics has been explained in a number of ways. Some researchers have found a decrease in the weight and length of poultry intestines (Visek, 1978 and Postma ***et al***., 1999 as cited by Gaskins, 1996). This was explained by a reduction in enteric cell development in the intestinal lumen due to a lower degree of microbial contamination (Visek 1978 as cited by Gaskins, 1996). It was proposed that the energy and protein normally required for re-synthesizing the enteric cells is available for growth (muscle accretion) when using AGP's (Bedford, 2000b). This has been demonstrated in oloxenic (germ-containing) animals where the enteric cell turnover due to microbial presence was shown to be twice as high compared to axenic (germ-free) animals (Vanbelle ***et al***., 1990). Another explanation for the enhancement of FCR and growth rate of AGP's is the indirect effect on bile function. In their conjugated form bile salts are important, aiding in the digestion, emulsification and absorption of fats and lipids from the small intestine. In the intestinal lumen, the endogenous gastrointestinal microbiota extensively biotransform bile acids, through deconjugating or hydrolysing conjugated bile salts (Hayakawa, 1973). Feighner and Dashkevicz (1987) investigated the effect of antibiotics on the bacterial bile salt hydrolase activity and found that this was reduced with the AGP's bacitracin and efrotomycin, but not with a non-AGP polymyxin.

It has also been acknowledged that AGP's confer a prophylactic effect. This is a preventative inhibition of low level type infections, especially by pathogenic bacteria, brought about by administering sub-therapeutic in-feed and/or in-water antibiotics. The transformation of primary bile acids to secondary bile acids has been reported

to be catalyzed by members of ***Clostridium*** and ***Eubacterium*** (Hayakawa, 1973; Hirano ***et al***., 1981). Lithocholic acid, a secondary bile acid produced from chenodeoxycholic acid (Norman & Sjovall, 1960), is a known hepatotoxic (Leveille ***et al***., 1962) which causes inflammation of the intestinal epithelium, and impairs nutrient uptake (Eyssen, 1973). Competition for nutrients in the intestinal tract, degradation of host enzymes and reduction of the absorptive surface area by the intestinal microbiota play a role in reduced feed conversion efficiency (Van Immerseel ***et al.,*** 2002). In some instances these bacteria can elicit an immune response, which, as a side effect, causes reduction of appetite and catabolism of muscle protein (Bedford, 2000b). One of the mechanisms which underlies this phenomenon is the massive production of tumour necrosis factor alpha. Such a mechanism has been found associated with ***Clostridium perfringens*** which causes necrotic enteritis (Hofacre ***et al***., 1998).

Other benefits of improved feed efficiency include reduced manure, phosphate and nitrogen output. More manure means greater expenditure for the removal thereof, thus money is saved when manure output is reduced. The reduction in excretion of phosphorus in the form of phytate is related to feed consumption, which is less in broilers fed AGP's. Chicken feed contains two forms of phosphorus: inorganic and organic (phytate), the latter which is the storage form in plants. Phytate is poorly utilized by nonruminant animals, like chickens. The result is that much of the phytate phosphorus passes directly through the chicken and is excreted in the faeces. Nitrogen in the form of ammonia is typically considered an indoor air quality concern by poultry producers because the gas often accumulates inside poorly ventilated or poorly managed animal facilities. Ammonia is produced from the deammination of amino acids while amines are produced from the decarboxylation of amino acids. Both these processes, which are due to bacterial action, reduce the available nitrogen uptake in the form of amino acids. Improved nitrogen retention was found with the use of virginiamycin (Page, 2003). This was found as a result of reduced nitrogen excretion and increased protein retention in broiler carcass composition. Elevated levels of ammonia (25 ppm) were responsible for a reduction in body weight and egg production of broilers and layers, respectively (Reece ***et al***., 1980; Deaton ***et al***., 1984)

Some studies however, showed that the increase in performance gained with the use of AGP's were only significant with the presence of disease and/or when the diet was of poor quality (Rosen, 1995; Inborr, 2001). Ficken (1997) found that high levels of fish meal or wheat can lead to damage of the intestinal mucosa which can predispose the chicken to necrotic enteritis by ***Clostridium perfringens***. Necrotic enteritis is one of the major diseases of poultry which has been controlled by the addition of subtherapeutic antibiotics. The use of AGP's therefore reduces the requirement for therapeutic antibiotics (De Craene & Viaene, 1992). Administration of therapeutic antibiotics usually increases when combating an increase in mortality and morbidity. An increase in the use of therapeutic antibiotics was seen in Netherlands, Germany and France (Muirhead, 2002; Veterinary Medicines Directorate, 2002) when producers voluntarily stopped the use of AGP's.

1.2.1.2.3. Types of AGP's and their modes of action

Antibiotics function by altering or impeding certain properties of the bacterial cellular structure or metabolism, resulting in impaired growth or death. This can be done by interfering with either cell wall biosynthesis, cell membrane integrity, protein synthesis (30S or 50S), DNA replication and repair, transcription, energy metabolism or intermediate metabolism. Antibiotics used as growth promoters work by influencing the microbial population found in the gastro-intestinal tract (Muramatsu ***et al.***, 1994). Most growth promoting antibiotics which have been used in the poultry industry have a broad spectrum of activity, with their primary action against the Gram-positive microbiota. The rational is that the Gram-positive microbiota is generally associated with poorer health and performance (Bedford, 2000a). The problem with this is that bacteria which are considered beneficial, like those belonging to the genera ***Lactobacillus*** and ***Bifidobacterium***, are impacted upon. Since this practise has begun a wide range of antibiotics have been used (Table 1), although many of these subtherapeutic antibiotics have recently been disallowed due to concerns over antibiotic resistance (Butaye ***et al.***, 2003)

1.2.1.2.4. Antibiotic resistance

Concerns over acquired resistance to antibiotics have been around since these 'wonder drugs' were first discovered. Alexander Fleming, discoverer of penicillin, warned about the misuse of it in a New York Times article published in 1945. In this article he stated that 'microbes could be educated to resist penicillin'. In the late 1960's these concerns were further evident when in Britain a committee, led by Dr. David Swann, was tasked to evaluate the use of antibiotics. In 1969 the Swann Committee made three recommendations for the use of antibiotics in feed. These entailed that the antibiotics be of economic value, should have little or no therapeutic application in humans or animals and should not impair the efficacy of any prescribed therapeutic antibiotics through the development of resistance strains. Tylosin, which was specifically named in the report as an antibiotic which should not be added to feed without prescription, was accepted for use in 1975 by the European Union together with another macrolide, spiramycin.

In the mid 1970's studies were conducted to evaluate the concerns of antibiotic resistance as a result of antibiotics in feed. One such study, which monitored microbial resistance in chickens as well as the workers that came into contact with the antibiotic, was conducted by Levy ***et al***. (1976). The study showed that chickens fed tetracycline-supplemented feed possessed and/or accumulated almost entirely tetracycline-resistant organisms intestinally after only the first week. Within five to six months, 31.3 % of weekly faecal samples from farm workers contained more than 80 % tetracycline-resistant bacteria as compared to 6.8 % of the samples from the workers on a neighbouring farm. It was suggested that the rapid spreading of resistant bacteria was due to transferable plasmids conferring multiple antibiotic resistance. This study therefore showed that antibiotic resistance can be transferred to humans that come in contact with chickens and/or feed supplemented with antibiotics (Levy ***et al***., 1976).

Table 1. Growth-promoting antibiotics allowed for use in the European Community, both past and present (modified from Butaye ***et al.***, 2003).

Antibiotic group	Antibiotic	Synonyms:	Action on specific bacteria	Related therapeutics	Action Mechanism
Glycolipid	Bambermycin	moenomycin, flavophospholipol, flavomycin	Some G+ & some G-		Inhibition of cell w all synthesis by preventing transpeptidation
Cyclic peptide	Bacitracin[1]		Mainly G+	Bacitracin	Inhibition of cell w all synthesis
Ionophore	Monensin		Mainly G+ & anaerobic bacteria		Interference in the ionic balance, disintegration of the cytoplasmic membrane
	Salinomycin				
Streptogramin peptolides	Virginiamycin[1]		Narrow spectrum: G+ & some G-	Quinupristin dalfopristin	Inhibition of protein synthesis by stalling ribosome
Macrolide	Tylosin[1]		Mainly G+ (mycoplasma) & G- anaerobes	Erythromycin	Inhibition of protein synthesis by stalling ribosome
	Spiramycin[1]				
Oligosaccharide, orthosomycin	Avilamycin		Only G+	Everinomycin	Inhibition of protein synthesis by preventing elongation
Glycopeptide	Avoparcin[1]		Mainly G+	Vancomycin, teicoplanin, daptomycin & teicomycin	Inhibition of cell w all synthesis by preventing transglycosylation
	Ardacin[1]				
Elfamycin	Efrotomycin				Inhibition of protein synthesis
Quinoxaline	Olaquindox[1]		Mainly G- & some G+	Cyadox	Inhibition of DNA synthesis
	Carbadox[1]				

1- Antibiotics that have been disallowed for use in poultry in the EU.

More recent work also shows the link between the selection of antibiotic resistant bacteria in animals and workers who come into contact with AGP's in feed (Nadeau ***et al.,*** 1999; Van den Bogaard ***et al.***, 2001). Another concern is the development of cross-resistance for antibiotics that either work similarly or are structurally similar. This has been the case with the two streptogramins virginiamycin and quinupristin-dalfopristin (Table. 1) that are used against vancomycin resistant ***Enterococcus faecium***. Kieke ***et al.*** (2006) showed that human exposure to poultry reared with virginiamycin selected for ***Enterococcus faecium*** with cross-resistance to quinupristin-dalfopristin. Furthermore, resistance towards antibiotics as a result of poultry to human transmission, through the transmission of host independent ***Escherichia coli*** resistant clones and/or transference of plasmids carrying resistance elements, has been found by Van den Bogaard ***et al.*** (2001) to commonly occur.

1.2.1.2.5. Withdrawal of in-feed antibiotics

The first pro-active action, in the UK, was probably taken with the presentation of the Swann report in 1969. This report concluded that antibiotics exert a positive effect on growth and feed conversion but it cautioned on the general use of them by putting forward certain criteria. These criteria entailed that only those antibiotics used in growth promotion which appeared, on scientific grounds, to have no direct correlation and function with antibiotics administered to humans, should be selected. In the early 1980's, avoparcin was replaced by virginiamycin, which was the main in-feed antibiotic in 1986. In 2001 only 4 antimicrobial growth promotants (avilamycin, flavomycin, salinomycin and monensin) were still allowed in the European Community. Since then market pressure resulted in the present application of only salinomycin and monensin and that only because of their effectiveness against coccidiosis.

Sweden implemented the ban on in-feed antibiotics in 1986 and then fully abandoned the use of in-feed antibiotics in 1988. They showed that dependence on in-feed antibiotics can be broken by addressing broiler- and housing hygiene and through better feed formulations. Since then Swedish

poultry farmers have successfully maintained a generally good health status and have improved on growth rate and feed efficiency. This was achieved with the use of a number of alternatives which included organic acids, prebiotics (mainly oligosaccharide products), probiotics and feed enzymes (Inborr, 2001).

1.2.1.3. Alternatives to in-feed antibiotics

In light of the growing concerns surrounding antibiotic use in food animals, many researchers have been looking for alternatives to improve feed utilization and growth promotion as well as non-antibiotic prophylactics. These include probiotics, prebiotics, enzymes used to treat non-starch polysaccharides, diet acidifiers, fermented liquid feeding, nutraceuticals, minerals, novel antibodies, vaccination, and improved management and husbandry practices. In this work only the effects of probiotics, prebiotics and synbiotics will be discussed.

1.2.1.3.1. Probiotics

The concept of probiotics evolved at the turn of the 20th century from a hypothesis first proposed by the Nobel Prize winning Russian scientist Elie Metchnikoff (Bibel, 1988). He suggested that the long, healthy life of Bulgarian peasants resulted from their consumption of fermented milk products. He believed that when consumed, the fermenting bacillus (***Lactobacillus***) replaced the intestinal microbiota (believing that the intestinal population was toxic) of the colon, decreasing toxic microbial activities. It is now recognized that the intestinal microbial population is a dynamic environment where naturally occurring beneficial bacteria do also exist. It is thought that in a healthy intestinal environment these beneficial bacteria are dominant, while it is also believed that in an upset environment these bacteria can be introduced and/or nurtured to become dominant.

A probiotic is defined as “a live microbial food supplement that beneficially affects the host animal by improving its intestinal microbial balance” (Fuller,

1989). The probiotics which have been most widely used are lactic acid producers belonging to the genera ***Lactobacillus*** and ***Bifidobacterium***.

The beneficial effects of a balanced intestinal microbial population which can be attained and/or maintained by effective probiotic addition include inhibition of pathogens, modulation of the immune system, synthesis of vitamins, mucosal permeability, colonization resistance, production of metabolic fuel for enterocytes and improved digestion (Sanders & Gibson, 2006). The benefits resulting from the influence of ***Bifidobacteriumi*** species is known as the bifidogenic effect.

Probiotics belonging to different genera, have shown various beneficial effects in poultry. An enhanced broiler growth rate resulted with the in-feed addition of ***Bacillus coagulans*** strains (Cavazzoni ***et al.***, 1998). A ***Lactobacillus*** probiotic consisting of the strains ***L. acidophilus, L. fermentum, L. crispatus*** and ***L. brevi*** improved the FCR and weight gain of broilers (Jin ***et al.***, 1998) reared on a corn starch diet. Supplemented at 0.05 % and 0.10 % these cultures also significantly lowered the serum cholesterol levels and coliform counts of broilers compared to the control group fed on the same untreated corn starch diet. Lundeen (2001) also credited ***Lactobacillus*** with reducing ***Eimeria acervulina*** infection rates in poultry challenged with ***E. acervulina***. He attributed the reduced infection rate by this protozoan species, shown to be the infectious agent in coccidiosis, to an enhanced immune response triggered by the introduction of ***Lactobacillus***. In broilers, ***Bifidobacterium*** have been shown by Smirnov ***et al***. (2005) to change intestinal mucin dynamics. They suggested that changes to mucin dynamics may influence health, associated with gut function, and nutrient uptake. Although tested ***in vitro***, Gibson and Wang (1994b) found ***E. coli*** and ***Clostridium perfringens*** to be inhibited by ***Bifidobacterium infantis*** in co-culture experiments, while diminished growth was noticed for species belonging to the genera ***Salmonella***, ***Listeria, Campylobacter*** and ***Shigella***. The ***Bifidobacterium*** fermentation products, acetate and lactate, and the production of anti-microbial substances with broad spectrum activity were some of the mechanisms which were credited with this inhibition effect.

Competitive exclusion is another way in which probiotics can control pathogenic species. This has been used to control ***Salmonella*** and ***Campylobacter*** infection in young chicks (see 1.4.4). Nurmi and Rantala (1973) were the first to demonstrate this concept by feeding adult caecal and/or faecal digests to young chicks. They explained that this populates the virgin intestines with naturally occurring bacteria found in adult birds, thus making the adherence sites unavailable to opportunistic pathogenic bacteria. It was found that complex mixtures of intestinal contents where more effective against ***Salmonella*** colonization than single species or simple mixtures (Mead & Impey, 1987; Stavric & D'Aoust, 1993). Stern ***et al***. (2001) compared a mucosal competitive exclusion culture (MCE)(Stern ***et al.***, 1995) to a competitive exclusion culture (CE)(Aho ***et al***., 1989) and found that colonization by ***Salmonella*** Typhimurium and ***Campylobacter*** was reduced in the MCE treated birds. The reduced colonization by pathogens when using MCE compared to CE was related to their alternative preparation (Stern ***et al***., 1988; Shanker ***et al***., 1990). The MCE product has a stricter anaerobic preparation, with more intensive washing and scraping of the epithelium, together with a different culturing media and temperature (MCE = 35 ^{o}C; CE = 42 ^{o}C). Some countries, however, do not allow the addition of uncharacterised (unidentified) microbial additives to feed. This is problematic since defined mixtures have been reported to be less effective as CE agents and have shorter shelf lives than undefined mixtures (Hofacre ***et al***., 2000).

Micro-organisms exist in various micro-habitats found in the gastrointestinal tract, four of these were defined as follows by Freter, (1992): 1) The surface of epithelium cells; ***2***) the crypts of the ileum, caecum and colon; ***3***) the mucus gel that overlays the epithelium; and ***4***) the lumen of the intestine. It has been found that ***Campylobacter*** spp. are associated in chicken with the crypt mucus of caeca, without being attached to the microvilli (Beery ***et al.***, 1988; Meinersmann ***et al***., 1991).

Introducing a probiotic to an imbalanced site can only be truly effective if they remain resident. It is therefore important that these organisms survive the passage to the imbalanced site. The first step of colonization at this site is

considered to be adherence. The adherence in live hosts is determined by the difference in colony forming units (cfu's) between fed and faeces-collected organisms. This is sometimes misleading since it was shown that even dead, but intact, ***L. acidophilus*** cells were able to adhere as effectively as viable ***L. acidophilus*** cells to cultivated monolayers of intestinal tissue cells (Hood & Zoitola, 1988). This shows the necessity for investigating active metabolism when determining adherence. Species specificity is another factor influencing successful colonization of the host ***in vivo***. Mitsuoka (1969a; 1969b) showed that various biotypes of the same ***Lactobacillus*** and ***Bifidobacterium*** species reside in humans and animals.

By providing a suitable food source the beneficial component of the intestinal tract can be nurtured to affect various benefits. Food sources benefiting probiotics are called prebiotics.

1.2.1.3.2. Prebiotics

Prebiotics are generally defined as non-digestible food ingredients which beneficially affect the host by selectively stimulating one, or a number of residential bacteria, which are beneficial for the host (Gibson & Roberfroid, 1995). The prebiotic concept is based on the assumption that particular colonic micro-organisms, such as ***Bifidobacterium*** and ***Lactobacillus*** species, considered beneficial to the health of the human or animal host, may be selectively stimulated by indigestible but fermentable dietary carbohydrates (Cummings ***et al***., 2001). Bacteria which are fed a preferential substrate have a proliferative advantage over other bacteria.

Prebiotics are found in a number of dietary sources such as soybeans, Jerusalem artichokes, raw oats, unrefined wheat and unrefined barley. The prebiotic components in these are fructo-oligosaccharides (FOS), xylo-oligosaccharides, galacto-oligosaccharides and mannan-oligosaccharides.

As has been discussed, prebiotics are beneficial when they positively affect beneficial bacteria. It has been shown that ***Salmonella*** serovars were

inhibited ***in vitro*** by ***Bifidobacterium*** and ***Lactobacillus*** species when grown in media containing FOS as the sole carbon source (Oyarzabal ***et al.***, 1995). A four fold reduction of caecal ***Salmonella*** was observed by Bailey ***et al***. (1991) in chickens fed FOS through either their drinking water or feed. Prebiotics can therefore selectively modify the colonic microbiota. Acting somewhat differently Bio-Mos® containing mannan-oligosaccharide has also shown promise in suppressing enteric pathogens, modulating the immune response, and improving the integrity of the intestinal mucosa in studies with chickens (Iji ***et al***., 2001; Spring, 1999a, 1999b; Spring ***et al***., 2000). This has, however, been attributed to the capacity of these sugars to bind pathogenic organisms such as ***Salmonella enterica*** subsp. ***enterica*** (Oyofo ***et al.,*** 1989) and ***Escherichia coli*** or to stimulate the immune system. Cell receptors and the antigenic determinants of several pathogenic bacteria contain mannans (Castro et ***al.,*** 1994; de Ruiter et ***al.,*** 1994; Kagaya et ***al.,*** 1996). Some mannan-oligosaccharides are added to vaccines as adjuvants for their immune stimulation enhancing effect and prolonging of the immune response.

1.2.1.3.3. Synbiotics

The combination of a prebiotic and probiotic as a single administration is called a synbiotic. This concept has been tested in poultry and it was shown that a prebiotic that is administered with a probiotic gives a greater response than when administered separately. This improvement was seen against ***Salmonella*** colonization in 7-day-old chicks (Bailey ***et al.***, 1991). Another enhanced effect of synbiotic use, was a higher villi density, seen in histological indexes of the intestinal mucosa, of 21 day-old chickens (Pelicano ***et al.,*** 2005). Prebiotics, when administered on their own, will only be effective if the health promoting microbial species that are capable of utilizing this energy source are present in the digestive tract of the host. In the following section the nature of some prebiotics will be discussed.

1.2.2. Fructans: Levans and Inulins

1.2.2.1 General properties and structure

Fructan is a general term used to describe any carbohydrate in which one or more fructosyl-fructose links constitutes the majority of osidic bonds. In most plants reserve carbohydrates are stored as starch; however in 15 % of flowering plant species fructans are synthesised (Hendry, 1993). In contrast to starch, that is stored in plastids, fructans are synthesised, stored and hydrolysed in cell vacuoles (Hendry, 1993). Hendry and Wallace (1993) estimated that as much as one third of total vegetation on earth consists of plants containing fructans. Of these a large part is present in regions with seasonal drought or cold, hence in addition to carbon storage, fructans have been implicated in protecting plants against water deficit caused by drought and/or low temperatures (Hendry and Wallace, 1993). Fructans synthesised in nature are water soluble, non-reducing sugars and are linear or branched polymers of fructose molecules (Banguela & Hernández, 2006). In higher plants five major classes of structurally different fructans have been distinguished.

These include inulin, levan, mixed levan, inulin neoseries, and the levan neoseries (Vijn & Smeekens, 1999). These fructose polymers are generally formed from sucrose; a glucose molecule is therefore usually also found in the structure. Based on the linkage between fructose-fructose units and/or fructose-glucose units different types of fructans can be distinguished.

Inulin consists mainly of linear β(2-1)-linked D-fructosyl units and is β(2-1)-linked to sucrose ($G_{1\text{-}2}F_{1\text{-}2}F_n$), of which the smallest is the trisaccharide 1-kestose (Fig 1.1A). Levan consists primarily of linear β(2-6)-linked D-fructosyl units ($G_{1\text{-}2}F_{6\text{-}2}F_n$), of which the smallest is the trisaccharide 6-kestose (Fig 1.1B)(Han & Watson, 1992). Mixed levan consists of both β(2-1) and β(2-6)- linked fructosyl units and in this case the smallest molecule is the tetrasaccharide bifurcose. The inulin neoseries have a structure consisting of D-fructosyl units linked to both C_1 and C_6 of the glucose moiety

of the sucrose molecule (Shiomi, 1989). The trisaccharide neokestose (Fig. 1.1C) is the smallest molecule of this type and with further polymerisation the D-fructosyl units are β(2-1)-linked. The levan neoseries are polymers of predominantly β(2-6)-linked D-fructosyl units linked to either side of the glucose moiety of the sucrose molecule.

Fructo-oligosaccharides are polymers that consist of between 2 and 10 fructosyl units. The FOS, fructans and inulin-type oligosaccharides all contain two or more fructosyl units. All these fructose containing saccharides share an important attribute, which is the presence of β-osidic bonds. These bonds are not digested by the enzymes synthesised by animals or humans (α-glucosidase, maltase-isomaltase, sucrase), which are specific for α-osidic bonds (Roberfroid, 1996).

The enzymes that synthesize these fructans are found in both plants and microorganisms. In plants neokestose has been isolated from onions, while the yeast ***Xanthophyllomyces dendrorhous*** synthesises this trisaccharide extra-cellularly when grown on sucrose (Kilian ***et al***., 1996). In the next section the enzymes which produce these FOS are discussed.

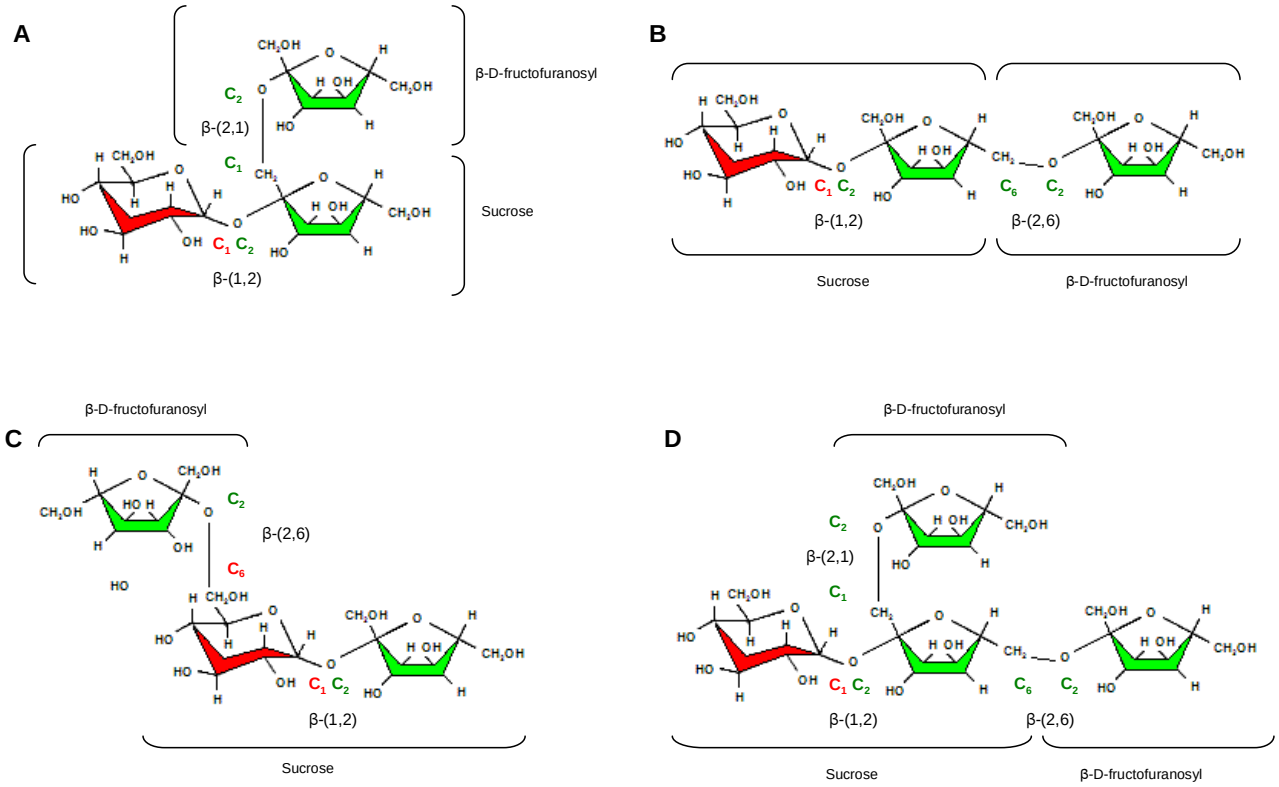

Figure 1.1. The structures of (A) 1-kestose, (B) 6-kestose, (C) neokestose and (D) bifurcose

1.2.2.2. Enzymes of fructan synthesis

Fructans are either synthesised by the transferase activity of β-fructofuranosidase or by β-fructosyltransferase (Anderson ***et al.***, 1969). The trisaccharides 1-kestose, 6-kestose and neokestose (Fig. 1.1) are examples of different reaction products, which are formed when a fructosyl moiety is transferred to various molecular locations on sucrose (Rehm ***et al.,*** 1998).

1.2.2.2.1. β-Fructofuranosidase

β-Fructofuranosidase, also known as invertase, catalyzes the hydrolysis of sucrose and related glycosides (Myrbäck, 1959). In plants, invertases exist in several isoforms with different biochemical properties and subcellular locations (Sturm, 1996; Tymowska-Lalanne & Kreis, 1998). They are located in the vacuole (acid invertase), cell wall (extracellular invertase) and the cytoplasm (alkaline or neutral invertase) of plants. Vacuolar and cell wall invertases cleave sucrose most efficiently between pH 4.5 and 5.0 and attack the disaccharide from the fructose residue (Unger ***et al***., 1992 as cited by Lee & Sturm, 1996). Historically, hydrolysis was supposed to be the primary action of invertases; however, it has been shown that even the purest β-fructofuranosidase preparations lead to formation of oligosaccharides (Myrbäck, 1959; Anderson, 1967). It was thus suggested that both hydrolysis and transfer are catalyzed by the same enzyme. The transfer activity dominates when there is a sufficient sucrose concentration, while the hydrolysis activity dominates when the substrate concentration is low, necessitating the liberation of utilisable carbon source (Myrbäck, 1959) With sufficient sucrose concentration β-fructofuranosidases are able to catalyze the formation of all three kestose isomers, 1-kestose, 6-kestose and neokestose (Rehm ***et al.,*** 1998). The simplest substrate for the production of fructans by invertases is sucrose. The alkaline invertases have been found to be sucrose specific, while neutral and acid invertases (β-fructofuranosidase) can hydrolyse other β-fructose-containing oligosaccharides such as raffinose and stachyose in addition to sucrose (Lee & Sturm, 1996).

1.2.2.2.2. β-Fructosyltransferase

The amino acids involved in sucrose hydrolyses are conserved among the plant invertases and fructosyltransferases, suggesting an analogous mechanism (Vijn & Smeekens, 1999). In plants, fructan is synthesised from sucrose by the action of two or more different fructosyltransferases. In contrast to plant fructosyltransferases bacterial fructosyltransferases are multifunctional enzymes capable of directly converting sucrose into FOS and fructans of a high degree of polymerisation. Bacterial fructosyltransferases are called levansucrases when levan is produced and inulosucrases when inulin-type fructans are produced. Bacterial fructosyltransferases can fructosylate a variety of substrates. These can be water (sucrose hydrolysis), sucrose (kestose synthesis), fructan (fructan polymerisation), glucose (sucrose synthesis), and fructose (bifructose synthesis). All known bacterial fructosyltransferases are either extracellular or cell bound proteins (Banguela & Hernández, 2006)

In plants, two types of fructosyltransferases are involved in the biosynthesis of fructans: sucrose:sucrose fructosyltransferase (EC 2.4.1.99) and 1^F, 6^F or 6^G–fructan:fructan fructosyltransferase (EC 2.4.1.100) (St. John ***et al.***, 1993; Rehm ***et al.***, 1998). Sucrose:sucrose 1-fructosyltransferase (1-SST) catalyses fructosyl transfer from one sucrose molecule to another, resulting in the formation of a trisaccharide (1-kestose) and glucose. Subsequently chain elongation is mediated by either 1^F- or 6^F –fructan:fructan fructosyltransferase (1-FFT and 6-FFT) to form inulins or levans, respectively (St. John ***et al.***, 1993; Roberfroid & Delzenne, 1998).

In the family ***Alliaceae*** (e.g. ***Allium cepa***, onion) and ***Asparagaceae*** (e.g. ***Asparagus officinallis***, asparagus) neokestose, the inulin-neoseries fructan (Fig. 1.1C), is synthesized by 6^G fructan:fructan fructosyltransferase (6G-FFT), which catalyses the transfer of the terminal fructosyl residue from 1-kestose to the glucose residue of sucrose via a β-(2→6) linkage (Vijn ***et al.***, 1998). Only 1-SST and 6^G-FFT are involved in the production of onion

neoinulins, while 1-FFT is additionally required in neoinulin production in asparagus (Ritsema ***et al.***, 2003).

1.2.2.3. Mechanisms of fructan synthesis

1.2.2.3.1. Plants

Plants posses a number of transfructosylases that act together to produce inulin, levan, mixed levan, inulin neoseries, and levan neoseries fructans. Different reaction mechanisms have been elucidated for the production of these various types of fructans (Fig. 1.2).

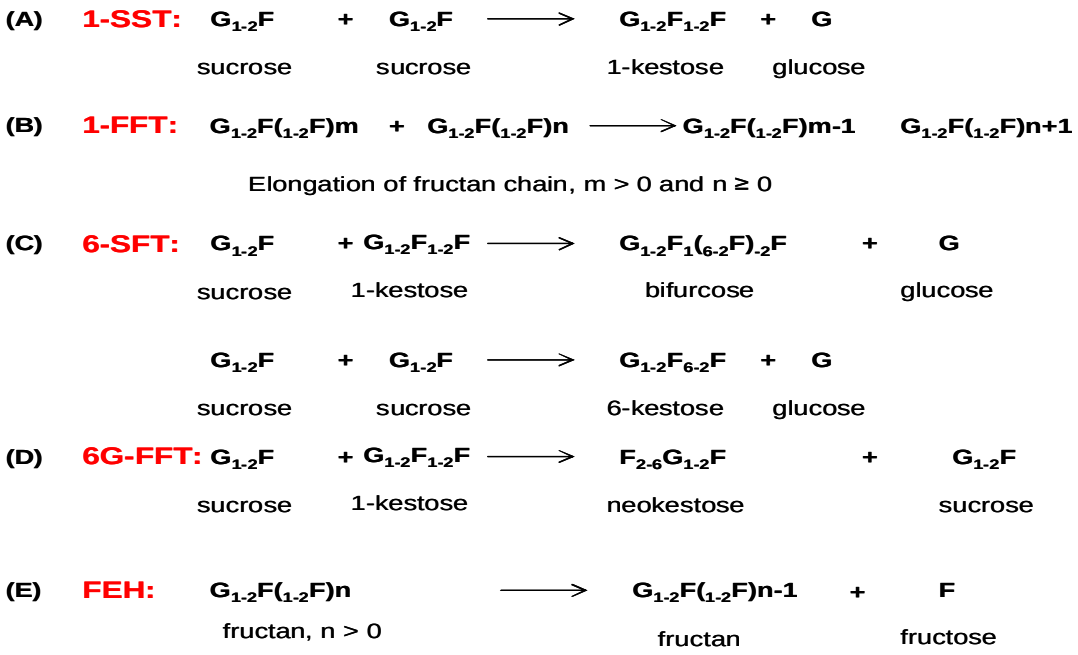

Figure 1.2. Enzymatic activities of different plant fructosyltransferases involved in fructan synthesis and of fructan exohydrolase, an enzyme involved in fructan degradation. FEH, Fructan exohydrolase (Vijn & Smeekens, 1999). See text for explanation of other abbreviations.

A model (Fig. 1.3) by Vijn & Smeekens (1999) best illustrates the cooperative action of these enzymes for the synthesis of various fructans from sucrose. In these mechanisms only levan can be produced without the initial production of 1-kestose (trisaccharide).

Inulin in the Jerusalem artichoke (***Helianthus tuberosus***) is synthesised by SST (Fig 1.2A) and 1-FFT (Fig 1.2B) which function independently. The only function of SST is to catalyze the formation of glucose and a trisaccharide (1-kestose) from sucrose, while further polymerization (i.e. increase in the degree of polymerisation (DP)) is achieved by FFT (Edelman & Jefford, 1968).

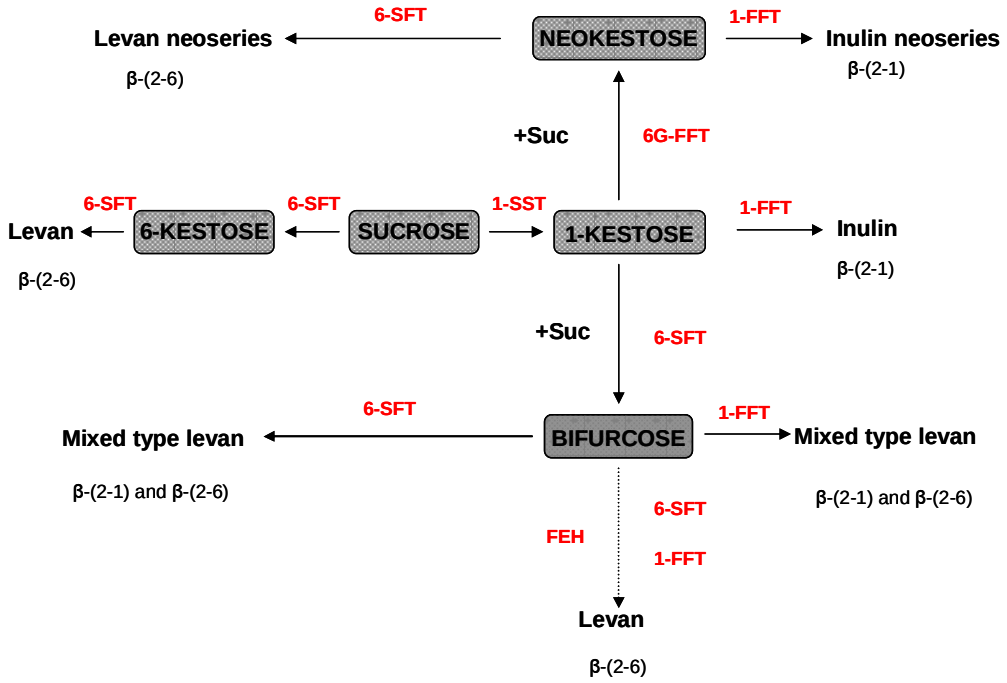

Figure 1.3. Model of fructan biosynthesis in plants proposed by Vijn and Smeekens (1999). The dotted arrow shows an alternative route for the production of levan (Wiemken ***et al***, 1995 cited by Vijn & Smeekens, 1999). FEH, Fructan exohydrolase. See text for explanation of other abbreviations.

Bifurcose (Fig. 1.1D), which is composed of both (2-1)-and (2-6)-linked β-D-fructosyl units linked to sucrose is produced in barley (Duchateau ***et al.***, 1995) by 6-SFT. Fructosyl from sucrose is attached to the glucose moiety of 1-kestose via a β-(2-6) linkage (Sprenger ***et al.***, 1995).

In the family ***Alliaceae*** (e.g. ***Allium cepa***, onion) and ***Asparagaceae*** (e.g. ***Asparagus officinallis***; asparagus) neokestose, the inulin-neoseries fructan, (Fig. 1C) is synthesized by 6^G fructan:fructan fructosyltransferase (6G-FFT), which catalyses the transfer of the terminal fructosyl residue from 1-kestose to

the glucose residue of sucrose via a β-(2→6) linkage (Vijn ***et al.***, 1998). Only 1-SST and 6^G-FFT are involved in the production of onion neoinulins, while 1-FFT is additionally required for neoinulin production in asparagus (Ritsema ***et al.***, 2003)

1.2.2.3.2. Micro-organisms

Fungal and bacterial inulins and levans are generally assumed to be synthesized without a trisaccharide as intermediate, but through sequential transfer of fructosyl residues from sucrose as fructosyl donor to the growing inulin and levan chains by inulosucrase (sucrose 1^F-fructosyltransferase) and levansucrase (sucrose 6^F-fructosyltransferase), respectively (Smeekens ***et al***., 1991).

Dickerson (1972) proposed the following mechanism for neo-inulin synthesis in the fungal plant parasite, ***Claviceps purpuria***.

$$\mathbf{F_{2\rightarrow1}G + F_{2\rightarrow1}G \rightarrow F_{2\rightarrow6}G_{1\rightarrow2}F + G} \qquad \mathbf{(1)}$$

$$\mathbf{F_{2\rightarrow1}G + F_{2\rightarrow6}G_{1\rightarrow2}F \rightarrow F_{2\rightarrow1}F_{2\rightarrow6}G_{1\rightarrow2}F + G} \qquad \mathbf{(2)}$$

In the first reaction the fructosyl moiety of one sucrose ($\mathbf{F_{2\rightarrow1}G}$) is transferred to the glycosyl moiety of another sucrose ($\mathbf{F_{2\rightarrow1}G}$) molecule forming neokestose ($\mathbf{F_{2\rightarrow6}G_{1\rightarrow2}F}$) and glucose (G). In the second reaction a tetrasaccharide is synthesized from neokestose and sucrose. In the above reactions numbers indicate the position of carbonyl carbon atoms and the arrows represent the direction of the glycosidic linkage (e.g., $F_{2\rightarrow1}G$ refers to sucrose). In addition to the two synthetic reactions above, hydrolyzing reactions also occur, and a hydrolysate like $F_{2\rightarrow6}G$ can act again as fructose donor and acceptor for the synthesis of neokestose and its tetra-oligomer. In some cases glucofructosides no larger than pentasaccharides (GF_4) are produced. This phenomenon has been explained by a limitation in their acceptor site, which is found in ***Fusarium oxysporum*** (Gupta and Bhatia, 1980), ***Aspergillus niger*** (Hidaka ***et al.***, 1988; Hirayama ***et al.***, 1989) and

Aureobasidium pullulans (Yun ***et al.,*** 1990,1992; Hayashi ***et al***., 1991). A good explanation of this limitation is described by Jung ***et al***. (1989), who suggested the following reaction mechanism for a transfructosylase, derived from ***Aureobasidium pullulans***:

$$GF_n + GF_n \rightarrow GF_{n-1} + GF_{n+1} \qquad (n=1\text{-}3)$$

According to this mechanism, the enzyme acts in a disproportionation type reaction, where one molecule (GF_n) serves as a donor and another (GF_n) acts as an acceptor. The products that can be formed by this mechanism are a trisaccharide (GF_2), tetrasaccharide (GF_3) and a pentasaccharide (GF_4).

Hidaka ***et al***. (1988) showed that S***accharomyces cerevisiae*** produces three trisaccharides: 1-kestose, 6-kestose and neokestose from sucrose. They attributed the formation of this variety of trisaccharides to the low regiospecificity for fructosyl transfer to the three primary alcohol groups of sucrose (Hidaka ***et al.***, 1988). Some fructosyltransferases from ***Aureobasidium*** spp. and ***A. niger***, however, have a higher regiospecificity (1-OH) to form only 1-kestose based fructans. Similarly, ***Xanthophyllomyces dendrorhous*** produces mainly neokestose from sucrose during growth (Kilian ***et al.,*** 1996). With ***Bacillus macerans***, GF_4 is exclusively produced, lacking GF_2 and GF_3 (Kim ***et al***., 1998). The production of GF_4 was also explained by a limitation in the acceptor site for a GF_4, while the lack in GF_2 and GF_3 was explained by these being better acceptors that sucrose. The next section deals with the hydrolysis of these substrates.

1.2.2.3.3. Fructan hydrolysis

Microbial levan and inulin are hydrolysed by extracellular endo- and exofructanases. The enzymes responsible for the hydrolysis of levan are called levanases. Exolevanases produce either levanbiose or fructose as end products. The fructose-releasing exolevanases attack the levan from the fructose end to produce monosaccharides. Except for levan they can also

hydrolyze inulin, raffinose and sucrose but with varying affinity. In contrast, levanbiose producing exohydrolases do not split β-(2-1) linkages of inulin, raffinose or sucrose (Murakami ***et al.***, 1990; Kang ***et al.***, 1999; Saito ***et al.***, 2003). The endolevanases and endoinulinases have absolute substrate specificity for levan and inulin, respectively. The products from these enzymes are a mixture of oligofructans of varying sizes due to the random hydrolsysis of the internal β-linkages.

In plants fructan hydrolysis is accomplished by a complex of fructan exohydrolases (FEHs). The inulin in chicory roots are degraded by 1-FEH, of which two isoforms have been identified (Van den Ende ***et al.***, 2001). The breakdown of branched graminan-type fructans containing both β-(2-1) and β-(2-6) fructosyl linkages is accomplished in wheat by 1-FEH's and 6-FEH's (Kawakami ***et al.***, 2005). In contrast to microbial exofructanases, plant FEH's are unable to hydrolyse sucrose.

1.2.2.4. Commercial sources of fructans

In nature FOS are widely distributed; they have been associated with 15 % of flowering plants that are mainly found in temperate and arid climates (Banguela ***et al.***, 2006; Mitsuoka ***et al.,*** 1987). Many micro-organisms produce β-fructofuranosidase and β-fructosyltransferase and are therefore also able to produce FOS (Myrbäck, 1959; Hayashi ***et al***., 1991).

Fructan-containing plant species are found in a number of mono- and dicotyledonous families such as ***Liliaceae, Amaryllidaceae, Gramineae*** and ***Compositae***. Parts of various fructan containing plant species are often eaten as vegetables (e.g. asparagus, garlic, leek, onion, artichoke, Jerusalem artichoke, chicory roots, etc.)(Van Loo ***et al***., 1995). Some other commonly eaten natural sources of FOS are banana, tomato, brown sugar and honey (Flamm ***et al***., 2001). The most suitable families for the extraction of fructans are ***Liliaceae, Amaryllidaceae*** and ***Compositae***. From these plants the storage organs, which include bulbs, tubers and tuberous roots, can be easily processed to purified products. The ***Gramineae*** (e.g. cereals: barley, wheat

and oat; forage grasses: ***Lolium*** and ***Fetusca***) are rich in fructan; these are present in the aerial parts of grasses, cereals and especially young seedlings, where the yield is up to 70% of their dry weight. The ***Gramineae*** are, however, not ideal for dietary fructan extraction (Fuchs, 1991).

In micro-organisms, fructosyl transferases (FTases) are mostly responsible for the production of FOS, however, fructofuranosidases can also be present. Several microbial sources have been screened for Ftases (Sangeetha ***et al***., 2005), of which most are fungal belonging to the genera: ***Aspergillus***, ***Aureobasidium***, ***Claviceps***, ***Fusarium***, ***Penicillium***, ***Phytophthora***, ***Scopulariopsis*** and ***Saccharomyces*** (Yun, 1996; Hayashi ***et al***., 2000; Wang & Rakshit, 2000).

While many bacteria belonging to the genera ***Bacillus***, ***Streptococcus***, ***Pseudomonas***, ***Erwinia*** and ***Actinomyces*** produce levan (Hendry & Wallace, 1993), the synthesis of inulin was thus far only reported for ***Bacillus sp***. 217C-11, ***Streptococcus mutans***, ***Lactobacillus reuterin*** and ***Leuconostoc citreum*** (Rosell ***et al,*** 1974; Wada ***et al,*** 2003; Van Hijum ***et al***, 2002; Olivares-Illana ***et al,*** 2002).

Fructans have found application in the food, nutraceutical and non-food industries. The food industry has been producing a synthetic fructan using the transfructosyl activity of β-fructosidase from ***Aspergillus niger***. The FOS (DP 3-5) is produced by the addition of 1-3 fructosyl groups through β-(2-1)-glycosidic linkage to a sucrose molecule (Crittenden & Playne, 1996; Fishbein ***et al.,*** 1988). In this process ***A. niger*** cells are entrapped in calcium alginate gels. Neosugar® is a product of this process. After a purification process involving decolourisation and desalination the product is called Neosugar G, whereas after removal of mono- and disaccharides it is called Neosugar P (Hidaka ***et al***., 1986; Mitsuoka ***et al***., 1987). Other fungi e.g. ***Aureobasidium pullulans***, ***Aspergillus japonicus***, ***A. oryzae*** and ***Penicillium citrinum*** show high yields (%) of FOS (Sangeetha ***et al***., 2005). In all these production processes the reaction mixture contains sucrose, glucose, fructose and FOS, which requires purification to enhance the FOS yield.

Three inulin-containing plant species are used in the food industry, i.e. agave (***Agave azul tequilana***; grown for production of tequila), Jerusalem artichoke (***Helianthus tuberosus***) and chicory (***Cichorium intybus***). Of these chicory is the most commonly used source for the extraction of inulin (Debruyn ***et al***, 1992). Raftiline® and Raftilose® are two Orafti products made from chicory roots with various degrees of purity. Raftiline® can contain 92-99% inulin with Raftilose® containing blends inulin and fructo-oligosaccharides with Raftilose® P95 containg 95% FOS (Gibson & Wang, 1994a).

In nature neokestose exists in plants such as onions and is produced by fungal enzymes (Yasuda ***et al***, 1986; Mitsuoka ***et al***., 1987; Kilian ***et al***, 1996). Neokestose is produced during growth of ***Xanthophyllomyces dendrorhous*** on sucrose and can therefore be purified from the culture supernatant. Neokestose was produced from whole cells by Kritzinger ***et al***. (2003) in a citrate phosphate buffered sucrose-containing solution.

1.2.3. The intestinal microbial community of the chicken

The intestinal microbiota changes from a relatively simple one early in life to a complex one in the adult chicken (Ochi ***et al***., 1964). The microbial composition of the intestinal tract, although continuous, is influenced by changes in available substrates, temperature, pH and redox potential.

1.2.3.1. The sections of the intestinal tract

Food that is ingested by chicken pass in sequence through their esophagus, crop, proventriculus, gizzard, duodenum, jejunum, ileum, caeca, colon and finally exits via the cloaca as faeces (Fig. 1.4).

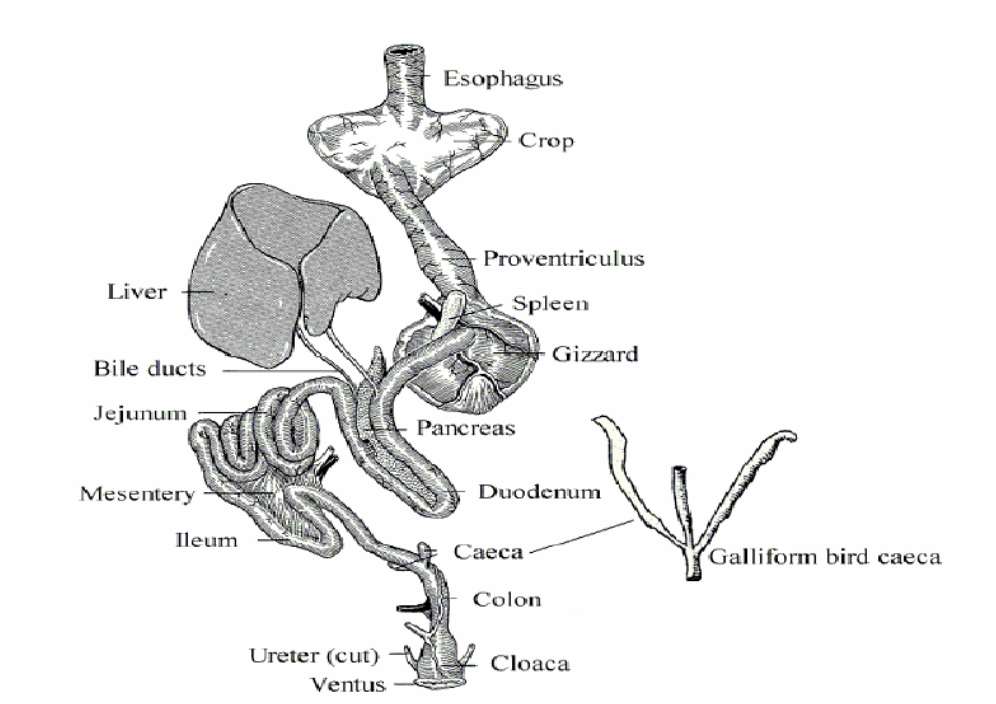

Figure 1.4. The mono-gastric intestinal tract of a chicken. (Procter and Lynch, 1993)

1.2.3.2. General characteristics of the gastro-intestinal tract

The difference between poultry compared to other mono-gastrics is that the former have a very rapid nutrient transit through their intestinal tract (Fig. 1.5). The longest residence occurs in the ileum (160-200 min) followed by the caecum (120 min).

Since chickens cannot grind their food, it is ingested whole and stored in the crop where a predominantly lactic acid semi-batch fermentation takes place. This decreases the pH and thus the crop content of healthy chickens is usually between pH 4-5 (Fig. 1.5). Small amounts of this fermented ingest, is continuously passed to the gizzard, which allows for the continuous flow of nutrients through the digestive tract. Acid production in the gizzard of the young chick is limited at first, but gradually increases (Rynsburger & Classen, 2007). This might be one of the reasons why competitive exclusion products are effective in young animals. This area has a low pH and fairly high reduction potential.

The array of digestive enzymes, the high oxygen tension and the presence of high concentrations of antimicrobial compounds such as bile salts in the duodenum further limit bacterial growth in this region of the gut. In the colon, bile acids that are not absorbed and recycled by the enterohepatic circulation are mostly deconjugated and 7-dehydroxylated to secondary bile acids (Tannock ***et al.***, 1989). Conjugation and the presence of hydroxyl groups give the primary bile acids a more hydrophilic character. Bile solubilizes lipids and can thus inactivate those organisms with a lipid envelope. Bile acids thus play a role in the regulation of the microbial composition of the intestinal tract. (Binder ***et al.***, 1975; Savage, 1977).

Further along the small intestine the environment changes and becomes more favourable for anaerobes because of the lower oxygen tension and the lower concentrations of enzymes and bile salts.

Microbiota 3 weeks	Subdivisions GI-tract		pH	Residence Time (min)	Microbiota Adult
Lactobacilli[1] Streptococci[1] Coliformi[1]	Crop		4.5-5.3	45	Lactobacilli[3] Streptococci[2] Coliformi[3]
Lactobacilli[1] Streptococci[1] Coliformi[1]	Proventriculus & Gizzard		2.0-4.5	70	Lactobacilli[3] Streptococci[2] Coliformi[3]
Lactobacilli[1] Streptococci[1] Coliformi[1]	Ileum		5.6-7.9	160-200	Lactobacilli[1] Streptococci[1] Coliformi[1]
Lactobacilli[3] Streptococci[1] Coliformi[1]	Caeca		5.8-6.8	120	Bacteroides[1] Bifidobacteria[1] Peptostreptococci[1] Clostridia[1] Propionibacteria[1] Eubacteria[1]
Lactobacilli[3] Streptococci[1] Coliformi[1]	Colon & Cloaca		6.3-7.7	30-50	Mixture of Ileal and caecal bacteria[3]

[1] Dominant [2] Predominant [3] Significant

Figure 1.5. The sub-division of the avian gastro-intestinal in relation to its microbial population: crop, proventriculus, ileum, caeca, colon and cloaca. (from Huyghebaert, 2003).

The caeca are filled with a thick viscous fluid containing no food particles. This organ has the highest viable count of 10^{11} CFU.g^{-1} contents and additionally the microbiota here is the most complex, which is due to the slow flow rate (Smith 1965a). The sections of the gastro-intestinal tract that are therefore important for the growth of ***Bifidobacterium*** spp. are the ileum and caecum.

1.2.3.3. The microbiota of the gastro-intestinal tract

The bacteria found in the GI-tract are essentially of two distinct populations, one which exists in intimate association with the epithelium of the tract (e.g. ***Lactobacillus*** spp.) and one which is mobile, occurring free or attached to digesta particles within the gastro-intestinal lumen. The microbial community develops from a pioneering one in the young chick to highly diversified one in the adult bird (Fig. 1.5)(Ochi ***et al***., 1964). It was found that the intestinal microbiota in the duodenum, middle intestine and rectum become established 9-13 days and of the caeca 25-32 days after feeding (Ochi ***et al***, 1964). The gastrointestinal tract harbours a wide variety of microbial species. Some of the more recent studies that describe the gastro-intestinal microbiota of the chicken have been done using molecular techniques (Apajalahti ***et al***., 2004). They used %G+C profiling and 16S rDNA techniques to analyse the community structure and identify individual species, respectively. Using these techniques they have found that only 10 % of the species found in the GI-tract represent previously known species, 35 % are unknown but belong to known genera, while 55 % are totally unknown. They found 640 different species belonging to 140 genera. Most of these microorganisms are anaerobes with many species able to hydrolyze polysaccharides to mono-saccharides, which are primarily fermented to short-chain volatile fatty acids, hydrogen, and carbon dioxide (Wolin ***et al***., 1997).

1.2.3.3.1. The crop

The crop is suitable for aerobes as well as facultative anaerobes. The main group are ***Lactobacillus*** spp. producing lactic- and acetic acids. ***Lactobacillus*** species isolated and characterised from the crop include ***L. salivarius***, ***L. fermentum*** and a type resembling ***L. salivarius*** (Fuller, 1973; Eyssen ***et al.***, 1965). Other species found in lower numbers in the crop include ***Escherichia coli*** (Barrow ***et al.***, 1988), ***Enterococcus faecalis*** subsp. ***liquefaciens*** and subsp. ***zymogenes***, ***E. avium, E. faecium*** and ***E. gallinarum***.

1.2.3.3.2. The proventriculus, gizzard and upper small intestine

In the proventriculus and gizzard microbial survival is dependent on acid tolerance since the pH can vary from 1-2 (Fig 1.2). The conditions prevailing here make this area unsuitable for ingested obligate anaerobes and other non-enteric organisms. The feed exiting the gizzard to the duodenum is therefore significantly depopulated of bacteria.

1.2.3.3.3. The ileum

Clostridium perfringens, known for its role in necrotic enteritis, has occasionally been isolated from this part of the small intestine, where it can metabolize fatty acids. The largest group present here are the ***Lactobacillaceae***, followed by the ***Clostridaceae***, ***Streptococcaceae*** and ***Bacteriodaceae*** (Fig. 1.6). Facultative anaerobes (***Streptococcus, Staphylococcus, Lactobacillus*** and ***E. coli***) are the predominant microbiota in the duodenal and ileal sections of the intestinal tract (Lu ***et al.*** 2003; Salanitro

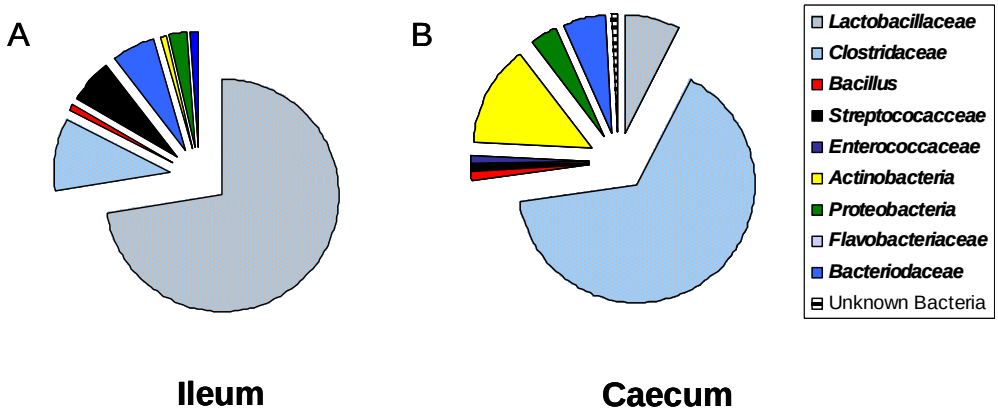

Figure 1.6. Composition of the microbial microbiota of the ileum and caeca of broiler chickens as determined using 16S rDNA community DNA library. (A) Bacterial composition of the ileum. (B) Bacterial composition of the caecum. (from Lu ***et al.***, 2003).

et al. 1978). Additionally, a large part (9-39 %) of the isolates belongs to the anaerobic genera of ***Eubacterium, Propionibacterium, Clostridium, Gemmiger*** and ***Fusobacterium***. Facultative anaerobes which have also been reported and are present in lower numbers include ***Enterobacteriaceae***, such as ***Citrobacter, Salmonella, Proteus*** and ***Klebsiella***. Smaller numbers of other organisms such as the aerobic ***Pseudomonas*** and yeasts are reported from time to time but are never present in high numbers.

1.2.3.3.4. The caecum

In poultry caeca the highest viable bacterial count and most complex microbiota exist (Huyghebaert, 2003). It was found that young broiler chickens, 1 to 14 days old, lack the established anaerobic microbiota found in older chickens. This was found to occur only later when a sufficiently anaerobic environment by facultative anaerobes was created (Salanitro ***et al.***, 1978). Similar banding patterns on denaturing gradient gel electrophoresis (DGGE) for 16S rDNA isolated from the crop, duodenum, ileum and caeca in 4-day-old chicken points to this succession (Van der Wielen ***et al***., 2002). ***Lactobacillus*** is such a genus consisting of facultative anaerobes, which are found in the GI-tract of chickens from an early age (Fuller, 1973).

Based on 16S rDNA analysis, Apajalahti ***et al***. (2004) determined that approximately 7 % of caecal bacteria belong to the ***Clostridaceae*** (Fig. 1.6B). Both Zhu ***et al***. (2002) and Lu ***et al***. (2003) reported ***Clostridaceae*** as the major component of the caecum making up between 50 % and 65 % of the population.

Saccharolytic bacteria of the caecum have been primarily associated with the production of acetic acid (Mead, 1989). However, chicken caeca also contain cellulolytic bacteria that are capable of producing acetate. Based on 16S rDNA analysis, 19 % of the caecal bacteria were ***Ruminococcus*** spp. which produce acetic and formic as their primary products (Apajalahti ***et al.***, 1998). Achen ***et al.*** (1998) showed that the caecum was the major colonization site of ***Campylobacter jejuni***, when broilers where orally inoculated and the digestive

tracts analyzed after 43 days. The caecum is also the site of the alimentary tract of poultry that is most readily colonized by ***Salmonella*** (Fanelli ***et al.,*** 1971).

Bifidobacterium spp. have been estimated by some researchers at 10^9 cfu per gram, which accounted for at least 9 % of the total counts (Barnes ***et al***, 1979; Barrow, 1992). Rada and Petr (2000; 2002) reported numbers in excess of 10^{10} cfu per gram in hen caeca, where they found these bacteria to predominate. High viable numbers of ***Lactobacillus*** are also found in the caecum (Sarra ***et al***., 1992; Smith, 1965a; Lu ***et al.,*** 2003)

The microbial content of the cloaca and the faeces depends on whether they contain material from the small intestine or from the caeca. Droppings from the latter are discharged roughly two to four times daily.

1.2.3.4. Factors influencing the intestinal microbiota

Age, food, environmental conditions and disease are some of the major factors that influence the intestinal microbial population. The intestinal tract of the young newborn chicken is essentially void of inhabitants. Thereafter, colonization by facultative anaerobes particularly ***Enterobacteriaceae*** and ***Streptococcus*** species, occurs rapidly along with ***Clostridium*** species. The initial colonizers are eventually displaced by ***Lactobacillus*** species as the dominant organisms in the crop and the small intestine. These facultative anaerobes create a favourable anaerobic environment, allowing for the colonization by obligate anaerobic bacteria (Barnes ***et al.***, 1980). Within 4-6 weeks after hatching the caecal microbiota is fairly stable (Smith, 1965b; Mead, 1989).

The composition of the intestinal microbiota of the chicken is influenced by the food it eats. Apajalahti ***et al.*** (2001) showed that wheat, corn and rye based diets each had their own characteristic effect on the intestinal microbial community. The %G+C profiling used by them was characteristic for each

species, and although not directly identifying the bacteria, it illustrated the effect of feed on the microbial composition. Wheat increased the proportion of the microbiota, falling within the 55 to 59 and 60 to 69 %G+C groups. Species of ***Propionibacterium*** belonging to the first and ***Bifidobacterium*** to the latter group are both represented by these %G+C ranges. Corn stimulated the microbiota towards the 25 to 30 %G+C group, which is indicative for species of ***Clostridium*** and ***Campylobacter***. Rye had an increasing effect on the 35 to 40 %G+C group, which represents species present in the ***Lactobacillus*** and ***Enterococcus*** genera. Wheat and barley diets, containing higher concentrations viscous non-starch polysaccharides, partly inhibited digestion and absorption, which resulted in enhanced microbial proliferation in the distal ileum (Dänicke ***et al.***, 1999). Feed composition is thus one factor determining the intestinal composition, whereas host dynamics and environmental factors were also found to influence this composition (Zhu ***et al.***, 2001; Apajalahti ***et al***., 2002). Given this, microbial population permutations are potentially limitless. Whilst some of the effects and capabilities of individual species may be understood in isolation, in a dynamic intestinal environment the roles that each species plays and the potential interactions with others, be it detrimental or beneficial to the host, are difficult to ascertain.

Volatile fatty acids have also been shown to affect the intestinal ***Enterobacteriaceae*** population of broiler chickens during growth. Barnes ***et al***. (1979) showed that there was a steady increase in the concentration of acetic, propionic and butyric acids in the caeca for the first 21 days after hatching, which corresponded with the gradual establishment of the caecal microbiota. Van der Wielen ***et al***. (2000) concluded that volatile fatty acids, especially in its undissociated form, are one of the mechanisms that are responsible for the reduction in the numbers of ***Enterobacteriaceae.*** The pH (pH 5.5-6.0), which determines the concentration of the undissociated fatty acids, was given as the reason for the reduction of the ***Enterobacteriaceae*** numbers in chickens and not in mice (pH 6.5–7.0)(Freter & Abrams, 1972 cited Van der Wielen ***et al***., 2000).

1.2.3.5. Influence of pathogenic and opportunistic bacteria on poultry performance.

In chickens, ***Campylobacter jejuni***, ***C. coli***, ***Escherichia coli***, ***Salmonella enterica*** and ***Clostridium perfringens*** are pathogenic micro-organisms that colonize the intestinal tract and have been isolated from the caeca of chickens. ***Campylobacter***, especially thermophilic species are responsible for foodborne bacterial gastroenteritis in humans (Taylor, 1992; Aydin ***et al***., 2001). ***Salmonella*** are human pathogens and can cause severe illness and even death (Tauxe, 1991).

It was found that newly hatched chicks are very susceptible to ***Salmonella*** infections (Smith 1965a), whereas their resistance increases after 5 days (Ziprin ***et al***., 1989). In poultry, ***Salmonella*** Typhimurium infections are responsible for increased mortality, especially of young chicks, in the post hatch period of 1 to 8 days (Gast & Beard, 1989). ***S***. Typhimurium susceptibility decreases with an increase in T-cell mediated immune response and through competitive exclusion, as a result of an increase in bacteria residing in the intestinal tract (Gast & Beard, 1989). When in-feed antibiotics were banned by Sweden in 1986 the main problem was necrotic enteritis, caused by ***Clostridium perfringens,*** in both its clinical and sub-clinical form (Inborr, 2001)

1.2.3.5.1. Fructo-oligosaccharides in broiler diets

Fructo-oligosaccharides (FOS) were tested in poultry by a number of investigators. Fructo-oligosaccharides have been reported to reduce susceptibility to ***Salmonella*** colonization in stressed broilers (Bailey ***et al***., 1991). Chicks that were fed FOS and then challenged with ***Campylobacter*** showed a reduction in infection where only 8 % of the chicks given the FOS were positive compared with 80 % of chicks on the control diet (Schoeni & Wong, 1994).

Waldroup ***et al***. (1993), indicated that improvements in weight gain and feed efficiency could be obtained with the addition of FOS in broiler diets. Furthermore, Ammerman ***et al***. (1988a; 1998b) and Terada ***et al***. (1994) suggested that feeding FOS to poultry may enhance their performance and may also be substituted for subtherapeutic levels of antibiotics. Rada ***et al. (***2001) found that the addition of 5 % (w/w) inulin for 1 week to the diet (40 % wheat, 26 % maize and 20 % soybean meal) of 1-week old laying hens improved the numbers of caecal ***Bifidobacterium*** spp. by 3-fold.

While not specific to poultry, FOS addition has also been shown to improve animal performance, reduce serum cholesterol, reduce disease-related diarrhea, alleviate constipation, reduce intestinal concentrations of putrefactive compounds, reduce tumors and inhance the immune response in a number of species (Hidaka ***et al***., 1986).

1.2.3.5.2. Fructo-oligosaccharides utilization by intestinal bacteria

Results on the fermentation of FOS by ***Enterobacteriaceae***, especially ***E. coli***, have been rather contradictory. FOS have been shown to be non-digestible in humans and animals (Oku ***et al.***, 1984) and have been shown to be utilized by only a few pathogenic and non-pathogenic intestinal bacteria in pure culture (Hidaka ***et al***., 1986). A number of investigators showed that FOS was utilized for growth by ***Klebsiella pneumoniae***, but not by ***E. coli*** (Mitsuoka ***et al.***, 1987; Bailey ***et al***., 1991; Wada, 1990; Kilian ***et al***., 2002). Some investigators found that ***E. coli*** was capable of growth on Raftilose® P95 (Wang & Gibson, 1993; Hartemink ***et al***., 1997) and Morisse ***et al.*** (1993) found that ***E. coli*** increased in rabbits fed a diet containing Neosugar. Hartemink ***et al***. (1997) however showed that growth rates of ***Klebsiella pneumoniae*** and ***E. coli*** on inulin were lower when compared to growth rates on Raftilose® P95. ***Proteus***, ***Bifidobacterium, Lactobacillus, Peptostretococcus*** and ***Enterococcus*** species were generally capable of growth on FOS, while ***Clostridium perfringens*** and ***Salmonella*** Typhimurium were not (Hartemink ***et al***., 1997; Kilian ***et al***., 2002; Bailey ***et al***., 1991). ***Bifidobacterium*** species produce mainly lactate and

acetate but not hydrogen or other gases (Biavati & Mattarelli, 2006). In FOS fermentation by faecal inocula, both ***in vivo*** and ***in vitro***, succinate, propionate and butyrate were found, which indicated that several other species utilized FOS (Fujita ***et al.,*** 1991 cited by Hartemink ***et al***., 1997; Wang & Gibson, 1993).

1.2.4. Experimental approach

The study entailed the production of the novel prebiotic, neokestose, from ***Xanthophyllomyces dendrorhous***. Neokestose was enhanced by column charcoal/celite chromatography and the product analyzed for purity. The aim of this study was to evaluate the effect of neokestose as a prebiotic on the intestinal microbial population of broilers ***in vitro.*** This was evaluated by testing various parameters, which included the growth of ***Bifidobacterium*** species on neokestose compared to inulin and their comparative effect on additional problematic microbial species. One day old Ross A1 broiler chickens were fed a balanced unmedicated diet for 5 weeks (35 days, when the microbial population is stable), where after the caeca was collected. A semi-defined test medium was infused with 10 % (w/v) caecal contents. The prebiotic effect was tested on media which contained either:(1) neokestose (2) glucose (3) inulin (commercial product) or (4) no additional carbohydrate. A bacterial quantification based on plate counts for total anaerobes, ***Bifidobacterium*** spp. ***Lactobacillus*** spp., ***Escherichia coli***, ***Salmonella*** spp. and ***Campylobacter*** on selective media as well as short chain fatty analysis (SCFA), was used to determine the effectiveness of neokestose as a prebiotic.

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CHAPTER 2
THE PRODUCTION AND PURIFICATION OF NEOKESTOSE

2.1 Abstract

Xanthophyllomyces dendrorhous, the teleomorph of the anamorph ***Phaffia rhodozyma***, produces neokestose during its vegetative growth on sucrose (Kilian ***et al***., 1996). Neokestose can be considered an inulin neoseries fructan. In this study neokestose was produced and then enriched. The subsequent aim of this work was to produce sufficient neokestose for testing the prebiotic effect on the caecal microbiota of broilers ***in vitro***. Conversion of sucrose using whole cells yielded between 55 and 58 % neokestose as a percentage of total sugar in the final mixture. Neokestose was produced at a maximum specific rate of 3.17 $g.g^{-1}.h^{-1}$ giving a maximum yield coefficient of 0.83 obtained with 220 $g.l^{-1}$ sucrose, 3.3 $g.l^{-1}$ cell concentration at pH 7 and 25 ^{o}C. The neokestose content was improved after purification on a carbon-celite column to an average purity of 78 %. In addition to neokestose, the mixture consisted of sucrose (~ 5 %), glucose (~1 %), fructose (~1%) and an unidentified FOS fraction (~16%).

2.2 Introduction

Neokestose is a trisaccharide, composed of 1 molecule of glucose and 2 molecules of fructose and belongs to the fructan polysaccharides. Fructans are linear or branched polymers of fructose molecules, whereas fructo-oligosaccharides (FOS) are polymers consisting of between 2-10 fructose units. In both plants and micro-organisms, sucrose is the basic building block for the synthesis of fructans and therefore fructose polymers are generally linked to a glucose molecule (Vijn & Smeekens, 1999). Based on their linkage to a glucose molecule as well as between the fructosyl units a few types can be distinguished: ***Inulin***, ***inulin neoseries***, ***levan***, ***mixed levan***, and ***levan neoseries***. Inulin consists of linear β(2-1)-linked D-fructosyl units ($G_{1\text{-}2}F_{1\text{-}2}F_n$), of which the smallest is the trisaccharide 1-kestose. ***Levan*** consists of linear β(2-6)-linked D-fructosyl units ($G_{1\text{-}2}F_{1\text{-}6}F_n$), of which the smallest is the trisaccharide 6-kestose. ***Mixed levan*** consist of both β(2-1) and β(2-6)- linked fructosyl units, in this case the smallest molecule is the tetrasaccharide bifurcose. The ***levan neoseries*** are polymers of predominantly β(2-1)-linked D-fructosyl units linked to either sides of the glucose moiety of the sucrose molecule. Neokestose ($F_{2\text{-}1}G_{6\text{-}2}F$)(Fig. 1.1C) is a ***inulin neoserie***s fructan, where D-fructosyl units are linked to the C_1 and C_6 of the glucose moiety. Additional D-fructosyl units are β(2-1) linked (**$F_{2\text{-}1}$**$F_{2\text{-}1}G_{6\text{-}2}F_{1\text{-}2}$**F**).

Fructo-oligosaccharides are synthesised in plants such as artichoke, chicory, onion, leek, garlic, asparagines, banana and wheat, while many other cereals contain smaller amounts (Mitsuoka ***et al.***, 1987). Neokestose, the smallest inulin neoseries fructan, has been isolated from vegetables such as onions, garlic and aspargines as well as cereals like oats, barley and rye (Slaughter ***et al***. (1993).

Extracting and purifying neokestose from these sources would be cumbersome and time consuming, whereas a more productive and efficient method is synthesising it with the yeast ***Xanthophyllomyces dendrorhous***. ***Xanthophyllomyces dendrorhous,*** the teleomorph of the anamorph ***Phaffia rhodozyma***, produces neokestose during its vegetative growth on sucrose

(Kilian ***et al***., 1996). Johnson ***et al***. (2003) stated that, although a common misconception, not all ***Phaffia rhodozyma*** strains should be designated ***X. dendrorhous***, since certain strains are unable to form basidia and basidiospores (Kuscera ***et al***., 1998). Whole cells of this yeast, produce neokestose extra-cellularly when grown in a sucrose containing medium (Kritzinger ***et al***., 2003). Residual sucrose as well as fructose, glucose and two as yet unidentified higher saccharides are also present in the supernatant during the synthesis of neokestose by ***X. dendrorhous***. The unidentified products are believed to be a tetra-glucofructan (GF_3) and a penta-glucofructan (GF_4) (Kritzinger ***et al***., 2003). Kritzinger e***t al***. (2003) demonstrated that the best conditions to obtain a high ratio of neokestose to mono- and disaccharides during the synthesis of neokestose from whole cells was with cells suspended in citrate-phosphate buffer containing sucrose at pH 7.00 and 25^{o}C.

Neosugar®, Raftalin® and Raftilose® are fructo-oligosaccharides that are commercially available. Neosugar® is a product synthesized from a 50-60% sucrose syrup by β-fructofuranosidase-producing ***Aspergillus niger*** cells immobilized on a column. Neosugar® in its final form is obtained by purifying the reaction mixture through both an active carbon column and an ion exchange column (Mitsuoka ***et al,*** 1987). Raftaline® and Raftilose®, however, are produced by hydrolysis from the extraction products of chicory roots.

Inulin with an average DP of 22 is obtained from the extraction of chicory roots and artichoke tubers. On average the two inulin hydrolysis products Raftiline® and Raftilose® differ only by 4 degrees of polymerisation (DP). Presently enzymatic hydrolysis products of inulin are the most common of all the fructo-oligosaccharides that are produced commercially. A enzymatic process by Cho ***et al***. (2001) described the production of FOS by a dual endo-inulinase system obtained from a ***Xanthomonas*** sp. and a ***Pseudomonas*** sp. FOS can also be produced via β-fructofuranosidase or β-fructosyltransferase synthesis from sucrose and not just through the hydrolysis of inulin (Anderson, 1967; Gupta and Bhatia, 1980; Van Balken ***et al***., 1991; Patel ***et al***, 1994).

Commercial FOS usually contain small quantities of mono- and disaccharides. FOS products, especially those that are derived through hydrolysis are of varying degrees of polymerisation. It is difficult to separate the different fructo-oligosaccharides because of their structural similarities and the absence of functional groups in these structures (Sims ***et al***., 1991; Crittenden & Doelle, 1993; Yun, 1996). Separation is usually done on activated carbon (charcoal) and celite using a linear ethanol gradient increasing from 0-50%. Bacon (1954) was the first to propose this method and this has since been extensively used (Crittenden & Doelle, 1993; Yun, 1996). It has, however, been stated by Sims et al. (1991) that it is difficult to achieve repeatable results with this method. Preparative HPLC (Crittenden & Doelle, 1993; Yun, 1996) is another method of separation. This technique is suitable only for small samples and not for production purposes.

In this chapter, neokestose was produced from sucrose with ***Xanthophyllomyces dendrorhous*** whole cells. Enrichment of neokestose was attempted with activated charcoal by using both column chromatography and a batch filtration method.

2.3 Material and Methods

2.3.1 Micro-organism

X. dendrorhous (UFS-0175) was obtained from the UFS Mircen culture collection. The yeast was maintained on yeast extract malt (YM) agar slants containing (l^{-1}):10 g sucrose, 5 g peptone, 3 g yeast extract, 3 g malt extract and 17 g agar.

2.3.2 Analytical methods

Growth was monitored by measuring the culture turbidity with a spectrophotometer at λ 690 nm. Dry biomass was determined gravimetrically in triplicate. Sugars were analysed by High Performance Liquid

Chromatography (HPLC) using a Biorad Aminex Carbohydrate HPX-42C column. Degassed deionised water containing 0.05 M CaEDTA served as eluent. The flow rate of the column was 0.6 ml. min^{-1} and the column temperature was 85 ^{o}C. A refractive index detector was used and 10-20 µl samples were automatically injected. The standards used for the quantification of products were analytical grade glucose, sucrose and fructose. Due to the unavailability of pure neokestose the concentration of the unknown oligosaccharide and neokestose were determined with sucrose as a standard.

2.3.3 Neokestose production

Kritzinger ***et al***. (2003) demonstrated, using a multifactorial experiment, that the best conditions for the synthesis of neokestose, was using a whole cell suspension in citrate-phosphate buffer containing 100 $g.l^{-1}$ sucrose at pH 7.00 and 25 ^{o}C. This resulted in a high ratio of neokestose to mono- and disaccharides.

Microbial biomass for use in the conversion of sucrose to neokestose was produced in a medium containing (per litre): 50 g sucrose, 5 g peptone, 3 g yeast extract and 3 g malt extract in shake flasks at 21 ^{o}C. Cultivations were terminated after 21 to 24 h, after which the biomass was harvested and then washed twice with 0.2 M citrate-phosphate buffer (pH 7). The cells were resuspended in 0.2 M citrate-phosphate buffer containing sucrose and incubated at 25 °C for 36-40 h. The production mixture was sampled regularly to evaluate the conversion of sucrose to neokestose. In this experiment the concentration of the sucrose in the citrate-phosphate buffer (pH 7) was varied between 100 $g.l^{-1}$ and 300 $g.l^{-1}$. The process was discontinued when the sucrose concentration dropped below 10 % (m/v) and the neokestose reached 50-60 %. The cells were removed from the suspension by centrifugation at 5000 g for 10 min at 4 ^{o}C. This cell-free suspension containing neokestose is hereafter called the supernatant. The sugar composition of supernatant solutions was analysed by HPLC. All

supernatant samples were kept at -20 ^{o}C. Supernatant samples were analysed, after which a decision to purify them was taken. Supernatants of differing sugar compositions were produced during this study and were numbered to simplify their identification.

2.3.4 Neokestose enrichment

In the enrichment of neokestose from the reaction product two methods were used: a chromatographic method and a batch filtration method.

2.3.4.1 Column chromatography

Neokestose was enriched by column chromatography using a mixture of activated charcoal and celite in a 1:1 (w/w) ratio as stationary phase. Approximately 5-10 grams of celite were first loaded onto the column to prevent blockage of the grid by activated carbon. A 45 cm x 5 cm column was modified and equipped with a fluid level sensing device to keep the level of

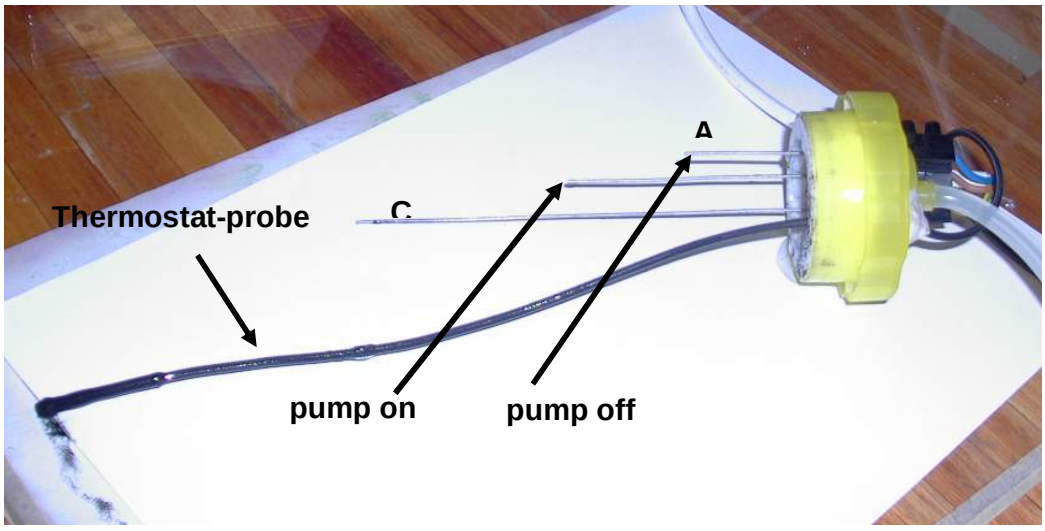

Figure 2.1. Modifications to the chromatographic column lid. There are three electrodes with the shortest (A) giving an "off" signal to the reservoir pump, the medium length electrode (B) providing an "on" signal and the longest (C) closing the current between CA and/or CB. The other modification was a thermostat probe which controlled a heat jacket surrounding the column.

mobile phase constant (Fig. 2.1) and a thermostat, which regulated the temperature during the final elution with ethanol at 60 ^{o}C. The thermostat was connected to a heating jacket which consisted of ribbon-like heating material. The column was packed with a mixture of 100 g activated carbon and 100 g celite. In order to remove some of the impurities found in the activated carbon the packed column was washed by rinsing with 5 l distilled water. Swallow and Low (1993) described the purification of 1-kestose by carbon-celite chromatography from beet medium invert syrup. This method was used in our first attempt to purify neokestose.

2.3.4.1.1 Purification by carbon:celite column chromatography:

Approach 1

A mixture of 100 ml reaction product (results Table 2.1) and 100 g activated charcoal was stirred for 17 h at 4 ^{o}C to allow the sugars to bind to the resin (Swallow & Low, 1993). The column was packed with 100 g activated carbon (reaction mixture) and 100 g celite. These two components were mixed uniformly before packing. The column was then eluted at room temperature in a series of steps: (1) 15 l H_2O, (2) 15 l 1 % EtOH and (3) 45 l 3 % EtOH. The remaining neokestose was then removed with (4) 2 l 50 % EtOH at 60 ^{o}C. One litre from each fraction was then concentrated by rotary evaporation under vacuum at 60 ^{o}C and analysed by HPLC. The final fraction was concentrated to 100 ml, analysed by HPLC and kept at -20 ^{o}C for further use.

2.3.4.1.2 Purification by carbon:celite column chromatography:

Approach 2

This method is a modification of method 1. The column was packed with 300 g activated charcoal-celite in a ratio of 1:1. A 250 ml aliquot of supernatant 1 (results Table 2.3) was loaded onto the column. The column was subsequently eluted with 10.75 l H_2O, followed by elution of the neokestose

with 1 l 50 % EtOH. The eluent in step 1 was captured in two fractions of 5 l and 5.75 l, respectively

In a further modification a smaller volume of 25 ml supernatant (composition 2 in Table 2.2) was used. After loading with the sample, the column was eluted with 4.2 l water and then with 1 l 50 % EtOH. All the fractions were analyzed by HPLC and final fractions were kept at -20 ^{o}C, until concentration by evaporation could be carried out as described previously.

2.3.4.2 Purification by carbon:celite batch filtration

This method was described by Kritzinger (1999). In this experiment, 25 ml of neokestose supernatant 2 (Table 2.2) was used. The supernatant was mixed with 200 g activated carbon-celite (1:1) with the consistency reduced to 4% with deionised H_2O, to enable stirring for 2 h at 4 ^{o}C. The mixture was then filtered through a sintered Pyrex filter (pore size 3) under vacuum. The filtrate was concentrated and analysed by HPLC. Following this, the mixture was washed with 4 l H_2O followed by 2 l 20 % EtOH.

2.4 Results

2.4.1 Synthesis of neokestose

During incubation of ***X. dendrorhous*** cells in buffer containing sucrose, neokestose was produced as the main product together with glucose, fructose, an unidentified tetra fructo-oligosaccharide (GF_3) (Kritzinger, 1999), and an unidentified fructo-oligosaccharide (GF_n).(Fig. 2.2). Neokestose reached a maximum concentration between 30 and 47 hours, after which its level gradually decreased. Furthermore, sucrose was rapidly consumed in the first 31 hours, whereafter the rate of utilisation leveled off. Neokestose is synthesized as sucrose is utilised. The production of glucose, which is produced as a by-product of fructo-oligosaccharide synthesis, is illustrated by the following reaction (Anderson ***et al***., 1969, Kim ***et al***., 1998).

$$F_{2\text{-}1}G + F_{2\text{-}1}G \rightarrow F_{2\text{-}6}G_{1\text{-}2}F + G$$

F denotes a fructosyl moiety while **G** denotes a glucosyl moiety in the above reaction, $F_{2\text{-}1}G$ is sucrose and $F_{2\text{-}6}G_{1\text{-}2}F$ neokestose. The glucose concentration reached its peak after 52 hours. Thereafter it declined gradually, which might be due to utilisation for cellular maintenance. GF_3 accumulated throughout the process.

After 96 hours 9 % (20.8 $g.l^{-1}$) of the initial sucrose (220 $g.l^{-1}$) remained unutilised. The maximum specific rate of neokestose production was 3.17 $g.g^{-1}.h^{-1}$, the maximum concentration of neokestose produced was 128.8 $g.l^{-1}$ and the maximum yield coefficient for neokestose production was 0.83

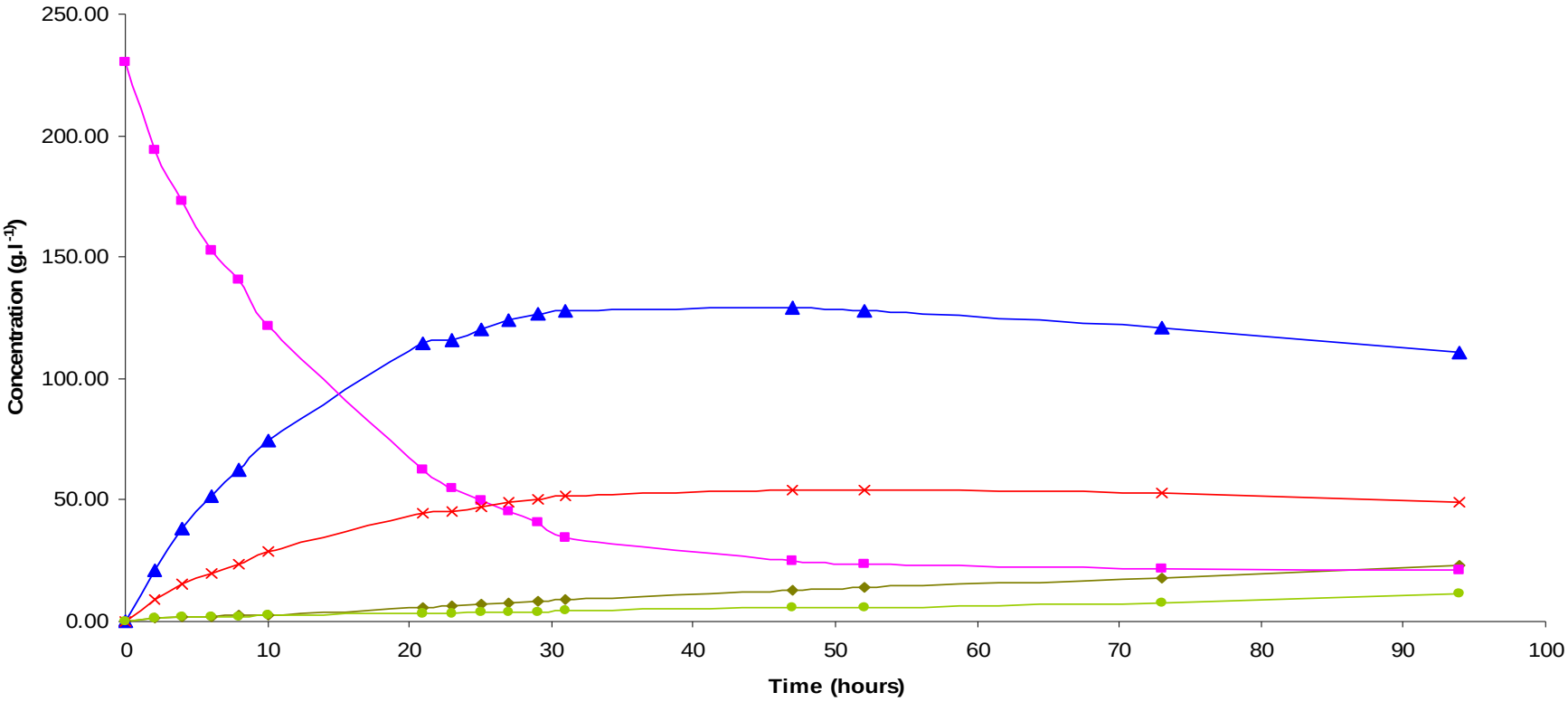

Figure 2.2. Production of neokestose (▲) from 220 g.l^{-1} sucrose (■) using 3.3 g.l^{-1} ***Xanthophyllomyces dendrorhous*** cells in citrate-phosphate buffer (pH 7), incubated at 25 ^{o}C. Glucose (x) accumulated up to 54 g.l^{-1} while GF_3 (♦) and fructose (●) accumulated as neokestose, sucrose and glucose started to decrease. The maximum specific rate of neokestose production was 3.17 g.g^{-1}.h^{-1}, the maximum concentration of neokestose produced was 128.8 g.l^{-1} and the maximum yield coefficient for neokestose production was 0.83.

2.4.2 Enrichment of neokestose with carbon:celite – Approach 1

2.4.2.1 Column chromatography – Run 1

The composition of the supernatant (Table 2.1) showed that, before the carbon:celite enhancement, neokestose made up 45.4 % of the total sugars. After eluting the column with 15 l H_2O, 15 l 1 % EtOH and 45 l 3 % EtOH the final 4 l 50 % EtOH eluent consisted of 81.3% neokestose. Analysis of the water and EtOH eluents showed that neokestose was present in all these fractions (data not shown). Of the 16.1 g neokestose contained in the 250 ml supernatant loaded on the column only 0.13 g was recovered (yield=0.81 %). Therefore enhancing the neokestose in the mixture through this method proved to be ineffective due to the low recovery achieved (Table 2.2).

Table 2.1. Sugar content in the culture supernatant and fraction 5 obtained by carbon:celite purification.

Sugar	Content (%) (m/m)	
	Culture supernatant	Purified fraction
GF_3	N.D.	N.D.
Neokestose	45.48	81.25
Sucrose	9.35	6.25
Glucose	31.95	6.25
Fructose	13.22	6.25

N.D. = not determined

Table 2.2. Purification obtained with carbon:celite through 15 l H_2O, 15 l 1 % EtOH, 45 l 3 % EtOH & 4 l 50 % EtOH elution.

Fraction	Description	Volume (l)	Total sugar (g)	neokestose (g)	Yield (%)	Purification factor
1	supernatant	0.25	35.40	16.10	100	1.00
5	50 % EtOH	4	0.16	0.13	0.81	1.79

[1]Fractions 2,3 & 4 where calculated but are not shown.

Neokestose made up 81.3 % of the total recovered saccharides in fraction 5. This is an improvement from the supernatant broth, in which neokestose made up only 45.5 % (m/m) of the total. This process proved to be extremely slow and tedious. The starting flow rate was measured at 145 $ml.h^{-1}$, while

the average flow rate was estimated at 88 $ml.h^{-1}$ for 35 days. The low recovery and long duration of this process prompted us to follow a different approach to neokestose purification.

2.4.3 Enrichment of neokestose by: Carbon:celite chromatography – Approach 2

2.4.3.1 Run 2

With this method one run took about 3½ days to complete. The starting flow rate was measured at 120-145 $ml.h^{-1}$ during step 1 (water elution), decreasing to 25-45 $ml.h^{-1}$ during step 2 (50 % EtOH elution).

The compositions of fractions collected during elution are shown in table 2.3. Fraction 2 (F_2) contained 90.5 % of the carbohydrates initially present in the supernatant (F_1), whereas F_3 contained only 1 %. Only neokestose was detected in the latter fraction. In the 2 l 50 % EtOH fraction (F_4), 7.6 % of the sugars were recovered, with neokestose, sucrose glucose and fructose respectively making up 80.2, 11.9, 2.9 and 5.2 % of the total sugars (Table 2.4). There was an over recovery of sucrose and fructose from the eluted fractions (F_2 to F_4; Table 2.4).

Table 2.3. Purification obtained with carbon:celite through 5 l H_2O, 5.75 l H_2O & 2 l 50 % EtOH elution.

Fraction	Description	Volume (l)	Total sugar (g)	neokestose (g)	Yield (%)	Purification factor*
F_1	supernatant	0.25	62.90	40.16	100.0	1.00
F_2	H_2O	5.00	56.91	34.27	85.3	0.94
F_3	H_2O	5.75	0.72	0.72	1.8	1.57
F_4	50 % EtOH	2.00	4.81	3.86	9.6	1.26

F_{1-4} are fractions 1, 2, 3 &4.
* GF_3 was not included in the calculation

It was possible that additional sucrose and fructose were produced, due to the hydrolysis of neokestose. This could also explain why only 96.7 % (F_2+F_3+F_4)

neokestose was recovered in the eluted fractions (Table 2.3). The peak corresponding to the GF_3 fraction was not calculated due to technical problems.

Table 2.4. Sugar content in the culture supernatant and fraction 4 obtained by carbon:celite purification.

Sugar	Content (%) (m/m)	
	Culture Supernatant	Purified fraction
GF_3	N.D.	N.D.
Neokestose	63.8	80.2
Sucrose	10.3	11.9
Glucose	23.3	2.9
Fructose	2.5	5.2

2.4.3.2 Run 3

The previous purification (Table 2.4) showed neokestose to be the main sugar present in F_3 and that only 4.81 g total sugar was recovered from F_4. This result seemed to point to the fact that the charcoal column (100 g activated carbon) had a limited binding capacity. It has been found by Bacon (1954) and Kritzinger (1999) that H_2O elution removed mainly the mono- and disaccharides. Based on this result it was decided to reduce the total sugar (supernatant) loaded onto the column and also to reduce the amount of water from 10 l to 4.2 l during this purification. The volume of the supernatant was reduced from 250 ml to 25 ml (Table 2.5), which contained 7.25 g total sugar.

Table 2.5. Sugar content in the culture supernatant and fraction 4 obtained by carbon:celite purification.

Sugar	Content (%) (m/m)	
	Culture Supernatant	Purified fraction
GF_3	9.2	15.2
Neokestose	51.1	72.1
Sucrose	11.1	8.2
Glucose	24.8	4.2
Fructose	3.9	0.3

This method showed an overall improvement in the purification of neokestose. Neokestose made up 72.1 % of the total sugars, which was lower than that reported for run 2 (Table 2.4), however, when this was compared to previous calculations where GF_3 was excluded, neokestosemade up 85.1 % of the

total. This was due to the inclusion of GF_3 into the total sugars in run 3. With this method, 61.7 % neokestose (Table 2.6) was recovered in the final EtOH fraction compared to 9.6 % with run 2. A better purification was achieved with run 3 (purification factor=1.41) than run 2 (purification factor=1.26).

Table 2.6. Purification obtained with carbon:celite through 4.2 l H_2O and 1 l 50 % EtOH elution.

Fraction	Description	Volume (l)	Total sugar (g)	neokestose (g)	Yield (%)	Purification factor*
F1	supernatant	0.025	7.25	3.71	100.0	1.00
F2	H_2O	4.20	2.98	0.80	21.6	0.53
F3	50 % EtOH	1.00	3.17	2.29	61.7	1.41

*GF_3 included in the calculation.

2.4.4 Retention of carbohydrates on an activated carbon:celite column

The rates of glucose, fructose, sucrose, neokestose and GF_3 elution was evaluated when eluting with water (Fig. 2.3). The constituents that eluted fastest had the lowest affinity for activated charcoal, when eluted with water and vice versa. Therefore both glucose and fructose had lower affinities for activated charcoal than the other constituents.

Neokestose and sucrose showed similar elution profiles with water. There was little or no elution of GF_3 from the column when water was used (Fig. 2.3). Activated carbon:celite chromatography will therefore increase the ratio of GF_3 to neokestose when water was used. The monosaccharides glucose and fructose are most readily removed.

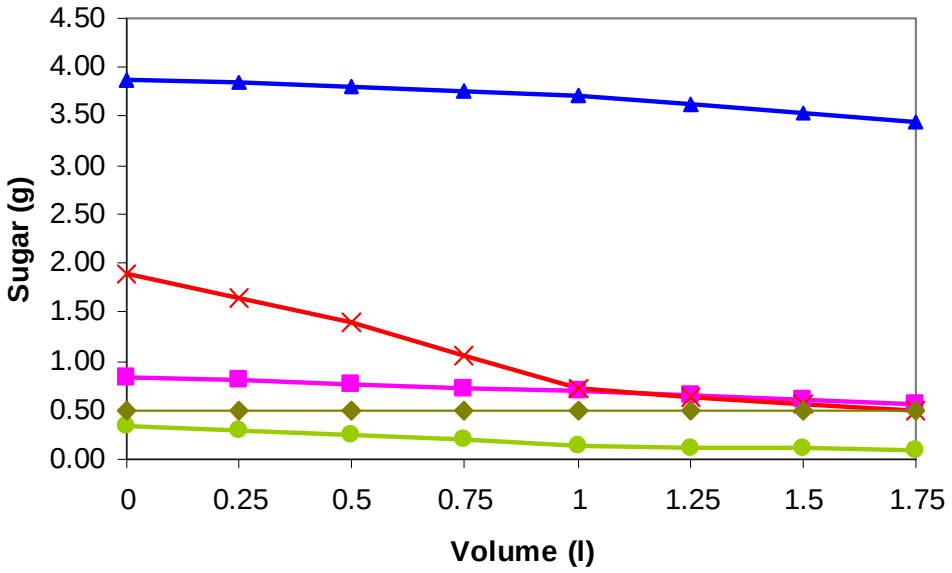

Figure. 2.3. Retention of carbohydrates on column during washing with deionised water. Neokestose (▲); glucose (x); sucrose(■); GF_3 (♦); fructose (●).

2.4.5 Purification by carbon:celite batch filtration – Approach 3.

2.4.5.1 Run 4

This method was extremely quick when compared to the column chromatography methods using approaches 1 and 2. The composition (Table 2.7) and purification factor (Table 2.8) of F_4 was similar to that of F_3 in run 3 (Tables 2.5 and 2.6). The neokestose yield was 8 % lower (56.7%) than achieved in F_3 run 3.

Table 2.7. Sugar content of fractions F_2 and F_4 obtained with carbon:celite purification.

Sugar	Content (%) (m/m)		
	Culture Supernatant	Purified fraction	Unbound fraction
GF_3	9.2	15.2	0.0
Neokestose	51.1	72.1	4.1
Sucrose	11.1	8.1	4.3
Glucose	24.8	4.4	79.6
Fructose	3.9	0.3	12.0

Table 2.8. Purification obtained with carbon:celite batch filtration through 4 l H_2O and 2 l 20 % EtOH elution.

Fraction	Description	Volume (l)	Total sugar (g)	Neokestose (g)	Yield (%)	Purification factor
F_1	supernatant	0.025	7.25	3.71	100.0	1.00
F_2	unbound	4	3.21	0.13	3.5	0.02
F_3	H_2O	4	ND	ND	ND	ND
F_4	20 % EtOH	2	2.92	2.10	56.7	1.41

ND – not determined

EtOH - ethanol

The unbound fraction F_2 yielded only 3.5 % neokestose, which meant that most of the neokestose was bound to the carbon:celite mixture, before being washed with 4 l H_2O (Table 2.8). The unbound sugar fraction was composed of 79.6 % glucose and 12 % fructose, confirming that monosaccharides were mostly removed during carbon:celite purification with water. Including GF_3 to calculate the composition of total sugars, neokestose made up 71.9 % of the total, whereas excluding GF_3 neokestose made up 84.7 % of the total sugars. Together neokestose and GF_3, i.e. the FOS, made up ~87% of the total sugars.

In the final fraction (20% ethanol) the ratio of GF_3:neokesose was 2.1:10, compared to 1.8:10 in the supernatant. Therefore the GF_3 increased as a percentage of the total when neokestose was enhanced by activated carbon-celite. In the final fraction 8.2% sucrose, 4.5% glucose and 0.3% fructose were still found.

2.4.6 Combined samples for in vitro growth studies

For the investigation of the prebiotic effect of neokestose, samples which had the highest neokestose to total sugar ratio were combined and the final composition was determined by HPLC. In this composition neokestose and GF_3 made up 82.6 % and 7.4 % of the total sugars, respectively (Table 2.9). Sucrose, glucose and fructose respectively made up 8.7 %, 1.2 % and 0.1 % of the total sugars.

Table 2.9. Composition of neokestose mixture for ***in vitro*** studies.

Composition of carbohydrates in mixture				
GF_3	neokestose	sucrose	glucose	fructose
7.4%	82.6%	8.7%	1.2%	0.1%

GF_3- unknown tetrasaccharide

2.5 Discussion

The yeast ***X. dendrorhous*** was grown in a YM medium to the late exponential growth phase where cells were harvested. These production cells, suspended in citrate-phosphate buffer (pH 7) and incubated at 25 ^{o}C with sucrose as the sole carbon substrate, produced high concentrations of neokestose (~51 % of the total sugars). This is similar to what Kritzinger ***et al***. (2003) found and the amount of neokestose compares well with values of 55-60 % (w/w) reported in literature for the commercial production of other fructo-oligosaccharides (Hang ***et al***., 1995; Yun, 1996). When enhancing this mixture for neokestose, synthesis should ideally be stopped when the ratio of neokestose:sucrose is at its highest. GF_3 which is synthesised during neokstose production is not removed as readily during water elution as the monosaccharides, sucrose and neokestose. This results in the enhancement of GF_3 in the final product due to the fact that both neokestose and GF_3 are removed during an ethanol elution. Care should therefor be taken with the ratio of neokestose:(GF_3) when deciding on the time to stop the synthesis of neokestose.

Both column chromatography (run 3) and the batch filtration (run 4) enhanced neokestose to 82 % or 90 % (Excluding GF_3) when the same water elution volumes were used. This compares well with the 87.5 % obtained by Kritzinger ***et al.***, 2003. The batch filtration method is quick when compared to the column chromatography method. This is due to the time required for setup and preparation of the column as well as the slow flow rate achieved by the latter method method. The filtration rate decreased with both methods when ethanol was used either to wash or to elute the sample. Both 20 % (approach 3) and 50 % ethanol (approach 2) were effective in eluting the neokestose in the final fraction.

The purification of neokestose by carbon:celite chromatography is a crude method for purifying neutrally charged carbohydrates. It was, however, possible to significantly enrich the product for neokestose, which made up

82.6 % of the final product. In the final product, sucrose and GF_3 were both present in large quantities, the removal of these from the supernatant would improve enhancement.

Similar to our findings, industrial production of FOS with microbial fructosyltransferases have been found to give a yield of 55-60 % which is limited due to the competitive inhibition by glucose (Yun, 1996). Recently it has been shown that high-content FOS can be obtained by purifying the reaction mixture or by the in-process removal of liberated glucose and the hydrolysis of the unutilized sucrose (Sangeetha ***et al***., 2005). High-content FOS of between 90-98 % have been produced with mixed enzyme systems which contained fructosyltransferase, fructofuranosidase and glucose oxidase or glucose dehydrogenase (Sangeetha ***et al***., 2005). High-content FOS mixtures differ in their fructan make-up, which depends on the enzyme and cell system used. For high content neokestose production, ***X. dendrorhous*** has thus far showed to be the best candidate when compared to other fungi like ***Penicillium citrinum*** which produces a mixture made up of 1-kestose (22 %), nystose (14 %) and neokestose (11 %)(Hayashi ***et al***., 2000).

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CHAPTER 3

VALIDATION OF ANAEROBIC CULTIVATION TECHNIQUES AND SELECTIVE MEDIA.

3.1 Abstract

Reviewing literature of intestinal microbial populations in chickens, the caeca where chosen for the source of the intestinal microbial population. New Hampshire layers were used to establish a technique for removal of caecae from the abdominal cavity. Furthermore various media for selective enumeration of different bacterial groups were evaluated on layers and broilers. This included testing anaerobic conditions that are necessary for enumeration of the total anaerobic and ***Bifidobacterium*** counts. Media were selected for the enumeration of ***Lactobacillus***, ***Bifidobacterium,*** coliforms and ***Salmonella***. For total anaerobic enumeration Brain Heart Infusion medium supplemented with 5% sheep blood, cysteine-HCl and vit K_1 was chosen. For enumeration of ***Lactobacillus*** and ***Bifidobacterium*** species LAMVAB and MTPY were chosen, respectively. MacConkey was chosen for the enumeration of coliforms. For ***Salmonella*** XLD was chosen. New Hampshire layers (17 weeks) were found to harbor ***Lactobacillus*** at 6.7 Log_{10} cfu g^{-1} and ***Bifidobacterium*** species at 8.3 Log_{10} cfu g^{-1}. In contrast ***Lactobacillus*** species were isolated from Ross A1 broiler chickens at levels ranging from 7.9 – 9.3 Log_{10} cfu g^{-1}, whereas ***Bifidobacterium*** species where absent. The total anaerobic count was estimated at 9.8 Log_{10} cfu g^{-1} for the layers and ranged from 9.2 – 10.2 Log_{10} cfu g^{-1} in broilers. The isolation procedure and incubation conditions that were used were adequate for the enumeration of ***Bifidobacterium*** and ***Lactobacillus*** species from the caeca of New Hampshire layers.

3.2 Introduction

The caecal intestinal microbial population of broilers develops from a facultative anaerobic to an obligate anaerobic one early in the chickens life (Smith, 1965; Mead, 1989). It has been found that the caecal population comprises the most complex community of the chicken digestive tract, and has been found to stabilize after approximately 4 weeks (Smith, 1965; Mead, 1989). The composition of the intestine is influenced by a number of factors, which include amongst others age, diet and environmental factors. Of all factors affecting the intestinal microbial population, diet was found to be the strongest individual determinant (Apajalahti ***et al***., 2001), whereas within the same diet the physical form of the feed also plays a role.

Engberg ***et al***. (2002) compared the effects of the same grain based diet, provided as either pellets or mash, on the microbiota in chickens. They found that the pH within the digestive tracts of pellet fed birds was significantly lower compared to that of mash fed birds. Volatile fatty acid (VFA) concentration was found to be significantly higher in the caeca of pellet-fed birds than in mash fed birds. Pellet-fed birds, also had larger numbers of coliform bacteria and enterococci in the ileum and a reduced number of ***Clostridium perfringens*** and ***Lactobacillus*** in the distal end of the digestive tract (caeca and rectum).

Various techniques have been used in the elucidation of the intestinal microbial population found in the caeca. The simplest method has been the culturing on various selective and differential medium, which is nonetheless dependant on the culturability of the microbiota. Lately the use of molecular based techniques has been extensively used in the analysis of the community structure of the various sections of the broiler intestinal tract (Apajalahti ***et al.***, 1998; Zhu ***et al.***, 2001; Lu ***et al.***, 2003).

Among the large number of species present in the intestine three groups have been distinguished by Mitsuoka (1990) as follows. A beneficial group, consisting of ***Bifidobacterium*** spp., ***Lactobacillus*** spp. as well as other lactic acid bacteria, thought to exert a positive effect on health. Total

Bifidobacterium counts have been determined by some researchers to account for at least 9% of the 10^9 cfu g^{-1} (Barnes ***et al***. 1979; Barrow, 1992), while Rada and Petr (2000; 2002) suggested numbers higher than 10^{10} cfu.g^{-1} in hen caeca, where these bacteria can predominate. The second group comprises ***Enterobacteriaceae*** and ***Clostridium*** sp, which are both considered detrimental to general health. The third group, comprising all other bacteria, is considered neutral

For this study our focus was on evaluating the total anaerobic count, ***Bifidobacterium***, ***Lactobacillus***, and ***Salmonella*** species as well as members from the ***Enterobacteriaceae***. ***Salmonella*** Typhimurium, ***Escherichia coli*** and ***Campylobacter jejuni*** are considered to be important food pathogens for humans, which can originate from poultry caeca (Achen ***et al***, 1998). High viable numbers of ***Lactobacillus*** spp. have been reported in the caecum (Sarra ***et al***., 1992; Smith, 1965; Lu ***et al.,*** 2003). According to Fanelli ***et al***. (1971) the caeca is the site most readily colonized by ***Salmonella*** in poultry.

It was decided to monitor the effect of neokestose on the caecal population ***in vitro*** over 24 hours. The basal peptone yeast-extract test medium (PY) from Holdeman ***et al***. (1977) was chosen, as it has previously been used to test the effects of probiotics in poultry (Patterson ***et al.***, 1997).

Hartemink and Rombouts (1999) compared a number of media for total anaerobic enumeration. They concluded Brain Heart Infusion (BHI) medium, supplemented with blood, to be a suitable medium for enumerating total anaerobic bacteria. In addition to this, they compared a number of dilution media and found that physiological salt solution gave the best results, while the addition of the reducing agent cysteine-HCl did not improve enumeration. Interestingly, these researchers also found no significant effect on reduction in cfu's when diluting and plating under aerobic versus anaerobic conditions.

MacConley and Eosine Methylene Blue are two media which are used for the the enumeration of ***Escherichia coli.*** Coliforms have been enumerated on MacConkey agar by Jin ***et al.*** (1998), Baron ***et al***. (1992) and Adami and

Cavazzoni (1996). On this medium ***E. coli*** forms pink to dark pink colonies with precipitation zones and a dry colony texture (Flournoy ***et al***., 1990).

De Man, Rogosa and Sharp Agar (MRS) and Rogosa Agar are two of the media that are most frequently used as ***Lactobacillus*** isolation media these were, however, found to be highly non-selective for ***Lactobacillus*** (Hartemink & Rombouts, 1999). An anaerobic ***Lactobacillus*** MRS medium supplemented with vancomycin and bromocresol green (LAMVAB) was developed by Hartemink ***et al***. (1997) and shown to be highly selective for the isolation of ***Lactobacillus*** from faeces. This modified MRS medium has an improved selectivity due to a low pH (5.0) and the addition of vancomycin (20 $mg.l^{-1}$). In addition, it contains cysteine-HCL to enhance anaerobic conditions and bromocresol green as pH indicator. Vancomycin was similarly used by Yuki ***et al***. (1999) in a modified ***Lactobacillus*** Selection (LBS) medium with lactitol substituting glucose as carbon source for the isolation of ***L. casei*** Shirota from faeces. It allows the detection and isolation of low numbers of ***Lactobacillus*** spp. in the presence of high numbers of other lactic acid bacteria (LAB) such as ***Streptococcus*** spp., ***Enterococcus*** spp. and ***Bifidobacterium*** spp. which are in general susceptible to vancomycin, although vancomycin-resistant enterococci may occur. LAMVAB medium was successfully used for the isolation of LAB from human colonic biopsies (Kontula ***et al.***, 2000). LAMVAB has a selective advantage over other ***Lactobacillus*** media (Hartemink & Rombouts, 1999); however, ***L. acidophilus*** and ***L. gasseri*** have been reported not to grow on it (Jackson ***et al.***, 2002). Both these species were found to be sensitive to vancomycin (Pacher & Kneifel, 1996).

Selective media for enumerating ***Bifidobacterium*** spp. from food, feed and animal origin have been reported (Charteris ***et al***., 1997; Rada ***et al***., 1999; Roy 2001; Leuschner ***et al***., 2003; Simpson ***et al***., 2004). In the intestinal environment, a shared habitat and similar biochemical properties make cultural selection and differentiation of ***Bifidobacterium*** spp. from ***Lactobacillus*** spp. difficult (Rada 1997). Similar to ***Lactobacillus*** spp., ***Bifidobacterium*** spp. also grow on the non-selective MRS and Rogosa agars, hence various media have been developed and used for the detection of ***Bifidobacterium*** species

(Muñoa & Pares, 1988; Beerens, 1991; Hartemink ***et al***., 1996; Pacher & Kneifel, 1996; Rada ***et al***., 1999). To improve selection of ***Bifidobacterium*** spp***.*** from the physiologically and/or ecologically related ***Enterobacteriaceae***, ***Enterococcus*** spp***.*** and ***Lactobacillus*** spp. antibiotic and non-antibiotic inhibitors, electives (specific carbohydrates) and acidic pH conditions have been used (Nebra & Blanch, 1999).

The susceptibility of ***Bifidobacterium*** spp. to various antibiotics which include β-lactams (penicillin G, ampicillin, methicillin, imipenam and cephalothin), aminoglycosides (kanamycin, neomycin sulfate, gentamicin, streptomycin and paromomycin sulfate), gram-positive spectrum (bacitracin, erythromycin, lincomycin and vancomycin), gram-negative spectrum (nalidixic acid, colistin and polymyxin-B-sulfate) and broad-spectrum antibiotics (nitrofurantoin, chloramphenicol and tetracycline)(Lim ***et al***., 1993; Yazid ***et al***., 2000), showed that ***Bifidobacterium*** spp. had the highest resistance to the gram-negative spectrum antibiotics and aminoglycosides. Media have been developed containing neomycin, paromomycin, naldixic acid and lithium chloride (NPNL) with various bases (Blood Liver-BL; MRS; Nutrient agar-N/A; TPY). The NPNL media were considered by many to be the reference medium for the isolation of ***Bifidobacterium*** spp. (Modler ***et al***., 1990; Lapierre ***et al***., 1992; Lim ***et al***., 1995; Tamime ***et al***., 1995). This medium, which is time consuming to prepare, requires careful measurement of ingredients and treatment of the NPNL antibiotics for consistent and reliable results (Laroia & Martin, 1991), additionally showing inhibition for some ***Bifidobacterium*** spp. (Pacher & Kneifel, 1996, Lim ***et al***., 1995). The topical antibiotic mupirocin, formerly termed pseudomonic acid A, was found to have excellent ***in vitro*** activity against ***Staphylococcus*** and most ***Streptococcus*** with less activity against other gram-positive and gram-negative bacteria (Parenti ***et al***., 1987). Additionally, Rada (1997) found that ***Bifidobacterium*** spp***.*** were resistant to this antibiotic, while ***Lactobacillus*** spp***.*** were susceptible.

In addition to antibiotics, methylene blue, lithium chloride (LiCl), crystal violet, dodecyl sulfate sodium salt, sodium sulfite, propionic acid/propionate and fuchsin (basic) are useful inhibitors used in ***Bifidobacterium*** selective media

(Nebra & Blanch, 1999; Biaviati & Mattarelli, 2006). Propionate (salt of propionic acid) has been used in media such as Propionate agar (PROP), Beerens-agar and Raffinose-***Bifidobacterium*** media (RB) as well as modified-trypticase-phytone-yeast extract medium (MTPY) and LiCl in MRS-agar with antibiotics (MRS-NN)(Hartemink ***&*** Rombouts, 1999).

Some components called electives improve the growth of ***Bifidobacterium*** species. These include riboflavin, nitrogenous bases and pyruvic acid, simple or complex carbohydrates such as raffinose, lactose, lactulose and oligosaccharides (Biavati & Mattarelli, 2006). It was found that the strictly anaerobic probiotic ***Bifidobacterium longum*** NCC2705 has no less than 19 permeases for the uptake of diverse carbohydrates (Parche ***et al***., 2007). Among them are permeases for lactose, maltose, raffinose and fructooligosaccharides these are commonly used prebiotic additive. Complex carbohydrates have been used in Acetylglucosamine-lactose agar (AL-agar) and Raffinose-***Bifidobacterium*** agar (RB) are media, which have used these as specific carbohydrates. Rada & Petr (2000) successfully enumerated ***Bifidobacterium*** spp. from hen caeca on modified (M) Wilkins-Chalgren (MW) and trypticase-phytone-yeast extract (MTPY) agars. These media were modified by the addition of mupirocin (antibiotic) and acetic acid for a final pH of 5.2.

In this chapter, New Hampshire layers were used to evaluate a technique for the removal of the caeca from the abdominal cavity. Furthermore, the three media requiring anaerobic incubation namely; LAMVAB, BHI-S and MTPY were used to determine caecal levels of ***Lactobacillus***, total anaerobes and ***Bifidobacterium***, respectively. These caecal levels were compared to those of RossA1 broilers.

3.3 Materials and Methods

3.3.1 Chickens

3.3.1.1 Layer chickens

New Hampshire chickens are a breed developed predominantly as layers. Two 14 week old New Hampshire layer hens from Glenn agricultural college near Bloemfontein were used for an initial trial. The chickens were kept at the animal research facility at the University of the Free State under supervision and after obtaining approval from the Ethics committee of the University of the Free State. The area measured 4 x 2 meters and the concrete floor was covered with wood shavings to serve as litter material. These chickens were fed an antibiotic free layer ration ***ad libitum*** for 3 weeks. The feed was formulated by Agridata Trust (ADT) and the following information was disclosed: 150 g kg^{-1} protein (minimum), 70 g kg^{-1} fibre (maximum), 27 g kg^{-1} calcium (minimum) and 6 g kg^{-1} phosphorus (minimum).

3.3.1.2 Broiler chickens

A Ross A1 broiler breed was used during this trial. Five day-old broilers were bought from a local supplier and raised at the animal research facility at the University of the Free State, near the laboratory. Artificial heat for 1 to 7 day old chicks was provided by infrared light. Food, donated and formulated by Meadow (Table 3.1) was given ***ad libitum***. The food composition was similar to that used by Jin ***et al.*** (1998). The condition of the chickens and their access to water were checked regularly. After 5 weeks three of these broilers were euthanased and their caeca removed.

Table 3.1. Composition of broiler chicken feed (Formulated by Meadow).

Ingredients	Composition (%)
Maize	64.93
Fishmeal	10.82
Gluten 60 (+/- 60% protein)[1]	5.41
Sunflower oil cake	10.82
Soya oil cake	5.41
Salt	0.16
Mono-and di-calcium phosphate (MDCP)[2]	0.72
Limestone Grit	1.08
Lysine	0.19
Methionine	0.12
Threonine	0.01
Premix	0.32

[1]Gluten 60 – A protein waste product of maize, after removal of starch, carbohydrates and fat.
[2]MDCP – (+21% P ; 17% Ca)

3.3.2 Caecal removal procedure

The chickens were euthanased with CO_2 and their condition checked after approximately 20 minutes. The lower abdominal region was then cleared of feathers and the area disinfected with alcohol. Scissors, forceps and tweezers were sterilized beforehand by autoclaving. A horizontal incision was made by cutting the skin just below the breastbone in the abdominal cavity. The small intestines were then removed through the hole. The caeca are attached by a membrane to the upper part of the rectum. The caeca were carefully separated from this membrane to the point were they connected the intestinal tract. The caeca then were jointly bound off with nylon string just below their connection to the GI-tract (Fig 3.1). Thereafter the caeca were cut from the GI-tract and placed into a sterile stomacher bag filled with medical CO_2. The bag was then placed into an anaerobic jar filled with medical CO_2 and closed. An anaerobic catalyst was activated and placed inside the anaerobic jar 10 minutes before caecal removal began. The anaerobic jar was only opened once inside the anaerobic cabinet. It took less than 2 minutes from removal of the caeca from the abdominal cavity until being

placed inside the anaerobic jar. The procedure took about 20 minutes from the time that the first incision was made.

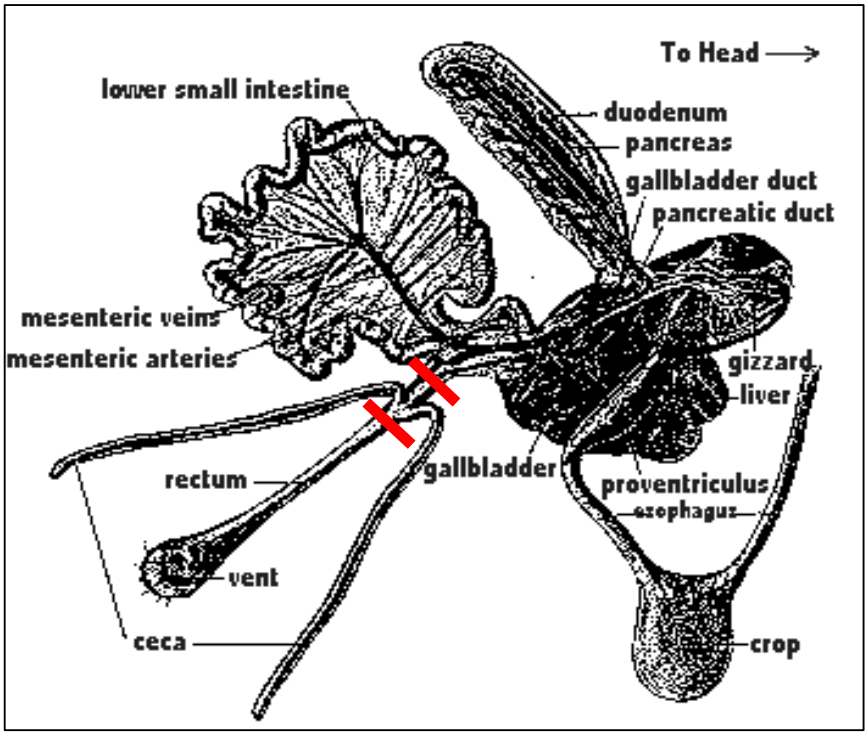

Figure 3.1. The caeca are two blunt ending sacs found in the lower gastrointestinal tract of chickens. Cuts made to remove the caeca are shown.

In the laboratory, the container with the caeca was put into an anaerobic chamber, which was tested and checked beforehand. Here the caeca were removed, cut open and the caecal contents together with some of the epithelial cells were scraped off. In this experiment the caecal contents from two layers were combined and mixed. For broilers experiments three sets of caeca were pooled and mixed.

3.3.3 Bacterial enumeration

3.3.3.1 Dilution medium

The initial caecal dilutions for broilers and layers were prepared as follows. For layers five gram caecal material was weighed off and a 10 % dilution (w/w) was prepared in phosphate buffered saline (PBS) containing cysteine-HCl (0.5 $g.l^{-1}$; pH 7.00). For broilers peptone yeast-extract (PY) basal medium was used for the initial 10 % dilution. The PY basal test medium contained (per litre): 2 g yeast extract; 2 g peptone; 0.1 g NaCl; 0.04 g K_2HPO_4; 0.04g KH_2PO_4; 0.01 g $MgSO_4$-$7H_2O$; 0.01 g $CaCl_2$-$6H_2O$; 2 g $NaHCO_3$; 0.5 g bile salts; 2 g Tween 80; 1 g resazurin; Haemin (0.05 g dissolved in 1 M NaOH), 10 ml cysteine-HCl (5%) and 10 µl Vit K_1 were filter sterilized (0.22 µm) into the medium after autoclaving. The medium was adjusted to pH 7.00 with 1M HCl. Both media were reduced prior to the mixing by vacating the available O_2 with high purity N_2 gas. These dilutions were then mixed in a stomacher bag for approximately 2 minutes until homogenous. This was then used to make a series of 10-fold dilutions (10^{-2} to 10^{-8}) in PBS+cysteine-HCl.

3.3.3.2 Enumeration medium and incubation conditions

From each of the dilutions 100 µl was spread plated on the following growth media. Brain Heart Infusion (BHI) medium, supplemented with 5 % defribinated sheep blood, vitamin K_1 and cystein-HCl (BHI+S) (Hartemink & Rombouts, 1999) was used for total anaerobes, which was incubated anaerobically at 37 ^oC for 48 h. For ***Lactobacillus*** spp. LAMVAB (Hartemink ***et al.***, 1997) and for ***Bifidobacterium*** spp. modified-trypticase-phytone-yeast extract (MTPY) (Rada & Petr, 2000) was also anaerobically incubated at 37^oC for 48 h and 72 h, respectively.

3.3.4 Anaerobic cultivation

Anaerobic cultivation was done inside an anaerobic work station (Forma Scientific, model # 901024). This station is equipped with a temperature adjustable incubator. The anaerobic atmosphere inside is created and maintained with two gas types. High purity N_2 gas was used for purging the chamber initially, while an anaerobic mixture consisting of 10% H_2, 10% CO_2 and 80% N_2 modulated the anaerobic environment. This chamber uses palladium catalyst and desiccant wafers to maintain strict anaerobiosis to less than 10 ppm O_2 (according to the specifications provided by the manufacturer). Possible air leaks were detected using a BBL GasPak disposable anaerobic indicator, which changes colour when the O_2 concentration reaches 0.5% (~ 5000 ppm). Additionally, agar plates containing 1 gram per liter resazurin (Sigma) were used to monitor anaerobic conditions. The agar plates were colourless when oxygen was absent but turned pink when O_2 was present. A set of two were placed inside the anaerobic cabinet, one was placed inside the incubator and the other on the working surface.

Incubation of plates in this workspace was continuously monitored with anaerobic indicators. As an alaternative to the anaerobic incubator anaerobic jars were available. These were used as a control measure to monitor anaerobiosis on MTPY medium.

3.3.4.1 Bacterial cultures

Bifidobacterium animalis ATCC 27536/DSM 20105 and ***Bifidobacterium gallinarum*** ATCC 33777/DSM 20670 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb (DSMZ). These strains, originally isolated from chickens, were used to test media and anaerobic culturing conditions. ***Lactobacillus salivarius*** (NCFB 1555), was obtained from the culture collection of the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State and used to test LAMVAB medium.

3.3.4.2 Bacterial maintenance medium

B. animalis and ***B. gallinarum*** were both grown on Reinforced Clostridial Medium base (RCA) (Biolab, Merck) containing 0.05 % cystein-HCl and incubated anaerobically. ***Lactobacillus salivarius*** was grown on MRS medium (Biolab, Merck).

3.4 Results

3.4.1 Bacterial enumeration for layers

The average total anaerobic count for three layer chickens on BHI-S medium was estimated at 9.8 $\log_{10}$ cfu per gram wet caecal material, with a 0.13 standard deviation. These numbers are similar to those reported by Hartemink and Rombouts (1999) for the total anaerobic count on BHI-S medium.

Bifidobacterium and ***Lactobacillus*** were present at 8.3 and 6.7 $\log_{10}$ cfu per gram wet caecal material, respectively. ***Bifidobacterium*** isolates tested gram positive and catalase positive and had characteristic ***Bifidobacterium*** morphology, often displaying Y-shapes typical of the genus (Fig 3.2).

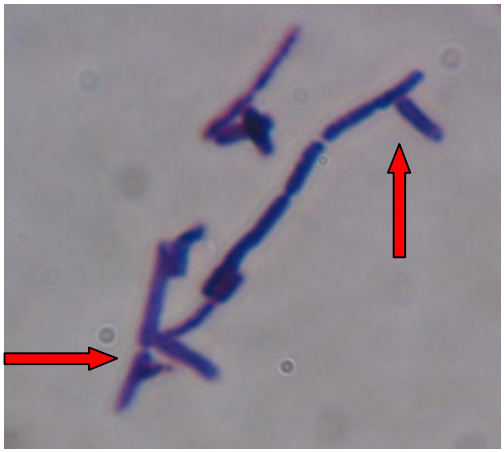

Figure 3.2. Picture taken with 1000x (oil) magnification of bacteria isolated from 17 week old layers on MTPY agar. The two bacteria shown by the red arrows both have the characteristic Y-shape of ***Bifidobacterium***.

The isolates also tested positive for fructose-6-phosphate phosphoketolase (F6PPK) activity. A positive F6PPK test is a direct and reliable characteristic distinguishing the ***Bifidobacteriaceae*** from related families (Scardovi, 1986; Tannock, 1999; Biavati & Mattarelli, 2006). The family ***Bifidobacteriaceae*** consists of the genus ***Bifidobacterium*** and ***Gardnerella*** with ***G. vaginalis*** the only species, which has so far only been isolated from the genital tract of mammals.

3.4.2 Bacterial enumeration for broilers

For broilers the total anaerobic count was similar on BHI-S medium to that reported by Hartemink & Rombouts (1999). The numbers ranged from 9.2-10.2 $\log_{10}$ cfu per gram wet caecal material for all the experiments.

Bifidobacterium spp. were absent in broilers, even when these plates were anaerobically incubated in anaerobic jars. Levels of ***Lactobacillus*** ranged from 7.9 to 9.3 $\log_{10}$ cfu per gram wet caecal material.

3.5 Discussion

The lack of ***Bifidobacterium*** species is probably not related to the feed composition given to the broilers, as similar compositions were used in experiments where ***Bifidobacterium*** were detected in high numbers by Xu ***et al***. (2003). They used the ***Bifidobacterium*** iodoacetate medium (BIM-25) described by Muñoa and Pares (1988). This medium was developed and tested for the enumeration of ***Bifidobacterium*** spp. from water samples as indicators of faecal contamination. Both Rada ***et al***. (1999) and Muñoa and Pares (1988) used a rich non-selective medium for the dilution of their samples, which had a resuscitation effect on ***Bifidobacterium.*** Another reason for the absence of ***Bifidobacterium*** spp. in these broilers could be the PY-basal dilution medium that was used. Even though PY-Basal medium does not contain any selective antibiotics, it did contain ox-bile. ***Bifidobacterium*** species, which included ***B. animalis***, have been found to be inhibited by bile salts, especially in a poor medium (Perrin ***et al***., 2000).

Chicken bile consists mainly of taurochenodeoxycholic acid (80%), taurocholic acid (15%) and tauroallocholic acid (5%) (Feighner & Dashkevicz, 1987). Bile is mainly found in the proximal part of the ileum while a lower concentration of these conjugated bile acids in the distal ileum and caeca allow for growth in these regions. Soy protein, which was a component of the broiler feed, has been shown to bind bile acids and aggregate them (Sugano ***et al.***, 1990).

Shimakawa ***et al***. (2003) observed that soy protein alleviated the inhibition of ***Bifidobacterium breve*** Yakult by bile. Some feed components could therefore protect bile sensitive ***Bifidobacterium*** species, as these have been reported to vary in their bile tolerance (Charteris ***et al***., 1998). Whether the bile present in the PY basal medium played a role in the low numbers of ***Bifidobacterium*** detected in the caeca of broilers was not established here. It may, therefore, be that these birds just possessed reduced numbers of ***Bifidobacterium*** compared to the layers used. In a study on Swedish broiler farms the single biggest determinant of variation in caecal community structure was found to be feed composition (Apajalahti ***et al***., 2001), while hygiene was said to have a small impact. Similar to our results, Lu ***et al***. (2003) found no ***Bifidobacterium*** spp. present in the caecum of Ross-Hybrid broilers when fed a commercial corn-soy diet. The importance of the age difference between these layers and broilers on the presence of ***Bifidobacterium*** spp. is difficult to evaluate since these birds were raised differently. This is important since it is unclear, how ***Bifidobacterium*** spp. are aquired at an early age and whether it is important that these establish in the GI-tract at an early age. It is known that succession occurs in GI-tract, with the caecum developing the most complex microbiota of the GI-tract in the maturing broiler chicken (Barnes ***et al***., 1972; Lu ***et al***., 2003). The anaerobic conditions and selective medium were found to be adequate for the enumeration of ***Bifidobacterium*** spp. from the caecum of layers. The absence of ***Bifidobacterium*** spp. in the caecum of broilers is, therefore, unlikely to be related to these factors.

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CHAPTER 4

IN VITRO POPULATION STUDY OF SELECTED BACTERIAL GROUPS FROM BROILER CAECAL MATERIAL

4.1 Abstract

Caecal contents from 5 week old RossA1 broilers were used to investigate the impact of specific carbohydrates over 24 hours on the intestinal microbiota. The ***in vitro*** experiments were done in basal peptone yeast (PY) medium (Holdeman ***et al.***, 1977) supplemented with commercial inulin, neokestose (composition, Table 4.1) or glucose. The basal medium without supplementation served as control. Samples were taken at 0, 12 and 24 hours, post infusing with 1% caecal material. Changes were investigated through plate count, pH and the volatile fatty acids acetic acid, butyric acid and propionic acid. Additionally the effect of the pathogens ***Campylobater jejuni, Escherichia coli*** and ***Salmonella*** Typhi was also evaluated on the over 24 hours on the intestinal microbial population. Selected microbial groups were enumerated using BHI supplemented with 5% sheep blood, cysteine-HCl and vitamin K_1 for total anaerobes, modified trypticase-phytone-yeast extract (MTPY) for ***Bifidobacterium*** spp., LAMVAB for ***Lactobacillus*** spp., MacConkey for ***Enterobacteriaceae,*** especially ***Escherichia. coli***, and XLD for ***Salmonella*** spp. The addition of inulin and neokestose resulted in higher numbers of ***Lactobacillus*** and lower numbers of ***Campylobacter jejuni***, ***E. coli*** and ***Salmonella*** Typhi ***in vitro*** over 24 hours compared to the control. No colonies developed on MTPY selective medium. The pH decreased for all the treatments to less than 4.5 after 24 hours. Acetic acid was the major VFA formed during the incubation, in addition to butyric and propionic acid. The reduction in pH is probably responsible for the negative effect on ***Campylobacter jejuni***, ***Escherichia coli*** and ***Salmonella*** Typhi. The effects of a bacteriocin like reuterin (***Lactobacillus reuteri***) or similar cannot, however, be excluded. Acetic acid, propionic acid and butyric acid all increased within 12 hours, whereas no major increase was detected thereafter.

4.2 Introduction

Fructo-oligosaccharides have various beneficial affects when administered to humans and animals and the basis of this health promoting process is the observation that these compounds stimulate the growth of beneficial bacteria but not that of potentially harmful intestinal inhabitants (Hidaka ***et al***., 1991).

Fructo-oligosaccharides are not digested in the animal small intestine but are fermented in the colon to short chain fatty acids (Oku ***et al***., 1984). The short chain fatty acids are absorbed by the host, resulting in an increase in available energy for the host and a decrease in pH in the intestine (McKellar & Modler, 1989, Delzenne ***et al***, 1995, Southgate, 1995, Bouhnic ***et al***, 1996, Campbell ***et al***., 1997, Djouzi & Andrieu, 1997).

The population of the gastro-intestinal tract of the chicken has been studied by a number of methods. Culture based techniques, which make use of plate counts on various selective media, have been used quite frequently while molecular based methods have been used recently to ascertain the diversity of the intestinal tract.

The major criticism against culture-based techniques is that only a fraction of the total microbial population can be cultured (Ricke & Pillai, 1999; Theron & Cloete, 2000; Amann ***et al.***, 1995; Felske ***et al.***, 1998; Lu ***et al***., 2003). This low recovery is believed to be due to the lack of understanding of the growth requirements of most bacteria, which therefore cannot be mimicked under laboratory conditions.

Apajalahti et al. (2001) PCR amplified and sequenced 100 randomly selected partial 16S rDNA fragments using universal PCR primers. They found that species of the genus ***Ruminococcus*** made up nearly 20 % of the total bacterial community in the caeca of the chickens analyzed. The second most abundant genus was ***Streptococcus***, followed by ***Bacteroides***, ***Clostridium***, ***Fusobacterium***, and ***Bifidobacterium***. No 18S eukaryotic sequences where found, which they suggested represented a minor proportion (<5%) of the total

16/18S rDNA. Studies of the culturable bacteria show that the predominant cultural bacteria in the chicken caecum are obligate anaerobes at a concentration of 10^{11} cfu per gram of caecal content (Barnes ***et al***., 1972; 1979). It was found by Mead (1989; cited by Lu ***et al***., 2003) that gram-positive cocci such as ***Peptostreptococcus*** made up 28% of the total culturable bacteria, with other bacteria included ***Bacteroidaceae*** (20%), ***Eubacterium*** spp. (16%), ***Bifidobacterium*** spp. (9%), budding cocci (6%), ***Gemmiger formicilis*** (5%), and ***Clostridium*** spp. (5%). A review by Rehman ***et al***. (2007) showed that anaerobes range from $10^{9.5}$ to $10^{10.9}$ cfu per gram content when poultry are raised on corn-soybean diets, while ***Bifidobacterium*** (<1% to 12%) and ***Lactobacillus*** (1% to 12%) were reported at similar levels.

A balanced intestinal microbial composition is generally an indication of good health, whereas a shift in this composition can point to the presence of a disease condition. ***Escherichia coli***, although normally present as a minor constituent of the functional microbiota, can proliferate during impaired disease resistance to such an extent that it causes colibacillosis. Such a disease condition in poultry normally results in a loss of production through increased mortality and/or morbidity. ***Campylbacter jejuni*** which is not considered a normal intestinal inhabitant of the chicken is reported as one of the most common causes of bacterial gastroenteritis in humans. This is caused by the consumption or handling of poultry meat infected by ***Campylobacter jejuni***.

The volatile fatty acids, acetic acid, propionic acid and butyric acid are metabolic fermentation products of the intestinal microbiota (Barnes ***et al***., 1979). Volatile fatty acids can be either in a dissociated or undissociated form of which the ratio is determined by the pH. Volatile fatty acids are in an undissociated form at a low pH and in a dissociated form at a high pH. Undissociated volatile fatty acids have been shown to inhibit ***Enterobacteriaceae*** (Freter & Abrams, 1972 cited Van der Wielen ***et al***., 2000).

In this study we investigated the effect of neokestose on the culturable microbial population in the caecum of broiler chickens ***in vitro***. The numbers of the (1) total anaerobes, (2) ***Bifidobacterium*** spp., (3) ***Lactobacillus*** spp.,(4) coliforms and (5) ***Salmonella*** spp. were enumerated and the pH and VFA determined. The change in the community profile with neokestose over 24 hours was compared to that of inulin and glucose. The prebiotic effect of neokestose, inulin and glucose were also compared with the addition of ***Campylobacter jejuni***, ***Escherichia coli*** and ***Salmonella*** Typhi to the caecal microbial population.

4.3 Material and Methods

4.3.1 Bacterial strains

Bifidobacterium animalis (isolated from chicken faeces)(ATCC27536/DSM 20105) and ***Bifidobacterium gallinarum*** (isolated from chicken caecum)(ATCC 33777/DSM 20670) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmb (DSMZ). These strains, originally isolated from chickens, were used as controls on MTPY medium. ***Lactobacillus salivarius*** (NCFB 1555), ***Escherichia coli*** (ATCC 25922) and ***Klebsiella pneumoniae*** (ATCC 9997) were supplied by the department of Food Science, University of the Free State. ***Salmonella*** Typhi (ATCC 19430) was obtained from Onderstepoort Veterinary Institute (OVI). ***Campylobacter jejuni***, isolated from chicken faeces, were supplied by Dr. Al Lastovica from the University of Cape Town.

4.3.2 Culture maintenance

B. animalis and ***B. gallinarum*** were maintained on medium 104: Peptone yeast extract glucose medium (PYG) was modified as described on the DSMZ website for the maintenance of ***Bifidobacterium gallinarum*** (Medium 104, n.d). ***Lactobacillus salivarius*** and ***Escherichia coli*** were maintained on MRS medium (Biolab, Merck) and Nutrient Agar (Biolab, Merck), respectively. ***Klebsiella pneumoniae*** and ***Campylobacter jejuni*** were maintained on Tryptic

Soy Agar (TSA; BBL) and Tryptose Blood Agar agar (TBA; Oxoid CM 233) respectively. ***Salmonella*** was grown and maintained in BHI broth (Biolab, Merck).

4.3.3 Media and cultivation conditions

4.3.3.1 *In vitro* test media

The basal peptone yeast extract (PY) test medium described by Holdeman ***et al***. (1977), was used for dilution and incubation of caecal material from broilers. The test medium contained per litre: 2 g yeast extract; 2 g peptone; 0.1 g NaCl; 0.04 g K_2HPO_4; 0.04g KH_2PO_4; 0.01 g $MgSO_4 \cdot 7H_2O$; 0.01 g $CaCl_2 \cdot 6H_2O$; 2 g $NaHCO_3$; 0.5 g bile salts; 2 g Tween 80; 1 g resazurin. These components were all added and dissolved in 950 ml deionised water the pH was adjusted to 7 with 1 M HCl and autoclaved. The following components filtered sterilized into the medium with a 0.22 µm syringe filter: 10 ml haemin solution (see below), 10 ml cysteine-HCl (5%) and 10 µl vitamin K_1. ***Haemin***: 50 mg was dissolved in 1 ml 1 N NaOH and made up to 100 ml with distilled water stored in at 4^{o}C. ***Vitamin K1***: 0.1 ml was dissolved in 20 ml 95% ethanol and then filter sterilized in a brown bottle and kept at 4^{o}C. After all the medium components were added to a 1 l Schott bottle equiped with a butyl rubber stopper, the liquid medium was reduced by vacating the available O_2 by cycling with N_2 gas through glasswool filled syringe units. Nitrogen gas was sparged through the liquid medium by drawing a vacuum and then pressurizing with nitrogen this was repeated 20 times. This medium which had a light brown colour, was stored in the anaerobic cabinet from which 32 ml aliquots were filtered into 100 ml serum bottles and stoppered.

4.3.3.2 Supplementation of PY basal test medium.

4.3.3.2.1 Carbohydrate supplementation.

Three different carbohydrate supplementations were used: (1) 10 $g.l^{-1}$ glucose, (2) 10 $g.l^{-1}$ commercial inulin (Inulin, colon support powder,

Bloomingdale, IL 60108), (3) 10 $g.l^{-1}$ neokestose mixture (composition given in table 4.1). The medium without added carbon served as control. The supplementation to the reduced PY medium was done by adding 4 ml of treatments 1 to 3 aseptically to 32 ml of reduced PY medium (C-PY). After the carbohydrates were added, the PY basal media was reduced by displacing the O_2 with N_2.

Table 4.1. Composition of Neokestose mixture used in caecal studies

Composition of neokestose mixture				
GF_3	Neokestose	Sucrose	Glucose	Fructose
7.4%	82.6%	8.7%	1.2%	0.1%

4.3.3.2.2 Caecal infusion, serial dilutions and sampling.

Caecal material from Ross A1 broiler chickens were obtained as described in chapter 3. Four gram wet caecal material from the combined caeca of 3 five week-old broilers was made into a 10% caecal slurry with 36 ml pre-reduced PY basal test medium (Holdeman ***et al***, 1977) which contained no added carbohydrates. The 10% caecal slurry was thoroughly homogenised inside a sterile stomacher bag by hand. For caecal studies 4 ml of a homogenised 10% caecal slurry was infused into 36 ml pre-reduced, C-PY medium. The sample bottle was incubated in a anaerobic cabinet at 37 ^{o}C. The bottle was swirled and 11 ml each was removed after 0 h 12 h and 24 h for analysis. A total volume of 1 ml was used for serial dilutions. The pH of the remaining 10 ml was measured, after which the broth was centrifuged at 4000 g for 5 min. The supernatant was removed and filter sterilized into 1.5 ml sterile eppendorf tubes for short chain fatty acid analysis. For butyric, acetic and propionic acid 0.85 ml of the filter sterilized supernatant was combined with 0.15 ml concentrated HOOC (methanoic acid). Serial dilutions were done in pre-reduced physiological salt solution containing 0.001 $g.l^{-1}$ resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt) and 0.5 $g.l^{-1}$ cysteine-HCl. The serial dilutions were done inside an anaerobic work station as described in chapter 3. Dilution series were spread plated onto the various media (4.3.3.3) using 0.1 ml for each dilution. On completion of the anaerobic serial plating,

the dilution series was removed from the anaerobic work station and further serial plating was done aerobically.

4.3.3.2.3 Pathogen addition

In one experiment the pathogens, ***C. jejuni***, ***E. coli*** and ***S.*** Typhi were added to the medium in addition to caecal material. To determine the level of substitution these bacteria were grown on their respective maintenance media. ***Escherichia coli*** and ***Salmonella*** Typhi were incubated at 35 ^{o}C for 24 hours and ***Campylobacter jejuni*** was incubated microaerophilicly for 48 hours at 35 ^{o}C. Colonies were picked up from the growth media with sterile swabs and suspended in saline solution to an absorbance of 0.5 at 420 nm. The level of addition for the various pathogens was as follows. From a standard 0.5 absorbance suspension (420 nm), 1 ml of a 10^{-1} dilution was used for ***Escherichia coli***, 1 ml of 10^{-2} dilution for ***Salmonella*** Typhi and 500 µl of 10^{-2} dilution for ***Campylobacter jejuni***. These were added to 4 g caecal material in a stomacher bag and 10 % caecal slurry was prepared with reduced PY media. From this caecal slurry 4 ml was added to 36 ml C-PY medium.

4.3.3.3 Enumeration media

For the total anaerobic count Brain Heart Infusion (BHI) medium, supplemented with 5 % defribinated sheep blood (Hartemink & Rombouts, 1999), vitamin K_1 and cystein-HCl was used (BHI+S). For ***Lactobacillus*** spp. LAMVAB (Hartemink ***et al.***, 1997) and for ***Bifidobacterium*** spp. modified-trypticase-phytone-yeast extract (MTPY) (Rada & Petr, 2000) was used. ***Campylobacter jejuni*** was enumerated on ***Campylobacter*** selective media (Fluka) to which 5% sheep blood and campylobacter selective supplement (Product 17775 from Fluka) were added. This supplement contained per vial (sufficient for 1000 ml medium): 25 ml Rifampin, 6.25mg Cefsulodin and 20000 units Polymyxin B sulfate. Xylose Lysine Deoxycholate (XLD) medium was used for ***Salmonella*** spp. and MacConkey agar for ***E. coli***.

4.3.3.4 Incubation of plates for enumeration of bacteria

All anaerobic media were incubated in an anaerobic cabinet fitted with a incubator. BHI+S medium was anaerobically incubated at 37 ^{o}C for 72 hours. ***Campylobacter*** medium was incubated at 42 ^{o}C for 48 hours in a O_2-deficient, CO_2-enriched atmosphere produced in an anaerobic jar with Anaerocult®C. MacConkey medium was incubated aerobically at 37 ^{o}C for 24 hours. XLD agar was incubated aerobically for 48 hours at 37 ^{o}C. LAMVAB medium was incubated anaerobically at 37 ^{o}C for 72 hours. MTPY medium was anaerobically incubated at 37 ^{o}C for 72 hours.

4.3.4 Chemical analysis

Volatile fatty acids were analysed with a Hewlett-Packard gas chromatograph equipped with a flame ionization detector. Acetic acid, methanoic acid, propionic acid, ***iso***-butyric acid ***n***-butyric acid, ***iso***-valeric acid and ***n***-valeric acids were used as standards.

4.4 Results

4.4.1 In vitro caecal study: effect of carbohydrate treatment on the caecal microbiota

4.4.1.1 Effect of carbohydrate addition on pH.

The pH of the PY-basal medium decreased for all the treatments during the 24 h of incubation (Fig. 4.1). The rate of pH reduction was highest for the first 12 hours, with only a small decrease occurring from 12 to 24 h. The control also showed a decline in pH from 0 h to 12 h, but the rate and extend of the decline was less than for the inulin, neokestose and glucose treatments. The lowest pH was recorded for the glucose treatment at both 12 and 24 h. The neokestose treatment had a lower pH after 12 hours than the inulin treatment but after 24 hours the pH values for the inulin and neokestose treatments were comparable.

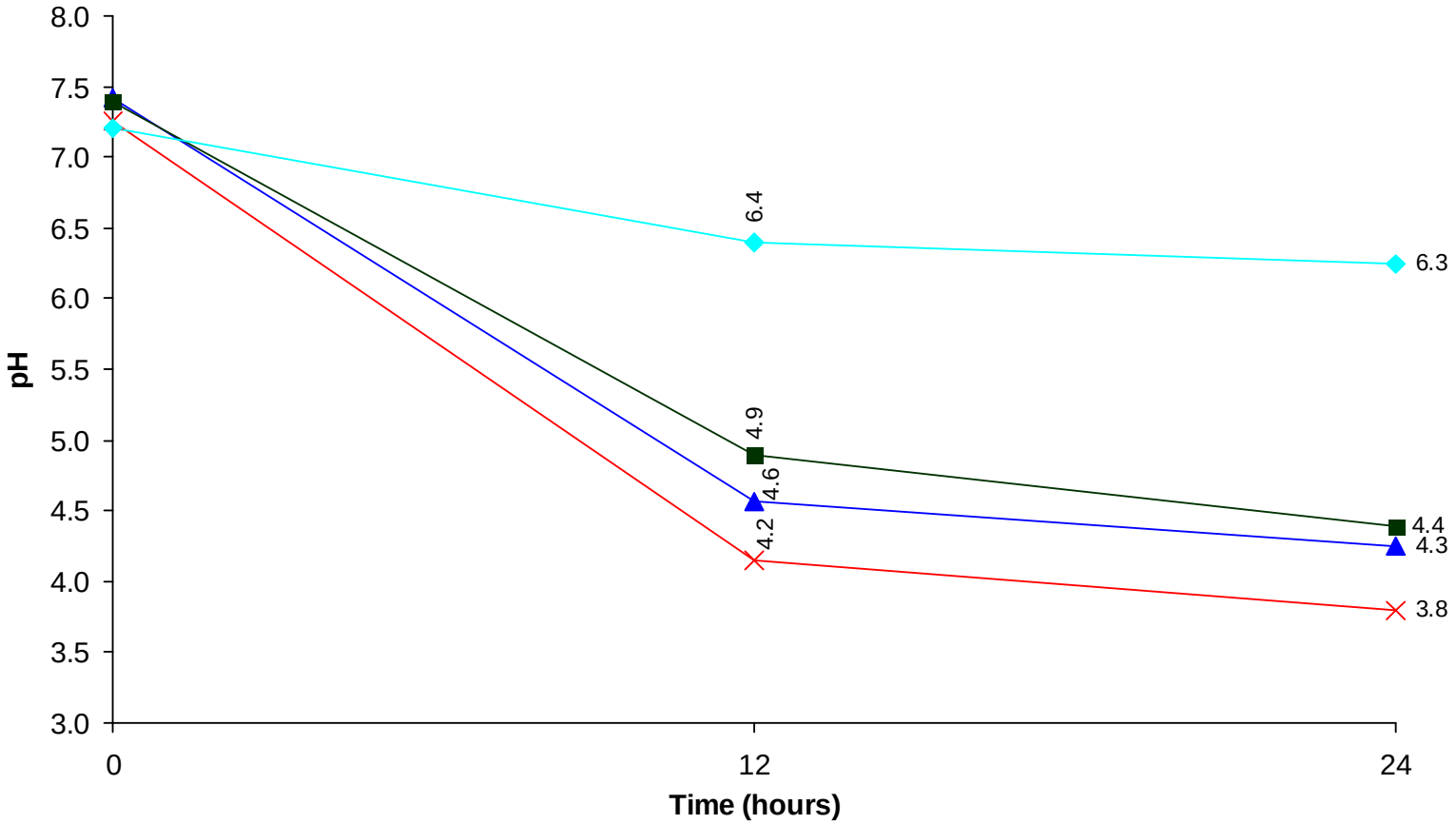

Figure 4.1. The effect of sugar supplementation on pH. Neokestose mixture (▲); Glucose (x) Inulin (■) and Control (♦) in PY basal media infused with chicken caecal contents ***in vitro***.

4.4.1.2 Microbial plate enumeration

The effect of carbon source on the numbers of selected bacterial groups done in triplicate is shown in figures 4.2, 4.3, 4.4 and 4.5.

4.4.1.2.1 Total anaerobic count on BHI+S

The mean for similar treatments was calculated and is shown in Fig 4.2. The various supplementations did not show a significant variation on the total count at the three sampling times. The average total anaerobic levels increased for all tests through the first 12 hours and remained at that level through to 24 hours. The 1 log cycle increase in the anaerobic count seen for the neokestose treatment from 0 h to 12 h was the highest of all the treatments. Both the inulin and glucose treatments showed a 0.7 log cycle increase with 0.4 log cycle increase for the control.

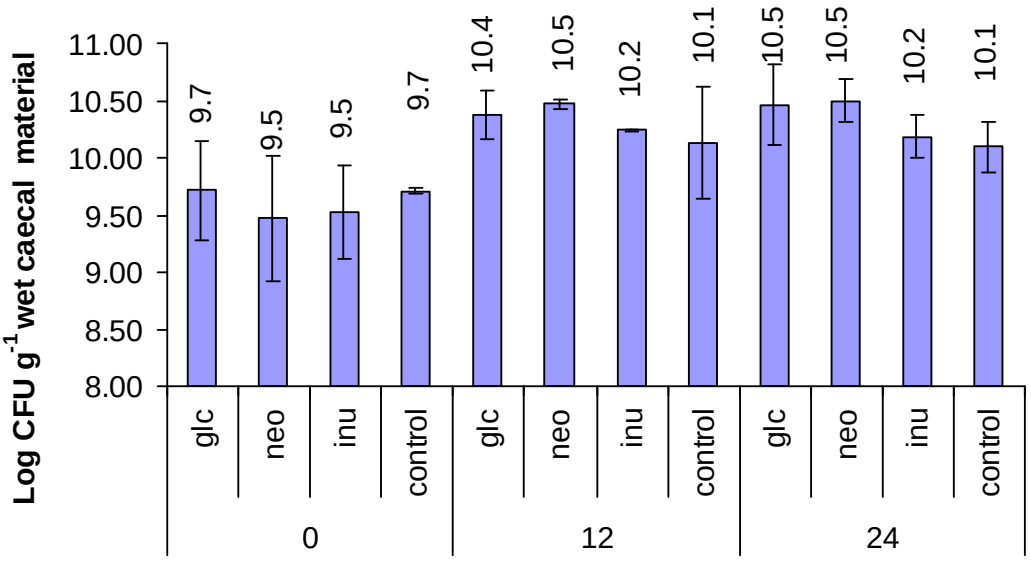

Figure 4.2. The effect of carbohydrate addition on the total anaerobic count at 0 h 12 h and 24 hours after caecal inoculation and enumerated on BHI+S. PY basal medium contained either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

4.4.1.2.2 *Lactobacillus* count on LAMVAB

The average ***Lactobacillus*** count increased by more than 1 log cycle over 24 hours (Fig. 4.3) for both the neokestose and glucose treatments, whereas it only increased by 0.4 and 0.1 log cycles for the inulin treatment and the control, respectively. Overall, increases were observed for all the treatments over the first 12 hours. During the last 12 hours the ***Lactobacillus*** level did not increase for the inulin and decreased on average by 0.3 log units for the control.

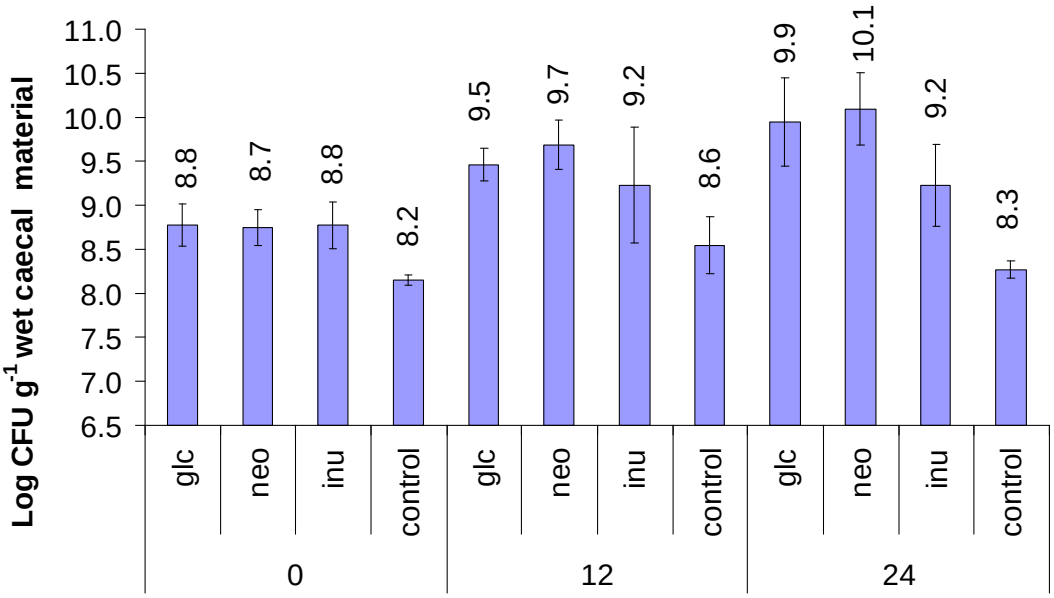

Figure 4.3. The effect of carbohydrate addition on the ***Lactobacillus*** count at 0, 12 and 24 hours after caecal inoculation enumerated on LAMVAB. PY basal medium contained either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

4.4.1.2.3 Coliform counts on MacConkey

MacConkey agar allows for the differentiation of ***Escherichia coli***, which generally appears as a large red colony surrounded by a turbid zone. This is due to the precipitation of bile acids as a result of decreasing pH. Coliform counts increased for all supplementations, including the control, after 12 hours (Fig. 4.4). The interval from 12 to 24 hours shows a decrease in coliform with all supplementations. Coliform counts for neokestose, inulin and control were similar at 24 hours.

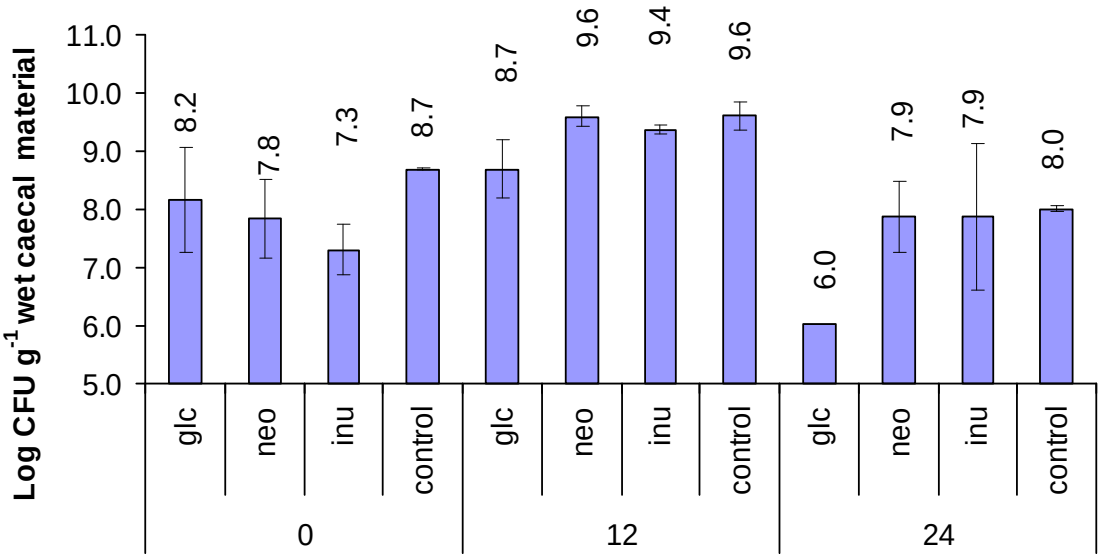

Figure 4.4. The effect of carbohydrate addition on the coliform count at 0, 12 and 24 hours after caecal inoculation and enumeration on MacConkey agar. PY basal medium contained either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

4.4.1.2.4 *Klebsiella* counts on XLD

Yellow colonies with precipitation zones around them were detected on this media. These have tentatively been identified as ***Klebsiella*** based on colony morphology on XLD agar. On average ***Klebsiella*** levels increased for all treatments and the control through the first 12 hours. The smallest increase in ***Klebsiella*** levels were detected for the glucose treatment (0.3 log units), whereas the neokestose treatment had the lowest average level (5.8 CFU g^{-1}) after 12 hours.. The highest average increase is seen for inulin and the control over the first 12 hours. All treatments showed a decrease in ***Klebsiella*** levels over 24 hours compared to the control. This differential agar also allows for the detection of ***Salmonella*** spp. which appear translucent, having the same colour as the culture medium and sometimes a characteristic black centre. No black centered colonies were detected on any of the treatments or control during these tests. <4.4 indicates that some colonies were present but were below the 25 to 250 cfu range normally counted.

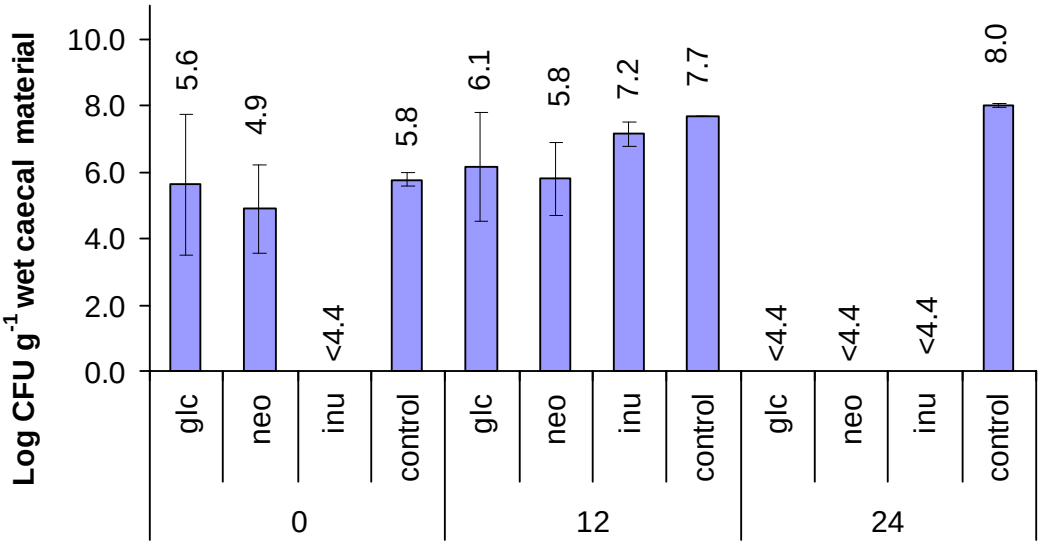

Figure 4.5. The effect of carbohydrate addition on ***Klebsiella*** count at 0, 12 and 24 hours after caecal inoculation and enumeration on XLD agar. PY basal medium contained either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

4.4.1.2.5 *Bifidobacterium* counts on MTPY

Growth on this medium was limited and sporadic throughout the trial, and interpretation of the effect of carbohydrate supplementation is thus difficult. No growth occurred in the majority of the cases. The media and anaerobic growth conditions were tested with ***Bifidobacterium animalis*** and ***B. gallinarum,*** both of which were able to grow anaerobically on MTPY medium. It is therefore unlikely that either the medium or the cultivation conditions were unsuitable to the growth of ***Bifidobacterium*** species. By contrast, ***Bifidobacterium*** species were isolated from 17 week old layers using the same medium (Chapter 3).

4.4.2 *In vitro* caecal study: Effect of carbohydrate addition on pathogens added to caecal contends.

Three bacterial isolates, ***Campylobacter jejuni*** (chicken isolate), ***Salmonella*** Typhi and ***E. coli,*** were added to freshly collected caecal material. The growth of these strains was evaluated ***in vitro*** with PY basal medium supplemented with the neokestose mixture or inulin. A treatment with all these bacterial isolates but no carbohydrate added served as control. Samples were taken after 0, 12 and 24 hours of incubation.

4.4.2.1 Effect on pH.

The addition of ***Salmonella*** Typhi, ***E. coli*** and ***C. jejuni*** to fructan-supplemented caecal contents resulted in similar trends in pH as previously reported for cultures without these additions (see section 4.4.1.1). All treatments resulted in a decline in pH, over 24 hours (Fig. 4.6). Overall the observed pH reduction was highest for the first 12 hours, whereas the pH decreased only minimally in the period from 12 to 24 hours. The control resulted in only a small decline in pH from 0 to 12 hours. The neokestose treatment resulted in a lower pH after 12 and 24 hours than the inulin treatment.

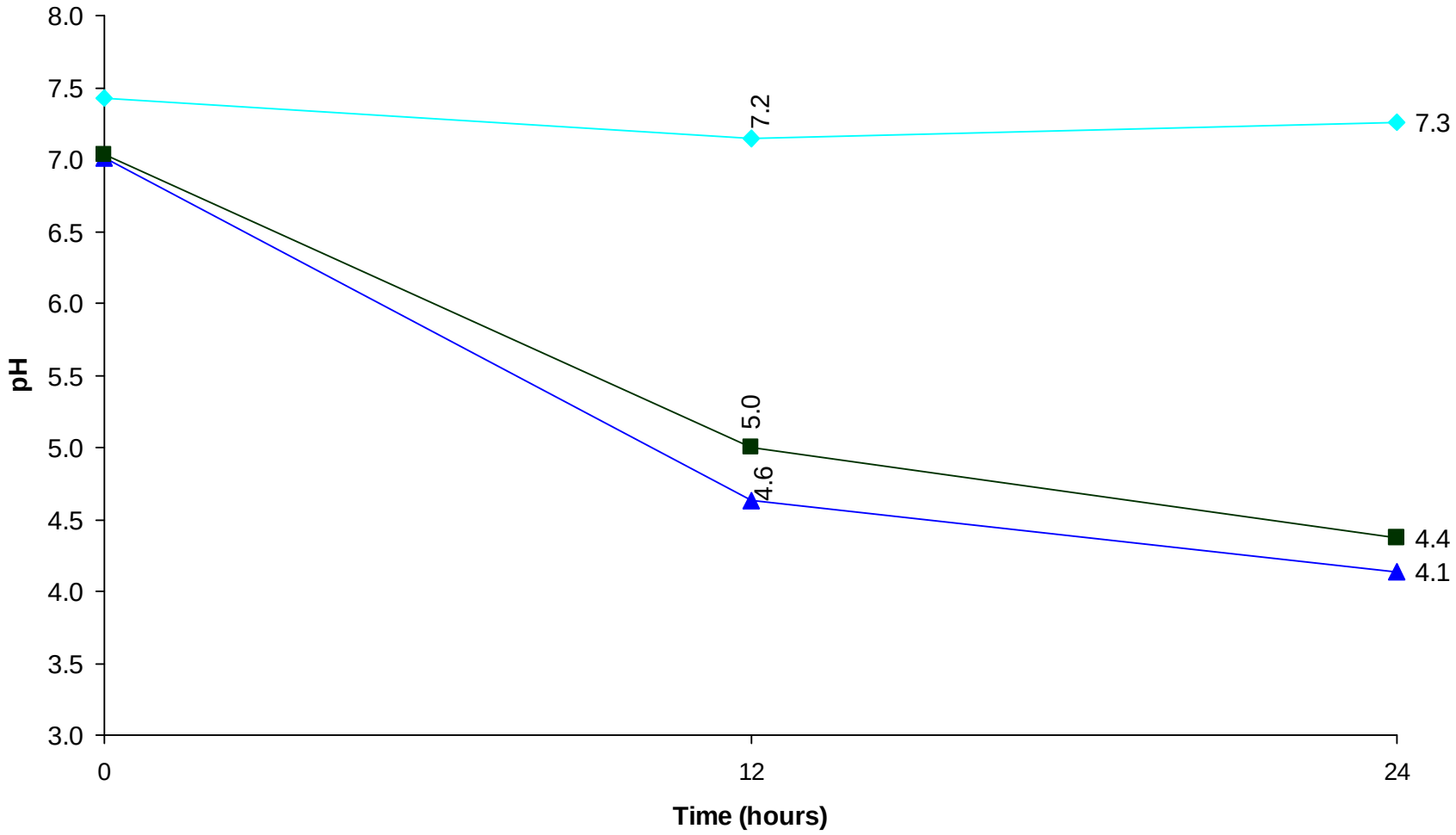

Figure 4.6. The effect of sugar supplementation on pH at 0, 12 and 24 hours. Neokestose mixture (▲); Inulin (■)and Control (♦) in PY basal media infused with chicken caecal contents with added ***S.*** Typhi, ***E. coli*** and ***C. jejuni in vitro***.

4.4.2.2 *Lactobacillus* count on LAMVAB

Lactobacillus increased almost 10 fold for both fructan treatments after the first 12 hours (Fig. 4.7), while further increasing for neokestose between 12 and 24 hours. These trends for the two fructan treatments and the control are similar to those found without pathogen addition (see section 4.4.1.2.2). The addition of ***C. jejuni***, ***Salmonella.*** Typhi and ***E. coli*** did not have a significant impact on ***Lactobacillus*** numbers for any of the treatments.

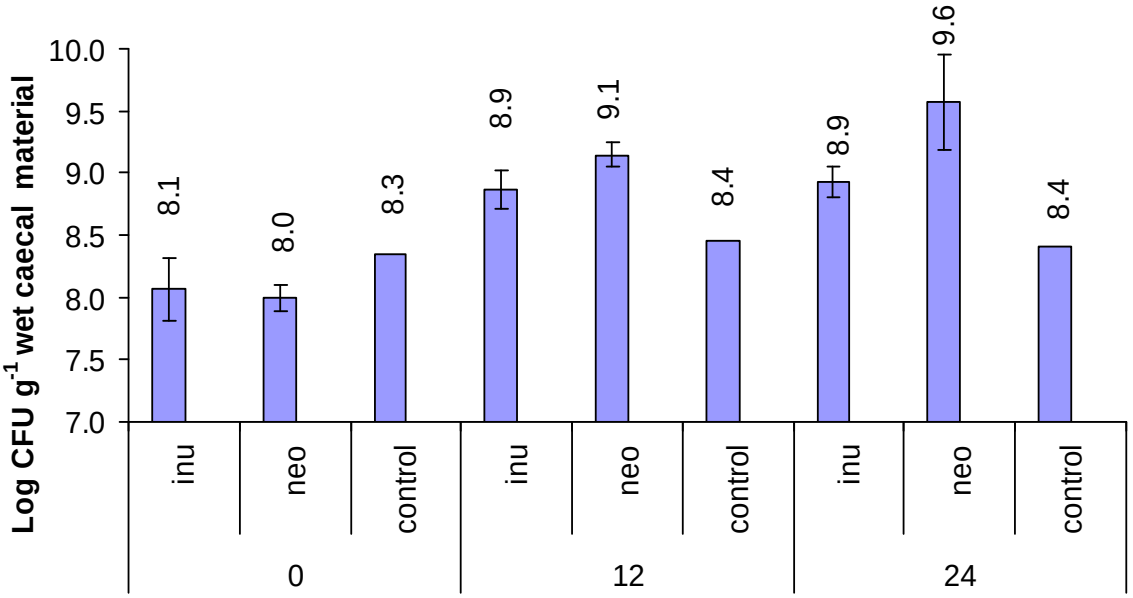

Figure 4.7. Effect of substrate and pathogen addition on the caecal ***Lactobacillus*** count ***in vitro*** over 24 hours. PY basal medium contained ***Salmonella*** Typhi, ***E. coli***, ***C. jejuni*** and either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

4.4.2.3 Coliform counts on MacConkey

Only the colonies displaying characteristic colony morphology for ***E. coli*** were counted. These were typically large red colonies, surrounded by turbid zones. More than 10 fold increases occured for all the supplementations over the first 12 hours (Fig. 4.8). The highest coliform levels were detected in the control at 12 and 24 hours. Numbers for the control were stable from 12 to 24 hours showing neither a significant increase nor decrease.

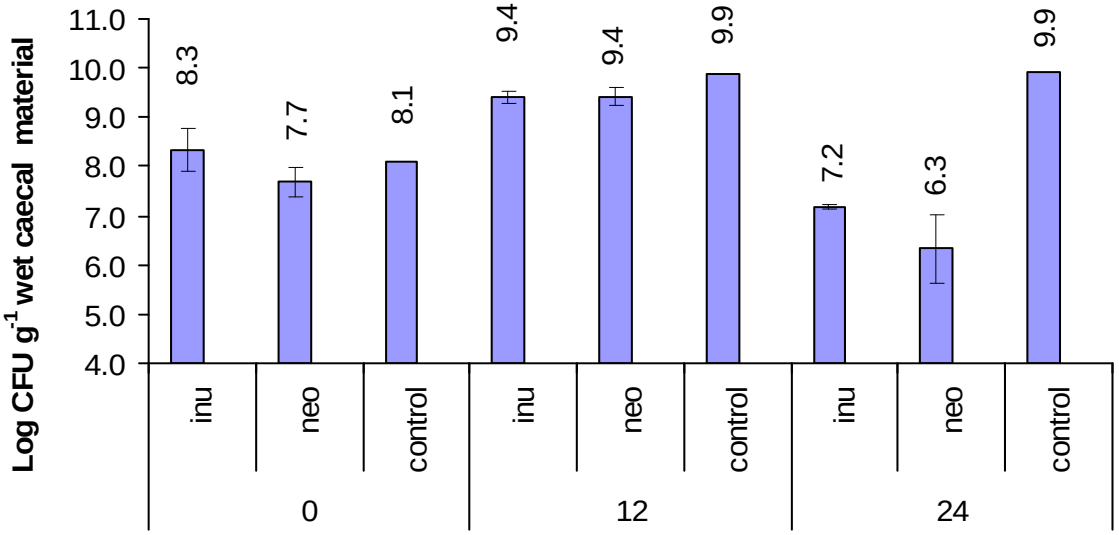

Figure 4.8. Effect of substrate and caecal addition on levels of challenged ***E. coli*** over 24 hours in ***in vitro***. PY basal medium contained ***Salmonella.*** Typhi, ***E. coli***, ***C. jejuni*** and either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

Both fructan supplementations showed similar coliform numbers after 12 hours, while after 24 hours there was a larger than 10 fold decrease for both fructan supplementations, compared to their initial numbers. In addition, the neokestose treatment had less coliform at 24 hours compared to both the inulin supplementation and control. No decreased coliform numbers were detected in the control during the 12 to 24 hour period as was detected in the control without pathogen addition (Fig. 4.4). This is probably related to the higher overall pH of 7.3 (Fig. 4.6) compared to 6.2 (Fig. 4.1) at 24 hours.

4.4.2.4 *Salmonella* counts on XLD

As with the MacConkey counts, here only the cfu's displaying characteristic ***Salmonella*** Typhi colony morphology on XLD agar were counted. These translucent colonies displaying black centres, characteristic for ***Salmonella*** Typhi, were not previously detected on XLD during any of the caecal experiments. Yellow colonies with clear precipitation zones were, indicative of ***Klebsiella***, not included in this result (Fig. 4.9).

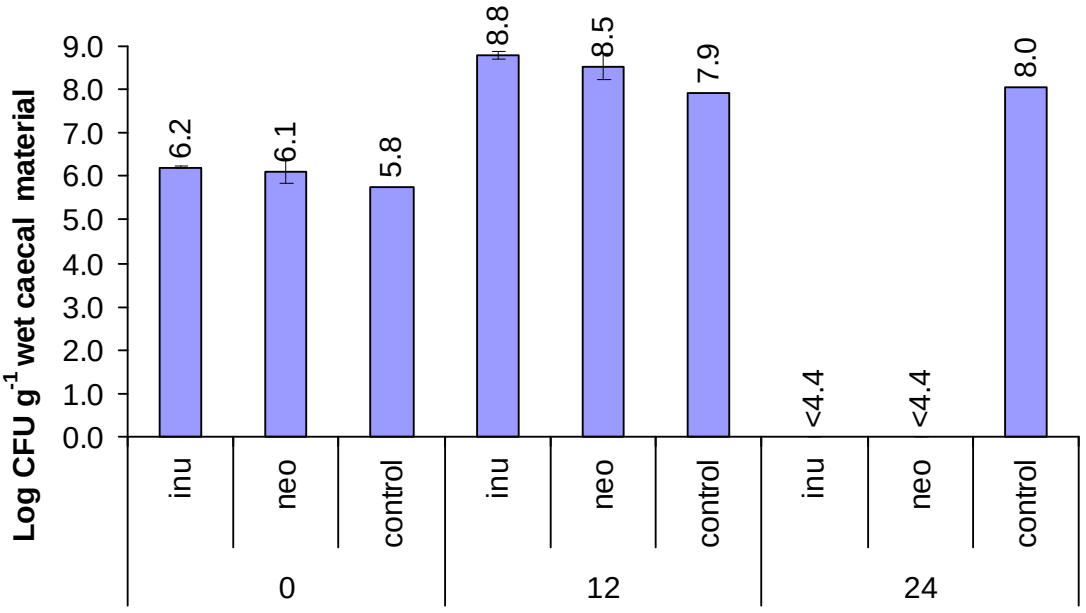

Figure 4.9. Effect of substrate and caecal addition on levels of ***Salmonella*** over 24 hours in ***in vitro***. PY basal medium contained ***Salmonella.*** Typhi, ***E. coli***, ***C. jejuni*** and either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control).

Overall there was an increase of more than 100 fold in the first 12 hours. The total ***Salmonella*** count at 12 hours for neokestose was slightly less when compared to that of inulin at the same time. At 24 hours ***Salmonella*** species were absent for both the fructan treatments, while levels for the control remained unchanged.

4.4.2.5 *Campylobacter* counts

The counts for ***Campylobacter jejuni*** were done on Campylobacter Selective Medium supplemented with Campylobacter Selective Supplement as described. Colonies that grew on this medium were considered being ***Campylobacter*** and counted as such. The results show that there was less than a 10 fold increase for in the first 12 hours (Fig. 4.10). After 24 hours no colonies were detected on any of the fructan supplemented media, while the numbers for the control remained unchanged.

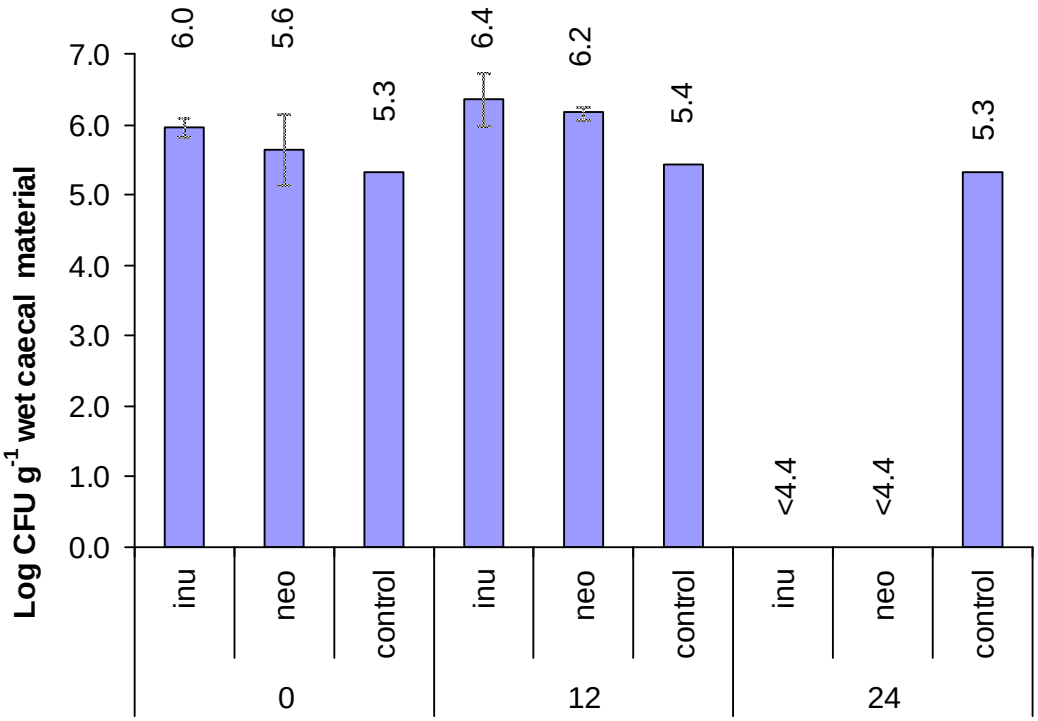

Figure 4.10. Effect of substrate and caecal addition on levels of ***Campylobacter jejuni*** over 24 hours in ***in vitro***. PY basal medium contained ***Salmonella*** Typhi, ***E. coli***, ***C. jejuni*** and either glucose (glc), neokestose mixture (neo), inulin (inu) or no carbohydrates (control)

4.4.3 Volatile fatty acid production

All samples were analysed for the volatile fatty acids acetic acid, butyric acid and propionic acid. We found that, with all the treatments as well as with the control, acetic acid increased from 0 to 24 hours (Fig. 4.11).

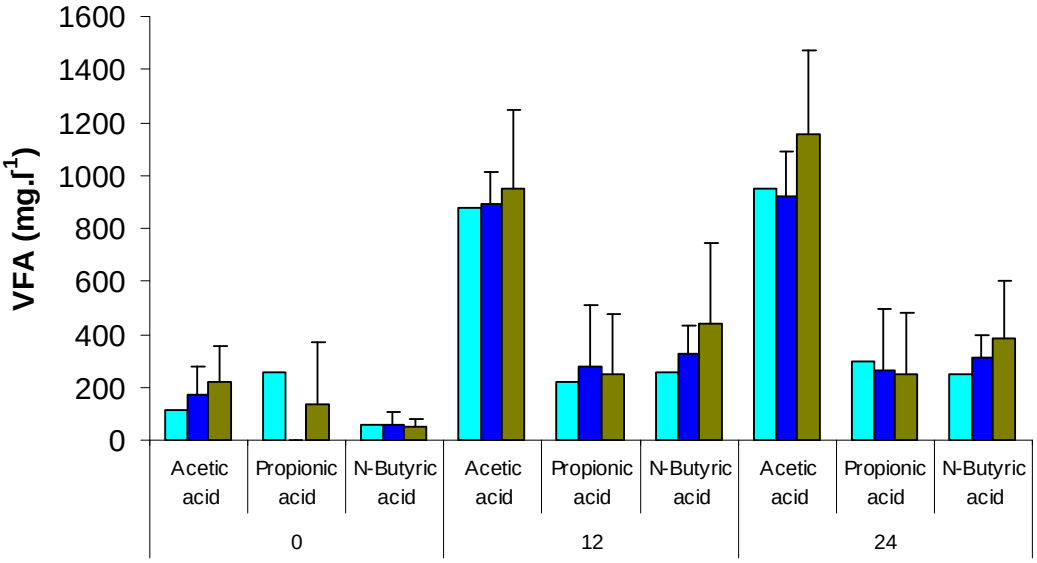

Figure 4.11. Volatile fatty acid (mg l^{-1}) levels at 0, 12 and 24 hours for inulin (■) and neokestose (■) compared to a control (■) which contained no added carbohydrate. These are averages of 3 results obtained for neokestose and inulin.

Butyric acid was detected in all samples of all treatments, but in lower concentrations than acetic acid. Propionic acid was not always detected, and when present, it was not specific to any particular treatment. Even the control samples, without any added carbohydrate, increased in both acetic and butyric acid, which suggests that acid production, was not associated with carbohydrate addition. Valeric acid (iso-valeric and n-valeric) was detected in all treatments, including the controls but was not quantified. The change in pH over the periods from 0-12 and 12-24 hours, ranged from 2.0 to 2.8 and

0.1 to 0.6 respectively for all the treatments. This would indicate that synthesis of SCFA was low during the last 12-24 hours.

4.5 Discussion

In these experiments the level of anaerobic bacteria in the caecum of broiler chickens was detected at average levels ranging from $1x10^{9.5}$ to $1x10^{9.7}$ CFU per gram wet caecal material. In literature these numbers are reported either as per gram wet weight (Jin ***et al***., 1998; Rubio ***et al***., 1998; Thitaram ***et al***., 2005a) or per gram dry weight (Xu ***et al***., 2003; Patterson ***et al***., 1997). In these cases the levels of anaerobic bacteria reported ranged from $1x10^{9.6}$ to $1x10^{10.4}$ CFU per gram wet caecal material and $1x10^{9.5}$ to $1x10^{10.6}$ CFU per gram dry caecal weight with higher numbers reported in younger birds (Jin ***et al***., 1998). The total anaerobic levels increased to between $1x10^{10.1}$ and $1x10^{10.5}$ CFU per gram wet weight for all the treatments including the control over 12 hours, whereas no significant increase was detected from 12 to 24 hours. No significant difference was detected between the anaerobic levels of the neokestose, glucose, inulin treatments and control at 0, 12 and 24 hours.

The ***Lactobacillus*** levels increased for all the carbohydrate supplementations in both the investigations over 24 hours, whereas the ***Lactobacillus*** levels remained unchanged for the control. The average ***Lactobacillus*** levels were higher for the neokestose treatment compared to the inulin treatment after 12 and 24 hours in both experiments. ***Lactobacillus*** levels increased by 14 to 16 fold and 7 to 11 fold for the neokestose and inulin treatments respectively. With both these experiments it was observed that ***Lactobacillus*** levels only increased in the first 12 hours for the inulin treatments, remaining unchanged through the next 12 hours. With neokestose the ***Lactobacillus*** level increased throughout the 24 hours incubation. This trend was similar to the results results found by Kilian ***et al***. (2002) for Raftilose 95 and neokestose.

The results for the coliforms showed that these bacteria are capable of utilizing components found in the rich PY-basal medium which contain

substantial amounts of potential carbon sources for growth. This is evident in the experiment without pathogen addition where both neokestose and inulin follow a similar trend to that of the control. In the experiment with pathogen addtion both inulin and neokestose resulted in the greater reduction of ***E. coli*** over 24 hours compared to both the initial levels as well as that of the control. This is in agreement with reductions of coliforms found when neokestose was used in ***in vitro*** experiments on human faecal material (Kilian ***et al***., 2002). The reduction of ***E. coli*** was also similarly seen for glucose. Glucose is, however, readily absorbed in the upper part of the gastro intestinal tract of the chicken (Riesenfeld ***et al***., 1980) and would not affect changes in the caecum. ***Klebsiella*** and ***Salmonella*** levels increased for al the treatments over 12 hours, which would suggest that they were able to utilize the supplemented carbohydrates, however, this increase was also seen in the control where no carbohydrate was added, which points to the utilization of media components for growth. After 24 hours all the treatments declined in ***Klebsiella*** as well as ***Salmonella*** levels compared to the control. No such effect was, however, detected with the ***Campylobacter jejuni*** levels which remained unchanged for the control over 24 hours. Both the inulin and neokestose treatments declined in ***C. jejuni*** levels in the period 12 to 24 hours.

No ***Bifidobacterium*** spp. were detected in the caeca of broilers, in contrast to layers. The reason for the absence of ***Bifidobacterium*** species in these broilers is not known. The occasional absence of ***Bifidobacterium*** spp. in broiler caeca was also reported by Lu ***et al***. (2003) and V. Rada (personal communication). ***Bifidobacterium*** numbers of $1x10^9$ CFU per gram wet caecal material, were found by Thitaram ***et al***. (2005a) and Patterson ***et al***. (1997) in 3-week old Ross and 4-week old Hubbard broiler breeds respectively, fed on a corn-soybean diet. The former study used a trans-oligosaccharide propionate agar (supplemented with acetic acid) TOS-A, for their ***Bifidobacterium*** enumeration, while the medium for the latter study was not disclosed. Mupirocin, not present in TOS-A, was shown to be necessary for the inhibition of related lactic acid bacteria by Thitaram ***et al.*** (2005b), and this could have lead to false high ***Bifidobacterium*** counts. In this study the probiotic effect of ***Bifidobacterium*** spp., on other intestinal species, effected by

neokestose was unfortunately not determinable. Apajalahti ***et*** al. (2004) found that corn, compared to wheat favoured, favoured low %G+C (20-34) microbes at the expense of the higher %G+C bacteria (65-69 % G+C). Even though the genus ***Bifidobacterium*** is found in a wide bracket (45-68), those members normally found in chickens, which include ***B. gallinarum*** (66), ***B. pullorum*** (67.5), ***B. thermophilum*** (60), ***B. pseudolongum*** subsp. ***globosum*** (59.5) and ***B. pseudolongum*** subsp. ***pseudolongum*** (64) fall into a higher %G+C bracket (Biavati & Mattarelli, 2006). In this study broilers were fed a corn-soy diet which could similarly have favoured low %G+C bacteria.

In this study we found that both acetic acid and butyric acid increased over 12 hours while propionic acid was found to be present variably. The volatile fatty acids formic, acetic, propionic and butyric acid are produced as end products by anaerobic intestinal microbial metabolism (Mead, 2000). It was found by Cummings (1981) that the production of these VFA were stimulated by adding fermentable prebiotics to feed. SCFA in their undissociated form were found to be bacteriostatic and/or bactericidal for gram-negative bacteria when tested ***in vitro*** (Thompson & Hinton, 1997). The proportion of undissociated SCFA was found to be increased with a reduction in caecal pH (Waldroup ***et al***., 1993). Van der Wielen ***et al***. (2000) proposed that, ***in vivo***, an increase in concentrations of acetate, propionate, and butyrate in the caeca, broiler chicken, effect the decrease in viable counts of members of the family ***Enterobacteriaceae***. In this study it was shown that caecal supplemented PY-basal medium decreased in pH when readily fermentable carbohydrates were added. Even though VFA's were also present in untreated controls, the pH did not go below 6.3. Similar to our findings, Freter & Abrams (1972) found the bactericidal and/or bacteriostatic effect to be minimal on ***Enterobacteriaceae***, when the pH was between 6.5 and 7.0. Siavoshian ***et al***. (1997), showed that butyrate, valerate and propionate increase cell differentiation and decrease cell proliferation in human colonic epithelial cell lines. This is similar to the effect obtained when growth-promoting antibiotics were administered to chickens (Bedford, 2000). The production of lactic acid could have been responsible for the higher reduction in pH seen with the

carbohydrate supplemented test. Concentrations of lactic acid were, however, not determined.

It has been proposed that the purported beneficial properties of ***Bifidobacterium*** spp. result from the fact that they produce vitamins (mainly of the B type), are effective stimulators of the immune response and can be used to restore the intestinal microbiota after antibiotic therapy (Hidaka ***et al***, 1986). They inhibit the growth of ***E. coli*** and ***C. perfringens*** either by the secretion of an inhibitory substance or by producing lactic acid, thereby lowering the pH (Gibson & Wang, 1994, Hartemink ***et al***., 1997, O'Riordan & Fitzgerald, 1998). The lower pH creates an environment which is undesirable for the growth of ***E. coli*** and ***C. perfringens***, resulting in the inhibition of the growth of these organisms (Gibson & Wang, 1994; Gibson ***et al.***, 1995, Tamime ***et al.***, 1995, Fujiwara ***et al.***, 1997). Our results similarly show the inhibition of ***E. coli***, ***C. jejuni***, ***Salmonella*** Typhi and ***Klebsiella*** with a reduction in pH below 6. These results show that neokestose compared to inulin was utilized better by lactic acid bacteria, seen by the lower pH and the higher level of ***Lactobacillus*** cultured at 12 and 24 hours. Considering experimental results which showed the physiological effect of smaller fructans to be more distinctive (Menne ***et al***., 2000; Kleesen ***et al***., 2001), neokestose promises to give improved results to that of inulin seen in poultry trials. The ***in vivo*** effect of neokestose on poultry performance has, however, to be evaluated since growth promotion apart from health is important for that industry.

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CHAPTER 5

GENERAL DISCUSSION AND CONCLUSION

Poultry is one of the major meat protein sources produced for the consumer market. The highly competitive nature and small profit margins of the market have led to extremely intensive rearing practices found in this industry. With such enormous numbers of animals cooped up in small spaces, it is important for pre-emptive approaches when dealing with various types of diseases. The use of in-feed antibiotics for chickens is one such approach which has been used regularly but has become unpopular, leaving a gap for alternatives. The inulin neoseries trisaccharide neokestose, is a prebiotic (Kilian ***et al***., 2002) which can possibly be used as an alternative.

The yeast ***Xanthophyllomyces dendrorhous*** was grown in a yeast-extract medium to the late exponential growth phase, from this phase whole cells were harvested. These production cells, suspended in citrate-phosphate buffer (pH 7) and incubated at 25 ^{o}C with sucrose as the sole carbon substrate, produced high concentrations of neokestose extracellularly (~51 % of the total sugars). This was similar to what Kritzinger ***et al***. (2003) found and the amount of neokestose compared well with the 55-60 % (w/w) found in literature for the commercial production of other fructo-oligosaccharides (Hang ***et al***., 1995; Yun, 1996). Large scale commercial production of neokestose should be evaluated with cheap unrefined sucrose containing syrup obtained from sugarbeet or sugarcane.

Carbon:celite chromatography is a method that is generally used for the separation of FOS (Yun, 1996). Both a column chromatography and a batch filtration method enhanced neokestose to 82 % or 90 % (excluding GF_3) when the same water elution volumes were used. This compared well with the 87.5 % obtained by Kritzinger ***et al.*** (2003). The batch filtration method is quick when compared to the column chromatography method, with the former being a lot more labour intensive. The purification of neokestose by carbon:celite chromatography is a crude method for purifying neutrally charged carbohydrates. It was, however, possible to significantly enrich the product for neokestose, which made up 82.6 % of the final product. This final product consisted of neokestose

(82.6 %), a GF_3 component (7.3 %), sucrose (8.7 %), glucose (1.2 %) and fructose (0.2 %). The major contaminating sugars were sucrose and GF_3. Using this product in chicken can have interesting results since sucrose, glucose and fructose can be metabolized in the crop. A further advantage is that the main bacterial group in the crop are the beneficial ***Lactobacillus*** spp. producing lactic- and acetic acids. Furthermore it is very likely that fructans, with a higher degree of polimerisation, which are co-synthesised during neokestose production, would have similar prebiotic properties. The necessity of separating GF_3 from neokestose when used prebiotically would therefore not be required. It is unlikely that these could be separated with activated caron:celite since it was shown that GF_3 absorbed more strongly onto activated charcoal, when eluted with water during carbon:celite chromatography, while both were removed with an ethanol elution step. The best enhancement of neokestose would then rather be achieved by using a supernatant which contained a high ratio of neokestose:GF_3. Recently it had been shown that high-content FOS can be obtained not only by purifying the reaction mixture but also by the in-process removal of liberated glucose and the hydrolysis of the unutilized sucrose (Sangeetha ***et al***., 2005). For high content neokestose production, ***X. dendrorhous*** has thus far showed to be the best candidate when compared to other fungi like ***Penicillium citrinum*** which produces a mixture made up of 1-kestose (22 %), nystose (14 %) and neokestose (11 %) (Hayashi ***et al***., 2000).

The prebiotic effect of this neokestose mixture was evaluated on the total anaerobic bacteria, ***Lactobacillus***, ***Bifidobacterium*** and coliform caecal microbial population of 5 week old broiler chickens ***in vitro***. This was compared to the effect of a commercial inulin product and glucose, whereas the additional effect of adding pathogenic ***E. coli***, ***Salmonella*** Typhi and ***C. jejuni*** strains was also evaluated on the beneficial component found in the caecum of broilers. Since it is known that succession occurs in GI-tract, with the caecum developing the most complex microbiota of the GI-tract in the maturing broiler chicken (Barnes ***et al***., 1972; Lu ***et al***., 2003) it would be interesting to see how feeding neokestose from

an early age effects the development of the GI-tract and how it would stand up against challenges of pathogenic ***E. coli***, ***Salmonella*** Typhi and ***Campylobacter jejuni***.

In these experiments the level of anaerobic bacteria in the caecum of broiler chickens was detected at average levels ranging from $1x10^{9.5}$ to $1x10^{9.7}$ CFU per gram wet caecal material. In literature these numbers are reported either as per gram wet weight (Jin ***et al***., 1998; Rubio ***et al***., 1998; Thitaram ***et al***., 2005a) or per gram dry weight (Xu ***et al***., 2003; Patterson ***et al***., 1997). In those cases the levels of anaerobic bacteria have been reported from $1x10^{9.6}$ to $1x10^{10.4}$ CFU per gram wet caecal material and $1x10^{9.5}$ to $1x10^{10.6}$ CFU per gram dry caecal weight with higher numbers reported in younger birds (Jin ***et al***., 1998). The total anaerobic level increased to between $1x10^{10.1}$ and $1x10^{10.5}$ CFU per gram wet weight for all the treatments including the control over 12 hours, whereas no significant increase was detected from 12 to 24 hours. No significant difference was detected between the anaerobic levels of the neokestose, glucose, inulin treatments and control at 0, 12 and 24 hours.

Both ***Lactobacillus*** and ***Bifidobacterium*** species, considered to be beneficial were found present in the caecum of 17 week old New Hampshire layers at levels of $1x10^{8.3}$ and $1x10^{6.7}$ CFU per gram wet caecal material, respectively. Day old broiler chickens were reared on commercial corn-soy diet for 5 weeks. In these 5 week old broilers levels of ***Lactobacillus*** ranged from $1x10^{7.9}$ to $1x10^{9.3}$ CFU per gram wet caecal material, while ***Bifidobacterium*** spp. were absent. The reason for the absence of ***Bifidobacterium*** species in these broilers is not known. The occasional absence of ***Bifidobacterium*** spp. in broiler caeca was also reported by Lu ***et al***. (2003) and V. Rada (personal communication). In some studies ***Bifidobacterium*** numbers of $1x10^{9}$ CFU per gram wet caecal material in 3-week old Ross and 4-week old Hubbard broiler breeds fed on a corn-soybean diet were found (Thitaram ***et al***., 2005a; Patterson ***et al***., 1997). One used a trans-oligosaccharide propionate agar supplemented with acetic acid (TOS-A), for its

Bifidobacterium enumeration, whereas other studies have used rich media with limited or no selective supplements. Mupirocin not present in TOS-A, was shown to be necessary for the inhibition of related lactic acid bacteria by Thitaram ***et al.*** (2005b). Another reason could have been that the ***Bifidobacterium*** spp. present required resuscitation, as was done by both Rada ***et al***. (1999) and Muñoa and Pares (1988) who used rich non-selective medium for the dilution of their samples. In this study the probiotic effect of ***Bifidobacterium*** spp., on other intestinal species, effected by neokestose was unfortunately not determinable. Apajalahti ***et al***. (2004) found that corn, compared to wheat favoured, favoured low %G+C (20-34) microbes at the expense of the higher %G+C bacteria (65-69). Even though the genus ***Bifidobacterium*** is found in a wide bracket (45-68), those members normally found in chickens which include ***B. gallinarum*** (66), ***B. pullorum*** (67.5), ***B. thermophilum*** (60), ***B. pseudolongum*** subsp. ***globosum*** (59.5) and ***B. pseudolongum*** subsp. ***pseudolongum*** (64) fall into a higher %G+C bracket (Biavati & Mattarelli, 2006). In this study broilers were fed a corn-soy diet which could similarly have favoured low %G+C bacteria.

The ***Lactobacillus*** levels increased for all the carbohydrate supplementations in both the investigations over 24 hours, whereas the ***Lactobacillus*** levels remained unchanged for the control. The average ***Lactobacillus*** levels were higher for the neokestose treatment compared to the inulin treatment after 12 and 24 hours in both experiments. ***Lactobacillus*** levels increased by 14 to 16 fold and 7 to 11 fold for the neokestose and inulin treatments respectively. With both these experiments it was observed that ***Lactobacillus*** levels only increased in the first 12 hours for the inulin treatments, whereas remaining unchanged through the next 12 hours. With neokestose the ***Lactobacillus*** level increased throughout the 24 hours incubation. This trend on ***Lactobacillus*** was similarly seen for Raftilose 95 and neokestose by Kilian ***et al***. (2002).

The results for the coliforms showed that these bacteria are capable of utilizing components found in the rich PY-basal medium which contain substantial

amounts of potential carbon sources for growth. This was evident in the experiment without pathogen addition where both neokestose and inulin followed a similar trend to that of the control. In the experiment with pathogen addtion both inulin and neokestose resulted in the greater reduction of ***E. coli*** over 24 hours compared to both the initial levels as well as that of the control. This is in agreement with reductions of coliforms found when neokestose was used in ***in vitro*** experiments on human faecal material (Kilian ***et al***., 2002). The reduction of ***E. coli*** was also similarly seen for glucose. Glucose is, however, readily absorbed in the upper part of the gastro intestinal tract of the chicken (Riesenfeld ***et al***., 1980) and would not affect changes in the caecum. ***Klebsiella*** and ***Salmonella*** levels increased for al the treatments over 12 hours, which would suggest that they were able to utilize the supplemented carbohydrates, however, this increase was also seen in the control where no carbohydrate was added, which points to the utilization of media components for growth. After 24 hours all the treatments declined in ***Klebsiella*** as well as ***Salmonella*** levels compared to the control. This effect was, however, not detected with ***Campylobacter jejuni*** levels which remained unchanged for the control over 24 hours. Both the inulin and neokestose treatments declined in ***C. jejuni*** levels in the period 12 to 24 hours.

The volatile fatty acids formic, acetic, propionic and butyric acid are produced as end products by anaerobic intestinal microbial metabolism (Mead, 2000). It was found by Cummings (1981) that the production of these VFA were stimulated by adding fermentable prebiotics to feed. In this study we found that both acetic acid and butyric acid increased over 12 hours while propionic acid was found to be present variably. SCFA were found to be bacteriostatic and/or bactericidal for gram-negative bacteria, in their undissociated form, when tested ***in vitro*** (Thompson & Hinton, 1997). The proportion of undissociated SCFA was found to be increased with a reduction in caecal pH (Waldroup ***et al***., 1993). Van der Wielen ***et al***. (2000) proposed that, ***in vivo***, an increase in concentrations of acetate, propionate, and butyrate in the caeca, broiler chicken, effect the

decrease in viable counts of members of the family ***Enterobacteriaceae***. In this study it was shown that caecal supplemented PY- basal medium decreased in pH when readily fermentable carbohydrates were added. Even though VFA's were also present in untreated controls the pH did not go below 6.3. Similar to our findings, Freter & Abrams (1972) found the bactericidal and/or bacteriostatic effect to be minimal on ***Enterobacteriaceae***, when the pH was between 6.5 and 7.0. Siavoshian ***et al***. (1997), showed that butyrate, valerate and propionate increase cell differentiation and decrease cell proliferation in human colonic epithelial cell lines. This is similar to the effect obtained when growth-promoting antibiotics were administered to chickens (Bedford, 2000).

The proposed beneficial properties of ***Bifidobacterium*** spp. result from the fact that they produce vitamins (mainly of the B type), are effective stimulators of the immune response and can be used to restore the intestinal microbiota after antibiotic therapy (Hidaka ***et al.***, 1986). They inhibit the growth of ***E. coli*** and ***C. perfringens*** either by the secretion of an inhibitory substance or by producing lactic acid, thereby lowering the pH (Gibson & Wang, 1994, Hartemink ***et al***., 1997, O'Riordan & Fitzgerald, 1998). The lower pH creates an environment which is undesirable for the growth of ***E. coli*** and ***C. perfringens***, resulting in the inhibition of the growth of these organisms (Gibson & Wang, 1994; Gibson ***et al.***, 1995, Tamime ***et al.***, 1995, Fujiwara ***et al.***, 1997). Our results similarly show the inhibition of ***E. coli*** and ***C. perfringens*** with a reduction in pH below 6. These results show that neokestose compared to inulin was utilized better by lactic acid bacteria, seen by the lower pH and the higher level of ***Lactobacillus*** cultured at 12 and 24 hours. Considering experimental results which showed the physiological effect of smaller fructans to be more distinctive (Menne ***et al***., 2000; Kleesen ***et al***., 2001), neokestose promises to give improved results which were seen with inulin in poultry trials. The ***in vivo*** effect of neokestose on poultry performance has however, to be evaluated since growth promotion apart from health is important for that industry.

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CHAPTER 6

SUMMARY

The inulin neoseries, trisaccharide, neokestose was produced by the yeast ***Xanthophyllomyces dendrorhous*** (***Phaffia rhodozyma*** Y4-3) during growth on sucrose. To produce neokestose, whole cells harvested from the late exponential growth phase were incubated for 36 to 40 h at 25 ^{o}C in 0.2 M citrate-phosphate buffer (pH 7) containing 220 g.l^{-1} sucrose. Neokestose made up about 50 % of this mixture, which was purified equally well by both a carbon:celite chromatography as well as a batch filtration process, when eluting with similar amounts of water followed by a 50 % ethanol elution step. A final product was combined from various purification runs which consisted of 82.6 % neokestose, 8.7 % sucrose, 7.6 % GF_3, 1.2 % glucose and 0.1 % fructose.

Lactobacillus and ***Bifidobacterium*** genera are considered part of the beneficial group in in the intestine of animal and man. ***Bifidobacterium*** levels were higher than ***Lactobacillus*** levels in the caeca of New Hampshire layers, whereas in this study only ***Lactobacillus*** species were found in broilers. The reason for the absence of the ***Bifidobacterium*** species in the caecum of broilers was not determined. The prebiotic effect was evaluated on 5 week old broiler caecal material ***in vitro*** over 24 hours based on the viable levels of the total anaerobic bacteria, ***Lactobacillus*** and coliforms. The prebiotic effect was also evaluated on viable levels of added ***Salmonella*** Typhi, ***Escherichia coli*** and ***Campylobacter jejuni***. Volatile fatty acids and pH were measured. The effect of neokestose on these groups was compared to that of inulin, a known prebiotic, and glucose. The total anaerobe and ***Lactobacillus*** levels increased over 24 hours for neokestose, inulin and glucose. Although there was no significant difference between the treatments higher levels were found for neokestose and glucose than for inulin. A decrease in the viable levels of ***E. coli***, ***S.*** Typhi and ***C. jejuni*** were seen over 24 hours. The production of acetic acid, butyric acid and propionic acid was not significantly different for the treatments and the control. The pH decrease over 24 hours for the treatments was significantly different from the control, which indicated that lactate (not measured) production was probably higher in the neokestose, inulin and glucose treatments. ***In vivo*** tests are,

however, required to fully evaluate the prebiotic and “bifidogenic” effect of neokestose for broilers.

CHAPTER 7

OPSOMMING

Die trisakkaried neokestose, wat deel uitmaak van die inulien neo-reeks, word geproduseer deur die gis ***Xanthophyllomyces dendrorhous*** (***Phaffia rhodozyma*** Y4-3) gedurende groei op sukrose. Selle wat verkry is vanaf die laat eksponensiële groeifase is gebruik vir die produksie van neokestose. Die selle is geïnkubeer by 25 ^{o}C vir ‘n periode van 36 tot 40 uur in ‘n 0.2 M sitraat-fosfaat buffer (pH 7) met 220 g.l^{-1} sukrose. Neokestose het ongeveer 50 % van hierdie mengsel beslaan. Suiwering met geaktiveerde koolstof:celite is ewe goed gedoen deur ‘n kromatografiese asook ‘n “batch” filtrasie proses, mits dieselfde volumes water en 50 % etanol aangewend wod gedurende die elluëringsstappe. Die finale produk wat ‘n samevoeging van verskeie suiwerings was het uit 82.6 % neokestose, 8.7 % sukrose, 7.6 % GF_3, 1.2 % glukose en 0.1 % fruktose bestaan.

Lactobacillus en ***Bifidobacterium*** genera word gesien as deel van die voordelige groep bakterieë wat aangetref word in die ingewandskanaal van mense en diere. ***Bifidobacterium*** vlakke was hoër as die ***Lactobacillus*** vlakke in die sekums van New Hampshire lê-henne, in vergelyking met braaikuikens waar daar slegs ***Lactobacillus*** spesies gevind is. `n Rede vir die afwesigheid van ***Bifidobacterium*** spesies in die sekum van braaikuikens kon nie vasgestel word nie. Die prebiotiese effek is geëvalueer op 5 weke oue braaikuikensekummateriaal ***in vitro*** oor ‘n tydperk van 24 uur en was gebasseer op die lewensvatbare vlakke van die totale anaërobe bakterië, ***Lactobacillus*** en koliforme. Die prebiotiese effek is ook addisioneel geëvalueer in die teenwoordigheid van lewensvatbare

Salmonella Typhi, ***Escherichia coli*** en ***Campylobacter jejuni*** wat toegevoeg is tot die sekummateriaal. Vir addisionelle evaluasie is vlugtige vetsure en pH ook gemeet. Die effek van neokestose op hierdie groepe is vergelyk met die van inulien, ‘n bekende prebiotikum, en glukose. Die totale anaërobiese en ***Lactobacillus*** vlakke het toegeneem oor 24 uur vir neokestose, inulien en glukose. Alhoewel daar geen beduidende verskille tussen die behandelings waargeneem is nie, was die vlakke vir beide neokestose en glukose hoër as vir inulien en die kontrole. ‘n Vermindering in die lewensvatbare vlakke van ***E. coli,*** ***Salmonella*** Typhi en ***C. jejuni*** is ook waargeneem oor 24 uur. Die produksie van asynsuur, bottersuur en propioonsuur het nie ‘n beduidende verskil getoon tussen die behandelings en die kontrole nie. Daar was wel `n beduidende verlaging in pH oor 24 uur teenoor die kontrole, wat daarop dui dat laktaat produksie (nie gemeet nie) waarskynlik hoër was in die neokestose, inulien en glucose behandelings. ***In vivo*** toetse is egter nodig om die prebiotiese en “bifidogeniese” effek ten volle te evalueer vir neokestose in braaikuikens.