

**THE EFFECTS OF DIETARY PHYTOSTEROL AND CHOLESTEROL
CONCENTRATION IN INFANT FORMULA ON CIRCULATING CHOLESTEROL
LEVELS, CHOLESTEROL ABSORPTION AND SYNTHESIS AS WELL AS OTHER
HEALTH BIOMARKERS USING NEONATE PIGLETS**

By

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A thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements for the degree of

Master of Science

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ABSTRACT

High cholesterol synthesis at infancy could lead to hypercholesterolemia later in life. However, high synthesis at infancy could be traced to low dietary cholesterol especially in the formula-fed infants because they consume diets high in phytosterol (PS), a known cholesterol absorption inhibitor. High PS levels are found in vegetable oil used in infant foods. Human milk contains significant amounts of cholesterol ranging from 0.26-0.28mmol/L, compared to the very low levels in infant formula (IF) which can be as low as 0.08mmol/L. Therefore, the objective of this study was to investigate the effect of IF containing different levels of PS and cholesterol on circulating cholesterol levels, cholesterol absorption and synthesis, and other related health biomarkers using neonate piglets as model for human infants. A total of 32 piglets were used with 8 piglets per group fed diets of the following composition: (i) high in PS; low in cholesterol (HiPSLoChol), (ii) high in PS; high in cholesterol (HiPSHiChol), (iii) low in PS; high in cholesterol (LoPSHiChol) and (iv) low in PS; low in cholesterol (LoPSLoChol). After 21 days of study, various tissues were collected for analysis. In plasma and liver, cholesterol levels were higher $p < 0.05$ in LoPSHiChol compared to LoPSLoChol diet group. Cholesterol synthesis levels in plasma, liver and distal intestine were also lower $p < 0.05$ in the LoPSHiChol diet group compared to the HiPSLoChol and LoPSLoChol diet groups. Circulating lipid profiles after 3wks on each diet were compared across different dietary treatments. No significant differences were observed in circulating total cholesterol levels across the treatments. However, circulating levels of LDL-C in HiPSHiChol and LoPSHiChol increased ($p < 0.05$) compared to the HiPSLoChol group. Plasma fatty acids shows higher ($P < 0.05$) docosahexanoic acid (DHA) in HiPSLoChol diet compared to LoPSHiChol diet group. Also, ratio of n-3 to n-6 fatty acids was higher ($P < 0.05$) in HiPSLoChol diet group compared to LoPSHiChol diet group. Beta and gamma

tocopherols were lower in LoPSHiChol and LoPSLoChol diet groups compared to the remaining two groups. Other health biomarkers parameters measured such as antioxidant capacity, apolipoprotein A1 and B, C-reactive protein, insulin, glucagon-like peptide 1, fructosamine and LDL particle size were not significantly different across diets. In conclusion, lowering dietary PS with an increase in cholesterol level in commercial IF may support efficient absorption of dietary cholesterol, while maintaining low cholesterol synthesis levels. These results are anticipated to help manufacturing industries in proper formulation of infant food to achieve a closer dietary benefit found in infants consuming human milk.

ACKNOWLEDGEMENT

My first appreciation goes to my supervisor, Dr. Peter Jones for believing in me, for his understanding and fatherly advices, you were not just my supervisor, you were my mentor and a role model. Thanks for been part of my dream to success.

To my amazing committee members, Dr. Mohammed Moghadasian and Dr. Trust Beta, I could not have chosen anyone better, your recommendations and reviews were so helpful and I appreciate every bit of it. To my lab members most especially Mark Miller Williams and Dr. Dylan Mackay, you were so helpful in putting me through in the methodologies and system set up. I appreciate you all.

Scholarship from International Graduate Students Entrance Scholarship (IGSES) is deeply acknowledged, a million thanks are not enough. To our collaborators at Enzymotec Ltd. and Fiddet Institute, thanks for your support and for giving me the opportunity to work on this very interesting project. The challenges brought the best in me.

To my friends far and near, thanks for being a good listener when I come ranting to you about my headaches, most exclusively the Olas (Opeyemi and Oyekunle), my MSc degree story is nothing without your name mentioned in it. Bolatito, Bridget, Oyin, Anthonia, Justice, Opeyemi, Anjalika, Jyoti, Semiu... just to mention but few, you guys were awesome. To my family members, of course I know you are heaven sent, thanks for putting me first and going extra mile just to see me smile.

Lastly but definitely not the least, my super man, most amazing husband in the world, Dr. Olumide Adegunna, since I met you, you have brought me nothing but joy, my achieving this was all part of your great plan for us. May heaven beautify the road that led me to you. Thanks for believing in me, I love you endlessly.

DEDICATION

I am dedicating this thesis to my maker, the almighty God, the beginning and the end, who saw me through the course of this program, who has been my place of comfort in distress, who gave hope when I lost it, to him alone be all the glory.

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LIST OF ABBREVIATIONS

ABTS	2,2-azino-di {3-ethylbenthiazoline sulphonate}
ANOVA	Analysis of variance
Apo A	Apolipoprotein A
Apo B	Apolipoprotein B
CDC	Cholesterol digested content
CRP	C-reactive protein
CVD	Cardiovascular disease
DHA	Docosahexaenoic acid
ELISA	Enzyme immune sorbent assay
FOX	Ferrous oxidation-xyleneol
GC	Gas chromatography
GC-FID	Gas chromatography-flame ionization detection
GLP	Glucagon-like peptide
HDL	High density lipoproteins
HM	Human milk
HPLC	High performance liquid chromatography
ICD	Ileal cholesterol digestibility

IDL	Intermediate density lipoprotein
IF	Infant formula
LDL	Low density lipoproteins
LOOH	Lipid hydroperoxide
MUFA	Monounsaturated fatty acids
NIP	Nutrition information panel
PS	Phytosterol(s)
PUFA	Polyunsaturated fatty acids
SEM	Standard error of the mean
TBARS	Thiobarbituric acid reactive protein
UV	Ultra-violet
VLDL	Very low density lipoproteins
VO	Vegetable oil

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

It has long been established that consumption of phytosterols (PS) lowers the absorption of cholesterol and PS are absorbed in very low amounts in the human body (1). PS, also known as plant sterols/stanols, occur naturally in vegetable oils (VO) which are used in the formulation of infant formula (IF). Cholesterol, a sterol compound present in the body cells, serves as a precursor of steroid compound and is an important part of the cell membrane. Cholesterol aids in the absorption of fat soluble vitamins and it is the main sterol produced by the body. However, it has also been established that cholesterol is necessary at infancy apparently because infancy is a period of rapid growth. Although little investigation has been done on the importance of cholesterol in infant's diets, cholesterol is present in higher proportion in breast milk; indeed, it is about three to five-fold higher compared to IF. Also, studies have shown that infants consuming breast milk have significantly higher lipid values than formula fed infants which could be important for proper lipid programming and metabolism in later life (2). However, this low cholesterol content in IF can be traced to a high PS content from VO used in supplementing infant food. Although, efforts have been made to mimic human breast milk, the relationship that exists between PS and cholesterol with regards to cholesterol absorption, synthesis and other health parameters within the context of the PS reduced VO in infants has not been documented.

1.2 Infant formula

Infant formula (IF) is the food provided as replacement for human milk (HM) for feeding of infants. Infants are referred to as newborn within the age of 0-12months. HM is the best food for infants because it contains the complete essential nutrients required for growth and development

of an infant. Notwithstanding, IF has been considered safe due to the current development in food technology and engineering. However, some dissimilarities still exist between nutrient composition and the growth pattern of infants fed with HM and those fed with IF (3,4). Many studies have shown that the cholesterol or fat contents of IFs are lower than that of HM (5–7). Notwithstanding, IF is designed to contain and deliver the nutrients found in HM which are macronutrients consisting of carbohydrates, protein and lipids and micronutrients consisting of vitamins and minerals. Protein serves as emulsifier of fat globules, however, with the fat content of HM higher than that of IF, it has been discovered that hydrolysis of triglyceride in HM is higher than that of IF due to low protein to fat ratio in HM compared to higher ratio in IF (8).

For several years, the purpose of producing IF has been to at least minimize the differences that exist between IF and HM, if not completely eliminate them. This intention has resulted in different modifications of the nutrients contained in IF but various constrains such as reactions between various complex components for example prebiotics and probiotics that occur in human breast, inability to specify the exact amount of nutrients to add or remove due to diversity of nutrients in various HM among others, have led to difficulties in proper formulation of nutrients in IF to mimic that of HM (9).

1.3 Cholesterol

Cholesterol is the most abundant sterol in human and animal milk. It is present in milk fat globule of HM. Cholesterol is the precursor of steroid hormone and is involved in growth regulation which is a crucial factor at infancy. Cholesterol is an integral component of cell membrane especially in the nerve cell walls. Also, cholesterol is the precursor of bile acids and bile steroid hormones (10,11). Cholesterol content in infant feeds is one of the major difference between IF and HM up to date, its composition is greatly higher in HM compared to IF (12). It

is also worthwhile to mention that the total body cholesterol pool size is dependant on dietary intake, de novo synthesis, adequacy of absorption and metabolism, gastrointestinal recirculation and excretion (13).

1.3.1 Cholesterol synthesis

Cholesterol in the body is either sourced from dietary cholesterol or synthesized by the liver and other organs. Synthesis rate of cholesterol is dependent on factors such as the amount of cholesterol in the body, presence of disease and the individual's activity rate. In humans, dietary cholesterol supplementation has been shown to reduce cholesterol synthesis (14). A study by Jones et al into the response of cholesterol biosynthesis to various dietary cholesterol level, showed a reduction in cholesterol synthesis with increase in dietary cholesterol (15). Reasons why cholesterol synthesis measurement is challenging to quantify in humans may include but not limited to the techniques for direct and indirect measurement which are complicated and hard to carry out.

1.4 Plant sterols

Plant sterols are bioactive compounds which perform the same function in plants as does cholesterol in animal. PS, unlike cholesterol cannot be manufactured by the human body and thus are supplemented in the diet and VO being its rich source (16). Steryl esters, steryl glycosides and free sterols are the major forms in which plant sterols exist and unlike in animal where cholesterol is the main sterol, plants utilize a wide range of sterols with the abundant species being sitosterol, campesterol and stigmasterol (17,18). According to Daniele et al, PS have a half-life average of 10 days which possibly contributes to their accumulation in the body which could lead to parenteral nutrition associated cholestasis in preterm infants (19). PS are similar to cholesterol in structure but are only different by their alkylation at position 24 as

shown in **Figure 1.1**. PS have a significant effect on cholesterol metabolism. The ability of PS to suppress cholesterol absorption is part of the factor that has led to their use as a food supplement. Meanwhile, this ability could be detrimental in infant's diets (20–22).

1.5 Cholesterol versus phytosterol metabolism

Cholesterol and PS are both sterols similar in structure with a comparable metabolism process. Because of this similarity in structure, PS could replace cholesterol in the cell membrane hindering some membrane functionality and enzymatic functions which are membrane bound (23). And even though the variability in both structures is minor, there is a wide gap in the absorption rate of these sterols. In the small intestine, absorption of PS is less than 2% compared to cholesterol absorption which is up to 50% (24). Digestion of cholesterol by enzymes takes place in the small intestine. Cholesterol is then absorbed by the intestinal cells; this absorption requires solubilisation of cholesterol into micelles (25,26). Total cholesterol content in the body is by accumulation from various sources such as absorption efficiency, de novo synthesis, recirculation by enterohepatic cell wall and dietary intake. Liver is the major and primary organ responsible for cholesterol regulation in the body. PS hinders the ability of cholesterol intake or absorption into the blood stream from diets by reducing greatly the solubility of cholesterol in the intestine which is the main site in which the inhibition takes place (27,28). It has been reported that at the very prime stage of an infant, the intestinal lipase responsible for lipid hydrolysis in the stomach is fully developed to perform its function (29). Therefore, level of cholesterol in the diet does impact the circulating levels in the body at infancy.

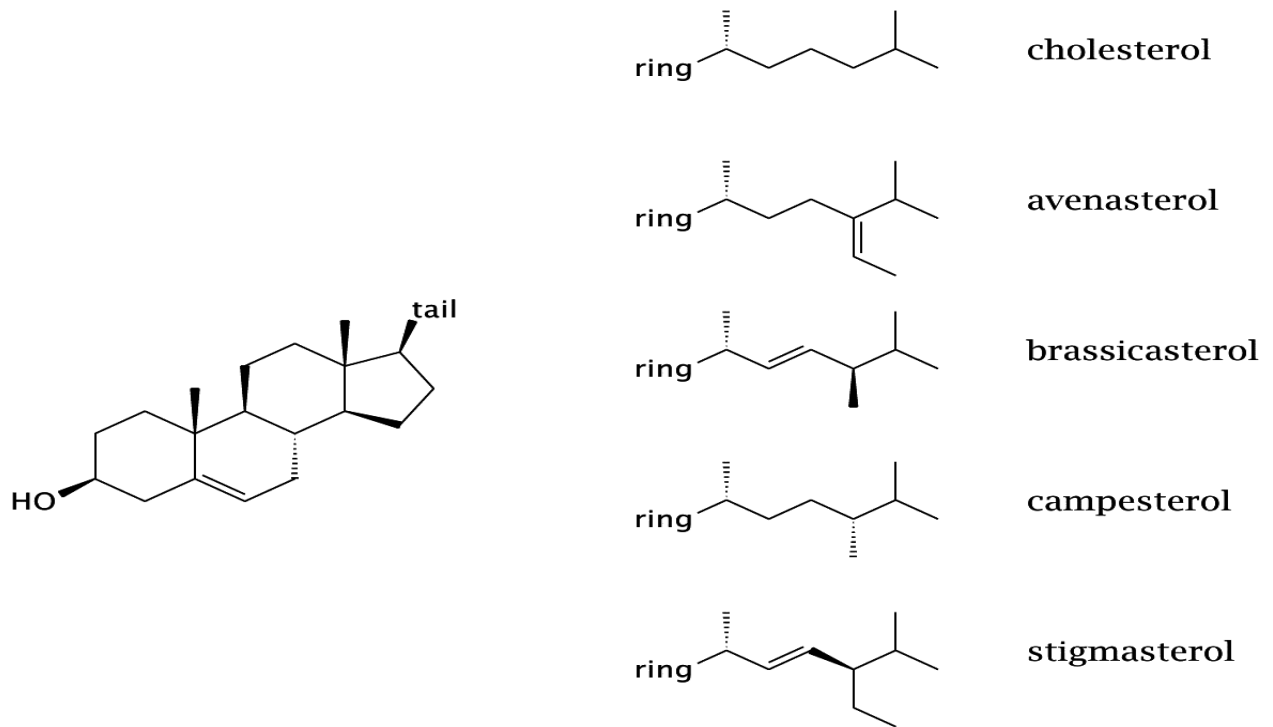


Figure 1 Structure of cholesterol and some phytosterols

<http://watcut.uwaterloo.ca/webnotes/Metabolism/cholesterolTransport.html>

1.6 Vegetable oils and infant formula

Vegetable oils are mainly triglycerides that are liquid at room temperature. They are also referred to as plant oils. Originally, IFs already contain a reasonable amount of milk fat. VO-based IF usually contains up to 4% residual milk fat but VOs tends to be richer in fat. Most of the VOs used in IF are soybean oil, palm oil, high oleic safflower oil, coconut oil, palm kernel oil, peanut oil and rapeseed oil (30). VO is mostly used by manufacturers in IF formulation for improved fatty acid profile to a level present in human milk (31). However, IF are high in PS which could be unfavourable in the cholesterol absorptivity of infants consuming IF.

1.7 Comparison between cholesterol in infant formula and breast milk.

Breastfeeding has been studied to correlate with high total cholesterol and LDL-C at infancy and lower levels in adulthood. This suggests a beneficial effect on cardiovascular health (32). It is important to note that larger percentage of infants are fed with IF; quite a large number of infants of ages between 0-6 months all over the world are IF dependent. Globally, especially in developed countries, there has been decline in breastfeeding rate, with less than 37% of infants being solely breastfed (33,34). Even though the main factor in IF production is to mimic the nutrient composition found in HM which is the fundamental standard of infant nutrition, there are still differences that exists in their nutrient levels. The cholesterol absorption of infants taking IF is still very low compared to that of infant taking HM (35). Commercially produced IF typically contains 2-4 mg/dl cholesterol whereas, the cholesterol level of HM ranges between 10-38 mg/dl (36). The cholesterol content of infant fed with HM is five times greater than that of infant fed with IF (37). Hodgson et al., (1996) studied the effect of low, moderate and high cholesterol intake at infancy on serum cholesterol levels. Results after 7-12yr shows a slight increase in mean serum cholesterol level of children fed with higher amounts but the increase

was insignificant. This increase was likely due to a wide range in the cholesterol level between individuals consuming HM affecting the mean value used (36).

Obesity has been known to relate with cholesterol level in human. HM which contains higher cholesterol and which is the gold standard of IF reduces incidence of obesity later in life (38). This calls for need to improve the cholesterol in IF to approximate the level found in HM. Also, Owen et al., (2008) reviewed that exclusive breastfeeding can be associated with low blood cholesterol later in life and therefore proper caution should be taken in the IF production with low cholesterol intake below the level found in HM (39). It has likewise been recommended that further research into the sterol composition of ingredients used in IF formulation should be performed to imitate the content found in HM (11). This in turn is the aim and objective of this experiment which is evaluating effect of using the newly developed PS reduced VO in formulation of IF to improve the blood cholesterol level of infants fed with IF to that found in infant consuming breast milk as suggested by the aforementioned authors.

1.8 Study rationale

Sales and promotion of IF in the past 2 decades has increased spontaneously and this trend is envisaged to continue especially among low and medium income parents (40–43). This drift has prevailed regardless of the effort to encourage and promote breastfeeding (44). However, insignificant breastfeeding has been associated with malnutrition leading also to high mortality especially in developing countries (45–47). This calls for an improvement in IF composition to the best it can be and a need for investigation into factors such as cholesterol absorption of infants consuming IF being low, also a necessity. From previous studies, improved cholesterol has been targeted by the supplementation of IF with milk fat globule membrane or cholesterol-

rich sources and even though an improved cholesterol level was observed, the improvement was insignificant probably due to high PS in VO that was present (48).

To our knowledge, there are few studies on the cholesterol composition of IF, none of these studies has investigated the concept of PS reduced VO, it is therefore essential to research into this in order to formulate the appropriate dietary cholesterol of infants consuming IF. In this study, piglets were used as a model for human infant because it has been established that piglets are useful in paediatric-related studies owing to the fact that unlike other animals used in studies, piglets have intestinal enzyme metabolism more similar to that of human infants (49).

1.9 Hypothesis

The main hypothesis of this study is that neonates fed diet with phytosterol-reduced vegetable oil will have a higher cholesterol absorption and a reduced cholesterol synthesis than those fed with diet high in phytosterol which is the level found in commercial infant formula.

2.0 Objectives of the research

The main goal of this research is to investigate the effects of varying concentrations of cholesterol and PS on cholesterol metabolism of infant formulas using neonate piglets as a model for the human infant.

The specific objectives of the research are:

1. To examine the effect of different ratios of cholesterol to PS on the sterol concentrations of the plasma, liver and ileal digesta.
2. To determine how these ratios, affect cholesterol absorption and synthesis in plasma, and liver of study animals.
3. To also test the effect of consuming these different sterol levels in the diet on additional health parameters including lipid profile, plasma fatty acids, vitamin A and E levels,

antioxidant capacity, apolipoprotein A and B levels, C-reactive protein levels, insulin levels, glucagon-like peptide 1 levels, fructosamine levels, as well as LDL particle size.

CHAPTER 2

(to be submitted soon to Journal of Lipid Research)

Modulating sterol concentrations in infant formula influences cholesterol absorption and synthesis in the neonatal piglet

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Running Title: Cholesterol and phytosterol effect on cholesterol concentrations

Abbreviations: PS-Phytosterols, DMI- Dry matter intake, NS-Not significant, GLM- General linear model.

Abstract

Objective: Cholesterol is an important component for infant growth and development. Human milk has relatively high concentrations of cholesterol, while vegetable-oil based infant formulas contain lower cholesterol concentrations and also contain plant sterols. Though the extent to which plant sterols in infant formulas impact cholesterol metabolism has not been fully elucidated, their cholesterol absorption inhibitory effect may result in early programming of elevated endogenous cholesterol synthesis with negative downstream effects on cholesterol metabolism and cardiovascular disease risk.

This study investigated the impact of dietary plant sterols on cholesterol metabolism using neonate piglets.

Methods: Thirty-two piglets were randomized to one of four groups fed milk-based formulas containing various combinations of sterols, based on human milk and infant formula sterol concentrations. Plasma and liver samples collected after 21 days of feeding were analysed for cholesterol, plant-sterols, and cholesterol synthesis markers. In addition, ileal digesta were analysed to determine apparent ileal cholesterol digestibility

Results: No differences were observed in growth, food intake or circulating cholesterol. However, ileal cholesterol digestibility was enhanced and cholesterol synthesis was reduced in the formula with high cholesterol and reduced plant-sterol concentrations

Conclusion: The results suggest that dietary sterol profile may facilitate cholesterol absorption and reduce endogenous cholesterol synthesis. This may have long-term benefits for cholesterol metabolism via early programming.

Keywords: cholesterol, plant sterols, infant formula, synthesis, absorption.

Introduction

Cholesterol is essential for life, especially during the rapid growth of infants as it is a structural element of the cell membrane lipid layer, a substrate for the production of steroid hormones, vitamin D and bile acids (50), crucial for proper brain development and myelin formation, and plays a key role in lipoprotein synthesis and metabolism (31). Moreover, studies suggest an influence of early nutrition on blood cholesterol levels and thus cardiovascular disease risk (CVD) during adulthood (24,51,52).

Human milk contains 90 to 150 mg/L cholesterol. In contrast to vegetable oil-based infant formulas contain a negligible amount of cholesterol which is mainly derived from residual milk fat present in the milk products added to the formulas as protein source (31), and also to the dairy fat, which contains approximately 40 mg/L which is added to dairy fat based infant formula (12). This higher cholesterol content results in about 3-5 times higher intake of cholesterol in breastfed infants compared to formula fed infants (31,53).

Formula fed infants consume vegetable oils which are included in infant formulas as the source of lipid. These vegetable oils, contain plant sterols that are structurally similar to cholesterol, and inhibit cholesterol absorption (31,54–56). Indeed, studies have shown that infants consuming breast milk display significantly higher cholesterol than do formula fed infants (37,57).

Phytosterols (PS) inhibit cholesterol absorption by competing in micelle formation. Although, they are barely absorbed by the adult human body, little is known about the importance of PS in infants' diets. The higher circulating lipid concentrations during infancy could be important for proper lipid programming and metabolism in later life (2). However, the long term effects of consuming diets relatively high in PS during infancy are yet to be explored (58).

Plasma cholesterol concentrations are determined by the balance between dietary cholesterol

absorption from the gastrointestinal tract and whole body endogenous cholesterol synthesis (59). All infants have a clearly demonstrated ability for endogenous cholesterol synthesis. Infants fed vegetable oil-based formulas, who do not receive optimal dietary cholesterol as compared to breast-fed infants synthesize more endogenous cholesterol than their breast-fed counterparts. The relatively low cholesterol content together with a higher PS content in vegetable oil-based infant formulas compared to human milk, potentially results in early programming of higher endogenous cholesterol synthesis with a possible negative long-term effect on cholesterol metabolism and CVD risk.

Although efforts have been made to mimic human breast milk composition by supplementation of infant formula with different cholesterol sources (59), the potential relationship between dietary PS and cholesterol concentrations with regards to cholesterol absorption from infant formulas has not been addressed. We hypothesized that a low PS modified vegetable oil should facilitate increased cholesterol absorption and lead to lower endogenous cholesterol synthesis. Therefore, the objective of this study was to investigate the effect of infant formulas containing different levels of PS and cholesterol on circulating cholesterol concentrations, apparent ileal cholesterol digestibility as well as endogenous cholesterol synthesis by using a neonate piglet as a model for the human infant.

Materials and methods

The impact of dietary PS and cholesterol concentration on cholesterol absorption and endogenous cholesterol synthesis was determined in neonate piglets given one of four dietary treatments following approval by Massey University's ethics committee.

Animals and diets

Thirty-two 7-day old male piglets were housed in purpose-built plastic metabolism crates in a temperature controlled room maintained at $28\pm 2^{\circ}\text{C}$ with a 16:8 h light: dark cycle. The piglets were initially weighed and randomly allocated to one of the four experimental diets such that there were eight piglets per treatment. The piglets were fed 345 g of prepared liquid formula per kg of body weight per day (60). The piglets were trained to drink using a bottle and teat. The piglets were acclimatized to their environment and diet over the first six days of the study during which time they were fed their daily ration over 17 meals given hourly from 06:00 h to 10:00 h. For the remainder of the experimental period (16 days) the piglets receive their daily food rations as 7 meals given every 2.5 h from 06:30 h to 21:30 h. From day 14 to 21, titanium dioxide (an indigestible marker) was also added to the prepared formula to provide a titanium dioxide concentration of 3 g/kg of dry matter. The daily formula ration was readjusted weekly based on the body weights of the piglets. Any formula that was not consumed was collected, dried and weighed for each piglet in order to determine dietary intake.

The dietary treatments (Table 1), prepared at the Food Pilot Plant (Massey University), contained different combinations of PS and cholesterol concentrations commensurate with those in human milk or standard vegetable oil based infant formulas. The "high PS" (HiPS) and "low cholesterol" (LoChol) refer to the levels in vegetable oils based infant formulas, while the "low PS" (LoPS, treated vegetable oils) and "high cholesterol" (HiChol, free cholesterol and cholesterol esters in levels and ratio similar to those in human milk) refer to levels closer to those observed in human milk. For diets containing LoPS, vegetable oil used in diets formulation was modified to contain very low PS concentration.

Sample collection and analysis

On day 22 of the study, piglets were fed their respective formula at hourly intervals starting at 0630 h. Seven hours after the start of feeding each piglet were anaesthetised using a cocktail of Xylazine, Zolazepam and Tiletamine, a blood sample was taken, and the piglets were then euthanized after which a liver tissue and ileal digesta were collected and stored at -80°C for analysis.

Determination of cholesterol and other sterols in plasma, ileal digesta and liver tissues

All standards, potassium hydroxide (KOH) salt and the internal standard were obtained from Sigma Aldrich Canada Ltd, Oakville, ON. Solvents were obtained from Fischer Scientific.

Sample extraction was performed according to Jones et al (61) with a few modifications. Sterols were extracted twice and dried under N₂ before being saponified with methanol-KOH, using 5 α -cholestane as an internal standard. The dried residue was re-suspended in hexane and 100 μ l of HMDS+TMCS+Pyridine (3:1:9) was added for derivatization. Extracted samples were then transferred to GC vials for analysis using a GC-FID with a SAC-5 fused silica capillary column 30m \times 0.25mm \times 0.25 μ m film thickness. An initial run temperature of 130°C was used for 2 min, before increasing the temperature to 270°C at 30°C/min, holding it at 270°C for 10 min, increasing the temperature to 290°C at 10°C/min holding for 9 min, then finally ramping the temperature up to 320°C at 40°C/min holding for 5 min. Helium was used as the carrier gas at a flow rate of 1.0ml/min. The injection volume was 1 μ l and the injector and detector temperatures of 280°C and 300°C, respectively.

Sterols were identified by comparing the retention time of the peaks in the sample with the retention time of known sterols in the external standard. Each sample was spiked with 200 μ g/ml of the internal standard in order to quantify the sterols in each sample.

Determining the titanium dioxide concentration in diets and ileal digesta

The titanium dioxide content of the diets and digesta were determined based on the method of Short et al. (1996) (62). Briefly, samples were ashed before being digested in 60% (v/v) sulphuric acid. The mixture was then incubated with 30% H₂O₂ and the absorbance read at 405 nm.

Calculations

Cholesterol flow rates at the terminal ileum were calculated as follows (units are mg.kg⁻¹ DM (dry matter)):

Ileal cholesterol flow (mg.kg⁻¹ DMI (dry matter intake)) =

Digesta cholesterol x Formula titanium dioxide/ Digesta titanium dioxide

The apparent ileal cholesterol digestibility value was calculated using the following equation (units are mg/kg DMI):

Apparent ileal cholesterol digestibility (%) =

(Formula cholesterol – Ileal cholesterol flow) / Formula cholesterol x 100

Statistical analysis

PROC UNIVARIATE (SAS, 2009) was used to test for normal distribution. Data were analyzed statistically using 1-way analysis of variance using GLM procedures (SAS, 2009). Where statistical ($P < 0.05$) effects were observed, individual means were compared using the Tukey test. In addition, specific contrast analyses were conducted between the groups for each dependent variable using Student's t-test.

Results are presented as means and standard error of the means. Results were deemed statistically different at $p < 0.05$.

Results

The piglets readily adapted to bottle feeding, gained weight and remained healthy throughout the trial. There was no occurrence of diarrhoea in any of the piglets at any time in the trial.

Food intake and body weight

The percentage of the weekly ration consumed and body weight of the piglets across treatment groups is presented in Table 2. No difference ($P < 0.05$) were observed for either percentage of the weekly ration consumed or piglet's body weight across treatment groups throughout the trial.

Plasma and liver PS levels

Total PS concentrations in the plasma and liver samples are shown in **Figure 1**. PS levels were significantly lower in plasma ($p < 0.01$) and liver ($p < 0.001$) samples for the animals fed the LoPS-LoChol and LoPS-HiChol infant formulas containing the lower concentrations of PS (0.94 and 1.00 mg/100ml in the plasma samples and 1.72 and 1.58 mg/100g in the liver samples for the LoPS-LoChol and LoPS-HiChol groups, respectively) compared to those fed the HiPS-LoChol and HiPS-HiChol formulas containing the higher concentrations of PS (4.14 and 5.03 mg/100ml in plasma samples and 8.36 and 7.72 mg/100g in liver samples for the HiPS-LoChol and HiPS-HiChol groups, respectively). The lower PS concentration for the piglets receiving the two low-PS infant formulas was mainly due to a lower liver and plasma concentration of campesterol and sitosterol, the major PS present in the formulas.

Plasma cholesterol concentrations

The circulating total cholesterol **concentrations** were not significantly different ($P < 0.05$) between the four treatment groups as shown in **Figure 2**.

Apparent ileal cholesterol digestibility and apparent ileal digestible cholesterol content

The apparent ileal digestibility of cholesterol was determined by comparing the sterol concentration in the formula with the unabsorbed cholesterol present at either the end of the small intestine. The apparent ileal cholesterol digestibility and the apparent ileal digested cholesterol content of the four test formulas determined in the neonate piglet are shown in **Figure 3**. Apparent ileal cholesterol digestibility ranged from 32%-85% across the different groups. Apparent ileal cholesterol digestibility was not different ($P < 0.05$) among the HiPS-HiChol, LoPS-LoChol and LoPS-HiChol groups but was higher for the latter treatments groups when compared to the HiPS-LoChol treatment group (73.0%, 66.30%, 85.0% vs 31.80%, respectively, $p < 0.05$).

Accordingly, the apparent ileal digestible cholesterol content was higher ($p < 0.05$) for the piglets fed either of the two the formulas containing the higher cholesterol content compared to the piglets receiving either of the two formulas containing the lower cholesterol content. Diets containing high cholesterol, HiPS-HiChol and LoPS-HiChol resulted in higher ($P < 0.01$) digestible cholesterol content values than the low cholesterol diets HiPS-LoChol and LoPS-LoChol.

Cholesterol synthesis precursor concentrations

The concentrations of the cholesterol synthesis precursors present in the plasma and the liver of the piglets receiving the four test formulas are shown in **Figure 4 and 5**. An overall difference ($p < 0.01$) across treatment groups was observed when the synthesis precursors were added together and when each precursor was normalized to the cholesterol level, i.e., the lathosterol to cholesterol ratio, and the desmosterol to cholesterol ratio as shown in **Figure 4a**. The combined plasma cholesterol precursor concentrations calculated as sum of the measured precursors desmosterol, lathosterol and lanosterol were the lowest for the LoPS-HiChol groups and was

significantly lower ($P < 0.05$) compared to all other groups (0.29 vs, 0.49, 0.41 and 0.36 mg/100ml in plasma for the LoPSHiChol, HiPSLoChol, HiPS-HiChol and LoPSLoChol, respectively), shown in **Figure 5a**. For the liver cholesterol precursors, overall differences ($p < 0.01$) across treatment groups were observed for both desmosterol and lathosterol concentrations (data not shown). A similar trend was observed when the latter concentrations were normalized to the cholesterol content (**Figure 4b**) as well as when expressed as the sum of total synthesis precursors. The total cholesterol precursor concentration (i.e. the sum of the desmosterol, lathosterol and lanosterol concentrations) determined in the liver was highest ($P < 0.001$) for the HiPS-LoChol groups when compared to all other groups 1.9 vs, 1.44, 1.09 and 1.05 mg/100gr for the LoPSHiChol, HiPSHiChol, LoPS-LoChol and LoPSHiChol, respectively, as shown in **Figure 5b**.

Discussion

This study showed that the naturally present levels of PS in the oils used in formula are sufficient to inhibit cholesterol absorption leading to enhancement of the cholesterol endogenous synthesis to satisfy the cholesterol requirement. This is the first study to address PS in formula as explaining the limited effect of cholesterol supplementation in formula fed infants.

Breast-fed infants consuming up to five fold higher amounts of cholesterol than formula-fed infants have been found to be less prone to obesity and related diseases such as cardiovascular diseases during adulthood, which has been in part attributed to exposure to dietary cholesterol early in life (63). Also, evidence from epidemiological studies relate initial breastfeeding, especially exclusive breastfeeding, with lower blood cholesterol levels during adulthood (64). As such, the importance of providing the growing infant with adequate dietary cholesterol cannot be overemphasized. Several studies report different approaches to improve circulating cholesterol

and metabolism in infants consuming infant formula by using various modifications including blending different oil sources or addition of cholesterol in modified infant formula all in an attempt to increase the cholesterol concentrations in infant formula (11,22,38,50,57,63,65).

However, the use of PS-reduced vegetable oil in an attempt to improve cholesterol absorption, in addition to cholesterol supplementation and its effects on cholesterol metabolism have not been previously investigated.

As expected, the piglets fed the formulas with lower PS concentration exhibited less PS in the plasma as well as in the liver tissue. Those results are in line with Mellies et al (66) who showed a direct correlation between the PS concentrations in the human milk and in the plasma of breastfed infants.

In the present work, the reduction in PS intake led to an increase in the absorption of cholesterol as revealed by the higher cholesterol digestibility in the piglets fed the formulas with the reduced PS. The enhancement of cholesterol absorption provides higher cholesterol availability from diet and enables lower cholesterol endogenous synthesis.

Apparent ileal cholesterol digestibility ranged from 32% to 85% across the test animals in this study. While there is a dearth of information relating to cholesterol digestibility of infant formulas in infants, the latter values were similar to those reported for the cholesterol absorption of mixed diets when determined in adult humans using techniques such as the dual isotope label method (67–70). Though the results are in line with the reported range, there was a significant difference observed between the regular infant formula diet and the formula with the high cholesterol and the low PS levels. Extrapolating the cholesterol digestibility to the potentially absorbed cholesterol reveals levels which are two times higher by just reducing the diet PS and can be up to 10 times higher by reducing the dietary PS and supplementing the formula with

cholesterol. Consequently, it would appear that by formulating an infant formula more similar to human milk, i.e. containing lower amounts of PS and greater amounts of cholesterol, cholesterol absorption can be greatly increased, thereby mitigating the need for significant endogenous cholesterol synthesis.

The nutritional requirement for, and the nutritional benefits of, cholesterol for infants have not been thoroughly elucidated. However, some evidence, suggests that high cholesterol intake in the young may beneficially affect cholesterol metabolism in later life (71).

The fact that plasma cholesterol and liver cholesterol concentrations were observed to be similar across the four feeding groups would suggest that a lower uptake of dietary cholesterol as seen for the “low cholesterol formulas” was compensated for by a greater synthesis of endogenous cholesterol. Indeed, the results suggest that a significant amount of cholesterol synthesis had occurred in the liver in response to differences in the amount of dietary cholesterol absorbed.

Cholesterol synthesis was not directly measured in the present study. Instead, three intermediates of cholesterol pathway (desmosterol, lathosterol and lanosterol) were determined as proxies for cholesterol synthesis. Circulating cholesterol precursor markers have been used in other studies examining cholesterol synthesis in vivo (72). In the present study, the latter synthesis precursors were suppressed in both plasma and liver by reducing the PS content and supplementing with cholesterol in infant food, and thus reducing cholesterol synthesis. Indeed, the liver concentration of total cholesterol synthesis precursors, which is almost twice in the control compared to other, support this increased synthesis of cholesterol in the case of the formulas with the lower cholesterol absorption. In liver, the individual cholesterol precursors, desmosterol, lanosterol and lathosterol, also differed between treatment groups to some extent, explaining the offsets in cholesterol synthesis. These offsets would suggest that a greater amount of endogenous

cholesterol synthesis had occurred in the piglets receiving the “low cholesterol, high PS” formula and for which the absorbed dietary cholesterol content was lower than for the two “high cholesterol” formulas. Moreover, the lathosterol/cholesterol ratio was only different between the “low cholesterol, high PS” formula and the “high cholesterol, low PS” formula, for which the difference in cholesterol absorption was hypothesized to be the most extreme. The results above are not inconsistent with the hypothesis that a lower absorption of cholesterol promotes a greater amount of endogenous cholesterol synthesis. This is evident most especially in the regular infant formula diet with Hi-PS and lo-Chol.

However, it should be noted that the concentration of cholesterol synthesis precursors is only a proxy for the cholesterol being synthesized since these compounds are only intermediates in the cholesterol synthesis pathway. Direct measures of synthesis would have been an asset in this study.

Our results showing that the sterols content, both phytosterols and cholesterol of the diet may affect the cholesterol metabolism and synthesis are consistent with other published studies. For example, Bayley et al, who investigated the effects of supplementing regular formula with cholesterol on de novo cholesterol synthesis in formula fed infants compared with breastfed infants, demonstrated higher fractional cholesterol synthesis rates in formula fed infants despite supplementation with cholesterol (57). In our study, the supplementation of cholesterol to a regular infant formula resulted in reduced cholesterol synthesis but to a lesser extent than the combination with the reduced PS contents. This therefore necessitates reduction of PS in infant formula to achieve a favorable cholesterol synthesis. Also, consumption of the newly developed formula of low PS and high cholesterol diet could be beneficial to infants in terms of cholesterol health, compared to a regular infant formula, with potential long-term effects.

Conclusion

In conclusion, our findings point at the importance of sterol profile in infant nutrition for short-term cholesterol balance, and this is speculated to enhance future studies on long term effect of cholesterol and PS concentrations in infant diets on health.

Table 1 Nutrient composition (per 100 g of air dry powder) of the four experimental infant formulas

/100g powder	HiPSLoChol	LoPSLoChol	HiPSHiChol	LoPSHiChol
Energy (kcal)	513	512	514	512
Protein (g)	12	12	11.9	11.9
Fat (g)	26.6	26.5	26.7	26.5
Carbohydrate (g)	56.5	56.3	56.4	56.4
Cholesterol* (mg)	22.2	24.2	85.6	84.2
PS (mg)	79.2	9.5	79.1	9.9

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 2 Mean daily dry matter intake and daily body weight gain for the piglets receiving the test infant formulas.

Formula	HiPSLoChol	HiPSHiChol	LoPSLoChol	LoPSLoChol	Overall	Overall	Overall
					SE ²	P-value	Signif. ³
Daily dry matter consumption (g)							
Week 1	133	129	133	129	8.2	0.962	NS
Week 2	162	152	161	155	9.6	0.864	NS
Week 3	201	188	198	191	11.8	0.857	NS
% consumed ⁴	98	96	98	97	0.8	0.229	NS
Daily body weight gain (g)							
Week 1	96	78	91	85	5.3	0.141	NS
Week 2	128	118	122	120	8.2	0.839	NS
Week 3	150	128	149	145	9.2	0.371	NS
Weeks 1-3	124	108	121	117	7.2	0.439	NS

¹*n*=8.

²Overall standard error of the mean.

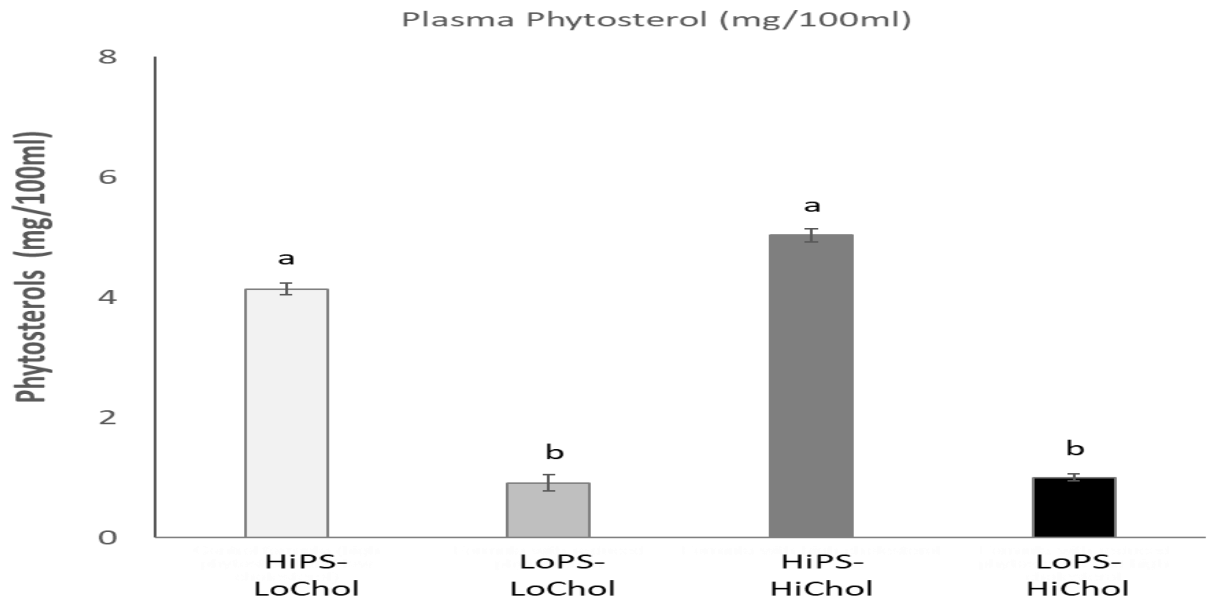
³Overall statistical significance. NS, Not significant, $P \geq 0.05$.

⁴% consumed was calculated as the amount of infant formula dry matter consumed over the trial period divided by the amount of infant formula dry matter given to each piglet over the trial period.

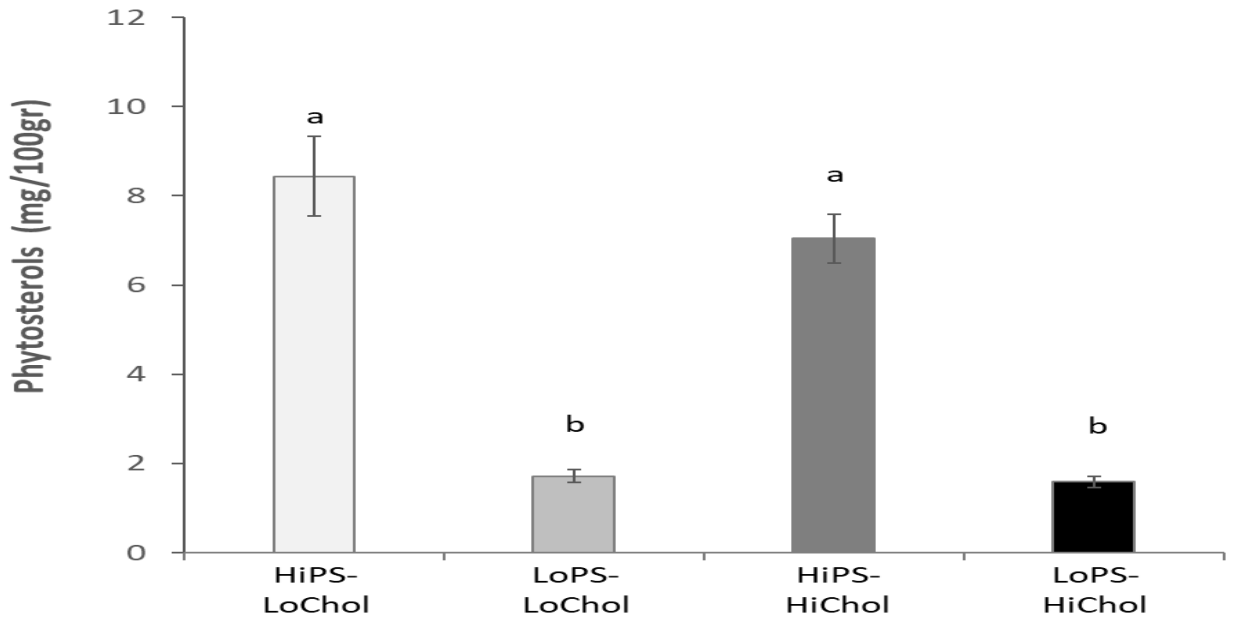
HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Figure 2 Plasma and liver PS levels

A. Plasma PS



B. Liver PS (mg/100gr wet weight)



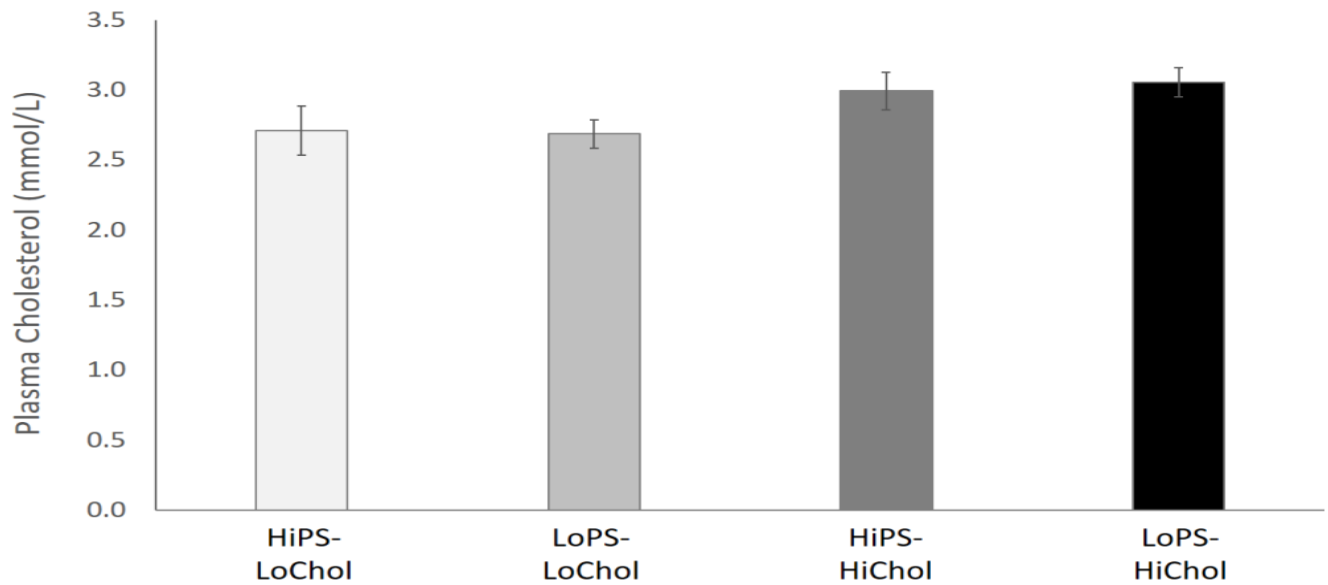
n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Figure 3 Plasma cholesterol concentration in the piglets receiving the test infant formulas



n=8

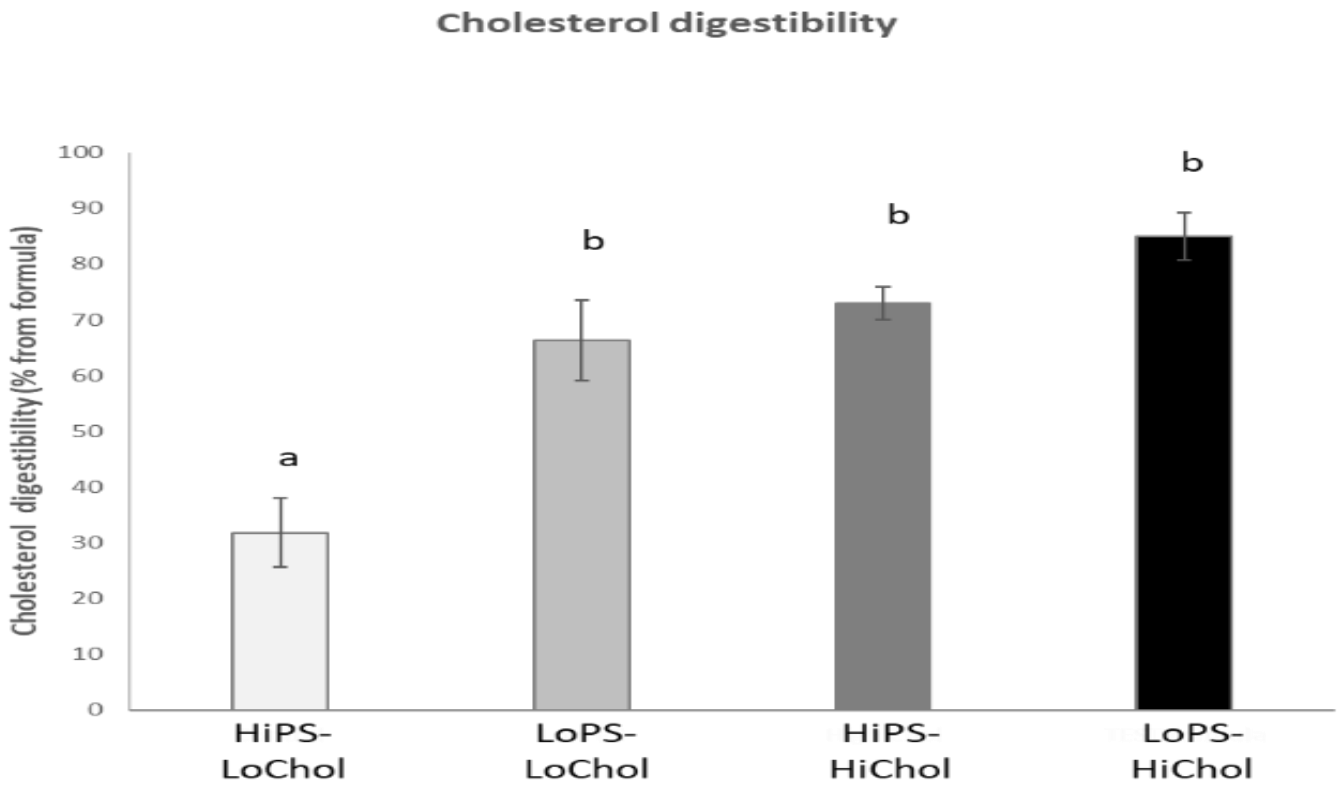
Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

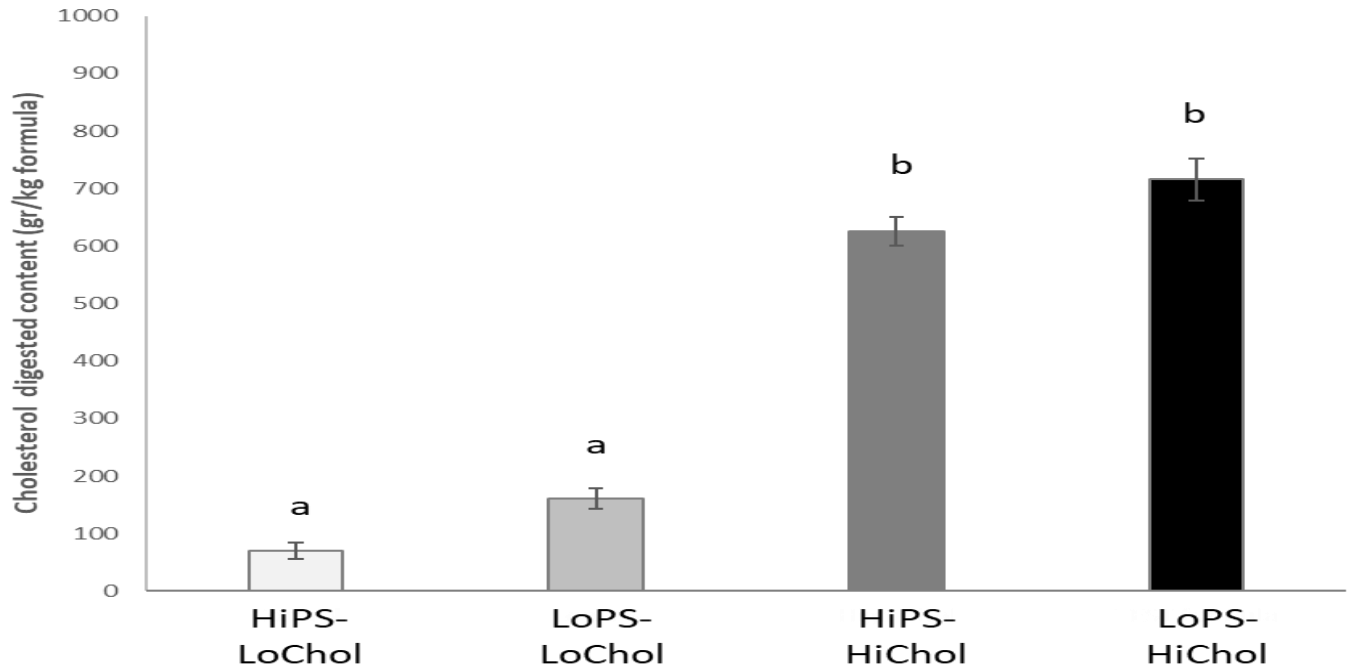
HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Figure 4 Apparent ileal cholesterol digestibility (%) and apparent ileal digestible cholesterol content (mg/kg air dry weight)

A. Apparent ileal cholesterol digestibility



B. Apparent ileal digestible cholesterol content



n=8

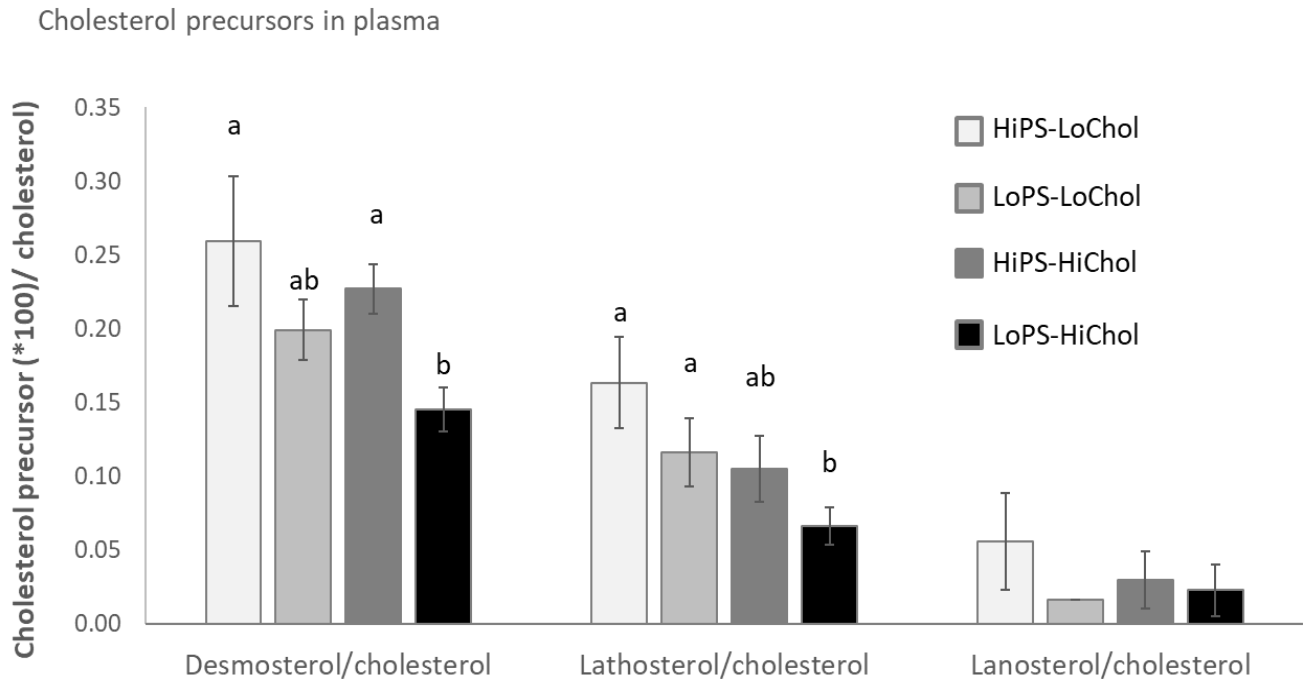
Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

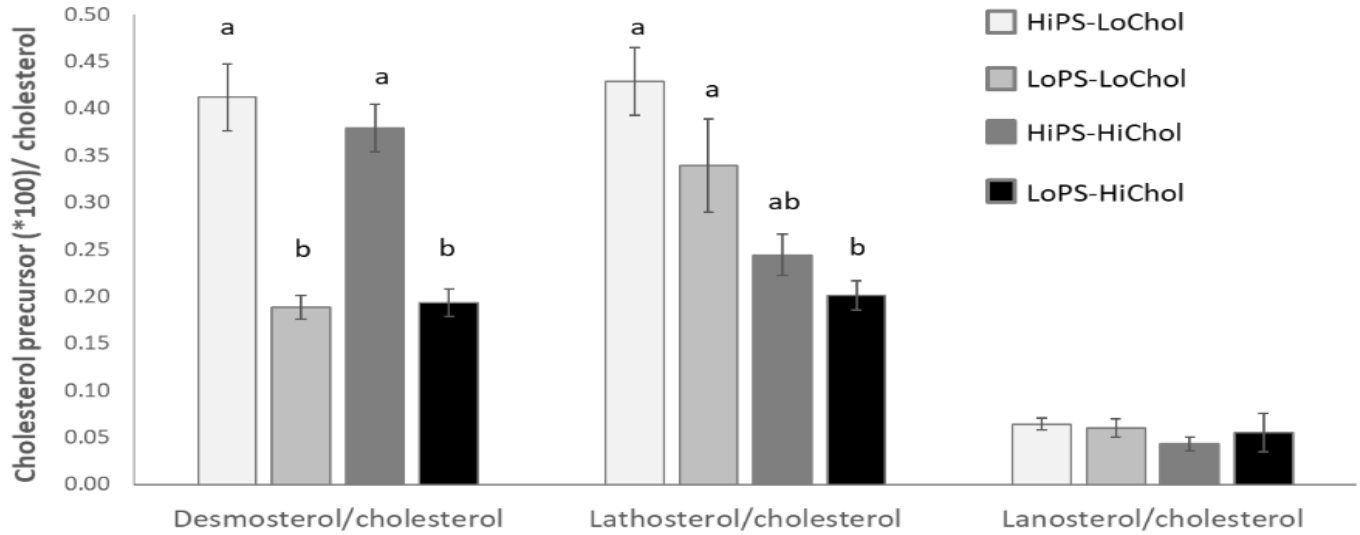
HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Figure 5 Endogenous cholesterol synthesis in the plasma and liver of the piglets receiving the test infant.

A. Plasma cholesterol synthesis



B. Liver cholesterol synthesis



n=8

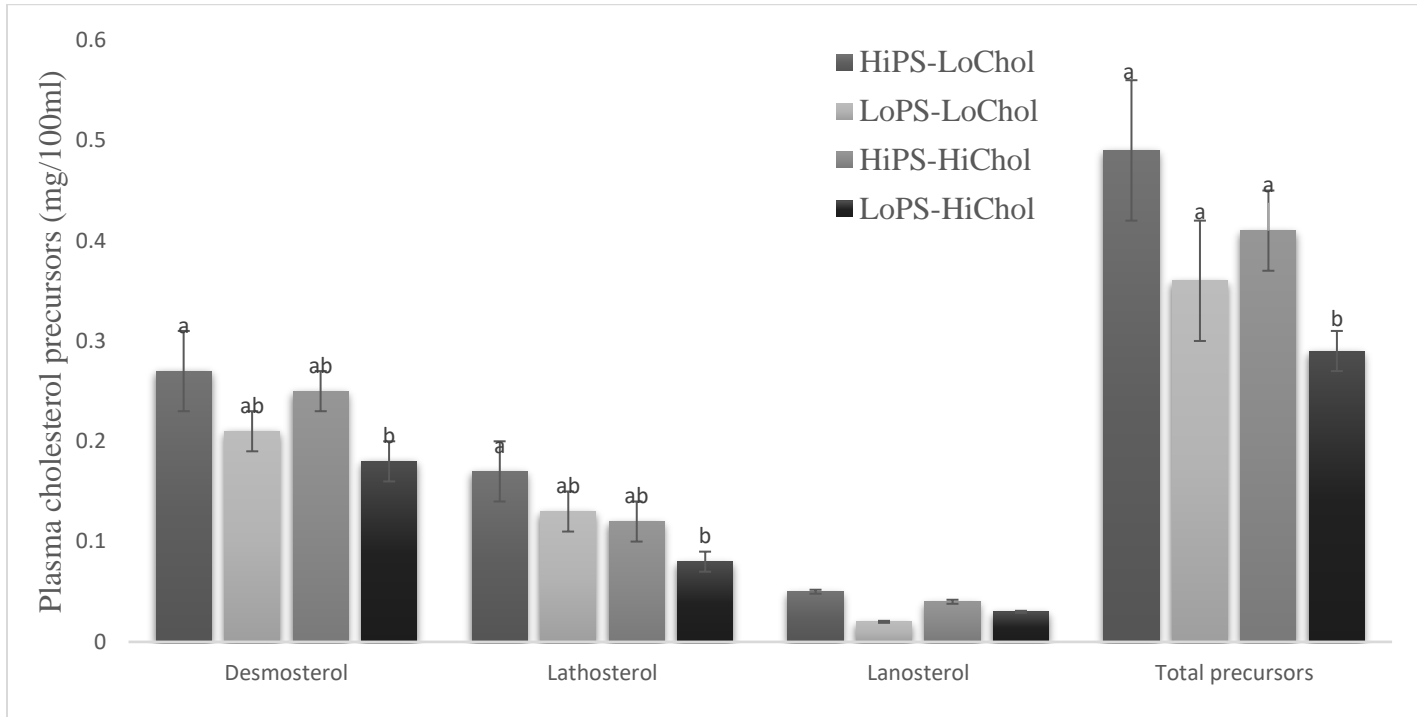
Data are presented as mean \pm SEM (Standard error of the mean).

Means without a common letter differ ($P < 0.05$).

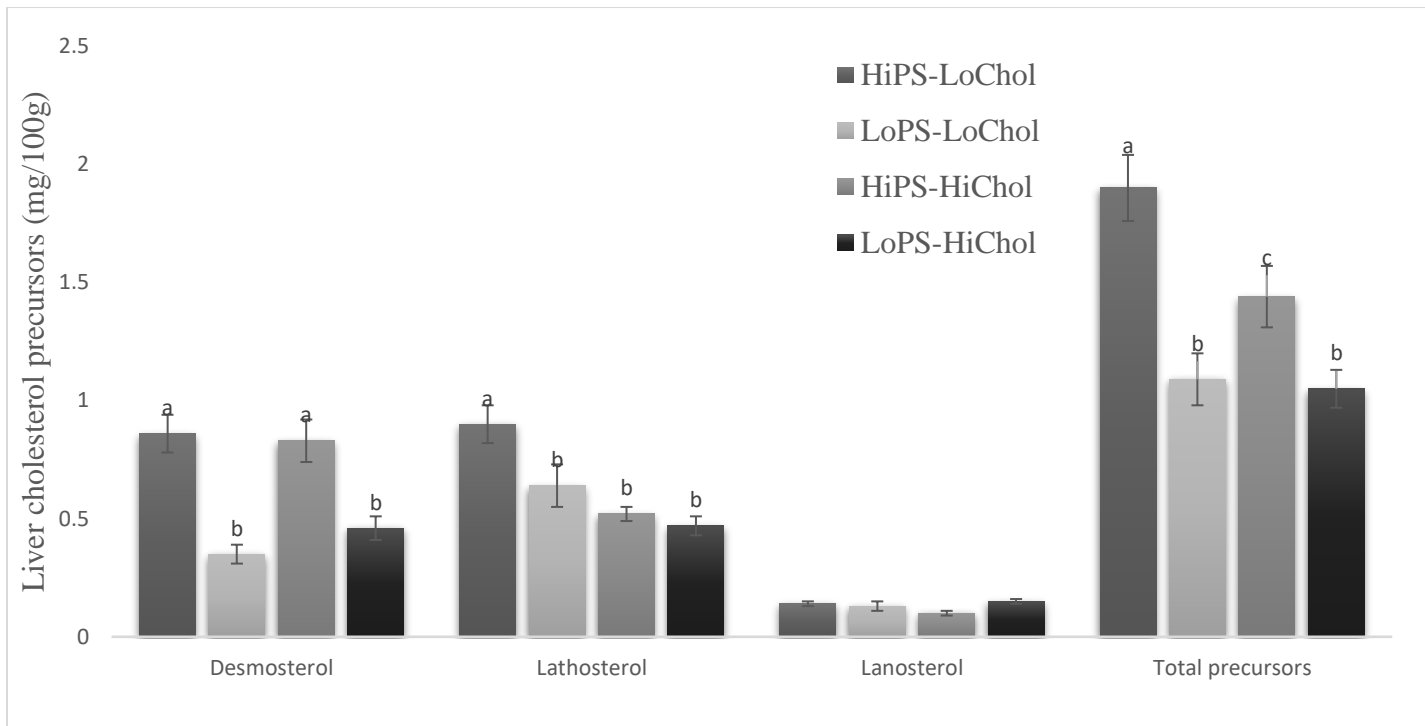
HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Figure 6 Cholesterol precursors in the plasma and liver of the piglets receiving the test infant

A. Plasma cholesterol precursors



B. Liver cholesterol precursors



n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

BRIDGE TO CHAPTER 3

In Chapter 2, the effect of consuming the regular commercial IF with high PS and low cholesterol versus other modified formulas for sterols on circulating cholesterol levels, cholesterol synthesis and absorption was demonstrated in the plasma, liver, distal intestine and ileal digesta. Since studies have shown that dietary modification of cholesterol and PS could influence health biomarkers, this chapter further explores the effect of these formulas most especially the modified formulas on some other health biomarker parameters which is believed could be influenced when sterols are modified in the body. These measured health biomarker parameters include lipid profile, plasma fatty acid levels, vitamins A and E levels, antioxidant capacity, apolipoprotein A and B levels, C-reactive protein levels, insulin levels, glucagon-like peptide 1, and fructosamine levels as well as LDL particle size. Results in this chapter show insignificant effects of diets modified with PS and or cholesterol on some health parameters measured.

CHAPTER 3

Effect of dietary modifications of cholesterol and phytosterol in infant formula on health-related biomarkers in piglet model.

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Abstract

The focus of every infant formula (IF) manufacturer is to mimic same nutritional value found in human milk (HM) while maintaining proper health in infants. Therefore, vegetable oils (VO) are usually added to infant food to provide a suitable fatty acid profile for infants. However, VO are elevated in levels of plant sterols (PS) which have long been known to inhibit cholesterol absorption. The levels of cholesterol in human milk is up to five-fold higher than in IF. However, a new process has been developed to reduce the amount of PS present in the VO that is incorporated into IF. The effect of consuming IF diets modified for cholesterol and PS most especially using PS-reduced VO, on health-related biomarkers is yet to be investigated. Therefore, the aim of this study was to test the health effects of consuming different levels of PS and cholesterol in diets using piglets as model for human infants. A total of 32 piglets were used with 8 piglets per group using the 4 following diet compositions: i) high in PS; low in cholesterol (HiPSLoChol), ii) high in PS; high in cholesterol (HiPSHiChol), iii) low in PS; high in cholesterol (LoPSHiChol) and iv) low in PS; low in cholesterol (LoPSLoChol). After 21 days of study, blood samples were collected and tested for lipid profile, plasma fatty acid levels, vitamins A and E levels, antioxidant capacity, apolipoprotein A and B levels, C-reactive protein levels, insulin levels, glucagon-like peptide 1, and fructosamine levels as well as LDL particle size. Comparison of the different diets yielded no effect ($p < 0.05$) in most of the health biomarker parameters measured except in tocopherols and fatty acids. In alpha tocopherol, an increase ($p < 0.01$) was observed in LoPSHiChol and LoPSLoChol diet groups when compared to the HiPSHiChol diet group. However, sterol effects cannot be assumed here as there was higher vitamin E in the diet composition of LoPSHiChol and LoPSLoChol than in the HiPSHiChol diet. Fatty acids also resulted in increase in LoPSHiChol (regular IF) diet group compared to HiPSLoChol diet groups. Therefore, it can be concluded that modification of VO to improve

sterol composition in IF may not affect an infant's health. This study will serve as insight to help in proper formulation of infant foods.

Keywords: Cholesterol, phytosterol, health biomarkers, vegetable oil, infant formula.

Introduction

Cholesterol is present in body cells, serving as a precursor to steroid compounds as well as serving as an important part of cell membranes. Cholesterol also aids in the absorption of fat soluble vitamins and is the main sterol produced by the body. Phytosterols (PS), also known as plant sterols/stanols, occur naturally in vegetable oil (VO) and are part of the composition of infant formula (IF). PS are absorbed in very low amounts in the human body and are known for their inhibiting actions on cholesterol absorption. Although limited investigations have been done on the importance of cholesterol in infant's diet, it has been established that cholesterol is necessary in infancy apparently because infancy is a period of rapid growth. The major discrepancies between breast milk and IF lie in the fatty acid composition, complex lipid structure, and importantly cholesterol composition (12). Cholesterol is present in a higher proportion in breast milk. Indeed, cholesterol is about three to five-fold higher in breast milk, compared to levels in infant formula. Lipid composition in mother's milk is dependent on mother's nutrition whereas the composition in IF depends on the formulation (12). Also studies have shown that infants consuming breast milk have significantly higher lipid values than formula fed infants which could be important for proper lipid programming and metabolism in later life (2). However, this low cholesterol content in infant formula is speculated to be due to a high PS content from VO used in supplementing infant food. In the present study, VO was modified for PS content. However, various lipids that exist in human milk are known to inflect functionality of some lipoproteins and fatty acid metabolism, gastrointestinal functions and some other health biomarkers which could affect the infant's health (73). In this regard, it is crucial that all various health related biomarker parameters be measured in our study to establish the action of these sterol modifications on the overall health of infants consuming diets with varying levels of cholesterol and PS.

Materials and methods

The animal control study trial aspect of this study was conducted at Massey Institute of Food Science and Technology, Massey University, Private Bag 11222, Palmerston North, New Zealand. The impact of dietary PS and cholesterol concentration on some health-related biomarkers was determined in neonate piglets given one of four dietary treatments following approval by Massey ethics committee.

Animals and diets

Thirty-two 7-day old male piglets were housed in purpose-built plastic metabolism crates in a temperature controlled room maintained at $28\pm 2^{\circ}\text{C}$ with a 16:8 h light: dark cycle. The piglets were initially weighed and randomly allocated to one of the four experimental diets such that there were eight piglets per treatment. The piglets were fed 345 g of prepared liquid formula per kg of body weight per day (60). The piglets were trained to drink using a bottle and teat and were fed their daily ration over 17 meals given hourly from 06:00 h to 10:00 h over the first six days for acclimatization and then as 7 meals given every 2.5 h from 06:30 h to 21:30 h for the remainder of the experimental period. From day 14 to 21, titanium dioxide was also added to the prepared formula to provide a titanium dioxide concentration of 3 g/kg of dry matter. The daily formula ration was readjusted weekly based on the body weights of the piglets. Any formula that was not consumed was collected, dried and weighed for each piglet.

The dietary treatments (Table 1), prepared at the food pilot plant (Massey University), contained different combinations of PS and cholesterol concentrations commensurate with those in human milk or standard vegetable oil based infant formulas. The "high PS" (HiPS) and "low cholesterol" (LoChol) refer to the levels in vegetable oils based infant formulas, while the "low PS" (LoPS, treated vegetable oils) and "high cholesterol" (HiChol, free cholesterol and

cholesterol esters in levels and ratio similar to those in human milk) refer to levels closer to those observed in human milk.

Sample collection and analysis

On day 22 of the study, piglets were fed their respective formula at hourly intervals starting at 0630 h. Seven hours after the start of feeding each piglet were anaesthetised using a cocktail of Xylazine, Zolazepam and Tiletamine, a blood sample taken fixed and stored at -80°C for analysis.

Lipid profile

Serum levels of triglycerides, total cholesterol, LDL-C, HDL-C and glucose were analysed (VITROS 350 instrument (Ortho-Clinical Diagnostics, a Johnson & Johnson Company, Raritan, NJ, USA) which employs multilayer slide dry chemistry technology as described by Sblendorio (74) and expresses results in mmol/L.

Plasma fatty acids

Fatty acids were extracted from plasma using one-step fatty acids methylation method with heptadecanoic acid as internal standard (75). Samples were analyzed as methyl esters by gas chromatography (Agilent, CA) and flame ionization detection with a capillary column of 30m×0.25mm×0.24µm. Quantification was done by comparing retention times with authenticated standards. All fatty acids were expressed as percentage of total identified fatty acids.

Plasma fat soluble vitamins A and E

All solvents used were HPLC grade obtained from Fisher Scientific. Vitamin A and E standards were obtained from Sigma Aldrich, CA.

The two compounds examined, retinol and tocopherols were extracted using method described by Bieri et al. (76)

Reverse phased HPLC was used with methanol-water (39.5:3.5, v/v) as eluent at flow rate of 1.25ml/min. Detection in plasma samples was done by UV detector. Excitation was set at 294nm and emission at 360nm. Analyte concentrations were calculated from standard curve.

Antioxidant capacity

All reagents, standards and samples were equilibrated to room temperature prior to use. Also, all control, standard and samples were assayed in duplicates. 100 µl of standard dilutions and 1-100 µl of serum samples were added in standard and sample wells respectively. 100 µl of working solvent was then added to control, standard and samples in the well. Plates containing the wells were then mixed gently and incubated for 2 hours in an orbital shaker protected from sunlight. Outputs were measured at 450nm on microplate reader. Samples with higher reading values than the highest standard value were re-diluted with the appropriate buffer and re-analyzed, concentration was then calculated by multiplying the output value with dilution factor.

Total antioxidant capacity

Colorimetric measurement of samples ability to inhibit oxidation of ABTS[®] (2, 2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS⁺(77). Data are expressed as mM Trolox equivalent.

Ascorbic acid concentration

A colorimetric method for quantification of oxidized ascorbic acid at 346 nm (78). The assay measures both ascorbic acid and dehydroascorbic acid. Ascorbic acid is oxidized to dehydroascorbic acid by ascorbate oxidase added during the analysis.

Lipid hydroperoxides concentration

Ferrous oxidation – xylenol orange (FOX2) method was used to analyze the concentration of lipid hydroperoxides in plasma (79). Data are reported as mM hydrogen peroxide equivalent.

8-Isoprostane concentration

Cayman chemicals' kit from Ann Arbor, Michigan 48108 USA (Item No. 516351) for 8-Isoprostane was used to quantify total content of 8-isoprostane concentration. The results are expressed in pg/ml.

Thiobarbituric acid reactive substances (TBARS) assay

R&D Systems kit from Minneapolis, USA (Cat No. KGE013) was used to quantify TBARS concentrations in μM . The use of conjugated enzyme activity for assessing and measuring the targeted product was employed in this process.

Apolipoprotein A1&B, CRP, insulin, GLP-1 and fructosamine

Apo A1, Apo B, CRP, Insulin, GLP-1 and fructosamine respectively, were measured using commercial sandwich Enzyme-Linked Immune Sorbent Assay (ELISA) technology kits specific for porcine from My Biosource INC. San Diego, CA. Absorbance was measured on Varian Cary 50 Bio UV-visible spectrophotometer (Agilent Technologies) at instructed wavelength(s).

Sample concentration of each parameter was quantified using standard curve obtained from serial dilution of the standard stock from each corresponding ELISA kit.

LDL particle size

Lipoprint LDL subfractions kit consisting of precast high-resolution polyacrylamide gel tubes, a loading gel solution containing lipophilic dye and buffer salt, and Lipoprint system used were obtained from Quantimetrix corporation (Redondo Beach, CA)

This method uses electrophoresis to separate lipoprotein particles into lipoprotein bands on a polyacrylamide gel based on the particle size as outlined by Muniz (80). After the electrophoresis is completed, the various stained lipoprotein bands in the sample were identified by their mobility using VLDL-C as the starting point and HDL-C as reference point at the bottom of the gel. The relative area for each lipoprotein band is determined and multiplied by total cholesterol concentration of the sample (independently measured in mmol/L) to yield the amount of cholesterol for each band in mg/dL.

Statistical analysis

Results are represented by means and standard error of the means. Analysis of variance was used in comparing the difference between means performed by least square difference. Results were deemed statistically different at $p < 0.05$

Results

No significant differences were observed in food intake or body weight of the piglets across treatment groups throughout the trial. **Table 1** shows ingredients and nutrient composition of each diet treatment used in the study, **Table 2** shows intake and body weight of the study piglets.

Lipid profile

The lipid profile of piglets consuming the different diets is shown in Figure 6 below. No significant effect of dietary cholesterol and PS level on triglycerides, very low-density lipoprotein (VLDL-C) cholesterol or high-density lipoprotein (HDL-C) cholesterol concentrations was observed. However, for LDL-C, HiPSHiChol and LoPSHiChol diet groups were significantly higher than LoPSLoChol and HiPSLoChol diet groups at $P < 0.05$.

Plasma fatty acids

Plasma fatty acid compositions in terms of total saturated fatty acids (SFA), total

monounsaturated fatty acids (MUFA) and total polyunsaturated fatty acids (PUFA) were not affected by the dietary PS and cholesterol concentration modification. However, the ratio of n-3 to n-6 fatty acids was significantly lower in the HiPSLoChol group compared to LoPSHiChol group. Also, Docosaehaenoic acid (DHA) was lower ($P<0.05$) in the LoPSLoChol diet compared to HiPSLoChol diet.

Fat soluble vitamins A and E

No significant differences were detected in retinol levels after dietary cholesterol and PS consumption in the piglets. However, alpha tocopherol levels, a fat-soluble vitamin was lower ($P<0.05$) in HiPSHiChol group compared with LoPSLoChol and LoPSHiChol group. Beta and gamma tocopherol levels were also reduced ($p<0.001$) in low PS (LoPSLoChol and LoPSHiChol) diet groups when compared with high PS (HiPSLoChol and HiPSHiChol) diet groups

Antioxidant capacity

Changes in the PS and cholesterol level across diets did not influence the antioxidant capacity factors such as ascorbate, total antioxidant capacity, TBARS or 8 isoprostane in our study (Table 5). However, high total sterols in the diet influenced LOOH levels because HiPSHiChol diet group was higher ($p<0.05$) in LOOH levels than in LoPSLoChol group. The inability to detect significant changes in the antioxidant measures could have been due to heterogeneity among piglets or high sensitivity of the antioxidant compounds in samples to react with environment could easily influence the result.

Plasma Apo A1, Apo B, CRP, insulin, GLP-1, fructosamine and LDL particle size levels across treatments

No significant effects of the treatment were detected in these factors measured.

Discussion and conclusion

Interest in quality of lipids in infant's nutrition is currently growing especially because it is a major determinant on long term health (73). This study shows the effect of dietary modification of cholesterol and PS in IF on lipids and certain health related parameters in piglets. The basis of this study is particularly due to potential effect of sterols modification on health (73). Lipid metabolism occurs in several pathways through intracellular and extracellular modification. The interactions between these pathways are essential for lipid regulation. HDL-C and LDL-C are the two major forms of cholesterol in the body, however, changes in cholesterol significantly influences LDL-C value than HDL-C, triglycerides and VLDL-C as seen in our study. This is in line with studies from Ballesteros et al which evaluated effect of dietary cholesterol on health biomarkers with a 4-week dietary cholesterol supplementation in a paediatric population. Here, no significant effect was observed in any of these lipid parameters measured when compared with diets with no cholesterol, likewise as carried out in our study, they also measured the Apo A1 and B concentrations as well as lipoprotein sub fractions. Even though the dietary intervention, study design and study subject was different from ours, dietary cholesterol intake failed to yield a significant effect in any of these measures as well (81). Also, breastfeeding has been studied and well documented to correlate with high total cholesterol and LDL at infancy (37,82–85,6,7,86) and lower levels in adulthood which suggests a beneficial effect on cardiovascular health (32). A study by Bayley et al (57), which compared cholesterol and LDL-C concentration in infant fed human milk with infants fed regular formula with added cholesterol gave a significant increase in circulating cholesterol and LDL-C level in infants fed human milk containing 3 fold higher cholesterol. High LDL-C in breast fed infants has been attributed to presence of high cholesterol in human milk. This is also in line with our study as LDL-C and total cholesterol levels were higher in our high cholesterol diet LoPSHiChol when compared

with HiPSLoChol diet. This signifies that not only the addition of cholesterol to the regular infant formula but also reducing the PS content could plausibly be collectively responsible for the dietary sterol metabolism response found in breast-fed infants with formula-fed infants.

Dietary lipids facilitate the absorption of fat soluble vitamins and in addition, provide essential fatty acids (87,88). This necessitates the need for fatty acids and vitamins A and E measurement in this study. Despite the discrepancies in the lipid composition in human milk and IF, there is considerable information which shows that the ratio of essential and non-essential fatty acids in the diet contributes to the total fatty acids in the cell membranes (89). These fatty acids are, however, crucial in various cell functioning. Although not part of this subject, the role of PUFAs in central nervous system development is emerging and an essential part of the new science. This justifies the reason VO is being used to replace milk fat in IF formulation, it maintains similar fatty acids profiles found in human milk (90). Interestingly in this study, dietary sterol modification did not affect the plasma fatty acids concentrations in the modified diets (HiPSHiChol, LoPSHiChol and LoPSLoChol), there was rather increase in DHA in LoPSHiChol diet when compared to the regular formula diet HiPSLoChol. The increase detected in alpha tocopherol level is believed to be due to different concentrations in the diet and not due to a sterol modification effect as there was higher vitamin E in the diet composition of LoPSHiChol and LoPSLoChol than in the HiPSHiChol diet.

In summary, this study demonstrates that modifying sterols especially to levels found in our study (viz PS and cholesterol levels of 79.2 and 24.5 or 9.6 and 24.3 or 78.8 and 85.2 or 9.9 and 84.5 mg/100gr respectively), does not necessarily influence health. This suggests a lower sensitivity of health parameters measured in this study to sterol modifications in piglets used as a model for human infant.

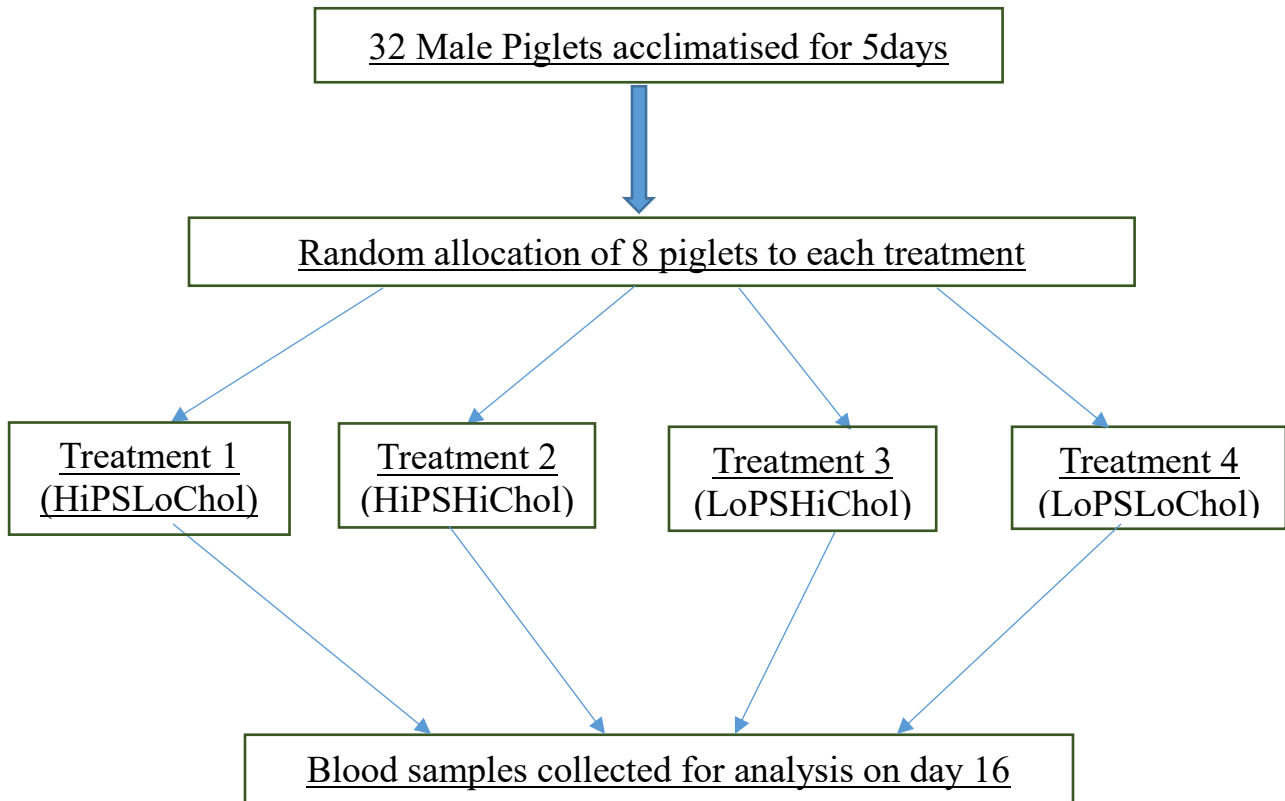


Figure 7 Study design for the treatments

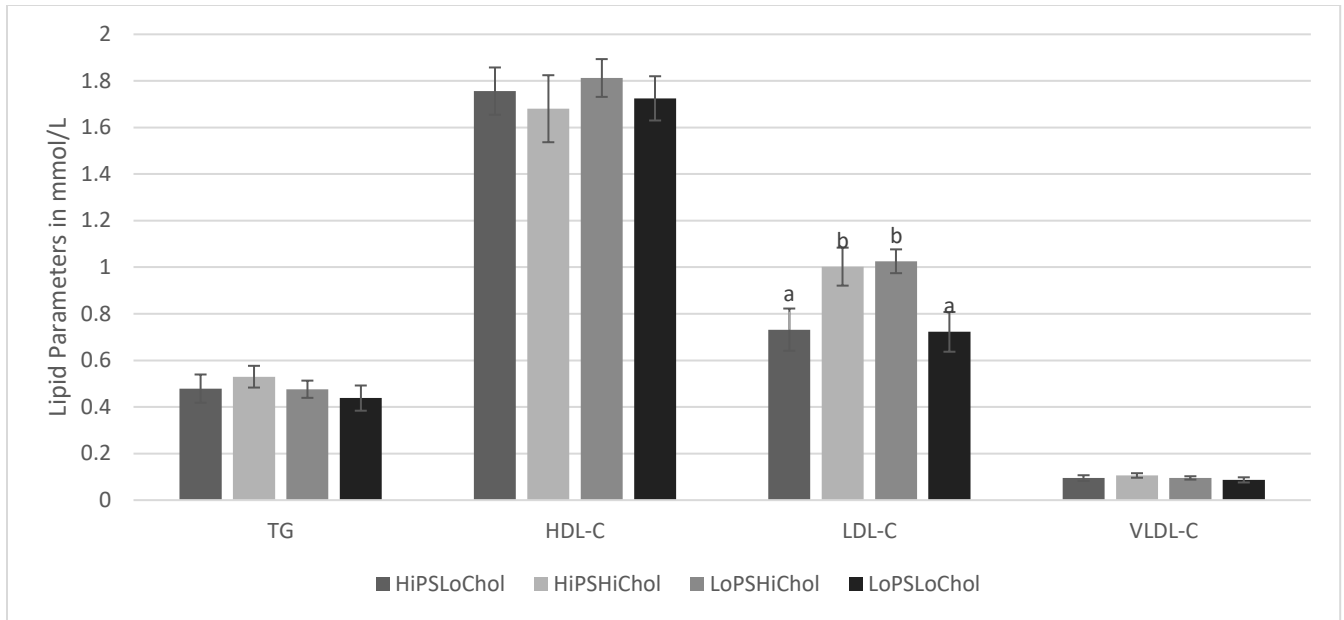


Figure 8 Lipid levels across the dietary treatments

TG: Triglycerides, HDL-C: High density lipoproteins concentrations, LDL-C: Low density lipoproteins concentration, VLDL-C: Very low density lipoproteins concentration

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 3 Plasma fatty acids levels

	HiPSLoChol	HiPSHiChol	LoPSHiChol	LoPSLoChol
Total saturated fat	43.85±0.66	43.34±0.45	43.34±1.34	42.66±0.62
Total MUFA	25.28±0.75	25.13±0.54	25.90±1.01	27.78±1.55
Total PUFA	30.87±0.39	31.53±0.38	30.76±1.08	29.56±0.97
C18:3n-3	1.16±0.12	1.12±0.05	1.10±0.13	1.52±0.28
C22:6n-3	1.15±0.08 ^a	1.13±0.10 ^{ab}	0.99±0.06 ^{ab}	0.94±0.07 ^b
C20:4n-6	4.54±0.14	4.73±0.29	4.64±0.27	4.44±0.32
C18:2n-6	23.07±0.36	23.55±0.54	23.06±0.86	21.79±0.98
PUFA/MUFA	1.23±0.04	1.26±0.04	1.20±0.06	1.10±0.09
n-3/n-6	0.13±0.02 ^a	0.15±0.02 ^{ab}	0.18±0.01 ^b	0.14±0.02 ^{ab}

SFA= Saturated fatty acids, MUFA=Monounsaturated fatty acids, PUFA= Polyunsaturated fatty acids, ALA= Alpha linolenic acid, DHA= Docosahexaenoic acid, AA= Arachidonic acid, and LA= Linoleic acid.

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 4 Plasma vitamins A and E levels

	HiPSLoChol	HiPSHiChol	LoPSHiChol	LoPSLoChol
Alpha Tocopherol (µg/mL)	10.10±0.62 ^{ab}	9.2203±0.7866 ^a	12.3435±0.77565 ^b	11.5385±0.62227 ^b
Beta and Gamma Tocopherol (µg/mL)	0.29±0.03 ^a	0.26±0.02 ^a	ND ^b	ND ^b
Retinol (µg/mL)	0.12±0.01	0.11±0.03	0.14±0.02	0.14±0.03

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 5 Antioxidant capacity measures across dietary treatments

	HiPSLoChol	HiPSHiChol	LoPSHiChol	LoPSLoChol
Ascorbate (µM)	66.91±12.30	77.29±11.36	117.67±17.93	101.32±31.59
Total antioxidant (mM Trolox Eq)	2.11±0.21	2.50±0.17	2.40±0.15	2.50±0.15
TBARS (µM)	2.87±0.23	2.73±0.20	2.50±0.22	3.10±0.29
LOOH (µM H₂O₂ Eq)	1.39±0.41	1.84±0.35	1.22±0.31	0.77±0.31
8-Isoprostane (pg/mL)	50.27±7.35	56.44±8.64	63.51±13.29	53.89±11.08

TBARS= Thiobarbituric acid reactive substances, LOOH= lipid hydroperoxides.

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 6 Plasma apo A1, Apo B, CRP, insulin, GLP-1 and fructosamine levels across treatments.

	HiPSLoChol	HiPSHiChol	LoPSHiChol	LoPSLoChol
Apo A1 (µg/dL)	127.17±6.48	129.46±6.92	124.35±5.34	139.78±8.32
Apo B (mg/dL)	6.49±0.38	6.93±0.53	6.98±0.27	6.72±0.16
CRP (ng/mL)	33.98±3.86	29.79±3.21	37.27±5.08	33.60±3.079
Insulin (ng/mL)	79.49±1.94	76.03±1.74	79.35±2.33	76.93±4.30
GLP-1 (ng/mL)	0.29±0.11	0.37±0.12	0.32±0.09	0.38±0.13
Fructosamine (ng/mL)	5.64±0.42	5.40±0.45	5.61±0.48	5.45±0.65

Apo A1= Apolipoprotein A1, Apo B= Apolipoprotein B, CRP= C-reactive protein, GLP-1= Glucagon-like peptide-1.

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

Table 7 Small LDL particle size levels across treatments

Reference range		HiPSLoChol	HiPSHiChol	LoPSHiChol	LoPSLoChol
≤22	VLDL-C	9.00±0.76	10.13±0.69	9.13±0.77	8.63±0.65
≤23	IDL-C	8.25±0.86	10.63±1.05	9.00±1.28	7.88±1.60
≤15	IDL-B	3.38±0.75	4.88±0.91	4.75±0.53	3.00±0.60
≤25	IDL-A	2.13±0.44	2.88±0.64	3.63±0.60	2.50±0.50
≤57	LDL-1	8.75±1.19	9.00±1.48	11.75±1.01	7.75±1.05
≤30	LDL-2	10.75±1.41	12.63±0.71	13.63±0.63	11.13±0.72
≤6	LDL-3	7.00±0.38	7.75±0.75	7.38±0.73	7.63±0.75
≤0	LDL-4	2.00±0.63	2.63±0.90	2.25±0.70	3.13±0.79

Reference range for each IDL and LDL parameter is the normal level stated for human in the kit's manual. IDL= intermediate density lipoprotein and LDL=low density lipoprotein.

n=8

Data are presented as mean ± SEM (Standard error of the mean).

Means without a common letter differ (P<0.05).

HiPSLoChol means high phytosterol and low cholesterol diet, LoPSLoChol means low phytosterol and low cholesterol diet, HiPSHiChol means high phytosterol and high cholesterol diet, LoPSHiChol means Low phytosterol and high cholesterol diet.

CHAPTER 4

Discussion and conclusion

This study provided insight to health effects of modifying PS and cholesterol in IF, especially as it pertains to the synthesis and absorption of cholesterol. To achieve this, neonate piglets were used as a model for human infants. This is because piglets have intestinal enzyme metabolism maturation similar to that of human infants unlike other animals used for studies. They have also been considered useful in pediatrics related studies for their commensurate physiological and nutritional needs with that of humans infants (49,91).

The major finding in this study is the high sensitivity of the neonates to slight changes in dietary sterols in terms of cholesterol synthesis which has been speculated to have an effect in later life (64). The newly formulated infant formula with low PS and high cholesterol lowers cholesterol synthesis in all the tissues measured, namely; plasma, liver and distal intestine. To our knowledge, this is the first work that uses reduction in PS to eliminate the inhibition effect of PS on cholesterol absorption which thus, raises cholesterol synthesis level in infants consuming IF most especially VO-based formulas. Furthermore, our study shows the dose effect of both PS and cholesterol in a single diet on some health biomarkers. The present study provides more evidence that lowering dietary PS would raise the blood cholesterol level. This is considered an improvement in infant's formula as it raises the level of cholesterol closer to levels found in human milk which is considered standard food for an infant.

In conclusion, our findings can be used in addressing issues about cholesterol levels in pediatric nutrition in the following context:

It is concluded that modifying sterols in infant formula by lowering the PS and increase

cholesterol content of regular IF could be beneficial in preventing upregulation of cholesterol synthesis as well as improving dietary cholesterol absorption. Also, this modification as seen in our study does not affect infant's health in terms of other health related biomarkers.

Future direction

The effect of this formula or treatment should be investigated in human infants with high sample size or high number of subjects to establish the significant effect of this newly developed formula within a physiologically feasible population.

Strengths and weaknesses

The study was properly blinded to the researcher to eliminate possible bias. Relevant tissues were collected after the trial with conduction of various relevant tests to properly monitor effect of the diets on relative organs of the piglets.

Small sample size which could reduce the statistical power of the study and the use of animal to model for human infant could be considered a weakness in this study.

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