EFFECTS OF FEEDING A FERMENTED PRODUCT ON THE FAECAL MICROFLORA AND EGG COMPOSITION IN LAYING HENS

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By

ELIZABETH LAW FANG LIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

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Dedicated to

My late mother Mdm. Goh Choo Moi who is my inspiration forever

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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Chairman: **Associate Professor Loh Teck Chwen, PhD**

Faculty: **Agriculture**

The thesis studied the characteristics of a fermented product (FP) and the effects of the FP on layers. Two experiments were conducted to achieve the objectives above. Experiment I (Chapter IV) was conducted to evaluate the composition and consistency of the finished fermented product (FP). The nutritive value and chemical compositions of the product were evaluated after the process of fermentation. In parallel, microbiological analyses, including lactic acid bacteria (LAB) and *Enterobacteriaceae* counts were also performed. The fermentation process was repeated three times under the same running conditions and using the same amounts of raw materials. The physical, chemical and microbiological properties of FP obtained in this study showed that FP had consistent chemical and microbiological characteristics. The presence of essential fatty acids (linoleic acid 24%, linolenic acid 1.1%, eicosapentaenoic acid 3.6%, docosapentaenoic acid 0.7%, docosahexaenoic acid 2.0%) and a lime-flavored aroma represent additional value-added attributes to the product.

Experiment II (Chapter V) was carried out to investigate the effects of feeding FP to layers (Babcock B380) on faecal microflora, essential fatty acid and cholesterol levels in eggs and plasma. A total of 96, 13-week-old Babcock B380 pullets were used in this study. They were randomly assigned to four numerically equal groups with eight replicates per treatments, three birds per replicates. All the birds were housed in individual cages. Diet I (Cont), had no FP, Diet II (FP3) contained 3% (w/w) FP (30g FP/kg diet), Diet III (FP6) contained 6% (w/w) FP (60g FP/kg diet) and Diet IV (FP9) contained 9% (w/w) FP (90g FP/kg diet). The study lasted 16 weeks inclusive of two weeks of adjustment period and a 14-week experimental period. Weekly feed intake and egg production were recorded. Fresh faecal droppings were collected immediately and cultured for LAB and *Enterobacteriaceae* and pH and VFA production were also measured. Blood plasma cholesterol and fatty acid profiles were assayed at the end of the experiment. Results obtained from this study showed that FP reduced ($P<0.05$) the faecal *Enterobacteriaceae* and faecal pH. Higher ($P<0.05$) counts of faecal LAB in layers fed with FP were observed. However, FP did not enhance ($P>0.05$) the egg production and egg mass but ($P<0.05$) decreased the egg weight slightly. Feed intake and feed conversion ratio (FCR, feed intake/egg mass) were not affected ($P>0.05$). However, egg yolk cholesterol and plasma cholesterol concentrations were reduced ($P<0.05$) in the FP fed laying hens compared to the control laying hens. The n-6: n-3 ratio in the egg yolk (control=7.9, FP9=6.1) and plasma (control=10.46, FP9=6.54) from the FP fed laying hens were decreased compared to the control laying hens. Moreover, FP was able to increase ($P<0.05$) the polyunsaturated fatty acid (PUFA): saturated fatty acid (SFA) ratio, total n-3 PUFA and DHA

concentrations in egg yolk and plasma. In summary, the results of the dietary manipulation demonstrated the effectiveness of FP in shifting the microflora composition of the avian GIT towards a beneficial balance. In addition, it has the ability to increase the unsaturated fatty acid (UFA) content to a more healthy proportion in eggs.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan ijazah Master Sains

KESAN PEMBERIAN PRODUK HASIL FERMENTASI KE ATAS MIKROORGANISMA TINJA DAN PENILAIAN KOMPOSISI TELUR DALAM AYAM PENELUR

Oleh

ELIZABETH LAW FANG LIN

Januari 2006

Pengerusi: Profesor Madya Loh Teck Chwen, PhD

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Tujuan utama kajian ini dijalankan adalah untuk mengkaji ciri-ciri produk hasil fermentasi dan kesan pemberiannya ke atas ayam penelur. Sebanyak dua eksperimen dilaksanakan untuk mencapai objektif di atas. Eksperimen I (Bab IV) memberi tumpuan terhadap komposisi dan keseragaman produk hasil fermentasi (FP). Nilai pemakanan dan kandungan kimia FP telah ditentukan. Sejajar dengan tujuan ini, analisis mikrob termasuk penentuan bakteria asid laktik (LAB) dan *Enterobacteriaceae* turut dijalankan. Proses fermentasi telah diulang sebanyak tiga kali dengan menetapkan keadaan operasi dan kuantiti bahan mentah yang sama. Kajian ini menunjukkan FP mempunyai ciri-ciri fizikal, kimia dan mikrobiologi yang konsisten. Kewujudan asid lemak perlu seperti asid linoleik (24%), asid linolenik (1.1%), asid eikosapentaenoik (EPA, 3.6%), asid dokosapentaenoik (DPA, 0.7%) dan asid dokosahexaenoik (DHA, 2.0%) dengan haruman limau nipis merupakan nilai tambahan produk.

Eksperimen II (Bab V) telah dijalankan untuk mengkaji kesan pemberian FP ke atas ayam penelur terhadap mikroflora tinja, tahap asid lemak perlu dan kolesterol di dalam telur dan plasma darah. Sebanyak 96 ekor ayam penelur Babcock B380 yang berumur 13 minggu telah digunakan dalam kajian ini. Kesemua ayam ditempatkan di dalam sangkar berasingan. Ayam-ayam ini dibahagikan kepada empat kumpulan rawatan, setiap kumpulan mengandungi lapan replikasi di dalam manakala setiap replikasi menggunakan tiga ekor. Diet yang diberi adalah seperti berikut: Diet kawalan, FP3 (30g PHF/kg diet), FP6 (60g PHF/kg diet) dan FP9 (90g PHF/kg diet). Eksperimen II dijalankan selama 16 minggu, dua minggu pertama adalah minggu penyesuaian dan 14 minggu berikutnya sebagai tempoh eksperimen sebenar. Pengambilan makanan dan hasil pengeluaran telur direkodkan setiap minggu. Tinja dipungut untuk pengkulturan LAB dan *Enterobacteriaceae*, penilaian pH dan penghasilan asid lemak meruap. Pada akhir ekperimen, pengambilan darah dijalankan untuk menentukan kandungan kolesterol plasma darah dan asid-asid lemak. Keputusan yang didapati menunjukkan FP ini dapat mengurangkan populasi ($P<0.05$) *Enterobacteriaceae* tinja dan merendahkan ($P<0.05$) pHnya. Pengkulturan tinja menunjukkan kumpulan yang menerima diet FP mempunyai mikroorganisma LAB yang lebih banyak ($P<0.05$) jika dibandingkan dengan kumpulan kawalan. Walau bagaimanapun, ayam penelur yang menerima diet FP didapati tidak berupaya ($P>0.05$) meningkatkan pengeluaran telur dan jisim telur, malahan meyusutkan berat telur. Pengambilan makanan dan kadar penukaran makanan juga didapati tidak menunjukkan perbezaan ($P>0.05$) bagi keempat-empat kumpulan. Tetapi, penurunan terhadap tahap kolesterol di dalam kuning telur dan plasma kolesterol dikesan ($P<0.05$) hasil daripada pemberian FP. Ayam

yang diberi FP menghasilkan telur (kawalan=7.9, FP9=6.1) dan plasma (kawalan=10.46, FP9=6.54) dengan nisbah asid lemak n-6:n-3 yang lebih rendah jika dibandingkan dengan kumpulan kawalan. FP dapat mempertingkatkan nisbah antara asid lemak politaktepu (PUFA) dengan asid lemak tepu, kandungan asid lemak politaktepu n-3 dan kandungan DHA di dalam kuning telur. Secara keseluruhannya, keputusan menunjukkan FP berkesan dalam meningkatkan prestasi ayam penelur. Sebagai kesimpulan, kaedah manipulasi pemakanan yang dipraktikkan dalam ujian ini dapat mengubah mikroorganisma dalam salur makanan ke aras keseimbangan yang lebih baik. Tambahan pula, FP dapat meninggikan kandungan asid lemak tak tepu pada telur ke tahap yang boleh memanfaatkan kesihatan pengguna.

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I certify that an Examination Committee has met on, 17th January 2006 to conduct the final examination of Elizabeth Law Fang Lin on her Master of Science thesis entitled "Effects of Feeding a Fermented Product on the Faecal Microflora and Egg Composition in Laying Hens" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

_____

ELIZABETH LAW FANG LIN

Date:

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LIST OF ABBREVIATIONS

ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
atm	atmosphere
BW	body weight
C12:0	lauric acid
C14:0	myristic acid
C15:0	pentadecanoic acid
C16:0	palmitic acid
C16:1 n-9	palmitoleic acid
C17:0	heptadecanoic acid
C17:1	heptadecenoic acid
C18:0	stearic acid
C18:1 n-9	oleic acid
C18:2 n-6	alpha-linoleic acid
C18:3 n-3	linolenic acid
C20:0	arachidic Acid
C20:1 n-9	eicosenoic acid
C20:2 n-6	eicosadienoic acid
C20:4 n-6	arachidonic acid
C20:5 n-3	eicosapentaenoic acid
C22:0	behenic acid
C22:5 n-3	docosapentaenoic acid
C22:6 n-3	docosahexaenoic acid

C24:0	lignoceric acid
CF	crude fibre
CFU	colony forming unit
CH_3	methane
cm	centimetre
CO_2	carbon dioxide
CP	crude protein
^oC	degree Celsius
DHA	docosahexaenoic acid
DM	dry matter
DPA	docosapentaenoic acid
EDTA	ethylenediamino tetraacetic acid
EE	ether extract
EMB	eosin-methylene-blue
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
FCR	feed conversion ratio
FP	fermented product
g	gram
g/b/d	gram per bird per day
GE	gross energy
GIT	gastrointestinal tract
GLC	gas liquid chromatography
GRAS	generally regarded as safe
H_2O_2	hydrogen peroxide

HPLC	high performance liquid chromatography
H_2SO_4	sulphuric acid
Kcal	kilocalories
KOH	potassium hydroxide
LAB	lactic acid bacteria
LDL	low-density lipoprotein
µl	microlitre
µg	microgram
mg	milligram
ml	milliliter
min	minute
mM	millimole
MUFA	monounsaturated fatty acids
MRS	DE Man, ROGOSA and SHAPE
N	nitrogen
n-3	Omega-3
n-6	Omega-6
NaCl	sodium chloride
Na_2CO_3	sodium carbonate
NaOH	sodium hydroxide
NH_3	ammonia
PUFA	polyunsaturated fatty acids
URT	upper respiratory tract
SAS	statistical analysis system
SE	standard error

SEM	standard error of mean
SFA	saturated fatty acids
Sig.	Significant
spp.	species
Subsp	Subspecies
UFA	unsaturated fatty acids
VFA	volatile fatty acids
v/w	volume per weight
VLDL	very low-density lipoprotein
v/v	volume per volume
W.	*Weisella*
w/v	weight per volume
w/w	weight per weight

CHAPTER I

GENERAL INTRODUCTION

Over the last two decades, antibiotics are popularly used as growth enhancers especially in intensive animal production. Antibiotic-based growth promoters have undoubtly improved animal performance and health status. However, the excessive use of antibiotics may cause pathogenic bacteria in food animals to develop antibiotic resistance. Excessive use of antibiotics may also result in antibiotic residue accumulation in animal products, which can be harmful to consumers (Mikkelsen and Jensen, 2000; Demecková *et al*., 2002; Loh *et al*., 2003b). Furthermore, cross-resistance to therapeutic antibiotics often occurs if the therapeutic antibiotic and the growth-promoting antibiotics belong to the same class of drugs. This in turn reduces the possibility of treatment in diseased animals and human populations. As a consequence, the usage of antibiotics as growth enhancers is prohibited in many countries (Kawashima, 2004). Therefore, there is a need to replace or partially replace the usage of antibiotic to sustain the growth and health of the animal in animal production.

At present, the improvement in biotechnology has led to a development of new range of natural feed additives. These additives included probiotics, prebiotics, enzymes, specific carbohydrates, organic acids, and recently fermented products (FP). Lactic acid bacteria (LAB) is the commonly used starter culture in food and feed fermentation (Leroy and de Vuyst, 2004; Chapter II). It has been suggested that the LAB in the FP played an important role in preventing the

adherence, establishment, replication and virulence of enteropathogens (Daly *et al*., 1993; Jin *et al*., 1998a,b; Patterson and Burkholfer, 2003) through the production of antimicrobial metabolites such as organic acids, bacteriocin and hydrogen peroxide (Leroy and Vuyst, 2004).

Direct feeding of FP has been shown to have beneficial effects in animal studies (Loh *et al*., 2003a,b). The pathogenic *Enterobacteriaceae* in faeces was decreased in conjunction with an increase in the beneficial faecal LAB among FP fed animals. Similar results on the bactericidal effects of fermented feed on the faecal pathogenic microflora such as *Salmonella* and *Escherichia coli* have also been reported by other studies (Urlings *et al*., 1993; Mikkelsen and Jensen, 2000; van Winsen *et al*., 2001, 2002; Beal *et al*., 2002; Demecková *et al*., 2002; Brooks, 2003; Canibe and Jensen, 2003; Brooks *et al*., 2003; Heres *et al*., 2003a,b). However, the characteristic of the FP with a high fish content used in this study has not been previously reported. Moreover, the use of this FP in the diet of layers has not been documented. Therefore, the general objective of this study was to study the characteristics of the FP fermentation and the effects of feeding on layers. Two experiments were conducted to achieve the above objective:

i. The microbial properties and chemical compositions of the FP.
ii. Effects of feeding the FP on faecal microflora, essential fatty acid and cholesterol levels in eggs and plasma in laying hens**.**

CHAPTER II

LITERATURE REVIEW

2.1 Probiotics

2.1.1 Definition and characteristics

The term "Probiotic" refers to substances produced by one microorganism that are able to stimulate the growth of other microorganism(s), aptly described by its Greek meaning of "pro live" (Ouwehand *et* al., 1999; Naidu *et al*., 1999). The term "substances" is imprecise and would include even antibiotics. Therefore, Fuller (1989) defined probiotics as mono- or mixed cultures of live microorganisms which, when applied to animal or man, affect the host beneficially by improving the properties of the indigenous microflora (Shimakawa *et al*., 2003; Coeuret *et al*., 2004). This definition stressed the importance of live microorganisms that improved the health status of either man or animal (Havenaar and Huis in't veld, 1992). Therefore the term 'probiotics' is restricted to products which contain live microorganisms such as freeze-dried cells or in a FP, improve the health status of man or animals (which can include growth promotion of animals) and can have its effect in the mouth or gastrointestinal tract (GIT), the upper respiratory tract (URT) or in the urogenital tract (Havenaar and Huis in't veld, 1992).

In 2001, the joint Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) expert consultation on heath

and nutritional properties of powder milk with live LAB, redefined probiotics as "live microorganisms which, when administered in adequate amounts (as part of food), confer a health benefit on the host". This again highlighted the importance of microbe viability in this most recent definition. The FAO/WHO recognized that probiotics should be capable of exerting health benefits on the host through growth and/or activity in the body (FAO/WHO, 2001). In order to exert positive health effects, the microorganisms need to be viable, active and abundant in the concentration of at least 10^6 cfu/g of bacteria on expiry. This is because at least 10^8-10^9 cells should always be available as the minimum therapeutic dose per day (Shimakawa *et al*., 2003; Coeuret *et al*., 2004).

Ideally, a microorganism should meet a number of predefined criteria in order to be considered as a probiotic (Collins *et al*., 1998). The desirable characteristics of probiotics are shown in Table 2.1. Table 2.2 shows the common microorganisms used in probiotic products. Adherent probiotics strains are desirable because they have a greater ability to establish themselves and survive in the GIT environment, thus colonizing the intestine to enhance their probiotic effects (Lee and Salminen, 1995; Kheadr *et al*., 2004). Furthermore, it should be technologically suitable for their incorporation into food products from a common dairy processing, fermentation technology or pharmaceutical manufacturing protocols. Furthermore, it must be able to survive in industrial applications (on a commercial scale) at high numbers in the product until the end of shelf life, through and following consumption (Rogelj, 1994; Stanton *et al*., 1998; Catherine *et al*., 2003).

Table 2.1: Desirable characteristics of probiotics

• Of human origins • Generally regarded as safe (GRAS) status • Desirable metabolic activity • Production of antagonistic antibacterial profiles • Proliferation and/or colonization on the location where it is active • No pathogenic, toxic, allergic, mutagenic or carcinogenic reaction by the probiotics itself, its fermentation products or its cell components • Genetically stable, no plasmid transfer • Easy and reproducible production • Viable during processing and storage • Potential vehicle for the delivery of recombinant proteins and peptides in a site-specific fashion to the human GIT

Source: Havenaar and Huis in't Veld (1992); Collins *et al*., (1998).

Table2.2: Microorganisms used in probiotics for farm animals

Lactobacillus species	*Bifidobacterium* species	Other LAB	Non-lactics
Lactobacillus acidophilus	*Bifidobacterium adolescentis*	*Entrococcus faecalis*	*Bacillus cereus*
Lactobacillus casei	*Bifidobacterium animalis*	*Entrococcus faecium*	*Bacillus subtilis*
Lactobacillus crispatus	*Bifidobacterium brevis*	*Lactococcus lactis*	*Bacillus toyoi*
Lactobacillus gallinarum	*Bifidobacterium infantis*	*Leuconostoc mesenteroides*	*Bacillus mesentericus*
Lactobacillus gasseri	*Bifidobacterium lactis*	*Pediococcus acidilactici*	*Bacillus licheniformis*
Lactobacillus johnsonii	*Bifidobacterium longum*	*Pediococcus pentosaceus*	*Bacillus natto*
Lactobacillus plantarum	*Bifidobacterium bifidum*	*Sporolactobacillus inulinus*	*Saccharomyces cerevisiae*
Lactobacillus reuteri	*Bifidobacterium pseudolongum*	*Streptococcus thermophilus*	*Aspergillus oryzae*
Lactobacillus rhamnosus			*Candida pintolopesii*
Lactobacillus delbrueckii			*Propionibacterium freudenreichii*
Lactobacillus fermentum			
Lactobacillus brevis			
Lactobacillus helveticus			

Source: Fuller (1999); Holzapfel, (1997).

2.2 The environmental-friendly probiotics: Lactic acid bacteria

2.2.1 Definition and characteristics

Lactic acid bacteria (LAB) is a group of bacteria commonly used as probiotics (Schillinger, 1999; Fooks and Gibson, 2002). They are bacteria that ferment sugar, such as glucose, predominantly to lactic acid (lactate) as the major end product (Liu, 2003). They are gram-positive, non-sporing, non-motile, catalase-negative and non-aerobic organisms (Wessels *et al*., 2004). The LAB stains are generally mesophilic but can grow at temperatures as low as 5^{o}C or as high as 45 ^{o}C. The majority of strains grow well at pH 4-4.5. Some are active at pH 9.6 and others at pH 3.2. They can tolerate a lower intracellular pH than many other bacteria, thus they are more resistant to acidic conditions (Adams and Nicolaides, 1997). The LAB strains are generally weak proteolytic and lipolytic. They require preformed amino acids, purine and pyrimidine bases and B vitamins for growth (Caplice and Fitzgerald, 1999).

The main genera of LAB includes *Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Enterococcus, Leuconostoc, Aerococcus, Carnobacterium, Tetragenococcus, Oenococcus, Weissella* and *Vagococcus* (Adams and Nicolaides, 1997; Stiles and Holzapfel, 1997; Wessels *et al*., 2004). They play an essential role in foods, feeds, silages and beverage fermentations given that a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, wine,

meat, fish, silage and vegetable products (Lindgren and Dobrogosz, 1990; Liu, 2003; Schnürer and Magnusson, 2005). They have traditionally been used as natural biopreservatives of foods and feeds. In fact, they are present naturally in food as long as people have eaten fermented foods (Rolfe, 2000). The LAB contributes to the flavour and texture changes in food and feed fermentation. It also had a preservative effect resulting in an increase in the shelf life of the transformed product apart from eliminating the pathogenic microorganisms (Wessels *et al*., 2004; Schnürer and Magnusson, 2005).

2.2.2 Lactobacilli

The genus *Lactobacillus* comprises about 50 species. They are facultative anaerobic, gram-positive bacteria; catalase negative, chemo-organotropic, typically non-motile, non-sporulating and non-pigmented mesophilic, but some species showed slightly yellow pigmentation in caryophanon (Kandler and Weiss, 1986). They tolerate temperature ranges between 2ºC to 53ºC, with the optimum for most strains at about 30 to 40ºC. The optimal sustenance pH for growth is usually 5.5-6.2 and growth generally occurs at five or less. The growth rate is often reduced at neutral pH or alkaline conditions.

The genus *Lactobacillus* is one of the largest groups of lactic acid bacteria used in the food fermentation process and is therefore of great economical importance. It is normally found in the dairy products, meat and fish products, water, sewage, beer,

wine, fruits and fruit juices, pickled vegetables, sauerkraut, silage, sour dough, and mash (Lindgren and Dobrogosz, 1990; Hansen, 2002; Liu, 2003; Schnürer and Magnusson, 2005). Lactobacilli strains vary in their fermentation process, hydrogen peroxide (H_2O_2) and bacteriocin production. It is important because these different features make them a versatile group suitable for growing in different conditions (Mombelli and Gismondo, 2000). Lactobacilli also constitute a significant portion of the human intestinal microflora, and their association with the general state of human health is a subject of intensive investigation (Sharpe 1981; Salminen *et al.,* 1996).

2.3 Fermented product

2.3.1 Introduction of fermented product (FP)

Fermentation has been performed, as an art, for many centuries and it is an important part of the microbiology-based industry. It is one of the oldest technologies used for food preservation (Motarjemi, 2002; Prajapati and Nair, 2003). Moreover, production of modified foods and beverages involving fermentations has been practiced for approximately 10, 000 years before the existence of microorganisms was recognized. Through the ages, fermentation has had a major impact on nutritional habits and traditions, on culture and on the commercial distribution and storage of foods (Holzapfel, 2002), where refrigeration is not available for the safe keeping of foods.

Fermentation is a desirable process dependent on the biochemical changes through the action of enzymes elaborated by microorganisms for the production of a range of metabolites (Nout and Motarjemi, 1997; Jay, 2000). Metabolites suppress the growth and survival of undesirable microflora in the feedstuff. Biochemically, fermentation is the metabolic process in which carbohydrates as well as some non-digestible poly- and oligosaccharides are partially oxidized with the release of the energy in the absence of any external electron acceptors. In broad terms, fermentation involves the use of microorganisms to carry out enzyme-catalyzed transformations of organic matter contributing to the development of characteristic properties in taste, aroma, texture, visual appearance, shelf life and safety (Nout and Motarjemi, 1997; Holzapfel, 2002). Enzymes indigenous to the raw materials may play an important role in enhancing these characteristics (Hammes, 1990a,b). Through trial and error, traditional skill gradually evolved and techniques were developed to control the technical parameters during fermentation processes. Experience has also shown that back-sloping, or the inoculation of raw materials with a residue from a previous batch, accelerates the initial phase of fermentation and results in the promotion of desirable changes during the fermentation process (Holzapfel, 2002).

Recent developments in biotechnology in developing countries has led to a variety of FP, which included beverages (alcoholic and non-alcoholic), fermented meat, fish, vegetables, dairy products and condiments which are produced from both edible and inedible raw materials (Motarjemi, 2002; Rolle and Satin, 2002). This

has led to a better standardized FP with desirable characteristics such as improved texture and flavour production, better storage properties, and enhanced nutritional value.

The fermented feeds have been used successfully as animal feed in developed countries. Their application has been extensively reported (Beal, *et al*., 2002; van Winsen *et* al., 2001, 2002; Demecková, *et al*., 2002, Heres *et al*., 2003a,b, 2004). The FP is a value added products that have been anaerobically fermented and whose physical, chemical and biological characteristics have been modified by the activity of microorganisms. They are generally appreciated for attributes such as pleasant flavour, aroma and texture (Holzapfel, 2002). It contains high numbers of LAB and yeast, a low pH and high concentration of lactic acid (Geary *et al*., 1996; Mikkelsen and Jensen, 1997; Brooks *et al*., 2001). It is known to contain specific microbial metabolites such as alcohol; lactic acid, propionic acid, acetic acid, carbon dioxide (CO_2), H_2O_2 and bacteriocins (Holzapfel, 2002). These derived products, which could be named "tertiary metabolites", can play a significant role in the biological activities of FP.

Food ingredients chosen for fermentation could be of animal or plant origins contained not only a variety of nutrients for animal feeding but also for the microbial growth and metabolism during fermentation (Nout, 2001). The action of microorganisms during the preparation of cultured foods has been shown to improve the quality, availability and digestibility of some dietary nutrients (Alm,

1982). In general, biochemical activity may result in the desirable effect of microbial activity in the FP. Microbial enzymes can improve food digestion in the animal's GIT, and thus increase nutrient uptake by breaking down carbohydrates, lipids, proteins, and other food components (Nout, 2001).

2.3.2 LAB as starter cultures in fermented food industries

The general name "lactic acid bacteria" (LAB) has been given to bacteria, which formed at least 50% of lactic acid at the end of carbohydrate catabolism, by conversion of carbon source (Klander, 1983; Condon, 1987). A starter culture may be defined as a preparation or material containing large numbers of variable microorganisms. They accelerate the fermentation process. Being adapted to the substrate, a typical starter culture facilitates improved control of a fermentation process and predictability of its products (Holzapfel, 1997). In addition, starter cultures facilitate control over the initial phase of a fermentation process. Modern starter cultures comprised of either single or multiple strains of LAB, specifically for their adaptation to a substrate or raw material. Those best adapted to the food substrate and to technical control parameters, eventually dominate the process and initiate rapid acidification of the raw material through the production of organic acids. The production of antimicrobial substances, acetic acid, ethanol, aroma compounds, bacteriocins, exopolysacharides, and several enzymes are crucial to the antimicrobial properties of LAB derived FP (Leroy and Vuyst, 2004). Furthermore, selected strains may enhance the general benefits of spontaneous fermentation such

as improved protein digestibility and micronutrient bioavailability, and contribute more specifically to biological enrichment through the biosynthesis of vitamins and essential amino acids (Nout and Mortarjemi, 1997; Holzapfel, 2002).

The LAB, which include the genera *Lactococcus, Streptococcus, Lactobacillus, Pediococcus, Leuconostoc, Enterococcus,* and *Propionibacterium* (Nettles and Barefoot, 1993; Holley and Blaszyk, 1998), played an essential role in food fermentations. The LAB as well as *Micrococcaceae* strains are important microorganism used as starter cultures in meat fermentation (Papamanoli *et al.,* 2003). Several LAB spp. are associated with meat and fish fermentations (Lücke, 2000; Ross *et al*., 2002). *Lactobacillus sakei, Lactobacillus curvatus* and *Lactobacillus plantarum* (Hammes and Hertel, 1998; Holley and Blaszyk, 1998; Hugas *et al*., 2003) have been determined to be superior starter cultures for meat fermentations. *Leuconostoc mesenteroides* frequently dominates the early stage of most spontaneous fermentations. Thermophilic LAB such as *Lactobacillus bulgaricus*, *Lactococcus lactis,* and/or *Lactobacillus Helveticus* and the coccus-shaped bacterium *Streptococcus thermophilus* with an optimum temperature around 40^{o}C, is used in the manufacturing of fermented foods and milk (Thunell and Sandine, 1985; Caplice and Fitzgerald, 1999). Plant materials containing fermentable sugar provide suitable substrates for the yeast species such as *Saccharomyces, Candida, Torula,* and *Hansenula*. Although the growth rate of these yeasts is lower than that of bacteria, such as *Lactobacillus mesenteroides*,

strain of *Saccharomyces cerevisiae* eventually dominates most spontaneous alcoholic fermentations as in the production of beer and wine (Holzapfel, 2002).

The LAB are well known for their acidification of the FP (Hammes *et al*, 1990). They improve safety, shelf life and stability of the product and inhibit the growth of spoilage and pathogenic bacteria (Lucke, 2000; Holley and Blaszyk, 1998; Caplice and Fitzgerald, 1999) through the competition for nutrients and the presence of inhibitors including organic acids, H_2O_2 and bacteriocins (Ray, 1992). For example, Daly *et al*. (1993) demonstrated the inhibition of the food bone pathogens *Pseudomonas fluorescens, Streptococcus aureus* and *Clostridium perfringens* by inoculation with the starter *Streptococcus diacetylactis*. Most of these genera have one thing in common: they produce lactic acid during their growth in the food products (*Propionibacteria* is the exception). The result is that most cultured foods taste sour (Gilliland, 1985a). The common starter cultures used in the fermented product are summarized in Table 2.3.

Table 2.3: Fermented foods, beverages and their associated LAB

Types of fermented products	Types of LAB
Dairy product	
-Cheese	*Lactococcus lactis, Lactobacillus delbruekii, Lactobacillus helveticus, Lactobacillus casei, streptococcus thermophilus*
-Yoghurt	*Lactobacillus delbruekii, streptococcus thermophillus*
-Fermented milk	*Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus johnsonii*
Fermented meats	
-Fermented sausage	*Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus alimentarius, Pediococcus pentosaceus*
-Fermented fish products	*L. alimentarius, Carnobacterium piscicola*
-Fermented pork products	*Pediococcus cerevisiae, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus salivarius, Pediococcus pentosaccus*
Fermented vegetables	
-Sauerkraut	*Leucnostoc mesenteroides, Lactobacillus plantarum, Lactobacillus brevis, Pediococcus acidilactici*
-Pickles	*Leuconostoc mesenteroides, Pediococcus cerevisiae, Lactobacillus brevis, Lactobacillus plantarum*
-Kimchi	*Leuconostoc mesenteroides, Lactobacillus plantarum, Lactobacillus brevis*
-Fermented olive	*Leuconostoc mesenteroides, Lactobacillus pentosus, Lactobacillus plantarum*
-Fermented vegetables	*Pediococcus acidilactici, Pediococcus pentosaceus, Lactobacillus plantarum, Lactobacillus fermentum*
Fermented cereals	
-Sourdough	*Lactobacillus sanfransiscensis, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus amylovorus, Lactobacillus reuteri, Lactobacillus pontis, Lactobacillus panis, Lactobacillus alimentarius, Weisella cibaria*
Alcoholic beverages	
-Wine	*Oenococcus oeni*
-Rice wine	*Lactobacillus sakei*

Source: Leroy and de Vuyst, (2004).

2.3.3 Lactic acid bacterial (LAB) fermentation

Lactic acid fermentation is a natural process regulated by LAB. These bacteria are naturally present in raw materials, or derived from a starter culture (Nout and Motarjemi, 1997). The LAB fermentation is also a method of food or feed preservation in which spoilage and pathogenic organisms, such as yeast, molds, enterobacter and clostridia are inhibited. The inhibitory effect on the growth of spoilage microorganism is mainly due to undissociated acid molecules. On the other hand, this is also a result from the lowered oxidation-reduction potential and competition for essential nutrients, through the production of inhibitory compounds (Bonestroo *et al.,* 1993).

The LAB are usually facultative anaerobes. Through the fermentation process, a carbohydrate is degraded (or fermented) into two-triose carbon molecules. These are further, degraded to a number of 1-, 2-, 3- and 4- carbon compounds. The characteristic end products of bacterial fermentation are mainly lactic acid (Vereecken and van Impe, 2002), some may produce acetic acid, formic acid, lactic acid, succinic acid, propionic acid, butyric acid, ethanol, isopropanol, butanol, acetylmethylcarbinol (acetoin) and CO_2, (Bottazzi, 1988).

The LAB can be divided further into two subgroups: the homolactic and the heterolactic fermenter. The homofermentative group comprised of *Lactococcus, Pediococcus, Enterococcus, Streptococcus* and some Lactobacilli (Ross *et al*.,

2002). The homolactic acid or simple LAB degrade glucose, via the Embden-Meyerhof pathway to convert one mole of glucose into two moles of lactate (Caplice and Fitzgerald, 1999). In homofermentative lactic acid fermentation, the predominant product is lactic acid, which has a limited preservation effect that is dependent on the pH value (Bottazzi, 1988; Holzapfel, *et al*., 1995).

The heterolactic fermenters are sometimes referred to as mixed lactic acid fermenters. These organisms may catabolize glucose by one of three degradation pathways (Embden-Meyerhof, Pentose Shunt or the Entner-Doudoroff process) to yield characteristic end products comprising of lactic acid plus acetic and formic acids, ethyl alcohol (ethanol), CO_2 and glycerol. Members of this group include *Leuconostoc, Weisella,* and some lactobacilli (Ross *et al*., 2002). The homofermentative and heterofermentative pathways are shown in Figure 2.1.

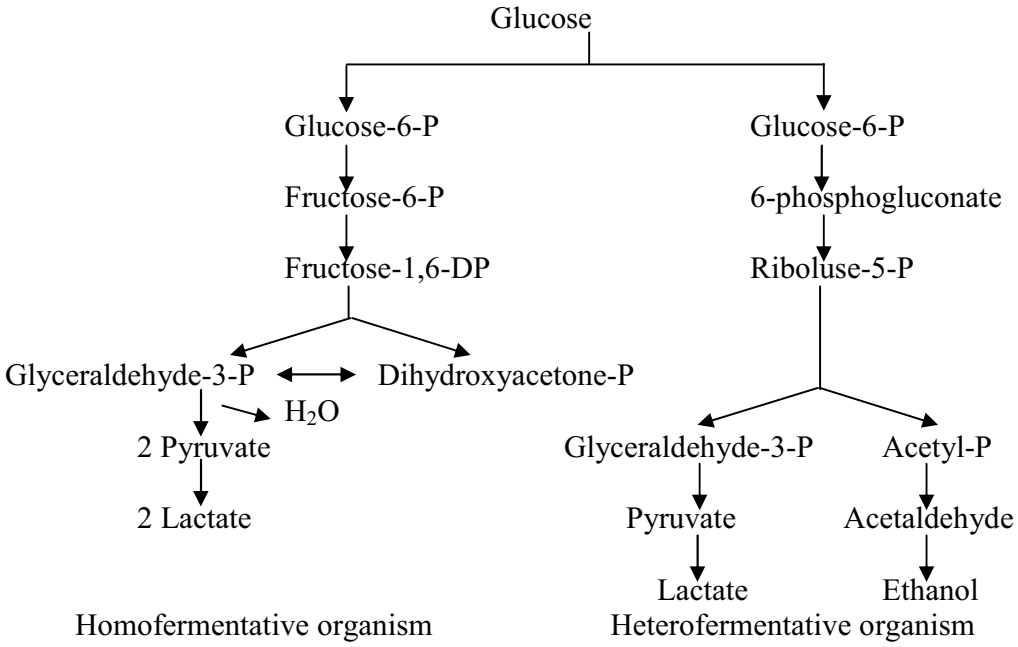

Figure 2.1: Simplified major pathways of glucose fermentation in LAB. Source: Compiled from Caplice and Fitzgerald, (1999); Liu, (2003).

On the other hand, a single bacteria species (such as *Lactobacillus brevis, Lactobacillus fermentum, Lactobacillus reuteri, Leuconostoc mesenteroides*) may exhibit both homolactic and heterolactic acid fermentation and they are known as facultative heterofermenters. These microorganisms usually ferment hexoses homofermentatively into lactic acid (Vuyst and Vandamme, 1994). However under special conditions, substrates are converted to lactic acid, CO_2 and ethanol (or acetic acid). Acetic acid production occurs under conditions where NAD^+ can be regenerated without the formation of ethanol, for example through the reduction of fructose or molecular oxygen. Pentoses are fermented into lactic and acetic acid via a phosphoketolases. The controlling factor is influenced by the pH and substrate

present for bacterial activity (Ross *et al*., 2002). The examples of the homofermentative and heterofermentative LAB are given in Table 2.4.

Table 2.4: Homo- and heterofermentative LAB

Homofermentative		Heterofermentive	
Lactobacillus spp.		*Lactobacillus*	
Lactobacillus acidophilus	*Lactobacillus alimentarius*	*Lactobacillus brevis*	*Lactobacillus buchneri*
Lactobacillus bulgaricus	*Lactobacillus casei*	*Lactobacillus cellobiosus*	*Lactobacillus coprophilus*
Lactobacillus coryniformis	*Lactobacillus curvatus*	*Lactobacillus fermentum*	*Lactobacillus hilgardii*
Lactobacillus delbrueckii	*Lactobacillus Helveticus*	*Lactobacillus sanfrancisco*	*Lactobacillus trichoides*
Lactobacillus jugurti	*Lactobacillus jenseii*	*Lactobacillus fructivorans*	*Lactobacillus pontis*
Lactobacillus lactis	*Lactobacillus leichmannii*		
Lactobacillus plantarum	*Lactobacillus salivarius*	*Leuconostoc* spp.	
		Leuconostoc cremoris	*Leuconostoc dextranicum*
Pediococcus spp.		*Leuconostoc lactis*	*Leuconostoc mesenteroides*
Pediococcus acidilactici	*Pediococcus cerevisiae*	*Leuconostoc gelidum*	*Leuconostoc carnosum*
Pediococcus pentosaceus	*Pediococcus damnosus*	*Leuconostoc citreum*	*Leuconostoc fallax*
Pediococcus dextrinicus	*Pediococcus inopinatus*	*Leuconostoc argentinum*	
Pediococcus parvulus		*Leuconostoc pseudomesenteroides*	
Tetragenococcus spp.		*Carnobacterium* spp.	
Tetragenococcus halophilus	*Tetragenococcus muriaticus*	*Carnobacterium divergens*	*Carnobacterium mobile*
		Carnobacterium gallinarum	*Carnobacterium pisicola*
Streptococcus spp.			
Streptococcus bovis	*Streptococcus thermophilus*	*Weisella* spp.	
		Weisella confusa	*Weisella hellenica*
Lactococcus spp.		*Weisella halotolerans*	*Weisella kandleri*
Lactococcus Lactis	*Lactococcus garvieae*	*Weisella minor*	*Weisella viridescens*
Lactococcus plantarum	*Lactococcus raffinolactis*	*Weisella paramesenteroides*	
Vagococcus spp.		*Oenococcus* spp.	
Vagococcus fluvialis	*Vagococcus salmoninarum*	*Oenococcus oeni*	

Source: Jay, (2000).

2.3.4 Fermentation end products

The LAB produce a wide variety of antimicrobial compounds during fermentation (de Vuyst and Vandamme, 1994; Caplice and Fitzgerald, 1999), including low molecular weight metabolites such as CO_2, H_2O_2, diacetyl, organic acids (lactic acid, acetic acid, formic acid, phenyllactic acid, caproic acid), alcohols and high molecular weight metabolites included polysaccharides, fatty acid and bacteriocins (Adam and Nicolaides, 1997; Vereecken and van Impe, 2002; Leroy and de Vuyst, 2004). Recent observations, however, confirmed that a number of metabolites, such as acetic acid (end product of heterofermentative fermentation), H_2O_2, and bacteriocins, produced during the fermentation process, exhibit antimicrobial properties, which may contribute to the feed safety of lactic fermented products (Holzapfel, 2002). Obviously, each antimicrobial compound produced during fermentation provides an additional hurdle for pathogens and spoilage bacteria to overcome before they can survive and/or proliferate in a food, from time of manufacture to time of consumption. This antimicrobial activity can contribute in a number of ways towards improving the quality of fermented foods, for example, through the control of pathogens, extending shelf life and improving sensory qualities (Sullivan *et al*., 2002). Therefore, lactic fermented products in particular are considered to be safe and wholesome. Acidification of pH values to less than 4.2 indicates a safer FP as pathogenic bacteria could not survive at this pH (Holzapfel, 2002).

2.3.4.1 Organic acids

Organic acids are generally thought to exert their antimicrobial effect by interfering with the maintenance of cell membrane potential, inhibiting active transport, reducing intercellular pH and inhibiting a variety of metabolic functions (Doores, 1993). Organic acids, which show strong anti-bacteria effects in the dissociated form at lower pH values, are particularly effective in inhibiting gram-negative bacteria, such as pathogens (Holzapfel, 2002). One good example is propionic acid produced by *Propionibacterium freudenreichii* subsp. *shermanii*, given its antimicrobial action against microorganisms including yeast and moulds (Daeschel, 1989). Propionic and acetic acids are produced in trace amounts by a wide variety of LAB. Most LAB are able to convert malate to lactate without the formation of pyruvate as an intermediate compound, which is commonly known as malolactic fermentation (Kunkee, 1991). Beside malate, most LAB can catabolise citrate (Liu, 2003).

Oxygen availability undoubtedly plays an important role in the control of the production of acetic acid in *Leuconostocs* (Lucey and Condon, 1986). Although acetic acid is widely used as food additive, it is not usually derived from LAB (Rubin, 1978; Adams and Hall, 1988). Acetic acid contributes to the aroma and prevents mould spoilage in sourdough (Messens and de Vuyst, 2002). It has the ability to inhibit yeast, moulds and bacteria (Caplice and Fitzgerald, 1999). Commercial production of lactic acid by fermentation began in 1881 and today 50%

of industrial lactic acid is produced by fermentation using *L. delbrueckii*. Lactic acid is the major metabolite produced by LAB during the end of carbohydrate catabolism. It is responsible for significant pH changes in their growth environment. Lactic acid is produced particularly from pyruvate by lactic dehydrogenase in order to regenerate pyridine nucleotides necessary for sugar breakdown.

Lactic acid can be formed either via the Embden-Meyerhof pathway, the bifidus pathway or by the 6-phosphogluconate or pentose phosphate pathway (Kandler, 1983; Condon, 1987). Lactic acid concentrations found in many fermented products could be sufficient to impact their observed microbiological stability. However, the effects of other inhibitory systems are difficult to define (Earnshaw, 1992). On the other hand, Tramer (1996) observed a profound inhibitory effect of lactic acid in fermented products on a variety of gram-negative spoilage bacteria. Some fermented foods rely on the antimicrobial action of these acids in combination with lactic acid. It is known that acetic acid has a synergistic effect against bacteria in the present of lactic acid (Rubin, 1978; Adams and Hall, 1988).

2.3.4.2 Other anti-microbial metabolites

The other antimicrobial products from LAB fermentation included Bacteriocin, H_2O_2, CO_2, ethanol, Diacetyl 2,3-butanedione. Bacteriocins are polypeptide anti-microbial protein or protein complexes antagonistic against bacteria competing with the producer organism (Klaenhammer, 1988; Adam and Nicolaides, 1997;

Holzapfel, 2002). Proteolytic enzymes present in food substrate are capable of inactivating bacteriocins. Bacteriocins may be produced early or late in the growth cycle and can be either cell bound or released extracellularly. Bacteriocins are ribosomally synthesized antimicrobial compound that are produced by many different bacteria species including many members of LAB (Klaenhammer, 1988; Jack *et al*., 1995). Nisin is produced by *Lactococcus lactis* subsp. *Lactis* (Tagg *et al*., 1976). Bacteriocinogenic LAB have been shown to effectively inhibit the growth of most gram-positive foodborne pathogens such as *Streptococcus Aureus, clostridium, bacillus spp.,* and *Listeria monocytogenes* (Holzapfel *et al*., 1995; Adam and Nicolaides, 1997; Caplice and Fitzgerald, 1999; Holzapfel, 2002). Inhibition of *Escherichia coli* and *Salmonella* are observed under conditions that disrupt the outer membrane of target organisms at low pH and high salt concentrations (Ross *et al*., 2002).

The H_2O_2 is produced during the aerobic growth stage. Hydrogen peroxide (H_2O_2) has a strong oxidizing effect on membrane lipids and cellular proteins (Condon, 1987; Caplice and Fitzgerald, 1999). The H_2O_2 is produced by a wide variety of catalase negative LAB (Condon, 1983; Thomas and Pera, 1983). The roles of LAB-derived H_2O_2 in food preservation is limited because of the many complex-controlling factors such as the availability of oxygen and the presence of varying levels of superoxide dismutase and catalase (Simonetti *et al.,* 1982).

The CO_2, formed from heterolactic fermentation can directly create an anaerobic environment. It is toxic at high concentration to aerobic food microorganisms such as moulds. Moulds and oxidative gram-negative bacteria are most susceptible while lactobacilli and yeast are highly tolerant. CO_2 acts on cell membranes and has the ability to reduce intracellular pH thereby inhibiting the growth of foodborne pathogens (Adam and Nicolaides, 1997; Caplice and Fitzgerald, 1999; Holzapfel, 2002). Ethanol is a well established anti-microbial compound. It may contribute significantly in the early stage of natural fermentation when heterofermenters are most active. However the fermentation condition will change from the initial aerobic to anaerobic as the oxygen is being utilized by the bacteria. Thus the concentration being produced by heterofermentative LAB at the initial stage is low so that the contribution is minimal (Adam and Nicolaides, 1997).

Diacetyl (2,3-butanedione) is produced by strains within all genera of LAB (Lindgren and Dobrogosz, 1990) (Figure 2.2). It is a product of citrate metabolism and is responsible for the production of aroma and flavour in the product. Low concentration of Diacetyl (2,3-butanedione) can be an effective anti-microbial, particularly at low temperature. It is active against *Aeromonas hydrophilia, Bacillus* spp*., Enterobacter aerogenes, Escherichia coli, Mycobacterium tuberculosis, Pseudomonas* spp*., Salmonella* spp*., Staphylococcus aureus and Yersinia enterocolitica.* The mode of action is believed to be due to interference with the utilization of arginine (Caplice and Fitzgerald, 1999).

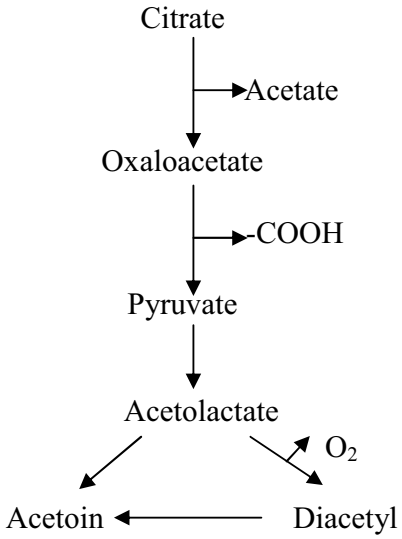

Figure 2.2: The general metabolic pathway for the generation of Diacetyl by LAB
Source: Hansen, 2002; Liu, 2003.

2.3.5 Application of fermented products in animal feeds

Fermented products (FP) are one of the probiotic supplements used as animal feed additives. Loh *et al*. (2003a,b) reported that feeding FP and fermented fruits containing *Lactobacillus*-probiotics to rats and piglets successfully reduced the *Enterobacteriaceae* and increased the LAB population in the GIT. The reduction of faecal *Enterobacteriaceae* as a result of feeding lactobacilli probiotics in pigs of different age groups have also been reported by other workers (Mikkelsen and Jensen, 1998; van Winsen *et al*., 2002; Demecková *et al*., 2002). It is possible to reduce the faecal *Enterobacteriaceae* counts by using fermented feed or lactic acid (Cole *et al*., 1968). Heres *et al*. (2003a) reported that broilers fed fermented liquid feeds had very much higher resistance to *S. enteritidis* challenge. It was found that a

total of 10^7 cfu/g of *Salmonella* was needed to infect chickens fed with fermented liquid feeds whereas only 10^3 cfu/g was sufficient to infect the negative control birds.

2.4 Gut microflora and probiotics

The gastrointestinal microflora consists of hundreds of different types of microorganisms and is biologically important to the host. These microorganisms have significant impacts on animal health, growth development, performance, waste management and even food borne pathogens (Frankin *et al*., 2002; Dibner and Richards, 2005). The intestinal microflora population is a complex ecosystem composed of a species-diverse group of microflora, predominantly gram-positive bacteria (Dibner and Richards, 2005). For farm animals, the most important purpose in using FP, which contain probiotics is the idea that they influence the gut beneficial microflora resulting in growth promotion of overall GIT health while suppressing pathogenic organisms in young and growing animals. It helps in pre-digestion of anti-nutritional factors, such as phytic acid, glucosinolates, trypsin inhibitors, lectin and non-starch polysaccharides (dietary fibre).

The most commonly used probiotics are strains of LAB (e.g., *Lactobacillus, Bifidobacterium* and *Streptococcus*). Directly fed microbes (lactobacilli-probiotics) have been utilized to improve the health status and performance by modulating and/or maintaining the gut microflora (Holzapfel *et al*., 1998; Jin *et al*., 1998a,b,

2000). The LAB are able to colonize and thrive in the host as they are able to resist gastric acid, bile salts and pancreatic enzymes. The LAB adhere to intestinal mucosal and readily colonize the intestinal tract. They are considered important components of the gastrointestinal flora and are harmless in a healthy animal (Silva *et al*., 1987).

2.4.1 Microbial population and the gut ecosystem

Compared to the colonic microflora, the gut microflora undergoes distinct quantitative variation and seems to be a good qualitative indicator of the distal colonic microflora (Holzapfel *et al*., 1998). In poultry, the ceca are the major site of microbial digestion of carbohydrates and proteins leaving the small intestine. Poultry caecum are colonized by abundant of bacterial flora. Table 2.5 shows the estimated populations of microflora in the alimentary canal of poultry.

Table 2.5: Microflora population of the GIT and faeces in chickens

Number of viable microorganisms/gram of contents[a]	Stomach		intestine		Caecum	Faeces
	Crop	Gizzard	Upper	Lower		
Total	6	6	8-9	8-9	8-9	8-9
Anaerobes	3	5-6	<2	<2	8-9	7-8
Enterobacteriaceae[b]	6	2	1-2	1-3	5-6	7-8
Steptococci	2	<2	4	3-5	6-7	6-7
Lactobacillus spp.	5-6	2-3	8-9	8-9	8-9	6-7
Clostridium perfringens	<2	<2	<2	<2	1-2	2

[a] Expressed as log $_{10}$ of the number of organisms cultured.
[b] Mainly *Escherichia coli.*
Source: Hirsh, (1990).

2.4.2 Functions of the intestinal flora

The intestinal microflora plays an important role in the development of organs, tissues and the immune system (Holzapfel *et al*., 1998). The microflora also provides both nutritional constituents and protection to the host animals in the form of fermentation end products such as volatile fatty acids (VFA), amino acids and vitamins (Salminen *et al*., 1998). The most important factor that determines the composition of the gut microflora is the ability of it to compete for limiting nutrients. For those which are unable to compete being effectively eliminated from the system.

LAB especially *Lactobacillus* spp. are traditionally included in probiotic products to help protect against the disruption of the colonic flora which cause by the pathogens, dietary antigens or other harmful substances (Fook and Gibson, 2002). These bacteria produce antimicrobial compounds prevent the colonization of pathogens (Dibner and Richards, 2005). Gut microflora stimulate the development of the intestinal host defense, including the mucus layer with its system of immune cells that underlie the epithelium. This mucus layer segregates both normal and pathogenic microbes away from the animal epithelium tissues (Dibner and Richards, 2005).

2.4.3 Factors modulating the avian gut microflora

The avian gut microflora comprised of a dynamic population mix which is constantly affected by intrinsic and extrinsic factors. Stress, age, feeding programmes and diets can have an effect on the intestine microflora, usually with a reduction of lactobacilli levels (Jin *et al*., 1998a,b, 2000; Timmerman *et al*., 2004). There had been many attempts to manipulate the intestinal microflora in order to maximize the performance and production of the host animal. The common method is through dietary supplements in order to obtain the desirable microflora (Holzapfel *et al*., 1998). Many products including antibiotics, organic acids, probiotics, prebiotics, trace minerals, enzymes, herbs and spices, fermented feed and others are used with the goal of altering the microflora for the benefit of animal health and production (Jin *et al*., 1998a,b, 2000; Loh *et al*., 2003a,b; Dibner and Richards, 2005). Antibiotics have been used widely for the past few decades. However, the development of bacteria resistance has prohibited the use of antibiotics in European countries (Patterson and Burkholder, 2003; Dibner and Richards, 2005). Therefore, non-therapeutic alternatives such as fermented liquid feeds have been successfully used to modulate the avian and swine gut microflora (Mikkelsen and Jensen, 1998; van Winsen *et al*., 2002; Demecková *et al*., 2002) without any adverse effect.

2.4.4 Roles of VFA on the caecal microflora

The VFA such as acetate, butyrate and propionate are highly prevalent anions of the by-products of commensal bacteria in chicken intestines. They are known to stimulate gut epithelial cell proliferation and villus size, thereby increasing the absorptive surface area. In poultry, caecal bacteria ferment available nutrient to a mixture of 2-5-carbon VFA, ammonia (NH_3), CO_2 and methane (CH_3). The VFA are absorbed and utilized by the animal, contributing significantly to the energy supply of the animal (Annison *et al*., 1968; Johansson *et al*., 1998). The nutritional significance of poultry caeca is limited due to its small volume. However, the undissociated forms of VFA played an important role in reducing the number of pathogenic bacterial species in the caecum (van der Wielen *et al*., 2001).

Barnes *et al*. (1980) reported that caecal VFA concentrations are indicators of anaerobe growth. Nisbet *et al*. (1994, 1996) have confirmed this fact. There was a negative correlation between caecal VFA concentrations, especially propionic acid, in 3-day-old chicks with the establishment of anaerobe caecal microflora and protection against *Salmonella typhimurium* colonization. Because of its antibacterial properties, propionic acid was proposed as a prophylactic treatment for control of salmonella infection in poultry.

2.5 The nutrient composition of eggs

Eggs are one of the few foods that are used throughout the world. It is an important part of the human diet due to its nutritional significance (Gonzalez-Esquerra and Leeson, 2000; Milinsk *et al*., 2003; Carrillo-Dominguez *et al*., 2005). Eggs are rich in high quality protein. It is an important source of essential fatty acids, iron, phosphorus, trace minerals, vitamin A, E, K and the B vitamins, including B_{12} and other vital nutrients (Surai and Sparks, 2001; Kim *et al*., 2004; Chowdhury *et al*., 2002, 2005). The yolk contributes most or all of the fat, iron, vitamin A value, thiamine and calcium, protein and riboflavin. Most of the protein and riboflavin is in the albumin (Cook and Briggs, 1973). However, people often limit their egg consumption on grounds that the high cholesterol content of eggs is often associated with coronary heart disease (Simopoulos, 2000; Bragagnolo and Rodriguez-Amaya, 2003; Carrillo-Dominguez *et al*., 2005). The reported values for nutrient compositions of egg as cited by Watkins (1995) is presented in Appendix 1 (page 125).

2.5.1 Fatty acid composition and cholesterol content of eggs

The fatty acid and cholesterol content of egg yolks from several studies are summarized in Tables 2.6 and 2.7. A 100 g edible portion of whole egg contains 3.35 g saturated fatty acids (SFA), 4.46 g monosaturated fatty acids and 1.45 g polyunsaturated fatty acids (PUFA). The predominant SFA is palmitic acid,

whereas oleic and linoleic acids are the major monounsaturated and PUFA, respectively.

2.5.3 Effects of dietary manipulation on the nutritional content of eggs

Many factors influence the concentration of nutrients in eggs. These include age, breed and strain of hens, diets and others. The types and levels of lipid in the diet of the hens influence the fatty acid composition of yolk lipid significantly (Milinsk *et al*., 2003; Carrillo-Dominguez *et al*., 2005). Studies have shown that palmitic and stearic acids are not affected by dietary fatty acid alterations. The total amount of palmitic and stearic acids in yolk lipids is generally between 30 and 38%, respectively (Privett *et al*., 1962). The concentration of total SFA ranged from 40% to 44% (palmitic, stearic and myristic) and 56-60% are unsaturated fatty acids (oleic, linoleic). Recently increasing number of work is focusing on increasing the n-3 fatty acids content of eggs (Meluzzi *et al*., 2000; Bean and Leeson, 2003). Since the chicken is monogastric, much of the dietary lipid is assimilated directly with minimal modification. The summary of the type of dietary intervention and their effects on the fatty acid and cholesterol contents in the egg is as depicted in Tables 2.6 and 2.7.

Table 2.6: Reported values for saturated, monosaturated and polysaturated fatty acid content of egg yolk as a result of dietary interventions

Breeds	Supplements/ Diets	Fatty acids (%)			References
		SFA	MUFA	PUFA	
Single Comb White Leghorn	5% seal blubber oil	35.94	47.93	15.88	Schreiner *et al.*, 2004
Single Comb White Leghorn	5% mixture of seal blubber oil and tallow	36.12	48.84	15.04	Schreiner *et al.*, 2004
Single Comb White Leghorn	5% tallow	36.24	50.05	13.71	Schreiner *et al.*, 2004
Single Comb White Leghorn	5% tallow	34.14	50.14	13.15	An *et al.*, 1997
Single Comb White Leghorn	5% mixture of Safflower oil and palm oil	35.92	35.26	28.66	An *et al.*, 1997
Single Comb White Leghorn	5% Crude safflower phospholipids	36.24	35.14	28.74	An *et al.*, 1997
Single Comb White Leghorn	5% purified safflower phospholipids	36.22	38.80	24.95	An *et al.*, 1997
Isa Brown	30% Chia (*Salvia hispanica*) diet	29.71	35.63	34.29	Ayerza and Coates, 1999
Red Lohman	3.2% Canola oil	32.21	47.33	20.39	Milinsk *et al.*, 2003
Red Lohman	3% Flaxseed oil	31.82	47.04	20.99	Milinsk *et al.*, 2003
Red Lohman	3% Soybean oil	33.13	44.61	22.15	Milinsk *et al.*, 2003
Red Lohman	2.9% Sunflower oil	32.48	41.88	25.45	Milinsk *et al.*, 2003
White Lohman	3.2% Canola oil	37.73	42.88	20.39	Milinsk *et al.*, 2003
White Lohman	3% Flaxseed oil	33.64	41.50	24.26	Milinsk *et al.*, 2003
White Lohman	3% Soybean oil	33.04	39.86	27.00	Milinsk *et al.*, 2003
White Lohman	2.9% Sunflower oil	38.32	33.29	28.17	Milinsk *et al.*, 2003
White Leghorn	*Camelina sativa* seed oil	33.14	41.50	21.44	Rokka *et al.*, 2002

SFA: saturated fatty acids
MUFA: monosaturated fatty acids
PUFA: polyunsaturated fatty acids

Table 2.7: Reported values for cholesterol content of egg yolk

Breeds	Supplements	Cholesterol (mg/100g)	References
Hy-Line® W-36 White Loghorn	0.125% Culpric sulfate pentahydrate	820	Pesti and Bakalli, 1998
Hy-Line® W-36 White Loghorn	0.250% Culpric sulfate pentahydrate	770	Pesti and Bakalli, 1998
Isa Brown	30% Chia (*Salvia hispanica*) diet	35%	Ayerza and Coates, 1999
Single Comb White Leghorn	5% tallow	1320	An *et al*., 1997
Single Comb White Leghorn	5% mixture of Safflower oil and palm oil	1280	An *et al*., 1997
Single Comb White Leghorn	5% Crude safflower phospholipid	1270	An *et al*., 1997
Single Comb White Leghorn	5% purified safflower phospholipid	1280	An *et al*., 1997
Barred Plymouth Rock	-	1330	Maurice *et al*., 1994
New Hampshire	-	1520	Maurice *et al*., 1994
Rhode Island Red	-	1300	Maurice *et al*., 1994
White Leghorn	-	1150	Maurice *et al*., 1994
Isa Brown	0.03% Lovastatin	1123	Kim *et al*., 2004
Isa Brown	0.06% Lovastatin	1159	Kim *et al*., 2004
Isa Brown	0.03% Simvastatin	1079	Kim *et al*., 2004
Isa Brown	0.06% Simvastatin	1064	Kim *et al*., 2004
Isa Brown	0.03% Pravastatin	1074	Kim *et al*., 2004
Isa Brown	0.06% Pravastatin	1083	Kim *et al*., 2004

2.6 The digestion and absorption of fatty acids in monogastrics

The assimilation and absorption of fatty acids involve a series of integrated and coordinated steps as illustrated in figure 2.3. The initial step in lipid digestion is emulsification, which is achieved partly vigorous mechanical mixing in the stomach (Ros, 2000). The next step is micellar solubilisation with the help of bile. In monogastrics, the post digestion mixed micelle contains mainly monoacylglycerol, lyphophospholipids and fatty acids (Gurr and Harwood, 2002). Transfer of free fatty acids to the micellar phase take place gradually during the transit of the digesta through the small intestine which is facilitating by the polar detergency of bile and increasing pH of the digestive environment in the small intestine. The enterocytes along the epithelium of small intestines are responsible for the absorption of luminal fatty acids and lipids. The digested lipids pass from the mixed micelles into the enterocytes by passive diffusion (Ros, 2000). Luminal fatty acids are absorbed as free fatty acids or esterified to 2-monoacylglycerol. After acylation, the majority of fatty acids are incorporated into triacylglycerol via the monoacylglycerol pathway or the phosphatidic acid pathway (Levin *et al*., 1992).

The final step is chylomicron formation. Chylomicrons are the largest lipoproteins. They are secreted through the basolateral membrane of the enterocytes, enter the lymphatic capillaries of the intestine microvilli and eventually reach the systemic circulation (Ros, 2000). As for the short and medium chain fatty acids, it is widely accepted that C2:0 to C12:0 fatty acids are transported to the liver via the portal

system and C12:0 to C24:0 via the lymphatic system (Akoh, 1998). The liver is an important site for the elongation and desaturation of many fatty acids (Li *et al*., 1992). It generate energy by metabolizing those fatty acids with chain lengths lesser than 12 carbons (Gurr and Harwood, 2002).

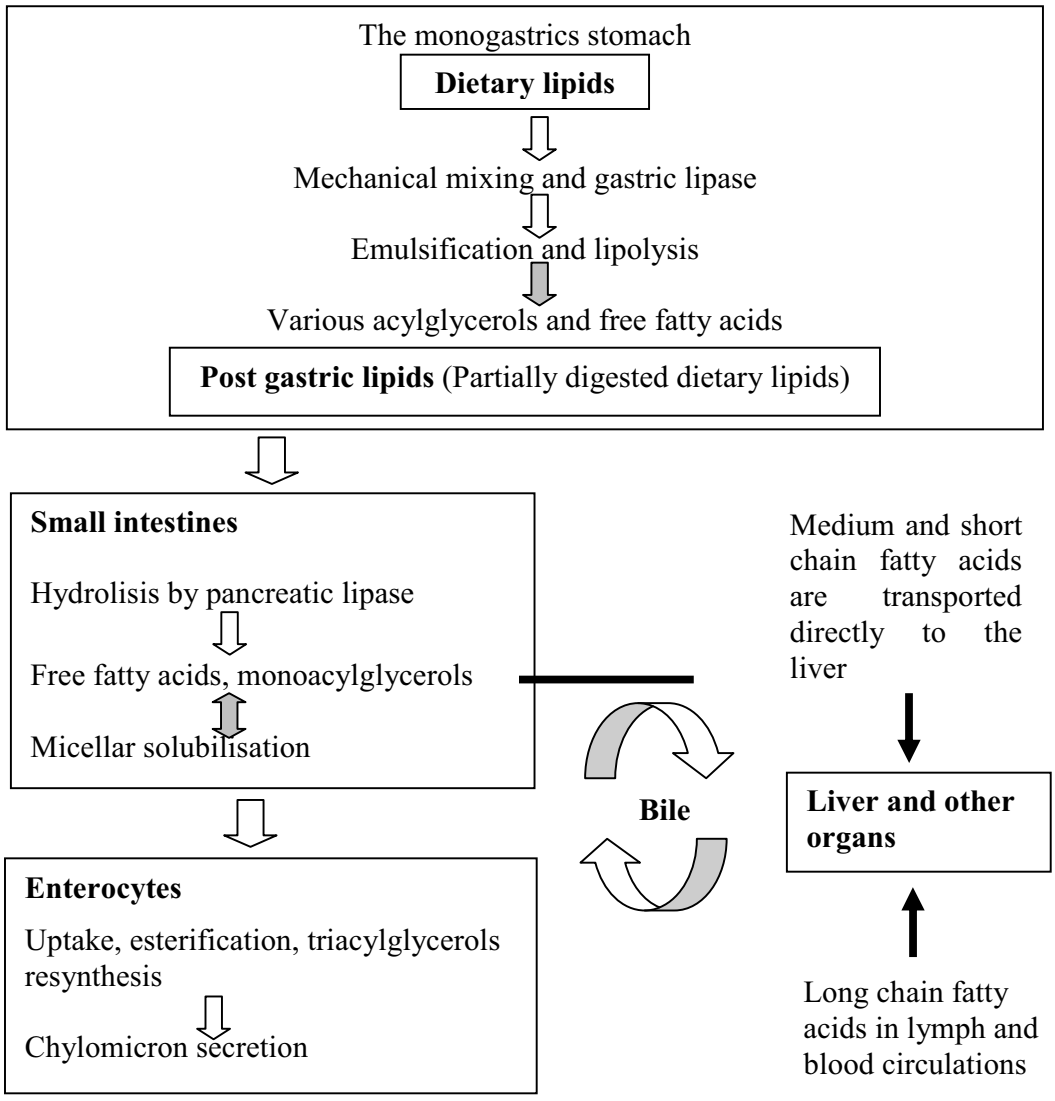

Figure 2.3: Simplified schematics showing the digestion, assimilation and transport of fatty acids in monogastrics
Source: compiled from Ros, 2000; Gurr and Harwood 2002.

2.7 Literature summary

The practice of feeding antibiotics have been very successfully adopted and become an integral part of developing nutritional strategies for all farm animals. However, the use of antibiotic growth promoters has come under increasing scrutiny because of the issue of antibiotic resistance in certain bacteria and residues in animal products. This can be harmful to consumers and has lead to growing public concern about the potential risk of antibiotic cross-resistance from animal products to humans. Currently, alternatives such as probiotic, prebiotic, organic acid, fermented liquid feed have been proven to improve the health status and growth performance of the farm animals. The health of the animal is much influenced by the intestinal micro-flora. The symbiotic relationship between the host and its beneficial intestinal micro-flora became the critical component in the development of good nutritional strategies. The *in-vivo* studies by Loh *et al*. (2003a,b) have demonstrated a promising result as mentioned in Section 2.3.5 (page 26). The FP produced in this study (Chapter III) was a combination of probiotic, prebiotic, organic acids with the addition of the essential fatty acids such as docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA). A product which comprises a combination of different types of alternative may exceed the results of a single alternative and can lead to significant improvements in animal production. This study aimed to investigate the effects of FP on layer performance and egg quality, which has not been previously reported.

CHAPTER III

GENERAL APPROACH OF THE STUDY AND EXPERIMENTAL PROCEDURES

3.0 General research approach

This study was conducted in two phases. Phase I was the microbiological and physico-chemical characterization of the FP. The fermentation process was repeated three times under the same experimental conditions and using the same amount of raw materials to validate the stability of the end product. This product was then used in Phase II to feed Babcock B380 pullets.

The FP was incorporated at 3% (w/w), 6% (w/w) and 9% (w/w) in the diet based on earlier studies by Loh *et al*., (2003a,b). In this phase, faecal sampling was collected at fortnightly intervals right after a two-week of adjustment period. Faecal characteristics such as faecal LAB and *Enterobacteriaceae*, pH and VFA were evaluated. Total feed intake, egg production and egg weight were recorded. Eggs were randomly selected for cholesterol and fatty acids profile analyses during the last five weeks of the trial. At the end of the trial, five birds from each treatment were randomly selected and sacrificed for blood plasmid lipid analyses.

3.1 The production of fermented product (FP)

The fermentation process was performed according to the method described by Loh *et al*. (2003a). The raw materials consisted of 9% (w/w) lime, 1% (v/w) molasses, 53.5% (w/w) rice bran, 35% (w/w) fish (*Rastrelliger kanagurta*), 1% (v/w) vinegar, and 0.5% (w/w) starter cultures. The starter culture was a combination of LAB and was supplied by Jia Yi Nutritional Sdn. Bhd (Klang, Malaysia). Lime was blended with molasses and the mixture was then fermented at ambient temperature (28±2^{o}C) for five days under aerobic conditions. The mixture was then poured into a closed 50-liter solid fermenter together with rice bran, raw fish, vinegar and starter culture. The fermenting mixture was mixed hourly in order to achieve homogenous mixing. The fermentation process was carried out for seven days at 35-38^{o}C. Fermentation was considered completed after seven days. The completed FP was allowed to cool to room temperature prior sampling. The final product was a dry-brownish mash with a distinctive lime-flavoured aroma. The FP was stored in vacuum-sealed black plastic bag until use.

3.2 Proximate analysis

3.2.1 Dry matter (DM)

Dry matter was determined as per AOAC (1990) procedures. Porcelain crucibles were soaked and cleaned with detergent. They were then rinsed with distilled water and dried in the oven at 105^{o}C. The dried crucibles were cooled in a dessicator and weighed to obtain W_1, the dry net weight of crucible. One gram of sample was placed in the pre-weighed empty crucible and the weight of sample and crucible was recorded (W_2). The crucibles were covered with lids and kept in the oven at 105^{o}C until there were no further changes in weight.

After 24 hours of drying, the crucibles were removed from the oven and cooled to room temperature for five to ten minutes. Subsequent cooling took place in the dessicator for 10-15 min before weighing W_3 (weight of crucible + dry sample) was taken. Each sample determination was done in triplicates. The moisture and DM were calculated by using the following formula:

$$\text{Dry matter, \%} = \frac{W_3 - W_1}{W_2 - W_1} \times 100$$

Where, W_1 = Dry weight of crucible

W_2 = Weight of fresh sample + crucible

W_3 = Weight of crucible + dry sample

% Moisture = 100 - DM %

3.2.2 Ash

The dried sample from DM analysis was utilized for ash determination. The crucible with the sample was covered and ignited in the muffle furnace at 600^{o}C for four hours until white, light gray residue was obtained. The crucible and their ash content were weighed immediately after they had cooled down in a dessicator. Each sample was analysed in triplicates. The ash content was calculated using the following formula:

$$\text{Ash, \%} = \frac{\text{Ash weight}}{\text{Sample dry weight}} \text{ X } 100$$

3.2.3 Crude protein

Crude protein (CP) was determined using the 2000 Digestion system and 2400 Kjeltec Auto Analyzer. The digestion system is a part of the Kjeltec system (FOSS TECATOR AB, Höganäs, Sweden). One g of sample was placed in a digestion tube to which one tablet of selenium oxide catalyst (BDH Laboratories, Poole, U.K) was added to facilitate digestion. Twelve mL of concentrated sulphuric acid (H_2SO_4) (Merck®, KGaA, Darmstadt) was then added. The tubes were placed in a tube rack and secured on to the digester. Digestion was carried out at 220^{o}C for 30 min, followed by 380 ^{o}C (40 min) and finally 420 ^{o}C for 40 min. Digestion was completed when the mixture solution was totally colourless. After digestion, the

tubes were placed in the 2400 Kjeltec machine. Two digestion tubes containing 12mL of concentrated sulphuric acid (Merck®, KGaA, Darmstadt) and selenium oxide catalyst (BDH Laboratories, Poole, U.K) were included with each batch of sample to serve as machine blanks. Another two digestion tubes containing 12mL of concentrated sulphuric acid (Merck®, KGaA, Darmstadt), selenium oxide catalyst (BDH Laboratories, Poole, U.K) and one mL of one molar ammonium sulphate (Appendix 2c) acted as standard.

Twenty-five mL of receiver solution (Appendix 2a) was dispensed into the titration vessel from the tank by the pump. Simultaneously, dilution water was dispensed into the digestion tube from the water tank automatically by the pump. Five mL of alkali (40% NaOH) (Appendix 2b) was then dispensed into the sample tube. The sample was subjected to steam distillation. The liberated NH_3 gas was condensed and trapped in the condenser and delivered to the titration vessel containing the receiver solution. The liberated NH_3 was then titrated with 0.2N hydrochloride acid (Merck®, KGaA, Darmstadt) until the end point was reached. The CP content was expressed as 6.25 x Nitrogen (N) content.

3.2.4 Crude fiber

Sintered glass crucibles were soaked in Decon 90 and then scrubbed thoroughly. The crucibles were rinse thoroughly with tap water before drying in the oven at 60^{o}C overnight. The Fibertec 2010 Hot Extractor (FOSS TECATOR AB, Höganäs,

Sweden) was used to determine crude fiber (CF). One g of sample was placed in a sintered glass crucible and secured with a crucible holder placed in the Hot Extraction Unit. Two to four drops of anti-foam agent (Dekalin) were added after solution R1 (1.25% H_2SO_4) was dispensed automatically into the sintered glass crucible. Sample mixture was then allowed to boil for 30 min. The samples were then drained using vacuum pump. Remaining samples were flushed with water until the washings were no longer acidic. Samples were vacuum drained as dry as possible between washing. After that, 150mL solution R2 (1.25% NaOH) together with two to four drops of Dekalin was added to the drained sample. The sample mixture was allowed to be re-boiled for 30 min. After boiling, the drain valve and vacuum pump were turned on to discard the solution from the sintered glass crucible. The residue in the sintered glass crucible was cleaned with water with the aid of a vacuum pump. Lastly, the residue was washed with acetone and vacuum-drained as dry as possible between washings.

The residue in sintered glass crucibles were then dried in oven at 105^{o}C overnight, after which they were removed with tongs and cooled down to room temperature in a dessicator and weighed (W_1). The contents of the sintered glass crucible were then ignited in a muffle furnace at 550^{o}C for four hours, until light gray residue was obtained and weighed (W_2). The CF content of the sample was calculated as follows:

$$CF, \% = \frac{W_1 - W_2}{\text{Sample dry weight}} \times 100$$

Where, W_1 = Sintered glass + residues weight after drying at 105°C

W_2 = Sintered glass + ash weight after ashing at 550°C

3.2.5 Ether extracts (Crude fat)

The 2050 Soxtec Auto Analyzer was used to determine the ether extract (EE) content. Decon 90 (Decon Laboratories Ltd., Sussex, U.K) was used to soak the aluminium flask before scrubbing. The flasks were then rinsed thoroughly with the tap water before oven drying at 60°C. One gram of sample was placed in a cellulose extraction thimble and covered with defatted cotton plug on top of the sample. The thimbles were transferred to the thimble support holder and inserted into the extraction unit. The aluminium flask was then transferred with tongs from the dessicator and weighed (W_1). Then, the aluminium flask was filled with petroleum ether (BDH Laboratories, Poole, U.K) at 3/4 of total volume and connected to the extractor. The extractor was set at 117°C for 20 min of extraction, 10 min for dripping the solvent with fat, 20 min for drying and three min for recovery. During recovery, the valve was closed and the condensed solvent was transferred to the collection vessel. Finally, the aluminium flask was then dried with the fats in oven at 105°C for 30 min and cooled down to room temperature in a dessicator and weighed (W_2).

$$EE, \% = \frac{W_2 - W_1}{\text{Sample dry weight}} \times 100$$

Where, W_1 = Weight of empty aluminum flask

W_2 = Weight of aluminium flask with fat

3.2.6 Gross energy (GE) content

The GE content of samples was determined using an adiabatic bomb calorimeter fitted with digital temperature recorder (1241 Adiabatic Calorimeter, Parr Instrument Co., Moline, III, USA). The amount of heat, that is released when a substrate is completely oxidized in a bomb calorimeter, is called GE. The heat of combustion is usually measured in calories. Enthalpy of combustion of the hydrocarbons typically followed the following chemical reaction.

$$C_xH_yO_z + ½[\ 2x + ½y - z]\ O_2 \rightarrow xCO_2 + ½y\ H_2O$$

The energy equivalent of the apparatus set up was obtained by combusting a known amount of benzoic acid (6.318 kcal/g, Merck KGaA, Darmstadt, Germany) prior to sample runs. A total of 0.5 – 0.7 g (measured to 4 decimal places) of benzoic acid (or sample) was made into a pellet using a pelleting machine. The pellet was placed in a fuel cup and held in a wire cradle suspended from the top of the bomb vessel. Ten cm of nickel alloy fuse wire (NO.45C10 Parr Instrument Co. Moline, IL, USA)

was then mounted on top of the pellet completing the electrical circuit within the oxygen bomb asSEbly. Oxygen was charged into the sealed bomb at 25 atm to reduce the formation of nitric acid during combustion, which was then placed within the water jacket inside the calorimeter.

Ignition leads were attached to the bomb and the water jacket temperature was allowed to stabilize and the initial temperature (T_1) was recorded. The bomb was then ignited electrically. The maximal temperature was obtained after five to six min and recorded (T_2). After that, the oxygen bomb was removed and the high-pressure gas within the bomb was vented slowly. The residue content was washed with distilled water and the washing was collected in a conical flask for titration using Na_2CO_3 (0.4%) solution (3.84 g/L, Hamburg Chemicals Ltd., UK). Acid formation energy (one mL = one cal) was calculated based on the titration volume and the un-combusted fuse was measured for calculation of caloric value of combusted fuse later (one cm = 2.3 cal). The energy equivalent of the bomb calorimeter was calculated as followed:

$$\text{Energy equivalent of bomb (S)} = \frac{(W \times E) + C_1 + C_2}{\Delta T}$$

After the energy equivalent of the bomb has been obtained, the sample runs could commence. Procedures were similar as described for the benzoic acid. The GE content was then calculated based on the following equation as described below

$$GE\ (kcal/g) = \frac{(S \times \Delta T) - C_1 - C_2}{W}$$

Where, S = energy equivalent of bomb (kcal/^{o}C)

E = energy content of benzoic acid (6.318 kcal/g)

C_1 = acid formation energy (from titration volume)

C_2 = energy generated by combusted fuse

ΔT = Final temperature, T_2 – initial temperature, T_1

W = sample weight

3.3 pH determination

Ten percent (w/v) of the sample was mixed homogenously with deionized distilled water in a universal bottle. The pH of the samples was measured with a Mettler-Toledo pH meter with glass electrode (Mettler-Toledo LTD., England). The pH meter was calibrated using standard buffer solutions (Merck®, KGaA, Darmstadt) at pH 4 and pH 7.

3.4 Bacteriological analyses

Ten percent (w/v) of sample was suspended in sterile peptone water (Appendix 3a) and incubated for an hour prior to further ten-fold series dilutions (v/v). In order to obtain a homogeneous sample, the mixtures were vortexed prior to sampling.

Enumerations of LAB were performed on MRS-agar (*Lactobacillus*-Agar DE Man, ROGOSA and SHAPE) (Merck®, KGaA, Darmstadt) (Appendix 3b). The plates were incubated in anaerobic jar at 30°C for 48 h. *Enterobacteriaceae* were counted on EMB-agar (Eosin-methylene-blue Lactose Sucrose Agar) (Merck®, KGaA, Darmstadt) (Appendix 3c) and incubated aerobically for 24 h at 37°C. Numbers of colony forming units (cfu) were expressed as $\log_{10}$ cfu per gram. All samples were performed in triplicates.

3.5 Fatty acid profile determination

3.5.1 Total lipid extraction

Lipids were extracted from the samples using the method of Rajion (1985) as modified by Goh (2002). A weighed amount of sample was homogenized (Ultra Turrax T5 FU homogenizer, IKA Germany) in 40mL of chloroform-methanol (2:1, v/v) in a 50mL stoppered ground-glass extraction flask. The tube was then flushed with N gas before stoppering and shaken vigorously. The mixture was then filtered through a No. 1 Whatman paper (Whatman Int. Ltd., Maidstone, England) into a 250mL separating flask after standing for 12 hours. Five mL of chloroform-methanol 2:1 (v/v) was used to wash the residue left over on the filter paper. Ten mL of normal saline solution (0.9% w/v NaCl) was added to facilitate phase separation. The mixture was then shaken vigorously for one minute and left to stand for four hours. The lower phase solution within the separation flask was collected in

a round bottom flask after completely the phase separation. The liquid phase was then evaporated using rotary evaporation (Heidolph GmBH, Germany) at 70-75^{o}C. Upon drying, the total lipid extract was weighed and then transferred to a capped methylation tube by re-diluting it with fresh chloroform-methanol 2:1 (v/v), making up to five mL.

3.5.2 Preparation of fatty acid methyl esters (FAME)

A known amount of heneicosanoic acid (21:0) (Sigma Chemical Co., St. Louis, Missouri, USA) was added to each sample as an internal standard to quantify individual fatty acid concentration within the sample. The lipid extract was then air-dried on a heating block (40^{o}C) under a steady stream of purified N.

Saponification was done using two mL of 0.66N methanolic potassium hydroxide (KOH) (R & M Chemicals, Essex, U.K.). The saponifying agent was added into the methylation tube after the sample had dried. The methylation tube was flushed with purified N, stoppered and heated in a boiling water bath for 15 min with occasional shaking. The mixture was allowed to cool to room temperature at the end of the 15 min. Two mL of 14% methanolic boron trifluoride (Sigma Chemical Co., St. Louis, Missouri, USA) was added into the methylation tube to facilitate for transmethylation. The mixture was reheated in the boiling water bath for another 30 min with occasional shaking. The sample mixture was allowed to cool to room temperature after the transmethylation process was completed.

Four mL of distilled water and four mL of petroleum ether (BDH Laboratories, Poole, U.K) were then added. The mixture was vortexed for one min before being centrifuged at 1500 G for ten min to hasten phase separation. The upper petroleum phase was transferred to a fresh test tube and washed with one mL of distilled water. The upper phase from this test tube was transferred to a second test tube, which contains 0.5 g of anhydrous sodium sulphate (R & M Chemicals, Essex, U.K.) to dry the sample. Finally, the petroleum ether containing FAME was transferred to a five mL screw-capped vial (Kimble Glass Inc., USA), and flushed with purified N before being stored at four ^{o}C until analysis by gas-liquid chromatography (GLC).

3.5.3 Gas liquid chromatography

FAME separation was performed using a Supelco SP-2330 (Supelco, Inc., Bellefonte, PA), fused silica capillary Column (30m, 0.25mm ID, 0.20μm film thickness) in a 5890 Hewlett-Packard Gas-Liquid Chromatograph (Hewlett-Packard, Avondale, PA). High purity N was the carrier gas at 40 mL/min. High purity hydrogen and compressed air were used for the flame ionization detector. The column temperature was set at the range of 100-190^{o}C with 7.2^{o}C/min increment rate to facilitate optimal separation. The injector and detector were programmed at 220^{o}C and 220^{o}C, respectively. A HP-3993A Integrator (Hewlett-Packard, Avondale, PA) was used to integrate peak areas. Peak areas were expressed as % total fatty acid or absolute fatty acid concentration using a

programmed PC running Microsoft Excel. The identification of the peaks was made by comparison of the equivalent chain lengths (ECL) with those of authentic fatty acid methyl esters (Sigma Chemical Co., St. Louis, Missouri, USA).

3.6 Total cholesterol determination

Half a gram of sample was weighed and transferred into a methylation tube. Ten mL of 2.14M Ethanolic KOH solution (Merck®, KGaA, Darmstadt) was added. The mixture was homogenized thoroughly before the tube was cap-sealed. The mixture was then held for one hour in water bath at 70°C with occasionally shaking. Mixture was then allowed to cool to room temperature.

Upon cooling, the mixture was transferred into a fresh glass centrifuge tube. Ten mL of petroleum ether, five mL of saturated NaCl solution and five mL of distilled water were added into the glass centrifuge tube. Mixture was vortex-mixed for one minute and then centrifuged at 1500 G for 15 min. The upper layer was transferred into a second test tube and re-extracted twice with 10 mL of petroleum ether (BDH Laboratories, Poole, U.K). The mixture was re-centrifuged and the upper petroleum phase was transferred into capped methylation tube and dried under N gas until only one mL of the original volume was left.

The cholesterol methyl ester was transferred into a four mL screw-capped vial (Kimble Glass Inc., USA) and drying continued under a stream of purified N gas.

After drying, the screw-capped vial containing the dried cholesterol extract was flushed with N gas; cap sealed and kept frozen at -20^{o}C until GLC analysis. Prior to GLC analysis, one mL of chloroform-methanol 2:1 (v/v) was added into the vial containing the cholesterol extract to reconstitute the cholesterol methyl ester.

Cholesterol methyl ester was separated using a Agilent (Agilent Technologies, Inc. USA), fused silica capillary column (30m, 0.32mm ID, 0.25µm film thickness) in a Hewlett-Packard Model 6890N GLC. High purity N was the carrier gas at 33.5-mL/min. High purity hydrogen and compressed air were used for the flame ionization detector in the GLC. The oven temperature was set at 310^{o}C. The injector and detector were programmed at 325^{o}C and 325^{o}C, respectively. Peak area was determined using a HP-3993A Integrator (Hewlett-Packard, Avondale, PA). A programmed PC running Microsoft Excel 2000 (Microsoft Corp., Redmond, USA) was used to express the peak areas as absolute amount of detected cholesterol. Quantification of the cholesterol concentration was done based on a standard cholesterol curve at 2, 4, 6, 8, 10 mg/mL.

3.7 Volatile fatty acid (VFA) determination

The total VFA contents of the faeces were determined using GLC method as described by Minato and Kudo (1985). One gram of sample was added into a test tube containing one mL of 24% metaphosphoric acid (BDH Laboratories, Poole,

U.K). The acidic mixture was left at room temperature overnight and then centrifuged at 10, 000 G for 20 min at four ^{o}C. The supernatant was then collected in a two mL screw-capped vial (Kimble Glass Inc., USA). The internal standard 20mM 4-methyl-butyric acid (Sigma Chemical Co., St. Louis, Missouri, USA) was added to the supernatant and stored at -20^{o}C until analysis by GLC. VFA separation was done on a Quadrex 007 Series (Quadrex Corporation, New Haven, CT 06525 USA) bonded phase fused silica capillary column (15m, 0.32mm ID, 0.25µm film thickness) in a 6890N Hewlett-Packard GLC (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector. The purified N gas as the carrier gas with a flow rate of 60mL/min. The injector and detector temperature were programmed at 230^{o}C and 230^{o}C, respectively. The column temperature was set at the 200^{o}C in an isothermal run. Commercial standards of acetic, propionic, butyric, isobutyric, valeric, isovaleric and 4-methyl-n-valeric acids from Sigma (Sigma chemical Co., St. Louis, Missouri, USA) were used as authentic standards to identify the peaks. Determination of the peak areas was performed as described earlier in Section 3.5.3 in this chapter.

3.8 Plasma Total cholesterol

Plasma total cholesterol was quantified using a cholesterol diagnostic kit (Randox®, UK), based on the cholesterol enzymatic endpoint method as described by Loh *et al*. (2002). Ten µL of plasma was diluted with 40µL of distilled water in a clean micro-centrifuged tube. A total of 7.5µL of solution was mixed with 750µL of

cholesterol determination reagent in a clean micro-centrifuged tube. The micro-centrifuge tubes were then incubated for ten min at 20°C to 25°C. Within the following 60 min, the absorbance value was read at 500nm using spectrophotometer (GENESYS™, Spectronic® 20, USA). The net absorbance value of tested samples at 500nm must fall within the range of the cholesterol standard curve. The cholesterol standard curve was obtained by preparing 0, 4, 8, 16 and 20 µg of cholesterol standard (200mg/dL) (Randox®, UK) in 750µL of cholesterol determination reagent. The solution was mixed thoroughly and the absorbance read at 500nm within 60 min using a spectrophotometer (GENESYS™, Spectronic® 20, USA).

CHAPTER IV

THE MICROBIAL PROPERTIES AND CHEMICAL COMPOSITIONS OF THE FERMENTED PRODUCT

4.1 Introduction

Fermentation is one of the oldest technologies used for food and feed preservation (Motarjemi, 2002; Prajapati and Nair, 2003; Jay, 2000). Over the centuries, the fermentation process has been evolved and refined extensively to produce a diversified range of products. Today, a variety of fermented products are produced using this technology both in industrialized and at the household level in developing countries, in small-scale food industries as well as in large enterprises (Nout, 2001).

It is possible to obtain a large variety of fermented products by selecting different types and compositions raw materials, starter cultures and fermentation conditions (Hansen, 2002). Fermentation depends on the biological activity of microorganisms, which can suppress the growth and survival of undesirable microflora in foodstuffs. Therefore, the actions of microorganisms during the preparation of cultured foods play an important role in improving the safety of feeds by removing their natural toxic components, or by preventing the growth of disease-causing microbes (e.g. *Enterobacteriaceae*). Fermentation with LAB cultures has been shown to be able to improve the quality, availability and digestibility of dietary nutrients (Nout, 2001;

Holzapfel, 2002; Ross *et al*., 2002). LAB improve protein digestibility and micronutrient bioavailability of fermented feed through the biosynthesis of vitamins and essential amino acids (Holzapfel, 2002). Thus, they play an essential role in food and feed fermentation and are well known for their acidification of the product (Hammes *et al*., 1990b; Ross *et al*., 2002).

Generally fermented products contain high numbers of lactic acid bacteria and yeast, a low pH and high concentration of lactic acid (Geary *et al*., 1996; Mikkelsen and Jensen, 1997; Brooks *et al*., 2001). The physical, chemical and biological characteristics have been modified by the activity of microorganisms. Microorganisms, by virtue of their metabolic activities, contribute to the development of characteristic properties such as taste, aroma, texture, visual appearance, shelf life and safety of the product (Holzapfel, 2002; Jay, 2000). It is important to define and standardize the composition of raw materials and conditions of fermentation process in order to maintain the quality of the end product.

During the early ages of civilization, fermented products are mainly used for human consumption. The beneficial LAB present in the products are well documented for its antimicrobial compounds such as bacteriocin, as a biopreservative for food product (Ross, *et al*., 2002; Leroy and Vuyst, 2004; Schnürer and Magnusson, 2005). Product derived from LAB fermentation has the potential in the prevention of various human diseases (Minamiyama, 2003; Kheadr *et al*., 2004). Recently, fermented liquid feed has been used as a natural and environmental friendly feed

additive as well as growth enhancer in animal diets. Fermented liquid feed has also shown promising results in enhancing the performance of animals (Mikkelsen and Jensen, 1998; Brooks *et al.,* 2001; Demeckova *et al*., 2002; Heres *et al*., 2003a,b, 2004). However, fermented liquid feeds are tedious to prepare and apply. The solid form FP investigated in this study was designed to overcome these limitations. Most of the studies on fermentation have been done on animal by-product (Kamra and Srivastava, 1994), agricultural by-product (Joshi and Sandhu, 1996) or slaughterhouse and household wastes (Fransen *et al*., 1998; Kherrati, *et al*., 1998; El Jalil *et al*., 2001). Up to date, none of the fermentation studies done on agricultural products involved the addition of marine fish in order to enhance the nutritive value of the final product. In the present study, fish was used to increase the levels of PUFA as PUFA had been used successfully to manipulate and increase the n-3 levels in animal products for livestock production (Gonzalez-Esquerra and Leeson, 2000; Milinsk *et al*., 2003; Visentainer *et al*., 2005).

Thus, the objective of this study was to evaluate the physico-chemical composition and nutritive value of the product after the process of fermentation.

4.2 Materials and methods

4.2.1 Preparation of FP

Preparation of FP was described earlier in Chapter III. The fermentation process was repeated three times under the same experimental conditions using the same raw materials to validate the stability of the end product.

4.2.2 Chemical and microbiological analyses

Proximate analyses, GE determination, pH, fatty acids determination, total lactobacilli and *Enterobacteriaceae* counts were performed as described previously in Chapter III. All analyses were performed in triplicates.

4.2.3 Statistical analyses

The numbers of colony forming units (cfu) of LAB and *Enterobacteriaceae* in the FP were expressed as $\log_{10}$ cfu per gram. The CP, CF, EE, DM and ash in FP were expressed as percentages. Fatty acids concentration was expressed both in absolute amount (mg/100g) and percentage of total fatty acids. All data were analyzed by ANOVA and Duncan's Multiple Range test using SAS (SAS®, 1991). All values were expressed as mean ± standard error (SE) and tested at $P<0.05$.

4.3 Results

4.3.1 Microflora of the FP

The total lactobacilli counts (Table 4.1) increased significantly ($P<0.05$) from an average of 10^4 cfu/g in the fermentation mix at Day 0 to 10^6 cfu/g in the product at Day 12. The total lactobacilli counts were similar in the final products although each batch started with significantly ($P<0.05$) different lactobacilli counts at Day 0. The *Enterobacteriaceae* counts decreased significantly ($P<0.05$) from Day 0 at 10^3 cfu/g to only 10^1 cfu/g in the product by Day 12. The *Enterobacteriaceae* counts (Table 4.1) were not significantly ($P>0.05$) different across batches in the initial stage and in the product.

Table 4.1: Total lactobacilli and *Enterobacteriaceae* counts in raw materials and finished FP

Microorganisms Log_{10}(cfu/g)	Fermentation time	Batch 1 (n=3)	Batch 2 (n=3)	Batch 3 (n=3)
Total Lactobacilli	Day 0	$4.28^{a,x}$ ±0.01	$4.40^{b,x}$ ±0.01	$4.37^{b,x}$ ±0.02
	Day 12	$6.26^{a,y}$ ±0.03	$6.25^{a,y}$ ±0.11	$6.41^{a,y}$ ±0.02
Enterobacteriaceae	Day 0	$3.87^{a,x}$±0.01	$3.87^{a,x}$ ±0.02	$3.83^{a,x}$ ±0.03
	Day 12	$0.83^{a,y}$ ±0.16	$0.87^{a,y}$ ±0.20	$1.26^{a,y}$ ±0.41

The results are presented as mean values ± SE.
[a,b] Values with different superscripts within a row differ significantly at $P<0.05$ due to batch effects;
[x,y] Values with different superscripts within a column differ significantly at $P<0.05$ due to day effects;

4.3.2 Physico-chemical characteristics of the FP

4.3.2.1 Proximate analyses

The DM, CF, ash, CP, EE and GE contents of the three batches of FP were similar (P>0.05) across the batches except pH (P<0.05) (Table 4.2). The pH ranged from 4.21 to 4.75.

Table 4.2: Physico-chemical characteristics of the FP

Parameters	Batch 1 (n=3)	Batch 2 (n=3)	Batch 3 (n=3)	Sig.
pH	4.75^{a} ± 0.02	4.21^{b} ± 0.01	4.55^{c} ± 0.01	*
GE, Kcal/kg	4490.97 ± 32.20	4509.98 ± 8.70	4415.69 ± 156.00	ns
(%, DM basis)				
DM	90.69 ± 0.83	92.65 ± 0.07	92.18 ± 0.23	ns
Crude fiber	0.07 ± 0.01	0.06 ± 0.01	0.07 ± 0.01	ns
Crude proteins	19.97 ± 0.10	19.00 ± 0.33	19.21 ± 0.22	ns
Crude fat	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	ns
Ash	1.02 ± 0.01	0.99 ± 0.01	1.00 ± 0.02	ns

The results are presented as mean values ± SE.
* P<0.05; ^{ns} No significant difference

4.3.2.2 Fatty acid concentration

Fatty acid concentration (Table 4.3) were not significantly different ($P>0.05$) between batches. In general, about one third of the FP's fatty acid content was made up of SFA. The SFA comprised of lauric, myristic, pentadecanoic, palmitic, heptadecanoic, stearic, arachidic, behenic and lignoceric acids. The remaining fatty acids content of FP was made up of total UFA, which comprised of both PUFA and MUFA. Generally, the n-6 fatty acids seemed to be the dominant PUFA species, constituting almost a quarter of the total fatty acids thus giving a n-6: n-3 ratio of about 3.5.

Table 4.3: Fatty acid concentration in FP of different batches

Fatty Acids	Batch 1 Mean ± SE (mg/100g)	%	Batch 2 Mean ± SE (mg/100g)	%	Batch 3 Mean ± SE (mg/100g)	%
C12:0	6.2±1.1	0.1	5.9±0.8	0.1	6.1±1.4	0.1
C14:0	200.6±13.1	2.9	193.7±2.36	2.9	212.3±22.6	2.9
C15:0	28.5±9.0	0.4	26.9±4.3	0.4	30.7±11.6	0.4
C16:0	1444.8±121.0	20.8	1393.5±170.0	20.9	1525.0±31.0	20.8
C16:1 n-9	270.6±21.6	3.9	259.5±34.8	3.9	287.8±34.7	3.9
C17:0	30.2±7.2	0.4	28.7±5.8	0.4	32.3±9.8	0.4
C17:1	40.7±7.1	0.6	38.4±5.6	0.6	43.1±10.1	0.6
C18:0	294.3±20.2	4.2	282.1±37.5	4.2	311.7±29.0	4.2
C18:1 n-9	2216.8±387.0	31.9	2138.9±237.0	32.0	2342.7±231.0	31.9
C18:2 n-6	1671.4±349.0	24.1	1613.2±167.0	24.1	1764.2±246.0	24.0
C18:3 n-3	73.3±12.5	1.1	71.3±5.6	1.1	76.7±10.1	1.0
C20:0	49.7±7.3	0.7	48.8±6.1	0.7	52.2±3.9	0.7
C20:1 n-9	35.2±4.1	0.5	34.6±4.2	0.5	37.0±1.2	0.5
C20:4 n-6	86.3±19.9	1.2	81.3±15.8	1.2	91.9±28.2	1.3
C22:0	24.1±2.7	0.3	22.6±3.4	0.3	25.4±0.9	0.3
C24:0	36.5±4.2	0.5	35.4±4.9	0.5	41.2±1.6	0.6
C20:5 n-3, EPA	246.9±19.8	3.6	230.6±22.4	3.5	261.8±31.4	3.6
C22:5 n-3, DPA	46.4±6.8	0.7	44.4±8.2	0.7	50.1±10.8	0.7
C22:6 n-3, DHA	141.9±18.1	2.0	132.3±13.7	2.0	152.1±25.2	2.1
Total SFA	2114.8±149.0	30.5	2037.5±256.0	30.5	2237.0± 102.0	30.5
Total UFA	4829.4±690.0	69.5	4644.5±509.0	69.5	5107.4± 350.0	69.5
Total MUFA	2563.3±382.0	36.9	2471.5±282.0	37.0	2710.6± 189.0	36.9
Total PUFA n-3	508.4±31.4	7.3	478.6±48.5	7.2	540.7± 57.4	7.4
Total PUFA n-6	1757.7±334.0	25.3	1694.5±182.0	25.4	1856.1± 218.0	25.3
Overall total	6944.2±802.0		6682.0±765.0		7344.4± 255.0	
n-6 : n-3 ratio	3.5		3.5		3.4	
UFA: SFA ratio	2.3		2.3		2.3	
PUFA: SFA ratio	1.1		1.1		1.1	

The results are presented as mean values ± SE.
All values within a row were not significantly different from each other (P>0.05).

4.4 Discussion

4.4.1 Microflora of the FP

The fermentation process performed in this study demonstrated a significant reduction of harmful bacteria such as *Enterobacteriaceae*, which includes *Salmonella* spp. and *Escherichia coli* in the end product compared to the raw materials. In parallel, the population of LAB increased significantly in the finished product compared to the raw materials. The results obtained in this study are in agreement with those of Mikkelsen and Jensen, (2000), van Winsen *et al*. (2002), Demecková *et al*. (2002), Loh *et al*. (2003a,b) and Heres *et al*. (2004). The use of potentially beneficial bacteria such as LAB have been demonstrated to inhibit the *in-vitro* growth of many enteric pathogens including *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli*, *Clostridium perfringens* and *Clostridium difficile* (Lindgren and Dobrogosz, 1990; de Vuyst and Vandamme, 1994; Forestier *et al.,* 2001). Furthermore, the *in-vivo* studies of feeding fermented feed improved the microbial balance in the GIT of animals (Urlings *et al*., 1993; Mikkelsen and Jensen, 2000; Van Winsen *et al.,* 2002; Demecková *et al*., 2002; Loh *et al*., 2003a,b; Heres *et al*., 2004). It was reported that the beneficial LAB present in the product produced a range of antimicrobial metabolites such as bacteriocins, ethanol, H_2O_2, and organic acids during fermentation (Ross *et al*., 2002; Chapter II). Organic acids helped to reduce the pH value to a range of 4-5, which was detrimental for deleterious microbe (Østergaard *et al*., 1998; Christine *et al*., 1999; Brooks, 2003;

Beal *et al*., 2002). This in turn, provided a suitable environment to promote the growth and sustenance of the lactic acid-producing microflora (Owen and Mendoza, 1985; Urlings *et al*., 1993; Langhendries *et al*., 1995; van Winsen *et al*., 2002; Loh *et al*., 2003a,b).

The undissociated, more hydrophobic form of the organic acids diffuses over the cell membrane. It dissociates inside the cell thus releasing H^+ ions that acidify the cytoplasm and reducing the intracellular pH. This in turn inhibits the active transport and a variety of metabolic functions (Doores, 1993; Ross *et al*., 2002). In addition to the pH effect, the undissociated acid collapses the electro-chemical proton gradient, causing bacteriostasis and finally death of susceptible bacteria (Axelsson *et al*., 1989 as cited by Schnürer and Magnusson, 2005) such as *Enterobacteriaceae* in the FP. The reduction of *Enterobacteriaceae* in the FP was important to the product safety as hazardous microorganisms were inhibited. This indicated a safe product and a successful fermentation process. This product may be used safely in the animal feed industry for optimal growth performance and as a prophylaxis against infectious agents such as *Salmonellosis*.

4.4.2 Physico-chemical characteristics of the FP

The DM, CF, CP and ash content of the three batches of FP were similar. These results indicated that the FP could be produced at consistent quality under a reproducible fermentation process. A high content of beneficial long-chain fatty

acids (PUFA) was another important characteristic of this product. These included notably linolenic, linoleic, arachidonic, EPA, DPA and DHA which are now regarded as nutritionally important fatty acids (Hansen, 1986; Lauritzen *et al*., 2001; Elmore *et al*., 2005).

The presence of n-3 long chain PUFA in the product was a direct result of incorporation of marine fish (*Rastrelliger kanagurta*) as one of the raw materials. Marine fish are rich in n-3 fatty acids because their diet is made up of zooplankton and phytoplankton, a rich source of PUFA (Kyle, 2002). The long chain n-3 fatty acids are prized for the beneficial effects to human health and feedstuffs with added n-3 is definitely more advantageous over conventional feeds. This could offer an added advantage, which helps to improve the content of these fatty acids in animal and animal products when the FP is being fed to livestock. However, the long–chain PUFA particularly the members of n-3 fatty acids are susceptible to lipid oxidation. Surprisingly, these PUFA especially EPA, DPA and DHA were detected in substantial amounts in the FP. The EPA was found to be the highest among all the n-3 long chain fatty acids in the FP. Besides the high content of n-6 PUFA detected in the FP was probably a direct result of the incorporation of more than 50% rice bran in the fermentation mix. Linoleic acid (the predominant n-6 fatty acid in the product) is the major fatty acid present in the lipid fraction of the rice bran (Ito and Simpson, 1996; Rouanet *et al*., 1993). Furthermore, rice bran contained high levels of several phytochemicals that have been claimed to be a good source of natural antioxidants (Krings *et al*., 2000; Kanaya *et al*, 2004; Chen and Bergman, 2005;

Iqbal *et al*, 2005). Among these natural antioxidants are vitamin E, tocopherols, tocotrienols and the γ-oryzanol fraction, which are thought to exert anti-oxidative and protective effects on fatty acids in animal tissues (Qureshi and Peterson, 2001; Kanaya, *et al*, 2004). In addition, rice bran has been shown to improve the storage stability of foods (Iqbal, *et al*., 2005; Chen and Bergman, 2005). Thus, it may be acted as a natural antioxidant during fermentation thereby improved the storage stability of FP and helped to prevent the oxidation of long-chain PUFA in FP.

4.5 Conclusions

The microbiological, physical, chemical properties of FP obtained in this study showed that the FP was of highly consistent quality and reproducible. It was rich in LAB and had a low pH (4.5). Furthermore, the presence of essential PUFA such as linoleic, linolenic, arachidonic, EPA, DPA and DHA in the FP was another important characteristic of this product. The FP was a dry-brownish mash with distinctive lime-flavoured aroma. This might have an added advantage to overcome the problem of unpleasant odour in feed such as fishmeal, as off-flavoured products could be a significant market disadvantage. Based on the results obtained in the present study, FP has a great potential as an animal feed with additional source of essential fatty acids compared to commercial feed. Results obtained from this study are essential for the large-scale FP production for animal feed applications in the future.

CHAPTER V

EFFECTS OF FEEDING THE FERMENTED PRODUCT ON FAECAL MICROFLORA, ESSENTIAL FATTY ACID AND CHOLESTEROL LEVELS IN EGGS AND PLASMA OF LAYING HENS

5.1 Introduction

The common method used to improve the productivity of animals is through the application of feed additives. Addition of growth promoting antibiotics is the most common approach. However, the extensive use of antibiotics as growth enhancer worldwide may lead to the development of antimicrobial resistance (tolerance) against pathogenic bacteria species (Mikkelsen and Jensen, 2000). This may hamper the effectiveness of treatment in infected humans in the event of any occurrence in human infections due to animal pathogens. Recently, fermented feed was suggested as alternatives to the antibiotic growth promotants (Mikkelsen and Jensen, 2000; Van Winsen *et al.,* 2002; Demecková *et al*., 2002; Loh *et al*., 2003a,b). Therefore, it has become an important and rapidly expanding segment of the animal feed market in developed countries. This is one of the avenues to reduce or even eliminate the need for antibiotics in feeds (Miles, 1997). Feeding fermented liquid and fermented fruits to animals resulted in the reduction of coliform numbers in the digestive tract (Mikkelsen and Jensen, 1998; Brooks *et al.,* 2001; Demeckova *et al*., 2002; van Winsen *et al*., 2001, 2002; Canibe and Jensen, 2003; Højberg *et al*.,

2003; Heres *et al*., 2003a,b, 2004; Loh *et al*., 2003a,b). In addition, it has been reported to result in the increase in feed intake post-weaning and thus increase post-weaning growth rate in piglets (Mikkelsen and Jensen, 1998). It also has been reported that feeding fermented solid product (Loh *et al*., 2003a,b) has a similar effect as feeding fermented liquid feed (Mikkelsen and Jensen, 1998; Brooks *et al.,* 2001; Demeckova *et al*., 2002; van Winsen *et al*., 2001, 2002; Canibe and Jensen, 2003; Højberg *et al*., 2003; Heres *et al*., 2003a,b, 2004). However, there is no information on the effects of feeding fermented solid products to layers on their egg quality. Furthermore, the fermented solid product in this experiment was designed to contain essential PUFA, as these fatty acids are important for their nutritive value. Additionally, the fatty acid composition in the animal product can be manipulated through dietary manipulation (Caston *et al*., 1994; Gonzalez-Esquerra and Leeson, 2000; Elmore *et al*., 2005; Carrillo-Domínguez *et al*., 2005; Visentainer *et al*., 2005). Thus, the objective of this study was:

i To study the effects of different level of FP inclusions in the diet of layers on the population of *Enterobacteriaceae* and LAB, pH and VFA composition in faeces

ii To evaluate the effects of FP supplementation on the essential fatty acid and cholesterol content in eggs and plasma

iii To investigate the effects of FP on the laying performance in layers

5.2 Materials and methods

5.2.1 Experimental birds

A total of 96 13-week old Babcock B380 pullets with an average body weight of 1.19 kg ± 0.01 (BW ± SE) were used in this study. All the birds were housed in individual wire cages. Each cage was 36 x 30 x 35cm^3 (width x depth x height). They were randomly allotted to four numerically equal groups, with eight replicates per treatment, three birds per replicate. The feeding period lasted for 16 weeks, commencing when the pullets were 13-weeks of age and ended when they were 29-weeks of age. Individual body weight was measured at the beginning and at the end of the experiment (refer Appendix 6). Water and feed were supplied *ad libitum*. The birds were raised under natural light circumstances. This feed was given early in the morning (8am) and topped up in the afternoon (5pm). Feed intake for each treatment groups was measured weekly. The experimental feeds were provided in mash form.

5.2.2 Diets

The FP used in the present study was fermented with LAB cultures (Chapter III). Isocaloric and isonitrogenous diets were formulated using the FeedLIVE software (Live informatics Co. Ltd, Thailand). Four experimental diets were used. Diet 1 (Control) without the fermented product acted as the negative control, Diet 2 (FP3)

contained 3% (w/w) FP (30g FP/kg diet), Diet 3 (FP6) contained 6% (w/w) FP (60g FP/kg diet) and Diet 4 (FP9) contained 9% (w/w) FP (90g FP/kg diet). The composition of the diets and their nutrient composition are presented in Tables 5.1 and 5.2.

Table 5.1: Composition of pullet diets (from 13-18 weeks of age)

Ingredient, %	Control	FP3	FP6	FP9
Corn	60.15	58.65	56.81	54.97
Palm oil	2.50	2.20	1.94	1.70
Soybean meal 44%	31.30	30.10	29.20	28.28
L-lysine	0.20	0.20	0.20	0.20
DL-methionine	0.20	0.20	0.20	0.20
MDCP	3.00	3.00	3.00	3.00
Limestone	1.10	1.10	1.10	1.10
Salt	0.40	0.40	0.40	0.40
Vitamin premix *	0.08	0.08	0.08	0.08
Mineral premix **	0.07	0.07	0.07	0.07
Choline chloride	1.00	1.00	1.00	1.00
FP	-	3.00	6.00	9.00
Total (%)	100.00	100.00	100.00	100.00
Calculated values				
ME (Cal/kg)	2923.27	2923.98	2923.21	2923.56
Crude protein, %	18.89	18.81	18.84	18.86
Analyzed values, %				
Crude protein	18.70	18.75	18.80	18.80
Fatty acids, %				
SFA	35.09	32.48	31.28	30.59
MUFA	33.75	34.85	35.40	35.75
PUFA	31.16	32.67	33.32	33.66
Total n-3 PUFA	1.63	3.93	5.06	5.73
Total n-6 PUFA	29.53	28.74	28.25	27.94
DHA	0.00	0.86	1.28	1.53

*The vitamin-premix provides the following amounts per kg of diet: Vitamin A 50.0 MIU, Vitamin D_3 8.0 MIU, Vitamin E 200g, Vitamin B_2 20.0g, Vitamin B_6 20.0 g, Vitamin B_{12} 1.0g, Vitamin K_3 10.0g, Biotin 0.8g, Panthothenic acid 80.0g, Folic acid 10.0g, Niacin 130.0g, Anticaking agent 20.0g and Antioxidant 0.2g.
**The mineral premix provides the following amounts per kg of diet: Mn 20.0g, Fe 80.0g, Zn 80.0g, Cu 10.0g, Co 0.2g, I 0.3g and Se 0.3g.
FP: Fermented product

Table 5.2: Composition of layer diets (from 19 weeks to 29 weeks of age)

Ingredient (%)	Cont	FP3	FP6	FP9
Corn	60.60	58.55	57.00	54.30
Palm oil	1.55	1.35	1.05	0.97
Soybean meal 44%	25.50	24.75	23.60	23.38
L-lysine	0.06	0.06	0.06	0.06
DL-methionine	0.16	0.16	0.16	0.16
MDCP	2.50	2.50	2.50	2.50
Limestone	8.00	8.00	8.00	8.00
Salt	0.50	0.50	0.50	0.50
Vitamin premix *	0.07	0.07	0.07	0.07
Mineral premix **	0.06	0.06	0.06	0.06
Choline chloride	1.00	1.00	1.00	1.00
FP	-	3.00	6.00	9.00
Total (%)	100.00	100.00	100.00	100.00
Calculated values				
ME (Cal/kg)	2726.09	2726.37	2726.54	2726.43
Crude protein, %	16.22	16.29	16.23	16.49
Analyzed values, %				
Crude protein	16.20	16.30	16.20	16.41
Fatty acids, %				
SFA	31.46	30.21	29.42	29.40
MUFA	32.84	34.51	35.20	35.65
PUFA	35.70	35.28	35.38	34.95
Total n-3 PUFA	1.87	4.36	5.50	6.05
Total n-6 PUFA	33.83	30.91	29.88	28.89
DHA	0.00	0.97	1.41	1.63

*The vitamin-premix provides the following amounts per kg of diet: Vitamin A 50.0 MIU, Vitamin D_3 8.0 MIU, Vitamin E 200g, Vitamin B_2 20.0g, Vitamin B_6 20.0 g, Vitamin B_{12} 1.0g, Vitamin K_3 10.0g, Biotin 0.8g, Panthothenic acid 80.0g, Folic acid 10.0g, Niacin 130.0g, Anticaking agent 20.0g and Antioxidant 0.2g.

**The mineral premix provides the following amounts per kg of diet: Mn 20.0g, Fe 80.0g, Zn 80.0g, Cu 10.0g, Co 0.2g, I 0.3g and Se 0.3g.

FP: Fermented product

5.2.3 Sample collection and chemical analyses

A total of three grams of faecal samples were collected from each replicate of each experimental group at 15, 17, 19, 21, 23, 25, 27 and 29 weeks of age during the experimental period. Fresh faecal samples were collected immediately after defecation and placed in different sterile universal bottle to determine the faecal pH, faecal VFA, numbers of LAB and *Enterobacteriaceae.* These parameters were analyzed according to the methods outlined previously in Chapter III.

During the production period (from 21 weeks to 29 weeks of age), the eggs were collected daily in the morning, labeled accordingly and transported to the laboratory of the Animal Science Department, Universiti Putra Malaysia for analyses. The numbers of egg produced including all the normal and abnormal eggs from the different treatment groups were recorded and only normal eggs were weighed accordingly.

Between 25 to 29 weeks of age, eight eggs per treatment were randomly selected and analyzed for egg yolk cholesterol and egg yolk fatty acid profiles. The eggs were broken manually and the albumin discarded. The egg yolk was then homogenized gently without foaming for 30 seconds by hand. About 0.3-0.5 g of liquid egg yolk was used for fatty acid analyses. The sample was homogenized in 40 mL of chloroform-methanol (2:1, v/v) in a 50 mL stoppered ground-glass test tube. The tube was gassed with N, stoppered and then shaken vigorously before

being allowed to stand for 12 hours. The procedures hereafter were similar to those described for total fatty acid extraction in Chapter III. The remaining liquid yolk was subjected to total cholesterol extraction immediately following the procedure for cholesterol determination as described in Chapter III.

At the end of the experiment, five birds from each treatment were randomly selected and sacrificed. The birds were fasted for 12 hours prior to blood collection. Blood samples from each bird were collected into three mL Ethylenediamino tetraacetic acid (EDTA) vacutainer tubes (Becton Dickinson, New Jersey, USA). Plasma was separated by centrifuging the blood at 1000 G for 10 min. The plasma was then transferred into a micro centrifuge tube using a Pasteur pipette and stored under -20^{o}C until analyses. One mL of plasma was allocated for total cholesterol analyses as outlined in Chapter III. The remaining volume was stored for fatty acid profiles analyses as described in Chapter III.

5.2.4 Statistical analysis

The datasets were analyzed using the one-way analysis of variance (ANOVA) in accordance to a Completely Randomised Design model (SAS®, 1991). Significant differences between treatments means were separated using the Duncan's Multiple Range test at $P<0.05$.

5.3 Results

5.3.1 Faecal *Enterobacteriaceae* counts

The faecal *Enterobacteriaceae* counts (Table 5.3 and Appendix 6) for all the treatment groups were similar (P>0.05) at the beginning of the experiment (15 weeks of age). However, the faecal *Enterobacteriaceae* counts for the FP9 groups were significantly lower (P<0.05) compared to the control groups from 17 weeks of age onwards. The faecal *Enterobacteriaceae* from FP6 fed group was significantly lower (P<0.05) compared to the control group except at 15 and 19 weeks of age. Similarly, the FP3-fed group had significantly lower (P<0.05) faecal *Enterobacteriaceae* counts compared to the control group except at 15 and 29 weeks of age.

Table 5.3: Effects of treatment diets on faecal *Enterobacteriaceae* counts

Age (weeks)	Treatments				Sig.
	Cont Mean ± SE (log_{10} cfu/g)	FP3 Mean ± SE (log_{10} cfu/g)	FP6 Mean ± SE (log_{10} cfu/g)	FP9 Mean ± SE (log_{10} cfu/g)	
15	7.23±0.07	7.03±0.09	6.96±0.12	6.83±0.06	ns
17	7.90^{a}±0.05	6.79^{b} ±0.09	7.45^{b}±0.08	7.38^{b}±0.12	*
19	6.35^{a}±0.09	6.11^{b}±0.06	6.55^{b}±0.14	6.03^{b}±0.12	*
21	6.83^{a} ±0.09	5.81^{b}±0.07	5.52^{c}±0.07	4.85^{d}±0.05	*
23	6.25^{a} ±0.03	5.72^{b}±0.11	5.56^{b}±0.07	4.96^{c}±0.11	*
25	6.49^{a}±0.09	5.89^{b}±0.12	5.34^{c}±0.12	4.62^{d}±0.08	*
27	6.32^{a}±0.09	5.75^{b}±0.09	5.00^{c}±0.07	4.64^{d}±0.09	*
29	5.92^{a}±0.09	5.69^{a}±0.13	5.24^{b}±0.08	4.64^{c}±0.08	*

The results are presented as mean values ± SE.
Values with different superscripts within rows differ significantly at P<0.05;
^{ns} No significant difference
* P<0.05

5.3.2 Faecal LAB counts

The faecal LAB counts (Table 5.4 and Appendix 7) for all the treatment groups were similar (P>0.05) at the beginning of the experiment (15 weeks of age). The faecal LAB counts between the control and FP3 fed group were not significantly different (P>0.05) at 17, 21, 25, 27 and 29 weeks of age. However, the faecal LAB counts of the FP6 and FP9 fed groups were significantly (P<0.05) higher compared to the control group except in the initial weeks (15 weeks of age).

Table 5.4: Effects of treatment diets on faecal LAB counts.

Age (weeks)	Treatments				Sig.
	Cont Mean ± SE (log_{10} cfu/g)	FP3 Mean ± SE (log_{10} cfu/g)	FP6 Mean ± SE (log_{10} cfu/g)	FP9 Mean ± SE (log_{10} cfu/g)	
15	6.16±0.11	6.06±0.11	6.25±0.04	6.06±0.10	ns
17	6.20^{a}±0.06	6.24^{a}±0.02	6.31^{b}±0.07	6.60^{c}±0.07	*
19	6.50^{a}±0.05	6.74^{b}±0.08	6.73^{b}±0.03	7.05^{c}±0.07	*
21	5.91^{a}±0.10	6.17^{b}±0.09	6.42^{c}±0.07	6.56^{c}±0.07	*
23	5.90^{a}±0.09	6.13^{b}±0.06	6.44^{c}±0.05	6.71^{d}±0.09	*
25	5.73^{a}±0.09	5.75^{a}±0.08	6.28^{b}±0.05	6.53^{c}±0.08	*
27	5.98^{a}±0.06	6.05^{a}±0.06	6.48^{b} ±0.08	6.65^{b}±0.09	*
29	5.78^{a}±0.08	5.98^{a}±0.11	6.31^{b}±0.07	6.60^{c}±0.10	*

The results are presented as mean values ± SE.
Values with different superscripts within a row differ significantly at P<0.05;
^{ns} No significant difference
* P<0.05

5.3.3 Faecal pH

The faecal pH (Table 5.5 and Appendix 8) of birds at 15 weeks of age for all the experimental groups was not significant different (P>0.05). Significant differences (P<0.05) in the faecal pH between FP9 fed group and control group were observed throughout the experimental weeks except at 15 weeks of age. In general, the faecal pH of the FP9 group remained the lowest (P<0.05) throughout the experimental period.

Table 5.5: Effects of treatment diets on faecal pH.

Age (weeks)	Treatments				Sig.
	Cont	FP3	FP6	FP9	
	Mean ± SE	Mean ± SE	Mean ± SE	Mean ± SE	
15	6.73±0.07	6.85±0.05	6.71±0.02	6.73±0.02	ns
17	6.81^{ab}±0.03	6.85^{a}±0.05	6.71^{b}±0.04	6.55^{c} ±0.04	*
19	6.90^{a}±0.06	6.79^{ab}±0.09	6.70^{ab}±0.07	6.68^{b} ±0.04	*
21	7.00^{a}±0.05	7.03^{a}±0.09	6.85^{ab}±0.06	6.72^{b}±0.06	*
23	7.57^{a}±0.05	7.52^{a}±0.15	7.40^{ab}±0.08	7.19^{b}±0.03	*
25	7.09^{a}±0.06	6.99^{a}±0.06	6.92^{a}±0.05	6.65^{b}±0.11	*
27	7.17^{a}±0.07	6.85^{b}±0.08	6.95^{b}±0.08	6.89^{b}±0.06	*
29	7.06^{a}±0.04	6.86^{a}±0.07	6.88^{a}±0.05	6.59^{b}±0.12	*

The results are presented as mean values ± SE.
Values with different superscripts within rows differ significantly at P<0.05;
^{ns} No significant difference
* P<0.05

5.3.4 Faecal volatile fatty acid (VFA) concentrations

The most abundant VFA (Table 5.6) found in the faeces for all groups was acetic acid. The acetic acid production was about 60% higher than other branched chain VFA production. Branched short-chain fatty acids (iso-butyric, iso propionic, valeric and iso-valeric acids) were detectable only in minute proportions.

Table 5.6: Effects of treatment diets on faecal VFA (%)

Age (weeks)	Parameters	Treatments				Sig.
		Cont Mean±SE	FP3 Mean±SE	FP6 Mean±SE	FP9 Mean±SE	
15	Acetic acid	77.84^{a}±5.84	81.49^{ab}±4.71	90.75^{b}±1.71	89.74^{b}±1.31	*
	Propionic acid	5.59^{ab}±1.79	10.23^{a}±3.09	1.84^{b}±0.17	2.96^{b}±0.92	*
	Butyric acid	5.27^{ab}±0.96	5.68^{a}±1.39	3.05^{ab}±0.68	2.48^{b}±0.32	*
	Other VFA^{x}	11.30^{a}±3.45	2.60^{b}±0.53	4.36^{b}±1.39	4.82^{b}±1.17	*
17	Acetic acid	79.88^{a}±1.46	66.66^{b}±3.43	68.52^{ab}±3.95	56.76^{ab}±4.39	*
	Propionic acid	7.94^{a}±2.14	6.30^{a}±1.58	6.01^{a}±1.04	27.07^{b}±9.62	*
	Butyric acid	1.80^{a}±0.61	5.59^{b}±0.52	4.10^{b}±0.59	3.58^{b}±0.50	*
	Other VFA^{x}	10.38^{a}±0.55	21.45^{a}±2.80	21.37^{a}±2.89	12.59^{a}±4.54	ns
19	Acetic acid	92.99^{a}±1.40	88.12^{a}±4.40	68.31^{b}±4.49	72.97^{b}±6.67	*
	Propionic acid	2.36^{a}±0.98	1.33^{a}±0.36	2.88^{a}±1.10	7.05^{a}±5.45	ns
	Butyric acid	1.07^{a}±0.36	0.92^{a}±0.26	2.66^{b}±0.63	1.42^{a}±0.19	*
	Other VFA^{x}	3.58^{a}±1.21	9.63^{ab}±3.85	26.15^{c}±4.56	18.56^{bc}±1.68	*
21	Acetic acid	78.82^{a}±1.84	76.12^{a}±3.46	78.51^{a}±3.75	81.32^{a}±3.05	ns
	Propionic acid	3.15^{a}±0.19	10.97^{b}±7.37	4.18^{a}±1.33	8.48^{ab}±1.51	*
	Butyric acid	5.89^{a}±0.83	4.80^{a}±0.93	6.06^{a}±1.26	4.49^{a}±0.93	ns
	Other VFA^{x}	12.14^{a}±1.68	8.11^{ab}±1.37	11.25^{ab}±2.82	5.71^{b}±1.48	*
23	Acetic acid	92.15^{a}±1.23	84.23^{a}±4.59	92.51^{a}±1.83	89.50^{a}±1.75	ns
	Propionic acid	3.72^{a}±0.57	7.08^{b}±1.70	3.39^{a}±1.03	3.64^{a}±0.38	*
	Butyric acid	1.30^{a}±0.26	3.14^{a}±1.13	1.33^{a}±0.47	1.60^{a}±0.40	ns
	Other VFA^{x}	2.83^{a}±0.60	5.55^{a}±2.39	2.77^{a}±0.58	5.26^{a}±1.05	ns
25	Acetic acid	82.31^{a}±3.85	78.98^{a}±3.21	80.15^{a}±4.74	88.18^{a}±2.86	ns
	Propionic acid	6.68^{a}±1.69	10.14^{a}±2.27	10.34^{a}±3.33	4.26^{a}±1.77	ns
	Butyric acid	3.49^{ab}±1.05	5.60^{a}±1.04	4.20^{ab}±1.15	1.85^{b}±0.75	*
	Other VFA^{x}	7.52^{a}±1.72	5.28^{a}±1.36	5.31^{a}±1.56	5.71^{a}±1.61	ns
27	Acetic acid	89.61^{a}±0.74	84.77^{a}±3.04	84.92^{a}±2.16	84.78^{a}±2.64	ns
	Propionic acid	4.64^{a}±0.45	8.67^{a}±1.84	6.43^{a}±0.61	8.12^{a}±1.91	ns
	Butyric acid	1.36^{a}±0.20	2.76^{a}±0.90	2.32^{a}±0.46	2.28^{a}±0.66	ns
	Other VFA^{x}	4.39^{ab}±0.36	3.80^{b}±0.48	6.33^{a}±1.26	4.82^{ab}±0.54	*
29	Acetic acid	79.72^{a}±3.55	81.10^{a}±1.84	82.35^{a}±1.90	81.50^{a}±4.99	ns
	Propionic acid	9.99^{a}±2.19	7.48^{a}±1.42	7.71^{a}±1.28	7.22^{a}±2.90	ns
	Butyric acid	4.35^{a}±1.01	2.90^{a}±0.97	2.45^{a}±0.74	4.55^{a}±2.01	ns
	Other VFA^{x}	5.94^{a}±1.24	8.52^{a}±1.69	7.49^{a}±1.08	6.73^{a}±1.90	ns

The results are presented as mean values ± SE.
Values with different superscripts within a row differ significantly at P<0.05;
^{ns} No significant difference,
^{x} iso-propionic, iso-butyric, valeric and iso-valeric
* P<0.05

5.3.5 Egg yolk fatty acid profiles

The total fatty acid concentration of the egg yolk from the different treatment groups is summarized in Tables 5.7 (Appendices 10a-10e). The SFA of eggs in all groups was lower than the total UFA at the ratio of about 35:65 (Table 5.7). The PUFA n-3 levels in the egg yolk from all the FP treated groups were significantly higher ($P<0.05$) compared to the control group. This was contributed mainly by higher ($P<0.05$) concentration of DHA in laying hens fed with FP, which resulted in a lower n-6: n-3 ratio.

Table 5.7: Effects of treatment diets on egg yolk fatty acid compositions (average from 25 to 29 weeks of age)

Fatty Acid	Cont Mean ±SE (mg/100g)	%	FP3 Mean ±SE (mg/100g)	%	FP6 Mean ±SE (mg/100g)	%	FP9 Mean ±SE (mg/100g)	%	Sig.
C14:0	78.3^{a}± 2.2	0.4	82.9^{ab}± 1.6	0.4	82.3^{ab}± 2.1	0.4	86.9^{b}± 1.8	0.4	*
C16:0	4783.3^{a}± 6.39	25.9	4863.0^{ab}± 65.9	25.9	4910.8^{ab}± 78.4	25.8	5015. 0^{b}± 62.5	25.7	*
C16:1 n-9	575.2^{a} ± 13.9	3.1	550.9 ^{ab}± 14.9	2.9	517.8^{b}± 14.4	2.7	560.5^{ab}± 18.3	2.9	*
C18:0 ^{ns}	1438.7± 20.9	7.8	1476.6± 28.2	7.9	1421.0± 27.8	7.5	1438.8± 20.9	7.4	ns
C18:1 n-9 ^{ns}	8208.5± 110.0	44.5	8231.3± 127.0	43.8	8294.2± 147.0	43.6	8393.2± 131.0	43.1	ns
C18:2 n-6	2477.7^{a}± 58.1	13.4	2656.7^{b}± 48.3	14.1	2785.0^{bc}± 70.3	14.7	2943.5^{c}± 52.7	15.1	*
C18:3 n-3	55.3^{a}± 1.4	0.3	58.2^{a}± 1.4	0.3	58.9^{a}± 1.7	0.3	67.8^{b}± 1.4	0.3	*
C20:1 n-9	69.6^{a}± 1.4	0.4	65.4^{ab}± 1.7	0.3	61.9^{b}± 1.2	0.3	64.6^{b}± 2.1	0.3	*
C20:2 n-6	36.5^{ab}± 0.8	0.2	36.4^{ab}± 1.0	0.2	36.0^{a}± 0.9	0.2	38.8^{b}± 0.9	0.2	*
C20:4 n-6	414.8^{a}± 7.5	2.2	409.1^{a}± 7.2	2.2	401.8^{ab}± 7.9	2.1	385.1^{b}± 5.8	2.0	*
DPA, C22:5 n-3	163.1^{a}± 5.2	0.9	132.2^{b}± 5.0	0.7	110.3^{c}± 3.9	0.6	85.6^{d}± 3.9	0.4	*
DHA, C22:6 n-3	151.9^{a}± 3.8	0.8	245.9^{b}± 6.7	1.3	325.9^{c}± 8.5	1.7	402.7^{d}± 6.3	2.1	*
Total SFA ^{ns}	6300.2± 80.4	34.1	6422.5± 9.1	34.1	6414.1± 103.0	33.7	6540.6± 78.3	33.6	ns
Total UFA	12152.4^{a}± 160.0	65.9	12386.1^{a}± 164.0	65.9	12591.7^{ab}± 213.0	66.3	12941.6^{b}± 168.0	66.4	*
Total MUFA ^{ns}	8853.2± 119.0	48.0	8847.7± 133.0	47.0	8873.9± 151.0	46.7	9018.2± 143.0	46.3	ns
Total PUFA n-3	370.2^{a}± 7.5	2.0	436.3^{b}± 8.4	2.3	495.0^{c}± 11.6	2.6	556.1^{d}± 8.3	2.9	*
Total PUFA n-6	2928.9^{a}± 62.0	15.9	3102.1^{ab}± 50.1	16.5	3222.8^{b}± 77.9	17.0	3367.3^{b}± 56.0	17.3	*
Overall Total ^{ns}	18452.6± 239.0		18808.6± 252.0		19005.8± 313.0		19482.2± 240.0		ns
n-6 : n-3 ratio		7.9		7.1		6.5		6.1	
UFA : SFA ratio		1.9		1.9		2.0		2.0	
PUFA : SFA ratio		0.5		0.6		0.6		0.6	

Values with different superscripts within a row differ significantly at P<0.05;

^{ns} No significant difference

* P<0.05

5.3.6 Egg yolk cholesterol concentrations

The egg cholesterol levels (Table 5.8 and Appendix 11) were not significantly different (P>0.05) between groups at 25 weeks of age. However, egg cholesterol levels for the rest of the weeks were significantly different (P<0.05). A lower (P<0.05) cholesterol level in eggs was observed in the FP9 fed group compared to the control group except at 25 weeks of age.

Table 5.8: Effects of treatment diets on egg yolk cholesterol concentrations in birds from 25 to 29 weeks of age.

Age (weeks)	Treatments				Sig.
	Cont Mean±SE (mg/100g)	FP3 mean±SE (mg/100g)	FP6 Mean±SE (mg/100g)	FP9 mean±SE (mg/100g)	
25	1364.8±38.9	1360.5±52.1	1324.3±58.9	1285.3±76.0	ns
26	1243.0^{ab}±55.8	1287.8^{a}±68.7	1101.3^{bc}±66.6	1058.4^{c}±47.0	*
27	1271.2^{a}±78.6	1219.3^{a}±54.3	1161.5^{ab}±52.6	1045.9^{b}±20.5	*
28	1387.3^{a}±83.4	1302.0^{ab}±42.7	1178.2^{bc}±61.0	1068.3^{c}±42.5	*
29	1241.9^{a}±42.2	1165.2^{ab}±59.5	1194.5^{a}±48.6	1035.9^{b}±34.7	*

The results are presented as mean values ± SE.
Values with different superscripts within a row differ significantly at P<0.05;
^{ns} No significant difference
* P<0.05

5.3.7 Plasma fatty acid profiles

After 16 weeks of treatment (inclusive of two weeks of adjustment period), only DHA concentration in the plasma (Table 5.9) from FP6 and FP9 groups were significantly higher ($P<0.05$) compared to the control group. This resulted in higher PUFA n-3 levels in the plasma of the FP treated groups compared to the control group. This lowered the n-6: n-3 ratio from 10.5 to a more beneficial value of 6.5. In general, more than one third of the plasma fatty acids content was made up of SFA (Table 5.9). This comprised mainly of palmitic, myristic and stearic acids. The remaining fatty acids were made up of UFA, which comprised both PUFA and MUFA.

Table 5.9: Effects of treatment diets on plasma fatty acid compositions of birds at 29 weeks of age

Fatty Acid	Control Mean±SE (µL/100mL)	%	FP3 Mean±SE (µL/100mL)	%	FP6 Mean±SE (µL/100mL)	%	FP9 Mean±SE (µL/100mL)	%	Sig.
C14:0	2.29±0.39	0.30	1.78±0.32	0.28	2.62±0.54	0.29	1.87±0.41	0.29	ns
C16:0	205.40±24.40	26.89	174.61±29.40	27.53	233.12±48.20	26.23	173.13±39.20	26.91	ns
C16:1 n-9	9.52±1.72	1.25	7.07±1.35	1.11	11.77±2.39	1.32	7.46±8.09	1.16	ns
C18:0	65.12±8.09	8.52	58.64±7.77	9.25	76.68±16.20	8.63	54.20±12.60	8.42	ns
C18:1 n-9	292.16±40.10	38.25	240.30±40.80	37.88	349.86±72.80	39.36	238.30±60.60	37.04	ns
C18:2 n-6	144.66±16.60	18.94	111.60±19.60	17.59	158.47±36.40	17.83	124.04±24.30	19.28	ns
C18:3 n-3	1.83±0.26	0.24	1.49±0.28	0.23	2.24±0.65	0.25	1.60±0.28	0.25	ns
C20:1 n-9	1.63±0.19	0.21	1.20±0.19	0.19	1.78±0.58	0.20	1.01±0.31	0.16	ns
C20:2 n-6	1.25±0.13	0.16	0.97±0.14	0.15	1.44±0.436	0.16	0.97±0.24	0.15	ns
C20:4 n-6	23.84±2.71	3.12	20.12±2.58	3.17	26.00±4.55	2.93	19.03±3.74	2.96	ns
C24:1	1.91±0.59	0.25	1.37±0.54	0.22	0.98±0.13	0.11	1.48±0.61	0.23	ns
DPA, 22:5 n-3	6.19±1.38	0.81	5.16±0.84	0.81	5.70±1.42	0.64	3.68±1.37	0.57	ns
DHA, 22:6 n-3	8.08[a]±0.85	1.06	10.01[a]±1.36	1.58	18.12[b]±2.99	2.04	16.61[b]±2.81	2.58	*
Total SFA	272.80±32.60	35.71	235.04±37.30	37.05	312.41±64.20	35.15	229.20±52.00	35.62	ns
Total UFA	491.07±64.20	64.29	399.28±66.70	62.95	576.37±120.00	64.85	414.17±95.90	64.38	ns
Total Monoenes	305.22±42.50	39.96	249.94±42.80	39.40	364.40±75.70	41.00	248.25±63.70	38.59	ns
Total PUFA n-3	16.11±2.41	2.11	16.65±2.33	2.63	26.06±4.92	2.93	21.89±4.10	3.40	ns
Total PUFA n-6	168.50±19.40	22.06	131.72±22.30	20.77	184.47±41.30	20.76	143.07±28.20	22.24	ns
Overall Total	763.88±96.70		634.32±104.00		888.78±185.00		643.37±148.00		ns
n-6 : n-3 ratio		10.46		7.91		7.08		6.54	
U : S ratio		1.80		1.70		1.84		1.81	
P : S ratio		0.68		0.63		0.67		0.72	

Values with different superscripts within row differ significantly at P<0.05;
[ns] No significant difference
* P<0.05

5.3.8 Plasma cholesterol

The plasma cholesterol concentrations (Figure 5.1) from all of the FP treated groups were significantly lower ($P<0.05$) compared to the control group at the end of the experiment (29 weeks of age). The birds from FP9 group had the lowest ($P<0.05$) cholesterol concentration among the experimental groups.

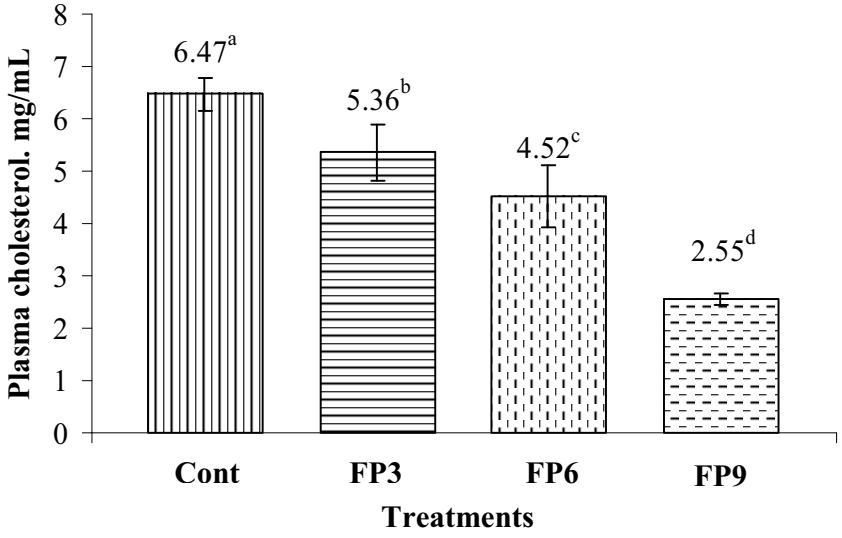

Figure 5.1: Effects of treatment diets on plasma cholesterol concentrations (mg/ml). (Error bars = SE; Bars with different superscripts differ significantly at $P<0.05$

5.3.9 Layer Performance

The average daily feed intake (g), hen-day egg production (%), egg mass (g), egg weight (g) and feed conversion ratio (g feed intake/g egg mass) are shown in Figures 5.2 and 5.3, Tables 5.10 and 5.11 and Figure 5.4, respectively. There was no significant difference ($P>0.05$) in average feed intake, egg production, egg mass and feed conversion ratio among the experimental groups. However, the FP9 fed hens had lower ($P<0.05$) egg weights compared to the control hens at 25, 26 27 and 28 weeks of age. The average initial and final body weight of the experimental groups are presented in Appendix 12.

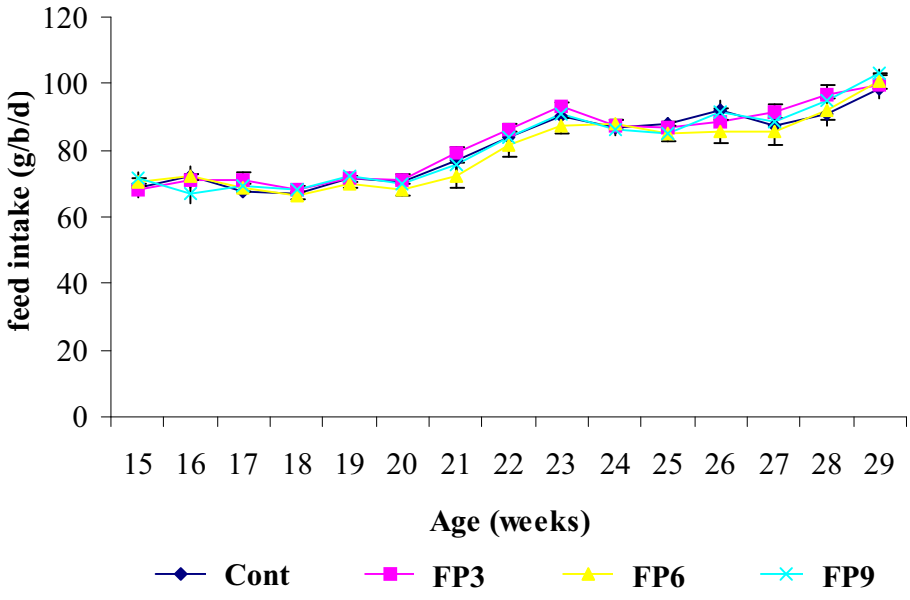

Figure 5.2: Effects of treatment diets on daily feed intake.
(Errors bars = SE)

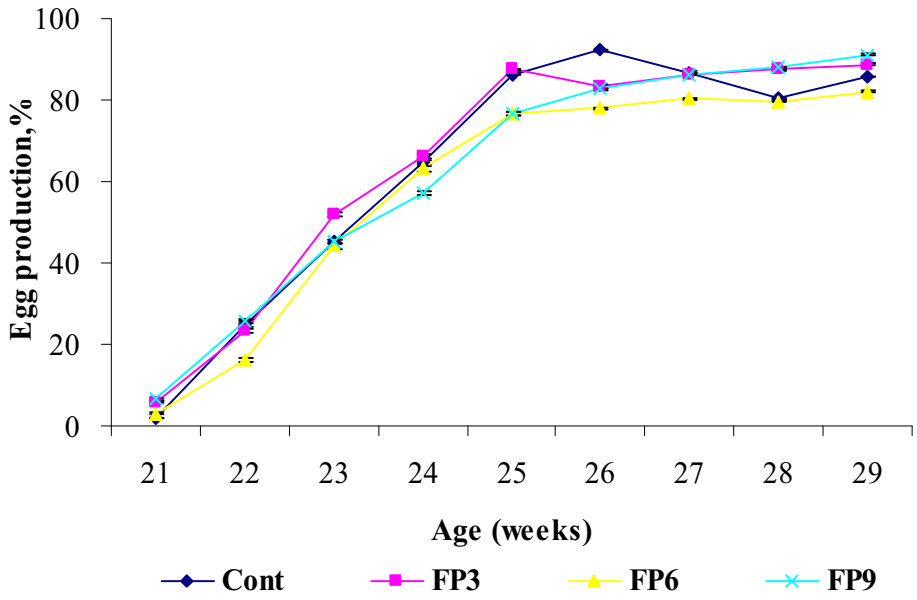

Figure 5.3: Effects of treatment diets on egg production.
(Errors bars = SE)

Table 5.10: Effects of treatment diets on egg mass

Age (weeks)	Treatments			
	Cont Mean (g) ± SE	FP3 Mean (g) ± SE	FP6 Mean (g) ± SE	FP9 Mean (g) ± SE
21	9.1±2.7	7.3±1.2	15.3±4.0	9.3±1.8
22	25.6±5.4	26.4±5.2	28.3±6.8	34.9±4.2
23	37.3±3.7	37.1±3.6	35.0±2.9	38.0±4.1
24	43.6±2.4	36.1±3.6	37.8±3.9	37.3±4.2
25	47.8±1.8	46.0±2.0	44.7±2.0	41.6±2.6
26	49.7±1.2	44.8±2.2	44.6±2.2	44.2±2.6
27	50.1±1.5	46.9±2.1	46.5±2.2	47.3±2.3
28	46.7±2.3	47.5±1.3	46.2±2.2	47.0±1.6
29	51.8±1.9	49.5±1.6	48.8±1.6	49.9±1.3

The results are presented as mean values ± SE.
All values within a row were not significantly different from each other (P>0.05).
egg mass = (egg weight x egg production)/100

Table 5.11: Effects of treatment diets on egg weight

Age (weeks)	Treatments				Sig.
	Cont Mean (g) ± SE	FP3 Mean (g) ± SE	FP6 Mean (g) ± SE	FP9 Mean (g) ± SE	
21	43.0±2.0	43.1±1.9	42.3±2.8	41.5±2.2	ns
22	47.8±1.2	47.8±0.6	45.5±0.9	46.1±1.0	ns
23	49.4±0.8	49.0±0.7	49.2±0.5	48.0±0.8	ns
24	52.0±0.9	51.1±0.7	51.5±0.6	50.1±0.6	ns
25	52.3±0.7	52.1±0.6	52.8±0.7	50.7±0.6	ns
26	53.8^{ab}±0.6	52.6^{ab}±0.6	54.3^{a}±0.7	51.9^{b}±0.7	*
27	54.8^{ab}±0.6	53.7^{b}±0.5	55.4^{a}±0.5	53.2^{b}±0.5	*
28	54.3^{ab}±0.6	54.2^{ab}±0.6	55.7^{a}±0.8	53.3^{b}±0.7	*
29	54.5^{a}±0.9	55.2^{ab}±0.6	56.9^{b}±0.7	54.3^{a}±0.7	*

The results are presented as mean values ± SE.
Values with different superscripts within a row differ significantly at P<0.05;
^{ns} No significant difference
* P<0.05

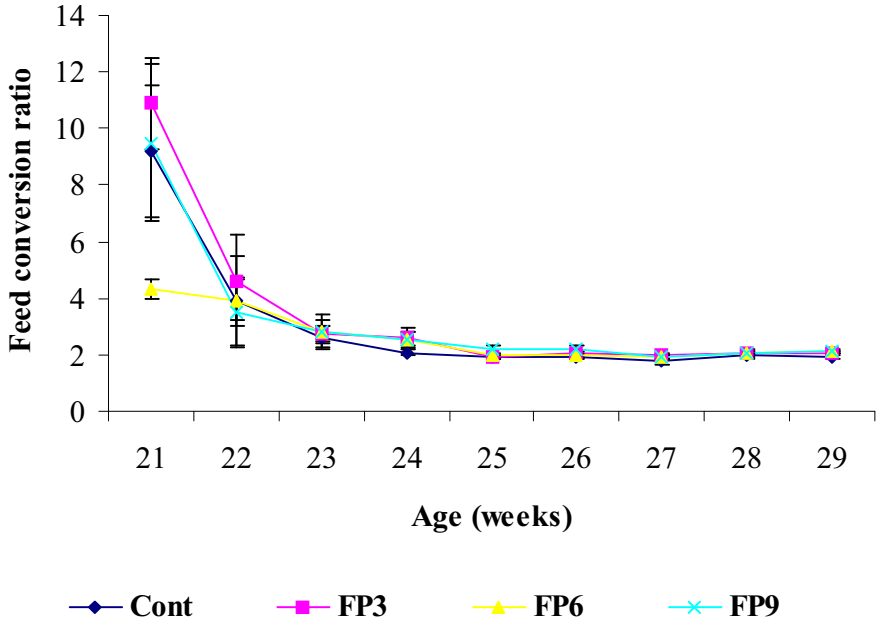

Figure 5.4: Effects of treatment diets on feed conversion ratios (g feed intake/g egg mass). (Errors bars = SE)

5.4 Discussion

5.4.1 Faecal characteristics

5.4.1.1 Faecal *Enterobacteriaceae* counts

The FP in the present study was produced using the LAB as the starter culture. Both *in-vitro* and *in-vivo* studies of LAB have been shown to have a bactericidal effect on harmful bacteria (Lindgren and Dobrogosz, 1990; de Vuyst and Vandamme, 1994; Forestier *et al*., 2001; Foo *et al*., 2003). Oral administration of FP (6%, w/w and 9%, w/w) rich in these beneficial bacteria has been shown to be able to reduce the faecal *Enterobacteriaceae* count. This was attributed to the reduction in faecal pH as a result of FP supplementation and increased proliferation of the faecal LAB in the present study. The faecal *Enterobacteriaceae* populations reflected the *Enterobacteriaceae*-LAB balance in the GIT of the host animal. Several *in-vivo* studies reported that the numbers of *Enterobacteriaceae* in the GIT of the fermented feed fed groups were lower compared to the control group (van Winsen *et al*., 2001; 2002, Canibe and Jensen, 2003; Højberg *et al*., 2003 Loh *et al*., 2003a,b). This could be due to the fact that intestinal LAB is able to produce antimicrobial compounds such as bacteriocin, VFA's and non-VFA, which are bacteriostatic against pathogenic bacteria (Adam and Nicolaides, 1997; Jin *et al*., 1998b; Scheinbach, 1998; Ross, *et al*., 2002; Holzapfel, 2002). The undissociated organic acid decreased the pH when it passed through the cell wall of the bacteria (Beal *et*

al., 2002; Immerseel *et al*., 2003). In the internal environment of the bacteria cell, the molecules will be dissociated and decreased the internal pH. This in turn stopped the enzymatic processes and collapsed the proto motive force. Growth is inhibited because the cell must spend energy trying to maintain internal pH and this energy cannot be used for other metabolic processes. This probably resulted in less numbers of *Enterobacteriaceae* that could pass the crop and gizzard, which would reduce the probability of *Enterobacteriaceae* reaching the caecum and be excreted through the faeces (Heres *et al*., 2003a,b).

5.4.1.2 Faecal LAB counts

In the present study, laying hens fed diets supplemented with FP (6%, w/w and 9%, w/w) had higher faecal LAB counts compared to the control group. The acidifying effect of the FP on the GIT provided a suitable environment for the growth of LAB (Loh *et al*., 2003a,b) as reflected in the LAB population in the faeces. The LAB are important components for a balanced microflora in the GIT. Feeding fermented liquid feed has been reported to increase the total number of lactobacilli in the GIT of treated animals (van Winsen *et al*., 2001; Canibe and Jensen, 2003; Højberg *et al*., 2003). However, there were also reports citing that fermented liquid feed had no effect on the lactobacilli populations in the GIT (van Winsen *et al*., 2002; Demecková *et al*., 2002). Loh *et* al. (2003a,b) reported that a solid form of fermented feed increased the faecal LAB of treated animals. This may be due to the fact that liquid fermented feed is more unpredictable and less consistent compared

to solid fermented feed (van Winsen *et al*., 2002; Demecková *et al*., 2002). Therefore, these conditions diminished the efficiency of fermented liquid feed compared to solid fermented feed and FP on improving the LAB in the GIT. The improvement in faecal LAB count in the present study was only observed after 2 weeks of experiment which was evidently faster than the study by Jin *et al*. (1998b) for *Lactobacillus*-fed broilers where the effect was seen after 30 days of experiment.

5.4.1.3 Faecal pH and volatile fatty acids (VFA)

Feeding FP (9%, w/w) to the birds was observed to decrease the faecal pH compared to the control group. This confirmed earlier reports (Heres *et al*. 2003a,b, 2004; Loh *et al*., 2003a,b). The FP lowered the crop pH to pH 4.5 (Cox *et al*., 1972) and therefore lowered the pH in GIT, resulting in a lower faecal pH. A number of factors may account for the beneficial effect of FP and these may act independently or synergistically. It was suggested that the FP reduced pH in the GIT thereby enhanced the activity of VFA. The VFA are known for their bacteriostatic effects on pathogenic bacteria in the GIT of chicken (Jin *et al*., 1998b; Izat *et al*., 1990; Mchan and Shotts, 1992; Hume *et al*., 1993a; Nisbet *et al*., 1996; Ricke, 2003). In the present study, the concentration of the acetic acid was the highest VFA found in the faeces of all groups. Acetic acid was also found to be the dominant VFA present in the ileum and cecal of chicken in earlier studies (Jin *et al*., 1998b; Choct *et al*., 1999; Immerseel *et al*., 2003). Propionic acid was metabolized and reabsorbed

before reaching the intestine (Hume *et al*., 1993b). Thus this explained the low proportion of propionic acids found in the faeces of all groups. It is presumed that the presence of organic acids in the feed together with the production of VFA and non-VFA in the GIT resulted in the more acidic faeces in the FP fed hens.

5.4.2 Egg nutritive value

5.4.2.1 Egg yolk fatty acid profiles

In the present study, feeding FP (6%, w/w and 9%, w/w) rich in n-3 and n-6 fatty acids to laying hens had resulted in the increased n-3 and n-6 fatty acid contents in the egg yolk. The FP (3% w/w, 6% w/w and 9%, w/w) was able to decrease the n-6: n-3 ratio and increase PUFA: SFA ratio in the eggs. These two parameters are currently used to determine the nutritional quality of the lipid fraction in foods (Ansorena and Astiasarán, 2004). The level of total n-3 and DHA in egg yolk was found to be higher in FP (3% w/w, 6% w/w and 9% w/w) fed hens compared to control hens. This observation is in agreement with the findings by several authors who fed the n-3 product to the laying hen successfully enhanced the n-3 fatty acids in the eggs (Ferrier *et al*., 1992; Ayerza and Coates, 1998; Milinsk *et al*., 2003; Carrillo-Domínguez *et al*., 2005). Enrichment of eggs with long-chain n-3 and n-6 PUFA had been accomplished through the supplement of hen diet with marine algae, fish and seed oil that were rich in n-3 and n-6 PUFA (Ferrier *et al*., 1992, Ayerza and Coates, 1998; Milinsk *et al*, 2003; Carrillo-Domínguez *et al*., 2005). It

is interesting to note that although the FP used in this study contained a higher level of EPA than DHA, the resulting level of DHA in the yolk was much higher as EPA was not detected in the egg yolk. These differences in incorporation rates of EPA and DHA into the egg yolk had been previously reported (Baucells *et al*., 2000; González-Esquerra and Leeson, 2000; Carrillo-Domínguez *et al*., 2005). The biochemical differences of EPA and DHA, both in their mechanisms for assimilation and metabolic conversion, are responsible for the different rates at which they are incorporated into the egg yolk (González-Esquerra and Leeson, 2000). Moreover, EPA is one of the precursors of DHA, as they are normally converted to DHA by chain reactions of desaturation and elongation (Gurr *et al*., 2002).

5.4.2.2 Egg yolk cholesterol concentrations

The egg yolk cholesterol content was affected by the level of FP supplementation (9%, w/w). Cholesterol reduction observed in this study was probably related to decreased cholesterol synthesis in the liver. In laying hens, the liver is the major site of cholesterol synthesis (Qureshi and Peterson, 2001). Laying hens usually synthesis cholesterol more than the body requirements (Luhman *et al*., 1990). The synthesized cholesterol was then secreted into the blood stream, carried by the very low density lipoprotein (VLDL) particles across the ovarian membranes and subsequently deposited in the developing yolks through the oocyte vitellogenesis receptor (March and Millan, 1990; Elkin *et al*., 1999). Thus, for the laying hen, a

major excretion pathway of cholesterol through the plasma transportation seems to be the production of egg yolk cholesterol. A reduction in cholesterol synthesized in the liver will result in lowered plasma and egg cholesterol. This is in agreement with the findings in the present study where egg yolk cholesterol and the plasma cholesterol were lowered compared to the control groups.

5.4.3 Plasma fatty acid profiles

It is well known that the fatty acids found in the plasma closely reflected the fatty acids composition of the diet offered. In the present study, the FP was able to increase the total n-3 fatty acid and decrease the total n-6 content of the plasma lipids, thus lowering the plasma n-6: n-3 ratio from 10.5 to a more beneficial value of 6.5. This was related to the high n-3 PUFA in the FP, due to the incorporation of marine fish. This is in agreement with the findings of Nash *et al*. (1995) who managed to increase plasma n-3 fatty acids in laying hens by feeding herring meals. In general, fatty acid synthesis in the laying hens usually occurred in the liver (Luhman *et al*., 1990). The desaturation and elongation of enzymes in the liver of the laying hens could elongate and desaturate the dietary sources of n-3 fatty acids to form the long-chain n-3 PUFA such as DHA (Watkins, 1995; González-Esquerra and Leeson, 2000; Gurr *et al*., 2002). This metabolite is then transported through the plasma and deposited in the egg yolk. Thus, feeding 6% (w/w) and 9% (w/w) FP to the laying hen increased the DHA concentration in the plasma. The high

plasma DHA was therefore postulated to contribute to the increased yolk DHA level in the present study.

5.4.4 Plasma cholesterol

In the present study, plasma cholesterol was significantly lower in all the FP fed hens compared to the control hens at the end of the experiment. Feeding 3% (w/w), 6% (w/w), and 9% (w/w) of FP significantly reduced the plasma cholesterol concentration by 17%, 30% and 61%, respectively. The reductions were greater than those observed in earlier reports (Jin *et al*., 1998a; Chowdhury *et al*., 2005). Feeding 0.1% (w/w) of Lactobacillus-probiotic to broiler significantly decreased the serum cholesterol by 17% (Jin *et* al, 1998a). Feeding a diet rich in n-6 PUFA to the laying hens significantly decreased the serum cholesterol concentrations by 11% to 43% (Chowdhury *et al*., 2005). The reduction of serum cholesterol might be due to a reduction in the synthetic enzyme activity. An increased PUFA had been known to lower regulate hydroxy-3-methylglutaryl coenzymes A (HMG Co-A) activity, the rate-limiting enzyme that determines the level of plasma cholesterol in animals (Fernandez *et al*., 1990).

Another possible explanation might be due to the presence of *Lactobacillus*-probiotic in the FP. Directly fed FP increased the LAB in GIT as reflected in the faecal LAB counts. This bacteria cell was reported to be able to assimilate the cholesterol and deconjugate the bile salt in the intestine (Jin *et al*., 1998a; Akalin *et*

al., 1997) and making it unavailable for absorption into the blood stream. The deconjugated bile salt will be excreted through the faeces. This in turn stimulates an increase in requirement for cholesterol as a precursor for the synthesis of new bile salts that are required for lipid metabolism (Jin *et al*., 1998a; Farnworth and Mainville, 2003; Chowdhury *et al*., 2005). This may explain the reduction of the plasma cholesterol in the FP fed hens.

5.4.5 Layer performance

The FP used in the present study was rich in LAB, had a lower pH level and contained beneficial long-chain PUFA. Supplementation of FP at 3% (w/w), 6% (w/w) and 9% (w/w) in layer diets did not affect feed intake, egg production, egg mass and FCR. There was no mortality recorded during the experimental period. The FP produced in the present study contained high levels of n-3 fatty acids. However, feeding higher levels of FP (9%, w/w) significantly decreased the egg weight compared to control group (0% FP). Changes in egg weight and egg mass associated with dietary n-3 fatty acids have been reported previously (Leeson *et al*., 1998; Gonzalez-Esquerra and Leeson, 2000; Schreiner *et al*., 2004). Feeding higher levels of n-3 rich products such as (6%, v/w) menhaden oil (Gonzalez-Esquerra and Leeson, 2000) and (5%, v/w) seal blubber oil (Schreiner *et al*., 2004) decreased the egg mass. A decrease in circulating triglycerides of birds due to n-3 consumption could limit the availability of lipids for yolk formation (Gonzalez-Esquerra and Leeson, 2000). This might be due to the lower plasma triglycerides, a result of the

hypotriglyceridemic effect of n-3 fatty acids (Schreiner *et* al., 2004). This could explain the lower egg weight and egg mass found in the birds fed FP in the present study.

5.5 Conclusions

The present experiment demonstrated that the addition of 3% (w/w), 6% (w/w) and 9% (w/w) FP to the layers diet did not affect the feed intake, egg production, egg mass and FCR. However, the 6% (w/w) and 9% (w/w) FP diets were able to reduce the *Enterobacteriaceae* population (log_{10} cfu/g) in faeces as well as increasing the faecal LAB population (log_{10} cfu/g). Feeding 9% (w/w) of FP was able to reduce the level of faecal pH in the present study. This indicated that FP has the potential to be added in animal feed to promote better health performance without adversely affecting production parameters. Furthermore, feeding 6% (w/w) and 9% (w/w) of FP resulted in a decrease in plasma (mg/ml) and egg cholesterol (mg/100g) concentrations. It was demonstrated in the present study that the essential fatty acids, especially DHA in the plasma and egg were increased due to the feeding of FP. In addition, the n-6: n-3 ratio was decreased with an increased in the PUFA: SFA ratio to a more beneficial balance. Finally, it can be concluded that feeding 6% and 9% FP to the laying hens enhanced the egg's nutritive quality.

CHAPTER VI

GENERAL DICUSSION AND CONCLUSIONS

6.1 General discussion

In the present study, LAB was used as the starter culture for fermentation. It is one of the most commonly used starter culture for food and feed fermentation (Leroy and de Vuyst, 2004; Chapter II). The fermentation process and characteristics of FP were reported in Chapters III and IV, respectively. The present findings demonstrated that the physico-chemical characteristics of the FP after fermentation process were quite constant. Furthermore, fermentation increased the numbers of LAB and decreased the numbers of *Enterobacteriaceae* in the product compared to the raw materials. LAB produced antimicrobial metabolites such as organic acids during fermentation, and also reduced the pH of the raw materials. The undissociated acids diffuse over the cell membrane and acidified the cytoplasm, inhibited a variety of cell metabolic functions (Ross *et al*., 2002) thus suppress the proliferation of *Enterobacteriaceae*. Indeed, numerous *in-vitro* studies on the preparation of fermented feed and study on different genera of probiotic bacteria and their metabolites had been reported to have bactericidal/bacteristatic effects on the pathogenic and spoilage bacteria (Lindgren *et al*., 1990; McHan and Shotts, 1992; de Vuyst and Vandamme, 1994; Forestier *et al.,* 2001; Demecková *et al*., 2002; Heres *et al*., 2004).

Another important characteristic of FP was the presence of beneficial n-3 and n-6 PUFA. These fatty acids are now regarded as nutritionally beneficial fatty acids (Elmore *et al*., 2005). Feedstuff with added n-3 and n-6 PUFA are definitely more advantageous over conventional feeds as it improved beneficial n-3 PUFA in eggs (Ayerza and Coates, 1999; Schreiner *et al*., 2004; Carrillo-Domínguez *et al*., 2005).

The FP (6%, w/w and 9%, w/w) diets significantly improved the LAB and decreased the *Enterobacteriaceae* counts in the GIT as reflected in the faecal bacteria counts. Similarly, directly fed fermented liquid feed with LAB has been shown to increase the LAB and decrease the pathogenic bacteria in the GIT of the animals (van Winsen *et al*., 2001, 2002; Demecková *et al*., 2002; Canibe and Jensen, 2003; Højberg *et al*., 2003; Heres *et al*., 2003a,b, 2004). Feeding fermented solid feed provided a similar effect as feeding fermented liquid feed in promoting the growth of intestinal LAB and eliminating the intestinal *Enterobacteriaceae* (Loh *et al*., 2003a,b). Oral administration of FP in this study was believed to have an acidifying effect on the GIT. FP reduced the pH in the GIT thus suppressed the growth of *Enterobacteriaceae* (Loh *et al*., 2003a,b). Furthermore, the lactobacilli have a strong inhibitory effect in preventing the adherence, establishment and replication of enteropathogens (Jin *et al*., 1998a). They competed against pathogenic bacteria for nutrient, binding and receptor sites (Gallaher *et al*., 1996; Fujiwara *et al*., 1997; Kailasapathy and Chin, 2000). This in turn decreased the survivability of *Enterobacteriaceae* in the GIT (Heres *et al*., 2003a,b) and resulted in lesser excretion of *Enterobacteriaceae* in faeces.

In addition, the faecal VFA played an important role in enhancing the bactericidal effects on *Enterobacteriaceae* (Jin *et al*., 1998b). They are the major end product of microbial carbohydrate fermentation in the intestinal tract of the animal among which, acetic, propionic and butyric acids are dominant (Johansson *et al*., 1998). In this study, the concentration of acetic acid was the highest VFA found in the faeces. The higher level of acetic acid in combination of low pH in the GIT forms an improved bactericidal barrier (Marounek *et al*., 1999; van der Wielen *et al*., 2000, 2001; Heres *et al*, 2003a). The faecal *Enterobacteriaceae* counts reported in Chapter V after FP (6% w/w and 9%, w/w) feeding were lower than published figures for *Enterobacteriaceae* in the gut. This could be related to the bactericidal effects of antimicrobial substance present in the FP and pH reduction due to VFA production in the faeces. Overall, these supported the proliferation of LAB. The LAB are always considered as one of the important components of the gastrointestinal flora (Salminen *et al*., 1999; Shimakawa *et al*., 2003; Coeuret *et al*., 2004). These microorganisms have significant impacts on animal health, growth development, performance and pathogens. Van Winsen *et al*, (2002) and Loh *et al*, (2003a,b) reported that fermented feed was able to increase the faecal LAB under the stressful condition. The mechanisms involved could be associated with the ability of intestinal LAB to stimulate the immune system (de Simon *et al*., 1993; Scheinbach, 1998) in laying hens. However, the underlying mechanism of immune stimulation in the present study is not well understood. Overall, the FP modified the microorganism in the GIT. This was reflected in the faecal microflora which has shifted towards to a beneficial balance.

The LAB presence in the FP could be responsible for the cholesterol-lowering effect observed in the present study. This confirmed earlier *in-vitro* and *in-vivo* reports by other workers (Zacconi *et al*., 1992; Akalin *et al*, 1997; du Toit *et al*., 1998; Kawase *et al*., 2000; Pereira and Gibson, 2002). The LAB was able to assimilate cholesterol or to coprecipitate cholesterol with deconjugated bile acids in the intestine and making it unavailable for absorption into the blood stream (Gilliland *et al*., 1985b; Buck and Gilliland, 1994). Since cholesterol is used in the production of bile acids, enhanced catabolism and excretion of bile acids is reflected in the reduced plasma and egg cholesterol levels. Another explanation for the hypocholesterolaemic effect of the FP diet is the high PUFA content in the FP. This enabled the FP to decrease the synthesis of lipid components in the liver of the laying hens. Laying hens usually synthesized cholesterol more than their body requirement via *de-novo* synthesis in the liver (Luhman *et al*., 1990), which is then secreted into the blood stream through the plasma transportation and deposited in the rapidly developing follicle (March and Millan, 1990; Elkin *et al*., 1999). Increased PUFA had been known to down regulate Hydroxy-3-methylglutaryl coenzyme A (HMG Co-A) activity, the rate-limiting enzyme that determines the level of plasma cholesterol in animals (Fernandez *et al*., 1990). Changes in egg yolk cholesterol are generally preceded by the changes in plasma cholesterol levels (Sim *et al*., 1984). This may explain the reduction of the plasma and egg cholesterol in the FP fed hens.

Dietary inclusion of n-3 PUFA altered the fatty acid profiles in the eggs significantly (Ayerza and Coates, 1998; Gonzalez-Esquerra and Leeson, 2000; Meluzzi, *et al*., 2000; Milinsk *et al*., 2003; Carrillo-Domínguez *et al*., 2005). Eggs are a rich source of protein and it is an important source of essential PUFA and other essential nutrients as reported in Appendix 1. Because chicken is a monogastric animal, much of the dietary lipid is assimilated directly with minimal modification. Chickens are able to desaturate and elongate the fatty acids through the desaturation and elongation enzymes which are synthesized by the liver. Thus, the long chain (C20-22) fatty acids of the n-3 series can be synthesized from the dietary precursor, alpha-linolenic acid albeit slowly and deposited in eggs (Wood *et al*., 2003). The enrichment of foods with beneficial n-3 PUFA has been extensively studied. This is due to its importance in the maintenance of human health by protecting against metabolic disease such as cardiovascular diseases (Ferrier *et al*., 1992; Ayerza and Coates, 1999; Schreiner *et al*., 2004).

n-3 PUFA was detected in substantial amounts in the FP. Feeding FP (9%, w/w) to the laying hens increased the total n-3 PUFA and DHA concentrations in eggs and plasma compared to the control group. These n-3 PUFA were readily incorporated into the egg yolk in a high proportion through plasma transportation. The n-3 PUFA exert beneficial effects on lipoprotein metabolism and modulate eicosanoid biosynthesis in the laying hens (Watkins, 1995). Effectively, the FP was able to lower the n-6: n-3 ratio in the eggs and plasma compared to the control group. In addition, the FP resulted in an increase of the PUFA: SFA ratio both in plasma

(control=0.68 to FP9=0.72) and egg (control=0.5 to FP9=0.6) as these two factors are currently used to determine the nutritional quality of the lipid fraction in food (Ansorena and Astiasarán, 2004).

6.2 Conclusions

Based on the experiments conducted herein, it could be concluded that the inclusion of FP up to 9% (w/w) in the diets of layers did not have any detrimental effects on the growth and laying performance. In fact, the FP (9%, w/w) successfully improved the nutritive value of eggs, increased the faecal LAB, and decreased the potentially pathogenic *Enterobacteriaceae* in laying hens. It is well known that the health performance of the animal is much dependent on the equilibrium between the beneficial microflora in the GIT. The FP helps to promote the symbiotic relationship between the host and its intestinal micro-flora. The results obtained from the study strongly demonstrated the consistency, efficiency and stability of the FP in enhancing the animal performance and nutrition. This indicated that FP has the potential to partially replace antibiotics in the feed and further promote green agricultural products in Malaysia.

6.3 Recommendations

Results from this study have shown that it is possible to alter the cholesterol and essential fatty acid concentrations in the plasma and egg yolk through the supplementation of FP in the diet. Lipoprotein metabolism plays an important role in determining the fat content and composition of the yolk. The VLDL is one of the major lipoprotein transporting triacylglycerol from the liver to extra hepatic tissues such as adipose tissues or deposited in the egg. Furthermore, the metabolism of VLDL in laying hens is influenced by age, diet and nutritional state. Thus, future studies should focus on the VLDL metabolism and lipoprotein lipase activity from different laying stages as age factors needed to be explored in order to understand the mechanism of fat deposition in laying hens. Another interesting aspect to be included is the organoleptic studies on eggs to determine the effects of FP on egg flavour and colour.

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APPENDICES

Appendix 1: Reported values for nutrient composition of eggs

	Egg		
Nutrients and units	Whole	White	Yolk
Proximate			
Solids, g	13.47	4.6	8.81
Calories	184	19	64
Crude protein (CP) (N X 6.25), g	6.6	3.88	2.74
Total lipids, g	6	-	5.8
Ash, g	0.55	0.26	0.29
Lipids			
Fatty acids, g			
Saturated, total	2.01	-	1.95
C8:0	0.027	-	0.027
C10:0	0.082	-	0.08
C12:0	0.027	-	0.026
C14:0	0.022	-	0.022
C16:0	1.37	-	1.31
C18:0	0.462	-	0.459
C20:0	0.022	-	0.022
Monounsaturated, total	2.53	-	2.5
C14:1	0.005	-	0.005
C16:1	0.214	-	0.211
C18:1	2.31	-	2.28
Polyunsaturated, total	0.73	-	0.72
C18:2	0.66	-	0.65
C18:3	0.011	-	0.014
C20:4	0.055	-	0.051
Cholesterol, g	0.213	-	0.213
Lecithin, g	1.27	-	1.22
Cephalin, g	0.253	-	0.241

Nutrients and units	Egg		
	Whole	White	Yolk
Vitamins			
A, IU	264	-	260
D, IU	27	-	27
E, mg	0.88	-	0.87
B_{12}, ug	0.48	-	0.48
Biotin, ug	11	2.58	8.35
Choline, mg	237	0.46	238
Folic acid, mg	0.023	0.006	0.026
Inositol, mg	5.94	1.52	4.35
Niacin, mg	0.045	0.035	0.01
Pantothenic acid, mg	0.83	0.09	0.73
Pyridoxine, mg	0.065	0.008	0.057
Riboflavin, mg	0.18	0.11	0.07
Thiamine, mg	0.05	0.004	0.048
Minerals, mg			
Calcium	29.2	3.8	25.2
Chlorine	96	66.1	29.9
Copper	0.033	0.009	0.024
Iodine	0.026	0.001	0.024
Iron	1.08	0.053	1.02
Magnesium	6.33	4.15	2.15
Manganese	0.021	0.002	0.019
Phosphorus	111	8	102
Potassium	74	57	17
Sodium	71	63	9
Sulfur	90	62	28
Zinc	0.72	0.05	0.66
Amino Acids, g			
Alanine	0.38	0.24	0.14
Arginine	0.42	0.23	0.19
Aspartic Acid	0.65	0.4	0.25
Cystine	0.15	0.11	0.05
Glutamic acid	0.85	0.52	0.33
Histidine	0.16	0.09	0.07

Nutrients and units	Egg		
	Whole	White	Yolk
Amino Acids, g			
Glycine	0.22	0.14	0.08
Isoleucine	0.36	0.21	0.15
Leucine	0.57	0.33	0.24
Lysine	0.45	0.25	0.2
Methionine	0.21	0.15	0.06
Phenylalanine	0.35	0.23	0.12
Proline	0.26	0.15	0.11
Serine	0.5	0.27	0.23
Threonine	0.32	0.18	0.14
Tryptophan	0.11	0.07	0.04
Tyrosine	0.28	0.16	0.12
Valine	0.43	0.27	0.16

Source: Cotterill and Glauert, (1979) as cited by Stadelman and Cotterill (1995)

Appendix2: Preparation and calculation of chemicals for analysis of crude protein (CP)

a. Receiver solution

Approximately ten gram of boric acid was weighed into a one litre volumetric flask and topped up to one litre with distilled water. One hundred ml of bromocresol green solution (100mg in 100ml methanol) was mixed homogeneously with 70ml of methyl red solution (100mg in 100ml methanol) as indicator solution. The boric acid described above was added to this indicator solution.

b. Alkali

Four hundred gram of sodium hydroxide (NaOH) was dissolved in one liter of distilled water in a volumetric flask.

c. Standard (Ammonium sulphate, $(NH_4)_2SO_4$)

Molecular weight,$(NH_4)_2SO_4$= 132.14

N content in $(NH_4)_2SO_4$ = $N/(NH_4)_2SO_4$*100

= 28/132.14*100

= 21.19%

Protein content of $(NH_4)_2SO_4$= 21.19*6.25

=132.4375%

The purity of $(NH_4)_2SO_4$ is 99.5%, therefore protein content = 0.995*132.4375

= 131.77%

1M of $(NH_4)_2SO_4$ = 132g/L

1mL of $(NH_4)_2SO_4$, Protein = 0.132/mL*131

=17.3%

Appendix 3: Preparation of buffer and agar for microbiological analyses

a. Peptone water

Approximately 25g of powdered peptone water was weighed and transferred into a one litre biker. One litre of distilled water was added into the biker. After powdered peptone water well dissolved, it was divided into the Duran bottle and autoclave at 121^{o}C for 15min.

b. *Lactobacillus*-Agar DE Man, ROGOSA and SHAPE (MRS) agar

Approximately 66.2g of powdered agar was weighed and transferred into one litre conical flask. One liter of distilled water was added into the conical flask. The powdered agar was vortex until well dissolved. The prepared agar was placed into an autoclave. The operating condition was set at 118^{o}C for 15min.

MRS agar, a media introduced by De Man, *et al* (1960) for the cultivation and isolation of *Lactobacillus* species from all types of materials. The MRS culture media contained polysorbate, acetate, magnesium and manganese, which were known to act as special growth factors for lactobacilli.

c. Eosin-Methylene-blue Lactose Sucrose Agar (EMB) agar

Approximately 36g of powdered EMB agar was weighed and transferred into one litre conical flask. One litre of distilled water was added into the conical flask. The powdered agar was stirred until well dissolved. The prepared agar was placed into an autoclave. The operating condition was set at 121^{o}C for 15min.

EMB agar is a selective agar for the enumeration of pathogenic *Enterobacteriaceae*. The lactose and sucrose contained in this medium allowed lactose and sucrose negative *Salmonella* and *Shigella* to be distinguished from lactose positive coliform organisms and lactose negative, sucrose positive accompanying flora (e.g. *Proteus vulgaris*, *Citrobacter*, *Aeromonas hydrophilia*). The growth of undesired accompanying microorganisms, particularly gram-positive bacteria, was largely inhibited by the dyes present in the medium. The appearances of colonies and microorganisms are shown in Appendix3C.

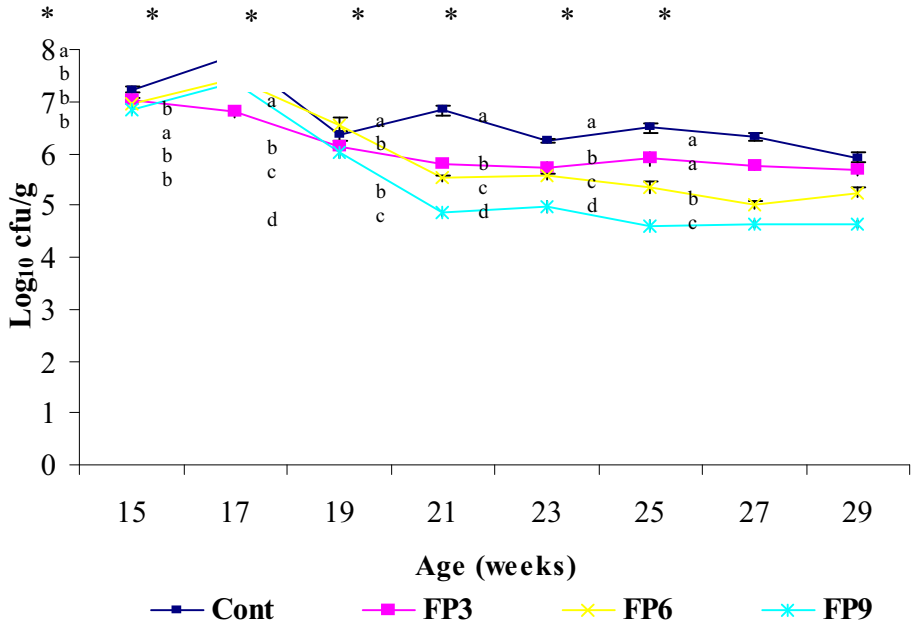
Appendix 4: The appearances of colonies and microorganisms

Appearance of colonies	Microorganism
Translucent, amber coloured	*Salmonella*, *Shigella*
Greenish, metallic sheen in reflected light, blue-black center in transmitted light	*Escherichia coli*
Colonies are larger than those of *E. coli*, mucoid, confluent, gray-brown center in transmitted light	*Enterobacter*, *Klebsiella* and others

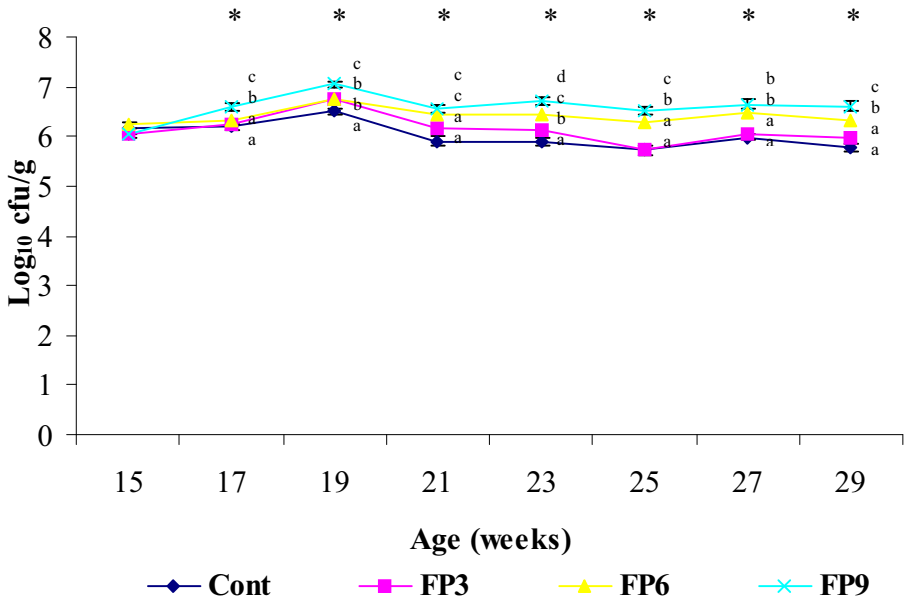
Source: Merck®, KGaA, Damstadt, Microbiological manual, 2000.

Appendix 5: Preparation of glassware and chemical for gas chromatography

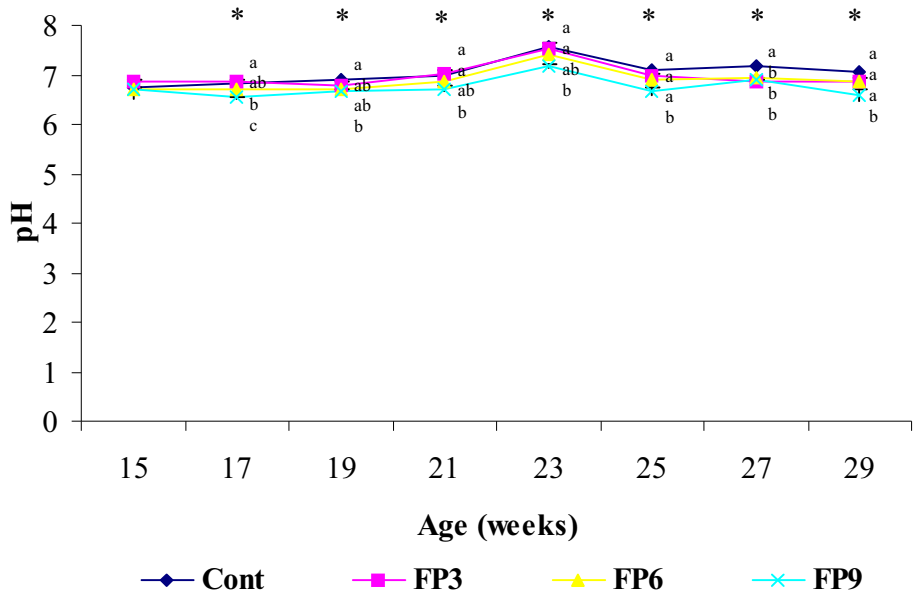
All chemicals, solvents and laboratory supplies used for total lipid extraction, VFA extraction and cholesterol extraction and preparation of cholesterol methyl esters and fatty acid methyl esters were of analytical grade. Teflon type lining materials were used to avoid contact of samples, solvents and chemical supplies with rubber or fat derivatives. Ten mg/L of buthylated hydroxytoulene (2,6-di-tert-butyl-p-cresol) (Sigma Chemical Co., St. Louis, Missouri, USA) was added into the solvent (petroleum ether, chloroform and chloroform methanol, from Merck® KGaA, Darmstadt, Germany) as antioxidant to prevent the destructive oxidation of UFA during experimental manipulations and storage. Decon 90 (Decon Laboratories Ltd., Sussex, U.K) was used to soak the glassware before scrubbed and then rinse thoroughly with the tap water. Finally, all the washed glassware were soaked in distilled water for overnight and re-rinsed the next day before dried in the oven at 60^{o}C.



Appendix 6: Effects of treatment diets on faecal *Enterobacteriaceae* counts [$\log_{10}$ cfu/g). (Errors bars = SE, * P<0.05, ^{a,b,c,d} points with different alphabet notation within a week differ significantly at P<0.05).



Appendix 7: Effects of treatment diets on faecal LAB counts (log_{10} cfu/g). (Errors bars = SE, * P<0.05, [a,b,c,d] points with different alphabet notation within a week differ significantly at P<0.05).



Appendix 8: Effects of treatment diets on faecal pH. (Errors bars = SE, * P<0.05, [a,b,c] points with different alphabet notation within a week differ significantly at P<0.05).

Appendix 9: Preparation of VFA standards

a. Preparation of external standard

The VFA standard was prepared according to the procedure of Cottyn and Bouque (1968) modified by Minato and Kudo (1985) as follows:

A. 11.45 ml acetic acid (AA), 7.46 ml propionic acid (PA), 9.27 ml iso-butyric acid (IsBA) and 9.19 ml butyric acid (BA) were put in different 100 ml volumetric flasks

and distilled water was added to make up to 100 ml. The concentrations of the stock solutions are 2M for AA and 1M for PA, IsBA and BA.

B. 1.097 ml iso-valeric acid (IsoVA), 1.088 ml valeric acid (VA) and 1.262 ml 4-methyl-n-valeric acid (4MV; internal standard) were put in different 100 ml volumetric flasks. One drop of phenolphthalein indicator was added the acid which was then neutralized by the addition of 2M NaOH (between 3 to 5 ml) and distilled water was added to make up to 100ml. Neutralization is reached when the colourless solution changes to pink. The concentrations of IsoVA, VA and 4MV are 0.1M.

1 ml of each of the acid from A 10 ml of each of the acid from B were added to a 100 ml volumetric flask and diluted with distilled water to make up to 100 ml. The final concentration of the acetic acid is 20 mM (0.02M) and that of the other acids is 10 mM (0.01M), respectively.

b. Preparation of internal standards

20 mM (0.02M) 4-methly-n-valeric acid was prepared by diluting 20ml of 0.1M stock solution of 4-methyl-n-valeric acid from B with distilled water to make up to 100ml.

Appendix 10a: Effects of treatment diets on egg yolk fatty acid compositions in birds at 25 weeks of age

Fatty Acid	Cont Mean ±SEM (mg/100g)	%	FP3 Mean ±SEM (mg/100g)	%	FP6 Mean ±SEM (mg/100g)	%	FP9 Mean ±SEM (mg/100g)	%
C14:0	72.5^{a}± 2.7	0.4	74.7^{a}± 2.4	0.4	78.7^{ab}± 3.1	0.5	86.7^{b}± 4.7	0.5
C16:0	4284.0^{a}± 142.0	26.0	4375.5^{ab}± 165.0	25.8	4534.8^{ab}± 106.0	26.6	4781.0^{b}± 142.0	26.4
C16:1 n-9	539.1^{ab}± 22.9	3.3	469.9^{a}± 34.3	2.8	588.9^{b}± 41.1	3.4	559.7^{ab}± 30.2	3.1
C18:0 ^{ns}	1274.4± 32.7	7.7	1380.1± 70.1	8.1	1265.2± 28.4	7.4	1313.8± 45.1	7.2
C18:1 n-9 ^{ns}	7406.4± 311.0	44.9	7317.0± 339.0	43.1	7366.9± 95.6	43.1	7655.5± 268.0	42.2
C18:2 n-6	2068.3^{a}± 117.0	12.5	2402.3^{ab}± 134.0	14.1	2364.2^{ab}± 131.0	13.8	2766.1^{b}± 150.0	15.3
C18:3 n-3	45.8^{a}± 2.9	0.3	52.7^{a}± 3.4	0.3	56.5^{a}± 3.5	0.3	70.4^{b}± 4.9	0.4
C20:1 n-9 ^{ns}	63.1± 2.6	0.4	57.5± 2.4	0.3	63.5± 1.1	0.4	64.0± 4.0	0.4
C20:2 n-6 ^{ns}	33.6± 6.2	0.2	31.8± 1.5	0.2	32.2± 5.4	0.2	39.2± 8.1	0.2
C20:4 n-6	415.2^{ab}± 25.4	2.5	422.1^{a}± 28.2	2.5	358.3^{bc}± 12.5	2.1	350.8^{c}± 11.5	1.9
DPA, C22:5 n-3	131.6^{a}± 7.9	0.8	105.3^{a}± 13.4	0.6	100.8^{b}± 18.9	0.6	51.4^{c}± 9.5	0.3
DHA, C22:6 n-3	156.9^{a}± 9.5	1.0	292.3^{b}± 22.8	1.7	282.4^{b}± 26.6	1.7	391.8^{c}± 19.8	2.2
Total SFA	5630.9^{a}± 172.0	34.1	5830.3^{ab}± 217.0	34.3	5878.7^{ab}± 128.0	34.4	6181.5^{b}± 179.0	34.1
Total UFA ^{ns}	10860.1± 361.0	65.9	11151.1± 427.0	65.7	11213.6± 208.0	65.6	11949.0± 397.0	65.9
Total MUFA ^{ns}	8008.6± 321.0	48.6	7844.5± 354.0	46.2	8019.3± 107.0	46.9	8279.3± 269.0	45.7
Total PUFA n-3	334.4^{a}± 10.4	2.0	450.4^{bc}± 31.6	2.7	439.6^{b}± 26.0	2.6	513.6^{c}± 16.6	2.8
Total PUFA n-6	2483.6^{a}± 122.0	15.1	2824.4^{ab}± 142.0	16.6	2722.5^{ab}± 143.0	15.9	3116.9^{b}± 161.0	17.2
Overall Total ^{ns}	16491.0± 526		16981.4± 640.0		17092.4± 326.0		18130.5± 563.0	
n-6 : n-3 ratio		7.4		6.3		6.2		6.1
UFA : SFA ratio		1.9		1.9		1.9		1.9
PUFA : SFA ratio		0.5		0.6		0.5		0.6

Values with different superscripts within row differ significantly at P<0.05;
^{ns} No significant difference

Appendix 10b: Effects of treatment diets on egg yolk fatty acid compositions in birds at 26 weeks of age

Fatty Acid	Cont Mean ±SEM (mg/100g)	%	FP3 Mean ±SEM (mg/100g)	%	FP6 Mean ±SEM (mg/100g)	%	FP9 Mean ±SEM (mg/100g)	%
C14:0 [ns]	80.8± 1.8	0.4	82.4± 3.4	0.5	77.8± 3.6	0.4	84.7± 1.6	0.4
C16:0 [ns]	4707.4± 97.9	25.9	4651.4± 136.0	25.8	4643.7± 98.1	25.6	4874.0± 52.9	25.8
C16:1 [ns] n-9	568.1± 23.5	3.1	583.1± 37.6	3.2	497.6± 31.7	2.7	546.3± 37.4	2.9
C18:0 [ns]	1462.2± 11.8	8.0	1382.8± 46.4	7.7	1416.9± 46.1	7.8	1358.0± 38.0	7.2
C18:1 [ns] n-9	8120.4± 164.0	44.6	7890.5± 229.0	43.7	7952.2± 193.0	43.8	8043.9± 82.3	42.6
C18:2 n-6	2392.7[a]± 63.7	13.2	2528.9[a]± 115.0	14.0	2582.3[a]± 152.0	14.2	2922.6[b]± 89.0	15.5
C18:3 n-3	56.4[a]± 1.9	0.3	54.3[a]± 3.1	0.3	55.4[a]± 1.8	0.3	68.5[b]± 1.7	0.4
C20:1 [ns] n-9	69.5± 1.8	0.4	65.0± 2.3	0.4	62.3± 2.6	0.3	69.8± 3.7	0.4
C20:2 n-6	35.5[ab]± 0.6	0.2	34.4[a]± 1.3	0.2	36.0[a]± 5.0	0.2	42.9[b]± 1.9	0.2
C20:4 n-6	396.4[ab]± 11.9	2.2	412.8[a]± 13.6	2.3	384.4[ab]± 17.4	2.1	362.6[b]± 10.3	1.9
DPA, C22:5 n-3	158.5[a]± 6.8	0.9	112.3[b]± 11.5	0.6	101.0[c]± 17.3	0.6	81.8[c]± 11.5	0.4
DHA, C22:6 n-3	146.5[a]± 9.5	0.8	252.5[b]± 9.9	1.4	328.9[c]± 15.5	1.8	406.5[d]± 13.3	2.2
Total SFA [ns]	6250.4± 9.3.	34.4	6116.7± 181.0	33.9	6138.5± 125.0	33.8	6316.7± 48.4	33.5
Total UFA [ns]	11944.0± 239.0	65.6	11933.7± 332.0	66.1	11999.9± 248.0	66.2	12544.8± 31.0	66.5
Total MUFA [ns]	8758.0± 183.0	48.1	8538.6± 257.0	47.3	8512.0± 207.0	46.9	8659.9± 110.0	45.9
Total PUFA n-3	361.5[a]± 15.7	2.0	419.0[b]± 21.9	2.3	485.2[b]± 9.6	2.7	556.8[c]± 6.7	3.0
Total PUFA n-6	2789.1[a]± 66.9	15.3	2941.7[ab]± 124.0	16.3	2966.7[ab]± 167.0	16.4	3285.2[b]± 96.9	17.4
Overall Total [ns]	18194.4± 331.0		18050.4± 511.0		18138.4± 364.0		18861.4± 48.0	
n-6 : n-3 ratio		7.7		7.0		6.1		6.0
UFA : SFA ratio		1.9		2.0		2.0		2.0
PUFA : SFA ratio		0.5		0.6		0.6		0.6

Values with different superscripts within row differ significantly at $P<0.05$;
[ns] No significant difference

Appendix 10c: Effects of treatment diets on egg yolk fatty acid compositions in birds at 27 weeks of age

Fatty Acid	Cont Mean ±SEM (mg/100g)	%	FP3 Mean ±SEM (mg/100g)	%	FP6 Mean ±SEM (mg/100g)	%	FP9 Mean ±SEM (mg/100g)	%
C14:0 [ns]	72.3± 1.9	0.4	74.4± 2.3	0.4	71.4± 2.4	0.4	74.3± 1.9	0.4
C16:0 [ns]	4754.0± 45.0	25.8	4909.3± 58.4	25.8	4657.8± 147.0	25.6	4941.4± 119.0	26.0
C16:1 n-9	543.0[a]± 13.5	2.9	511.2[a]± 25.1	2.7	432.6[b]± 17.9	2.4	428.6[b]± 7.9	2.3
C18:0 [ns]	1432.8± 25.8	7.8	1446.2± 22.9	7.6	1352.5± 52.7	7.4	1439.1± 13.3	7.6
C18:1 n-9	8089.4[ab]± 68.7	43.9	8401.6[a]± 142.0	44.2	7760.0[b]± 252.0	42.6	7997.8[ab]± 65.4	42.0
C18:2 n-6	2647.1[a]± 67.7	14.4	2749.5[a]± 71.3	14.5	2937.9[ab]± 85.9	16.1	3097.7[b]± 154.0	16.3
C18:3 [ns] n-3	58.3± 2.5	0.3	57.2± 3.1	0.3	56.2± 5.6	0.3	62.5± 3.0	0.3
C20:1 n-9	65.2[a]± 1.3	0.4	66.1[a]± 5.9	0.3	54.1[b]± 1.4	0.3	52.3[b]± 0.9	0.3
C20:2 n-6	35.2[ab]± 5.8	0.2	39.7[a]± 7.6	0.2	37.3[b]± 1.7	0.2	37.2[b]± 1.3	0.2
C20:4 [ns] n-6	403.1± 6.6	2.2	390.6± 13.1	2.1	391.9± 13.6	2.2	399.7± 3.0	2.1
DPA, 22:5 n-3	156.6[a]± 1.7	0.9	131.9[b]± 7.3	0.7	112.4[bc]± 11.4	0.6	98.9[c]± 8.7	0.5
DHA, 22:6 n-3	155.1[a]± 4.5	0.8	224.7[b]± 11.5	1.2	339.2[c]± 20.0	1.9	393.4[d]± 13.3	2.1
Total SFA [ns]	6259.1± 58.5	34.0	6429.9± 79.8	33.8	6081.7± 199.0	33.4	6454.7± 112.0	33.9
Total UFA [ns]	12153.0± 126.0	66.0	12572.6± 118.0	66.2	12121.7± 387.0	66.6	12568.0± 163.0	66.1
Total MUFA	8697.6[ab]± 66.4	47.2	8978.9[a]± 137.0	47.3	8246.7[b]± 268.0	45.3	8478.6[b]± 66.7	44.6
Total PUFA n-3	370.0[a]± 7.3	2.0	413.8[a]± 13.3	2.2	507.9[b]± 31.6	2.8	554.8[b]± 18.0	2.9
Total PUFA n-6	3050.2[a]± 78.8	16.6	3140.1[a]± 8.1	16.5	3329.8[ab]± 100.0	18.3	3497.3[b]± 156.0	18.4
Overall Total [ns]	18412.1± 177.0		19002.5± 187.0		18203.4± 571.0		19022.8± 262.0	
n-6 : n-3 ratio		8.2		7.6		6.6		6.3
UFA : SFA ratio		1.9		2.0		2.0		2.0
PUFA : SFA ratio		0.5		0.6		0.6		0.6

Values with different superscripts within a row differ significantly at $P<0.05$;
[ns] No significant difference

Appendix 10d: Effects of treatment diets on egg yolk fatty acid compositions in birds at 28 weeks of age

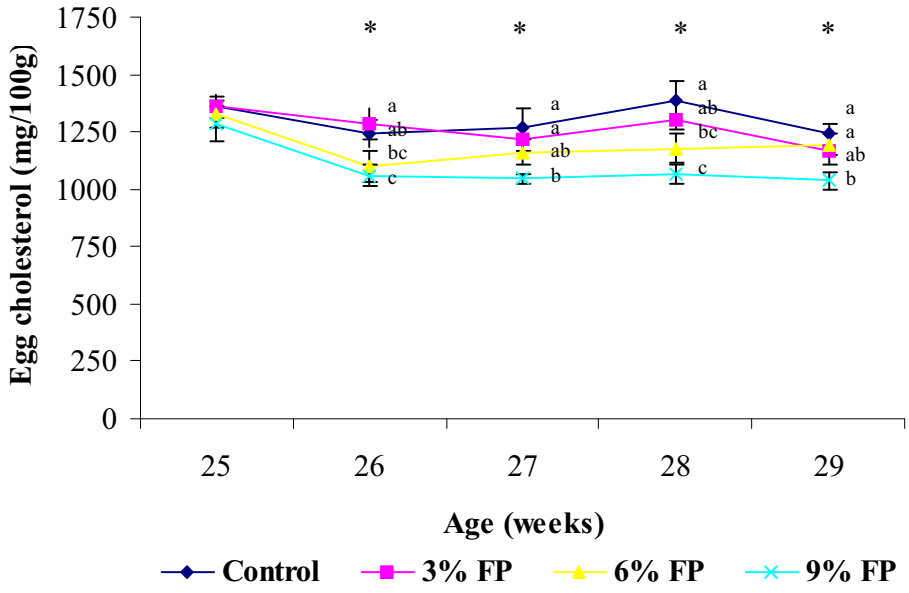
Fatty Acid	Cont Mean ±SEM (mg/100g)	%	FP3 Mean ±SEM (mg/100g)	%	FP6 Mean ±SEM (mg/100g)	%	FP9 Mean ±SEM (mg/100g)	%
C14:0 [ns]	75.3± 8.5	0.4	86.2± 2.2	0.4	85.7± 5.3	0.4	88.5± 2.8	0.4
C16:0 [ns]	5183.3± 62.1	26.1	5080.3± 87.4	26.0	5242.6± 165.0	25.7	5090.8± 187.0	25.1
C16:1 [ns] n-9	581.7± 41.7	2.9	569.6± 30.7	2.9	526.8± 28.1	2.6	597.3± 36.6	2.9
C18:0 [ns]	1532.0± 32.3	7.7	1519.6± 66.0	7.8	1517.9± 83.2	7.4	1535.8± 52.7	7.6
C18:1 [ns] n-9	8841.8± 94.0	44.5	8611.6± 239.0	44.0	9055.0± 292.0	44.4	8973.4± 366.0	44.3
C18:2 [ns] n-6	2685.0± 148.0	13.5	2748.0± 79.8	14.0	2894.7± 159.0	14.2	2869.0± 115.0	14.2
C18:3 [ns] n-3	57.1± 3.0	0.3	61.6± 1.4	0.3	60.8± 4.2	0.3	65.2± 2.9	0.3
C20:1 n-9	71.0[a]± 3.1	0.4	63.4[ab]± 2.5	0.3	59.9[b]± 2.6	0.3	58.8[b]± 3.3	0.3
C20:2 [ns] n-6	36.5± 2.1	0.2	35.6± 5.9	0.2	33.4± 1.3	0.2	33.9± 0.7	0.2
C20:4 [ns] n-6	440.2± 16.5	2.2	411.6± 12.4	2.1	434.7± 19.2	2.1	420.8± 13.7	2.1
DPA, C22:5 n-3	181.0[a]± 15.3	0.9	159.7[a]± 3.8	0.8	112.1[b]± 4.3	0.6	92.3[c]± 17.5	0.5
DHA, C22:6 n-3	165.3[a]± 8.6	0.8	226.6[b]± 7.3	1.2	356.4[c]± 12.5	1.7	431.6[d]± 5.5	2.1
Total SFA [ns]	6790.6± 62.8	34.2	6686.2± 150.0	34.2	6846.2± 253.0	33.6	6715.1± 236.0	33.1
Total UFA [ns]	13059.5± 153.0	65.8	12887.7± 270.0	65.8	13533.7± 450.0	66.4	13542.1± 479.0	66.9
Total Monoenes [ns]	9494.6± 122.0	47.8	9244.6± 218.0	47.2	9641.7± 320.0	47.3	9629.4± 400.0	47.5
Total PUFA n-3	403.4[a]± 23.9	2.0	448.0[a]± 9.6	2.3	529.3[b]± 17.6	2.6	589.1[b]± 23.0	2.9
Total PUFA [ns] n-6	3125.2± 159.0	15.7	3159.6± 85.9	16.1	3329.4± 175.0	16.3	3289.8± 123.0	16.2
Overall Total [ns]	19850.1± 208.0		19573.9± 417.0		20380.0± 696.0		20257.2± 701.0	
n-6 : n-3 ratio		7.7		7.1		6.3		5.6
UFA : SFA ratio		1.9		1.9		2.0		2.0
PUFA : SFA ratio		0.5		0.5		0.6		0.6

Values with different superscripts within a row differ significantly at P<0.05;
[ns] No significant difference

Appendix 10e: Effects of treatment diets on egg yolk fatty acid compositions in birds at 29 weeks of age

Fatty Acid	Cont Mean ±SEM (mg/100g)	%	FP3 Mean ±SEM (mg/100g)	%	FP6 Mean ±SEM (mg/100g)	%	FP9 Mean ±SEM (mg/100g)	%
C14:0 [ns]	90.4± 4.1	0.5	95.7± 0.7	0.5	96.0± 4.1	0.5	100.1± 3.5	0.5
C16:0	4987.5[a]± 113.0	25.8	5237.5[ab]± 45.0	25.9	5422.8[b]± 92.9	25.8	5358.7[b]± 90.9	25.6
C16:1 n-9	644.1[a]± 37.4	3.3	610.7[a]± 15.9	3.0	527.6[b]± 9.1	2.5	670.6[a]± 31.2	3.2
C18:0	1492.3[a]± 60.6	7.7	1642.2[b]± 60.1	8.1	1542.5[ab]± 36.3	7.3	1531.6[ab]± 11.3	7.3
C18:1 n-9	8584.3[a]± 173.0	44.4	8821.7[ab]± 136.0	43.6	9230.6[b]± 126.0	43.9	9203.0[b]± 93.1	43.9
C18:2 n-6	2595.3[a]± 112.0	13.4	2823.0[ab]± 81.3	14.0	3145.3[c]± 100.0	15.0	3039.8[bc]± 46.7	14.5
C18:3 n-3	58.7[a]± 3.4	0.3	66.0[ab]± 2.5	0.3	66.1[ab]± 2.5	0.3	72.7[b]± 1.5	0.3
C20:1 [ns] n-9	79.1± 2.8	0.4	74.0± 2.5	0.4	68.3± 3.2	0.3	78.0± 4.8	0.4
C20:2 [ns] n-6	40.7± 5.4	0.2	42.7± 7.2	0.2	40.2± 1.6	0.2	41.0± 5.3	0.2
C20:4 n-6	419.0[ab]± 16.6	2.2	409.9[ab]± 13.6	2.0	436.8[a]± 8.6	2.1	387.4[b]± 8.1	1.8
DPA, C22:5 n-3	187.7[a]± 11.8	1.0	148.2[b]± 6.5	0.7	121.8[b]± 10.1	0.6	79.1[c]± 13.2	0.4
DHA, C22:6 n-3	135.5[a]± 6.9	0.7	239.3[b]± 10.1	1.2	327.1[c]± 7.8	1.6	388.9[d]± 13.8	1.9
Total SFA	6570.2[a]± 158.0	34.0	6975.4[b]± 98.1	34.5	7061.2[b]± 91.6	33.6	6990.4[b]± 101.0	33.4
Total UFA	12744.4[a]± 270.0	66.0	13235.5[a]± 148.0	65.5	13963.9[b]± 190.0	66.4	13960.4[b]± 66.0	66.6
Total MUFA	9307.4[a]± 202.0	48.2	9506.4[ab]± 127.0	47.0	9826.6[bc]± 121.0	46.7	9951.6[a]± 69.0	47.5
Total PUFA n-3	382.0[a]± 14.5	2.0	453.5[b]± 13.3	2.2	515.0[c]± 10.0	2.4	540.6[c]± 14.6	2.6
Total PUFA n-6	3014.3[a]± 126.0	15.6	3232.9[ab]± 93.0	16.0	3582.1[c]± 109.0	17.0	3427.2[bc]± 50.0	16.4
Overall Total	19314.6[a]± 420.0		20211.0[b]± 198.0		21025.1[b]± 264.0		20950.7[b]± 134.0	
n-6 : n-3 ratio		7.9		7.1		7.0		6.3
UFA : SFA ratio		1.9		1.9		2.0		2.0
PUFA : SFA ratio		0.5		0.5		0.6		0.6

Values with different superscripts within a row differ significantly at $P<0.05$;
[ns] No significant difference



Appendix 11: Effects of treatment diets on egg yolk cholesterol concentrations in birds from 25 to 29 weeks of age. (Errors bars = SE, * P<0.05, ^{a,b,c} points with different alphabet notation within a week differ significantly at P<0.05).

Appendix 12: Initial and final body weight of birds

Age (weeks)	Treatments			
	Cont mean±SE	FP3 mean±SE	FP6 mean±SE	FP9 mean±SE
15	1.20±0.01	1.19±0.01	1.19±0.01	1.17±0.01
29	1.67±0.02	1.66±0.02	1.68±0.02	1.64±0.02

All values within a row were not significantly different (P>0.05) from each other.

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The author, Elizabeth Law Fang Lin was born on March 3, 1979 in Muar, Johore. She completed her primary, secondary and high school education in Johor. She was offered to do a degree study in the Faculty of Agriculture, Universiti Putra Malaysia, in year 2000 and graduated in 2003. After graduation, she was awarded a Graduate Research Assistantship by the Ministry of Science, Technology and Environment Malaysia to pursue her Master of Science Degree in Animal Nutrition under the chairmanship of Associate Professor Dr. Loh Teck Chwen at the Department of Animal Science, Faculty of Agriculture, Universiti Putra Malaysia in April 2003. During the time of study, she won a silver medal in the Exhibition of invention, Research and innovation organized by UPM in year 2005.

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