To the Graduate Council :

I am submitting herewith a dissertation written by Patsy Ann Francis entitled "Gastrointestinal Fluxes as a Function of Enzymatic Treatment of Non-Starch Polysaccharides in Rye-based diets and its Effects on Selected Microflora and Broiler Performance." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Animal Science.

K. R. Robbins, Major Professor

We have read this dissertation and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

Gastrointestinal Fluxes as a Function of Enzymatic Treatment of Non-Starch Polysaccharides in Rye-based diets and its Effects on Selected Microflora and Broiler Performance

A Dissertation
Presented for the
Doctor of Philosophy
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Patsy Ann Francis
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DEDICATION

This dissertation is dedicated to my mother

Mrs. Doreen Mc Rae

who died on March, 02, 1998

She made every sacrifice in order that I receive education

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ABSTRACT

Three experiments were conducted utilizing male broilers to determine the effect of Avizyme® on non starch polysaccharides in rye-based diets. Avizyme® which contains 2500 units of xylanase and 800 units of protease/g was added to the experimental diets at the rate of 0, 0.05 and 0.1%. Feed efficiency, weight gain and water consumption were measured as indices of performance, whereas lactobacilli, total pathogenic clostridia and *Salmonella typhimurium* populations assessed gastrointestinal health and potential food safety hazards. Microflora were determined in the crop, jejunum, ileum and cecum. Volatile fatty acids (VFA) determinations in the distal ileum assessed fermentation activity. Interactions of VFA with microflora, gut mucosa and performance were assessed. DNA, RNA and protein of mucosal tissue were determined as indices of gut morphology and cellular activity.

The addition of Avizyme® to rye-based diets consistently improved feed efficiency and weight gain which was equal to that observed in corn-based diets ($P < 0.05$). Water : feed consumption ratio declined significantly with the addition of the enzyme to the rye-based diets ($P < 0.05$). Performance was unaffected by the carrier state in birds challenged with *Salmonella typhimurium*.. Acetate concentration in rye-based diets

supplemented with Avizyme was 5 fold higher than that observed in the corn-based diets ($P < 0.03$). That increase was positively correlated ($r = .66$) with increased mucosal mass in the rye-based diets. Mucosal protein:DNA and RNA:DNA ratios were significantly higher during week one in birds fed rye-based diets ($P < 0.01$).

Lactobacilli populations were relatively stable throughout the experimental period irrespective of treatment. Clostridia declined with the addition of Avizyme in birds not challenged with *Salmonella typhimurium*. When birds were challenged, clostridia populations remained stable and acetate levels were low. *Salmonella typhimurium* tended to increase with the addition of Avizyme and persisted longer and at a higher level in rye based diets supplemented with the enzyme ($P < 0.05$). *Clostridium spp.* were higher in the cecum regardless of grain type but *Salmonella typhimurium* was recovered primarily in the cecum and crop.

(Key words: Fermentation, mucosa, DNA, *Salmonella,* clostridia, broilers.

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PART 1

INTRODUCTION

General Background

Cereal grains and legumes generally comprise about 60% and 20% respectively, of poultry rations. They account for about 70-75% and 20%, respectively of the total caloric intake. Grains commonly used in these diets include corn, rye, wheat, barley and rice. The complex physiochemical properties of the non-starch polysaccharides (NSPs) in several of these grains are thought to be the main negative determinant of nutritional value. Avian or mammalian enzymes are capable of cleaving the glycosidic bonds α-1,2 in sucrose, β-1,4 in lactose, α-1,4 and 1,6 in starch or glycogen as well as α-1,1 in trehalose. All other glycosidic bonds are resistant to digestive enzymes but may be cleaved by microbial enzymes. Additionally, there is evidence that the soluble structural polysaccharides contained primarily in the endosperm and inner aleurone cell wall of some grains contribute to the increased viscosity of the gastrointestinal tract (Almirall et al.,1995; Annison, 1991; Bedford, 1995; Friesan et al., 1992 and Wagner and Thomas, 1978). The increased viscosity is thought to create an unfavorable gastrointestinal environment. However, the net effect of this complex interaction of NSPs with other components within the gastrointestinal environment is dependent on the variability in quantity and

type of these polymers. The variability within the cereal may also be influenced by variety and environmental growing conditions (Aastrup, 1979; Campbell et al., 1989; Willingham et al., 1960).

The principal NSPs are β-glucans in grains such as sorghum, barley and oats, while arabinoxylans are the major NSPs in wheat and rye. Generally, β-D-glucans are comprised of glucose units bonded by β-1,3 and β-1,4 linkages, and arabinoxylans have a β-1,4 xylose backbone with arabinose side chains. Arabinoxylan complexes also contain D-glucoronic acid or its 4-O methyl ether, acetic, coumaric and ferulic acids. The structure of the major NSPs in barley, rye, and wheat are detailed in **Fig 1.** Polysaccharide structures frequently found in feed ingredients of plant origin are detailed in **Fig 2.**

The behavior of NSPs in the aqueous gastrointestinal environment will be influenced by their association with other cell wall components since they exist closely bound to other polysaccharides and non-carbohydrate materials. These structures result in polymers of varying solubilities which impact the rheological properties of the gut environment. It has been postulated that changes in the rheological properties of the gut contents affect lipid, protein and starch digestion and absorption, fecal moisture as

well as gut microflora (Bedford, 1995; Choct et al. 1996). The interaction of several of these factors may result in reduced feed efficiency and growth rate, problems with litter management, debilitating diseases to the animal and possibly result in zoonoses.

It has been reported that the physiochemical properties of NSPs are responsible for the depressed digestibility of protein, starch and fat in broilers (Choct and Annison ,1990; Fengler and Marquardt, 1988). In their review Smits and Annison (1996) reported that several researchers had demonstrated that NSPs present in cereal grains increased viscosity in the gastrointestinal tract. This has been positively correlated with decreased feed efficiency, development of necrotic enteritis, undesirable microflora and increased fermentation acids in the gastrointestinal tract. The supplementation of those diets with penicillin and terramycin improved feed conversion and growth rate and altered volatile fatty acid production in broilers. The mode of action of these antibiotics appeared to be associated with the suppression of some gram positive organisms which ferment soluble NSPs. Exogenous enzymes have also been demonstrated to alleviate the problems associated with feeding diets high in NSPs, but their mode of action has been primarily associated with partial depolymerization

of NSPs thereby reducing intestinal viscosity. Whether or not decreased viscosity results in decreased mean retention time and consequently increased feed intake has not been clearly established. It is clear that chickens are able to adapt to some increases in gut viscosity since their response to partially depolymerized NSPs is equal to that observed when fed corn-soy based diets. Therefore, some of the antinutritive effects of NSPs may be attributed to stearic interferences, however microfloral interactions and immunologic factors may also be contributing factors.

The changes occurring in the intestinal environment are complex and may present opportunities for noncommensal organisms to become established. The depolymerization process makes available more L-arabinose, D-xylose, phenolic, acetic and other acids which may serve as substrates for fermentation and thereby improve the metabolizable energy of the diet. However, there are several pathogenic organisms capable of utilizing L-arabinose and D-xylose and other compounds as their sole source of carbon a nd they may proliferate when presented with more favorable conditions.

Research Objectives

The focus of this dissertation was to examine the interaction of NSPs in rye

and corn -soybean based diets and an exogenous depolymerizing enzyme complex, Avizyme® on various gastrointestinal parameters in order to gain some insight into factors associated with improved bird performance as well as changes occurring in the populations of total lactobacilli, a nali-dixic acid resistant strain of *Salmonella typhimurium* and total pathogenic clostridia. Three experiments were conducted. The focus of Experiment **1** was the effect of a commercial enzyme, Avizyme® in rye and corn-soybean based diets on performance, water consumption, fermentation in the ileum, changes in gastrointestinal mucosa and microflora. Experiment **2** focused on the susceptibility of broilers challenged with a nalidixic acid resistant strain of *Salmonella typhimurium* under conditions described in Experiment **1**. Its aim was to determine shedding patterns and persistence of that organism. Experiment **3** examined the effect of a challenge with a nalidixic acid resistant strain of *Salmonella typhimurium* under field conditions to determine the shedding pattern and the effect of fasting on the recovery of that organism after slaughter in the crop, ileum and cecum.

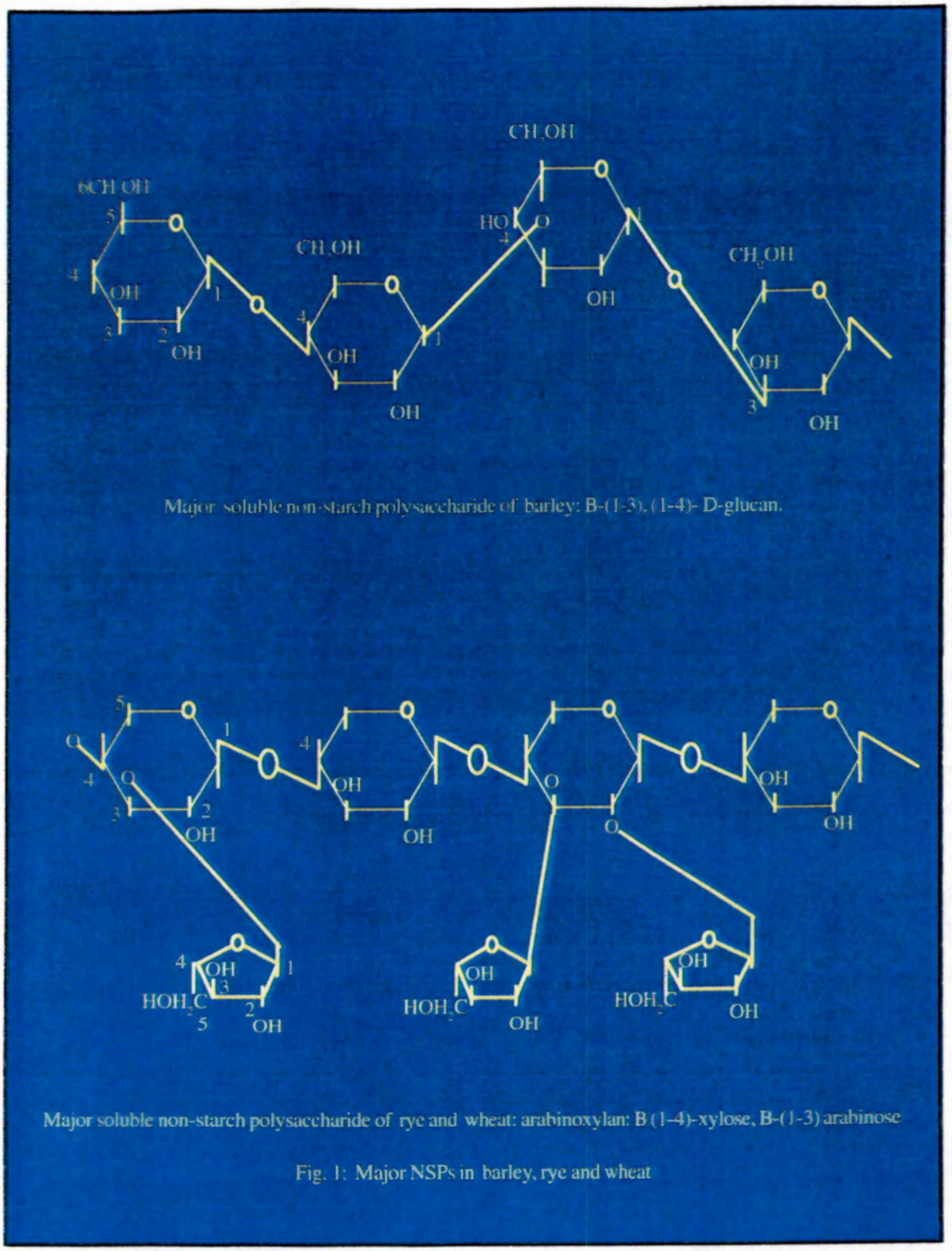
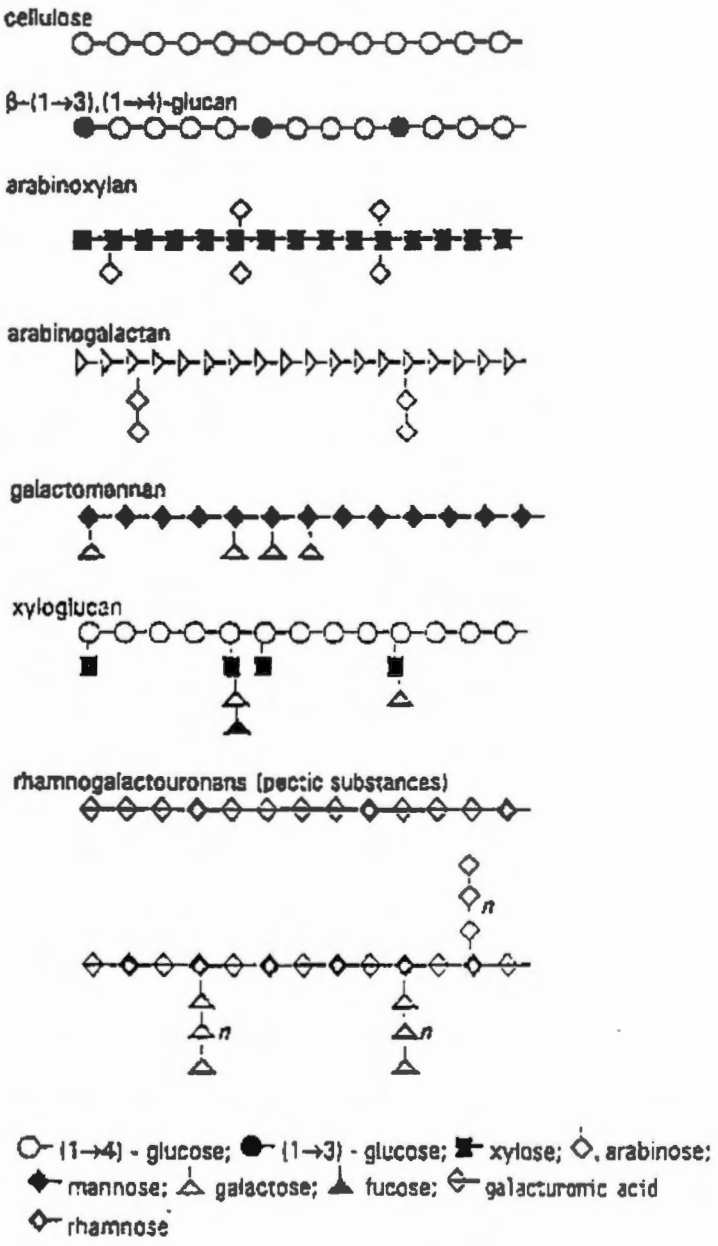


Fig. 1: Major NSPs in barley, rye and wheat



*Bedford, 1993. J. Appl. Poultry Res. 2: 85-92

Fig. 2: Polysacharides commonly found in feeds of plant origin*

PART 2
LITERATURE REVIEW

Utilization of feeds with low levels of NSPs

The intestinal system of the hatching chicken is anatomically complete (Chambers and Grey, 1979; Overton and Shoup, 1964), but the absorptive surface and rate of enterocyte proliferation increases after hatching (Cook and Bird, 1973; Moran, 1985). This is reported to be primarily due to cellular hyperplasia (number of enterocytes per villus) and not cellular hypertrophy (increases in protein:DNA) (Uni et al.,1995). It has been demonstrated that the presence of feed in the gastrointestinal tract stimulates villus growth in the young chick, involving the generation of cells in the crypts of liberkuhn, which attain maturity while ascending the shaft (Baranylova and Holman, 1976; and Moran, 1985). Uni et al. (1995) reported the greatest absorptive surface in the duodenum (number of villi per surface area). However, during the 7 to 14 day post hatch period there were greater increases in villi volume in the jejunum followed by that in the ileum.

Noy and Sklan (1995a), also reported low amylase, trypsin and lipase activity at day 4 post hatch which increased 100, 50 and 20 fold respectively by day 21. These data support earlier work conducted by Krogdahl and Sell (1989). However their work indicated that pancreatic lipase may be limiting

in some diets. Green and Kellog (1987) reported that bile secretion was relatively low during the first weeks of life. This was supported by Inarrea et al. (1989) and may explain why supplementation of broiler diets with bile acids significantly improved fat digestion (Kussaibati et al., 1982; Polin et al.,1980; Polin and Hussein, 1982). Noy and Sklan (1995b) also reported that during the period 4 to 21 days post hatch digestion of starch was greater than 85%. They also reported that nitrogen digestion increased from 78% to 90% from day 4 to day 21 while fatty acid digestion was over 85%. It had been previously shown by Reisenfeld et.al. (1980) that over 95% of starch was digested in the jejunum of 12 day old birds. Uni et al. (1998), also reported that the sucrase-maltase complex is lowest in the duodenum and highest in the jejunum and ileum. The activity of this complex increased two to four fold two days after hatch and stabilized by day fourteen. Alkaline phosphatase activity which is associated with active and mature mucosal enterocytes was higher in the duodenum than in the jejunum and ileum and also stabilized by day fourteen.

Digestion and absorbtion of starch and protein in standard corn-soybean diets do not appear to be limited whereas the gastrointestinal enzymes and absorptive surfaces increase to their optimum levels post hatch.

However, there appears to be some questions concerning optimal fat digestion.

Utilization of feeds with high levels of NSPs

Feedstuffs commonly used in the poultry industry include corn, barley, rye, wheat and soybean-meal. These feedstuffs contain variable amounts of soluble and insoluble NSPs. The composition of some of these feedstuffs are outlined on **page 85**.

There is considerable evidence that NSPs depress growth in chickens. Several theories have been advanced to explain the efficiency with which rations containing high levels of NSPs are utilized by poultry. Many researchers base their argument on the assumption that changes in transit and mean retention time may alter feed utilization by one of the following three mechanisms viz.,

1. Increased residence time of nutrients in the gastrointestinal tract increases contact with digestive enzymes and absorptive cells, thereby increasing nutrient utilization.
2. Effecting changes in the microbial population which may affect nutrient utilization in positive or deleterious ways.
3. Altering feed intake.

It has been hypothesized that the rate of passage of digesta through the gastrointestinal tract is a good measure of gastrointestinal function. Its best measure is MRT in the entire tract or any of its segments (Warner, 1981). Generally, mean retention time increases with age and body weight. Bolton (1955) observed increased digestibility of pentosans by mature chickens. Shires et al.(1987) reported MRT of 309 and 388 minutes for broilers fed corn and canola or corn and soybean respectively. The corn-soy diet contained more soluble NSPs. Wasburn (1991), compared MRT of 4 and 8 week old chickens fed a corn -soy diet and reported transit times of 174 and 237 minutes respectively. This supports earlier claims that MRT increased with age. Van Der Klis et al.(1993a) however reported a decreased transit time in 3 to 5 week old birds fed an indigestible soluble carbohydrate carboxy methyl cellulose (CMC). The length of the gastrointestinal tract increased from 139.2 cm to 181.3 cm while the 50% retention time increased from 4.58 to 7.2 hours as CMC was increased from 0 to 2%. Concurrently, body weight gain and feed intake decreased, while water intake, feed:gain ratio and water:feed ratio increased. Protais et al. (1996), however suggests that the reduction in feed intake is not consistent. These data support the theory that MRT is a better indicator of feed

utilization than transit time.

Fincher and Stone (1986) reported that 30 to 60 g/kg DM, β 1-3, 1-4 glucan in barley resulted in growth depression in broilers. The high level of arabinoxylan in rye and wheat, approximately 10% and 8% of dry matter respectively, also depressed growth in broilers (Antoniou et al., 1981; Ward and Marquardt, 1987). Annison (1991) demonstrated a high negative correlation between the AME (apparent metabolizable energy) of wheats and their soluble NSP content. Friesan et al. (1992) reported a nitrogen corrected AME (AMEn) in young broiler chicks of 3380 kcal/kg when a conventional wheat -soy diet was fed. However, when barley, rye and oats replaced 70% wheat in the diet. AMEn was reduced to 2446, 2300 and 2577 kcal/kg respectively. These findings were supported by earlier work (Misir and Marquardt, 1978a b; Boros et al., 1985; Classen et al.,1985).

There are several theories pertaining to the reduced AME of diets containing NSPs. The β linkage in many NSPs results in supercoiling of the molecules creating stearic hindrance to enzymatic action. Most NSPs are also part of the cell wall and associate closely with other polysaccharides, proteins and lignin (Fincher and Stone, 1986; Selvendran et al., 1987). The arabinoxylans of wheat are thought to be linked to lignin through a phenolic

acid ester bond as well as closely associating with wheat proteins in a covalent bond (Fincher and Stone, 1974). At a given pH, some NSPs, for example, pectin, may have a high charge density because of the presence of acidic groups. Some cations may become chelated with these groups rendering them unavailable. Further, these charged groups may become associated with lipid micelles or the glycocalx surfaces of the gut. It is thought that this binding may interfere with lipid digestion and enzyme function. These aspects have not been adequately investigated.

It has been demonstrated that the newly hatched chicken's ability to digest starch, protein and lipids is relatively high. However, the addition of NSPs to the diet significantly lowers overall digestibility. Fengler and Marquardt (1988) comparing the effect of a wheat-based diet (52%) to wheat-based diets plus 15, 30 and 60 g/kg crude pentosans (arabinose and xylose), reported reduced digestibility of C18:0 (.76, .74, .60, and .69 respectively). Choct and Annison (1990) also reported lipid digestibility of .93, .87, .76, and .69 when 0, 20, 25 and 40 g water extracted pentosans/kg diet were added to a control sorghum - soybean diet. These researchers also reported digestibilties of .66 and .95 for C18:0 and C18:2, respectively, when 30 g pentoses/kg diet were fed.

Several researchers have reported increased viscosity and sticky droppings in chickens fed NSPs. They postulated that the decreased AME, growth rate and feed efficiency were primarily due to the increased viscosity (Edney et al.,1989; Friesan et al., 1991,1992; Peterson and Aman, 1989). Foregut viscosities of 21- day- old broiler chicks ranged from 4.5 centipoise units (cps) on corn - soybean based diets to 45, 50, 225 and more than 1000 cps for wheat, triticale, barley and rye -soybean diets, respectively (Bedford et al., 1991; Bedford, 1993; Bedford, 1995). Several theories have been advanced regarding the mode of action of increased viscosity within the gastrointestinal tract. The solubility of NSPs in aqueous solution is dependent on several factors including structure, size, linkages with other molecules, presence of charged groups and concentration. Edwards et al.(1988), using an in vitro model demonstrated that convection transport of glucose and sodium were greatly inhibited by guar gum. They concluded that intestinal contractions which create turbulence and convection currents thus mixing lumenal contents and bringing material from the center of the lumen close to the mucosal epithelium, may be impaired. It has also been shown that gel forming gums and pectin result in thickening of the unstirred water layer which is adjacent to the intestinal mucosa (Johnson and Gee,

1981; Flourie et al., 1984). There are also reports that feeding rats various gelling agents resulted in increased proliferation of enterocytes within the jejunum and ileum, which changed the morphology of villi and microvilli and decreased the activity of epithelial enzymes (Johnson et al., 1984; Johnson and Gee, 1986). Sakata (1987) demonstrated that short chain fatty acids resulting from fiber fermentation stimulate epithelial cell proliferation. The increased enterocyte turnover may increase maintenance requirement of the bird. Ikegami et al. (1990) reported that several viscous indigestible polysaccharides induced significant increases in pancreatic amylase, protease and lipase, bile acids, mucosal DNA and RNA in rats. Simultaneously, there were increases in the length and weight of the pancreas, small and large intestine as well as the cecum. There was also decreased digestibility of protein and fat. Similar findings were reported in chickens (Almirall et al.,1995; Brenes et al.,1993). The inclusion of rye and guar gums have also been found to have a rachitogenic effect as well as an overall growth depressing effect. However, the inclusion of barley has only been found to elicit an overall growth depressing but no rachitogenic effect (Grammar et al.,1982; Ray et al.,1982). This is thought to interfere with vitamin D3 utilization and therefore calcium homeostasis (MacAuliffe et al.,

1976, 1979). Van Der Klis et al. (1993 a, b) also determined that carboxymethyl cellulose interferes with mineral absorption from the gastrointestinal tract. These adaptations require additional energy, reduce nutrient utilization and therefore must have an impact on the overall net growth depression. It has been reported that increased viscosity within the gastrointestinal tract changes the microfloral population and fermentation patterns, which are thought to be at least partially responsible for the deleterious effects observed. Choct et al. (1992) reported that reduced digestibility of long chain fatty acids was less pronounced in caecectomized chickens and inferred an interaction between cecal microorganisms, wheat pentosans and viscosity.

In addition to functioning as the primary site of digestion and absorption, the gastrointestinal tract presents physical and chemical barriers to the translocation of organisms, toxic metabolites and other injurious substances present within the tract. The immune system which is a component of this barrier is relatively immature at hatching. Jeurissen et al.(1989) and Schat and Myers (1991) reported that B and T cells, leucocytes and plasma cells producing IgM, IgG or IgA were barely detectable by day five. However considerable increased activity was

observed from day 14 through 28 in the Meckel's diverticulum and cecal tonsils, as well as in Peyer's patches and intestinal mucosa. The concentration of IgA , the primary type of antibody secreted in bile and intestinal tissue was reported to be very low at one day post hatch and increase slowly through day nine (Piquer et al., 1991). Four fold increases were detected from day nine through day 28.

An immature immune system coupled with the developing digestive and absorptive capacity of the gastrointestinal tract implies that young poultry would be more susceptible to bacteremias as a result of enteric problems caused by increased indigestibility of feedstuffs and increased gut viscosity. It has been demonstrated that changes in the microbiota of the gastrointestinal tract as a result of diet are significant (Adams, 1980; Hungate, 1984; Stutz and Lawton, 1984). The intestinal tract of chickens is colonized primarily by gram positive bacteria as soon as eggs are hatched (Smith,1965) and within 7 to 14 days a stable population is established. Smith (1965) examined changes occurring in the intestinal flora of the young chick and reported that clostridia, streptococci and enterobacteria were the primary organisms present in the gastrointestinal tract by day fourteen. Morishita et al. (1982) also reported that the predominant

organisms found from days 5 through 14 were as follows.

1. Crop and Jejunum - *Lactobacillus acidophilus, Lactobacillus salivarius* and bifidobacteria. Streptococci, clostridia, proteus, *Escherichia coli* and other bacteria were present in low numbers.

2. Cecum - *Escherichia coli,* proteus, clostridia and other enterobacteria were most common while lactobacilli were present at lower levels. Total viable counts were estimated at 11 $\log_{10}$ CFU/g of intestinal sample. Barnes et al. (1972) reported that lactobacilli were the only organisms normally present in the duodenum and small intestine at levels greater than 3 $\log_{10}$ CFU/g in birds two to six weeks old. There was a general failure to consistently isolate *C. perfringens* from the cecum and it was concluded that the organism was not a commensal. Eight different types of nonsporing anaerobes were recovered accounting for 10 $\log_{10}$ CFU/g of intestinal contents. Gram negative anaerobes were about one quarter of the population. These findings generally confirm work of Sofos et.al. (1984) and Salinitro et al.(1978) who reported that total aerobes, anaerobes, coliforms, lactobacilli, bifidobacterium and clostridia generally increased while streptococci decreased with age and distally along the gastrointestinal tract. Yeast, molds and clostridia were generally present at less than 3 $\log_{10}$

CFU/g of intestinal sample. A *Veillonella spp.* which has been associated with increases in volatile fatty acid production in cecal contents in poultry has also been isolated (Hinton et al.,1992).

Several factors including the external environment, presence of feed in the gastrointestinal tract and rate of passage influence the type and population of microorganisms present (Adams, 1980; Hungate, 1984; Stutz and Lawton, 1984). Wagner and Thomas (1978) reported that the ileal anaerobe counts of 4 day old chicks fed diets containing rye or pectin were two or three logs greater than chicks fed corn-soybean diets. Canzi et al.(1994) demonstrated that there was no difference in cecal microflora when either a cellulose, lignocellulose or fiber free diet was fed to Sprague Dawley rats. However, guar gum and pectin significantly increased bifidobacteria as well as clostridia. Cellulose and hemicellulose diets also significantly decreased microbial counts of 7α dehydroxylating cholic and chenodeoxycholic microbes when compared to diets containing guar gum or pectin. It was also found that the population of spore formers in chicks fed rye was 6 to 7 logs greater than chicks fed corn-soybean diets. Cecal butyric acid concentration was 4.65 mM compared to .22 mM for corn-soybean diets. The addition of penicillin to diets containing rye or pectin reduced

butyric acid and spore-formers by about 5 logs. They therefore suggested that a *Clostridium* species may be implicated in this problem. MacAulifle and McGinnis (1971) and Misir and Marquardt (1978 a, b) also demonstrated that the negative effects of excessive microbial proliferation associated with feeding diets high in NSPs was reduced by feeding penicillin. Choct et al. (1992, 1996) reported no response to penicillin when feeding 30g arabinoxylan/kg diet and 150 mg/kg penicillin. However, there was a significant increase in the digestibility of C20 and C20:1 and a non significant increase in feed to gain ratio.

Another effect associated with reduced growth rate when soluble NSPs are fed is the hydrolysis of taurine and glycine conjugated bile acids by bacterial enzymes (Hayakawa,1973; Hylemon and Stellwag, 1976). The hydrolysis is reported to be catalysed only by bacterial enzymes known as bile salt hydrolases (Hayakawa,1973; Hylemon and Stellwag, 1976). This activity is expressed by several microorganisms which colonize the gastrointestinal tract (Gilliland and Speck,1977, Hayakawa and 1973, Hylemon and Stellwag, 1976). Bacteria capable of deconjugating bile acids have been isolated attached to epithelial cells, unattached, as well as in cell free supernatant from intestinal contents (Norman and Widstrom, 1964).

Species and strains from the genera *Clostridium, Lactobacillus, Peptostreptococcus, Bifidobacterium, Fusobacterium, Eubacterium, Streptococcus and Bacteroides* have been shown to catalyze this reaction. However, the bile salt hydrolase activity has been partially purified only from *Clostridia perfringens* and *Bacteroides fragilis* (Aries and Hill, 1970; Stellwag and Hylemon, 1976). Cholyltaurine hydrolase activity was higher in rye based diets than in corn-soy or sucrose-soy diets. The activity of this enzyme increased distally from the crop to the cecum and indicates bacterial activity. Body weight of nine day old birds on rye diets in these studies was correspondingly lower (Feighner and Dashevicz, 1988). Campbell et al. (1983) observed improved apparent lipid digestibility (ALD) in rye diets by the addition of sodium taurocholate as well as a high correlation between reduced viscosity and improved ALD. Hock et al. (1997), observed a synergistic effect when zinc bacitracin and xylanase were added to a wheat based diet. *E. coli*, streptococci, lactobacilli as well as clostridia and anaerobic cocci decreased. It is thought that the deconjugation of bile salts reduces enterohepatic circulation thereby reducing fat digestion.

Several researchers have demonstrated that necrotic enteritis, a disease caused by *Clostridia perfringens* is increased when diets high in

NSPs are fed (Branton et al.,1987; Riddel and Kong, 1992). Riddel and Kong (1992) also reported significant mortality and typical pathologies associated with this disease in birds fed rye, wheat or oats and challenged with 6 to 9 $\log_{10}$CFU/g *C. perfringens*. Birds fed a corn-soy diet had a significantly shorter shedding time and mortality. However, Branton et al. (1996) were unable to demonstrate in vitro that the proliferation of *Clostridia perfringens* was stimulated by the presence of NSPs. *Clostridia perfringens* was suppressed by high levels of NSPs in that study. It is therefore inconclusive whether NSPs , viscosity or some combination of these and other factors increase *Clostridia perfringens* in chickens.

Bacterial infection in poultry and food safety

The gastrointestinal tract of chickens is colonized by an array of microorganisms. Some of those organisms promote gut health and feed utilization while others become pathogenic. Pathogenic organisms isolated from poultry include *Clostridia perfringens, Escherichia coli, Erysipelothrix rhisiopathiae, Pasturella multocida, Mycobacterium avium, Salmonella spp. and Serpulina spp. (*Porter, 1998). Poultry may also serve as reservoirs of microorganisms pathogenic to humans, while exhibiting no harmful symptoms in the animal. Several of the aforementioned organisms

have been implicated in human diseases or have zoonotic potential. This review will concentrate on clostridia, salmonellae and lactobacilli.

The genus Clostridium consists of rod shaped cells, 0.3-2.0 x 1.5-20 μm, anaerobic, gram positive bacilli which produce spores. They are commonly isolated from soil and fresh water and many pathogenic and nonpathogenic clostridia are normal inhabitants of the gastrointestinal tract. The classification of *Clostridia botulinum* is based on the antigenic responses of the seven identified strains A-G. Types A, B, E and F, have been associated with the disease in humans while types C and D are mainly responsible for animal diseases. Food-borne botulism results from ingestion of preformed toxins in adults and preformed toxins or spores in children (Hauschild,1989). *Clostridia perfringens* is the principal obligate anaerobe in the normal chicken gastrointestinal tract (Johansson and Sarles, 1948; Shapiro and Sarles 1949). *Clostridia perfringens* Types A-E produce toxins in the gastrointestinal tract which may be absorbed into the blood stream resulting in toxemias. Other *Clostridia spp.* are invasive as well as produce potent toxins which damage cells in the gut resulting in ulcerative enteritis, e.g, histotoxic *Clostridia colinum* (Quinn et al., 1994). This organism has been isolated from chickens, turkeys, pheasants and captive quail and

results in a highly fatal enteric disease (Berkoff, 1985; Ononiwu et al.,1978; Bickford, 1985). *Clostridia perfringens* types A or C, the cause of necrotic enteritis, has also been isolated from chickens (Al-Sheikhly and Truscot, 1977; Riddel and Kong, 1992).

The incidence of diseases in the USA human population associated with *C. perfringens* is very low. Two large outbreaks were reported by the FDA during the period 1985 to 1994 (CDC,1997). Conversely, the incidence of salmonellosis is very high occurring second only to campylobacteriosis.

There are more than 2000 serotypes of salmonella which have been classified by somatic (O), flagellar (H), and capsular (K) antigens (Keush and Thea,1993). Generally, they are facultative, non-spore forming , gram negative rods. These organisms are generally considered invasive and may produce enterotoxins. Salmonella infections may become systemic resulting in bacteremias which may be accompanied by enteric lesions. The lesions are most prominent in the cecum and are usually associated with pullorum disease, fowl typhoid and paratyphoid infections.

Symptoms of pullorum disease and fowl typhoid are similar and are caused by *Salmonella pullorum* and *Salmonella gallinarium,* respectively.

These organisms cause severe systemic diseases in chickens, turkeys, ducks and other birds. Breeding flocks in the USA are serologically monitored for these diseases through the National Poultry Improvement Plan and there is a policy of mandatory disease reporting and quarantine of affected flocks (Anonymous,1994). The incidence of these diseases has been significantly reduced as a result of this program. In addition to causing severe debilitation and death, birds recovering from pullorum disease and fowl typhoid may become chronic carriers and transmit infection to progeny through eggs (transovarian transmission). Infection may also spread via feces of infected progeny (Pomeroy and Naganja, 1991; Holt and Porter, 1993). Although these organisms are generally non flagellated, nonmotile and host specific to birds, they have been isolated from a number of domestic mammals and may be motile and produce flagella in defined cultures (Holt and Chaubal, 1997).

The majority of serotypes of salmonella are thought to belong to the paratypoid group which is usually described as serotypes other than S. *pullorum, S. gallinarium and S. arizona* (Ashton,1990). That group which includes *S. typhimurium, S .enteritidis, S. montevideo* and *S. heidelberg,* are motile, and infect a wide range of animals, including humans, resulting in

debilitating diseases and death (Brown et al., 1976; Holt and Porter, 1993). Paratyphoid infections may be transmitted by several routes including fecal-oral, fecal soiling of eggs and feed which may become contaminated from several sources including mice. Organisms in this group may colonize the intestinal tact, invade the gut wall and become established in internal organs (Brown et al.,1976). Adult birds may become carriers, harboring the organism but not exhibiting clinical signs. *Salmonella enteritidis* has the unique distinction of also being transmitted through the transovarian route (Gast and Beard, 1990).

The most common *Salmonella spp.* isolated from the human and nonhuman populations are *S. typhimurium*, S. *newport, S. heidelberg, S. infantis, S. agona, S. enteritidis, S. saint-paul, S. senftenberg, S. anatum, and S. derby* (D'Aoust, 1989). In 1997, of the 8576 laboratory confirmed cases of food-borne illness, 2205 were salmonellosis. Further, 21% of the confirmed cases were hospitalized and of the 36 deaths, 13 were associated with salmonella (CDC, 1998). Additionally, an increasing percentage of *S. typhimurium* isolates from humans in England and Wales have been identified as a highly virulent DT104 strain. Food borne transmission of DT104 has been associated with several meats including poultry

(Anonymous, 1996). This strain of *S. typhimurium* is resistant to five antibiotics commonly used to treat infection in the human population (Wall et al., 1996; Ramos et al., 1996).

Plasmids containing drug resistant genes are known to be involved in conferring resistance within and across different genera of bacteria (Le Clerc, 1996). However, chromosomal transfer of drug resistance is also reported to occur. High mutation frequencies among *E. coli* and pathogenic salmonella have also been observed (Wall et al., 1995,1996). There are clearly emerging problems with the use of antibiotics and drug resistant, virulent, pathogenic microorganisms. Therefore, changes in the gastrointestinal environment which mediate changes in its microflora need to be carefully examined.

Feedstuffs containing high levels of NSPs create viscous conditions in the gut which alter gut microflora. Antibiotics, probiotics and exogenous enzymes have been used as ameliorants. Some antibiotics have reduced the deleterious effects of NSPs but since their use is associated with multiple drug resistance their use is ill-advised. Probiotics and other direct-fed microbials e.g cultures of *Lactobacilli acidophilus, Lactobacilli lactis* and *Bifidobacteriim bifidum* or the inclusion of feedstuffs which promote the

growth of these organisms have been shown to effect desirable gut conditions (Jin et al.1997; Patterson et. al., 1997). Dietary lactose, some volatile fatty acids, and other defined and undefined cultures have been shown to control or reduce the incidence of several pathogens including *S. typhimurium* in chickens (Corrier et al., 1995; Chateau et al., 1993). The effectiveness of lactic acid bacteria against pathogens has been attributed to the production of bacteriocidal substances including bacteriocins, organic acids and hydrogen peroxide (Vincent et al.,1959; Sorrels and Spec, 1970; Juven et al., 1988).

Pioneering work on the use of normal gut microflora for the control of pathogens was conducted by Nurmi and Rantala (1973) as well as Snoeyenbos et al. (1986). This concept, termed competitive exclusion, is aimed at innoculation of the gastrointestinal tract of chickens with organisms which would preclude pathogens by several mechanisms. Several researchers have since tried to determine the best combination of organisms to administer and ways to preserve and improve the efficacy of competitive exclusion products (Pivnick et al., 1982; Hinton et al., 1991; Ziprin and Deloach, 1993, Byrd et al., 1998). Variable results have been obtained by several researchers (Watkins and Kratzer, 1984; Yeo and Kim, 1997). The

application of this concept to large scale field operations continues to be studied since the best time of colonization is within the first 48 hours post hatch (Cox et al., 1990; Bailey et al., 1991; Wierup et al., 1988). Currently the efficacy of competitive exclusion products are being tested in ovo (Edens et al., 1997).

Exogenous enzymes and NSPs

It is well documented that the application of exogenously derived enzymes from various bacteria and yeasts to diets containing high levels of NSPs result in improved digestibility and overall bird performance. Starch digestibility accounted for up to 35% of the improvement in available metabolic energy when a xylanase was fed (Carre, 1992). Fat and protein digestibility were improved by 35% and 30%, respectively, in that study.

Supplementation of the diet with appropriate enzymes reduces intestinal viscosity by partially degrading NSPs (Almirall et al., 1995; Ferket, 1993; Petterson and Aman,1989). Digestibility studies (Annison, 1992; Salih et al., 1991), indicate that these enzymes decrease the solubility of pentosans and β- glucans thereby increasing the rate of digestion and absorption as well as reducing fecal moisture. Ward (1996) reported up to 85 % reduction in intestinal viscosity with 10-20 % overall bird

improvement. This was supported by Bedford et al. (1991) and Bedford, (1993 and 1995), who reported a 10% improvement in digestible amino acids as intestinal viscosity decreased. The foregut viscosities reported in those studies were comparable to the 1.5-4.5 centipoise (cps) determined in 21 day old broiler chicks fed a corn-soy diet. Almirall et al.(1995) reported significant improvement of trypsin, amylase and lipase activity when enzymes were included in diets with high NSPs. Similar reports were made by Rotter et al.(1990) and Friesan et al. (1991).

The partial depolymerization of NSPs by the addition of exogenous enzymes results not only in the release of glucose but also of other sugars such as L-arabinose and D-xylose (Chesson, 1987). Both pentose sugars are absorbed from the gastrointestinal tract (Wagh and Waibel 1967a,b; Schutte,1990; Schutte et al.,1992). The rate of absorption of hexose sugars was twice that of the pentose sugars xylose and arabinose in the jejunum and ileum. The absorption rates of hexose and pentose sugars were similar in the cecum; however, xylose was absorbed at a faster rate than arabinose (Savory and Mitchell, 1991). Schutte et al. (1991) also demonstrated that about 15% of the ingested D-xylose and L-arabinose was excreted in the urine. The decrease in ME reported by Wagh and Waibel (1966) when

feeding arabinose and xylose in chick diets may therefore be explained by the urinary losses observed by Schutte et al., (1991). It has also been reported that the ileal flow of volatile fatty acids was significantly increased in pigs given xylose or arabinose. It can therefore be deduced that part of these monosaccharides was being fermented. Microbial fermentation was also evidenced by increased caecal weight and increased VFA. Schutte et al. (1992) concluded that some energy is available to the bird from the absorption and metabolism of pentoses.

Utilization of pentoses by microorganisms

Several pathogenic organisms including *S. typhimurium*, *C. perfringens* and *E. coli* can utilize D-xylose as their sole carbon source (Mortlock and Old, 1979; Lee et al., 1982; Mishra et al., 1993). The fermentation of xylose by bacteria under anaerobic conditions yields a variety of products. Several strains of lactobacilli will convert xylose into a mixture of ethanol, acetic and lactic acid and acetone (Flickenger, 1980). Under anaerobic conditions ethanol, acetate, butyrate as well as lactate may be produced by several genera including *Clostridia* and *Salmonella*. Several factors influence the relative amounts of end products formed during the fermentation of pentoses and include pH, temperature, nutritional

factors and redox potential (Misha et al.,1993). Reporting on kinetic studies, Lee et al. (1982) reported the presence of a low affinity permease for L-arabinose within *S. typhimurium*; however, a binding protein was isolated. Although kinetic studies indicate only a low affinity transport system for D-xylose, a binding protein which is usually associated with a high affinity transport system and a high energy phosphate compound was also isolated. Shamanna and Sanderson (1979), conducted several experiments to determine the utilization of various sugars by *E. coli* and *S. typhimurium.* They reported an inducible D-xylose transport system in *S. typhimurium*. All other pentoses in that study could not be utilized as a sole carbon source. Activities of D-xylose isomerase and D- xylulokinase were induced in medium with D-xylose levels as low as 0.01%. D- xylose accumulation in *S. typhimurium* by means of the inducible system and resulted in an accumulation of at least 40 fold concentration inside the cell when compared to the medium. That system also resulted in the accumulation of smaller quantities of L-arabinose within the organism. It is therefore possible for *S. typhimurium* and other salmonellae to proliferate under inducible conditions.

The interactions of microflora, NSPs and exogenous enzymes have

not been well examined. There are only a few reports indicating a reduction in total VFA in the ileum but increased in the ceca when diets high in NSPs are supplemented with enzymes (Choct et al., 1996). Lazaro et al. (1998) also indicated that rye based diets supplemented with 858U B-glucanase and 864U of xylanase resulted in increased butyric acid in the cecum. The effect of increased fermentable pentoses on the interaction of lactobacilli, clostridia and salmonellae has not been examined.

Gastrointestinal mucosa and dietary fiber

Data from several research efforts indicate that the gastrointestinal tract attains maturity between days 14 and 21 post hatch (Uni et al., 1995; 1998). Those researchers also demonstrated that primarily hyperplastic growth (decrease protein:DNA) occurs in the first week of life followed by hypertrophic growth. Increased cellular activity was also observed during that time. Generally, RNA:DNA, RNA:protein and protein:DNA ratios are used as indices of tissue activity, ribosomal capacity, and cell size respectively. Mucosal enzymatic and lymphoid activity have been previously described and increases with age, whereas tissue activity decreases (Uni et al. 1996).

The gastrointestinal epithelium is multifunctional. It serves as a

barrier to some macromolecules as well as performing immunological and enzymatic functions (Bienenstock, 1994; Dibner, 1996). Several researchers have demonstrated that the fermentation of dietary fiber results in the production of metabolites and or products which affect gastrointestinal mucosa directly or indirectly. Biogenic amines (particularly polyamines) produced by lumenal bacteria are thought to stimulate small intestine and colonic mucosa (Osborne et al. 1989; Seidel et al. 1985). Several researchers have also demonstrated that the stimulatory effect VFA on epithelial cells in the presence (Sakata, 1987) or absence (Koruda et al. 1990) of gastrointestinal microbes. Lynch et al. (1994) reported decreased protein: DNA ratio (hyperplastic growth), in the jejunum and colon of rats fed triacetin. It was suggested that the trophic effects observed may have been as a direct result of elevated levels of circulating acetate. Crypt depth and villus height were unaffected by feeding short chain fatty acids (SCT) in that study although there was an increase in cell number (decreased protein:DNA). RNA and protein concentration were also higher in the SCT group. The researchers therefore suggested that enterally administered triacetin elicited a trophic effect on mucosal cells which resulted in the proliferation of numerous smaller sized cells. Other data had

previously demonstrated that parenteral nutrition with SCT elicited trophic effects on small and large bowel mucosa (Sakata, 1987; Karlstad et al. 1992). Frankel et al.(1994) demonstrated that trophic effects of SCT are mediated by the autonomic nervous system in association with increased jejunal gastrin. Local trophic effects were observed in the colon. However, in that study there was an increase in mucosal DNA together with increased villus height, surface area and crypt dept, without significantly affecting protein. Hyperplastic and maybe some hypertrophic growth are implied.

It has been demonstrated that rye-based diets and isolated wheat arabinoxylans increase the concentration of VFA in the intestinal lumen (Choct et al. 1996; Wagner and Thomas, 1978). Whether or not this increase is beneficial or detrimental to the animal depends on the physiological status of the gastrointestinal tract. Enhanced proliferation of gastrointestinal mucosa requires energy and nutrients thereby increasing the animal's maintenance requirements. However, if increased circulating VFA concentrations ameliorates a diseased or stressed state and is associated with increased uptake and utilization of nutrients, benefits may be derived. VFA are thought to contribute about 20 - 30% of the maintenance requirement of omnivores and herbivores (Bergman, 1990).

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PART 3

Experiment 1

Effect of Avizyme in rye or corn-based diets on broiler performance, morphological changes, microbiological and fermentation changes in the gastrointestinal tract

Abstract

One hundred eighty, male, one-day-old, Hubbard broiler birds were randomly assigned to either rye or corn-based diets containing three levels of Avizyme® to assess changes in fermentation, water consumption, effect on performance and microbial population in the gastrointestinal tract. Enzyme addition reduced water to feed consumption ratio in rye diets ($P < 0.03$), feed to gain ratio, and increased cumulative weight gain ($P < 0.03$) to a greater extent in rye than in corn-based diets. Total pathogenic clostridia and lactobacilli were consistently greater in birds fed rye diets ($P < 0.02$), but salmonellae were only higher in week six and remained at very low levels throughout the experimental period. Enzyme addition reduced total colony forming units in the ceca but not in the ileum or jejunum of birds on both grain types ($P < 0.02$) with clostridia declining at a greater rate in the corn diets.

Acetate concentration was greater and increased with addition of Avizyme® in rye diets ($P < 0.001$), but neither grain source nor enzyme addition affected the concentrations of isobutyrate, isovalerate or propionate in digesta samples.

Ileal mucosal weight was significantly greater throughout the

experimental period in birds fed rye based diets ($P < 0.05$). There was a signifcant correlation ($r = 0.66$) between the acetate levels in rye based diets and mucosal weight ($P < 0.001$). DNA/mg of wet ileal mucosal tissue was generally unchanged throughout the experimental period; whereas hyper-plastic growth (protein :DNA) was observed during weeks 1-3. Elevated RNA:DNA ratios (cellular activity) were observed in week one and stabilized at a lower level throughout the remainder of the experimental period.

These data indicate that the addition of Avizyme® generates more digestible and fermentable substrates which improves bird performance and reduces litter moisture as well as the proliferation of total pathogenic clostridia. Trophic effects on ileal mucosa were observed in birds on rye-based diets but the gastrointestinal tract adapted to increased levels of fermentable substrates and fermentation products.

(Key words: Broilers, rye, Avizyme®, *Salmonella*, fermentation, mucosa)

Introduction

The high level of soluble and insoluble fibers in several cereal grains limits their use as feedstuffs in poultry diets. Generally, broilers lack the appropriate array of enzymes required to depolymerize complex non-starch

polysaccharides (NSPs), thus presenting the bird with an unsuitable environment for the optimum utilization of feed nutrients (Almirall et al.,1995; Bedford et al.,1991). Additionally, the increased viscous environment predisposes the gastrointestinal tract to colonization by opportunistic and sometimes pathogenic microorganisms. Some of these organisms have zoonotic potential and are therefore a food safety concern. Gastrointestinal development and health may also be impaired as some non-commensals colonize and necrotize mucosal tissue (Bayer et al., 1977; Branton et al., 1997).

Addition of exogenously derived enzyme preparations to diets high in NSPs partially depolymerizes NSPs, thereby releasing several encapsulated nutrients within the cell wall matrix and endosperm of cereal grains. The consequent fermentation of pentose sugars and release of other substrates released yields higher levels of volatile fatty acids (VFA) which are multifunctional. Depending on species VFA contribute between 5% and 80% of maintenance requirements (Bergman, 1989). Specific values are not available for the chicken but are expected to be within the range of 23-30%, as reported for pigs. VFA are also associated with increased blood flow and trophic effects in the gastrointestinal tract of nonruminants (Bergman 1990;

Jacobs, 1983). These effects, increased nutrient availability and uptake, and decreased viscosity are likely explanations for the improved performance observed in broilers fed exogenous enzymes in diets with high levels of NSPs. VFA also exert bacteriostatic and bacteriocidal effects on several pathogenic microorganisms (Singh et al., 1985; Thompson and Hinton, 1997). Chain lengths of less than eight carbons and unsaturated fatty acids appear to be effective (Fay and Farias. 1975; Sheu and Freese, 1973). These effects are dependant on the pH of the environment as well as the organism's ability to combat this stressor through homeostatic mechanisms including lowering internal pH , producing compatible solutes, heat shock proteins or pumping out hydrogen ions (Russell, 1992).

It has been reported that diets high in NSPs predispose poultry to microbes which produce butyric acid and are capable of necrotic activity and. It has also been demonstrated that the magnitude of trophic effects on gastrointestinal mucosa occur in the following order: butyric > propionic > acetic (Sakata,1987).

The purpose of **Experiment 1** was to determine changes occurring in the beneficial and pathogenic microflora and their interaction with viscosity and fermentable fibers. *Lactobacillus spp.* are beneficial commensals and

predominate in the gastrointestinal tract (Fuller, 1978; Axelsson et al., 1989). Changes in their population are proposed to be an index of gut health and changes in gastrointestinal microbial population. Total pathogenic clostridia were determined since previous associations with diets high in NSPs were reported. The purpose of that determination was to establish the effect of the enzyme complex on the population of clostridia as well as changes occurring in volatile fatty acids. *Salmonella spp.* were also determined since it is hypothesized that proliferation is enhanced under conditions of increased pentose availability. Many of these organisms are also pathogenic to the bird and have zoonotic potential. They are therefore of concern in the food industry. Gastrointestinal morphology was also examined since it has been demonstrated that increased fiber in nonruminanant diets elicits trophic effects on gut mucosa as a result of increased VFA. Additionally, organisms such as salmonellae and clostridia also adversely alter gut mucosa.

Materials and Methods

1. General Management

One hundred eighty, one-day-old, Hubbard male broiler birds were randomly assigned to eighteen experimental units and housed in a Petersime

brooder battery. On day one, the temperature was set at 32°C and subsequently reduced over the next three weeks until 23°C was achieved. At three weeks of age, birds were transferred to Petersime grower batteries. Each pen within the battery was equipped with a device to facilitate water measurement.

Starter and growing-finishing rations which were fed ad libitum from hatch to three weeks and four to seven weeks, respectively, were formulated to be isocaloric and isonitrogenous and are described in **Tables 2** and **3**. Diets were formulated to meet or exceed NRC requirements (NRC, 1994). Individual birds were wing banded at the beginning of the trial and weighed each week. Feed consumption, water intake and body weight gain were measured each week.

2. Microbiological Media and Methods

Each week one bird was randomly selected from each treatment, killed by cervical dislocation and one gram of the contents of the proximal jejunum, distal ileum and cecum were aseptically removed for analyses. A fractional factorial sampling scheme was used (SAS, 1995).

A: *Salmonella spp.* and lactobacilli

Total lactobacilli and salmonellae were determined by placing 1g of intestinal contents in 9 ml of buffered peptone water, followed by serial dilutions in peptone water and surface plating 100 μl on the following media. Lacobacillus were determined on dEMann rogosa sharpe (MRS, Difco) agar, (DeMan et al. 1960). The MRS media was acidified to pH 5.4. Several isolates of a pure culture of *lactobacillus spp.* were plated on the MRS media in order to identify the organisms on that medium. *Salmonella spp.* were determined on xylose lysine tergitol (XLT4, Difco) agar, (Miller et al. 1991). All plates were incubated at 37^{0}C and checked at 24 and 48 hours. Samples deemed negative for *Salmonella spp.* by presumptive methods were enriched in Hajna's tetrathionate broth (Difco), (D'Aoust et al. 1993), incubated for 48h and replated on XLT4 and incubated at 37^{0}C for 24 to 48 hours. Typical colonies were confirmed on lysine iron agar (LIA, Difco) and triple sugar iron (TSI, Difco) agar slants.

B: Total pathogenic clostridia

One gram of intestinal contents was placed in 9 ml vials of peptone water plus 1% sodium thioglycollate. All samples were flushed with nitrogen during this process. After serial dilution in reduced sodium

thioglycollate and peptone water, total pathogenic clostridia were determined (Atlas, 1995) by surface plating 50μl on Clostrisel agar (BBL). in an anaerobic chamber. Clostrisel agar is a highly selective medium for cultivation of pathogenic clostridia from wounds, fecal specimens and soil (Atlas, 1993). Selective ingredients include sodium formaldehyde sulfoxylate, sodium azide and neomycin sulphate. Simultaneously, plating was done on *Clostridium perfringens* agar, OPSP (Oxoid) as a positive control. The anaerobic chamber was fitted with a palladium catalyst system (Coy Laboratory Products, Detroit MI) which reduced trace oxygen in the chamber. The catalyst was changed twice weekly. An analyzer (Coy Laboratory Products) capable of detecting 0 to 2000 ppm oxygen was used to monitor oxygen levels in the chamber. Oxygen levels were kept lower than 200 ppm in the chamber. The reduced chamber environment was maintained by utilizing a premixed gas consisting of 90% nitrogen, 5% carbon dioxide and 5% hydrogen. Routine flushing of the chamber was conducted to remove oxygen which may have been introduced through the port. Plates which were placed in glass jars with sealable lids and vacuum sealed before leaving the chamber were incubated anaerobically at 37^{0}C for 48 hours. Typical colonies were confirmed using the catalase test (Blazevic, 1975).

3. Analysis of ileal volatile fatty acids

Distal ileal samples were determined for total acetic, isobutyric, butyric, valeric and isovaleric volatile fatty acids using a gas chromatographic method modified by Playne (1985). The parameters for this analysis are detailed in **Table 4.** In synopsis, 1.5 mL of supernatant were combined with 300 μL of 25% metaphosphoric acid (5:1) and allowed to stand at room temperature for 30 min and then centrifuged. One μL of the sample was then injected into a Hewlett Packard model 5890 gas chromatograph equipped with a HP-FFAP 10-m x 1μm capillary column, with cross-linked polyethylene glycol-TPA packing and a flame ionizing detector.

4. Measurement of ileal mucosal mass

Following cervical dislocation 10 cm sections of the distal ileum were removed and mucosal scrapings were obtained from each section, as described by Jacobs and Schneeman (1981). Following weight determination of the mucosal and tissue mass, the mucosal scrapings were stored at -80° C until analyzed. DNA and RNA were determined by modification of the methods described by Wannemacher et al. (1965). Protein was determined using the method of Lowry et al. (1951). These

procedures are detailed in **appendix 1**. Briefly, 5 ml of phosphate buffered saline were added to the gut mucosa and homogenized with a Polytron (Brinkman Instruments, NY). Trichloroacetic acid was added to 714μL of the homogenate and supernatant was discarded. Lipid extraction was performed using potassium acetate, chloroform, ether and ethanol. Following air drying overnight potassium hydroxide was added to sample .

Standard curves were established for protein (Bovine Albumin fraction V, Sigma), DNA (Calf thymus, Sigma) and RNA (Baker's yeast type XI, Sigma) and absorbance read on a UV-VIS spectrophotometer (Shimadzu Model # 160). Protein, DNA and RNA were read at 650 nm, 490 nm and 260 nm respectively.

5. Experimental design and statistical analysis

Birds were randomly assigned to six experimental treatments consisting of rye or corn plus 0%, 0.05% and 0.1% Avizyme ®. Avizyme ® 1300 was obtained from Finfeeds International, and contained 2500 units of xylanase and 800 units of protease/g. The statistical model consisted of a completely randomized design with 2 x 3 factorial arrangement of treatments. Microbiological sampling was conducted using a fractional factorial scheme (SAS, 1995). The experimental data were analyzed by the

Mixed Model Procedure of SAS (1994,1995). Differences among treatments were determined using least significant difference. Log $_{10}$ transformations were done on microbial results prior to statistical analyses.

Results

Avizyme addition to rye diets resulted in improved cumulative feed:gain ratio (FCR) throughout the experimental period. A significant improvement in FCR was observed from week three through seven ($P < 0.01$) in rye-based diets. The significant improvement in FCR in Avizyme supplemented corn-based diets observed during weeks two and three was less than that observed in the rye based diets (**Table 5**). Birds on rye based diets with Avizyme performed as well as birds on corn based diets.

There was also significant improvement in cumulative weight gain ($P < 0.03$), in birds fed rye supplemented with Avizyme from weeks three through seven (**Table 6**). This performance was equal to or greater than that observed in birds fed corn based diets. In this study, enzyme addition to rye based diets also decreased ($P < 0.01$), water:feed consumption ratio **(Table 7).**

The levels of acetate in ileal digesta samples were significantly higher ($P < 0.05$), in rye-based diets vs corn-based diets **(Table 8, Fig. 1)** and

increased through week seven. Although not significant, isobutyrate plus butyrate and isovalerate tended to be about 40-60 % higher in rye diets, but did not change with time.

Total pathogenic clostridia and lactobacilli were greatest ($P< 0.01$), in the cecum **(Fig. 2)**. *Lactobacillus* population within treatment remained relatively constant throughout the experimental period, whereas, total pathogenic clostridia declined at a faster rate ($P< 0.01$), in corn than in rye based diets **(Figs. 3, 4, 5)**. The population of total pathogenic clostridia in the caecum also declined ($P< 0.01$), with the addition of Avizyme **(Fig. 6).** The incidence of salmonellae differed between rye and corn-based diets and decresed over time. *Salmonella spp.* were not isolated by either direct plating or enrichment techniques on day one of the trial but were recovered by enrichment during the experimental period. Salmonellae were found 100% and 67.0 % in birds fed rye and corn-based diets respectively at week six and 12.5% and 0% respectively at week seven **(Fig. 7).**

Mucosal weights increased from week one through week six and declined in week seven in birds on either grain type. However, mucosal weights were significantly higher ($P < 0.05$) in birds fed rye based diets throughout the experimental period **(Fig. 8).** The amount of DNA/mg of

wet mucosa was generally unchanged throughout the experimental period while protein:DNA ratios were higher ($P < 0.01$), with the addition of enzyme in rye based diets in week one compared to corn based diets **(Table 9).** There was a significant reduction of protein:DNA beyond week two. RNA:DNA ratios were elevated in week one in birds fed either grain type however a significant increase was observed with enzyme addition to rye based diets during that time, **(Table 10).** A significant correlation ($P < 0.001$, $r = 0.66$) was observed between grain type and acetate levels. There were no significant correlations between acetate and protein:DNA or RNA:DNA in this study.

Discussion and Conclusions

The improved performance in birds fed rye-based diets supplemented with Avizyme was anticipated since corn contains lower levels of arabino-xylans (Annison, 1991; Petterson et al., 1990; Smits and Annison, 1996). These results are consistent with the general trend in improvement observed by Bedford et al. (1991) who conducted a feeding trial utilizing 0 and 0.2% pentosanase (*Trichderma longibrachiatum*). They reported gain:feed ratios of 0.60 vs 0.65 and cumulative weight gains of 376 g vs 420 g, respectively, in 21 day old broilers fed a 58% rye-based diet. Similarly, Pettersen et al.,

(1993) reported improved performance of broilers fed a 58% rye-based diet plus xylanase and β- glucanase. Lazaro et al., (1998) also reported improved weight gain (776 g vs 923 g), respectively and feed:gain (1.93 vs 1.69), respectively, in broilers fed a rye-based diet with 0 or 500 ppm enzyme (858 β-glucanase and 864 units xylanase), during a 25 day starter period. Boros et al., (1995) reporting on a 42 day trial feeding a 60% rye-based diet supplemented with 1500 units of xylanase and 1000 units of cellulase (*Trichoderma reesei*), observed live weight gain and feed:gain of 2331g and 1.99, respectively, which were slightly less than that observed in the current trial 2500 units of xylanase and 800 units of protease were utilized. The reduction in water:feed consumption observed in this experiment is supported by previous research (Campbell et al., 1983; Choct et al.,1992; Fengler et al., 1988; Friesan et al., 1991; Vranjes and Wenk, 1995). This reduction in water consumption has been highly correlated with reduced vent pasting and litter moisture as well as improved litter conditions. The majority of studies addressing the problem of reduced performance in broilers fed diets with high levels of NSPs were conducted during the starter phase implying that major benefits would be derived during that time. It is also known that gut structures and function are

maturing during the first two weeks of life. Further, it is thought that since intestinal viscosity decreases with age, the efficacy of the enzyme would also decrease as the bird matures(Almirall et al.,1995 and Petersen et al.,1993). The findings in this study demonstrate that major benefits are obtained after week three, and are consistent with those reported in a summary of several experiments with wheat-based diets conducted by Bedford and Morgan (1996). Therefore, in addition to being grain specific, enzyme supplementation needs to be continued throughout the experimental period.

A significant increase in acetate over the experimental period was observed in rye-based diets supplemented with enzyme. This result is inconsistent with those of Choct et al. (1996), who reported a significant reduction in total VFA in the ileum when 40g/kg soluble NSPs were added to a basal sorghum-soybean diet (comparable to a 60% rye diet) and supplemented with 0.1% Avizyme 1300. In that study there may have been losses due to the chromatographic conditions used. Zin et al., (1998) reported total VFA values of 18.07 μmol/mg in a standard corn soybean diet, which is consistent with the results of this study. They reported increased total VFAs in the cecum. Lazaro et al. (1998) also reported that

there was a tendency for acetic acid to increase in rye diets with enzyme supplementation, however, the level of enzyme used may have been too low to elicit a significant response. Wagner and Thomas (1978) conducted a study using diets containing 48% rye and reported cecal butyric acid concentrations of 4.65 and 0.22 μmol/mL in rye and corn-based diets respectively. These results are similar to those observed in the present study.

The significant reduction observed in the population of total pathogenic clostridia in rye-based diets supplemented with Avizyme indicates that unfavorable conditions existed for their proliferation as the experiment progressed. Several researchers have reported that diets high in wheat or rye supported the growth of *Clostridia perfringens* with a concomittant increase in butyric acid (Branton et al.,1997 and Riddel and Kong, 1992). In an earlier study Branton et al. (1996) were unable to elicit a growth response in *Clostridia perfringens* in vitro when wheat extracts were added to a medium. Vranjes and Wenk (1996) also reported reduced neutral detergent fiber (NDF) and acid detergent fiber (ADF) degradability when an antibiotic was fed in diets high in NSPs. It is therefore clear that the effect of improved fiber digestibility is associated with microbial fermentation and other factors. The current research does not indicate increased butyric acid

synthesis which is usually associated with the presence of *Clostridium spp.* ((Branton et al.,1997). Further, the decline in total pathogenic clostridia with enzyme addition also indicates that several *Clostridium spp.* are incapable of sustained growth on soluble depolymerized NSPs. The increase in acetate may be associated with the fermentation by lactobacilli and other bacteria present in the gastointestinal tract. Zin et al., (1998) administered 9 Log_{10} CFU/ml *Lactobacillus acidophilus* or a mixture of *Lactobacillus spp.* to broilers fed a corn-soybean diet and reported a significant increase in total VFA. They reported 58.35 vs 80.09 μmol/mg of cecal and 18.07 vs 23.53 μmol/mg in ileal samples when feeding control or spiked diets. Acetic acid accounted for 49-70% of the total VFA. There was a reduction in pH (6.88 vs 6.53) in the cecum but no significant decrease in pH in the ileum. There was also a significant increase in the population of bifidobacteria (log 7.76 vs 8.5). Singh et al. (1985) reported that coliforms decreased and lactobacilli increased when 0.625g/kg sodium diacetate was added to the diet. It could therefore be concluded that the addition of Avizyme improved performance by at least some depolymerization of the soluble NSPs in rye, which provided fermentable substrates increasing the levels of acetate. Increased acetate may have contributed to improved energy retention and

bacteriocidal activity on specific organisms, including clostridia, which is associated with gastrointestinal diseases. *Salmonella spp.* may be controlled by high levels of acetate since they persisted at very low levels throughout the study. The increase in acetate in the current study is several times that reported by Zin et al. (1998). It may therefore be inferred that a similar or greater decrease in pH is expected.

Several researchers have established that short chain fatty acids (SCFA) are bacteriostatic and bacteriocidal in vitro, provided that sufficient undissociated acid molecules are present and in contact with the organism for a defined period (Salmond et al., 1984 and Young and Foegeding, 1993). The mode of action remains unclear since little work has been conducted in this area. In his review on their mode of action, Russell (1992) explained that SCFA may serve as uncouplers decreasing the proton motive force across the cell membrane with consequent acidification of the cytoplasm. Kashket (1986) also reported that *Clostridium acetobutylicum* had a lower phosphotransferase activity when acetate accumulated within the cell. Cell exhaustion is thought to result from increased utilization of ATP to maintain homeostasis. Growth of several organisms including *Clostridium* spp. may also be inhibited by anion accumulation. Acetate may therefore have elicited

a bacteriocidal effect on some organisms, while improving energy retention. The possibility of bacteriocins, hydrogen peroxide or other mechanisms of control of pathogenic organisms by lactobacillus or other beneficial species should be further investigated. The contribution of pentose sugars and VFA to improved energy retention due to the partial depolmerization of NSPs in rye should also be quantified in order to formulate poultry rations in a cost effective manner.

The observed increased ileal mucosal weight in birds fed rye based diets throughout the experimental period may be attributed to the trophic effects of acetate, since it explains 66% of the variability. Several researchers observed similar effects (Jacobs and Schneeman , 1980; Jacobs, 1983; Koruda et al., 1990; Karlstad et al.,1992; Frankel et al., 1994; Lynch et al., 1994). Komai et al. (1982) postulated that the trophic effects were mediated only in the presence of gut microorganisms however Sakata (1987) demonstrated trophic effects in germ free rats which indicates independence of microbes. It is clear however that microorganisms facilitate the production of VFA through fermentative processes associated with the ingestion of high fiber diets. Increased mucosal weight may also be associated with microbial load in rye based diets. Droleskey et al. (1995) reported extensive colonization of cecal mucosal

epithelium within and between the crypts in 3 day old chicks administered a continuous flow culture consisting of 29 microorganisms. This may be partially responsible for increased mucosal weight.

The protein:DNA and RNA:DNA ratios observed in this experiment are generally similar to those previously reported in rats and chicks (Witlock, 1982; Jacobs, 1983; Karlstad, 1992). The elevated protein:DNA and RNA:DNA observed in rye based diets during week one may be indicative of gut maturation and adaptation to the viscous gastrointestinal environment. These indices of cellular activity also increase with enzyme addition and may indicate hyperplastic growth as well as increased cell turnover. Several researchers have reported hyperplastic growth and increased cellular activity in rats fed various fiber types or triacetin (Jacobs and Schneeman 1980; Jacobs, 1981; Jacobs, 1983; Lynch et al., 1994). The higher population of microorganisms associated with rye-based diets may also be implicated in this process since they persist longer in these diets. Witlock (1982) reported that wet and dry weights of *Eimeria brunetti* infected ceca were significantly increased, indicating cellular increase rather than an edematous condition. In that study cecal protein and DNA increased uniformly in response to the infection. There are also conflicting

increased uniformly in response to the infection. There are also conflicting reports on increases in specific indices of cellular hyperplasia or hypertrophy (Allen et al.,1973 ; Fernando, 1974). Several theories have been advanced to explain increases in mucosal mass and include increases in collagen, proliferation of cells (hyperplastic growth), increase in cell size (hypertrophic growth), increases in gut associated lymphoid tissue and microbial mass or any combination of these factors. In the present experiment the protein:DNA ratio decreases over the first three week indicates hyperplastic growth which stabilized throughout the remainder of the experiment. The increased mucosal mass together with that index indicates hypertrophic growth throughout the remainder of the experiment. RNA:DNA stabilized in week two and is an indication of decreased cellular activity which is consistent with gastrointesinal development in the bird. These findings are in agreement of those of Dibner et al., 1996 ;Uni et al. 1998). The findings in this study indicate that performance as well as litter conditions improve on the addition of Avizyme to diets with high levels of NSPs Fermentation in the gut increased levels of acetate which may have contributed to improved energy retention as well as inhibit some pathogens Adaptation to the changed GIT environment also occurs as birds mature.

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APPENDIX 1

TABLES AND FIGURES EXPERIMENT 1

Table 1. Non starch polysaccharide content of some ingredients (% DM)

Item	Soluble NSPs	Insoluble NSPs	Total NSPs	Main NSPs (%DM)
Wheat	2.4	9.0	11.4	Arabinoxylan-6.05 , B-D-Glucan- 0.5 Cellulose- 2.0
Rye	4.6	8.6	13.2	Arabinoxylan-8.9 , B-D-Glucan-1.0 Cellulose-1.3
Barley	4.5	12.2	16.7	Arabinoxylan-3.3 , B-D-Glucan-7.6 Cellulose 3.6
Sorghum				Arabinoxylan-2.8, B-D-Glucan-0.1
Maize				Arabinoxylan-4.2, B-D-Glucan-0.1 , Cellulose-2.6
Soybean meal	13.9	16.4	30.3	Arabinoxylan-3.6, Cellulose 4.6, Pectin-14.0
Rape seed meal	11.3	34.8	46.1	Complex polymer

Englyst, 1989; Annison, 1991; Englyst et al., 1992; Haddam and Aman, 1987; Chang et al., 1989; Carre, 1992.

TABLE 2. Composition of experimental starter diets %

Ingredient	1	2	3	4	5	6
Rye	59.85	-	59.75	-	59.66	-
Corn	-	58.2	-	58.1	-	58.0
Soybean meal	26.30	37.12	26.13	37.12	26.13	37.12
Fishmeal	5.0	0.0	5.0	0.0	5.0	0.0
Vegetable fat[1]	6.0	.40	6.0	.40	6.0	.40
DL-Methionine	.25	.29	.25	.29	.25	.29
Dicalcium Phosphate	.97	1.8	.97	1.8	.97	1.8
Limestone	.82	1.2	.82	1.2	.82	1.2
Vit-Min premix[2]	1.0	1.0	1.0	1.0	1.0	1.0
Salt	.15	.15	.15	.15	.15	.15
Avizyme ®	0.0	0.0	0.05	0.05	.10	.10
Analysis Calculated						
ME, kcals/kg	2900	2900	2900	2900	2900	2900
CP, %	23.0	23.0	23.0	23.0	23.0	23.0
Calcium%	1.0	1.0	1.0	1.0	1.0	1.0
Phosphorous%	.45	.45	.45	.45	.45	.45

1. Vegetable fat supplied 9020 kcal ME /kg
2. Vitamin Mineral premix supplied a minimum of 0.08% Cu, 0.00441% I, 1.00% Fe, 1.00%Mn, 0.003% Se, . 075% Zn and also supplied per kg 800,000 IU vitamin A, 299,200 ICU vitamin D_3, 2992 IU Vitamin E, 2.2 mg Vitamin B_{12}, 165 mg Menadione, 19.8 mg Biotin, 5500 mg Choline, 99 mg Folic acid, 6600 mg Niacin , 1100 mg Pantothenic acid, 440 mg B_6, 660 mg riboflavin, 110 mg Thiamine.

TABLE 3. Composition of experimental growing -finishing diets %

Ingredient	1	2	3	4	5	6
Rye	66.18	-	66.09	-	65.99	-
Corn	-	64.87	-	64.77	-	64.67
Soybean meal	18.54	29.86	18.56	29.88	18.58	29.90
Fishmeal	5.0	-	5.0	-	5.0	-
Vegetable fat[1]	7.4	1.08	7.4	1.11	7.4	1.15
DL-Methionine	.11	.14	.11	.14	.11	.14
Dicalcium phosphate	1.0	1.8	1.0	1.8	1.0	1.8
Limestone	.84	1.3	.84	1.3	.84	1.3
Vit-Min premix[2]	1.0	1.0	1.0	1.0	1.0	1.0
Salt	.15	.15	.15	.15	.15	.15
Avizyme •	0.0	0.0	.05	.05	.10	.10
Calculated Analysis						
ME, kcal/kg	3000	3000	3000	3000	3000	3000
CP, %	20	20	20	20	20	20
Calcium%	1.0	1.0	1.0	1.0	1.0	1.0
Phosphorous%	.45	.45	.45	.45	.45	.45

1. Vegetable fat supplied 9020 kcal ME /kg
2. Vitamin Mineral premix supplied a minimum of 0.08% Cu, 0.00441% I, 1.00% Fe, 1.00%Mn, 0.003% Se, .075% Zn and also supplied per kg 800,000 IU vitamin A, 299,200 ICU vitamin D_3, 2992 IU Vitamin E, 2.2 mg Vitamin B_{12}, 165 mg Menadione,19.8 mg Biotin, 5500 mg Choline, 99 mg Folic acid, 6600 mg Niacin , 1100 mg Pantothenic acid, 440 mg B_6, 660 mg riboflavin, 110 mg Thiamine.

TABLE 4. Operating Parameters for Gas Chromatographic Analysis

Item	Setting
Carrier gas (He) flow rate	35 cc/min
Injector temperature	200 °C
Detector temperature	250 °C
Initial oven temperature	80 °C
Final oven temperature	160 °C
Temperature program rate	10°/min
Sample volume injected	1 μl
Split/splitless ratio	7:1
Lower detectable limit	0.1μmol/ml
Internal Standard	2 ethyl butyrate

TABLE 5: Effect of Avizyme on cumulative feed:gain in broilers

	Rye			Corn		
	Enzyme concentration%					
Week	0	.05	.10	0	.05	.10
1	1.44[b]	1.30[b]	1.43[ab]	1.66[a]	1.57[ab]	1.55[ab]
2	1.38[b]	1.34[b]	1.26[b]	1.66[a]	1.41[b]	1.39[b]
3	1.70[ab]	1.51[b]	1.44[b]	1.78[a]	1.60[b]	1.46[b]
4	1.84[a]	1.63[b]	1.58[b]	1.77[a]	1.69[ab]	1.58[b]
5	1.99[a]	1.72[b]	1.69[b]	1.85[ab]	1.83[ab]	1.75[b]
6	2.09[a]	1.80[b]	1.82[b]	1.91[b]	1.88[b]	1.82[b]
7	2.19[a]	1.86[b]	1.96[b]	1.99[ab]	1.96[b]	1.91[b]

[ab] Least squares means (n = 10, reduced by 1 bird/week), with different superscripts within rows are significantly different ($P < 0.01$) SEM ± .071

TABLE 6: Effect of Avizyme on cumulative weight gain (g) in broilers

	Rye			Corn		
	Enzyme concentration %					
Week	0	.05	.10	0	.05	.10
1	197	242	233	191	195	197
2	428	519	556	370	413	423
3	694[b]	886[b]	967[a]	682[b]	725[b]	731[b]
4	1068[b]	1268[a]	1406[a]	1114[b]	1177[b]	1147[b]
5	1613[b]	1930[a]	2083[a]	1626[b]	1649[b]	1741[b]
6	2264[b]	2560[a]	2756[a]	2223[b]	2229[b]	2383[b]
7	2929[b]	3217[a]	3310[a]	2801[b]	2683[b]	3286[a]

[ab]Least squares means (n = 10, reduced by 1 bird/week), with different superscripts within rows are significantly different (P < 0.03) SEM ± 68.08

TABLE 7: Effect of Avizyme on water:feed consumption ratio in broilers

	Rye			Corn		
	Enzyme concentration %					
Week	0	.05	.10	0	.05	.10
1	2.77^{a}	2.46^{ab}	2.26^{b}	2.32^{b}	2.26^{b}	2.57^{b}
2	3.05^{a}	2.31^{b}	2.02^{b}	2.39^{b}	2.04^{b}	2.14^{b}
3	2.63^{a}	1.80^{b}	1.89^{b}	1.72^{b}	1.61^{b}	1.90^{b}
4	3.40^{a}	3.27^{ab}	2.92^{b}	2.74^{b}	2.48^{b}	1.92^{c}
5	2.09^{a}	1.95^{ab}	1.62^{b}	1.45^{b}	1.61^{b}	1.09^{c}
6	2.62^{a}	2.41^{a}	1.76^{b}	1.62^{b}	1.84^{b}	2.00^{b}
7	2.82^{a}	2.90^{a}	2.26^{b}	1.97^{b}	2.07^{b}	2.18^{bc}

^{abc} Least squares means (n = 10, reduced by 1 bird/week), with different superscripts within rows are significantly different ($P < 0.01$) SEM ± .15

TABLE 8: Effect of Avizyme on Ileal VFA in broilers over 7 weeks (umol/ml)

	Rye			Corn		
	Enzyme concentration %					
VFA	0	.05	.10	0	.05	.10
Acetate	80.67[c]	118.43[a]	103.42[b]	20.15[d]	18.43[d]	20.13[d]
Isobutyrate	4.23	3.41	3.88	1.92	1.81	2.33
Butyrate	.98	1.66	1.17	.83	.38	1.04
Isovalerate	1.40	2.54	1.49	.40	.35	.92
Propionate	.45	.98	.05	.62	.40	.08

[abcd] Least squares means (n = 6), with different superscripts within rows are significantly different ($P < 0.05$)
SEM ± 2.39 - 3.07 (range for level x acid)

TABLE 9: Effect of Avizyme on protein:DNA ratio of gut mucosa in broilers fed rye or corn-based diets

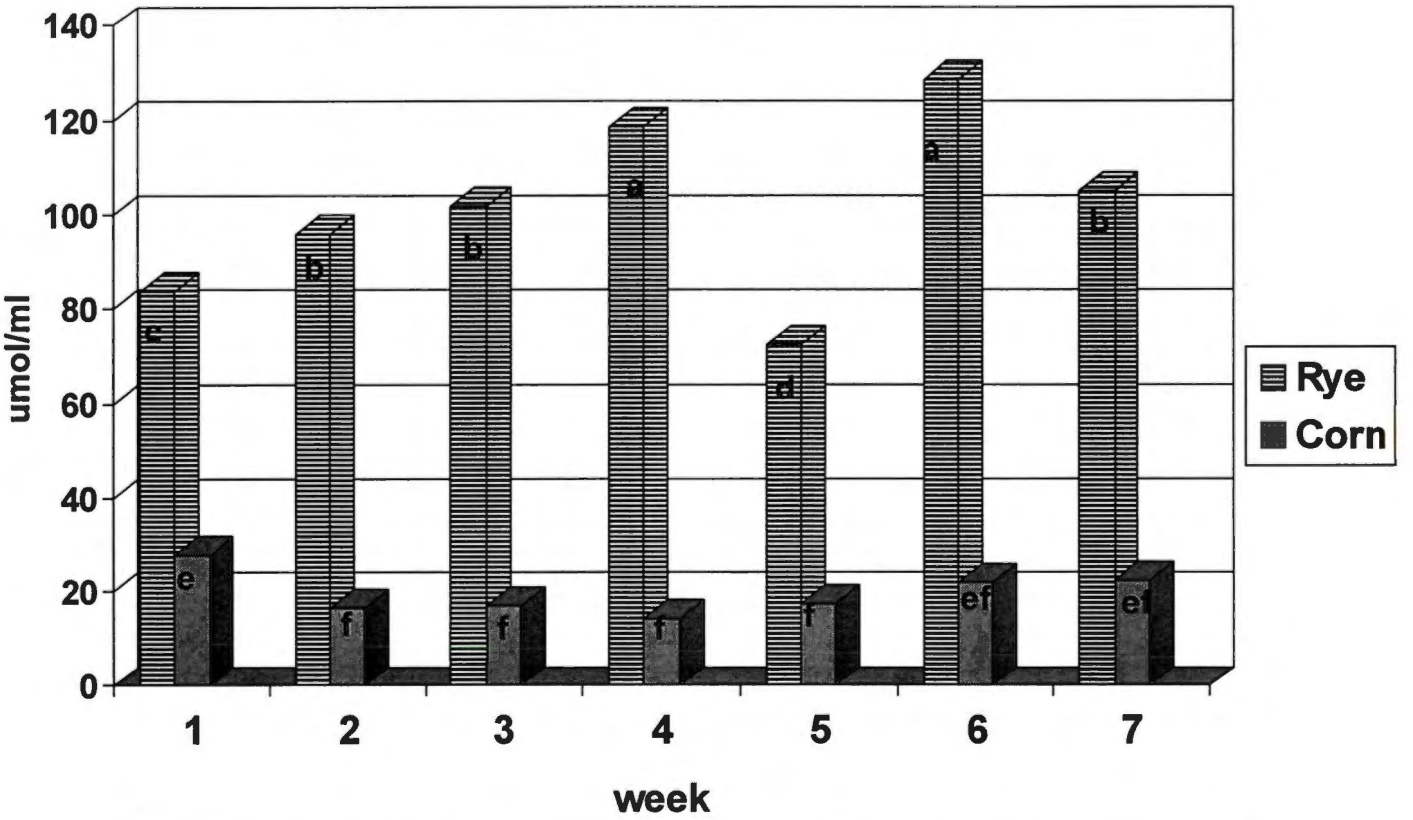
	Rye			Corn		
	Enzyme concentration %					
Week	0	.05	.10	0	.05	10
1	37.81^{c}	96.85^{a}	76.68^{b}	50.96^{c}	36.79^{c}	46.22^{c}
2	34.21^{b}	44.08^{ab}	56.30^{a}	42.37^{ab}	57.72^{a}	44.14^{ab}
3	23.36^{ab}	34.49^{ab}	50.56^{a}	19.78^{ab}	46.01^{ab}	33.88^{ab}
4	30.64	30.93	30.61	32.19	32.90	45.52
5	28.53	33.23	33.47	20.38	31.30	30.88
6	22.30	22.08	19.90	19.11	31.57	22.11
7	33.62	24.06	27.39	26.03	26.01	27.96

^{abc} Least squares means (n = 6) with different superscripts within rows are significantly different (P < 0.01) SEM ± 6.23 - 7.62 (range for level x enzyme)

TABLE 10: Effect of Avizyme on RNA:DNA ratio of gut mucosa in broilers fed rye or corn -based diets

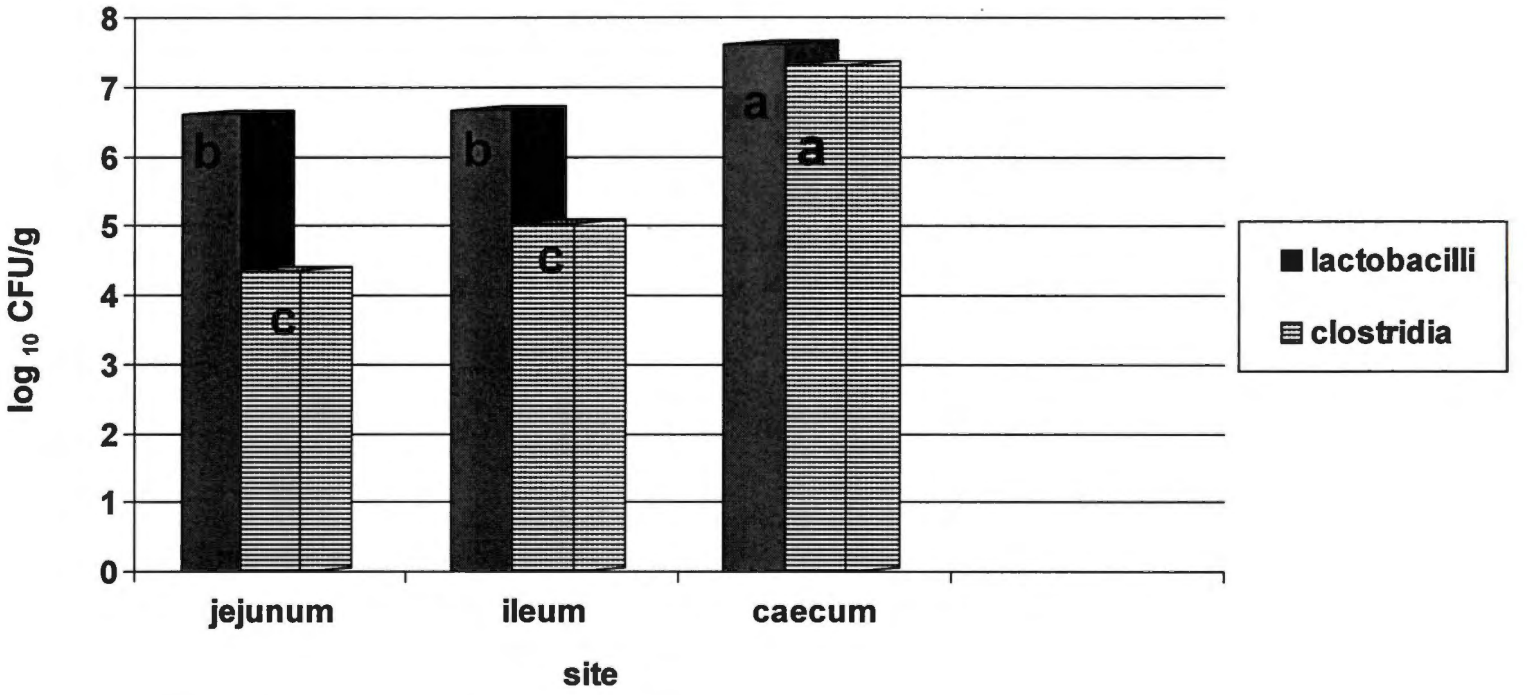
	Rye			Corn		
	Enzyme concentration %					
Week	0	.05	.10	0	.05	.10
1	5.26[d]	9.96[ab]	11.63[a]	9.54[bc]	6.30[d]	8.10[c]
2	2.29	1.32	1.95	1.33	2.69	1.65
3	1.69	2.18	2.23	3.19	2.45	2.01
4	2.48	2.13	3.05	3.04	2.04	1.55
5	3.44	2.15	2.50	1.99	1.78	1.87
6	1.80	1.27	1.88	1.38	1.09	1.54
7	2.40	1.82	2.94	2.92	2.97	2.45

[abcd] Least square means with different superscripts within rows are significantly different ($P < 0.01$) SEM ± 0.63 - 0.78 (range for level x enzyme)



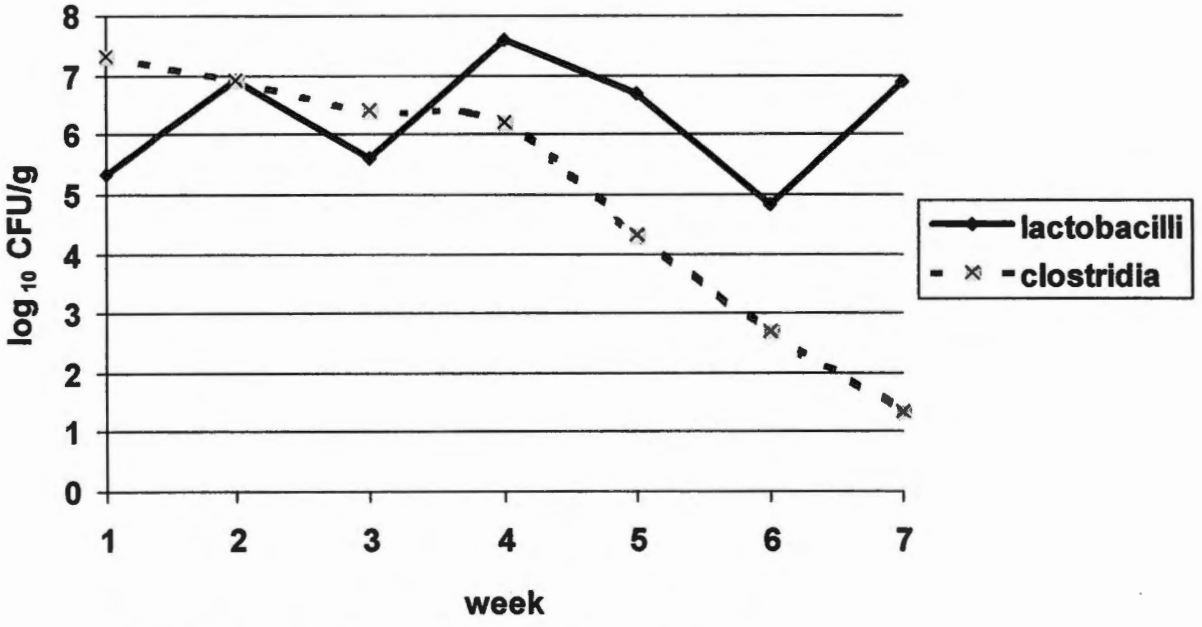
[a-f] Least square means (n = 6) across bars with different superscripts are significantly different (P<0.01) SEM ± 4.41-4.89 (range for acid x week)

Fig 1: Effect of time on ileal Acetate levels in broilers fed rye or corn -based diets



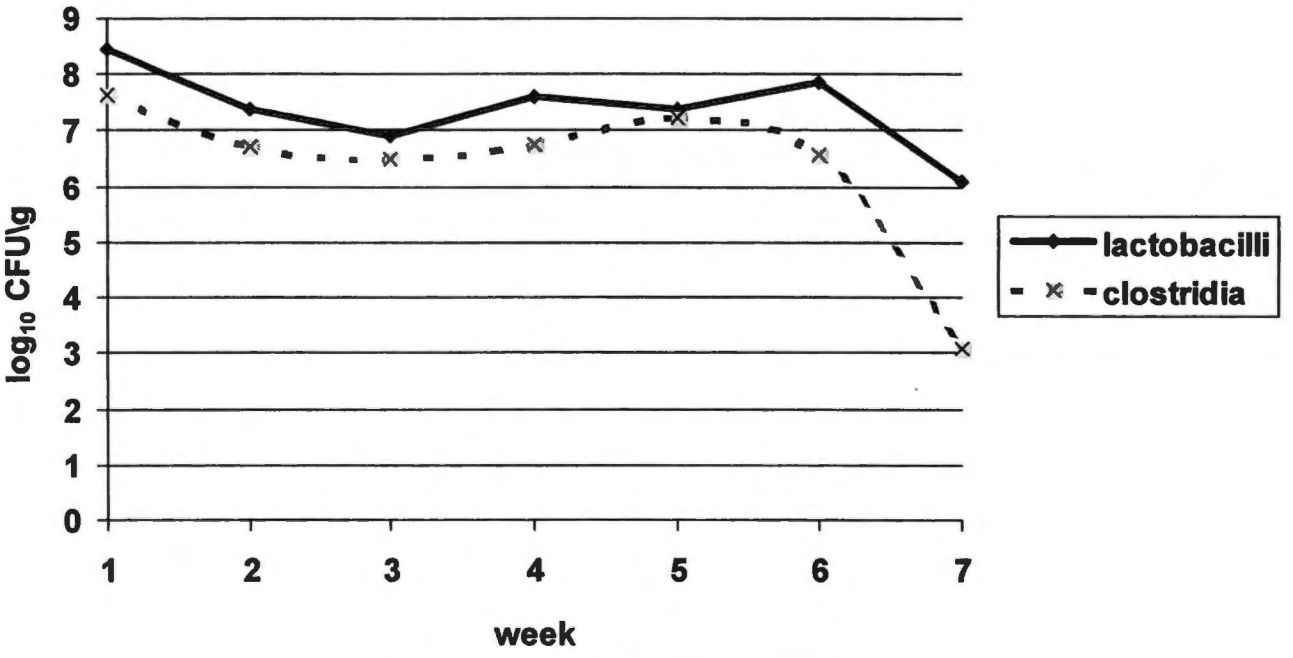
[abcd] Least square means with different superscripts are significantly different ($P < 0.01$) SEM ± 0.24 - 0.29 (range for organism x site). Data are averaged over grain type and enzyme level

Fig 2: Effect of site on intestinal lactobacilli and total pathogenic clostridia in broilers fed rye or corn-based diets



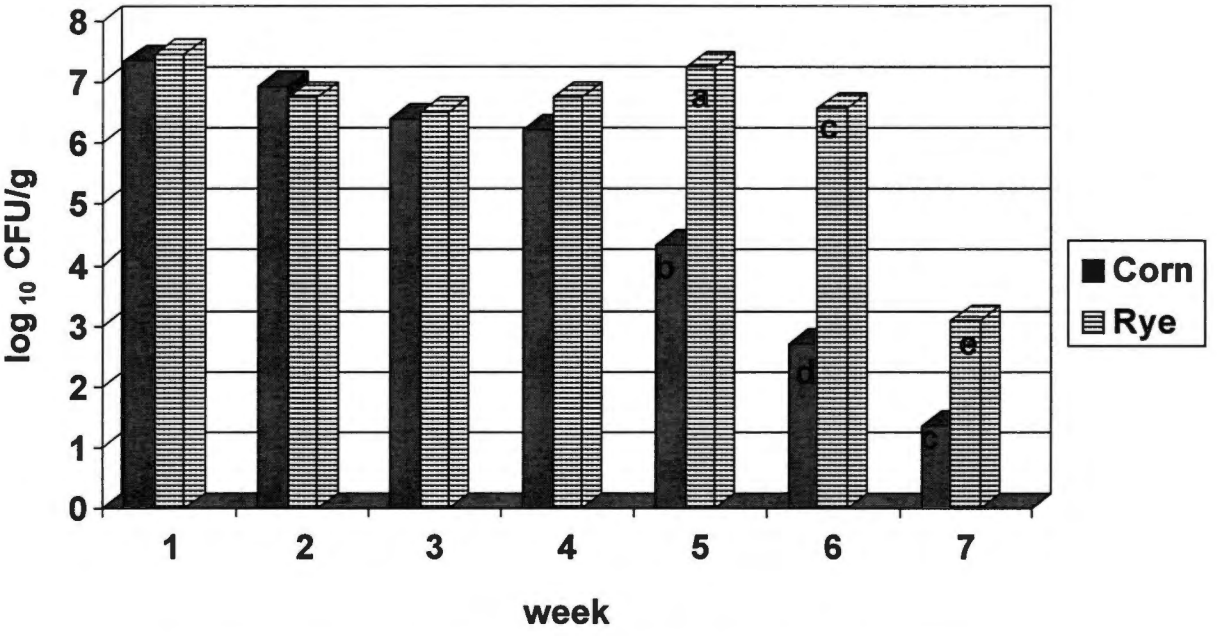
Data arre averaged over the ileum, cecum and lejunum

Fig 3: Effect of time on population of lactobacilli and clostridia in broilers fed corn-based diets



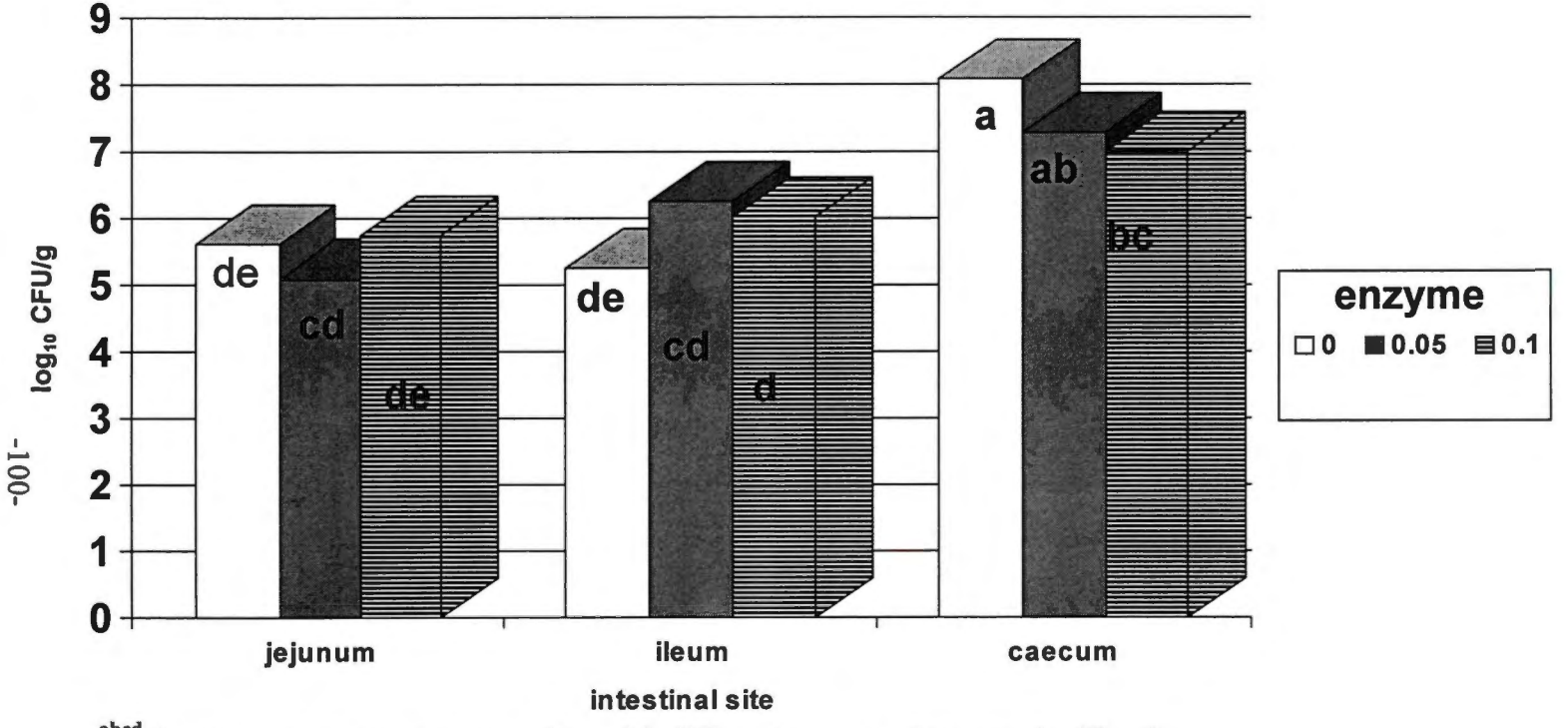
Data averaged over ileum, jejunum and cecum

Fig 4: Effect of time on population of lactobacilli and clostridia in broilers fed rye-based diets



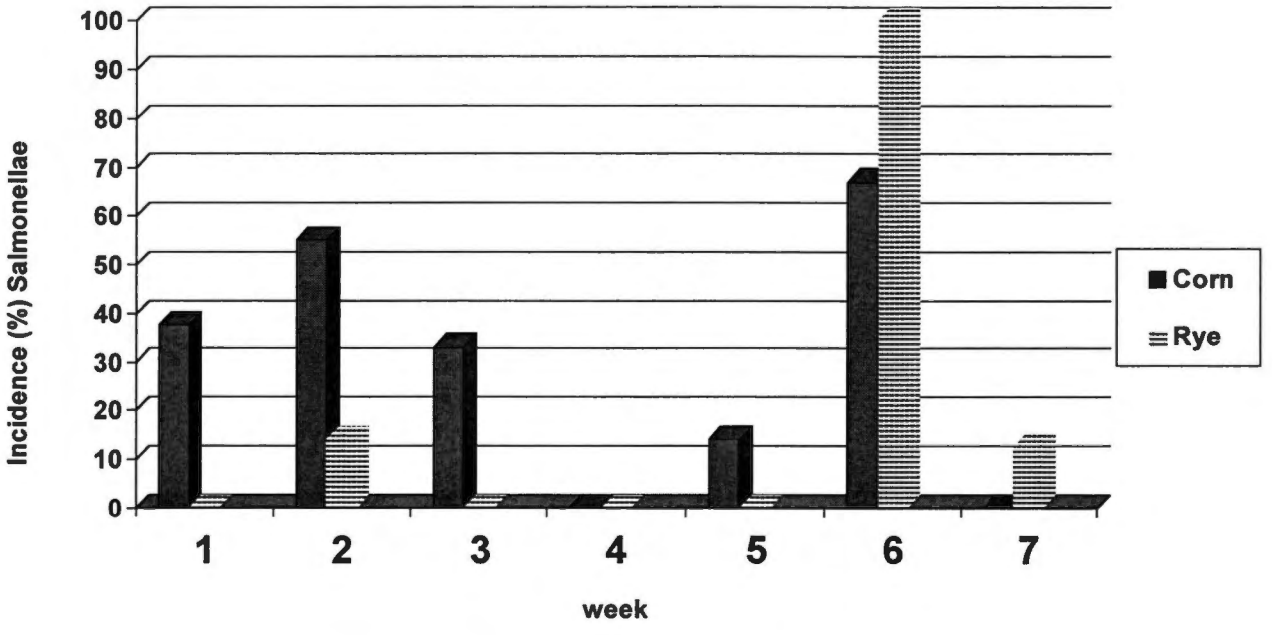
[abcde] **Least squares means with different superscripts are significantly different (P < 0.02) SEM ± 0.40 - 0.67 (range for organism x week)**

Fig 5: The effect of time on total pathogenic clostridia in broilers fed rye and corn -based diets



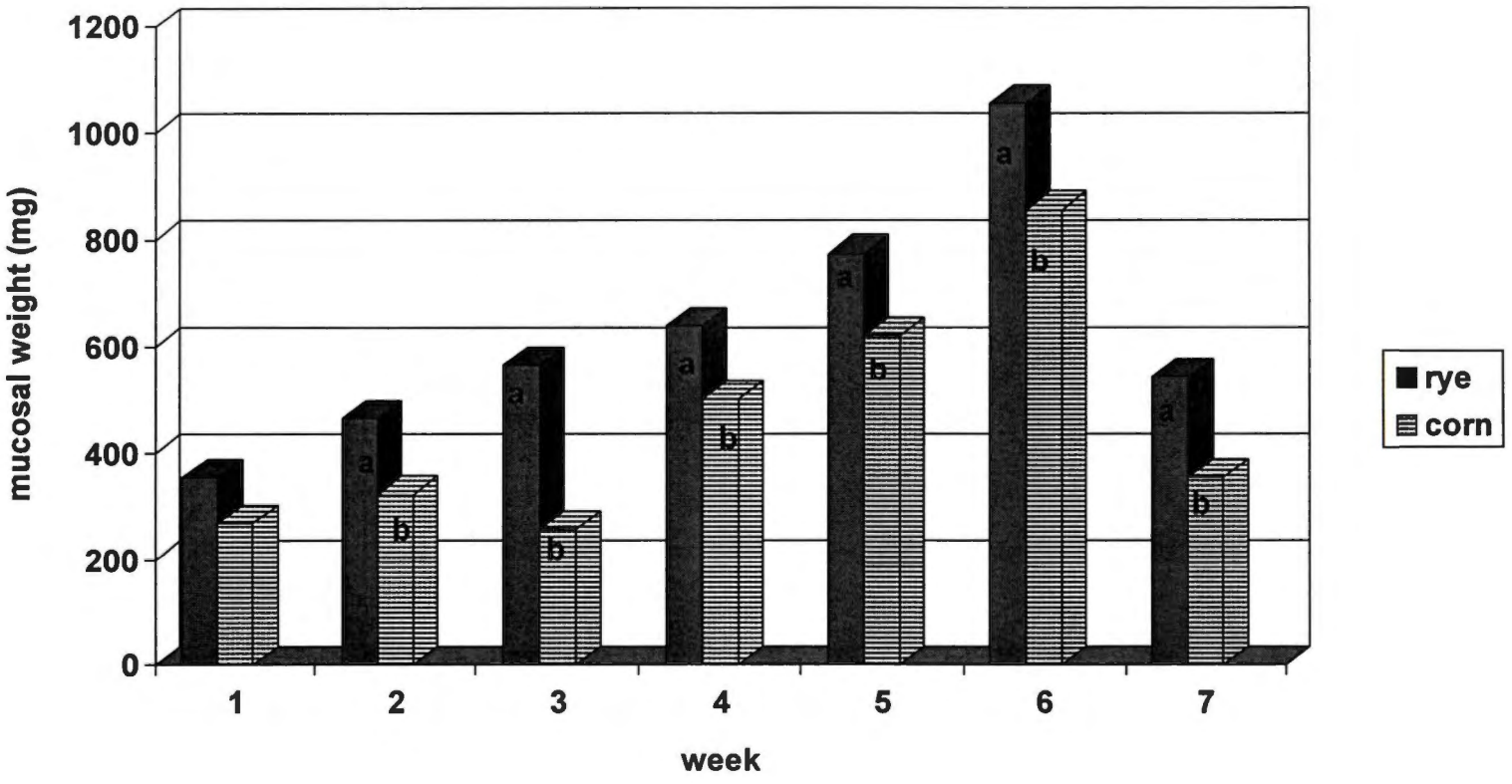
[abcd] Least squares means across sites with different superscripts are significntly different ($P < 0.05$) SEM ± 0.37 - 0.56 (range for enzyme x site)

Fig 6: Effect of Avizyme on total pathogenic clostridia on site in broilers fed rye or corn-based diets



Data are salmonellae recovered as a % of all birds tested (n =126) within treatment x week (n = 9)

Fig 7: Effect of grain type on the incidence of *Salmonella spp.* in the gastrointestinal tract of broilers



[ab] Least squares means (n = 9) with different superscripts within week are significantly different (P < 0.05) SEM± 5.93 -24.25 (range for week x mucosa) . Data are expressed/ 10 cm of ileum

Fig 8: Effect of rye and corn based diets on mucosal weight (mg) in broilers

PART 4

Experiments 2 and 3

Effects of Avizyme in rye or corn-based diets and response to a challenge with *Salmonella typhimurium* on broiler performance, microbiological and fermentation changes in the gastrointestinal tract

Abstract

Two experiments were conducted utilizing male broilers to determine the effect of Avizyme® on non starch polysaccharides in rye-based diets. Avizyme® which contains 2500 units of xylanase and 800 units of protease/g was added to the experimental diets at the rate of 0 and 0.1%. Feed efficiency, weight gain and water consumption were measured as indices of performance, whereas lactobacilli, total pathogenic clostridia and *Salmonella typhimurium* populations assessed gastrointestinal health and potential food safety hazards. Microflora were determined in the crop, ileum and cecum. Volatile fatty acids (VFA) determinations in the distal ileum assessed fermentation activity. Interactions of VFA with microflora, gut mucosa and performance were assessed.

The addition of Avizyme® to rye-based diets consistently improved feed efficiency and weight gain which was equal to that observed in corn-based diets ($P < 0.05$). Water : feed consumption ratio declined significantly with the addition of the enzyme to the rye-based diets ($P < 0.05$). Performance was unaffected by the carrier state in birds challenged with *Salmonella typhimurium.*

Lactobacilli populations were relatively stable throughout the

experimental period irrespective of treatment.. When birds were challenged with *Salmonella typhimurium*, clostridia populations remained stable and acetate levels were low. *Salmonella typhimurium* tended to increase with the addition of Avizyme and persisted longer and at a higher level in rye based diets supplemented with the enzyme ($P < 0.05$). *Clostridium spp.* were higher in the cecum regardless of grain type but *Salmonella typhimurium* was recovered primarily in the cecum and crop.

(Key words: Fermentation, mucosa, DNA, *Salmonella,* clostridia, broilers)

Introduction

In Experiment **1** it was observed that the addition of Avizyme to rye-based diets improved performance of birds which was equal to that of corn based diets. It was also noted that the predominant volatile fatty acid (VFA) produced was acetate and it was concluded that the VFA mediated bacteriocidal effects on the population of total pathogenic clostridia as well as trophic effects on gastrointestinal mucosa. In that experiment the level of salmonellae was very low and did not facilitate the testing of the theory that members of that genus would persist under conditions of high levels of pentose sugars. Therefore, birds were challenged with a nalidixic acid resistant strain of *Salmonella typhimurium* to assess that interaction.

It has been demonstrated that common sources of contamination by *Salmonella spp.* include feed, feces, rats and dust. Low challenge doses (< 10 cells) in the crop are sufficient to cause salmonellosis (Nurmi and Rantala, 1973). The presence of lactobacilli and other beneficial organisms is thought to exert control of pathogenic species through the production of lactic acid, VFA, hydrogen peroxide and bacteriocins. *Lactobacillus reuteri, L. salivarius and L. animales* are the three predominant species of lactobacilli present in chickens (Edens et al. 1997). In addition to killing mechanisms previously mentioned, *Lactobacillus reuteri,* produces reuterin (3, hydroxypropionaldehyde) which has broad spectrum antimicrobial activity at low levels (Edens et al. 1997). It has also been demonstrated that birds challenged with *Salmonella typhimurium* develop asymptomatic carrier states which serve as a source of contamination during processing. The lymphoid system as well as the liver and other organs become infected during establishment of the carrier state. The practice of fasting birds overnight before processing reduces gastrointestinal contents however the crop may become infected since its pH rises and birds ingest litter. Experiment **2** focused on microbiological and fermentation changes occurring in birds challenged with a nalidixic acid resistant strain of

Salmonella typhimurium. Enumeration of Lactobacilli and total pathogenic clostridia were also done on ileal and cecal digesta. Experiment **3** examined the effect of the challenge and fasting on the persistence of *Salmonella typhimurium* in the crop, Ileum and cecum under field conditions.

Materials and Methods

1. General Management

One hundred twenty, one-day-old, Hubbard male broiler birds were randomly assigned to twelve experimental units and housed in a Petersime brooder battery in Experiment two. On day one, the temperature was set at 32°C and reduced over the next three weeks until 23°C was achieved. At three weeks of age, birds were transferred to Petersime grower batteries. Each pen within the battery was equipped with a device to facilitate water measurement.

Starter and growing-finishing rations which were fed ad libitum from hatch to three weeks and four to six weeks, respectively, were formulated to be isocaloric and isonitrogenous and are described in **Tables 1** and **2**. Diets were formulated to meet or exceed NRC requirements (NRC,1994). Individual birds were wing banded at the beginning of the trial and weighed each week. Feed consumption, water intake and body weight gain were

determined each week.

In Experiment three, sixty, one-day-old, Hubbard male broiler birds were randomly assigned to six experimental units and housed in 4' x 4' pens with wood shavings. The temperature regime was previously described in Experiment **2.** Starter and growing-finishing rye based diets utilized in this trial were supplemented with 0.1% Avizyme and were previously described as diet **3, Tables 1 and 2** in Experiment **2.** These diets were fed ad libitum from hatch to three weeks and four to seven weeks, respectively, and were formulated to be isocaloric and isonitrogenous. Individual birds were wing banded at the beginning of the trial and weighed each week. Feed consumption and body weight gain were also determined each week.

2. Microbiological Challenge

A strain of *Salmonella typhimurium* 798 (#4232) acquired from the USDA Animal Disease Control Center was used in this experiment. Two hundred μl of inoculum were added to trypticase soy broth (BBL), containing 50 μg/ml nalidixic acid and incubated for 24 to 48 hours at 36.5°C in a shaking incubator. The sample was then serially diluted in peptone water, plated on XLT4 (Difco) agar with and without 50 μg/ml nalidixic acid, and incubated at 37°C for 24 to 48 hours. Typical colonies were aseptically

removed from several plates and incubated in trypticase soy broth containing 50 μg/ml nalidixic acid and incubated for 24 to 48 hours at 36.5°C, followed by plating on XLT4 with and without 50 μg/ml nalidixic acid and incubation. This process was repeated several times until a population of 9 $\log_{10}$ CFU/ml of broth was achieved (Draughon, 1997). Typical colonies were confirmed as *Salmonella typhimurium* using the Triple sugar iron (TSI , Difco) and Lysine iron agar (LIA , Difco) biochemical tests. The population of *Salmonella typhimurium* in the culture used as a challenge for the birds in experiments **2** and **3** was determined as 9 $\log_{10}$ CFU/ml using the aforementioned protcol.

3. Microbiological media and methods

In Experiment **2** the ileum and cecum of twelve birds were examined for the presence of *Salmonella typhimurium* before the challenge culture was administered using XLT4 agar with and without 50 μg/ml nalidixic acid. In Experiment **3** the crop, ileum and cecum of six birds were examined for the organism. The protocol used was previously described. All procedures pertaining to the care and treatment of the experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC), University of Tennessee, Knoxville.

Approximately 1ml of broth containing the nalidixic acid resistant strain of *Salmonella typhimurium* was administered by gavage using sterile syringes and calgon tubes in Experiment **2.** In Experiment **3,** one ml of the challenge culture was administered in 5 g of feed, which was consumed within 10 min. Three days after the challenge, one bird from each replicate in each treatment was killed by cervical dislocation and the contents of the ileum and caecum examined for colonization by *Salmonella typhimurium.*

Subsequent to colonization with the nalidixic acid resistant strain of *Salmonella typhimurium,* one bird was randomly selected from each treatment on weeks 1, 3, 5 and 6, killed by cervical dislocation and one gram of the contents of the distal ileum and caecum were aseptically removed for the following analyses. In experiment three the crop, ileum and cecum were examined on weeks 1,3, and 6 (before and after a 10 hour fast).

A: *Salmonella typhimurium* and lactobacilli

Total lactobacilli and *Salmonella typhimurium* were determined by placing 1g of intestinal contents in 9 ml of buffered peptone water; followed by serial dilutions in peptone water and 100 μl was surface plated on deMann rogosa sharpe (MRS, Difco) agar acidified to pH 5.4 and xylose lysine tergitol (XLT4) agar respectively. All plates were incubated at 37^0C

and checked at 24 and 48 hours. Samples deemed negative for *Salmonella typhimurium* by presumptive methods were enriched in tetrathionate and selenite cystine broths, incubated for 48 hours, plated on XLT4 and incubated at 37^{0}C for 24 to 48 hours. Typical colonies were confirmed on LIA and TSI slants.

B: Total pathogenic clostridia

One gram of intestinal contents was placed in 9 ml vials of peptone water plus 1% sodium thioglycollate. All samples were flushed with nitrogen during this process. After serial dilution in reduced sodium thioglycollate and peptone water, total pathogenic clostridia were determined by surface plating 50μl on Clostrisel agar in an anaerobic chamber. Clostrisel agar is a highly selective medium for cultivation of pathogenic clostridia from wounds, fecal specimens and soil (Atlas, 1993). Selective ingredients include sodium formaldehyde sulfoxylate, sodium azide and neomycin sulphate. Simultaneously, plating was done on *Clostridium perfringens* agar, OPSP (Oxoid) as a positive control. The anaerobic chamber was fitted with a palladium catalyst system (Coy Laboratory Products, Detroit MI) which reduced trace oxygen from the chamber. The catalyst was changed twice weekly. An analyzer (Coy Laboratory Products) capable of detecting 0 to

2000 ppm oxygen was used to monitor oxygen levels in the chamber. Oxygen levels were kept below 200 ppm in the chamber. The reduced chamber environment was maintained by utilizing a premixed gas consisting of 90% nitrogen, 5% carbon dioxide and 5% hydrogen. Routine flushing of the chamber was done to remove oxygen which may have been introduced through the port. Plates were placed in glass jars, vacuum sealed before leaving the chamber and incubated anaerobically at 37°C for 48 hours. Typical colonies on the Clostrisel agar were confirmed using the catalase test (Mac Faddin, 1980).

4. Analyses of ileal Volatile Fatty Acids

Distal ileal samples were determined for total acetic, isobutyric, butyric, valeric and isovaleric volatile fatty acids using a gas chromatographic method modified by Playne (1985). The parameters for this analysis were previously detailed in **Table 4.** In synopsis, one and one half millilitres of supernatant were combined with 300 μl of 25% metaphosphoric acid (5:1) and allowed to stand at room temperature for 30 min and then centrifuged. One μl of the sample was then injected into a Hewlett Packard model 5890 gas chromatograph equipped with a HP-FFAP 10-m x 1μm capillary column, with cross-linked polyethylene glycol-TPA packing and a flame ionizing

detector.

Results

Experiment 2

The performance of birds on rye diets or rye diets supplemented with 0.1% of enzyme was significantly improved, as evidenced by the cumulative feed:gain ratio (2.17 vs 1.76) respectively, at week six ($P < 0.01$). This effect was not evident in the corn based diets which were similarly supplemented, **(Table 3).** Cumulative weight gain also improved from week five ($P < 0.04$), **(Table 4).** Overall water:feed consumption ratio was also reduced ($P< 0.04$), by the addition of the enzyme, **(Table 5).** The populations of total pathogenic clostridia and lactobacilli were stable throughout the experimental period in rye or corn based diets, however, the population of clostridia was higher in the rye-based-diets. *Salmonella typhimurium* increased with the addition of the enzyme in rye based diets, ($P < 0.02$), although it was not significantly different from the level in corn-based diets, **(Table 6).** *Salmonella typhimurium* was also significantly higher in the caecum regardless of grain type, **(Fig. 1).** Total pathogenic clostridia persisted at higher population densities ($P < 0.05$), in rye based diets and was also higher in the cecum **(Table 7 and Fig. 1).** *Salmonella typhimurium* was

not recovered by direct plating in week seven but was recovered throughout the experiment in birds on both grain types by enrichment techniques **(Table 7, Figs. 2 , 3)**. The incidence of *Salmonella typhimurium* was higher in rye-based diets than in the corn based diets in week five ($P < 0.05$), **(Fig. 2 and 3)**. Acetate concentration was significantly higher in rye based diets, however it was at its lowest level in week five ($P < 0.05$), **(Table 8 and Fig. 4)**.

Experiment 3

There was no significant difference observed in the performance of birds fed rye and challenged with *Salmonella typhimurium* and birds not challenged, **(Table 9)**. Colonization by this organism was significantly higher in the cecum and ileum in week **1,** ($P<0.05$) and persisted in the crop and cecum throughout the experiment, **(Fig 5, 6)**. No recovery was made in the unchallenged birds.

Discussion and Conclusion

Experiments 2 and 3

The improved performance observed in birds fed rye and Avizyme was similar to that previously reported in Experiment one ($P < 0.01$). Similarly, water:feed consumption ratio declined ($P < 0.04$). These results

clearly demonstrate that an enzyme preparation containing 2500 units of xylanase and 800 units of protease is effective in improving bird performance when added to rye-based diets. Reports by several researchers including Bedford et al. (1996), Lazaro et al. (1998) and Vranjes and Wenk, (1995), are in general agreement with these findings.

Birds challenged with a broth containing 9 $\log_{10}$ CFU/ml *Salmonella typhimurium* resulted in colonization of approximately 5 $\log_{10}$ CFU/g and 3 $\log_{10}$ CFU/g of cecal and ileal contents respectively. This phenomena is fairly well documented (Cox et al., 1972; Chambers et al., 1997; Xu et al., 1988). Colonization of the gastrointestinal tract (GIT) may be limited by transit time and pH of the various sections. Reported ranges of pH in the GIT are as follows: crop (4.1 - 5.0); proventriculus (4.4); ventriculus (2.6); small intestine (5.8 - 6.4); large intestine (6.3) and caecum (6.0 - 7.4), (Farner, 1943 and McNab, 1973). These levels may be altered by diet. Although the concentration of volatile fatty acids is higher in the cecum, it is more likely to be colonized by *Salmonella typhimurium* since the pH is usually between 6.5 and 7.5 and the pKa of acetate is 4.8. Therefore the dissociated form of the molecule is more likely to be predominant. This observation is supported by the work of Jayne-Williams and Fuller (1971)

and Thompson and Hinton (1997). The population of this organism in rye-based diets supplemented with Avizyme is significantly higher than that in the unsupplemented diets. This may be the result of increased uptake and utilization of pentose sugars released as a result of depolymerization, or increased uptake of pentose sugars together with uptake and utilization of acetate or other products of mixed acid fermentation by other organisms within the gastointestinal tract. These activities may have led to the decreased levels of acetate observed with higher populations of *Salmonella typhimurium*. Increased pentose uptake by *Salmonella typhimurium* under inducible conditions has been reported (Shamanna and Sanderson, 1979). Competitive inhibition of acetate- producing *Veillonella* and other genera may also explain the low levels of acetate in rye based diets which had a relatively high numbers of salmonellae. Hinton et al. (1993) reported on the control of *Salmonella spp.* by a mixed culture of *Veillonella* spp. which had been isolated from adult chickens. Inhibition was thought to be achieved through the production of high levels of acetate by these species. It was previously reported that *Veillonella* spp. metabolized lactate and produced acetate and propionate as end products (Johns, 1951a,b). Overall the population of *Salmonella typhimurium* decreased significantly over time in

both sites, while the population of total pathogenic clostridia remained relatively constant. Results observed in the ileum may be an interaction of viscosity and rate of passage, since there is no difference in grain type. The determination of the complex interaction of the metabolism of pentose sugars and viscosity in a mixed population of *Salmonella typhimurium*, lactobacilli, clostridia and other predominant species in the gastrointestinal tract requires a more extensive model. It is however clear that organisms capable of producing and utilizing acetate are the main effectors of change.

Birds in Experiment **3** performed as well as those in earlier trials. There is clearly no apparent deleterious effects on health nor performance of birds challenged with *Salmonella typhimurium*. However, birds maintained a carrier state throughout the experimental period. The incidence of the organism was higher in the crop and cecum, than that in the ileum after the 10 hour fasting period. Previous reports suggest that the crop is a significant reservoir of salmonellae and serves as a source of contamination during processing (Chambers et al., 1998; Corrier et al., 1998; Hinton et. al., 1998). In those studies it was demonstrated that pH increased from 3.64 to 5.14 during a 4-8 hour fast. Lactic acid was significantly reduced and *Salmonella typhimurium* increased from 3 to 13%. It is however generally agreed that

the ceca is the primary reservoir for the organism (Barrow et al., 1988; Brownell et al., 1969; Xu et al., 1988). The absence of *Salmonella typhimurium* in birds fed rye and Avizyme without the challenge indicates a low probability of infection in an environment free of the organism. Diets high in NSPs with or without enzyme supplementation result in litter with a higher moisture content than corn-based diets. That environment therefore presents more favorable growing conditions for several organisms than dry litter. It has been reported that salmonella persisted to a greater extent in moist (A_w 0.90 - 0.95) than in dry litter (A_w 0.79- 0.84). Harry et al. (1973) also reported that high litter moisture affected the efficacy of methyl bromide gas when used to sanitize poultry houses. In that study there was a reduction of over 99% of the *Salmonella typhimurium* except when it was applied to wet litter. Therefore, continuous monitoring must be done for this organism when rye or other grains high in NSPs are fed. Addition of the enzyme reduces water intake in rye diets but the feces have a higher water content than birds on corn-based diets. The litter moisture will therefore be higher and may enhance the proliferation of pathogenic organisms.

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Appendix 2

Tables and Figures
Experiments 2 and 3

TABLE 1. Composition of experimental starter diets %

Ingredient	1	2	3	4
Rye	59.85	-	59.66	-
Corn	-	58.2	-	58.0
Soybean meal	26.30	37.12	26.13	37.12
Fishmeal	5.0	0.0	5.0	0.0
Vegetable fat[1]	6.0	.40	6.0	.40
DL-Methionine	.25	.29	.25	.29
Dicalcium Phosphate	.97	1.8	.97	1.8
Limestone	.82	1.2	.82	1.2
Vit-Min premix[2]	1.0	1.0	1.0	1.0
Salt	.15	.15	.15	.15
Avizyme®	0.0	0.0	.10	.10
Analysis Calculated				
ME, kcals/kg	2900	2900	2900	2900
CP, %	23.0	23.0	23.0	23.0
Calcium, %	1.0	1.0	1.0	1.0
Phosphorous, %	.45	.45	.45	.45

1. Vegetable fat supplied 9020 kcal ME /kg
2. Vitamin Mineral premix supplied a minimum of 0.08% Cu, 0.00441% I, 1.00% Fe, 1.00%Mn, 0.003% Se, . 075% Zn and also supplies per kg 800,000 IU vitamin A, 299,200 ICU vitamin D_3, 2992 IU Vitamin E, 2.2 mg Vitamin B_{12}, 165 mg Menadione, 19.8 mg Biotin, 5500 mg Choline, 99 mg Folic acid, 6600 mg Niacin , 1100 mg Pantothenic acid, 440 mg B_6, 660 mg riboflavin, 110 mg Thiamine.

TABLE 2. Composition of experimental growing -finishing diets %

Ingredient	1	2	3	4
Rye	66.18	-	65.99	-
Corn	-	64.87	-	64.67
Soybean meal	18.54	29.86	18.58	29.90
Fishmeal	5.0	5.0	-	5.0
Vegetable fat[1]	7.4	1.08	7.4	1.15
DL-Methionine	.11	.14	.11	.14
Dicalcium phosphate	1.0	1.8	1.0	1.8
Limestone	.84	1.3	.84	1.3
Vit-Min premix[2]	1.0	1.0	1.0	1.0
Salt	.15	.15	.15	.15
Avizyme ®	0.0	0.0	.10	.10
Calculated Analysis				
ME, kcal/kg	3000	3000	3000	3000
CP, %	20	20	20	20
Calcium, %	1.0	1.0	1.0	1.0
Phosphorous, %	.45	.45	.45	.45

1. Vegetable fat supplied 9020 kcal ME /kg
2. Vitamin Mineral premix supplied a minimum of 0.08% Cu, 0.00441% I, 1.00% Fe, 1.00%Mn, 0.003% Se, .075% Zn and also supplied per kg 800,000 IU vitamin A, 299,200 ICU vitamin D_3, 2992 IU Vitamin E, 2.2 mg Vitamin B_{12}, 165 mg Menadione,19.8 mg Biotin, 5500 mg Choline, 99 mg Folic acid, 6600 mg Niacin , 1100 mg Pantothenic acid, 440 mg B_6, 660 mg riboflavin, 110 mg Thiamine.

Table 3: Effect of Avizyme on cumulative feed:gain in broilers

	Rye		Corn	
	Enzyme concentration %			
Week	0	.10	0	.10
1	1.61[ab]	1.48[b]	1.56[ab]	1.79[a]
2	1.61[ab]	1.48[b]	1.56[ab]	1.75[a]
3	1.63[ab]	1.42[b]	1.61[ab]	1.76[a]
4	1.82[a]	1.54[b]	1.67[ab]	1.88[a]
5	2.00[a]	1.65[b]	1.76[ab]	1.89[ab]
6	2.17[a]	1.76[b]	1.86[b]	1.91[b]

[ab] Least squares means (n = 10, reduced by 1 bird/week) with different superscripts within rows are significantly different ($P < 0.01$) SEM ± .088

Table 4: Effect of Avizyme on cumulative weight gain (g) in broilers

	Rye		Corn	
	Enzyme concentration %			
Week	0	.10	0	.10
1	161	182	163	147
2	323	338	315	298
3	615	719	627	548
4	939[ab]	1079[a]	1065[ab]	899[b]
5	1341[b]	1552[a]	1602[a]	1456[ab]
6	1805[c]	2214[a]	2116[ab]	1996[b]

[ab c] Least squares means (n = 10, reduced by 1 bird/week) with different superscripts within rows are significantly different ($P < 0.04$) SEM ± 66.08

Table 5: Effect of Avizyme on water:feed consumption ratio in broilers

	Rye		Corn	
	Enzyme concentration %			
Week	0	.10	0	.10
1	2.40[a]	1.94[b]	1.62[c]	1.61[c]
2	2.43[a]	2.18[ab]	1.98[b]	1.84[b]
3	2.41[a]	2.14[a]	1.70[b]	1.73[b]
4	2.43[a]	2.26[a]	1.76[b]	2.00[ab]
5	2.58[a]	2.07[b]	1.76[bc]	1.62[c]
6	2.46[a]	2.05[b]	1.92[bc]	1.66[c]

[ab c] Least squares means (n = 10, reduced by 1 bird/week) with different superscripts within rows are significantly different (P < 0.04) SEM ± .12

TABLE 6: Effect of Avizyme on population of *Salmonella typhmurium*, lactobacilli and clostridia in broilers fed rye and corn -based diets

	Log_{10}CFU/g of digeata					
	lactobacilli		clostridia		*Salmonella typhimurium*	
Enzyme%	Rye	Corn	Rye	Corn	Rye	Corn
0.0	9.4	8.8	7.8	7.2	2.2^{b}	2.2^{b}
0.1	9.2	9.1	7.4	6.4	3.5^{a}	2.8^{ab}

[ab] Least squares means (n = 36) with different superscripts are significantly different (P< 0.02) SEM ± .027 Data averaged over ileum and cecum (n =24)

Table 7. Effect of time on population of *Salmonella typhimurium*, lactobacilli and clostridia in broilers fed rye and corn-based diets

	lactobacilli		clostridia		*Salmonella typhimurium*	
Week	Rye	Corn	Rye	Corn	Rye	Corn
1	9.21	9.15	6.73	6.58	3.21	3.24
3	9.54	9.23	8.29[a]	7.14[b]	3.67	3.30
5	9.11	8.31	7.87[a]	6.70[b]	1.63	0.98
6	8.02	7.80	6.20	6.23	-	-

[ab] Least squares means (n = 12) with different superscripts within row are significantly different (P < 0.05) SEM ± 0.32 - 0.37 (range of week x organism)

Data averaged over ileum and cecum (n = 12)

Table 8: Effect of time on acetate and butyrate plus isobutyrate in ileal digesta of birds fed rye or corn-based diets (umol/ml)

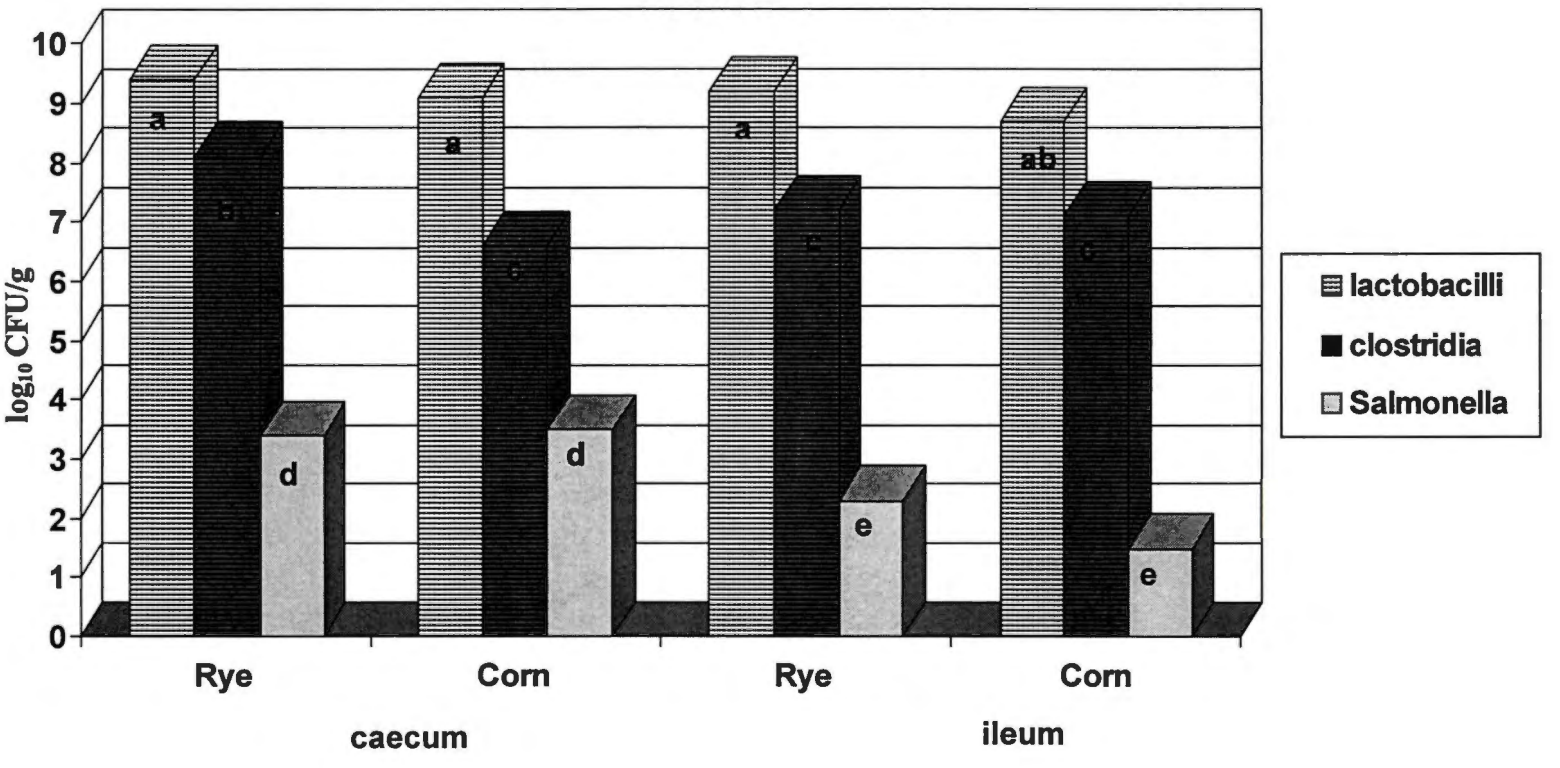
	Acetate		Isobutyrate +Butyrate	
Week	Rye	Corn	Rye	Corn
1	34..78	22.08	4.77	2.55
3	39.69[a]	16.05[b]	3.70	1.94
5	22.75[b]	63.35[a]	2.62	5.44
6	111.35[a]	42.96[b]	5.97	3.68

[ab] Least squares means (n = 12) with different superscripts within rows are significantly different (P < 0.05) SEM ± 7.05

Table 9: The effect of 0.1%Avizyme on cumulative feed:gain ratio a nd weight gain in birds fed rye based diets with (S+) and without (S-) *Salmonella typhimurium*

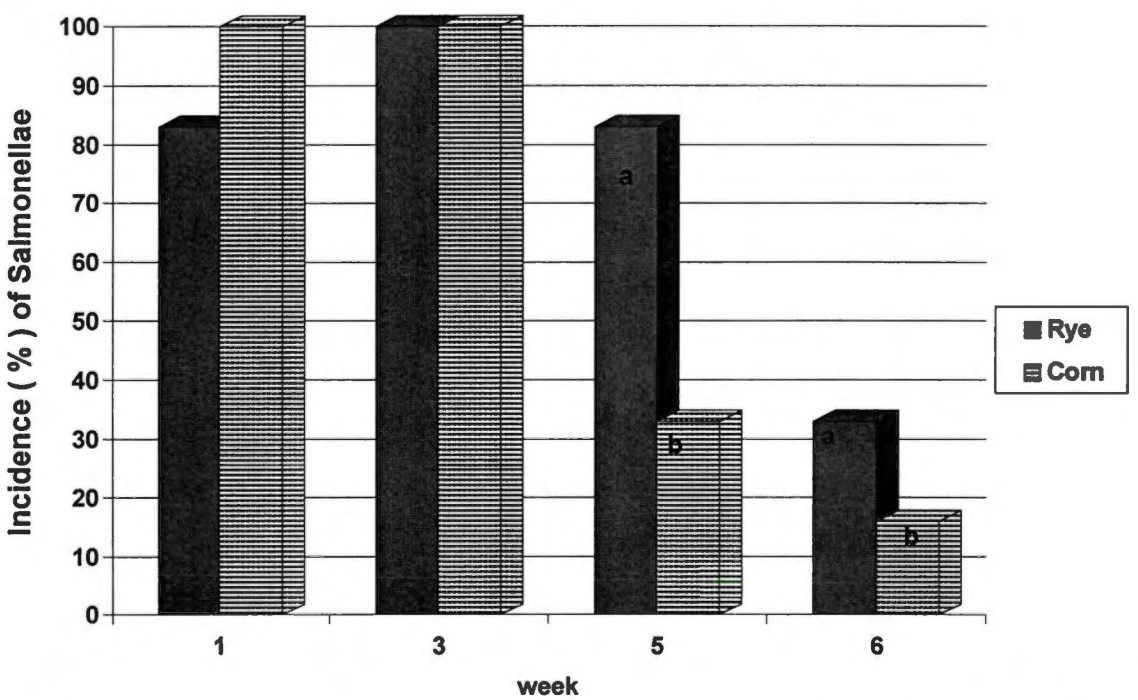
	Feed:Gain		Weight gain (g)	
Week	S+	S-	S+	S-
1	1.57	1.55	181	173
2	1.59	1.80	339	369
3	1.46	1.74	684	690
4	1.63	1.58	1119	1082
5	1.88	1.75	1687	1669
6	1.99	1.91	2167	2225

Least squares means (n = 10, reduced by 1 bird/week) are not different (P > 0.05)
SEM ± .05 (FCR); SEM ± 34.52 (Gain)



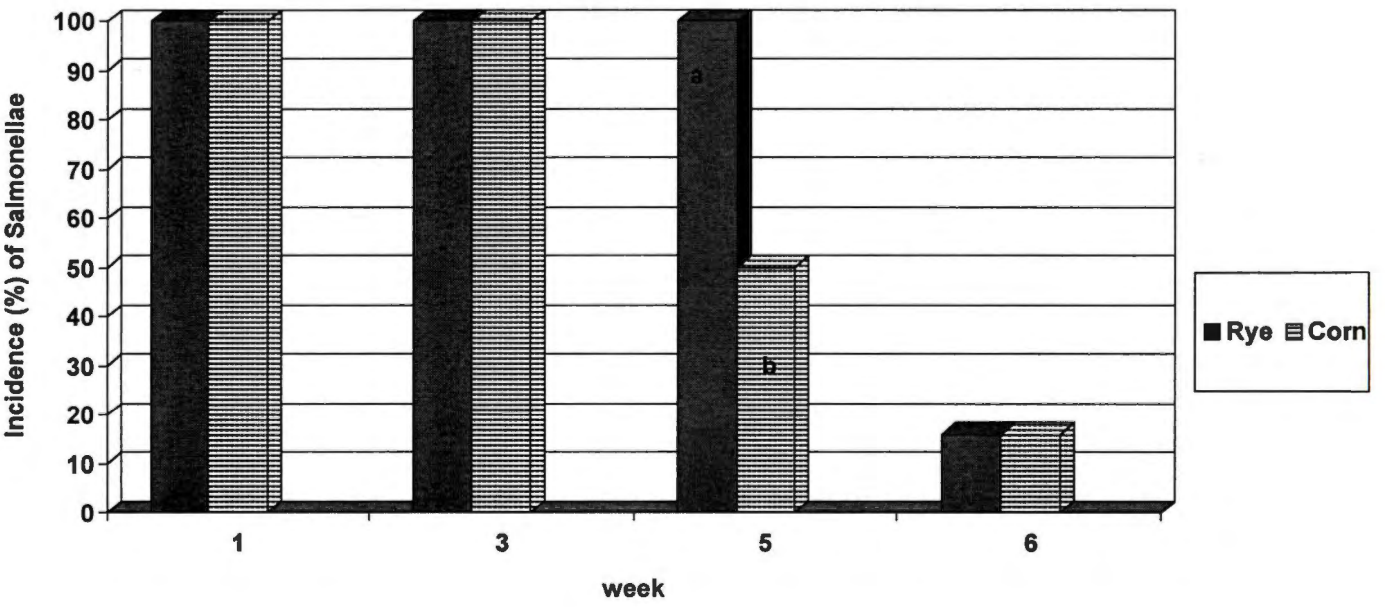
[abcde] Least squares means (n = 12) with different superscripts across columns are significantly different
(P < 0.02) SEM ± 0.28

Fig 1: Effect of site on *Salmonella typhimurium*, clostridia and lactobacilli in broilers fed rye or corn-based diets



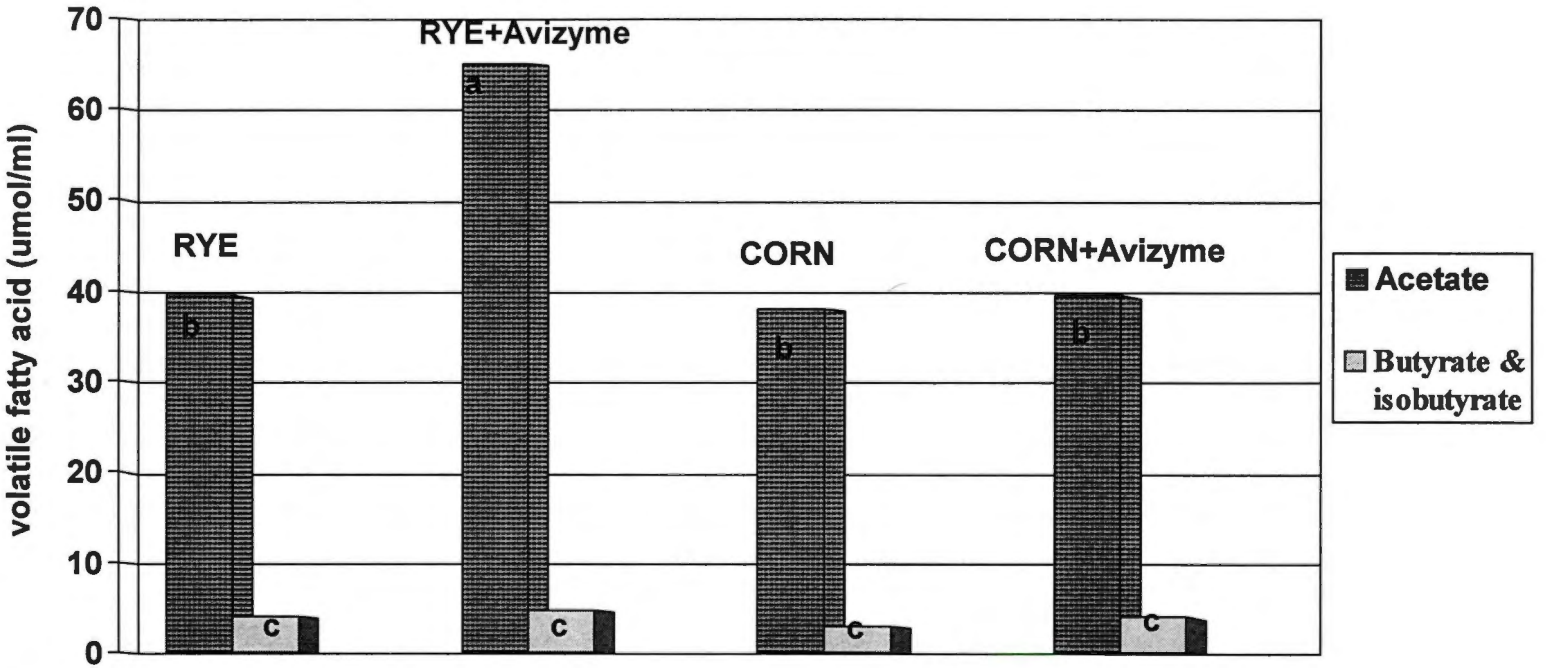
[ab] **Least squares means (n = 6) with different superscripts are significantly different (P < 0.05) SEM ± 11.8. Incidence is expressed as a % of total number of birds tested**

Fig 2 The effect of time on *Salmonella typhimurium* in Ileal digesta of broilers fed rye or corn-based diets



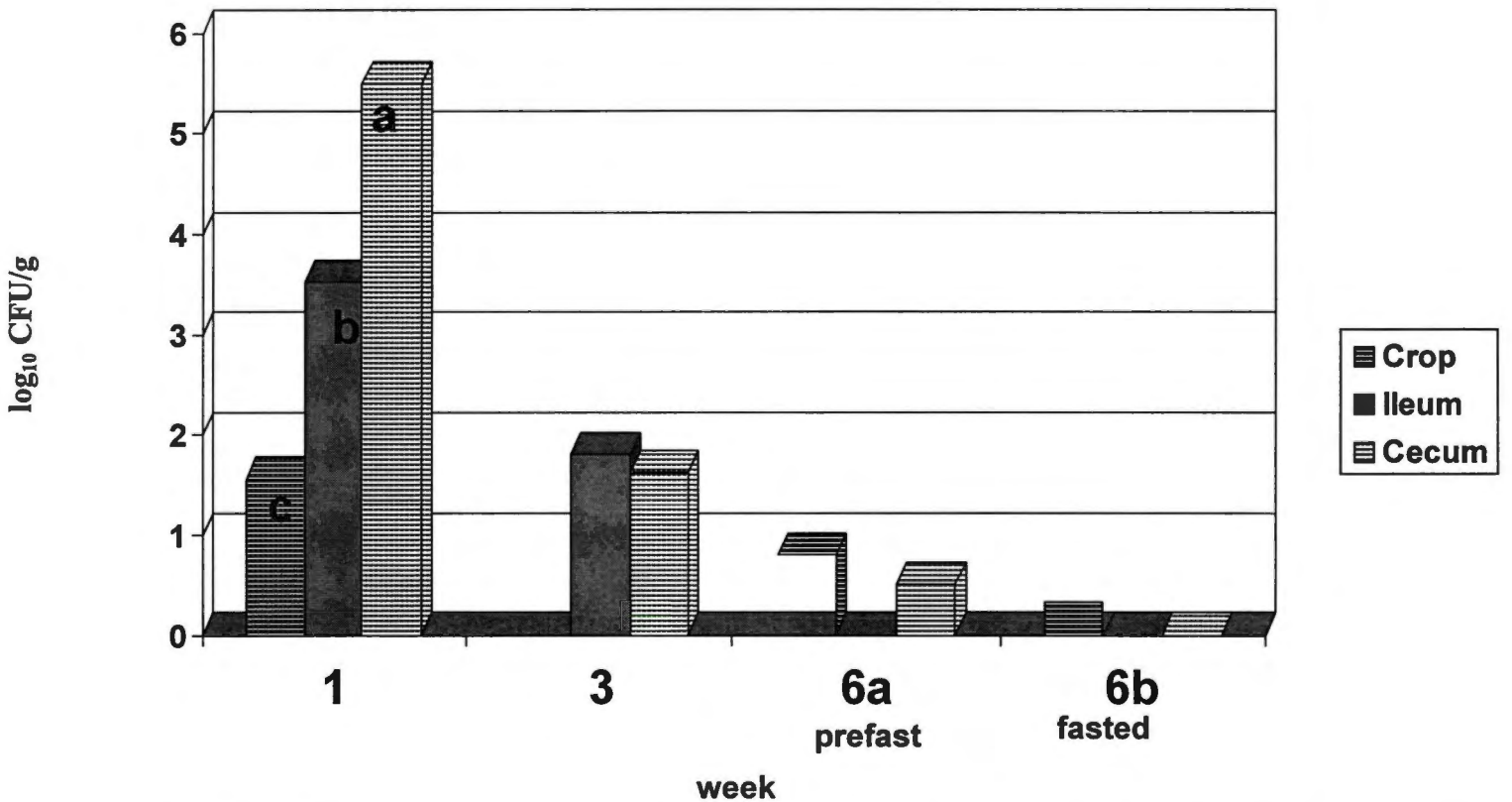
[ab] **Least sqares means (n =6) with different superscrits are significantly different (P< 0.05) SEM ± 11.8 Incidence is expressed as a % of total number of birds tested**

Fig 3: The effect of time on *Salmonella typhimurium* in the cecum of broilers fed rye or corn-based diets



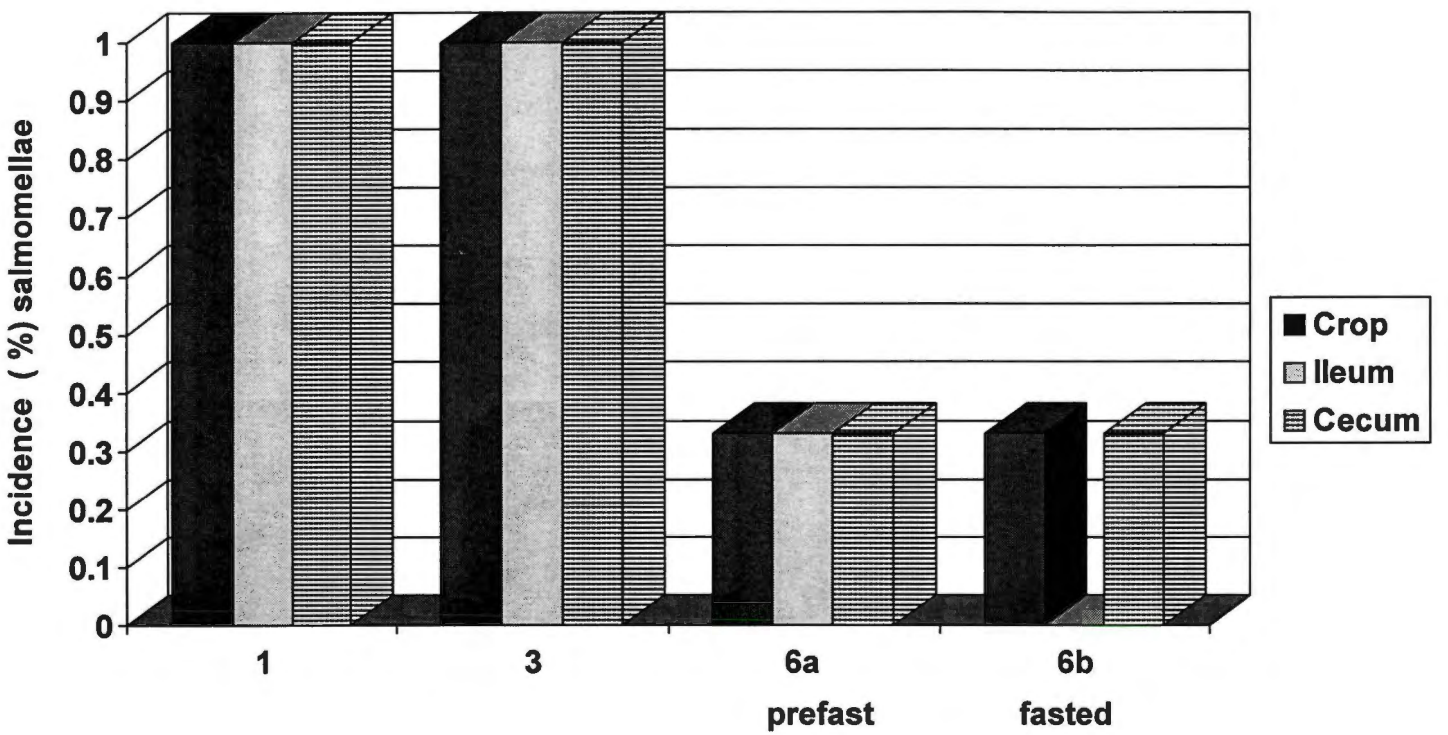
[abc] Least squares means (n =36) with different superscripts across bars are significantly different (P< 0.05) SEM ± 4.98

Fig 4: Effect of Avizyme on ileal acetate and butyrate +isobutyrate in broilers fed rye or corn-based diets



[abc] Least squares means (n = 9) with different superscripts within time are significantly different (P < 0. 05) SEM ± 0.41

Fig 5 : Effect of site on the population of *Salmonella typhimurium* in broilers fed rye-based diets with Avizyme



Least squares means (n = 9) within time are not different (P > 0.05) SEM ± 0.21
Incidence is expressed as a % of total number of birds tested

Fig 6. Effect of site and time on the incidence of *Salmonella typhimurium* in broilers fed rye -based diets and Avizyme

APPENDIX 3

DNA, RNA AND PROTEIN PROCEDURES

DNA, RNA AND PROTEIN ANALYSES

Samples: Gut sample + 5 ml saline (thaw , dissect muscle from sample, add water to sample to equal 5 g).

Note: Always make a blank.
Make standard solutions for DNA, RNA AND PROTEIN.

1. Homogenize sample for 30-45 sec; Remove .714ml standard and sample and place in 12 ml polypropylene tubes.

2. Add .286 ml of 35% TCA to all tubes. Store the remainder of the sample at -70°C.

3. Add 4 ml of 10% TCA to each tube.

4. Vortex (approximately for 30 sec).

5. Centrifuge 3000 x g (4000 rpm) for 10 min.

6. Discard supernatant.

LIPID EXTRACTION

7. Add 4 ml of 2% potassium acetate in a 4:1 ethanol water. Vortex, Centrifuge at 3000 x g (4000 rpm), for 10 min.

8. Discard supernatant.

9. Extract with 4 ml of 3:1 ethanol:chloroform. Vortex, centrifuge at 3000 x g (4000 rpm) for 10 min.

10. Discard supernatant.

11. Extract with 4 ml of 3:1 ethanol : ether. Vortex , Centrifuge at 5000 x g (10,000 rpm) for 10 min.

RNA, DNA AND PROTEIN ANALYSES

12. Discard supernatant.

13. Air dry precipitate under hood- be sure hood is nearly closed for best draft.

Following day:

14. Add 3 ml of .3M KOH to each sample , vortex, incubate for 1h at 37^{0}C.

15. Remove .25 ml for protein analysis.

RNA ANALYSIS:

16. Remove 1 ml of the solution at 14 and add 1 ml of .6M PCA , vortex , centrifuge at 5000 x g (10,000 rpm) for 10 min. Collect supernatant.

17. Wash DNA pellet with 3.0 ml of .2 M PCA , vortex centrifuge at 5000 x g for 10 min. Add supernatant to that collected in step 16.

18. Measure RNA absobance at 260nm (Use UV and quartz cuvettes).

DNA ANALYSIS:

19. Add 2.0 ml of 0.8 M PCA to pellet obtained in step 17 above, vortex , heat at 90^{0}C for exactly 15 mins, cool, cap, vortex and centrifuge at 5000 x g for mins. Save supernatant .

20. Wash precipitate from step 19 with 1 ml 0.8 M PCA , vortex, centrifuge at 5000 x g for 10 min. Add supernatant to that collected in step 19.

21. To combined supernatant, add 1 ml of 0,04% cold Indole and 1.0 ml of concentrated HCL. Vortex and heat at 100^{0}C for exactly 10 min. Cool tubes in running water.

22. Extract water layer (top layer) twice with 4 ml of chloroform each time and centrifuge at 5000 x g. Discard lower layer. Read absorbance at 490nm.

PROTEIN ANALYSIS:

Make fresh **alkaline copper sulphate solution** each day.
1. 50 ml of 0.1 M NaOH plus 1.0 g $Na_2 CO_3$
2. 0.5 ml of 2% (w/v) NaK tartrate.
3. 0.5 ml of 1% (w/v) cupric sulphate

Procedure:

1. Add .75 ml of water to .25 ml of KOH protein solution.

2. To the 1ml solution above add 6.0 ml of alkaline copper sulphate solution. Allow to stand at room temperature for 10 min. After stirring.

3. Add .3ml of phenol reagent and stir. Allow solution to stand at room temperature for not less than 30 min.

4. Determine absorbance at 650nm.

Vita

Patsy Ann Francis was born in Georgetown, Guyana, where she graduated from the Guyana School of Agriculture in 1977 and the University of Guyana in 1986, with a diploma and BSc. in Agriculture respectively. She has served as a livestock extension agent, manager of several livestock enterprises and an agricultural training institution. She has also served as a lecturer at the University of Guyana and several colleges in Guyana. In 1988, she was awarded a LASPAU scholarship to pusue the MSc. in animal science at the University of Tennessee. That program was successfully completed in 1990. In 1995, while teaching at the University of Guyana, she was awarded a Fulbright scholarship to pursue the PhD in animal Science . She was awarded that degree, with a concentration in nutrition in May, 1999. She returned to Guyana where she will serve the University and the country as a whole.