THE PREBIOTIC EFFECT OF ENZYMATICALLY-RELEASED BIOACTIVE COMPONENTS OF CANOLA MEAL FIBRE ON GUT HEALTH AND GROWTH PERFORMANCE OF MONOGASTRIC ANIMALS

By

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ABSTRACT

Canola meal (**CM**) contains a significant amount of non-starch polysaccharides (**NSP**) which with the use of enzyme technology could be hydrolyzed to low-molecular-weight polysaccharides and oligosaccharides. We hypothesized that enzymatically-released bioactive components of CM fibre would have a prebiotic effect on gastrointestinal microbiota of monogastric animals. A variety of carbohydrases were evaluated in the series of in vitro studies, and the most effective enzyme combination was used in the study. The blend of a multi-carbohydrase preparation containing pectinase and xylanase activities showed the ability to hydrolyze 47.9% of CM NSP in vitro and improved NSP digestibility of broilers fed diets containing CM. The enzyme blend was used to produce enzymatically-modified CM (**ECM**, lab scale) and ECM solubles (**ECMS**, pilot scale), and their prebiotic properties were evaluated.

The results of the broiler chicken study showed that replacing CM with ECM significantly improved the overall apparent total tract digestibility of NSP. *Lactobacillus spp.* was present in much larger counts ($P < 0.01$) in ileal and cecal digesta of birds fed the ECM diets when compared to the Control treatment. The AMEn value of ECM was 31% higher than that of CM. There was no difference in the standardized ileal amino acid digestibility of CM and ECM. Supplementation diets with ECMS improved growth performance, increased total SCFA in ileal digesta, and provided favorable conditions for the proliferation of *Lactobacillus spp.* and *Enterococcus spp.* in ileum and ceca of broilers.

The effect of ECM and ECMS supplementation on growth performance and gut health of weaned piglets was investigated. No effect of ECM was observed; however, ECMS positively affected the intestinal pH and proliferation of beneficial gut microbiota.

In conclusion, modification of CM fibre with carbohydrases significantly changed its

structure and enhanced its bioactivity. Processing of CM developed throughout this project generated novel products, ECM and ECMS, which when fed to broilers and pigs, demonstrated their ability to enhance the diversity of the gut microbiota population and improve nutrient utilization. Therefore, the enzymatic modification of CM can create value-added feed additives, which can improve animal health and support antibiotic-free feeding programs in monogastric animals.

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This thesis was prepared following a manuscript format and is composed of five manuscripts. Some parts of this thesis had been presented as posters or oral presentations at the conferences including: Animal Nutrition Conference of Canada, May 9-11, 2023, Montreal; Poultry Science Association Annual Meeting, July 11-15, 2022, San Antonio; Animal Nutrition Conference of Canada, May 11-12, 2022, Saskatoon; Poultry Science Association Annual Meeting, July 19-22, 2021, online; Annual Canadian Poultry Research Forum, June 21-23, 2021, online; Animal Nutrition Conference of Canada, May 10-14, 2021, online; Poultry Science Association Annual Meeting, July 20-22, 2020, online; Animal Nutrition Conference of Canada, May 26-28, 2020, online. The manuscripts are listed as follows:

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2. **Niu Y.**, Rogiewicz A., Shi L., Patterson R., Slominski B. A. The Effect of Enzymatically-modified canola meal on growth performance, nutrient utilization, and gut health and function of broiler chickens. Anim. Feed Sci. Technol. Under review.

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5. **Niu Y**., Rogiewicz A., Ferreira J., Patterson R., Nyachoti M., Slominski B. A. The effect of enzymatically-modified canola meal on growth performance and gut health of weaned piglets. Under preparation and to be submitted to the Journal of Animal Science.

CONTRIBUTIONS OF AUTHORS

This thesis was prepared by Yanxing Niu and was reviewed, revised, and approved by the internal examiners including Drs. Anna Rogiewicz, Bogdan A. Slominski (co-advisor), Martin Nyachoti (committee member), and Robert Duncan (committee member).

All manuscripts (Chapter Three to Chapter Seven) had been revised and approved by the co-authors, and the contributions of all authors are described below:

Chapter Three: Manuscript I. Dr. Anna Rogiewicz, Dr. Bogdan A. Slominski, and Yanxing Niu conceptualized and designed the study. Yanxing Niu and Lan Shi performed the experiments. Yanxing Niu and Dr. Anna Rogiewicz analyzed the data. Yanxing Niu drafted the manuscript. Yanxing Niu, Drs Anna Rogiewicz and Bogdan A. Slominski, and Rob Patterson of CBS Bio Platforms revised the manuscript.

Chapter Four: Manuscript II. Dr. Anna Rogiewicz, Dr. Bogdan A. Slominski, and Yanxing Niu conceptualized and designed the study. Yanxing Niu and Lan Shi performed the experiments. Yanxing Niu and Dr. Anna Rogiewicz analyzed the data. Yanxing Niu drafted the manuscript. Yanxing Niu, Drs. Anna Rogiewicz and Bogdan A. Slominski, and Rob Patterson of CBS Bio Platforms edited and revised the manuscript.

Chapter Five: Manuscript III. Drs. Anna Rogiewicz and Bogdan A. Slominski, and Yanxing Niu conceptualized and designed the study. Yanxing Niu performed the experiments. Yanxing Niu and Dr. Anna Rogiewicz analyzed the data. Yanxing Niu drafted the manuscript. Yanxing Niu, Drs Anna Rogiewicz and Bogdan A. Slominski, and Rob Patterson of CBS Bio Platforms edited and revised the manuscript.

Chapter Six: Manuscript IV. Drs. Anna Rogiewicz and Bogdan A. Slominski, and Yanxing Niu conceptualized and designed the study. Yanxing Niu performed the experiments. Yanxing Niu

and Dr. Anna Rogiewicz analyzed the data. Yanxing Niu drafted the manuscript. Yanxing Niu, Drs. Anna Rogiewicz and Bogdan A. Slominski, and Rob Patterson (CBS Bio Platforms) edited and revised the manuscript.

Chapter Seven: Manuscript V. Dr. Anna Rogiewicz, Dr. Bogdan A. Slominski, and Dr. Martin Nyachoti, Yanxing Niu conceptualized and designed the study. Yanxing Niu and Dr. Joao Ferreira performed the experiments. Yanxing Niu and Dr. Anna Rogiewicz analyzed the data. Yanxing Niu drafted the manuscript. Yanxing Niu, Dr. Anna Rogiewicz, Dr. Bogdan A. Slominski, Dr. Martin Nyachoti, and Rob Patterson (CBS Bio Platforms) edited and revised the manuscript.

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AA	amino acids
ADF	acid detergent fibre
ADFI	average daily feed intake
ADG	average daily gain
AIAAD	apparent ileal amino acid digestibility
AID	apparent ileal digestibility
AOAC	Association of Official Analytical Chemists
ATTD	apparent total tract digestibility
AMEn	nitrogen corrected apparent metabolizable energy
BCFA	branched chain fatty acids
BWG	body weight gain
CA	cellulase A
CB	cellulase B
CC	cellulase C
CD	cellulase D
CM	canola meal
CM+E	enzymatic modification processing
CM+E+NaN_3	enzymatic modification processing with NaN_3
DF	dietary fibre
DM	dry matter
ECM	enzymatically-modified canola meal
ECMS	enzymatically-modified canola meal solubles

FCR ..... feed conversion ratio

FI ..... feed intake

FOS ..... fructo-oligosaccharides

GE ..... gross energy

G: F ..... gain to feed ratio

GIT ..... gastrointestinal tract

HPLC ..... high-performance liquid chromatography

Inv ..... invertase

IAA_{end} ..... basal ileal endogenous amino acid losses

M ..... mannanase

MOS ..... manno-oligosaccharides

N ..... nitrogen

NDICP ..... neutral detergent insoluble crude protein

NDF ..... neutral detergent fibre

NE ..... net energy

NSP ..... non-starch polysaccharides

P ..... phosphorus

PA ..... pectinase A

PB ..... pectinase B

PCR ..... polymerase chain reaction

Pr ..... protease

qPCR ..... quantitative real-time polymerase chain reaction

SBP ..... sugar beet pulp

SCFA ..... short chain fatty acids

SFH ..... sunflower hulls

SIAAD ..... standardized ileal amino acid digestibility

SID ..... standardized ileal digestibility

SSF ..... solid-state fermentation

STTD ..... standardized total tract digestibility

TDF ..... total dietary fibre

VFA ..... volatile short chain fatty acids

XA ..... xylanase A

XB ..... xylanase B

XC ..... xylanase C

XOS ..... xylo-oligosaccharides

CHAPTER ONE : GENERAL INTRODUCTION

1.1 Background

Canola meal (**CM**), the co-product of canola processing industry, is the second largest plant protein and commonly used as a protein source in monogastric animal diets worldwide (Canola Council of Canada, 2019). Canola meal is rich in protein and dietary fibre (**DF**). Total dietary fibre (**TDF**) of CM varies from 25.8 to 41.1% on a dry matter (**DM**) basis and its content would depend on the cultivar, seed color, hull thickness, seed processing, etc.(Khajali and Slominski, 2012). The DF of CM includes non-starch polysaccharides (**NSP**), lignin with associated polyphenols, glycoprotein and minerals that are bound to the fibre components (Slominski et al., 2012; Lannuzel et al., 2022). Among all components, NSP are the most abundant constituents of DF and account for 53-80% of TDF of CM with only around 10% or less being water-soluble (Slominski and Campbell, 1990; Adewole et al., 2016; Lannuzel et al., 2022).

Non-starch polysaccharides are composed of cellulose and non-cellulosic polymers, including arabinans, arabinogalactans, arabinoxylans, mixed-linked β-glucans, galactans, galactomannans, mannans, xyloglucans, xylans and pectic polysaccharides (Slominski and Campbell, 1990; Khajali and Slominski, 2012; Do et al., 2020). Arabinans, arabinogalactans, xyloglucans, galactans, galactomannans, and pectic polysaccharides are predominant in CM. The NSP of CM are poorly digestible by poultry with the apparent total tract digestibility (**ATTD**) varying from 2.1 to 8.6% (Slominski and Campbell, 1990; Slominski et al., 1994; Kocher et al., 2000; Meng et al., 2005; Meng and Slominski, 2005). In weaned pigs, NSP are more digestible, with 10.0% being digested at the ileal level and partly fermentable, resulting in 49.0% of total tract NSP digestibility (Omogbenigun et al., 2004).

1.2 Research gap

Application of carbohydrases in CM included diets is a practical approach to improve the nutritive value of CM (Slominski, 2010; Pirgozliev et al., 2022). It can be achieved by facilitating the hydrolysis and depolymerization of NSP and eliminating nutrient encapsulating effect of cell walls thus increasing their solubilization and allowing for a more effective lower gut fermentation to improve energy utilization and proliferation of beneficial bacteria (Slominski, 2010; 2011). The complexity of NSP would require diversified enzyme preparations for effective NSP depolymerization. This is because the targeting of complex linkages within the cell wall structure would require many activities acting in concert as opposed to a single enzyme, the application of which would result in minimal NSP depolymerization and only on the surface of the cell wall. Therefore, only the most complex enzyme combinations, containing a variety of activities, would be required for the effective NSP depolymerization (Slominski, 2017).

Enzymatic modification of CM can be a method to increase its nutritive value. In general, enzymatic modifications of feed ingredients have been proven to have some beneficial effect on nutrient digestibility, growth performance, and antinutritional concerns (Omogbenigun et al., 2004; Meng et al., 2005; Jakobsen et al., 2015; Loman and Ju, 2016; Drazbo et al., 2018). By incubating CM with enzymes, NSP will be depolymerized with the release of prebiotic-type components, including low-molecular-weight polysaccharides and oligosaccharides, which may facilitate the proliferation of beneficial bacteria important for gut health such as *Bifidobacterium* and *Lactobacillus*, thereby decreasing the abundance of pathogens such as *Escherichia coli*, *Clostridium perfringens*, *Campylobacter*, *Salmonella*.

To date, studies have focused on growth performance, nutrition digestibility, enzyme addition, and recommended dietary formulations for CM, with little focus on the bioactive components of CM fibre.

1.3 Hypotheses and objectives

1.3.1 Hypothesis

Enzymatically-released bioactive components of CM fibre have prebiotic effects when fed to young monogastric animals.

1.3.2 Objectives

The overall objective of this thesis was to investigate the effect of enzymatically-released bioactive components of Canola (*Brassica napus L.*) meal fibre on gut health and growth performance of monogastric animals.

The specific objectives were:

1. To screen multi-carbohydrase preparations in vitro for their ability to depolymerize NSP and to evaluate the effects of the most effective enzyme combinations on NSP digestibility and growth performance of broiler chickens fed diets containing high inclusion levels of CM.

2. To develop enzymatically-modified CM (**ECM**), evaluate its nutritive value for broiler chickens, and compare its effect with the diet supplemented with the same enzyme cocktail.

3. To investigate the dynamics of physicochemical processes and carbohydrate profile during the ECM production and analyze the standardized ileal digestibility (**SID**) of amino acids (**AA**) and nitrogen corrected metabolizable energy of final ECM product for broiler chickens.

4. To evaluate the prebiotic effect of enzymatically-modified CM solubles (**ECMS)** on gut health and growth performance of broilers chickens.

5. To evaluate the effect of ECM and ECMS on gut health and growth performance of weaned piglets.

1.4 Thesis structure

Chapters of this thesis are structured as follows:

- Chapter Two provides information about chemical composition and nutritive value of CM, methods towards improvement of nutritive value of CM for monogastric animals, definition of DF components and their role in animal nutrition.
- Chapters Three to Seven present the studies on the effect of enzymatically-released bioactive components of CM fibre on gut health and growth performance of monogastric animals.
- Chapter Eight summarizes and concludes the thesis.

In this research, three approaches were used to obtain the enzymatically-released bioactive components of CM fibre. First one included direct exogenous enzymes addition to the diet to facilitate the breakdown of the fibre components and release of bioactive components in the digestive tract of the animal (Chapters Three and Four). In the second approach, CM was pre-treated with specific enzymes to produce ECM (Chapters Four, Five, and Seven). In the third approach, the water-soluble fraction of ECM was separated to produce ECMS (Chapters Six and Seven).

In Chapter Three, a variety of multi-carbohydrate enzyme preparations had been evaluated in vitro to determine their efficacy in NSP depolymerization. The most effective enzyme combination used in broiler chicken diet improved apparent total tract digestibility of NSP in vivo. In Chapter Four, the selected enzyme blend was used to produce ECM. The effect of ECM or enzyme blend supplementation on growth performance, nutrient utilization and gut health of

broiler chickens was evaluated. The production conditions of ECM and the physicochemical dynamics and carbohydrate profile during the production were explored to obtain the optimal quality of the final product in Chapter Five. In addition, the nutritive value characteristics, including the nitrogen corrected apparent metabolizable energy (**AMEn**) and SID of AA of ECM and CM, were determined and presented. It was demonstrated that ECM added to the diet could improve growth performance, nutrient digestibility, and gut health of broiler chickens, and enzymatic modification increased AMEn value of CM by 31%. Therefore, the method for scaling-up of the ECM production was explored and it resulted in the production of ECMS solubles. In Chapter Six, the effect of ECMS supplementation on growth performance, nutrient digestibility, and gut health of broiler chickens was evaluated. Moreover, the effects of enzymatically-released bioactive components of CM fibre that are present in ECM and ECMS on growth performance, nutrient utilization, and gut health of weaned piglets were investigated and presented in Chapter Seven.

CHAPTER TWO : LITERATURE REVIEW

2.1 Background

Canola is one of the most important and valuable crops in Canada and contributes 29% to Canada's overall grain and oilseed revenue (Canola Council of Canada, 2019; Wells and Slade, 2021). The production of canola has grown steadily to approximately 20 million metric tonnes since its introduction (So and Duncan, 2021). Canola is the internationally recognized term for *Brassica* species containing no more than 2% erucic acid in the oil and less than 30 µmol/g of glucosinolates in the meal. Currently, with the efforts and improvement of breeding, commercial canola contains less than 20 µmol/g of glucosinolates and less than 1% erucic acid (So and Duncan, 2021).

Canola meal (**CM**) is the co-product of canola processing industry. Canola meal is rich in protein and dietary fibre (**DF**), especially non-starch polysaccharides (**NSP**) (Adewole et al., 2016; Radfar et al., 2017). Canola protein accounts for over 40% of CM on a dry matter (**DM**) basis and is composed of well-balanced amino acids (**AA**) and a high content of methionine and cysteine. Total dietary fibre (**TDF**) accounts for 35-42% of CM on a DM basis.

Dietary fibre has historically been regarded as an antinutritional factor due to its adverse effects on nutrient utilization and energy digestibility for poultry and swine (Slominski et al., 2006; Agyekum and Nyachoti, 2017). However, recently it has drawn a special attention due to its beneficial effect on gut health of monogastric animals (Jha et al., 2019). Particularly, the NSP as the most important constituents of TDF are of interest. In CM, NSP account for more than a half of the dietary fibre, of which only about 10% are water-soluble and less than 10% can be digested by broilers (Slominski and Campbell, 1990; Khajali and Slominski, 2012; Lannuzel et al., 2022).

Application of carbohydrases in CM included diets is a practical approach for improving the nutritive value of CM (Omogbenigun et al., 2004; Meng et al., 2006; Slominski et al., 2006; Slominski, 2011; Long et al., 2020). It can be achieved by the hydrolysis of NSP, thus increasing their solubilization which, in turn, allows for a more effective lower gut fermentation. This would improve the energy utilization, facilitate the releasing of nutrients encapsulated by the cell wall structure, and increase NSP digestibility. The enzymatic hydrolysis of some NSP might eliminate their anti-nutritive properties and release prebiotic-type components, including low-molecular-weight polysaccharides and oligosaccharides, which may facilitate the proliferation of beneficial bacteria for gut health. Enzymatic hydrolysis of DF components and subjecting CM to fermentation are important means to add value to CM.

2.2 Chemical composition of canola meal

2.2.1 Factors that influence the chemical composition of canola meal

Canola quality can result from any one of three species, including *Brassica napus* L., *Brassica. rapa* (*campestris*) L. (with the three subspecies *oleifera*, *trilocularis*, and *dichotoma*), and *Brassica juncea* (L) containing low glucosinolates (< 30μmol/g in meal) and low erucic acid (< 2% in oil) in North America (Daun et al., 2011). In Europe and Asia, rapeseed with similar quality is called "double zero", "zero-zero" or "double low" (Bell, 1984; P. Li et al., 2020; So and Duncan, 2021). *Brassica napus* is the mostly cultivated species. As glucosinolates have a negative impact on palatability and toxic effects on many livestock species (Zhu et al., 2018), CM is regarded as a better feed protein source than traditional rapeseed.

The chemical composition of CM depends on the quality of canola seeds and the processing method applied during the oil extraction process (Watts et al., 2021). The chemical composition and quality of canola quality *B. napus* seeds can vary due to the type of cultivars and different

agronomic growing conditions; therefore, the efforts in canola breeding are continuous, mainly on improving yield, and the quality and quantity of oil. However, due to the large volume of CM co-produced during the oil extraction there is a demand for its upcycling. When meal is utilized as a feed ingredient for livestock, the quality of canola seed towards the nutritive value of its oil-free portion is very much considered in the canola breeding programs (Rathod and Solanki, 2020; So and Duncan, 2021). The breeding efforts aimed at increasing protein content, decreasing the content of fibre, phenolic acids and phytate, and decreasing glucosinolates further remain major goals in rapeseed breeding (Wittkop et al., 2009; Ton et al., 2020). Because of its small seed size, the seed coat of canola, which accounts for 16% of seed weight, contributes to a significant proportion of the seed (Slominski et al., 2012). The dark coloured seed coat of canola is the major cause of the high fibre and antinutritional factors content of rapeseed meal as black hulls are relatively thick (Wittkop et al., 2009). It was reported that yellow-seeded *B. napus* and *B. juncea* canola contains more protein and less fibre than black-seeded canola due to the lower contribution of the hull fraction to the seed mass and lower fibre content of hulls (Slominski et al., 2012).

Prepress solvent extraction process is the most prevalent technique for industrial canola seed processing (Pirgozliev et al., 2022). The heat-preconditioned flaked canola seeds are expeller pressed to obtain part of the oil, and then the pressed cake is extracted with hexane to obtain the residual oil and meal. Expeller processing is another method that is also popular around the world (Gaber et al., 2018). The preconditioned canola seeds are expeller pressed to recover the oil and the residual canola cake or meal is used. Seeds are pressed once, but also can be pressed twice to recover more oil. The oil removal is not as effective as in the solvent extraction process. Therefore, expeller pressed CM contains higher amount of oil than the conventional solvent extracted CM.

According to Canadian Oilseed Processors Association (2020), expeller pressed canola should contain minimum 10% of oil, while solvent extracted CM should contain more than 2% of oil.

Although the industry has already successfully produced oil and meal from canola seed, the improvement of the oil and meal quality through advanced processing technique is still an ongoing effort (Watts et al., 2020). The technologies that are under investigation include aqueous enzyme extraction, supercritical fluid extraction, dehulling and microwave processing. The last two techniques are normally intervened into traditional processes.

In canola seed, most of the oil is embedded in oil bodies and presented in the kernel of seeds (Niu et al., 2015). In order to extract canola oil as much as possible and to produce high quality oil, a variety of enzymes were studied (Niu et al., 2012). It was reported that canola oil obtained from the enzyme-assisted aqueous extraction process contained higher level of γ, and δ tocopherols compared with traditional solvent extraction (Latif et al., 2008). This method also increased the quality of canola protein.

Supercritical fluid extraction method uses supercritical fluid as solvent to extract oil from canola seeds or cake. Some gases, such as carbon dioxide (CO_2), propane, can exhibit both the solvating characteristics of a liquid and the permeability of a gas. The most popularly used supercritical solvent is CO_2, due to its relatively low critical temperature (30°C), pressure (7 MPa), safety, and reasonable price (Gaber et al., 2018; Watts et al., 2020). However, the cost associated with this processing is still too high for broad application in the canola industry, and it is used exclusively for extracting high-value products.

As hulls of canola seeds contribute most of the fibre, researchers attempted to improve the quality of CM by dehulling (Khajali and Slominski, 2012). Removal of the hulls can be achieved before or at the end of seed processing. Dehulling of seeds before processing needs specific

equipment that can break the seeds and separate the hull from the kernel. Tail-end dehulling of the meal can be achieved by air classification or sieving (Mejicanos et al., 2016). It has been reported that hull removal can increase the apparent total tract digestibility **(ATTD**) and standardized total tract digestibility (**STTD**) of phosphorus of CM in growing pigs (Mejicanos et al., 2018). Microwaving is used as an alternative method for the precondition or cooking of canola seeds. It has been reported that oil obtained from microwave treatment contains more phytosterols and tocopherols, and hence demonstrates better oxidative stability (Niu et al., 2015).

2.2.2 Chemical composition of canola meal

The reported chemical composition of CM is shown in Table 2.1. As an important plant protein source for animals, CM contains more than 40% of crude protein on a DM basis. The AA of canola are well balanced and high in sulfur-containing AA – methionine and cysteine, which are relatively higher than those in other available plant proteins, including legumes. In canola seed, approximately 90% of proteins are storage proteins with cruciferin (11S globulin) and napin (2S albumin) being the most abundant. Structural oil body proteins such as oleosins, caleosins and metabolic proteins are also present (Chmielewska et al., 2020). Canola meal contains antinutritional compounds, including glucosinolates, phytates and phenolic compounds (Bell, 1993; Khajali and Slominski, 2012; Wickramasuriya et al., 2015).

Table 2.1 Reported chemical composition of canola quality meal (g/kg, dry matter basis)

Component	*B. napus*, black[1]	*B. napus*, yellow[1]	*B. juncea*, yellow[1]	Meal from Canadian processing facilities[2]			Solvent extracted CM[3]	Expeller canola meal[3]
				Mean	Min.	Max.		
Crude protein	438	498	474	417	402	437	420	390
Ether extract	18	16	17	35	26	43	32	124
Ash	73	70	72	75	71	80	73.0	79
TDF	301	241	258	379	347	424	382	421
NDF	-	-	-	294	269	339	290	305
NSP	202	187	200	219	207	233	229	-
Simple sugars	2.0	3.0	3.0	2.9	1.7	4.5	17.6	-
Sucrose	88	102	92	61	57	70	63	-
Oligosaccharides	31	25	36	29	24	32	25	-
Phosphorus (**P**)	13.0	12.4	12.3	11.2	10.1	12.2	11.7	10.9
Phytate P	7.8	8.0	7.8	7.4	7.0	7.7	7.3	-
Glucosinolates (μmol/g)	27.1	17.1	17.2	4.6	1.9	9.7	3.6	10.6

[1]Slominski et al., 2012;

[2] Adewole et al., 2016;

[3] Canola Council of Canada, 2019

Historically, glucosinolates have been regarded as the first antinutritional factors for rapeseed. Based on their primary amino acid precursors, glucosinolates can be divided into aliphatic (derived from alanine, leucine, isoleucine, methionine, or valine), aromatic (derived from phenylalanine) and indole (derived from tryptophan) glucosinolates (Halkier and Gershenzon, 2006). The content and compositions of glucosinolates vary among different varieties, origin, plant and harvesting conditions (Tripathi and Mishra, 2007). Gluconapin, progoitrin, and 4-hydroxyglucobrassicin are the major glucosinolates of *B. napus* rapeseed, while sinigrin and gluconapin are the predominant glucosinolates of *B. juncea* (Niu et al., 2015). Glucosinolates are

biologically inert and deemed innocuous when intact, but they can lead to bitter taste of rapeseed, especially sinigrin and progoitrin. Glucosinolates can be degraded by endogenous enzyme (myrosinase), microorganisms, and heat treatment (Khajali and Slominski, 2012). Their degradation products such as isothiocyanates, nitriles, thiocyanates, and oxazolidithione are biologically active and can have detrimental biological effects on animals. They can adversely affect growth performance and thyroid function and cause endocrine disturbance (Tripathi and Mishra, 2007).

Different animal species have different tolerance levels for glucosinolates. Pigs are more sensitive to glucosinolates compared to poultry (0.78 vs 5.4 μmol/g of diet) (Tripathi and Mishra, 2007). A relatively higher tolerance level of 2.0-2.5 μmol/g for swine was also reported (Bell, 1993). Glucosinolates have a more severe effect on laying hens and turkeys than in broilers (Khajali and Slominski, 2012). Ruminants have a tolerance level of around 4.22 μmol/g of dietary glucosinolates (Tripathi and Mishra, 2007). Due to advances in plant breeding, the content of glucosinolates in canola has significantly been decreased to less than 10 μmol/g in most of the commercial CM. When used in animal nutrition, this level would be further diluted once CM is incorporated into the diets (Rogiewicz and Slominski, 2019). Therefore, the presence of glucosinolates is not a major concern related to the utilization of rapeseed/CM by the feed industry in recent years.

Phytate, or phytic acid, is myo-inositol (1,2,3,4,5,6) hexakisphosphoric acid which is naturally present in the form of a salt with minerals, such as calcium, magnesium. Phytate is accumulated during seed maturation and represents 60 - 90% of the total phosphorus (**P**) content of the seed (Kumar et al., 2010). In CM, phytate is present in the form of IP6 (myo-inositol hexakisphosphate) and IP5 (myo-inositol pentakisphosphate) (Chmielewska et al., 2020). Phytate

can have negative effects on animal performance by binding minerals and proteins, thus reducing their absorption (Khajali and Slominski, 2012). Compared to most cereal grains and soybean meal, CM is relatively high in P and phytate P (White and Veneklaas, 2012). Phytate can be hydrolyzed by phytase to release inorganic P and inositol thus improving the utilization of P and growth performance (Kumar et al., 2019).

Canola meal is high in phenolic compounds, mainly polyphenols and condensed tannins, when compared with other plant protein ingredients. It contains up to five times higher phenolic acids than soybean meal (Naczk et al., 1998). Phenolic acids in CM are present in free, esterified and insoluble-bound forms with sinapic acid and sinapine being the predominant ones (Shahidi and Naczk, 1992; Niu et al., 2013). Complex polyphenols with molecular weights between 500 to 3000 Da are classified as condensed or hydrolysable tannins. Rapeseed meal contains around 1% of phenolic acids and up to 6% of tannins. Phenolic compounds tend to decrease palatability of diet because they contribute to bitter, astringent and unpleasant flavor or taste. In addition, phenolic compounds can interact with minerals and proteins and hence decrease their availability (Khajali and Slominski, 2012; Chmielewska et al., 2020).

Canola meal has higher content of TDF compared with soybean meal, and as a result, it is considered as a fibre source in some cases. Traditionally, DF was regarded as an antinutritional factor, but recently, the opinion of DF in CM has been undergoing re-evaluation.

2.3 Nutritive value of canola meal for monogastric animals

Although the potential of CM to be used as a food or functional protein source is undergoing an intensive exploration, it is primarily used in the feed industry.

2.3.1 Nutritive value of canola meal for poultry

The production of poultry is increasing rapidly around the world. Canola meal has been proven to be a good protein source for poultry, mainly due to the favourable content of methionine, which is the first limiting AA for many species of poultry. Diets should be formulated based on the digestible level of AA and metabolizable energy contents to realize the nutritional potential of CM. The recommended inclusion levels of CM are 20% for broilers, turkeys, and laying hens, and 10% for ducks, geese, and quail (Canola Council of Canada, 2019; Rogiewicz and Slominski, 2019). When CM is included at higher levels in poultry diets, it might cause difficulties with a proper balance of energy.

Gorski et al. (2017) investigated how growth performance of broiler chickens was affected when birds were fed starter diets that included 0, 10, 20, 30 or 40% of CM from 2 to 21 days of age, and grower diets containing 0, 10, 20 or 30% of CM from 21 to 37 days of age. The results showed that the inclusion of up to 30% of CM in diets during the grower period (21 to 37d) had no significant effect on growth performance. However, when CM inclusion exceeded 10% in the starter period, it had a significant negative effect on body weight gain (**BWG**) and feed intake (**FI**). High level of CM (above 20%) had a negative effect on the overall growth performance. According to Hanna et al. (2018), inclusion of 25% of conventional CM in the diet had no negative effect on the growth performance of crossbred chicks. However, a recent *Salmonella Typhimurium* challenge study showed that inclusion of 40% of CM in diet did not have any adverse effect on the growth performance of broiler chickens (Yadav et al., 2021).

One recent study investigated the effect of feeding laying hens with diets containing *B. napus* and *B. juncea* extruded-expelled cake and solvent-extracted meal on laying performance, egg quality, and nutrient digestibility. The results demonstrated that including *Brassica* cakes or

meals up to 20% had no adverse effects on the performance or egg quality of hens and increased the monounsaturated fatty acids of eggs (Oryschak et al., 2020).

Due to the high content of DF, CM has a relatively lower energy value for poultry compared with soybean meal and animal protein ingredients. The nitrogen corrected apparent metabolizable energy (**AMEn**) of mash CM, soybean meal, and meat and bone meal determined with Ross 308 broiler chickens were 1881, 2404, and 3535 kcal/kg (7.87, 10.06, and 14.79 MJ/kg) on a DM basis, respectively, while the AMEn of pellet CM, soybean meal, and meat and bone meal were 2017, 2361, and 3401 kcal/kg (8.44, 9.88, and 14.23 MJ/kg) on a DM basis (Khalil et al., 2021). The processing method and cultivar are also the drivers of metabolizable energy content of CM. Pre-press solvent extraction of CM provides less energy than expeller pressed meal/cake because of the lower oil content. Apparent metabolizable energy of solvent extracted meal of *B. napus* and *B. juncea* were 1973 and 2184 kcal/kg (8.26 and 9.14 MJ/kg), respectively, while the extruded-expelled cake from these types were 2730 and 2763 kcal/kg (11.42 and 11.56 MJ/kg) for laying hens (Oryschak et al., 2020). Watts et al. (2021) recently compared the chemical composition and AMEn of 10 single-cultivars and commercial rapeseed meals for broiler chickens and found that processing could be a more critical factor in affecting the AMEn content than the cultivar.

The AMEn value of CM varies among species of poultry (Table 2.2). It was reported that the AMEn of CM, on a DM basis, was 1590 - 2041 kcal/kg (6.65 - 8.54 MJ/kg) for broilers (Adewole et al., 2017b), 2089 - 2278 kcal/kg (8.74 – 9.53 MJ/kg) for turkeys (Kozlowski et al., 2018), 2186 kcal/kg (9.15 MJ/kg) for ducks (Hong et al., 2001), 2065 kcal/kg (8.64 MJ/kg) for guinea fowls, and 2084 kcal/kg for quails (Mandal et al., 2005).

The reported standardized ileal digestibility (**SID**) of AA of CM for poultry is shown in Table 2.2. The average standardized ileal amino acid digestibility (**SIAAD**) of CM ranges between 72.5 to 85.6% (Adewole et al., 2017b; Kozlowski et al., 2018; Agyekum and Woyengo, 2022). Compared to soybean meal, SID of AA of CM is relatively lower. Kim et al. (2012) compared the SIAAD of CM with soybean meal and demonstrated that the average SIAAD of CM was 78.8% while that of soybean meal was 88.0%, with the total SID of AA of CM being lower than those of soybean meal. However, similar values for the SID of methionine, cystine, glutamic acid of soybean meal and CM were also reported (Perttilä et al., 2021).

In addition, the variation of SID of AA CM in broilers is greater than that of soybean meal or fish meal (Kim et al., 2012). The amino acid digestibility of CM is affected by seeds quality and processing conditions (Table 2.2). According to Kozlowski et al. (2018), the coefficients of SID of cystine of black-seeded *B. napus*, and yellow-seeded *B. napus* and *B. juncea* for turkeys at 21 – 28 days of age were 0.71, 0.68, 0.55, respectively. The SID of AA of expeller pressed CM is significantly higher than those of solvent-extracted CM (Agyekum and Woyengo, 2022). The study on the SID of AA of CM from different processing plants and forms (mash and pellet) from same plants showed that SID of AA varies among processing plants and forms of CM (Adewole et al., 2017b).

Table 2.2 Nitrogen corrected apparent metabolizable energy (AMEn) and standardized ileal digestibility (SID) of amino acids of canola quality meal for poultry

Item	Solvent extracted meal[1]	Expeller pressed meal[1]	Canadian source meal[2]	*B. napus*, black[3]	*B. napus*, yellow [3]	*B. juncea*, yellow[3]
Species		Ross 308 broilers			Tukey	
AMEn, kcal/kg DM	1608	1994	1590-2041	2144	2218	2273
Standardized ileal digestibility (SID) of amino acids, %						
Arg	86	92	86-91	86	87	85
His	83	90	53-65	69	66	66
Ile	75	84	75-85	75	70	72
Leu	79	87	81-87	78	76	76
Lys	76	86	77-83	76	73	67
Met	86	91	87-93	79	84	75
Phe	79	87	80-87	80	76	77
Thr	66	77	71-80	73	73	69
Val	70	80	72-82	73	68	71
Trp	89	92	-	-	-	-
Ala	78	87	81-87	78	72	74
Asp	71	86	73-80	80	76	75
Cys	73	82	68-76	71	68	55
Glu	87	92	87-90	84	84	80
Gly	76	85	76-83	77	74	73
Pro	71	80	74-80	74	71	70
Ser	68	78	73-81	77	77	73
Tyr	77	85	85-97	75	75	75

[1] Agyekum and Woyengo, 2022

[2] Adewole et al., 2017b

[3] Kozlowski et al., 2018

2.3.2 Nutritive value of canola meal for swine

Pigs have a highly sensitive sense of smell and taste, thus the flavor of their diets affects FI (Kittawornrat and Zimmerman, 2011; Canola Council of Canada, 2019). Due to the presence of glucosinolates, phenolic compounds and fibre, CM can negatively affect the palatability of the diet. However, due to the generally low level of glucosinolates in CM, it does not cause major concerns for the application of CM in swine diets anymore. Intensive research has been conducted to develop nutritional strategies towards increasing CM inclusion levels in swine diets and to investigate the effects of CM on the growth performance and gut health of pigs.

King et al. (2001) evaluated the effect of diets when replacing commonly used protein sources with solvent-extracted CM on the growth performance of weaning pigs, grower/finisher pigs, and lactating sows. The BWG and FI of weaner pigs (20 to 42d) were not significantly affected, while feed efficiency was improved for diets containing up to 25% of CM. For grower/finisher pigs (63 to 140 d), the replacement of CM had no significant effect on growth performance, carcass quality and weight of the thyroid gland. Diets with up to 20% of CM had positive effects on feed intake of sows. In another study, Velayudhan and Nyachoti (2017) investigated the effect of CM inclusion in sow diets and concluded that inclusion of up to 30% of CM had no negative effects on the performance of sows and suckling piglets.

Heyer et al. (2021) fed weaned pigs with 20% of solvent-extracted CM and extruded CM and found that the inclusion of CM had no significant effect on growth performance. Hong et al. (2020) fed nursery pigs (weaned at 21 days of age) with corn/soybean meal-based diets containing 0, 10, 20, 30, or 40% of conventional CM (from day 0 to day 42) in three phases to investigate the effect of diets on growth performance and gut health. Diets were formulated to provide the same digestible AA, net energy, and phosphorus contents. Results demonstrated that there was an

increase in BWG, FI and gain to feed ratio, improvement in gut microbial composition, and reduction in immune response as the CM inclusion rate increased up to 20%. With CM inclusion over 20%, growth performance of pigs decreased to a similar level as that of 0% CM diet. Hong et al. (2021) also found that inclusion of 20% CM increased average daily gain (**ADG**) and FI of weaned piglets and had the potential to reduce white blood cell counts of pigs orally challenged with *Escherichia coli*. Another study on nursey pigs showed that partial replacement of soybean meal with 20% of CM in either corn- or wheat- based diet had no significant effect on growth performance of piglets, but significantly reduced digestibility of energy and protein (Mejicanos et al., 2017). It could be concluded that inclusion of CM up to 20% will have no negative effect on growth performance of pigs. Provided that diets are carefully formulated based on digestible amino acid and net energy contents, even 40% of CM could be included in diets for piglets.

The precise diet formulation for swine requires accurate values of net energy (**NE**) of feed ingredients. In this context, the measurement of NE requires an advanced research technique that is laborious and costly; thus, prediction formulas are commonly used. According to Woyengo and Zijlstra (2021), the predicted NE value of CM did not differ from that of the determined NE value, while Heo et al. (2014) reported that NE value of *B. napus* yellow and *B. juncea* yellow meals measured using the direct determination technique through the indirect calorimetry chamber was about 5.9% higher than the value predicted based on chemical composition. The NE of CM varies from 1720 to 2457 kcal/kg (7.20 - 10.28 MJ/kg) among different sources (Table 2.3), which might reflect the difference in canola seed processing conditions. According to Heyer et al. (2021), extrusion had no significant effect on digestible energy and NE of CM for weaned pigs.

Table 2.3 Net energy of canola quality meal for swine

NE	*B. napus*				*B. juncea*
	CM[1]	CM[2]	CM[3]	CM, expelled[3]	CM[1]
kcal/kg DM	1720	2156 - 2457	1890	2351	2140

[1] Woyengo and Zijlstra, 2021

[2] Kim et al., 2018

[3] National Research Council, 2012

The reported SID of AA of CM for pigs are shown in Table 2.4. The average SID of AA of Canadian CM obtained from different processing plants and in different forms (mash vs pellet) for pigs ranges between 74.7 to 80.7% (Adewole et al., 2017a). Heyer et al. (2021) demonstrated a range of SID of AA between 83.2 to 87.9%. The coefficients of SID of methionine and cysteine differ among different processing plants (Adewole et al., 2017a). Extrusion of canola seeds might increase SID of AA, but it depends on the processing conditions (Heyer et al., 2021).

Table 2.4 Standardized ileal digestibility (SID) of amino acids of canola quality meal for growing pigs (%)

	Rapeseed expeller meal[1]	Rapeseed meal[1]	Canadian CM[2]	Expeller-pressed CM[3]	*B. napus* CM[4]	Extruded CM[4]
Indispensable amino acids						
Arg	87.3	85.3	84.7-89.0	83.1	86.9	92.2
His	81.1	81.4	58.7-70.3	81.7	85.9	88.9
Ile	79. 7	77.0	76.1-83.9	74.3	80.3	84.0
Leu	79.9	74.5	79.1-83.3	78.8	82.6	86.7
Lys	72.6	73.0	74.6-80.0	73.2	83.1	85.8
Met	86.3	83.5	81.2-89.5	83.9	88.2	92.7
Phe	82. 8	76.3	79.4-84.8	78.0	82.9	86.9
Thr	70.1	70.1	69.0-76.8	67.6	79.2	81.8
Val	75.1	73.5	73.5-81.9	70.5	77.7	82.8
Trp	76.8	75.6	-	83.9	86.4	89.4
Dispensable amino acids						
Ala	75.3	75.3	76.2-82.0	72.1	82.4	85.8
Asp	73.5	69.4	69.8-77.3	72.0	78.5	84.9
Cys	75.2	73.1	59.2-81.9	72.7	78.5	79.4
Glu	84.6	94.0	84.1-88.5	84.3	87.0	90.7
Gly	79.8	80.4	72.7-82.0	63.6	79.0	82.2
Pro	-	-	76.9-87.9	57.2	76.7	79.1
Ser	69.6	71.0	71.2-78.9	70.6	81.7	84.5
Tyr	76.1	75.8	74.2-81.3	75.1	84.5	87.8

[1] P. Li et al., 2020

[2] Adewole et al., 2017a

[3] Seneviratne et al., 2010

[4] Heyer et al., 2021

2.4 Dietary fibre

The definition of DF changes over time and differs among different organizations and countries (Trowell, 1988; Macagnan et al., 2016). In general, DF refers to edible part of plants or analogous carbohydrates that cannot be digested by digestive enzymes in animals (Anderson et al., 2009; Macagnan et al., 2016; Jha et al., 2019).

2.4.1 Definition of dietary fibre

The term "dietary fibre" was initially introduced by Hipsley in 1953 to define the non-digestible constituents of plants that make up the plant cell wall, which consist of cellulose, hemicellulose and lignin. In 1976, Trowell et al. (1988) broadened the definition to "The remnants of plant cells resistant to hydrolysis by the alimentary enzymes of man". It contains "cellulose, hemicellulose, gums, lignins, unavailable nitrogen, trace elements associated with fibre zinc and chromium".

The most acceptable and recently referenced is the Codex definition which was adopted in June 2009 by the Codex Alimentarius Commission (Beta and Camire, 2019; McCleary, 2019). This definition includes three categories of carbohydrate polymers "that are not hydrolyzed by the endogenous enzymes in the small intestine of humans": edible carbohydrate polymers, carbohydrate polymers modified from food raw material, and synthetic carbohydrate polymers which have been shown to have a physiological effect of benefit to health. It also includes lignin and other compounds intimately associated with plant polysaccharides, such as waxes, protein fractions, phytates, etc. (Beta and Camire, 2019). It is worth noting that the first category of the Codex definition is similar to the definition proposed by Trowell in 1976.

2.4.2 Analysis of dietary fibre

The official and classic method for DF analysis was the enzymatic-gravimetric method of AOAC International (AOAC Method 985.29). This method measures the alcoholic insoluble fibre, also known as high-molecular-weight DF, after removing starch and protein by enzymatic treatments. Later, AOAC 991.43 method was adapted for analyzing the soluble and insoluble fibre (Beta & Camire, 2019). However, these two methods are inadequate to determine oligosaccharides and protein compounds that are associated with plant polysaccharides.

In 2007, McCleary (2007) published a new method, approved by AOAC (2009.01), which determines high-molecular-weight DF by enzymatic-gravimetric method, and low-molecular-weight DF by high-performance liquid chromatography (**HPLC**). Then, McCleary et al. (2012) developed 2009.01 method into the 2011.25 method, which distinguishes between soluble and insoluble high-molecular-weight DF. Recently, the method was updated again by decreasing the time for incubation with pancreatic a-amylase and amyloglucosidase from 16 to 4 h to better mimic human digestive transit time, using glycerol as an internal standard for HPLC (McCleary, 2019). However, the protein fractions associated with plant polysaccharides are not included. To address this, Slominski et al. (1994) proposed a method that measures the NSP, lignin with associated polyphenols, minerals and fibre-bound protein (glycoproteins). In this method, NSP are measured in both the sample and the residue collected following the neutral detergent fibre (**NDF**) analysis. It allows for the correction of losses of neutral detergent soluble NSP. In addition, the procedure includes the measurement of protein present in the NDF residue to quantify the protein-containing fibre fraction referred to as glycoprotein or neutral detergent insoluble crude protein. This fraction can be composed of cell wall protein and advanced glycation end products of Maillard reaction between AA and reducing sugars. Thus, this method allows to calculate the DF content of feed

ingredients by including NSP content, cell wall-inserted protein, minerals, lignin with associated polyphenols, and Maillard reaction products. In addition, the measurement of NSP by GLC quantifies each component sugar, thus provides valuable qualitative information as to the type of polysaccharides present in the sample. Apart from a holistic determination of DF, it can be separated into water-soluble polysaccharides, water-insoluble polysaccharides, and lignin.

2.4.3 Nutritive value of dietary fibre

Dietary fibre present in feeds is composed of lignin, a range of polysaccharides other than starch, and other substances which are not digestible by monogastric animals, i.e., resistant starch, advanced glycation end products of Maillard reaction, gums, mucilage, waxes, polyphenols other than lignin, cell wall proteins, galacto- and fructo-oligosaccharides, and minerals. Lignin, as a structural component of plant cell walls, is composed of polyphenolic residues and negatively influence the biodegradability of plant-derived feeds (Slominski et al., 1994; Beta and Camire, 2019).

Dietary fibre was historically recognized as an antinutritional factor in monogastric animal nutrition, but recently it has attracted attention because of its potential beneficial effects (Anderson et al., 2009; Patel and Goyal, 2012; Slominski, 2017; Zhao et al., 2020). This is mostly driven by the end of the era of using antibiotics for accelerating the growth of healthy animals, and by the needs for alternative bioactive components that would support growth, intestinal health and immunity of animals, and would support the food safety by preventing entering infections while also mitigating pathogen shedding. Advancement in research techniques and knowledge of the gut microbiome and gut health supports greater understanding of bioactivity of DF components and their functional properties. Such progress allows for research on the dietary strategies towards manipulations of the fermentation processes in the hindgut using the raw materials, bioactive

components, or enzymes to improve colonization of beneficial bacteria and exclusion of enteric pathogens. Dietary fibre is highly resistant to endogenous enzymes in the small intestine. However, as a main substrate for bacterial fermentation it may exert the prebiotic properties due to its positive effects on microbiota composition and proliferation, production of short chain fatty acids (**SCFA**) and the control of enteric bacterial pathogens in the large intestine (Jha et al., 2019).

2.4.3.1 Non-starch polysaccharides

Among the many DF fractions present in feedstuffs, NSP have been identified as the main components of fibre that impair the digestibility of nutrients; however, NSP derivatives have been appreciated for their prebiotic-type properties beneficial for monogastric animals (Slominski and Campbell, 1990; Kiarie et al., 2010; Sasaki and Kohyama, 2012). Fibre-rich feed ingredients would contain significant amounts of NSP, such as rhamnogalacturonans, galactomannans, mannans, arabinoxylans, β-glucans, arabinans, arabinogalactans, pectic polysaccharides and others. They can be divided into cellulose and non-cellulosic polysaccharides which often referred to as hemicellulose, pectins, mucilage and gums. In relation to the NSP properties that are important in animal nutrition, they can also be divided into water-soluble (β-glucan, arabinoxylans) and water-insoluble (cellulose, xylans, xyloglucans, galactomannans, pectins, arabinogalactans) polysaccharides (Slominski et al., 1994; Rodica et al., 2010).

The NSP content and profile of component sugars vary among the feed ingredients, and various types of polysaccharides may be rationalized from the component sugar profile (Meng et al., 2005; Knudsen, 2014). In cereal grains arabinoxylans would predominate although significant amounts of β-glucans and cellulose are also present. The relatively high concentration of uronic acids along with glucose residues indicates that pectic-type substances and cellulose are the major cell wall constituents of soybean and canola. Arabinose and galactose not associated with pectic

substances would derive from arabinan and arabinogalactan, while xylose indicates the presence of xylan and xyloglucan.

In cereal grain-based diets, β-glucan and arabinoxylan cause the main concerns in monogastric animals nutrition. Barley is high in β-glucans (4.3%, DM) with more than 80% being water-soluble (3.6%, DM). Compared with other cereals, rye and wheat contain relatively high amounts of arabinoxylans (8.1% and 8.9%). More water-soluble arabinoxylans are present in rye (3.4%) than wheat (1.8%). While in corn, an important energy source, soluble arabinoxylan accounts for only 0.1% of the total arabinoxylan content of 5.2% (Choct, 1997). Water-soluble NSP are also present in plant-based protein supplements. Soybean meal contains about 21% of NSP (DM basis) with pectic polysaccharides predominating and only 6.4% being water-soluble (Knudsen, 2014).

Water-soluble NSP can increase the viscosity of digesta, intervening or slowing down the mixing of digestive enzymes and nutrients and thereby inhibiting digestion and absorption. In addition, the water-soluble NSP would highly likely increase endogenous secretion of water, proteins, electrolytes and lipids, and enlarge the organ of gastrointestine (Englyst et al., 1983; Choct et al., 2010; Sinha et al., 2011). The water-insoluble NSP are often inert in the digestive tract function and have little effect on nutrient absorption (Choct, 1997). However, they contribute the bulk of the diets, fast the passage rate of digesta, have the nutrient encapsulating effects, and dilute the dietary energy. Due to the aforementioned properties, NSP historically were recognized as antinutritional factors or fractions repersenting very little nutritive value. However, recent research showed that some NSP or products of their hydroylsis exert the prebiotic properties and may have important effects on gut health by stimulating proliferation of beneficial bacteria, or attracting pathogenic bacteria away from the

intestinal binding sites, thus preventing any potential disturbance in secretion, digestion, and nutrient absorbtion processes by mucosa.

2.4.3.2 Nutritional value of water-soluble dietary fibre

Water-soluble DF of feed ingredients, which mainly include β-glucans and arabinoxylan of cereals grains, or gums/mucilage of flax, might express a high water-holding capacity, thus increasing digesta viscosity in the gut and having a negative effect on nutrient utilization. The high viscosity of soluble DF inhibits the digestion and absorption of nutrients and decreases feed intake due to increased satiety (Agyekum and Nyachoti, 2017). Not all soluble DF components are viscous. Water-soluble DF components, particularly NSP are the substrates that can be fermented by the bacteria in the hindgut of monogastric animals, hence providing some energy. Therefore, the use of the exogenous carbohydrases may facilitate the hydrolysis and fermentation of long-chain polysaccharides. Microbial fermentation of DF produces SCFA, branched chain fatty acids (**BCFA**), and various gases like carbon dioxide, and methane. Additionally, DF may induce the growth and activity of beneficial microorganisms such as *Bifidobacterium* and *Lactobacillus*. The SCFA produced by gut microbiota can then reduce the intestinal pH and serve as energy sources for epithelial cells proliferation. In addition, SCFA are water-soluble and once absorbed into the blood stream boost the activation of B cells and production of immunoglobulin G and A. Some bacterial strains produce antimicrobial factors or stimulate the immune system by signaling dendritic cells (Pourabedin and Zhao, 2015).

2.4.3.3 Nutritional value of water-insoluble dietary fibre

Water-insoluble DF components of feed ingredients include cellulose, hemicellulose, part of pectin, and lignin with associated polyphenols or tannins and would primarily be considered as diluents of nutrients. They are fermented slowly, and only to some extent (more so in pigs than

poultry), in the hindgut of monogastric animals. In pigs high inclusion of water-insoluble DF generally reduces growth performance, increases digesta passage in the gastrointestinal tract (**GIT**), reduces the exposure time of feed to digestive enzymes, increases colon motility, and decreases energy density, nutrients digestibility and transit time of digesta (Agyekum and Nyachoti, 2017). The decreased digestibility of nitrogen due to presence of water-insoluble DF may lead to mechanical erosion of the gut epithelium, and thus lead to more endogenous nitrogen and amino acid losses. However, when included in the low amounts may prevent the prolonged time of digesta in GIT thus reducing the overgrowth of bacteria in the small intestine.

Certain types of water-insoluble DF present at proper level will not negatively affect the nutrient digestibility but might increase the feed intake and, therefore, improve growth performance. Gutierrez et al. (2013) reported that the addition of up to 40% insoluble low-fermentable fibre from corn to corn/soybean meal-based diet had no negative effect on growth performance of growing pigs. Water-insoluble DF express similar properties in poultry. When water-insoluble DF is used in moderate amounts, this may improve growth performance, nutrient digestibility and gizzard development and function, increase gizzard weight and size, and gastrointestinal tract health (Jiménez-Moreno et al., 2016). Many studies demonstrated that water-insoluble DF increases gizzard weight and size that is highly likely due to the fact that insoluble DF tends to stimulate the development of muscular layers of gizzard. In addition, insoluble DF increases the secretion of endogenous enzymes and the intensity of the intestinal refluxes (Svihus, 2011).

The results of research about the effect of DF on growth, development, and physiological processes of monogastric animals is presented in Table 2.5.

Table 2.5 The effect of dietary fibre on growth, development and physiological processes of monogastric animals

Subjects	Dietary fibre	Main outcome	Reference
Female broilers	oat hulls, rice hulls,	Insoluble DF improved ADG and feed conversion ratio (**FCR**).	Jiménez-Moreno et al., 2016
Female broilers	sunflower hulls (**SFH**)	Moderate amounts of insoluble DF (2.5%) improved gizzard development and increased total tract apparent retention of DM, organic matter, N, soluble ash, and ether extract.	Jiménez-Moreno et al., 2019
Pullets	cereal straw or sugar beet pulp (**SBP**)	Inclusion of 2% of straw benefited the development of the GIT without adverse effect on growth performance. SBP had negative effect on growth performance.	Guzmáan et al., 2015
Pullets	cereal straw, SBP, or SFH	Inclusion of 2% fibre improved ADG, ADFI, and energy efficiency. SFH tend to be more beneficial than straw or SBP.	Guzman et al., 2015
Turkeys infected with *Salmonella*	wood shavings	Wood shavings improved FCR of turkeys by 8%. It was also beneficial for gizzard and intestinal development.	Santos et al., 2006
Barrows	wheat bran	Increasing the inclusion of fibre affected the digestibility of protein, ether extract and energy.	Wilfart et al., 2007
Pigs	wheat bran, rapeseed meal, and soya hulls	High inclusion of fibre negatively impacted ADG, FCR and digestibility of organic matter, energy and N. It also influenced microbiota composition.	Le Sciellour et al., 2018
Gilts	Soluble DF	Supplementation with SDF shortened the puberty time and increased the following reproductive performance.	Zhuo et al., 2017

2.4.4 Dietary fibre of canola meal

2.4.4.1 Dietary fibre of canola meal

Canola seed is relatively small compared with other oil seeds, and the seed coat accounts for about 10-20% of the whole seed and about 30% of CM (Naczk et al., 1998; Jia et al., 2012; Carre et al., 2016; Asad et al., 2017). The seed coat consists of outer and inner layers. A typical seed coat structure of the *Brassicaceae* consists of four distinct layers, epidermal layer, palisade layer, pigment layer and aleurone layer (Bellani et al., 2002; Moise et al., 2005) with the epidermal and palisade layers consisting of the outer integument and the pigment and aleurone layers form the inner integument. The epidermal layer contains mucilage, pectic polysaccharides (Jiang and Deyholos, 2010). Below it is the palisade layer which is typified by tangential and radial walls. The third layer, inner integument, is also known as pigment layer, contains polyphenols. The aleurone layer is derived from endosperm and contains lipid droplets, proteins, and polysaccharides. Therefore, canola hulls are rich in pectin, cellulose, hemicellulose, lignin, and polyphenols (Rommi et al., 2014). Hulls include 95% of the lignin, 80% of the acid detergent fibre (**ADF**) and 73% of the NDF of the entire seed (Carre et al., 2016).

In the black-seeded *B. napus* fractionation studied by Slominski et al. (2012), the hull fraction accounted for around 16% of the total seed mass, and it contributed 51% of DF to the total seed fibre. Fibre components accounted for 57% of the hull fraction while 10% in the seed embryo fraction. Dietary fibre of hull and embryo is composed of NSP (55 and 59%, respectively), lignin with associated polyphenols and minerals (37 and 19%, respectively) and glycoprotein (8 and 22%, respectively). Similarly, in CM the NSP are the predominant fibre components (approximately 67%) followed by lignin with associated polyphenols and minerals (approximately 26%) and

glycoprotein (approximately 7%). In addition, the composition of fibre in black-seeded *B. napus* is different from that of yellow-seeded *B. napus* and *B. juncea* (Slominski et al., 2012).

Carbohydrates (48% of the meal) of the oil-free, dehulled rapeseed meal contain bound lipids, 80% alcohol soluble fraction including low-molecular-weight polysaccharides, and 80% alcohol insoluble fractions. The 80% alcohol insoluble polysaccharides (29% of the oil-free dehulled meal) contain 14.5% of pectin, 7% of cellulosic residue, 2% of arabinan, and 1% of arabinogalactan (Siddiqui and Wood, 1977).

Although dehulling would appear to be beneficial for the quality of CM, it is not popular in the industry due to the small seed size, difficulty with hull removal, loses of oil, and high cost of processing. Therefore, DF content of CM is relatively higher than that of soybean meal, the latter one representing the meal after front-end seed dehulling.

In canola processing and animal nutrition fields, researchers and industry typically use NDF, ADF or even crude fibre to characterize the fibre content. The holistic determination of TDF and its components is more appropriate as it reflects the structure of plant cell wall and effects of heat treatment during seed processing. The variation in TDF content of CM exists and it depends on the breed, seed color, hull thickness, processing parameters, etc. (Table 2.6). Yellow-seeded CM derived from *B. napus* or *B. juncea* contains less lignin and polyphenols which contribute the lower level of TDF compared with the meal from black-seeded canola (Slominski et al., 2012).

Table 2.6 Composition of dietary fibre of canola quality meal (g/kg, dry matter basis)

Type of samples	NSP	NDICP[1]	Ash	L & P[2]	TDF	Reference
Brown-seeded meal	178	35	10	80	302	Slominski et al., 1994
Yellow-seeded meal	215	22	4	32	273	Slominski et al., 1994
B. napus, black-seeded meal	202	21	7	71	301	Slominski et al., 2012
B. napus, yellow-seeded meal	187	15	3	37	241	Slominski et al., 2012
B. juncea, yellow-seeded meal	200	17	3	39	258	Slominski et al., 2012
Commercial CM from 2011-2014	209-233	45-60	-	93-116	374-411	Adewole et al., 2016
Commercial CM from eleven plants	207-228	41-75	-	97-121	347-424	Adewole et al., 2016

Note: [1] NDICP refers to neutral detergent insoluble crude protein

[2] L & P refers to lignin and polyphenols

2.4.4.2 Non-starch polysaccharides of canola meal

Non-starch polysaccharides are the predominant constituents of TDF, and account for 53-80% of TDF of CM. On a DM basis, CM contains 18-23% of NSP (Slominski et al., 2012; Adewole et al., 2017b; Radfar et al., 2017), of which only around 10% are water-soluble (Slominski and Campbell, 1990; Khajali and Slominski, 2012; Lannuzel et al., 2022).

In CM, cellulose, arabinans, arabinogalactans, xyloglucans, galactans, and pectic polysaccharides predominate. The latter fraction has been considered to include diverse types of polysaccharide structures based on linked units of rhamnose, galacturonic acid, galactose, and

arabinose (i.e., rhamnogalacturonans, galacturonans, arabinans). It should be noted that the structural features of polysaccharides can be oversimplified by omitting interpolymeric linkages within the cell wall. It is known that linear 5-linked arabinans are most prevalent in the side chains of rhamnogalacturonan I, although they can also be found as part of arabinogalactan glycoproteins. As well, considerable amounts of arabinogalactans I and II are also present in the cell wall as side chain structures of rhamnogalacturonan I or attached to arabinogalactan proteins. There is also strong evidence that both xyloglucan and heteroxylan are intensively crosslinked to the cell wall matrix (Low et al., 2020).

The digestibility of NSP varies significantly depending on the type of cultivars, inclusion level of CM, and the age and species of monogastric animals (Table 2.7). The total tract NSP digestibility varies from 2.1 to 8.6% (Slominski and Campbell, 1990; Slominski et al., 1994; Kocher et al., 2000; Meng et al., 2005; Meng and Slominski, 2005) in broilers or laying hens. When uronic acids residue is not included in NSP calculation, the ileal NSP digestibility could be as high as 16.3% for insoluble NSP (Kocher et al., 2000). Pigs can digest and utilize NSP better than poultry because of the well-developed large intestine. The ceca of poultry are short and not all the chyme will pass through them. The total tract NSP digestibility for weaned pigs could be as high as 49% while ileal NSP digestibility would be 10% (Omogbenigun et al., 2004).

Table 2.7 Digestibility of non-starch polysaccharides (NSP) of canola meal (CM)

Type of samples	Inclusion ratio, %	Animal	NSP digestibility, %	Reference
Commercial CM	40	Pullets	2.6[a]	Slominski and Campbell, 1990
		Caecectomised hens	2.1[a]	
Brown-seeded CM	46.4	Laying hens	3.4[a]	Slominski et al., 1994
Yellow-seeded CM	37.8	Laying hens	8.6[a]	
Solvent extracted CM	7	Broilers	6.3[a]	Meng et al., 2005
Solvent extracted CM	30	Broilers	7.6[a]	Meng and Slominski, 2005
Solvent extracted CM	35	Broilers	[1]Insoluble 16.3[b], soluble 9.3[b]	Kocher et al., 2000
Solvent extracted CM	6.11	Weaned pigs	10.1[b]/48.9[a]	Omogbenigun et al., 2004

[a]Apparent total tract NSP digestibility

[b]Apparent ileal NSP digestibility

[1] exclude uronic acids.

2.5 Means to improve the nutritive value of canola meal

2.5.1 Application of exogenous enzymes for canola meal-based diets

The application of exogenous enzymes is a practical approach for improving the nutritive value of feedstuffs (Meng et al., 2005; Slominski, 2010; Rad-Spice et al., 2018). It can be achieved by addition of carbohydrases, phytase and other enzymes to animal diets. Phytase is widely used to release P from phytate, thus increasing P utilization and reducing pollution caused by over usage of P (Slominski, 2011; Dailin et al., 2018). Carbohydrases can target DF components, including cell wall NSP. Although the mechanism towards the benefits of carbohydrase supplementation is

quite understood, it still remains an interesting subject for further investigations (Slominski, 2010; Aftab and Bedford, 2018). Their benefits may be achieved by facilitating the hydrolysis and depolymerization of NSP, thus increasing their solubilization and allowing for a more effective lower gut fermentation. This, in turn, would improve energy utilization and the release of nutrients encapsulated by the cell wall structure, therefore improving their digestibility. In addition, enzymatic hydrolysis of NSP would release the prebiotic-type components, including low-molecular-weight polysaccharides, oligosaccharides, disaccharides, and simple sugars, which may facilitate the proliferation of beneficial bacteria for gut health, such as *Bifidobacterium* and *Lactobacillus*. Consequently, these enzyme hydrolysis products may indirectly prohibit the growth of certain pathogenic species by increasing intestinal lumen acidity through an increase in SCFA production in the gut. In addition, certain enzyme hydrolysis products may attract microbes away from the intestinal binding sites by a means of competitive exclusion, thereby reducing colonization and disease and allowing the mucosa to perform its function of secretion, digestion and nutrient absorption and be beneficial for the overall health of animals (Slominski, 2010; 2017; Aftab and Bedford, 2018; Helm et al., 2020).

A variety of enzymes are available for the feed industry and the choice of enzymes depends on the type of dietary substrates (Slominski, 2010; 2011; 2017). As diets are mixtures of ingredients, and the cell wall structure of these ingredients is complex, choosing the right enzymes is not easy. Targeting a number of linkages within the cell wall structure would require many activities acting in concert, as opposed to a single enzyme activity, the application of which would result in minimal NSP depolymerization, and most likely could be limited to the surface of the cell walls. Therefore, only the most appropriate enzyme combinations, containing a variety of activities

that can target the 'real' substrate, would be required for the effective NSP depolymerization (Niu et al., 2022).

In earlier studies, carbohydrases improved BWG, FCR, AMEn, and ATTD of nutrients in poultry fed CM-based diets. The supplementation of multi-carbohydrases increased metabolizable energy of CM from 1,943 to 2,249 kcal/kg for broilers, from 2,088 to 2,196 kcal/kg for turkeys, and from 2,792 to 2,976 kcal/kg for growing pigs (Jia et al., 2012; Kozlowski et al., 2018; P. Li et al., 2020). Multi-carbohydrase addition improved the energy value of CM and canola quality *B. juncea* meal and increased BWG and FCR (Jia et al., 2012; Radfar et al., 2017). Improvements in apparent ileal digestibility (**AID**) of minerals, protein and AA in broiler chickens were also reported by Moss et al. (2018). According to Li et al.(2020), multi-enzyme supplementation (including carbohydrase and protease activities) of diets containing solvent-extracted or expeller-pressed double low rapeseed meal increased the metabolizable energy value, ATTD of ether extract, NDF, and ADF of diets, SID of crude protein and all AA except phenylalanine, and metabolizable energy of ingredients. Pirgozliev (2022) reported that xylanase supplementation enhanced feed efficiency but did not improve FI of diets containing 200 g/kg of rapeseed meal. However, in some studies, enzyme supplementation had no significant effect on growth performance and nutrient utilization in poultry. The overview of the growth performance and nutrient utilization in poultry and swine fed CM diets supplemented with exogenous carbohydrases is presented in Table 2.8. The lack of improvement in growth performance parameters of animals fed diets containing CM can be attributed to the fact that the enzymes were ineffective in targeting the fibre components of CM because they were not substrate-specific, thus less effective. It could also be assumed that even if products of enzymatic hydrolysis of NSP were released when fermented in the hindgut, they would only facilitate the SCFA production. This, however, might

not be a sufficient contribution to the metabolizable energy production and improvements in growth performance.

Moss et al. (2018) reported that CM diets supplemented with xylanase had no significant effect on growth performance and AID of macro-minerals, protein and AA, while when fortified with phytase, an improvement in AID of minerals, protein and AA was observed. Mandal et al. (2005) investigated the effect of enzyme supplementation on AMEn of test ingredients for chickens, guinea fowl and quail and found that supplementation of diets with enzyme preparations containing amylase and polysaccharidase activities had no significant effect on AMEn of rapeseed meal but increased AMEn of sunflower meal. This phenomenon may be due to the complexity and specificity of the NSP of CM.

Table 2.8 Growth performance and nutrient utilization in poultry and swine fed canola meal diets supplemented with exogenous carbohydrases

Animal	Enzyme	Growth performance	Other outcomes	Reference
Broilers	Multi-carbohydrase	Increased BWG and gain to feed (**G: F**) ratio	Increased AMEn	Radfar et al., 2017
Broilers	Multi-carbohydrase	Increased BWG and G: F	Increased AMEn, AID of starch and protein, and ATTD of NSP	Meng et al., 2005
Broilers	Multi-carbohydrase	No significant effect	No significant effect on NSP digestibility	Kocher et al., 2000
Broilers	Xylanase	No significant effect	No significant effect on AID of maco-minerals, protein and amino acids.	Moss et al., 2018
Broilers	Xylanase +phytase	No significant effect	Significantly increased AID of Ca, P, Zn, Na, protein and amino acids.	Moss et al., 2018
Turkeys	Multi-carbohydrase	Increased G: F	Increased SCFA production	Kozlowski et al., 2018
Sows	Multi-carbohydrase +phytase	Reduced the sow body weight loss	Improved P digestibility	Velayudhan et al., 2018
Guinea fowl, quail	Multi-carbohydrase	-	No significant effect on AMEn	Mandal et al., 2005

The effect of carbohydrases on gut function and health has recently been attracting the attention of both scientific community and feed industry (Zduńczyk et al., 2013; 2020). It has been suggested that the prebiotic-type products of enzymatic hydrolysis of NSP may improve hindgut fermentation and stimulate gut development and health of animals. In their studies, enzyme supplementation influenced the cecal bacterial enzymes by increasing activities of α-glucosidase, α-galactosidase, and β-galactosidase and decreasing the activity of β-glucuronidase in the gut of turkeys fed CM diet (Zduńczyk et al., 2013). Carbohydrase supplementation of poultry diets has also been reported to increase the abundance of *Bifidobacterium spp.* and total SCFA content in cecal under certain circumstances. Xylanase addition increased the abundance of *Bifidobacterium* in ceca and the cecal digesta concentration of butyric acid when birds were fed the wheat-based diets for 35 d (Morgan et al., 2022) or when birds were at 42 days of age instead of 11 or 21 days of age (Lee et al., 2017). In some studies, carbohydrase supplementation had no significant effect on selected beneficial bacteria and total SCFA contents in cecal and ileal digesta (Zduńczyk et al., 2013; González-Ortiz et al., 2019; Craig et al., 2020). Karunaratne et al. (2022) reported that the supplementation of β-glucanase led to a decrease in ileal SCFA concentration in laying hens fed a 73% hulless barley based diet, while it had no effect on the ileal SCFA concentration in laying hens fed a wheat based diet.

2.5.2 Enzymatic pre-treatment of feed ingredients

The enzyme technology has been widely used for improving of the nutritive value of feed ingredients and their diets. Due to the relatively short feed transit time and unfavorable pH in poultry GIT as digestion process progresses, enzymes cannot fully express their potential. The technology based on 24 - 48 hours liquid incubation of feed ingredients with multiple enzyme activities can be utilized to release fibre encapsulated nutrients, including protein and fat, thereby

improving their digestibility. Derivatives from enzymatic hydrolysis of NSP might also exert some prebiotic properties. In this type of target-specific processing, the conditions such as time of incubation, temperature, pH, range of activities and concentration of enzyme preparations used can be optimized to ensure efficient outcomes. This type of processing could be advantageous in improving the value of fibre-rich plant protein sources which are difficult to digest by young animals such as growing poultry or weaned pigs.

It was demonstrated that enzymatic treatment using protease and carbohydrase can increase the extractability of dry matter, protein and carbohydrates (Fischer et al., 2001). In another study, enzyme broths from *Aspergillus niger*, *Aspergillus aculeatus* and *Trichoderma reesei* fermentations containing cellulase, xylanase, pectinase, and galactosidase activities was used to investigate their effects on soy flour carbohydrates. The results showed that xylanase and pectinase strongly influenced the carbohydrate conversion with the significant release of soluble carbohydrates (Loman and Ju, 2016). It has also been reported that enzyme treatment can mitigate the antinutritional effects and improve growth performance (Li et al., 2020)

Research has also focused on degrading polysaccharides into more accessible source for microbial fermentation derivatives. Research showed that citric acid (10 g/L) pre-treatment and cellulase supplementation (10 FPU/g) were able to increase the release of monosaccharides of soybean meal (Islam et al., 2018). Dumitru et al. (2018) evaluated the efficiency of producing fermentable sugars from feed materials and found that field peas can produce more available sugars through enzymatic hydrolysis than corn and field beans.

During liquid enzymatic incubation of feed ingredients, the spontaneous fermentation can occur, and can further improve their nutritive value. In one study, xylanase in concert with the enzyme preparation obtained from *Aspergillus aculeatus* were evaluated with the objective of

decreasing the NSP contents of feed ingredients, including wheat, barley, wheat bran and soybean meal. It was observed that during the process, spontaneous fermentation occurred. Enzymatic treatment increased NSP solubility, decreased total NSP and reduced the solution viscosity (Christensen et al., 2007). In another study, nine enzyme or enzyme combinations were used in the liquid incubation of rapeseed cake. Only one enzyme blend containing β-glucanase, xylanase, and pectinase activities significantly decreased the total and insoluble NSP contents. Carbohydrate derivatives released from rapeseed cake were fermented and lactic acid and acetic acid were produced during the process (Jakobsen et al., 2015). Phytase enzyme has also been used to improve the nutritional value of rapeseed meal. The result showed that the phytase treatment along with spontaneous fermentation slightly increased crude protein and crude fibre, but significantly decreased glucosinolates and phytate P contents. In addition, using this product in experimental diets significantly increased the final body weight of turkeys (Drazbo et al., 2018).

2.5.3 Fermentation

Fermentation, as a traditional technology in the food industry, has also been applied in the feed industry to improve the nutritional values of feed ingredients. In such studies, a variety of microorganisms was used for CM fermentation, including yeast, lactic acid bacteria, fungi, etc. (Ashayerizadeh et al., 2017; 2018; Wu et al., 2021). The extent of effects on the nutritive value of fermented CM varied depending on the species of microorganisms, fermentation condition and drying method. Overall, the effect of processing was mainly associated with the reduction in glucosinolates, phytic acid, and total carbohydrates contents and the release of soluble carbohydrates.

In the solid-state fermentation (**SSF**) of CM using *Aureobasidium pullulans*, *Neurospora crassa*, and *Trichoderma reesei* organisms inoculated individually or in combination, it was

demonstrated that such treatments can increase protein level, and reduce crude fibre, ADF, NDF and total glucosinolates contents (Alhomodi et al., 2021). Similarly, the reduction in phytic acid, total glucosinolates and soluble carbohydrate contents of CM, along with the reduced protein molecular weight and increased microbial counts in CM subjected to SSF process was observed when *Aspergillus sojae*, *Aspergillus ficuum* and their co-cultures were used. (Olukomaiya et al., 2020). *Aspergillus ficuum* inoculum used in 72 h SSF of CM resulted in increased protein by 25%, and reduced phytic acid by 67% (Nair and Duvnjak, 1990). Rapeseed meal that was inoculated and fermented with *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Aspergillus niger* improved growth performance, antioxidant capacity and meat quality of broiler chickens (Ashayerizadeh et al., 2017; 2018).

Bakers' yeast *Saccharomyces cerevisiae* was also used for CM fermentation. After SSF, CM was soaked in water and the dry residue was used in the study with Nile tilapia fish. This treatment removed glucosinolates completely and reduced phytic acid content by 18%. Replacing soybean meal with this type of yeast-fermented CM at the rate of 0, 25, 50, 75 and 100% for a 60-day Nile tilapia trial demonstrated that no significant differences was observed in BWG, FI, survival rate and FCR for all diets. However, the replacement of SBM with fermented CM above 75% had a negative effect on nutrient digestibility and utilization (Plaipetch and Yakupitiyage, 2014).

Aljuobori et al. (2014a) collected lactic acid bacteria from traditionally fermented foods and studied their ability to modulate CM composition and value. The *Lactobacillus salivarius* was the most efficient lactic acid bacteria that demonstrated the ability to decrease the total glucosinolates and crude fibre contents of CM by 38% and 16%, respectively. When the value of fermented CM was evaluated as a feed ingredient for broiler chicken raised under the heat stress,

no negative effects was noted when up to 30% of fermented CM was fed to broiler chickens from 29 to 35 days of age (Aljuobori et al., 2017). Moreover, the digestibility of protein, methionine, cysteine, arginine, aspartic acid, serine and glutamic acid improved significantly (Aljuobori et al., 2014b)

2.6 Conclusion

Canola meal is a valuable protein source for monogastric animals with high level of dietary fibre. Dietary fibre was recognized as an antinutritional factor in monogastric animal nutrition, but it has recently attracted attention for its potential beneficial effects on gut health. Research shows that the dietary fibre of CM is poorly digestible in poultry and partly fermentable in swine. The application of exogenous enzymes and enzyme pre-treatment or fermentation of feed ingredients were strategies that may improve the nutritive value of canola meal, especially canola meal fibre.

Pre-treatment of canola meal with carbohydrase enzymes through liquid incubation can provide carbohydrases with a longer time and better conditions for enzymes to aid in the depolymerization of canola meal fibre compared to adding enzymes directly as dietary supplements. However, research about enzymatic modification of canola meal is limited, with a focus on its effect on the chemical composition and growth performance of animals. The study of the effect of enzyme pre-treated canola meal on gut health in monogastric animals is rare. By incubating canola meal with enzymes, non-starch polysaccharides (NSP) will be depolymerized, releasing prebiotic-type components, including low-molecular-weight polysaccharides and oligosaccharides, which may be beneficial for the gut health of monogastric animals. Therefore, this thesis will investigate the effect of enzymatically-released bioactive components of canola meal fibre on gut health and growth performance of monogastric animals.

CHAPTER THREE : MANUSCRIPT I

The effect of multi-carbohydrase preparations on non-starch polysaccharides degradation and growth performance of broiler chickens fed diets containing high inclusion levels of canola meal

3.1 Abstract

Although canola meal (**CM**) contains a significant amount of dietary fibre, including high concentrations of non-starch polysaccharides (**NSP**), it is still a valuable source of protein for poultry diets. Supplementation of CM-containing poultry diets with multi-carbohydrases is an effective approach to improve the nutrition value of CM. The enzymatic degradation of NSP will contribute to the production of NSP hydrolysis products, which may improve gut health and growth performance. A series of in vitro incubation studies was carried out to determine if various carbohydrase preparations can target NSP of canola (*Brassica napus* L.) meal. The most effective enzyme combination was studied further in a growth performance and NSP utilization trial (1-20 d of age) with Ross 308 broiler chickens. Broilers were assigned to 3 dietary treatments each consisting of 8 cages of 5 birds and were fed control corn/CM-based starter and grower diets (Control) or diets supplemented with an enzyme blend at two levels: 0.3 and 1.5 g/kg. Starter (1-10 d) and grower (11-20 d) diets contained 270 and 290 g/kg of CM, respectively. In the in vitro studies, the highest NSP depolymerization was observed for a combination of two pectinase-like enzymes fortified with xylanase. No further improvement with other enzyme preparations was observed. In comparison to the Control diets, birds fed the diet supplemented with 1.5 g/kg of multi-carbohydrase cocktail showed higher ($P<0.05$) body weight gain during the grower phase and the entire trial. The carbohydrase enzyme combination significantly increased the coefficient of apparent total tract NSP digestibility. Our research suggests that the administration of a carbohydrase cocktail in diets with high inclusion of CM can improve growth performance and NSP utilization in broiler chickens.

Key words: canola meal, non-starch polysaccharides, multi-carbohydrase enzyme, pectinase, in vitro depolymerization

3.2 Introduction

Conventional, pre-press solvent extracted canola (*Brassica napus* L.) meal (**CM**), is the second largest feed protein ingredient worldwide and commonly used in poultry diets (Watts et al., 2020). Even though CM is relatively high in dietary fibre, particularly non-starch polysaccharides (**NSP**), it is still a valuable source of protein for poultry (Rogiewicz and Slominski, 2019; Watts et al., 2021). It contains over 400 g/kg of crude protein (dry matter basis) with well-balanced amino acids (**AA**) and high content of sulfur AA (Khajali and Slominski, 2012). When diets for broiler chickens, turkeys, or laying hens are formulated based on nitrogen-corrected apparent metabolizable energy (**AMEn**) and ileal digestible amino acid contents, CM can be successfully used at 150-200 g/kg of the diet (Adewole et al., 2016; Rogiewicz and Slominski, 2019).

Non-starch polysaccharides, the vital constituents of plant cell walls, are the most diverse carbohydrates (Meng et al., 2005). They are not only difficult to digest but also interfere with the digestion and absorption of nutrients such as protein, fat, and minerals, due to their encapsulation and/or interaction with protein and trace minerals (Debon and Tester, 2001; Meng et al., 2005). The content of NSP is higher in CM than in most plant protein sources due to a relatively high proportion of hulls in the small canola seeds (Pustjens et al., 2013). On a dry matter (**DM**) basis, CM contains 200-230 g/kg of NSP (Adewole et al., 2017b; Radfar et al., 2017), of which only around 10% can be digested by broilers (Slominski and Campbell, 1990; Khajali and Slominski, 2012).

Non-starch polysaccharides are composed of cellulose and non-cellulosic polysaccharides; the latter often being referred to as hemicelluloses and pectic substances. In CM, arabinans, arabinogalactans, xyloglucans, galactans, galactomannans, and pectic polysaccharides predominate. The latter fraction has been considered to include diverse types of polysaccharide

structures based on linked units of rhamnose, galacturonic acid, galactose, and arabinose (i.e., rhamnogalacturonans, galacturonans, arabinans). It should be noted that the structural features of polysaccharides can be oversimplified by omitting interpolymeric linkages within the cell wall. It is known that linear 5-linked arabinans which are found as part of arabinogalactan glycoproteins are dominant in the side chains of rhamnogalacturonan I. As well, substantial amounts of arabinogalactans I and II are attached to rhamnogalacturonan I or arabinogalactan proteins. There is also a strong evidence that both xyloglucan and heteroxylan are intensively crosslinked to the cell wall structure (Low et al., 2020).

The complexity of NSP would require diversified enzyme preparations for effective NSP depolymerization. This is because the targeting of a number of linkages within the cell wall structure would require many activities acting in concert as opposed to a single enzyme, the application of which would result in minimal NSP depolymerization, and only on the surface of the cell wall. Therefore, only the most complex enzyme combinations, containing a variety of activities, would be required for effective NSP depolymerization. Therefore, the development and evolution of "tailor-made" enzyme mixtures to enable more effective utilization of complex diets by poultry would be valuable.

Due to the complexity and specificity of the NSP of CM, it was necessary to explore the effect of the new-generation enzymes expressing different activities to be used in concert. In earlier in vitro studies, multi-carbohydrase supplementation resulted in depolymerization of NSP of CM (Meng et al., 2005) and it was demonstrated that the enzymatic degradation of NSP can generate some energy, and thus increase the metabolizable energy content of CM for poultry. The results of earlier in vivo studies demonstrated that the value of AMEn of CM increased following multi-carbohydrase supplementation from 7.87 to 9.30 MJ/kg for broilers and from 8.74 to 9.19 MJ/kg

for turkeys (Kozlowski et al., 2018; Rad-Spice et al., 2018). Improvements in growth performance, dietary AMEn and NSP utilization in broilers fed diets containing CM were also reported (Meng et al., 2005; Gallardo et al., 2017; Radfar et al., 2017; Toghyani et al., 2017; Moss et al., 2018). In some studies, however, the enzyme supplementation had no noticeable effect on the growth performance of broiler chickens fed CM-based diets (Meng and Slominski, 2005; Mushtaq et al., 2007).

Therefore, the objectives of this study were to screen the multi-carbohydrase preparations for their ability to depolymerize NSP of CM in vitro and to evaluate the effects of the best enzyme combination on NSP degradation and growth performance of broiler chickens fed diets containing high inclusion levels of CM.

3.3 Materials and methods

3.3.1 Materials

The test CM was produced by pre-press solvent extraction process from black-seeded *B. napus* canola and was obtained from Bunge Canola Processing Plant, Altona, Canada. Prior to analyses, CM was ground to pass through a 1mm sieve.

The evaluated enzymes, defined by their main activities as follow: **PA** (pectinase A, 443,061 U/g), **PB** (pectinase B, 425,773 U/g), **CA** (cellulase A, 38,115 U/g), **CB** (cellulase B, 42,512 U/g), **CC** (cellulase C, 38,280 U/g), **CD** (cellulase D, 27,360 U/g), **XA** (xylanase A, 22,965 U/g), **XB** (xylanase B, 60,763 U/g), **XC** (xylanase C, 24,561 U/g), and **M** (mannanase, 10,709 U/g) were provided by CBS Bio Platforms Inc., Calgary, Alberta, Canada. Enzymes were evaluated against the control treatment, where CM was incubated in water without enzyme supplementation.

3.3.2 In vitro evaluation of the efficacy of carbohydrase enzymes

An in vitro incubation study was carried out to determine if various multi-carbohydrase enzymes contain appropriate activities to target NSP present in CM. Enzyme preparations were evaluated individually and in combinations with CM as a substrate.

In vitro incubation was carried out using the procedures described by Slominski and Campbell (1990) for the determination of NSP by gas-liquid chromatography (component sugars) and colorimetry (uronic acids). In brief, duplicate samples of CM (100 mg) were boiled at 100°C with 2 mL of dimethyl sulphoxide for 1 h to disperse starch. After cooling to 45°C, 3 mL of 0.1 M sodium acetate buffer pH 5.2 or 2 ml of buffer and 1 ml of test enzyme solution were added and were followed by the addition of 3 mL of a solution of starch-degrading enzymes (amylo-glucosidase and amylase). The amount of the test enzyme preparation was calculated in grams per kilogram of the substrate. Test enzymes were dissolved in sodium acetate buffer. The mixtures were incubated at 45°C for 16 h. After this time, 42 mL of 95% ethanol was added and was let stand for 1 h at room temperature. The solution was then centrifuged for 10 min and the supernatant containing the ethanol-soluble enzyme hydrolysis products was discarded. Following drying, the residue was hydrolyzed in 12 M sulfuric acid, and the sugars were converted to alditol acetates and analyzed by gas-liquid chromatography. Component sugars were separated using Varian CP 3380 gas chromatograph (Agilent Technology, Canada). Uronic acids were determined using the procedure described by Scott (1979).

The degree of cell wall polysaccharide degradation was determined by a reduced recovery of NSP constituent sugars when compared to those of the Control treatment. The most effective enzyme combination was selected for further in vivo evaluation.

3.3.3 Evaluation of the effect of the multi-carbohydrase preparation on NSP utilization and growth performance of broiler chickens fed corn-CM diets

One-day-old male Ross 308 broiler chickens were used to evaluate the effects of diets containing high levels of CM and supplemented with the select carbohydrase combination on digestibility of NSP and growth performance.

The animal experimental procedures followed the guidelines of the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Manitoba.

Birds were provided by a local hatchery and were held in an electrically heated battery brooder (Super Brooders, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR) under a controlled environment. After arrival, birds were randomly assigned into 3 experimental treatments, with 5 birds/cage and 8 cages per treatment. Birds were fed starter (1 -10 d of age) and grower (10 - 20 d of age) mash diets. The birds had ad libitum access to feed and water. Diets were formulated to meet the Ross 308 requirement (Aviagen, 2019), except the lower metabolizable energy (95% of the recommendation) to make the diets more sensitive to enzyme effects. Chromic oxide (Cr_2O_3) was used as an indigestible marker for the calculation of the coefficient of NSP digestibility. The composition of the diets is presented in Table 3.1. Canola meal was included at the maximum level that allowed for the reasonable balance of nutrients. Starter and grower diets contained 270 and 290 g/kg of CM, respectively. Diets were fed without enzyme supplementation (Control) or were supplemented with the selected enzyme cocktail at two levels – 0.30 and 1.50 g/kg of complete feed.

Body weight and feed intake were recorded at the end of each phase (days 10 and 20) with cages as the experimental units. On day 20, excreta samples were collected, immediately frozen at

-20°C, and then freeze dried. Duplicate samples of diets and excreta were analyzed for chromium and NSP contents.

Body weight and feed intake were recorded at the end of each phase (days 10 and 20) with cages as the experimental units. On day 20, excreta samples were collected, immediately frozen at -20°C, and then freeze dried. Duplicate samples of diets and excreta were analyzed for chromium and NSP contents.

3.3.4 Chemical analysis

The chemical composition of CM was analyzed (Table 3.2). Standard AOAC (2005) methods were used for crude protein (AOAC 990.03), dry matter (945.15), ether extract (920.39), total phosphorus (965.17), and ash (942.05) determination (Association of Official Analytical Chemists, 2005). Neutral detergent fibre (**NDF**) was determined using an Ankom fibre analyzer (Ankom Technology, Macedon, NY, USA) according to the methodology of Van Soest et al. (1991) with "a heat stable amylase and expressed inclusive of residual ash" (Udén et al., 2005). Total dietary fibre (**TDF**) and its components were analyzed using the procedure described by Slominski and Campbell (1994). Phytate P was determined using the method described by Haug and Lantzsch (1983). Starch was analyzed using the Megazyme Total Starch Kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland). Glucosinolates were determined using the method described by Slominski and Campbell (1987). Simple sugars, sucrose, raffinose, and stachyose were analyzed by gas-liquid chromatography (Slominski et al., 1994).

All samples of diet and excreta were ground to pass through a 1mm sieve. For chromium determination, ashed samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES Vista, Varian, USA).

Table 3.1 Composition of diets for broilers (g/kg, as-fed basis)

Ingredient	Starter (1-10d)	Grower (11-20d)
Corn	500	510
Soybean meal	94.6	53.6
Canola meal	270	290
Fish meal	50.0	50.0
Canola oil	46.9	61.3
Calcium carbonate	11.5	9.20
Mono calcium phosphate	7.00	6.60
L-Lysine HCL	1.90	1.30
DL-Methionine	0.10	-
Mineral Premix[a]	5.00	5.00
Vitamin Premix[b]	10.0	10.0
Cr_2O_3	3.00	3.00
	1000	1000
Calculated composition		
Metabolizable energy (kcal/kg)	2850	2945
Crude protein	230	215
Calcium	9.60	8.70
Non-phytate Phosphorus	4.80	4.35
Lysine	14.4	12.9
Methionine	5.60	5.10
Methionine + cysteine	10.8	9.90
Threonine	9.70	8.80
Analyzed composition (g/kg)		
Crude protein	233	226
Ash	67.5	65.0
Total phosphorus	8.31	7.92
Non-phytate phosphorus	5.52	5.00

[a] Provided per kg of diet: 70 mg Mn, 80 mg Zn, 80 mg Fe, 10 mg Cu, 0.3 mg Se, 0.5 mg I, 337 g Na.

[b] Provided per kg of diet: 8250 IU vitamin A, 3000 IU vitamin D3, 30 IU vitamin E, 0.13 mg vitamin B12, 2 mg vitamin K3, 6 mg riboflavin, 40.3 mg niacin, 1301 mg choline, 4 mg folic acid, 0.25 mg biotin.

Table 3.2 Chemical composition of canola (*Brassica napus* L.) meal used in the study (g/kg, as-is basis)

Component	Canola meal
Dry matter	915
Crude protein	388
Ether extract	29.3
Carbohydrates	
Simple sugars[a]	4.92
Sucrose	78.4
Raffinose Family Oligosaccharides[b]	34.4
Starch	3.02
Fibre fractions	
Neutral detergent fibre (NDF)	256
Total dietary fibre (TDF)	341
Non-starch polysaccharides (NSP)	213
NSP component sugars	
Arabinose	42.9
Xylose	16.6
Mannose	3.70
Galactose	15.1
Glucose	65.7
Uronic acids	65.9
Ash	65.9
Total phosphorus (P)	11.7
Phytate P	5.92
Non-phytate P	5.78
Glucosinolates (μmol/g)	1.51

[a] Includes fructose and glucose

[b] Includes raffinose and stachyose.

Total NSP of diet and excreta samples were analyzed using the method described by Englyst and Cummings (1984) with some modifications (Slominski and Campbell, 1990) by gas-liquid chromatography (component sugars) and colorimetry (uronic acids).

3.3.5 Calculations and statistical analysis

In the growth performance study, the coefficient of apparent total tract NSP digestibility were calculated by the following equations (Meng et al., 2005):

Coefficient of apparent total tract NSP digestibility = $\{1 - [(Cr_2O_{3\ g/kg\ diet}/Cr_2O_{3\ g/kg\ excreta}) \times (NSP_{\ g/kg\ excreta}/\ NSP_{\ g/kg\ diet})\}$ Equation 3.1

Where Cr_2O_3 is chromic oxide.

All the statistical analysis was conducted using the SAS program (version 9.4, SAS Institute Inc., Cary, NC, USA). In vitro study was set up as a completely randomized design and data were analyzed using the ANOVA procedure. Means were separated by Duncan's multiple range tests.

Data collected during the growth performance study were analyzed as a completely randomized design using MIXED procedure. Outliers were removed using the UNIVARIATE procedure, where an observed value greater than a 1.5-interquartile range away from the 25th quartile or the 75th quartile was considered to be an outlier. For the MIXED procedure, the treatment was a fixed factor, and the cage was the experimental unit and a random factor. Treatment means were compared using Tukey's honestly significant difference.

All statements of significance were based on $P < 0.05$.

The figure was made with R studio (Version 1.4.1106) software with ggplot2 (Version 3.3.3).

3.4. Results

3.4.1 In vitro evaluation of the efficacy of various carbohydrases on CM NSP depolymerization

The chemical composition of CM was determined and is presented in Table 3.2. Protein and dietary fibre are the main components of CM and account for more than 300 g/kg. Non-starch polysaccharides contribute to more than half of dietary fibre. The total NSP content of CM averaged 213 g/kg with uronic acids (65.9 g/kg), glucose (65.7 g/kg), arabinose (42.9 g/kg), xylose (16.6 g/kg) and galactose (15.1 g/kg) accounting for the major component sugars. Rhamnose and mannose were also present but in small quantities.

In vitro hydrolysis of NSP of CM by PA and PB, which are the two enzymes that had demonstrated some good efficacy towards NSP of CM in our earlier research (Slominski et al., 2006), is presented in Table 3.3. Depolymerization of NSP was observed following pectinases addition as evidenced by reduced recoveries of NSP component sugars, including arabinose, xylose, galactose, and uronic acids. Incubation with PA and PB individually depolymerized NSP of CM significantly. When PA and PB were used in combination, they degraded NSP a little bit further. As the concentration of enzymes increased, NSP depolymerization increased but the results were statistically significant between the two pectinases only when both were added at the levels of 5 and 25 g/kg of CM. Therefore, adding PA and PB at a level of 10 g/kg of CM for NSP depolymerization appeared to be an optimal approach as increasing the enzyme level to 25 g/kg of CM did not depolymerize NSP markedly ($P > 0.05$). In summary, the blend of PA and PB at a level of 10 g/kg decreased the recovery of arabinose by 49.9%, xylose by 31.9%, galactose by 22.5%, glucose by 8.52%, and uronic acids by 9.71%, and the total NSP of CM by 20.7%.

In an attempt to achieve further NSP depolymerization, more enzymes were evaluated individually (Table 3.4) and in combination with a blend of PA and PB (Table 3.5). As presented in Table 3.4, five enzymes, CA, CC, CD, XB, and M, had notable effects on NSP depolymerization ($P < 0.05$). More specifically, incubation of CM with CC, CD, XB, and M significantly decreased the recovery of arabinose and glucose derived from NSP. As well, the decrease of galactose recovery was substantially affected by CA, CC, CD, and XB ($P < 0.05$). All the five enzymes significantly reduced the recovery of xylose but had no significant effect on the level of uronic acids.

As a follow up, eight enzymes were combined with PA and PB (Table 3.5). Among them, three enzymes CC, XB, and M were able to remarkably degrade NSP of CM when compared to the blend of PA and PB ($P < 0.05$). The highest NSP depolymerization was observed for XB in combination with PA and PB ($P < 0.05$), and no further improvement with other enzyme preparations was observed. Therefore, this combination was selected for further evaluation.

The profiles of NSP hydrolysis products of CM incubated with XB, PA+PB, and a combination of PA+PB+XB are presented in Figure 3.1. The use of combination XB with PA and PB resulted in the release of more glucose, however, no further improvements were observed for other sugars. Arabinose, glucose, and uronic acids accounted for about 80% of the component sugars of NSP hydrolysis products of CM incubated with PA+PB+XB. The most abundant sugar was arabinose, which accounted for 39.6% of NSP hydrolysis products. The cocktail of PA+PB+XB exerted the best effectiveness in the NSP depolymerization, thus it was selected for the in vivo evaluation with broiler chickens.

Table 3.3 In vitro hydrolysis of non-starch polysaccharides (NSP) of canola (*Brassica napus* L.) meal by pectinase-like preparations (PA, PB, or PA+PB) at different levels (g/kg)

Enzyme	Component sugar					Total NSP[1]
	Arabinose	Xylose	Galactose	Glucose	Uronic Acids	
Control[2]	42.9[a]	16.6[a]	15.1[a]	65.7[a]	65.9[a]	213[a]
PA[3]	31.7[b]	13.9[b]	13.7[b]	65.1[a]	58.8[b]	189[b]
PB[4]	29.4[b]	12.5[bc]	13.7[b]	65.0[a]	61.2[b]	188[b]
PA+PB 0.5[5]	27.4[b]	12.0[cd]	13.0[bc]	62.6[ab]	60.3[b]	180[bc]
PA+PB 1.0[6]	21.5[c]	11.3[cd]	11.7[d]	60.1[b]	59.5[b]	169[cd]
PA+PB 2.5[7]	19.9[c]	10.7[d]	12.3[cd]	62.7[ab]	57.1[b]	168[d]
SEM	1.54	0.461	0.313	1.17	1.22	3.32
p value	< 0.001	0.001	0.002	0.086	0.024	0.001

[a-d] Means within a column with no common superscripts differ significantly (P<0.05).

[1] Include arabinose, xylose, galactose, mannose, glucose, and uronic acids.

[2] None.

[3] PA (Pectinase A) was added at a level of 10 g/kg of canola meal.

[4] PB (Pectinase B) was added at a level of 10 g/kg of canola meal.

[5] PA and PB were both added at a level of 5 g/kg of canola meal.

[6] PA and PB were both added at a level of 10 g/kg of canola meal.

[7] PA and PB were both added at a level of 25 g/kg of canola meal.

Table 3.4 In vitro hydrolysis of non-starch polysaccharides (NSP) of canola (*Brassica napus* L.) meal by cellulase (CA, CB, CC, or CD), xylanase (XA, XB, or XC), and mannanase (M) (g/kg)

Enzyme	Component sugar					Total NSP[1]
	Arabinose	Xylose	Galactose	Glucose	Uronic Acids	
Control[2]	42.9[a]	16.6[a]	15.1[ab]	65.7[a]	65.9[ab]	213[a]
CA[3]	40.9[ab]	14.1[cd]	13.7[c]	62.3[abc]	63.0[b]	199[b]
CB[3]	44.0[a]	14.9[bc]	16.0[a]	64.8[ab]	68.8[a]	214[a]
CC[3]	36.7[c]	12.6[d]	12.2[d]	57.5[d]	65.9[ab]	190[b]
CD[3]	37.5[bc]	13.3[d]	13.0[cd]	59.5[cd]	64.3[ab]	192[b]
XA[3]	44.3[a]	15.6[ab]	15.0[ab]	65.6[a]	67.6[ab]	213[a]
XB[3]	37.2[c]	12.6[d]	13.1[cd]	60.0[cd]	63.6[ab]	190[b]
XC[3]	43.0[a]	13.8[cd]	14.2[bc]	60.6[bcd]	65.2[ab]	202[ab]
M[3]	36.8[c]	13.0[d]	13.9[bc]	60.2[cd]	62.8[b]	193[b]
SEM	1.08	0.416	0.382	1.29	1.60	4.00
P value	0.002	0.001	0.001	0.011	0.245	0.005

[a-d] Means within a column with no common superscripts differ significantly ($P < 0.05$).

[1] Include rhamnose arabinose, xylose, galactose, mannose, glucose and uronic acids.

[2] None.

[3] CA (Cellulase A), CB (Cellulase B), CC (Cellulase C), CD (Cellulase D), XA (Xylanase A), XB (Xylanase B), XC (Xylanase C), and M (Mannanase) were added by 10 g/kg of canola meal separately.

Table 3.5 In vitro hydrolysis of non-starch polysaccharides (NSP) of canola (*Brassica napus* L.) meal by cellulase (CA, CB, CC, or CD), xylanase (XA, XB, or XC), and mannanase (M) combined with pectinase-like enzymes (PA+PB) (g/kg)

Enzyme	Component sugar					Total NSP[1]
	Arabinose	Xylose	Galactose	Glucose	Uronic Acids	
Control[2]	42.9^{a}	16.6^{a}	15.1^{a}	65.7^{a}	65.9^{a}	213^{a}
PA+PB[3]	21.5^{bc}	11.3^{bc}	11.7^{bc}	60.1^{b}	59.5^{bc}	169^{b}
PA+PB+CA[3]	22.8^{b}	11.3^{bc}	11.6^{c}	58.2^{bc}	56.2^{c}	165^{bcd}
PA+PB+CB[3]	23.7^{b}	11.4^{bc}	12.4^{b}	57.1^{bc}	61.1^{b}	171^{b}
PA+PB+CC[3]	18.0^{d}	10.3^{de}	10.2^{ef}	56.1^{c}	57.8^{bc}	157^{d}
PA+PB+CD[3]	21.8^{bc}	11.2^{bc}	11.3^{cd}	57.7^{bc}	59.3^{bc}	166^{bc}
PA+PB+XA[3]	23.4^{b}	11.6^{b}	11.6^{c}	58.1^{bc}	58.0^{bc}	168^{bc}
PA+PB+XB[3]	16.7^{d}	8.97^{f}	9.89^{f}	50.5^{d}	56.2^{c}	147^{e}
PA+PB+XC[3]	19.5^{cd}	10.5^{cd}	10.6^{def}	56.4^{c}	62.1^{ab}	164^{bcd}
PA+PB+M[3]	17.5^{d}	9.56^{ef}	10.9^{cde}	56.6^{c}	59.5^{bc}	159^{cd}
SEM	0.929	0.262	0.238	0.958	1.24	2.50
P value	<0.001	<0.001	<0.001	<0.001	0.007	<0.001

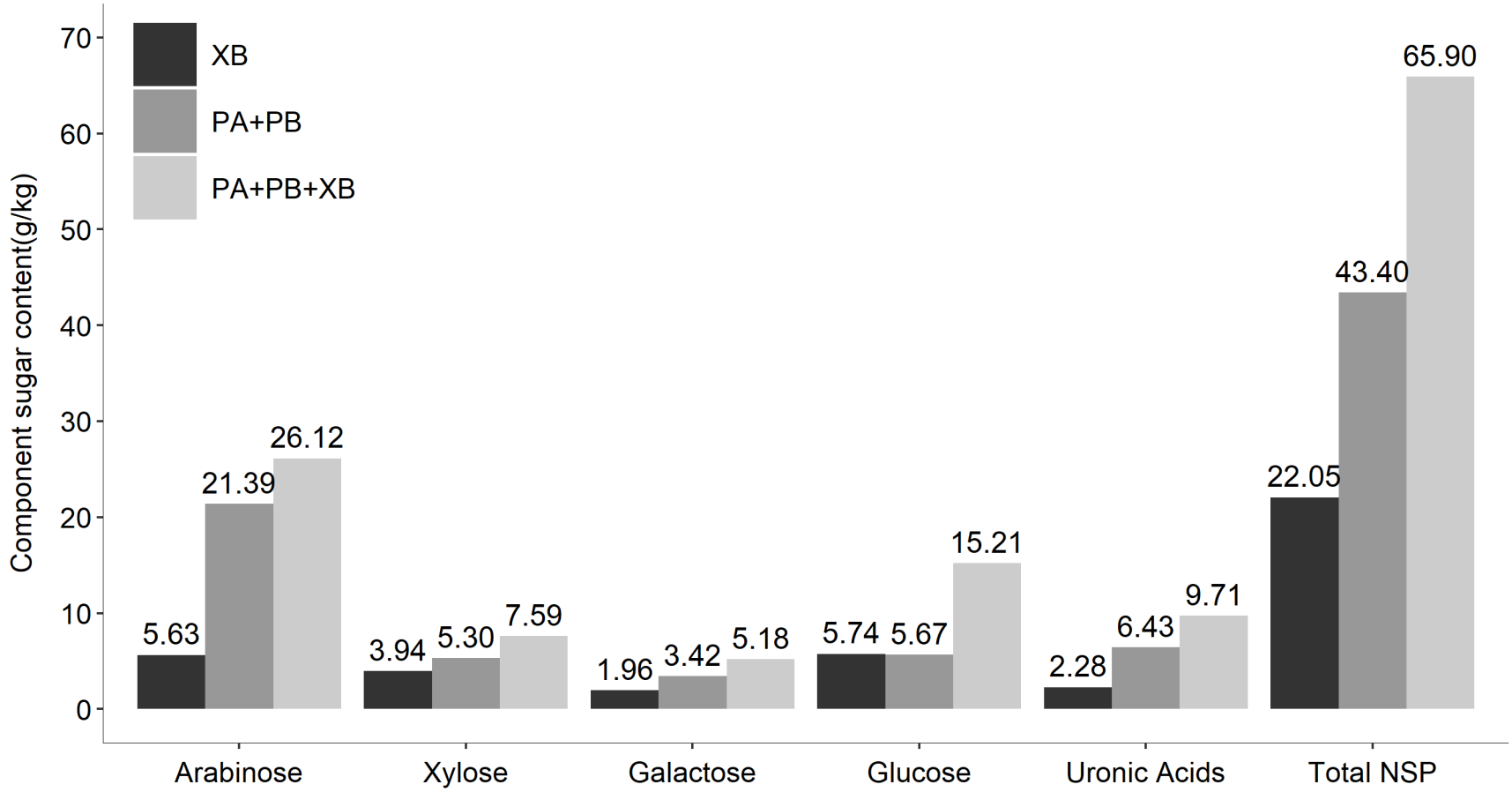
[a-f] Means within a column with no common superscripts differ significantly (P<0.05).

[1] Includes rhamnose, arabinose, xylose, galactose, mannose, glucose and uronic acids.

[2] None.

[3] PA (Pectinase A), PB (Pectinase B), CA (Cellulase A), CB (Cellulase B), CC (Cellulase C), CD (Cellulase D), XA (Xylanase A), XB (Xylanase B), XC (Xylanase C), and M (Mannanase) were added by 10 g/kg of canola meal separately.

Figure 3.1 Component sugars of non-starch polysaccharides (NSP) hydrolysis products of canola (*Brassica napus* L.) meal incubated with different carbohydrase enzymes



3.4.2 Evaluation of the effect of the multi-carbohydrase preparation on growth performance and coefficient of apparent total tract NSP digestibility in broiler chickens fed CM-based diets

The growth performance of broilers (1 - 20 d of age) is shown in Table 3.6. The body weight gain (**BWG**) of birds from the Control treatment did not achieve the Ross 308 performance due to the reduced level of the metabolizable energy in the diet, that was designed for allowing to express the effect of enzyme supplementation. Neither feed intake (**FI**) nor feed conversion ratio (**FCR**) was impacted ($P > 0.05$) by enzyme supplementation during the entire growth period. However, BWG of broilers fed diets with 1.5 g/kg of the enzyme combination was greater ($P < 0.05$) than the Control diet during the grower (10 - 20d) period and the entire trial (1 - 20d). No significant difference was observed between diets containing different concentrations of enzymes.

The coefficients of apparent total tract NSP digestibility in broiler chickens fed diets supplemented with or without enzyme cocktails are presented in Table 3.7. The combination of carbohydrase enzymes had a significant effect on NSP depolymerization, while no difference ($P > 0.05$) was observed between the low and high dietary concentration of enzymes. Only 1.4% of total NSP was degraded in birds fed the Control diet. A substantial increase in the coefficient of total tract NSP digestibility was observed in both treatments with low and high concentrations of carbohydrase (19.8%, and 18.4%, respectively). As there was no significant difference in NSP utilization caused by the concentration of the enzyme cocktail in the diet, it was concluded that using the low concentration of the enzyme cocktail would be reasonable.

Table 3.6 Growth performance (FI, BWG in g/bird, and FCR g/g) of broiler chickens (1 to 20 d) fed diets supplemented with a carbohydrase cocktail

Diet	Starter (1-10d)			Grower (10-20d)			Overall (1-20d)		
	FI	BWG	FCR	FI	BWG	FCR	FI	BWG	FCR
Control[1]	249	220	1.13	813	565[b]	1.44	1060	786[b]	1.35
Low concentration enzyme, 0.30 g/kg in complete feed	244	219	1.11	823	583[ab]	1.41	1065	802[ab]	1.33
High concentration enzyme, 1.50 g/kg in complete feed	256	224	1.15	844	605[a]	1.40	1097	829[a]	1.33
SEM	3.94	5.67	0.017	11.63	8.20	0.014	14.19	12.02	0.011
p value	0.108	0.831	0.393	0.165	0.008	0.121	0.139	0.050	0.199

[a-b] Means within a column with no common superscripts differ significantly (P<0.05).

[1] The composition of the diet is in Table 3.1.

Table 3.7 Effect of a multi-carbohydrase cocktail on coefficient of apparent total tract non-starch polysaccharides (NSP) digestibility in broiler chickens

Diet	Coefficient of apparent total tract NSP digestibility
Control[1]	0.014[b]
Low concentration enzyme, 0.30 g/kg in complete feed	0.198[a]
High concentration enzyme, 1.50 g/kg in complete feed	0.184[a]
SEM	0.023
P value	<0.001

[a-b] Means within a column with no common superscripts differ significantly (P<0.05).

[1] The composition of the diet is in Table 3.1.

3.5 Discussion

The test CM used in the study contained 388 g/kg of crude protein, 1.51 μmol/g of glucosinolates, 341 g/kg of TDF, and 213 g/kg of total NSP (Table 3.2). Its chemical composition characteristics are within the range reported for Canadian CM, with an exception for glucosinolates content, which in the test CM was lower than that reported earlier (Adewole et al., 2016; Canola Council of Canada, 2019). Due to the low glucosinolate content, it is unlikely that the test CM used in the current study at high levels (i.e., 270 and 290 g/kg for starter and grower diets, respectively) would cause any negative effects associated with their antinutritional properties. Based on the chemical composition, CM used in the current study represents overall a good quality feed ingredient for poultry.

Non-starch polysaccharides contain cellulose and non-cellulosic polymers, including arabinans, arabinoxylans, mixed-linked β-glucans, mannans, xyloglucans, xylans, and pectic polysaccharides (Slominski and Campbell, 1990; Khajali and Slominski, 2012; Do et al., 2020). More specifically, in meal derived from the seed of *B. napus*, arabinans, xyloglucans, xylans, and pectic polysaccharides are the main components of non-cellulosic polymers (Pustjens et al., 2013), which was confirmed in the analyzed NSP profile of test CM used in the current study. Pectic polysaccharides of CM consist of structural elements homogalacturonan and rhamnogalacturonan with the side chains containing arabinose, galactose, and xylose residues (Nunes and Kumar, 2018; Corral-martinez et al., 2019).

The fundamental role of carbohydrase enzymes is breaking down the high-molecular-weight polysaccharides to simple sugars, disaccharides, oligosaccharides, and low-molecular-weight polysaccharides (Meng et al., 2005). It has been suggested that the mode of action of carbohydrase supplements in the poultry diet is to reduce viscosity (Aftab and Bedford, 2018),

release nutrients encapsulated within the plant cells, provide oligomers for lower gut fermentation and the production of short chain fatty acids with increased energy utilization, and to improve gut health by modulating gut microbiota and stimulating the proliferation of beneficial microbiota (prebiotic effect) (Slominski, 2011).

In this study, the efficacy of carbohydrases was evaluated in vitro by incubating different enzyme preparations with CM and analyzing the recovery of NSP. Therefore, the reduced recoveries of NSP component sugars indicated which enzymes targeted the polysaccharides composed of arabinose, xylose, galactose, glucose, and uronic acid residues. This is not always clear as many polysaccharides may be composed of the same sugar residue. Glucose is the best example as it may derive from cellulose and some non-cellulosic polysaccharides, including mixed-linked β-glucans, xyloglucans, and pectins. Evaluated carbohydrases are produced utilizing strains that express multiple activities thus, in addition to the main activity (i.e., pectinase, xylanase, or cellulase) they may contain a variety of other (i.e., xylanase, cellulase, mannanase, β-glucanase or amylase) or side activities that are expressed in lower level. In other words, the enzymes expressing the pectinase, xylanase, or cellulase activities would contain some other activities. As an example, mannanase used in the current study did not only demonstrate mannanase activities towards NSP of CM but also showed a significant effect on arabinose, xylose, and glucose containing polysaccharides. When all enzymes were used as individual preparations, arabinose and xylose residues appeared to be effectively targeted, while uronic acids residues of pectic polysaccharides were the most difficult to show any significant reduction in recovery. When cellulase, xylanase, and mannanase preparations were combined with pectinases PA and PB, no further depolymerization was observed for CA, CB, CD, XA, and XC ($P > 0.05$). That would indicate that these enzymes targeted similar structures of NSP as PA and PB. However, three

enzymes XB, CC, and M which degraded NSP further by combining with PA and PB, can work complementarily to depolymerize the polysaccharide structures. These results clearly demonstrate that the combination of enzymes would be more effective in NSP depolymerization than single-activity enzymes as reported (Slominski, 2011; Pedersen et al., 2017). The efficacy of the enzyme combinations depends on the specific characteristics of each enzyme product which are related to the origin of organisms used for enzyme production (Nunes and Kumar, 2018). In addition, because of the complexity of plant cell walls, the main activities of enzymes do not always play their parts in degrading polysaccharides as the enzyme activity measurement procedures often require specific substrates which can have different properties than the polysaccharides of feed ingredients. As an example, pectinases developed by the industry for fruit processing and juice clarification are totally ineffective in depolymerization of pectic polysaccharides of CM and soybean meal (Slominski, unpublished).

Compared to CM, 60.9% of arabinose, 43.4% of xylose, 31.0% of galactose, 16.3% of glucose, and 13.0% of uronic acids of NSP were released due to the activity of the enzyme cocktail PA+PB+XB. This means that polysaccharides containing arabinose residues are the easiest to be hydrolyzed while the pectic polysaccharides containing uronic acids residues are most difficult to be degraded. This result is coherent with the outcome when enzymes were incubated individually with CM. In this regard, the results of the current study further support the earlier report that a significant amount of arabinose residues are terminal residues in cell wall polysaccharides of *B. napus* meal (Pustjens et al., 2013). The lower content of uronic acids residues in NSP hydrolysis products indicated that uronic acids are mainly present in the backbone of pectin which is difficult to act on. This phenomenon was also observed by Meng et al. (2005).

Although in vitro study showed that the enzyme cocktail effectively depolymerized NSP of CM, the result should be supported by in vivo evaluation in order to facilitate their validation. In the broiler chicken study, the enzyme cocktail effectively improved NSP digestibility. Without enzyme cocktail added (Control), only 1.4% of NSP of the diet containing 290 g/kg of CM was digested which confirms that NSP are poorly utilized by broilers. Earlier research demonstrated that NSP are also poorly digested by mature birds such as laying hens. When 400 g/kg of CM was included in the diet, only 2.3% of NSP was digested (Slominski and Campbell, 1990). Overall, with different types of CM and different levels of inclusion in broiler or laying hen diets, the apparent total tract digestibility of NSP varied from 2.1 to 16.3 (Slominski and Campbell, 1990; Slominski et al., 1994; Kocher et al., 2000; Meng et al., 2005; Meng and Slominski, 2005). Most of the research concluded that enzyme supplements in poultry diets significantly increased the NSP utilization, however, in some studies no effect was also reported (Kocher et al., 2000).

The effect of carbohydrase enzymes on growth performance of poultry fed diets containing CM varied in different studies. According to Mushtaq et al. (2007) and Meng and Slominski (2005), dietary multi-carbohydrase supplementation of CM diets did not significantly improve growth performance of broiler chickens. On the other hand, Chegeni et al. (2011) reported that dietary β-mannanase supplementation of a corn-soybean-CM-based diet improved BWG and FCR of broilers, while FI was not affected by enzyme supplementation. In the current study, however, supplementation of multi-carbohydrase enzymes significantly improved BWG of broilers during the grower (10 - 20d) period and the entire trial (1 - 20d), but it did not affect the FI and FCR ($P > 0.05$).

In conclusion, the present study demonstrated that the selected multi-carbohydrase cocktail significantly depolymerized non-starch polysaccharides in vitro and expressed similar effect in

vivo by improving body weight gain and coefficient of apparent total tract non-starch polysaccharides digestibility in broiler chickens. Taking into account the improvement of non-starch polysaccharides utilization and body weight gain together, the results confirm that non-starch polysaccharides degrading enzymes released nutrients (i.e., energy) from the fibre components and provided extra energy for broilers. Therefore, this study suggests that administration of a combination of carbohydrases in diets with high inclusions of canola meal can improve growth performance and non-starch polysaccharides utilization in broiler chicks.

CHAPTER FOUR : MANUSCRIPT II

The effect of enzymatically-modified canola meal on growth performance, nutrient utilization, and gut health and function of broiler chickens

4.1 Abstract

Enzymatically-modified canola meal (**ECM**) is a product obtained from the incubation of canola meal (**CM**) with a blend of specific carbohydrase enzymes. Incubation of CM with multi-carbohydrase enzymes resulted in a significant decrease in non-starch polysaccharides (**NSP**) content, production of potentially bioactive NSP hydrolysis products, and the spontaneous lactic acid bacteria fermentation. The latter resulted in the production of lactic acid, phytate hydrolysis, and the reduction in the product pH from 5.9 to 4.0. The main objective of this study was to evaluate the effects of ECM on growth performance, nutrient utilization, and gut health and function of broiler chickens. Four-day-old as-hatched Ross 308 broilers were assigned to 5 dietary treatments, each consisting of 8 cages of 8 birds. Birds were fed the starter (4-14d of age, 100 g/kg CM) and grower (14-21d of age, 200 g/kg of CM) Control diet, and Control diets supplemented with ECM (Low and High) or enzymes (E1 and E2). In the ECM-containing diets, ECM replaced either 50 g/kg (Low ECM diet) or entire CM (High ECM diet) of the Control diets. In the E1 and E2 diets, the enzyme blend was supplemented at 1.25 g/kg and 12.5 g/kg of the starter Control diet and 2.5 g/kg and 25 g/kg of the grower Control diet, respectively. Replacing CM with ECM did not affect growth performance, but significantly increased the apparent total tract NSP digestibility and nitrogen-corrected apparent metabolizable energy content. The ECM-containing diets decreased sialic acids secretion on day 21 and increased the abundance of *Lactobacillus* ($P < 0.01$) in ileum and ceca compared to Control, E1, and E2 during the whole trail. The abundance of *Escherichia coli* was significantly lower in ceca of birds fed the High ECM grower diets compared to the Control. Total short chain fatty acids in cecal digesta of birds fed High ECM starter diet significantly increased compared with the Control. Replacing CM with ECM improved nutrient digestibility and increased the presence of probiotic organisms which was not observed in the

enzyme supplemented diets. Therefore, ECM can improve gut health and could be used as a valuable feed ingredient or additive.

Key words: canola meal, enzymatically-modified canola meal (ECM), non-starch polysaccharides (NSP), broiler chicken, carbohydrase

4.2 Introduction

Canola meal (**CM**) is a co-product of the oil-crushing industry and is commonly used in poultry diets as a valuable protein source. The use of CM in poultry diets still concerns nutritionists due to its high dietary fibre content associated with low nutrient digestibility and low available energy content. Based on research done by Adewole et al. (2016), fibre components of average Canadian CM contain, on a dry matter (**DM**) basis, 294 g/kg of neutral detergent fibre (**NDF**) and 379 g/kg of total dietary fibre (**TDF**), which includes 219 g/kg of non-starch polysaccharides (**NSP**), 54 g/kg of neutral detergent insoluble protein and 107 g/kg of lignin with associated polyphenols. Among the dietary fibre fractions present in plant feedstuffs, NSP have been identified as the main components that impair digestibility and absorption of nutrients, often due to nutrient encapsulating effect of the cell walls (Slominski and Campbell, 1990; Khajali and Slominski, 2012; Pirgozliev et al., 2022). It is well known that NSP of CM are highly insoluble in water and, thus, poorly digestible by broilers. The apparent total tract digestibility of NSP of CM-containing diets varies from 3.4 to 8.6% (Slominski and Campbell, 1990; Slominski et al., 1994; Kocher et al., 2000; Meng et al., 2005; Meng and Slominski, 2005). When diets were supplemented with carbohydrase preparations, the NSP digestibility improved in most cases from 6 to 14% only (Meng et al., 2005; Meng and Slominski, 2005). In addition to the low-water solubility, the conditions of the gastrointestinal tract, including pH and short digesta transit time (i.e., ~5 h), are

not ideal for the supplemental enzymes to exert their full potential in CM NSP depolymerization (Lannuzel et al., 2022; Chapter Five). This is the reason why in our earlier studies, the nitrogen-corrected apparent metabolizable energy (**AMEn**) values of CM (DM basis) for broiler chickens increased only from 8.18 to 8.72 MJ/kg and for turkeys from 9.12 to 9.39 MJ/kg following multi-carbohydrase supplementation (Kozlowski et al., 2018; Rad-Spice et al., 2018).

In our recent study, the potential of a new generation of multi-carbohydrate enzymes expressing activities towards degrading NSP of CM had been explored in vitro, with the most effective carbohydrase blend leading to depolymerization of around 40% of NSP and production of bioactive NSP-hydrolysis products (Niu et al., 2022). Therefore, the production of enzymatically-modified canola meal (**ECM**) was explored further to upcycle the NSP of CM into potentially bioactive and beneficial prebiotic components. It was hypothesized that modifying the NSP of CM with a multi-carbohydrase preparation would result in a value-added end-product, which would exhibit the prebiotic and probiotic properties for broiler chickens, promoting gut health and function. Hence, the main objective of this research was to evaluate the effects of ECM on growth of broiler chickens, ileal and cecal microbiota proliferation, concentration of short chain fatty acids (**SCFA**)**,** mucin production**,** and nutrients, including NSP utilization. The addition of multi-carbohydrase enzymes directly to the CM-containing diets was also included in this study to explore if any gut health and function effects were comparable or not to those of ECM.

4.3 Materials and methods

All research methods and procedures involving animals were conducted according to the guidelines of the Canadian Council on Animal Care. The animal care protocol for this study was approved by the Animal Care Committee of the University of Manitoba (F20 - 001/1, AC11558).

4.3.1 Materials

Canola meal was produced by commercial pre-press solvent extraction of Brassica napus canola seeds by Bunge Canola Processing Plant, Altona, Canada.

In our earlier studies, two pectinases, four cellulases, three xylanases, and one mannanase were tested in vitro to establish the enzyme combination that was most effective in NSP depolymerization of CM (Niu et al., 2022). Carbohydrase preparations, labeled herein as **PA** (Pectinase A, 443,061 U/g), **PB** (Pectinase B, 425,773 U/g), **XB** (Xylanase B, 60,763 U/g), and **Inv** (Invertase, 362,631 U/g) were provided by CBS Bio Platforms Inc., Calgary, Alberta, Canada. It must be emphasized that the pectinases used in the current study contained activities towards CM pectin and thus were different from the "classical" pectinases commonly used for fruit beverage processing.

The production of ECM was carried out using submerged liquid processing involving a continuous mixing (BDC 2002, Cafromo LabSolutions, Ontario, Canada) of 2 kg of CM in 8 L of distilled water and a select combination of carbohydrase preparations (PA, 8 g; PB, 8 g; XB, 8 g; I, 0.4 g) in a 15 L container at 40 °C for 48 h. The material was then frozen and freeze-dried. This procedure was repeated several times to produce enough material for in vivo evaluation. The final product was mixed and ground to pass through a 2 mm sieve and along with the CM subjected for analyses to determine their chemical composition. The chemical composition of ECM and CM is shown in Table 4.1.

Table 4.1 Chemical composition of canola (*Brassica napus* L.) meal (CM) and enzymatically-modified canola meal (ECM) (g/kg, as-is basis)

Component	CM	ECM
Dry Matter	915	915
Crude Protein	388	404
Ether Extract	29.3	32.3
Carbohydrates		
Simple Sugars[1]	4.9	4.3
Sucrose	78.4	0.8
Oligosaccharides[2]	34.4	ND[6]
Dietary Fibre Fractions		
Neutral Detergent Fibre (NDF)	256	219
Total dietary fibre (TDF)	341	281
Non-starch polysaccharides (NSP)	213	136
Arabinose	43.1	16.0
Xylose	14.8	5.7
Mannose	3.4	2.4
Galactose	14.1	8.6
Glucose	66.3	36.0
Uronic acids	71.8	66.9
Glycoproteins	50.9	45.4
Lignin and Polyphenols	76.6	99.6
NSP hydrolysis products[3]	-	77.8
Ash	65.9	68.5
Total phosphorus	11.7	12.0
Phytate phosphorus	5.9	ND
Non-phytate phosphorus	5.8	12.0
Lactic acid	ND	57.0
pH[4]	5.9	4.0
Lactobacillus spp.[5]	1.0	304

[1] Includes fructose and glucose.

[2] Includes raffinose and stachyose.

[3] Water-soluble and 80% EtOH-soluble NSP hydrolysis products.

[4] Measured in slurry with 4 times weight of water added and mixed for 30 mins.

[5] Measured by PCR and analyzed using the $2^{-\Delta\Delta CT}$ method by normalizing the expression of *Lactobacillus spp*. to that of total eubacteria and comparing it to CM.

[6] Not detected.

4.3.2 Birds and housing

As-hatched Ross 308 broiler chickens were used to evaluate the effects of ECM on growth performance, nutrient utilization, and gut health. The study was carried out at the Small Animal Research Facility of the Department of Animal Science, University of Manitoba, Canada. Birds were provided by a local hatchery and raised in electrically heated battery housing system (Super Brooders, Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR, USA) under a controlled environment. The temperature was monitored, daily recorded, and adjusted in response to the comfort of the birds. Birds had free access to water and feed.

4.3.3 Experimental diets

Birds were fed the Control starter diet throughout the 4-day pre-experimental period to allow for yolk sac absorption. On day 4, chicks were randomly allocated to 5 experimental treatments using 8 birds per cage and 8 replicate cages per treatment. Birds were fed starter (4-14 d of age) and grower (14-21 d of age) mash diets. The composition of the experimental diets is presented in Table 4.2. Isonitrogenous and isoenergetic starter and grower diets were formulated to meet Ross 308 nutrient requirement as per breeder recommendations (Aviagen, 2019), except the metabolizable energy which was reduced by 100 kcal/kg (0.42 MJ/kg) to make them more responsive to enzyme effects or ECM addition. Chromic oxide (3.0 g/kg) was used as an inert marker and was used to calculate the apparent total tract digestibility (ATTD) of NSP and nitrogen (N) and AMEn. The experimental design is presented in Table 4.3. Both the starter and grower diets contained CM at 100 and 200 g/kg, respectively, with ECM added to the Low ECM starter diet at 50 g/kg at the expense of conventional CM for the total of 100 g/kg, and at the expense of CM

to grower diets at 50 g/kg for the total of 200 g/kg as in the Control diets. Canola meal was fully replaced with ECM in High ECM diets to evaluate any potential negative effect of ECM on growth performance, nutrient digestibility, or indices of the gut health. Two enzyme-supplemented diets were employed to evaluate if enzyme supplementation of CM-containing diets had similar effect to inclusion of ECM which was subjected to 48 h submerge liquid processing, thus treatments were designed to use comparable activities of enzymes. The amount of enzyme used was similar to either that of enzymes used in the preparation of ECM in the High ECM diet (E1) or when taking into account the difference in the time of ECM incubation i.e., 48 h and the feed passage time in the chicken gut, i.e., 5 h (E2). It should be noted that the dosage of enzymes in experimental diets was much higher than that used in commercial practice, and it was applied here to prove the concept. Body weight and feed consumption were recorded at 4, 14, and 21 days of age. Average body weight gain (BWG), feed intake (FI), and feed conversion ratio (FCR) were used to determine performance and were calculated on a cage basis with each cage representing one replicate.

4.3.4 Sample collection

On days 13 and 20 of the study, excreta samples were collected, frozen, and freeze-dried for analyzing the ATTD of NSP and N, sialic acids content, and AMEn. On days 14 and 21 of the study, four birds per cage were randomly selected and euthanized using carbon dioxide for the collection of digesta from the small intestine (ileum) and ceca. The ileum was defined as the segment from Meckel's diverticulum to 4 cm proximal to the ileocecal junction. Ileal and cecal digesta samples were gently collected, pooled within the cage, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis. On day 21

of the trial, the pH of ileum and ceca content was measured (mean of three readings) using a pH meter with a microelectrode (Hanna Instruments Inc., Vila do Conde, Portugal).

4.3.5 Chemical analysis

Canola meal, ECM, diets, and dried excreta samples were ground to pass through a 1 mm sieve. Chemical components of CM and ECM were determined in duplicate. Crude protein (N x 6.25) was determined using the combustion method of nitrogen determination (AOAC 968.06) and TruSpecN nitrogen analyzer (Leco Corp., St. Joseph, MI, USA). Standard AOAC (2005) methods were used to analyze dry matter (945.15), ether extract (920.39), ash (923.03), and total phosphorus (965.17). Phytate phosphorus was determined following the method described by Haug and Lantzsch (1983). Sugars, including fructose, glucose, sucrose, raffinose, and stachyose were determined by gas-liquid chromatography using a 3% OV-7 column and a Varian 430 Gas Chromatograph (Agilent Technology, Mississauga, ON, Canada) as described by Slominski et al.(1994).The NDF was measured using an Ankom fibre analyzer (Ankom Technology, Macedon, NY, USA) and AOAC procedure 2002.04 (2005). Non-starch polysaccharides were measured by gas-liquid chromatography (component neutral sugars) using SP-2340 column and Varian CP-3380 Gas Chromatograph (Agilent Technology, Mississauga, ON, Canada) and by colorimetry (uronic acids) using a Biochrom Ultrospec 50 (Biochrom Ltd., Cambridge, UK) and the procedure described by Englyst and Cummings (1984) with some modifications (Slominski and Campbell, 1990). Total dietary fibre, glycoprotein, lignin, and polyphenols were analyzed according to the procedure of Slominski et al. (1994). The content of water-soluble NSP hydrolysis products in ECM was calculated from the difference between intact CM NSP and NSP after incubation with enzymes.

Duplicate samples of diets and excreta were analyzed for chromium, NSP, gross energy, and nitrogen. Crude mucin was extracted from excreta according to the procedure described by Lien et al. (1997) and total sialic acids were measured using the method described by Jourdian et al. (1971) with some modifications (Hejdysz et al., 2018). For chromium determination, ashed samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES Vista, Varian, USA) described in AOAC Method 985.01 (2005). Gross energy was measured by combustion of samples in a Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL, USA).

The concentration of SCFA, including acetic acid, propionic acid, butyric acid, valeric acid, branched chain fatty acids (**BCFA**, refers to iso-butyric, 2-methyl butyric, and iso-valeric) and lactic acid was analyzed according to the procedure described by Apajalahti et al. (2019) using pivalic acid (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard.

4.3.6 DNA extraction and quantitative real-time polymerase chain reaction (qPCR)

The diversity of the bacterial community in ileum and ceca was measured. Microbiota DNA was extracted from ileal and cecal digesta samples using an Invitrogen™ PureLink™ Microbiome DNA Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA) following the manufacturer's instructions. After extraction, the DNA samples were quantified by a spectrophotometer (NanoDrop-2000, Thermo Fisher Scientific Inc., Waltham, MA, USA) and diluted with ultrapure water to 0.75 ng/µL (Lunedo et al., 2014).

Pairs of primers used for quantification of different bacterial groups were selected according to previously published works (Walter et al., 2001; Rinttilä et al., 2004; Khan et

al., 2007; Guo et al., 2008; Zwielehner et al., 2009; Mejicanos et al., 2017) and obtained from Integrated DNA Technologies, Inc. (Coralville, IA, USA).

The bacterial groups studied, forward and reverse primer sequences, and annealing temperatures for the qPCR assay are listed in Table 4.4. All reactions consisted of 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories Ltd., ON, Canada), 0.5 µL of 10 pmole/µL of each primer, 2 µL of Nuclease-free water, and 2 µL of extracted DNA. The reactions were conducted in 96 well plates in CFX Connect ™ qPCR Detection System (Bio-Rad Laboratories Ltd., ON, Canada).

The results of qPCR were analyzed using the $2^{-\Delta\Delta C_T}$ method by normalizing the expression of the target bacterial groups to that of total eubacteria and comparing them to the Control treatment (Livak and Schmittgen, 2001). All extracted DNA samples were analyzed in duplicate.

Table 4.2 Composition of experimental diets (g/kg, as-fed basis)

Ingredient	Starter (4 - 14 d of age)			Grower (14 – 21 d of age)		
	Control	Low ECM	High ECM	Control	Low ECM	High ECM
Corn	516	520	523	510	514	527
Soybean Meal	295	293	291	192	190	180
Canola Meal (CM)	100	50.0	-	200	150	-
Enzymatically-modified CM (ECM)	-	50.0	100	-	50.0	200
Canola oil	36.2	35.1	34.5	50.2	49.4	46.2
Calcium carbonate	15.4	16.1	16.8	11.9	12.7	14.7
Mono calcium phosphate	13.0	11.6	10.2	12.5	11.1	6.8
L-Lysine HCL	3.2	3.2	3.2	2.9	2.9	2.9
DL-Methionine	1.9	1.9	1.9	1.4	1.4	1.4
Threonine	1.5	1.5	1.5	1.0	1.0	1.0
Mineral Premix [a]	5.0	5.0	5.0	5.0	5.0	5.0
Vitamin Premix [b]	10.0	10.0	10.0	10.0	10.0	10.0
Chromic oxide (Cr_2O_3)	3.0	3.0	3.0	3.0	3.0	3.0
Total	1000	1000	1000	1000	1000	1000
Calculated composition (g/kg unless specified)						
Metabolizable energy (MJ/kg)	12.3	12.3	12.3	12.5	12.5	12.5
Crude Protein	230	230	230	215	215	215
Calcium	9.6	9.6	9.6	8.7	8.7	8.7
Available Phosphorus	4.8	4.8	4.8	4.4	4.4	4.4

Digestible Lysine	12.8	12.8	12.8	11.5	11.5	11.5
Digestible Methionine	5.1	5.1	5.1	4.7	4.7	4.7
Digestible Methionine + cysteine	9.5	9.5	9.5	8.7	8.7	8.7
Digestible Threonine	8.6	8.6	8.6	7.7	7.7	7.7
Digestible Arginine	13.7	13.7	13.7	12.3	12.3	12.3
Analyzed composition (g/kg unless specified)						
Crude Protein	225	226	234	223	218	219
Ash	70.5	63.7	59.2	56.7	58.1	55.7
Total Phosphorus	7.3	7.5	7.3	7.2	7.1	7.2
Non-phytate Phosphorus	5.1	5.3	5.4	4.8	4.9	4.9
Non-starch Polysaccharides (NSP)	102	99.6	93.6	110	105	94.0

[a] Provided per kg of diet: 70 mg Mn, 80 mg Zn, 80 mg Fe, 10 mg Cu, 0.3 mg Se, 0.5 mg I, 337 g Na.

[b] Provided per kg of diet: 8250 IU vitamin A, 3000 IU vitamin D3, 30 IU vitamin E, 0.13 mg vitamin B12, 2 mg vitamin K3, 6 mg riboflavin, 40.3 mg niacin, 1301 mg choline, 4 mg folic acid, 0.25 mg biotin.

Table 4.3 The experimental diets for broiler chickens

Treatment	Starter (4 - 14 d of age)		Grower (14 - 21 d of age)	
	Diet	Enzyme blend,[1] g/kg of diet	Diet	Enzyme blend[1], g/kg of diet
Control	Control (CM 100 g/kg)	0	Control (CM 200 g/kg)	0
Low ECM	ECM 50 g/kg + CM 50 g/kg	0.633[2]	ECM 50 g/kg +CM 150 g/kg	0.633[2]
High ECM	ECM 100 g/kg	1.25[2]	ECM 200 g/kg	2.50[2]
E1	Control + Enzyme blend	1.25[3]	Control + Enzyme blend	2.50[3]
E2	Control + Enzyme blend	12.5[3]	Control + Enzyme blend	25.0[3]

[1] The enzyme blend was composed of 327.9 g/kg of each of pectinase A and B (PA, PB), xylanase (XB), and 16.3g/kg of invertase (Inv).

[2] Represents the amount of enzyme blend used to produce Enzymatically-modified canola meal (ECM).

[3] Represents the amount of enzyme blend directly added in the diets.

Table 4.4 Paris of primers used for real-time PCR assay

Target groups	Primer sequence		AT (ºC)	References
	Forward	Reverse		
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC	60	Guo et al., 2008
Bacteroidetes	GGARCATGTGGTTTAATTCGATGAT	AGCTGACGACAACCATGCAG	60	Guo et al., 2008
Clostridium cluster IV	GCACAAGCAGTGGAG T	CTTCCTCCGTTTTGTCAA	56	Zwielehner et al., 2009; Mejicanos et al., 2017
Enterococcus spp.	CCCTTATTGTTAGTTGCCATCATT	ACTCGTTGTACTTCCCATTGT	61	Rinttilä et al., 2004; Mejicanos et al., 2017
E. coli	CAATTTTCGTGTCCCCTTCG	GTTAATGATAGTGTGTCGAAAC	55	Khan et al., 2007; Wealleans et al., 2017
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	58	Walter et al., 2001; Heilig et al., 2002
Bifidobacterium spp.	TCGCGTCCGGTGTGAAAG	CCACATCCAGCATCCAC	58	Rinttilä et al., 2004; Mejicanos et al., 2017
Total Eubacteria	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	60	Guo et al., 2008

4.3.7 Calculations and statistical analysis

Coefficient of apparent total tract digestibility of NSP and N (using NSP digestibility calculation as an example), and AMEn were calculated using the following equations (Hill et al., 1960; De Groote et al., 1971; Meng et al., 2005):

$$\text{Coefficient of ATTD of NSP (\%)} = 1 - \frac{{}^{Cr_2O_{3\%diet}}/_{Cr_2O_{3\%excreta}}}{{}^{NSP_{\%diet}}/_{NSP_{\%excreta}}}$$

Equation 4.1

$$\text{AME}_\text{n}(\text{MJ/kg}) = GE_{MJ/kg\ diet} - \left[GE_{MJ/kg\ excreta} \times \frac{Cr_2O_{3\ \text{g/kg}\ diet}}{Cr_2O_{3\ \text{g/kg}\ excreta}} + 0.0344 \times \left(N_{g/kg\ diet} - N_{g/kg\ excreta} \times \frac{Cr_2O_{3\ \text{g/kg}\ diet}}{Cr_2O_{3\ \text{g/kg}\ excreta}}\right)\right]$$

Equation 4.2

Where **GE** is gross energy, Cr_2O_3 is chromic oxide, and 0.0344 is the energy equivalent of uric acid nitrogen.

Statistical analysis was conducted by the SAS program (version 9.4, SAS Institute Inc., Cary, NC). The animal study was designed as a completely randomized design. The data were analyzed using the Mixed procedure with the treatment as a fixed factor and the cage as a random factor. Means were separated by Tukey's honestly significant difference. All statements of significance were based on $P < 0.05$. All figures were made with R studio (Version 1.4.1106) software with ggplot2 (Version 3.3.3).

4.4 Results

4.4.1 Determination of chemical composition of ECM

As shown in Table 4.1, incubation of CM with enzyme preparations blend (PA, PB, XB, and Inv) resulted in a decrease of NSP content, on an as-is basis, from 213 to 136 g/kg, and TDF content from 341 to 281 g/kg, and a production of water-soluble NSP hydrolysis products, 77.8 g/kg. During the incubation, spontaneous fermentation occurred which is reflected in 304-fold increasing abundance of *Lactobacillus spp.*, production of 57.0 g/kg of lactic acid, and decreasing the pH of ECM slurry.

The processing of CM positively affected the phosphorus availability, as the phosphorus bound to the phytate molecule has not been detected in ECM. Sucrose and galactooligosaccharides (raffinose and stachyose) decreased dramatically.

4.4.2 The effect of dietary treatments on growth performance of broiler chickens

The growth performance of broilers fed the starter (4-14 d of age) and grower (14-21 d of age) diets is shown in Table 4.5. Replacing CM with ECM did not influence growth performance of birds significantly while carbohydrase preparation supplementation affected FI and BWG of broilers during the starter period (4 – 14 d of age). Birds fed the E2 starter diet had higher FI ($P = 0.002$) compared with the Control and the E1 diets. Body weight gain of broilers fed the E2 starter diet was greater ($P = 0.008$) than birds fed the Control and E1 starter diets, while no significant difference ($P > 0.05$) was observed during the grower period and the entire trial. Despite differences in FI and BWG, the FCR was not significantly affected by dietary treatments.

4.4.3 The effect of dietary treatments on nutrient utilization in broiler chickens

Apparent total tract digestibility of NSP and N and AMEn of diets supplemented with ECM or carbohydrases are demonstrated in Table 4.6. The NSP present in the Control diet were poorly

digestible, i.e., only 3.7% in the starter and 5.3% in the grower diet were digested. The multi-carbohydrase, either directly supplemented or applied through the enzymatic modification of CM, significantly improved the coefficient of ATTD of NSP ($P < 0.001$ during each phase).

During the starter period, birds fed the High ECM diet digested significantly more NSP when compared with birds from the Low ECM group, while no significant difference was carried throughout the grower period. The concentration of ECM in diets showed a significant effect on ATTD of NSP only during the starter period, while the level of multi-carbohydrase blend in diets had no effect on the NSP digestibility. A significant increase in ATTD of N was observed in birds fed the High ECM starter diet when compared with the Control diet ($P = 0.011$). However, the ATTD of N during the grower phase was not impacted by experimental treatments.

The replacement of CM with ECM increased the dietary AMEn value, while supplementation with the multi-carbohydrase did not affect it. Starter diets containing ECM were higher ($P < 0.001$) in AMEn than the Control, while only the grower High ECM diet was higher ($P < 0.001$) in AMEn than the Control.

Table 4.5 Growth performance of broiler chickens (4 to 21 d) fed diets supplemented with enzymatically-modified canola meal (Low ECM and High ECM) or multi-carbohydrase blends of enzymes (E1 and E2)

Diet[1]	Starter (4 – 14 d of age)			Grower (14 – 21 d of age)			Overall (4 – 21 d of age)		
	FI (g/bird)	BWG (g/bird)	FCR (g/g)	FI (g/bird)	BWG (g/bird)	FCR (g/g)	FI (g/bird)	BWG (g/bird)	FCR (g/g)
Control	379[b]	310[b]	1.23	532[ab]	381	1.40	912[ab]	693	1.32
Low ECM	396[ab]	329[ab]	1.21	533[ab]	388	1.38	929[ab]	717	1.30
High ECM	388[ab]	323[ab]	1.21	507[b]	355	1.43	893[b]	676	1.32
E1[2]	386[b]	316[b]	1.22	520[ab]	370	1.41	914[ab]	690	1.32
E2[3]	407[a]	341[a]	1.19	548[a]	390	1.41	952[a]	730	1.32
SEM	6.11	8.24	0.021	11.68	11.15	0.02	16.5	17.4	0.010
P value	0.002	0.008	0.156	0.046	0.077	0.286	0.041	0.060	0.260

[a-b] Means within a column with no common superscripts differ significantly ($P < 0.05$).

[1]The composition of diets is presented in Table 4.2.

[2] E1- Starter diet supplemented with 1.25 g/kg of multi-carbohydrase; Grower diet supplemented with 2.5 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

[3] E2 - Grower diet supplemented with 12.5 g/kg of multi-carbohydrase; Grower diet supplemented with 25.0 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

Table 4.6 Coefficient of apparent total tract digestibility (ATTD) of non-starch polysaccharides (NSP) and nitrogen (N), and nitrogen corrected apparent metabolizable energy (AMEn) contents of diets supplemented with enzymatically-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2) fed to broilers

	Starter (4 – 14 d of age)			Grower (14 – 21 d of age)		
Diet[1]	Coefficient ATTD of NSP	AMEn, MJ/kg	Coefficient ATTD of N	Coefficient ATTD of NSP	AMEn, MJ/kg	Coefficient ATTD of N
Control	0.037[c]	12.5[b]	0.710[b]	0.053[b]	12.3[b]	0.717
Low ECM	0.163[b]	12.8[a]	0.735[ab]	0.158[a]	12.6[b]	0.723
High ECM	0.226[a]	13.0[a]	0.745[a]	0.189[a]	13.0[a]	0.723
E1[2]	0.176[ab]	12.5[b]	0.723[ab]	0.166[a]	12.5[b]	0.723
E2[3]	0.186[ab]	12.5[b]	0.726[ab]	0.172[a]	12.4[b]	0.732
SEM	0.014	0.060	0.007	0.013	0.168	0.010
P value	<0.001	<0.001	0.011	<0.001	<0.001	0.889

[a-c] Means within a column with no common superscripts differ significantly ($P < 0.05$).

[1]The composition of diets is presented in Table 4.2

[2] E1- Starter diet supplemented with 1.25 g/kg of multi-carbohydrase; Grower diet supplemented with 2.5 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

[3] E2 - Grower diet supplemented with 12.5 g/kg of multi-carbohydrase; Grower diet supplemented with 25.0 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

4.4.4 The effect of dietary treatments on gut health of broiler chickens

The measurement of sialic acids, the components of intestinal mucins, was used to investigate the effect of ECM inclusion or multi-carbohydrase supplementation on endogenous nutrient losses and gut integrity in broilers fed experimental diets. The sialic acids content in excreta of broiler chickens fed diets supplemented with ECM or multi-carbohydrase preparation on days 14 and 21 are presented in Table 4.7. Replacement of CM with ECM did not significantly decrease the secretion of sialic acids on day 14, while it remarkably decreased their secretion on day 21. However, with the increased ECM replacement, a significant reduction of sialic acids secretion was detected on day 14, but no difference between High and Low ECM was observed on day 21. The effect of enzyme supplementation on sialic acids secretion was not noted on days 14 and 21. The high ECM diet significantly decreased sialic acids secretion compared with the E1 diet on days 14 and 21, while it only significantly decreased the secretion of sialic acids compared with the E2 diet on day 21.

As it is presented in Table 4.7, the pH of ileal and cecal digesta of broiler chickens fed diets containing ECM or supplemented with carbohydrases on day 21 was not affected by dietary treatments.

Table 4.7 Sialic acids content in excreta and pH of ileal and cecal digesta of broiler chickens fed diets supplemented with enzyme-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2)

Diet[1]	Sialic acids, mg/100g excreta		pH at day 21	
	Starter (4 – 14 d)	Grower (14 – 21 d)	Ileal digesta	Cecal digesta
Control	24.2[ab]	34.3[a]	8.02	6.26
Low ECM	28.9[a]	19.1[b]	8.00	6.29
High ECM	22.0[b]	16.7[b]	7.96	6.31
E1[2]	27.3[a]	30.1[a]	7.97	6.04
E2[3]	26.2[ab]	31.1[a]	7.94	6.05
SEM	1.33	1.90	0.05	0.101
P value	0.006	<0.001	0.742	0.159

[a-b] Means within a column with no common superscripts differ significantly ($P < 0.05$).

[1] The composition of diets is presented in Table 4.2 .

[2] E1 - Starter diet supplemented with 1.25 g/kg of multi-carbohydrase; Grower diet supplemented with 2.5 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

[3] E2 - Grower diet supplemented with 12.5 g/kg of multi-carbohydrase; Grower diet supplemented with 25.0 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

The abundance of selected bacterial communities in ileum and ceca is presented in Table 4.8 and Table 4.9, respectively. The Firmicutes, Bacteroidetes, *Enterococcus spp.*, and *Bifidobacterium spp.* were detected in ileal digesta, however, their abundance was not different between treatments, with exception of the abundance of Firmicutes in birds fed the Low ECM diet. It was significantly lower than the High ECM and E2 diets on day 21. The abundance of *Lactobacillus spp.* was significantly higher ($P < 0.001$) in ileal digesta of birds fed starter (d 14) and grower (d 21) diets containing ECM than those fed the Control and enzyme-supplemented diets. The same effect was observed in the cecal contents. The contents of ceca were additionally subjected to the measurement of the fold change of *Clostridium cluster IV* and *Escherichia coli*. On day 21, the abundance of *Escherichia coli* was lower ($P = 0.002$) in cecal digesta of broilers fed the High ECM diet than that of the Control group.

Cecal digesta of birds fed the E2 diet was significantly richer in *Bacteroidetes* than that of Low ECM, E1, and Control diets, while the abundance of *Enterococcus spp.* was significantly higher in cecal digesta of birds fed the E1 diet than in the High ECM diet on day 21. The fold change of other selected bacterial communities other than *Lactobacillus spp.* was not affected by diets in this study.

Table 4.8 Fold change[1] of selected ileal bacteria communities of broiler chickens fed diets[2] supplemented with enzyme-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2) at days 14 and 21.

		Control	Low ECM	High ECM	E1[3]	E2[4]	SEM	P value
Day 14	Firmicutes	0.97	1.34	1.10	1.34	1.24	0.17	0.458
	Bacteroidetes	1.08	1.29	1.09	1.20	1.15	0.36	0.993
	Enterococcus spp.	1.10	1.49	0.93	1.33	1.11	0.33	0.777
	Lactobacillus spp.	0.95^{b}	58.9^{a}	62.3^{a}	0.89^{b}	1.22^{b}	6.23	<.001
	Bifidobacterium spp.	1.07	0.71	0.73	1.03	1.54	0.35	0.393
Day 21	Firmicutes	1.16^{ab}	0.72^{b}	1.40^{a}	1.21^{ab}	1.37^{a}	0.16	0.027
	Bacteroidetes	1.01	1.06	1.42	0.84	1.01	0.29	0.685
	Enterococcus spp.	1.13	0.77	0.32	1.50	1.46	0.45	0.333
	Lactobacillus spp.	1.12^{b}	95.7^{a}	113.1^{a}	2.03^{b}	7.26^{b}	16.7	<.001
	Bifidobacterium spp.	0.93	0.39	0.48	0.84	0.97	0.26	0.389

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1] The fold change was measured using a $2^{-\Delta\Delta C_T}$ method by normalizing the target gene of selected bacteria to the reference gene of total eubacteria and comparing to the Control treatment.

[2] The composition of the diet is in Table 4.2.

[3] E1- Starter diet supplemented with 1.25 g/kg of multi-carbohydrase; Grower diet supplemented with 2.5 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

[4] E2 - Grower diet supplemented with 12.5 g/kg of multi-carbohydrase; Grower diet supplemented with 25.0 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

Table 4.9 Fold change[1] of selected cecal bacterial communities of broiler chickens fed diets[2] supplemented with enzyme-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2) at days 14 and 21

		Control	Low ECM	High ECM	E1[3]	E2[4]	SEM	P value
Day 14	Firmicutes	1.04	0.93	0.88	0.98	1.07	0.09	0.537
	Bacteroidetes	0.95	1.61	1.61	1.21	1.06	0.38	0.654
	Clostridium cluster IV	1.06	1.32	1.36	1.34	1.55	0.17	0.392
	Enterococcus spp.	1.06	0.77	0.31	1.19	1.00	0.30	0.288
	Escherichia coli	1.08	0.85	0.41	1.02	1.04	0.19	0.054
	Lactobacillus spp.	1.10^{b}	254^{a}	322^{a}	3.17^{b}	1.85^{b}	26.8	<.001
	Bifidobacterium spp.	1.14	0.90	0.84	1.17	1.24	0.23	0.634
Day 21	Firmicutes	1.02	0.86	0.83	0.91	0.92	0.05	0.094
	Bacteroidetes	0.93^{b}	1.01^{b}	1.58^{ab}	1.26^{b}	2.08^{a}	0.20	0.001
	Clostridium cluster IV	1.09	1.24	1.01	1.38	1.26	0.13	0.270
	Enterococcus spp.	0.91^{ab}	1.09^{ab}	0.04^{b}	1.39^{a}	0.40^{ab}	0.34	0.037
	Escherichia coli	1.00^{a}	0.61^{ab}	0.04^{b}	0.64^{ab}	0.66^{a}	0.16	0.002
	Lactobacillus spp.	0.96^{b}	95.4^{a}	76.1^{a}	13.4^{b}	18.2^{b}	13.6	<.001
	Bifidobacterium spp.	0.99	1.34	1.10	1.09	1.23	0.18	0.640

$^{a-b}$ Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1] The fold change was measured using a $2^{-\Delta\Delta C_T}$ method by normalizing the target gene of selected bacteria to the reference gene of total eubacteria and comparing to the Control treatment.

[2] The composition of the diet is in Table 4.2.

[3] E1- Starter diet supplemented with 1.25 g/kg of multi-carbohydrase; Grower diet supplemented with 2.5 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

[4] E2 - Grower diet supplemented with 12.5 g/kg of multi-carbohydrase; Grower diet supplemented with 25.0 g/kg of multi-carbohydrase. The enzyme supplement contained Pectinase A, Pectinase B, Xylanase B, and Invertase.

Short chain fatty acids in ileal digesta of broiler chickens fed experimental diets on day 14 and day 21 are shown in Figure 4.1. Lactic and acetic acids were predominant in the ileal contents. Acetic acid accounted for 19 - 26% of total SCFA on day 14 and 30 - 45% of total ileal SCFA on day 21. Lactic acid accounted for 64 - 73% of total SCFA on day 14 and 40 - 51% of total ileal SCFA on day 21. The high level of lactic acid in the ileal digesta observed on day 14 was not maintained on day 21.

As is presented in Figure 4.2, the acetic, butyric, and propionic acids dominated in the ceca contents. Acetic acid accounted for 76 - 79% of total SCFA on day 14 and 81 - 85% of total SCFA on day 21. Butyric acid accounted for 15 - 17% of total cecal SCFA on day 14 and 10 - 12% of total cecal SCFA on day 21, and propionic acid accounted for 5 - 8% of total cecal SCFA on days 14 and 21. A significant difference in total SCFA was observed between cecal digesta of birds fed the High ECM and the Control starter diets on day 14. No increase in the SCFA concentration between days 14 and 21 in both segments of the gastrointestinal tract (**GIT**) was observed.

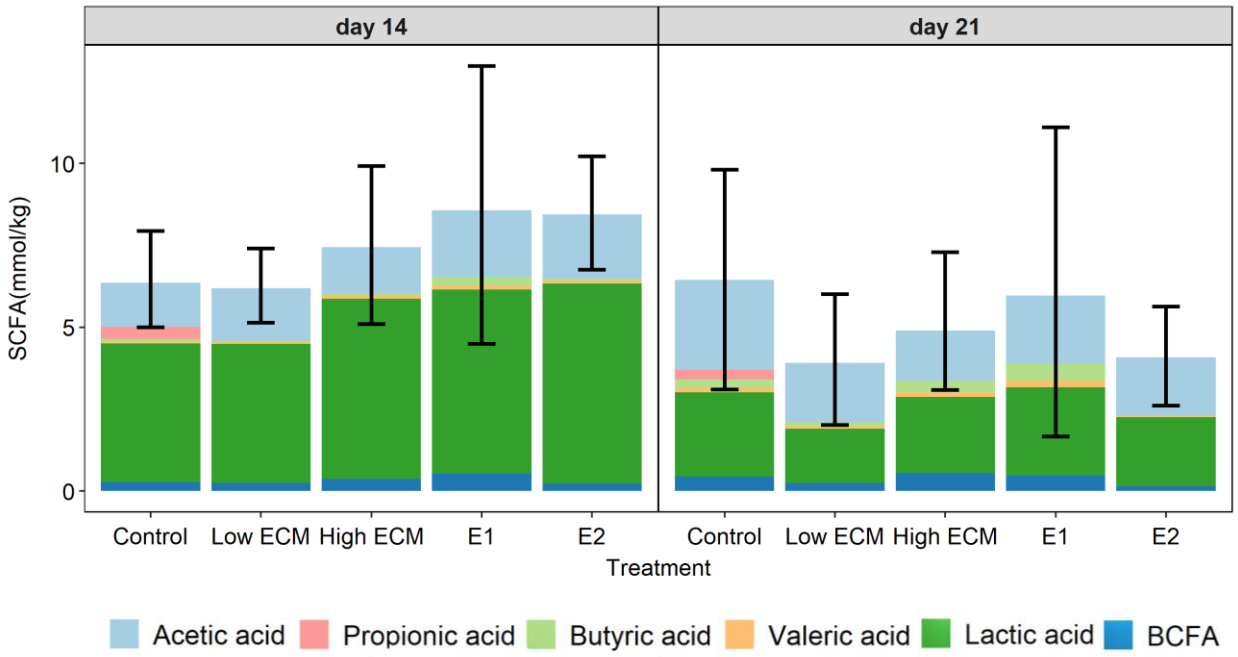


Figure 4.1 Short chain fatty acids (SCFA) content in ileal digesta of broiler chickens fed diets supplemented with enzyme-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2) at days 14 and 21 (mmol/kg of fresh digesta). Data are means with standard deviations represented by vertical bars. BCFA refer to branched chain fatty acids including iso-butyric, 2-methyl butyric, and iso-valeric.

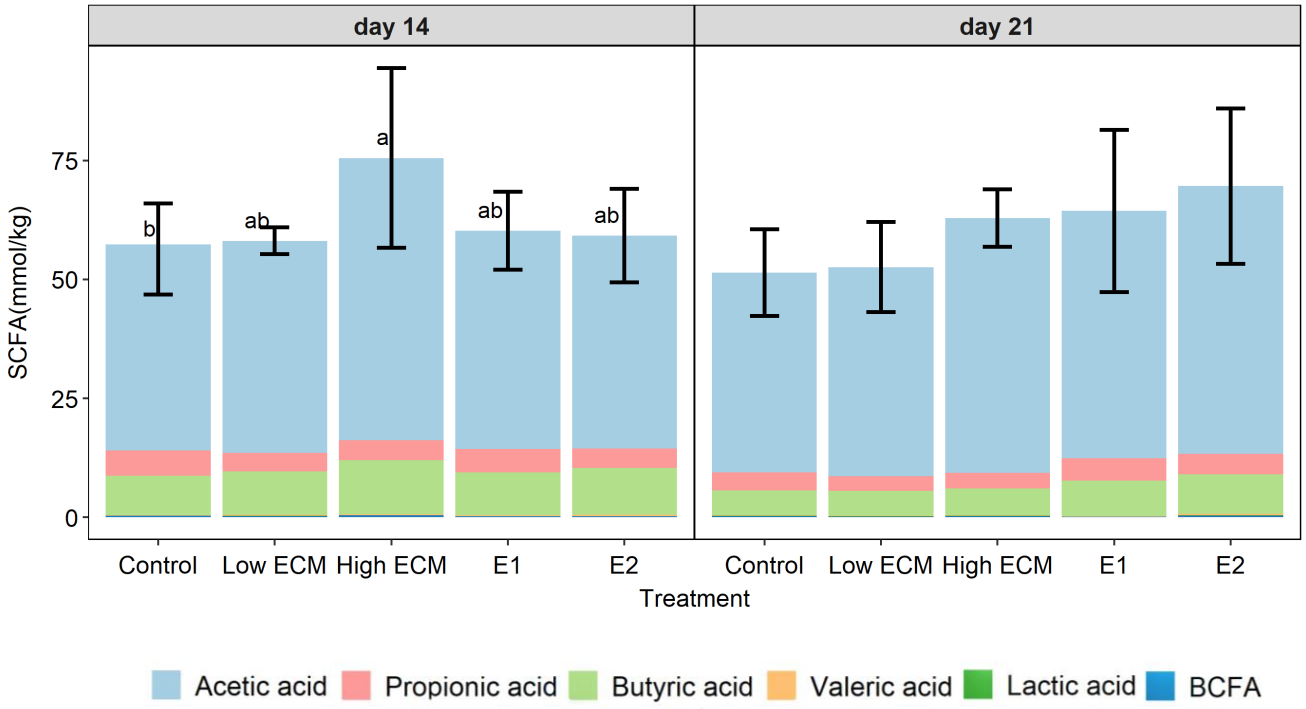


Figure 4.2 Short chain fatty acids content in cecal digesta of broiler chickens fed diets supplemented with enzyme-modified canola meal (Low ECM and High ECM) or carbohydrases (E1 and E2) at days 14 and 21 (mmol/kg of fresh digesta). Data are means with standard deviations represented by vertical bars. Means with no common superscript letters within a subgrouping differ significantly ($P < 0.05$). BCFA refer to branched chain fatty acids including iso-butyric, 2-methyl butyric, and iso-valeric.

4.5 Discussion

Enzymatic modification of CM changed its chemical composition, particularly the composition of dietary fibre, sugars, and phosphorus. When compared to the intact CM, incubation with the enzyme preparation resulted in reduced contents of TDF by 17.7%, NDF and NSP by 14.4%, and 36.5%, respectively, and production of water-soluble NSP hydrolysis products. It means that the reduction of TDF was mainly attributed to the degree of NSP depolymerization thus production of NSP hydrolysis products. Non-starch polysaccharides in CM include cellulose and non-cellulosic polysaccharides, i.e., pectic polysaccharides, arabinans, arabinogalactans, galactomannans, xylans and xyloglucans (Slominski et al., 2012; Do et al., 2020). The procedure of NSP determination involves the measurement of constituent sugars dissociated from the polysaccharide chains. Therefore, the decreased recovery of components sugars would indicate the effect of carbohydrase action and incubation. As demonstrated in our recent research (Niu et al., 2022), the selected multi-carbohydrase cocktail significantly depolymerized NSP in vitro, and it was reflected in the NSP profile of ECM obtained from scaled up processing of CM. It indicated that NSP has been depolymerized into potentially bioactive components, which would include various oligosaccharides, and low-molecular-weight polysaccharides, which, when fed to animals, should exert some prebiotic properties.

During the production of ECM, the content of all analyzed component sugars of NSP was reduced, with the lowest recovery of arabinose. It is in agreement with research by Meng et al. (2005) and Pustjens et al. (2013) in which the highest enzymatic depolymerization of arabinose of CM was observed. It can be due to the specific structure of NSP of CM where arabinose residues are mainly in terminal branches of polysaccharides (Pustjens et al., 2013).

When compared with intact CM, sucrose level in ECM dropped from 78.4 to 0.8 g/kg, while raffinose and stachyose, the main oligosaccharides of CM, were completely hydrolyzed, therefore not detected in ECM. This would be attributed to the action of invertase and, to some extent, to the effect of spontaneous fermentation that occurred during the incubation of CM with enzymes.

In processing of ECM, we allowed for much longer incubation time (i.e., 48 h) than that when enzymes are fed directly as dietary supplements. Spontaneous fermentation was promoted by not implementing any preservatives to facilitate lactic acid bacteria growth, lactic acid production, and a significant decrease in the slurry pH. During liquid enzymatic incubation of feed ingredients, spontaneous fermentation had been observed. In one study, xylanase in concert with the enzyme preparation obtained from *Aspergillus aculeatus* increased NSP solubility, decreased total NSP, reduced the solution viscosity of feed ingredients, including wheat, barley, wheat bran and soybean meal, and spontaneous fermentation was observed (Christensen et al., 2007). Jakobsen et al. (2015) reported that pretreat rapeseed cake with carbohydrase blend can decrease the total and insoluble NSP contents and lead to the production of lactic and acetic acids. In addition, some positive properties of fermented canola meal products have been recently reported (Drazbo et al., 2018; 2019; Wu et al., 2021). Fermentation of rapeseed meal resulted in increased protein level, reduced crude fibre, ADF, NDF, phytate-phosphorus and total glucosinolates contents (Drazbo et al., 2018; Alhomodi et al., 2021), and improved growth performance, antioxidant capacity and meat quality of broiler chickens (Ashayerizadeh et al., 2017; 2018; Wu et al., 2021).

As presented in Table 4.1, phytate phosphorus was not detected in ECM, while in CM, over 50% of total phosphorus is associated with phytic acid. This result indicated that the

enzymatic modification process during the production of ECM would improve the phosphorus availability of CM. The enzyme blend used in the study did not contain phytase preparation, however, the hydrolysis of phytic acid could be catalyzed by endogenous CM phytase and bacteria involved in the spontaneous fermentation process that occurred during the production of ECM (Lei et al., 2013). Therefore, it can be concluded that modification of CM with enzymes would not only change the composition and structure of dietary fibre but would also improve the availability of phosphorus.

The nutritional value of ECM for poultry and the effect of ECM and carbohydrase enzymes on growth and microbiota status were evaluated in this study with broiler chickens. No significant differences in FI, BWG, and FCR between the two ECM-containing diets and the Control diet were observed during the starter and grower phases and the entire trial.

Non-starch polysaccharides of CM are generally poorly digestible in broilers. When diets were supplemented with carbohydrase enzymes, the NSP digestibility improved in most cases (Meng et al., 2005; Meng and Slominski, 2005). In the current study, the replacement of CM with ECM and supplementation of carbohydrase enzymes improved dietary ATTD of NSP ($P < 0.001$) in both starter and grower periods. As presented in Table 4.2, the content of NSP in diets containing ECM was lower due to enzymatic modification of CM. However, NSP of diets containing ECM were further digested. It might represent the benefit of the depolymerization of NSP and production of low molecular weight NSP which are more accessible for enzymes and microorganisms.

Although it is believed that exogenous carbohydrases can break down NSP, release nutrients and improve the energy of diets, the increase of AMEn in diets is not always observed or statistically significant when enzymes are added to the broiler diets (Kocher et al., 2000; Meng et al., 2005; Mushtaq et al., 2007; Amerah et al., 2017; Pirgozliev et al., 2022). Results of the current

study showed a similar tendency towards increased metabolizable energy content of diets supplemented with carbohydrase, yet such changes were not significant ($P > 0.05$). However, the substitution of CM with ECM significantly increased AMEn during the starter period. Overall, the High ECM diets were significantly higher in AMEn than other diets.

In the present study, the replacement of CM with ECM significantly decreased the secretion of sialic acids in the grower phase. Sialic acids are components of the cellular mucin of the GIT and are indicators of endogenous secretion when determined in digesta or excreta (Cowieson et al., 2003). Sialic acids, together with fucose, galactose, N-acetylgalactosamine, and N-acetylglucosamine, account for about 80% of the total mucin molecule by weight (Bansil and Turner, 2006; MacMillan et al., 2019) and are regarded as glycan markers of mucin secretion (Miner-Williams et al., 2009). Feed composition and supplementation with enzymes may influence the secretion of sialic acids (Cowieson et al., 2003; Hejdysz et al., 2018; Vila et al., 2018; dos Santos et al., 2019; Kubiś et al., 2020). No significant effect of dietary fibre on sialic acids secretion in GIT of broiler chickens fed the corn-rice bran-soybean meal diet was reported by dos Santos et al. (2019), but a significant effect of water-soluble NSP and raffinose on sialic acids secretion increasing was observed in broilers fed diets containing different lupin species (Hejdysz et al., 2018). Dietary supplementation with phytase was reported to decrease sialic acids secretion and endogenous losses of minerals and amino acids in broiler chickens (Cowieson et al., 2003). According to Kubiś et al. (2020) and Vila et al. (2018), carbohydrase enzyme supplementation did not affect the sialic acids secretion. Likewise, in the present study multi-carbohydrase enzymes did not impact the secretion of sialic acids, and the observed decrease in sialic acids secretion was most likely influenced by the lower NSP level in the ECM diet.

Although the pH value of ECM slurry was remarkably lower than CM and lactic acid was present in ECM, ECM containing diet did not lower the pH of ileal and cecal digesta. As lactic acid is easily absorbed by upper gut, no reduction of pH in ileum and ceca of birds fed ECM diets in comparison to Control treatment could be expected (Kim et al., 2015).

The intestinal microbiota of chickens, including commensal, symbiotic, and pathogenic microorganisms, plays an important role in the gut health and immune system of the host. Among them, Firmicutes, Bacteroidetes, and Proteobacteria are dominant (Wei et al., 2013). As the gut microbiota is complex and diversified, only the most relevant microorganisms were subjected for measurement of the abundance in the current study. Firmicutes and Bacteroidetes were selected because they are the most abundant phyla in the gut microbiota of chickens (Li et al., 2016). *Enterococcus spp.* is a group of commensal bacteria that are commonly found in GIT and associated with the production of lactic acid (Silva et al., 2012; Han et al., 2016). *Lactobacillus spp.* and *Bifidobacterium spp.* are prevalent organisms in gut microbiota and have beneficial effects on host animals. *Clostridium cluster IV* which is associated with fibre digestion and butyrate production (Ricke et al., 2020) and *Escherichia coli* are typically not highly abundant in the ileum of healthy birds; therefore, they were measured only in the cecal contents. In this study, no significant differences were observed in the abundance of *Lactobacillus spp.* and *Escherichia coli* between Control and both enzyme-supplemented diets, while the increased abundance of *Lactobacillus spp.* in ileum and ceca and a lower abundance of *Escherichia coli* in ceca was observed in birds fed ECM diets which is highly likely due to the probiotic and prebiotic effects of ECM. In addition, the presence of lactic acid may also support proliferation of *Lactobacillus spp.* and suppress the growth of *Escherichia coli* (Byrd et al., 2001; Sugiharto and Ranjitkar, 2019).

The gut microorganisms can ferment undigested feed components or mucins and produce SCFA, which can provide energy for intestinal epithelium cells, reduce the pH of the gut, maintain intestinal barrier integrity and thus improve gut health (Dalile et al., 2019). In poultry research, the SCFA are analyzed mainly in the ceca, as intensive fermentation occurs there. However, the physiological processes related to the action of feed additives could also be expressed in the ileum, therefore in the current study, both the cecal and ileal contents were subjected for measurement of SCFA. As demonstrated in Figure 4.1 and Figure 4.2, butyric, acetic, and propionic acids were predominant in cecal digesta which is consistent with the results of other studies with poultry (Toghyani et al., 2017; Kheravii et al., 2018; Konieczka et al., 2019; Shabani et al., 2019; Adewole, 2020). The level of lactic acid in cecal digesta was low, which would agree with the results of research by Xue et al. (2017) and Kim et al. (2020). The lactate content belongs to the top two fatty acids produced in the ileum (Ptak et al., 2015; González-Ortiz et al., 2019), which would agree with the ileal digesta fatty acids profile in this study. As birds were growing (from d 14 to 21), no obvious changes in the SCFA content of cecal digesta were observed. The total SCFA concentration of ileal digesta on day 21 was lower than that on day 14. This result is similar to the study that monitored ileal SCFA on days 1, 7, 14, 21, and 42 (Liao et al., 2020), where despite the tendency toward the increase in total SCFA as birds grow, SCFA content decreased on day 21 when compared with day 14.

Despite the significant fold change in *Lactobacillus spp.* in both ileum and ceca, no effect of ECM on the SCFA concentration was observed when compared to the Control treatment, except for higher acetate concentrations in the ceca of birds fed the High ECM diet on day 14. No relationship was observed between the relatively high abundance of *Lactobacillus spp.* and the concentration of lactic acid as it could be produced by various other microorganisms in the gut. It

should be emphasized that the concentration of SCFA in ileal and cecal digesta illustrates the difference between the production and the absorption of SCFA. As SCFA produced in the GIT are absorbed fast and used effectively for the gut cell proliferation, poor repeatability in the measurement of concentration of SCFA in the ileal and cecal digesta has been observed (González-Ortiz et al., 2019; 2020). Supplementation of poultry diets with carbohydrase has been reported to increase the abundance of *Bifidobacterium spp.* and total SCFA content in cecal under certain conditions or could depend on the type of feed used, thus substrate for enzyme (Morgan et al., 2022). In the current study, carbohydrase enzyme supplementation had no significant effect on selected beneficial bacteria and total SCFA content in cecal and ileal digesta, similarly to that reported earlier by González-Ortiz et al.(2019). Although enzyme supplementation can produce prebiotic type compounds for poultry, the effect, due to their relatively small amount, on the production of SCFA and growth of beneficial microbiota may be inadequate.

In conclusion, the incubation of canola meal with a blend of select enzymes resulted in a significant depolymerization of non-starch polysaccharides. Although replacing canola meal with its enzymatically-modified counterpart did not improve the growth performance of broilers, it positively affected gut health by modulating mucin secretion, microbiota community, and cecal concentration of short chain fatty acids. Addition of enzymatically-modified canola meal to the broiler chicken diet increased the presence of probiotic organisms and prebiotics in the gut and had more pronounced effect on the gut environment than the dietary enzyme supplementation. Enzyme supplementation improved nutrient digestibility, due to their high concentration, but this strategy demonstrated somewhat lower potential towards modulating the gut microbiome when compared to the enzymatically-modified canola meal. Therefore, enzymatically-modified canola meal could be used as a value-added product.

CHAPTER FIVE : MANUSCRIPT III

Enhancing the Nutritive Value of Canola Meal for Broiler Chickens through Enzymatic Modifications

5.1 Abstract

Enzymatic modification of canola (*Brassica napus* L.) meal (**CM**) is a potential way to enhance its nutritional value as it can depolymerize non-starch polysaccharides (**NSP**) and mitigate its potential antinutritive properties. Based on previous studies, pectinase A (**PA**), pectinase B (**PB**), xylanase B (**XB**), and invertase (**Inv**) were used for the enzymatic modifications. The highest NSP depolymerization ratio was obtained when 4 g/kg of each PA, PB, and XB and 0.2 g/kg of Inv were used during incubation. During the enzymatic modification (**CM+E**) of CM, changes in pH, simple sugars, sucrose, oligosaccharides, and NSP contents were monitored and compared to a processing procedure without enzyme addition (CM Control) or with the addition of a bacteriostat, sodium azide (**CM+E+NaN_3**). The results showed that spontaneous fermentation occurred during the incubation. After 48-hour incubation, the pH of the slurry decreased, lactic acid was produced, phytate disappeared and the concentration of simple sugars decreased significantly. The NSP of the slurry was continuously depolymerized by the enzyme blend. The chemical composition and nutritive value of enzymatically-modified CM (**ECM**) were evaluated. Ross 308 broilers were randomly assigned to 18 cages of 6 birds each for the determination of standardized ileal digestibility (**SID**) of amino acids (AA) and nitrogen-corrected apparent metabolizable energy (**AMEn**) assay. A corn/soybean meal-based basal diet formulated to meet Ross 308 breeder recommendations and two test diets contained 70% of the basal diet and 30% of CM or ECM, respectively, were fed to Ross 308 from 13 to 17 d of age. From 1 to 12 d of age, birds were fed the basal diet. Chromic oxide was included in all diets as an indigestible marker. No significant difference ($P > 0.05$) was observed between SID of AA of CM and ECM. The AMEn value of ECM was 2118.0 kcal/kg on a dry matter basis which was 30.9% higher ($P < 0.05$) than that of the CM.

Key words: carbohydrase, enzymatically-modified canola meal, lactic acid, nitrogen-corrected apparent metabolizable energy, non-starch polysaccharides, standardized ileal digestibility of amino acids

5.2 Introduction

Canola meal (**CM**) is a co-product of the canola oil processing industry and the second most abundant feed protein supplement (Canola Council of Canada, 2019) which, on a dry matter (**DM**) basis, contains 20-23% of non-starch polysaccharides (**NSP**) (Adewole et al., 2017b; Radfar et al., 2017) that are almost undigestible by poultry (Slominski and Campbell, 1990; Khajali and Slominski, 2012) and partly fermentable by swine (Long et al., 2020).

Supplementation of diets with carbohydrases is a practical way to improve the nutritive value of CM. (Slominski, 2010). In our previous study, a carbohydrase blend that demonstrated high efficiency in NSP depolymerization was selected using the in vitro and in vivo experimentation (Niu et al., 2022). It has been hypothesized that the enzymatic hydrolysis of NSP would eliminate their anti-nutritive properties and release the prebiotic-type components, including low-molecular-weight polysaccharides and oligosaccharides, which in a manner similar to prebiotics, may facilitate the proliferation of beneficial bacteria for gut function and health bacteria such as *Bifidobacterium* and *Lactobacillus* thereby decreasing the abundance of pathogens. Such enzyme hydrolysis products may indirectly prohibit the growth of certain pathogenic species by increasing intestinal lumen acidity through an increase in lactic acid production and by competitive exclusion of pathogens from the binding sites of the gastrointestinal tract (Slominski, 2010; Helm et al., 2020). However, the complexity of NSP and the short transit time of digesta in both poultry and swine may be the reasons why dietary supplemental enzymes cannot fully

perform their function. Therefore, enzymatic modification of CM becomes a promising means to enhance its nutritive value.

Carbohydrase modification has been proven to be able to increase the solubility of NSP in feed ingredients and may improve their nutritive value. Soy flour carbohydrates incubated with carbohydrase broths from *Aspergillus niger*, *Aspergillus aculeatus*, and *Trichoderma reesei* fermentations containing cellulase, xylanase, pectinase, and galactosidase activities significantly increased solubility of carbohydrates (Loman and Ju, 2016). Enzymatic hydrolysis of brewers' spent grain using carbohydrases and proteases resulted in a reduction of arabinose and xylose in the insoluble residues, which are likely to originate from arabinoxylans (Robertson et al., 2011). Nine enzymes or enzyme combinations were used to improve the nutritional value of rapeseed cake. During the process of incubation, spontaneous fermentation occurred, the abundance of lactic acid bacteria *Enterobacteriaceae* and yeasts increased, and acetic acid and lactic acid were produced. Only one enzyme blend containing β-glucanase, xylanase, and pectinase activities significantly decreased the total and insoluble NSP contents. When phytase was included, the reduction of phytate phosphorous content was pronounced (Jakobsen et al., 2015). Our earlier study had shown that replacing CM with enzymatically-modified CM (**ECM**) improved the apparent total tract digestibility of NSP and nitrogen-corrected apparent metabolizable energy (**AMEn**) of diets and increased the abundance of *Lactobacillus spp.* in ileal and cecal digesta of broiler chickens (Chapter Four).

Although carbohydrase enzymes have been shown to depolymerize NSP (Jakobsen et al., 2015), the dynamics of pH, sugars, and NSP changes during enzymatic treatment have not been reported. In addition, whether spontaneous fermentation could enhance NSP depolymerization has not been explored. More importantly, the influence of enzymatic treatment on standardized ileal

digestibility (**SID**) of amnio acids (**AA**) and AMEn content has not been reported even though these parameters are vital for feed formulation. Therefore, this study aimed to optimize the production of enzymatically-modified CM, monitor the dynamics of pH, simple sugars, sucrose, oligosaccharides, and NSP changes during the process, lactic acid production, phytate disappearance, and to evaluate their effects on the nutritive value of ECM for broiler chickens.

5.3 Materials and methods

The animal care protocol for this study was approved by the Animal Care Committee of the University of Manitoba (F20 - 001/1, AC11558).

5.3.1. Materials

The CM used for ECM production and the animal trial was produced from black-seeded *B. napus* canola by pre-press solvent extraction process and was obtained from Bunge Canola Processing Plant, Altona, Manitoba. Canada.

Carbohydrase preparations **PA** (Pectinase A, 443,061 U/g), **PB** (Pectinase B, 425,773 U/g), **XB** (Xylanase B, 60,763 U/g), and **Inv** (Invertase, 362,631 U/g) were provided by CBS Bio Platforms Inc., Calgary, Alberta, Canada.

5.3.2 Optimization of ECM production

A small-scale pilot study was carried out to determine the optimal concentration of carbohydrases for targeting the NSP of CM and the production of ECM. The efficiency of the processing was demonstrated by reduced recoveries of total NSP compared to CM control.

The test towards the optimization of production was performed in 1 L flasks with 200 g of CM, 800 mL of distilled water, and test amount of enzyme preparations (as in Table 5.1) for 48 h at 40°C in the incubator shaker (New Brunswick Benchtop Incubator Shaker, I24, Eppendorf

Biotools, Canada). After 48 h, samples were collected, immediately frozen at -20°C, and then freeze-dried.

5.3.3 Dynamics of pH, sugars, and NSP changes during ECM production

The study of dynamics of pH, simple sugars, sucrose, galactooligosaccharides, and NSP changes during ECM production was performed in 100 mL flasks with 10 g of CM and 40 mL of distilled water (**CM Control**), or 10 g of CM, 40 mL of distilled water and optimized concentration of carbohydrases (**CM+E**), or 10 g of CM, 40 mL of distilled water, optimized concentration of carbohydrases and 0.05% of NaN_3 (**CM+E+NaN_3**) for 8, 16, 24, 36, and 48 h at 40°C with constant mixing (Islam et al., 2018). After each interval, the pH of the samples was measured immediately, and then the samples were frozen at -20°C and freeze-dried for further analyses.

5.3.4 Preparation of ECM for animal studies

The ECM production was carried out in a 15 L container with 2 kg of CM, 8 L of distilled water, and the optimized concentration of carbohydrases. The incubation was carried out in a water bath for 48 h at 40°C with continuous mixing (BDC 2002, Cafromo LabSolutions, Ontario, Canada). During the incubation, pH was monitored, and the concentration of lactic acid was measured (0, 8, 18, 24, 42, and 48 h). This procedure was repeated several times to produce sufficient amounts of material for the animal trial. After each incubation run, the samples were frozen, freeze-dried, and then combined and ground to pass through a 2 mm sieve.

Table 5.1 Concentrations of individual enzymes in enzyme blends used for in vitro pilot incubations

Treatment	Enzyme, g/kg of canola meal			
	Pectinase A	Pectinase B	Xylanase B	Invertase
Control (no enzyme)	0	0	0	0
Pectinase+xylanase+invertase - 1	1	1	1	0.1
Pectinase+xylanase+invertase - 2	2	2	2	0.2
Pectinase+xylanase+invertase - 3	3	3	3	0.2
Pectinase+xylanase+invertase - 4	4	4	4	0.2

5.3.5 Standardized ileal amino acid digestibility and AMEn assay

The diets for the assay included a corn/soybean meal-based basal mash diet (Table 5.2) that was formulated to meet Ross 308 breeder recommendations (Aviagen, 2019) and 2 test diets containing 70% of the basal diet and 30% of CM or ECM, respectively. Chromic oxide (Cr_2O_3) was used as an indigestible marker. One-day-old as-hatched Ross 308 broiler chickens were obtained from a local hatchery. After arrival, birds were weighed, divided into 18 cages of 6 birds each, and raised in electrically heated Super Brooders (Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR, USA) under a controlled environment. The temperature was monitored daily and adjusted according to the breeder recommendations. Birds were fed the basal diet from 1 to 12 d of age. On day 13, the basal diet and 2 test diets were randomly assigned to the cages (6 cages of each). On day 17, excreta samples were collected, immediately frozen at -20°C, and then freeze-dried. On the same day, all birds were euthanized using carbon dioxide. Ileal digesta was collected by gently squeezing the contents of the ileum from Meckel's diverticulum to a point 4 cm proximal to the ileocecal junction into sample bags. The samples were frozen immediately after collection and then freeze-dried.

Table 5.2 Composition of the basal diet (g/kg, as-fed basis)

Ingredient	Basal diet
Corn	545.0
Soybean meal	368.9
Vegetable oil	33.6
Calcium carbonate (Limestone)	15.4
Monocalcium phosphate (Biofos)	13.0
L-Lysine	2.5
DL-Methionine	2.0
Threonine	1.6
Mineral Premix [a]	5.0
Vitamin Premix [b]	10.0
Cr_2O_3	3.0
	1000
Calculated composition	
Metabolizable energy (kcal/kg)	3000
Crude protein	230.0
Calcium	9.6
Available phosphorus	4.8
Digestible Lysine	12.8
Digestible Methionine	5.2
Digestible Methionine + cysteine	9.5
Digestible Threonine	8.6
Digestible Arginine	13.7
Analyzed composition (g/kg)	
Crude protein	230.0
AMEn (kcal/kg)	2920

[a] Provided per kg of diet: 70 mg Mn, 80 mg Zn, 80 mg Fe, 10 mg Cu, 0.3 mg Se, 0.5 mg I, 337 g Na.

[b] Provided per kg of diet: 8250 IU vitamin A, 3000 IU vitamin D3, 30 IU vitamin E, 0.13 mg vitamin B12, 2 mg vitamin K3, 6 mg riboflavin, 40.3 mg niacin, 1301 mg choline, 4 mg folic acid, 0.25 mg biotin.

5.3.6 Chemical analysis

Canola meal, ECM, diets, excreta, and ileal digesta samples were ground to pass through a 1 mm sieve before analysis. Samples were analyzed in duplicate.

Standard AOAC (2005) methods were used for dry matter (945.15), crude protein and nitrogen (968.06), ether extract (920.39), ash (923.03), and total phosphorus (965.17) analysis. Neutral detergent fibre (**NDF**) was measured according to AOAC procedure 2002.04 by an Ankom fibre analyzer (Ankom Technology, Macedon, NY, USA). Sugars, total dietary fibre (**TDF**), NSP, and water-soluble NSP were analyzed as described by Slominski et al. (1990; 1994). Phytate phosphorus was determined according to the procedure of Haug and Lantzsch (1983). Lactic acid was analyzed using the Megazyme L-Lactic Acid Assay Kit (Megazyme International Ireland Ltd., Co. Wicklow, Ireland).

Chromium of diet, excreta, and ileal digesta samples were analyzed by inductively coupled plasma atomic emission spectroscopy (ICP-AES Vista, Varian, USA) using AOAC 985.01 method (2005). The gross energy of diets and excreta samples was determined by combustion of samples in a Parr adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, IL). The nitrogen content of excreta and diets was determined for AMEn calculations. Amino acids of ingredients, diets, and ileal digesta samples were analyzed by the AOAC method (994.12).

5.3.7 Calculations and statistical analysis

The nitrogen corrected apparent metabolizable energy (AMEn) was calculated using the following equations (Hill et al., 1960; De Groote et al., 1971):

$$\mathrm{AME_{n\ diet}(kcal/kg)} = GE_{kcal/kg\ diet} - GE_{kcal/kgexcreta} \times \frac{Cr_2O_{3\%diet}}{Cr_2O_{3\%excreta}}$$

$$- 8.22 \times \left(N_{g/kg\ diet} - N_{g/kg\ excreta} \times \frac{Cr_2O_{3\%diet}}{Cr_2O_{3\%excreta}} \right)$$

Equation 5.1

AMEn of ingredients = (AMEn of test diet -0.7× AMEn of basal diet)/0.3

Equation 5.2

Where **GE** refers to gross energy, Cr_2O_3 refers to chromic oxide, and **N** refers to nitrogen.

The apparent ileal AA digestibility (**AIAAD**) and Standardized ileal AA digestibility (**SIAAD**) of diets and ingredients were calculated by the following equations (Stein et al., 2005; Nalle et al., 2010; Adewole et al., 2017a):

$$\text{AIAAD of diet (\%)} = \left(1 - \frac{Cr_2O_{3\%diet} \big/ Cr_2O_{3\%\ ileal\ digesta}}{AA_{\%diet} \big/ AA_{\%\ ileal\ digesta}}\right) \times 100$$

Equation 5.3

SIAAD of diet (%) = AIAAD + (basal IAA_{end}/AA_{diet}) × 100

Equation 5.4

Where basal **IAA_{end}** refers to basal ileal endogenous AA loss which was determined previously with a nitrogen-freed diet in our laboratory.

SIAAD of ingredients (%) =

(SIAAD of test diet ×AA of test diet-0.7×SIAAD of basal diet ×AA of basal diet)

/ (0.3× AA of the ingredient)

Equation 5.5

All the statistical analysis was performed by the SAS program (version 9.4, SAS Institute Inc., Cary, NC) using the MIXED procedure. Treatment means were compared using Tukey's honestly significant difference. Linear and quadratic effects of enzyme concentration were analyzed by orthogonal polynomial contrasts.

All statements of significance were based on $P < 0.05$.

The figures were made with R studio (Version 1.4.1106) software with ggplot2 (Version 3.3.3).

5.4 Results

5.4.1 Optimization of ECM production

The effect of enzyme concentration (Table 5.1) on total NSP and NSP component sugar contents of canola meal after 48h incubation is presented in Table 5.3. The total NSP of canola meal incubated with enzyme cocktail decreased with the increasing concentration of the enzyme blend and was significantly lower than that of the Control treatment. The content of total NSP and their component sugars including arabinose, xylose, galactose, glucose, and uronic acids were linearly and quadratically reduced with increasing enzyme concentration ($P < 0.05$). When adding 4 g/kg of each of PA, PB, and XB, 47.9% of total NSP of CM was depolymerized. As for the NSP component sugars, 73.3% of arabinose, 68.8% of xylose, 45.3% of galactose, 50.5% of glucose, and 27.4% of uronic acid residues were enzymatically released from the NSP of CM.

Table 5.3 Effect of different concentration of enzyme blends on the content of total non-starch polysaccharides (NSP) and their component sugars after 48h incubation of canola (*Brassica napus* L.) meal (dry matter, g/kg)

Treatment[1]	Component sugar					Total NSP[2]
	Arabinose	Xylose	Galactose	Glucose	Uronic Acids	
Control (no enzyme)	46.4[a]	16.4[a]	15.5[a]	78.3[a]	82.5[a]	242.6[a]
Pectinase+xylanase+invertase - 1	25.7[b]	7.9[b]	11.6[b]	52.2[b]	72.1[b]	172.4[b]
Pectinase+xylanase+invertase - 2	19.9[c]	7.8[b]	10.7[c]	43.9[c]	73.4[b]	158.2[c]
Pectinase+xylanase+invertase - 3	17.0[c]	6.3[c]	9.8[d]	40.1[d]	62.3[bc]	140.6[d]
Pectinase+xylanase+invertase - 4	12.4[d]	5.1[c]	8.5[e]	38.7[d]	59.8[c]	126.5[e]
SEM	0.795	0.252	0.143	0.652	1.465	1.790
P value						
Model	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	<0.0001
Linear	0.0002	0.0016	<0.0001	0.0007	<0.0001	<0.0001
Quadratic	<0.0001	0.0004	<0.0001	<0.0001	0.0005	<0.0001

[a-e] Means within a column with no common superscripts differ significantly ($P < 0.05$).

[1] The concentration of enzyme cocktail is in Table 5.1.

[2] Includes small amounts of rhamnose and mannose.

5.4.2 Dynamics of pH, sugars, NSP and lactic acid changes during ECM production

The pH changes in the incubation slurry are shown in Figure 5.1. It demonstrates that the decreasing rate of pH value in the slurry of CM Control treatment was slower than that of CM+E treatment. The changing rate of pH value in CM+E+NaN_3 treatment was similar to that of CM Control treatment before 32 h, then the pH value dropped rapidly. After 48 h incubation, the pH values of CM Control, CM+E, and CM+E+NaN_3 treatment were 5.38, 4.02, and 4.14, respectively.

The dynamics of carbohydrate contents during CM incubation are illustrated in Figure 5.2. It demonstrates that simple sugars decreased dramatically after 48 h of incubation in all treatments although their dynamics were quite different in CM+E and CM+E+NaN_3 and indicative of their increased production due to enzyme addition and much slower utilization by bacteria in the presence of NaN_3. Therefore, the CM+E+NaN_3 treatment had the slowest speed of simple sugars disappearance and their highest concentration after 48 h incubation. Before 32 h, the content of simple sugars in CM+E treatment was always higher than that of CM Control treatment, while it was up to 2.5 g/kg in both treatments after 32 h. Simple sugars increased significantly between 0 to 8 h and decreased remarkably after 8 h in CM+E treatment; while in CM+E+NaN_3 treatment, simple sugars increased significantly from 0 to 8 h, then stayed constant at a relatively high level until 32 h of incubation. For the CM Control treatment, simple sugars increased between 8 to 16 h and then decreased after 16 h. The contents of sucrose and galactooligosaccharides were identical for CM+E and CM+E+NaN_3 treatments and decreased dramatically after 8 h of incubation.

Figures 5.2 and 5.3 show the dynamics of NSP contents during CM incubation. As illustrated in Figure 5.2, the total NSP was fairly stable in CM Control treatment, while in CM+E and CM+E+NaN_3 treatments, total NSP decreased as the incubation progressed. In addition, the total NSP of CM+E treatment was lower than that of CM+E+NaN_3 after 48 h of incubation.

Arabinose and glucose residues contributed to the top two component sugars of NSP depolymerization (Figure 5.3).

The dynamics of lactic acid changes during ECM production are shown in Figure 5.4. It clearly demonstrates that lactic acid was produced continuously during the incubation.

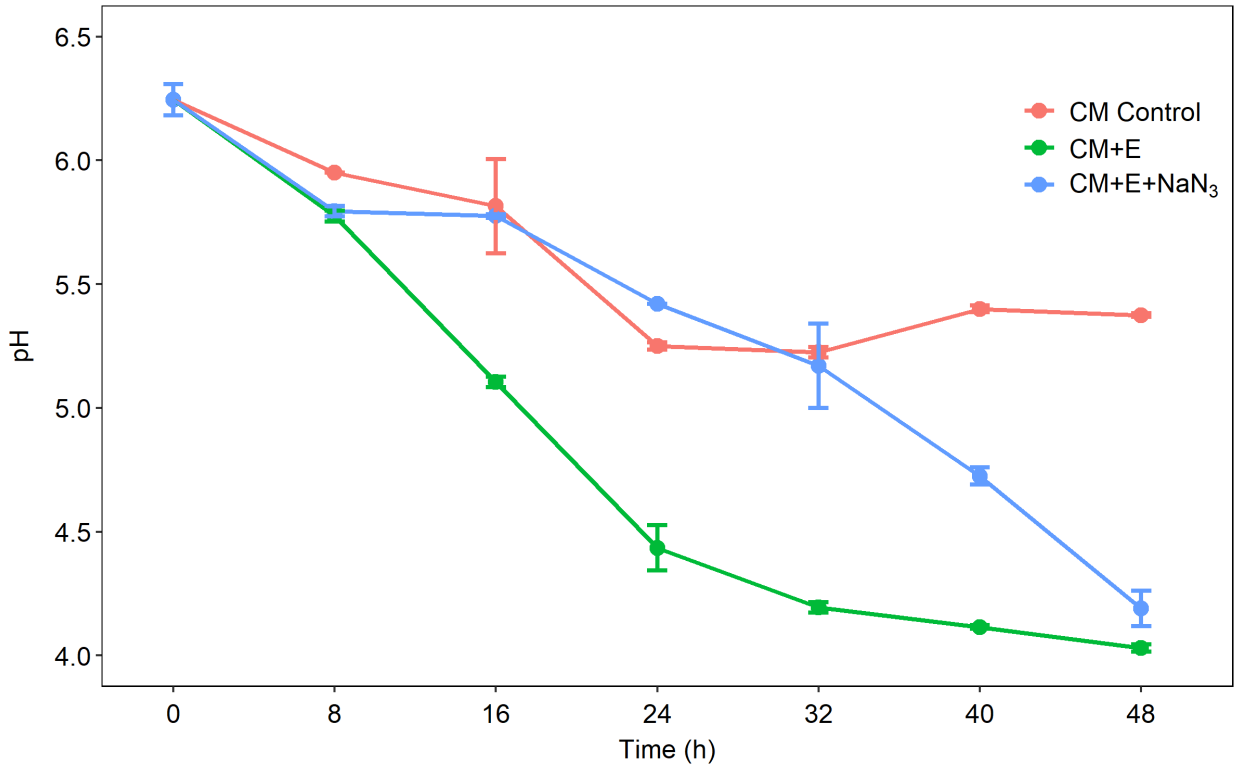


Figure 5.1 Dynamics of pH changes during canola (*Brassica napus* L.) meal incubation. CM Control: canola meal incubated in water; CM+E: canola meal and enzyme blend incubated in water; CM+E+NaN_3: canola meal, enzyme blend, and 0.05% of NaN_3 incubated in water. Data are means with standard deviations represented by vertical bars.

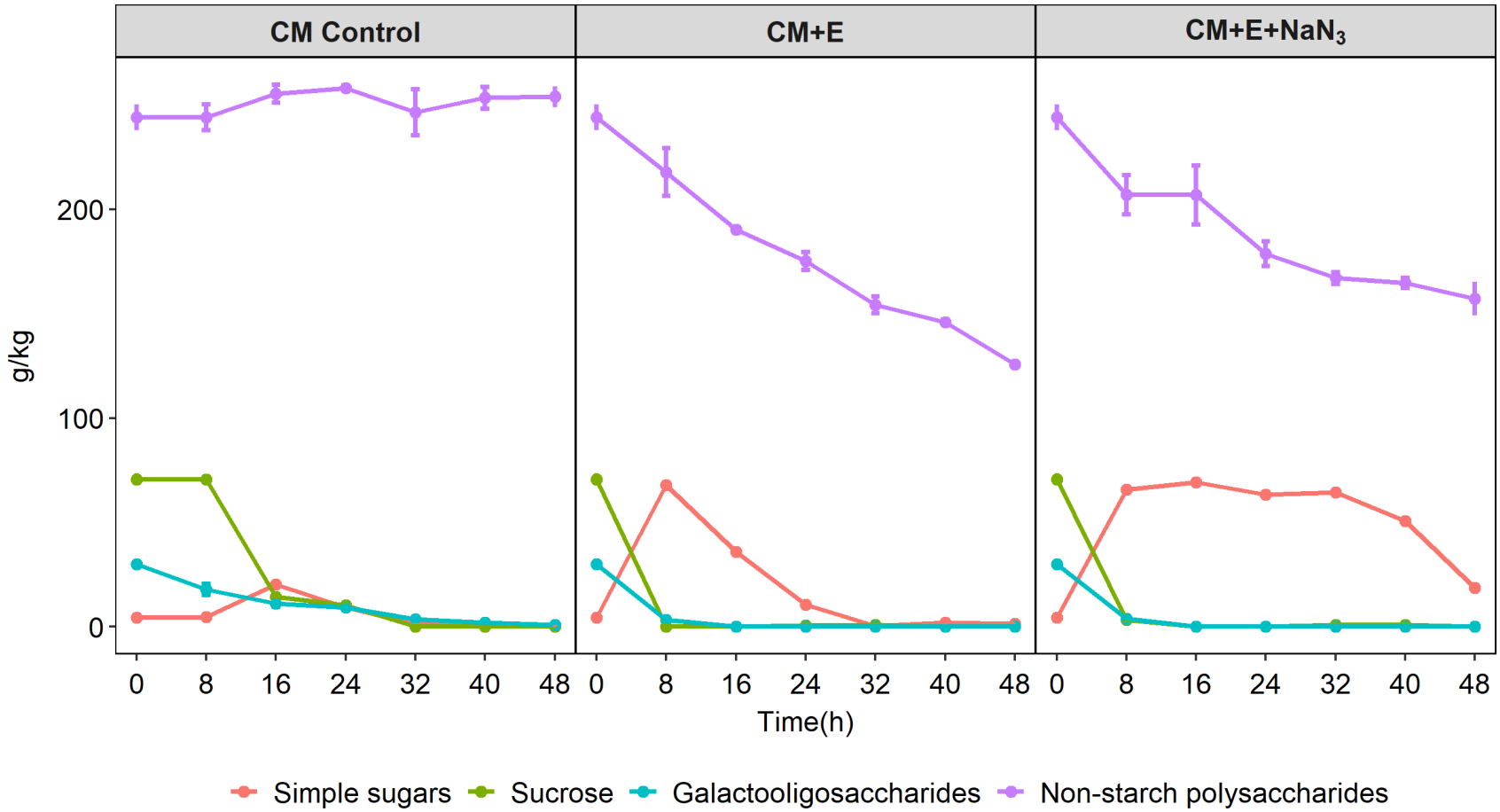


Figure 5.2 Dynamics of carbohydrate changes during canola (*Brassica napus* L.) meal incubation (g/kg). CM Control: canola meal incubated in water; CM+E: canola meal and enzyme blend incubated in water; CM+E+NaN_3: canola meal, enzyme blend, and 0.05% of NaN_3 incubated in water. Data are means with standard deviations represented by vertical bars. Galactooligosaccharides include raffinose and stachyose.

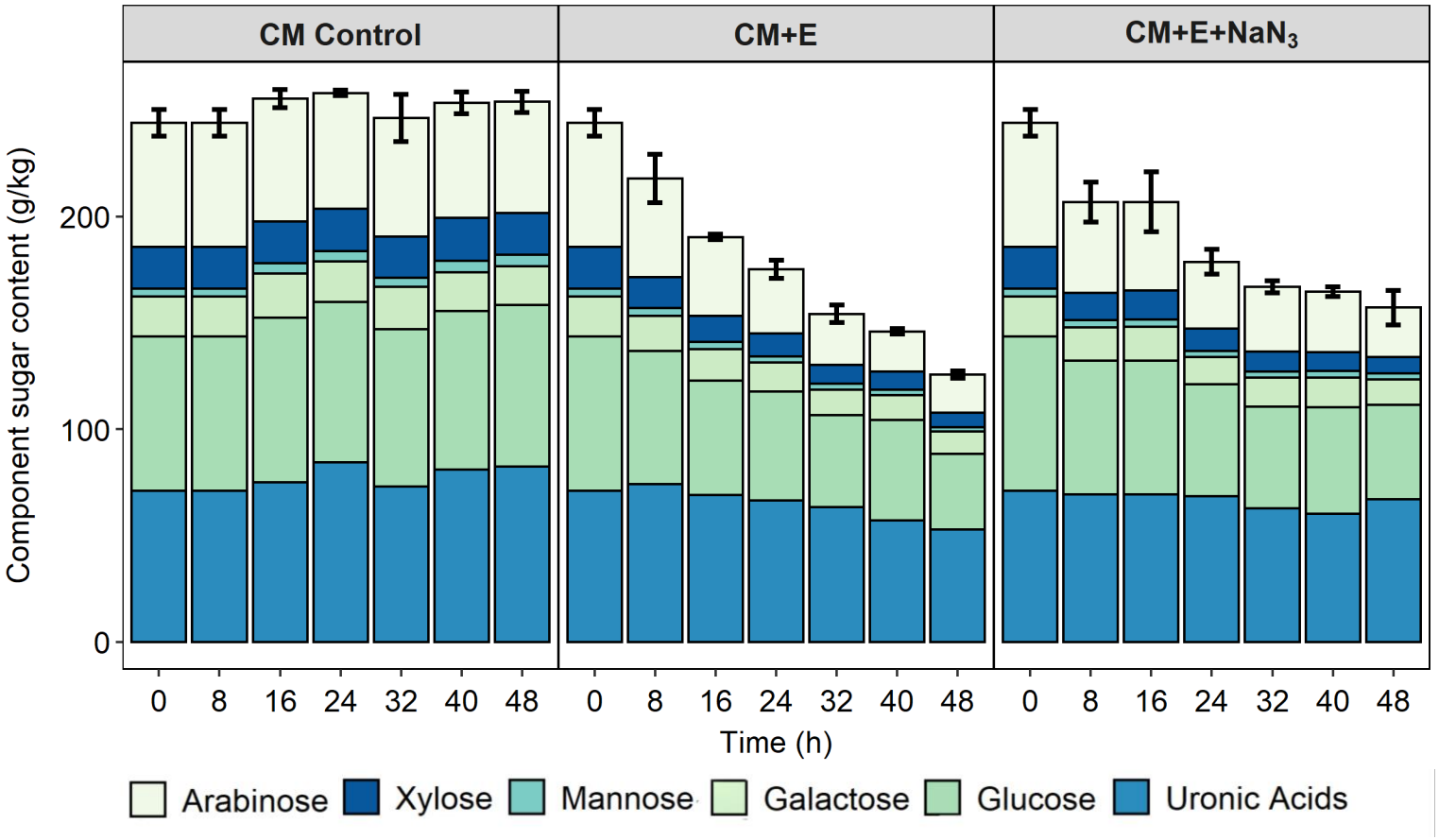


Figure 5.3 Dynamics of non-starch polysaccharides (NSP) component sugars changes during canola (*Brassica napus* L.) meal incubation (g/kg). CM Control: canola meal incubated in water; CM+E: canola meal and enzyme blend incubated in water; CM+E+NaN_3: canola meal, enzyme blend, and 0.05% of NaN_3 incubated in water. Data are means of component sugars with standard deviations of total NSP represented by vertical bars.

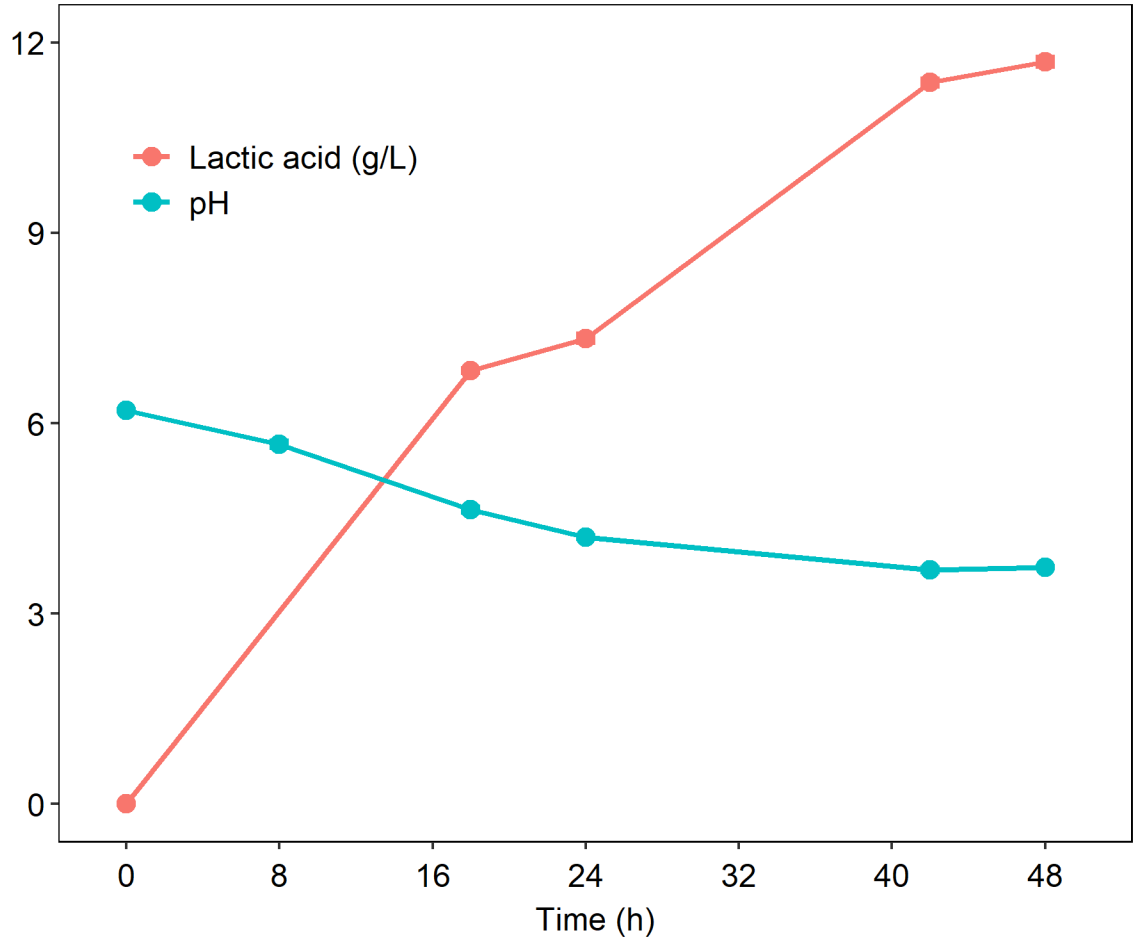


Figure 5.4 Content of lactic acid and pH during enzymatically-modified canola meal production.

5.4.3 Chemical composition of CM and ECM

The chemical composition and AA content of CM and ECM are presented in Table 5.4 and 5.5, respecitively. Compared with CM, ECM contained less NSP, NDF, TDF, phytate phosphorus, sucrose, and galactooligosaccharides (i.e., raffinose and stachyose). Protein and TDF appeared to be the main components of both ECM and CM.

The concentration of total NSP averaged 235.8 g/kg in CM; and 9.2% of these polysaccharides were water-soluble before enzyme modification. The main NSP component sugars in CM were uronic acids (85.0 g/kg), glucose (72.3 g/kg), arabinose (46.9 g/kg), xylose (15.5 g/kg), and galactose (13.6 g/kg). Enzyme modification decreased the content of NSP by 41.8% which accounted for 98.6 g/kg of DM. To be more specific, 69.3% of arabinose, 62.6% of xylose, 39.1% of glucose, 34.6% of galactose, and 26.7% of uronic acid residues were released following NSP depolymerization. Arabinose, glucose, and uronic acid residues accounted for 33.0%, 28.7%, and 23.1% of the NSP disappearance. In spite of the decreasing total NSP of ECM, the content of water-soluble NSP of ECM increased from 21.7 g/kg to 24.8 g/kg (DM basis) following the enzymatic modification.

Lactic acid was produced, and the availability of phosphorus was improved following modification as no phytate was detected in ECM. The crude protein and total AA content of ECM were identical to those of CM.

Table 5.4 Chemical composition of canola (*Brassica napus* L.) meal and enzymatically-modified canola meal (ECM) (g/kg, dry matter basis)

Component	CM	ECM
Dry matter	915.0	947.0
Crude protein	429.0	436.7
Ether extract	32.0	29.1
Carbohydrates		
Simple sugars[1]	4.6	4.2
Sucrose	72.9	0.8
Galactooligosaccharides[2]	33.0	ND[3]
Fibre fractions		
Neutral detergent fibre (NDF)	252.9	188.5
Total dietary fibre (TDF)	364.0	254.8
Glycoprotein	50.3	39.0
Lignin and polyphenols	77.8	78.6
Non-starch polysaccharides (NSP)	235.8	137.2
NSP component sugars		
Arabinose	46.9	14.4
Xylose	15.5	5.8
Mannose	2.5	1.9
Galactose	13.6	8.9
Glucose	72.4	44.0
Uronic acids	85.0	62.3
Water-soluble NSP	21.7	24.8
Lactic acid	ND	58.4
Ash	72.0	72.5
Total phosphorus (P)	12.8	11.3
Phytate P	6.5	ND

[1] Includes fructose and glucose.

[2] Includes raffinose and stachyose.

[3] Not detected.

Table 5.5 Analyzed amino acid content of test ingredients used in the study (g/kg, dry matter basis)

Amino acids	CM[1]	ECM[2]
Arginine	22.5	21.4
Histidine	10.8	10.7
Isoleucine	16.3	16.7
Leucine	28.6	29.1
Lysine	22.3	21.0
Methionine	7.3	7.5
Phenylalanine	16.7	16.8
Threonine	17.0	17.0
Valine	20.7	21.2
Alanine	17.9	18.9
Aspartic Acid	28.9	29.7
Cystine	9.8	10.1
Glutamic Acid	74.3	73.8
Glycine	21.1	21.5
Proline	25.3	25.7
Serine	15.9	15.7
Tyrosine	10.6	10.6
Total analyzed AA	365.9	367.7

[1] Canola meal

[2] Enzymatically-modified canola meal

5.4.4 Standardized ileal digestibility of amino acids and AMEn contents of CM and ECM

The SID of AA of CM and ECM are shown in Table 5.6. No difference ($P > 0.05$) was observed between the SID of both individual and total AA of CM and ECM. The SID of individual AA of CM ranged from 70.0% to 88.1%, while that of ECM ranged from 71.4 to 90.4%. The lowest SID was for isoleucine in CM and ECM while the highest SID of AA in CM and ECM were valine and arginine, respectively.

Table 5.6 also shows the standardized ileal digestible AA contents (DM basis) of CM and ECM for broilers. No differences ($P > 0.05$) were observed between the standardized ileal digestible AA contents of CM and ECM, except cystine. ECM had a higher standardized ileal digestible cystine content. The total analyzed digestible AA contents in CM and ECM were 281.2 and 287.0 g/kg, respectively.

As presented in Figure 5.5, the AMEn value of ECM ($P < 0.05$) averaged 2118 kcal/kg and was significantly higher than that of CM (DM basis). The enzymatic modification increased the AMEn value by 30.9%.

Table 5.6 Standardized ileal digestibility (SID) of amino acids (AA) and standardized ileal amino acid contents of canola (*Brassica napus* L.) meal (CM) and enzymatically-modified canola meal (ECM) for broiler chickens

	SID of AA, %				Standardized ileal AA content, g/kg, DM			
	CM	ECM	SEM	P value	CM	ECM	SEM	P value
Arginine	86.0	90.4	2.55	0.227	19.3	19.4	0.57	0.988
Histidine	81.2	82.2	2.49	0.790	8.8	8.8	0.27	0.955
Isoleucine	70.0	71.4	3.54	0.774	11.4	11.9	0.58	0.528
Leucine	77.6	79.8	2.77	0.590	22.2	23.2	0.80	0.387
Lysine	74.0	72.8	2.79	0.770	16.5	15.3	0.60	0.186
Methionine	79.6	81.3	3.28	0.711	5.8	6.1	0.24	0.401
Phenylalanine	79.5	81.7	3.00	0.620	13.3	13.7	0.50	0.545
Threonine	74.3	73.9	0.55	0.914	12.6	12.6	0.43	0.914
Valine	88.1	89.8	1.69	0.502	18.2	19.0	0.35	0.143
Alanine	77.5	77.6	2.35	0.990	13.9	14.7	0.43	0.227
Aspartic Acid	70.7	75.0	2.80	0.307	20.4	22.3	0.82	0.145
Cystine	74.2	77.7	1.45	0.120	7.3	7.8	0.14	0.021
Glutamic Acid	83.5	83.4	1.65	0.966	62.0	61.5	1.23	0.781
Glycine	74.5	77.6	2.52	0.391	15.7	16.7	0.53	0.217
Proline	72.2	73.5	1.81	0.642	18.3	18.9	0.46	0.376
Serine	79.8	76.9	2.50	0.426	12.7	12.1	0.40	0.294
Tyrosine	86.7	88.8	3.16	0.653	9.2	9.4	0.34	0.653
Total AA	76.8	78.0	2.42	0.730	281.2	287.0	8.86	0.651

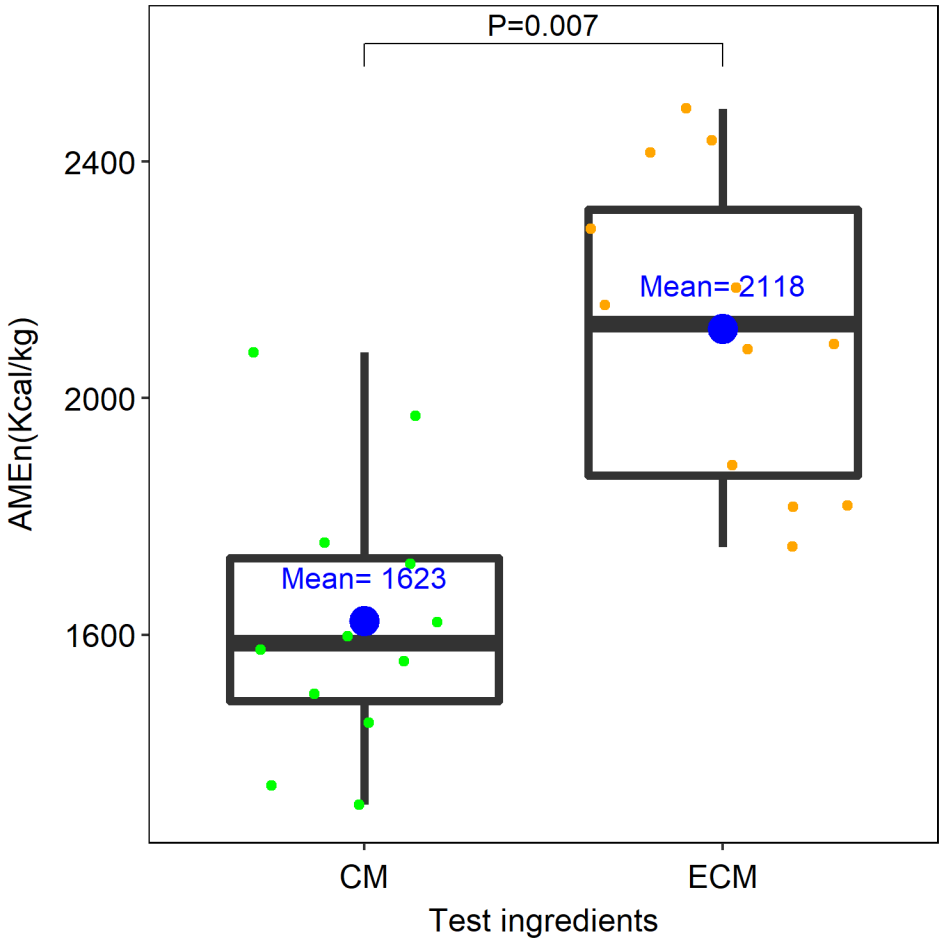


Figure 5.5 Nitrogen corrected apparent metabolizable energy (AMEn) of test ingredients (dry matter basis) for broiler chickens expressed by a boxplot. CM: canola (*Brassica napus* L.) meal; ECM: enzymatically-modified canola meal.

5.5 Discussion

The enzyme blend used in this study demonstrated its ability to significantly depolymerize the NSP of CM (Niu et al., 2022). In this context, the enzyme concentration would appear to be of importance for any enzyme-catalyzed reaction (Mussatto et al., 2008). In the enzyme cocktail used in the current study, invertase was also used to hydrolyze the galactooligosaccharides, raffinose, and stachyose (Ford, 1979; Omogbenigun et al., 2004). This was achieved when 0.2 g/kg of Inv was used, hence the concentration of Inv was maintained at 0.2 g/kg in the high enzyme concentration treatments.

An optimized concentration of the enzyme blend resulted in the depolymerization of 47.9% of NSP. It was reported that around half of the NSP of *B. napus* black-seeded canola comes from the hull fraction which would be difficult to depolymerize due to the high concentration of lignin and polyphenols (Slominski et al., 2012). Therefore, the concentration of Pectinase+xylanase+invertase - 4 used in ECM production would appear sufficient for NSP depolymerization of the embryo fraction of CM.

During incubation, the dynamics of changes in pH, sugars, and NSP of three treatments were investigated. The CM+E treatment reflects the dynamics during the production of ECM. In the CM+E+NaN_3 treatment, NaN_3 was added to inhibit the growth of microorganisms during incubation (Islam et al., 2018). The CM Control treatment is the control treatment which reflects the dynamics during the incubation without any enzyme or preservative added.

A significant decrease of simple sugar contents after 8 h of incubation of CM+E and 16 h of incubation of CM Control indicated that a natural and spontaneous fermentation occurred during the process. Such fermentation was most likely initiated by microorganisms (i.e., lactic acid bacteria) present in the feed or feed ingredients as was earlier observed in liquid feeds including

wheat, barley, wheat bran, or soybean meal (Christensen et al., 2007; Jorgensen et al., 2010). In the CM+E+NaN_3 treatment, relatively stable levels of simple sugars between 8 to 32 h of incubation demonstrated some bacteriostatic effect of sodium azide, although their decrease after 32 h would be indicative that sodium azide could not prevent the proliferation of bacteria during the entire period of incubation.

The lower sucrose concentration and higher simple sugar concentration observed at 8 hours compared to 0 hours in CM+E and CM+E+NaN_3 treatments are likely the result of enzymatic hydrolysis of sucrose by Inv, which can break sucrose down into glucose and fructose. In CM Control treatment, the sucrose content decreased due to the emergence of bacteria involved in fermentation. In the CM+E+NaN_3 treatment, the content of simple sugars remained relatively stable until 32 h. As NSP depolymerization occurred in the CM+E+NaN_3 treatment, it is unlikely that simple sugars were the major products of this process during incubation.

In general, the pH value of CM slurry was around 5.8 (Newkirk and Classen, 1998), which is similar to the pH values of the CM Control and CM+E+NaN_3 slurries prior to 16 h of incubation. The decreased pH values of the three treatments indicated that organic acids were produced during the incubation. The dynamics of lactic acid during ECM production confirmed it. This phenomenon was observed when a carbohydrase combination containing β-glucanase, xylanase, and pectinase activities was incubated with rapeseed cake and liquid feed containing soybean meal, wheat, and barley (Christensen et al., 2007; Engberg et al., 2009; Jorgensen et al., 2010). The final pH of CM+E and CM+E+NaN_3 treatments was lower than that of CM Control, which indicated that products of NSP depolymerization were metabolized by microorganisms in the slurry and generated more acids.

The constant level of NSP in the CM Control slurry during 48h of incubation indicated that the microorganisms could not utilize NSP as an energy source, as the enzymes secreted by such organisms did not contain any specific activities towards CM NSP. This demonstrated that natural fermentation alone cannot depolymerize the NSP of CM as reported in earlier studies (Jakobsen et al., 2015; Sugiharto and Ranjitkar, 2019; Wu et al., 2021). When the enzyme cocktail was used for the incubation, the NSP of CM were depolymerized. Although the enzyme cocktail used in this study was very specific and able to degrade NSP, not all the carbohydrases used in some earlier studies were capable to depolymerize NSP. Jakobsen et al. (2015) used nine enzymes or enzyme combinations to increase the solubility of NSP in rapeseed cake. Only one of the enzyme preparations decreased the total NSP content significantly.

The lower content of sucrose, galactooligosaccharides, and NSP in ECM compared to CM was consistent with the dynamics results of sugars and NSP. Although total NSP content decreased in ECM, the content of water-soluble NSP increased. This result proved that carbohydrases had the capability to improve the solubility of NSP (Christensen et al., 2007; Staack et al., 2019; Vangsøe et al., 2020). It also indicated that low-molecular-weight NSP were formed during the incubation. The lower concentration of sugars and phytate phosphorus and the production of lactic acid was also observed in the previous reports when spontaneous fermentation was observed in liquid feeds or feed ingredients (Engberg et al., 2009; Drazbo et al., 2018). However, the NSP contents were not affected in the above studies because no exogenous enzymes were added (Engberg et al., 2009; Drazbo et al., 2018). Although the enzyme blend used in the current study did not contain phytase, the hydrolysis of phytic acid could be catalyzed by bacteria involved in the fermentation process that occurred during the production of ECM (Lei et al., 2013). Therefore,

it can be concluded that modification of CM with enzymes would not only change the composition and structure of carbohydrates but would also improve the availability of phosphorus.

The enzymatic NSP modifications in the current study increased AMEn by 30.9% but had no significant effect on the SID of individual and total AA. However, it should be noted that the test ingredients were not the sole protein source for the SID of AA assay. This method improves the palatability of test diets for amino acid digestibility measurement (Fan and Sauer, 1995). As SID of AA is more additive than AID of AA in a mixed diet, the SID of AA of the test ingredients was calculated (Stein et al., 2005). Although the SEM value for SID of AA of soybean meal and fish meal may be less than 2.0, the SEM value for SID of AA of CM was normally higher than 2.0 as reported earlier (Kim et al., 2012; Ndou and Woyengo, 2020). Therefore, the SEM value in this study would appear reasonable.

To the best of our knowledge, the effect of enzymatic modification on the SID of AA and AMEn has not been reported. However, studies with exogenous enzyme supplementation have been conducted. In earlier reports, supplementation of CM-based diets with multi-carbohydrases increased metabolizable energy from 1,866 to 1,955 kcal/kg for broilers, from 2,088 to 2,196 kcal/kg for turkeys, and from 2,792 to 2,976 kcal/kg for growing pigs (Kozlowski et al., 2018; Rad-Spice et al., 2018; P. Li et al., 2020). According to Li et al. (2020), multi-enzyme supplementation, including carbohydrase and protease activities, of diets containing solvent-extracted or expeller-pressed double low rapeseed meal increased the metabolizable energy value, apparent total tract digestibility of ether extract, NDF, and acid detergent fibre of diets, and SID of all AA except phenylalanine. As no protease was added in the current study for the production of ECM, no significant difference between SID of AA of ECM and CM would appear reasonable.

The effect of fermentation on SID of AA and AMEn contents was reported in some earlier studies. Results showed that solid-state fermentation of rapeseed meal using *Lactobacillus acidophilus*, *Bacillus subtilis*, and *Saccharomyces cerevisiae* significantly improved AMEn and SID of alanine, valine, isoleucine, leucine, tyrosine, lysine, arginine, and phenylalanine of rapeseed meal (Wu et al., 2020). However, a lack of improvement in SID of total AA by fermentation of soybean meal was also reported (Upadhaya and Kim, 2015). Similarly, fermentation and enzyme treatment of cottonseed meal had no obvious effect on the true AA digestibility (Tang et al., 2018).

Treatments with enzymes have been investigated to increase the digestible protein and to decrease the antinutritional components of plant-based protein sources. It was reported that using xylanase and an enzyme product obtained from *Aspergillus aculeatus* to pre-treat liquid feed including wheat, barley, wheat bran, and soybean meal decreased the pH value and total NSP contents significantly (Christensen et al., 2007). Pretreated rapeseed cake with phytase was reported to be able to increase the final body weight of turkeys and had positive effects on intestinal histomorphology (Drazbo et al., 2018).

In conclusion, incubation of canola meal with 4 g/kg of each of pectinase A, pectinase B, and xylanase B and 0.2 g/kg of invertase for 48 h at 40°C contributed to depolymerization of 47.9% of non-starch polysaccharides of canola meal. The dynamics of changes in pH, carbohydrate and non-starch polysaccharides contents during the process indicated the effectiveness of the enzyme blend towards depolymerization of non-starch polysaccharides of canola meal. The enzymatic modification of canola meal was realized in increasing AMEn by 30.9% and water-soluble non-starch polysaccharides by 14.3%, and remarkably decreasing non-starch polysaccharides when compared to its intact counterpart. No significant effect on standardized ileal amino acid digestibility between enzymatically-modified canola meal and its control treatment indicates that

the nutritive value of protein was neither compromised nor improved by the incubation and drying. Results suggest that enzymatic modification can be an effective method of improving the nutritive value of canola meal.

CHAPTER SIX : MANUSCRIPT IV

Prebiotic properties of enzymatically-modified canola meal solubles for broiler chickens

6.1 Abstract

Enzymatically-modified canola (*Brassica napus* L.) meal solubles **(ECMS)** is a product obtained following spray-drying of water-soluble fraction of canola meal (**CM**) incubated with a specific multi-carbohydrase cocktail and protease. The ECMS product would be expected to have prebiotic effects in broilers as it would contain non-starch polysaccharides (**NSP**) hydrolysis products, including low-molecular-weight polysaccharides and oligosaccharides. When compared to CM, ECMS contained higher levels of crude protein, ash, phosphorus, and less intact NSP, neutral detergent fibre (**NDF**), phytate phosphorus, sucrose, and raffinose and stachyose. The objective of this study was to evaluate the effect of ECMS supplementation on growth performance, nutrient digestibility, and gut function and health of broiler chickens. Ross 308 broiler chickens were assigned to 3 dietary treatments with 6 birds per cage and 8 replicates per treatment. The birds were raised under a controlled environment for 14 days. The 3 experimental diets were a corn/soybean meal Control diet, a Low ECMS diet containing 1g/kg of ECMS, and a High ECMS diet containing 20 g/kg of ECMS. The results showed that the High ECMS diet significantly increased feed intake and body weight gain compared to the Low ECMS and the Control diet. No obvious differences were observed in feed conversation ratio, pH of ileal and cecal digesta, sialic acids secretion, and apparent total tract digestibility of NDF and nitrogen between birds fed the different diets. The abundance of *Lactobacillus spp*. and *Enterococcus spp.* were significantly higher in both ileal and cecal digesta of birds fed the High ECMS diet compared to the Control diet. Total short-chain fatty acids (**SCFA**) in the ileal digesta of birds fed ECMS diets significantly increased compared to birds fed the Control diet. In conclusion, ECMS significantly modulated gut microbiota, increased

SCFA production in the ileum, and improved the growth performance of broilers. Therefore, ECMS could be considered as a prebiotic in broiler chickens nutrition.

Key words: enzymatically-modified canola meal solubles (ECMS), prebiotic, broiler chicken, gut health, carbohydrase

6.2 Introduction

The broiler chicken industry is one of the most advanced and developed sectors of agriculture and food production. According to FAO (2020), over 70 billion broilers are slaughtered annually and their production is predicted to continue to grow. Driven by advancements in genetics, nutrition, technology, and management practices, the broiler chicken produced today is a healthy and affordable protein source in human nutrition. Over the past 60 years, the aforementioned advances have increased growth rate, meat yield, feed efficiency, and dramatically decreased slaughter age (Hartcher and Lum, 2020). In commercial broilers, the feed conversion ratio ranges from 1.4 to 2.8 (Sood et al., 2020) which means that birds have a strong ability to efficiently convert dietary nutrients to their body weight. Regardless of the genetic potential for fast growth, advanced nutrition and health management have been implemented towards efficient broiler chicken production. In recent years, great attention has been paid to maintaining and improving the health status of the gastrointestinal tract. In particular, it has become challenging when antibiotic growth promoters were no longer allowed and/or recommended to be excluded as feed additives in broiler chicken nutrition. It led to decreased growth performance, increased losses in morbidity and mortality, increased production costs (Reuben et al., 2021), resulting in greater risks related to food safety (Al-Khalaifah, 2018; Rinninella et al., 2019). To

mitigate this problem, various nutritional strategies have been proposed, along with the improvements of animal welfare and biosecurity. These would include the use of prebiotics in poultry diets, thus stimulating various research approaches, including the development of safe and efficient bioactive components of dietary fibre that exert the properties beneficial for poultry health and food safety.

The term prebiotic was initially defined by Gibson and Roberfroid in 1995 and was defined as 'a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host' (Bindels et al., 2015). The prebiotic effect refers to 'the beneficial physiological outcome that arises from the modulation of the composition and/or activity of the gut microbiota through the metabolization of a nondigestible compound' (Bindels et al., 2015). In general, the products that are used as prebiotics are non-digestible carbohydrates and include fructo-oligosaccharides (**FOS**), galacto-oligosaccharides, manno-oligosaccharides (**MOS**), inulin, xylo-oligosaccharides (**XOS**), or isomalto-oligosaccharides (Pourabedin and Zhao, 2015; Ricke et al., 2020; Reuben et al., 2021).

Although the mechanism that can fully elucidate how prebiotics exert their function in poultry is not clear yet, the beneficial effect of prebiotics in poultry has been well documented in the literature. It has been reported that prebiotics can maintain intestinal microbiota balance, suppress intestinal pathogens, improve nutrient digestibility, stimulate the immune system, and may even improve growth performance. Including yeast-derived carbohydrates (MOS) in the diet of birds infected with *Eimeria tenella* significantly improved body weight gain (**BWG**), feed intake (**FI**), feed conversion ratio (**FCR**), and

decreased oocyte level (Chand et al., 2016). Worawong et al. (2022) investigated the effect of various prebiotics on the cecal short-chain fatty acids (**SCFA**) of broilers and noticed that the effect of prebiotic supplementation was highly diverse, given that out of 14 studies conducted to date, only 6 studies showed some positive effects.

Enzymatically-modified canola meal solubles (**ECMS**) is a spray-dried product obtained from the supernatant of canola (*Brassica napus* L.) meal (**CM**) incubated in water with a multienzyme cocktail of xylanase, pectinase, invertase, protease, and other enzyme site activities. We hypothesized that ECMS would possess some prebiotic properties due to the presence of bioactive components released from enzymatic hydrolysis of non-starch polysaccharides (**NSP**), including low-molecular-weight polysaccharides, oligosaccharides or disaccharides.

The objective of this study was to evaluate the effect of ECMS supplementation on growth performance, nutrient digestibility, and any potential prebiotic effects on gastrointestinal microbiota abundance, and gut function and health of broiler chickens.

6.3 Materials and methods

6.3.1 Materials

Canola meal was provided by Bunge Canola Processing Plant, Altona, Canada, and was produced by pre-press solvent extraction process from black-seeded *B. napus* canola.

Commercial enzymes, **PA** (Pectinase A, 443,061 U/g), **PB** (Pectinase B, 425,773 U/g), **XB** (Xylanase B, 60,763 U/g), **Inv** (Invertase, 362,631 U/g), and **Pr** (Protease, 1,082,614 U/g) were obtained from CBS Bio Platforms Inc., Calgary, Alberta, Canada.

6.3.2 Production of ECMS

ECMS was produced by continuous mixing of CM in distilled water containing a mixture of carbohydrases and protease (4 g/kg of PA, 4 g/kg of PB, 4 g/kg of XB, 4 g/kg of Pr, and 0.2 g/kg of Inv) for 48 h at 40°C. The pH of the slurry was monitored throughout the process. After incubation, the slurry was centrifuged for 10 min at 3000rpm after which time the supernatant was collected and spray-dried (flow rate 2 L/h; inlet temperature 215°C, outlet temperature 73-78°C) to obtain the ECMS preparation (Figure 6.1).

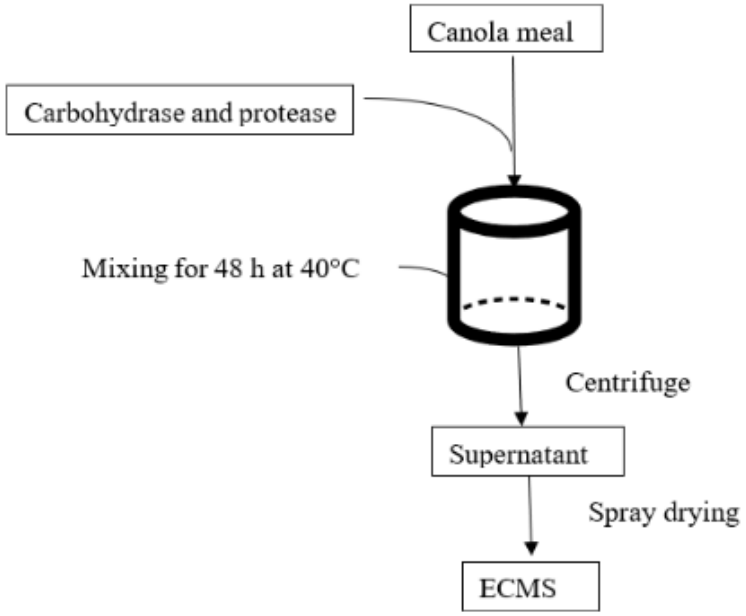


Figure 6.1 Flow chart of the production of enzymatically-modified canola meal solubles (ECMS)

6.3.3 Animals, experimental design, diets, and sample collection

As-hatched Ross 308 broiler chickens were used to evaluate the effect of ECMS addition on growth performance and gut health of poultry. The animal care protocol for this study followed the guidelines of the Canadian Council on Animal Care and was approved by the Animal Care Committee of the University of Manitoba (F20-001/1,

AC11558). One-day-old birds were purchased from a local hatchery and raised in electrically heated Super Brooders (Alternative Design Manufacturing and Supply, Inc., Siloam Springs, AR) under a controlled environment for two weeks (1-14 d of age) and were fed ad libitum. After arrival, birds were randomly assigned into 3 experimental dietary treatments, with 6 birds per cage and 8 replicate cages per treatment. Experimental starter diets were formulated to meet the Ross 308 breeder recommendations (Aviagen, 2019). All diets were fed in mash form. Due to the unknown nature of this novel product, Low and High dietary levels of ECMS were investigated. Birds were fed a corn/soybean meal Control diet, a Low ECMS diet containing 1 g/kg of ECMS, and a High ECMS diet containing 20 g/kg of ECMS (Table 6.1). Chromic oxide (3.0 g/kg) was used as an inert marker.

Table 6.1 Composition of experimental diets (g/kg, as-fed basis)

Ingredient	Control	Low ECMS	High ECMS
Corn	545.0	544.0	545.9
Soybean meal	368.9	368.9	348.0
ECMS	0	1.0	20.0
Canola oil	33.6	33.6	33.6
Calcium carbonate	15.4	15.4	15.4
Mono calcium phosphate	13.0	13.0	13.0
L-Lysine	2.5	2.5	2.5
DL-Methionine	2.0	2.0	2.0
Threonine	1.6	1.6	1.6
Mineral Premix [a]	5.0	5.0	5.0
Vitamin Premix [b]	10.0	10.0	10.0
Cr_2O_3	3.0	3.0	3.0
	1000	1000	1000
Calculated composition (g/kg unless specified)			
Metabolizable energy (kcal/kg)	3000	3000	3000
Crude protein	230.0	230.0	230.0
Calcium	9.6	9.6	9.6
Available phosphorus	4.8	4.8	4.8
Digestible Lysine	12.8	12.8	12.8
Digestible Methionine	5.2	5.2	5.2
Digestible Methionine + cysteine	9.5	9.5	9.5
Digestible Threonine	8.6	8.6	8.6
Digestible Arginine	13.7	13.7	13.7
Analyzed composition (g/kg)			
Crude protein	230.0	230.1	230.3

[a] Provided per kg of diet: 70 mg Mn, 80 mg Zn, 80 mg Fe, 10 mg Cu, 0.3 mg Se, 0.5 mg I, 337 g Na.

[b] Provided per kg of diet: 8250 IU vitamin A, 3000 IU vitamin D3, 30 IU vitamin E, 0.13 mg vitamin B12, 2 mg vitamin K3, 6 mg riboflavin, 40.3 mg niacin, 1301 mg choline, 4 mg folic acid, 0.25 mg biotin.

Body weight and feed disappearance were recorded on day 14 to calculate the body weight gain, feed intake, and feed conversion ratio. On day 14, excreta samples were collected, frozen immediately, and freeze-dried. All birds were euthanized using carbon dioxide, and the contents of ileum and ceca were collected. The pH of ileum and ceca content was measured (mean of three readings) using a portable pH meter with microelectrode HI9126 (HANNA Instruments, Romania). The collected samples were snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

6.3.4 DNA extraction and quantitative real-time polymerase chain reaction (qPCR)

The selected the bacterial groups in ileum and ceca was measured. Microbiota DNA of ileal and cecal digesta were extracted by an Invitrogen™ PureLink™ Microbiome DNA Kit (Thermo Fisher Scientific Inc.) following the manufacturer's instructions. After extraction, the concentration of DNA in the samples was measured by a spectrophotometer (NanoDrop-2000, Thermo Fisher Scientific, Waltham, MA) and diluted to 0.75 ng/µL (Lunedo et al., 2014).

The selected bacterial groups studied and the forward and reverse primers for the real-time PCR are listed in Table 6.2. The real-time PCR assay was conducted in 96 well plates in CFX Connect™ Real-Time PCR Detection System (Life Science Research, Bio-Rad, Canada) with all reactions consisting of 2 µL of extracted DNA, 0.5 µL of 10 pmole/µL of each primer, 5 µL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), and 2 µL of Nuclease-free water.

The results of qPCR were analyzed by the $2^{-\Delta\Delta C_T}$ method by normalizing the expression of the target bacterial groups of that of total eubacteria as the reference gene and comparing those to the Control treatment (Livak and Schmittgen, 2001). All extracted DNA samples were analyzed in duplicate.

Table 6.2 Paris of primers used for real-time PCR assay

Target groups	Primer sequence		AT (ºC)	References
	Forward	Reverse		
Firmicutes	GGAGYATGTGGTTTAATTCGAAGCA	AGCTGACGACAACCATGCAC	60	Guo et al., 2008
Bacteroidetes	GGARCATGTGGTTTAATTCGATGAT	AGCTGACGACAACCATGCAG	60	Guo et al., 2008
Clostridium cluster IV	GCACAAGCAGTGGAG T	CTTCCTCCGTTTTGTCAA	56	Zwielehner et al., 2009; Mejicanos et al., 2017
Enterococcus spp.	CCCTTATTGTTAGTTGCCATCATT	ACTCGTTGTACTTCCCATTGT	61	Rinttilä et al., 2004; Mejicanos et al., 2017
E. coli	CAATTTTCGTGTCCCCTTCG	GTTAATGATAGTGTGTCGAAAC	55	Khan et al., 2007; Wealleans et al., 2017
Lactobacillus spp.	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	58	Walter et al., 2001; Heilig et al., 2002
Bifidobacterium spp.	TCGCGTCCGGTGTGAAAG	CCACATCCAGCATCCAC	58	Rinttilä et al., 2004; Mejicanos et al., 2017
Total Eubacteria	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	60	Guo et al., 2008

6.3.5 Analytical procedures

Canola meal, ECMS, diets, and dried excreta samples were ground to pass through a 1 mm sieve and analyzed in duplicate. The chemical composition of CM and ECMS was determined. Dry matter (945.15), crude protein and nitrogen (968.06), amino acids (994.12), ether extract (920.39), ash (923.03), neutral detergent fibre (**NDF,** 2002.04) and total phosphorus (965.17) were analyzed according to standard AOAC (2005) methods. Total and water-soluble NSP were measured by gas-liquid chromatography (component neutral sugars) using SP-2340 column and Varian CP-3380 GC (Agilent Technology, Ontario, Canada) and colorimetry (uronic acids) using Biochrom Ultrospec 50 (Biochrom Ltd., Cambridge, UK) and the procedure described by Englyst and Cummings (1984) with some modifications (Slominski and Campbell, 1990). Total dietary fibre (**TDF**) was measured and calculated based on the procedure described by Slominski et al. (1994). Simple sugars (i.e., fructose and glucose), sucrose, raffinose, and stachyose were measured by gas-liquid chromatography using a 3% OV-7 column and a Varian 430 GC (Agilent Technology, Ontario, Canada) as described by Slominski et al., (1994). Phytate phosphorus was determined following the method described by Haug and Lantzsch (1983). The NSP hydrolysis products in CM and ECMS were measured by hydrolyzing samples in 12 M sulfuric acid and analyzing the content of sugars according to Slominski and Campbell (1990) and then subtracting NSP, simple sugars, and sucrose from the total component sugar content.

Duplicate samples of diets and excreta were analyzed for chromium, NDF, and nitrogen. Ashed samples were analyzed for chromium content by inductively coupled plasma atomic emission spectroscopy (ICP-AES Vista, Varian, USA) according to AOAC (2005) Method 985.01. Crude mucin was extracted from excreta following procedure described by Lien et al. (1997) and

total sialic acids were measured using the methodology described by Jourdian et al. (1971) with some modifications (Hejdysz et al., 2018).

The concentration of SCFA in ileal and cecal digesta was measured according to Apajalahti et al.(2019) using pivalic acid (Sigma-Aldrich, St. Louis, MO, USA) as an internal standard.

6.3.6 Calculations and statistical analysis

Apparent total tract digestibility (**ATTD**) of NDF and nitrogen (**N**) were calculated using the following equations (Meng and Slominski, 2005):

$$\text{ATTD (\%)} = \left(1-\frac{{}^{Cr_2O_3\ \%diet}/_{Cr_2O_3\ \%excretra}}{{}^{NDF\ or\ N\ \%diet}/_{NDF\ or\ N\ \%excretra}}\right) \times 100$$

Where Cr_2O_3 is chromic oxide.

Equation 6.1

All the statistical analysis was conducted by the SAS program (version 9.4, SAS Institute Inc., Cary, NC). The broiler study was set up as a completely randomized design and analyzed using the Mixed procedure with the cage as an experimental unit. Means were separated by Tukey's honestly significant difference. All statements of significance were based on $P < 0.05$. All figures were made with R studio (Version 1.4.1106) software with ggplot2 (Version 3.3.3).

6.4 Results

6.4.1 Production and chemical composition of ECMS

Changes in pH during the ECMS production are shown in Figure 6.2. The pH value of the slurry decreased from 6.20 to 3.72 at the end of the incubation. The chemical composition and amino acid content of ECMS and CM are presented in Table 6.3 andTable 6.4. Compared to CM, ECMS contained more crude protein, ash, phosphorus, and simple sugars, and less NSP, NDF, phytate phosphorus, sucrose, raffinose, and stachyose.

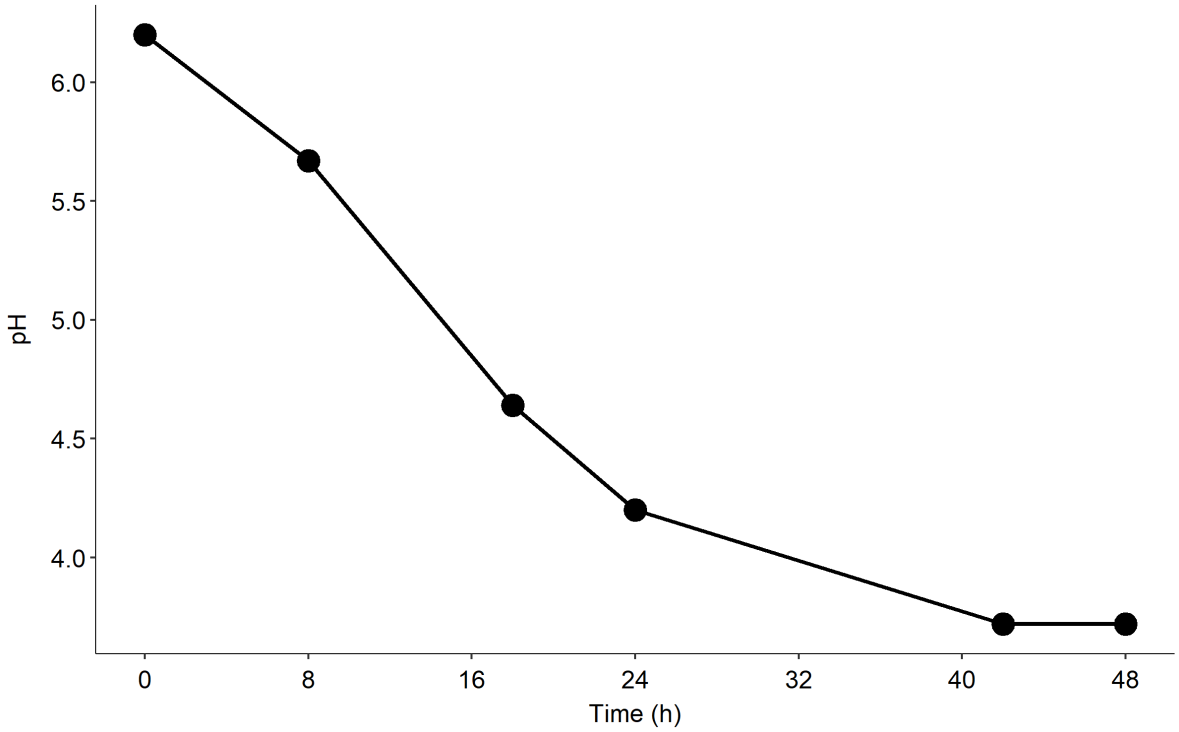


Figure 6.2 Dynamics of pH changes during enzymatically-modified canola meal solubles (ECMS) production.

Table 6.3 Chemical composition of canola (*Brassica napus* L.) meal (CM) and enzymatically-modified canola meal solubles (ECMS) (g/kg, dry matter basis)

Component	CM	ECMS
Dry matter	915.0	930.7
Crude protein	429.0	507.0
Ether extract	32.0	10.7
Carbohydrates		
Simple sugars[1]	4.6	47.3
Sucrose	72.9	1.0
Raffinose family oligosaccharides[2]	33.0	ND[3]
Fibre fractions		
Neutral detergent fibre (NDF)	252.9	ND
Total dietary fibre (TDF)	364.0	41.1
Non-starch polysaccharides (NSP)	235.8	41.1
Component sugars of NSP		
Arabinose	46.9	12.8
Xylose	15.5	1.7
Mannose	2.5	1.8
Galactose	13.6	6.7
Glucose	72.4	3.2
Uronic acids	85.0	15.0
Water-soluble NSP	21.7	41.1
NSP hydrolysis products	ND	158.7
Ash	72.0	113.9
Total phosphorus (P)	12.8	20.3
Phytate P	6.5	ND

[1] Includes fructose and glucose.

[2] Includes raffinose and stachyose.

[3] Not detected.

Table 6.4 Analyzed amino acid content of canola (*Brassica napus* L.) meal (CM) and enzymatically-modified canola meal solubles (ECMS) (g/kg, dry matter basis)

	CM	ECMS
Arginine	22.5	24.4
Histidine	10.8	13.8
Isoleucine	16.3	18.2
Leucine	28. 6	32.6
Lysine	22.3	25.9
Methionine	7.3	8.9
Phenylalanine	16.7	19.4
Threonine	17.0	20.2
Valine	20.7	23.2
Alanine	17.9	21.1
Aspartic Acid	28.9	33.4
Cystine	9.8	11.9
Glutamic Acid	74.3	90.8
Glycine	21.1	28.3
Proline	25.3	31.0
Serine	15.9	18.3
Tyrosine	10.6	11.7

The content of intact NSP in ECMS (41.1 g/kg) decreased by 82.6% compared to CM (235.8 g/kg DM), with the component sugars arabinose, galactose, glucose, and uronic acid accounting for 31.2, 16.3, 7.7, and 36.3% of the total NSP of ECMS, respectively. As ECMS represents the water-soluble fraction of the enzymatically-modified CM, ultimately all intact NSP in ECMS are soluble in water.

Crude protein content of ECMS averaged 507.3 g/kg (DM basis) which is slightly higher than that of CM (429.2 g/kg DM). Phytate phosphorus was not detected in ECMS, while total phosphorus was substantially higher than that of CM.

6.4.2 The effect of ECMS supplementation on growth performance, nutrient digestibility, and gut function and health of young broiler chickens

Ross 308 broiler chickens assigned to three dietary treatments, the Control, Low ECMS, and High ECMS were fed for two weeks (1-14 d of age). The composition of experimental diets is shown in Table 6.1.

The growth performance and ATTD of NDF and N are shown in Table 6.5. Birds fed the High ECMS diet had significantly higher FI and BWG compared to the Control and Low ECMS treatments, while no difference was observed for FCR and ATTD of NDF and N.

The pH values of ileal and cecal digesta and sialic acids contents of excreta of broiler chickens fed experimental diets are presented in Table 6.6. Dietary addition of ECMS did not significantly impact the secretion of sialic acids, and the pH values of ileal and cecal contents.

Table 6.5 Growth performance and apparent total tract digestibility (ATTD) of neutral detergent fibre (NDF) and nitrogen (N) of broiler chickens (1 to 14 d) fed diets containing enzymatically-modified canola meal solubles (ECMS)

Diet[1]	Feed intake (g/bird)	Body weight gain (g/bird)	FCR (g/g)	ATTD (%)	
				NDF	N
Control	435.9[b]	371.4[b]	1.18	9.9	71.9
Low ECMS	436.1[b]	365.5[b]	1.19	7.0	70.0
High ECMS	467.9[a]	404.8[a]	1.16	11.2	71.3
SEM	8.27	9.77	0.02	2.04	0.77
P value	0.015	0.019	0.251	0.319	0.245

[a-b] Means within a column with no common superscripts differ significantly (P < 0.05).

[1] The composition of the diet is in Table 6.1.

Table 6.6 The pH value in ileal and cecal digesta and sialic acids contents in broiler chickens fed diets containing enzymatically-modified canola meal solubles (ECMS) on day 14

Diet[1]	pH of ileal digesta	pH of cecal digesta	Sialic acids, mg/100g excreta
Control	7.5	5.3	31.3
Low ECMS	7.6	5.4	30.6
High ECMS	7.4	5.5	30.5
SEM	0.06	0.05	8.60
P value	0.416	0.164	0.785

[1]The composition of the diet is in Table 6.1.

The fold changes of selected ileal and cecal bacterial groups in broiler chickens fed experimental diets on day 14 are presented in Table 6.7. Real-time PCR was used to measure the abundance of Firmicutes, Bacteroidetes, *Enterococcus spp.*, *Lactobacillus spp.*, and *Bifidobacterium spp.* in ileal digesta, and additional *Clostridium cluster IV* and *E. coli* in cecal digesta. No significant difference was observed between Firmicutes, *Clostridium cluster IV,* and *E. coli* in the ileal and cecal digesta of birds fed experimental diets. The *Lactobacillus spp.* population was significantly higher in the ileal and cecal digesta of birds fed the High ECMS diet compared to those fed the Low ECMS and Control diets. In addition, the abundance of *Lactobacillus spp.* was higher ($P < 0.05$) in the ileal digesta of birds fed the Low ECMS diet than the Control diet. The abundance of *Enterococcus spp.* in ileal and cecal digesta of birds fed the High ECMS diet was higher ($P < 0.05$) than those of the Control group. The abundance of *Enterococcus spp.* was also higher in the cecal digesta of birds fed the Low ECMS diet than the Control diet. Bacteroidetes and *Bifidobacterium spp.* in ileal digesta of birds fed the High ECMS diet was significantly lower than the Control diet, while in cecal digesta it was significantly higher than the Control diet. In addition, the abundance of Bacteroidetes was remarkably higher in the cecal digesta of birds fed the High ECMS diet than the other diets while it was significantly lower in the ileal digesta of birds fed the high ECMS diet than the Control diet.

Table 6.7 The fold change of selected ileal and cecal bacteria communities in broiler chickens fed diets containing enzymatically-modified canola meal solubles (ECMS)[1]

Selected bacteria		Control[2]	Low ECMS[2]	High ECMS[2]	SEM	P value
Ileal digesta	Firmicutes	1.10	1.39	1.40	0.177	0.4044
	Bacteroidetes	1.05^{a}	0.40^{ab}	0.25^{b}	0.197	0.0194
	Enterococcus spp.	0.99^{b}	5.21^{ab}	8.57^{a}	1.805	0.0249
	Lactobacillus spp.	1.05^{c}	39.72^{b}	80.31^{a}	8.236	<.0001
	Bifidobacterium spp.	1.10^{a}	0.34^{ab}	0.21^{b}	0.0029	0.0029
Cecal digesta	Firmicutes	1.01	1.09	1.14	0.058	0.2981
	Bacteroidetes	1.05^{b}	1.22^{b}	5.21^{a}	0.419	<.0001
	Clostridium cluster IV	1.04	1.27	0.95	0.150	0.3079
	Enterococcus spp.	1.02^{c}	11.14^{b}	19.78^{a}	1.915	<.0001
	E. coli	0.97	1.72	1.13	0.276	0.1567
	Lactobacillus spp.	1.06^{b}	17.96^{b}	59.64^{a}	10.538	0.0024
	Bifidobacterium spp.	1.01^{b}	1.43^{ab}	1.87^{a}	0.221	0.0419

[a-c] Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1] The fold change was measured using a $2^{-\Delta\Delta C_T}$ method by normalizing the target gene of selected bacteria to the reference gene of total eubacteria and comparing to the Control treatment.

[2] The composition of the diet is in Table 6.1.

The concentration of SCFA in ileal and cecal digesta of broilers fed experimental diets is shown in Figure 6.3. Total SCFA in the ileum was significantly higher in birds fed ECMS-containing diets while no significant difference was observed in the total cecal SCFA content. In detail, the higher level of total SCFA in ileal digesta of birds fed ECMS-containing diets is attributed to the higher level of acetic acid and lactic acid compared to the Control diet. Butyric acid in the ileal digesta of birds fed the Low ECMS diet was significantly higher than birds fed the Control diet. The most important SCFA in ileal digesta were acetic acid and lactic acid while in the cecal contents they would be acetic and butyric acids. The concentration of propionic acid in the ceca of birds fed the Low ECMS diet was significantly lower than that of birds fed the High ECMS diet.

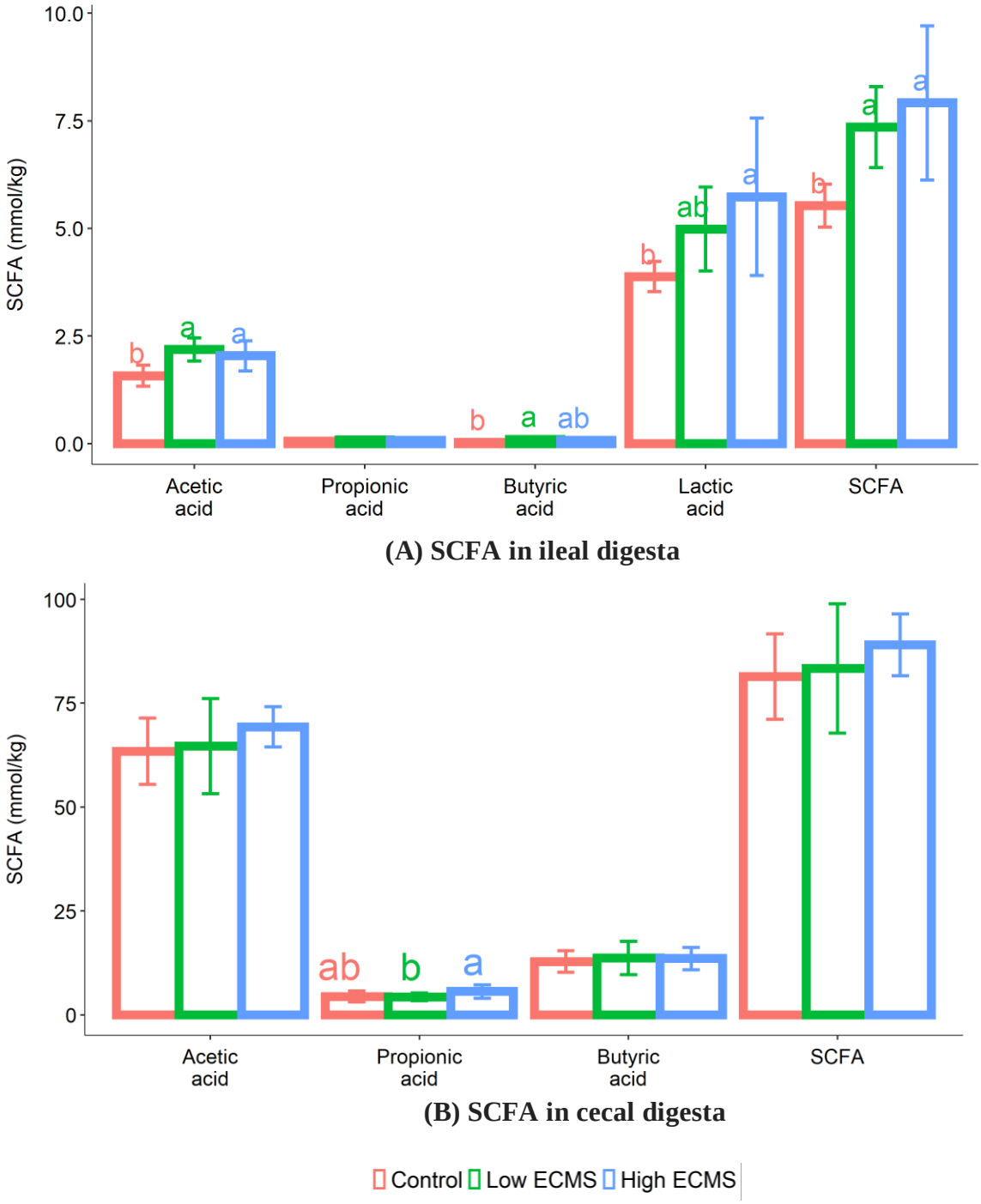


Figure 6.3 Short chain fatty acids (SCFA) in ileal (A) and cecal (B) digesta of broiler chickens fed experimental diets on day 14 (mmol/kg of fresh digesta). Valeric acid, isobutyric acid, and isovaleric acid are also included in total SCFA. The diet formulation is shown in Table 6.1. Data are means with standard deviations represented by vertical bars. Means with no common superscript letters within a subgrouping differ significantly ($P < 0.05$)

6.5 Discussion

In the earlier studies of this thesis, a natural and spontaneous fermentation was observed during the enzymatic treatment of CM (Chapter Four and Five). The changes in the pH of the slurry during ECMS production also confirmed the occurrence of such fermentation. It has been reported that the combination of enzymatic treatment and spontaneous fermentation can increase NSP solubility, and decrease the total NSP and phytate phosphorus contents (Christensen et al., 2007; Jakobsen et al., 2015; Drazbo et al., 2018).

The dried ECMS preparation was further used in the animal study. This preparation derived from the processing of enzymatically-modified CM (ECM) that was subjected to centrifugation and spray-drying of the water-soluble ECM fraction. The crude protein content was high in ECMS reaching 507 g/kg (DM basis) and was higher than that of CM by 18.2%. The ash and total phosphorus of ECMS increased by 58.2% and 58.6% compared to CM, respectively. This was an effect of the release of encapsulated minerals by enzymatic treatment, as well as their increased solubility at lower pH induced by the fermentation.

The main difference between ECMS and CM was observed within the fibre fractions. As the test material was water-soluble, it is understandable that NDF was not detected, while NSP accounted for a small amount of water-soluble fraction only. The top three component sugars of NSP in ECMS were uronic acids, arabinose, and galactose, which differed from the order of component sugars present in CM, i.e., uronic acid, glucose, and arabinose. However, significant amounts of NSP hydrolysis products were present in ECMS.

It was hypothesised that NSP hydrolysis products of ECMS would possess the potential to become prebiotics and be beneficial in improving the gut microbiota diversity and gut function. Therefore, the broiler chicken study was conducted to investigate the effect of low and high dietary

inclusion of ECMS on growth performance, nutrient digestibility, and gut health of broiler chickens and to assess the prebiotic properties of ECMS. The abundance of selected bacteria and key metabolites i.e., SCFA in the ileal and cecal digesta of broilers were analyzed.

The abundance of Firmicutes, Bacteroidetes, *Enterococcus spp.*, *Lactobacillus spp.*, and *Bifidobacterium spp.* were measured in ileal digesta, while additional *Clostridium cluster IV* and *E. coli* were measured in cecal digesta. Except Firmicutes, Bacteroidetes are the most common phyla in the chicken ceca (Li et al., 2016). Enterococci are a group of commensal bacteria that are commonly found in the gastrointestinal tract of healthy animals and are associated with the production of lactic acid as they belong to peripheral genera of lactic acid bacteria (Silva et al., 2012; Han et al., 2016). Strains of *Lactobacillus spp.* and *Bifidobacterium spp.* are commonly used as probiotics in animal and human nutrition and have a positive impact on host animals (Markowiak and Ślizewska, 2018; Al-Khalaifa et al., 2019; Ren et al., 2019). In addition, it was reported that *Lactobacillus* was the most abundant genus in the small intestine including duodenum, jejunum, and ileum (Liao et al., 2020). *Clostridium cluster IV* is related to fibre digestion and butyrate production which plays an important role in the beneficial physiological and immunological function of SCFA (Bindels et al., 2015; Ricke et al., 2020). *Escherichia coli* was detected to represent pathogenic strains.

The abundance of *Lactobacillus spp*. and *Enterococcus spp.* were higher in both ileal and cecal digesta of birds fed ECMS diets, especially in those fed a diet containing 20 g/kg of ECMS. As *Lactobacillus spp*. and *Enterococcus spp.* both belong to lactic acid bacteria, the higher content of lactic acid in the ileal digesta of birds fed the High ECMS diet confirmed their presence and indicated their rich abundance in the ileum. Lactobacilli also produce acetic acid (Bindels et al., 2015) which level was higher in the ileal digesta of birds fed ECMS-containing diets than in the

Control diet. *Bifidobacterium*, although mainly produces acetic acid, belongs also to the group of lactic acid-producing bacteria. For this reason, in this study an increase of the *Lactobacillus* population in ileum could cause the reduction of the *Bifidobacterium* population, because there was no need for compensatory production of bifidobacterial lactic acid. This relationship can be confirmed by the highest ileal lactic acid production in the High ECMS diet where the *Lactobacillus* was highly abundant ($P < 0.001$) while *Bifidobacterium* population was lower when compared to the Control ($P < 0.05$). Such relationship was not observed in cecal microbiota, whereas in the High ECMS treatment both *Lactobacillus* and *Bifidobacterium* were statistically more abundant in the ceca when compared to the Control. Such shift can contribute to the beneficial effects of ECMS on gut health of the host animal.

Bacteroidetes which represents the most common phyla in the chicken ceca were significantly higher in the High ECMS treatment when compared to the Control. This may contribute to the improvement in growth performance due to their role in aiding the digestion of complex molecules of poultry diets (Wexler, 2007). In the ileal microbiota, the higher abundance of this phyla in the Control group than in the High ECMS treatment may be attributed to less stable microbiota when compared to ceca. It can be emphasized that the majority of gut microbiota is present in the ceca and reaches 10^{11} to 10^{12} cells/g, while in the ileum the bacterial numbers were only up to 10^{8} cells/g. However, microbiota populations of the small intestine, which is a major compartment of nutrient digestion and absorption, would be of importance and would reflect the effect of dietary treatments on the physiological processes. It is known, however, that ceca are the main sites for bacterial fermentation, and when compared to ileum it could be characterized by more stable microbial community.

The key beneficial metabolites of gut microorganisms are SCFA which can decrease intestinal pH, provide energy for intestinal epithelium cells, and have a positive effect on physiological, metabolic, and immunological functions (Bindels et al., 2015; Dalile et al., 2019; Do et al., 2020). SCFA are saturated fatty acids with one to six carbons with acetic acid, propionic acid, butyric acid, and lactic acid predominating (Dalile et al., 2019). In some studies, however, lactic acid measurements are not always included (Czerwiński et al., 2010; Konieczka et al., 2019; Markowiak-Kopeć and Śliżewska, 2020).

Acetic acid, lactic acid, and total SCFA contents of ileal digesta of birds fed diets containing ECMS were significantly higher than those in birds fed the Control diet. Although there was a difference observed in butyric acid in the ileal digesta of birds fed different diets, butyric acid accounted for less than 1.4 % of total SCFA, so its effect could be negligible as the difference in the total contents of SCFA was mainly attributed to the levels of acetic and lactic acids.

The increased amount of total ileal SCFA contents is believed to induce lower pH in the gut (Shim et al., 2005). However, it was not observed in this study. As well, no obvious differences were observed in sialic acids secretion, and ATTD of NDF and N between birds fed different diets. However, the diet containing 20 g/kg of ECMS significantly improved FI and BWG compared to the diet containing 1 g/kg of ECMS and the Control diet.

Prebiotics have attracted great attention due to their beneficial effects on gut health and the emergence of antibiotic resistance (Ricke et al., 2020). Supplementation of wheat/soybean-based diet with 0.025% and 0.01% of XOS showed no significant effect on growth performance and SCFA concentration in ceca on day 14, and significantly decreased ileal digestibility of DM, N, and energy in broilers (Craig et al., 2020). Refined functional carbohydrates including MOS, β-glucan, and D-mannose increased BWG, FI and reduced colonization of *Campylobacter spp.* in

the ceca of broilers (Froebel et al., 2019). A positive effect on FI was also observed when MOS (5 g/kg) was supplemented in corn/soybean meal-based diets. In addition, the pH values of ileal digesta were not significantly affected by MOS or FOS supplementation (Al-Khalaifa et al., 2019). Microencapsulated probiotics and prebiotics (β-mannose and FOS) supplementation demonstrated a positive effect on average daily gain, Lactobacilli counts, and serum immune function in broiler chickens (Wang et al., 2018). The effect of various prebiotics on the cecal SCFA contents of broilers showed great variations. Out of 14 studies, 6 studies showed significant positive effects, while 2 studies demonstrated negative effects of prebiotics on the concentration of total cecal SCFA. Overall, this meta-analysis concluded that prebiotic supplementation had no significant effect on the concentration of cecal SCFA (Worawong et al., 2022). Thus far, the modulation of microbiota is the most common effect of prebiotics in broiler chickens while other beneficial effects may or may not be that evident.

In conclusion, the water-solubles separated from enzymatically-modified canola meal were higher in crude protein and lower in dietary fibre when compared to their intact counterparts of canola meal. The main effect of the enzymatic modification was realized by depolymerization of non-starch polysaccharides thus releasing various non-starch polysaccharides hydrolysis products that exert prebiotic properties. When included in the diet, especially at the level of 20 g/kg, enzymatically-modified canola meal solubles contributed to significant proliferation of the beneficial bacteria, increased SCFA production in the ileum, and improved growth performance of broiler chickens. Therefore, enzymatically-modified canola meal solubles could be used as a prebiotic preparation in broiler chicken nutrition.

CHAPTER SEVEN : MANUSCRIPT V

The effect of enzymatically-modified canola meal on growth performance and gut health of weaned piglets

7.1 Abstract

Weaning is considered the most critical and challenging period for piglets. Enzymatic modification of canola (*Brassica napus* L.) meal (**CM**) can depolymerize non-starch polysaccharides (**NSP**) and release low-molecular-weight polysaccharides and oligosaccharides which due to their prebiotic properties can be beneficial for weaned piglets. The effect of two products, enzymatically-modified canola meal (**ECM**) and water-solubles isolated from the enzymatically-modified canola meal (**ECMS**), on growth performance and gut health of weaned piglets were investigated. Seventy-two weaned piglets (half male and half female; body weight 6.94 ± 0.57 kg) were randomly assigned to 3 dietary treatments with 8 replicate pens in a randomized complete block design. A two-phase feeding program (phase I, 1-21d and phase II, 21-28 d after weaning) was carried out in this 28-d feeding study. During phase I, pigs were fed a corn/soybean meal Control diet containing 50 g/kg of CM, and two experimental diets containing ECM or ECMS replacing 20 g/kg of CM. During phase II, all pigs were fed the same corn/soybean meal-based diet to investigate the after-effect of the phase I experimental diets. The results showed that experimental diets had no significant effect on growth performance. The ECMS diet significantly decreased the pH value of the colon contents and increased the population of *Bifidobacterium* in the cecum and colon. After ECM and ECMS were withdrawn from the diet, the pH in cecum of the ECM group become significantly lower than in the Control group. In conclusion, dietary supplementation with ECMS can decrease intestinal pH and stimulate proliferation of beneficial gut microbiota. Therefore, its prebiotic properties can be utilized in weaned pigs nutrition.

Key words: canola meal, enzymatical modification, weaned piglet, non-starch polysaccharides (NSP), prebiotic.

7.2 Introduction

Weaning is considered the most critical and challenging period for piglets. During this period, pigs are facing separation from the sows, mixing litters, a new hierarchy dispute, a change from a liquid to a solid format of feed, a new environment, transportation, etc. (Pluske et al., 2003). Consequently, a reduction in appetite and body weight gain can be observed. In more severe cases, a decrease in body weight and an increase in the incidence of diseases or disorders, such as post-weaning diarrhea, may occur (Pluske et al., 1997; Mahfuz et al., 2022). Traditionally, antibiotics were used as growth promoters during this period to mitigate the negative effect of dysbiosis. However, their use has been banned in most jurisdictions because of concerns about antibiotic resistance (de Lange et al., 2010; Heo et al., 2013; Faccin et al., 2020). In this context, strategies to help pigs overcome the challenges during this period has been subject of intense research attract the interest of researchers. Among these, adding feed additives, such as prebiotics, is regarded as a possible way to improve performance during the post-weaning period (Azad et al., 2020). A prebiotic refers to 'a nondigestible compound that, through its metabolization by microorganisms in the gut, modulates composition and/or activity of the gut microbiota, thus conferring a beneficial physiological effect on the host' (Bindels et al., 2015).

Canola (*Brassica napus* L), was developed to have low levels of erucic acid (< 2%) and glucosinolates (< 30 μmol/g) (Rathod and Solanki, 2020; Ton et al., 2020). Due to the success in plant breeding, canola meal (**CM**), the co-product of oil extraction from canola seed, is the second ranked worldwide feed protein ingredient which is utilized as an economical feedstuff in swine nutrition (Canola Council of Canada, 2019; Long et al., 2020). Canola meal contains 347 - 424 g/kg of total dietary fibre (**TDF**) on a dry matter (**DM**) basis (Adewole et al., 2016). The TDF of CM includes non-starch polysaccharides (**NSP**, 207–228 g/kg DM), lignin and polyphenols (97–

121 g/kg DM), and neutral detergent insoluble crude protein (41.0–75.0 g/kg DM). Non-starch polysaccharides are predominant dietary fibre fractions in CM.

Enzymatic modification of CM dietary fibre could facilitate the break down of the long polysaccharide chain of NSP towards releasing low-molecular-weight polysaccharides, and oligosaccharides which may have prebiotic effects on monogastric animals. In our previous study, enzymatically-modified CM (**ECM**) and enzymatically-modified CM solubles (**ECMS**) have demonstrated prebiotic properties and had a positive effects on gut health and growth performance of broiler chickens (Chapters Four and Six). The potential of NSP hydrolysis products produced from 80% ethanol-insoluble CM to maintain fluid balance in Enterotoxigenic *Escherichia coli* (ETEC) infected piglets was investigated by Kiarie et al. (2008) using an *in-situ* model, who found that these products had a positive effect on the net absorption of fluid and solutes during infection. However, the knowledge about their effect on growth and health of weaned piglets is still scarce.

The objective of this study was to evaluate if supplementation of diets with ECM and ECMS will influence the growth performance, intestinal pH, volatile fatty acids (**VFA**) in the cecum and colon contents, and intestinal microbial populations of weaned piglets. It was hypothesized that ECM and ECMS could have prebiotic effects on weaned piglets.

7.3 Materials and methods

All research procedures and methods relating to animals were performed according to the protocols that were reviewed and approved by the University of Manitoba Animal Care Committee (F18-037/1/2), and pigs were cared for according to the guidelines of the Canadian Council on Animal Care (CCAC, 2009).

7.3.1 Materials

Prepress solvent-extracted CM was produced from black-seeded *B. napus* canola seed and provided by Bunge Canola Processing Plant, Altona, Canada.

Enzymes, **Pr** (Protease, 1,082,614 U/g), **PA** (Pectinase A, 443,061 U/g), **PB** (Pectinase B, 425,773 U/g), **XB** (Xylanase B, 60,763 U/g), and **Inv** (Invertase, 362,631 U/g) were obtained from Canadian Bio Platforms Inc., Calgary, Alberta, Canada.

7.3.2 Production of ECM and ECMS

The production of ECM was performed by constantly mixing CM, distilled water, and enzyme cocktail (4 g/kg of PA, 4 g/kg of PB, 4 g/kg of XB, and 0.2 g/kg of Inv) at 40°C for 48h using an overhead stirrer (BDC 2002, Cafromo LabSolutions, Ontario, Canada) and then freeze-dried.

To produce the ECMS preparation, CM was mixed with distilled water and a mixture of carbohydrases and protease (4 g/kg of PA, 4 g/kg of PB, 4 g/kg of XB, 4 g/kg of Pr, and 0.2 g/kg of Inv) for 48 h at 40°C. After incubation, the slurry was centrifuged for 10 min at 3000rpm after which time the supernatant was collected and spray-dried (flow rate 2 L/h; inlet temperature 215°C, outlet temperature 73-78°C) to obtain the ECMS preparation.

7.3.3 Animals, housing, diet, and experimental design

Seventy-two piglets (half male and half female, TN 70 × TN Tempo, Winnipeg, MB, Canada) with initial average body weights of 6.94 ± 0.57 kg were assigned to 3 treatments with 8 replicate pens (4 pens with 3 barrows per pen and 4 pens with 3 gilts per pen) according to sex and body weight in a randomized complete block design. The pigs were weaned at 21 days of age and obtained from the Glenlea Research Station at the University of Manitoba. After weaning, the piglets were transported to T.K. Cheung Centre for Animal Science Research facility at

Department of Animal Science and fed experimental diets without acclimatization period which allows assessing the effect of ECM and ECMS during the challenging period. Piglets were raised in pens with plastic covered expanded sheet-metal flooring. Feed and water were provided ad libitum.

Experimental diets were formulated to meet NRC requirements (NRC 2012). The experiment was divided into Phase I (1 to 21 days post-weaning) and Phase II (22 to 28 days post-weaning). In Phase I, pigs were fed one of 3 experimental mash diets which consisted of a corn/soybean/CM diet (Control), the ECM and ECMS diets in which 2% of CM in the Control diet was replaced with ECM or ECMS, respectively (Table 7.1). In Phase II, all the pigs were fed the same corn/soybean meal diet (Table 7.1) to test the post-effect of the Phase I diet.

Titanium dioxide was added to the experimental diets at 0.3% as an indigestible marker. The performance of piglets was monitored and the fecal score of each pen was recorded daily on a scale from 1 to 5 (Holanda and Kim, 2021). Body weight and feed intake were recorded weekly with pen as the experiment unit.

Table 7.1 Composition of experimental diets (g/kg, as-fed basis)

Ingredient	Phase I			Phase II
	Control	ECM	ECMS	
Corn	604.4	604.4	604.4	661.8
Soybean meal	220.0	220.0	220.0	250.0
Fish meal	60.0	60.0	60.0	30.0
CM	50.0	30.0	30.0	0
ECM	0	20.0	0	0
ECMS	0	0	20.0	0
Vegetable oil	20.0	20.0	20.0	14.5
Limestone	10.5	10.5	10.5	9.1
Monocalcium phosphate	5.8	5.8	5.8	8.5
Salt (NaCl)	8.0	8.0	8.0	6.1
Vit-Min Premix[a]	10.0	10.0	10.0	10.0
Lys-HCl	5.3	5.3	5.3	4.2
DL-Met	1.3	1.3	1.3	1.3
L-Thr	1.4	1.4	1.4	1.4
L-Trp	0.3	0.3	0.3	0.1
Titanium dioxide	3.0	3.0	3.0	3.0
	1,000	1,000	1,000	1,000
Calculated composition (g/kg unless specified)				
Net energy (kcal/kg)	2503	2503	2503	2510
Total calcium	8.2	8.2	8.2	7.0
STTD phosphorus	3.9	3.9	3.9	3.7
SID crude protein	185.0	185.2	186.2	169.4
SID Lys	14.2	14.2	14.2	12.3
SID Methionine + cysteine	7.4	7.4	7.4	6.8
Analyzed composition (g/kg)				
Crude protein	219.0	220.0	220.0	206.0
Dry matter	887.6	889.8	895.0	898.8

[a] Provided per kg of diet: vitamins A, 2,200 IU; vitamin D_3, 220 IU; vitamin E, 16 IU; vitamin K, 0.5 mg; thiamine, 1.0 mg; riboflavin, 3.5 mg; niacin, 30 mg; pantothenic acid, 10 mg; vitamin B12, 0.02 mg; folic acid, 0.3 mg; Cu, 6 mg as copper sulfate; I, 0.14 mg as calcium iodate; Fe, 100 mg as ferrous sulfate; Mn, 4 mg as manganese oxide; Se, 0.3 mg as sodium selenite; Zn, 100 mg as zinc oxide; biotin 0.05 mg.

7.3.4 Sample collection

Fresh feces from each pen were collected on days 19 to 20 and days 26 to 27 and stored at -20°C until oven-dried. The two days feces samples were pooled by pen and dried at 60°C for 120 h in a forced-air oven for apparent total tract nutrient digestibility (**ATTD**) measurement.

One pig close to the average pen body weight was selected on day 21 and one pig close to the average body weight of all the piglets for this trial was selected on day 28 from each pen for digesta sample collection. Selected pigs were injected with stresnil: xylazine (2:4 mg/kg) and euthanized by a captive bolt to collect jejunal, ileal, cecal, and colonic digesta. Jejunal digesta samples were taken 2 m away from the ileocecal junction. Ileal digesta samples were taken 20 cm proximal to the ileocecal junction. Samples of colonic digesta were collected from the distal end of the colon. Each sample was mixed prior to pH measurement in duplicate, and then immediately snap-frozen in liquid nitrogen and stored at -80°C until further analysis.

7.3.5 DNA extraction and quantitative real-time polymerase chain reaction (qPCR)

Microbiota DNA of cecal and colonic digesta was extracted according to the manufacturers' guide (Pub. No. MAN0014266) using an Invitrogen™ PureLink™ Microbiome DNA Kit (Thermo Fisher Scientific Inc.). After extraction, the DNA concentration of the samples was measured by a spectrophotometer (NanoDrop-2000, Thermo Fisher Scientific, Waltham, MA) and diluted with ultrapure water to 0.75 ng/µL(Lunedo et al., 2014). Quantitative real-time polymerase chain reaction was performed in 96 well plates in duplicate using CFX Connect ™ Real-Time PCR Detection System (Life Science Research, Bio-Rad, Canada). The forward and reverse primer sequences of selected bacterial groups were designed according to previously published works (Table 6.2). The results of qPCR were analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001).

7.3.6 Analytical procedures

Diet, ingredient, and fecal samples were ground to pass through a 1 mm sieve before analysis. All samples were analyzed in duplicate. Standard AOAC (2005) methods were used to measure dry matter (945.15), ether extract (920.39), nitrogen (**N**) and crude protein (968.06), ash (923.03), total phosphorus (965.17), and **NDF** (neutral detergent fibre, 2002.04). Simple sugars (including fructose and glucose), sucrose, raffinose, and stachyose were analyzed according to the method of Slominski et al. (1994). Total dietary fibre was analyzed according to Slominski et al. (1994). Total and water-soluble NSP were measured following the procedures described by Englyst and Cummings (1984) with some modifications (Slominski and Campbell, 1990). The NSP hydrolysis products were measured by hydrolyzing samples in 12 M sulfuric acid and analyzing the content of sugars according to Slominski and Campbell (1990) and then subtracting NSP, simple sugars, and sucrose from the total component sugar content. Phytate phosphorus was determined following the method described by Haug and Lantzsch (1983). Titanium was measured using an inductively coupled plasma atomic emission spectroscopy (ICP-AES Vista, Varian, USA) according to the procedures of Lomer et al (2000).

Volatile fatty acids were analyzed according to the method described by Liao et al. (2020) with some modifications. Briefly, 0.5 g of thawed samples were mixed with 1.0 mL of 2.5% metaphosphoric acid solution and 0.5 mL of internal standard solution (2-ethylbutyric acid). The mixture was put in the fridge for 30 min, vortexed intermittently, and then centrifuged for 15 min at 14,000 ×g at 4°C. The supernatant was used to analyze VFA including acetic acid, propionic acid, butyric acid, isobutyric acid, valeric acid, and isovaleric acid by gas chromatography (Scion 436 GC, Netherlands).

7.3.7 Calculations and statistical analysis

The apparent total tract digestibility (**ATTD**) of NDF, DM, and N was calculated using the following equation (Meng and Slominski, 2005):

$$\text{ATTD of Nutrients (\%)} = [1-(Nu_f \times Ti_d)/(Nu_d \times Ti_f)] \times 100$$

Equation 7.1

where Nu_f refers to nutrient concentration in feces; Nu_d refers to nutrient concentration in diets; Ti_f refers to marker concentration in feces; Ti_d refers to marker concentration in diets.

All the statistical analysis was conducted using the SAS program (version 9.4, SAS Institute Inc., Cary, NC). Data were analyzed using the Mixed procedure with the dietary treatment as the fixed factor and sex as the block. Each pen was regarded as an experimental unit. Means were separated by Tukey's honestly significant difference. All statements of significance were based on $P < 0.05$. All figures were created using ggplot2 (Version 3.3.3) in R studio (Version 1.4.1106).

7.4 Results

7.4.1 Chemical composition of ECM and ECMS

The chemical composition and amino acid profile of CM, ECM, and ECMS were determined and are demonstrated in Table 7.2 and Table 7.3.

Table 7.2 Chemical composition of canola (*Brassica napus* L.) meal (CM), enzymatically-modified canola meal (ECM) and enzymatically-modified canola meal solubles (ECMS) (g/kg, dry matter basis)

Component	CM	ECM	ECMS
Dry matter	915.0	947.0	930.7
Crude protein	429.0	436.7	507.0
Ether extract	32.0	29.1	10.7
Carbohydrates			
Simple sugars[1]	4.6	4.2	47.3
Sucrose	72.9	0.8	1.0
Raffinose family oligosaccharides[2]	33.0	ND[3]	ND
Fibre fractions			
Neutral detergent fibre (NDF)	252.9	188.5	ND
Total dietary fibre (TDF)	364.0	254.8	41.1
Non-starch polysaccharides (NSP)	235.8	137.2	41.1
Component sugars of NSP			
Arabinose	46.9	14.4	12.8
Xylose	15.5	5.8	1.7
Mannose	2.5	1.9	1.8
Galactose	13.6	8.9	6.7
Glucose	72.4	44.0	3.2
Uronic acids	85.0	62.3	15.0
Water-soluble NSP	21.7	24.8	41.1
NSP hydrolysis products	ND	84.2	158.7
Ash	72.0	72.5	113.9
Total phosphorus (P)	12.8	11.3	20.3
Phytate P	6.5	ND	ND

[1] Includes fructose and glucose.

[2] Includes raffinose and stachyose.

[3] Not detected.

Table 7.3 Analyzed amino acid content of canola (*Brassica napus* L.) meal (CM), enzymatically-modified canola meal (ECM) and enzymatically-modified canola meal solubles (ECMS) (g/kg, dry matter basis)

	CM	ECM	ECMS
Arginine	22.5	21.4	24.4
Histidine	10.8	10.7	13.8
Isoleucine	16.3	16.7	18.2
Leucine	28.6	29.1	32.6
Lysine	22.3	21.0	25.9
Methionine	7.3	7.5	8.9
Phenylalanine	16.7	16.8	19.4
Threonine	17.0	17.0	20.2
Valine	20.7	21.2	23.2
Alanine	17.9	18.9	21.1
Aspartic Acid	28.9	29.7	33.4
Cystine	9.8	10.1	11.9
Glutamic Acid	74.3	73.8	90.8
Glycine	21.1	21.5	28.3
Proline	25.3	25.7	31.0
Serine	15.9	15.7	18.3
Tyrosine	10.6	10.6	11.7
Total analyzed amino acids	365.9	367.7	433.1

Canola meal, ECM, and ECMS contain 429, 437, and 507 g/kg of crude protein on a DM basis, respectively. ECM had lower content of sucrose, NDF, TDF, NSP, and phytate P compared with CM. The highest level of crude protein, simple sugars, ash, total phosphorus, and lowest NDF, TDF, and NSP was observed in ECMS. Phytate phosphorus was not detected in ECMS and ECM.

The differences in the content of dietary fibre and its hydrolysis products are well pronounced between ECM and ECMS. ECMS contained 41.1 g/kg of water-soluble NSP and 158.7 g/kg of NSP hydrolysis products, whereas ECM contained 24.8 g/kg of water-soluble NSP and 84.2 g/kg of NSP hydrolysis products.

7.4.2 Growth performance and nutrient digestibility

The effects of ECM and ECMS supplementation on growth performance and ATTD of NDF, DM, and N of weaned piglets are shown in Table 7.4 and Table 7.5. Experimental diets had no effect ($P > 0.05$) on the average daily gain (**ADG**), average daily feed intake (**ADFI**), and gain to feed ratio (**G:F**) weekly and during the whole experimental period. However, the trend in the ADFI from day 21 to 28 showed a slight difference ($P= 0.085$), indicating better intake in the control treatment compared to the ECM and ECMS treatments. Similar trend was observed in the G:F ratio in the third week of the experiment ($P=0.065$). There was a relatively high standard error of the mean (SEM) in ADG and G:F, especially among pigs in the first phase of the study, highlighting the challenging impact of transitioning to the new diet. The experimental diets had no effect on the ATTD of NDF, DM, and N.

Table 7.4 Effect of experimental diets[1] on growth performance of weaned piglets

Item	Control	ECM	ECMS	SEM	P- value
Body weight, kg					
Day 0	6.97	7.01	6.88	0.189	0.881
Day 7	7.06	7.35	7.09	0.220	0.545
Day 14	8.68	9.22	8.88	0.285	0.410
Day 21	11.88	12.40	12.01	0.329	0.530
Day 28	15.93	16.58	16.03	0.409	0.490
Average daily gain, g/d					
Day 0 to 7	26.1	54.0	59.3	20.34	0.174
Day 7 to 14	236.0	269.4	237.4	14.94	0.202
Day 14 to 21	446.9	437.0	448.2	16.69	0.871
Day 21 to 28	612.5	589.1	565.0	30.90	0.543
Day 0 to 28	330.4	337.4	327.5	11.35	0.502
Average daily feed intake, g/d					
Day 0 to 7	96.1	98.5	92.2	15.03	0.916
Day 7 to 14	252.0	284.4	275.2	14.37	0.222
Day 14 to 21	537.6	610.1	576.4	26.29	0.133
Day 21 to 28	850.7	799.6	744.6	34.07	0.085
Day 0 to 28	434.1	448.2	422.1	13.05	0.111
Gain to feed ratio, g/g					
Day 0 to 7	0.25	0.48	0.59	0.063	0.073
Day 7 to 14	0.91	0.95	0.88	0.032	0.358
Day 14 to 21	0.83	0.72	0.80	0.022	0.065
Day 21 to 28	0.74	0.74	0.79	0.052	0.649
Day 0 to 28	0.75	0.76	0.77	0.013	0.805

[1] The Composition of experimental diets is in Table 7.1.

Table 7.5 Effect of experimental diets[1] on apparent total tract digestibility of nutrients (%)

Item	Control	ECM	ECMS	SEM	P value
Phase I					
NDF	43.3	46.7	49.3	2.51	0.258
DM	78.0	77.6	79.1	0.68	0.318
N	71.8	68.9	71.4	1.37	0.265
Phase II					
NDF	43.2	47.6	48.3	2.24	0.193
DM	81.7	82.0	80.0	0.46	0.843
N	74.3	75.3	74.5	0.90	0.679

[1] The Composition of experimental diets is in Table 7.1.

7.4.3 Concentration of VFA and pH in digestive tract content

The pH value of jejunal, ileal, cecal, and colonic digesta, and VFA concentration in cecal and colonic digesta are presented in Figure 7.1 and Table 7.6.

On day 21 after weaning, a lower pH value ($P < 0.05$) was observed in the colonic digesta of the ECMS group when compared to that of the Control and ECM groups. Diets had no effect ($P > 0.05$) on total VFA concentration in cecal and colonic digesta, whereas the value of colonic VFA in ECMS treatment was higher than that of Control treatment. The concentration of butyric acid in the cecal VFA of ECMS treatment was higher ($P < 0.05$) than in ECM.

Table 7.6 Effect of experimental diets[1] on volatile short chain fatty acids (VFA) in digestive contents

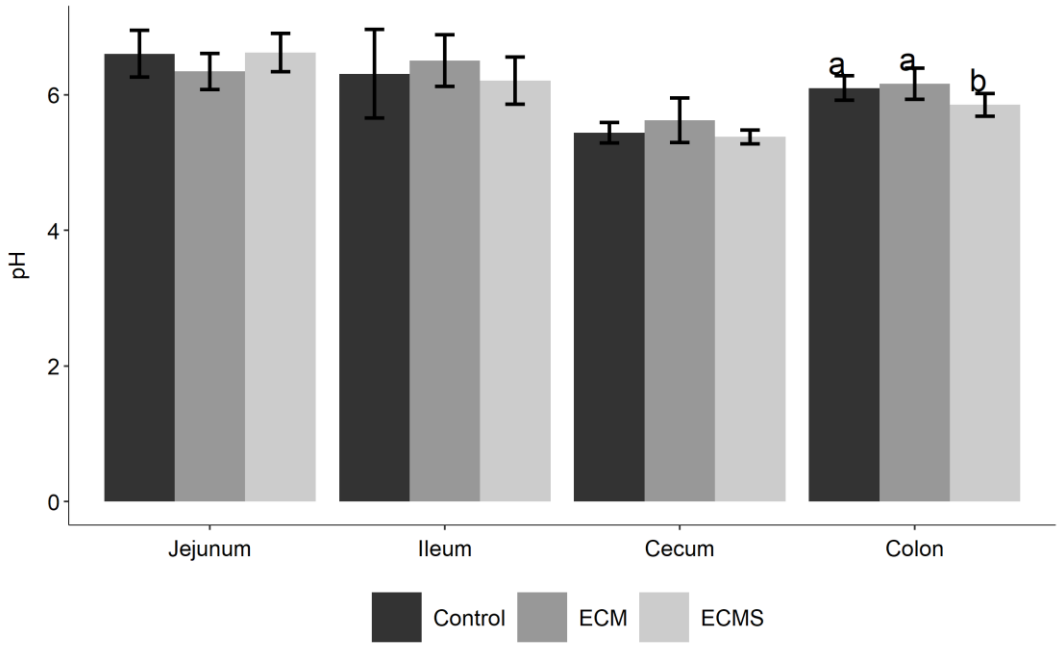
Item	Control	ECM	ECMS	SEM	P value
Day 21 Cecal VFA, mmol/kg					
Acetate	67.19	63.86	65.56	4.658	0.865
Propionate	35.48	35.68	39.42	2.992	0.571
Butyrate	24.34[ab]	19.15[b]	27.19[a]	1.854	0.015
Valerate	5.00	4.47	5.27	0.881	0.761
BCFA	0.58	0.76	0.53	0.102	0.261
VFA	132.71	124.92	137.29	8.373	0.581
Day 21 Colonic VFA, mmol/kg					
Acetate	72.36	70.83	76.05	4.641	0.719
Propionate	39.22	40.48	44.57	4.001	0.608
Butyrate	24.62	27.16	30.05	2.616	0.358
Valerate	8.01	7.50	8.00	1.065	0.920
BCFA	8.37	10.13	7.84	1.089	0.316
VFA	151.95	156.11	169.82	12.039	0.556
Day 28 Cecal VFA, mmol/kg					
Acetate	57.84	68.98	65.74	5.757	0.466
Propionate	32.67[b]	48.92[a]	44.15[ab]	5.913	0.034
Butyrate	24.03	23.61	22.74	2.674	0.942
Valerate	10.62	7.70	5.50	1.671	0.104
BCFA	0.72	0.82	0.71	0.157	0.849
VFA	116.51	145.46	140.15	14.400	0.226
Day 28 Colonic VFA, mmol/kg					
Acetate	62.95[a]	50.65[b]	64.52[a]	3.662	0.027
Propionate	38.50	30.82	37.01	3.474	0.275

Butyrate	18.45	16.89	17.22	2.214	0.871
Valerate	6.56	4.64	5.43	0.722	0.192
BCFA	7.62	5.97	6.43	0.821	0.362
VFA	134.08	108.97	130.61	9.413	0.149

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1] The Composition of experimental diets is in Table 7.1.

(A) Day 21



(B) Day 28

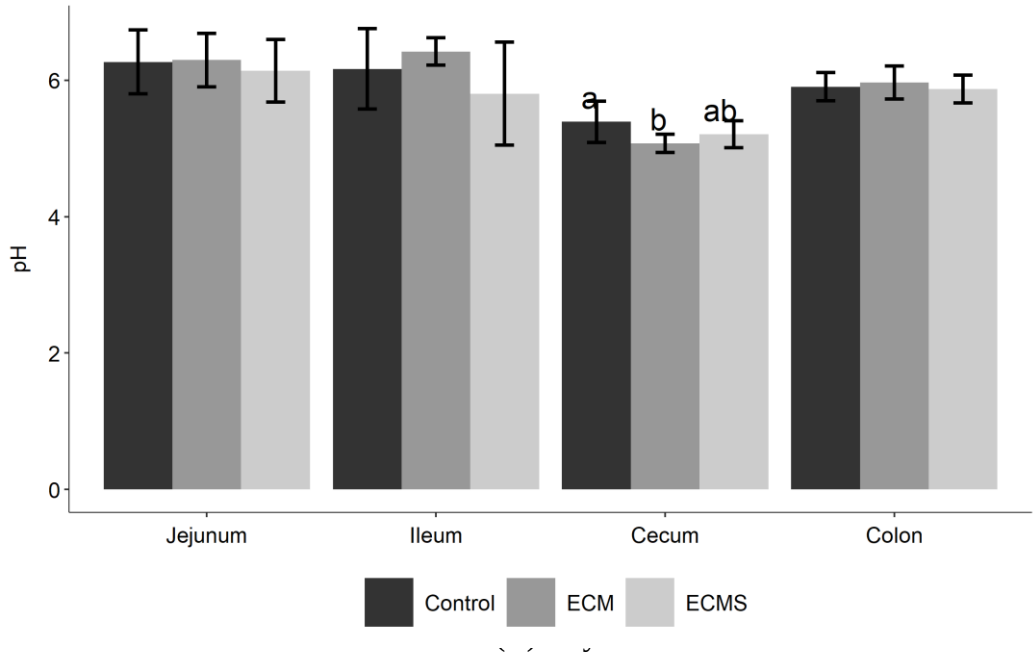


Figure 7.1 Effect of experimental diets on pH in content of digestive tract segments on days 21 and 28 of post weaning piglets. Data are means with standard deviations represented by vertical bars. Means with no common superscript letters within a subgrouping differ significantly ($P < 0.05$)

On day 28, the pH value in the cecal digesta of pigs from the ECM group was lower ($P < 0.05$) than pigs in the Control group, while no effect of experimental diets on the pH value of jejunal, ileal, and colonic digesta was noticed. The propionate concentration in the cecum was higher ($P < 0.05$) in the ECM group than in the Control. The total content of VFA in cecal digesta of ECM treatment was numerically higher than in the Control group, although the difference was not significant. In the colon, the concentration of acetate in ECM treatment was lower ($P < 0.05$) than in the Control and ECMS treatments. No difference ($P > 0.05$) was observed between total colonic VFA among treatments.

On day 28, the pH values in the content of jejunum, ileum, cecum, and colon of pigs from all the experimental treatments were numerically lower than those observed on day 21, except for the colon digesta of ECMS treatment where the pH did not fluctuate over time. The pH value of cecal digesta on both days 21 and 28 was negatively related to the total VFA. That is to say, the treatment with the highest content of VFA had the lowest pH and the treatment with the lowest VFA content had the highest pH value.

In both cecal and colonic digesta, the most abundant VFA were acetate, propionate, and butyrate. Branched chain fatty acids (**BCFA**) which include iso-butyrate and iso-valerate were the least present VFA in cecal digesta with a concentration of less than 1 mmol/kg, while their concentration in colonic digesta was similar with or even higher than the concentration of valerate.

7.4.4 The fold changes of selected cecal and colonic bacteria community

The effect of experimental diets on the fold changes of selected cecal and colonic bacteria community is shown in Table 7.7.

Table 7.7 Effect of experimental diets[1] on fold change[2] of selected cecal and colonic bacteria community

Item	Control	ECM	ECMS	SEM	P value
Day 21 Cecum					
Firmicutes	0.98	1.03	0.95	0.161	0.9370
Bacteroidetes	1.08	1.23	1.18	0.159	0.7909
Lactobacillus spp.	1.03	1.10	1.37	0.317	0.6670
Bifidobacterium spp.	1.00[b]	2.15[b]	6.08[a]	0.682	<0.0001
Enterococcus spp.	1.09	1.35	1.05	0.187	0.4670
Clostridium cluster IV	1.09	0.83	1.02	0.120	0.2993
Day 21 Colon					
Firmicutes	1.06	1.09	0.92	0.110	0.5266
Bacteroidetes	1.01	1.06	0.89	0.152	0.7343
Lactobacillus spp.	1.03	0.66	1.11	0.138	0.0704
Bifidobacterium spp.	0.99[b]	1.40[b]	10.65[a]	1.719	0.0007
Enterococcus spp.	1.06	1.39	1.02	0.205	0.3688
Clostridium cluster IV	1.09	0.86	0.60	0.202	0.2223
Escherichia coli	1.04	0.47	0.54	0.236	0.1677
Day 28 Cecum					
Firmicutes	1.01	1.08	1.04	0.097	0.8874
Bacteroidetes	1.06	1.28	1.27	0.206	0.7031
Lactobacillus spp.	0.97	0.96	1.04	0.120	0.8840
Bifidobacterium spp.	1.09	0.87	0.37	0.252	0.1442
Enterococcus spp.	0.98	1.67	0.89	0.385	0.1234
Clostridium cluster IV	1.07	1.45	1.64	0.181	0.1043
Day 28 Colon					
Firmicutes	1.07	0.85	0.91	0.114	0.3245

Bacteroidetes	0.99	0.98	0.87	0.100	0.6658
Lactobacillus spp.	1.05	0.85	0.68	0.122	0.1265
Bifidobacterium spp.	1.05[a]	0.70[ab]	0.34[b]	0.238	0.0499
Enterococcus spp.	1.07	1.12	0.98	0.245	0.9204
Clostridium cluster IV	1.03	1.03	1.21	0.112	0.4024
Escherichia coli	1.00	1.42	1.52	0.383	0.2780

[a-b] Means within a row with no common superscripts differ significantly ($P < 0.05$).

[1] The Composition of experimental diets is in Table 7.1.

[2] Measured by qPCR and analyzed using the $2^{-\Delta\Delta C_T}$ method by normalizing the expression of selected bacteria to that of total eubacteria and comparing it to the Control treatment.

The abundance of Firmicutes, Bacteroidetes, *Lactobacillus spp.*, *Bifidobacterium spp.*, *Clostridium cluster IV,* and *Enterococcus spp.* were measured in cecal and colonic digesta. Additionally, *E. coli* was analyzed in colonic digesta. No significant differences were observed in the fold changes of Firmicutes, Bacteroidetes, *Enterococcus spp.*, *Lactobacillus spp.*, *Clostridium cluster IV,* and *E. coli* in cecal and colonic digesta between different treatments on 21 and 28 days. *Bifidobacterium spp.* abundance was higher ($P < 0.01$) in cecal and colonic digesta of piglets fed the ECMS diet than the ECM and Control diets on day 21. However, the abundance of *Bifidobacterium spp*. was remarkably lower ($P < 0.05$) in colonic digesta of ECMS treatment on day 28 than the Control treatment.

7.5 Discussion

Canola meal when compared to soybean meal was traditionally considered a poor protein and energy source for swine because of a relatively low digestibility of key amino acids and a higher amount and complex structure of fibre which are poorly digested (Mejicanos et al., 2016; Lannuzel et al., 2022). Recently, as the nutrient balance of swine diet moved towards using more precise values for net energy and digestible amino acids, it has been accepted that CM could be a

valuable feed ingredient for swine nutrition providing that diets are properly formulated (Landero et al., 2011; Wang et al., 2017; Canola Council of Canada, 2019; Hong et al., 2020). Moreover, the inclusion of 20% CM in corn/soybean meal-based diets for weaned pigs challenged with *E. coli* improved growth performance and tended to reduce the inflammatory response (Hong et al., 2021). Therefore, CM was included in the diet during the Phase I period to facilitate the comparison between ECM and ECMS.

Enzymatical modification of CM reduced the fibre content and depolymerized part of the carbohydrate matrix to release of NSP hydrolysis products. The NSP hydrolysis products, including low-molecular-weight polysaccharides and oligosaccharides, are not quantified because it would require more advanced analytical technology and methodology. Research evaluating the properties of soybean meal and CM NSP hydrolysis products showed that these products exert a beneficial effect during enterotoxigenic *E. coli* infection by maintaining fluid balance (Kiarie et al., 2008).

Weaned piglets are faced with critical nutritional, psychological, and environmental challenges (Pluske et al., 1997). Piglets are separated from the sow, litters are mixed, they are transported to a different environment, and the solid diet becomes their only source of nutrients. To reduce the stress caused by the weaning process, a complex diet is usually offered in the first two to three weeks post weaning. Complex diets are formulated with highly digestible and palatable ingredients (whey permeate, lactose, plasma, sugar, soy protein concentrate, etc.), avoiding or reducing ingredients with anti-nutritional factors, such as soybean meal. In addition, the design of most of the studies include feeding weaned piglets with a commercial diet for one to two weeks and then experimental diets are introduced (Wang et al., 2017; Koo et al., 2020; Guo et al., 2022). In this study, weaned piglets were fed diets with corn, soybean, and fish meal as

primary sources of protein and energy without acclimatization period. This approach was expected to create a less than optimal weaning conditions so as to give room to express the impact of ECM and ECMS preparations on piglet health and performance. Feeding newly weaned piglets experimental diets without the acclimatization period is also practiced in research when such application is justified. Hong et al. (2020) fed weaned piglets with corn/soybean meal-based diets containing 15% of animal source protein with or without CM. During the first week, the ADG and ADFI were lower than 100 g/d. Higher ADG (76-105 g/d) and ADFI (118-161 g/d) were also observed in CM diets containing 30% of animal source protein (Mejicanos et al., 2020).

In this study, the post-weaning ADG were lower than expected. Piglets should gain around 100, 200, and 400 g/d in the first, second, and third weeks (Pluske et al., 2003). In the current study during the first week after weaning, the ADG of piglets was less than 100 g/d and the feed intake was low in all treatments. Moreover, body weight loss was observed in several piglets during the first week. The low ADG and ADFI during the first week are highly likely due to the protein ingredients of the diets as well as to the stress related to weaning and relocation of animals. The protein source of the Phase I diets contained soybean meal, fish meal, CM, ECM, or ECMS, thus ingredients that can be challenging for newly weaned pigs. Except for fish meal, other animal protein sources, such as dried whey and animal plasma, which are preferred for weaned piglets due to their superior palatability and high protein digestibility were not included (Solà-Orio et al., 2011). Therefore, the palatability and digestible nutrients of the diets were anticipated to be poorer when compared with the commercial diets or the complex diets for nursery piglets. Similar results were reported by Hong et al. (2020) and Whang et al. (2000). Whang et al. (2000) fed weaned piglets with highly digestible starter diets with animal protein as major protein source and low digestible starter diets with plant source protein as the main protein source. For the first 7 days of

postweaning, the piglets fed a low digestible diet lost body weight. In addition, the difference in the feed quality during the starter period did not influence the entire production period. Overall results indicate that inclusion of ECM or ECMS was not efficient in overcoming the stress and diet-related challenges after piglets were weaned.

Both ECM and ECMS are CM modified by carbohydrases and high in protein, so ATTD of NDF, N, and DM were estimated. Results showed that ECM and ECMS replacement had no obvious effect on ATTD of N and DM, which has also been demonstrated previously when isomalto-oligosaccharides were included in a corn/soybean meal-based diet (Wu et al., 2017).

The pH value of the cecal or colonic digesta of piglets was lowered when ECMS was added to the diet (Phase I) and in the Phase II treatment where the ECM was withdrawn from the diet. The lower intestinal pH value is regarded as beneficial for intestinal well-being (Heo et al., 2013; Bindels et al., 2015). The more acidic environment will be favorable for proliferation of the beneficial bacteria and will supress the overgrowth of pathogens. In this study, the pH values of jejunal, ileal, cecal, and distal colonic chyme were 6.14 to 6.62, 5.80 to 6.51, 5.08 to 5.62, and 5.82 to 6.16, respectively. In addition, the pH value in the jejunum, ileum, cecum, and colon of pigs on day 28 was lower than that on day 21. The pH value in the jejunum, ileum, and cecum is consistent with the reported literature (Shim et al., 2005; Heo et al., 2014) whereas the pH value of distal colonic chyme was lower than the weaned piglets fed with fructo-oligosaccharide supplemented diets but was similar with that of piglets fed with CM involved diets (Mejicanos et al., 2020; Hong et al., 2021).

It was anticipated that ECM and ECMS would stimulate the proliferation of beneficial bacteria, increase the production of VFA and thus reduce the pH value. Although the high abundance of *Bifidobacterium spp.* in cecal and colonic digesta of piglets fed the ECMS diet was

observed, a significant increase of VFA concentration in cecal or colonic digesta was not observed. Volatile fatty acids with acetate, propionate, and butyrate as the main components are the key metabolites that provide energy for the intestinal epithelial cell, regulate gut endocrine functions and reduce intestinal pH, and have beneficial effects on host physiology, metabolism and immune response (Bindels et al., 2015; Martin-Gallausiaux et al., 2021). Butyrate and propionate are identified to play a critical role in the beneficial effect of VFA. In the present study, when the piglets were fed diets supplemented with ECM or ECMS, the concentration of butyrate in cecal VFA of ECMS treatment was significantly higher than ECM. However, after CM, ECM and ECMS were withdrawn from the diet, the propionate concentration in the cecum was higher in the ECM group than in the Control group. It should be highlighted that the concentration of VFA in digesta represents the difference between the production and absorption of VFA.

It is worth noticing that the treatments with a significantly lower pH in the colon or cecum did not have a significantly higher concentration of VFA, which had also been observed when the different level of folate was supplemented in a corn-soybean meal-based diet for weaned piglets. This may be due to the presence of the low pKa lactate (pKa = 3.86) (Heo et al., 2014; Baxter et al., 2019). The pH in the large intestine is related to the diet composition, especially the fermentable fibre that can produce VFA and lactic acid (Heo et al., 2014).

Supplementation of diets for weaned piglets with a variety of prebiotic preparations has been a recommended strategy supporting the antibiotic free feeding programs (Markowiak and Ślizewska, 2018). The fructo- oligosaccharide (**FOS**) and inulin are being used as prebiotic feed additive. Shim et al. (2005) investigated the effect of FOS on growth performance and gut health of weaned pigs. The result showed that a diet supplemented with 3% FOS significantly decreased the pH value in the cecum and proximal colon but did not affect the pH of the distal colon and

feces, and had no significant influence on growth performance (Shim et al., 2005). Another study where FOS was included in the diets for piglets challenged with the Enterotoxigenic *E. coli,* showed that FOS supplementation significantly increased the concentration of acetic acid, propionic acid, and butyric acid, the abundance of *Bacillus* and *Bifidobacterium*, and reduced the populations of *E. coli* in the cecal digesta (Liu et al., 2020). Dietary inulin supplementation had no significant influence on growth performance, but it significantly increased the concentrations of acetic and butyric acid, proliferation of the population of *Lactobacillus*, and decreased the population of *E. coli* (Wang et al., 2020).

In conclusion, replacing canola meal with enzymatically-modified canola meal or enzymatically-modified canola meal solubles had no significant effect on the growth performance of weaned piglets. Weaned piglets fed diets including enzymatically-modified canola meal solubles had significantly lower pH values in the colon and higher population of *Bifidobacterium* in the cecum and colon than that of the Control group. Although enzymatically-modified canola meal supplementation did not show significant effects during Phase I, it significantly decreased the pH value in the cecum after canola meal and enzymatically-modified canola meal were withdrawn from the diet. Therefore, enzymatically-modified canola meal solubles demonstrated more pronounced prebiotic effects in weaned piglets than the enzymatically-modified canola meal. Supplementation of diets with enzymatically-modified canola meal solubles can provide benefits for the piglets after weaning. The prebiotic properties of enzymatically-modified canola meal still require further investigation.

CHAPTER EIGHT : CONCLUSION AND FUTURE STUDIES

8.1 General discussion

The fact that nutrition plays an important role not only in the health and growth of animals, but also in enhancing gut health and function, via modulation of gut microbiota is without question. Further, there is evidence that various dietary fibre components of feed ingredients and fibre breakdown products can have a profound positive impact on gut health and function in monogastric animals. Release of fibre-derived prebiotics can be achieved by utilization of the advanced exogenous enzyme.

Dietary fibre (**DF**) was historically regarded as an antinutritional factor for monogastric animals, but with the progress in research techniques and methods, and advanced understanding of its nature and function in monogastric animal nutrition it regained the attention for its benefits to animal health and growth. The DF profile of feedstuffs is diverse, thus detailed measurements of DF components, particularly non-starch polysaccharides (**NSP**), both soluble and insoluble allow for creating its comprehensive profile and determine the most efficient means to utilize or enhance its bioactive properties.

Canola (*Brassica napus* L.) meal is an important protein source for monogastric animals with high level of dietary fibre. In this project, a detailed chemical composition of canola meal (**CM**) was determined, and a broad selection of multi-carbohydrases was evaluated towards improving of the nutritive value of CM as well as adding value to it by releasing various bioactive components. The effects of the released bioactive components on growth performance and gut health of broiler chickens and weaned piglets were investigated.

Three approaches were used in this project to release the bioactive components from CM fibre. In the first method it was achieved by supplementing the exogenous carbohydrases directly in the CM diets. In the second approach, CM was subjected to 48 h submerge liquid processing with preparations expressing multiple enzyme activities and was dried to produce enzymatically-modified CM (**ECM**). Thirdly, the water-soluble bioactive products of enzymatic hydrolysis of CM dietary fibre were separated from water-insoluble fraction of ECM and spray-dried towards producing ECM solubles (**ECMS**).

A variety of carbohydrase preparations were tested in vitro for their ability to depolymerize NSP of CM and to release potentially bioactive products of enzymatic hydrolysis of CM fibre (Chapter Three). Due to the complexity of CM NSP, its depolymerization requires enzymes that are specific to the main and side chain structures of NSP. The in vitro method involved incubation of CM with the various enzyme preparations.

The degree of cell wall polysaccharide degradation was determined by a reduced recovery of NSP constituent sugars when compared to those of the Control treatment where enzymes were not applied. The most effective enzyme combination was selected for further in vivo evaluation.

The in vitro evaluation showed that the enzyme cocktail which included the multi-carbohydrase preparations identified based on their main activity profile as Xylanase B, Pectinase A and Pectinase B had the strongest ability to depolymerize NSP of CM. The select multi-carbohydrase cocktail was evaluated in the growth performance and the nutrient digestibility study with broiler chickens. As the efficiency has been demonstrated in vitro and in vivo, the select enzyme cocktail was used throughout the entire project to release the bioactive compounds derived from CM fibre.

In Chapters Three and Four, the effect of enzyme supplementation on growth performance and nutrient digestibility was investigated. In both studies, enzyme supplementation significantly improved total tract NSP digestibility and growth performance of broiler chickens. In addition, the effect of enzyme supplementation of diet containing CM on the gut health indices is presented in Chapter Four. Results showed that enzyme supplementation significantly increase the abundance of Bacteroidetes on day 21 but had no significant influence on the production of short chain fatty acids (**SCFA**) and pH value of ileal and cecal digesta.

The effect of enzyme supplementation on the growth performance and gut health of poultry is not always consistent among different studies. In the study by Wiśniewska et al. (2023), supplementation of CM-containing diets with a multicarbohydrase preparation (containing cellulase, mannanase, galactanase, xylanase, glucanase, amylase, and protease activities) significantly improved feed conversion ratio (**FCR**), total tract neutral detergent fibre (**NDF**) degradation, but had no significant effect on average feed intake (**FI**) and body weight gain (**BWG**), nitrogen corrected apparent metabolizable energy (**AMEn**), excretion of sialic acids and SCFA in cecal digesta of broilers. However, according to Olukomaiya et al. (2021), diets containing xylanase had no significant effect on FCR, BWG, FI and apparent ileal digestibility of crude protein and amino acids. Toghyani et al. (2017) reported that supplemented diets including CM with carbohydrase significantly improved final body weight, FCR and ileal energy digestibility, but had no impact on cecal SCFA of broiler chickens. The results presented in Chapter Three and Four indicate that although the enzyme cocktail could depolymerize CM NSP in the gut of broilers, its beneficial effect on gut health is still limited, which is highly likely due to the short transit time of digesta in broilers. To mitigate this limitation, the CM enzymatic modification process has been developed and ECM has been produced.

The production of ECM was performed by continuous mixing of CM and carbohydrase cocktail with water for 48 h at 40°C followed by freeze-drying of the slurry. During the incubation, the NSP of CM were undergoing through the continuous depolymerization, and over the time the spontaneous fermentation occurred, and the pH value of the slurry decreased. Compared with CM, ECM contains less NSP, NDF, total dietary fibre, phytate phosphorus, sucrose, and oligosaccharides (including raffinose and stachyose). The soluble NSP in ECM is 14.3% higher than that of in CM.

The effect of supplementation of broiler chicken diet with ECM on their growth and health was investigated and is presented in Chapter Four. The nutritive value, i.e., standardized ileal digestibility (**SID**) of amino acids (**AA**) and AMEn of ECM for broilers, were determined and is presented in Chapter Five. The AMEn value of ECM is 30.9% higher than that of CM. The increased AMEn value for ECM supplemented diets were also observed earlier (Chapter Four). The results from the two studies confirm that enzymatic treatment significantly increased AMEn of CM. In the Chapter Four, the total tract digestibility of nitrogen in ECM treatment were higher than the Control, with significant difference observed on day 14 between the High ECM diet and the Control. However, no significant improvement was observed on day 21. The SID of AA of ECM with sample collected on day 17 showed no significant improvement compared to CM.

In order to produce a larger volume of ECM for animal studies, CM was incubated under the same conditions as applied to the preliminary in-lab processing. Several attempts were undertaken to facilitate the spray-drying process that was intended to be utilized herein. Due to the physical properties of the slurry, the drying process was not efficient. Therefore, the procedure was modified, and water-soluble fraction (syrup) was separated from the solids using

centrifugation. Additionally, protease (0.4%) was introduced into the enzyme cocktail to facilitate solubility of nutrients. The syrup was spray-dried to obtain ECMS.

The prebiotic effect of ECM and ECMS on broiler chickens was studied in the Chapter Four and Six. Both ECM and ECMS supplementation could modulate gut microbiota, increase the production of SCFA, but had no significant effect on pH value of ileal and cecal digesta. Specifically, both ECM and ECMS increased the relative abundance of *Lactobacillus spp.* in ileal and cecal digesta which is beneficial for gut health. In addition, the effect of ECMS in broiler chicken diet is more pronounced because significant improvement on gut microbiota and SCFA was observed with 20 g/kg of supplementation, while the effect on SCFA was not significant when 5% of ECM were supplemented.

In the Chapter Seven, the supplementation of ECM and ECMS on weaned piglets were investigated. The results showed that the pigs fed diets supplemented with ECMS had relatively lower pH value in colonic digesta and proliferated the population of *Bifidobacterium* in cecum and colon. However, ECM supplementation did not show significant effect on gut health of weaned piglets. In addition, ECM and ECMS supplementation had no significant effect on growth performance of weaned piglets. In the Control diet of this study, 5% of CM was included. The inclusion of CM in the experimental diets may alleviate the influence of ECM and ECMS on weaned piglets.

Based on the results of the five studies, the effects of supplementing diets with ECM or ECMS on broilers and weaned piglets differed in terms of their impact on growth performance, intestinal pH, and microbiota groups although ECMS showed prebiotics on both broiler chickens and weaned piglets. In detail, ECM or ECMS supplementation had a significant effect on growth performance and no obvious effect on intestinal pH of broiler chickens, while the effect of their

supplementation was more pronounced on intestinal pH and limited on growth performance of weaned piglets. The effect of the same or similar feed additive on pigs and broiler chicks were reported. Olukosi et al. (2007) investigated the effect of graded levels of xylanase on the growth performance of growing pigs and broiler chickens. The results showed that xylanase supplementation had no effect on growth performance of pigs but significantly improved the weight gain of broilers. Kipper et al.(Kipper et al., 2020) summarized the performance responses of β-mannanase supplementation on broilers and pigs based on 30 broiler studies and 20 swine studies and concluded that β-mannanase supplementation improved FCR of broilers but improved both weight gain and FCR of pigs.

It is mostly likely that differences in the response of broilers and swine to ECM and ECMS attribute to the differences in digestive physiology and diet formulation. Although broilers and pigs are all monogastric animals, the structures of their gastrointestinal tract are distinct. Broilers have the crop and gizzard which are specific in poultry. Moreover, the large intestine in broilers and pigs is also different. The pair of ceca in broilers sited at the junction of ileum and colon as elongated blind sacs is a unique characteristic of the avian digestive tract. Ceca are kind of like bypass of digestive system of poultry that specific type of material will enter. In addition, the empty of ceca is not as often as the excretion of feces, so the retention time of digesta in the ceca of broilers will usually be much longer than ordinary defecation (Svihus et al., 2013; 2014). The cecum of the pig is a sac that connects the ileum and colon which is weigh much shorter than colon. Therefore, ceca provide great circumstance for the performance of gut microbiota in broilers, while in pigs, cecum and colon are both important segments for gut microbiota and the production of SCFA (Pluske et al., 2018). Moreover, the feed transit time in pigs is about five times more than that in broilers (Olukosi et al., 2007). Another difference is the diet formulation which is obvious

because the diets are formulated to meet the requirement of weaned piglets and broilers respectively. More importantly, the inclusion ratio of ECM in broilers is higher than that in weaned piglets. The supplementation of ECM in swine diet is 20g/kg in Chapter Seven, while the supplementation of ECM in broilers is up to 200 g/kg in Chapter Four. Therefore, the low supplementation of ECM (20 g/kg) in swine diet may lead to its limited effect on the gut microbiota of swine.

The supplementation of ECMS in broilers and weaned piglets was comparable with 1 g/kg and 20 g/kg in broilers (Chapter Six) and 20 g/kg in weaned piglets (Chapter Seven). In both studies, ECMS showed significant influence on the population of selected bacteria. Supplementation of 20 g/kg of ECMS significantly increased the abundance of *Lactobacillus spp*. and *Enterococcus spp.* in both ileal and cecal digesta of birds and stimulated the growth of *Bifidobacterium spp.* and *Bacteroidetes* in cecal digesta of broilers. The most obvious improvement was the population of *Lactobacillus spp*. However, the supplementation of 20 g/kg of ECMS only improved the proliferation of *Bifidobacterium spp*. in cecal and colonic digesta of weaned piglets. It has been observed that the abundance of *Lactobacillus* decreased significantly after weaning (Heo et al., 2013; Pluske et al., 2018). It was reported that the relative abundance of *Lactobacillus* in feces declined from 12.25 to 1.76% after 21 days of weaning (Chen et al., 2017). The relatively stable abundance of *Lactobacillus* when ECMS was supplemented may lie in the high relative abundance of *Lactobacillus* in gut microbiota.

Based on the results presented in chapters Four, Six and Seven, it could be concluded that ECMS had more pronounced prebiotic effect on both broilers and weaned piglets than ECM. Both preparations are in fact very different in the chemical composition and physicochemical properties. The biggest difference between ECM and ECMS is that ECMS is only water-soluble fraction of

ECM with the insoluble fibre fraction excluded. As the depolymerization of NSP increased the solubility of carbohydrates and released low-molecular-weight polysaccharides, oligosaccharides, and disaccharides, the soluble and more fermentable compounds became concentrated in ECMS, thus expressed the prebiotic effect on monogastric animals.

In conclusion, the carefully selected, target-specific enzyme cocktail demonstrated the ability to release bioactive components from CM fibre. Both when used directly in feed or when used in the submerge liquid processing, the enzyme cocktail initiated a beneficial effect on monogastric animals. The extended incubation of CM with target-specific enzyme is advantageous in releasing bioactive components and leads towards the production of value-added novel feedstuffs in form of enzymatically-modified CM or water-soluble fraction of the latter. The production of ECM/ECMS is a promising way to facilitate the utilization of CM in the feed industry and to support the antibiotic free feeding programs.

8.2 Summary

The summary remarks and conclusions drawn from the present study are as follows:

1. Carefully evaluated and designed multi-carbohydrase cocktail hydrolyzed 47.9% of CM NSP in vitro. Such high NSP depolymerization was achieved by using a combination of two pectinase enzymes fortified with xylanase. In addition to their main activities, the above enzymes contained a variety of side activities beneficial in NSP hydrolysis. No further improvement with other enzyme preparations was realized.

2. Birds fed the diet supplemented with 1.5 g/kg of multi-carbohydrase cocktail showed significantly ($P < 0.05$) higher body weight gain during the grower phase (11 - 20 d of age) and the entire trial (1 - 20 d of age). In addition, multi-carbohydrase preparation improved total tract NSP digestibility significantly.

3. Replacing CM with ECM significantly improved digestibility of NSP, dietary AMEn, and relative abundance of *Lactobacillus spp.* in ileal and cecal digesta.

4. Compared with the direct in-feed enzyme supplementation, inclusion of ECM in the diets significantly increased the dietary AMEn during the starter phase (4 to 14 d of age) and relative abundance of *Lactobacillus spp.* in ileal and cecal digesta during the entire trail (4-21 d of age). Therefore, ECM could be considered as a high-value feed additive and/or feed component.

5. No significant differences were observed between standardized ileal digestibility of individual amino acids of CM and ECM. ECM contained higher ($P < 0.05$) standardized ileal digestible cystine than CM.

6. The AMEn value of ECM ($P < 0.05$) was 30.9% higher than that of CM.

7. The use of diet supplemented with 20 g/kg of ECMS significantly increased feed intake and body weight gain of broiler chickens when compared to 1 g/kg of ECMS addition and the Control diet.

8. The abundance of *Lactobacillus spp.* and *Enterococcus spp.* were significantly higher in both ileal and cecal digesta of birds fed the High ECMS diet (20 g/kg) compared to the Control diet. Total SCFA content in ileal digesta of birds fed ECMS diet significantly increased as well. Therefore, ECMS due to its prebiotic attributes can be recommended for broiler chicken nutrition.

9. Replacing CM with ECM or ECMS did not affect growth performance of weaned piglets. When compared to the Control treatment, significantly lower pH value in colon, and higher population of *Bifidobacterium* in cecum and colon were observed in piglets fed diet supplemented with ECMS. Therefore, the ECMS preparation, due to its prebiotic properties can be used to support the health of weaned piglets. The effect of ECM still requires a further investigation.

8.3 Future studies

The present research demonstrated the potentials of ECM/ECMS products as prebiotics in monogastric animal nutrition. However, more studies are still needed before they can be applied by the feed industry. In my opinion, the future research directions are as follows:

1. Optimize the protocols for ECMS production;

2. Investigate the prebiotic effect of ECM/ECMS in disease challenged trials and in on-farm trials (e.g., animals raised without antibiotics);

3. Explore the economic and environmental impacts of ECM/ECMS at the farm gate.

CHAPTER NINE : LITERATURE CITED

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