Factors affecting the production of fermented moist feed for chickens and effects on the gastrointestinal environment.

by

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Abstract

Fermented feed technology is a biosafe method of feed preparation and fermentation improves feed quality. Factors affecting lactic acid production in fermented moist feed for chickens and the influence of fermented feed on the gastrointestinal environment of chickens were investigated.

Water quality was observed to affect lactic acid production in fermentation of cereals (maize, sorghum, wheat and barley). The presence of calcium carbonate in the fermentation mixture significantly ($P<0.01$) reduced lactic acid production from maize, sorghum and wheat. Grain particle size did not significantly affect feed fermentation as biosafe concentrations of lactic acid (>150mmol/L) were obtained from fermentation of coarse grain particle sizes. Differences in polyphenols content of red and white sorghum varieties did not have a significant effect on 24-hour lactic acid production from sorghum for any LAB used (*Lactobacillus plantarum* (SLP), *Lactobacillus farciminis*, *Pediococcus acidilactici* (PA1) *L. plantarum* NCIMB 41229).

In vivo experiments on the application of *Lactobacillus plantarum* NCIMB 41229 (of porcine origin) as a feed fermenter and in water for chickens showed a marked reduction of *Salmonella* colonisation between dosing through fermented feed and through water. Dosing through water had a modest response while dosing through fermented feed had a significant effect on the birds' resistance to *Salmonella* colonisation (40% vs 75% of birds resistant). Molecular-based analysis of gut microbial species diversity showed that banding patterns for fermented feed treatments were closely related (>60%). The application of *Lactobacillus plantarum* NCIMB 41229 to chickens through fermented feed could be a better way of controlling *Salmonella* in chickens than through water. *Lactobacillus plantarum* NCIMB 41229 could be used as effective inoculant for the production of fermented moist feed for chickens.

Keywords: Cereals; chickens; fermented moist feeds; gastrointestinal environment; lactic acid bacteria.

FREQUENTLY USED ABBREVIATIONS

AGP	Antimicrobial growth promoters
CP	Crude protein
DM	Dry matter
DNA	Deoxyribonucleic acid
FCR	Feed conversion ratio
FLF	Fermented liquid feed
1 FTU	The amount of enzyme which releases 1 mmol inorganic orthophosphate/ minute under the following conditions (pH 5.5, 37 °C, 0.0061 M sodium phytate) (see Engelen *et al* . 1994).
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal
GLM	General linear model
HPLC	High-performance liquid chromatography
IFLF	Inoculated fermented liquid feed
LAB	Lactic Acid Bacteria
LF	Liquid feed
LF1	*Lactobacillus farciminis* CNCM MA 67/4 R
SLP	*Lactobacillus plantarum* Medipharm
LP2	*Lactobacillus plantarum* NCIMB 41229
MRS	Mann Rogosa Sharpe (broth/agar)
NSP	Non-starch polysaccharides
OD	Optical density
PA1	*Pediococcus acidilactici* CNCM MA 18/5 M
PCR	Polymerase chain reaction
PBS	Phosphate Buffered Saline
RISA	rRNA Intergenic Spacer Analysis

RNA	Ribonucleic acid
SCFA	Short-chain fatty acids
SFLF	Spontaneous fermented liquid feed
SPF birds	Specific pathogen free birds

CHAPTER 4

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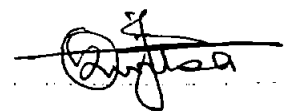
Signed: 

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CHAPTER 1

GENERAL INTRODUCTION

1.1 RATIONALE FOR FEEDING MOIST FEEDS TO CHICKENS

The chicken's gastrointestinal tract (GI) tract undergoes rapid morphological, cellular and molecular changes to accommodate a rapid transition to external nutrients at the end of incubation (Uni 2006). At hatch, the chicks GI tract passes through rapid structural and functional developments (Uni *et al.* 1998). Approximately 2 to 5 % of hatchlings do not survive the critical post-hatch period adjustments and many survivors exhibit stunted growth, poor feed utilisation and meat yield (Uni & Ferket 2004). Nutrient supply immediately after hatch is a critical factor in intestinal development (Uni *et al.* 1998; Uni 2006; van den Brink & van Rhee 2007) and limits problems of post-hatch adjustments (Uni & Ferket 2004). Rapid GI tract development after hatch is also essential for optmisation of digestive function and underpins efficient growth and development as well as a full expression of genetic potential for production traits (Mitchell & Moreto 2006; Mai 2007).

The micro- and macrostructure of the diet, as determined by processing, has large effects on gut function and digestive processes in poultry (Svihus 2006). In broiler chickens, diet structure and conformation during the starter phase plays an important role in the functional development of the fore-gut (Mai 2007).

Early access to semi-moist diets for day-old chicks stimulates GI tract development and prevents dehydration during transport (van den Brink & van Rhee 2007). Significantly ($P<0.05$) greater development of the proventriculus and gizzard of chickens fed wet feeds, compared with dry feeds has been reported (Yasar & Forbes 1999).

Several studies have shown that wet feeding increases the feed intake and growth rate of chickens (Yalda & Forbes 1995; Yalda & Forbes 1996; Yasar & Forbes 1999; Forbes 2003; Mai 2007). Diets containing 700g water/kg feed increased dry matter (DM) approximate digestibility from 0.65 for dry feed to 0.73 for female broilers (Yalda & Forbes 1995). Feed intake, body weight gain and carcass weights were also significantly increased for male broilers, while cockerels of the laying strain had significantly increased in body weight gain. Yalda and Forbes (1996), found that DM digestibility of feed was significantly increased from 634 g/kg for dry food to 659 g/kg for freshly soaked feed and 664 g/kg for feed soaked for 1 hour before mixing and feeding. They concluded that the improved growth rate and feed conversion efficiency (FCE) resulted from improvements in the proportion of feed absorbed from the digestive tract.

The moistening capacity of the crop of chicks during the first weeks of life is also believed to be a limiting factor for the optimal functioning of the gut when standard solid diets are fed (Mai 2007). Yasar and Forbes (1999), attributed the beneficial effects of wet feeding to decreased viscosity of gut contents, greater development of the layer of villi in the digestive segments and reduced crypt cell proliferation in the crypts of the epithelium. Fermented moist feeds for chickens could therefore be useful in improving GI tract development. It could also be quite important for food and environmental safety.

1.2 SCOPE OF THE STUDY

One of the main objectives of the present study was to evaluate factors that could be affecting fermentation of cereals for poultry diets. The aim for this part of the study was to investigate how manipulating such factors could optimise the production of organic acids (primarily lactic acid) and lower pH of the cereal medium. Understanding how the water mineral contents used in fermentation, the particle size of the cereals used and differences in anti-nutrient contents of sorghum varieties may influence the concentration of these two parameters after 24 hour fermentation with different lactic acid bacteria (LAB) is the subject of Chapters **3**, **4** and **5**.

In **Chapter 5**, the influence of lactic acid fermentation on the concentration of anti-nutrients for varieties used was not considered. A detailed study of the starch structure of the cereals used and how this may impact on starch hydrolysis and fermentation end metabolites was also beyond the scope of this study.

Another objective of this study **(Chapter 6)** was to examine the application of *Lactobacillus plantarum* NCIMB 41229 (a lactobacillus isolated from pigs) as a feed fermenter and in drinking water for chickens at different post-hatch periods. It was hypothesised that;

- Provision of fermented feeds or LAB in drinking water for chickens at periods corresponding to landmark phases of gut-associated lymphoid tissue development might upregulate immune response and the bird's ability to resist on enteropathogens challenge.

- Using fermented feeds rather than LAB in drinking water may be a better means of improving gut health in chickens.

1.3 OUTLINE OF THE THESIS

Section 2.0 reviews relevant literature on bacterial fermentation of feed substrates with particular emphasis on fermented feeds for chickens. It examines how feed fermentation can influence gastrointestinal ecophysiology. In **Section 3.0**, the effect of water mineral contents, cereal type and LAB spp used on outcome lactic acid fermentation were investigated using graded concentrations of calcium carbonate in sterile distilled water, four cereals and three LAB. The results of the effect of LAB used and particle size on fermentation of sorghum and maize for poultry feed are described in **Section 4.0**. A comparison of two sorghum grain varieties with different polyphenols contents in lactic acid fermentation for poultry diets is presented in **Section 5.0**.

The application of *L. plantarum* NCIMB 14229 as a feed fermenter and in drinking water for chickens is the subject of **Section 6.0**. The outcome of the results obtained from the different experiments in this study compared with other results from literature are analysed in a concluding discussion in **Section 7.0**.

Chapter 2

LITERATURE REVIEW

2.1 Bacterial fermentation of feed substrates for chickens

2.1.1 Fermented feed technology

Food fermentation is one of the oldest ways of food processing and preservation. Man has known the use of microbes for preparation of food products for thousands of years and all over the world a wide range of fermented foods and beverages contribute significantly to the diet of many people (Achi 2005).

The use of liquid feeds in animals has created an opportunity for recycling of liquid co-products from the human food industry especially in pig nutrition (Scholten *et al.* 1999; Brooks *et al.* 2003a). This has considerably reduced the need for alternative methods of disposal of these products, like drying, disposal to land fill or burning (reviewed by Scholten *et al.* 1999).

However, liquid feeds have the potential to serve as potent reservoirs of enteropathogens unless steps are taken to prevent their introduction and proliferation during storage and feeding (Beal *et al.* 2002). Brooks *et al.* (2001), also stated that liquid-feeding systems can easily become contaminated. They further observed that the development of computerised liquid-feeding systems capable of feeding pigs *ad libitum* has rekindled interest in the possibility of liquid feeding for weaner pigs. This, in addition to recent developments in the use of lactic acid bacteria in the accelerated fermentation of feed substrates for animal feeding as well as reducing the possibility of

contamination by enteropathogens (Beal *et al.* 2002; Beal *et al.* 2005), has provided a good basis for improvement in pig nutrition. It is also having much promise in other farm animal species especially poultry (Heres *et al.* 2003b; Skrede *et al.* 2003) and aquaculture (Refstie *et al.* 2005).

According to Beal *et al* . (2002), lactic acid bacterial fermentation of feeds provides a feed that has a pH of 3.8-4.0 and contains 150-250 mmol/L lactic acid. A similar range of fermented feed pH has been reported by Geary *et al* . (1996) (3.8-4.2), Christensen *et al.* (2007) (3.6-4.2), Scholten *et al* . (1999) (3.5-4.5) and Moran *et al* . (2006)(less than 3.8). The synergistic effect of a high lactic acid concentration and low pH is believed to act in concert to give fermented feeds their antimicrobial activity. This enables them to withstand contamination by pathogens like *Salmonella* spp (Geary *et al.* 1996; van Winsen *et al.* 2001a; Beal *et al.* 2002; van Winsen *et al.* 2002), *Campylobacter* spp (Heres *et al.* 2004), and coliforms (Russell *et al.* 1996).

The mechanism of action of fermented feeds and fermented co-products in controlling enteropathogens both *in vitro* and *in vivo* has been reviewed extensively (Brooks *et al.* 1996; Scholten *et al.* 1999; Hansen *et al.* 2000; van Winsen *et al.* 2001a; Beal *et al.* 2002; Demeckova *et al.* 2002; Hojberg *et al.* 2003; Boesen *et al.* 2004; van Immerseel *et al.* 2004; Beal *et al.* 2005; Moran *et al.* 2006). The antimicrobial effects of lactic acid are believed to be exerted by the ability of the undissociated acid to gain entry into the cell, disrupt pH homeostasis and consequently cause nucleic acid and protein damage (Beal *et al.* 2002). According to Moran (2001), the low pH, dissociation constant (pKa value), and the molar concentration are factors that determine the inhibitory activity of lactic and acetic acid in fermented feed. While inside the cell, the acid dissociates and causes a drop in pH. This stops enzymatic processes and causes the proton motive force

to collapse. The anion may also destroy the cell wall resulting in cell death (van Winsen *et al.* 2001a; van Winsen *et al.* 2001b). However, Alakomi *et al.* (2000) earlier stated that disruption of the outer membrane by acids could involve both dissociated and undissociated forms. As indicated, the likely action on the outer membrane of *Salmonella* could be protonation of anionic components such as carboxyl and phosphate groups. This consequently weakens the molecular interactions between outer membrane components thus increasing its permeability.

In a recent review by Brooks (2008) on fermented liquid feeds for pigs, increasing feed cost, withdrawal or reduction of antimicrobial growth promoters (AGP) in feeds and quality assurance programmes related to *Salmonella* in pig meat were given as reasons why producers should adopt liquid feeding. However, he indicated that the success of liquid feeding depended on;

- Microbial fermentations and selection of LAB capable of generating lactic acid levels above 100mM that can significantly reduce numbers of enteropathogens and the incidence of *Salmonella*.
- Batch fermentation of the cereal portion of feeds with inoculants capable of generating high lactic acid concentrations to give more consistent results of fermentation.
- Fermentations that could preserve the feed, improve the availability of nutrients, reduce the level of anti-nutrients and have LAB with probiotic properties.

Meanwhile, the three principal components involved in the fermentation process are the fermenting micro-organisms, the feed substrate and the enabling environment for fermentation (Figure 2.1).

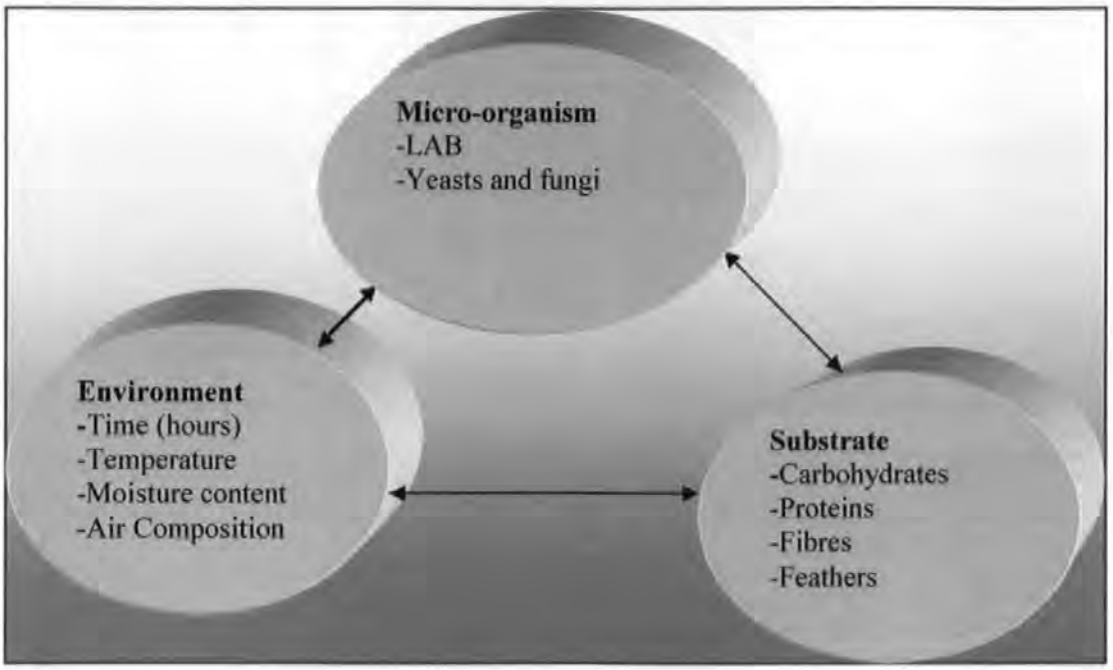


Figure 2.1: Interactions in the fermentation medium. Fermentation conditions influence rate of fermentation, type of microbe and substrate quantity and quality affects the medium.

The present review, examines bacterial fermentation of feed substrates with reference to chickens. Emphasis will be placed on the interplay of factors affecting feed fermentation and their relationship with the quality of feed. The effects of fermented moist feeds on the chicken gut as well as food and environmental safety will also be reviewed.

2.1.2 Influence of micro-organism and feed substrate on fermentation

The selection of LAB for feed fermentation to meet desired feed and production objectives has been highlighted in previous reviews (Brooks *et al* . 2003b; Brooks in press). The choice of feed substrates to obtain high numbers of LAB (10^9 cfu/g feed) and levels organic acids (>150 mM) or a consistent fermentation product has also been researched (Canibe *et al* . 2007a; Lyberg *et al* . 2008; Olstorpe *et al* . 2008) or reviewed (Brooks in press). Fermentation objectives that have influenced the use of LAB have centred on;

- Selection for rapid production of organic acids (mainly lactic acid) to ensure biosafety (e.g. Missotten *et al.* 2007; Missotten *et al.* 2008)
- Selection for homolactic fermentation to improve feed palatability
- Breakdown of anti-nutrients and increased bioavailability of nutrients (e.g. Bertsch *et al.* 2003; Brooks *et al.* 2003a; Oboh 2006; Skrede *et al.* 2007; Lyberg *et al.* 2008; Okpako *et al.* 2008).

A summary of how some of these objectives relate is shown in Table 2.1.

Table 2.1: Effect of micro-organisms and feed substrates on fermentation

Fermentation type	**Substrate**	**pH**	**Lactic acid or effects on diet**	**Acetic acid or effects on diet**	**Ethanol or effects on diet**	**Source**
L. plantarum, *P. pentosaceus*, Yeasts	Wheat and wheat by-products	<3.8 4.53	<60† 22.21*	<10† 22.42*	<10† 9.12*	(Moran *et al.* 2006) (Beal *et al.* 2005)
Spontaneous fermentation. (LAB and Yeasts)	Barley	4.30	34.43*	27.34*	10.74*	(Beal *et al.* 2005)
Lactobacillus brevis	Soybean white flakes	4.8	Elimination of indigestible carbohydrates and lowered trypsin inhibitor activity			(Refstie *et al.* 2005)
Lactobacillus sp (strain AD_2), *L. plantarum* (AM4).	Barley and wheat and barley whole meal flours	-	Significant reduction in phytic acid, dietary fibre and β-glucans (33.5-18.4 g/kg in barley), alpha-amylase activity in barley			(Skrede *et al.* 2001; Skrede *et al.* 2002; Skrede *et al.* 2003; Skrede *et al.* 2007)
Lactobacillus acidophilus	Sesame seed meal	-	Phytic acid reduced to below detectable limits and tannin contents reduced from 20 to 10 g/Kg			(Mukhodhyay & Ray 1999)
Lactobacillus plantarum	Complete diets of cereals and soybean meal	3.6	Reduction in feed dry matter and insoluble non-starch polysaccharides, increased viscosity of feed			(Christensen *et al.* 2007)
Unspecified in vitro fermentation (LAB and yeasts)	Pig grower diet	4.9-5.3	Reduced contents of total and free lysine (g/kg crude protein, threonine and methionine			(Canibe *et al.* 2007c)
LAB fermentation	Phytic acid in cereals	-	Increase in apparent bioavailability of Phosphorus, Calcium, Magnesium and Copper			(Brooks *et al.* 2001; Brooks 2008)
Kocuria rosea	Poultry feathers (fermented feather meal)	-	Improved content and availability of amino acids, lysine 3.46%, histidine 0.94%, methionine 0.69%.			(Bertsch *et al.* 2003; Bertsch & Coello 2005)
Aspergillus nigeir and *L. rhamnosus*	Cassava peel meal	-	Increase in proteins (24.4%), ash (7.52%), crude fibre (10.62%) and decrease in cyanide (7.35 mg/kg)			(Okpako *et al.* 2008)
Saccharomyces cerevisae and *Lactobacillus spp*	Cassava peel meal	-	Increase in protein content (21.5%) and decrease in cyanide (6.2 mg/kg), and phytate (789.7 mg/100g).			(Oboh 2006)

† g/kg dry matter, *mmol/L, WDG-wet wheat distiller's grain

The advantages of fermenting feeds can be summarised from the table as follows;

- Reduction in the level of anti-nutrients within the feed.

- Improved bioavailability of minerals (e.g. P, Ca, Mg and Cu).
- Increase in protein contents (lysine, histidine and methionine).
- Breakdown of indigestible carbohydrates.

2.1.3 Influence of fermentation length and conditions

The length of steeping feed ingredients, the type of feed substrates and fermentation conditions influence the quality of the fermentation product. Steeping time has been related to its effects on the activity of endogenous enzymes and the breakdown of anti-nutrients within the grain. According to Choct *et al* . (2004a) the effects on growth and feed intake for weaner pigs resulting from steeping of feed for 15 hours might be related to the release and activation of endogenous enzymes in the grain. The activation of these enzyme systems within the grain can act on cell wall structures in a similar way to exogenous feed enzymes (Choct *et al.* 2004b). In reviewing the effect of steeping in liquid feeding systems, Brooks *et al* . (1996), indicated that phytases that were naturally present in the pericarp of some grains (like cereals) could be activated by soaking. They also stated that soaking feed for 8-16 h before feeding increased the bioavailability of phosphorus, calcium, magnesium and copper. In another study (Lyberg *et al.* 2008), the phytase activity for a cereal grain mix of wheat, barley and triticale was 1382 FTU /kg DM and inositol hexaphosphate bound-phosphorus and total phosphorus were 2.2 and 3.7 g/kg DM. After fermentation, dietary inositol hexaphosphate was completely degraded to release phosphorus. The fermentation of carbohydrate-rich cereal components of the diet separately and combining them with the protein-rich components just before feeding is necessary (Beal *et al.* 2002; Brooks *et al.* 2003b; Beal *et al.* 2005; Moran *et al.* 2006; Canibe *et al.* 2007a; Brooks 2008). It was observed that fermenting the protein rich components produced undesirable end-products, such as

biogenic amines, which could affect the palatability of fermented liquid feed (Canibe *et al.* 2007a). Some studies have also reported the degradation of free amino acids added to diets during fermentation (as reviewed by Brooks *et al.* 2003b; Handoyo & Morita 2006; Canibe *et al.* 2007b). However, Niven *et al.* (2006) demonstrated that the loss of lysine from fermented liquid pig feed was due to metabolism of lysine by *E. coli* present in the feed rather than its utilisation as an energy source by LAB. It was observed that inoculation of feed with LAB and 50 mM lactic acid at the beginning of fermentation resulted in lysine levels remaining unaltered after 72 h fermentation. The addition of acid reduced or eliminated the *E. coli* and allowed the lysine to remain intact during the fermentation process.

The main goal of fermentation is a high lactic acid concentration (>150mmol/L) and a low pH (<4.5). Temperature affects fermentation rate and low temperatures may yield insufficient quantities of fermentation end-products. Fermentation of a cereal grain mix at 10°C produced 8.6 gl^{-1} of lactic acid compared with 13.6 gl^{-1} at 20°C (Lyberg *et al.* 2008). At low temperatures yeast predominates and produces ethanol (Brooks 2008). Insufficient lactic acid concentration with 24 hour fermentation cycles which are more practical on farms may be the case at low temperatures. Furthermore, spontaneous fermentation of a cereal grain mix at 10°C required 7 days for the pH to drop to 4.0 compared with 5 days for 15 and 20°C. Prolonged fermentation also results in considerable variation in species composition of fermented pig feed (Olstorpe *et al.* 2008). However, fermentation at 30°C seems ideal as at 35 and 40°C there was no significant effect on lactic acid concentrations and acetic and butyric acid and ethanol concentrations were significantly increased (Beal *et al.* 2005). Some of the effects of incubation time and temperature on fermented feeds properties are presented in Table 2.2.

Table 2.2: Effect of fermentation length and conditions

Incubation time (hours)	Temperature (°C)	pH	Lactic acid or effects on diet	Acetic acid or effects on diet	Ethanol or effects on diet	Source
24	-	3.75	54.5†	Yeast population increases 10-fold		(Moran *et al.* 2006)
48	-	3.65	Yeasts population stabilizes and coliforms eliminated mainly by backslopping			
24	-	4.69	11.68*	17.22*	6.81*	(Beal *et al.* 2005)
48	-	4.34	31.92*	27.55*	10.62*	
72	-	4.21	46.14*	30.75*	12.79*	
-	30	4.47	30.16*	16.42*	11.69*	
-	35	4.41	25.29*	26.57*	9.78*	
-	40	4.36	28.64*	32.89*	8.42*	
48	20	4.2	115*	$D_{value\ (min)}$-250		(Beal *et al.* 2002)
72	20	3.9	164*	$D_{value\ (min)}$-164		
96	20	3.8	167*	$D_{value\ (min)}$-137		
48	30	3.8	161*	$D_{value\ (min)}$-45		
72	30	3.8	196*	$D_{value\ (min)}$-38		
96	30	3.8	203*	$D_{value\ (min)}$-34		
1	Room	-	Increase in apparent digestibility of phosphorus			(Lyberg *et al.* 2005)
8		4.2				(Christensen *et al.* 2007)
24		3.6				
0[a]	10	-	ND[b]	5.5[c]		(Olstorpe *et al.* 2008)
0[a]	15	-	ND[b]	5.5[c]		
0[a]	20	-	ND[b]	5.5[c]		
3[a]	10	-	ND[b]	ND[c]		
3[a]	15	-	ND[b]	5.3[c]		
3[a]	20	-	2.1[b]	5.6[c]		
5[a]	10	-	ND[b]	3.8[c]		
5[a]	15	-	2.1[b]	4.6[c]		
5[a]	20	-	3.1[b]	4.9[c]		
7[a]	10	-	2.0[b]	4.5[c]		
7[a]	15	-	2.1[b]	6.2[c]		
7[a]	20	-	2.1[b]	5.7[c]		
0	20	-	<3.0±0.00[c]	5.0±1.27[d]	3.9±0.00[b]	(Canibe *et al.* 2007c)
6	20	-	<3.0±0.00[c]	6.0±0.12[d]	<3.2±0.21[b]	
24	20	-	8.1±0.75[c]	7.1±0.56[d]	3.6±0.16[b]	
48	20	-	9.5±0.34[c]	6.7±0.87[d]	3.7±0.95[b]	
0	20	-	ND	4.7±0.03	ND	
6	20	-	ND	4.9±0.16	1.9±0.11	
24	20	-	ND	5.9±0.42	6.8±0.14	
48	20	-	91.2±27.66	20.9±6.21	15.5±1.31	
17-19[a]	10		1.8	10.4	1.2	(Lyberg *et al.* 2008)
17-19[a]	15		1.9	10.4	1.2	
17-19[a]	20		2.2	10.5	1.1	
17-19[a]	10		3.3[b]	7.2[c]	2.4[d]	
17-19[a]	15		3.2[b]	5.9[c]	2.3[d]	
17-19[a]	20		4.8[b]	7.4[c]	2.0[d]	
8	10	48% of total barley phytate degraded in fermentation				(Carlson & Poulsen 2003)
8	20	55% of total barley phytate degraded in fermentation				
8	10	52 % of total wheat phytate degraded in fermentation				
8	20	62% of total wheat phytate degraded in fermentation				
2	38	72 % of total phytate degraded in fermentation in 2 h				

†g/kg dry matter, *mmol/L, $D_{value\ (min)}$-decimal reduction time (minutes) of *Salmonella* in fermented feed. [a]days, [b]yeasts counts (cfu/g feed), [c]LAB counts (cfu/g feed), [d]Enterobacteriaceae counts (cfu/g feed), [d]moulds.

2.1.4 Liquid to feed ratios

An important aspect of a successful liquid feeding regime is the liquid to feed ratio of the diet. This affects the dry matter content of diet and may also have implications for the intake and organic acid concentration of the feed. Research to confirm the ideal dry matter content of liquid diets is limited (Choct *et al.* 2004a). In pigs, a wide range of liquid to feed ratios (3:1, 4:1) (Choct *et al.* 2004a), (2:1) (Demeckova *et al.* 2002; Choct *et al.* 2004a; Xuan Dung *et al.* 2005), (2.5:1)(Russell *et al.* 1996; Boesen *et al.* 2004), (3.5:1)(Geary *et al.* 1996) have been used. In chickens, these ratios have been reduced to 1.3:1 (Yasar & Forbes 1999) and 1.4:1 (Heres *et al.* 2003a; Heres *et al.* 2003b; Heres *et al.* 2003c; Heres *et al.* 2003d; Heres *et al.* 2004). However, the dry matter (DM) concentration of feed has been shown to have little overall effect on the pattern of microbial activity (Geary *et al.* 1996). Meanwhile, increasing water to feed ratios improved both DM and energy digestibility of diets for pigs. However, since in commercial practice with pigs liquid to feed ratios can vary from 2:1 to 7:1 (Choct *et al.* 2004a), performance is likely to be affected by DM intake.

Table 2.3: A summary of the effects of liquid to feed ratios on diets and performance

Liquid to feed ratio	Type of operation	Remarks	Source
2:1, 3:1,4:1	Experimental trial	No significant effect on growth and performance parameters but FCR* of liquid diets higher ($P<0.05$) than dry weaner pig diets.	(Choct *et al.* 2004b)
1.63: 1 to 3.25:1	Experimental trial	Digestibility coefficient increase from 0.791 to 0.829 with increase in liquid to feed ratio in pigs.	(Barber *et al.* 1991)
1.5:1 to 2.25:1	Experimental trial	Feed intake, weight gain and carcass weights of chickens not significantly affected.	(Yalda & Forbes 1995)
2.1:1 to 5:1	Commercial farms	Good results with pigs.	(Choct *et al.* 2004b)
1.5:1 to 3:1	Experimental trial	No significant effect on pig performance	(Hurst 2002)
†DM 149 to DM 255	Experimental trial	Little effect on microbial activity, DM intake, weight gain or DM FCR of pigs	(Geary *et al.* 1996)

*FCR- feed conversion ratios; †Diet dry matter concentrations (g/kg diet).

2.1.5 Controlled fermentation using starter cultures

Successful fermentation results have also been found to be dependent on the type of fermentation adopted. A brief definition of the methods of fermentation is given in Table 2.4. Spontaneous (Beal *et al.* 2005), backslopping (Moran *et al.* 2006), inoculated or controlled fermentations (e. g. Christensen *et al.* 2007; Canibe *et al.* 2008) have been investigated as means of feed fermentation with varying results. Spontaneous fermentation has been discouraged (Brooks *et al.* 2003b; Brooks 2008) because in this system yeast, which can tolerate low pH and a low temperature, can predominate. Yeast fermentation of starch will result in alcohol and carbon dioxide production. The production of CO_2 represents a loss of feed dry matter and energy value. Such feeds could be unpalatable due to 'off' flavours resulting in reduce feed intake. Secondly, spontaneous fermentation may not guarantee a rapid build-up of lactic acid in the feed, which is necessary for biosafety of the feed and to limit the utilisation of feed nutrients like added synthetic lysine by pathogens (Niven *et al.* 2006). Lastly, since feed ingredients differ in their load of natural microflora, spontaneous fermentation of the same raw material at different times results in inconsistent end-products.

Backslopping has been practiced on many farms (Beal *et al.* 2002). The limitations of this method has recently been highlighted in the review by Brooks (2008). In addition to these limitations, additions of fresh feed to a dynamic fermenting medium could have adverse implications on microbial balance and the ability of the feed to resist enteropathogens. Temperature shifts during addition that are outside the optimal range of particular pathogens could provoke the secretion of cold-shock proteins (Beal *et al.* 2002). Such cold shock proteins could increase pathogen tolerance to lactic acid in feed fermented at 20 °C compared with 30°C.

Table 2.4: Definitions of types of fermentation

Type of fermentation	Definition	Source
Spontaneous	Fermentation through the action of indigenous microflora present in the feed	(Brooks 2008)
Backslopping	A proportion of a previous fermentation is retained as an inoculum for fresh feed	(Moran *et al.* 2006).
	Consecutive microbial re-inoculation with micro-organisms from the previous batch	(Häggman & Salovaara 2008)
Inoculated	Fermentation resulting from inoculation of feed with selected lactic acid bacteria	(Brooks 2008)
Controlled	Fermentation resulting from inoculation of feed with selected lactic acid bacteria under controlled environmental conditions (e.g. temperature)	(e.g. Beal *et al.* 2002)

Controlled fermentation or inoculated fermented liquid feed would appear preferable for production of moist chicken feeds because more predictable results could be obtained. Selection for LAB that produce lactic acid rapidly, with high 24 h lactic acid (>150 mM) contents (Brooks 2008), should be the primary objective. The selection of LAB for other factors, such as probiotic properties is beyond the scope of this review.

2.1.6 Conclusion

A successful application of fermented moist feeds in chicken feeding depends on the ability to select the right balance of LAB and feed substrates capable of producing repeatable fermentation results. Meanwhile, the resistance of such feeds to enteropathogen contamination during short storage, and their capability to reduce pathogen colonisation in the gut of chickens, could have far-reaching implications for improved food and environmental safety.

2.2 Bacterial fermentation in the GI tract of chickens: Influence of fermented feeds and fermentable carbohydrates.

2.2.1 Introduction

Enteric infection with pathogenic bacteria and the subsequent clinical expression of disease occurs frequently in young animals, including children (Montagne *et al.* 2003). These diseases have often brought about significant economic losses in animal production and high rates of infant mortality. In order to solve this problem in animal production, antibiotics have been included in animal feeds either at sub-therapeutic levels (acting as growth promoters-AGPs), or at therapeutic levels, to treat disease. As growth promoters, they reduce competition between the GI microflora and the host for nutrients (Dibner & Richards 2005). The effects of such competition have often been at the cost of animal performance.

Unfortunately, there has been considerable concern over the use of AGPs, because their long term and extensive use in animal production has resulted in selection for survival of resistant bacteria species or strains (Doyle 2001; Montagne *et al.* 2003; Khaksefidi & Rahimi 2005). Genes encoding for this resistance could also be transferred to other previously susceptible bacteria, thereby posing a threat to both animal and human health (Montagne *et al.* 2003). For instance, in swine production, collibacillosis induced by *E coli* has become increasing difficult to treat because of the build-up of antibiotic resistance by many strains of these bacteria (Harvey *et al.* 2005). This has necessitated stringent rules on the use of in-feed antibiotics and has resulted in a complete ban of their use in animal feeds in the EU (Wilkie *et al.* 2005; Williams *et al.* 2005). Furthermore, the reality that AGPs are being curtailed by market actions, if not

legislative actions, has led to new urgency in the search for replacements (Dibner & Richards 2005).

Acceptable alternatives to antibiotics should enhance the natural defence mechanisms of animals, so as to avoid possible complications resulting from renewed bacterial resistance to such alternatives. Interest in the role of commensal gut microflora within the GI tract is presently growing, particularly due the advantage which the fermentative properties of beneficial gut microflora could confer on gut health (Williams *et al.* 2001; Gidenne & Licois 2005; Richards *et al.* 2005; Williams *et al.* 2005).

According to Lan *et al.* (2005a) the establishment of dominant bacterial communities within the GIT is affected by dietary and host-related factors in chickens. Dietary manipulation of fermentation in the hind gut, by the use of feed additives, or the choice of dietary raw materials, can improve colonisation resistance exerted by commensal flora to exclude enteric pathogens thereby improving gut health (Montagne *et al.* 2003).

This review examines the influence of fermented feeds and fermentable carbohydrates on bacterial fermentation in the GI tract of chickens and their relation to gut health.

2.2.2 Microbial fermentative activities and their relation to gut health

The primary functions of the gastrointestinal (GI) tract have traditionally been perceived to be limited to the digestion and absorption of nutrients and electrolytes, and to water homeostasis (Ramakrishna *et al.* 1990; Fasano & Shea-Donohoue 2005). Excretion of waste products of metabolism and toxic substances and safe containment of micro-organisms present (Cummings 1983) are also functions of the GI tract.

However, a better understanding of microbial activity within the GI tract and the introduction of molecular techniques in studies of gut microbial ecology (Vaughan *et al.* 1999), has brought about renewed interest in gut function in relation to microbial activities. The microbial population in the small intestines competes with the host animal for nutrients (van der Klis & Jansman 2002). Comparisons between germ-free and conventionally raised chicken (Muramatsu et al. reviewed by van der Klis & Jansman 2002) have indicated that the production performance of germ free birds was higher than that of conventionally raised birds. Van der Klis and Jansman, (2002), attributed the lower performance of conventionally raised chickens to highly digestible diets to four factors;

1) Competition for nutrients
2) Lower endogenous secretions
3) Lower concentrations of bacterial reaction products
4) Better health status, which means that less nutrients are needed for immune response and acute phase reactions.

Dibner and Richards, (2005) made similar observations. They reported that the GI microflora compete with the host for other nutrients, stimulate rapid turnover of absorptive epithelial cells, require an increased rate of mucus secretion by intestinal goblet cells, and stimulate immune system development and inflammatory responses. However, Snel *et al.* (2002) indicated that the GI microflora is important for the normal development of gut morphology and functioning. They summarised that several studies showed germ free animals to have enlarged caeca, a thinner mucosa and shorter villi and crypts. Germ free animals also had physiological abnormalities such as reduced intestinal motility, lower body temperature and, a poorly developed immune system. All these characteristics were quickly restored after addition of normal microflora to the

gut. Gut microflora could also benefit the host by the fermentation of poorly digestible feed ingredients (van der Klis & Jansman 2002; Lan *et al.* 2005a), and the production of short chain fatty acids (SCFA) (Lan *et al.* 2005a). According to Williams *et al* ., (2001) about 68 % of the energy value of fermented carbohydrates can be metabolised into volatile fatty acids (SCFA). All SCFA can contribute to the energy supply of the host (Lan *et al.* 2005b). Therefore, energy availability to the host animal fed low energy diets containing potentially fermentable substrates could be increased by microbial fermentation. It is now realised that one of the principal functions of the GI tract includes the salvage of energy and nutrients through its symbiotic relationship with gut microflora (Williams *et al.* 2001).

For the most part, dietary breakdown takes place through physical and enzymatic digestion by the host animal. The most important part of hydrolysis by enzymes takes place in the small intestines. However, a larger proportion of digestion, which takes place by microbial fermentation in non-ruminants, occurs in the large intestine (Ewing & Cole 1994; Williams *et al.* 2001). Furthermore, fermentation in non-ruminant animals occurs to the largest extent in the large intestines (caecum and colon), mainly due to the longer transit time of the diet in this part of the GI tract (Williams *et al.* 2001). In chicken particularly, a major portion of microbial fermentation is concentrated in the caecum (Barnes *et al.* 1980).

Therefore, improvements in fermentative activities within the gut will depend on the inclusion of ingredients that can escape the host's digestive enzymes in the small intestines and be available for fermentation by microflora in the large intestines. According to Collins and Gibson, (1999) and Williams *et al* . (2001) the large intestines still receive a constant supply of dietary residues, which are undigested in the upper GI

tract (especially resistant starch and non-starch polysaccharides) as well as host enzymes and other endogenous materials such as mucins, sloughed epithelial cells and bacterial lysis products. Montagne *et al.* (2003), stated that a major proportion of the dietary non-starch polysaccharides (NSPs) leave the small intestines nearly intact, and is fermented in the large intestine by commensal microflora. It is well established that the fermentative activities and diversity of gut microbes are dependent on the substrates available for their digestion (e.g. Gibson 1998; Salminen *et al.* 1998; Rastall & Maitin 2002; Konstantinov *et al.* 2004; Lan *et al.* 2005b; Rada *et al.* 2008; Reid 2008). These activities could have positive and negative consequences on the GI tract and the host animal, depending on whether fermentation is of carbohydrate or proteinaceous substances (Williams *et al.* 2005). For instance Armstrong *et al.* (1992) reported that the diet at weaning may be important in determining the pathways of caecal bacterial metabolism in the adult rat. In pigs, Dong *et al* ., (1996) have demonstrated that the presence of excess proteins in the large intestines can lead to increased ammonia production in the colon and this causes considerable problems of diarrhoea in early weaned piglets. Nyachoti *et al* . (2006) also observed that although piglet performance may suffer when dietary crude protein (CP) is reduced by 4 or more percentage units from 23%, low-CP diets did maintain enteric health by lowering toxic microbial metabolites such as ammonia. Indeed, several potential pathogens are protein-fermenters, and would therefore be more likely to multiply in conditions which favour protein fermentation (reviewed by Williams *et al.* 2005).

Fermentation of carbohydrates leads to the production of principally short chain fatty acids (SCFA) resulting in uptake of ammonia as a source of nitrogen for microbial growth (Stewart *et al.* 1993; Williams *et al.* 2000). The production of SCFA (mainly acetate, propionate and butyrate) from fermentation of non-hydrolysable oligo- and

polysaccharides improves gut epithelial cell proliferation, thereby increasing intestinal tissue weight, with changes in mucosal morphology (Le Blay *et al.* 2000; Fukunaga *et al.* 2003). As observed by Lan *et al.* (2005a) several mechanisms are involved in the growth-stimulating role of SCFA on animal intestines. For example, collagenous and non-collagenous protein syntheses in mucosa are both stimulated by butyrate. According to them, butyrate may affect intestinal muscles by directly acting at the molecular level on myocytes. Growth of the intestinal epithelium, which is the largest mucosal surface of the human body (Fasano & Shea-Donohoue 2005) and in chicken (Yegani & Korver 2008), is very important since it is heavily implicated in intestinal permeability through regulation by the intestinal epithelial barrier and its intercellular tight junctions. Furthermore, Fasano and Shea-Donohue (2005), observed that the regulation of intestinal permeability is crucial in the pathogenesis of gastrointestinal diseases (leaky gut) which is mainly involved with trafficking of macromolecules between the gastrointestinal environment and the host through the barrier mechanism. Therefore, the contribution of butyrate and other SCFA to intestinal development is vital to the barrier mechanism. The barrier is important in regulating the leaking out of electrolytes and water from the host to the intestinal lumen. In the large intestines, SCFA also stimulate the absorption of water and sodium (Roediger & Moore 1981) thereby limiting the risk of diarrhoea. Lan *et al.*, (2005b) also stated that it appears SCFA, particularly butyric acid, can contribute significantly to the health of the colon mucosa.

The pathogenesis of gastrointestinal diseases has been observed to be strongly dependent on the activities of mostly intestinal pathogenic bacteria. Pathogenic bacteria can have their harmful effects either through mucosal invasion, or production of toxin, or both (Snel *et al.* 2002). The principal species of pathogens, on which most attention

has been focused in enteric infections are the gram negative species including *Salmonella* spp, *Campylobacter* spp and *Escherichia coli* (Snel *et al.* 2002). On the other hand, some specific intestinal microflora especially the lactic acid-producing bacteria particularly members of the genera *Lactobacillus* and *Bifidobacterium* have been associated with beneficial effects for the host animal (Snel *et al.* 2002; Lan *et al.* 2005a). These health promoting bacteria are nutritionally referred to as probiotic bacteria. Probiotic bacteria are known to play a vital role in inhibiting the establishment of pathogenic bacteria (Zhu *et al.* 2002), through the phenomenon known as competitive exclusion first referred to as colonisation resistance by Van der Waaj *et al.* (reviewed by Williams *et al.* 2001). An illustration of the potential activities of beneficial bacterial cells as well as the mechanisms by which microflora can contribute to intestinal health of animals and man has been given by Snel *et al.* (2002) (Table 2.5 and Figure 2.2 respectively).

Table 2.5: Mechanisms by which the microflora can contribute to intestinal health of animals and man (Source: Snel *et al.* 2002).

Growth promotion

- improvement of mucosal architecture
- degradation of unfermentable substrates into digestible components

Improvement of intestinal and general health

- breakdown of cytotoxic substances
- production of vitamins

Suppression of pathogens

- competition for nutrients
- competition for adhesion sites at the mucosal epithelium
- stimulation of intestinal motility
- stimulation of the immune system
- production of volatile fatty acids
- production of antimicrobial substances

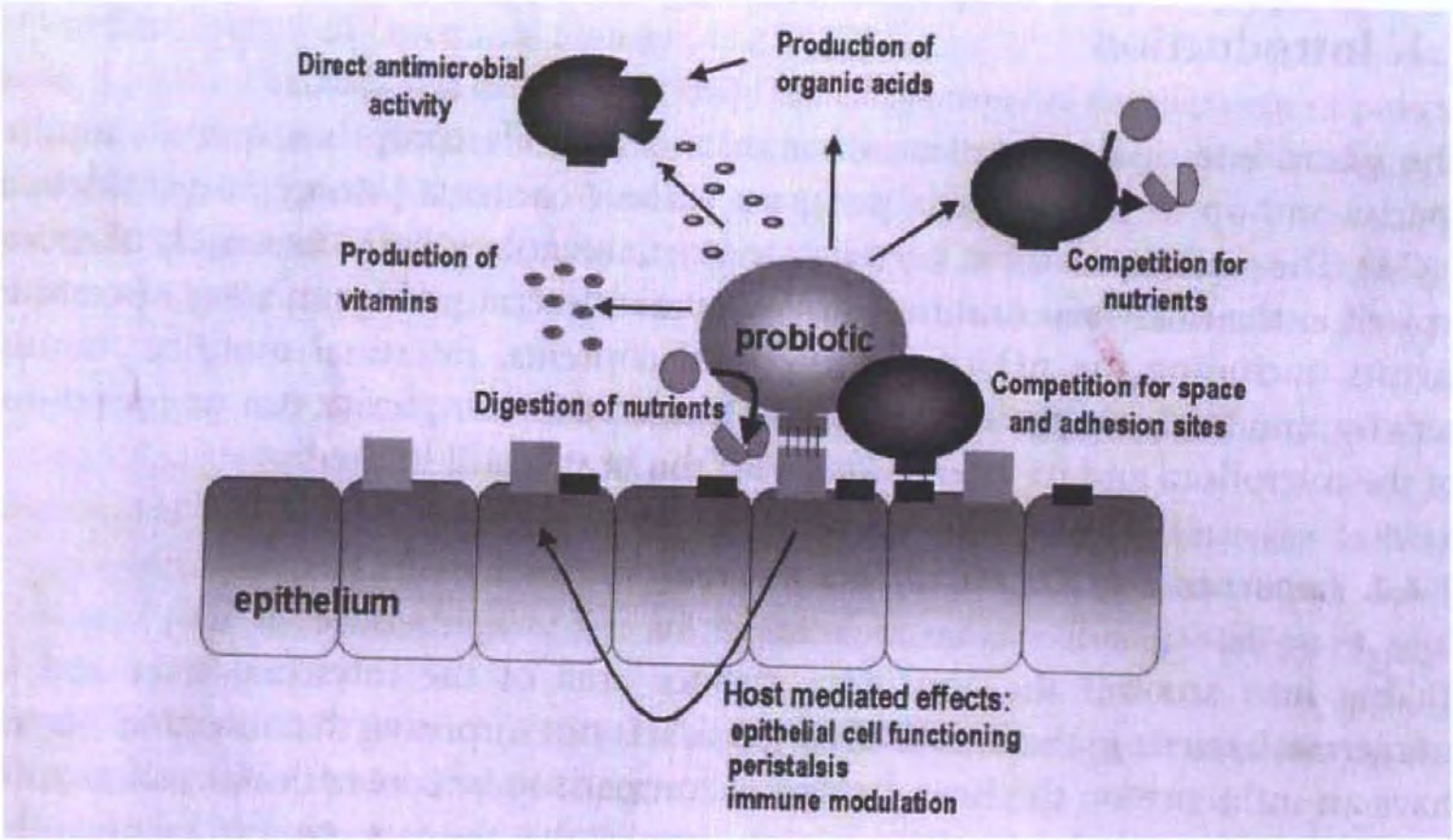


Figure 2.2: Schematic representation of the potential activities of a beneficial bacterial cell in the intestinal tract. Bacteria express activities towards other bacteria such as the production of antimicrobial components and competition for space and nutrients. Next to that, there is a strong interaction with epithelial cells resulting in an influence on the host physiology (Snel *et al.* 2002).

The role of gut commensal micro-organisms in preventing colonisation by pathogens and dietary influences on colonisation resistance has been the subject of some studies in non-ruminants. For instance in chicken, (e. g. Fernandez *et al.* 2000a; Fernandez *et al.* 2000b; Fernandez *et al.* 2002; Hampson *et al.* 2002; van Immerseel *et al.* 2004; Wilkie *et al.* 2005; de los Santos *et al.* 2008), in rabbits, (e.g. Gidenne *et al.* 2000; Gidenne *et al.* 2004; Gidenne & Licois 2005), in pigs, (e. g. van Winsen *et al.* 2002; Boesen *et al.* 2004; Harvey *et al.* 2005; Nyachoti *et al.* 2006) and *in vitro* (Marounek *et al.* 1999; Wilson *et al.* 2005). Some of the principal results from these studies could be summarised as follows;

1. Additions of exogenous enzymes to chicken diets were observed to reduce susceptibility to enteropathogens such as *Salmonella*, *Campylobacter* and *Brachyspira intermedia*.
2. High fibre intake significantly reduced morbidity and mortality resulting from acute diarrhoea in rabbits and was associated with higher fermentative activity and lower caecal pH.
3. Moderate levels of dietary fibre could be beneficial for gut health especially young non-ruminant animals.
4. Commensal bacteria from porcine GI tract controlled weaned pig disease induced by enterotoxigenic *E. coli* in competitive exclusion studies.
5. Some feed additives such as Caprylic acid reduced enteric infections from *Campylobacter jejuni* in broilers or Lasalocid increased the molar proportion of propionate in cultures of chicken caecal contents.

Protective measures against infectious agents in the gut can be achieved through modest dietary interventions that could improve the metabolism and numbers of beneficial bacteria. These dietary benefits are improved by feeding fermented feeds as they

contain lactobacilli that produce enzymes and can also have a probiotic effect on the gut.

2.2.3 Influence of fermented and acidified feeds on the GI tract

Protection of human health is now of prime concern for food producers with the aim of eliminating food borne pathogens from food animals and their products (La Ragione *et al.* 2001; La Ragione & Woodward 2003). This has necessitated a paradigm shift from emphasis on productive efficiency to one of public security in animal agriculture, which is justified by the increasing concern over the link between antibiotic resistance in human therapy and use of antibiotics at therapeutic and growth promoting levels in animal nutrition. According to La Ragione *et al.* (2001), the poultry industry, has addressed this challenge by interventions to reduce the burden of zoonotic pathogens through improved hygienic methods, vaccination and competitive exclusion.

Fermented moist feeds have a key role in meeting these challenges as the low pH and high acid concentrations improve biosafety as has been discussed in the first part of this review. Although the use of fermented feeds has had a large impact on commercial pig farms, it has not yet been adopted in commercial chicken production. There is some scope for its use in small holder production systems and for free range chickens; particularly in warm, wet climates in tropical countries. In tropical countries, daily ambient temperatures of 25-30 °C could permit inoculated fermentations in feeds to reach acceptable levels of lactic acid (>150 mmol/L) and low pH values (<4), within 24 hours.

Other reasons for the adoption of fermented feeds in such areas could be;

1) In hot climates, wet feeding increases feed intake and growth rates of chickens (Forbes 2003).
2) Some unconventional tropical seeds have great potential to be used for livestock feeding but are limited by their content of anti-nutritional factors (Iyayi *et al.* 2007). Feed fermentation has been shown to improve bioavailability of minerals (Brooks *et al.* 2001) by degrading anti-nutrients such as phytic acid (e.g. Skrede *et al.* 2007) and trypsin inhibitor (e.g. Refstie *et al.* 2005), tannins (Mukhodhyay & Ray 1999) and might be used to improve the feeding value of such seeds.
3) Furthermore, environmental pollution resulting from excretion of unutilized compounds like phytate phosphorus from intensive poultry farms is becoming an issue of great concern (Rama Rao *et al.* 2007)
4) In particularly hot and humid areas, there are greater tendencies for feed spoilage and pathogen contamination during storage. Fermentation could improve biosafety and reduce the risks of contamination of poultry flocks and the human food chain.

These factors, in addition to the health benefits that feeding moist feeds can confer on chickens, are the factors that could favour the adoption of fermented feeds in chicken nutrition practice. Several studies have indicated the modes of action and benefits of feeding fermented feeds on the gastrointestinal tract ecophysiology (Table 2.6). The consensus is that fermented feeds;

1. Improve intestinal microbial and physiological balance
2. Stimulate the gut immune system
3. Lower gut pH thereby improving the barrier function of the gut to pathogen

Table 2.6: Possible modes of action of fermented feeds

Property of feed	Mode of action	Consequences on welfare of gut and host and/or offspring	Source
Acidity	Low pH inhibits growth of undesirable microbes such as Coliforms and *Salmonella*	1. Coliforms are the main producers of toxic amines and ammonia which are harmful for the gastrointestinal mucosa 2. Reduction in competition for nutrients between coliforms and the host animal helps in growth promotion	Russell *et al* ., (1996) Scholten *et al* ., (1999) van Winsen *et al* ., (2001a) Demeckova *et al* ., (2002)
	Stimulates protein digestion by reducing gastric pH and low gastric pH is needed to increase activity of pepsin	1.Positive influence on the apparent total tract digestibility of crude protein and energy 2.Better utilisation of the diet	Scholten *et al* ., (1999)
	Both lactic acid & SCFA elevate pancreatic secretions in pigs	Improves lipid digestion*	Harada *et al* ., (1986) Scholten *et al* ., (1999)
	Production of higher concentrations of butyric acids in middle part of small intestines of weaned piglets	Butyric acid is beneficial to mucosal structure and has a trophic effect on mucosal epithelial cells*	Scholten *et al* ., (1999)
Lactic acid bacteria content	Probiotic effect	1. Improve intestinal microbial balance 2. Stimulation of immune system	Fuller, (1989) Scholten *et al* ., (1999) Snel *et al* ., (2002) Gill and Rutherford, (2001) Demeckova *et al* ., (2002) (reviewed)
		Increased expression of mucin mRNA and mucin glycoprotein in jejenum of chicken	(Smirnov *et al.* 2005)
	Enhancement of mucosal immunity against coccidiosis in broiler chickens	Improve resistance to *Eimeria acervulina* Resistance to Eimeria infections and higher (P<0.001) Eimeria-specific antibodies	(Dalloul & Lillehoj 2006) (reviewed) (Dalloul *et al.* 2003; Lee *et al.* 2006; Lee *et al.* 2007b)
	Pediococcus acidilactici	Also protection against the negative growth effects associated with coccidiosis	(Lee *et al.* 2007a),

Liquid nature of feed	Reduction in physical digestion of liquid feed as opposed to dry feed Less energy is spent on digestion	Higher daily weight gain because of more rest and sleeping	Scholten *et al* ., (1999)
	Limiting effects of anti-nutrients on mink	Improved digestibility and energy utilisation	(Skrede *et al.* 2007)
	Increase in daily feed intake in piglets	Higher daily weight gain, pigs reach slaughter weight 15-21 days earlier	Hurst *et al* ., (2000b), (Hurst 2002)
	Decrease in levels of villus tip erosion and disruption of brush border in pigs as opposed to dry fed pigs	Increased surface area for nutrient absorption*	Hurst *et al* ., (2000a; Hurst *et al.* 2000b),(Hurst 2002)
Unclear	1. Higher mitogenic activity of colostrums from sows fed fermented feed as opposed to dry feed 2. Reduction in the probability of introduction of *Campylobacter* in broiler flocks 3. Delayed excretion of *Lawsonia intracellularis* in pigs	1a. Accelerates maturation of piglet GI tract and better protection by maintaining the integrity of the intestinal mucosa 1b. Enhanced neonatal immunity 2. Possible improvement in flock health 3. Limited prevalence and severity of diarrhoea	1 a & b-Demeckova *et al* ., (2002) 2- Heres *et al* ., (2003b) 3. Boesen *et al* ., (2004)

* included by the author

Studies on acidified feeds have concentrated on the effect of acidified feeds on the susceptibility of the host animal to pathogenic microflora. In competitive exclusion studies in chickens, it was observed that SCFA may be responsible for the observed reduction of *Salmonella* in the caeca of broiler chickens (Snel *et al.* 2002). Indeed, Heres *et al.*, (2004) stated that acidification of feed may be a tool to reduce *Campylobacter* and *Salmonella* carriage in broiler chickens. Hinton and Linton, (1988) and Humphrey and Lanning, (1988), reported that the inclusion of formic acid in chicken feed reduced the number of *Salmonella* positive caeca in a laboratory-scale experiment and in a small feed trial. For pigs, Boesen *et al.* (2004) observed that a standard dry diet supplemented with lactic acid reduced pathological lesions when animals were examined 4 weeks after experimental challenge with *Lawsonia intracellularis*.

However, studies in which organic acids have been added to dry feeds to mimic the effects of fermented feeds have been inconclusive with regards to their effect in the large intestines. With chicken, studies have shown that the addition of SFCA to feed resulted in higher concentrations of such acids in the upper part of the intestinal tract (i.e. crop and gizzard) (Hume *et al.* 1993a; Thompson & Hinton 1997). Furthermore, the chemical fate of ^{14}C-propionic acid was followed in broilers fed with radio-labelled propionate and results indicated that the label could only be traced in the foregut and not lower in the gut or caeca (Hume *et al.* 1993b) suggesting it is absorbed across the gut.

In vitro comparisons of the microbial catabolic capacity in digesta from the gastrointestinal tract of pigs fed fermented liquid feed (FLF) and dry feeds conducted by Hojberg *et al* ., (2003) are similar to these findings. FLF reduced large intestinal total

counts of anaerobic bacteria in general and LAB specifically as well as microbial activity as determined from the concentration of ATP and SFCA in digesta. They concluded that the availability of readily fermentable carbohydrates is more likely to become a limiting factor for activity and growth of the microbiota in the large intestines of pigs fed FLF compared with pigs fed dry feed.

However, an interesting route in research on the contribution of fermented feeds to gut health could be the addition of readily fermentable carbohydrates or prebiotics to fermented feeds at the time of feeding animals. The working hypothesis for such research would be in the implications of increased beneficial microbial activity within the large intestines on gut health and the health of the host animal.

2.2.4 Fermentation of carbohydrates and dietary fibre components.

Most of the carbohydrates that may be fermented in the hind gut of non-ruminants are classified as dietary fibre (Montagne *et al.* 2003; Jozefiak *et al.* 2004). Bach Knudsen (2001) described the term dietary fibre use in recent animal literature to mean cell wall or storage non-starch polysaccharides together with non-carbohydrate components including lignin, protein, fatty acids and waxes to which dietary fibre are intricately bound. This consists of 700-900 g/kg of plant cell wall (Bach Knudsen 2001). According to Montagne *et al.*, (2003), the term dietary fibre in the nutritional context includes any polysaccharide reaching the hind gut and so includes resistant starch (RS) and soluble and insoluble non-starch polysaccharides. The classification of carbohydrates which are non-digestible by non-ruminant endogenous enzymes is shown in Table 2.7.

Table 2.7: Classification of carbohydrates which are non-digestible by non-ruminant endogenous enzymes (after Bach Knudsen, 1997; Englyst *et al* ., 1992).

Category	Monomeric residues	Sources
Polysacharides (dietary fibre)		
Resistant starch		
Physical inaccessible(RS1)	Glucose	Partly milled grains and seeds
Resistant starch granules	Glucose	Raw potato, banana
Retrograded starch	Glucose	Heat-treated starch products
Non-starch polysaccharides (NSP)		
Cell wall NSP		
Cellulose	Glucose	Most cereals and legumes
Mixed linked β-glucans	Glucose	Barley, oats, rye
Arabinoxylans	Xylose, arabinose	Rye, wheat, barley
Arabinogalactans	Galactose, arabinose	Cereal co-products
Xyloglucans	Glucose, Xylose	Cereal flours
Rhamnogalacturans	Uronic acids, rhamnose	Hulls of pea
Galactans	Galactose	Soya bean meal, sugar-beet pulp
Non-cell wall NSP		
Fructans	Fructose	Rye
Mannans	Mannose	Coconut cake, palm cake
Pectins	Uronic acids, rhamnose	Apple, sugar-beet pulp
Galactomannans	Galactose, mannose	Guar gum
Oligosaccharides (prebiotics)		
α-Galacto-oligosaccharides	Galactose, glucose, fructose	Soya bean meal, peas, rapeseed meal
Fructo-oligosaccharides	Fructose, glucose	Cereals, feed additives, Jerusalem artichokes
Transgalacto-oligosaccharides	Galactose, glucose	Feed additives, milk products

Source: Montagne *et al* ., (2003).

The role of bacteria in metabolising available carbohydrates from the diet has been to obtain energy for their growth and maintenance (Snel *et al.* 2002). However, recent interest in hind gut fermentation of carbohydrates has been focused on microbial fermentation of potentially prebiotic carbohydrates. Attention has also focussed on factors that can influence the potential fermentability of such carbohydrates as well as host related factors in the establishment of dominant microbiota in the GIT. The most important factors influencing fermentability of dietary fibre (DF), include the source of the DF, solubility, degree of lignification, processing, the level of inclusion in the diet, intestinal transit time, the age and weight of the animal and microbial composition (reviewed by Montagne *et al.* 2003).

Given that the amount and composition of substances reaching the large intestines can be readily modified by diet, it is probably the carbohydrate fraction that is most important in terms of bacterial substrates (reviewed by Snel *et al.* 2002). The extent to which these non-digestible carbohydrates could change the microbial community of the broiler caecum, either in terms of the species detected or its activity, needs to be investigated further and then related to its effect on gut health of the animal itself (Jozefiak *et al.* 2004; Lan *et al.* 2005b). This is because the potential energy contribution resulting from carbohydrate fermentation in other non-ruminant species like pigs have been measured by analysing arterial and portal blood together with portal blood flow (Gäbel 1995) as compared with chicken.

In chicken, this method may be used but it could be difficult to use a catheter due to the narrow diameter of the portal vein (Jozefiak *et al.* 2004). However, to some extent, the effects of diet on the micro-organisms and their capacity to generate SCFA are measurable using *in vitro* techniques (Hojberg *et al.* 2003; Lan *et al.* 2005b).

The main products of fermentation of DF are SCFA, predominantly acetate, propionate and butyrate, lactate and succinate, as well as water, various gases (carbon dioxide, hydrogen, methane) and bacterial cell biomass (Montagne *et al.* 2003). The free SCFA produced are absorbed by the colon (Ruppin *et al.* 1980), and energy is conserved while the osmotic load is reduced (Williams *et al.* 2001). SCFA are important fuels for large intestinal colonocytes, with butyric acid being the most important (Heneghan 1988). In poultry, after fermentation, the protonated forms of SCFA are transported through the caecal epithelial membranes (Jozefiak *et al.* 2004). Although the importance of fermentation end-products to the host animal still remains unclear (Jozefiak *et al.* 2004),

most researchers agree that it is the SCFA fraction of the fermented end-products that has the most impact on the health of the gut and host animal.

SCFA lower gut pH and at such environmental conditions they have been capable of inhibiting the growth of some intestinal bacterial pathogens such as *Enterobacteriacae* (McHan & Shotts 1993), *Salmonella* (McHan & Shotts 1992; McHan & Shotts 1993) and *Clostridium* species (Hentges 1992; McHan & Shotts 1993), *Escherichia coli* and *Clostridium difficile* in pigs, (e.g. Prohaska 1986; May *et al.* 1994) and *E. coli* in rabbits, (e.g.Gidenne & Licois 2005) and pathogens of chicken (Marounek *et al.* 1999). For instance, adding butyric acid-impregnated microbeads to chicken feed brought about a significant decrease in colonisation of chicken caeca by *Salmonella enteritidis* although no effect was observed for the liver and spleen (van Immerseel *et al.* 2004). Increases in the population of micro-organisms by the carbohydrate fraction of the diet are also thought to stimulate increased production of SCFA (same as SCFA) and this process uses up ammonia as a source of nitrogen (Williams *et al.* 2001). The negative effects of excess NH_3 production to the health of the gut have already been highlighted. For weaned piglets, Hogberg and Lindberg, (2004), reported that an increase in dietary content of cereal non-starch polysaccharides and supplementation with fibre degrading enzymes influenced the distribution of organic acids in the ileum, indicating a shift in dominating bacteria.

SCFA also contribute considerably to energy supply in the host animal. As earlier stated 68 % of the energy value of fermented carbohydrate can reappear in the form of completely metabolisable short chain fatty acids (Williams *et al.* 2001). According to Soergal (reviewed by Williams *et al.* 2001) such a preservation of metabolic energy has

major implications for the maintenance of the colonic bacterial population, the metabolic needs of the colonic epithelium and for the energy salvage from malabsorbed carbohydrate by means of colonic SCFA absorption. In chickens, Lan *et al.* (2005a) stated that if dietary energy is provided in the form of substrates that are easily digested by the animal itself, the efficiency of energy utilisation may be reduced in the presence of GI tract microflora. However, SCFA concentrations in avian species are governed by a variation in evolutionary adaptations of the GI tract (Jozefiak *et al.* 2004). Avian species that naturally feed on more fibrous diets could be more able to make use of the advantages of prebiotic diets. For example, birds such as the ostrich may obtain as much as 75 % of their energy from SCFA resulting from caecal fermentation (Jozefiak *et al.* 2004).

Studies on the use of fermentable carbohydrates as bifidogenic agencies in the GI tract have been involved mostly with the use of oligosaccharides. According to Crittenden and Playne (1996), effective bifidogenic doses appear to vary among different oligosaccharide types. Oligosaccharides that have been indicated to have bifidogenic and/or general prebiotic properties are lactulose, lactosucrose, fructo-oligosaccharides (Crittenden & Playne 1996), and soybean oligosaccharides (Crittenden & Playne 1996; Refstie *et al.* 2005).

2.2.5 Conclusion

The search for replacements to in-feed antibiotics in animal nutrition highlighted the fact that gut health can be improved by dietary modulation amongst other factors (environmental and genetic). The present review recognises the contribution of

fermented feeds and fermentable carbohydrates in modulating the gut environment. Fermented feeds are seen as containing organic acids (principally lactic acid), which perform the important function of modifying the gut environment. The complex interactions between fermentable carbohydrates and gut micro-organisms have implications on the health of the gut and the host animal. This could be directly through colonisation resistance to pathogenic bacteria offered the host animal by the growing population of beneficial bacteria adhering to the gut mucosal surface and the stimulation of host local immunity. It could also be mediated by the increased production of end-products of bacterial fermentation mainly SCFA. SCFA have a trophic effect on GI mucosa and may improve absorption and retention of electrolytes and water by the non-ruminant gastrointestinal tract. However, some of the interactions underlying dietary composition and microbial fermentation and their relation to gut and animal health still need to be elucidated.

2.3 Effect of feed substrates and fermentation on the incidence of *Salmonella*

Poultry and/or poultry products contaminated with *Salmonella* are a major cause of human food borne disease (Swaggerty *et al.* 2005; McCrea *et al.* 2006). In chickens, just as in pigs (Lo Fo Wong *et al.* 2002), it can be deduced that two aspects are involved in the relation between the feed and the establishment of *Salmonella* infection; 1) feed as a potential source of introduction of infection and 2) the role of the feed in the establishment of infection in the chicken.

The effects of feeds on the incidence of *Salmonella* or Enterobacteriaceae in animals are related to the feed structure or method of processing (Hansen 2004; Mikkelsen *et al.* 2004; Canibe *et al.* 2005; Hedemann *et al.* 2005) and the choice of feed ingredients

(Beal *et al.* 2003; Hansen 2004). In a series of five studies, Hansen (2004), has demonstrated that gastric ulceration and *Salmonella* incidence were significantly reduced in pigs fed coarsely ground diets compared with finely ground and pelleted feed. It was observed that the barrier function of the stomach was improved when coarsely ground diets were fed and this reduced *Salmonella* colonisation of the lower parts of the GI tract. It was indicated that the consistency of stomach contents was improved by coarse diets and this created a pH gradient that decreased flow of gastric digesta to small intestines. Increased retention of digesta caused an increase in numbers of primarily LAB which increased organic acid production and reduced *Salmonella* colonisation. The addition of 10% sugar beet pulp to feed or meal feed containing 69 % wheat were observed to significantly reduce the relative risk of a pig being positive to *Salmonella* to 0.40 and 0.19 respectively compared with 0.56 for potato protein concentrate and zinc gluconate supplemented feed. Canibe *et al* . (2005) also observed that feeding coarse diets to pigs decreased (P=0.03) the number of Enterobacteriaceae in the caecum compared with standard pelleted diets.

The effect of feed on the incidence of *Salmonella* after the stomach seems to be related to the ability of the feed to alter mucin dynamics. Using a pig intestine organ culture model, Hedemann *et al.* (2005), demonstrated that *Salmonella* adherence to illeal tissue was increased ($P<0.05$) with pelleted feeds compared with non-pelleted feeds (60% less adherence). They concluded that pelleted feeds increased secretion of mucins that were capable of binding *Salmonella* consequently facilitating colonisation of the GI tract.

The effects of feed structure on *Salmonella* incidence could be exerted through the ability of such feeds to change the physicochemical and microbial properties of feed in

the GI tract. Coarsely ground unpelleted feeds increased significantly the gastric concentrations of undissociated lactic acid compared with fine pelleted feeds (Mikkelsen *et al.* 2004). Micronutrients such as copper salts increased the death rate of *Salmonella* in liquid food substrates 10-fold (Beal *et al.* 2003). In chickens, bacterial cationic peptides were involved in the upregulation of the avian innate immune response and provided protection against extraintestinal *Salmonella* infections (Kogut *et al.* 2007). Purified β-glucans used as feed additives significantly decreased the incidence of *Salmonella enterica* serovar Enteritidis organ invasion in immature chickens (Lowry *et al.* 2005).

Fermented feeds have been reported to improve the barrier function of the crop and gizzard against the passage of *Salmonella* (Heres *et al.* 2003d). Studies on *Salmonella* transmission in chickens farms (inoculated with 10^3 cfu *Salmonella*) have been conducted by Heres *et al.* (2003c). The between-chicken, maximum likelihood of transmission of *Salmonella* (reproduction ratio) was reduced in fermented feed treated groups (1.3) compared with groups receiving dry feed (∞). In fermented liquid pig feed, the threshold lactic acid concentration that is required to prevent the growth of *Salmonella* spp is ≥75 mmol/L (Beal *et al.* 2002). However, nutritional influences on *Salmonella* incidence may not be as simple as presented here. The GI tract is a complex medium with several pathogens having different nutrient requirements, ecological niches and patterns of metabolism (Pluske *et al.* 2002). These patterns may overlap with different dietary treatments so a greater understanding of how feed structure or processing influences pathogenic bacteria in the chicken will help tackle enteric diseases in an era without AGPs.

2.4 Modulation of the immune system by lactic acid bacteria

The GI tract affords prevention against enteric infections by secreting defensins and secretory IgA, while phagocytes and M-cells continually survey the microflora of the lumen and lamina propria for potential pathogens (as reviewed by Donoghue *et al.* 2006). In many poultry operations, transfer of microflora from hen to her offspring is non existent because chicks are raised separately from the parent stock (Snel *et al.* 2002; Donoghue *et al.* 2006). The GI tract of the newly hatched chicks is sterile (Donoghue *et al.* 2006) and delayed development of beneficial intestinal microflora increases the risk of colonisation by pathogens like *Salmonella* (Snel *et al.* 2002).

At hatch, the chick's immune system is immature and it relies on innate effector mechanisms and maternal antibodies primarily IgY transmitted from the hen via the yolk (Klipper *et al.* 2004; Beal *et al.* 2006). At this stage, the chick is vulnerable to a number of pathogens until the adaptive immune system matures to produce efficient immune responses (Bar-Shira *et al.* 2003; Beal *et al.* 2006). For instance, chicks are most susceptible to *Salmonella* infection during the first 4 days post-hatch (Wells *et al.* 1998). However, functional maturation of the gut-associated lymphoid tissue in the post-hatch chick occurs in two stages or waves and requires two weeks (Bar-Shira *et al.* 2003). The primary wave during the first week post-hatch and a secondary wave during the second week. Maturation of these intestinal cellular immune responses is a prerequisite for humoral responses (Bar-Shira *et al.* 2003). Furthermore, the development of an adaptive immune system depends on the arrival and replication of specialised leukocytes at specific locations in the gut especially B and T lymphocytes (Beal *et al.* 2006). This occurs during the second week post-hatch (Bar-Shira *et al.*

2003). Therefore, strategies that could upregulate gut immune system development within this post-hatch period might improve immunocompetence against specific antigens throughout the chick's life.

It is widely agreed that microbial components, commensal bacteria and/or probiotics enhance the development of the immune system (Dong *et al.* 1996; Clancy 2003; Koenen *et al.* 2004a; Koenen *et al.* 2004b; Haghighi *et al.* 2005; Nava *et al.* 2005; Smirnov *et al.* 2005; Donoghue *et al.* 2006; Farnell *et al.* 2006; Haghighi *et al.* 2006). Indeed, interest in their immuno-modulatory properties dominates contemporary literature (Clancy 2003; Alvaro *et al.* 2007). Clancy proposed the term "immunobiotics" to describe bacteria that promote health through driving mucosal immune mechanisms.

In mice, a dose of 10^9 *L. rhamnosus* HN001 in live and heat-killed preparations enhanced phagocytotic activity of blood and peritoneal lymphocytes in mice (Gill & Rutherfurd 2001). It has been demonstrated that avian immune response may be potentiated by stimulation with killed probiotic bacteria (Farnell *et al.* 2006). Haghighi *et al* . (2005) observed that treatment of broiler chickens with probiotics did not enhance serum IgM and IgG responses to bovine serum albumins (BSA). However, immunisation with tetanus toxoid (TT) had a significant effect ($P \leq 0.001$) on the appearance of anti-TT IgA and IgG in the gut. Manipulation of intestinal microbiota by administration of probiotics induced production of natural systemic antibodies in unimmunised chickens (Haghighi *et al.* 2006). They also observed significantly more IgA antibodies reactive to TT, alpha-toxin and BSA in intestinal contents of probiotic treated chickens than untreated control chickens. Manipulating the intestinal microbial

balance early in the chick's life is thus important for food and environmental safety. It also affects intestinal cell dynamics and the overall performance of the bird.

A research idea that could be developed from the foregoing facts is;

- Can probiotic application at day-old and one week of age upregulate the development of gut associated lymphoid structures and promote mucosal T-cell function in chicks? OR
- Can LAB with putative probiotic properties applied through fermented feeds or in water to day-old and 7 days post-hatch chicks have different impacts on GALT development or resistance to colonisation by specific antigens?

Understanding this question might provide vital clues on strategies to improve efficiency in chicken production and food safety.

Chapter 3

Experiment 1: Effect of water hardness, cereal type and micro-organism on short chain fatty acid production and viscosity of fermented feeds

3.1 Introduction

The phasing out of antimicrobial growth promoters (AGPs) from poultry diets in Europe and recent efforts to reduce or remove AGPs in other parts of the world (North America) will have far reaching implications on chicken gut microbial profiles in such areas (Yegani & Korver 2008).

Currently, there is an active search for alternatives to AGPs in animal feeding. This includes the use of probiotics, organic acids, prebiotics, minerals and fermented feeds (Knarreborg *et al.* 2002). Fermented Liquid Feed is considered to be a good method of replacing AGP in pigs (Knarreborg *et al.* 2002; Kobashi *et al.* 2008). Fermented feeds are characterised by high numbers of lactic acid bacteria and high concentrations of lactic acid (Heres *et al.* 2003a). In chickens, organic acid content of fermented feeds has been reported to improve foregut barrier function against pathogens by increasing acidity and lowering the pH of the crop (Heres *et al.* 2003b). Changes in pH have been demonstrated to alter the physiology of *Salmonella enterica* subsp. *enterica* serovar. Typhimurium (*Salmonella* Typhimurium) and indicated as a determinant factor in their survival in poultry GI systems (Dunkley *et al.* 2008).

An important element in the search for replacements to AGPs, is a better understanding of factors that affect the production of lactic acid and consequently pH during fermentation. Since LAB spp are the main source of lactic acid, modulation of their metabolic activities

will affect the acidity and pH of the feed. The first part of this study investigated the effect of water hardness on the fermentation process. In addition to water hardness, the effect on viscosity of the fermented feed is also investigated. This is because changes in the chemical and physical properties of the grains brought about by grinding and fermentation may have far reaching effects on the physiology of the gastrointestinal tract. According to Anguita *et al.* (2006), alterations in the solubility, viscosity and hydration properties of digesta may affect the physiology of the gastrointestinal tract. A subsidiary objective of this study was to look at the effect of water quality, grain type and micro-organism on viscosity of fermented feed.

3.2 MATERIALS AND METHODS

3.2.1 Experimental design

The experiment was design as a 4 x 3 x 5 factorial experiment with four cereals (sorghum, barley, wheat and maize), three LAB species (*Pediococcus acidilactici* (PA1) or *Lactobacillus plantarum* (SLP) or *Lactobacillus farciminis* (LF1)) and five concentrations of $CaCO_3$ in sterile distilled water. There were three replicates per treatment.

3.2.2 Fermentation and sample collection

Barley, maize and wheat were obtained locally (Edwin Tucker and Sons, StonePark, Ashburton, Devon) while sorghum was the white variety (*Sorghum bicolour* L. Moench) acquired from the World Foods Shop, Plymouth. Grains were hammer-milled through a 3.0 mm screen and weighed into 100 g sachets for subsequent irradiation with 25 KGy γ-

radiation from ^{60}Co. Irradiation of grains was undertaken by Becton and Dickinson, Plymouth, UK.

Feed samples were mixed with sterile distilled water containing different concentrations of calcium carbonate (T0-0g/l, T25-0.01g/l, T50-0.02g/l, T75-0.03g/l or T100-0.04g/l), at least four hours before usage. A grain to water ratio of 1:1.4 was used in this study as recommended by Heres *et al.* (2003). The mixture was inoculated with 0.01 ml of an overnight culture of De Man, Rogosa and Sharpe (MRS) broth containing ca 10^9 cfu/ml one of three LAB *spp* (*Pediococcus acidilactici* (PA1), *Lactobacillus plantarum* (SLP) or *Lactobacillus farciminis* (LF1)) and incubated at 30 °C. After 24 hour fermentation, the pH was measured using a pH electrode (pH 213 microprocessor pH meter, Hanna instruments, Portugal) and 0.5ml samples were collected in Eppendorf tubes and immediately frozen and kept at -20 °C until analysis for sugar and organic acids.

3.2.3 Analysis for short chain organic acids and sugars

Organic acids and simple sugars were analysed using High Performance Liquid Chromatography (HPLC) according to the method of Niven *et al.* (2004) using a Varian metacarb 87H column (Serial N° 05524314, USA). Elution was performed using dilute sulphuric acid (5mmol/L) at a flow rate of 0.5ml/min. The volume of samples injected was 20µL and analysis time was 30.5 min per sample. Detection of sugars and organic acids was conducted by refractometry and signals were recorded using the Chromeleon information management systems software version 6.20 SP2 Build 541 (Dionex corporation, U,K).

To each sample, 20µL of 7 % (v/v) sulphuric acid was added to denature dissolved proteins and shift the acid dissociation equilibrium towards complete protonation of organic acids. Samples were mixed for 30 seconds using a vortex mixer and centrifuged at 13000 rpm for 10 minutes. The supernatant was extracted using 1ml polypropylene disposable syringes (Fisher Scientific, SZR-150-011Q) and filtered through 0.45µm NYL polypropylene syringe filters (Whatman International Ltd, Maidstone, England) to eliminate any particulate material still present. Standards containing three concentrations of analytical grade lactic acid (300, 150 and 75 mmol/L), acetic acid (100, 50 and 25 mmol/L), maltose (100, 50 and 25 mmol/L), glucose (50, 25 and 12.5 mmol/L) and fructose (50, 25 and 12.5 mmol/L) were run before and after every six subsequent samples.

3.2.4 Determination of viscosity

The viscoelastic properties of the liquid fraction of the fermented feed samples were determined using a Brookfield digital viscometer (Model DV-III). Feed samples were centrifuged in 300 ml centrifuge bottles at 8000 rpm for 5 minutes. The supernatant was decanted and the viscosity determined at a shear rate of 42.5 sec-1 and temperature of 40^0C as described by Bedford and Classen, (1993). Approximately 8.4 ml of supernatant was used. The spindle (type SC4-37) was housed in a cabinet containing running warm water at $40\ ^0$C.

3.2.5 Data analysis

Data were analysed by analysis of variance using Minitab (release 15.0). Differences between means were determined using the Tukey's test (Zar 1999). Probability values $\leq$ 0.05 were considered to be statistically significant.

3.3 RESULTS

3.3.1 Effect of water hardness, cereal type and micro-organism on lactic acid fermentation.

Sugar and organic acid concentrations and pH after 24 hours were significantly affected by cereal type (Table 3.1). Wheat had a pH above 4 after fermentation at 30 °C for 24 hour and this was significantly higher (P<0.001) than the pH of other cereals which were all below 4. Wheat had a significantly higher (P<0.001) maltose concentration and this was 9.5, 33 and 35 times higher than the maltose concentration in barley, maize and sorghum. Lactic acid production from barley was significantly higher (P<0.001) than any other cereal type and each cereal was significantly different from the other in lactic acid production.

Table 3.1: Effect of cereal type on 24-hour lactic acid fermentation (mmol/L), (n=45).

Cereal type	pH	Maltose*	Glucose*	Fructose*	Lactic acid*	Acetic acid*
Sorghum	3.59^{a}	9.13^{a}	49.60^{b}	23.80^{b}	292.33^{c}	11.58^{b}
Barley	3.64^{a}	36.58^{b}	84.99^{c}	82.88^{c}	403.17^{d}	25.15^{c}
Wheat	4.35^{b}	346.16^{c}	14.84^{a}	7.34^{a}	168.62^{a}	4.62^{a}
Maize	3.63^{a}	10.49^{a}	101.13^{d}	27.70^{b}	204.89^{b}	10.05^{b}
SED	**0.02**	**4.05**	**3.83**	**2.12**	**7.13**	**1.63**
P-value	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.01**

^{abcd} significant difference between means bearing different letters in the same column. *mmol/L. SED-standard error of the difference, n=number of observations per mean.

Lactic acid concentration was significantly (P<0.01) affected by the presence of $CaCO_3$ in the fermentation mixture (Table 3.2). There was no significant effect of water mineral content on acetic acid production. However, there was a mixed response in fermentation end-products with different levels of $CaCO_3$ in the fermenting medium. There were no significant effect of $CaCO_3$ concentration on lactic acid production except the concentration with 0.2g/l of $CaCO_3$ which had a significantly lower value (227.02 mmol/L).

Table 3.2: Effect of water quality on lactic acid fermentation of four cereals (mmol/L), (n=36).

$CaCO_3$ in water†	pH	Maltose*	Glucose*	Fructose*	Lactic acid*	Acetic acid*
0	3.76^{bc}	113.12^{c}	66.05^{b}	38.82^{b}	317.78^{c}	12.36
0.01	3.82^{ab}	97.59^{b}	80.24^{c}	37.50^{ab}	255.82^{b}	13.08
0.02	3.85^{a}	95.42^{b}	54.19^{a}	31.81^{a}	227.02^{a}	12.18
0.03	3.85^{a}	114.33^{c}	66.30^{b}	36.54^{ab}	273.77^{b}	12.82
0.04	3.75^{c}	82.50^{a}	46.43^{a}	32.47^{ab}	261.86^{b}	13.81
SED	**0.02**	**4.53**	**4.28**	**2.37**	**7.97**	**1.82**
P-value	**<0.05**	**<0.05**	**<0.05**	**<0.05**	**<0.01**	**0.93**

^{abc} significant difference between means bearing different letters in the same column. *mmol/L. SED-standard error of the difference. † Quantity of calcium carbonate in sterile distilled water (g/l), n= number of observations per mean.

Maltose, glucose, organic acid concentrations and pH were significantly affected by the micro-organism used in fermentation (Table 3.3). SLP had significantly lower pH value ($P<0.001$) and higher fructose ($P<0.001$) and lactic acid ($P<0.01$) concentrations than the other LAB fermentations. PA1 produced significantly ($P<0.001$) more acetic acid than LF1 and PA1.

Table 3.3: Effect of micro-organism on lactic acid fermentation of four cereals (mmol/L), (n=60).

Micro-organism	pH	Maltose*	Glucose*	Fructose*	Lactic acid*	Acetic acid*
LF1	3.88^{a}	105.59^{a}	64.03	40.31^{a}	241.41^{a}	7.81^{a}
PA1	3.86^{a}	91.97^{b}	60.10	38.69^{a}	260.15^{b}	20.83^{b}
SLP	3.67^{b}	104.21^{a}	63.80	27.28^{b}	300.20^{c}	9.91^{a}
SED	**0.02**	**3.51**	**3.32**	**1.84**	**6.17**	**1.41**
P-value	**<0.001**	**<0.01**	**0.46**	**<0.001**	**<0.01**	**<0.001**

^{abc} significant difference between means bearing different letters in the same column. *mmol/L. SED-standard error of the difference. PA1-*Pediococcus acidilactici*, LF1-*Lactobacillus farciminis*, SLP-*L. plantarum*, n=number of observations per mean.

There were significant ($P<0.01$) cereal type x water hardness interactions for pH, sugar and lactic acid concentration (Table 3.4). There was no significant cereal type x water hardness interaction in acetic acid production. The presence of $CaCO_3$ in the fermentation mixture significantly reduced ($P<0.01$) lactic acid production in all the cereals except barley. Acetic acid production was not significantly affected by water hardness in fermentation of any cereal type. A significant effect ($P<0.001$) of water hardness on maltose concentration was only observed with wheat. There were no significant differences between water treatments

Table 3.4: Effect of water hardness and cereal type on pH, sugar and organic acid concentrations (mmol/L), (n=9).

Cereal type	$CaCO_3$ in water*	pH	Maltose	Glucose	Fructose	Lactic acid	Acetic acid
Sorghum	**0**	3.58	14.76	60.25^{b}	29.52	388.85^{a}	13.35
	0.01	3.59	9.38	63.60^{b}	25.80	272.01^{b}	12.38
	0.02	3.60	7.75	61.54^{b}	23.75	250.48^{b}	8.50
	0.03	3.58	6.21	33.97^{b}	21.17	277.22^{b}	10.12
	0.04	3.59	7.56	28.64^{a}	18.75	273.10^{b}	13.53
	P-Value	1.0	1.0	<0.05	0.75	<0.001	0.9986
Barley	**0**	3.58^{a}	42.32	59.70^{a}	75.91	326.65^{a}	20.00
	0.01	3.61^{ab}	35.69	105.15^{b}	88.21	431.84^{c}	25.35
	0.02	3.72^{ab}	33.70	72.50^{a}	77.14	356.24^{ab}	25.66
	0.03	3.76^{b}	36.96	88.89^{ab}	90.15	506.53^{d}	27.54
	0.04	3.55^{a}	34.24	98.71^{b}	82.98	394.56^{bc}	27.21
	P-Value	<0.05	1.0	<0.05	0.25	<0.01	0.9954
Wheat	**0**	4.18^{a}	383.18^{a}	11.11	8.53	283.48^{d}	3.99
	0.01	4.42^{b}	332.27^{b}	14.63	7.35	110.42^{ab}	5.19
	0.02	4.45^{b}	333.96^{b}	16.27	7.35	98.46^{a}	4.73
	0.03	4.50^{b}	403.48^{a}	21.67	9.15	160.52^{bc}	5.76
	0.04	4.18^{a}	277.91^{c}	10.53	4.30	190.25^{c}	3.44
	P-Value	<0.001	<0.001	0.9995	1.0	<0.001	1.0
Maize	**0**	3.69	12.20	133.15^{b}	41.30^{b}	272.15^{c}	12.08
	0.01	3.64	13.01	137.58^{b}	28.65^{ab}	209.02^{b}	9.42
	0.02	3.62	6.29	66.43^{a}	18.98^{a}	202.91^{ab}	9.82
	0.03	3.54	10.66	120.67^{b}	25.70^{ab}	150.82^{a}	7.86
	0.04	3.67	10.27	47.83^{a}	23.85^{a}	189.55^{ab}	11.06
	P-Value	0.16	1.0	<0.001	<0.05	<0.05	0.9998
SED		0.05	9.05	8.56	4.75	15.94	3.65
Interaction	**P-value**	<0.001	<0.001	<0.001	0.003	<0.001	0.659

^{abcd} significant difference between means bearing different letters in the same column and cereal type. SED-standard error of the difference. *Quantity of $CaCO_3$ in sterile distilled water (g/l), n=number of observations per mean.

in glucose concentration of wheat whilst the differences between water treatments in the other cereals were all significant (P<0.05). There were significant cereal type x LAB treatment interactions in pH, sugar and organic acid concentrations (Table 3.5). Lactic acid

concentrations resulting from fermentation of sorghum ($P<0.05$) was significantly lower with LF1 fermentations than with SLP or PA1.

Table 3.5: Effect of cereal type and micro-organism on pH, sugar and organic acid concentrations (mmol/L), (n=15).

Cereal type	Micro-organism	pH	Maltose	Glucose	Fructose	Lactic acid	Acetic acid
Sorghum	**LF1**	3.72^{a}	6.643	48.33	28.38^{a}	264.61^{a}	7.82^{a}
	PA1	3.57^{b}	7.634	43.25	29.84^{a}	308.57^{b}	20.43^{b}
	SLP	3.47^{b}	13.118	57.22	13.17^{b}	303.81^{b}	6.48^{a}
	P-Value	<0.001	0.9997	0.62	<0.01	<0.05	<0.01
Barley	**LF1**	3.65^{ab}	36.451	81.47	92.37^{a}	444.12^{a}	12.37^{a}
	PA1	3.71^{a}	33.054	90.12	78.53^{b}	346.61^{b}	42.80^{b}
	SLP	3.57^{b}	40.238	83.38	77.73^{b}	418.77^{a}	20.28^{a}
	P-Value	<0.05	0.9968	0.977	<0.05	<0.001	<0.001
Wheat	**LF1**	4.40^{a}	366.49^{a}	13.15	8.64	112.39^{a}	4.32
	PA1	4.52^{a}	317.64^{b}	13.96	7.27	141.57^{a}	3.31
	SLP	4.12^{b}	354.35^{a}	17.41	6.11	251.92^{b}	6.23
	P-Value	<0.001	<0.001	1.0	0.9999	<0.001	0.9966
Maize	**LF1**	3.73^{a}	12.76	113.16	31.85^{a}	144.51^{a}	6.70^{a}
	PA1	3.65^{a}	9.57	93.06	39.12^{a}	243.87^{b}	16.79^{b}
	SLP	3.51^{b}	9.13	97.18	12.11^{b}	226.29^{b}	6.65^{a}
	P-Value	<0.05	1.0	0.1131	<0.001	<0.001	<0.05
SED		0.04	7.01	6.63	3.68	12.35	2.83
Interaction	**P-value**	<0.001	<0.001	0.023	<0.001	<0.001	<0.001

[ab] significant difference between means bearing different letters in the same column and cereal type.
SED- standard error of the difference. PA1-*Pediococcus acidilactici*, LF1-*Lactobacillus farciminis*, SLP-*L. plantarum*, n=number of observations per mean.

Fermentation of wheat with SLP produced significantly more lactic acid than fermentation with LF1 or PA1. Maize fermentation with LF1 produced significantly less lactic acid than the other two LAB fermentations. Fermentation of wheat ($P<0.001$) and maize ($P<0.05$) with SLP resulted in significantly lower pH values than LF1 or PA1. Sorghum fermented with LF1 had significantly higher pH (3.72) ($P<0.001$) than sorghum fermented with other LAB fermentations. Maize, sorghum and barley fermented with PA1 produced significantly more acetic acid. Inoculant had no effect on acetic acid concentration in wheat fermentation.

There were significant (P<0.01) micro-organism type x water hardness interactions for pH, sugar and lactic acid concentration (Table 3.6). There were no significant quadratic or

Table 3.6: Effect of micro-organism and water hardness on pH, sugar and organic acid concentrations (mmol/L), (n=12).

LAB sp	$CaCo_3$ in water (g/l)*	pH	Maltose	Glucose	Fructose	Lactic acid	Acetic acid
LF1	**0**	3.76^{a}	155.68^{a}	70.82^{ab}	36.64	242.63^{ab}	6.50
	0.01	3.91^{b}	103.97^{b}	83.78^{b}	42.59	236.51^{ab}	9.41
	0.02	3.88^{ab}	92.48^{b}	45.84^{a}	38.78	219.80^{b}	4.67
	0.03	3.94^{b}	91.29^{b}	67.88^{ab}	44.21	273.06^{a}	7.87
	0.04	3.90^{b}	84.52^{b}	51.82^{a}	39.33	235.05^{ab}	10.58
	P-Value	<0.05	<0.001	<0.01	0.9809	<0.05	0.862
PA1	**0**	3.87	92.57	66.01^{ab}	54.55^{a}	329.37^{a}	22.72
	0.01	3.90	93.71	86.70^{b}	42.62^{ab}	275.89^{b}	20.84
	0.02	3.87	93.31	52.78^{a}	31.49^{b}	224.71^{c}	17.68
	0.03	3.85	100.43	53.61^{a}	30.63^{b}	226.16^{c}	20.38
	0.04	3.82	79.85	41.40^{a}	34.17^{b}	244.65^{bc}	22.55
	P-Value	0.8311	0.3639	<0.01	<0.001	<0.05	0.9563
SLP	**0**	3.65^{abc}	91.11^{a}	61.32^{ab}	25.25	381.35^{a}	7.85
	0.01	3.64^{b}	95.08^{a}	70.25^{ab}	27.30	255.07^{b}	9.00
	0.02	3.79^{a}	100.49^{a}	63.93^{ab}	25.15	236.57^{b}	14.19
	0.03	3.74^{ab}	151.26^{b}	77.42^{b}	34.79	322.10^{c}	10.21
	0.04	3.53^{c}	83.13^{a}	46.06^{a}	23.91	305.90^{c}	8.30
	P-Value	<0.05	<0.001	<0.01	0.3506	<0.05	0.7904
SED		0.04	7.84	7.42	4.11	13.81	3.16
Interaction	P-value	<0.001	<0.001	0.004	<0.001	<0.001	0.167

^{abc} significant difference between means bearing different letters in the same column and micro-organism.
SED- standard error of the difference. PA1-*Pediococcus acidilactici*, LF1-*Lactobacillus farciminis*, SLP-*L. plantarum*.
*Quantity of $CaCO_3$ in sterile distilled water (g/l), n=number of observations per mean.

linear effects in acetic acid production. The presence of $CaCO_3$ in the fermentation mixture had a significant effect (P<0.01) on lactic acid concentration with SLP or PA1. Fermentation without $CaCO_3$ significantly increased lactic acid concentration for these LAB. The presence of $CaCO_3$ significantly (P<0.001) reduced maltose concentrations in LF1 fermentations but did not significantly increase lactic acid concentrations in LF1 fermentations without $CaCO_3$ in the mixture.

3.3.5 Effect of water hardness, grain type and micro-organism on viscosity of fermented feed supernatant.

Viscosity was significantly (P<0.001) affected by cereal type (Figure 3.1). Barley and wheat had significantly higher supernatant viscosities than sorghum and maize. There were no significant differences between micro-organisms (Figure 3.2) or water treatments (Figure 3.3) in supernatant viscosities.

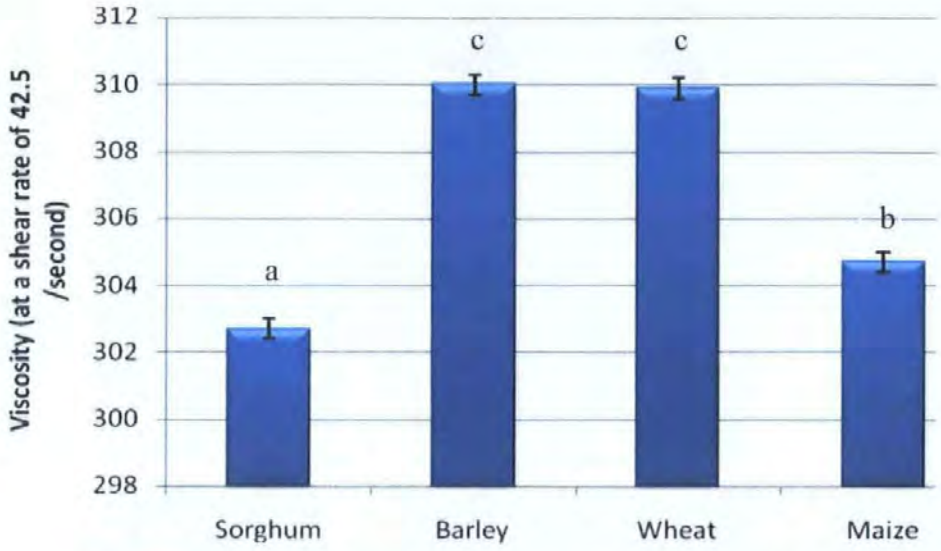


Figure 3.1: Effect of cereal type on viscosity of fermented feed (P-value-<0.001), (n=45).

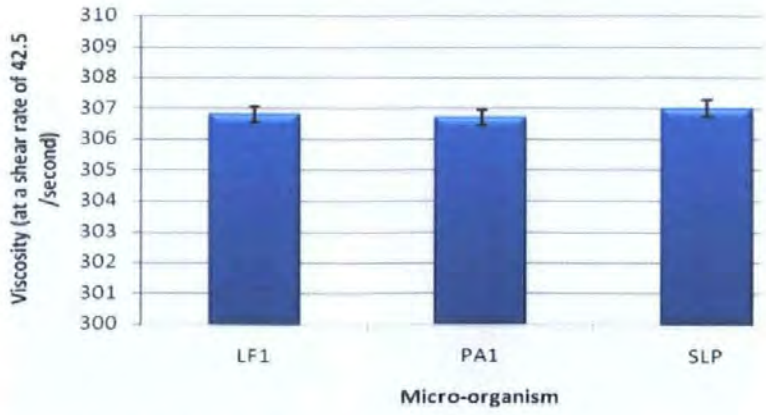


Figure 3.2: Effect of micro-organism on viscosity of fermented feed (P-value-0.60), LF1-*Lactobacillus farciminis*, PA1-*Pediococcus acidilactici* and SLP-*Lactobacillus plantarum*, (n=60).

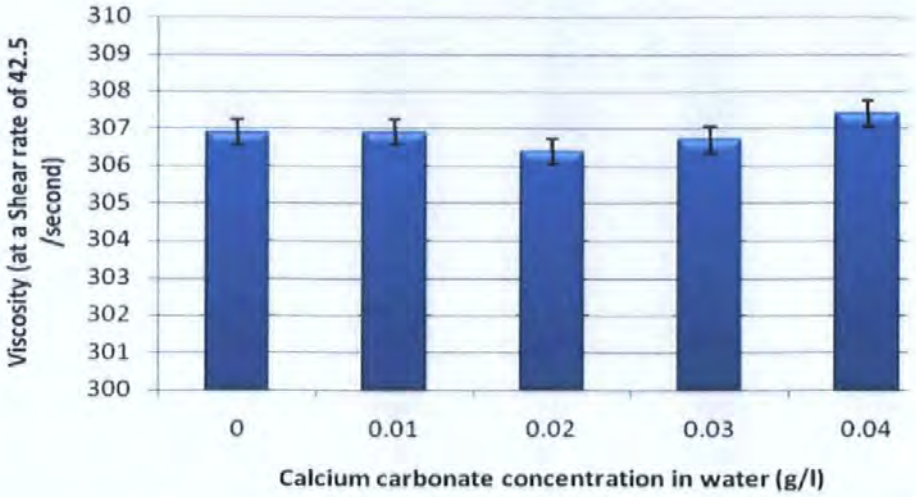


Figure 3.3: Effect of water hardness on viscosity of fermented feed (P-value-0.089), (n=36).

The cereal type x micro-organism and cereal type x water hardness interactions were highly significant (P<0.001) as shown in Table 3.7. There were no linear effects in supernatant viscosity between micro-organisms in sorghum and barley fermentations. Fermentation of wheat with PA1 resulted in a significantly (P<0.001) lower supernatant viscosity than other micro-organisms. A significantly lower maize supernatant viscosity was obtained with LF1 than PA1 or SLP.

Table 3.7: Effect of cereal type, micro-organism and water hardness on viscosity of fermented feeds

Parameter	Sorghum	Barley	Wheat	Maize
Micro-organism*				
LF1	303.0	309.4	311.99[a]	303.1[a]
PA1	302.8	310.6	307.5[b]	306.0[b]
SLP	302.4	310.1	310.3[a]	305.1[b]
P-value	**0.9952**	**0.3839**	**<0.001**	**<0.05**
$CaCO_3$ in water†				
0	303.2	309.6	309.1	305.8[bc]
0.01	302.9	310.7	311.4	302.5[a]
0.02	302.7	310.0	309.1	303.9[b]
0.03	302.7	309.6	311.2	303.4[b]
0.04	302.2	310.3	308.8	308.1[c]
P-value	**0.9976**	**0.9675**	**0.061**	**<0.001**

* SED-0.52 and n=15, Interaction P-value- <0.001; †SED-0.67 and n=9, Interaction P-value-<0.001. LF1- *Lactobacillus farciminis*, SLP-*L. plantarum*, PA1-*Pediococcus acidilactici*. †Quantity of $CaCO_3$ in sterile distilled water (g/l).

A significant interaction (P=0.014) in supernatant viscosity was also observed between micro-organisms and water treatments (Table 3.8). There were no significant linear effects on supernatant viscosity between water treatments in LF1 and SLP fermentations.

Table 3.8: Effect of water hardness and micro-organism on viscosity of fermented feeds, (n=12).

$Caco_3$ in water*	LF1	PA1	SLP
0	306.2	307.0^{ab}	307.5
0.01	307.1	306.9^{ab}	306.6
0.02	306.6	305.4^{a}	307.3
0.03	306.4	307.0^{ab}	306.8
0.04	307.8	307.0^{b}	306.8
P-value	**0.526**	**<0.05**	**0.9712**

SED-0.58, Interaction P-value- 0.014. *Quantity of $CaCO_3$ in sterile distilled water (g/l).
LF1= *Lactobacillus farciminis*, SLP= *L. plantarum*, PA1= *Pediococcus acidilactici*, n=number of observations per mean.

3.4 DISCUSSION

3.4.1 pH

The pH values reported here are similar to that (3.8) of Beal *et al.* (2002) for fermented liquid pig feed incubated at 30 °C. The reduction in pH is very important for the ability of the feed to withstand contamination by enteropathogens. Brooks *et al.* (2001) stated that acidifying feed to a pH of less than 2.5 would eliminate bacteria and yeast. They also indicated that with a pH greater than 4.5, coliforms and *Salmonella* could proliferate.

Despite the fact that the total acid concentration of fermented barley was almost double that of sorghum and maize, its pH was still slightly higher (3.64) than those of maize (3.63) and sorghum (3.59). This may be related to a higher buffering capacity in barley than maize or sorghum. The results for the pH of wheat and barley presented here are in

agreement with studies by Beal *et al.* (2005) who found that wheat samples (4.53) had significantly higher ($P<0.001$) pH values than barley samples (4.30).

3.4.2 Sugars

The concentration of maltose was significantly higher with wheat than other cereals. Wheat maltose concentration was 9.5 times higher than maltose with barley and over 30 times higher than with maize or sorghum. Starch hydrolysis of raw wheat has been reported (Anguita *et al.* 2006) to be higher than raw maize and barley at the end of SI incubation (simulation of pancreatic digestion; pancreatin; pH 7; duration 4h).

Another factor implicated in the variation of the simple sugars concentration of the fermented feeds is the utilisation of these sugars by the LAB spp present for the production of lactic and acetic acid. This is mostly the case for wheat with very high concentrations of maltose but lowest overall mean concentration of glucose, lactic and acetic acids. This result is in conformity with the findings of Charalampopoulos *et al.* (2002) who reported that low metabolite density in fermented cereal-based substrates coincided with a relatively low consumption of the available carbohydrates although it was mainly glucose in their study.

The variation in the concentration of the different sugars used in the study of the LAB spp could be explained by the fact that sugar fermentation by lactic acid bacteria is strain specific. This has also been highlighted by Charalampopoulos *et al.* (2002) using probiotic lactic acid bacteria incubated in malt, wheat and barley substrates. They indicated that each lactobacillus (*Lactobacillus acidophilus, L. plantarum, L. reuteri and L. fermentum*) had a

specific preference for one or more sugars. Brooks *et al.* (2002) also found that the poorest performing substrate in fermentation of five sugars (glucose, fructose, maltose, sucrose and lactose) with five LAB was fructose producing 121-150 mmol/L lactic acid for 72 h and 30 °C fermentation compared with lactose (171-188 mmol/L).

3.4.3 Organic acids

The results for lactic acid fermentation of barley and wheat are higher than those reported by Beal *et al.* (2002). The difference in results could be attributed to the differences in the dry matter content of the fermenting medium. They used a feed to water ratio of 1 feed to 2.5 water, more practical for pig nutrition whereas in the current study 1 feed to 1.4 water thought to be ideal for chicken feeds was used. The organic acid concentrations here are also higher than results from natural fermentation of wheat and barley reported by Beal *et al* (2005). They attributed their low values to the fact that spontaneous fermentation was inconsistent and could not result in the production of the desired lactic or other short chain fatty acid concentrations in the feed.

One of the objectives of the present study was to investigate the effect of water hardness on feed fermentation. The results presented here clearly demonstrate that the presence of $CaCO_3$ in the culture mixture affects the production of lactic acid except for barley where the absence of $CaCO_3$ did not result in higher lactic acid production. This trend was not the same with acetic acid. Logically, the calcium carbonate dissolved and/or suspended in the fermenting medium should have acted like a buffer by neutralising the growing acidity and permitting the LAB spp to produce more thereby delaying autolysis. However, it was not the case for this study. The mineral content might therefore have influenced the metabolism of the fermenting microbes in a way that reduced lactic acid production. Wee

et al. (2006) cited $CaCO_3$ as one of the factors affecting lactic acid production under culture conditions. A lower concentration of $CaCO_3$ in the fermentation medium has been shown to enhance lactic acid production in fermentations with *Lactobacillus delbrueckii* NCIM 2025 (Bhatt & Srivastava 2008).

3.4.4 Viscosity

In this study, the cereal type was observed to be the only factor that significantly affected the supernatant viscosity. These differences in supernatant viscosity may be attributed to intrinsic differences in the physical and chemical properties of the grains. The non-starch polysaccharide components of feed ingredients especially barley and wheat are known to increase intestinal viscosity and reduce nutrient utilisation in chickens (e.g. Razdan & Pettersson 1996; Smits *et al.* 1997; Saki 2005; Ponte *et al.* 2008). The use of feed enzymes on barley and wheat-based diets to reduce non-starch polysaccharides in such diets and intestinal viscosity in chickens has been extensively reported (e. g. Gracia *et al.* 2004; Lazaro *et al.* 2004; Medel *et al.* 2004; Meng *et al.* 2005; Wang *et al.* 2005; Meng *et al.* 2006; Garcia *et al.* 2008). The general response of the chickens to enzyme supplementation in such diets has been;

- Reduction in feed and intestinal viscosity
- Reduced feed passage rate and increased nutrient retention
- Improved feed conversion efficiency and weight gain
- Reduced relative weights of digestive organs
- Improvements in intestinal characteristics

Enzyme producing *Lactobacillus* strains have also been used to reduce intestinal viscosity for chickens fed barley and wheat-based diets. For instance, reduction in chicken intestinal fluid viscosity by 21 to 46% through the use of β-glucanase-producing *Lactobacillus*

strains in barley-based diets has been reported to improve intestinal characteristics and reduce feed passage rate by 2.2 hours when 50% of the administered marker is excreted (Sieo *et al.* 2005).

3.5 CONCLUSION

The presence of $CaCO_3$ in the culture mixture reduces lactic acid production in the cereals used for fermentation in the current study except with barley. There was no significant effect of $CaCO_3$ in the culture medium on pH and acetic acid production. Lactic acid production was significantly affected by cereal type or LAB used in fermentation. Feed viscosity was not affected by microbial fermentation or water mineral content for the specific grain particle size used in this study.

Chapter 4

EXPERIMENT 2: EFFECT OF MICRO-ORGANISM AND PARTICLE SIZE ON FERMENTATION OF SORGHUM AND MAIZE FOR POULTRY FEED

4.1 INTRODUCTION

Grain sorghum is widely used as a food cereal in many parts of Africa, Asia and the semi-arid tropics world-wide (Elkhalifa & El-Tinay 2002; Osman 2004; Fombang *et al.* 2005; Ragaee *et al.* 2006). In Africa, India and China, it is only superseded by rice and wheat as a cereal for human consumption (Elkhalifa & El-Tinay 2002). In addition to being a staple food for humans, it is also used as a feed for animals (Peiris *et al.* 1998; Elkhalifa & El-Tinay 2002; Balogun *et al.* 2005; Huang *et al.* 2005) and as an industrial raw material (Elkhalifa & El-Tinay 2002). In the semi-arid tropics it is more popular than maize because it grows well with limited water and under temperature stress (Osman 2004). Maize on the other hand has been used in many parts of the world as a feed ingredient in poultry nutrition (e.g. Huang *et al.* 2005; McNaughton *et al.* 2007; Rama Rao *et al.* 2007; Yu *et al.* 2007).

Provision of dry diets containing such cereals as the main energy substrates has been the conventional feeding method used for chickens. However, it has been demonstrated that soaking the feed increases nutrient availability or, alternatively, reduces particle size with consequent increase in surface area of the diet for action of the animal's digestive enzymes (Choct *et al.* 2004a). Although the success of a feeding method such as liquid feeding is highly dependent on the manner in which the grains are initially processed (Choct *et al.* 2004b), addressing the nutrient requirement for physiological development

of the animal is as important as the type of grain and the way in which the grain was processed, especially during milling.

Apart from the texture of the feed and its nutrient value, the ability of the feed to remain free of pathogens during short storage and handling for liquid feeds and to change gut microbial activity towards improved gut health is of critical importance to food and environmental safety. Consequently, striking a balance between the need for a good milled feed, cost of feed associated with extra milling and an appropriate concentration of organic acid (mainly lactic acid) in the feed is important for animal productivity, biosafety and economic reasons.

According to Beal *et al.* (2002), to prevent the growth of *Salmonella* Typhimurium DT104:30 in liquid feeds, a threshold lactic acid concentration of 75 mmol/L is required in the feed. However, due to the practical advantages of fermenting the carbohydrate-rich cereal component of the diet separately and combining it with the protein-rich components just before feeding (Beal *et al.* 2002; Beal *et al.* 2005; Moran *et al.* 2006; Canibe *et al.* 2007; Brooks 2008), it is desirable to have a higher lactic acid concentration (ca >150 mmol/L) in the cereal component so as to minimise the dilution effect to the acid concentration of the feed and pH when mixed with the protein-rich component at feeding.

Several factors are thought to affect the production of lactic acid in cereal fermentation. Among these factors are, fermentation temperature , cereal substrate type and strain of LAB (Charalampopoulos *et al.* 2002) and proportion of pre-fermented feed used in backslopping (Moran *et al.* 2006). With the same cereal substrate and LAB, a key factor

that might influence lactic acid production is the particle sizes produced at milling, which could affect the amount of sugars available for microbial enzymatic fermentation. Furthermore, Anguita *et al.*, (2006) concluded that technological processing of ingredients promotes higher starch hydrolysis in addition to increasing the amount of soluble non-starch polysaccharides and modifications in the physicochemical properties depending on the nature of the feed ingredients. According to Williams *et al.*, (2005) by assessing potential fermentability of a large number of ingredients, it is possible to make an informed choice as to which substrates are most suited for inclusion in a diet. This is true not only for the ingredients, but also for the fermenting microbes especially with the development of accelerated fermentation of animal feed substrates using lactic acid bacteria. With these points in mind, the aim of this study was to investigate the effect of particle size and micro-organism on fermentation of sorghum and maize for poultry feed.

4.2 MATERIALS AND METHODS

4.2.1 Experimental design

The study conducted as a 4 x 3 x 4 factorial design with three factors:

Factor 1: Particle Size (Coarse, Medium, Fine and Very fine); Factor 2: Control treatments without LAB and LAB treatments (*Pediococcus acidilactici* (PA1) or *Lactobacillus plantarum* (SLP)); Factor 3: Incubation time (0, 4, 8, and 24 hours). All treatments with both grains were replicated three times.

4.2.2 Particle size determination

Raw sorghum was milled in a hammer mill to pass through a 3mm screen while equal quantities of raw maize were milled either through 6 or 3 mm screens and mixed manually to give a uniform mixture of particle sizes. Both grains were separated into coarse, medium, fine and very fine particles sizes using a Retisch flask shaker (Endecotts LTD London, England) with a stack of sieves. The sieve apertures were 2.5 mm, 850 µm, and 500 µm, from the first to the last sieve and ending in a pan with the very fine particles. Samples (185 ± 10g) were placed on the sieves (diameter 200mm) for each cycle and the sieving done for a period of 10 minutes at amplitude of 80. Samples of each particle size were weighed to 100 g sachets for subsequent irradiation. Irradiation of sorghum and maize was conducted with 25 kGy γ-radiation from ^{60}Co by Becton and Dickinson, Plymouth, UK. Maize was obtained from Edwin Tucker and Sons, Ashburton, Devon while Sorghum was the white variety (*Sorghum bicolour* L. Moench) acquired from the World Foods Shop, Plymouth.

4.2.3 Fermentation and sample collection

Feed samples were mixed with sterile distilled water at a ratio of 1:1.4 as recommended by Heres *et al.* (2003). The mixture was inoculated with 0.01 ml of an overnight culture of De Man, Rogosa and Sharpe (MRS) broth concentration containing one of two ca 10^9 cfu/ml LAB *spp* (PA1 or SLP). These were incubated at 30 °C simultaneously with a control treatment without lactic acid bacteria. Samples were stirred for 1-2 minutes and sub-samples removed aseptically from each beaker at the beginning of the fermentation (0 hours) and at 4, 8, 24 hours after fermentation. The samples were used to measure the

pH using a pH electrode (pH 213 microprocessor pH meter, Hanna instruments, Portugal) and 0.5ml samples were collected for sugar and organic acid analysis and immediately frozen in Eppendorf tubes and kept at -20 °C until analysis.

4.2.4 Analysis for short chain organic acids and sugars

Organic acids and simple sugars were analysed by High Performance Liquid Chromatography (HPLC) according to the method of Niven *et al.* (2004) described in chapter 3 section 3.2.3.

4.2.5 Data analysis

Data were analysed using the general linear model procedure (GLM) of analysis of variance using Minitab (release 15.0) according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \delta_{ij}$$

Where Y_{ij} is the observed dependent variable; μ is the overall mean; α_i is the effect due to particle size; β_j is the effect due to lactobacilli used; $(\alpha\beta)_{ij}$ is the interaction between particle size and lactobacilli used and δ_{ij} is the random error. Data for the different time periods and grains (maize or sorghum) were analysed separately and least square means with pooled standard error of the means (SEM) were obtained. Differences between means were determined using the Tukey's test (Zar 1999). Probability values ≤ 0.05 were considered to be statistically significant.

4.3 RESULTS

4.3.1 Maize fermentation

After 24 hour fermentation the pH had dropped significantly more (P<0.001) in the LAB fermentations (Table 4.1) than the control treatment. Fermentation of maize with SLP resulted in a significantly lower (P<0.001) pH (3.56) than fermentation with PA1 (pH 3.71). As expected, total fermentable sugars were significantly higher (P=0.002) in the control treatment than the LAB treatments. Maize fermented with SLP had a significantly lower (P=0.002) total fermentable sugar concentration than maize fermented with PA1. Lactic acid production from SLP fermented maize was significantly higher (P=0.003) than the production from PA1. However, acetic acid production from maize fermented with PA1 was significantly higher (P<0.001) than the concentration obtained with SLP.

Table 4.1: Effect of micro-organism used for maize fermentation on 24 hour pH, total sugars and organic acid concentrations (mmol/L), (n=12).

	pH	Total sugars*	Lactic acid	Acetic acid
Control	5.77^{a}	227.67^{a}	15.66^{a}	2.64^{a}
P. acidilactici (PA1)	3.71^{b}	107.48^{b}	167.57^{b}	14.51^{b}
L. plantarum (SLP)	3.56^{c}	80.42^{c}	206.17^{c}	4.54^{a}
SED	**0.014**	**6.72**	**10.51**	**1.60**
P-Value	**<0.001**	**0.002**	**0.003**	**<0.001**

^{abc} significant difference between means bearing different letters in the same column. *Total sugars are the sums of maltose, glucose and fructose concentrations. n=number of observations per mean.

4.3.1.1 Variation in fermentable sugars and lactic acid with time.

There was a consistent increase in the glucose concentration from 25.92 ± 0.96 mmol/L at 0 hour fermentation to 145.99 ± 2.70 after 24 hour fermentation in the control treatment (Figure 4.1). The linear increase in fructose concentration (39.92 mmol/L), was a third of the increase observed with glucose for the same period (24 h). Maltose

decreased in concentration from 81.89 ± 4.45 mmol/L at 0 h to 26.15 ± 1.07 mmol/L at 24 h. Within the first 4 hours of fermentation, the highest decrease in maltose concentration was recorded.

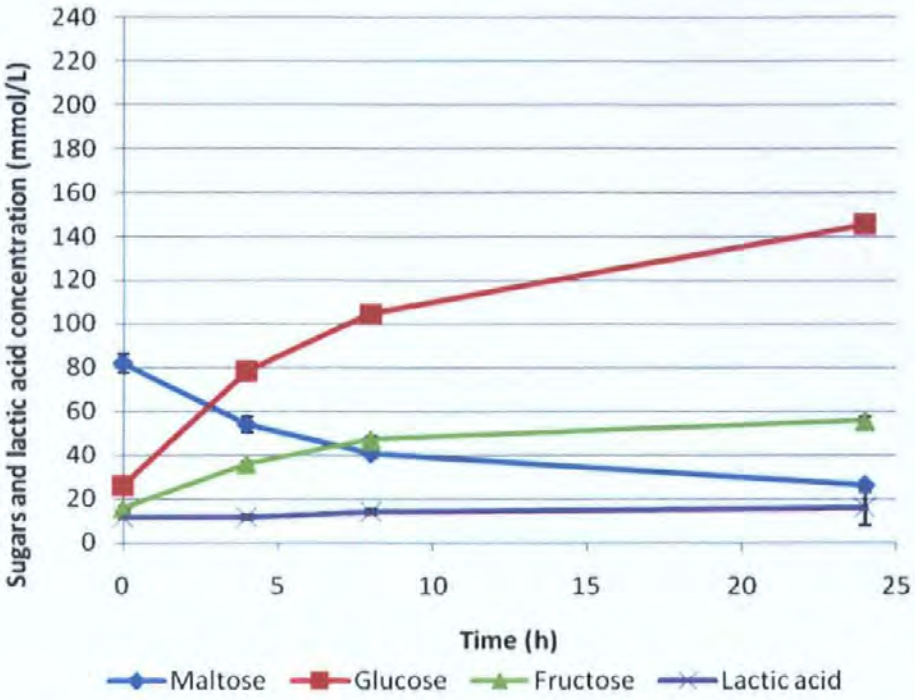


Figure 4.1: Effect of maize fermentation without LAB on concentrations of fermentable sugars and lactic acid (mmol/L).

The initial rapid increase in lactic acid production resulting from fermentation with PA1 (Figure 4.2) compared with SLP (Figure 4.3) for the first 8 hours of fermentation was not maintained until 24 hour fermentation. The concentration of lactic acid was consequently higher for SLP fermented maize (206.17 ± 7.43) (Mean ± SEM) than fermentation with PA1 (167.57 ± 7.43). The concentrations of glucose increased from 27.22 ± 1.14 at 0 h to 84.19 ± 2.55 at 8 h while fructose increase from 12.08 ± 0.78 to 38.35 ± 1.35 for SLP fermentation (Figure 4.3). The concentrations of these sugars also increased for fermentation with PA1. The concentration of maltose was observed to decrease throughout the fermentation period in LAB treatments.

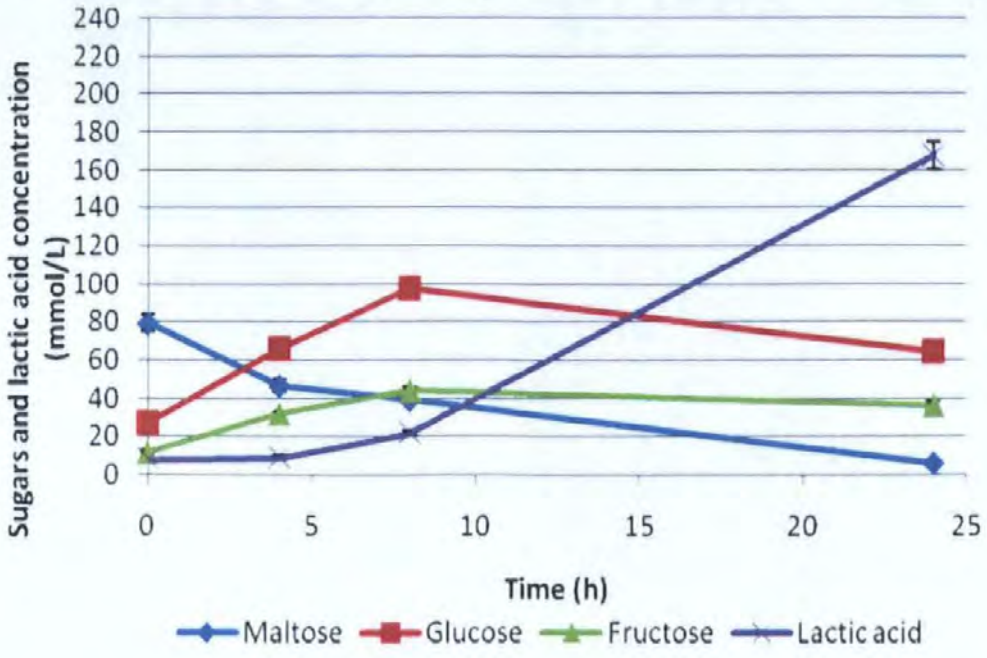


Figure 4.2: Effect of maize fermentation with *P. acidilactici* (PA1) on concentrations of fermentable sugars and lactic acid (mmol/L).

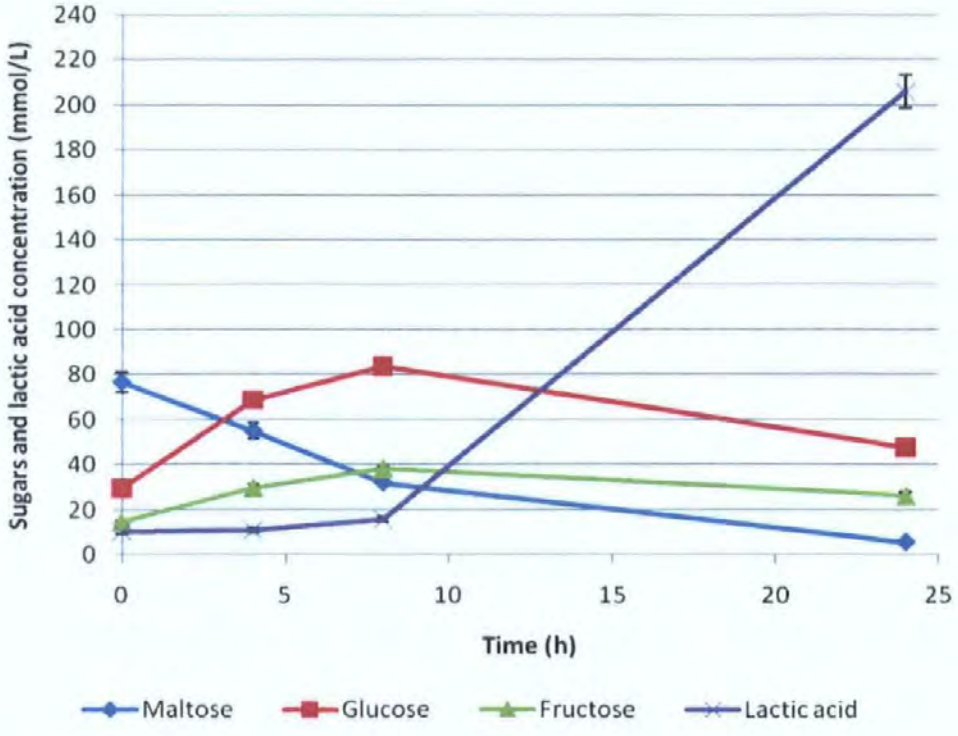


Figure 4.3: Effect of maize fermentation with *L. plantarum* (SLP) on concentrations of fermentable sugars and lactic acid (mmol/L).

Due to large differences in pH, fermentable sugars and organic acid concentrations between the different time periods especially between 0 and 24 hours for the respective LAB and particle sizes, these variables will be discussed on a sampling time basis.

4.3.1.2 Time 0 hours

There were no significant quadratic (P=0.107) or linear effects between particle sizes or between LAB treatments (Table 4.2) in maltose concentration. However, a significant particle size x LAB treatment interaction (P<0.004) was obtained in glucose concentration. The effect due to particle size in glucose concentration was significant in the SLP (P<0.03) treatments. Coarse and medium particle sizes were significantly lower in glucose concentration than the fine and very fine sizes for this treatment (SLP).

There were no significant particle size x LAB treatment interactions in the fructose (P=0.083) and total fermentable sugar (Maltose+glucose+fructose) concentrations (P=0.097). There was no significant particle size or treatment effect on the total fermentable sugars. However, there was a significant effect on the control treatment (P<0.04) between particle sizes or LAB treatments for comparisons of the fine (P<0.03) particle sizes for fructose.

Table 4.2: Effect of particle size and micro-organism used for maize fermentation on 0 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	76.31	105.62	83.83	0.49
	Medium	72.19	75.51	61.98	0.99
	Fine	85.34	69.29	79.06	0.97
	Very fine	93.71	69.29	83.34	0.73
	P-Value	0.96	0.21	0.84	**0.107**
Glucose	**Coarse**	24.50	25.81	23.80^{a}	1.00
	Medium	22.29	26.23	20.14^{a}	0.5369
	Fine	28.02	28.42	36.20^{b}	0.1648
	Very fine	28.85	28.42	38.28^{b}	0.0664
	P-Value	0.43	1.0	<0.03	**0.004**
Fructose	**Coarse**	19.32^{a}	14.09	16.97	0.2845
	Medium	13.95^{ab}	14.00	12.41	0.999
	Fine	$^{A}17.46^{ab}$	$^{B}10.12$	$^{AB}16.98$	0.034
	Very fine	12.12^{b}	10.12	12.72	0.14
	P-Value	**0.04**	**0.80**	**0.64**	**0.083**
Total	**Coarse**	120.13	145.52	124.60	0.8621
	Medium	108.43	115.74	94.53	0.9523
	Fine	130.82	107.82	132.24	0.8883
	Very fine	134.68	107.82	134.34	0.8168
	P-Value	**0.84**	**0.39**	**0.32**	**0.097**

^{abc} significant difference between means bearing different letters in the same column and parameter.
^{AB}significant difference between means bearing different letters in the same row
*Standard error of the difference-Maltose (12.59), Glucose (2.72), Fructose (1.94) and Total (15.15). n=number of observations per mean.

There were no significant interactions or between treatment effects in pH values of LAB treatments or particles sizes (Table 4.3).

Table 4.3: Effect of particle size and micro-organism used for maize fermentation on 0 hour pH, (n=3).

Particle size	Control	PA1	SLP	P-Value
Coarse	5.55	5.43	5.49	0.6366
Medium	5.62	5.55	5.51	0.6366
Fine	5.59	5.53	5.61	0.9946
Very fine	5.73	5.61	5.62	0.5211
P-Value	0.08	0.12	0.48	0.524

*Standard error of the difference - 0.054, n=number of observations per mean.

4.3.1.3 Time 4 hours

The particle size x LAB treatment interactions for maltose, glucose and total fermentable sugars were not significant (Table 4.4). There were also no significant effects between particles sizes or LAB treatments in the maltose concentration. Particle size had a linear effect on the glucose concentration in all LAB treatments. Glucose concentration in the fine and very fine particle sizes in the control ($P<0.02$), PA1 ($P<0.05$) and SLP ($P<0.01$) treatments were all significantly higher than the concentration obtained with the coarse and medium particle sizes. The effect due to particle size on the total fermentable sugars in the control treatment was significant ($P=0.003$). Particle size x treatment interactions in pH and lactic acid production were not significant (Table 4.5). The presence of LAB had a linear effect on pH ($P=0.02$) for the coarse particle size in the SLP treatment. Particle size reduction also had a linear effect ($P=0.02$) on lactic acid concentration in this same treatment.

Table 4.4: Effect of particle size and micro-organism used for maize fermentation on 4 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	61.90	67.93	62.35	1.00
	Medium	37.46	48.30	54.74	0.83
	Fine	47.62	35.58	44.82	0.98
	Very fine	68.99	35.58	58.42	0.08
	P-Value	0.12	0.10	0.81	**0.065**
Glucose	**Coarse**	65.00^{a}	51.91^{a}	46.44^{a}	0.19
	Medium	57.89^{a}	55.75^{a}	55.72^{a}	1.00
	Fine	90.36^{b}	78.94^{b}	82.37^{b}	0.80
	Very fine	100.57^{b}	78.94^{b}	90.99^{b}	0.08
	P-Value	<0.02	<0.05	<0.01	**0.275**
Fructose	**Coarse**	$^{A}45.54^{a}$	$^{A}34.62$	$^{B}30.94$	0.02
	Medium	29.73^{bc}	29.34	29.94	1.00
	Fine	40.21^{ac}	32.37	34.56	0.59
	Very fine	27.87^{b}	32.37	23.48	0.41
	P-Value	0.003	0.94	0.15	**0.031**
Total	**Coarse**	172.44^{ac}	154.46	139.73	0.57
	Medium	125.08^{a}	133.38	140.41	0.995
	Fine	178.19^{ac}	146.89	161.75	0.63
	Very fine	197.43^{bc}	146.89	172.88	0.08
	P-Value	0.003	0.95	0.55	**0.104**

^{abc} significant difference between means bearing different letters in the same column and parameter.
^{AB}significant difference between means bearing different letters in the same row
*Standard error of the difference-Maltose (9.85), Glucose (6.36), Fructose (3.63) and Total (14.95), n=number of observations per mean.

Table 4.5: Effect of particle size and micro-organism used for maize fermentation on 4 hour pH and lactic acid concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	$^{A}5.73^{a}$	$^{A}5.63$	$^{B}5.61$	0.02
	Medium	5.67^{bc}	5.63	5.55	1.00
	Fine	5.69^{ac}	5.57	5.59	0.59
	Very fine	5.77^{b}	5.59	5.53	0.41
	P-Value	0.003	0.94	0.15	**0.322**
Lactic acid	**Coarse**	10.15	9.14	5.69^{a}	0.98
	Medium	8.07	9.42	8.80^{a}	1.00
	Fine	13.92	9.61	9.03^{a}	0.96
	Very fine	13.25	9.58	20.05^{b}	0.75
	P-Value	0.88	1.00	0.02	**0.113**

^{abc} significant difference between means bearing different letters in the same column and parameter. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.05) and Lactic acid (3.57), n=number of observations per mean.

4.3.1.4 Time 8 hours

Significant particle sizes x treatment effects were observed on the concentrations of all sugars (Table 4.6) after 8 hours of fermentation. There was a significant treatment effect on maltose (P=0.0023), glucose (P<0.001), fructose (P=0.004) and total fermentable sugars (P<0.001) concentrations for the very fine particle sizes. Particle size had a significant effect on the concentration of sugars in all treatments. In the control treatment, coarse and very fine particle size fermentations resulted in significantly higher (P=0.002) maltose concentrations than medium and fine particle size fermentations. Fine particle size fermentation with PA1 resulted in a significantly lower (P=0.004) maltose concentration (21.30 ± 3.29) (Mean ± SEM) than medium (31.02 ± 3.29), Coarse (51.75± 3.29) and very fine (53.19± 3.29) particle size fermentations. Maltose in the coarse (45.05 ± 3.29) and very fine (29.56 ± 3.29) particles sizes in SLP treatment were significantly higher (P=0.031) than the medium (27.23 ± 3.29) and fine

Table 4.6: Effect of particle size and micro-organism used for maize fermentation on 8 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	53.03^{a}	51.75^{a}	45.05^{a}	0.845
	Medium	28.58^{b}	31.02^{a}	27.23^{bc}	1.00
	Fine	29.13^{b}	21.3^{b}	27.07^{bc}	0.861
	Very fine	$^{A}52.57^{a}$	$^{A}53.19^{a}$	$^{B}29.56^{ac}$	0.0023
	P-Value	0.002	0.004	0.031	**0.005**
Glucose	**Coarse**	79.96^{a}	79.30^{a}	67.36^{a}	0.8301
	Medium	76.28^{a}	75.07^{a}	77.97^{a}	1.00
	Fine	$^{A}127.31^{b}$	$^{B}95.08^{a}$	$^{A}111.01^{b}$	0.0071
	Very fine	$^{A}135.87^{b}$	$^{A}142.08^{b}$	$^{B}80.40^{a}$	<0.001
	P-Value	<0.001	<0.001	0.012	**<0.001**
Fructose	**Coarse**	56.93^{a}	57.54^{a}	46.43^{a}	0.1965
	Medium	40.59^{bc}	41.26^{b}	41.55^{a}	1.00
	Fine	52.61^{ac}	40.85^{b}	44.08^{a}	0.1434
	Very fine	$^{A}38.51^{b}$	$^{A}39.39^{b}$	$^{B}21.34^{b}$	0.0064
	P-Value	0.041	0.011	<0.001	**0.004**
Total	**Coarse**	189.91^{a}	188.59^{a}	158.84^{ab}	0.3457
	Medium	145.44^{b}	147.34^{a}	146.75^{ab}	1.00
	Fine	$^{A}209.05^{a}$	$^{B}157.24^{a}$	$^{AB}182.16^{a}$	0.011
	Very fine	$^{A}226.95^{a}$	$^{A}234.66^{b}$	$^{B}131.30^{b}$	<0.001
	P-Value	0.0423	0.0316	0.0128	**<0.001**

^{abc} significant difference between means bearing different letters in the same column and parameter. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-Maltose (4.66), Glucose (7.215), Fructose (3.81) and Total (12.07), n=number of observations per mean.

(27.07 ± 3.29) particle sizes. Particle size reduction led to a linear increase in glucose concentration. Exceptions were observed with the medium sized particles in the control and PA1 treatments and the very fine particle sizes in the SLP treatments.

There were significant particle size x LAB treatment interactions for pH (P=0.002) and lactic acid concentrations (P<0.001) (Table 4.7). pH values in LAB treatments were all significantly lower than (P<0.04) than values obtained with the control treatment. The pH of fine (5.04 ± 0.05) and very fine (4.96 ± 0.05) particles sizes in the PA1 treatment were significantly lower (P=0.033) than values obtained with the coarse (5.33 ± 0.05) and medium (5.29 ± 0.05). These values were also lower (P<0.04) than the mean pH values for all the particle sizes on the SLP treatment. Lactic acid concentrations for the particle sizes on the PA1 treatment were higher than control treatment concentrations for the coarse (P=0.0138), medium (P=0.002) and fine particle sizes (P=0.0043). The concentrations of lactic acid in PA1 fermentations for the coarse (22.77 ± 2.07) and fine (27.47 ± 2.07) particle size, were significantly higher (P=0.0138 and P=0.0043) than corresponding fermentations (14.65 ± 2.07 and 14.08 ± 2.07) in the SLP treatment. Lactic acid production resulting from fermentation of very fine particles sizes with PA1 was significantly lower (P=0.0159) than the concentration obtained with the larger particle sizes.

Table 4.7: Effect of particle size and micro-organism used for maize fermentation on 8 hour pH and lactic acid (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	$^{A}5.76$	$^{B}5.33^{a}$	$^{B}5.51$	0.005
	Medium	$^{A}5.71$	$^{B}5.29^{a}$	$^{B}5.46$	0.03
	Fine	$^{A}5.75$	$^{C}4.96^{b}$	$^{B}5.36$	0.003
	Very fine	$^{A}5.78$	$^{C}5.04^{b}$	$^{B}5.29$	<0.04
	P-Value	0.999	0.033	0.11	**0.002**
Lactic acid	**Coarse**	$^{A}10.52^{ab}$	$^{B}22.77^{ab}$	$^{A}14.65$	0.0138
	Medium	$^{A}10.38^{a}$	$^{B}25.06^{b}$	$^{AB}15.01$	0.002
	Fine	$^{A}13.74^{ab}$	$^{B}27.47^{b}$	$^{A}14.08$	0.0043
	Very fine	21.05^{b}	12.98^{a}	19.70	0.2617
	P-Value	0.0469	0.0159	0.7393	**<0.001**

^{abc} significant difference between means bearing different letters in the same column and parameter. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.07) and Lactic acid (2.93), n=number of observations per mean.

4.3.1.5 Time 24 hours

Particle size x LAB treatment interactions were observed to be significant (P<0.001) for maltose, glucose and total fermentable sugars concentrations whilst for fructose it was not significant (Table 4.8). Fermentable sugar concentrations in LAB treatments were lower (P<0.02) than control treatment concentrations. The concentrations of glucose (70.09 ± 5.40) and fructose (10.15 ± 4.02) for the very fine particle sizes in the SLP treatment were significantly lower (P<0.04) than corresponding values with all other fermentations. There was a linear effect (P≤0.002) of particle size on glucose concentration within all the treatments.

Table 4.8: Effect of particle size and micro-organism used for maize fermentation on 24 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	14.41^{a}	4.77	7.01	0.12
	Medium	$^{A}20.55^{a}$	$^{B}3.02$	$^{B}3.88$	<0.006
	Fine	$^{A}31.53^{b}$	$^{B}6.34$	$^{B}6.25$	<0.001
	Very fine	$^{A}38.10^{b}$	$^{B}10.48$	$^{B}5.65$	<0.001
	P-Value	<0.05	0.40	0.995	**<0.001**
Glucose	**Coarse**	$^{A}101.58^{a}$	$^{B}24.82^{a}$	$^{B}23.40^{a}$	<0.001
	Medium	$^{A}103.88^{a}$	$^{B}28.11^{a}$	$^{B}31.34^{a}$	<0.001
	Fine	$^{A}185.88^{b}$	$^{B}79.60^{b}$	$^{B}69.18^{b}$	<0.001
	Very fine	$^{A}192.61^{b}$	$^{B}125.81^{c}$	$^{C}70.09^{b}$	<0.001
	P-Value	<0.001	0.002	<0.002	**<0.001**
Fructose	**Coarse**	$^{A}70.61^{a}$	$^{AB}50.30^{a}$	$^{B}47.23^{a}$	0.016
	Medium	$^{A}50.11^{b}$	$^{B}28.07^{b}$	$^{B}25.92^{b}$	0.03
	Fine	$^{A}58.65^{ab}$	$^{B}35.27^{ab}$	$^{B}21.58^{b}$	0.016
	Very fine	$^{A}43.16^{b}$	$^{A}33.32^{ab}$	$^{B}10.15^{b}$	0.02
	P-Value	<0.05	0.03	<0.04	**0.127**
Total	**Coarse**	$^{A}186.61^{a}$	$^{B}79.89^{ab}$	$^{B}77.65$	<0.001
	Medium	$^{A}174.54^{a}$	$^{B}59.20^{a}$	$^{B}61.15$	<0.001
	Fine	$^{A}276.06^{b}$	$^{B}121.21^{b}$	$^{B}97.00$	<0.001
	Very fine	$^{A}273.87^{b}$	$^{B}169.61^{b}$	$^{C}85.89$	<0.001
	P-Value	<0.001	<0.001	0.299	**<0.001**

^{abc} significant difference between means bearing different letters in the same column and parameter. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-Maltose (3.02), Glucose (7.63), Fructose (5.68) and Total (13.44), n=number of observations per mean.

Particle size x LAB treatments interactions in the pH (P=0.312) and acetic acid concentration (P=0.194) were not significant (Table 4.9). However, there was a significant interaction in the lactic acid concentration (P<0.001). All LAB treatments had mean pH values ranging from 3.50 to 3.80 whilst the control treatment had values ranging from 5.70 to 5.90. The differences between LAB treatments and the control treatment were significant (P<0.004). The mean pH range of 3.50 to 3.64 observed in the PA1 treatment was lower (P<0.004) than the range of 3.66 to 3.80 obtained in the SLP

Table 4.9: Effect of particle size and micro-organism used for maize fermentation on 24 hour pH and organic acid concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	$^{A}5.90^{a}$	$^{B}3.80^{a}$	$^{C}3.64^{a}$	0.003
	Medium	$^{A}5.75^{b}$	$^{B}3.71^{ab}$	$^{C}3.55^{b}$	<0.004
	Fine	$^{A}5.70^{b}$	$^{B}3.67^{b}$	$^{C}3.54^{b}$	<0.0063
	Very fine	$^{A}5.71^{b}$	$^{B}3.66^{b}$	$^{C}3.50^{b}$	<0.004
	P-Value	0.0012	0.005	0.003	**0.312**
Lactic acid	**Coarse**	$^{A}7.82$	$^{B}175.89$	$^{B}245.09^{a}$	<0.001
	Medium	$^{A}13.37$	$^{B}132.72$	$^{B}200.49^{ab}$	<0.004
	Fine	$^{A}20.80$	$^{B}162.68$	$^{B}234.26^{a}$	<0.001
	Very fine	$^{A}20.66$	$^{B}199.00$	$^{B}144.85^{b}$	<0.003
	P-Value	1.00	0.1266	0.01	**0.001**
Acetic acid	**Coarse**	2.55	11.96	5.57	0.1879
	Medium	2.66	10.23	4.92	0.4618
	Fine	$^{A}3.49$	$^{C}21.56$	$^{B}5.07$	0.0014
	Very fine	$^{A}1.84$	$^{C}14.30$	$^{B}2.59$	<0.05
	P-Value	1.00	0.0573	0.998	**0.194**

^{abc} significant difference between means bearing different letters in the same column and parameter. ^{ABC} significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.029), Lactic acid (21.02) and Acetic acid (3.2), n=number of observations per mean.

treatment. Reduction in particle size tended to decrease the pH within all treatments ($P \leq 0.005$). LAB treatments all had significantly higher ($P<0.004$) lactic acid concentrations than the control treatment. The choice of LAB used in fermentation did not affect the concentration of lactic acid for any particle size. While there were no differences between particle sizes in lactic acid concentration within the control and PA1 treatments, coarse particles in SLP treatment produced significantly ($P=0.01$) more lactic acid (245.09 ± 14.86) than the very fine particles (144.85 ± 14.86). Acetic acid production from fine and very fine particles sizes in the PA1 treatment were significantly higher ($P<0.05$) than the control and SLP treatments.

4.3.2 Sorghum fermentation

The pH after 24 hour fermentation dropped significantly more (P=0.045) in the LAB treatments (Table 4.10) than the control treatment. Fermentation of sorghum with SLP resulted in a significantly higher pH than fermentation with PA1. As expected, total fermentable sugars was significantly higher (P<0.001) in the control than in the LAB treatments. There was no significant difference between the LAB treatments in the total fermentable sugar concentrations. Lactic acid production from SLP fermented sorghum was significantly higher (P<0.001) than the production from PA1. However, acetic acid production from sorghum fermented with PA1 was significantly higher (P<0.001) than the concentration obtained with SLP and the concentration in the control treatment.

Table 4.10: Effect of micro-organism used for sorghum fermentation on 24 hour pH, total sugars and organic acid concentrations (mmol/L), (n=12).

	pH	Total sugars*	Lactic acid	Acetic acid
Control	6.06^{a}	167.19^{a}	13.23^{a}	5.42^{a}
PA1	3.42^{c}	46.81^{b}	240.00^{b}	33.07^{b}
SLP	3.51^{b}	33.95^{b}	302.73^{c}	10.62^{a}
SED	**0.04**	**7.28**	**8.64**	**4.92**
P-Value	**0.045**	**<0.001**	**<0.001**	**<0.001**

^{abc} significant difference between means bearing different letters in the same column. *Total sugars are the sums of maltose, glucose and fructose concentrations, n=number of observations per mean.

4.3.2.1 Variation in fermentable sugars and lactic acid with time.

The was a linear increase in glucose concentration from 20.87 ± 0.77 mmol/L at 0 hour fermentation to 114.51 ± 3.67 mmol/L after 24 hour fermentation in the control treatment (Figure 4.4). There was an increase in fructose concentration (14.69 ± 0.83 for 0 h to 36.00 ± 1.36 mmol/L for 24h), a quarter of the increase obtained with glucose.

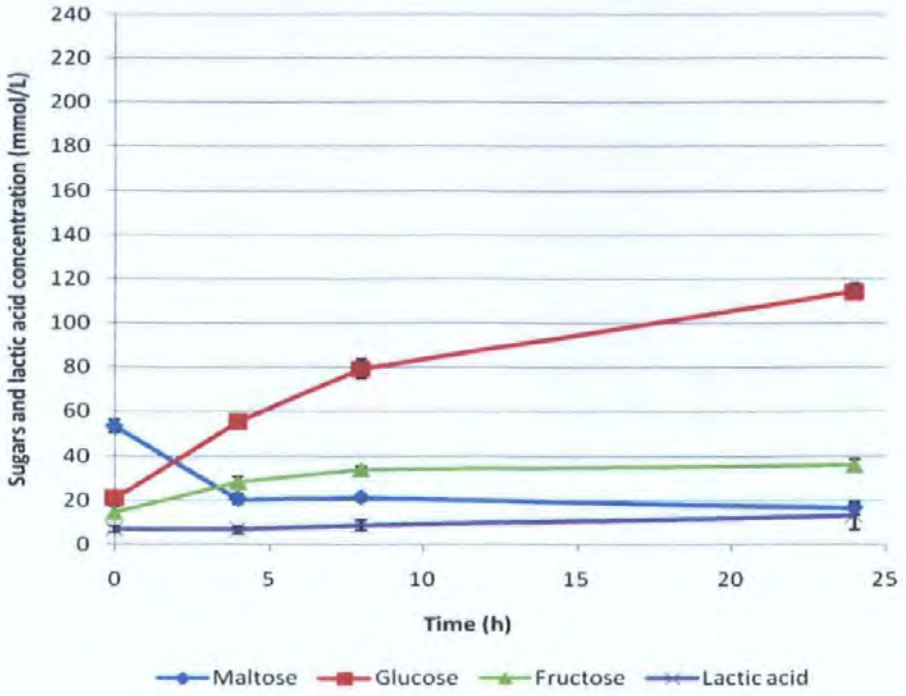


Figure 4.4: Effect of sorghum fermentation without LAB on concentrations of fermentable sugars and lactic acid (mmol/L).

Maltose decreased in concentration from 53.49 ± 2.74 mmol/L to 16.69 ± 2.87 mmol/L during this period with the highest change taking place within the first 4 hours of fermentation.

PA1 (Figure 4.5), produced more lactic acid within 8 hours of fermentation (46.60 ± 2.37mmol/L) than SLP (21.38 ± 2.37 mmol/L) (Figure 4.6). However, between 8 and 24h fermentation, the increase in lactic acid production from SLP was higher (increase of 281 mmol/L) than from PA1 (increase of 193 mmol/L). While the concentration of maltose was observed to decrease throughout fermentation for both LAB, the glucose and fructose concentrations increased during 8 hours of fermentation and then decreased drastically in the 24 h samples.

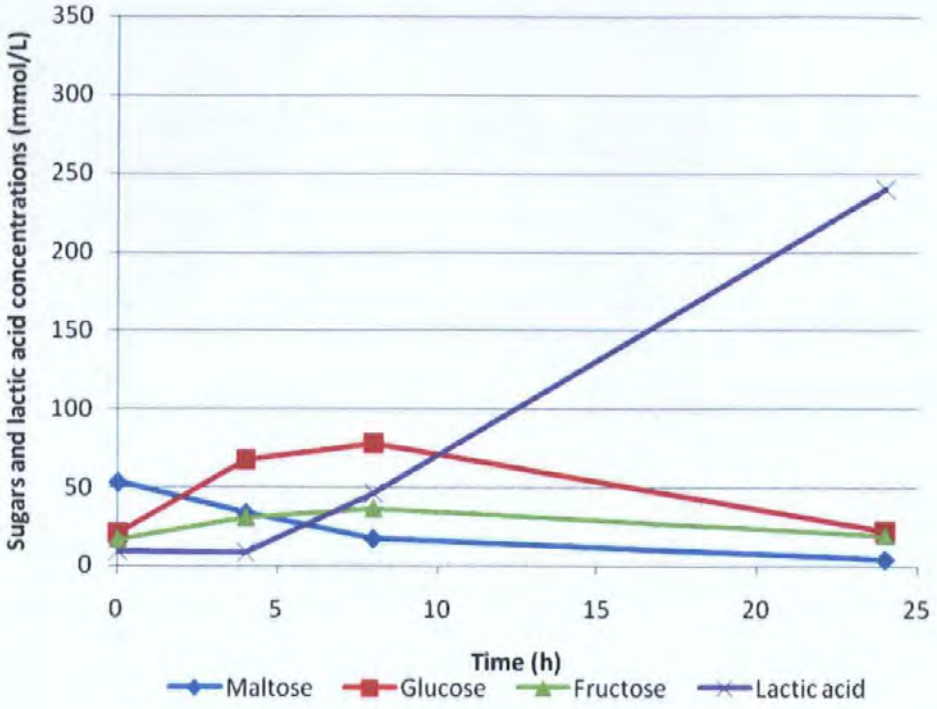


Figure 4.5: Effect of sorghum fermentation with *P. acidilactici* (PA1) on concentrations of fermentable sugars and lactic acid (mmol/L).

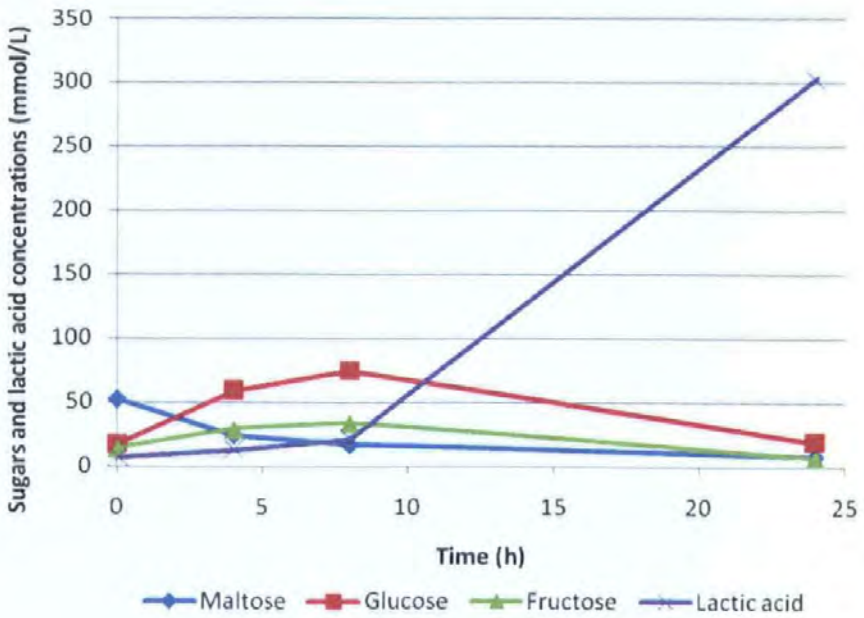


Figure 4.6: Effect of sorghum fermentation with *L. plantarum* (SLP) on concentrations of fermentable sugars and lactic acid (mmol/L).

4.3.2.2 Time 0 hours

There was no significant (P=0.105) particle size x LAB treatment interaction (Table 4.11) in maltose concentration. However, a significant interaction (P<0.001) was obtained for the concentrations of glucose (P<0.009) and fructose (P<0.004). The effect due to particle sizes in maltose concentration was significant in the control (P=0.05) and PA1 (P=0.006) treatments. No significant treatment effects were observed in the maltose concentration. The variation in glucose concentration between particle sizes was significant (P<0.05) for the LAB treatments. Significant variation in fructose (P<0.001) between particle sizes were observed in the SLP treatment.

Table 4.11: Effect of particle size and micro-organism used for sorghum fermentation on 0 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	50.60^{ab}	48.02^{ab}	52.57	1.00
	Medium	41.91^{a}	36.75^{a}	40.61	0.999
	Fine	69.84^{b}	56.03^{ab}	60.94	0.811
	Very fine	51.62^{ab}	71.99^{b}	54.83	0.316
	P-Value	0.05	0.006	0.318	**0.105**
Glucose	**Coarse**	20.36	19.42^{ab}	13.02^{ab}	0.091
	Medium	17.78	16.06^{a}	11.06^{a}	0.174
	Fine	24.59	22.23^{ab}	26.31^{c}	0.692
	Very fine	20.74	24.52^{b}	19.40^{bc}	0.385
	P-Value	0.091	0.016	0.035	**0.009**
Fructose	**Coarse**	13.77	15.07	7.76^{a}	0.093
	Medium	13.64	13.30	7.27^{a}	0.208
	Fine	17.85	19.35	20.31^{b}	0.991
	Very fine	13.48	19.40	20.05^{b}	0.177
	P-Value	0.699	0.167	<0.001	**0.004**
Total	**Coarse**	83.13^{ab}	82.51^{ab}	73.34^{ab}	0.998
	Medium	73.32^{a}	66.11^{a}	59.54^{a}	0.959
	Fine	112.28^{b}	97.61^{ab}	107.56^{b}	0.938
	Very fine	85.84^{ab}	116.45^{b}	94.28^{ab}	0.16
	P-Value	0.03	0.002	0.004	**0.084**

^{abc} significant difference between means bearing different letters in the same column and parameter
^{AB} significant difference between means bearing different letters in the same row
*Standard error of the difference-Maltose (7.74), Glucose (2.05), Fructose (2.46) and Total (10.08), n=number of observations per mean.

There were no significant quadratic or linear effects in pH values between treatments or particles sizes (Table 4.12).

Table 4.12: Effect of particle size and micro-organism used for sorghum fermentation on 0 hour pH, (n=3).

Particle size	Control	PA1	SLP	P-Value
Coarse	6.02	6.06	6.13	0.843
Medium	5.94	5.98	6.02	0.971
Fine	6.12	6.03	6.01	0.866
Very fine	5.99	5.91	5.97	0.978
P-Value	0.236	0.446	0.385	**0.264**

*Standard error of the difference- 0.064, n=number of observations per mean.

4.3.2.3 Time 4 hours

The particle sizes x LAB treatment interaction in all fermentable sugars were significant ($P<0.001$) (Table 4.13). Significant linear effects ($P<0.008$) between particle sizes in the maltose concentration were observed in the PA1 treatment. A linear effect ($P<0.001$) of particle size on glucose concentration was only obtained in the SLP treatment. Fermentable sugars in the very fine particle sizes for the PA1 treatment were all significantly higher ($P<0.01$) than concentrations for the control and SLP treatments. There were no significant differences between treatments in fermentable sugar concentration for other particle sizes.

Table 4.13: Effect of particle size and micro-organism used for sorghum fermentation on 4 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	24.79	26.71^{a}	28.13	1.00
	Medium	15.15	12.05^{a}	17.77	0.9988
	Fine	20.97	30.61^{a}	24.13	0.9338
	Very fine	$^{A}21.37$	$^{B}65.74^{b}$	$^{A}26.49$	<0.001
	P-Value	0.934	<0.008	0.898	**<0.001**
Glucose	**Coarse**	53.33	57.74^{a}	46.36^{a}	0.8226
	Medium	54.40	48.75^{a}	43.34^{a}	0.8462
	Fine	62.93	56.21^{a}	66.54^{ab}	0.8934
	Very fine	$^{A}52.90$	$^{C}108.22^{b}$	$^{B}81.80^{b}$	0.017
	P-Value	0.91	<0.001	<0.001	**<0.001**
Fructose	**Coarse**	29.52^{ab}	29.80^{b}	25.80^{a}	0.9832
	Medium	23.29^{a}	17.00^{a}	16.76^{a}	0.6164
	Fine	36.53^{b}	31.26^{b}	40.39^{b}	0.1816
	Very fine	$^{A}23.52^{a}$	$^{C}45.14^{c}$	$^{B}32.06^{ab}$	0.012
	P-Value	0.013	0.02	0.004	**<0.001**
Total	**Coarse**	107.64	114.25^{a}	100.29^{ab}	0.9921
	Medium	92.84	77.80^{a}	77.87^{a}	0.9859
	Fine	120.43	118.08^{a}	131.06^{b}	0.9992
	Very fine	$^{A}97.79$	$^{B}219.10^{b}$	$^{A}140.35^{b}$	<0.001
	P-Value	0.588	<0.001	0.014	**<0.001**

^{abc} significant difference between means bearing different letters in the same column and parameter
^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-Maltose (6.54), Glucose (6.46), Fructose (3.08) and Total (12.76), n=number of observations per mean.

Particle size x LAB treatment interactions in pH and lactic acid production were not significant (Table 4.14).There were also no significant differences between treatments in pH and lactic acid concentrations.

Table 4.14: Effect of particle size and micro-organism used for sorghum fermentation on 4 hour pH and organic acid concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	6.04	6.07^{a}	6.12^{a}	0.9281
	Medium	5.94	5.92^{ab}	5.88^{b}	0.9904
	Fine	6.11	6.00^{ab}	5.98^{ab}	0.3899
	Very fine	5.92	5.87^{b}	5.89^{b}	0.9979
	P-Value	0.056	0.038	0.008	**0.224**
Lactic acid	**Coarse**	4.40	6.02	5.64	1.00
	Medium	4.72	7.61	6.90	0.999
	Fine	9.81	4.38	10.27	0.9655
	Very fine	8.43	16.01	14.49	0.8438
	P-Value	0.981	0.315	0.687	**0.58**

^{abc} significant difference between means bearing different letters in the same column and parameter ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.054) and Lactic acid (4.42), n=number of observations per mean.

However, the presence of LAB in the fermenting medium had a significant effect ($P<0.04$) on particle size pH and particle size reduction had no effect on lactic acid concentrations.

4.3.2.4 Time 8 hours

The particle size x LAB treatment effects in glucose and fructose concentrations (Table 4.15) continued to be significant after 8 hours of fermentation. Particle size had a significant effect ($P<0.04$) on the concentration of sugars in all treatments except for maltose in the control treatment. Particle size reductions led to a linear increase in glucose and total fermentable sugar concentrations except for the medium sized particles.

Table 4.15: Effect of particle size and micro-organism used for sorghum fermentation on 8 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	18.17	22.31^{ab}	14.62^{ab}	0.886
	Medium	11.35	7.25^{a}	8.62^{a}	0.999
	Fine	20.97	13.99^{ab}	16.32^{ab}	0.935
	Very fine	35.14	26.87^{b}	27.86^{b}	0.832
	P-Value	0.083	0.02	0.019	**0.553**
Glucose	**Coarse**	54.49^{a}	59.67^{a}	56.10^{a}	1.00
	Medium	65.84^{a}	50.04^{a}	53.01^{a}	0.972
	Fine	62.93^{a}	61.99^{a}	85.32^{ab}	0.741
	Very fine	134.70^{b}	142.95^{b}	105.76^{b}	0.154
	P-Value	<0.001	<0.001	0.02	**0.036**
Fructose	**Coarse**	28.98^{ab}	34.36^{a}	30.15^{ab}	0.983
	Medium	25.67^{a}	22.83^{a}	20.24^{a}	0.982
	Fine	36.53^{ab}	35.67^{a}	47.06^{c}	0.351
	Very fine	$^{AB}44.74^{b}$	$^{A}53.86^{b}$	$^{B}37.69^{bc}$	0.05
	P-Value	0.01	0.02	0.032	**0.009**
Total	**Coarse**	101.64^{a}	116.34^{a}	100.87^{ab}	0.999
	Medium	102.86^{a}	80.12^{a}	81.87^{a}	0.986
	Fine	120.43^{a}	111.65^{a}	148.70^{bc}	0.861
	Very fine	214.58^{b}	223.68^{b}	171.31^{c}	0.185
	P-Value	0.001	<0.001	0.035	**0.035**

^{abc} significant difference between means bearing different letters in the same column and sugar type. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-Maltose (4.75), Glucose (12.20), Fructose (4.44) and Total (10.25), n=number of observations per mean.

However, differences in concentration between the medium and coarse particle sizes in all the treatments were not significant for all the sugars. Apart from the significant

differences (P=0.05) between treatments in fructose concentration for the very fine particle sizes, there was no significant treatment effect in all the sugars.

There were significant particle size x treatment interactions in pH (P<0.001) and lactic acid concentrations (P<0.001) (Table 4.16). pH values in LAB treatments were all significantly lower (P<0.001) than values obtained in the control treatment. pH values ranging from 5.08 to 5.33 were obtained in the PA1 treatment and these were significantly higher (P<0.001) than the range of 5.58 to 5.74 observed in the SLP treatment. Treatments effects on pH were also reflected by higher lactic acid concentrations in the PA1 treatment especially for the fine (P=0.012) and very fine (P<0.001) particle sizes. Reduction in particle size increased lactic acid production significantly (P<0.001) in the PA1 treatment.

Table 4.16: Effect of particle size and micro-organism used for sorghum fermentation on 8 hour pH and organic acid concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	$^{A}6.07^{a}$	$^{C}5.25^{a}$	$^{B}5.63^{a}$	<0.001
	Medium	$^{A}5.91^{a}$	$^{C}5.08^{b}$	$^{B}5.58^{a}$	<0.001
	Fine	$^{A}6.11^{a}$	$^{C}5.33^{a}$	$^{B}5.83^{b}$	<0.001
	Very fine	$^{A}5.82^{b}$	$^{C}5.09^{b}$	$^{B}5.74^{b}$	<0.001
	P-Value	0.03	0.04	0.05	**<0.001**
Lactic acid	**Coarse**	5.34	29.26^{a}	19.00	0.059
	Medium	5.08	27.95^{a}	15.57	0.082
	Fine	$^{A}9.81$	$^{B}38.28^{a}$	$^{A}18.83$	0.012
	Very fine	$^{A}14.77$	$^{B}90.89^{b}$	$^{A}32.12$	<0.001
	P-Value	0.951	<0.001	0.402	**<0.001**

^{abc} significant difference between means bearing different letters in the same column and sugar type. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.043) and Lactic acid (6.70). n=number of observations per mean. .

4.3.2.5 Time 24 hours

Particle size x treatment interactions were observed to be significant for glucose (P=0.01) and total fermentable sugar (P=0.006) concentrations whilst for fructose and maltose there was no significant interaction (Table 4.17). LAB treatments all had significantly lower (P<0.001) glucose and total fermentable sugar concentrations than the control treatment. The fermentable sugar concentration in LAB treatments after 24 hour fermentation was not significantly affected by particle size except for fermentation of fructose in the PA1 treatment (P=0.016). Significant particle size x treatment interactions in the pH (P=0.009), lactic (P<0.001) and acetic acid concentration (P=0.026) were also observed after 24 hour fermentation (Table 4.18). All LAB treatments had pH values ranging from 3.25 to 3.63 that were significantly higher (P<0.001) the range of 5.94 to 6.31in the control treatment.

Treatment effects on pH were reflected by higher (P<0.002) lactic acid concentrations in LAB treatments (197.08 to 401.87 mmol/L) as opposed to the control treatment (8.35 to 23.55 mmol/L). Twenty four hour lactic acid concentrations from coarse particle size fermentations in LAB treatments were not significantly different from concentrations in the medium and fine particle size fermentations. Acetic acid production from the fine particle sizes in the PA1 treatment was significantly higher (P<0.001) than the control and SLP treatments.

Table 4.17: Effect of particle size and micro-organism used for sorghum fermentation on 24 hour fermentable sugar concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
Maltose	**Coarse**	11.39	3.65	3.28	0.998
	Medium	8.88	3.41	3.09	1.0
	Fine	13.85	4.92	20.99	0.701
	Very fine	^{A}32.65	^{A}6.22	^{B}3.06	0.05
	P-Value	0.192	1.00	0.56	**0.100**
Glucose	**Coarse**	^{A}90.88^{a}	^{B}12.36	^{B}13.09	<0.001
	Medium	^{A}100.22^{a}	^{B}20.09	^{B}10.41	<0.001
	Fine	^{A}101.98^{a}	^{B}16.18	^{B}22.79	<0.001
	Very fine	^{A}164.95^{b}	^{B}41.50	^{B}32.09	<0.001
	P-Value	<0.001	0.237	0.634	**0.01**
Fructose	**Coarse**	^{A}33.16^{ab}	^{B}15.65^{ab}	^{B}6.50	0.006
	Medium	^{A}30.20^{a}	^{B}11.79^{a}	^{B}2.45	0.003
	Fine	^{A}44.90^{b}	^{B}23.82^{ab}	^{B}11.89	<0.001
	Very fine	^{A}35.72^{ab}	^{A}27.63^{b}	^{B}6.16	<0.001
	P-Value	0.032	0.016	0.415	**0.178**
Total	**Coarse**	^{A}135.43^{a}	^{B}31.66	^{B}22.87	<0.001
	Medium	^{A}139.30^{a}	^{B}35.29	^{B}15.95	<0.001
	Fine	^{A}160.73^{a}	^{B}44.92	^{B}55.67	<0.001
	Very fine	^{A}233.32^{b}	^{B}75.35	^{B}41.31	<0.001
	P-Value	<0.002	0.169	0.271	**0.006**

^{abc} significant difference between means bearing different letters in the same column and sugar type. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-Maltose (8.12) Glucose (10.37) Fructose (3.86) and Total (14.56), n=number of observations per mean.

Table 4.18: Effect of particle size and micro-organism used for sorghum fermentation on 24 hour pH and organic acid concentrations (mmol/L), (n=3).

Parameter	Particle size	Control	PA1	SLP	P-Value
pH	**Coarse**	$^{A}6.04^{ab}$	$^{B}3.49$	$^{B}3.63$	<0.001
	Medium	$^{A}5.94^{a}$	$^{B}3.43$	$^{B}3.33$	<0.001
	Fine	$^{A}6.31^{b}$	$^{B}3.50$	$^{B}3.58$	<0.001
	Very fine	$^{A}5.94^{a}$	$^{B}3.25$	$^{B}3.50$	<0.001
	P-Value	0.002	0.085	0.10	**0.009**
Lactic acid	**Coarse**	$^{A}8.35$	$^{B}233.54^{a}$	$^{B}272.46^{ab}$	<0.001
	Medium	$^{A}8.92$	$^{B}197.08^{a}$	$^{B}211.55^{a}$	<0.001
	Fine	$^{A}12.09$	$^{B}216.21^{a}$	$^{C}325.02^{b}$	<0.001
	Very fine	$^{A}23.55$	$^{B}313.15^{b}$	$^{C}401.87^{c}$	<0.002
	P-Value	0.999	0.005	0.008	**<0.001**
Acetic acid	**Coarse**	3.70	25.64^{a}	10.04	0.546
	Medium	9.16	17.37^{a}	9.25	0.999
	Fine	$^{A}2.61$	$^{B}62.80^{b}$	$^{A}13.78$	<0.001
	Very fine	6.21	26.47^{a}	9.43	0.654
	P-Value	0.999	0.042	1.00	**0.026**

^{abc} significant difference between means bearing different letters in the same column and sugar type. ^{AB}significant difference between means bearing different letters in the same row. *Standard error of the difference-pH (0.074) Lactic acid (17.28) and Acetic acid (9.84). n=number of observations per mean.

4.4 DISCUSSION

Apart from high numbers of lactic acid bacteria, other desirable properties of fermented liquid feeds are low pH (3.5-4.5) (Geary *et al.* 1996; Scholten *et al.* 1999; Christensen *et al.* 2007) and a high lactic acid concentration (>150 mmol/L)(Geary *et al.* 1996). 24 hour fermentation pH values for LAB treatments in this study in both grains were within this pH range. The pH values in this study are similar to those reported by Moran *et al.* (2006), who obtained pH values below 3.80 using backslopping with pre-fermented feed after 24 hours fermentation. This is vital, as an important advantage of the pH of the feed lies in its ability to improve resistance to enteropathogenic contamination.

According to Brooks *et al.*, (2001), coliforms and *Salmonella* will thrive when undesirable fermentation results in a pH greater than 4.5. Working on the effect of temperature on the growth and persistence of *Salmonella* in liquid pig feed, Beal *et al.* (2002), reported that the microbial population initially increased more rapidly in co-inoculated feed incubated at 30 °C compared with 20 °C. They indicated that once the lactic acid concentrations reached ca. 75 mmol/L and the pH dropped below 4.5 the microbes were killed more rapidly.

In order for fermentation to achieve the > 75 mmol/L lactic acid concentration to resist *Salmonella spp* growth as observed in pig feed (Beal *et al.* 2002), a higher concentration of ca > 150 mmol/L lactic acid in the fermented cereal-based component should be the goal. In the present study, this goal was achieved for all four particle sizes and LAB treatments by 24 hours of fermentation for both cereals. Beal *et al.* (2005), stated that in liquid pig feed substrates where competing micro-organisms could involve enteropathogens, it is imperative to have a rapid build-up of lactic acid in the medium. According to Moran *et al.*, (2006), the duration of exposure of coliforms to low pH and/or high lactic acid concentrations needs to be recognised as an important factor in their exclusion from fermented feed. Earlier, Hansen (2004) indicated that if the risk of *Salmonella* infections in growing-finishing pigs is to be reduced, it is important to obtain a low gastrointestinal pH and a high concentration of organic acids as quickly as possible after intake of the feed. Therefore, the factors that might bring about a rapid drop in pH and/or rapid increase in lactic acid production within the feed are very important for the biosafety of the feed prior to and at feeding.

From the results of the current study, significant reductions in the pH of maize and sorghum for LAB treatments were evident after 8 hours of fermentation. The reduction in particle size was related to a reduction in pH of maize although this trend was not observed with the 8 h lactic acid fermentation. A low pH is required for organic acids to remain in the undissociated form (Hansen 2004). This undissociated form of the acid is required for the antimicrobial property of the feed.

PA1 was observed to produce lactic acid at a faster rate than SLP. However, the fact that the 24 h fermentation pH values for maize fermented with SLP were significantly lower than the values for PA1 indicates that the initial rapid lactic acid production from PA1 was not maintained until 24 hours. Furthermore, lactic acid concentrations in SLP fermentations for both grains were generally higher than corresponding values for PA1 after 24 hours fermentation. The significant increase in acetic acid production from PA1 compared with SLP in this study clearly depicts a higher ratio of lactic to acetic acid concentrations for PA1. Charalampopoulos *et al.* (2002) indicated that *L. plantarum* NCIMB 8826 isolated from human saliva had a homofermentative pattern for cereal-based substrates with significant depletion of glucose, fructose, maltose and sucrose. They also observed that the growth of *L. acidophilus* NCIMB 12116 was associated with the production of lactic acid and comparably significant amounts of acetic acid. However, high acetic acid in feed could have adverse effects on palatability and feed intake of chickens. Unpublished data in pigs (Moran and Brooks) demonstrates that acetic acid concentrations above 30 mM reduced feed intake particularly in young pigs.

Glucose was increased significantly with time in the control treatment while fructose was not altered and maltose was reduced. This is an indication that there was endogenous enzyme hydrolysis of maltose. Amylolytic enzymes in sorghum malt have been shown to produce higher amount of fermentable sugars than are required for breakdown by microbial fermentation (Michodjehoun-Mestres *et al.* 2005). However, only microbial breakdown was involved in the utilisation of glucose and fructose. This is because glucose and fructose concentrations in the control treatment are at least twice their concentrations in the LAB treatments after 24 hours fermentation. The variation in the utilisation of glucose and fructose by LAB in this study could be attributed to the fact that sugar fermentation by lactic acid bacteria is strain specific. A similar observation has been made by Charalampopoulos *et al.* (2002) using probiotic lactic acid bacteria incubated in malt, wheat and barley substrates. They indicated that each lactobacillus (*Lactobacillus acidophilus, L. plantarum, L. reuteri and L. fermentum*) had a specific preference for one or more sugars. In a mixture of five sugar substrates (Lactose, Maltose, Sucrose, Glucose and Fructose) preferential use of certain substrates by different LAB has been demonstrated (Brooks *et al.* 2002).

It was evident from the current study, that although the availability of glucose was related to the particle size, final lactic acid concentrations after 24 hour fermentations in all LAB treatments were not related to the particle size. Therefore, small initial differences in fermentable sugar concentrations at the start of fermentation may not be important especially for 24 hour fermentations. Indeed, production of lactic acid from the coarse particle sizes was not significantly different from the smaller particle sizes (except the very fine particle sizes).

Higher acid content did not always correspond to a lower pH value in this study. An overall mean total acid concentration of 273.07 mmol/L resulting from fermentation of sorghum with PA1 had a pH of 3.42 whilst fermentation with SLP had a pH of 3.51 for a total acid concentration of 313.35 mmol/L (Table 4.10). The lack of a linear relationship between pH and acid concentration was also observed with the particle sizes in SLP fermentation of maize. The total acid concentration of the coarse particle size, though higher than any other particle size in the treatment, had a significantly higher (P=0.003) pH value. This observation could be related to the buffering capacity of the coarse particle size which could buffer the excess acid resulting in a resistance to drop in pH.

A reduction in size particle could increase the surface area for amylolytic enzyme action and result in a rapid fermentation of glucose and fructose. The relation between particle size and sugar availability has been highlighted by Anguita *et al.*, (2006) who reported that reduction increased hydrolysis of starch especially for raw cereals. However, Tester *et al.*, (2006) pointed out that whilst the size and shape of the starch granules is clearly a controlling factor in the hydrolysis of native starches with amylases, factors which control the accessibility of the enzyme to the interior of the granule also regulate hydrolysis. A rapid build-up of fermentation end metabolites will also depend on whether microbial fermentative capacity can handle immediate increases in fermentable sugars concentration resulting from hydrolysis of starch.

Based on the results of the current study and the following reasons it is proposed that larger grain sizes could be better for fermentation and inclusion into moist poultry diets:

1. Coarse particle sizes in this study produced comparable or higher lactic acid concentrations in most treatments, suggesting that moderate grain processing may be enough to permit production of biosafe levels of lactic acid in fermented feed for chickens.
2. Secondly, Mai (2007), demonstrated that feeding wet and coarsely ground diets improved feed intake, feed conversion and growth rate in broilers. This effect was pronounced during the starter phase and was associated with improvements in the functional development of the foregut (proventriculus-gizzard system).
3. Grain processing to small particle sizes of cereals like the fine and very fine sizes in this study could have important implications for both the diet and cost of feed for the farmer.
4. Increased particle size, feeding whole wheat or corn-based diets, reducing non-starch polysaccharides, and reducing levels of animal-based proteins in the diet seemed to help reduce the incidence of necrotic enteritis in broiler chickens (Dahiya *et al.* 2006).
5. Non-pelleted rolled barley or wheat increased both firmness and dry matter percentage of the stomach content of growing pigs compared with ground feed (Nielsen & Ingvartsen 2000). A higher firmness of the stomach content coincided with a lower score of gastric lesions.

However, the use of coarse grains in fermentation for moist poultry diets may be more relevant in feeding programs where batch fermentation is practiced and cycles of 24 hour feeding are strictly adhered to.

Chapter 5

EXPERIMENT 3: LACTIC ACID FERMENTATION OF TWO SORGHUM VARIETIES FOR POULTRY DIETS IS NOT AFFECTED BY THEIR POLYPHENOL CONTENTS.

5.1 Introduction

Grain sorghum is widely used as a human food and animal feed cereal in many parts of Africa, Asia and the semi-arid tropics world-wide (Elkhalifa & El-Tinay 2002; Osman 2004; Balogun *et al.* 2005; Michodjehoun-Mestres *et al.* 2005; Ragaee *et al.* 2006; Vieira-Dalode *et al.* 2008). It is recognised that differences in the colour of sorghum varieties are due mainly to their concentration of polyphenol compounds (mainly tannins). It is suggested that tannins may have a deleterious effect on methanogenesis in the gut (Woodward *et al.* 2001; de Oliveira *et al.* 2007). In ruminants, this has the advantage of reducing ruminal methane emissions (Makkar 2003; Puchala *et al.* 2005). While this property of tannins may be useful in reducing one of the greenhouse gases, it is necessary to know how it may influence lactic acid bacterial fermentation of tropical cereals for liquid feeds. Although, lactic acid fermentation has been reported to eliminate anti-nutritional factors in soybean meal (Refstie *et al.* 2005), little is known about the lactic acid fermentation of sorghum grain varieties with different polyphenol contents. The use of sorghum as a food resource is limited because tannins or polyphenols in some sorghum grain cultivars interferes with bioavailability of nutrients (Reed *et al.* 1987; Mahgoub & Elhag 1998).

As indicated by Brooks, (2008) feed fermentation could be effective means by which levels of anti-nutrients and mycotoxins could be reduced in feeds. Indeed, enzymatic methods (fermentation and malting) for phytic acid removal from sorghum grain cultivars have been found to be more effective than physical extraction methods

(milling, soaking and heating) (Mahgoub & Elhag 1998). Furthermore, environmental pollution resulting from excretion of unutilized compounds like phytate phosphorus from intensive poultry farms has been of great concern in recent times (Rama Rao *et al.* 2007). Therefore lactic acid fermentation of sorghum for production of moist poultry diets could reduce excretion of unutilized compounds like phytate phosphorus and environmental pollution.

A better understanding of the influence of variety on the fermentation of grain sorghum may also be valuable for making an informed choice in terms of fermentation under situations where raw material availability may vary with tannin content and season. The present study was designed to compare the fermentation of red and white sorghum with LAB for inclusion as the fermented component of moist poultry diets.

5.2 MATERIALS AND METHODS

5.2.1 Experimental design

The study conducted as a 2 x 5 x 4 factorial design with three factors:

Factor 1: Sorghum variety (white and red sorghum); Factor 2: Control treatment without LAB and LAB treatments (*Pediococcus acidilactici* (PA1) or *Lactobacillus plantarum* (SLP) or *Lactobacillus plantarum* NCIMB 41229 (LP2) or *Lactobacillus farciminis* (LF1); Factor 3: Incubation time (0, 4, 8, and 24 hours). There were three replicates per treatment.

5.2.2 Sample preparation

Raw red and white sorghum grains (*Sorghum bicolour* L. Moench) obtained from Northern Nigeria, were hammer-milled through a 3 mm screen and weighed into 50 g sachets and for subsequent irradiation with 25 KGy γ-radiation from ^{60}Co. Irradiation of the sorghum was undertaken by Becton and Dickinson, Plymouth, UK.

5.2.3 Fermentation and sample collection

Sorghum samples (red or white sorghum) were mixed with sterile distilled water at a ratio of 1:1.4 as recommended by Heres *et al.*(2003). The mixture was inoculated with 0.05 ml of an overnight culture of De Man, Rogosa and Sharpe (MRS) broth concentration containing one of four ca 10^9 cfu/ml of LAB; (SLP, PA1, LF1 and LP2). The LAB treatments were incubated at 30 °C simultaneously with a control treatment without lactic acid bacteria. Samples were stirred for 1-2 minutes and subsamples removed aseptically from each beaker at the beginning of the fermentation (0 hours) and at 4, 8, and 24 hours after fermentation.

These samples were used to measure the pH using a pH electrode (pH 213 microprocessor pH meter, Hanna instruments, Portugal) and 0.5ml samples were collected for sugar and organic acid analysis and immediately frozen in Eppendorf tubes and kept at -20 °C until analysis was conducted.

5.2.4 Analysis for short chain organic acids and fermentable sugars

Organic acids and simple sugars were analysed by High Performance Liquid Chromatography (HPLC) according to the method of Niven *et al.* (2004) described in chapter 3 section 3.2.3.

5.2.5 Extraction of phenolic compounds

Phenolic compounds extracted from red and white sorghum grains using a modification of the procedure described by Chavan *et al.* (2001). Duplicates of 5 g of each sorghum variety were used for the extraction. Samples were extracted with 50 ml of 70% acetone with 1% concentrated hydrochloric acid and homogenized for 1 min before centrifugation at 3350 rpm for 10 min at 20°C. The supernatant was poured in a volumetric flask and the extraction procedure was repeated with the residue twice for each sample. The repeated collections gave an extraction solution of about 150 ml of reddish brown solute. Solvents were kept in a fridge at below 4 °C until the evaporation procedure was done.

Solvents were evaporated to 30 ml in round bottom volumetric flasks using a rotary evaporator (Rotavapor-R, Büchi, Switzerland) with a speed ranging from 1-5 (equivalent to 60-180 rpm) at 40°C in a water bath. Samples were transferred to plastic beakers and methanol was used to remove the dry residue at the base of the flasks. The resulting solution was frozen at -65°C for 24 hours. After freezing, they were freeze-dried at – 50°C for 96 hours and the weight of the residue was determined by subtraction of the weight of the centrifuge tubes.

5.2.6 Data analysis

Data were analysed using the general linear model procedure (GLM) of analysis of variance using Minitab (release 15.0) according to the following general model:

$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \delta_{ij}$

Where Y_{ij} is the observed dependent variable; μ is the overall mean; α_i is the effect due to sorghum variety; β_j is the effect due to lactobacilli used; $(\alpha\beta)_{ij}$ is the interaction between sorghum variety and lactobacilli used and δ_{ij} is the random error. Data obtained for changes in sugars and lactic acid concentration with time for each micro-organism were analysed separately and least square means with pooled standard error (SEM) were obtained. Differences between means were determined using the Tukey's test (Zar 1999). Initial individual and total concentrations of reducing sugars for the two varieties at the beginning of fermentation (0 hour samples) were compared using a paired t-test. Probability values ≤ 0.05 were considered to be statistically significant.

5.3 RESULTS

5.3.1 Phenolic contents

The mean phenolic contents of red and white sorghum were 5.53 ± 0.48 and 4.05 ± 0.80 g/100g grain respectively.

5.3.2 Fermentable sugars

The initial concentration (mmol/L) of maltose, glucose, fructose and the total sugars in the sorghum grains at the beginning of fermentation (0h) is summarised in Table 5.1.

The initial concentration of maltose was significantly higher in white sorghum than in red sorghum (58.90± 3.61 and 45.40 ± 3.61; P<0.002). There was no significant difference between red and white sorghum in the concentration of glucose, fructose or total fermentable sugars.

After 24 hour fermentation, there was no significant difference in maltose and fructose concentration between varieties (Table 5.2). As expected, the concentration of fermentable sugars in the control treatment was significantly higher than the value observed with the LAB treatments. For the glucose concentration it was about 9 times the concentration obtained with the LAB treated samples after 24 hour fermentation.

For the concentrations of glucose, a significant difference (P=0.001) was only observed between varieties in the control treatment with no LAB (lactic acid bacteria). There were no significant differences (P>0.05) between LAB treatments in maltose and glucose concentrations after 24 hours fermentation. However, for both varieties, fermentation with strains of *L. plantarum* (SLP and LP2) resulted in a significantly lower fructose concentration than for other LAB treatments.

5.3.3 Organic acids

The concentration of lactic and acetic acid acids is presented in Table 5.3. Lactic acid concentration was significantly greater (P<0.001) in all LAB treatments than in the control. There were no significant differences in lactic acid concentration after 24 h fermentation between LAB treatments for either sorghum varieties.

Table 5.1: Comparisons for the initial concentrations of fermentable sugars (mmol/L) at the beginning of fermentation (0 hour), (n=15).

Grain Variety	Maltose	Glucose	Fructose	Total sugars†	pH
Red Sorghum	45.40	29.52	18.09	93.01	5.74
White Sorghum	58.90	27.23	15.86	101.99	6.01
SED*	3.61	3.29	1.53	4.55	0.04
P-value	0.002	0.496	0.167	0.069	<0.001

*Standard error of the difference. †Total sugars are the sums of maltose, glucose and fructose concentrations, n=number of observations per mean.

PA1 produced significantly (P<0.05) more acetic acid than the other micro-organisms in white sorghum. With red sorghum, a significantly lower value was only observed when fermentation was done with LF1. The pH of all LAB treated sorghum samples were significantly lower (P<0.001) after 24h than their controls. There were no significant differences (P>0.05) in pH after 24 h between all LAB treated sorghum samples.

Table 5.2: Fermentable sugar concentrations (mmol/L) after 24h fermentation of red and white sorghum with different lactic acid bacteria, (n=3).

	Maltose		**Glucose**		**Fructose**	
Micro-organism	Red sorghum	White sorghum	Red sorghum	White sorghum	Red sorghum	White sorghum
Control	^{a}10.58	^{a}13.89	^{a}104.84^{A}	^{a}120.72^{B}	^{a}35.27	^{a}34.47
SLP	^{b}2.70	^{b}3.52	^{b}16.57	^{b}16.73	^{c}4.00	^{c}7.19
PA1	^{b}2.52	^{b}3.62	^{b}12.84	^{b}11.80	^{b}21.93	^{a}25.31
LF1	^{b}2.49	^{b}3.41	^{b}19.02	^{b}18.36	^{b}21.06	^{b}21.74
LP2	^{b}3.36	^{b}3.50	^{b}15.78	^{b}13.97	^{c}6.87	^{c}6.07
s.e.m.	0.25	0.23	0.74	0.65	0.80	0.71

^{abc} significant difference between means bearing different letters in the same column (P<0.05). ^{AB}significant difference between means bearing different letters in the same row and parameter (P=0.001), n=number of observations per mean.

Table 5.3: Organic acid production (mmol/L) and pH after 24h fermentation of red and white sorghum with different lactic acid bacteria, (n=3).

	Lactic acid		**Acetic acid**		**pH**	
Micro-organism	Red sorghum	White sorghum	Red sorghum	White sorghum	Red sorghum	White sorghum
Control	7.25^{c}	12.18^{b}	1.32^{a}	5.37^{bc}	5.67^{b}	5.88^{b}
SLP	312.3^{a}	313.65^{a}	10.13^{b}	9.70^{bc}	3.49^{a}	3.22^{a}
PA1	203.67^{b}	264.07^{a}	14.88^{b}	20.65^{a}	3.65^{a}	3.41^{a}
LF1	261.3^{a}	246.18^{a}	2.61^{a}	3.99^{b}	3.64^{a}	3.34^{a}
LP2	273.8^{a}	264.98^{a}	11.6^{b}	10.21^{c}	3.58^{a}	3.25^{a}
s.e.m.	6.37	5.66	0.60	0.54	0.18	0.16

^{abc}Significant difference between means bearing different letters in the same column ($P<0.05$).
There was no significant effect ($P>0.05$) of variety on any of the parameters tested, n=number of observations per mean.

5.3.4 Variation in fermentable sugars and lactic acid concentrations with time.

Time series data for variation in fermentable sugars during fermentation for the control treatment (Figure 5.1) and LAB treatments (Figures 5.2-5.5) shows a sharp decrease in maltose concentration within the first four hours of fermentation for both varieties of sorghum. The decrease in maltose concentration during this period was also characterised by an expected rise in the glucose concentration in all treatments. This increase was more pronounced in the control treatment than LAB treatments.

After 8 hours fermentation concentrations of lactic acid in white sorghum were higher than in red sorghum for all treatments. The highest lactic acid concentration (Figure 5.3) was obtained from white sorghum fermented with PA1 (51.2 ± 1.77 mmol/L), which was twice the concentration of lactic acid produced with red sorghum (28.76 ± 1.77mmol/L). Production of lactic acid resulting from 8 h fermentation with SLP (Figure 5.2) was 31.08 ± 1.45 mmol/L and 18.73 ± 1.45 mmol/L for white and red sorghum respectively.

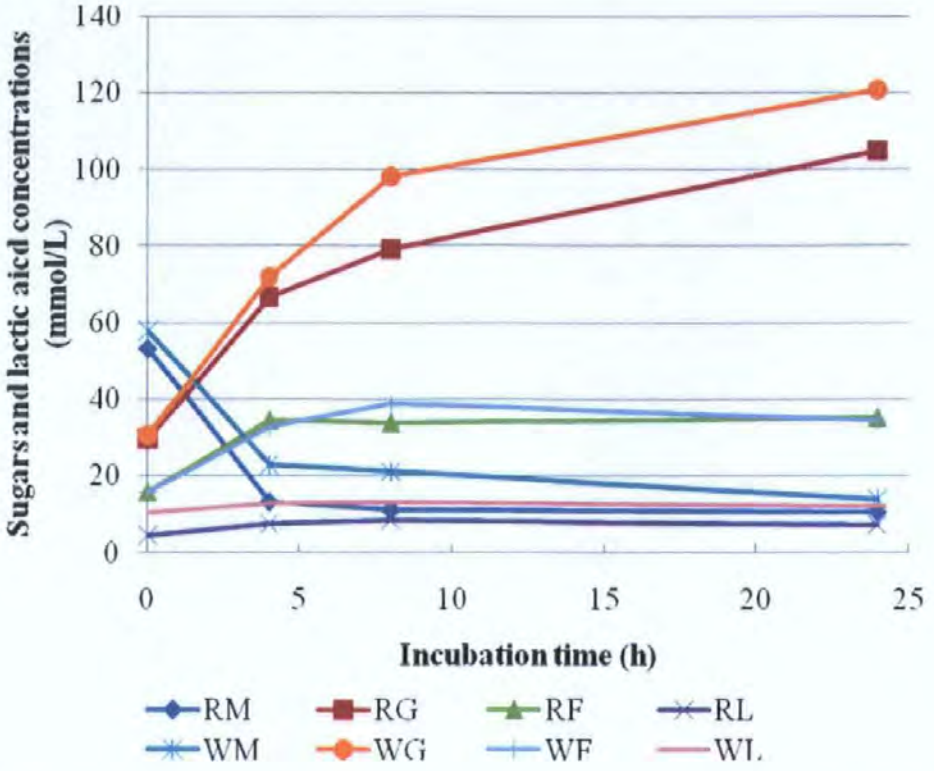


Figure 5.1: Effect of sorghum variety on concentrations (mmol/L) of maltose (M), Glucose (G), Fructose (F) and lactic acid (L) following incubation without fermenting microbes (R-Red sorghum and W-White sorghum).

When LP2 was used in fermentation, the difference in lactic acid production between red (25.46 ± 1.45 mmol/L) and white sorghum (30.26 ± 1.77 mmol/L) was less (Figure 5.5). The difference between varieties for fermentation with LF1 was 6.16 mmol/L with white sorghum having a higher value of 17.35 ± 1.45 mmol/L lactic acid content. These differences between varieties were significant for fermentation with SLP ($P=0.0004$) and PA1 ($P<0.0001$) and not significant with LF1 ($P=0.153$) and LP2 ($P=0.554$).

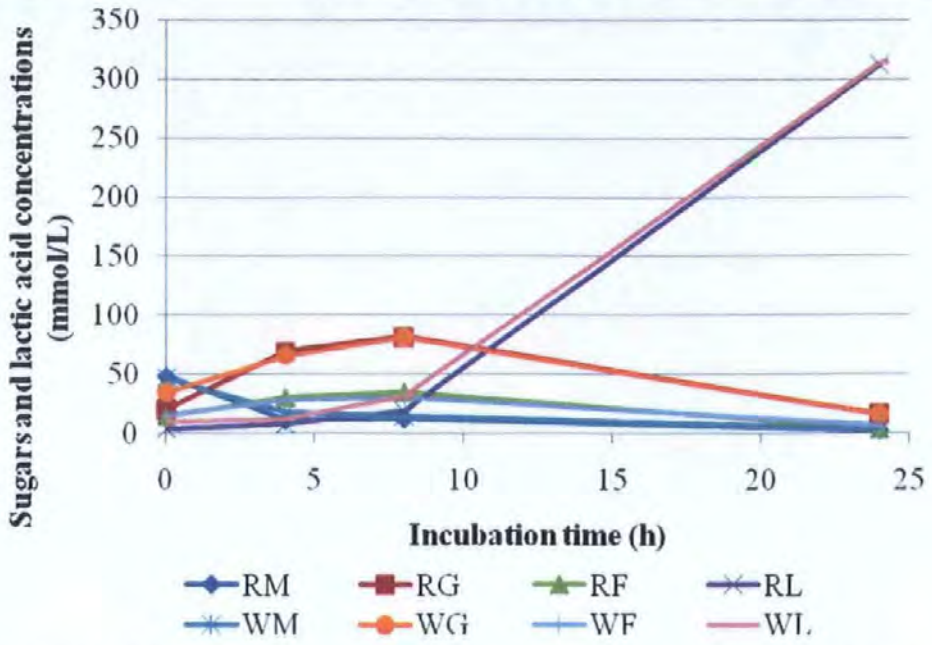


Figure 5.2: Effect of sorghum variety on concentrations (mmol/L) of maltose (M), Glucose (G), Fructose (F) and lactic acid (L) fermented with *L. plantarum* (SLP) (R-Red sorghum and W-White sorghum).

When rate of change in lactic acid concentration between 8 and 24 hours was calculated, more rapid increases in lactic acid production were obtained with red sorghum than white sorghum in all LAB treatments except when fermentation was undertaken with PA1 (174.91 and 212.87 mmol/L for red and white sorghum). The increases associated with fermentation of red sorghum were 293.57, 250.11 and 248.34 mmol/L for SLP, LF1, and LP2 respectively. Corresponding values for white sorghum were 282.57, 228.83 and 234.72 mmol/L respectively.

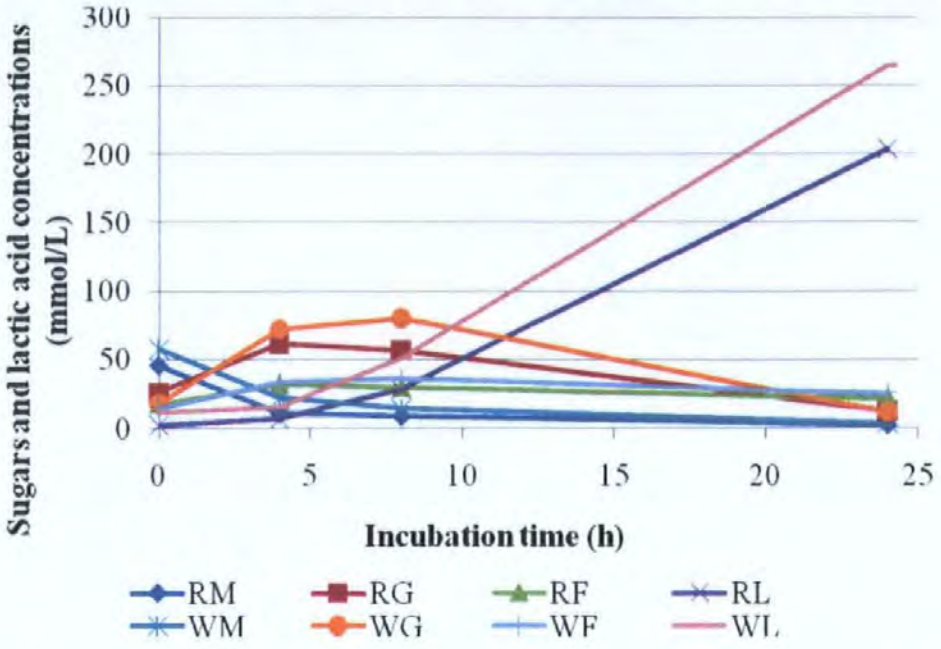


Figure 5.3: Effect of sorghum variety on concentrations (mmol/L) of maltose (M), Glucose (G), Fructose (F) and lactic acid (L) following fermentation with *P. acidilactici* (PA1) (R-Red sorghum and W-White sorghum).

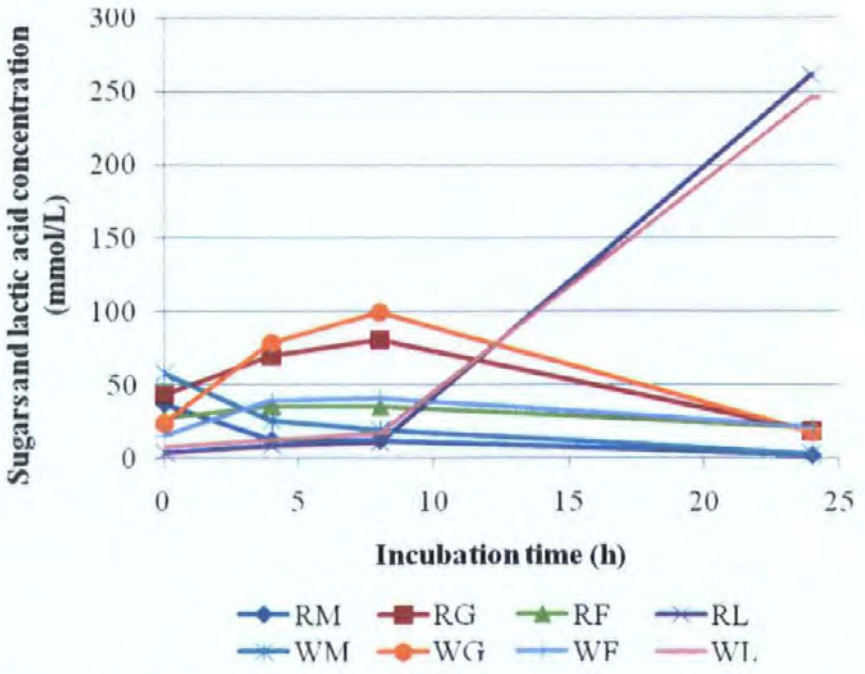


Figure 5.4: Effect of sorghum variety on concentrations (mmol/L) of maltose (M), Glucose (G), Fructose (F) and lactic acid (L) fermented with *L. farciminis* (LF1) (R-Red sorghum and W-White sorghum).

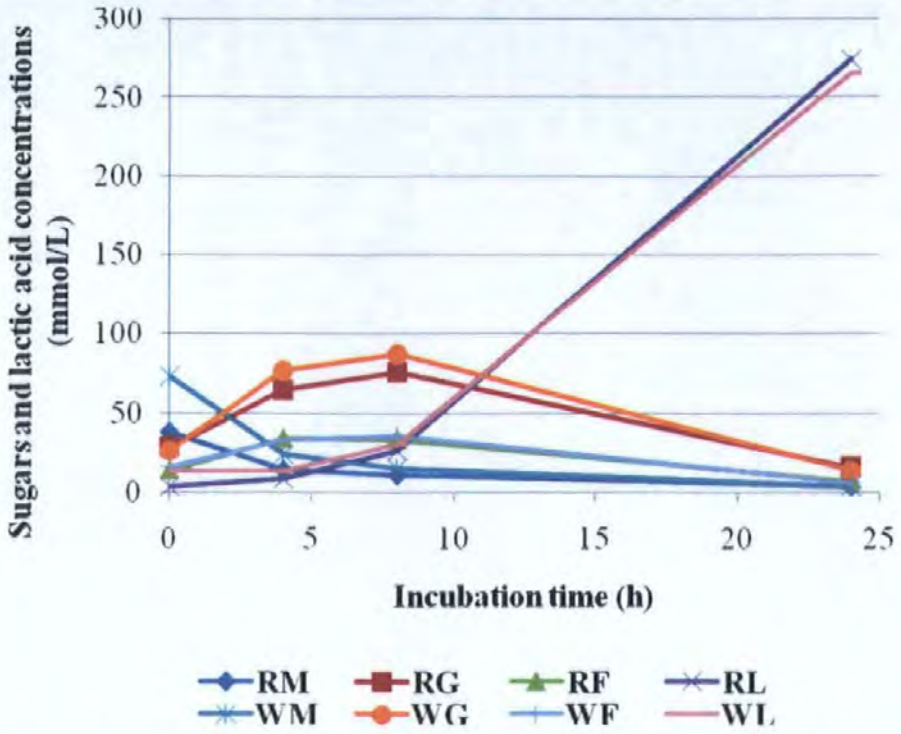


Figure 5.5: Effect of sorghum variety on concentrations (mmol/L) of maltose (M), Glucose (G), Fructose (F) and lactic acid (L) fermented with *L. plantarum* NCIMB 41229 (LP2) (R-Red sorghum and W-White sorghum).

5.4 DISCUSSION

After 24 hour incubation white sorghum had a significantly higher (P=0.0011) glucose concentration than red sorghum in the control treatment. This suggests that starch hydrolysis was affected by differences in phenolic content. A similar observation has been made by Kock *et al.* (1985) who reported that polyphenols in the testa and pericarp of sorghum seed had an inhibitory effect on enzyme activity during hydrolysis of starch. Furthermore, sorghum grain cultivar differences have been observed for changes in diastatic activity (combined α and β-amylase activities) (Ahmed *et al.* 1996) and enzyme activity during germination (Butler *et al.* 1984; Ogbonna *et al.* 2004). Ahmed *et al.* (1996) suggested that the inhibitory effect on enzyme activity in sorghum results from the formation of insoluble complexes between tannins and the enzymes.

Starch hydrolysis may also have been affected by varietal differences in starch structure. The crystalline organisation of the starch granules within the white sorghum grain could make them easier to break down when compared with the starch granules in red sorghum. This concept has been reviewed by Tester *et al.* (2004) who stated that it is widely known that the shape and size of starch granules may be related to their botanical source. Meanwhile, Tester *et al.* (2006), stated that although the size and shape of granules are clearly controlling factors, factors implicated in the control of accessibility to the interior of the starch granules for the enzymes also regulate hydrolysis. Enzymatic processes resulting in the breakdown of starch and disaccharides to glucose may therefore be more pronounced in white than red sorghum.

There were no significant differences in glucose or fructose concentration between sorghum varieties in the LAB treatments after 24 hour fermentation. This indicates that differences in polyphenol contents and probably starch structure did not have a significant effect on the availability of fermentable sugars for LAB fermentation. The presence of LAB amylolytic enzymes in the LAB treatments might be responsible for additional hydrolysis of starch and minimizing varietal differences in phenol contents and starch structure.

Prolonged steeping could also have deactivated the inhibitory effects of phenolic compounds and released enzymes for starch hydrolysis and consequently fermentable sugars for microbial fermentation. The content of certain phenolic compounds in barley has been shown to decrease significantly during steeping (Lu *et al.* 2007). High moisture storage of high tannin sorghum deactivates tannins and improves digestibility in chickens (Mitaru *et al.* 1983) and pigs (Mitaru *et al.* 1984a; Mitaru *et al.* 1984b).

Mitaru *et al* (1984a) also demonstrated that soaking of high and low tannin sorghum grains in 30 % (wt/wt) distilled water for 20 days at 25 °C improved the digestibilities of dry matter, energy, protein and amino acids in high but not in low tannin sorghum diets for pigs. The greater increase in production of lactic acid for red sorghum between 8 and 24 hours in this study are in line with these results on soaking length on phenolic contents. Apart from deactivating phenols, prolonged soaking may also have affected the activity of endogenous enzymes in both grain varieties. Choct *et al.* (2004a) suggested that effects on growth and feed intake for weaner pigs resulting from steeping of feed for 15 hours might be related to the release and activation of endogenous enzymes in the grain.

The activation of these enzyme systems within the grain can act on cell wall structures in a similar way to exogenous feed enzymes (Choct *et al.* 2004b). In reviewing the effect of steeping in liquid feeding systems, Brooks *et al.* (1996), indicated that phytases that were naturally present in the pericarp of some grains (like cereals) could be activated by soaking. They also stated that soaking feed for 8-16 h before feeding increased the bioavailability of phosphorus, calcium, magnesium and copper. Bioavailability of these minerals during this period might have had a beneficial effect on microbial activities resulting in a faster breakdown of fermentable sugars.

The LAB used in this study, may also vary in their ability to access substrates (i.e. produce enzymes of varying hydrolytic capacity) and their susceptibility to polyphenols. The *L. plantarum* strains used in this study utilised significantly more fructose than the other LAB. Preferences in the utilisation of sugars by different LAB has been reported by Charalampopoulos *et al.* (2002) and Brooks *et al.* (2002). On the other hand, tannins

are antimicrobial so high tannin in the red sorghum could have inhibited LAB at first before they became adapted to the tannins. Differences between white and red sorghum cultivars in fermentation characteristics in spontaneous fermentation to produce non-alcoholic beverage have been reported (Bvochora *et al.* 1999).

However, the concentration of fermentable sugars in both varieties was observed to decrease with fermentation time between 8 and 24 hours for LAB treatments.

These results are not in accordance with the observations of Michodjehoun-Mestres *et al.* (2005) and Vieira-Dalode *et al.* (2008) who found that total sugar concentrations increased significantly with time for 24 hour fermentation despite increases in microbial counts. Michodjehoun-Mestres *et al.* (2005) attributed this increase to the amylolytic activities associated with malting of sorghum which favoured the production of higher amounts of fermentable sugars than required for microbial metabolism during fermentation. However, titratable acidity expressed as g/kg lactic acid, dry weight reported by Vieira-Dalode *et al.*(2008), for 24 hour fermentation of red sorghum with LAB was between 62.5 and 66.3 g/kg and by Michodjehoun-Mestres *et al.* (2005) for lactic acid expressed in mg/g dry basis (14.1-18.5) in spontaneous fermentation. The values reported by Vieira-Dalode *et al.* (2008) were comparable with the values obtained in this study (36.66-56.46g/kg dry weight) but higher than values obtained by Michodjehoun-Mestres *et al.* (2005).

The more pronounced decrease in maltose concentration in the LAB treatments than control treatments, may suggest that more than one agency is implicated in the hydrolysis of maltose in the LAB treatments. In the control treatments, endogenous

amylolytic enzymes of the grain seem to be solely responsible. The large difference in glucose concentration between the LAB treatments and the control treatments for both varieties indicates that LAB produces amylolytic enzymes that are responsible for the removal of the accumulating glucose during the fermentation process. Although, the fructose concentration in LAB treatments decreased considerably between 8 and 24 hours fermentation (Figures 5.2 and 5.5), the most important sugars implicated in fermentation in this study were observed to be maltose and glucose (Figures 5.2 - 5.5). Michodjehoun-Mestres *et al.* (2005), also found that the most important sugars implicated in spontaneous fermentation of a raw red sorghum grain beverage "*gowe*" were maltose and glucose. Brooks *et al.* (2002) also indicated that the poorest performing substrate in

fermentation of five sugars (glucose, fructose, maltose, sucrose and lactose) with five LAB was fructose producing 121-150 mmol/L lactic acid for 72 h and 30 °C fermentation compared with lactose (171-188 mmol/L). While chemical analyses of the distribution of sugars and the starch structure of both sorghum varieties could have permitted a better understanding of our in our results, these analyses were beyond the scope of our study.

5.5 CONCLUSIONS

The results of this study suggest that the *Lactobacillus spp* used were not affected by the phenolic content of sorghum as there were no significant differences in lactic acid production between sorghum varieties. However, possible differences in starch structure (hydrolysis) and phenolic contents between varieties could be confounding in their

influence on LAB fermentation. The most important fermentable sugars for sorghum varieties in this study were maltose and glucose.

Fermentation of both varieties of sorghum with lactic acid bacteria produced a low pH medium (pH$\leq$ 3.65) that had a high lactic acid concentration ($\geq$ 203.67 mmol/L). Therefore, both grain varieties may be successfully fermented for inclusion in moist feed for poultry.

Chapter 6

EXPERIMENT 4: APPLICATION OF *LACTOBACILLUS PLANTARUM* NCIMB 41229 IN WATER AND FEED (AS A FEED FERMENTER) FOR CHICKENS.

6.1 INTRODUCTION

The importance of gut health associated with a well-balanced gut microflora has been recognised as a precondition for cost-efficient and environmentally-friendly livestock production (Steiner 2006). Furthermore, it is now widely agreed that a healthy gut is essential for the efficient absorption of nutrients by the animal. Various approaches have been used to improve gut health and animal performance more recently, with emphasis on 'environmentally-friendly' methods that do not depend on AGPs. Microbial fermentation of feeds, using lactic acid bacteria with probiotic potential, is regarded as one of the biosafe methods by which gut and host health can be improved. These feeds known as Fermented Liquid Feeds have been described by Heres *et al.* (2003b), as moistened feed with a high number of lactobacilli, a high concentration of lactic acid and a pH of <4.5. Not only do these feeds resist enteropathogens contamination prior to feeding (Beal *et al.* 2002; Beal *et al.* 2005), but also serve as vehicles by which large amounts of organic acids (>150 mmol/L) and lactobacilli (approximately 10^9 cfu/g) (Heres *et al.* 2003a), can be delivered to the bird.

In poultry, fermented feeds have been demonstrated to improve the acidic barrier function in the upper gastrointestinal tract of chickens (Heres *et al.* 2003d). Feeding fermented feeds resulted in a faster reduction of *Salmonella* in the birds. This reduced *Salmonella* colonisation of the duodenum and the lower parts of the gastrointestinal (GI) tract. The acidity of the crop and gizzard seems to be the most important single factor resisting the passage of *Salmonella* through the upper part of the gut. The age at which fermented feeds, or lactobacilli with probiotic potential, are introduced to the birds could also be important because the post-hatch period is of major immunological significance to the chick. The chick is most susceptible to pathogens like *Salmonella* during the first 4 days post-hatch (Wells *et al.* 1998). The rapid development and functional maturation of the gut-associated lymphoid tissue (GALT) during this time is critical in providing protection and for the chick's survival soon after hatch.

However, in the growing chick, functional maturation of the GALT is biphasic (Bar-Shira *et al.* 2003). The first phase-during the first week post-hatch, and the second phase during the second week. The second phase is also characterised by a marked increase in the intestinal lymphocyte population. Further evidence suggests that maturation of cellular immune responses in the intestinal milieu is a prerequisite for the initiation of humoral responses (Bar-Shira *et al.* 2003). This is in agreement with an earlier report by Mast and Goddeeris, (1999) who indicated that the vaccination of chicks at day-old did not activate the B-cell response resulting in anti-body production. They attributed this to the idea that the structural organisation of secondary immune organs of late embryonic and neonatal chickens are not well developed compared with chickens at 7 and 14 days of age, respectively.

The rudimentary organisation of the secondary immune organs early in the chicks' life is critical because lack of contact between chicks and the hen in modern production can result in a delayed development of beneficial intestinal microflora and subsequently an increased susceptibility of young chicks to colonisation by *Salmonella* and/or other pathogens (Snel *et al.* 2002). More recently, Beal *et al.* (2004) observed that the age at primary infection of the chicken with *Salmonella enterica* serovar Typhimurium influences persistence of infection and subsequent immunity to re-challenge. They indicated that uninfected birds and birds infected at one week of age produced a stronger antibody response to re-challenge, but were slower to clear *Salmonella* from the gut than the older-primed groups which expressed a stronger T-lymphocyte response.

Although little is known about the process of induction of immune response in the chicken gut (Haghighi *et al.* 2005), it is hypothesised (Bar-Shira *et al.* 2003) that exposure to environmental antigens initiates the initial activation of lymphocytes and NK cells, which reside mainly in the intraepithelial lymphocytes compartment of the newly hatched chicks leading to GALT maturation. Therefore, substances that can upregulate maturation of the gut immune architecture (GALT), which is a prerequisite for initiation of humoral immune responses, could be vital for enteral protection of the growing chick during the immediate post-hatch era.

It is widely agreed that commensal bacteria in the intestine play an important role in the development of immune response (Dong *et al.* 1996; Clancy 2003; Koenen *et al.* 2004; Haghighi *et al.* 2005; Nava *et al.* 2005; Donoghue *et al.* 2006; Farnell *et al.* 2006;

Haghighi *et al.* 2006). These bacteria are known to interact with cells of the GALT notably B-1 cells involved with the production of natural antibodies (Haghighi *et al.* 2006). Furthermore, the demonstration that probiotic bacteria may exert their effect through activation and regulation of the common mucosal system, provides a framework for the selection of both isolate and clinical targets, and the definition of optimal management strategies (Clancy 2003).

The study reported here was designed to:-

- Examine the effect of administration of *Lactobacillus plantarum* NCIMB 41229 in water or fermented feed in the control of *S.* Typhimurium infection in poultry.
- Evaluate changes in gut microbial composition and circulating antibodies associated with the method and age of administration of *Lactobacillus plantarum* NCIMB 41229 delivered to chickens in fermented feeds or drinking water.

6.2 MATERIALS AND METHODS

6.2.1 Experimental design

One hundred and two specific pathogen free birds (SPF, SPAFAS) were randomly allocated to six treatments of 17 per treatment as follows:

- Birds with no *Lactobacillus plantarum* NCIMB 41229 application in water or feed (CON).

- Oral gavage (single dose) of birds with 10^7 (cfu ml^{-1}) organisms which were washed and reconstituted from a 24h culture of *Lactobacillus plantarum* NCIMB 41229 on day 1 of age (GAV).

- Birds treated with cells from a 24h culture of *Lactobacillus plantarum* NCIMB 41229 in 100 ml De-Man-Rogosa-Sharp (MRS) broth washed with PBS and reconstituted in 100 ml PBS and mixed in 10 litres of drinking water (to obtain approximately 10^7 cfu ml^{-1}) from day 1 of age (WAT1).

- Birds fed wet mash fermented with *Lactobacillus plantarum* NCIMB 41229 prepared by inoculating wet feed (1 feed: 1.2 water) and fermenting for 24h at 30°C (to obtain approximately10^9 cfu ml^{-1}) prior to feeding from day 1 of age (FEED1).

- Birds treated with cells from a 24h culture of *Lactobacillus plantarum* NCIMB 41229 in 100 ml MRS broth washed with PBS and reconstituted in 100 ml PBS and mixed in 10 litres of drinking water (to obtain approximately10^7 cfu ml^{-1}) from day 7 of age (WAT7).

- Birds fed wet mash fermented with *Lactobacillus plantarum* NCIMB 41229 prepared by inoculating wet feed (1 feed: 1.2 water) and fermented for 24h at 30°C (to obtain approximately10^9 cfu ml^{-1}) prior to feeding from day 7 of age (FEED7).

6.2.2 Feed fermentation

A commercial diet (unmedicated Saracen chick crumbs obtained from J & W Attlee, Parsonage Mills, Dorking, Surrey, RH 14 1 EL) with the composition shown in Table 6.1 was used for this study. Fermented wet mash was prepared by inoculating wet feed (1 feed: 1.2 water) with cells from a 24 hour culture of *Lactobacillus plantarum* NCIMB 41229 re-suspended in PBS (0.1 M, pH 7.2). The 24 hour culture was prepared by the inoculation of 20 ml MRS broth with *Lactobacillus plantarum* NCIMB 41229 to give a concentration of 10^9 cfu ml^{-1} of broth. The fermented culture was centrifuged in 50 ml centrifuge tubes at 4000 rpm for 10 minutes at a temperature of 20 °C. The *L plantarum* pellet was re-suspended in the same volume of PBS and used to inoculate wet mash at a volume of 0.1% of the quantity of dry feed in the mixture to give an inoculum of 10^6 cfu/g dry feed. The resultant feed was incubated at 30°C in polythene bags for 24 hours prior to feeding. The pH of fermented and wet feeds was recorded daily using a pH electrode (pH 213 microprocessor pH meter, Hanna instruments, Portugal).

6.2.3 Housing and feeding

Newly hatched chicks were obtained from an SPF White Leghorn flock (SPAFAS). Chicks were housed in negative pressure rooms on wood shavings and fed experimental diets and provided with water *ad libitum*. The brooding period was two weeks and brooding temperatures were maintained at 21°C. Fresh feed was provided in circular tray feeders on a daily basis and trays were washed and disinfected on a weekly basis.

Table 6.1: Percentage composition of feed used in the study (% Dry matter basis).

Quantity of feed (In descending order)	Composition (%)	Proximate composition (%)	
Wheat	54.5	Oil	4.0
Hipro soya	16.7	Protein	18.5
Barley	10	Fibre	3.2
Minerals	2.7	Lysine & Methionine	0.4
Peas	2.5	Vitamin A (iu/kg)	10000
Fishmeal	1.5	Vitamin D3 (iu/kg)	3000
Vegetable fat*	1.2	Vitamin E (iu/kg)	15
Vitamins	0.75	Moisture	14.0
Methionine	0.13		
Lysine	0.03	Copper (mg/kg)†	25

*Vegetable fat contains BHT as an anti-oxidant. † Copper was added as cupric sulphate.

Fresh water and feed was provided daily. Water was provided in conical 10 litre capacity drinkers and all drinkers were washed daily prior to watering and *L. plantarum* in 100 ml PBS was added for the water treated birds. Feed intake was recorded daily while chicks were observed twice daily and weighed on a weekly basis. The experiment was undertaken in accordance with UK Home Office approved procedures (Animal Scientific procedures act, 1986) and approved by the local ethics committee of Veterinary Laboratory Agency, Weybridge, U.K.

6.2.4 Challenge with *Salmonella*

On day 14 of the experiment, seventeen birds were randomly selected and cloacally swabbed and their faecal contents streaked on Brilliant Green Agar (BGA) to ensure they were not infected with any *Salmonella* strains. Ten birds per treatment were wing-tagged for identification purposes.

At 15 days of age all birds were dosed with 10^6 cfu ml^{-1} of *S.* Typhimurium (Sal1344 nal^r) by oral gavage according the method described by La Ragione *et al.*, (2000). Oral gavage was conducted using a dosing catheter of size 4.5fg and length 60 mm (Harvard Apparatus Limited, USA). Prior to dosing with *Salmonella* birds were dosed with 105 % Sodium bicarbonate to instantaneously neutralise the acidity of the crop. This was done to by-pass the acid barrier imposed by the upper gut to coliforms and permit access of the introduced *S.* Typhimurium to the lower gut environment.

The *S.* Typhimurium (SL1344 nal^r) used for the challenge was a nalidixic acid resistant derivative obtained from Veterinary Laboratories Agency (VLA), Weybridge, UK. Cloacal swabs were taken once before challenge and at least twice a week after challenge for a period of 4 weeks. Swabs were plated onto selective media, incubated and then plates examined for *Salmonella* Typhimurium SL1344 nal^r and Lactic acid bacteria (LAB).

6.2.5 Enumeration of *S.* Typhimurium and Lactic acid bacterial shedding

All media used for the experiment were obtained from Oxoid (Basingstoke, UK). A semi-quantitative method where swabs were weighed before and after swabbing was used to determine the microbial counts per gram of cloacal faecal material on the swabs. Viable counts of microbes were determined using the standard drop method (Miles & Misra 1938) by plating 1 in 10 dilutions made in PBS (0.1 M, pH 7.2) on Brilliant Green Agar (BGA) supplemented with nalidixic acid (15µg ml^{-1}) for *S.* Typhimurium and MRS agar for LAB counts. All colonies were visualized using the Petri Viewer

MK2 camera and enumerated with the Sorcerer Image Analysis System software version 2.2 (Perceptive Instruments Ltd 20063, UK).

BGA plates were incubated overnight, aerobically at 37°C and MRS plates were incubated in anaerobic jars, for 48 hours, at 37°C. Samples showing negative for *S.* Typhimurium were further enriched in Selenite broth for 24 hours, or a further 6 days if not positive after 24 hours enrichment. All enrichments that were found to be positive for *Salmonella* were considered to contain 1 x 10^1 colony forming units.

6.2.6 Post-mortem enumeration of *S.* Typhimurium and Lactic acid bacteria

Two post-mortem samplings were undertaken when the birds were 4 and 6 weeks of age. Post-mortem enumeration of *S.* Typhimurium and Lactic acid bacteria was conducted according to the method described by La Ragione *et al.*, (2004). Six birds per treatment per post-mortem sampling were euthanized by cervical dislocation, and liver, spleen, ileum and caeca were removed aseptically from each bird and separately placed in sterile 1 oz MacCartney glass bottles containing sterile PBS.

Samples of tissue were weighed and homogenized and serial 10 fold dilutions were made of each homogenate in PBS (0.1 M, pH 7.2). The viable counts of *S.* Typhimurium in homogenates were determined using the Miles and Misra method (Miles & Misra 1938) by plating drops of appropriate dilutions made in PBS on BGA supplemented with nalidixic acid (15 μg ml^{-1}). One ml of residual homogenate was added to 10 ml Selenite enrichment broth and incubated for 24 h at 37°C and

sub-cultured on BGA supplemented with nalidixic acid. If not positive after the first enrichment in Selenite broth, the enriched sample media were further sub-cultured after 6 days incubation on BGA supplemented with nalidixic acid. Viable counts of LAB for the ileum and caeca were determined by plating on MRS agar and incubating in anaerobic jars for 48 hours at 37 °C.

6.2.7 Determination of *Salmonella* load in litter material

The degree of contamination of the room environment by *S.* Typhimurium was determined by collection of 5 samples of litter material from different locations in each room as shown in Figure 6.1. Position 1 was always the entrance to the room. Samples were collected in MacCartney bottles and 1 g of each sample was added to PBS to obtain 1 in 10 dilutions. Further dilutions were plated on BGA supplemented with nalidixic acid to determine the *S.* Typhimurium load as described previously. Negative samples were also enriched and plated as previously described in section 6.2.5.

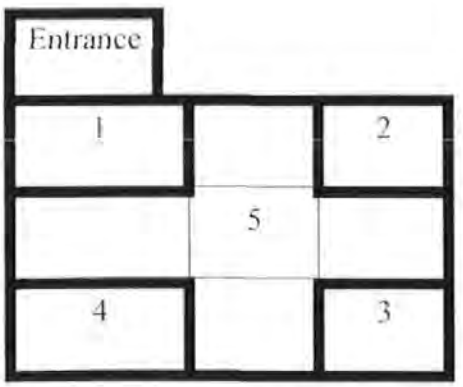


Figure 6.1: Room floor space showing sampling points for each negative pressure isolator.

6.2.8 Estimation of species (Risatype) diversity using rRNA Intergenic Spacer Analysis (RISA).

The rapid estimation of bacterial species diversity was conducted as described by Borneman and Triplett, (1997) and Acinas *et al.*, (1999). For DNA extraction from faecal samples, 1.5ml of fresh lysozyme and Tris EDTA (TE) mix (50mg lysozyme/ml in TE) was added to faecal samples in Falcon tubes and incubated at 37°C for 30 minutes (TE= 10 Tris/Cl, 1mM EDTA and adjusted to a pH of 8.0). One hundred and five µl of lysis solution (50mM Tris/Cl pH 8, 25mM EDTA, 3% SDS, 1.2% PVP) was added to the mixture after incubation and gentle mixing. 1.2 ml of warmed (60°C) extraction solution (10mM Tris/Cl pH 8, 1mM EDTA, 0.3M Na acetate, 1.2% PVP) in a water bath was added to the resultant mixture and mixed gently.

An equal volume of ice cold phenol (Sigma molecular grade, pre-equilibrated with TE) was added and the mixture was kept on ice for 10 minutes. Chloroform was added to the mixture to the brim of the Falcon tubes in a fume cupboard and gently mixed to homogenise the mixture.

The homogenate was spun at 4000rpm in a Hettich centrifuge (Rotina 46, Tuttlingen, Germany) for 10 minutes to obtain a clear upper layer of extracted mixture. This upper layer was removed to a fresh tube on ice, taking care that no protein that precipitated at the interface was collected in the process. The lower chloroform phase was carefully discarded and a proportion of 0.54 of the total volume of ice cold isopropanol was added to the extracted supernatant and left for 10 minutes to precipitate the DNA.

To pellet the precipitated DNA, the mixture was spun at 4000rpm for 10 minutes. The supernatant was removed after centrifugation and re-extracted if necessary. Pellets were washed by adding 1ml 70% ethanol before re-spinning and discarding the supernatant. This procedure was repeated twice to purify the DNA pellet and the pellets dried within the open Falcon tubes using a vacuum pump for 10 minutes.

Pellets were dissolved in a volume of 200µl TE and left overnight at 4°C or until amplification of the DNA using the Polymerase chain reaction (PCR).

6.2.8.1 Spectrophotometric assay.

The quantity of DNA in ng/µl in the samples was determined using a Nanodrop® ND-1000 spectrophotometer at a wavelength of 230 nm. Distilled water was used as a blank and TE was also used to re-zero the spectrophotometer since DNA pellets were re-suspended in TE.

6.2.8.2 PCR amplification

For PCR amplification, a solution of 100 µl was prepared from the samples and TE to obtain a concentration of 10 ng/µl of DNA. Two µl of each sample was placed in PCR Eppendorf tubes and to each tube 18 µl Qiagen kit solution mixture consisting of 10 µl master mix (PCR buffer), 6 µl of PCR grade water, 1 µl of primer B1055, and 1 µl of primer 23SOR was added. The sequence of bases in primers used were as described by Acinas *et al.* (1999) and primers were obtained from Eurofins, London, U.K.

The primer solutions constituted the primers resuspended in TE to a concentration of 100pM/µl. Samples were amplified in a PCR thermalcycler (TECHNE, Model TC-312) for a period of 4 hours. The thermalcycle conditions were 95°C for 5 minutes, for cycle 1, 35 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and a final extension of 72°C for 10 minutes.

6.2.8.3 Preparation of Metaphor Agarose gels

Fifteen microlitres of the resulting PCR products were electrophoresed at 70 volts on a pre-cast, 2% 'metaphor' agarose gel (Lonza, Rockland ME, USA), containing ethidium bromide (7g of Agarose gel in 350 ml of 1x TBE and 175 µl of ethidium bromide) and visualized using a UV transilluminator (Universal Hood II, BIO-RAD Laboratories, Segrate, Milan, Italy) and Quantity 1 BIO-RAD Software version 4.6.3 (California, USA). PCR products were simultaneously run with a calibration ladder (Bioline HyperLadder II Batch # H2 K2-1008).

6.2.9 Antibody response

6.2.9.1 Protein concentration from whole cell *Salmonella* antigen

Salmonella Typhimurium Sal 1344 nal^r was plated on sheep blood agar and incubated at 37 °C overnight. A loop of culture from overnight plates was used to inoculate 20 ml of LBG broth and incubated at 37°C overnight in a bench top shaker. The resultant inoculum was centrifuged at 4000 rpm for 10 minutes to remove the supernatant and

re-suspended in 20 ml PBS. Bacteria were killed by placing the suspension in a water bath at 65°C for 30 minutes. The protein concentration was estimated using a commercial assay (Bio-Rad Dc protein Assay), following the manufacturer's instructions. A standard curve was obtained using dilutions of Bovine Serum Albumin (2 mg, 1.5, 0.5, and 0.25). Test samples were diluted 1 in 5 and 1 in 10 for the assay. In flat bottom plates (Nunc, Denmark), 5 µl of standards and samples were plated in triplicate.

The plates were left to incubate on the bench for 15 minutes and then read at a wavelength of 630 nm on the plate reader. The protein concentration of the whole *Salmonella* bacteria in the samples was calculated using a standard curve derived from the results of the BSA standards. The standard curve was defined by a linear regression as follows 0.25 (average row b M^+) 0.5 (average row c M^+) 1 (average row d M^+) 1.5 (average row e M^+) 2 (average row b M^+). The result obtained was 0.961164 (approximately 1) to show a linear fit in the dilution concentrations of protein. The concentration of the 1/5 and 1/ 10 dilutions in mg ml^{-1} were estimated from the standard curve.

6.2.9.2 Antibody determination using Enzyme Linked Immunosorbent assay (ELISA).

The ELISA technique was conducted in a series of steps depicted as coating antigen phase, blocking step, primary and secondary antibody reaction and the developing phase.

Coating antigen

The whole cell antigen preparation was diluted to 5 μg ml^{-1} and 100 μl of this was added to each well of microtitre plates (Polysorb, Nunc). Plates were incubated overnight at room temperature for use the next day or stored at 4 °C for up to 4 weeks before use.

Blocking Step

Plates were washed three times by filling wells with ELISA wash (0.1 M PBS pH 7.2, 0.5 % (v/V) Tween-20) and blocked with tissue paper. Two hundred microlitres of 3 % (w/v) dried skimmed milk in ELISA wash were added to each well and plates were incubated for 30 minutes at 37°C. Plates were washed three times with ELISA wash and blocked with tissue paper.

Primary antibody reaction

Dilutions of test samples were made using ELISA wash solution. One hundred microlitres of each dilution was added to each well according to the sample labels on the wells. Test controls without antigen, or test sample, or conjugate, were also included on each plate to serve as controls. Plates were incubated at 37°C for 1 hour. After incubation, plates were washed three times by filling wells with ELISA wash and blocked with tissue paper.

Secondary antibody reaction

One hundred microlitres of species-specific anti-immunoglobulins conjugated with horseradish peroxidase were added, at the manufacturers recommended dilution, to each well except the conjugate control wells. Plates were incubated at 37°C for 30 minutes. After incubation, plates were washed three times as above.

Developing

Tetramethyl Benzidine (TMB) substrate (sigma) was added (100 µl) to wells and incubated at room temperature in the dark for 10 minutes. The reaction was stopped after 10 minutes by adding 50 µl of 10 % (v/v) sulphuric acid to all the wells.
Absorbencies at 450 nm were read using a spectrophotometer.

Interpretation of ELISA results

The principle of this method is that bound HRP-conjugated antibodies will cause the substrate to turn blue. Therefore, the intensity of the colour (optical density) correlates to the levels of specific antibodies in the samples. A higher concentration of bound antibodies will give a higher A_{450} ELISA reading.

6.2.10 Statistical analyses

Statistical analyses of results were done using the General linear models procedure of Minitab® (Minitab 15) according to the following general model:

$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \delta_{ij}$

Where Y_{ij} is the observed dependent variable; μ is the overall mean; α_i is the effect due to treatment; β_j is the effect due to tissue for *Salmonella* counts or time of post-mortem for lactobacilli counts; $(\alpha\beta)_{ij}$ is the interaction between treatment and tissue or time of post-mortem and δ_{ij} is the random error. The results obtained for measurements in weight gain, feed intake and fermented feed end-metabolite concentrations were analysed separately and least square means with pooled standard error (SEM) or standard error of the difference (SED) were obtained. Data transformations prior to statistical analysis on microbial counts and percentage positive or negative samples were conducted according to the method described by Zar, (1999). Means were compared using the pairwise comparisons of Tukey's test. Data on microbial diversity of caecal contents were analysed using the Plymouth Routines In Multivariate Ecological Research (PRIMER 6, Primer-E Ltd, Plymouth Marine Laboratory, Plymouth, UK) software package. Hierarchical clustering (CLUSTER), ordination by non-metric multidimensional scaling (MDS) and permutation-based hypothesis testing (ANOSIM) were used to analyse differences between treatments (Clarke & Warwick 2001).

6.3 RESULTS

6.3.1 pH, sugar and organic acid concentration of fermented feeds

The mean pH of the feed used in treatments FEED1 and FEED7 (Table 6.2) was the same for both feeds at the beginning of the incubation period. However, after 24 hour incubation the mean pH of FEED7 was significantly lower ($P<0.04$) than the pH of FEED1. There were no significant differences between FEED1 and FEED7 in the concentration of all fermentation end-metabolites measured (Table 6.3).

Table 6.2: Mean daily pH of feed used for SPF birds provided with *L. plantarum* in fermented feeds treatments, (n=39).

	Incubation time	
Treatment	0 hour	24 hours
FEED1	5.94	4.42
FEED7	5.94	4.39
SED	0.012	0.013
P-value	0.931	0.040

n=number of observations per mean.

Table 6.3: Average concentrations of sugar and organic acid of fermented feed used for the study (mmol/L), (n=10).

Treatment	Maltose	Glucose	Fructose	Lactic acid	Acetic acid	Total acid
FEED1	29.63	31.53	26.80	214.19	22.02	236.21
FEED7	31.52	27.61	28.11	228.00	20.16	248.16
SED	3.93	5.03	4.16	28.89	4.04	29.40
P-value	0.636	0.447	0.757	0.639	0.65	0.69

n=number of observations per mean

6.3.2 Feed intake

Feed intake (Table 6.4) increased from week 1 to week 6. The differences in feed consumption between the treatments in week 1 were not significant. For weeks 2 and 3,

feed consumption (g/bird/day) was significantly higher (P<0.001) for the fermented feed treatments than the other treatments. In week 5, although the fermented feed treatments still had significantly higher consumption values, birds of WAT1 treatment consumed significantly lower feed than birds on all other treatments.

The consumption of feed during the last week of the study (week 6) for CON birds remained unchanged at 59 grams/bird/day as in week 5. Corresponding values for GAV and WAT7 water treated birds showed an increase from 60.55 to 74.97 and 65.68 to 73.86 respectively from week 5 to 6. There were no significant differences between the three treatments (CON, GAV and WAT7) in week 6 for daily feed consumption. Meanwhile, birds on WAT1 treatment maintained their significantly lower feed consumption when compared with all other treatment groups. The fermented feed treatment groups also maintained the significantly higher consumption patterns when compared with all other treatments.

Table 6.4: Mean daily feed consumption (grams/bird/day dry feed basis[z]) of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed.

Weeks	1	2	3*	4*	5*	6[†]
CON	23.18[a]	24.03[a]	44.00[a]	48.79[a]	59.30[a]	59.09[ab]
GAV	17.28[a]	20.92[a]	43.19[a]	48.67[a]	60.55[a]	74.97[b]
WAT1	21.05[a]	20.20[a]	42.91[a]	47.10[a]	52.18[b]	51.91[a]
WAT7	20.27[a]	23.41[a]	47.00[a]	56.76[a]	65.68[a]	73.86[b]
FEED1	16.95[a]	51.32[b]	74.93[b]	124.32[b]	135.76[c]	151.25[c]
FEED7	18.52[a]	50.15[b]	77.20[b]	120.74[b]	132.16[c]	151.40[c]
SEM	3.03	3.66	5.77	7.44	2.65	2.65
P-value	0.682	<0.001	<0.001	<0.001	<0.001	<0.001

*number of observations for weeks 1 and 2 were 17 birds in all treatments then from week 3 10 birds with exception of D with 9 birds. [†] Week 6 is constituted of data for the last four days of the study.
[abc] significant difference between means bearing different letters in the same column.
[z] feed consumption for FEED1 and FEED7 were multiplied by a factor of 1/1.2 (0.833) to convert values to dry feed basis

6.3.3 Weight gain

Results for daily weight gain (DWG, g/bird/day) are presented in Table 6.5. The results show a steady increase in DWG for all the treatments from week 1 to week 5. The DWG for the first week ranged from 7.65 for CON to 9.50 g/bird/day for FEED1 birds. The differences between these treatments were significant (P<0.006). There was no significant difference between the GAV and FEED1 birds.

Table 6.5: Mean daily weight gain (grams/bird/day) of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed.

Weeks	1	2	3*	4*	5*
CON	7.65^{a}	10.24^{a}	12.86^{a}	16.14^{a}	17.46^{a}
GAV	8.46^{ac}	10.46^{a}	13.06^{a}	16.71^{a}	16.90^{a}
WAT1	7.93^{a}	10.55^{a}	13.80^{a}	16.77^{a}	18.10^{ab}
WAT7	7.82^{a}	10.19^{a}	14.37^{a}	16.23^{a}	18.71^{ab}
FEED1	9.50^{bc}	9.73^{a}	15.23^{a}	16.00^{a}	22.14^{b}
FEED7	8.82^{a}	10.26^{a}	14.19^{a}	16.54^{a}	18.44^{ab}
SED	0.54	0.92	0.89	1.06	1.54
P-value	0.006	0.966	0.107	0.969	0.025

*SED between D and other treatments for weeks 3= 0.914, 4=1.088, 5=1.58 and number of observations for weeks 1 and 2 were 17 birds in all treatments then from week 3 10 birds with exception of D with 9 birds. ^{abc} significant difference between means bearing different letters in the same column.

There were no significant differences between treatments in the DWG of birds for weeks 2, 3 and 4. However, significant differences were observed in week 5 with birds on the CON and GAV treatments having lower (P<0.025) values than FEED1 birds. There were no significant differences between the other treatments.

6.3.4 *Salmonella* shedding

The percentage of birds not shedding *Salmonella* is presented in Figure 6.2. There was a linear increase in percentage not shedding from the CON treatment to the GAV, WAT1 and WAT7 birds and FEED1 and FEED7 birds, respectively.

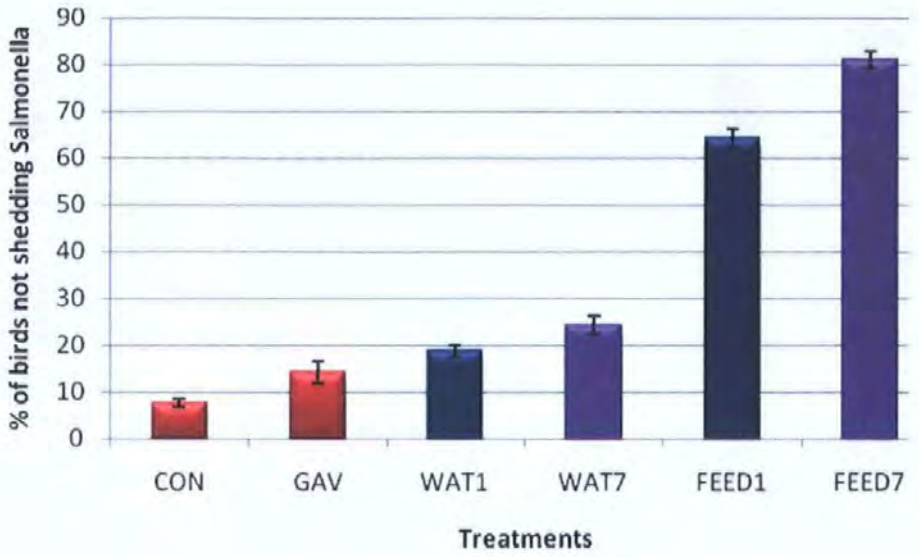
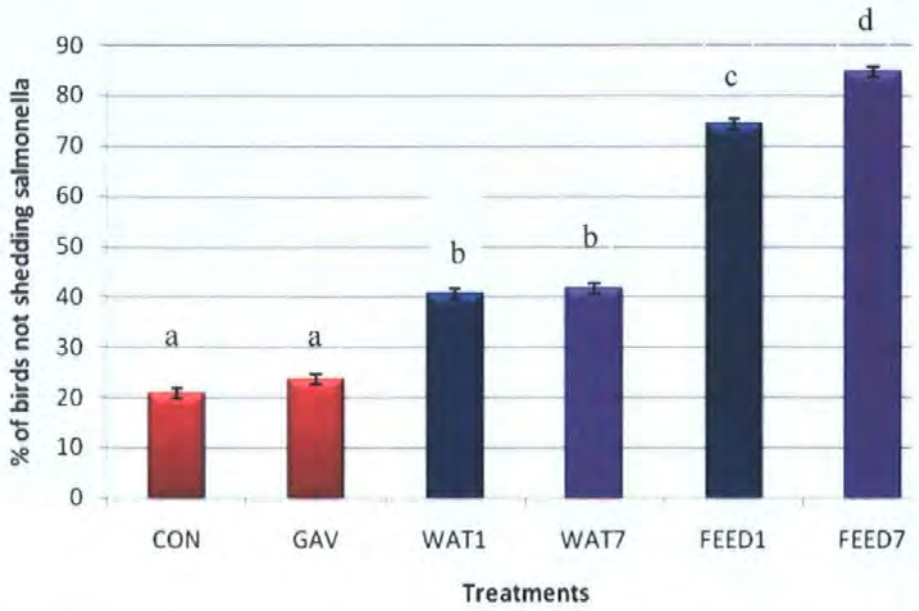


Figure 6.2: Percentage of birds provided with *L. plantarum* by oral gavage, or in water or fermented feed not shedding *S.* Typhimurium nal^r in faeces during the study, (n=90).

When the results of the percentages of birds not shedding *Salmonella* were transformed (Figure 6.3), it was observed after data analysis that the percentage of birds not shedding *Salmonella* was significantly highest for FEED7 (84.68 % ± 1.82) (Mean ± SEM). This value was higher than the mean obtained with birds of FEED1 (74.4 % ± 1.90). There were no significant differences between means for *Salmonella* counts for the water treatments although the value for WAT7 birds was slightly higher (41.69% ± 1.24) than the value for WAT1 birds (40.78% ± 2.28). The percentage of birds not

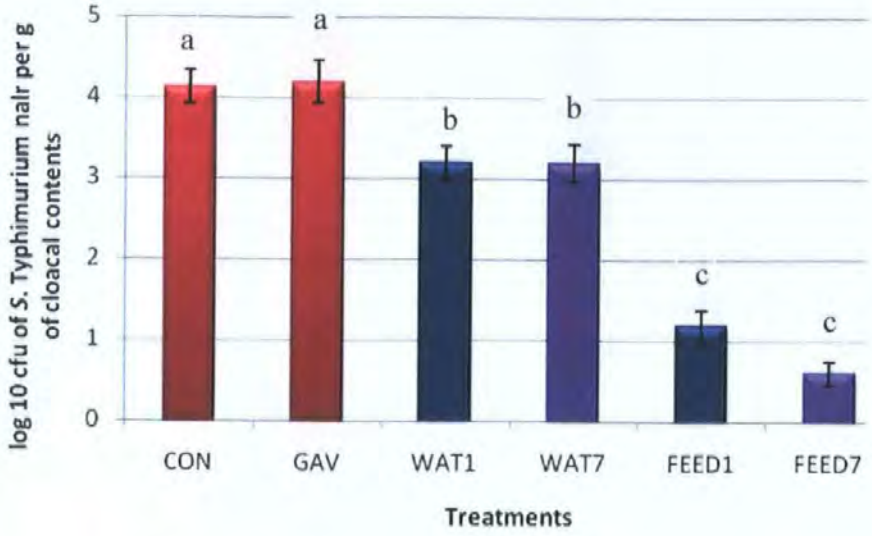
shedding for the water treatments was significantly higher ($P<0.001$) than the mean values for the CON treatment (20.85% ± 1.66) and the GAV treatment (23.77% ± 0.79).



[abcd] significant difference between histograms bearing different letters. (Untransformed standard errors presented).

Figure 6.3: Percentage of birds provided with *L. plantarum* by oral gavage, or in water or fermented feed not shedding *S.* Typhimurium nal^r during the study, (n=90).

Data presented in Figure 6.4 shows the *Salmonella* Typhimurium counts (log_{10} cfu per gram of cloacal contents) from swabs of birds provided with *L. plantarum* in fermented feeds and through drinking water. Mean cloacal *Salmonella* counts was respectively 1.2 ± 0.19 and 0.61 ± 0.14 log_{10} cfu per gram of cloacal contents for birds on FEED1 and FEED 7. These counts for the fermented feed treatments were significantly lower than counts obtained with the water treated birds which were also significantly lower than the CON (4.13 ± 0.21 log_{10} cfu per gram of cloacal contents) and GAV treatments (4.2 ± 0.26 log_{10} cfu per gram of cloacal contents).



[abcd]significant difference between histograms bearing different letters.

Figure 6.4: *Salmonella* Typhimurium nal[r] counts of SPF birds provided with *L. plantarum* by oral gavage or in water or fermented feed during the study, (n=90).

6.3.4.1 Variation in *S.* Typhimurium counts with days post-challenge

Time series data for the variation in *S.* Typhimurium counts in days post-challenge (Figure 6.5) of birds showed *S.* Typhimurium counts in the fermented feed treatments to be consistently lower than the other treatments. The important findings illustrated in Figure 6.5 are that;

1) *Salmonella* shedding varied from day to day.
2) *Salmonella* shedding was consistently lower in birds fed fermented feeds.
3) From day 7 *Salmonella* shedding was no lower in birds receiving LAB in water than the controls.

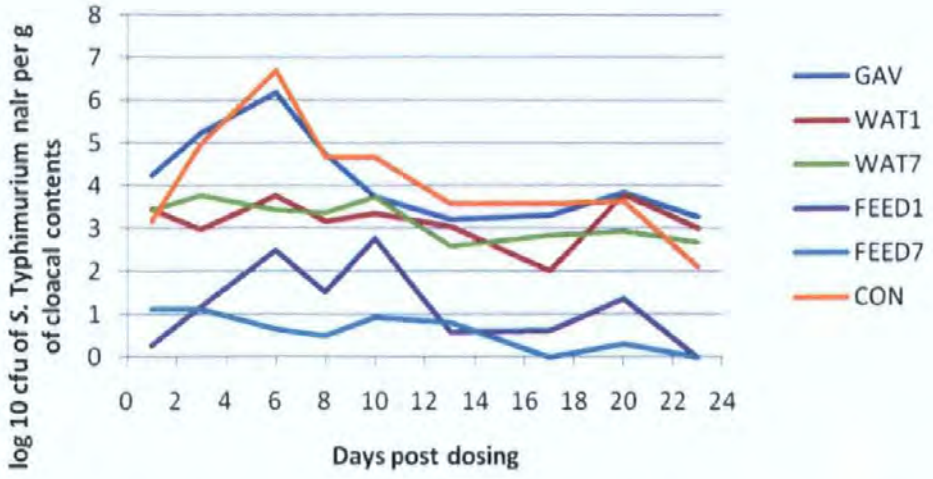


Figure 6.5: Variation in *Salmonella* Typhimurium nal^r counts with days post dosing of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed, (n=10).

The variation in *Salmonella* counts as reflected by the method, or time of administration of lactobacilli, is shown in Table 6.6. There was no significant difference in the method and time interaction (P=0.12). However, there was a highly significant (P<0.001) difference between the methods of administration. The mean cloacal *Salmonella* count for treatments where *L. plantarum* was administered in water was significantly higher than mean counts for administration of the microbes through feed. When the time of administration of lactobacilli was considered, there was no significant difference (P=0.137) between administration in feed or water on day 1 and administration on day 7 although administration on day 7 recorded numerically lower counts than administration on day 1.

Table 6.6: Variation in *Salmonella* Typhimurium nal^r counts with the method, or time, of administration of *L. plantarum* to SPF birds (log10cfu/g of cloacal contents).

Time		Method		
Day 1	Day 7	Water	Feed	SEM
2.19	1.91	3.19	0.91	0.14
P=0.137		P<0.001		P=0.12*

* P- value for method and time interactions

6.3.5 Colonisation and invasion of SPF birds dosed with *Salmonella* Typhimurium nal^r and provided with *L. plantarum* in fermented feed and drinking water.

6.3.5.1 Caecum

Two weeks after challenge with *Salmonella* only birds in the fermented feed treatments were free from caecal colonisation (Table 6.7). While one bird was free from colonisation in FEED1 birds out of the 6 birds slaughtered for the first *post-mortem*, two birds were not colonised for FEED7 birds.

Apart the GAV treatment, which had one sample that was detected to be positive after the first plating, positive samples for the water and control treatments were all found to be positive at the first plating. In the first enrichment 2 caecal samples were found to be positive for FEED7 birds while for FEED1 there was 1 positive sample ineach enrichment.

In the second post-mortem (Table 6.7) the number of birds sampled after four weeks post challenge for the birds sampled found to be negative for caecal *Salmonella* were 1 out of 6 for WAT1 birds and 3 & 2 for FEED1 and FEED7 birds respectively.

6.3.5.2 Liver

The colonisation of the liver with *Salmonella* is illustrated also shown in Table 6.7. 3 and 4 birds were not colonised by *Salmonella* for FEED 1 and FEED 7 respectively in the first post-mortem. There was 1 bird each negative for colonisation of the liver for the GAV and WAT1 birds. All 6 birds randomly sampled from WAT7 and CON treatments had livers that were colonised by *Salmonella*.

At the second post-mortem, a higher number of birds were negative for liver colonisation (n=25) than the first-first post-mortem (n=9) (Table 6.7). All birds in the CON treatment were negative for colonisation followed by 5 birds each for the water treatments. The fermented feed treatments had 4 and 3 for FEED1 and FEED7 treatments respectively. More than 50% of the colonisation with the fermented feed treatments was obtained only after the samples had been enriched in Selenite broth.

6.3.5.3 Ileum

Ileal colonisation by *Salmonella* for the fermented feed treatments in the first *post-mortem* (Table 6.7) was more or less similar to the other tissues. These treatments had 3 birds each with ileums not colonised by *Salmonella* for birds sampled. Birds in WAT1 had 5 birds with ileal colonisation while all birds in the other treatments (CON, GAV and WAT7) had ileums colonised by *Salmonella*. However, except for WAT7 birds (one week before challenge) a greater proportion of all lactobacillus treated birds showed ileal colonisation only after samples had been enriched in Selenite broth at least once. This was not the case for the CON and the GAV treatments in which much of the colonisation was detected in the first plating.

In the second post-mortem (Table 6.7), there was a threefold increase in the total number of birds sampled (20) that were not carrying ileal *Salmonella* when compared with the birds sampled in the first post-mortem (7 birds). 19 of the 20 birds sampled whose ileums were not colonised by *Salmonella* were fed *L. plantarum* in water or feed. The fermented feed treatments accounted for more than 50% of these negative ileal samples (10 birds) while for WAT7 birds there were 6 negative ileal samples.

6.3.5.4 Spleen

The absence of colonisation of the spleen by *Salmonella* in the first *post-mortem* (Table 6.7) was observed only for 8 birds in the fermented feed treatments. FEED1 birds had a higher number negative spleen samples (5 birds) while three birds on FEED7 had negative spleen samples.

With the second *post-mortem*, the colonisation of the spleen by *Salmonella* was also considerably reduced for the total number birds sampled (Table 6.7) when compared with the first post-mortem. While 8 out of 36 birds had spleens that were not colonised by *Salmonella*, the corresponding number for the second *post-mortem* was 31 out of 36 birds. The GAV birds had the highest number (50%) of birds whose spleens were colonised by *Salmonella* while the CON and FEED7 birds both had 1 bird each with a spleen colonised by *Salmonella*.

A summary of the total number of birds found to be negative for the respective tissues or tissue contents irrespective of treatment is shown in Table 6.7. High increases in clearance of *Salmonella* between the first and second post-mortem from the tissues or

tissue contents after challenge of birds sampled were observed with the spleen and liver while the clearance rate was not so high in intestinal tissues especially the caecum.

6.3.6 Influence of administration of *L. plantarum* in fermented feed or drinking water on tissue counts *Salmonella* for SPF birds.

The *Salmonella* counts for the first post-mortem samples are presented in Table 6.8. Data were compared for treatments between rows and tissues within rows. When means within rows were compared, it was observed that *Salmonella* counts in the caecum for the control treatment (5.72 ± 0.23) were significantly higher ($P<0.02$) than the counts for the spleen (2.54 ± 0.23) and the liver (2.08 ± 0.23). *Salmonella* counts for the ileal contents were not significantly different from those of all the other tissues. However, the caecal *Salmonella* counts were significantly higher ($P=0.01$) in the GAV and *L. plantarum* in water treated birds than ileal counts. Caecal counts for the GAV and WAT1 birds were also higher than the corresponding counts for the liver. Though means within rows for the fermented feed treatments between the tissues varied, this variation was not significant ($P>0.05$). When the means between rows (within columns) were compared, caecal and ileal *Salmonella* counts for the fermented feed treatments were significantly lower than corresponding counts for the other treatment groups. In the liver, there were no differences between the treatments. For the spleen, counts for FEED7 birds were not significantly different from the other treatments groups. However, FEED1 birds had significantly lower ($P=0.0012$) spleen counts all other treatments.

Table 6.7: Number of birds positive for *S.* Typhimurium nal^r following *post-mortem* of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed.

			Liver			Spleen			Ileum			Caecum		
Treatments		**N**	**P1**	**E1**	**E2**	**P1**	**E1**	**E2**	**P1**	**E1**	**E2**	**P1**	**E1**	**E2**
PM1	**CON**	**6**	4	2	0	2	4	0	6	0	0	6	0	0
	GAV.	**6**	4	1	0	6	0	0	5	1	0	5	1	0
	WAT1	**6**	5	0	0	6	0	0	1	2	2	6	0	0
	WAT7	**6**	6	0	0	6	0	0	5	1	1	6	0	0
	FEED1	**6**	1	1	0	1	0	0	1	1	1	3	1	1
	FEED7	**6**	2	1	0	2	0	1	1	2	0	2	2	0
PM2	**CON**	**6**	0	0	0	1	0	0	2	3	0	2	2	2
	GAV.	**6**	3	1	0	3	0	0	4	1	1	5	1	0
	WAT1	**6**	1	0	0	0	0	0	1	2	0	4	1	0
	WAT7	**6**	0	1	0	0	0	0	0	0	0	6	0	0
	FEED1	**6**	1	1	0	0	0	0	0	0	0	2	1	0
	FEED7	**6**	1	1	1	1	0	0	0	1	1	1	3	0
N° of negative tissues			**PM1**	**PM2**		**PM1**	**PM2**		**PM1**	**PM2**		**PM1**	**PM2**	
	CON	**12**	0	6		0	5		0	1		0	0	
	GAV.	**12**	1	2		0	3		0	0		0	0	
	WAT1	**12**	1	5		0	6		1	3		0	1	
	WAT7	**12**	0	5		0	6		0	6		0	0	
	FEED1	**12**	4	4		5	6		3	6		1	3	
	FEED7	**12**	3	3		3	5		3	4		2	2	
	Total	**36**	**9**	**25**		**8**	**31**		**7**	**20**		**3**	**6**	

P1-first plating, E1-first enrichment, E2-second enrichment, PM1-first *post-mortem*, PM2-second *post-mortem*.

Table 6.8: Mean *Salmonella* Typhimurium nal^r counts for the first post-mortem tissues (after 4 weeks) of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed (log_{10} cfu/g tissue) and dosed with *Salmonella*, (n=6).

Treatments	Tissues				Tissue Mean (n=24)	Tissue SEM	P-value
	Caecum	Ileum	Spleen	Liver			
CON	$^{A}5.72^{a}$	$^{AB}4.26^{b}$	$^{B}2.54^{a}$	$^{B}2.08^{a}$	3.64^{a}	0.28	0.02
GAV	$^{A}6.07^{a}$	$^{B}2.74^{ab}$	$^{AB}3.96^{a}$	$^{B}2.16^{a}$	3.73^{a}	0.28	0.01
WAT1	$^{A}5.64^{a}$	$^{B}2.18^{ab}$	$^{AB}3.80^{a}$	$^{B}1.56^{a}$	3.29^{a}	0.28	0.006
WAT7	$^{A}5.21^{a}$	$^{B}2.02^{ab}$	$^{AB}4.42^{a}$	$^{AB}3.29^{a}$	3.73^{a}	0.28	0.02
FEED1	$^{A}2.24^{b}$	$^{A}0.79^{a}$	$^{A}0.62^{b}$	$^{A}0.40^{a}$	1.0^{b}	0.28	0.798
FEED7	$^{A}2.06^{b}$	$^{A}0.96^{a}$	$^{A}1.73^{ab}$	$^{A}0.76^{a}$	1.37^{b}	0.28	0.99
Treatment Mean (n=36)	$^{A}4.48$	$^{BC}2.14$	$^{B}2.84$	$^{C}1.70$			
Treatment SEM	0.23	0.23	0.23	0.23		0.56*	0.018*
P-value	0.001	0.012	0.0012	0.064			

Means within the same column $^{(ab)}$ or row $^{(AB)}$ bearing different letters differ significantly (P<0.05). *SEM and P-value for the Treatment x tissue interactions, n=number of observations per mean.

The *Salmonella* counts for the second post-mortem samples are shown on Table 6.9. Data were compared for treatments between rows and tissues within rows. When the means within rows were compared, it was observed there were no significant differences between the tissues were observed for all the treatments.

Separation of means between rows for caecal *Salmonella* counts shows that counts for the CON and GAV birds were significantly higher (P=0.012) than the counts for the fermented feed treated birds but not different from the water treatments groups. The water treatment birds were not significantly different in caecal *Salmonella* counts from the fermented feed treated birds.

Table 6.9: Mean *Salmonella* Typhimurium nal^r counts for the second post-mortem tissues (after 6 weeks) of SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed (log_{10} cfu/g tissue) and dosed with *Salmonella*, (n=6).

Treatments	Tissues				Tissue Mean	Tissue	
	Caecum	Ileum	Spleen	Liver	(n=24)	SEM	P-value
CON	$^{A}2.30^{a}$	$^{A}2.28$	$^{A}0.72$	nd†	1.32^{b}	0.26	0.21
GAV	$^{A}4.08^{a}$	$^{A}2.47$	$^{A}1.91$	$^{A}1.84$	2.58^{a}	0.26	0.26
WAT1	$^{A}2.26^{ab}$	$^{A}1.04$	nd	$^{A}0.39$	0.92^{b}	0.26	0.24
WAT7	$^{A}2.65^{ab}$	nd	nd	$^{A}0.16$	0.70^{b}	0.26	0.11
FEED1	$^{A}1.08^{bc}$	nd	nd	$^{A}0.72$	0.45^{b}	0.26	0.99
FEED7	$^{A}0.95^{bc}$	$^{A}0.34$	$^{A}0.47$	$^{A}0.65$	0.60^{b}	0.26	1.00
Treatment Mean (n=36)	$^{A}2.22$	$^{B}1.02$	$^{B}0.63$	$^{B}0.52$			
Treatment SEM	0.21	0.21	0.21	0.21		0.51*	0.172*
P-value	0.012	0.34	0.57	0.64			

Means within the same column $^{(abc)}$ or row $^{(AB)}$ bearing different letters differ significantly (P<0.05). *SEM and P-value for the Treatment x tissue interactions. †nd: not detected, n=number of observations per mean.

6.3.7 Effect of feeding SPF birds with *L plantarum* on environmental colonization by *Salmonella* for birds challenged with *S.* Typhimurium.

There were no significant differences (P=0.281) between the treatments in the *Salmonella* counts in the litter material (Figure 6.6). However, it was observed that the *Salmonella* counts in all treatments where *L. plantarum* was provided in fermented feed or water were numerically lower than the CON or GAV treatments. The lowest value was obtained from litter material collected from FEED7 birds (0.63 log_{10} cfu g^{-1} of litter). This was followed by FEED1 birds (1.11 log_{10} cfu g^{-1} of litter).

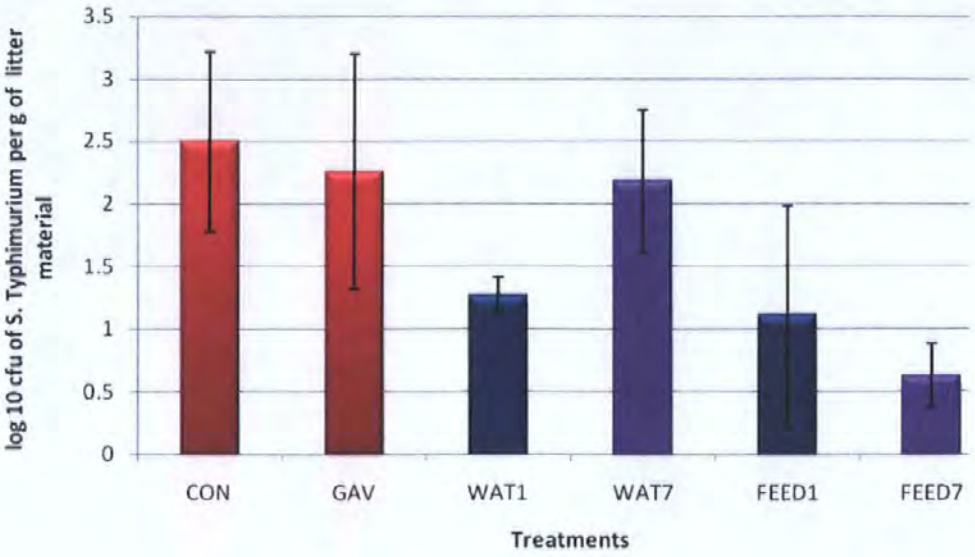
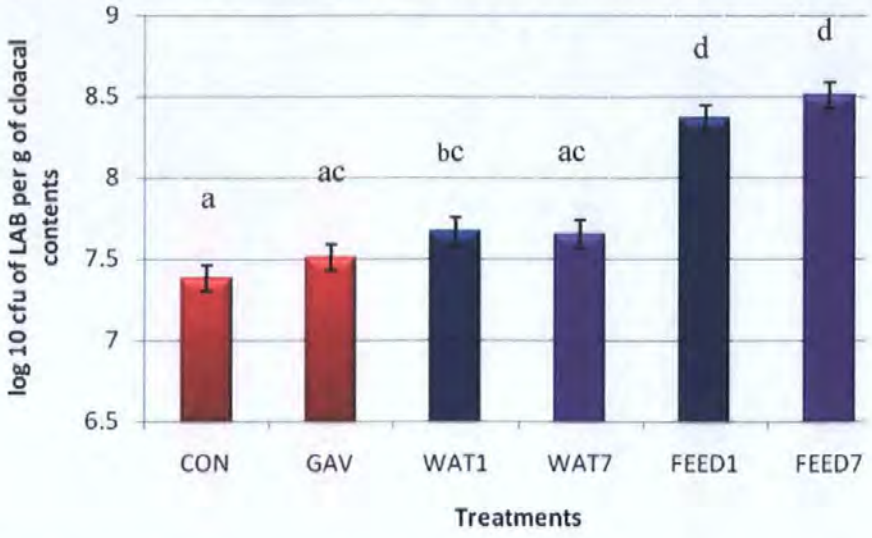


Figure 6.6: *S.* Typhimurium load in the room environment for SPF birds provided with *L. plantarum* by oral gavage, or in water or fermented feed, (n=5).

6.3.8 Cloacal lactobacilli counts

The LAB counts in faeces of birds provided with *L. plantarum* in fermented feed and through drinking water are shown in Figure 6.7. The LAB counts for the CON treatment (7.38 $\log_{10}$ ± 0.078 cfu/g cloacal contents) was significantly lower ($P<0.05$) than the counts for the fermented feed treatments (8.37 $\log_{10}$ ± 0.074 for FEED1 and 8.51 $\log_{10}$ ± 0.078 cfu/g cloacal contents for FEED7 birds), and WAT1 birds (7.67 $\log_{10}$ ± 0.087 cfu/g cloacal contents). There were no significant differences between the mean counts of the CON treatment and the counts for the GAV treatment (7.51 $\log_{10}$ ± 0.078 cfu/g cloacal contents) and WAT7 birds (7.65 $\log_{10}$ ± 0.087 cfu/g cloacal contents).



[abcd]significant difference between histograms bearing different letters.

Figure 6.7: LAB counts in faeces of birds provided with *L. plantarum* by oral gavage or in water or fermented feed, (n=90).

6.3.9 Influence of administration of *L. plantarum* (SLP) in fermented feed or drinking water on intestinal counts of LAB for SPF birds.

There was a significant ($P<0.002$) increase in the overall mean caecal LAB counts between treatments (Table 6.10). Separation of means between the treatments showed three levels with the CON treatment birds having the lowest level for mean counts while the next three treatments (GAV and water treatments) occupied the second level and the fermented feed treatments the highest level. Ileal counts showed a similar trend with significantly higher LAB counts being observed with the fermented feed treatments although these means were only different from the means of WAT7 birds and the CON birds. The counts for the *post-mortem* periods between columns within the same intestinal tissue were not significantly different for the caecal counts while for the ileal counts, a significant ($P<0.001$) difference was observed between the first and the second *post-mortem* counts for WAT1 birds.

Table 6.10: Comparisons of post-mortem LAB counts of intestinal contents of SPF birds provided with *L. plantarum* (SLP) by oral gavage, in water or fermented feed (log_{10} cfu/g caecal or ileal contents).

	Caecum			Ileum		
Treatments	PM1[†]	PM2	Mean	PM1	PM2	Mean
CON	7.46^{A}	8.29^{A}	7.87^{a}	9.89^{A}	8.31^{A}	8.70^{a}
GAV	8.88^{A}	8.39^{A}	8.64^{b}	9.39^{A}	8.76^{A}	9.08^{ab}
WAT1	8.86^{A}	8.30^{A}	8.59^{b}	9.11^{A}	8.81^{A}	8.96^{ab}
WAT7	8.80^{A}	8.39^{A}	8.59^{b}	9.06^{A}	8.06^{B}	8.56^{a}
FEED1	9.56^{A}	9.53^{A}	9.5^{c}	9.71^{A}	9.20^{A}	9.45^{b}
FEED7	9.45^{A}	9.56^{A}	9.50^{c}	9.71^{A}	9.16^{A}	9.46^{b}
SEM	0.07	0.07	0.12	0.08	0.08	0.14
P-value			0.002			0.003
Interaction			0.002*			0.58*

Means within the same column $^{(abc)}$ or row $^{(AB)}$ bearing different letters differ significantly ($P<0.05$). [†]PM1 and PM2 refer to first and second post mortem samples. *P-value for treatment x time interactions

6.3.10 Microbial diversity of the caecal microflora

RISA analysis (Figure 6.8) of microbiota of the caecal contents on 2% metaphor gels shows changes in operative taxonomic units (risatypes or bands) between treatments.

The similarity matrix (dendrogram) between treatments (Figure 6.9) shows a high level similarity between FEED1 and FEED 7 and a low level similarity with the CON, WAT1, WAT7 and GAV treatments.

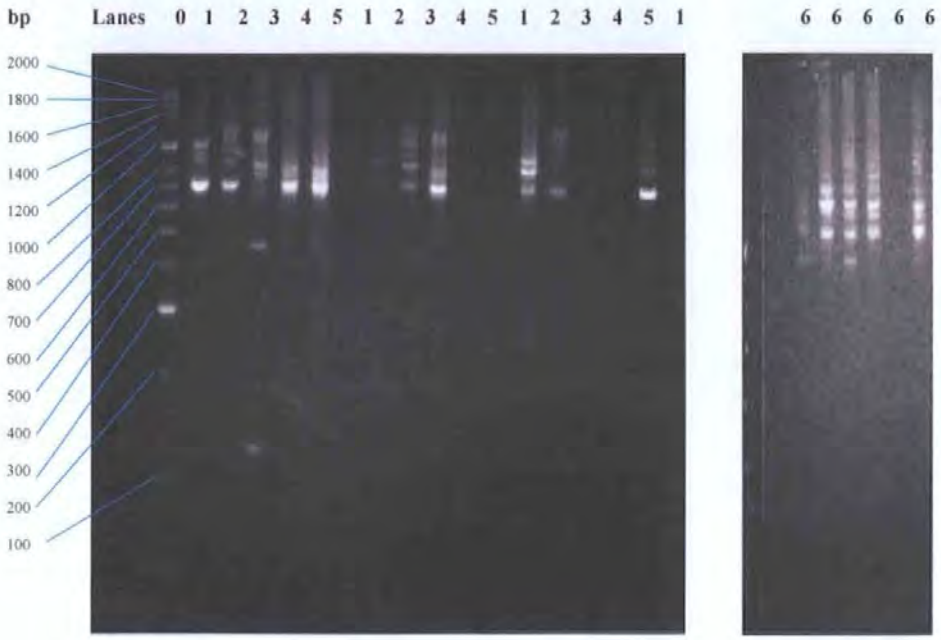


Figure 6.8: Metaphor 2% gel fragments from RISA analyses of caecal microflora for SPF birds provided with *L. plantarum* 41229 by oral gavage or in water or fermented feed (Lane 0-Ladder II, 1-GAV, 2-WAT1, 3-WAT7, 4-FEED1, 5-FEED7and 6-CON).

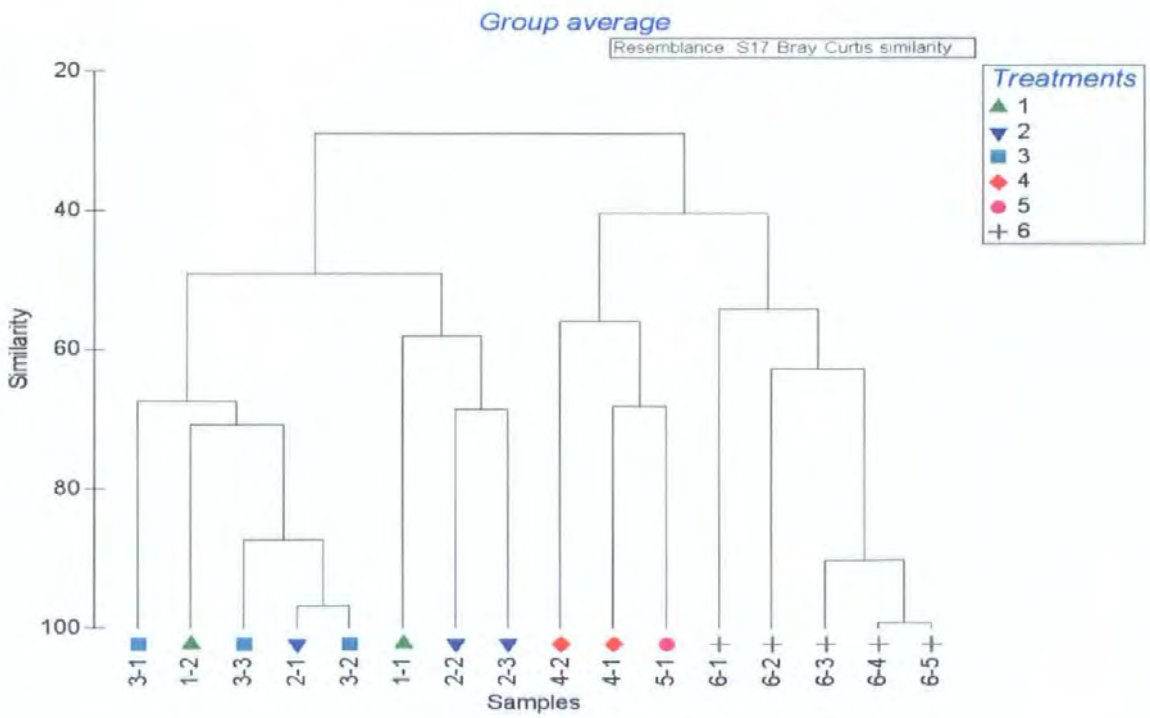


Figure 6.9: Resemblance: S17 Bray Curtis similarity dendrogram between treatments samples (1-GAV, 2-WAT1, 3-WAT7, 4-FEED1, 5-FEED7 and 6-CON).

Two dimensional non-metric multidimensional scaling (MDS) (Figure 6.10) of S17 Bray Curtis similarity (Clarke & Warwick 2001) showed that the banding patterns of replicates in CON treatment were identical in most cases. These were separated from WAT1, WAT7 and FEED1 and FEED7 treatments. FEED1 and FEED7 were closely related in their banding patterns.

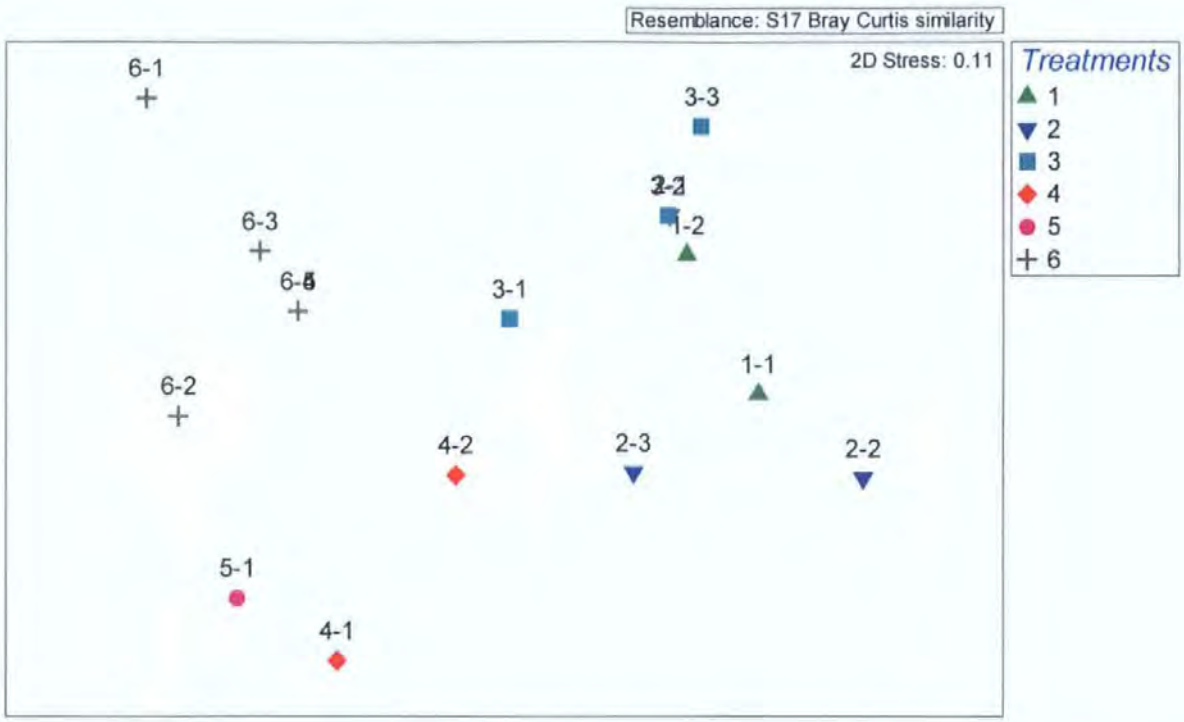


Figure 6.10: Two dimensional multidimensional scaling ordinations based on Bray Curtis similarities (2D Stress-0.11); (1-GAV, 2-WAT1, 3-WAT7, 4-FEED1, 5-FEED7 and 6-CON)

One way analysis of similarity or dissimilarity percentages within and between treatments (Clarke & Warwick 2001) is shown in Table 6.11. FEED1 and FEED7 treatments had the lowest dissimilarity percentage of 40.4 %. GAV, WAT1 and WAT7 also had dissimilarity percentages ranging from 40.68 to 41.71 %.

Table 6.11: Within treatment similarity (SIMPER) and between treatment dissimilarity percentages.

Group	**Similarity (%)**	**Between group**	**Dissimilarity (%)**
1	57.80	1-2	41.71
2	56.80	1-3	40.68
3	72.07	2-3	41.66
4	60.77	1-4	67.36
5	-	2-4	67.49
6	68.46	3-4	60.17
		1-5	85.77
Group	**Treatment**	2-5	85.04
1	GAV	3-5	74.45
2	WAT1	4-5	40.40
3	WAT7	1-6	74.06
4	FEED1	2-6	74.55
5	FEED7	3-6	66.61
6	CON	4-6	60.44
		5-6	57.90

6.3.11 Antibody response

The circulating antibody responses against *Salmonella* in chickens (Table 6.12) reveal a difference (P=0.03) between treatments in reaction of the sera for birds aged 6 weeks (third sampling). In the first two samples, corresponding to birds at 2 and 4 weeks of age, observed differences between the treatments were not significant. The CON birds' response (0.16 ± 0.009) was significantly higher than the response obtained with birds provided with *L. plantarum* in feed or in drinking water. There were no significant differences between the CON and the GAV treatments.

Table 6.12: Application of *L. plantarum* NCIMB 41229 by oral gavage, or in water or fermented feed on plasma *Salmonella* antibody Elisa optical density results

	Time of bleeding birds				
Treatments	**28/01**	**08/02**	**22/02**	**SEM**	**P-value**
CON	$^{AB}0.05$	$^{A}0.03$	$^{B}0.16^{b}$	0.012	0.002
GAV	0.04	0.03	0.11^{ab}	0.012	0.34
WAT1	0.06	0.05	0.05^{a}	0.012	1.00
WAT7	0.06	0.05	0.07^{a}	0.012	1.00
FEED1	0.07	0.08	0.04^{a}	0.012	0.98
FEED7	0.02	0.05	0.03^{a}	0.012	1.00
SEM	0.009	0.009	0.009	0.02*	0.003*
P-value	0.88	0.86	0.03		

Means within the same column $^{(ab)}$ or row $^{(AB)}$ bearing different letters differ significantly (P<0.05). *SEM and P-value for the Treatment x time interactions

When sampling periods were compared, a significant difference (P=0.002) was only observed in the CON treated birds between the birds aged 4 weeks (second sampling) and birds aged 6 weeks (third sampling).

6.4 DISCUSSION

The results of current study show significant differences between treatments in the reduction of *Salmonella* colonisation in birds. Dosing birds with *L. plantarum* through fermented feed had a higher effect on the birds' resistance to colonisation. Dosing by oral gavage (single dose) had an insignificant effect on resistance to colonisation while through water there was a modest response. Fermented feed treatments were very effective in controlling colonisation by *Salmonella*. The prime concern of food producers is on the protection of human health through elimination of food borne

pathogens from food animals and their products (La Ragione *et al.* 2001; La Ragione & Woodward 2003). This concern finds its justification in the use of fermented feeds from

our results. A significant reduction in susceptibility of broilers chickens to *Campylobacter jejuni* colonisation with or without concurrent inoculation of *Salmonella* Enteritidis determined by cloacal swabbing for fermented liquid feeds has also been reported by Heres *et al.* (2003b). It was not clear how the acid barrier function imposed by the increased acidity and lower pH of the upper gut resulting from feeding fermented feeds to their birds was by-passed during inoculation with *Salmonella* in their studies. However, the mean percentages of birds shedding *Salmonella* for the fermented feed treatments reported in the current study were lower than the mean percentages obtained in their studies for a period of 10-13 days after inoculation (50% for 4.2 $\log_{10}$ cfu/ml *Salmonella* in a co-inoculation with *Campylobacter jejuni* and 100% for 6.8 $\log_{10}$ cfu/ml *Salmonella*).

The probable absence of a mechanism to reduce the acidity and increase the pH of the crop and gizzard prior to challenge with *Salmonella* within their study, compared with this study could have reduced the number of coliforms that gained access to the lower parts of the gut. Indeed, Heres *et al.* (2003d) demonstrated a sharper decrease of *Salmonella* in the anterior parts of the gastro-intestinal tract in chickens fed with fermented feed than in chickens fed dry feed due to improvement in the barrier function of the crop and gizzard resulting from the consumption of fermented feed. They further stated that while the reduction of *Salmonella* was fully realised in the crop and gizzard, this was not the case for the lower intestinal compartments which did not show a substantial effect on the reduction of *Salmonella*.

The counts for cloacal shedding of *Salmonella* throughout the study also revealed a sharper decrease for the fermented feed treated birds when compared with the control and GAV birds. The counts for the birds receiving *L. plantarum* in water were also significantly higher than the control groups indicating that the small proportion of birds that were positive for *Salmonella* shedding in the water and fermented feed treatments were carrying a lower number of *Salmonella* per gram of cloacal contents than the control and oral gavage treated birds. This observation is important in the light of food safety and environmental control of *Salmonella* on chicken farms. This observation corroborated the results obtained from environmental samples collected for the current study. Although there were no significant differences between treatments in *Salmonella* counts (Figure 6.6), the mean *Salmonella* load in the rooms where lactobacilli treated birds were housed was observed to be numerically lower than counts for the control and oral gavage treatments. As observed by (Heres *et al.* 2003c) the between-chickens transmission which they quantified by calculating the reproduction ratio and a transmission rate parameter were reduced in fermented feed treated groups. They concluded that FLF could reduce transmission of *Salmonella* in chicken flocks although this will not prevent the occurrence of major outbreaks.

While it is logical that the level of infection of the birds may not prevent the occurrence of outbreaks, food and environmental safety concerns will require a better prevention, or control, of enteropathogen shedding to the environment even if it is only down to numbers. Therefore, measures that would resist a rise, or maintain *Salmonella* shedding at constantly low levels for a longer period, could be considered improved practices. Time series data for the variation of *Salmonella* Typhimurium nal^r counts with days

post-dosing (Figure 6.5) reflects the fact that fermented feeds were better able to control (FEED1) or reduce (FEED7) cloacal *Salmonella* shedding for a greater part of the study. The results also showed a very high infection peak for the oral gavage and control treatments after 6 days post dosing. These peaks from day 1 to 6 post dosing for these treatments had the highest gradients for the counts for shedding of *Salmonella* in this study. The resistance to colonisation of the birds by *Salmonella* for the fermented feed treatments was also demonstrated by the results obtained from the organ homogenates for the birds of these treatments (Table 6.7). An important point from these results is that resistance to *Salmonella* colonisation was down to the application of lactobacilli within the treatments. This resistance is reflected to the fact that a higher proportion of the organ homogenates from birds on the control and oral gavage were positive during the first plating especially for the ileal and caecal contents. This was also the case for the first post-mortem results of the water treatment birds. These findings are in line with the observations of Heres *et al.* (2003a) who stated that the number of enrichments needed to detect *Salmonella enteritidis* for broilers in their experiment indicated a lower level of colonisation.

The results were even clearer when the organ homogenate *Salmonella* counts per gram of tissues or tissue contents for the various treatments (Table 6.8 and 6.9) were analysed statistically after base 10 logarithmic transformations. The significantly lower counts for caecal *Salmonella* in the fermented feed treatments for birds sampled in the two *post-mortems* largely reflects the ability of these treatments to control the exponential phase of *Salmonella* growth in the current study. The lower non significant *Salmonella* counts in the other tissues for the fermented feed treatments in the first *post-mortem* also highlights' this point.

A number of points could also arise from these findings.

1) The inherent ability of the birds to resist invasion of *Salmonella* could have played a part in natural clearance.

2) The water consumption patterns of the birds provided with *L. plantarum* in water could have permitted them to amass a large population of lactobacilli within the gut by the second post-mortem.

3) It is possible that high numbers of lactic acid bacteria are necessary to give the bird sufficient potential to resist invasion by *Salmonella*. Indeed, Heres *et al.* (2003d) reported that high numbers of lactobacilli fed in fermented liquid feed for broilers did not prevent the multiplication of *Salmonella* that had reached the caecum. They then concluded that unless there are specific lactobacilli that have specific negative interactions with *Salmonella*, it is questionable if probiotics alone could prevent colonisation by *Salmonella* in the caeca.

4) The organic acid contents of the fermented feed treatments alone was not enough to provide the birds with resistance to infection. Heres *et al.* (2004) also found that acidified feed had no impact on the susceptibility of chickens to *Salmonella* infection in their studies and that their results were consisitent with previous studies. This led them to the conclusion that organic acids may have insufficient potential for a total control of *Salmonella*. Furthermore, van Winsen (2002) stated that the role of an undissociated form of faecal volatile fatty acids on significant reduction in numbers of Enterobacteriaceae in fermented feed fed pigs could not be demonstrated because of a significantly higher pH of the faeces of pigs fed fermented feed compared with faeces of pigs fed conventional feeds.

Aside from the large numbers of lactobacilli and organic acids, there could also be issues surrounding the vigour or better still the metabolic status of the lactobacilli present in the feed or water. While there were substrates to fuel the metabolic activity of the *L. plantarum* of the fermented feeds during feeding, there could have been no substrates to permit the same organisms in water to maintain their metabolic status of substrate breakdown for energy. Microbial growth also depends on factors such as pH, temperature and accumulation of metabolic end-products (Charalampopoulos *et al.* 2002). This could affect their vigour and strength of production of bacteriocins while in the gut of the birds. Furthermore, the crude protein fraction (18.5% DM basis) of the diet used for this study might have buffered the acid produced to permit further production by the lactobacilli. Perhaps, the antimicrobial effect due to lactobacilli and acid content of fermented feed is additive. The undissociated organic acid enters into the cytoplasm of the enteropathogens and disrupts the internal pH. This weakens the resistance of pathogens prior to subversion by lactobacilli of their carbon utilisation. According to Collins *et al.* (2008) fundamental changes in metabolism occurred when lactic acid and probiotic cell free cell supernatants (CFCS) were added to a culture medium containing *S.* Typhimurium. They further stated that the presence of lactate and CFCS (derived from *L. plantarum*) had pH independent antimicrobial activity as well as regulatory effects on the phosphotransferase system transport, *de novo* amino acid synthesis, chemotaxis, efflux and the central metabolism of the pathogen. However, they proposed that the mode of action could be a large influx of the lactate produced by the probiotic inducing gluconeogenesis in the exponential phase of the *S. enteric* serovar Typhimurium thereby affecting their growth at near neutral and low pH. Gut pH may also have caused an effect of the ability of *Salmonella* to adhere to the intestinal epithelial cells of the chickens. Letho and Salminen (1997) observed that inhibition of

Salmonella Typhimurium adhesion to a monolayer of Caco-2 cells was higher at a pH of 4.7 (% adhesion-7.1) than a pH of 6.9 (39.8%) and 7.5 (44.1%).

From the foregoing discussion it is apparent that fermented liquid feed is an effective means through which large amounts of very viable lactobacilli can be administered to chickens. Comparisons of cloacal *Salmonella* counts in our study showed a marked difference between the combined effect of administration of lactobacilli in water and administration through fermented feeds (Table 41). The magnitude of this difference and the fact that the method and time of administration interaction was not significant (P=0.12), suggests, that aside from numbers of lactobacilli, other factors are also implicated in the resistance of birds to *Salmonella* colonisation for FEED1 and FEED7 birds. The age at which fermented feed or lactobacilli are administered to the birds seems to be a factor that needs to be considered in the birds' resistance to colonisation.

This view is strengthened by the results obtained from analysis of the combined effect of the time of administration of lactobacilli to birds in water or fermented feed. The combined effect of administration at 7 days of age had lower cloacal *Salmonella* counts compared with administration at 1 day of age. It would be logical to think that the birds that received lactobacilli in water or fermented feed on day 1 of age might have had an advantage of numbers over the birds receiving the same lactobacilli 7 days later. This would have made the combined effect on *Salmonella* numbers in the day 1 treated birds to be quite significantly lower than birds treated 7 days after. However, in the current study, though the difference was not significant, the reverse was the case.

These results might lead us to the suggestion that the birds receiving the lactobacilli for the first time at 7 days age could have had some physiological fitness characteristics that are absent from birds having a first time dose at 1 day of age. A possible explanation for this could be found in the differences in the development of the intestinal immune structures of birds at the time of initial application of the lactobacilli. As stated in the introduction of this chapter, commensal bacteria like the lactobacilli used in this study could play an important role in the intestinal immune response development (Dong *et al.* 1996; Clancy 2003; Koenen *et al.* 2004; Haghighi *et al.* 2005; Nava *et al.* 2005; Donoghue *et al.* 2006; Farnell *et al.* 2006; Haghighi *et al.* 2006). It is also known that the development of the GALT is biphasic and not only is it biphasic, but also the maturation of cellular immune responses in the intestinal milieu is the precondition a prerequisite for the initiation of humoral responses (Bar-Shira *et al.* 2003). On the basis of this information our study had as one of its aims to introduce *L. plantarum* NCIMB 41229 isolated from the digestive tract of pigs with putative probiotic properties (Demeckova 2003) to the digestive tract of chickens at periods that corresponded to the two landmark phases of GALT development of the chicken (day 1 and day 7 of age). This was done to have an indication if application at this time would have an effect on the bird's ability to fight an enteropathogens challenge. Based on our results from the combined effect on *Salmonella* counts for birds receiving lactobacilli from seven days of age, we suggest that one of the factors contributing in improving resistance to *Salmonella* in birds could be related to ripeness of the intestinal immune architecture at the time of initial stimulation by the immune modulating organism. It is possible that the lactobacilli might have also helped in activating the gut mucosal system through stimulation of gut antigen-presenting cells to both promote protection and to switch

regulatory mechanisms (Clancy 2003) better in birds aged 7days treated birds than birds aged 1 day.

Mast and Goddeeris, (1999) working on vaccination of chickens also found that activation of B-cell response resulting in antibody production was not achieved for broiler chickens vaccinated at one day of age as opposed to 7 and 14 days of age. They supported the idea that this was related to the immune function of the late embryonic and neonatal chickens not being well developed due to incomplete structural organisation of their secondary immune organs. Bar-Shira *et al.* (2003) also concluded that the lack of antibody response in young chicks is primarily due to immaturity of T lymphocytes.

However, optical densities (OD) for whole *Salmonella* bacteria antibody response in this study, does not seem to have an age related response to introduction of lactobacilli in feed and water. While there were no significant differences between treatments for the first two sampling times, the control treatment had a higher (P=0.009) OD than the lactobacilli treated birds. Although the OD of the oral gavage treatment was not significantly higher than these treatments, it also had a higher numerical value.

The reason for these results was not clear although it there might have been more response for fermented feeds if intestinal mucosal secretory IgA levels had been measured rather than circulatory plasma levels of antibodies. The explanation for this is that more stimulation of the intestinal mucosal systems in the lactobacilli treatments might have caused the chickens' immune resources to be diverted to the intestinal environment at the detriment of circulatory antibodies. However, this aspect was

beyond the scope of the present study. Perhaps, these results can be explained by the findings of Broom *et al.*, (2006) who found that while *Enterococcus faecium* SF68 did not affect gastrointestinal bacterial populations, it tended to reduce serum IgG, suggesting an immunomodulatory role for this bacterium in weaned piglets. Haghighi *et al.*, (2005) observed that treatment of broiler chickens with probiotics did not enhance serum IgM and IgG responses to bovine serum albumins (BSA). However, immunisation with tetanus toxoid (TT) had a significant effect ($P \leq 0.001$) on the appearance of anti-TT IgA and IgG in the gut. Manipulation of intestinal microbiota by administration of probiotics induced production of natural systemic antibodies in unimmunised chickens (Haghighi *et al.*, 2006). They also observed significantly more IgA antibodies reactive to TT, alpha-toxin and BSA in intestinal contents of probiotic treated chickens than untreated control chickens. Furthermore, Lallès *et al.* (2007) indicated that it is probable that commensal microbiota could protect against pathogens, while directing and determining important functions of the host. They recommended that future research efforts should be geared towards a deeper understanding of these interactions so as to generate an integrated view of gut health determinants and make projections in terms of diet composition and formulation. According to Klasing (2007), understanding the nuances between nutrition and immunity is necessary for optimizing bird health and productivity. He indicated that this understanding will be an important contributor in meeting consumers conflicting demands for more natural production and improved animal welfare.

Changes in gut microflora of the caecal content as determined by RISA analysis in the current study (Figures 6.8-6.10) indicates that fermented feed influenced the caecal microbial structure (Risatypes) in a way that was different from the water and control

treatments. The combined effect of organic acid content and lactobacilli in the fermented feed may explain the difference in caecal microbial community structure in the fermented feed treatments. Other studies have indicated that dietary manipulation changes microbial community structure in chickens (e.g. Poole *et al.* 2004; Wise & Siragusa 2007). Wise & Siragusa, (2007) observed changes in chicken GI tract microbial community structure using the PCR-based 16S rDNA technique as result of withdrawal of AGPs from the diet. They concluded that understanding the quantitative taxonomic shifts of the chicken gut microbiota is relevant in light of the withdrawal of AGPs from animal feeds. However, supplementation of layer diets with maize and barley-pea silages had a minor effect on the composition of intestinal microflora of the hens (Steenfeldt *et al.* 2007). Minor changes in gut microbial composition with supplementation of hens with silages could be because supplements were not enough to steer gut microbial composition and activity compared with feeding fermented feeds.

There was a significant increase in feed consumption (Table 6.4) in the fermented feed treatments. The difference in feed consumption between the fermented feed treatments and other treatments in the current study could not be explained. It is possible that feed wastage with the fermented feeds was higher as compared with the other birds treatments fed dry feeds for the circular tray feeders used in the current study. Forbes (2003) also observed that considerable feed wastage resulting from wet feeding of poultry was due to initial difficulty in designing a trough suited for wet food.

However, the daily weight gains (Table 6.5) for the fermented feed treatments throughout the study were comparable or higher than the weight gain of the other

treatments also suggesting that nutrient utilisation might have been improved by fermentation.

Feed fermentation has also been shown to significantly reduce feed levels of total and soluble dietary fibre in cereal whole meals flours (Skrede *et al.* 2003; Christensen *et al.* 2007). This is important for nutrient utilisation in the gastrointestinal tract as it has been suggested that effects of fermentation on digestibility and faecal characteristics are attributable to the reduction in levels of total and soluble β-glucans in barley and wheat during fermentation (Skrede *et al.* 2002; Skrede *et al.* 2003). Lactic acid fermentation also eliminates indigestible carbohydrates and anti-nutritional factors in feed legume meals such as soybean meals (Refstie *et al.* 2005). Although there could be loss of dry matter resulting from the breakdown of these substrates, Skrede *et al.*,(2001) have argued that the increase in nutritional value as a result of fermentation greatly exceeded the loss of dry matter which they calculated to be a maximum 13.3 g kg^{-1} of fermented whole meal flour.

Although, Skrede *et al.* (2003) found that fermentation of barley and wheat with β-glucan-degrading lactic acid bacteria can improve growth and early feed:gain ratio in broiler chickens, the likely mechanisms of action of the *L. plantarum* used for fermentation in this study may be different from their lactobacillus strain obtained from Norwegian rye. Differences may also be attributed to differences in the diets used and the fact that specific pathogen free birds rather than commercial broilers were used for this study. Perhaps the decarboxylation of free amino acids of the diet by the fermenting microbes might have altered the feed: gain ratio. In fact, some authors have advocated the fermentation of the carbohydrate fraction of the diet before addition of the protein

components prior to feeding (Beal *et al.* 2002; Beal *et al.* 2005; Moran *et al.* 2006; Canibe *et al.* 2007). Furthermore, Canibe *et al.* (2007) indicated that such a strategy, could reduce the time available for the dietary microflora to decarboxylate free amino acids present if complete diets were fermented. They also recommended fermenting the cereal grains as a way of avoiding microbial decarboxylation of free amino acids which could improve palatability and feed intake although this required further investigation.

Chapter 7

7.0 Concluding Discussion

Today, there is a global paradigm shift from emphasis on productive efficiency to one of public security in animal agriculture. Nothing demonstrates this change better than consumer concerns over the use of antibiotic growth promoters in monogastric nutrition. These concerns are justified due to the build-up of antibiotic resistance by many micro-organisms. Fermented feeds enhance the natural defence mechanisms of the animal and can improve gut and host health (Demeckova *et al.* 2002). This has important implications for food safety and the environment since the provision of biosafe methods of food production will reduce the need for costly drugs to fight enteric diseases and drug resistance.

The studies described in the first part of this thesis (Chapters 3, 4 and 5) were designed with the principal aim of examining the dynamics of fermentation of cereals for use in chicken feed. Factors affecting microbial fermentation that have not been researched previously such as water quality on substrate utilisation, particle size and sorghum grain varietal influences on lactic acid fermentation are discussed. Microbial influences on the fermentation products notably simple sugars; organic acids (primarily lactic acid) and feed viscosity are reported.

Data presented in Chapter 3 indicate that the presence of $CaCO_3$ in the culture mixture reduces lactic acid production in cereal fermentation. Cereals used in fermentation were observed to exhibit different degrees of buffering capacity. Barley with a significantly higher lactic acid concentration than maize and sorghum was observed to have a slightly

high pH (3.64) than maize (3.63) and sorghum (3.59). The significance of pH changes or a lower pH is known to influence the lactic acid produced to remain in its undissociated form (Moran 2001; Hansen 2004) as it is this form that is believed to give fermented feeds their antimicrobial properties.

Microbial fermentation does not seem to significantly change the viscosity of fermented cereals used in this study. The importance of this observation lies in the influence of viscosity of the intestinal contents of chickens on nutrient utilisation. Increases in feed and digesta viscosity have been demonstrated to reduce nutrient utilisation (e. g. Smits *et al.* 1997). Digesta viscosity in chickens is correlated with feed form and structure. Several studies have demonstrated that smaller feed particle sizes in chicken increase digesta viscosity and reduce nutrient utilisation (e. g. Yasar 2003).

Chapter 4 compared fermentation of different maize and sorghum grain particle sizes. Although, the results and their significance have been discussed within the chapter, it is important to note that large particle fermentation resulted in comparable and biosafe lactic acid concentrations after 24 hour fermentation of maize and sorghum as small particles. The practical implications of these findings are that;

- using larger particle sizes means potential savings in processing (energy) and feed costs
- several studies have reported improved feed intake, gastric conditions and growth performance for chickens fed coarse diets when compared with fine diets (e. g. Yasar 2003; Mai 2007; Amerah *et al.* 2008).

Results obtained in Chapter 5 demonstrate that fermentation of sorghum varieties is not affected by differences in polyphenol content. However, the red sorghum variety which had higher polyphenol content had a slower initial build-up of lactic acid than white sorghum but after 24 hours lactic acid concentrations in red sorghum were higher than white sorghum. Possible reasons and mechanism underlying these changes are not fully understood as the contents of polyphenols were not monitored during the fermentation process. However, several reports have demonstrated that soaking decreases the content of anti-nutrients (Mitaru *et al.* 1983; Mitaru *et al.* 1984; Brooks *et al.* 2001; Lu *et al.* 2007). It is suggested that differences in polyphenol contents between varieties, should not be ruled out as possible factors initially impeding the rapid fermentation of red sorghum. These factors may not have provoked differences in sugar hydrolysis between varieties as simple sugar accumulation in red sorghum after prolong soaking and/or fermentation may be the reason for a higher lactic acid in red sorghum after 24 hours.

Sorghums have great potential to be used for chicken feeding but may be limited by their content of anti-nutritional factors. It is known that, approximately 600-700 g/kg of plant-Phosphorus is present as phytate (Pirgozliev *et al.* 2008). Furthermore, a negative linear relationship between grain phytate content and weight gain of chicks has been reported (Salarmoini *et al.* 2008). Dietary phytase improves nutrient metabolism and growth performance of broilers (Centeno *et al.* 2007; Pirgozliev *et al.* 2008). Feed fermentation has been shown to improve bioavailability minerals (Brooks *et al.* 2001) by degrading anti-nutrients such as phytic acid (e.g. Skrede *et al.* 2007) and trypsin inhibitor (e.g. Refstie *et al.* 2005), tannins (Mukhodhyay & Ray 1999) and might be used to improve the feeding value of sorghum.

The framework under which these experiments were designed was that not much is known about fermentation characteristics of maize and sorghum for use as fermented feeds compared with barley and wheat (e.g. Skrede *et al.* 2003; Pedersen *et al.* 2005). Results of this study suggest that maize and sorghum can be effectively fermented as components of fermented poultry diets. These results could be important to fermentation of feed in semi-arid tropical regions of the world, where sorghum is produced. This is especially so in Africa where intensification of livestock production is gaining ground at a faster rate.

In Chapter 6, the application of lactic acid bacteria isolated from porcine GI tract as a feed fermenter and in water for chickens was investigated. It was designed with the aim of understanding the relationship between lactic acid bacteria in fermented feeds and in water as methods used in optimising bird health through their ability to resist colonisation by pathogens. It was hypothesized that;

- provision of fermented feeds or LAB in drinking water for chickens at periods corresponding to landmark phases of gut-associated lymphoid tissue development might upregulate immune response.
- using fermented feeds rather than LAB in drinking water may be a better means of improving gut health in chickens.

The results demonstrate that;

- *S.* Typhimurium counts in infected birds was reduced by administration of *L. plantarum* in feed and water.
- providing *L. plantarum* through fermented feed was better through feed than water.
- provision of *L. plantarum* in feed and water on day 7 of age reduced of *Salmonella* shedding better than day 1 of age.

- serum antibody augmentation for whole *Salmonella* bacteria was not observed in LAB treatments when compared with controls.

The significance of these results has been discussed within chapter 6, but it is important to state that the reasons for lower serum responses to *Salmonella* challenge in LAB treatments are not known. However, the diversion of immune resources to the intestinal lamina propria in LAB treated birds cannot be ruled out as a possible explanation for the low circulatory plasma levels of *Salmonella* antibodies. Administration of probiotics to chickens did not enhance serum IgM and IgG responses to bovine serum albumins (Haghighi *et al.* 2005). However, immunisation with tetanus toxoid (TT) had a significant effect ($P \leq 0.001$) on the appearance of anti-TT IgA and IgG in the gut.

Provision of *L. plantarum* for birds at 7 days of age was better because their immune system was more capable of responding to the immunomodulatory effect of the micro-organisms than birds at 1 day of age. As indicated by Mast and Goddeeris, (1999) vaccination of chicks at day-old did not activate the B-cell response resulting in anti-body production. They attributed this to the idea that the structural organisation of secondary immune organs of late embryonic and neonatal chickens are not well developed as opposed to chickens at 7 and 14 days of age, respectively.

The results of this study indicate that *L. plantarum* NCIMB 41229 of porcine origin could be used to improve gut health in chicken. Studies have shown that this bacterial strain can be used as starter cultures for pig feed fermentation and have putative probiotic properties (Demeckova 2003). Four strains of *Lactobacillus fermentum* isolated from poultry and one strain isolated from pigs were also observed to adhere to the epithelial cells from both

swine and chicken (Lin *et al.* 2007). It is possible that the bacterial strain used for birds in the current study could also exert a probiotic effect. *L. plantarum* strains (REB1 and MLBPL1) have been reported to have the ability to adapt to different environmental conditions as demonstrated by their ability to change metabolic pathways according to the media used (Plumed-Ferrer *et al.* 2008). However, this aspect was beyond the scope of this study and further research on this area is recommended.

7.1 Future work

Fermented feed technology could provide general opportunities for enhancing nutritive value and reducing anti-nutrient effects by fermenting these raw materials. This is good for little known tropical cereals and legumes (sorghum, millets, cottonseed meal, rubber seed meal etc) with a wide range of anti-nutrients and little known nutritive value. In most African countries (e.g. Cameroon) heat treatment is used to reduce the effect of anti-nutrients in feeds. In the process other nutrients could be denatured by the heat treatment. Feed fermentation could be a better way of reducing such anti-nutrients without denaturing other nutrients and this worth further investigation.

A better understanding of how the starch structure is related to cereal fermentation is also necessary to permit an informed choice on modifications in feeding and perhaps plant breeding programmes to improve animal feed fermentation.

The influence of the age at which fermented feed or LAB in water or probiotics are first introduced to the birds on gut immune modulation especially relating to landmark phases of the GALT maturation needs to be further investigated.

References

ACHI, O. K. (2005). The potential for upgrading traditional fermented foods through biotechnology. *African Journal of Biotechnology* **4**(5), 375-380.

ACINAS, S. G. ANTON, J. & RODRIGUEZ-VALERA, F. (1999). Diversity of Free-Living and Attached Bacteria in Offshore Western Mediterranean Waters as Depicted by Analysis of Genes Encodibng 16S rRNA. *Applied and Environmental Microbiology* **65**(2), 514-522.

AHMED, S. B. MAHGOUB, S. A. & BABIKER, B. E. (1996). Changes in tannin and cyanide contents and diastic activity during germination and the effect of traditional processing on cyanide content of sorghum cultivars. *Food Chemistry* **56**(2), 159-162.

ALAKOMI, H. L. SKYTTA, E. SAARELA, M. MATTILA-SANDHOLM, T. LATVA-KALA, K. & HELANDER, I. M. (2000). Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Applied and Environmental Microbiology* **66**(5), 2001-2005.

ALVARO, E. ANDRIEUX, C. ROCHER, V. RIGOTTIER-GOIS, L. LEPERCQ, P. SUTREN, M. GALAN, P. DUVAL, Y. JUSTE, C. & DORE, J. (2007). Composition and metabolism of the intestinal microbiota in consumers and non-consumers of yogurt. *British Journal of Nutrition* **97**, 126-133.

AMERAH, A. M. RAVINDRAN, V. LENTLE, R. G. & THOMAS, D. G. (2008). Influence of particle size and xylanase supplementation on the performance, energy utilisation, digestive tract parameters and digesta viscosity of broiler starters. *British Poultry Science* **49**(4), 455-462.

ANGUITA, M. GASA, J. MARTIN-ORUE, S. M. & PEREZ, J. F. (2006). Study of the effect of technological processes on starch hydrolysis, non-starch polysaccharides solubilization and physicochemical properties of different ingredients using a two-step in vitro system. *Animal Feed Science and Technology* **129**(1-2), 99-115.

ARMSTRONG, E. F. EASTWOOD, M. A. EDWARDS, C. A. & BRYDON, W. G. (1992). The effect of weaning diet on the subsequent colonic metabolism of dietary fibre in the adult rat. *British Journal of Nutrition* **68**, 741-751.

BACH KNUDSEN, K. E. (2001). The nutritional significance of "dietary fibre" analysis. *Animal Feed Science and Technology* **90**(1-2), 3-20.

BALOGUN, R. O. ROWE, J. B. & BIRD, S. H. (2005). Fermentability and degradability of sorghum grain following soaking, aerobic or anaerobic treatment. *Animal Feed Science and Technology* **120**(1-2), 141-150.

BAR-SHIRA, E. SKLAN, D. & FRIEDMAN, A. (2003). Establishment of immune competence in the avian GALT during the immediate post-hatch period. *Developmental & Comparative Immunology* **27**(2), 147-157.

BARBER, J. BROOKS, P. H. & CARPENTER, J. L. (1991). The effects of water to food ratio on digestibility, digestible energy and nitrogen retention of a grower ration *British Society of Animal Production* **52**, 601-615.

BARNES, E. M. IMPEY, C. S. & COOPER, D. M. (1980). Manipulation of the crop and intestinal flora of the newly hatched chick. *American Journal of Clinical Nutrition* **33**, S2426-S2433.

BEAL, J. D. NIVEN, S. J. BROOKS, P. H. & GILL, B. P. (2005). Variation in short chain fatty acid and ethanol concentration resulting from the natural fermentation of wheat and barley for inclusion in liquid diets for pigs. *Journal of the Science of Food and Agriculture* **85**(3), 433-440.

BEAL, J. D. NIVEN, S. J. CAMPBELL, A. & BROOKS, P. H. (2002). The effect of temperature on the growth and persistence of Salmonella in fermented liquid pig feed. *International Journal of Food Microbiology* **79**(1-2), 99-104.

BEAL, J. D. NIVEN, S. J. CAMPBELL, A. & BROOKS, P. H. (2003). The effect of copper on death rate of *Salmonella typhimurium* DT104:30 in food substrates acidified with organic acids. *Letters in Applied Microbiology* **38**, 8-12.

BEAL, R. K. POWERS, C. DAVISON, T. F. & SMITH, A. L. (2006). Immunological Development of the avian gut. In *Avian Gut Function in Health and Disease* (Ed G. C. Perry), pp. 85-103. Wallingford, U. K.: CAB International.

BEAL, R. K. WIGLEY, P. POWERS, C. HULME, S. D. BARROW, P. A. & SMITH, A. L. (2004). Age at primary infection with *Salmonella enterica* serovar Typhimurium in the chicken influences persistence of infection and subsequent immunity to re-challenge. *Veterinary Immunology and Immunopathology* **100**(3-4), 151-164.

BEDFORD, M. R. & CLASSEN, H. L. (1993). An *in vitro* assay for prediction of broiler intestinal viscosity and growth when fed rye-based diets in the presence of exogenous enzymes. *Poultry Science* **72**, 137-143.

BERTSCH, A. ALVAREZ, R. & COELLO, N. (2003). Nutritional evaluation of *Kocuria rosea* fermented feather meal as an alternative protein source in poultry feed. *Revista Cientifica-Facultad De Ciencias Veterinarias* **13**(2), 139-145.

BERTSCH, A. & COELLO, N. (2005). A biotechnological process for treatment and recycling poultry feathers as a feed ingredient. *Bioresource Technology* **96**(15), 1703-1708.

BHATT, S. M. & SRIVASTAVA, S. K. (2008). Lactic Acid Production from Cane Molasses by *Lactobacillus delbrueckii* NCIM 2025 in Submerged Condition: Optimization of Medium Component by Taguchi DOE Methodology. *Food Biotechnology* **22**(2), 115-139.

BOESEN, H. T. JENSEN, T. K. SCHMIDT, A. S. JENSEN, B. B. JENSEN, S. M. & MOLLER, K. (2004). The influence of diet on *Lawsonia intracellularis* colonization in pigs upon experimental challenge. *Veterinary Microbiology* **103**(1-2), 35-45.

BORNEMAN, J. & TRIPLETT, E. W. (1997). Molecular microbial diversity in soils from eastern Amazonia: Evidence for unusual microorganisms and microbial population shifts associated with deforestation. *Applied and Environmental Microbiology* **63**(7), 2647-2653.

BROOKS, P. H. (2008). Fermented Liquid Feed for Pigs. In *CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources* pp. 1-18. http://www.cababstractsplus.org/cabreviews/.

BROOKS, P. H. BEAL, J. D. & NIVEN, S. J. (2003a). Liquid feeding of pigs I. Potential for reducing environmental impact and improving productivity. In *Review paper presented at the coneference on Effect of genetic and non-genetic factors on carcass and meat quality of pigs* pp. 7-22
Siedlce, Poland.

BROOKS, P. H. BEAL, J. D. NIVEN, S. J. & CAMPBELL, A. (2002). Fermented Liquid Feed for Pigs: Potential for Improving Productivity and Environmental Impact. In *DEFRA Project Number LS0812*
p. 178. Plymouth: University of Plymouth.

BROOKS, P. H. BEAL, J. D. NIVEN, S. J. & DEMECKOVA, V. (2003b). Liquid feeding of pigs: II Potential for improving pig health and food safety. In *Review paper presented at the coneference on Effect of genetic and non-genetic factors on carcass and meat quality of pigs.* pp. 23-40. Siedlce, Poland.

BROOKS, P. H. GEARY, T. M. MORGAN, D. T. & CAMPBELL, A. (1996). New developments in liquid feeding. *Pig Veterinary Journal* **36** 43-64.

BROOKS, P. H. MORAN, C. A. BEAL, J. D. DEMECKOVA, V. & CAMPBELL, A. (2001). Liquid feeding for young piglet. In *The Weaner Pig, Nutrition and Management* Eds M. A. Varley & J. Wiseman), pp. 153-178. Oxon, UK: CABI Publishing.

BROOM, L. J. MILLER, H. M. KERR, K. G. & KNAPP, J. S. (2006). Effects of zinc oxide and Enterococcus faecium SF68 dietary supplementation on the performance, intestinal microbiota and immune status of weaned piglets. *Research in Veterinary Science* **80**(1), 45-54.

BUTLER, L. G. RIEDL, D. J. LEBRYK, D. G. & BLYTT, H. J. (1984). Interaction of proteins with sorghum tannin: Mechanism, specificity and significance. *Journal of American Oil Chemists' Society* **61**(5), 916-920.

BVOCHORA, J. M. REED, J. D. READ, J. S. & ZVAUYA, R. (1999). Effect of fermentation processes on proanthocyanidins in sorghum during preparation of Mahewu, a non-alcoholic beverage. *Process Biochemistry* **35**(1-2), 21-25.

CANIBE, N. HOJBERG, O. BADSBERG, J. H. & JENSEN, B. B. (2007a). Effect of feeding fermented liquid feed and fermented grain on gastrointestinal ecology and growth performance in piglets. *Journal of Animal Science* **85**(11), 2959-2971.

CANIBE, N. HOJBERG, O. HOJSGAARD, S. & JENSEN, B. B. (2005). Feed physical form and formic acid addition to the feed affect the gastrointestinal ecology and growth performance of growing pigs. *Journal of Animal Science* **83**(6), 1287-1302.

CANIBE, N. MIETTINEN, H. & JENSEN, B. B. (2008). Effect of adding *Lactobacillus plantarum* or a formic acid containing-product to fermented liquid feed on gastrointestinal ecology and growth performance of piglets. *Livestock Science* **114**(2-3), 251-262.

CANIBE, N. VIRTANEN, E. & JENSEN, B. B. (2007b). Effect of acid addition to pig liquid feed on its microbial and nutritional characteristics. *Livestock Science* **108**(1-3), 202-205.

CANIBE, N. VIRTANEN, E. & JENSEN, B. B. (2007c). Microbial and nutritional characteristics of pig liquid feed during fermentation. *Animal Feed Science and Technology* **134**(1-2), 108-123.

CARLSON, D. & POULSEN, H. D. (2003). Phytate degradation in soaked and fermented liquid feed--effect of diet, time of soaking, heat treatment, phytase activity, pH and temperature. *Animal Feed Science and Technology* **103**(1-4), 141-154.

CENTENO, C. ARIJA, I. VIVEROS, A. & BRENES, A. (2007). Effects of citric acid and microbial phytase on amino acid digestibility in broiler chickens. *British Poultry Science* **48**(4), 469-479.

CHARALAMPOPOULOS, D. PANDEIELLA, S. S. & WEBB, C. (2002). Growth studies of potentially probiotic lactic acid bacteria in cereal-based substrates. *Journal of Applied Microbiology* **92**, 851-859.

CHAVAN, U. D. SHAHIDI, F. & NACZK, M. (2001). Extraction of condensed tannins from beach pea (*Lathyrus maritimus* L.) as affected by different solvents. *Food Chemistry* **75**(4), 509-512.

CHOCT, M. SELBY, E. A. D. CADOGAN, D. J. & CAMPBELL, R. G. (2004a). Effect of liquid to feed ratio, steeping time, and enzyme supplementation on the performance of weaner pigs. *Australian Journal of Agricultural Research* **55**(2), 247-252.

CHOCT, M. SELBY, E. A. D. CADOGAN, D. J. & CAMPBELL, R. G. (2004b). Effects of particle size, processing, and dry or liquid feeding on performance of piglets. *Australian Journal of Agricultural Research* **55**(2), 237-245.

CHRISTENSEN, P. GLITSØ, V. PETTERSSON, D. & WISCHMANN, B. (2007). Fibre degrading enzymes and *Lactobacillus plantarum* influence liquid feed

characteristics and the solubility of fibre components and dry matter *in vitro*. *Livestock Science* **109**(1-3), 100-103.

CLANCY, R. (2003). Immunobiotics and the probiotic evolution. *Fems Immunology and Medical Microbiology* **38**(1), 9-12.

CLARKE, K. R. & WARWICK, R. M. (2001). *Change in Marine Communities: An approach to Statistical Analysis and Interpretation*. Plymouth, U.K.: PRIMER-E Ltd.

COLLINS, J. COLDHAM, N. RASTALL, R. GIBSON, G. WOODWARD, M. & LA RAGIONE, R. M. (2008). Subversion of *Salmonella* Typhimurium carbon utilization by probiotic bacteria. In *4th Annual Scientific Meeting of the Med-Vet-Net project* p. 52. St Malo, France.

COLLINS, M. D. & GIBSON, G. R. (1999). Probiotics, prebiotics, and synbiotics: approaches for modulating the microbial ecology of the gut. *American Journal of Clinical Nutrition* **69**, S1052-S1057.

CRITTENDEN, R. G. & PLAYNE, M. J. (1996). Production, properties and applications of food-grade oligosaccharides. *Trends in Food Science & Technology* **7**(11), 353-361.

CUMMINGS, J. H. (1983). Fermentation in the human large intestine: evidence and implications for health. *Lancet* **i**, 1206-1209.

DAHIYA, J. P. WILKIE, D. C. VAN KESSEL, A. G. & DREW, M. D. (2006). Potential strategies for controlling necrotic enteritis in broiler chickens in post-antibiotic era. *Animal Feed Science and Technology* **129**(1-2), 60-88.

DALLOUL, R. A. & LILLEHOJ, H. S. (2006). Poultry coccidiosis: recent advancements in control measures and vaccine development. *Vaccines* **5**(1), 143-163.

DALLOUL, R. A. LILLEHOJ, H. S. SHELLEM, T. A. & DOERR, J. A. (2003). Enhanced mucosal immunity against *Eimeria acervulina* in broilers fed a Lactobacillus-based probiotic. *Poultry Science* **82**(1), 62-66.

DE LOS SANTOS, F. S. DONOGHUE, A. M. VENKITANARAYANAN, K. DIRAIN, M. L. REYES-HERRERA, I. BLORE, P. J. & DONOGHUE, D. J. (2008). Caprylic acid supplemented in feed reduces enteric *Campylobacter jejuni* colonization in ten-day-old broiler chickens. *Poultry Science* **87**(4), 800-804.

DE OLIVEIRA, S. G. BERCHIELLI, T. T. PEDREIRA, M. D. S. PRIMAVESI, O. FRIGHETTO, R. & LIMA, M. A. (2007). Effect of tannin levels in sorghum silage and concentrate supplementation on apparent digestibility and methane emission in beef cattle. *Animal Feed Science and Technology* **135**(3-4), 236-248.

DEMECKOVA, V. (2003). *Benefits of fermented liquid diets for sows, and their piglets*. PhD, University of Plymouth.

DEMECKOVA, V. KELLY, D. COUTTS, A. G. P. BROOKS, P. H. & CAMPBELL, A. (2002). The effect of fermented liquid feeding on the faecal microbiology and colostrum quality of farrowing sows. *International Journal of Food Microbiology* **79**(1-2), 85-97.

DIBNER, J. J. & RICHARDS, J. D. (2005). Antibiotic Growth Promoters in Agriculture: History and Mode of Action. *Poultry Science* **84**, 634-643.

DONG, G. ZHOU, A. YANG, F. CHEN, K. WANG, K. & DAO, D. (1996). Effect of dietary protein levels on bacterial breakdown of protein in the large intestine, and diarrhoea in early piglets. *Acta Veterinaria et Zootechnica Sinica* **27**, 293-302.

DONOGHUE, A. M. FARNELL, M. B. COLE, K. & DONOGHUE, D. J. (2006). Mechanisms of pathogen control in the avian gastrointestinal tract. In *Avian Gut Function in health and Disease* (Ed G. C. Perry), pp. 138-155. Wallingford, U.K.: CABI International.

DOYLE, E. M. (2001). Alternatives to antibiotic use for growth promotion in animal husbandry. In *Food Research Institue Report funded by National Pork Producers Council* p. 15. Wisconsin-Madison, USA: University of Wisconsin-Madison.

DUNKLEY, K. D. CALLAWAY, T. R. CHALOVA, V. I. ANDERSON, R. C. KUNDINGER, M. M. DUNKLEY, C. S. NISBET, D. J. & RICKE, S. C. (2008). Growth and genetic responses of *Salmonella* Typhimurium to pH-shifts in an anaerobic continuous culture. *Anaerobe* **14**(1), 35-42.

ELKHALIFA, A. E. O. & EL-TINAY, A. H. (2002). Effect of cysteine on bakery products from wheat-sorghum blends. *Food Chemistry* **77**(2), 133-137.

ENGELEN, A. J. VAN DER HEEFT, F. C. RANDSDORP, P. H. G. & SMIT, E. L. C. (1994). Simple and rapid determination of phytase activity. *Journal of the Association of Official Analytical Chemists* **77**, 760-764.

EWING, W. N. & COLE, D. J. A. (1994). *The Living Gut: An introduction to Micro-organisms in Nutrition.* Ireland Context.: Dungannon.

FARNELL, M. B. DONOGHUE, A. M. SANTOS, F. S. L. BLORE, P. J. HARGIS, B. M. TELLEZ, G. & DONOGHUE, D. J. (2006). Upregulation of oxidative burst and degranulation in chicken heterophils stimulated with probiotic bacteria. *Poultry Science* **85**(11), 1900-1906.

FASANO, A. & SHEA-DONOHOUE, T. (2005). Mechanisms of Disease: the role of intestinal barrier function in the pathogenesis of gastrointestinal autoimmune diseases. *Nature Clinical Practice,Gastroenterology & Hepatology* **2**(9), 416-422.

FERNANDEZ, F. HINTON, M. & VAN GILS, B. (2000a). Evaluation of the effect of mannan-oligosaccharides on the competitive exclusion of *Salmonella* Enteritidis colonization in broiler chicks. *Avian Pathology* **29**(6), 575-581.

FERNANDEZ, F. HINTON, M. & VAN GILS, B. (2002). Dietary mannan-oligosaccharides and their effect on chicken caecal microflora in relation to *Salmonella* Enteritidis colonization. *Avian Pathology* **31**(1), 49-58.

FERNANDEZ, F. SHARMA, R. HINTON, M. & BEDFORD, M. R. (2000b). Diet influences the colonisation of Campylobacter jejuni and distribution of mucin carbohydrates in the chick intestinal tract. *Cellular and Molecular Life Sciences* **57**(12), 1793-1801.

FOMBANG, E. N. TAYLOR, J. R. N. MBOFUNG, C. M. F. & MINNAAR, A. (2005). Use of [gamma]-irradiation to alleviate the poor protein digestibility of sorghum porridge. *Food Chemistry* **91**(4), 695-703.

FORBES, J. M. (2003). Wet foods for poultry. *Avian and Poultry Biology Reviews* **14**(4), 175-193.

FUKUNAGA, T. M. SASAKI, Y. ARAKI, T. OKAMOTO, T. YASUOKA, T. TSUJIKAWA FUJIYAMA, Y. & BAMBA, T. (2003). Effects of the soluble fibre pectin on intestinal cell proliferation, fecal short chain fatty acid production and microbial population. *Digestion* **67**, 42-49.

FULLER, R. (1989). A review: probiotics in man and animals. *Journal of Applied Bacteriology* **66**, 365-378.

GÄBEL, G. (1995). Transport of short-chain fatty acids in the ruminant forestomach. In *Physiological and clinical Aspects of Short-Chain Fatty Acids.* Eds J. H. Cummings, J. L. Rombeau & T. Sakata), pp. 133-147. Cambridge: Cambridge University Press.

GARCIA, M. LAZARO, R. LATORRE, M. A. GRACIA, M. I. & MATEOS, G. G. (2008). Influence of enzyme supplementation and heat processing of barley on digestive traits and productive performance of broilers. *Poultry Science* **87**(5), 940-948.

GEARY, T. M. BROOKS, P. H. MORGAN, D. T. CAMPBELL, A. & RUSSELL, P. J. (1996). Performance of weaner pigs fed *ad libitum* with liquid feed at different dry matter concentrations. *Journal of the Science of Food and Agriculture* **72**(1), 17-24.

GIBSON, G. R. (1998). Dietary modulation of the human gut microflora using prebiotics. *British Journal of Nutrition* **80**, S209-S212.

GIDENNE, T. & LICOIS, D. (2005). Effect of high fibre intake on resistance of the growing rabbit to an experimental inoculation with an enteropathogenic strain of *Escherichia coli*. *Animal Science* **80**, 281-288.

GIDENNE, T. MIRABITO, L. JEHL, N. PEREZ, J. M. ARVEUX, P. BOURDILLON, A. BRIENS, C. & DURPERRAY, J. (2004). Impact of replacing starch by digestible fibre, at two levels of lignocellulose, on digestion, growth and digestive health of the rabbit. *Animal Science* **78**, 389-398.

GIDENNE, T. PINHEIRO, V. & FALCAO E CUNHA, L. (2000). A comprehensive approach of the rabbit digestion: consequences of a reduction in dietary fibre supply. *Livestock Production Science* **64**(2-3), 225-237.

GILL, H. S. & RUTHERFURD, K. J. (2001). Viability and dose-response studies on the effects of the immunoenhancing lactic acid bacterium *Lactobacillus rhamnosus* in mice. *British Journal of Nutrition* **86**(2), 285-289.

GRACIA, M. I. FLORES, A. MCCARTNEY, E. CORTES, M. & SANCHEZ, J. (2004). Efficacy of enzyme supplementation in laying hens fed diets based mainly on barley. *Poultry Science* **83**, 317-317.

HÄGGMAN, M. & SALOVAARA, H. (2008). Microbial re-inoculation reveals differences in the leavening power of sourdough yeast strains. *LWT - Food Science and Technology* **41**(1), 148-154.

HAGHIGHI, H. R. GONG, J. GYLES, C. L. HAYES, M. A. SANEI, B. PARVIZI, P. GISAVI, H. CHAMBERS, J. R. & SHARIF, S. (2005). Modulation of Antibody-Mediated Immune Response by Probiotics in Chickens. *Clinical and Diagnostic Laboratory Immunology* **12**(12), 1387-1392.

HAGHIGHI, H. R. GONG, J. H. GYLES, C. L. HAYES, M. A. ZHOU, H. J. SANEI, B. CHAMBERS, J. R. & SHARIF, S. (2006). Probiotics stimulate production of natural antibodies in chickens. *Clinical and Vaccine Immunology* **13**(9), 975-980.

HAMPSON, D. J. PHILLIPS, N. D. & PLUSKE, J. R. (2002). Dietary enzyme and zinc bacitracin reduce colonisation of layer hens by the intestinal spirochaete *Brachyspira intermedia*. *Veterinary Microbiology* **86**(4), 351-360.

HANDOYO, T. & MORITA, N. (2006). Structural and Functional Properties of Fermented Soybean (Tempeh) by Using *Rhizopus oligosporus*. *International Journal of Food Properties* **9**(2), 347-355.

HANSEN, C. F. (2004). *Choice of dry feed influences gastric conditions, incidence of Salmonella and performance in growing-finishing pigs* PhD Dissertation, The Royal Veterinary an Agricultural University.

HANSEN, L. L. MIKKELSEN, L. L. AGERHEM, H. LAUE, A. JENSEN, M. T. & JENSEN, B. B. (2000). Effect of fermented liquid food and zinc bacitracin on microbial metabolism in the gut and sensoric profile of *m. longissimus dorsi* from entire male and female pigs. *Animal Science* **71**, 65-80.

HARADA, E. NIIYAMA, M. & SYUTO, B. (1986). Comparison of pancreatic exocrine secretion via endogenous secretion by intestinal infusion of hydrochloric acid in anesthesized piglets. *Japanese Journal of Physiology* **36**, 843-858.

HARVEY, R. B. ANDERSON, R. C. GENOVESE, K. J. CALLAWAY, T. R. & NISBET, D. J. (2005). Use of competitive exclusion to control enterotoxigenic strains of *Escherichia coli* in weaned pigs. *Journal of Animal Science* **83**(13_suppl), E44-47.

HEDEMANN, M. S. MIKKELSEN, L. L. NAUGHTON, P. J. & JENSEN, B. B. (2005). Effect of feed particle size and feed processing on morphological characteristics in the small and large intestine of pigs and on adhesion of *Salmonella enterica* serovar Typhimurium DT12 in the ileum *in vitro*. *Journal of Animal Science* **83**(7), 1554-1562.

HENEGHAN, J. B. (1988). Alimentary tract physiology: interactions between the host and its microbial flora. In *Role of the Gut Flora in Toxicity and Cancer* (Ed I. R. Rowland), pp. 39-78. London: Academic Press.

HENTGES, D. J. (1992). Gut flora and disease resistance. In *Probiotics:The Scientific Basis* (Ed R. Fuller), pp. 51-67. London: Chapman and Hall.

HERES, L. ENGEL, B. URLINGS, H. A. P. WAGENAAR, J. A. & VAN KNAPEN, F. (2004). Effect of acidified feed on susceptibility of broiler chickens to intestinal infection by *Campylobacter* and *Salmonella*. *Veterinary Microbiology* **99**(3-4), 259-267.

HERES, L. ENGEL, B. VAN KNAPEN, F. DE JONG, F. WAGENAAR, J. A. & URLINGS, H. A. P. (2003a). Fermented liquid feed reduces susceptibility of broilers for *Salmonalla enteritidis*. *Poultry Science* **82**, 603-611.

HERES, L. ENGEL, B. VAN KNAPEN, F. WAGENAAR, J. A. & URLINGS, B. A. P. (2003b). Effect of fermented feed on the susceptibility for *Campylobacter jejuni* colonisation in broiler chickens with and without concurrent inoculation of *Salmonella enteritidis*. *International Journal of Food Microbiology* **87**(1-2), 75-86.

HERES, L. URLINGS, H. A. P. WAGENAAR, J. A. & DE JONG, M. C. M. (2003c). Transmission of *Salmonella* between broiler chickens fed with fermented liquid feed. *Epidemiology and Infection* **132**, 107-116.

HERES, L. WAGENAAR, J. A. VAN KNAPEN, F. & URLINGS, B. A. P. (2003d). Passage of *Salmonella* through the crop and gizzard of broiler chickens fed with fermented liquid feed. *Avian Pathology* **32**, 173-181.

HINTON, M. & LINTON, A. H. (1988). Control of *Salmonella* infections in broiler chickens by the acid treatment of their feed. *The Veterinary Record* **123**, 416-421.

HOGBERG, A. & LINDBERG, J. E. (2004). Influence of cereal non-starch polysaccharides and enzyme supplementation on digestion site and gut environment in weaned piglets. *Animal Feed Science and Technology* **116**(1-2), 113-128.

HOJBERG, O. CANIBE, N. KNUDSEN, B. & JENSEN, B. B. (2003). Potential Rates of Fermentation in Digesta from the Gastrointestinal Tract of Pigs: Effect of Feeding Fermented Liquid Feed. *Applied and Environmental Microbiology* **69**(1), 408-418.

HUANG, K. H. RAVINDRAN, V. LI, X. & BRYDEN, W. L. (2005). Influence of age on the apparent ileal amino acid digestibility of feed ingredients for broiler chickens. *British Poultry Science* **46**(2), 236-245.

HUME, M. E. CORRIER, D. E. AMBRUS, S. HINTON, J. A. & DELOACH, J. R. (1993a). Effectiveness of dietary propionic acid in controlling *Salmonella typhimurium* colonisation in broiler chicks. *Avian Diseases* **37**, 1051-1056.

HUME, M. E. CORRIER, D. E. IVIE, G. W. & DELOACH, J. R. (1993b). Metabolism of [14C] propionic acid in broiler chickens. *Poultry Science* **72**, 786-793.

HUMPHREY, T. J. & LANNING, D. G. (1988). The vertical transmission of salmonellas and formic acid treatment of chicken feed: A possible strategy for control. *Epidemiology and Infection* **100**, 43-49.

HURST, D. (2002). *The influence of liquid feeding on gastrointestinal adaptation, growth and performance in the growing pig*. PhD thesis, University of London.

HURST, D. LEAN, I. J. & HALL, A. D. (2000a). The effects of liquid feed on the small intestine mucosa and performance of finishing pigs at different water to feed ratios. In *Proceedings of the British Society of Animal Science* p. 161. Scarborough: British society of Animal Science.

HURST, D. LEAN, I. J. & HALL, A. D. (2000b). The effects of liquid feed on the small intestine mucosa and performance of piglets at 28 days postweaning. In *Proceedings of the British Society of Animal Science* p. 162. Scaborough: British Society of Animal Science.

IYAYI, E. A. KLUTH, H. & RODEHUTSCORD, M. (2007). Effect of heat treatment on antinutrients and precaecal crude protein digestibility in broilers of four tropical crop seeds. *International Journal of Food Science and Technology* **43**(4), 610-616.

JOZEFIAK, D. RUTKOWSKI, A. & MARTIN, S. A. (2004). Carbohydrate fermentation in the avian ceca: a review. *Animal Feed Science and Technology* **113**(1-4), 1-15.

KHAKSEFIDI, A. & RAHIMI, S. (2005). Effect of probiotic inclusion in the diet of broiler chickens on performance, feed efficiency and carcass quality. *Asian-Australasian Journal of Animal Sciences* **18**, 1153-1156.

KLASING, K. C. (2007). Nutrition and the immune system. *British Poultry Science* **48**(5), 525-537.

KLIPPER, E. SKLAN, D. & FRIEDMAN, A. (2004). Maternal antibodies block induction of oral tolerance in newly hatched chicks. *Vaccine* **22**(3-4), 493-502.

KNARREBORG, A. MIQUEL, N. GRANLI, T. & JENSEN, B. B. (2002). Establishment and application of an *in vitro* methodology to study the effects of organic

acids on coliform and lactic acid bacteria in the proximal part of the gastrointestinal tract of piglets. *Animal Feed Science and Technology* **99**(1-4), 131-140.

KOBASHI, Ŷ. OHMORI, H. TAJIMA, K. KAWASHIMA, T. & UCHIYAMA, H. (2008). Reduction of chlortetracycline-resistant *Escherichia coli* in weaned piglets fed fermented liquid feed. *Anaerobe* **14**(4), 201-204.

KOCK, J. L. F. GROENEWALD, E. G. KRUGER, G. H. J. ELOFF, J. N. & LATEGAN, P. M. (1985). Extraction of polyphenols and hydrolysis of birdproof sorghum starch. *Journal of the Science of Food and Agriculture* **36**(11), 1140-1144.

KOENEN, M. E. KRAMER, J. VAN DER HULST, R. HERES, L. JEURISSEN, S. H. M. & BOERSMA, W. J. A. (2004a). Immunomodulation by probiotic lactobacilli in layer- and meat-type chickens. *British Poultry Science* **45**(3), 355-366.

KOENEN, M. E. VAN DER HULST, R. LEERING, M. JEURISSEN, S. H. M. & BOERSMA, W. J. A. (2004b). Development and validation of a new *in vitro* assay for selection of probiotic bacteria that express immune-stimulating properties in chickens *in vivo*. *Fems Immunology and Medical Microbiology* **40**(2), 119-127.

KOGUT, M. H. GENOVESE, K. J. HE, H. LI, M. A. & JIANG, Y. W. (2007). The effects of the BT/TAMUS 2032 cationic peptides on innate immunity and susceptibility of young chickens to extraintestinal *Salmonella enterica* serovar Enteritidis infection. *International Immunopharmacology* **7**(7), 912-919.

KONSTANTINOV, S. R. AWATI, A. SMIDT, H. WILLIAMS, B. A. AKKERMANS, A. D. L. & DE VOS, W. M. (2004). Specific Response of a Novel and Abundant Lactobacillus amylovorus-Like Phylotype to Dietary Prebiotics in the Guts of Weaning Piglets. *Applied and Environmental Microbiology* **70**(7), 3821-3830.

LA RAGIONE, R. M. CASULA, G. CUTTING, S. M. & WOODWARD, M. J. (2001). *Bacillus subtilis* spores competitively exclude *Escherichia coli* O78:K80 in poultry. *Veterinary Microbiology* **79**(2), 133-142.

LA RAGIONE, R. M. NARBAD, A. GASSON, M. J. & WOODWARD, M. J. (2004). *In vivo* characterisation of *Lactobacillus johnsonii* FI 9785 for use as adefined competitive exclusion agent against bacterail pathogens in poultry. *Letters in Applied Microbiology* **38**, 197-205.

LA RAGIONE, R. M. SAYERS, A. R. & WOODWARD, M. J. (2000). The role of flagella and fimbriae in the colonisation, invasion and persistence of *E. coli* 078:K80 in the day-old chick model. *Epidemiology and Infection* **124**, 351-363.

LA RAGIONE, R. M. & WOODWARD, M. J. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype Enteritidis and *Clostridium perfringens* in young chickens. *Veterinary Microbiology* **94**(3), 245-256.

LALLÈS, J.-P. BOSI, P. SMIDT, H. & STOKES, C. R. (2007). Weaning -A challenge to gut physiologists. *Livestock Science* **108**(1-3), 82-93.

LAN, Y. VERSTEGEN, M. W. A. TAMMINGA, S. & WILLIAMS, B. A. (2005a). The role of the commensal gut microbial community in broiler chickens. *World's Poultry Science Journal* **61**, 95-104.

LAN, Y. WILLIAMS, B. A. TAMMINGA, S. BOER, H. AKKERMANS, A. ERDI, G. & VERSTEGEN, M. W. A. (2005b). *In vitro* fermentation kinetics of some non-digestible carbohydrates by the caecal microbial community of broilers. *Animal Feed Science and Technology* **123-124**(Part 2), 687-702.

LAZARO, R. LATORRE, M. A. MEDEL, P. GRACIA, M. & MATEOS, G. G. (2004). Feeding regimen and enzyme supplementation to rye-based diets for broilers. *Poultry Science* **83**(2), 152-160.

LE BLAY, G. BLOTTIERE, H. M. FERRIER, L. LE FOLI, E. C. BONNET, J. P. GALMICHE & CHERBUT, C. (2000). Short-chain fatty acids induce cytoskeletal and extracellular protein modifications associated with modulation of proliferation on primary culture of rat intestinal smooth muscle cells. *Digestive Disease Science* **45**, 1623-1630.

LEE, S. LILLEHOJ, H. S. PARK, D. W. HONG, Y. H. & LIN, J. J. (2007a). Effects of *Pediococcus*- and *Saccharomyces*-based probiotic (MitoMax®) on coccidiosis in broiler chickens. *Comparative Immunology, Microbiology and Infectious Diseases* **30**(4), 261-268.

LEE, S. H. LILLEHOJ, H. PARK, D. W. DALLOUL, R. HONG, Y. H. & LIN, J. (2006). Influence of pediococcus-based probiotic on coccidiosis in broiler chickens. *Poultry Science* **85**, 122-123.

LEE, S. H. LILLEHOJ, H. S. DALLOUL, R. A. PARK, D. W. HONG, Y. H. & LIN, J. J. (2007b). Influence of Pediococcus-based probiotic on coccidiosis in broiler chickens. *Poultry Science* **86**(1), 63-66.

LEHTO, E. M. & SALMINEN, S. J. (1997). Inhibition of *Salmonella typhimurium* adhesion to Caco-2 cell cultures by *Lactobacillus* strain GG spent culture supernate: only a pH effect? *Fems Immunology and Medical Microbiology* **18**(2), 125-132.

LIN, W.-H. YU, B. JANG, S.-H. & TSEN, H.-Y. (2007). Different probiotic properties for *Lactobacillus fermentum* strains isolated from swine and poultry. *Anaerobe* **13**(3-4), 107-113.

LO FO WONG, D. M. A. HALD, T. VAN DER WOLF, P. J. & SWANENBURG, M. (2002). Epidemiology and control measures for *Salmonella* in pigs and pork. *Livestock Production Science* **76**(3), 215-222.

LOWRY, V. K. FARNELL, M. B. FERRO, P. J. SWAGGERTY, C. L. BAHL, A. & KOGUT, M. H. (2005). Purified [beta]-glucan as an abiotic feed additive up-regulates the innate immune response in immature chickens against *Salmonella enterica* serovar Enteritidis. *International Journal of Food Microbiology* **98**(3), 309-318.

LU, J. ZHAO, H. CHEN, J. FAN, W. DONG, J. KONG, W. SUN, J. CAO, Y. & CAI, G. (2007). Evolution of phenolic compounds and antioxidant activity during malting. *Journal of Agricultural and Food Chemistry* **55**(26), 10994-11001.

LYBERG, K. OLSTORPE, M. PASSOTH, V. SCHNÜRER, J. & LINDBERG, J. E. (2008). Biochemical and microbiological properties of a cereal mix fermented with whey, wet wheat distillers' grain or water at different temperatures. *Animal Feed Science and Technology* **144**(1-2), 137-148.

LYBERG, K. SIMONSSON, A. & LINDBERG, J. E. (2005). Influence of phosphorus level and soaking of food on phosphorus availability and performance in growing-finishing pigs. *Animal Science* **81**, 375-381.

MAHGOUB, S. E. O. & ELHAG, S. A. (1998). Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. *Food Chemistry* **61**(1-2), 77-80.

MAI, A. K. (2007). *Wet and Coarse diets in broiler nutrition: Development of the GI tract and performance*. PhD Thesis, Wageningen University and Research Centre.

MAKKAR, H. P. S. (2003). Effect and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. . *Small Ruminant Research* **49**, 241-256.

MAROUNEK, M. SUCHORSKA, O. & SAVKA, O. (1999). Effect of substrate and feed antibiotics on in vitro production of volatile fatty acids and methane in caecal contents of chickens. *Animal Feed Science and Technology* **80**(3-4), 223-230.

MAST, J. & GODDEERIS, B. M. (1999). Development of immunocompetence of broiler chickens. *Veterinary Immunology and Immunopathology* **70**, 245-256.

MAY, T. MACKIE, R. I. FAHEY, G. C. CREMIN, J. C. & GARLEB, K. A. (1994). Effect of fiber source on short-chain fatty acid production and on the growth and toxin production by *Clostridium difficile*. *Scandinavian Journal of Gastroenterology* **29**, 916-922.

MCCREA, B. A. TONOOKA, K. H. VANWORTH, C. BOGGS, C. L. ATWILL, E. R. & SCHRADER, J. S. (2006). Prevalence of *Campylobacter* and *Salmonella* species on Farm, after Transport and at processing in specialty market poultry. *Poultry Science* **85**, 136-143.

MCHAN, F. & SHOTTS, E. B. (1992). Effect of feeding selected short-chain fatty acids on the *in vivo* attachment of *Salmonella typhimurium* in chick ceca. *Avian Diseases* **36,** 139-142.

MCHAN, F. & SHOTTS, E. B. (1993). Effect of short-chain fatty acids on the growth of *Salmonella typhimurium* in an *in vitro* system. *Avian Diseases* **37**, 396-398.

MCNAUGHTON, J. L. ROBERTS, M. RICE, D. SMITH, B. HINDS, M. SCHMIDT, J. LOCKE, M. BRYANT, A. ROOD, T. LAYTON, R. LAMB, I. & DELANEY, B. (2007). Feeding performance in broiler chickens fed diets containing DAS-59122-7 maize grain compared to diets containing non-transgenic maize grain. *Animal Feed Science and Technology* **132**(3-4), 227-239.

MEDEL, P. PASTRANA, L. MENDEZ, J. MCCARTNEY, E. & GRACIA, M. I. (2004). Enzyme supplementation of laying hens fed diets containing barley and wheat. *Poultry Science* **83**, 398-399.

MENG, X. SLOMINSKI, B. A. CAMPBELL, L. D. GUENTER, W. & JONES, O. (2006). The use of enzyme technology for improved energy utilization from full-fat oilseeds. Part I: Canola seed. *Poultry Science* **85**(6), 1025-1030.

MENG, X. SLOMINSKI, B. A. NYACHOTI, C. M. CAMPBELL, L. D. & GUENTER, W. (2005). Degradation of cell wall polysaccharides by combinations of carbohydrase enzymes and their effect on nutrient utilization and broiler chicken performance. *Poultry Science* **84**(1), 37-47.

MICHODJEHOUN-MESTRES, L. HOUNHOUIGAN, J. DOSSOU, J. & MESTRES, C. (2005). Physical, chemical and microbiological changes during natural fermentation of « gowe », a fermented beverage from sprouted or non sprouted sorghum (*Sorghum bicolor)* in West-Africa. *African Journal of Biotechnology* **4**(6), 487- 496.

MIKKELSEN, L. L. NAUGHTON, P. J. HEDEMANN, M. S. & JENSEN, B. B. (2004). Effects of Physical Properties of Feed on Microbial Ecology and Survival of *Salmonella enterica* Serovar Typhimurium in the Pig Gastrointestinal Tract. *Applied and Environmental Microbiology* **70**(6), 3485-3492.

MILES, A. A. & MISRA, J. S. (1938). The estimation of the bactericidal power of the blood. *Journal of Hygiene, Cambridge* **38**, 732-749.

MISSOTTEN, J. A. M. GORIS, J. MICHIELS, J. VAN COILLIE, E. HERMAN, L. DE SMET, S. DIERICK, N. A. & HEYNDRICKX, M. (2008). Screening of isolated lactic acid bacteria as potential beneficial strains for fermented liquid pig feed production. *Animal Feed Science and Technology* **In Press, Corrected Proof**, 17p.

MISSOTTEN, J. A. M. MICHIELS, J. GORIS, J. HERMAN, L. HEYNDRICKX, M. DE SMET, S. & DIERICK, N. A. (2007). Screening of two probiotic products for use in fermented liquid feed. *Livestock Science* **108**(1-3), 232-235.

MITARU, B. N. REICHERT, R. D. & BLAIR, R. (1983). Improvement of nutritive value of high tannin sorghums for broiler chickens by high moisture storage (reconstitution). *Poultry Science* **62**, 2065-2072.

MITARU, B. N. REICHERT, R. D. & BLAIR, R. (1984a). The Binding of Dietary Protein by Sorghum Tannins in the Digestive Tract of Pigs. *Journal of Nutrition* **114**(10), 1787-1796.

MITARU, B. N. REICHERT, R. D. & BLAIR, R. (1984b). Nutritive value of reconstituted sorghums for weanling pigs. *Journal of Animal Science* **58**, 1211-1215.

MITCHELL, M. A. & MORETO, M. (2006). Absorptive function of the small intestine: adaptations meeting demand In *Avian Gut Function in Health and Disease* (Ed G. C. Perry), pp. 43-64. Wallingford, U.K.: CAB International.

MONTAGNE, L. PLUSKE, J. R. & HAMPSON, D. J. (2003). A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Animal Feed Science and Technology* **108**(1-4), 95-117.

MORAN, C. A. (2001). *Development and benefits of liquid feeding through fermentation for the post-weaned pig.* PhD, University of Plymouth.

MORAN, C. A. SCHOLTEN, R. H. J. TRICARICO, J. M. BROOKS, P. H. & VERSTEGEN, M. W. A. (2006). Fermentation of wheat: Effects of backslopping different proportions of pre-fermented wheat on the microbialand chemical composition. *Archives of Animal Nutrition* **60**(2), 158-169.

MUKHODHYAY, N. & RAY, A. K. (1999). Effect of fermentation on the nutritive value of sesame seed meal in the diets for rohu, *Labeo rohita* (Hamilton), fingerlings. *Aquaculture Nutrition* **5**, 229-236.

NAVA, G. M. BIELKE, L. R. CALLAWAY, T. R. & CASTANEDA, M. P. (2005). Probiotic alternatives to reduce gastrointestinal infections: The poultry Experience. *Animal Health Research Review* **6**, 105-118.

NIELSEN, E. K. & INGVARTSEN, K. L. (2000). Effect of cereal type, disintegration method and pelleting on stomach content, weight and ulcers and performance in growing pigs. *Livestock Production Science* **66**(3), 271-282.

NIVEN, S. J. BEAL, J. D. & BROOKS, P. H. (2004). The simultaneous determination of short chain fatty acid, monosaccharides and ethanol in fermented liquid pig diets. *Animal Feed Science and Technology* **117**(3-4), 339-345.

NIVEN, S. J. BEAL, J. D. & BROOKS, P. H. (2006). The effect of controlled fermentation on the fate of synthetic lysine in liquid diets for pigs. *Animal Feed Science and Technology* **129**(3-4), 304-315.

NYACHOTI, C. M. OMOGBENIGUN, F. O. RADEMACHER, M. & BLANK, G. (2006). Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. *Journal of Animal Science* **84**(1), 125-134.

OBOH, G. (2006). Nutrient enrichment of cassava peels using a mixed culture of *Saccharomyces cerevisae* and *Lactobacillus spp* solid media fermentation techniques. *Electronic Journal of Biotechnology* **9**(1), 46-49.

OGBONNA, A. C. OBI, S. K. C. & OKOLO, B. N. (2004). Optimization of proteolytic activities in malting sorghum. *Process Biochemistry* **39**(6), 713-718.

OKPAKO, C. E. NTUI, V. O. OSUAGWU, A. N. & OBASI, F. I. (2008). Proximate composition and caynide content of cassava peels fermented with *Aspergillus nigeir* and *Lactobacillus rhamnosus*. *Journal of Food Agriculture and Environment* **6**(2), 251-255.

OLSTORPE, M. LYBERG, K. LINDBERG, J. E. SCHNURER, J. & PASSOTH, V. (2008). Population diversity of Yeasts and lactic acid bacteria in pig feed fermented with whey, wet wheat distillers' grains, or water at different temperatures. *Applied and Environmental Microbiology* **74**(6), 1696-1703.

OSMAN, M. A. (2004). Changes in sorghum enzyme inhibitors, phytic acid, tannins and *in vitro* protein digestibility occurring during Khamir (local bread) fermentation. *Food Chemistry* **88**(1), 129-134.

PEDERSEN, C. ROOS, S. JONSSON, H. & LINDBERG, J. E. (2005). Performance, feeding behaviour and microbial diversity in weaned piglets fed liquid diets based on water or wet wheat-distillers grain. *Archives of Animal Nutrition* **59**(3), 165-179.

PEIRIS, H. ELLIOTT, R. & NORTON, B. W. (1998). Substitution of sorghum grain for molasses increases the liveweight gain of steers given molasses-based diets. *Journal of Agricultural Science, Cambridge* **130**, 199-204.

PIRGOZLIEV, V. ODUGUWA, O. ACAMOVIC, T. & BEDFORD, M. R. (2008). Effects of dietary phytase on performance and nutrient metabolism in chickens. *British Poultry Science* **49**(2), 144-154.

PLUMED-FERRER, C. KOISTINEN, K. M. TOLONEN, T. L. LEHESRANTA, S. J. KARENLAMPI, S. O. MAKIMATTILA, E. JOUTSJOKI, V. VIRTANEN, V. & VON WRIGHT, A. (2008). Comparative Study of Sugar Fermentation and Protein Expression Patterns of Two Lactobacillus plantarum Strains Grown in Three Different Media. *Applied and Environmental Microbiology* **74**(17), 5349-5358.

PLUSKE, J. R. PETHICK, D. W. HOPWOOD, D. E. & HAMPSON, D. J. (2002). Nutritional influences on some major enteric bacterial diseases of pigs. *Nutrition Research Reviews* **15**(2), 333-371.

PONTE, P. I. P. LORDELO, M. M. S. GUERREIRO, C. I. P. D. SOARES, M. C. MOURÃ£O, J. L. CRESPO, J. P. CRESPO, D. G. PRATES, J. A. M. FERREIRA, L. M. A. & FONTES, C. M. G. A. (2008). Crop beta-glucanase activity limits the effectiveness of a recombinant cellulase used to supplement a barley-based feed for free-range broilers. *British Poultry Science* **49**(3), 347-359.

POOLE, T. L. GENOVESE, K. J. CALLAWAY, T. R. BISCHOFF, K. M. DONSKEY, C. J. & NISBET, D. J. (2004). Competitive exclusion of a glycopeptide-resistant Enterococcus faecium in the presence of vancomycin but not equivalent concentrations of tylosin or gentamicin. *Poultry Science* **83**(7), 1099-1105.

PROHASKA, L. (1986). Antibacterial mechanism of volatile fatty acids in the intestinal tract of pigs against Escherichia coli. *Zentralbl. Veterinarmed., Reihe B* **33**, 166-173.

PUCHALA, R. MIN, B. R. GOETSCH, A. L. & SAHLU, T. (2005). The effect of a condensed tannin-containing forage on methane emission by goats. *J. Anim Sci.* **83**(1), 182-186.

RADA, V. NEVORAL, J. TROJANOVÁ, I. TOMÁNKOVÁ, E. SMEHILOVÁ, M. & KILLER, J. (2008). Growth of infant faecal bifidobacteria and clostridia on prebiotic oligosaccharides in in vitro conditions. *Anaerobe* **14**(4), 205-208.

RAGAEE, S. ABDEL-AAL, E.-S. M. & NOAMAN, M. (2006). Antioxidant activity and nutrient composition of selected cereals for food use. *Food Chemistry* **98**(1), 32-38.

RAMA RAO, S. V. RAJU, M. V. L. N. & REDDY, M. R. (2007). Performance of broiler chicks fed high levels of cholecalciferol in diets containing sub-optimal levels of calcium and non-phytate phosphorus. *Animal Feed Science and Technology* **134**(1-2), 77-88.

RAMAKRISHNA, B. S. NANCE, S. H. ROBERTS-THOMSON, I. C. & ROEDIGER, W. E. W. (1990). The effects of enterotoxins and short chain fatty acids on water and electrolyte fluxes in ileal and colonic loops in vivo in the rat. *Digestion* **45**, 93-101.

RASTALL, R. A. & MAITIN, V. (2002). Prebiotics and synbiotics: towards the next generation. *Current Opinion in Biotechnology* **13**(5), 490-496.

RAZDAN, A. & PETTERSSON, D. (1996). Hypolipidaemic, gastrointestinal and related responses of broiler chickens to chitosans of different viscosity. *British Journal of Nutrition* **76**, 387-397.

REED, J. D. TEDLA, A. & KEBEDE, Y. (1987). Phenolics, fiber and fiber digestibility in the crop residue from bird resistant and non-bird resistant sorghum varieties. *Journal of the Science of Food and Agriculture* **39**(2), 113-121.

REFSTIE, S. SAHLSTROM, S. BRATHEN, E. BAEVERFJORD. G. & KROGEDAL, P. (2005). Lactic acid fermentation eliminates indigestible carbohydrates and

antinutritional factors in soybean meal for Atlantic salmon (*Salmo salar*). *Aquaculture* **246**(1-4), 331-345.

REID, G. (2008). Probiotics and prebiotics - Progress and challenges. *International Dairy Journal* **18**(10-11), 969-975.

RICHARDS, J. D. GONG, J. & DE LANGE, C. F. M. (2005). The gastrointestinal microbiota and its role in monogastric nutrition and health with emphasis on pigs: Current understanding, possible modulations, and new technologies for ecological studies. *Canadian Journal of Animal Science* **85**, 421-435.

ROEDIGER, W. E. W. & MOORE, A. (1981). Effect of short-chain fatty acid on sodium absorption in isolated human colon perfused through the vascular bed. *Digestive Diseases and Sciences* **26**, 100-106.

RUPPIN, H. BAR-MIER, S. SOERGEL, K. H. WOOD, C. M. & SCHMITT, M. G. J. (1980). Absorption of short-chain fatty acids by the colon. *Gastroenterology* **78**, 1500-1507.

RUSSELL, P. J. GEARY, T. M. BROOKS, P. H. & CAMPBELL, A. (1996). Performance, water use and effluent output of weaner pigs fed *ad libitum* with either dry pellets or liquid feed and the role of microbial activity in the liquid feed. *Journal of the Science of Food and Agriculture* **72**, 8-16.

SAKI, A. A. (2005). Effect of wheat and barley viscosity on broiler performance in Hamadan Province. *International Journal of Poultry Science* **4**(1), 7-10.

SALARMOINI, M. CAMPBELL, G. L. ROSSNAGEL, B. G. & RABOY, V. (2008). Nutrient retention and growth performance of chicks given low-phytate conventional or hull-less barleys. *British Poultry Science* **49**(3), 321-328.

SALMINEN, S. BOULEZ, C. BOUTRON-RUAULT, M.-C. CUMMINGS, J. H. FRANK, A. GIBSON, G. R. ISOLAURI, E. MOREAU, M.-C. ROBERFROID, M. & ROWLAND, I. (1998). Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* **80 Suppl.1**, S147-S171.

SCHOLTEN, R. H. J. VAN DER PEET-SCHWERING, C. M. C. VERSTEGEN, M. W. A. DEN HARTOG, L. A. SCHRAMA, J. W. & VESSEUR, P. C. (1999). Fermented co-products and fermented compound diets for pigs: a review. *Animal Feed Science and Technology* **82**(1-2), 1-19.

SIEO, C. C. ABDULLAH, N. TAN, W. S. & HO, Y. W. (2005). Influence of beta-glucanase-producing *Lactobacillus* strains on intestinal characteristics and feed passage rate of broiler chickens. *Poultry Science* **84**(5), 734-741.

SKREDE, A. SAHLSTROM, S. AHLSTROM, O. CONNOR, K. H. & SKREDE, G. (2007). Effects of lactic acid fermentation and gamma irradiation of barley on

antinutrient contents and nutrient digestibility in mink (*Mustela vison*) with and without dietary enzyme supplement. *Archives of Animal Nutrition* **61**(3), 211-221.

SKREDE, G. HERSTAD, O. SAHLSTROM, S. HOLCK, A. SLINDE, E. & SKREDE, A. (2003). Effects of lactic acid fermentation on wheat and barley carbohydrate composition and production performance in the chicken. *Animal Feed Science and Technology* **105**(1-4), 135-148.

SKREDE, G. SAHLSTRØM, S. SKREDE, A. HOLCK, A. & SLINDE, E. (2001). Effect of lactic acid fermentation of wheat and barley whole meal flour on carbohydrate composition and digestibility in mink (*Mustela vison*). *Animal Feed Science and Technology* **90**(3-4), 199-212.

SKREDE, G. STOREBAKKEN, T. SKREDE, A. SAHLSTRØM, S. SØRENSEN, M. SHEARER, K. D. & SLINDE, E. (2002). Lactic acid fermentation of wheat and barley whole meal flours improves digestibility of nutrients and energy in Atlantic salmon (*Salmo salar* L.) diets. *Aquaculture* **210**(1-4), 305-321.

SMIRNOV, A. PEREZ, R. AMIT-ROMACH, E. SKLAN, D. & UNI, Z. (2005). Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. *Journal of Nutrition* **135**(2), 187-192.

SMITS, C. H. M. VELDMAN, A. VERSTEGEN, M. W. A. & BEYNEN, A. C. (1997). Dietary carboxymethylcellulose with high instead of low viscosity reduces macronutrient digestion in broiler chickens. *Journal of Nutrition* **127**(3), 483-487.

SNEL, J. HARMSEN, H. J. M. VAN DER WIELEN, P. W. J. J. & WILLIAMS, B. A. (2002). Dietary strategies to influence the gastrointestinal microflora of young animals, and its potential to improve intestinal health. In *Nutrition and health of the gastrointestinal tract* Eds M. C. Blok, H. A. Vahl, L. de Lange, A. E. van de Braak, G. Hemke & M. Hessing), pp. 37-69. Wageningen, Netherlands: Wageningen Academic Publishers.

STEENFELDT, S. KJAER, J. B. & ENGBERG, R. M. (2007). Effect of feeding silages or carrots as supplements to laying hens on production performance, nutrient digestibility, gut structure, gut microflora and feather pecking behaviour. *British Poultry Science* **48**(4), 454-468.

STEINER, T. (2006). *Managing Gut Health: Natural Growth Promoters as a Key to Animal Performances*. Nottingham: Nottingham University Press.

STEWART, C. S. HILMAN, K. MAXWELL, F. KELLY, D. & KING, T. P. (1993). Recent advances in probiotics in pigs:observations on the microbiology of the pig gut. In *In. Recent Advances in Animal Nutrition* Eds P. C. Garnsworthy & D. J. A. Cole), pp. pp 197-220. Nottingham: Nottinghan University Press.

SVIHUS, V. (2006). The role of feed processing on gastrointestinal function and health in poultry. In *Avian function in health and disease* (Ed G. C. Perry), pp. 183-194. Wallingford, U. K.: CAB International.

SWAGGERTY, C. L. FERRO, P. J. PEVZNER, I. Y. & KOGUT, M. H. (2005). Heterophils are associated with resistance to systemic *Salmonella enteritidis* infections in genetically distinct chicken lines. *Fems Immunology and Medical Microbiology* **43**(2), 149-154.

TESTER, R. F. KARKALAS, J. & QI, X. (2004). Starch--composition, fine structure and architecture. *Journal of Cereal Science* **39**(2), 151-165.

TESTER, R. F. QI, X. & KARKALAS, J. (2006). Hydrolysis of native starches with amylases. *Animal Feed Science and Technology* **130**(1-2), 39-54.

THOMPSON, J. L. & HINTON, M. (1997). Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. *British Poultry Science* **38**, 59-65.

UNI, Z. (2006). Early development of small intestinal function. In *Avian Gut Function in Health and Disease* (Ed G. C. Perry), pp. 29-42. Wallingford, U.K.: CAB International.

UNI, Z. & FERKET, R. P. (2004). Methods of early nutrition and their potential. *World's Poultry Science Journal* **60**(1), 101-111.

UNI, Z. GANOT, S. & SKLAN, D. (1998). Posthatch development of mucosal function in the broiler small intestine. *Poultry Science* **77**(1), 75-82.

VAN DEN BRINK, M. & VAN RHEE, W. (2007). Early access to semi-moist diets for day old chicks stimulates gastrointestinal tract development and prevents dehydration during transport. In *World Poultry* pp. 17-19. Twilmij BV, Stroe, The Netherlands: World Poultry, http://www.twilmij.nl/UK/pages/documents/Semi-moistdiets.pdf.

VAN DER KLIS, J. D. & JANSMAN, A. J. M. (2002). Optimising nutrient digestion, absorption and gut barrier function in monogastrics: Reality or illusion? In *Nutrition and health of the gastrointestinal tract* Eds M. C. Blok, H. A. Vahl, L. de Lange, A. E. van de Braak, G. Hemke & M. Hessing), pp. 15-36. Wageningen, Netherlands: Wageningen Academic Publishers.

VAN IMMERSEEL, F. FIEVEZ, V. DE BUCK, J. PASMANS, F. MARTEL, A. HAESEBROUCK, F. & DUCATELLE, R. (2004). Microencapsulated short-chain fatty acids in feed modify colonisation and invasion early after infection with *Salmonella enteritidis* in young chickens. *Poultry Science* **83**, 69-74.

VAN WINSEN, R. L. KEUZENKAMP, D. URLINGS, B. A. P. LIPMAN, L. J. A. SNIJDERS, J. A. M. VERHEIJDEN, J. H. M. & VAN KNAPEN, F. (2002). Effect of

fermented feed on shedding of Enterobacteriaceae by fattening pigs. *Veterinary Microbiology* **87**(3), 267-276.

VAN WINSEN, R. L. LIPMAN, L. J. A. BIESTERVELD, S. URLINGS, B. A. SNIJDERS, J. A. M. & VAN KNAPEN, F. (2001a). Mechanism of *Salmonella* reduction in fermented pig feed. *Journal of the Science of Food and Agriculture* **81**, 342-346.

VAN WINSEN, R. L. URLINGS, B. A. P. LIPMAN, L. J. A. SNIJDERS, J. M. A. KEUZENKAMP, D. VERHEIJDEN, J. H. M. & VAN KNAPEN, F. (2001b). Effect of Fermented Feed on the Microbial Population of the Gastrointestinal Tracts of Pigs. *Applied and Environmental Microbiology* **67**(7), 3071-3076.

VAUGHAN, E. E. HEILIG, H. G. H. J. ZOETENDAL, E. G. SATOKARI, R. COLLINS, J. K. AKKERMANS, A. D. L. & DE VOS, W. M. (1999). Molecular approaches to study probiotic bacteria. *Trends in Food Science & Technology* **10**(12), 400-404.

VIEIRA-DALODE, G. MADODE, Y. E. HOUNHOUIGAN, J. JESPERSEN, L. & JAKOBSEN, M. (2008). Use of stater cultures of lactic acid bacteria and yeasts as inoculum enrichment for the production of gowe, a sour beverage from Benin. *African Journal of Microbiology Research* **2**(7), 179 -186.

WANG, Z. R. QIAO, S. Y. LU, W. Q. & LI, D. F. (2005). Effects of enzyme supplementation on performance, nutrient digestibility, gastrointestinal morphology, and volatile fatty acid profiles in the hindgut of broilers fed wheat-based diets. *Poultry Science* **84**(6), 875-881.

WEE, Y. J. KIM, H. O. YUN, J. S. & RYU, H. W. (2006). Pilot-scale lactic acid production via batch culturing of *Lactobacillus sp* RKY2 using corn steep liquor as a nitrogen source. *Food Technology and Biotechnology* **44**(2), 293-298.

WELLS, L. L. LOWRY, V. K. DELOACH, J. R. & KOGUT, M. H. (1998). Age-dependent phagocytosis and bactericidal activities of the chicken heterophil. *Developmental & Comparative Immunology* **22**(1), 103-109.

WILKIE, D. C. VAN KESSEL, A. G. WHITE, L. J. LAARVELD, B. & DREW, M. D. (2005). Dietary Amino acids affect intestinal *Clostridium perfrigens* populations in broiler chickens. *Canadian Journal of Animal Science* **85**, 185-193.

WILLIAMS, B. A. BOSCH, M. W. BOER, H. VERSTEGEN, M. W. A. & TAMMINGA, S. (2005). An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. *Animal Feed Science and Technology* **123-124**(Part 1), 445-462.

WILLIAMS, B. A. BOSCH, M. W. & VERSTEGEN, M. W. A. (2000). Changes in digesta NH_3 concentration related to fermentable carbohydrates in piglet diets. In *Proceedings of the British Society of Animal Science* p. 21. Scarborough: British Society of Animal Science.

WILLIAMS, B. A. VERSTEGEN, M. W. A. & TAMMINGA, S. (2001). Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutrition Research Reviews* **14**, 207-227.

WILSON, A. R. SIGEE, D. & EPTON, H. A. S. (2005). Anti-bacterial activity of *Lactobacillus plantarum* strain SK1 against *Listeria monocytogenes* is due to lactic acid production. *Journal of Applied Microbiology* **99**, 1516-1522.

WISE, M. G. & SIRAGUSA, G. R. (2007). Quantitative analysis of the intestinal bacterial community in one- to three-week-old commercially reared broiler chickens fed conventional or antibiotic-free vegetable-based diets. *Journal of Applied Microbiology* **102**(4), 1138-1149.

WOODWARD, S. L. WAGHORN, G. C. ULYATT, M. J. & LASSEY, D. K. R. (2001). Early indications that feeding *Lotus* will reduce methane emissions from ruminants In *Proceedings of the New Zealand Society of Animal Production* pp. 23-26. New Zealand: New Zealand Society of Animal Production.

XUAN DUNG, N. N. MANH, H. L. & OGLE, B. (2005). Effects of fermented liquid feeds on the performance, digestibility, nitrogen and plasma urea nitrogen (PUN) of growing-finishing pigs *Livestock Research for Rural Development* **17**(9), 1-8.

YALDA, A. Y. & FORBES, J. M. (1995). Food intake and growth in chickens given food in the wet form with and without access to drinking water. *British Poultry Science* **36**(3), 357-369.

YALDA, A. Y. & FORBES, J. M. (1996). Effects of food intake, soaking time, enzyme and cornflour addition on the digestibility of the diet and performance of broilers given wet food *British Poultry Science* **37**(4), 197-807.

YASAR, S. (2003). Performance, gut size and ileal digesta viscosity of broiler chickens fed with whole wheat added diet and the diets with different wheat particle sizes. *International Journal of Poultry Science* **2**(1), 75-82.

YASAR, S. & FORBES, J. M. (1999). Performance and gastro-intestinal response of broiler chickens fed on cereal grain-based foods soaked in water. *British Poultry Science* **40**(1), 65 - 76.

YEGANI, M. & KORVER, D. R. (2008). Factors Affecting Intestinal Health in Poultry. *Poultry Science* **87**(10), 2052-2063.

YU, B. WU, S. T. LIU, C. C. GAUTHIER, R. & CHIOU, P. W. S. (2007). Effects of enzyme inclusion in a maize-soybean diet on broiler performance. *Animal Feed Science and Technology* **134**(3-4), 283-294.

ZAR, J. H. (1999). *Biostatistical Analysis*. London, UK: Prentice-Hall International (UK)