GEORGIKON FACULTY, UNIVERSITY OF PANNONIA
DEPARTMENT OF ANIMAL SCIENCE

Group of Animal Physiology and Animal Nutrition

DOCTOR OF PHILOSOPHY (PhD) THESIS

Festetics Doctoral School

NUTRITIONAL MODULATION OF SELECTED INTESTINAL PHYSICO-CHEMICAL, HISTOLOGICAL AND MICROBIOLOGICAL PARAMETERS IN BROILERS

DOI:10.18136/PE.2018.689

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KESZTHELY, HUNGARY

2018.

NUTRITIONAL MODULATION OF SELECTED INTESTINAL PHYSICO-CHEMICAL, HISTOLOGICAL AND MICROBIOLOGICAL PARAMETERS IN BROILERS

Értekezés doktori (PhD) fokozat elnyerése érdekében

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List of abbreviations

AGP	Antibiotic growth promoters
BW	Body weight
CFU	Colony forming units
C. jejuni	*Campylobacter jejuni*
DPI	Day post infection
E. coli	*Escherichia coli*
FCR	Feed conversion ratio
GALT	Gut associated lymphoid tissue
GIT	Gastrointestinal tract
IEL	Intraepithelial lymphocytes
MALT	Mucosa associated lymphoid tissue
MBC	Minimal bactericidal concentration
MIC	Minimal inhibitory concentration
M+B	Maize-barley based diet
M+I	Inulin supplemented maize based diet
M+L	Lactose supplemented maize based diet
M+W	Maize-wheat based diet
M+WE	Enzyme supplemented maize-wheat based diet
sNDCs	Soluble non-digestible carbohydrates
NSPs	Non-starch polysaccharides
sNSPs	Soluble non-starch polysaccharides
PBS	Phosphate buffered saline
SCFAs	Short-chain fatty acids
SBM	Soybean meal
SEM	Standard error of the mean

1. ABSTRACTS

1.1. Abstract

NUTRITIONAL MODULATION OF SELECTED INTESTINAL PHYSICO-CHEMICAL, HISTOLOGICAL AND MICROBIOLOGICAL PARAMETERS IN BROILERS

The effects of various cereal grains and prebiotics were elucidated on selected intestinal characteristics associated with gut health conducting two boiler chicken experiments. Different mucus obtained from the small intestine of chickens fed maize based (M), maize-wheat based (M+W) or maize-barley based (M+B) diets were also tested on butyrate anti-*Campylobacter* activity *in vitro*.

In Trial I, a total of 54 one day-old Ross 308 broiler chickens were randomly divided into three isocaloric and isonitrogenous dietary groups: M, M+W and M+W diet with NSP-degrading enzyme supplementation (M+WE). Chickens were orally infected with 10^8 CFU *C. jejuni* on day 14 and were euthanized on 7, 14 and 21 days post infection (DPI). Colony forming units of *C. jejuni* of cecum and ileum, short-chain fatty acid (SCFA) concentration, pH values of the cecum, ileal histomorphology and viscosity of ileal chymus were measured. In Trial II, a total of 200 Ross 308 male chickens were kept in deep litter pens (n=40) and fed diets from day 1 to day 35 of life according to Ross technology (Aviagen, 2014a). Five isocaloric and isonitrogenous diets, differing in their soluble non-digestible carbohydrate (sNDC) content, were composed; M (containing maize as the only cereal), M+W, M+B and maize based supplemented either with 20 g/kg inulin (M+I) or 30 g/kg lactose (M+L). The following parameters were measured: growth performance, gut histology (morphology, goblet cell and IEL numbers), ileal viscosity, cecal SCFA concentration, pH, coliform and *Lactobacillus* counts in comparison to a maize based (control) diet.

In Trial I, the M+WE group had lower *C. jejuni* colonization 14 DPI, higher ileal viscosity, higher total SCFA concentrations in the cecum and enhanced ileal histomorphology compared to the M group. In Trial II, all of the diets tested decreased ileal crypt depth, muscle layer thickness and increased cecal coliform counts relative to the M group. Villus-crypt ratio increased only in the M+L group. Ileal digesta of chickens fed the M+W diet had the highest ileal viscosity and the highest cecal butyrate, valerate and total SCFA concentrations while the lowest pH was observed in cecal contents of chickens fed the M+I diet. Mucus obtained from chickens received different diets did not varied in their effect on butyrate anti-*Campylobacter* activity.

From the results of the study it can be concluded that diet composition can modify *C. jejuni* colonization depending on sampling time point post infection and this change may relate to ileal histomorphology and cecal pH and SCFA concentration. Various sNDC sources had beneficial gut health effects in Trial II, however some of the intestinal variables were dependent on the type of sNDCs.

1.2. Absztrakt

A TAKARMÁNYOZÁS HATÁSA EGYES FIZIKAI-KÉMIAI, SZÖVETTANI ÉS MIKROBIOLÓGIAI PARAMÉTEREK VÁLTOZÁSÁRA BROJLERCSIRKÉK BÉLCSŐVÉBEN

Munkám során két kísérletben vizsgáltam különféle gabonamagvak és prebiotikumok etetésének hatását brojlercsirkékben olyan paraméterekre, amelyek a bél egészségi állapotát jelzik. Az eredmények azt mutatják, hogy a takarmányozás hatással van a *C. jejuni* kolonizáció dinamikájára, ami összefüggésben állhatott a bélmorfológia, illózsírsav és pH változásával. Magas nem-emészthető szénhidrát tartalmú (búzával, árpával, inulinnal vagy tejcukorral kiegészített) tápok kedvező hatással voltak több mutatóra. Hasonló módon befolyásolták a csípőbél-nyálkahártya kriptamélységét, a vakbél pH értékét, valamint a vakbél coliform baktériumainak számát a kukorica alapú táphoz képest. Ezzel szemben a magas nem-emészthető szénhidrát tartalmú tápok a csípőbél viszkozitást, boholy/kripta arányt, vakbél illózsírsav koncentrációt változóan módosították.

1.3. Auszug

DIÄTETISCHE EINFLÜSSE AUF AUSGEWÄHLTE PHYSIKALISCH-CHEMISCHE, HISTOLOGISCHE UND MIKROBIOLOGISCHE PARAMETER IM DARMTRAKT VON BROILERN

Die Effekte der Fütterung verschiedener Getreidekörner und Präbiotika auf Gesundheitsmarker des Darms wurden in dieser Arbeit, auf zwei Studien untergliedert, untersucht. Die Ergebnisse implizieren, dass die Diät Einfluss auf die Dynamik der Kolonisation durch *C. jejuni* hatte. Diese Beobachtung war auf die Änderung der Darmmorphologie, der kurzkettigen Fettsäuren und des pH-Wertes zurückzuführen. Futtermittel mit hohem Gehalt an nichtverdaulichen Kohlenhydraten (ergänzt mit Weizen, Gerste, Inulin oder Laktose) beeinflussten verschiedene untersuchte Parameter positiv. Ebenso hatten diese Futtermittel im Vergleich mit maisbasierten Futtermitteln positive Auswirkung auf die Kryptentiefe der Ileummukosa und auf den pH-Wert des Caecum, sowie einen steigernden Effekt auf die Zahl der coliformen Bakterien im Caecum. Demgegenüber war die Wirkung der an nichtverdaulichen Kohlenhydraten reichen Futtermittel auf die Viskosität und auf das Zotten-Krypten-Verhältnis des Ileum, sowie auf die Konzentration der kurzkettigen Fettsäuren im Caecum nicht einheitlich. Desweiteren zeigten die Ergebnisse Zusammenhänge zwischen der Zahl der Becherzellen und der Zahl der intraepithelialen Lymphozyten, sowie der Höhe der Darmzotten.

2. INTRODUCTION

Poultry meat represents high biological value protein for a favourable price which confers a great popularity on a global level (Barroeta, 2007; Vaarst et al., 2015). The efficiency of poultry meat production has been greatly advanced in the last 50 years due to a huge genetic progress and nutritional optimization. The poultry sector has shown the largest increase relative to other food producing animals and probably poultry will become the most consumed meat in the near future (Conway, 2014; Zuidhof et al., 2014).

In order to ensure efficient and secure poultry meat production, the inclusion of antibiotic growth promoters (AGPs) in animal diets were common for a long time. However, the likelihood of antimicrobial resistance increased with the use of AGPs and thus the European Union have banned the AGPs since 2006 (Onrust et al., 2015). This restriction has led to increased incidences of intestinal diseases in poultry and to increased human health risk such as campylobacteriosis and salmonellosis (Ajuwon, 2016; Hao et al., 2014). Gastrointestinal dysbiosis have also emerged in livestocks and became one of the most challenging problem in broilers flocks (Ducatelle et al., 2015). Therefore, feed additives as substitutes for AGPs are seeked to ameliorate gut health of broilers and to support efficient and secure meat production. Control of the impaired gut function requires a detailed understanding of the interactions between nutrition, gut physiology and microbiota (Onrust et al., 2015; Pan and Yu, 2014).

The aims of this study was to assess various nutritional factors (cereal grain types; enzyme, inulin and lactose supplementation) on gut physiological, histological and microbiological characteristics in broiler chickens contributing to a more complete knowledge of the chicken intestinal ecosystem.

3. LITERATURE OVERVIEW

3.1. Basics of gut health

Gut health has a special importance in animal production due to high performance expectations and strict food safety regulations. The intestinal microflora and the adjacent intestinal wall, connecting intimately to each other, are the key elements which predominantly determine gut health (Jeurissen et al., 2002). The composition of the microbiota and their metabolites are important in the development of gut structure, immune response and serve as a barrier against harmful agents (Onrust et al., 2015). Not only the microflora, but the intestinal mucus and epithelial layer are crucial for the resistence to enteric diseases (Jeurissen et al., 2002; Mantle and Allen, 1989).

3.1.1. Microbes of the avian gut

The internal gut surface and gut ecosystem are very complex unity comprising more than 640 bacterial species, containes over 20 hormones, digests and absorbs the overwhelming majority of nutrients and requires 20% of the body maintainance energy (Choct, 2009). The intestinal microflora of broiler chickens consist of bacteria, fungi and protozoa, but predominantly bacteria reaching approximately 10^9 and 10^{11} CFU/g in the ileum and cecum, respectively (Yegani and Korver, 2008). The GIT of chickens at the first days of life inhabited by facultative aerobes as *Enterobacteriaceae*, *Lactobacillus*, and *Streptococcus*, later obligate anaerobes will become dominant. This trend is also true from proximal to distal direction in the gut lumen of chickens (Rinttilä and Apajalahti, 2013). Due to the high bacterial load it is not surprising that the cecum is the main site for fermentation in avian species (Józefiak et al., 2004). The microbial fermentation in the small intestine, which is the main site for digestive processes, entails a competition for nutrients between the host and the microbes. In contrast, the large intestine (cecum and colon) is already beyond the host digestion system and microbial fermentation will not lead to further energy losses for the host (Chan et al., 2013).

3.1.1.1. Fermentation products (short-chain fatty acids; SCFAs)

Feed components escaping the digestive process of the host can be metabolized by the microbiota in the large intestine. The major end products of bacterial fermentation, specially from fibre components, are SCFAs (Koh et al., 2016). These SCFAs cover acetate, butyrate, propionate, valerate and isovalerate. Usually in the chicken cecum, the relative amount of these SCFAs range with the order of appearance and influenced by diet composition (Józefiak et al., 2004; Molnár et al., 2015). Beside SCFAs, microbial fermentation produces lactate, however, it does not accumulate in the large intestine as some bacterial species convert it to SCFAs (Ríos-Covián et al., 2016). Some bacteria that are not able to utilize complex carbohydrates, benefit by substrate cross-feeding, using breakdown compounds produced by other bacterial groups. For example, some *Bifidobacterium* strains, lacking the ability to ferment inulin-type fructans, can thrive on mono- and oligosaccharides produced by primary inulin degraders (Rossi et al., 2005). Den Besten et al. (2013) proved that, among SCFAs, the main direction for bacterial cross-feeding is acetate to butyrate and in a smaller extent butyrate to propionate.

The SCFAs can be absorbed from the intestinal lumen into the blood system and thus, they serve as energy contributing to the total energy requirements of the chickens by 3-5% (Svihus et al., 2013). Short-chain fatty acids have several benefits also on gut health by functioning as energetic precursors for epithelial cells and for the metabolic processes in the host, providing antimicrobial potential, catalysing enzymatic processes in digestion, controlling gut functionality and modulating secretions of pancreatic and biliaric juices (Mroz et al., 2006). In humans, SCFAs are considered to play an important role in colonic health, for instance, reducing the risk of inflammatory bowel disease, irritable bowel syndrome, cancer and cardiovascular diseases (Chan et al., 2013; Hijova and Chmelarova, 2007). With an increase in SCFA concentration, luminal pH drops inhibiting the growth of pathogenic bacteria and improving the absorption of some nutrients (Macfarlane and Macfarlane, 2012). The selective antimicrobial effect of SCFAs is regarded to the dissipation of the proton motive force across

the bacterial cell membrane (Józefiak et al., 2004). At lower pH, SCFAs are found in undissociated form and they penetrate through the bacterial cell wall. Inside the cell, at higher pH values, SCFA changes into the dissociated form resulting in decreased intracellular pH whilst being entrapped (**Fig. 1**). Amongst SCFA, butyrate is thought to have the greatest protective role, as it fuels intestinal epithelial cells, increases mucus production, improves tight-junctions integrity, reduces inflammation and inhibits tumor cell progression (Ríos-Covián et al., 2016). Butyrate also showed the strongest anti-*Campylobacter* activity *in vitro* amongst SCFAs (Van Deun et al., 2008).

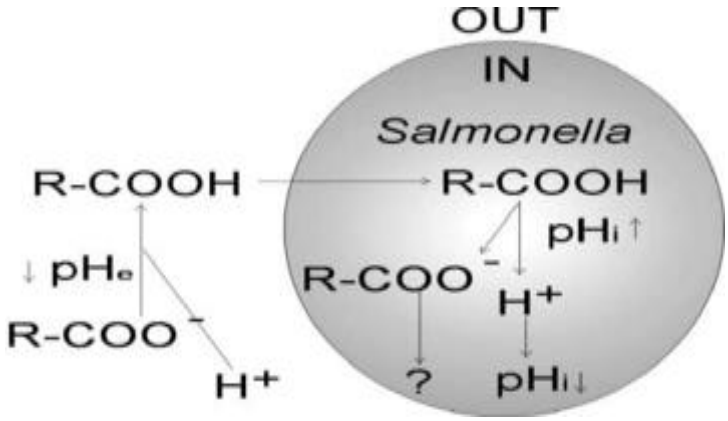

Fig. 1. Mechanism behind toxicity of short-chain fatty acids in *Salmonella spp.* pH_e = external pH; pH_i = internal pH (Source: Józefiak et al. (2004))

3.1.1.2. Thermophilic Campylobacters

Recently, *Campylobacter* infections are the leading cause of human bacterial gastroenteritis in the developed world (EFSA, 2011; Ghareeb et al., 2013). Disease in humans is mainly limited to enteritis and self-cured. However, campylobacteriosis in infants and in adults having immune deficiencies can be more severe with extraintestinal signs such as neurological defects (Laczai, 2008). Broiler chickens are generally considered as a natural host for *Campylobacter spp*. carrying these pathogens in their intestinal tract leading to carcass contaminations at slaughterhouses (**Fig. 2;** (Hermans et al., 2011b; Varga et al., 2007). *Campylobacter* prevalence reaches about 70% at slaughter age in broiler flocks in the EU (Hermans et al., 2011b). Amongst *Campylobacter spp*., *C. jejuni* is isolated predominantly from poultry (EFSA, 2011). Inadequate

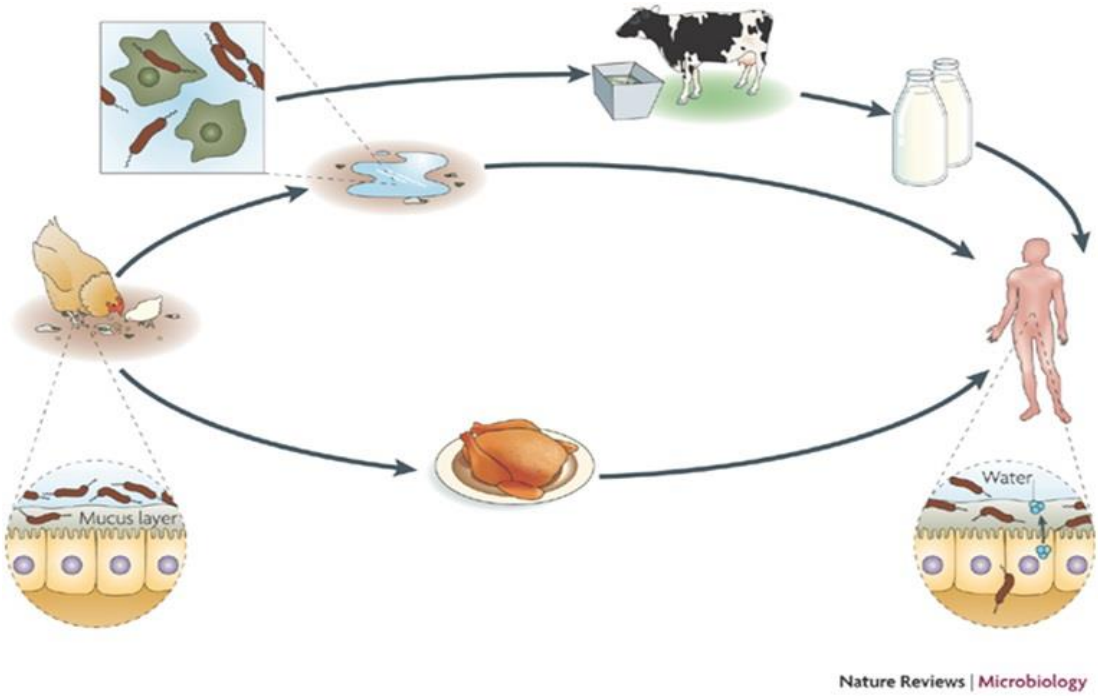

Fig. 2. Sources and consequences of *Campylobacter* infections (Source: Young et al., 2007)

cleaning and downtime of broiler houses may play dominant role in the high *Campylobacter* prevalence whereas flyes, wild birds, water, feed and equipments can also transmit the bacteria (Agunos et al., 2014). Broiler flocks become infected mostly at the age of 2 to 4 weeks old and subsequently they carry high bacterial numbers in their ceca (generally around 10^6 to 10^8 cfu/g) (Hermans et al., 2012). Decreasing the number of

Campylobacters in the chicken intestine at slaughter would reduce the risk of infections in humans (EFSA, 2011). Although many measures such as the use of biosecurity restrictions, feed additives, vaccines, antibiotics, pre- and probiotics have been studied, an overwhelmingly successful technique to reduce *Campylobacter* prevalence has not been found yet (Ghareeb et al., 2013; Hermans et al., 2011b). Further investigations are seeked to test promising candidates and to obtain reproducible results (Meunier et al., 2016). Some studies elucidated effective anti-*Campylobacter* feed additives, based on *in vitro* experiments, however they were uneffective *in vivo*. These contradictory results are explained with the protecting effect of the mucus (Hermans et al., 2010; Robyn et al., 2013). Butyrate showed reduced anti-*Campylobacter*

activity when chicken mucus was added to the medium (Van Deun et al., 2008). The role of mucus in *Campylobacter* colonization (chickens) and in the establishment of human enteric infections has become an intensive research area in recent years (Alemka et al., 2012).

3.1.1.3. Lactobacillus spp. and coliforms

Lactobacillus spp. considered beneficial for the host organism (Bucław, 2016). *Lactobacillus spp.* competes for nutrients and space, they produce lactate which lowers the intestinal pH. The promoting effect of soluble non-digestible carbohydrates (sNDCs) on intestinal *Lactobacillus* population is well known (Pan and Yu, 2014; Rebole et al., 2010; Rodríguez et al., 2012).

Elevated intestinal coliform and *E. coli* counts are generally associated with adverse health effects. These bacteria are often contrasted with *Lactobacillus* (Bucław, 2016). Rodríguez et al. (2012) and Walugembe et al. (2015) reported increased cecal coliform or *E. coli* numbers in case of diets containing high sNDC levels. Cecal coliform numbers were unchanged when chickens were fed maize-, wheat- or barley-based diets (Shakouri et al., 2009). Inulin supplementation reduced cecal *E. coli* counts in several studies or resulted in no alteration (Bucław, 2016). A diet rich in sNSP (pectin) resulted in higher cecal coliform load at 14 days of age without unfavourable effects on feed conversion ratio (Saki et al., 2010). These contradictory results may be a consequence of the complexity of cecal microbiota and therefore altered coliform counts could be interpreted as an indication for a microbial shift not necessarily as a sign for impaired gut health.

3.1.2. Intestinal structure

Main tasks of the intestinal wall involve absorption of nutrients and providing protection for the host organisms from unwanted substances such as large feed components, microorganisms or toxins (Jeurissen et al., 2002; Scanes and Pierzchala-Koziec, 2014). Small intestine comprises villi and crypts which increase the intestinal surface contributing to enhanced nutrient utilization. Proliferation of the epithelial cells take place in the crypts and thereafter epithelial cells migrate towards the tip of the villi whilst they undergo maturation (**Fig. 3.**, Jeurissen et al., 2002). Longer villi are generally associated with greater nutrient absorption, whereas deeper crypts indicate greater cellular turnover and tissue renewal (Olukosi and Dono, 2014). On the other hand, increased villus height and increased epithelial surface requires more maintainance energy (de Verdal et al., 2010). Alterations in the intestinal structure can relate to physico-chemical changes of the diet as well as to changes in the intestinal microbiota

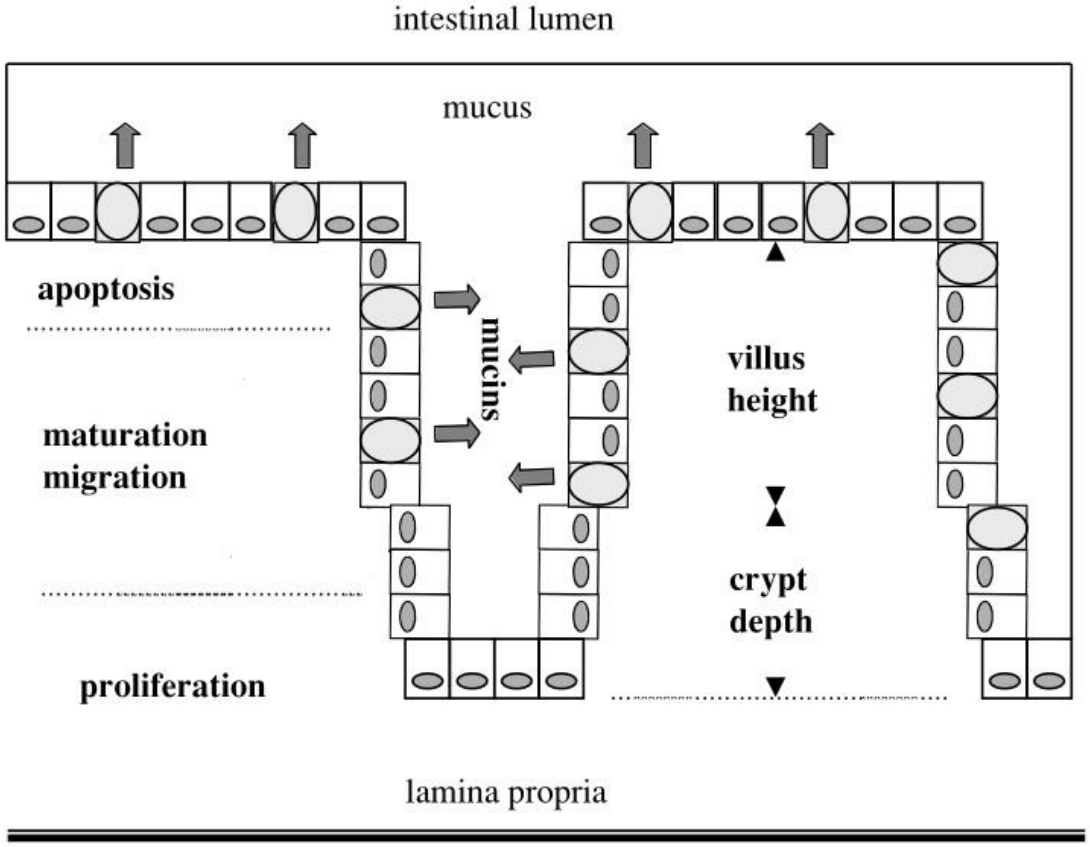

Fig. 3. A schematic representation of small intestinal integrity (figure modified from the original source: Jeurissen et al., 2002).

composition (Awad et al., 2006; Bucław, 2016; Rohe et al., 2014). Feeding wheat- or barley-based diets without NSP degrading enzyme supplementation caused a decrease in villus height in comparison to a maize-based diet (Shakouri et al., 2009), however other reports showed no differences (Amerah et al., 2008; Molnár et al., 2015) or demonstrated an increase in this parameter (Morales-López et al., 2010). It seems that the level of application and the NSP degrading enzyme supplementation are key points regarding the effects of wheat and barley supplemented diets (de Lange, 2000).

3.1.3. Intestinal mucus

Intestinal mucus, synthetized by specialized enterocytes, called goblet cells, is an important barrier in the gut acting as a physical fence (**Fig. 3**.), participates in bacterial clearance and displays antimicrobial activity (Alemka et al., 2012). Actually it provides the first defense line of the GIT which limits the number of bacteria that can reach the epithelium (Pelaseyed et al., 2014). The mucus layer is rather discontinuous in the small intestine, however, providing two layers in the large intestine. The basal layer is adjacent to the epithelium and largely sterile. The luminal layer is looser and consists of intestinal bacteria. Furthermore, the luminal layer of the mucus in the large intestine provides a unique microbial niche with distinct bacterial communities (Li et al., 2015). Nine candidate mucin genes have been recognized in the chicken; Muc1, Muc2, Muc4, Muc5ac, Muc5b, Muc6, Muc13, Muc16, and the bird-specific ovomucin. Of these, the predominant is Muc2 in the chicken large intestine (Smith et al., 2014). Both, microbial status and nutrition could alter mucin production (Brufau et al., 2015; Cheled-Shoval et al., 2014). The mucus layer become thicker as microbial diversity increases (Jakobsson et al., 2015). On the other hand, thicker mucus layer is often associated with decreased nutrient availability (Rahmatnejad and Saki, 2016). Fernandez et al. (2000) showed that diet composition altered the amount of mucin carbohydrate components in the chicken small and large intestine which was associated with reduced *Campylobacter* colonisation in xylanase supplemented M+W diet relative to the M group.

3.1.4. Gut-associated immune system

The intestine represents a major immune organ with several specialized lymphoid structures and cell types such as Peyer's patches, lymphoid follicles, tonsils and diffuse lymphoid tissues along the avian intestinal tract (Casteleyn et al., 2010; van Wijk and Cheroutre, 2009). Instead of highly structured lymph nodes, as it is in mammals, chickens have distinct lymphoid aggregates along the instestine (Smith et al., 2014). The mucosa associated lymphoid tissue (MALT) is well developed in most birds and it forms the first line of defense against harmful antigens that enters the respiratory or intestinal apparatus (Casteleyn et al., 2010; Matsumoto and Hashimoto, 2000). The gut associated lymphoid tissue (GALT) is actually the part of the MALT located in the intestinal tract (Liebler-Tenorio and Pabst, 2006). The GALT comprises the largest number of immune cells comparing to other tissues (Smith et al., 2014). In this way, the gut is inhabited by heterophils, macrophages, dendritic cells and natural killer cells, and also B and T lymphocytes. The proportions of these cell types vary widely depending on locality, microbial status and age. Further factors are contribute to the composition and surface phenotype of the gut associated immune cell populations; such as diet, host genetics and the presence of pathogenic microorganisms. In addition, the epithelial layers of the gut are populated with a highly spezialized group of lymphocytes, the so called intraepithelial lymphocytes (IELs; Smith et al., 2014). They form the front line of host defence against invading pathogens (Cheroutre et al., 2011). The cell composition of IELs includes T-lymphocytes and natural killer cells (Smith et al., 2014). They are responsible for rapid protective immunity, epithelial integrity and immune homeostasis (van Wijk and Cheroutre, 2009). Several studies are available which demonstrates the immunomodulatory potential of prebiotic feeding in chickens. For instance, Huang et al. (2015) showed the beneficial effect of inulin supplementation on intestinal immune function by elevated IgA and mucin mRNA levels. On the other hand, numerous forms of nutrient deficiencies can cause destruction in immune

function, including dietary protein, lysine, arginine, methionine, vitamin D, vitamin E or phosphorus (Korver, 2012).

3.2. Nutrition and gut health

In contrast to other food animals (e.g. swine, ruminants…) poultry has a shorter GIT and shorter transit time of digesta which will result in special features of the digestive process and microbiome composition (Pan and Yu, 2014). Great proportion of digestion and absorption of nutrients take place in the small intestine which consist of the duodenum, jejunum and ileum (Rinttilä and Apajalahti, 2013). The large intestine, mainly the cecum, is the place for water and electrolyte absorption. Herein, uric acid and carbohydrates are fermented into ammonia and short-chain fatty acids (SCFA) by intestinal microbiota (Svihus et al., 2013).

3.2.1. Cereal grains and fibre fractions of poultry diets

3.2.1.1. Maize, wheat and barley

Nowadays, maize is the main cereal grain of poultry diets in many part of the world. As substitutes, poultry diets contain wheat or barley in a lesser or larger extent depending on local climatic factors. In Central Europe dryer periods (300-350 mm annual precipitation) promote wheat/barley crop, whereas more rainfalls (450-550 mm annual precipitation) foster optimal maize crop yield (Antal, 2005; Schmidt, 2003). Barley is the least sensitive to cool and dry climatic conditions in comparison to maize or wheat (Blair, 2008). Accordingly, in dryer periods the price of wheat or barley decreases relative to the price of maize. So far, global warming may infer a growing importance of wheat/barley inclusion in poultry diets due to its favourable price over dry periods. Furthermore, maize is the major source for the increasing biofuel production (Manochio et al., 2017) and this can also influence crop costs.

Maize, wheat and barley serve as energy source in animal diets but they vary in some nutritional contents. Maize (Zea mays) consist the greatest amount of energy (ME=13.50 KJ/kg, or around) amongst cereal grains. Highly digestible starch, soluble polysaccharides and a high oil content

(3-4%) contributes to the high energy content of maize (Schmidt, 2003). The protein (8-10%) and fiber (2-3%) content of maize are relatively low. The proportion of maize in poultry diets is generally high (50-60%). Wheat (Triticum aestivum) exceeds other cereals in protein (14-15%), however its biological value is low due to low lysin and methionin content (Schmidt, 2003). Mostly starch constitutes the energy content of wheat (ME=12.50 KJ/kg) which is nearly as high as that in maize. Wheat has a higher soluble non-starch polysaccharide (NSP) fraction – notably arabinoxylans - in comparison to maize (Summers and Leeson, 2005). Barley (Hordeum vulgare) is considered as a medium energy grain (ME=11.1-12.5 KJ/kg) (McNab and Shannon, 1974; Ravindran et al., 1999), containing more fiber, more protein (11-12%) and less energy than maize (Blair, 2008; Schmidt, 2003). Barley has a substantial amount of NSPs, mainly in the form of β-glucan. It is worth to mention, that wheat has the highest variances in energy and protein content in comparison to maize or barley (Zijlstra et al., 2001).

3.2.1.2. Fibre fractions

The term crude fiber has been widely used in nutritional practice to describe the fiber content of feedstuffs. However, it underestimates the cell wall content and therefore is not an accurate category (Choct, 2015). Refering to true fiber, all indigestible organic components of cereal grains can be expressed as all NSPs plus the lignin content (**Fig. 4**). The term NSP stands for

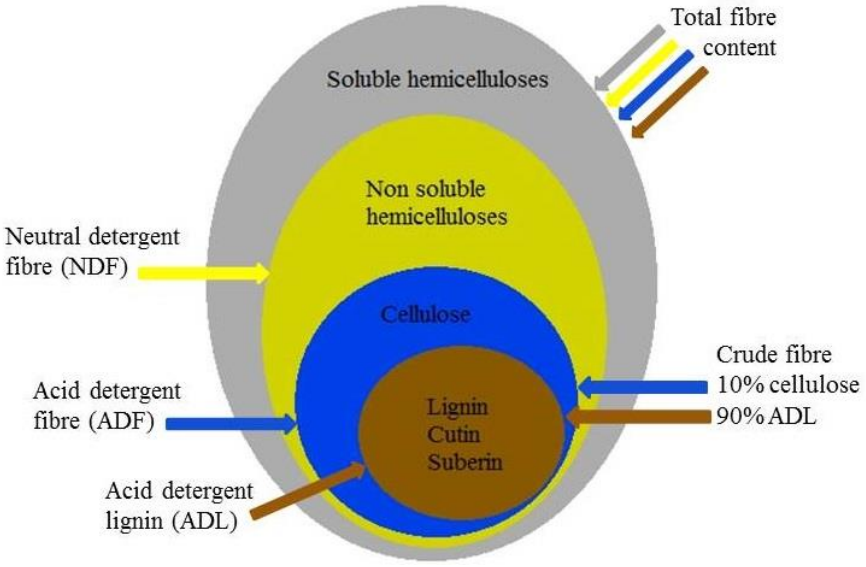

Fig. 4. Fibre fractions of cell wall components (AOAC, 1990)

all the molecules which are polysaccharides but differ from starch. Thus, NSPs cover cellulose and hemicelluloses (arabinoxylans, β-glucans, mannans, galactans, xyloglucans, fructans, pectin polymers, etc...) and its solubility is a key point regarding the chemico-physiological effects. Non-soluble NSPs have important roles in digestion as they stimulate gizzard motility, reduce the pH of gizzard and duodenum, whereas it ameliorates the digestibility of amino acids and starch (Svihus and Gullord, 2002). On the other hand, high amounts of fibre can reduce the efficiency of host enzymes, therefore the digestibility of nutrients could decrease. Soluble NSPs are also resist to host enzymes but serve as substrates for bacteria residing the gut. The main soluble NSPs are arabinoxylans and β-glucans found in wheat and barley, respectively. The NSP fractions of various cereal grains are shown in **Fig. 5**.

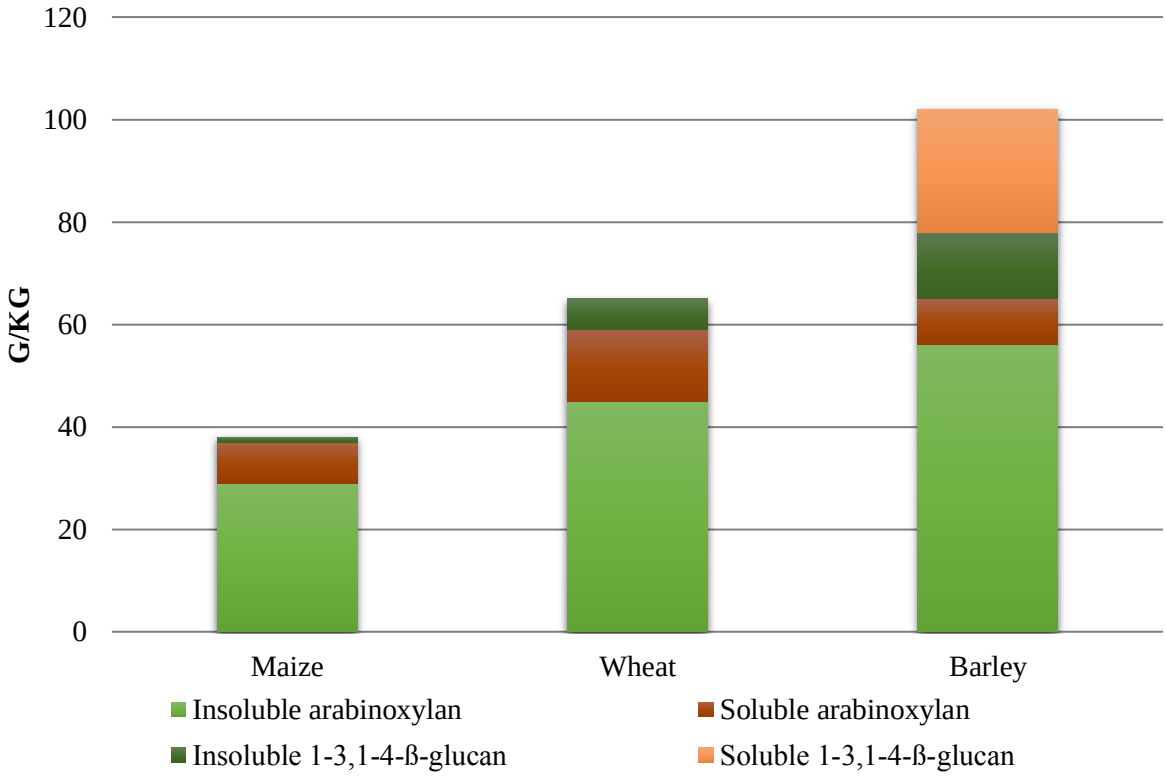

Fig. 5. NSP fractions of some cereal grains (Jeroch, 2013).

3.2.1.3. Effects on gut physiology

Soluble NSPs increase digesta viscosity and also slow down passage time of the chymus. As a consequence, microbial fermentation intensifies in the small intestine contributing to lowered

digestibility of nutrients. High digesta viscosity hinders antiperistaltic movements of the gut, further deteriorating digestive functions. Small intestinal microbial fermentation fosters bile acid deconjugation and a relative bile acid deficit will evolve (Choct, 2006; Józefiak et al., 2004). Furthermore, soluble NSPs decrease the availability of protein, fat and starch for digestive enzymes (Szigeti, 2003). In contrast, the large intestine is already beyond the host digestion system (see part 3.1.1.) and supporting substrates (soluble NSPs) for a stable and diverse microflora desirable in the cecum and colon. It seems that the effects of soluble NSPs acts with treshold-like mechanisms (de Lange, 2000). For instance, in practical application, a poultry diet containing more than 30-40% wheat can result in adverse gut health effects and depressed growth rate of broiler chickens. Application of NSP-degrading enzymes (xylanase, glucanase) in the diet can diminish the undesirable effects of soluble NSPs, while improves the prebiotic properties by producing more fermentable oligosaccharides (de Lange, 2000).

3.2.2. Feed additives

Beside genetic progression, optimization of poultry diets contributed to the high productivity of modern poultry rearing. Intensive poultry diets - consisting low amount of undigestible components - have been optimized for the requirements of the target species. Feed additives such as enzymes predominantly help to improve nutrient digestibilities and other feed additives supporting to maintain gut health which is of special importance since the ban of AGPs.

3.2.2.1. Enzymes

Exogenous enzymes have been applied lately in poultry diets in order to improve production characteristics (Slominski, 2011). Using supplemental enzymes in the diet targets at least one of the following points: 1) augment the animal's own supply; 2) diminishing the adverse effects of antinutritional factors, such as arabinoxylans, ß-glucans; 3) increasing the availability of specific nutrients for absorption and improve the energy value of feed ingredients; 4) modify gut microflora into a healthier state (Engberg et al., 2004; Ferket, 2011). The most important enzymes used in poultry diets are hydrolytic amylase, lipase, protease, phytase, and NSP-

degrading enzymes. In general, commercial enzyme products are a mixture of several different enzymes (Ferket, 2011). The NSP-degrading xylanases and ß-glucanases have been developed to counteract the antinutritional effects of NSPs over the past 30 years. Generally, NSP-degrading enzymes disrupt the polysaccharide chain into smaller units such as sugars and oligomers (Bedford, 2000). Beside reducing viscosity of the chyme, the application of NSP-degrading enzymes can reduce the nutrient encapsulating effect of cell walls, and thus can increase protein, starch and energy utilization (Slominski, 2011). Endoxylanase improves nutrient utilization also by the fact that increasing the passage rate of the chyme in the intestine, so decreasing the competition between the host and the microbiota living in the intestine (Choct et al., 1999). In addition, dietary supplementation of NSP-degrading enzymes can expand the variety of oligosaccharides that act as substrates for a more diverse microbiota (Santos et al., 2006).

3.2.2.2. Prebiotics (including inulin and lactose)

Gibson and Roberfroid (1995) introduced the term, 'prebiotics', giving a definition as „nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacterial species already resident in the colon, and thus attempt to improve host health." Prebiotics are selectively fermented into SCFA and lactate by beneficial bacteria which can effectively exclude the pathogenic ones through an altered intestinal milieau. In other words, the positive effects of prebiotics are achieved by „selectively feeding harmless bacteria at the expense of the harmful ones". The consequence of prebiotic feeding depend on the type and dose of substrates and also on the rate of fermentation by the intestinal bacteria (Dhama et al., 2014). Amongst prebiotics, non digestible oligosaccharides containing either xylose, fructose, galactose, mannose or glucose monomers seemed to be the most promising. Also, sNSPs as potential prebiotics have been investigated (Gibson and Roberfroid, 1995; Jozefiak et al., 2008).

Inulin is a storage carbohydrate in many plants and it is usually extracted from chicory (Chicorium intybus) roots and tubers of Jerusalem atrichoke (Helianthus tuberosus; Niness, 1999). Chemically, inulin is a polydisperse fructan, constituting fructose polymers and oligomers connecting with ß(2-1) bonds. Because inulin contains ß-glycosidic bonds, it resists to host digestive enzymes and inulin is functioning as substrate for healthy bacteria in the large intestine. Application of inulin has been widely tested in monogastric animals and it is considered to be one of the most efficient prebiotics. However, in many aspects the findings are inconclusive (Bucław, 2016; Kozłowska et al., 2016).

Lactose is a disaccharide found naturally in milk. Absorption of lactose in the intestine occurs in the form of monomers, glucose and galactose. This requires an enzyme called lactase (McReynolds et al., 2007). So far chickens do not secrete lactase in their intestine, lactose can be broken down only by intestinal microbial fermentation (Gülşen et al., 2002). Lactose is fermented to lactic acid and SCFA which may promote the colonisation of *Lactobacilli* (Hume et al., 1992). The literature on lactose feeding in poultry is relatively limited. Chicken experiments indicated an effect of lactose supplementation on growth performance (Douglas et al., 2003; Gülşen et al., 2002), ileal *Lactobacillus* number, cecal and gizzard pH (Jozefiak et al., 2008) and disease condition of necrotic enteritis (McReynolds et al., 2007). Only Van Der Wielen et al. (2002) studied cecal fermentation profile and reported increased lactate concentration whereas SCFA concentration were unchanged in case of feeding a lactose supplemented diet. Lactose is commonly used in the broiler industry as a component of prestarter diets, however the literature of the effect of lactose feeding is scarce.

4. OWN EXPERIMENTATIONS

4.1. Significance and aims of the study

The actual study is intended to investigate the influence of nutritional factors on selected gut characteristics in broilers in order to gain information on chicken gut health effects. As gut health intimately connect to the intestinal microbiota (Ducatelle et al., 2015), selected bacteria and bacterial fermentation products (SCFA) constituted one of the main focus of the experiments. Furthermore, histological changes of the intestine were assessed together with goblet cell and IEL numbers. Growth performance was also measured connecting gut variables to production data.

Two trials were performed. In Trial I *Campylobacters* bearing crucial human health significance were under spotlight. Most of the nutrition related *Campylobacter* experiments (Heres et al., 2004; Hermans et al., 2011a, 2010; Hilmarsson et al., 2006; Skånseng et al., 2010; Solís de los Santos et al., 2010, 2009; Van Deun et al., 2008; van Gerwe et al., 2010) assessed cecal *C. jejuni* colonization once during the trial period therefore little is known about the colonization dynamics of this bacterium altered by nutritional factors over a longer period. Our aim in Trial I was to investigate *C. jejuni* colonization in the broiler chicken intestine - using maize based (M) or maize-wheat based diets with (M+WE) and without (M+W) NSP-degrading enzyme supplementation - after artificial infection at multiple sampling time point. Beside *Campylobacter* counts in the ileum and cecum, ileal viscosity, histomorphology, cecal pH and SCFA concentrations were studied considering the link between *Campylobacter* colonisation and chicken gut health.

Trial II was conducted to confer gut health effects of diets containing different sNDC sources. Due to the climate change, the proportion of cereals can be shifted in poultry diets in the near future and it can have substantial consequences on the gut ecosystem. To prepare for such a challenge, detailed knowledge of the effects of different cereal grains (maize, wheat, barley) on

gut health is desirable. Similarly, promotion of a 'diverse, healthy' gut flora by nutrition is a hot topic owing to the ban of AGPs (Ducatelle et al., 2015). Although inulin has a widespread literature as a prebiotic, the results are inconclusive in many points (Bucław, 2016). In contrast, there is a gap of knowledge regarding the influence of lactose feeding on chicken gut health though lactose is a common component of poultry prestarter diets. Soluble carbohydrate components of diets which bypass the alimentary canal of the host without digestion can be potential nutrient sources for intestinal bacteria in the lower gut. Thus, it is important to have detailed knowledge on the type of sNDCs which have the most beneficial gut health effects without deteriorating production characteristics. The author hypothesized that different sNDCs may influence gut health dissimilarly and a comparison could provide useful information regarding its applicability in poultry diets. The gut health aspects of different sNDCs were tested in several experiments; however the present study is the first which test different sNDC sources on gut health characteristics using wheat, barley, inulin and lactose in parallel at the same time and location. Studies which compared the effects of wheat/barley based diets to maize based diets predominantly used wheat and barley composition in the diet at high proportion (55-68%; Amerah et al., 2008; Masey-O'neill et al., 2014; Morales-López et al., 2010; Rodríguez et al., 2012; Shakouri et al., 2009; Teirlynck et al., 2009a). However, in field conditions lower inclusion levels of wheat/barley are more common. Therefore, the current investigation deals with moderate levels of wheat/barley inclusion and in gradual elevation of these grains from starter to finisher diets (20/30% to 40/50%). In this way, this study is aimed to provide novel data which will be useful for the nutritional practice. The objective was to survey the influence of a M+W, maize-barley based (M+B), inulin and lactose supplemented maize-based (M+I and M+L) diets on growth performance, gut histology (morphology, goblet cell and IEL numbers), ileal viscosity, cecal SCFA concentration, pH, coliform and *Lactobacillus* counts in comparison to a M diet. The gut variables, goblet cell and IEL numbers were rarely investigated in nutritional studies using broiler chickens and hence these analyses

can provide novel results on intestinal barrier function. The combination of the selected gut characteristics may also point out novel relations not described previously.

Van Deun et al. (2008) has shown that the mucus reduced the anti-*Campylobacter* efficacy of butyrate by increasing the minimal bactericidal concentration (MBC) values in vitro. On the other hand, the effect of mucus types on butyrate sensitivity of *C. jejuni* was not yet investigated. For that reason in Trial II mucus were obtained from chickens fed the M, M+W, M+B diets to test the influence of mucus types on butyrate anti-*Campylobacter* effect *in vitro*.

4.2. Materials and methods

4.2.1. Animal welfare considerations

Trial I was approved by the Institutional Ethics Committee under the license number GZ 68.205/0227-II/3b/2011 (University of Veterinary Medicine, Vienna, Austria). Trial II was performed at the Georgikon Faculty of University of Pannonia (Keszthely, Hungary) so it was approved by the County Food Chain Safety and Animal Health Directorate of Zala County, Hungary (ZAI/100/1361-009/2013). All husbandry practices and euthanasia were performed with full consideration of animal welfare.

4.2.2. Trial I

4.2.2.1. Experimental design and diets

Fifty-four, day-old male and female broiler chickens (Ross 308) purchased from a commercial hatchery (Geflügelhof Schulz, Graz, Austria) were randomly divided into three groups. Chickens were kept in floor pens using wood shavings bedding and were fed *ad libitum* (**Fig. 6A**). Three diets – a maize based (M), a maize-wheat based (M+W) and a M+W diet supplemented with 135 mg kg^{-1} NSP-degrading enzyme (M+WE) - were supplied by Georgikon Faculty, University of Pannonia. The enzyme used in the M+WE diet was a Grindazym GP15000 product containing a combination of xylanase and glucanase. Diets were

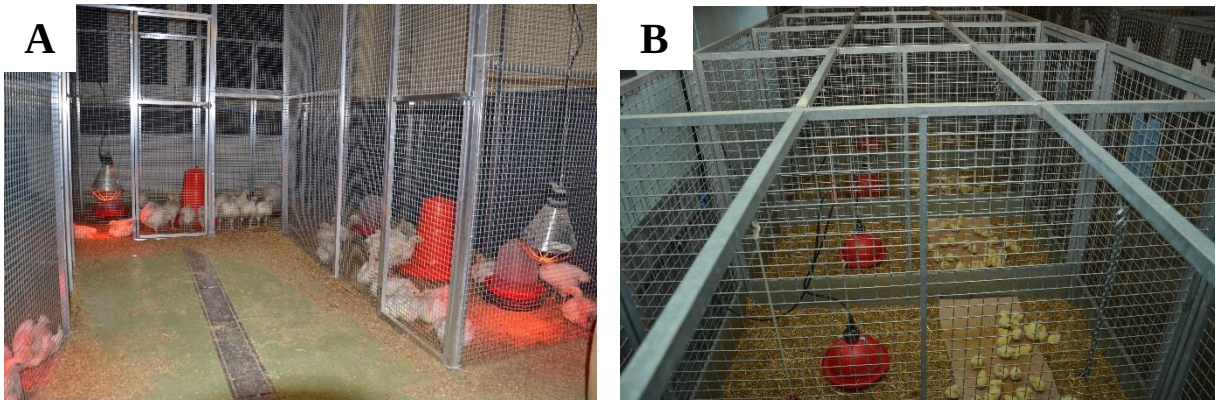

Fig. 6. Broiler chickens kept on wood shavings and on straw litter in Trial I (A) and II (B).

isocaloric and isonitrogenous and were prepared to meet the nutrient requirements of Ross 308 broilers (Aviagen, 2014a). Composition and nutrient contents of diets are shown in **Table 1**. Starter, grower and finisher diets were fed between days 1-10, days 11-24 and days 25-35, respectively. Each diet was fed one of the groups contained 18 chickens at the beginning of the experiment. Chickens were monitored daily for any adverse effects and clinical signs. Body weight of all chickens was measured on days 10, 24 and prior to euthanasia.

On days 1 and 14 of age, *Campylobacter* presence in chickens were tested by taking cloacal swabs which were direct-plated on *Campylobacter* Blood-Free Agar (CBFA; CM0739, OXOID, Hampshire, UK) for *Campylobacter* determination (42 °C, 48 hrs).

On day 14, the chickens were infected orally with 10^8 CFU *C. jejuni* using crop gavages. Chickens were killed 7, 14 and 21 days post infection (DPI) and bacteriological, histological and digesta samples were taken. At each time point 6 chickens per group were euthanized for sampling. The gut section ileum is referred as a part of the small intestine starting from the Meckel's diverticuli to the ileocecal junction.

Table 1. Composition of experimental diets in Trial I (g/kg)

Ingredient	Starter		Grower		Finisher	
	M	M+W	M	M+W	M	M+W
Maize	459	187	526	143	576	111
Wheat	0	300	0	300	0	400
Barley	0	0	0	100	0	100
Extracted soybean meal	285	229	317	236	253	181
Fullfat soybean	172	200	67	126	82	109
Corn gluten	10	10	0	0	0	0
Sunflower oil	30	30	50	55	50	60
L-Lysine	1	2	1	2	1	2
DL-Methionine	2	2	2	2	2	2
Limestone	17	17	15	15	15	15
MCP	16	15	14	13	13	12
Salt	3	3	3	3	3	3
Premix*	5	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000
Nutrient composition (calculated)						
AME_n (MJ/kg)	12.6	12.6	13.0	13.0	13.3	13.3
Crude protein	220.0	220.0	200.0	200.0	180.0	180.0
Crude fibre	33.0	33.0	30.4	33.6	30.0	32.0
Crude fat	84.2	85.3	85.5	96.0	88.8	97.2
Starch	312.7	331.5	349.0	351.0	376.7	390.0

Abbreviations: M – maize based diet; M+W – maize-wheat based diet;

*Premix was supplied by Visonka Kft. (Páhi, Hungary). The active ingredients contained in the vitamin-mineral premix were as follows (per kg of diet):

Starter and grower premix - Vitamin A - $2{,}4x10^6$ IU, Vitamin D3 - $8x10^5$ IU, Vitamin E – $1x10^4$ IU, Vitamin K3 – $4x10^2$ IU, Monenzin-Na $2x10^4$ mg, Phyzyme phytase - $2{,}5x10^4$ mg, Zn – $1{,}2x10^4$ mg, Cu – $3x10^3$ mg, Fe – $5x10^3$ mg, Mn – $1{,}8x10^4$ mg, Se – $6x10^1$ mg,

Finisher premix - Vitamin A - $9x10^5$ IU, Vitamin D3 - $3x10^5$ IU, Vitamin E – $3{,}75x10^3$ IU, Vitamin K3 – $1{,}5x10^2$ IU, Phyzyme phytase - $2{,}5x10^4$ mg, Zn – $1{,}2x10^4$ mg, Cu – $3x10^3$ mg, Fe – $5x10^3$ mg, Mn – $1{,}8x10^4$ mg, Se – $6x10^1$ mg,

4.2.2.2. Challenge organisms and Campylobacter enumeration

Reference strain *Campylobacter jejuni* NCTC 12744 was cultured in LB medium (Lennox L broth base, Invitrogen by Life Technologies Corporation, California, USA) at 42ºC for 48 h under microaerobic conditions using GENbox microaer bags (BioMerieux, Vienna, Austria). The *C. jejuni* bacteria were enumerated by preparing 10-fold dilutions in PBS (Gibco Life Technologies Corporation, California, USA) and plated on Campylosel agar (BioMerieux, Vienna, Austria), followed by microaerobic incubation at 42ºC for 48 h.

One gram of ileum (5 cm below to Meckel's diverticuli) and cecum including content and a piece of intestinal tissue were aseptically taken, homogenized and a 10-fold dilution series was made in PBS. One hundred µl of each dilution were inoculated onto Campylosel agars (BioMerieux, Vienna, Austria). Plates were incubated at 42ºC under microaerobic condition. Greyish, gleaming and bulging colonies were counted after 48 h of incubation and the presence of *Campylobacter* was confirmed by examining colonial morphology, motility and shape of the bacteria.

4.2.2.3. Analytical methods

Fresh ileal and cecal contents were diluted immediately after collection with distilled water (1:5) and vortexed manually by shaking for 1 minute. Measurement of the pH values were carried out with a SNEX electrode (pH200A Portable pH meter equipped with CS1068 SNEX pH Sensor, CLEAN Instruments, Sanghai).

To measure the ileal digesta viscosity, 2 g of digesta were frozen and stored at -80ºC. After thawing samples were centrifuged (12,000 G for 10 min) and the viscosity of the supernatant (0.5 ml) was measured using a Brookfield DV II+ viscometer (Brookfield Engineering Laboratories, Stoughton, MA, USA) at 25ºC with a CP40 cone and shear rate of 60-600s^{-1} (**Fig. 7**).

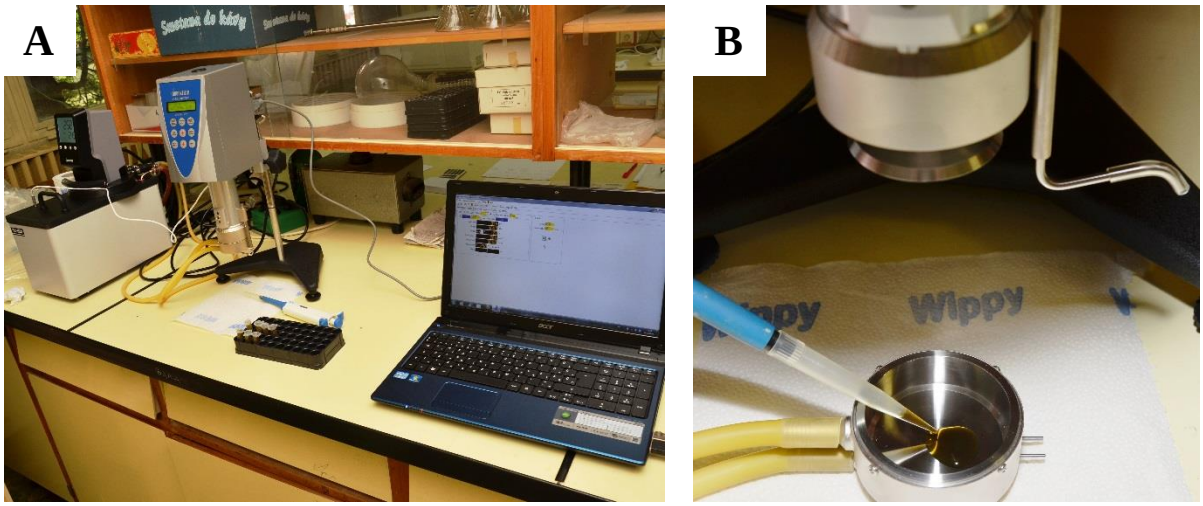

Fig. 7. Computer controlled viscosity measurement with a Brookfield DV II+ viscometer attached to a temperature controlled circulating water bath (A) and pipetting of 0.5 ml ileal supernatant into the cone of the viscosimeter(B).

For SCFA analyses 1 g of cecal content samples were frozen, and were stored at -80 ^{o}C and the analyses were prepared as described by Atteh et al. (2008). A standard SCFA mixture (20 mmol l^{-1}) of acetic, propionic, isobutyric, butyric, isovaleric, valeric acid was used for calibration as external standard.

One microliter of the ether phase extract was injected into a Gas Chromatograph (TRACE 2000, Thermo Scientific, USA). The instrument was equipped with a Nukol Fused Silica Capillary Column (30 m x 0.25 mm with a film thickness of 0.25 µm; Supelco, USA). The carrier gas was helium with a pressure of 83 kPa. The detector type was FID with a split injector (1:50). Injector and detector temperatures were 220 and 250 ^{o}C, respectively.

Tissue samples were taken from ileum close to the junction of Meckel's diverticulum for histomorphological examination. Samples were fixed in 5% buffered formalin. The processing consisted of serial dehydration, clearing and impregnation with wax. Tissue sections, 5 µm thick (three cross-sections) from each of 6 chickens per treatments, were cut by a microtome and were fixed on slides.

A routine staining procedure was carried out using hematoxylin and eosin. The slides were examined on an Olympus BX43F light microscope (Olympus Corporation, Tokyo, Japan) fitted

with digital video camera (Olympus DP-26) using Olympus Stream 1.7 software. The images were analyzed with ImageJ software (Version 1.47) developed by National Institutes of Health (Maryland, USA). A total of 10 intact, well-oriented crypt-villus units were selected in triplicate for each intestinal cross-section for all samples (**Fig. 8**). Apparent villus surface area were calculated as: (villus height * (apical transverse + basal transverse)/2)/10^6.

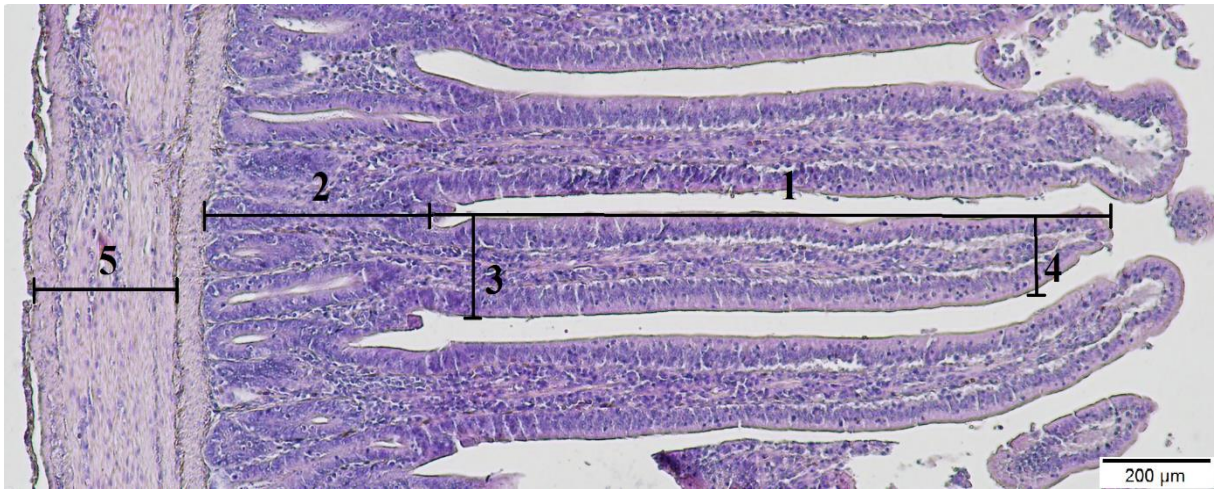

Fig. 8. Hematoxillin-eosin stained ileal cross section. Numbers (1-5) indicate measurements of histomorphology (1_villus height, 2_crypt depth, 3_basal transverse, 4_apical transverse, 5_muscle layer thickness)

4.2.2.4. Statistical analyses

All data were analysed by using SPSS 16.0 software. The arrangement of the results for viscosity, SCFA, pH and histomorphology data was regarded as a 3 x 3 general linear model, with dietary treatments and sampling time points as independent variables. Differences were considered significant at a level of $P \leq 0.05$.

Campylobacter counts were analyzed for diet and time effect separately by Kruskal-Wallis tests. Prior to statistical evaluation *Campylobacter* counts were scored on a scale from 1-6, respectively, ranging them as follows: (1) to $<10^{1,5}$, (2)$10^{1,5}$-10^3, (3)10^3-$10^{4,5}$, (4)$10^{4,5}$-10^6, (5)10^6-$10^{7,5}$, (6)$>10^{7,5}$.

4.2.3. Trial II

4.2.3.1. Chickens, housing and diets

In total, 200 Ross 308 day-old male chickens (39.8 ± 2.25 g) were obtained from a commercial hatchery (Gallus Company, Devecser, Hungary). Chickens were group housed on wheat straw litter in 10 metal floor pens (**Fig. 6B**; pen size: 2 m^2, 20 chickens in each) from day 1 of life until the end of the experimental period (day 35 of life). Computer controlled housing and climatic conditions were maintained according to the breeder suggestion (Aviagen, 2014b). Chickens received an artificial lighting regimen starting with 24 hours of light period at day 1 of life, then light hours were gradually decreased to 20 hours until day 8 of life, and 16 hours of light period were set from day 9 until day 35 of life. Upon arrival, chickens were randomly divided into five dietary treatment groups (n=40): maize based (M), maize-wheat based (M+W), maize-barley based (M+B), inulin supplemented (M+I) and lactose supplemented (M+L). Experimental diets, as mash form, were formulated to be isocaloric and isonitrogenous, and to meet the requirements of Ross 308 chickens (Aviagen, 2014a). A three phase feeding programme was used as chickens were fed starter (day 1 to 10 of life), grower (day 11 to 24 of life) and finisher (day 25 to 35 of life) diets. Detailed list of ingredients in the different diets are shown in **Table 2**. The M+W diets contained approximately 60% more soluble arabinoxylans whereas the M+B diets consisted of around 300% more sNDCs (mainly in the form of ß-glucans) compared to the M diet (**Table 3**). The M+I and the M+L diets were supplemented with 20 g/kg inulin and 30 g/kg lactose (UBM Group, Pilisvörösvár, Hungary), respectively. Water and feed were offered ad libitum throughout the whole experiment. Diets were free from NSP-degrading enzymes.

4.2.3.2. Feed analyses

Experimental diets were analysed for dry matter (ISO 6496), crude protein (ISO 5983-1:2005), crude fat (ISO 6492) and crude fibre (ISO 6865:2001) (**Table 4**). Acid (ADF) and neutral detergent fibre (NDF) were determined according to Van Soest and Wine (1967). The starch content was analysed by the polarimetric method in line with the European Directive 152/2009.

Table 2. Composition of experimental diets in Trial II (g/kg as fed basis)

Ingredient	Starter					Grower					Finisher				
	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L
Maize	456	172	244	417	398	519	138	207	480	461	567	88	150	522	505
Wheat	0	300	0	0	0	0	400	0	0	0	0	500	0	0	0
Barley	0	0	200	0	0	0	0	300	0	0	0	0	400	0	0
Inulin	0	0	0	20	0	0	0	0	20	0	0	0	0	20	0
Lactose	0	0	0	0	30	0	0	0	0	30	0	0	0	0	30
SBM	351	272	252	351	351	309	204	167	310	311	325	202	212	329	336
Fullfat soybean	99	162	200	108	112	79	164	200	87	90	14	109	138	20	14
Maize gluten meal	0	0	10	0	0	0	0	32	0	0	0	0	0	0	0
Sunflower oil	45	45	45	55	60	50	50	50	60	65	55	60	60	70	76
Limestone	18	18	18	18	18	15	16	16	15	15	15	15	15	15	15
MCP	16	15	15	16	16	14	13	13	14	14	13	12	12	13	13
L-LYS	1	2	2	1	1	1	2	2	1	1	0	2	1	0	0
DL-MET	4	4	4	4	4	3	3	3	3	3	2	3	3	2	2
L-THR	1	1	1	1	1	1	1	1	1	1	0	0	0	0	0
NaCl	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
$NaHCO_3$	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
Premix[1]	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Abbreviations: M – maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I – inulin supplemented maize based diet; M+L – lactose supplemented maize based diet; SBM – soybean meal; MET – methionin; CYS – cystein; THR – threonin.

[1]Premix was supplied by UBM Ltd. (Pilisvörösvár, Hungary). The active ingredients contained in the premix were as follows (per kg of diet):

Starter and grower premixes – retinyl acetate – 5.0 mg, cholecalciferol – 130 µg, dl-a-tocopherol – 91 mg, menadione – 2.2 mg, tiamin – 4.5 mg, riboflavin – 10.5 mg, piridoxin HCL – 7.5 mg, cyanocobalamin – 80 µg, niacin – 41.5 mg, pantothenic acid – 15 mg, folic acid – 1.3 mg, biotin – 150 µg, betaine – 670 mg, ronozyme np – 150 mg, monensin-Na – 110 mg (only grower), narasin – 50 mg (only starter), nikarbazin – 50 mg (only starter), antioxidant – 25 mg, Zn (as $ZnSO_4 \cdot H_2O$) – 125 mg, Cu (as $CuSO_4 \cdot 5H_2O$) – 20 mg, Fe (as $FeSO_4 \cdot H_2O$) – 75 mg, Mn (as MnO) – 125 mg, I (as KI) – 1.35 mg, Se (as Na_2SeO_3) – 270 µg;

Finisher premix - retinyl acetate – 3.4 mg, cholecalciferol – 97 µg, dl-a-tocopherol – 45.5 mg, menadione – 2.7 mg, tiamin – 1.9 mg, riboflavin – 5.0 mg, piridoxin HCL – 3.2 mg, cyanocobalamin – 19 µg, niacin – 28.5 mg, pantothenic acid – 10 mg, folic acid – 1.3 mg, biotin – 140 µg, l-ascorbic acid – 40 mg, betaine – 193 mg, ronozyme np – 150 mg, antioxidant – 25 mg, Zn (as $ZnSO_4 \cdot H_2O$) – 96 mg, Cu – 9.6 mg, Fe (as $FeSO_4 \cdot H_2O$) – 29 mg, Mn (as MnO) – 29 mg, I (as KI) – 1.2 mg, Se (as Na_2SeO_3) – 350 µg.

Table 3. Calculated nutrient analysis of experimental diets in Trial II (g/kg as fed basis)

Nutrient	Starter					Grower					Finisher				
	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L
AME_n (MJ/kg)	12.6	12.6	12.6	12.6	12.6	13.0	13.0	13.0	13.0	13.0	13.2	13.2	13.2	13.2	13.2
Crude protein	220	220	220	220	220	200	200	200	200	200	190	190	190	190	190
Crude fibre	31.5	32.4	37.7	31.3	31.1	30.6	31.2	38.1	30.3	30.1	28.7	29.5	30.9	28.4	28.1
Soluble arabinoxylan[1]	3.6	5.6	3.8	3.2	3.3	4.2	6.7	4.4	3.7	3.8	4.5	7.7	4.8	4.0	4.2
Soluble 1-3, 1-4-ß-glucan[1]	0	0	4.8	0	0	0	0	7.2	0	0	0	0	9.6	0	0
Crude fat	85.0	89.2	97.2	95.2	100.2	88.2	93.9	101.4	98.2	103.0	83.6	93.8	100.6	98.1	102.5
LYS	14.3	14.3	14.3	14.3	14.3	12.4	12.4	12.4	12.4	12.4	11.2	11.2	11.2	11.2	11.2
MET	7.2	7.2	7.2	7.2	7.2	6.2	6.2	6.2	6.2	6.2	5.4	5.4	5.4	5.4	5.4
MET + CYS	11.4	11.3	11.1	11.4	11.4	10.0	9.9	9.7	9.8	9.8	8.9	9.1	9.2	8.9	8.9
THR	9.4	9.4	9.4	9.4	9.4	8.3	8.3	8.3	8.3	8.3	7.4	7.4	7.4	7.4	7.4
Ca	10.5	10.5	10.5	10.5	10.5	9.0	9.0	9.0	9.0	9.0	8.5	8.5	8.5	8.5	8.5
Available P (%)	5.0	5.0	5.0	5.0	5.0	4.5	4.5	4.5	4.5	4.5	4.2	4.2	4.2	4.2	4.2

Abbreviations: M – maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I – inulin supplemented maize based diet; M+L – lactose supplemented maize based diet; LYS – lysin; MET – methionin; CYS – cystein; THR – threonin.

[1]Calculation was based on the report of Jeroch (2013).

Table 4. Analysed chemical composition of experimental diets (g/kg as fed basis)

Nutrient	Starter (day 1 to 10 of life)					Grower (day 11 to 24 of life)					Finisher (day 25 to 35 of life)				
	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L	M	M+W	M+B	M+I	M+L
Dry matter	897	895	894	894	893	888	896	894	887	885	887	898	893	885	884
Crude protein	221	222	219	221	220	208	208	209	208	207	196	199	194	195	195
Crude fat	87.0	85.1	94.8	97.1	101.2	88.2	91.2	105.1	97.9	102.0	85.1	92.2	99.5	99.2	104.2
Starch	303	301	295	271	266	348	360	328	331	325	354	343	320	319	324
Crude fibre	31.8	32.1	33.7	31.7	31.5	30.3	29.3	31.2	29.6	29.7	28.5	32.4	37.8	28.2	28.2
NDF	131	122	148	127	126	151	138	136	146	144	116	129	162	114	111
ADF	35.4	36.9	38.7	35.8	36	40.9	41.2	41.5	41.1	41.2	35.5	34.6	43.5	35.7	35.4

Abbreviations: M - maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I: maize based diet with inulin supplementation; M+L: maize based diet with lactose supplementation; NDF – neutral detergent fibre; ADF – acid detergent fibre;

4.2.3.3. Sample collection

Body weight of chickens was measured at day 1 and day 35 of life individually, and the average of body weights were calculated per treatments. Feed consumption was measured in each treatment groups over the trial and the amount of feed consumption per 1 kg BW gain (feed conversion ratio, FCR) was calculated for the whole period. On day 28 of life, 3 chickens were killed in each pen and mucus was collected from the small intestine as described by Van Deun et al. (2008). On day 35 of life, chickens were euthanized in each treatment group by bleeding out of the jugular vein under general anaesthesia induced by carbon dioxide. Immediately after killing, abdominal cavities of 12 chickens in each treatment group (6 chickens per pen) were opened and intestinal tracts were removed. From the ileum (10 cm distal to the Meckel's diverticulum), 1 cm long section of the intestine was excised and put into phosphate buffered formalin for histomorphological analysis. Two gram digesta samples were taken from the ileum (distal 1/3 part) for viscosity measurements and stored at -80^{o}C until analysis. Approximately 1.0 g digesta samples were collected into 2-mL Eppendorf tubes from the cecum for short-chain fatty acid analysis and 0.5 g digesta samples for pH measurement and for bacterial enumeration, respectively. Samples for bacterial enumeration were immediately put into -20^{o}C and SCFA samples were stored at -80^{o}C until laboratory analysis. In each dietary group, samples from 10 chickens (5 chickens per pen) were used for the analyses, except for pH measurement where the number of replicates were 12.

4.2.3.4. Analytical methods

The viscosity, SCFA and pH measurements were the same as described in the Trial I. The fixed tissue samples in formalin were dehydrated and embedded in paraffin wax. Five μm thick sections, in duplicate, were cut by a microtome and were fixed on slides. A routine staining procedure was carried out using hematoxylin and Periodic Acid-Schiff reagents (**Fig. 9**).

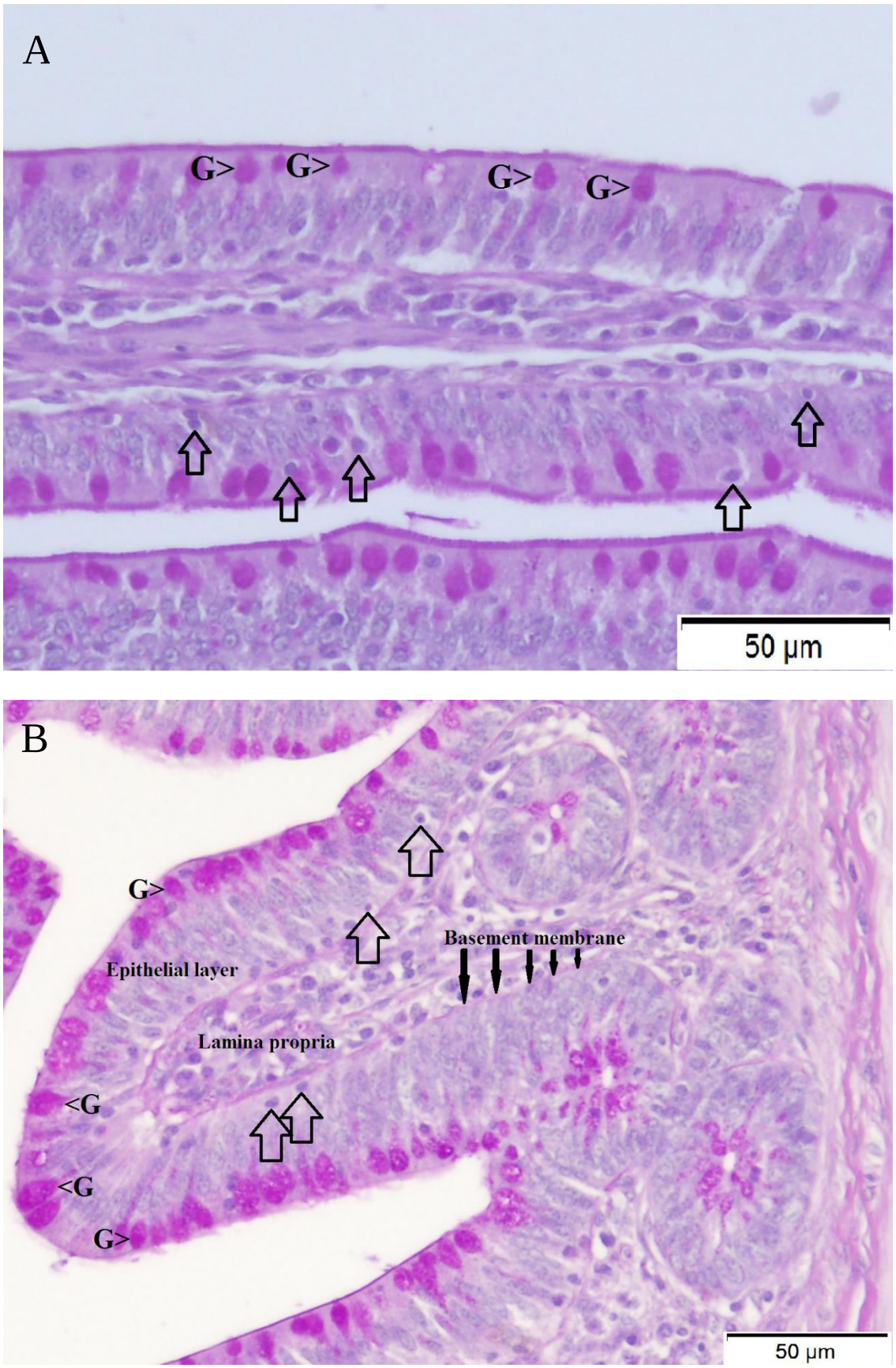

Fig. 9. Periodic acid-Schiff (PAS) staining of ileal (A) and cecal (B) cross sections. Mucus is seen in purple color mainly in goblet cells (G) at the apical region and intraepithelial lymphocytes (IELs) can be observed as small dark purple objects (⇧) with a surruonding bright area in the epithelial layer.

The slides were examined on an Olympus BX43F light microscope (Olympus Corporation, Tokyo, Japan) fitted with digital video camera (Olympus DP-26) using Olympus Stream 1.7 software. The images were analyzed with ImageJ software (Version 1.47) developed by National Institutes of Health (Maryland, USA). A total of 10 intact, well-oriented crypt-villus units were selected for each intestinal cross-sections at 4x magnifications. The principle for villus selection required villi covered by intact lamina propria. The measurements of villus height (from the apical end of the villus to the lamina muscularis mucosae), crypt depth, (from the onset of crypt to the lamina muscularis mucosae), basal transverse (villus width at the crypt-villus axis), apical transverse (villus width at the top region of the villi) and muscular layer thickness (tunica muscularis) were conducted. In Trial II, number of goblet cells and IELs were counted in the ileum and cecum under 20x magnification. Assessment were performed on randomly chosen intact villi parts at the length of 400 µm of villus epithelium and in 10 replicates per chicken.

Culturing techniques were used for the microbial enumeration of coliform and *Lactobacillus* counts from cecal digesta samples (**Fig. 10A, 10B**). Cecal digesta samples were thawed on ice, weighed and equivalent amounts of sterile phosphate buffered saline (PBS) were added to the samples and vortexed, in order to get a pipettable mixture. Tenfold serial dilutions were made in sterile PBS up to 10^9 and 100 µl suspensions were streaked out onto selective agar plates from each dilutions, in duplicate. *Lactobacillus* numbers were evaluated on MRS agar (Biolab, Hungary) followed by anaerobic incubation at 37°C for 48 h, whereas coliform counts were determined on MacConkey agar (Biolab), as red colonies, after aerobic incubation at 37°C for 24 h. Results were expressed as base-10 logarithm colony forming units (CFU) per gram of cecal digesta.

To study the effect of different mucus types on butyrate sensitivity of *C. jejuni*, one single strain (NCTC 12744) was chosen. Bacteria were thawed from -80°C and were streaked out onto

Campylosel agars (BioMerieux, Vienna, Austria). These plates were incubated microaerobically at 40°C for 48 h. Following incubation, two to three colonies were picked up and inoculated into 4 ml Preston broth (Oxoid, CM0689) containing *Campylobacter* selective supplement (Oxoid, SR0117). *Campylobacter* count of the suspensions were determined after

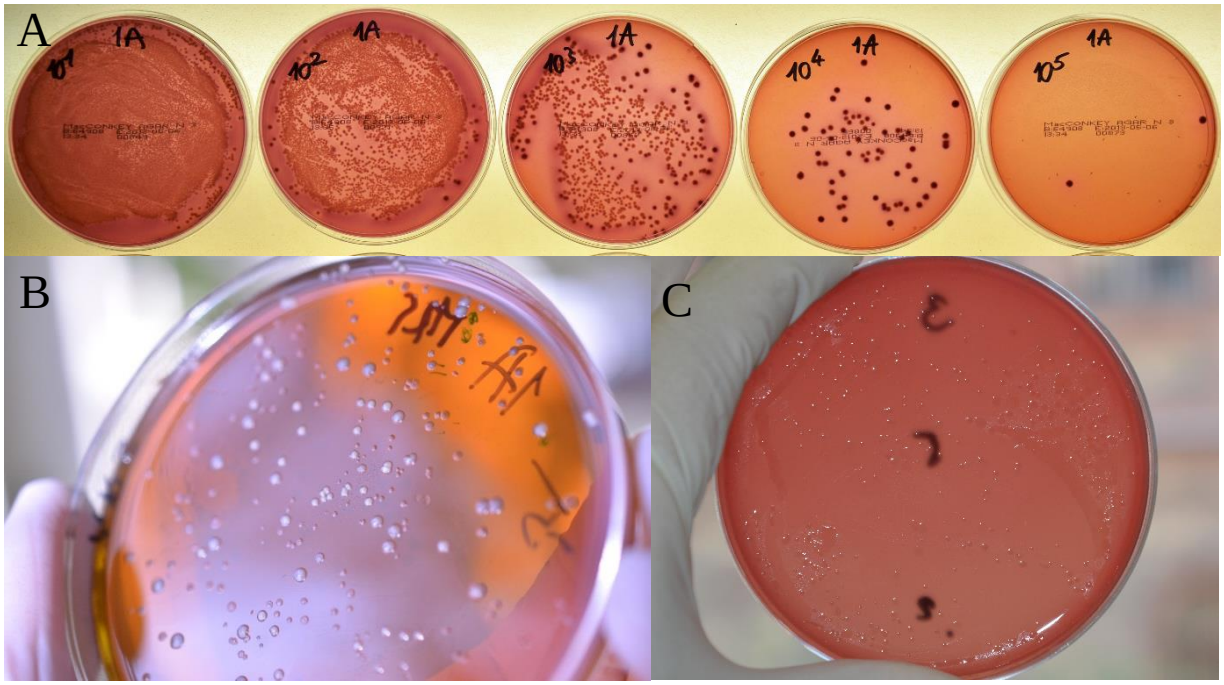

Fig. 10. Colony counting of coliform bacteria (A) on MacConkey agars after 24 hours of incubation using tenfold serial dilution (10^{-1} to 10^{-5}) from a cecal sample. Growth of *Lactobacillus spp.* on MRS selective agar (B). Growing of *Campylobacter jejuni* NCTC 12744 on Campylosel agar (C).

48 h of incubation and CFU numbers of bacteria was assessed after additional 48 h plating on Campylosel agars by plate counting (**Fig. 10C**). For microaerophil incubations Campygen sachets (Oxoid, CN0035) were applied. Solutions containing different concentrations of sodium butyrate in the range of 5 to 100 mmol/l (5, 7.5, 10, 15, 20, 30, 50, 100 mmol/l) were prepared by dissolving sodium butyrate in buffered Preston broth. The pH of each solution was set at 6.0 by adding the appropriate amount of concentrated HCl. Each solution were supplemented with 5 mg/ml chicken mucus by dissolving mucus in PBS without Preston Broth at pH 6.0. The protein content of the mucus was determined using the Bradford protein assay analysis (Bradford, 1976). Dilutions were inoculated with $6*10^3$ CFU/ml *C. jejuni* in 96-well plates in a final volume of 220 µl/well, in triplicate. After 48 h of incubation, CFU/ml values for the *C.*

jejuni strain was determined by plating in serial dilutions. For this, each suspension plus controls were serially diluted (10-fold) in PBS up to the 10^{-8}, in duplicate. From each dilution 100 µl were cultivated on Campylosel agars. *Campylobacter* colonies were counted after 48 h of culturing; minimal bactericide concentration (MBC) and minimal inhibitory concentration (MIC) values were determined from *Campylobacter* counts.

4.2.3.5. Statistical analyses

Statistical analyses were performed predominantly by SPSS 24.0 software. Data were assessed for normality prior to statistical analyses. The level of significance was set at $P < 0.05$. Comparison of dietary treatments were carried out by ANOVA. Differences between groups were determined by Duncan´s post hoc tests. Data which were not shown equal variances by Levene's test, were analyzed by Kruskal-Wallis test (ileal viscosity, cecal propionate and valerate concentrations).

Data obtained for testing the effect of different mucus types on butyrate sensitivity of *C. jejuni* were subjected to statistical analysis using R 2.14.0 software (http://cran.r-project.org/bin/windows/base/old/2.14.0/ on 14 December 2011). Relative inhibitions of *C. jejuni* NCTC 12744 caused by different concentrations of butyrate were determined as the ratio of CFU/ml values in butyrate-treated wells compared to those of the positive controls (no butyrate). Furthermore, decimal logarithm of relative inhibition was calculated for each dilution and analyzed. In order to reach the MIC value, a decimal logarithm of relative inhibition of -3 had to be gained to inhibit growth of the inoculated bacteria. Thus, a decimal logarithm of relative inhibition of -6 matched the requirements of MBC according to its definition. One and Two-way ANOVA was approved for test the mucus effect on relative inhibition.

4.3. Results

4.3.1. Trial I

4.3.1.1. Health status and growth performance

Chickens were in good health during the 35 days of experimental period. There were no significant differences in body weights between dietary groups.

4.3.1.2. Campylobacter enumeration

Colonization of *C. jejuni* in the chicken intestine is summarized in **Fig. 11**. Chickens fed the M diet had higher *C. jejuni* load both in the cecum and in the ileum ($P < 0.01$) compared to the M+WE diet 14 DPI. Chickens fed the M+W diet showed no significant difference in *C. jejuni* colonization compared to chickens fed either the M or the M+WE diet 14 DPI. No significant differences were found in the *C. jejuni* numbers between dietary treatments in the ileum or cecum at the other dates of sampling.

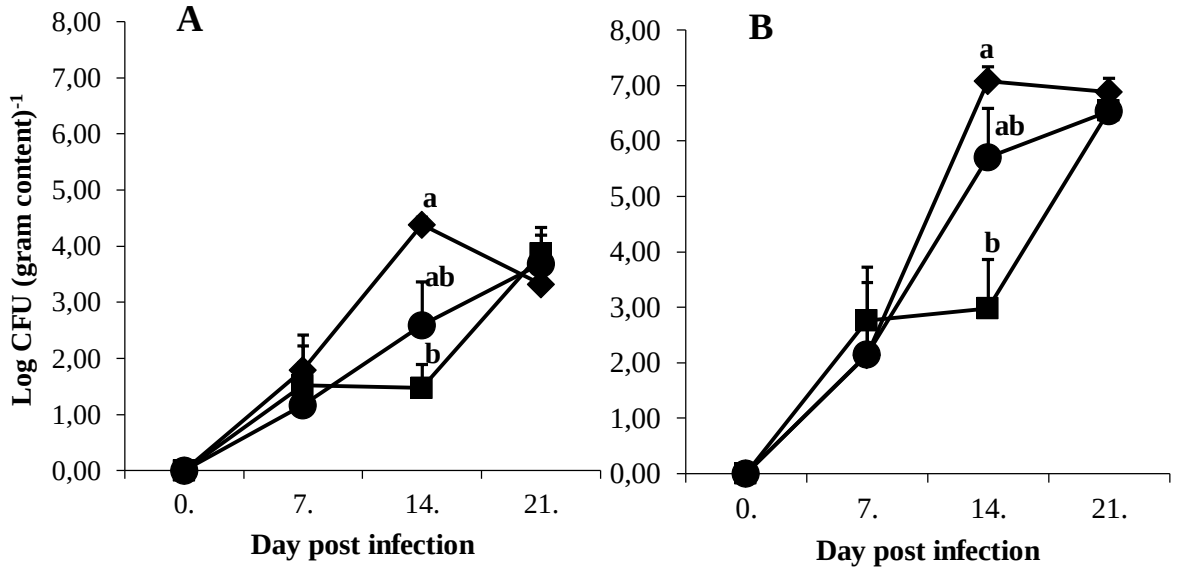

Fig. 11. The impact of feeding maize based (♦), maize-wheat based (M+W)(●) and enzyme supplemented M+W (■) diets on *C. jejuni* colonization dynamics in the ileal (**A**) and in the cecal (**B**) content of broiler chickens at different times post infection. Results are presented as the means of six chickens with SEM. Numbers of bacteria are expressed in logarithmic form of colony forming units (log CFU). Significant differences were labelled with different letters at the same sampling time points.

Irrespective of diets the chickens had higher *C. jejuni* loads in cecum and ileum at 7 DPI compared to 0 DPI ($P < 0.001$). Significantly higher *C. jejuni* numbers could be observed between 7 DPI and 14 DPI ($P < 0.001$) in the cecum of chickens fed both the M and M+W diets. *Campylobacter jejuni* numbers increased in the cecum of chickens fed the M+WE diet from 14 DPI to 21 DPI ($P < 0.001$), however no differences were found between 7 DPI and 14 DPI. Numbers of *C. jejuni* increased significantly in the ileum of chickens fed the M diet from 7 DPI to 14 DPI ($P < 0.001$). In case of the M+W diet the differences in the ileum were significant ($P < 0.05$) between 7 DPI and 21 DPI, while in the ileum of chickens fed M+WE diet showed a significant difference ($P < 0.001$) between 14 DPI and 21 DPI.

4.3.1.3. Ileal viscosity and histochemical studies

Time did not, however feeding different diets, have significant influence on the viscosity values of ileal content, without any interaction between the two factors (**Table 6**). Ileal viscosity was higher in chickens fed the M+W diet compared to chickens fed the M diet. Chickens fed the M+WE diet had lower ileal viscosity compared to chickens fed the M+W diet, though it showed values higher than in chickens fed the M diet. Diet and time significantly affected the villus height, crypt depth, muscle layer thickness and villus surface area. Diet and time interactions were not significant in any of the histomorphometric measures as shown in **Table 5**. In chickens fed the M+WE diet villus height and villus surface area were significantly increased compared to the M and M+W diets. Crypt depth and muscle layer thickness were also significantly increased by M+WE diet compared to chickens fed the M diet. A significant increase could be detected in villus height, crypt depth, muscle layer thickness and in villus surface area regardless diet treatments between 7 DPI and 14 DPI. Interestingly, a decrease was noticed in crypt depth between 14 DPI and 21 DPI. Villus height to crypt depth ratio was not affected by dietary treatments, however, it was increased significantly in chickens fed the M and M+WE diets from 14 DPI to 21 DPI.

Table 5. The effect of different diets on ileal histomorphology of broiler chickens at 7, 14 and 21 days post infection (DPI)[1]

Time	Dietary treatments[2]	Villus height (μm)	Crypt depth (μm)	Muscular layer thickness (μm)	Villus height/ Crypt depth ratio	Villus surface area (mm^2)
7DPI	M	640.8	114.0	78.3	5.7	0.75
	M+W	662.2	121.2	94.8	5.6	0.80
	M+WE	724.2	134.5	101.3	5.5	0.82
14DPI	M	792.3	140.9	115.0	5.6	1.04
	M+W	916.3	156.6	117.7	5.9	1.15
	M+WE	1071.4	176.1	130.0	6.1	1.38
21DPI	M	809.2	112.7	92.3	7.2	1.13
	M+W	767.3	138.5	118.1	5.6	1.01
	M+WE	991.0	138.5	122.3	7.1	1.32
	M	747.4^{b}	122.5^{b}	95.2^{b}	6.2	0.97^{b}
	M+W	781.9^{b}	138.7^{ab}	110.3^{ab}	5.7	0.98^{b}
	M+WE	928.8^{a}	149.7^{a}	117.9^{a}	6.3	1.17^{a}
7 DPI		675.7^{b}	123.2^{b}	91.5^{b}	5.6^{b}	0.79^{b}
14 DPI		926.7^{a}	157.8^{a}	120.9^{a}	5.9^{b}	1.19^{a}
21 DPI		855.8^{a}	129.8^{b}	110.9^{ab}	6.7^{a}	1.15^{a}
Pooled SEM[3]		25.4	3.9	3.7	0.14	0.04
		p-values				
Diet		<0.001	0.004	0.021	0.17	0.034
Time		<0.001	<0.001	0.002	0.002	<0.001
Diet x Time		0.29	0.77	0.83	0.070	0.46

[1]Values are means of six chickens.
[2]M – maize based diet; M+W – maize-wheat based diet; M+WE – maize-wheat based diet supplemented with NSP-degrading enzymes.
[3]SEM – standard error of the mean.
[a, b] Means within a column with different superscript letters are significantly different ($P < 0.05$).

4.3.1.4. The pH values and SCFA concentrations

Ileal pH values were not influenced either by time or by diet. On the other hand cecal pH was affected by both factors. Chickens fed the M+WE diet had lower pH in the cecum compared to the M diet 14 DPI and 21 DPI. This difference was only significant at the latter time. Postinfection time had a significant effect on cecal pH only in chickens fed the M diet. Significantly lower values were observed at 7 DPI compared to 14 or 21 DPI.

Cecal SCFA concentrations and pH values are shown in **Table 6**. Total SCFA and acetate concentrations were influenced by both diets and time, but no interaction between diet and time was found. Total SCFA and acetate concentrations were higher in M+WE diet compared to the other dietary treatments independently of sampling time point. Total SCFA and acetate production in the cecum increased significantly with the age of chickens from 7 DPI to 14 DPI.

Propionate concentrations were also influenced by both diets and time, but in this case diet to time interaction was also significant. Propionate concentrations were significantly higher in the M diet compared to the M+W or M+WE diets 14 DPI. Similarly to acetate a significant increase in propionate concentratios were found between the values measured at 7 DPI and those recorded at 14 or 21 DPI. Butyrate concentrations of the cecum were influenced only by dietary treatments with significant diet to time interaction. The highest butyrate concentrations were found in M+WE diet, which was significantly different from that of the M diet 21 DPI. Besides, higher butyrate concentration was detected in the M+W group in comparison to the M group 14 DPI. Only the age of chickens affected the butyrate to acetic acid ratio. Significantly lower ratio was found at 14 and 21 DPI compared to 7 DPI, respectively.

Valerate concentrations were altered only by the time; an increase was detected in valerate concentrations from 7 DPI to 14 and 21 DPI.

Table 6. The effect of dietary composition on ileal viscosity, cecal short-chain fatty acid concentrations (SCFA) and pH values of ileal and cecal contents in broiler chickens fed different diets at 7, 14 and 21 days post infection (DPI)[1]

Time	Dietary treatments[2]	Viscosity[3]	Acetate[4]	Propionate[4]	Butyrate[4]	Valerate[4]	Butyrate: Acetate ratio	Total SCFA[4]	pH	
		Ileum							Ileum	Cecum
7 DPI	M	2.8	47	2.3^{d}	29^{a}	0.8	0.66^{a}	80	6.8	6.1^{c}
	M+W	5.4	44	3.0^{cd}	22^{ab}	1.1	0.49^{ad}	71	6.3	6.8^{ac}
	M+WE	4.1	49	2.4^{d}	30^{a}	1.0	0.62^{ab}	83	6.9	6.3^{bc}
14 DPI	M	2.7	53	13^{a}	15^{b}	1.7	0.28^{cd}	85	6.3	7.1^{ab}
	M+W	7.6	56	6.9^{bd}	32^{a}	2.1	0.59^{ac}	99	6.7	6.9^{ac}
	M+WE	5.8	81	5.3^{bd}	27^{ab}	1.7	0.34^{bd}	116	6.5	6.6^{bc}
21 DPI	M	2.5	51	9.9^{ab}	14^{b}	2.1	0.27^{d}	79	6.6	7.5^{a}
	M+W	7.1	58	7.3^{bc}	22^{ab}	1.6	0.38^{ad}	90	6.2	6.6^{ab}
	M+WE	4.0	73	7.4^{bc}	33^{a}	1.8	0.46^{ad}	116	6.3	6.0^{c}
	M	2.69^{c}	50^{b}	8.1^{a}	19^{b}	1.5	0.41	81^{b}	6.6	6.9^{a}
	M+W	6.76^{a}	52^{b}	5.7^{b}	25^{ab}	1.6	0.48	86^{b}	6.4	6.8^{a}
	M+WE	4.64^{b}	67^{a}	5.0^{b}	30^{a}	1.5	0.47	105^{a}	6.5	6.3^{b}
7 DPI		4.0	47^{b}	2.6^{b}	27	0.98^{b}	0.59^{a}	78^{b}	6.6	6.4^{b}
14 DPI		5.4	64^{a}	8.2^{a}	24	1.8^{a}	0.40^{b}	101^{a}	6.3	6.8^{a}
21 DPI		4.6	60^{a}	8.2^{a}	23	1.8^{a}	0.37^{b}	95^{a}	6.5	6.8^{a}
	Pooled SEM[5]	0.32	0.32	0.56	1.3	0.08	0.03	3.1	0.08	0.09
			p-values							
	Diet	<0.001	<0.001	0.001	<0.001	0.95	0.33	<0.001	0.66	<0.001
	Time	0.089	<0.001	<0.001	0.19	<0.001	<0.001	0.0016	0.30	0.013
	Diet x Time	0.29	0.15	0.005	<0.001	0.096	0.0038	0.16	0.44	<0.001

[1]Values are means of six chickens.
[2]M – maize based diet; M+W – maize-wheat based diet; M+WE – maize-wheat based diet supplemented with NSP-degrading enzyme.
[3]Values are expressed in millipascal-second (mPa.s).
[4]µmol/g
[5]SEM – standard error of the mean.
[a-d] Means within a column with different superscript letters are significantly different ($P < 0.05$).

4.3.2. Trial II

4.3.2.1. Growth performance

Dietary treatments did not influence significantly the BW at 35 days of life (*P = 0.097*). The M group had 2323 ± 40.7 g final body weight. Chickens which received the M+W, M+B, M+I and M+L diets reached 2480 ± 47.9 g, 2377 ± 39.0 g, 2346 ± 54.1 g, and 2354 ± 40.4 g body weights at day 35 of life, respectively. Chickens consumed 3690 g, 3763 g, 3764 g, 3744 g and 3655 g feed in the M, M+W, M+B, M+I, and M+L groups over the trial period, respectively. The FCR values (day 1 to day 35) varied from 1.54 to 1.62 among all groups, M+W group having the lowest whereas the M, M+I groups having the highest values. The FCR of the M+B group was 1.61, and 1.59 for the M+L group.

4.3.2.2. Ileal viscosity and histological analyses

An increase (*P < 0.01*) in ileal viscosity was detected in chickens fed the M+W diet relative to the M diet, M+I chickens and to those fed the M+L diet (**Table 7**).

Dietary treatments had no significant effect on villus height (*P ≥ 0.05*) and on basal transverse (*P ≥ 0.05*), however all other histomorphological measures tested in this study varied among dietary groups. Chickens fed the M diet showed deeper crypt values compared to the other groups (*P < 0.05*). Besides, shallower crypts were observed in chickens received the M+L diet relative to the others (*P < 0.05*). Villus-crypt ratios were highest in chickens fed the M+L diet and it differed from all other dietary treatments (*P < 0.05*). Also, chickens fed the M+L diet had difference for apical transverse (*P < 0.05*), showing lower values than the other groups. Muscle layer thickness showed higher values in the M group comparing to the other ones (*P < 0.05*). No differences (*P ≥ 0.05*) were detected in goblet cell and IEL numbers in the ileum or cecum among dietary groups (**Table 8**).

Table 7. Effects of diets containing soluble non-digestible carbohydrates from different sources on ileal viscosity and on ileal histomorphology measures of male broiler chickens (day 35 of life)[1]

Dietary treatments[2]	Viscosity[3]	Villus height[4]	Crypt depth[4]	Villus-crypt ratio	Basal transverse[4]	Apical transverse[4]	Muscle layer thickness[4]
M	2.31[b]	1271	167[a]	7.83[b]	188	175[a]	248[a]
M+W	3.00[a]	1160	145[b]	8.09[b]	171	165[a]	199[b]
M+B	2.69[ab]	1110	147[b]	7.73[b]	173	177[a]	196[b]
M+I	2.31[b]	1057	147[b]	7.47[b]	166	165[a]	204[b]
M+L	2.35[b]	1148	129[c]	9.16[a]	155	144[b]	184[b]
Pooled SEM	0.064	26.1	2.7	0.172	3.0	2.7	4.9
P-value[5]	0.001	0.135	<0.001	0.013	0.076	0.001	<0.001

[1]Values are means of 10 chickens per treatments.
[2]M: maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I - maize based diet with inulin supplementation; M+L - maize based diet with lactose supplementation.
[3]mPa.s
[4]µm
[5]Viscosity was analysed by Kruskal-Wallis test.
[a-c]Means in each row with no common superscript letter are significantly different ($P < 0.05$). SEM, standard error of the mean.

Table 8. Effects of diets containing soluble non-digestible carbohydrates from different sources on ileal and cecal goblet cell and intraepithelial lymphocyte (IEL) numbers of male broiler chickens (day 35 of life)[1]

Dietary treatments[2]	Ileum		Cecum	
	Goblet cells	IEL	Goblet cells	IEL
M	24.4	20.9	21.3	10.1
M+W	23.4	19.0	17.1	10.6
M+B	25.4	19.5	19.2	8.9
M+I	24.9	21.7	19.8	7.5
M+L	26.0	21.5	21.5	10.1
Pooled SEM[3]	0.14	0.75	0.75	0.42
P-value	0.482	0.733	0.376	0.125

[1]Values are means of 10 chickens per treatments. Goblet cell and IEL numbers are expressed for 400 µm villus epithelium.
[2]M: maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I - maize based diet with inulin supplementation; M+L - maize based diet with lactose supplementation.
[3]Standard error of the mean.

4.3.2.3. Cecal pH and SCFA concentrations

Feeding the M+I diet resulted in the lowest cecal pH whereas chickens in the M group had the highest cecal pH (**Table 9**). Chickens received the M+W, M+B and M+L diets were in between differing from both, the M and the M+I diet ($P < 0.05$). No differences ($P \geq 0.05$) were found in acetate and propionate values in cecal content of chickens fed different diets; however, butyrate, valerate and total SCFA concentrations varied ($P < 0.05$) among dietary groups. There was an increase ($P < 0.05$) in butyrate concentration in chickens fed the M+W diet in comparison to all other dietary groups. Similarly, feeding the M+W diet resulted in the highest valerate concentration, showing a difference ($P < 0.05$) relative to chickens fed the M+B, M+I, and M+L diets. Chickens fed the M diet had higher valerate concentration compared to chickens received the M+L diet ($P < 0.05$). Total SCFA was highest in the M+W group differing ($P < 0.05$) from chickens fed the M, M+B and M+L diets. Besides, the M+I dietary group had higher total SCFA concentration relative to the M+L group ($P < 0.05$).

Table 9. Effects of diets containing soluble non-digestible carbohydrates from different sources on cecal pH and short chain fatty acid (SCFA) concentration of male broiler chickens (day 35 of life)[1]

Dietary treatments[2]	pH	Acetate[3]	Propionate[3]	n-Butyrate[3]	n-Valerate[3]	Total SCFA[3]
M	7.12[a]	43.7	11.3	17.3[b]	1.95[ab]	72.2[bc]
M+W	6.80[b]	45.1	8.9	36.1[a]	2.57[a]	94.6[a]
M+B	6.76[b]	41.2	8.0	20.7[b]	1.45[bc]	72.3[bc]
M+I	6.41[c]	51.0	7.2	21.4[b]	1.35[bc]	83.4[ab]
M+L	6.74[b]	37.6	7.2	15.7[b]	1.14[c]	61.9[c]
Pooled SEM	0.052	1.8	0.44	1.81	0.132	3.09
P-value[4]	<0.001	0.205	0.061	0.001	0.006	0.007

[1]Values are means of 10 chickens per treatments except for pH when 12 chickens were used in each group.
[2]M: maize based diet; M+W: wheat based diet; M+B: barley based diet; M+I - maize based diet with inulin supplementation; M+L - maize based diet with lactose supplementation.
[3]μmol/g
[4]Propionate and n-valerate were analysed by Kruskal-Wallis tests.
[a-c]Means in each row with no common superscript letter are significantly different ($P < 0.05$). SEM, standard error of the mean.

4.3.2.4. Cecal coliform and Lactobacillus numbers

Cecal coliform counts increased when chickens received the M+W, M+B, M+I and M+L diets in comparison to chickens fed the M diet (*P = 0.001*) (**Fig. 12**). There were no differences in cecal *Lactobacillus* counts (*P = 0.259*).

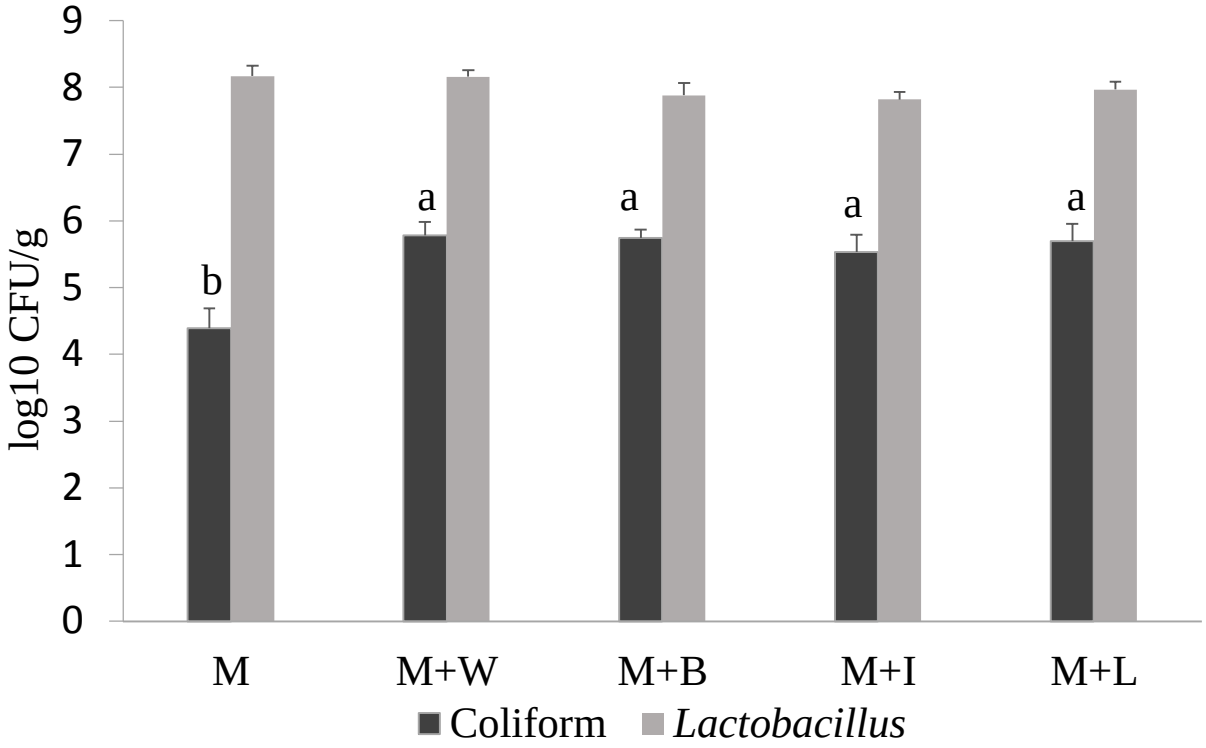

Fig. 12. Effects of diets containing soluble non-digestible carbohydrates from different sources on cecal coliform and *Lactobacillus* counts of male broiler chickens at 35 days of life. Results are presented as means of 10 chickens. Error bar represent standard error of the mean. Abbreviations: M - maize based diet; M+W – maize-wheat based diet; M+B – maize-barley based diet; M+I - maize based diet with inulin supplementation; M+L - maize based diet with lactose supplementation. Significant differences were marked with different letters (a, b) between dietary groups (*P < 0.01*).

4.3.2.5. Effect of different mucus types on butyrate sensitivity of C. jejuni

The MIC value of butyrate for *C. jejuni* NCTC 12744 was measured as 10 mM butyrate concentration at pH 6.0. The addition of mucus to the medium shifted the MIC value to 15 mM in each case, however this difference did not reach the level of significance. No differences were found among the protective effect of mucus samples originated from different dietary groups **(Fig. 13).**

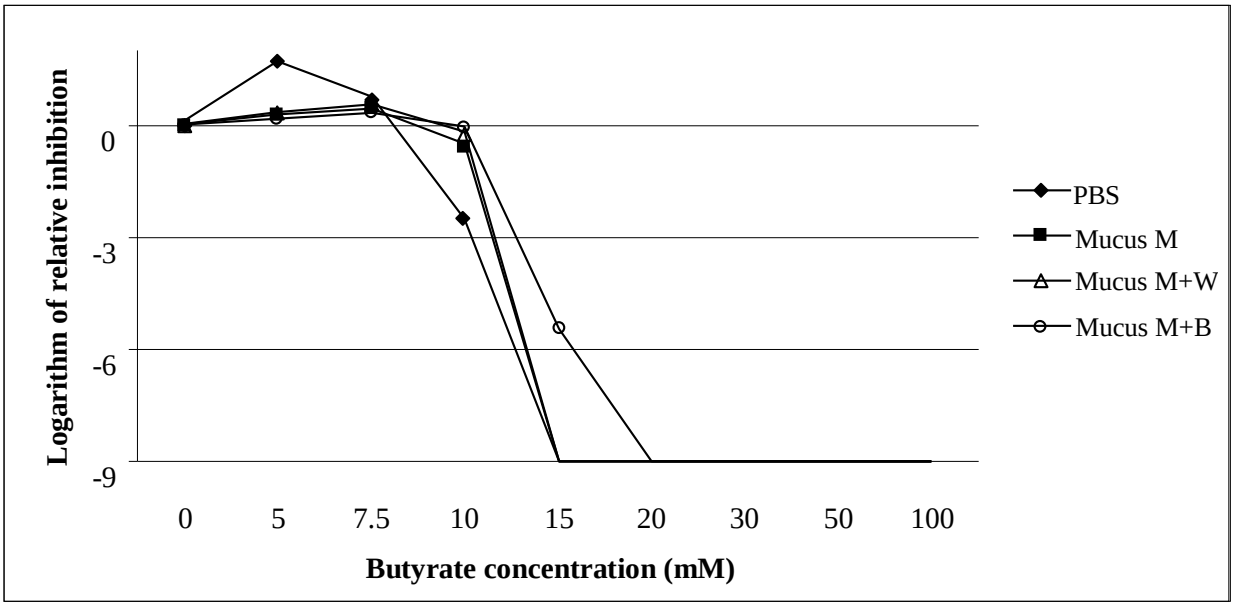

Fig. 13. Decimal logarithm of relative inhibition of *C. jejuni* 12744 caused by different concentrations of butyrate influenced by different mucus types. Relative inhibition was considered as the ratio of CFU values in butyrate-treated wells compared to those of the positive control (no butyrate). Mucus M = mucus collected from chickens fed the maize based diet. Mucus M+W = mucus collected from chickens fed the maize-wheat based diet, Mucus M+B = mucus collected from chickens fed the maize-barley based diet.

4.4. Discussion

4.4.1. Trial I

Comparing the effects of maize and M+W diets on intestinal physiology and microbiota is of special interest, as these cereal components are most commonly used in poultry diets. Besides their prebiotic effect, sNSPs - found at high rate in wheat, barley, rye - are thought to be mainly responsible for the increase in the viscosity of digesta (de Lange, 2000). Because of the rise in ileal viscosity, more opportunity is given for pathogenic bacteria to settle and to multiply in the intestine. For this reason, the effects of cereal type and enzyme supplementation on *C. jejuni* and on *Salmonella* colonization were investigated by several researchers. Teirlynck et al. (2009a, b) observed that chickens fed M+W diet had higher *Salmonella* colonization compared to chickens fed the M diet, describing it as a consequence of high sNSP content resulting in a shift of gut microbiota and alterations of gut morphology. Fernandez et al. (2000) found a positive relationship between the rise in small intestinal viscosity and an increase in cecal *C. jejuni* numbers in case of chickens fed a wheat-based (58.8%) diet in comparison with chickens fed maize-based diet. In our experiment, the high-viscosity M+W diet had no such influence on *Campylobacter* load compared to the M diet. Interestingly, Santos et al. (2008) found lower *Salmonella* prevalence connected with improved diversity of the microbial community in the turkey fed M+W (36.25–52%) diet compared to maize-based diet. Eeckhaut et al. (2008) observed that sNSP (arabinoxylooligosaccharides) supplementation had a time-dependent inhibition on *Salmonella* colonization in the chicken cecum. These contradictory results draw attention to the amount of sNSPs added to the diet at different age of the chicken as it modifies the influence of sNSPs on gut health. In the actual Trial, wheat/barley supplementation were gradual from starter to finisher diet which have resulted in 25-29% lower wheat/barley inclusion in the starter diet comparing to the cited works (Fernandez et al., 2000; Teirlynck et al., 2009a, 2009b). This finding corresponds to the suggestion of de Lange (2000) that the effects of sNSPs can have threshold-like mechanisms. Although M+W poultry diets can have adverse effects on

digestion and on gut health (de Lange, 2000; Teirlynck et al., 2009a), these diets can efficiently used with NSP-degrading enzyme supplementation. The beneficial effect of enzyme supplementation on the intestinal microflora composition and on related intestinal characteristics was demonstrated by several researchers. Enzyme supplementation of a wheat- and barley-based diet improved apparent digestibility of crude fat, increased the number of lactobacilli and bifidobacteria in the ileum and in the cecum of broilers, respectively (Rodríguez et al., 2012). In the study of Engberg et al. (2004), enzyme supplementation tended to decrease ileal and cecal *Clostridium perfringens* numbers in chickens fed a diet containing whole seed wheat. Fernandez et al. (2000) found reduced *Campylobacter* colonization in chickens fed enzyme supplemented wheat-based diet compared to maize-based and wheat-based diets, respectively in broilers at 28 days of age. Similarly, in our experiment, chickens fed the M+WE diet had lower *Campylobacter* counts at 28 days of age and had improved ileal histomorphology compared to chickens fed the M diet. In the study of Fernandez et al. (2000), lower *C. jejuni* numbers were associated with lower ileal viscosity. In our trial, the rate of *Campylobacter* colonization was not strongly correlated with ileal viscosity, as chickens fed M+WE diet showed significantly higher viscosity values than those fed the M diet. Instead of viscosity, other factors could be involved in altering *C. jejuni* colonization. As SCFAs have a marked bactericidal and bacteriostatic effect *in vitro* (Van Deun et al., 2008), the lower *C. jejuni* numbers, found in chickens fed the M+WE diet, may be explained by the higher SCFA concentrations in the cecum of these chickens. Short-chain fatty acids can provide its bactericidal and bacteriostatic attitude at lower pH due to the amount of undisassociated forms presented in this circumstance (Mroz et al., 2006; Van Deun et al., 2008). Van Deun et al. (2008) observed butyrate pH-dependent efficacy on *C. jejuni* in an *in vitro* study. Numerous studies demonstrated a correlation between cecal SCFA concentrations and microflora composition in different animals. Campbell et al. (1997) studied the cecal microflora composition, SCFA concentration and pH in rats fed fermentable oligosaccharides, and they

found higher bifidobacteria and total anaerob numbers together with higher SCFA concentrations and lower pH. The supplementation of both, a prebiotic (isomalto-oligosaccharides) and a multistrain probiotics (consisting of 11 *Lactobacillus* strains), increased cecal SCFA concentrations and cecal populations of lactobacilli, bifidobacteria, while *Escherichia coli* numbers were decreased at the same time (Mookiah et al., 2014). Reduced cecal *Salmonella* numbers were found concurrently with higher cecal SCFA concentrations in broilers fed with medium-chain fatty acids (Chotikatum et al., 2009). Although differences in SCFA concentrations and pH values in the cecum of chickens in our study fed the M and M+WE diets were more expressed 21 DPI compared to 14 DPI, no differences were found in *C. jejuni* colonization between these two treatments 21 DPI. This correlation suggests the limitation of the direct effect of SCFA and pH on *C. jejuni* colonization *in vivo*. The same issue was raised by Van Deun et al. (2008) who observed the inhibitory effect of chicken mucus on butyrate anti-*Campylobacter* activity. The propionate and butyrate concentrations showed an opposite tendency when comparing the M and the two wheat based diets. This may relate to differences in microbial cross-feeding phenomenons such as shifting the lactate-propionate pathway to lactate-pyruvate formation (Ríos-Covián et al., 2016). Stressors such as toxins and certain bacteria can impact the structure of the mucosa (Awad et al., 2006; Fasina et al., 2010). Decreased villus surface was detected by Fasina et al. (2010) in *Salmonella typhimurium*-infected chickens compared to non-infected ones. On the other hand, Xu et al. (2003) and Rehman et al. (2007) observed the increase of villus height in case of feeding the prebiotic inulin. The observation in the latter study was associated with increased bifidobacteria and lactobacilli numbers in the small intestine. Cao et al. (2013) reported greater ileal villus height in chickens fed a diet supplemented with the probiotic *Enterococcus faecium* compared to M chickens. Overall, these findings strongly hint the correlation between the composition of intestinal microflora and the histomorphological measures. Therefore, we suppose that the delayed peak in *C. jejuni* colonization in the M+WE diet group could be the result of an

improved intestinal microflora. This hypothesis is reinforced by the findings of Ghareeb et al. (2012), showing a reduced cecal colonization of *C. jejuni* after supplementation of drinking water of broilers with a probiotic feed additive.

Diets contained higher sNSP level (M+W and M+WE) showed beneficial effects on intestinal characteristics in our study only in case of enzyme supplementation compared to the diet contained lower sNSP level (maize-based). The reason for this can be the conversion of sNSPs into more fermentable oligosaccharides for bacteria by the NSP-degrading enzyme (de Lange, 2000). Up to now, only limited information is available in the literature about the influence of diet composition on *C. jejuni* colonization of the chicken gut. In the present study, it took 14 and 21 days for the applied *C. jejuni* strain to reach its colonization maximum in chickens fed the M and M+WE diet, respectively. The colonization results were independent of the gut section and were similar in the ileum and in the cecum, even though the level of colonization was lower in the ileum. The differences in *C. jejuni* colonization over time suggest the importance of sampling time point postinfection and point out the benefit of multiple sampling. The actual results indicate that differences in the tested diets contribute to *Campylobacter* colonization in broilers. In this context, the lower prevalence of *C. jejuni* in Northern European countries (EFSA, 2011) in which production is based on M+WE diets in comparison with Southern European countries could be mentioned.

4.4.2. Trial II

Soluble fibre fractions of feedstuffs and prebiotic feed additives can modify gut health, the gut morphology, the digestion and also the production traits of chickens in different ways (de Vries, 2015). In our trial, feeding isonitrogenous and isocaloric diets with different sNDCs failed to cause differences in the growth rate and final body weight.

Viscous polysaccharides (arabinoxylans, ß-glucans) can increase intestinal viscosity and decrease the digestibility of nutrients (de Lange, 2000; Jacob and Pescatore, 2012). The potential adverse effects, such as reduced BW by feeding wheat/barley is known from the literature (Shakouri et al., 2009; Jacob and Pescatore, 2012; Rodríguez et al., 2012). According to the results of Wang et al. (1992) negative relationship exist between intestinal viscosity and the BW of chickens. In our study, only the M+W diet increased intestinal viscosity, although this difference was relatively small in comparison to the other reports (Shakouri et al., 2009; Morales-López et al., 2010; Molnár et al., 2015). This relatively low changes in viscosity could be an explanation that in the present study feeding M+W and M+B diets without NSP-degrading enzymes did not result in significant differences in the production traits.

The structure of the intestine is also influenced by sNDCs, since increase in digesta viscosity could lead to epithelial cell losses and result in villus atrophy or enlarged crypts (Rahmatnejad and Saki, 2016). In this study neither M+W nor M+B diets decreased villus height. Feeding inulin supplemented diets, it could increase ileal villus height (Rebole et al., 2010; Nabizadeh, 2012). The unchanged villus height in the M+I group may relate to the unchanged cecal SCFA values relative to the M group, as increased villus heights are often caused by the trophic effect of cecal SCFA which is not restricted to the lower gut only (Montagne et al., 2003). Gülşen et al. (2002) fed chickens with a lactose supplemented (25 g/kg) diet and investigated the histological changes of intestinal villi. They did not detect changes of ileal villi on day 28 or 42 of life which corresponds to our findings. A decrease in crypt depth was observed when chickens fed the M+W, M+B, M+I or M+L diets compared to the M diet. Stem cells division take place in the crypts permitting renewal of villi (Bucław, 2016). Deeper crypts are associated with increased crypt-cell proliferation, faster cell turnover and increased water secretion. Alterations of the microbiota, the presence of stressors such as bacterial toxins can harm intestinal structure (Awad et al., 2006; Bucław, 2016) and therefore these factors might have contributed to the deeper crypt values observed in chickens fed the M diet in the present study.

Furthermore, higher villus height/crypt depth ratio was found in the M+L group relative to all other groups which was a result of the shallowest crypt values observed here. The thickness of muscle layer decreased in each high sNDC group relative to the M group. In one hand, changes in muscle layer thickness may relate to gut peristalsis and the rate of digesta passage (Chou et al., 2009). Thinner muscle layers were also reported as a result of antibiotic supplementation of diets due to a change of the microbiota and associated reduction in inflammation process (Ferket et al., 2002; Miles et al., 2006; Brufau et al., 2015). Diets supplemented with mannanoligosaccharides or ß-galactomannans could also reduce muscle layer thickness (Ferket et al., 2002; Brufau et al., 2015). Our results are in agreement with these findings. The reduced muscle layer thickness found in chickens fed high sNDC diets in the present study might be the result of a change in the microbiota composition of the small intestine.

Mucin secreted by goblet cells forms a chemical barrier on the epithelium by protecting the intestinal mucosa from chemical and mechanical damage (Khan, 2008). The present outcomes showed no differences in goblet cell and IEL numbers between dietary treatments. Physical abrasion and proteolytic breakdown of mucus gels are the main factors for intensified mucin production (Allen, 1981). Microbial changes, such as increasing numbers of Gram-negative bacteria may necessitate the need for more mucus production (Edens et al., 1997; Ferket et al., 2002). Teirlynck et al. (2009) reported more ileal and cecal goblet cells associated with mucosal damage and lymphocyte infiltration when chickens received wheat/rye (53%/5%) at high inclusion levels in comparison to a M diet. The literature is scarce regarding the effects of wheat, barley, inulin, and lactose supplementation on intestinal goblet cell and on IEL counts. Increased recovery of the mucus or increased lymphocyte infiltration were not observed in the actual experiment.

Cecum is the main site for bacterial fermentation in chickens due to its special habitat (Svihus et al., 2013). Bacteria metabolize sNDCs into SCFAs and lactate which consequently lowers

the pH (Rinttilä and Apajalahti, 2013). Reduced pH may inhibit the growth of acid-sensitive bacteria such as members of the family *Enterobacteriaceae* (van Der Wielen et al., 2000). Previous nutrition studies showed a 0.3-1.0 pH reduction in case of feeding sNDCs from various sources compared with maize based diet (Jozefiak et al., 2008; Shakouri et al., 2009; Molnár et al., 2015). In the present investigation, all dietary treatments resulted in lowered pH (0.32-0.71 reduction) relative to the M diet and cecal pH was even more reduced in the M+I group in comparison to the other sNDC diets. However, the cecal SCFA concentration in the M+W diet was numerically higher than in the M+I group, expected to cause the lower cecal pH. This inconsistency can originate from the different buffer capacities of the cecal contents and the differences in the lactic acid concentrations (Rebole et al., 2010), which were not measured here. Not only total SCFA concentration, but fermentation profiles differed among the dietary treatments as the M+W diet increased cecal butyrate concentration and the M+L diet reduced the valerate content in comparison to the M diet. Amongst SCFAs, butyrate draws special attention due to its high antimicrobial potential and its contribution to epithelial cell development (Van Deun et al., 2008; Rinttilä and Apajalahti, 2013). Elevated cecal butyrate concentrations were observed in association with lowered intestinal *Campylobacter* and *Salmonella* counts which support the beneficial gut health effect of feeding wheat supplemented diets (Meimandipour et al., 2010; Molnár et al., 2015). It is worthy to mention that pH plays an important role in the antimicrobial action of butyrate, as butyrate can penetrate the bacterial cell in undissociated form and at lower pH more undissociated molecules are present (Józefiak et al., 2004).

It is generally accepted that indigenous *Lactobacillus spp*. are considered beneficial bacteria as they positively contribute to microbial balance and gut health through competitive exclusion and through the production of lactic acid (Patterson and Burkholder, 2003; Rebole et al., 2010). Surprisingly, in our study, none of the high sNDC diets increased cecal *Lactobacillus* numbers. Instead, sNDC diets resulted a microbial shift towards a higher cecal coliform load relative to

the M group. Elevated intestinal coliform and *E. coli* counts are generally associated with adverse health effects. These bacteria are often contrasted with *Lactobacillus* (Bucław, 2016). On the other hand, the outcomes in some novel studies hinted a relation between higher intestinal *E. coli/Enterobacteriaceae* load and improved performance (van der Hoeven-Hangoor et al., 2013; Singh et al., 2014). In the present experiment higher cecal coliform numbers showed enhanced intestinal functions such as lower cecal pH or higher butyrate concentration. Increased cecal coliform load may point out an augmented bacterial fermentation in the tested sNDC groups due to higher substrate availability.

A previous study reported a reduction in anti-*Campylobacter* efficiency of butyrate *in vitro* (Van Deun et al., 2008) and accordingly some other chicken studies concluded that mucus protects *Campylobacter* from butyrate or from medium chain fatty acids (Hermans et al., 2010; Robyn et al., 2013). The present results are in contrast with the study of Van Deun et al. (2008) as mucus addition did not altered butyrate anti-*Camplobacter* activity considerable. No data are available on the effects of different mucus types on butyrate anti-*Camplobacter* activity. In this study no differences were observed in butyrate anti-*Camplobacter* activity between mucus obtained from chickens fed the control, M+W and M+B diets, however the exact compositions of these mucuse types were not investigated. Fernandez et al. (2000) showed that diet type (maize-based, wheat-based or wheat-based enzyme supplemented) influenced mucus composition of the chicken intestinal tract and it was correlated to altered *C. jejuni* colonization. The present outcomes suggest the importance of other factors such as cecal pH and butyrate concentration in the anti-*Camplobacter* efficacy of butyrate *in vivo*. Further studies including in vivo experiments needs to clarify the exact role of mucus and mucus composition as potential factors protecting *Camplobacter* in the chicken intestine.

In summary, different sNDC sources acted differently on some intestinal characteristics as higher villus-crypt ratio, lower cecal pH and higher butyrate concentration were found in

different dietary groups. On the other hand, some common features were observed as crypt depth, muscle layer thickness and cecal coliform numbers were altered in the same manner in all sNDC groups relative to the M diet. Overall, based on histomorphology, pH and SCFA data, the tested sNDC diets influenced the chicken gut health positively.

4.5. Conclusion

Two chicken trials provided information on gut characteristics associated with gut health testing the effects of cereal grains, NSP-degrading enzymes, inulin and lactose supplementations. Overall, diets containing higher proportions of soluble undigestible components (sNDC) were used as substrates for bacterial fermentation and were compared to M diets in both cases. Significant differences were revealed on ileal viscosity, histomorphology, cecal pH, SCFA concentration and on some microbiological measures. These changes were evaluated and discussed as beneficial gut health effects when chickens were fed higher sNDC contents. The enzyme supplemented maize-wheat (M+WE) based diet delayed *Campylobacter* colonisation in the gut 14 DPI. This finding can be applied in the feeding practice for increasing food safety in broiler meet production. Generally, slaughter time for broiler chickens is fallen to 28 to 42 days of life wheareas the time period for *Camplyobacter* colonisation is exposed between 14 and 21 days of life. Composing a diet which have a delaying effect on *Campylobacter* colonisation could reduce the *Campylobacter* load in the intestine at slaughter age, and thus can reduce the human health risk. Regarding gut health supporter diet formulation in poultry, the studied sNDC components can be potential candidates for contributing to an improved intestinal milieau. On the other hand, the choice of the exact amount and type of sNDC source needs to be further assessed to refine the optimal requirements. This is also necessary because the adverse impacts of exceeding amounts of sNDC are well known. As scientific reports predominantly investigated the effects of wheat/barley inclusion at high proportion, more studies using moderate inclusion levels (likewise here) of these grains should be conducted.

The use of NSP-degrading enzymes needs to be investigated paralelly as they may improve the valuable and mitigate the unfavourable effects of sNSPs. Wide-scaled experiments are welcomed on production characteristics to further assessing the applicability of sNDC sources. A better understanding on the relation of the different carbohydrate fractions and the factors like microbial colonization in the small intestine and ceca, metabolic effects of the microbial fermention products, digestibility of nutrients, intestinal development could help to develop nutritional strategies using these carbohydrates to enhance performance and gut health also in field conditions.

5. SUMMARY

Nutritional strategies to promote gut health and safe broiler meat production has come into prominence due to the emerging challenges related to the ban of antibiotic growth promoters. Also, there is a high demand on revealing effective nutrional strategies against the cause of most common bacterial zoonosis, *Campylobacter jejuni*. There is a gap of knowledge in nutritional studies regarding the dynamics of *Campylobacter* colonization post infection. Additionally the climate change can affect cereal production in the near future and wheat/barley may appear more frequently in poultry diet recipes as substitutes for maize. There is little information available on gut health effects of lactose feeding and on measures such as goblet cell and intraepithelial lymphocyte (IEL) numbers when chickens were fed various cereal grains. Therefore two broiler chicken trials were conducted to elucidate the impact of nutrition on selected intestinal characteristics associated with gut health. Furthermore, different mucus obtained from chickens fed maize based (M), maize-wheat based (M+W) and maize-barley based (M+B) diets were tested on butyrate anti-*Campylobacter* activity *in vitro*.

In Trial I, a total of 54 one day-old Ross 308 broiler chickens were randomly divided into three isocaloric and isonitrogenous dietary groups: M, M+W diet and M+W diet with NSP-degrading enzyme supplementation (M+WE). Chickens were orally infected with 10^8 CFU *C. jejuni* on day 14 and samples (n=6) were collected from the intestinal content on 7, 14 and 21 days post infection (DPI), respectively. Colony forming units of *C. jejuni* of cecum and ileum, short-chain fatty acid (SCFA) concentration, pH values of the cecum, ileal histomorphology and viscosity of ileal chymus were measured.

The objective of Trial II was to study the influence of a M+W, M+B and maize based diets supplemented with inulin (M+I) or lactose (M+L) on growth performance, gut histology (morphology, goblet cell and IEL numbers), ileal viscosity, cecal SCFA concentration, pH, coliform and *Lactobacillus* counts in comparison to a M diet. In total, 200 Ross 308 male

chickens were kept in deep litter pens (n=40) and fed their appropriate diets from day 1 to day 35 of life. Five isocaloric and isonitrogenous diets, differing in their soluble non-digestible carbohydrate (sNDC) content, were composed; M, M+W, M+B and maize-based supplemented either with 20 g/kg inulin (M+I) or 30 g/kg lactose (M+L).

In Trial I, the M+WE group had lower *C. jejuni* colonization 14 DPI, higher ileal viscosity, higher total SCFA concentration in the cecum and enhanced ileal histomorphology compared to the M group. In Trial II, all of the diets tested decreased ileal crypt depth, muscle layer thickness and increased cecal coliform counts relative to the M group. Villus-crypt ratio increased only in the lactose supplemented group. Ileal digesta of chickens fed the M+W diet had the highest ileal viscosity and the highest cecal butyrate, valerate and total short-chain fatty acid concentrations while the lowest pH was observed in cecal contents of chickens fed the inulin supplemented diet. The diet had no effect on ileal or cecal goblet cell and IEL numbers. *Lactobacillus* counts in the cecal content remained unchanged. Different mucus types did not varied in their effect on butyrate anti-*Campylobacter* activity.

Trial I showed that diet composition can modify *C. jejuni* colonization depending on sampling time point post infection and this change may relate to ileal histomorphology and cecal pH and SCFA concentrations. In Trial II, different sNDC sources acted differently on some intestinal characteristics such as viscosity, villus-crypt ratio, cecal pH, butyrate, valerate and total SCFA concentrations. These beneficial effects were not solely related to the sNDC proportion as the M+B diet consisted more sNDC than the M+W one. The *in vitro* mucus study suggested that mucus type does not play any role in butyrate anti-*Campylobacter* efficacy. Overall, it is hard to rank the tested sNDC sources based on their effects on gut health. On the other hand, some common features were demonstrated broadening our understanding on chicken gut health and nutrition.

6. NEW SCIENTIFIC RESULTS

6.1. New scientific results

1. This is the first study which assessed the influence of M+W and M+WE diets on *C. jejuni* colonization in multiple time points post infection. The M+WE diet delayed *C. jejuni* colonization which was indicated by the lowered bacterial numbers in the ileum and cecum 14 days post infection in comparison to the M diet.

2. Diets containing higher sNDCs from different sources (wheat, barley, inulin, lactose) decreased ileal crypt depth, cecal pH and increased cecal coliform counts collectively relative to the M diet. Amongst the tested sNDCs, only the M+W diet increased cecal butyrate and total SCFA concentrations.

3. Lactose supplemented maize-based diet increased ileal villus/crypt ratio and decreased cecal pH and cecal valerate concentration in broilers at slaughter age relative to the M diet.

4. The results of our *in vitro* studies demonstrated that mucus obtained from chickens received different diets did not influence anti-*Campylobacter* activity of butyrate.

6.2. Új tudományos eredmények

1. Elsőízben tanulmányoztuk kísérletünkben a búza alapú tápok hatását a *C. jejuni* kolonizációjára a fertőzést követően több időpontban. Kísérletünkben a xylanáz/glükanáz enzimmel kiegészített búza alapú táp késleltette a csípőbélben és a vakbélben mérhető *C. jejuni* kolonizációját, ami a fertőzést követő 14. napon mutatkozott meg alacsonyabb baktériumszámokban, a kukorica alapú táphoz hasonlítva.

2. Nem-emészthető szénhidrátokat magasabb arányban tartalmazó (búzával, árpával, inulinnal vagy tejcukorral kiegészített) tápok hasonló módon csökkentették a csípőbél nyálkahártya kriptamélységét, vakbélbeli pH értéket, valamint növelték a vakbél coliform baktériumainak számát a kukorica alapú táphoz viszonyítva. A vizsgált tápok a vakbélbeli *Lactobacillus* számokra nem voltak hatással. A vizsgált nem-emészthető szénhidrát források közül - az alkalmazott bekeverési arányban -, csak a búzával kiegészített takarmány növelte a vakbél butirát és össz-illózsírsav koncentrációit.

3. Vágókorú pecsenyecsirkében a tejcukorral kiegészített kukorica alapú táp megnövelte a csípőbélbeli boholy/kripta arányt, csökkentette a vakbél pH értékét és a vakbél valerát koncentrációját a kukorica alapú táphoz képest.

4. A bélnyálka *in vitro* kísérletünk azt mutatta, hogy a különböző takarmányozási csoportból származó bélnyálkák nem befolyásolják a butirát (vajsav) *Campylobacter*-ellenes hatását.

7. REFERENCES

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8. SCIENTIFIC PUBLICATIONS BY THE CONTRIBUTION OF THE AUTHOR

8.1. Publications related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

Molnár, A., Hess, C., Pál, L., Wágner, L., Awad, W.A., Husvéth, F., Hess, M. and Dublecz, K. (2015) **Composition of diet modifies colonization dynamics of *Campylobacter jejuni* in broiler chickens.** J. Appl. Microbiol. 118: (1) pp. 245-254. (IF: 2.479, Applied Microbiology and Biotechnology: Q2)

Awad, W.A., Smorodchenko, A., Hess, C., Aschenbach, J.R., **Molnár, A.,** Dublecz, K., Khayal, B., Pohl, E.E., and Hess, M. (2015) **Increased intracellular calcium level and impaired nutrient absorption are important pathogenicity traits in the chicken intestinal epithelium during *Campylobacter jejuni* colonization.** Appl. Microbiol. Biotechnol. 99:6431-6441. (IF: 3.337, Applied Microbiology and Biotechnology: Q1)

Awad, W. A., **Molnár, A.,** Aschenbach, J. R., Ghareeb, K., Khayal, B., Hess, C., Liebhart, D., Dublecz, K., Hess, M. (2015) ***Campylobacter* infection in chickens modulates the intestinal epithelial barrier function.** Innate Immun. 21:151-161. (IF: 3.271, Microbiology: Q2)

Oral presentations, published in proceedings:

Molnár, A., Dublecz, F., Pál, L., Wágner, L., Hess, C., Hess, M., Husvéth, F. and Dublecz, K. **The effects of various soluble non-digestible carbohydrates on gut health in broiler chickens.** Fiatal Biotechnológusok Országos Konferenciája "FIBOK 2018". Eötvös Loránd Tudományegyetem, Budapest, 2018.03.28-29.

Molnár, A., Dublecz, F., Pál, L., Wágner, L., Husvéth, F., Hess, C., Hess, M. and Dublecz, K. **Nem-emészthető szénhidrátok etetése brojlercsirkékben: a bél fiziko-kémiai, szövettani és**

egyes mikrobiológiai paramétereinek változása. XXIII. Ifjúsági Tudományos Fórum, Keszthely, 2017.05.26.

Dublecz, F., Pál, L., Wágner, L., Husvéth, F., Dublecz, K. and **Molnár, A. The effects of feeding maize or wheat based diets with or without xylanase supplementation on the gut morphology and microflora of broiler chick**s. Proceedings of the XXth European Symposium on Poultry Nutrition (ESPN). August 24-27, 2015. Prague, Czech Republic. P-141.

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Molnár, A., Hess, C., Pál, L., Wágner, L., Awad, W. A., Husvéth, F., Hess, M. and Dublecz, K. **Búza és kukorica alapú tápok hatása a *Campylobacter jejuni* kolonizációjára brojlercsirkékben.** MTA Akadémiai Beszámolók, Budapest, Hungary, 2013.

Molnár, A., *A Campylobacter* visszaszorításának lehetőségei brojlerállományokban. LIII. Georgikon Napok, Keszthely, Hungary, 2011.

8.2. Publications not related to the topic of the present dissertation

Full text papers in peer-reviewed journals:

B. U. Metzler-Zebeli, E. Magowan, M. Hollmann, M. E. E. Ball, **A. Molnár**, K. Witter, R. Ertl, R. J. Hawken, P. G. Lawlor, N.E. O'Connell, J. Aschenbach, and Q. Zebeli. **Differences in intestinal size, structure and function contributing to feed efficiency in broiler chickens reared at geographically distant locations.** Poult. Sci. (accepted 2017 Oct, last known IF: 1,908, Animal Science and Zoology: D1)

Kulcsár, A., Mátis, G., **Molnár, A.,** Petrilla, J., Wágner, L. Fébel, H., Husvéth, F., Dublecz, K. and Neogrády, Zs. (2017) **Nutritional modulation of intestinal drug-metabolizing cytochrome P450 by butyrate of different origin in chicken.** Res. Vet. Sci. 113: 25-32. (last known IF: 1.504, Veterinary: Q1)

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Metzler-Zebeli, B.U., Magowan, E., Hollmann, M., Ball, M.E.E., **Molnár, A.,** Lawlor, P.G., Hawken, R.J., O'Connell, N.E. and Zebeli., Q. (2017) **Assessing serum metabolite profiles as predictors for feed efficiency in broiler chickens reared at geographically distant locations.** Br. Poult. Sci. doi: 10.1080/00071668.2017.1362688 (IF: 0.884, Animal Science and Zoology: Q2)

Metzler-Zebeli, B.*, **Molnár, A.*,** Hollmann, M., Magowan, E., Hawken, R., Lawlor, P., and Zebeli., Q. (2016) **Comparison of growth performance and excreta composition in broiler chickens when ranked according to various feed efficiency metrics.** J. Anim. Sci. 94:2890-2899. (IF: 2.1, Animal Science and Zoology: D1)
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Oral presentations, published in proceedings:

Molnár, A., Hollmann, M., Zebeli. Q., Magowan, E., Hawken, R., Lawlor, P. and Metzler-Zebeli, B. (2015) Comparison of different feed efficiency traits in broiler chickens. Proceedings of the Society of Nutrition Physiology. March 10-12, 2015. Göttingen, Germany.

9. ACKNOWLEDGEMENT

The present thesis is based upon work conducted in CEPO and CEPI projects which were supported by the European Union in the frame of the Cross-border Cooperation Programme Austria-Hungary 2007-2013 and the INTERREG V-A Austria-Hungary Cooperation Programme 2014-2020.

I would like to express my gratitude to my consultant Professor Károly Dublecz who has been giving me the possibility, trust and support to this work. Also, Professor Michael Hess and Claudia Hess kindly helped me at my first steps as a researcher. I have learned a lot from them. I would especially like to thank Professor Ferenc Husvéth for his generous and devoted help in supervising manuscripts and conference materials. My personal gratitude goes to my friend Gábor Mátis who encouraged me to pursue my scientific aims and shared moral support.

My thanks and appreciation to the staff of CEPO and CEPI project: Angéla Vörös-Mezei, László Pál, Fanni Dublecz, László Wágner and all the other participants from the group of Animal Physiology and Nutrition (Georgikon Faculty, Keszthely) and from the Poultry Clinic (VetMedUni, Vienna). Special thanks is addressed to Anna Kulcsár (Physiology and Biochemistry Department, University of Veterinary Medicine, Budapest) for her help in the mucus investigation.

I'm indebted to my family whose value to me grows with the age. My personal belief is that the present work is a continuation of my mother's scientific carrier which she had to give up for her children.

Last but not least, I'm thankful for God because "The Lord is my Shepherd, I shall not want. He lets me lie down in green pastures; He leads me beside the still and quiet waters. He refreshes and restores my soul; He leads me in the paths of righteousness for His name's sake." Psalm 23.1-3.