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Effects on Productive performance, Organs weights and Caecal microbiota

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Abstract

In this experimental study broiler birds were fed in a randomized block design for 32 days on a control diet (C) based on wheat and soy bean meal and two experimental diets containing 2% (C2) and 4% ciona meal (C4) to examine the suitability of *Ciona intestinalis* in poultry feed as a source of fibre and its effects on growth performance, microbial composition and digestive traits. Luminal content was taken from caeca at day 12 and 32 and analysed for microbiota profile and short chain fatty acids (SCFA). Diversity and abundance of microbial population was determined by sequencing of amplicons generated from 16S rRNA (bacteria) genes. Effect of inclusion of ciona meal on growth performance and digestive traits were analysed by weighing the birds and visceral organs at different ages and comparing it with control diet. In addition, the analysis of different feeds and sticky dropping tests were also performed.

The results indicate that there was no significant effect ($P \geq 0.05$) of any dietary treatments on individual weight gain and feed intake while feed conversion ratio was higher for C4 diet than C2 diet. No difference in occurrence of sticky droppings were observed and all modules had good condition during assessment of litter scoring. Birds fed with C4 showed lower dry matter of excreta. No effect of any treatment on relative weight of different organs were observed except for liver and spleen, where an interaction between treatment and day was observed. There was no significant effect of treatments on lactic acid and SCFA concentrations.

The effect of diet on similarities and dissimilarities of microbial profile was tested with principal coordinate analysis (PCoA) and showed no clear pattern for any treatment. There was effect of age but no effect ($P \geq 0.05$) of any dietary treatment on caecal microbial composition except Lachnoclostridium, where effect of treatment was observed and an interaction between age and treatment for unclassified Lachnospiraceae1.

Generally, all birds stayed healthy but a decreased dry matter (DM) of excreta in C4 diets were observed. In conclusion, inclusion of *C. intestinalis* in poultry diets showed no clear effects as a source of fibre.

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List of abbreviations

C	Control
C2	Ciona 2%
C4	Ciona 4%
SCFA	Short chain fatty acid
GIT	Gastrointestinal tract
NSP	Non-starch polysaccharides
D	Days
NaCl	Sodium chloride
Na	Sodium
PCoA	Principal coordinate analysis
ME	Metabolizable energy
FCR	Feed conversion ratio
OTU	Operational taxonomic units
QIIME	Quantitative Insights into Microbial Ecology
Spp.	Species
LSM	Least square means
SEM	Standard error of means
DM	Dry matter
DNA	Deoxyribonucleic acid
PCR	Polymerase chain reaction

1. Introduction

Broiler production has undergone an enormous expansion and development in the last 50 years (Hetland et al., 2004). For the 1950's broiler chickens, it took approximately 100 days to reach a target weight of 1.8 kg, some 50 years later, the equivalent time needed was reduced to 32 days (Havenstein et al., 2003). The selection of broilers for increased growth rate may have unpremeditatedly resulted in changes in gastro-intestinal development during growth of the animal (Lan et al., 2005).

Gut microflora has significant effects on host nutrition, health, and growth performance (Barrow, 1992) by interacting with nutrient utilization and the development of gut system of the host. Compromised gut health results in poor immunocompetence and increased susceptibility to pathogens (Tottori et al., 1997). Globally, the main disease problems in broilers at early days of age are related to vertically transmitted infections such as salmonella, E. coli (Hafez, 2011) and enteric diseases that have pathognomonic lesions like coccidiosis and necrotic enteritis (Dekich, 1998). Optimization of performance by improving gut health has encouraged nutritionists to explore alternative feed management strategies to improve the health and digestive efficiency of broilers either by changing diet composition or using various dietary interventions such as supplementation of probiotics, prebiotics, organic acids and dietary fibre (De Lange et al., 2010). It is believed that these types of dietary interventions have the potential to improve gut health (Lindberg, 2014).

Dietary fibre in the poultry feed has mostly been considered as a diluent (Hetland et al., 2004) due to low digestibility. However, it has an important role in poultry diets. A minimum level has to be included to maintain normal physiological function in the digestive tract (Wenk, 2001). The physical structure of feed influences morphology and physiology of the gastrointestinal tract (GIT). In poultry fine particles can negatively affect the development of the foregut (gizzard) but decreased particle size enhances the access of digestive enzymes to substrates because of the increased surface area of feed particles. Depending upon the solubility of the fibre and its physicochemical properties, dietary fibre affects broilers in different ways i.e. organ size, feed intake, nutrient utilisation, gut health and growth performance (Sklan et al., 2003; Hetland et al., 2003; Amerah et al., 2009). In chickens, the caeca appear to be the main site for microbial degradation of dietary fibre and for absorption of the fermentation products (Thomas and Skadhauge, 1988). However, the dietary fibre can be broken down by the microbial flora permanently colonizing the gastrointestinal tract, resulting in various gases (H_2, CO_2, CH_4), lactic acid and short chain fatty acids (SCFA) (Bach Knudsen, 2001). The SCFA produced are rapidly absorbed from the gut lumen (Rechkemmer and Rönnau, 1988) and provide a significant amount up to 3-5% of metabolizable energy (ME) (Choct et al., 1992; Jørgensen et al., 1996; Jamroz et al., 2002).

Sea squirt (*Ciona intestinalis; C. intestinalis*) can be cultivated in the sea. Today, the harvested biomass in Sweden is mainly used for biogas production by the company Marine Biogas, however different areas of use are also explored (Marine Biogas, 2019). The thick mass of ciona consists of a protein-rich inner body and a fibre optic outer shell. The fibre in the outer shell consists largely of cellulose like substance (Larousse, 1967), making it interesting as a fibre source in poultry production, while the inner body could be interesting as a protein source.

1.1 Aim

The main objective of the present experiment was to test and evaluate the suitability of sea squirts (*C. intestinalis*) as a source of fibre in poultry feed by assessing changes in caecal bacteria composition, organ development and growth performance of broilers.

1.2 Hypothesis

Inclusion of sea squirts in the diet would improve gut health by stimulating beneficial caecal microbiota populations and SCFA production in broilers resulting in improved growth performance.

2. Literature review
2.1 *Ciona intestinalis* (Vase tunicate)

C. intestinalis is a solitary ascidian (Table 1) having a soft "tunic" that covers the body. Blood vessels, nerves and cells are scattered in this tunic (Larousse, 1967). It is whitish to almost transparent in colour and the organs under the tunic can be seen. *C. intestinalis* is sessile, filter feeding, non-colonial vase or tube-shaped invertebrate which grows about 120 mm high. A branching vascular system within the entire tunic, makes up about 60% of the animal's weight (Grzimek, 1972). It has two siphons where inhalant siphon is surrounded by eight lobes, while exhalant siphon by six lobes. Through inhalant siphon it pumps water in and filters out oxygen. *C. intestinalis* feeds on suspended organic materials and small organisms such as phytoplankton, zooplankton, oyster and mussel larvae and then pumps the water as waste out through the exhalent siphon (Larousse, 1967).

2.1.1 Geographical Distribution and habitat

There are about 1850 species of sea squirts of which 20 are more common to find on the Swedish west coast including *C. intestinalis*. Its native range is unknown, but it is well distributed throughout the world, including many European oceans (Ricketts et al., 1985).

It is commonly found in tidal waters and silty conditions in 0-500 meters or above 1000 feet depth but can also be found under cover near rocky shores, pilings, boat hulls etc.

Table 1. Scientific classification of *C. intestinalis*

Kingdom	Animalia
Phylum	Chordata
Subphylum	Tunicata
Class	Ascidiacea
Order	Enterogona
Suborder	Phlebobranchia
Family	Cionidae
Genus	Ciona
Species	*C. intestinalis*

(Linnaeus, 1767)

2.1.2 Reproduction

C. intestinalis is hermaphroditic like all other solitary or colonial sessile ascidians which possess all characters of vertebrates but does not self-fertilize because male and female parts of every individual does not mature at the same time. *C. intestinalis* has high rate of reproduction (>10,000 eggs per individual). It spawned eggs and sperm through exhalent siphon into the water. After 25 hours of fertilization a tadpole like larvae is formed that can survive for only a few days depending upon the temperature of surrounding and unless a suitable substrate is found to attach to. It then metamorphoses into an adult (Coleman, 1991). Self/non-self-recognition molecules are considered to play a key role in the process of interaction between sperm and the vitelline coat of the egg. It appears like self-incompatibility systems in flowering plants (Sawada et al., 2014). In Swedish waters the ciona reproduces twice a year in the surface layer (0-20 m) but only once per year in deeper and colder water.

2.1.3 The concept of ciona refinement

The concept of culturing and harvesting of ascidians from the sea is that it gives several environmental and economic benefits. *C. intestinalis* is easily broken down to a renewable

energy source in the form of biogas and the rest product, which is a rich source of nitrogen (5% of the dry weight) and phosphorous is a valuable fertilizer for agriculture. Due to high production it provides a cost-effective removal of nutrients as compared to conventional removal of nitrogen and phosphorous from marine through sludge processes. Moreover, their production needs no extra fertilizers or pesticides. Industrial processing of ascidians is easy because it consists only of soft tissues. The culture of *C. intestinalis* (20*200m) was estimated to hold 475 tons wet weight and this growth is 4-8 % per day (Marine Biogas, 2019). These qualities appear to make it a suitable source of fibre in the poultry diet industries.

2.2 Dietary fibres

Dietary fibre is a complex mixture of carbohydrate polymers that are associated with several non-carbohydrate components in a plant and constitutes a significant part of all plant feed stuff. Historically, the definition of dietary fibre and reasons for its determination and classification has been a matter of continuous debate both in research and practical application.

Dietary fibre has long history, its term originated from Hipsley (1953) who described it as a non-digestible constituent making up the plant cell wall. Latterly, that definition has seen several revisions, but a general agreement of the definition was stated by Codex Alimentarius Commission (2009). CODEX defines dietary fibre as carbohydrate polymers with ten or more monomeric units, which are resistant to hydrolysis by endogenous enzymes of human's small intestine. They have been derived from food raw material by physical, enzymatic or chemical tools and belong to the one of following categories: edible natural carbohydrate polymers, synthetic carbohydrate polymers or carbohydrate polymers (De Vries, 2011).

Now a days most researchers are using either physiological or chemical definitions. From physiological aspect, it is defined as the dietary components resistant to degradation by mammalian enzymes (Bach Knudsen, 2001). Chemically dietary fibre is defined as the sum of non-starch polysaccharides and lignin (Theander et al., 1994).

2.2.1 Classification systems of dietary fibres

There are several different classification systems for components of dietary fibre depending upon their role in the plant, type of polysaccharide, gastrointestinal solubility, site and product of digestion and physiological classification (Tungland and Meyer, 2002). There are two main classes of polysaccharides, starch and non-starch polysaccharides (NSP). Classification of polysaccharides based on differences in solubility places them into the following categories: cellulose, hemicellulose, pectin and lignin (Albersheim et al., 1984).

According to the water solubility, dietary fibres are divided into two main physiochemical groups (Table 2): the insoluble and soluble fibre (Bach Knudsen, 2001). Plants generally contain a mixture of both soluble and insoluble fibre, but amount and ratio of soluble and insoluble fibre depends upon species and maturity of the plant (Montagne et al., 2003). Amount, composition and physiological activity of dietary fibre depends upon various factors like production system, botanical belonging of plants, tissue type and maturity, harvest time and type of processing.

2.2.2 Cellulose

Cellulose is a carbohydrate, mainly consisting of glucose units linked together by β1-4 bonds (Rogers et al., 1980) and is particularly resistant to hydrolytic cleavage. Cellulose is one of the major polysaccharides in plant cell wall and has been considered an important part of diets because of its physiological and nutritional functions. Takehisa et al. (1979) reported that the passage time of digesta was shortened and faecal excretion increased in rats when 30%

cellulose content is added and Nahm and Carlson (1987) observed the same findings for broilers. The direct microscopic counts of caecal flora was increased in turkeys by dietary cellulose (Bedbury and Duke, 1983). Inclusion of 1.5-3.5% dietary cellulose enhanced growth and metabolizable energy retention of 7-15 day old chicks (Cao et al., 1998a; b; Cao, 2001). Some reports indicated that ratio of mucosal protein to DNA was increased by addition of dietary cellulose compared to fibre free diets, altering digestive physiology due to increase cell size and mucus production, suggesting that structural changes may be associated with functional changes (Farness and Schneeman, 1982).

Table 2. Classification of dietary fibre components based on water solubility/fermentability. Modified from the information of Dhingra et al. (2012)

Class	Component of dietary Fibre	Description
Water insoluble/less-fermented	Cellulose	Major structural component of cell wall, soluble in concentrated acid, insoluble in concentrated alkali
	Hemicellulose	Cell wall polysaccharide, soluble in alkali
	Lignin	Non-carbohydrate cell wall component, resistant to microbial degradation
Water soluble/well-fermented	Pectin	Component of cell wall, Gel forming with major component D-galacturonic acid
	Gum	Secreted at the site of plant injury
	Mucilage	Prevent desiccation of seed endosperm

2.2.3 Importance of dietary fibre

The fibre fraction with low digestibility was long considered of diluting or even anti-nutritive nature in poultry diets, as reviewed by (Mateos et al., 2012). Impact of dietary fibre is determined by the fibre physio-chemical properties such as cation exchange capacity, hydration properties, viscosity and organic compounds absorptive properties (Bach Knudsen, 2001). These properties are linked to the type of polymers that build up the cell wall and their intermolecular association (McDougall et al., 1996) and may differ between fibre sources.

Some experiments have shown that if insoluble fibre is included in poultry diets at moderate concentrations (2-3%), performance of birds will not be impaired until the nutrient concentration of the diet is reduced (Hetland and Svihus, 2001; Hetland et al., 2002). However, fibre must be included in the diet to maintain normal physiological functions in the digestive

tract (Wenk, 2001). Some of the nutritional and biological effects of dietary fibre are given below.

2.2.3.1 Effect on feed digestion

Solubility has deep effects on fibre functions. The soluble fibres can produce high viscosity of digesta in the small intestine, which lowers the passage rate and inhibit feed digestion and absorption (Smits and Annison, 1996). Furthermore, high viscosity decreases feed intake and passage rate, which creates a favourable environment for the proliferation of microbes in the intestine (Langhout, 1998), increasing the risk of diseases such as necrotic enteritis (Annett et al., 2002).

Insoluble fibre fraction in the monogastric diet has been considered as a diluent due to its little or no degradation by bacterial microflora in the chicken which makes its influence on quantity and composition of microflora relatively insignificant (Choct et al., 1996; Langhout, 1998). Diets high in insoluble fibre contain low energy values, to compensate this reduced nutrient concentration, birds tend to increase feed consumption (Rogel et al., 1987b; a).

2.2.3.2 Effect on organs development and gizzard activity

Birds show quick response to dietary fibre content by modifying intestinal length and weight. Increasing the insoluble fibre content of the diet has resulted in a decreased proventricular weight (Jiménez-Moreno et al., 2009), reduced length of the small intestine (Sklan et al., 2003; Amerah et al., 2009) and an increased gizzard organ weight and weight of gizzard contents (González-Alvarado et al., 2007, 2008; Amerah et al., 2009; Svihus, 2011). A more muscular and enlarged gizzard can improve digestion, as the feed is retained for a longer time in the upper digestive tract. Some fibre sources may cause pancreatic enlargement, leading to an increase in pancreatic secretions (Kratzer et al., 1967). It has been suggested that a large, well developed gizzard improves GIT motility, favours gastroduodenal refluxes, and increases cholecystokinin release, which in turn may stimulate the secretion of pancreatic enzymes (Duke, 1992; Svihus et al., 2004). In fact, the inclusion of dietary fibre consistently reduces pH in the gizzard, which benefits pepsin activation and solubility of the mineral sources (van der Aar et al., 1983; Guinotte et al., 1995).

Stimulation of muscular activity of gizzard by insoluble fibre is closely related to its physical structure. In broilers replacement of 50% ground wheat with ground oats increased gizzard weight to a similar extent as replacing 50% ground wheat with whole wheat, which indicates that a small amount of insoluble fibre (e.g., 10% of oat hulls of 50% oat) stimulate the gizzard at same extent as 50% whole wheat (Hetland et al., 2002). Rezaei et al. (2018) also found that the use of micronized wheat fibre (5g/kg feed) to quails significantly improves growth rate and feed efficiency while feed intake was not affected by the levels of fibre. As micronized wheat fibre content increased, the relative weight of gizzard and gastrointestinal tract significantly increased due to enhanced functioning, whereas liver relative weight significantly decreased because fibre may reduce liver lipid deposition and plasma lipid content in chickens fed ad libitum (Akiba and Matsumoto, 1982).

2.3 Caecal bacterial profile of chickens

The GIT of chickens contains a very complex microbiota, with over 600 different bacterial species from more than 100 bacterial genera (Torok et al., 2011). The bacterial communities originating from different sections of the chicken GIT are so different that it has been suggested that they should be considered as separate ecosystems (Wielen et al., 2002). The microbial

profiles of different GIT sections differ significantly between studies due to differences in bird genetics, age, sex, diet, health status, housing and analysis technique-imposed differences such as primers used, method sensitivity, DNA extraction protocol etc.

The caecum is a key region for bacterial fermentation of dietary fibres and main site for colonization by pathogens. The caeca in chickens carries a diverse and dynamic microflora and is by far the most densely colonized microbial habitat and its bacterial diversity is much higher than in the upper GIT. Chickens have two paired ceca, both harbouring similar bacterial communities (Stanley et al., 2015). It is estimated that one gram of caecal content contains 10^{11} bacteria (Mead, 1989).

Caecal microflora characterization started in the 1970s (Barnes, 1979) and since then, better analysis methods, including molecular techniques, have been used to address caecal microbial ecology (Yeoman et al., 2012). The cecum is mainly occupied by Clostridia followed by genera Lactobacillus and Ruminococcus (Gong et al., 2007). Most Clostridia detected in the caecum fall primarily into three main families, Clostridiaceae, Lachnospiraceae and Ruminococcaceae (Danzeisen et al., 2011). Enterococcaceae, Enterobacteriaceae and Bacteroidaceae are other reported abundant families in the caecal microbiota (Yin et al., 2010). Predominating species of bacteria at early days of healthy chickens are *Enterococcus spp., Lactobacillus spp.* and *Enterobacteriacae*, while *Eubacterium spp.* and *Bacteriodes spp.* establish themselves after two weeks of life. Obligate anaerobic microflora dominate from 7 days of age onwards (Barnes, 1972; Salanitro et al., 1974; Mead, 1997). At the species level, Stanley et al. (2015) found *Bacteroides fragilis, Lactobacillus crispatus (L; Lactobacillus), L. johnsonii, L. salivarius and L. reuteri* comprise more than 40% of the caecal microbiota within each sample using pyro-sequencing (DNA sequencing, determining the order of nucleotides in DNA) to determine and compare the phylogenetic profile. The cecum is also rich in unknown and unclassified bacterial residents (Stanley et al., 2013).

2.3.1 Analysis of microbial composition

In the past, standard culture methods were used to characterize microbial ecology by using commercial growth medias (Kirk et al., 2004). Major limitation of using culture technique is that all the microorganisms are not culturable (Hugenholtz, 2002). Culturing methods are time consuming, labour intensive and also need previous knowledge about the nutritional and growth requirements of bacteria's (Amann et al., 1995; Zoetendal et al., 2004). A wide variety of molecular techniques for describing and characterizing the phylogenetic and functional diversity of microorganisms have been developed. 16SrRNA sequencing methods are increasingly being applied to the characterization of microbial communities based on amplification of this gene and have led to a better appreciation of extant biodiversity (Sogin et al., 2006). However, the 16SrRNA -based techniques are known to have some limitations for the short-read lengths, sequencing errors, differences arising from the different regions chosen, and difficulties in assessing operational taxonomic units (Quince et al., 2009, 2011; Youssef et al., 2009; Huse et al., 2010). Use of the 16S rRNA gene to characterize caecal microbiota addresses many genus and species, but still does little to reveal comprehensive details of all caecal microbiota species.

2.4 Short chain fatty acids in the caeca

The caeca are paired blind pouches which together with the colon and rectum form the hind gut. The caeca account for only 1% of the chicken body weight (Redig, 1989). Caecectomy has not been shown to have any considerable effect on nutrient utilisation (Chaplin, 1989; Son et

al., 2000) but research has speculated that the caeca have several functions like water absorption, microbial degradation of some soluble carbohydrates, microbial synthesis of vitamins, degradation of nitrogenous products by majority of caecal organisms, as well as cholesterol digestion and absorption (Coates et al., 1968; McNab, 1973; JøRgensen et al., 1996; Jamroz et al., 2002).

Nutrients, such as undigested starch and protein, as well as fibre that bypass the small intestine will reach the caeca. Once digestive tract contents enter the caeca, they may be fermented by caecal microbiota to produce SCFA. Concentration of these SCFA varies from very low levels at early age (<15µmol g^{-1} at day one) to high levels at day 15 (acetate, 70µmol g^{-1}; propionate, 8µmol g^{-1}; butyrate, 24µmol g^{-1}) after which they then stabilize (van der Wielen et al., 2000).

The SCFA produced from fermentation includes acetate, butyrate, propionate, valerate, and isovalerate (Jamroz et al., 1998). The amount of energy accounted by the caeca is still uncertain, particularly for modern high-yielding poultry, and the effect of dietary composition also remains unclear, but some previous studies have reported that the caeca accounts for about 4% metabolizable energy, derived from SCFA (Sugahara et al., 2004). Subsequent work on caecal fermentation has estimated that the amount of energy from SCFA contributed to around 3-5% of the total energy needs of the chicken (Choct et al., 1992; Jørgensen et al., 1996; Jamroz et al., 2002).

2.5 The role of dietary fibre in gut health

In general, the inclusion of fibre in the diet has been shown to increase intestine functioning by influencing the nutrients absorption through its effect on luminal viscosity. It also modifies the composition and quantity of the gut microflora in the GIT of broilers and all types of poultry species (McHan and Shotts, 1993; Dunkley et al., 2007; Kalmendal et al., 2011).

Dietary fibre has a protective effect against a range of problems. Dietary fibre might reduce the incidence of ulcers by physically protecting the mucosa of GIT and might act as a barrier against infection by suppressing the growth of harmful microbial species such as *C. perfringes* (Kalmendal et al., 2011). Dietary fibre that leads to more acid mucin appears to favour the elimination of pathogens (Montagne et al., 2003) by increasing the potential of mucus to resist the attack of bacterial enzymes (Rhodes, 1989). In addition, Kalmendal et al, (2011) reported that the high levels of insoluble fibre in the diet was associated with significant decreases in colony counts of *Clostridium spp*. (cause of necrotic enteritis). Short-chain fatty acids resulting from fermentation processes have a bacteriostatic effect by decreasing the pH on some enteric bacteria, including *Salmonella typhimurium*, and seem not to affect beneficial bacteria, such *Lactobacillus spp*. (van der Wielen et al., 2000; Nisbet, 2002). Rezaei et al. (2018) found that the colony forming unit (CFU/g) of *Streptococci spp*. (a common cause of opportunistic infections) in ileal digesta was decreased with increasing micronized wheat fibre inclusion levels in the diet and should be considered in relation to its prebiotic properties.

3. Materials and methods

The experiment (diary number: C16/16) was approved by the ethical committee of the Uppsala region, Sweden.

3.1 Birds and facilities

The experiment was done at The Swedish Livestock Research Centre (Uppsala, Sweden) from November to December 2018. A total of 219 unsexed, day old broiler chickens (Ross 308) having initial body weight of 38.9±3.1gram/bird (mean±sd) were purchased from a commercial

broiler hatchery (Väderstad, Sweden). They were housed in an environmentally controlled room, and randomly placed in groups of nine in 24 modules (1.50×0.75 m). Modules were provided with solid floor and wood shavings, equipped with three nipple drinkers and an open trough feeder. Room temperature was decreased according to Ross manual from 33°C on day 0 to 23°C on day 24 and kept at 23°C until the chickens were euthanized at the age of 32 days. Chickens had continuous artificial light for the first 2 days(d); thereafter, the dark period was gradually increased to 6h at day 8 and remained so until the age of slaughter. Sex ratio was calculated as: Number of roosters/total number of birds in pen. The birds were managed according to regular routines with supervision twice a day. All possible off-sets were recorded in a log book. Group weighing of chickens occurred on arrival and thereafter weekly.

Table 3. Feed ingredients (%) in control and experimental diets, respectively

Ingredients	C	C2	C4
Wheat	61.2	61.0	60.8
Soybean protein	19.8	18.3	16.9
Wheat middling	10	10	10
Rapeseed oil	3.7	3.7	3.7
Limestone	2.4	2.2	2.1
Monocalcium phosphate	1.3	1.3	1.3
Ciona meal	0	2.0	4.0
Potato protein	0.4	0.4	0.4
Sodium chloride	0.3	0.1	0
Lysine	0.3	0.3	0.3
Methionine	0.3	0.3	0.3
Threonine	0.1	0.1	0.1
Sodium bicarbonate	0.1	0.1	0.1
Premix	0.2	0.2	0.2
Total	100	100	100

C=Control; C2=Ciona2%; C4=Ciona4%

3.2 Diets and feeding

The experiment was performed as a randomized block design with three feed treatments (Table 3) and eight replicates per treatment. The control feed was based on wheat and soy bean meal optimized according to the requirements of the chicken (NRC, 1994). In the experimental feeds, 2 and 4%, respectively, of *C. intestinalis* were included. The *C. intestinalis* was grown and harvested at the Swedish west coast and were boiled in water for at least 4 minutes before it was pressed/dewatered and dried in 70°C over-night. The dried ciona was milled with a hammer-mill before mixing with the other feed ingredients. The composition of ciona meal (Table 4) was analysed before the start of the trial (Eurofins, Sweden). All feeds were pelleted, and the experimental feeds were optimized to have the same nutritional content as the control feed. The same feed was used throughout the experiment and birds had free access to feed and water throughout the trial. The amount of consumed feed was calculated at each feed replenishment by weighing the residual feed.

3.3 Analytical evaluation of feeds

The chemical composition of all three diets were calculated prior to the start of feeding trial. After the trial the feeds were analysed (Table 5) for crude protein by the Kjeldahl method according to Nordic committee on feed analysis (2003), ether-extract according to Official Journal of European Communities (1994), non-starch polysaccharide analysis was done according to the Uppsala method (Theander et al., 1995). Dry matter (DM) of feed, excreta and ash content were analysed according to standard methods (AOAC, 1995).

Table 4. Analysed composition of *C. intestinalis*

Parameters		Amount (g/kg sample)
Crude Protein		421
Crude Fat		30.6
Sodium		38
Dietary fibre		200
	insoluble part	175
Fucose		2.5
	insoluble part	2
Arabinose		2.1
	insoluble part	1.9
Xylose		1.6
	insoluble part	1.3
Mannose		5.5
	insoluble part	3.5
Galactose		29.8
	insoluble part	15.5
Glucose		153
	insoluble part	145
Uronic acid		2.9
	insoluble part	1.9
Lignin		3.1

3.4 Sticky dropping, dry matter determination of excreta and litter scoring

Sticky droppings were recorded (scale 0–1; where 0 is no occurrence and 1 is occurrence) on day 7 and 13. The animal caretaker was instructed to not remove sticky droppings from the chickens. Excreta samples were collected for DM analysis at day 31 by covering the litter area with plastic foil for 2 hours and a minimum of 25g of excreta was collected per module. The bedding was assessed during the last week of the trial by dividing each module in four sections and applying a scoring with points between 0-4, where 0 represents completely dry and flaky; 1 is dry but not easy to move with boot; 2 leaves imprint of foot and can be shaped in a ball that easily falls apart; 3 Sticks to boots and can be formed in a firm ball; 4 is wet and sticky under hard crust (Butterworth et al., 2009).

3.5 Sampling of caecal content and mucosa

At day 12 and 32 of age, one chick per cage was randomly selected, weighed individually and killed by decapitation (d12) and by intravenous injection with sodium pentobarbital (d32). Aseptically, the ventral side of each bird was opened and whole digestive tract was taken out. The caeca were cut open with sterile scissors and tweezer was used to squeeze caecal content and the mucosal layer into separate sterile tubes, both for microbiota analysis and for SCFA.

The amount of digesta was very limited at day 12, so samples were only used for microbiota analysis. On day 32, 200-400mg of caecal content were taken . Samples taken for microbiota analysis were stored at −80°C until DNA extraction. Similarly, samples for SCFA were immediately stored in freezer at −20°C until analysis.

Table 5. Analysed chemical composition of feeds g/kg

Parameters	C	C2	C4
Dry matter	916	918	915
Ash	69.0	58.0	57.0
Crude protein	206	208	206
Crude fat	62.0	58.0	57.0
ME[1]	11.8	12.1	11.8
Sodium[2]	1.7	1.7	1.9
Dietary fibre	134	142	140
insoluble part	120	124	127
Fucose	0.7	0.5	0.6
insoluble part	0.7	0.5	0.6
Arabinose	25.9	27.5	25.2
insoluble part	22.6	22.1	22.1
Xylose	37.6	37.3	37.6
insoluble part	33	31.9	33.2
Mannose	6.5	6.7	5.9
insoluble part	5.9	5.9	5.4
Galactose	10.8	12.9	11.5
insoluble part	9	10.7	9.2
Glucose	36.7	39.9	41.6
insoluble part	34	36	39.7
Uronic acid	8.6	8.6	8.5
insoluble part	7.4	7.3	7.3
Lignin	6.6	9	8.5
Lysine	11.7	11.9	11.7
Methionine	5.7	5.3	5.3
Cysteine + Cystine	3.3	3.3	3.2

[1]*ME= Metabolizable energy MJ/KG (Calculated values) = 0.1551 x % CP + 0.3431 x % crude fat + 0.1669 x % starch + 0.1301 x % total sugar expressed as sucrose.* [2]*calculated values of sodium*

3.6 Growth performance

Feed consumption and body weight of chicks were recorded group wise and average feed intake, body weight gain and feed conversion ratio per chicken were calculated from these data by period (6, 12, 19, 27 and 32 days) and cumulatively. Birds that died during the experiment were weighed, and the data were used in the calculations of FCR.

3.7 Organs measurements

The proventriculus, gizzard, bursa of fabricious, spleen, heart and liver were excised, dried and weighed. Also, the digesta content of the gizzard was calculated as the difference between the weight of the full and the empty organ. Weight and length of complete intestinal tract were also recorded.

3.8 DNA extraction and PCR

DNA was extracted from each microbiota sample by using EZ1 Advanced XL Robot (NEBNextUltraDNA Library Prep Kit for Illumina NEB, USA). The bead beating tubes were prepared with 0.2g (200 mg) of silica bead beats (0.1mm). After thawing of samples for 30 minutes, 400μL of ASL buffer was added into the tube with sample. After homogenising, 120μL of sample was pipetted into the bead beating tube and 980μL of ASL buffer was added to make 1.1 ml of total volume. The samples were vortexed briefly and incubated in a heating block at 95°C for 5 minutes and instantly put on ice for 10 minutes. The bead beater (Techtum Lab AB, Uppsala, Sweden) was run for 3 minutes at 8000rpm (soft programme). Thereafter samples were centrifuged at 2500xg for 1 min. A volume of 20μL of proteinase kinase was added in the bottom of the sample tube, and 200μL of supernatant was added for further extraction by robot. The purification procedure (15 minutes) of robot was started to get the DNA. PCR was run for few samples for confirmation of DNA extraction and the conditions for PCR included denaturation at 98°C for 40s, followed by 30 cycles of 98°C for 30s and hybridization 58°C for 40s, elongation 72°C for 60s and final ending with 72°C for 7 min. Amplicons were visualized by electrophoresis.

3.9 Illumina sequencing data analysis

The extracted DNA samples were sent (Novogene lab) for illumina sequencing where all the data were processed by using HiSeq 2500 platform. Splitting of data and reads merging were done by using FLASH software. Data filtration and chimera removal were done in Quantitative Insights into Microbial Ecology (QIIME). The operational taxonomic units (OTUs) clustering and species annotation, including OTUs heatmap, GraPhlAn display, taxonomy tree and KRONA results were compiled by using Uparse, PyNast and Mothur software's. The OTUs identified as chloroplasts and mitochondria were removed from the dataset, since only bacteria were of interest. Mean values of the relative abundance of the refined OTUs were taken and sorted according to largest to smallest. Some OTUs which remained unclassified for further division were named as Lachnospiraceae 1-5, Ruminiclostridium 1-2, and Ruminococcaceae 1-2. The 15 OUTs with highest mean value of relative abundance was selected and statistically analysed individually. In addition, the sum of all remaining OTUs were calculated and named as "others". They were also statistically evaluated, although they were very small individually, but they made a big part of DNA all together.

3.10 SCFA concentration analysis

The concentrations of SCFA (acetic acid, propionic acid, butyric acid) were determined only on day 32 from the content of one cecum per experimental unit (module). The analysis of lactic acid and SCFA was performed according to Andersson and Hedlund (1983) by a high-performance liquid chromatography (HPLC).

3.11 Statistical analyses

All data was analysed as a completely randomized block design by the Proc Mixed in SAS version 9.4. The model included diet as a fixed factor and module as a random factor, the effect of sex ratio was tested in the models. Module served as experimental unit for performance data, sticky droppings, and DM of excreta. For relative organs weights/lengths and OTUs, the individual bird was representative for the module and considered as the experimental unit. Since these parameters were measured at two time points the data was analysed with repeated statement with unstructured co-variance. Results are presented as least square means (LSM) and standard error of means (SEM). P-values ≥0.05 were considered significant. To explore

and visualize the similarities and dissimilarities of microbial profile principal coordinate analysis (PCoA) was done by using Past3 software.

4. Results

4.1 Bird performance

The birds were evaluated for weight gain, accumulated feed intake and feed conversion ratios (Table 5). There was no effect (P > 0.05) of feeds and sex ratio on individual weight gain, feed intake and FCR, except for that FCR at day 32 was higher on Ciona 4% compared to Ciona 2%. On average (mean of day 7 and day 13) 8.7% of the birds had sticky droppings and there were no differences in occurrence between treatments. Moreover, no module scored more than 2 during assessment of litter scoring. Overall, seven birds died during the experiment at different ages with no specification to any treatment.

Table 5. Effect of experimental diets on accumulated body weight, accumulated feed intake and feed conversion ratio. Results are presented as least square means. SEM is the pooled standard error of mean

Parameters	C	C2	C4	SEM	P-value*
Body weight (g/bird)					
day 6	141.1	139.7	138.8	2.96	0.80
day 12	354.4	348.5	352.4	7.47	0.86
day 19	757.5	748.5	769.4	19.44	0.75
day 27	1506.5	1479.9	1485.8	34.51	0.85
day 32	2035.5	2031.0	1974.4	45.00	0.58
Feed intake (g/bird)					
day 6	106.7	105.0	101.4	2.78	0.21
day 12	382.8	373.6	374.4	10.37	0.79
day 19	922.8	905.1	927.6	23.43	0.78
day 27	1946.3	1915.1	1949.3	40.65	0.81
day 32	2740.4	2687.7	2717.4	51.56	0.77
Feed Conversion Ratio					
day 6	1.03	1.03	1.01	0.02	0.54
day 12	1.21	1.21	1.19	0.03	0.86
day 19	1.28	1.27	1.26	0.01	0.65
day 27	1.33	1.33	1.35	0.01	0.06
day 32	1.37[ab]	1.35[a]	1.41[b]	0.01	0.02

Different letters (ab, a, b) within row shows difference at p > 0,05. p-value=only for treatment effect*

4.2 Organs development

There was no significant effect of treatments on relative organs weight (g/kg) and intestinal length (cm/kg) while, day had significant effect on relative weight of all organs except spleen and bursa (Table 6). The relative weight of the heart, liver and the gastrointestinal organs decreased with age. There was both an effect of day and interaction between day and treatment for relative liver weight and spleen, since the day effect was so strong for the liver, the interaction was not clearly shown in the pairwise comparisons. The relative weight of spleen

for birds fed the control diets at day 12 was different from birds fed C2 and C4 diets at age 12 while there was no difference between treatments at age 32.

Table 6: Effect of treatment and age on relative weight (g/kg body weight) of different organs. Results are presented as least square means. SEM is the pooled standard error of mean

Organs	Age 12			Age 32			SEM	P-value		
	C	C2	C4	C	C2	C4		Treatment	Day	Treatment*Day
Heart	7.5	8.1	7.9	7.2	7.4	7.4	0.28	0.337	0.054	0.737
Liver	35.5[a]	39.2[a]	36.5[a]	27.2[b]	25.5[b]	24.9[b]	1.37	0.636	<0.0001	0.028
Spleen	0.7[b]	1.0[a]	1.0[a]	0.9[ab]	0.8[b]	0.8[b]	0.07	0.578	0.301	0.018
Bursa	1.8	2.0	2.1	2.3	2.3	2.3	0.22	0.690	0.074	0.905
SI length (cm/kg body weight)	310.1	310.9	323.4	88.7	83.3	92.0	11.89	0.666	<0.0001	0.918
SI weight	81.4	85.2	85.5	54.5	55.6	54.2	2.39	0.583	<0.0001	0.653
Proventriculus and gizzard	47.9	49.6	53.9	23.4	25.3	27.1	1.98	0.124	<0.0001	0.685
Gizzard	29.4	30.1	33.8	14.0	14.6	15.6	1.64	0.254	<0.0001	0.523
Empty Gizzard	7.5	7.0	7.2	4.6	4.3	4.4	0.39	0.578	<0.0001	0.969

Different letters (ab, a, b) within row shows difference at p > 0,05.

4.3 DM of excreta and SCFA

There was an effect of treatment on the dry matter of excreta. Birds fed diets with 4% inclusion of *C. intestinalis* showed lower excreta dry matter (Table 7). There was no effect of treatments on caecal SCFA concentrations or proportions of SCFA as shown in Table 7.

Table 7: Effect of diets on DM of excreta and organic acids at day 32, mmol/L and mol-%. Results are presented as least square means. SEM is the pooled standard error of mean

	Diets				
	C	C2	C4	Pooled SEM	P-value
DM	18.20[a]	17.93[a]	16.37[b]	0.619	0.031
mmol/L					
Lactic acid	3.05	2.85	2.10	0.780	0.506
∑ SCFA*	100.13	81.22	84.56	11.324	0.266
Acetic acid	74.05	59.55	62.25	8.923	0.269
Propionic acid	2.46	1.86	3.35	0.905	0.295
Iso-Butyric acid	3.34	2.33	2.40	0.566	0.164
n-Butyric acid	17.83	14.80	14.56	2.059	0.471
Iso-valeric acid	0.57	0.78	0.09	0.450	0.550
n-valeric acid	1.10	1.05	1.15	0.249	0.930
mol% SCFA					
Acetic acid	76.99	77.67	76.58	1.966	0.893
Propionic acid	2.61	2.54	3.94	0.864	0.254
i-Butyric acid	3.31	2.35	2.59	0.676	0.359

n-Butyric acid	15.29	15.39	15.43	1.289	0.997	
i-valeric acid	0.77	0.94	0.20	0.549	0.561	
n-valeric acid	1.07	1.15	1.30	0.241	0.659	

*Sum of all SCFA, Different letters (ab, a, b) within row shows difference at p > 0,05

4.4 Caecal bacterial profile

At early age major dominating phylum was firmicutes and genus clostridia. The most clostridia detected in the caecum fall primarily into three main families; Lachnospiraceae, Ruminococcaceae and Lactobacillacea (Figure 1). At the age of 32 days, main phylum were firmicutes and proteobacteria. The main detected families were Ruminococcaceae, Lachnospiraceae, Enterobacteriaceae and Lactobacillaceae (Figure 1.1). Caecum was also rich in many least abundant operational units. There was clear effect of age on microbiota profile. Ruminiclostridium 1, Coprococcus, Lachnospiraceae 5, Butyricicoccus were seen only on early age while Ruminococcaceae 1, Eubacterium, Ruminococcaceae 2, Escherichia-shigella were present only on the later stages of age. There was effect of treatment on Lachnoclostridium, birds fed C2 had higher relative abundance than birds fed the control at day 12. An interaction between age and treatment for Lachnospiraceae 1 (Table 8) was also observed, where birds fed C2 had higher relative abundance than birds fed the control at day 12, and they did also have a higher relative abundance than birds fed C2 at day 32. The PCoA graph showed that there was no clear pattern of any treatment on microbiota for both sampling times.

Table 8: Effect of treatment on microbiota profile (%). Results are presented as least square means. SEM is the pooled standard error of mean

	Age 12			Age 32			Pooled SEM	P-value		
OTU	C	C2	C4	C	C2	C4		Treatment	Age	T*Age
Faecalibacterium	21.30	4.11	26.39	28.16	23.53	31.79	5.84	0.074	0.021	0.347
Lachnospiraceae 1	7.52[b]	16.37[a]	11.73[ab]	10.07[ab]	7.15[b]	13.23[ab]	2.76	0.486	0.355	0.028
Eisenbergiella	5.02	7.34	5.39	4.55	5.29	4.50	1.15	0.333	0.260	0.796
Lactobacillus johnsonii	7.71	5.33	3.46	1.78	4.32	1.92	1.36	0.117	0.038	0.249
Lachnospiraceae 2	3.40	8.01	4.82	3.10	3.54	1.82	0.97	0.067	0.003	0.105
Anaerotruncus	1.84	3.04	3.34	1.18	1.94	1.54	0.55	0.233	0.020	0.611
Ruminococcus torques	2.16	2.38	1.78	1.29	2.20	1.15	0.36	0.156	0.029	0.500
Lachnospiraceae 3	2.73	1.89	1.59	2.20	1.83	1.76	0.49	0.409	0.644	0.633
Lachnospiraceae 4	1.38	1.94	1.89	1.07	1.73	0.90	0.45	0.510	0.097	0.504
Ruminiclostridium 1	1.55	2.16	1.22	.	.	.	0.51	.	.	.
Coprococcus	2.54	1.38	0.32	.	.	.	0.62	.	.	.
Lachnospiraceae 5	0.67	2.08	1.19	.	.	.	0.37	.	.	.
Ruminiclostridium 2	1.02	1.09	1.54	0.82	1.15	0.95	0.23	0.499	0.133	0.244
Lachnoclostridium	0.93[a]	1.40[b]	1.13[ab]	0.83[a]	1.24[ab]	0.85[a]	0.16	0.038	0.171	0.843
Butyricicoccus	1.08	1.58	0.65	.	.	.	0.26	.	.	.
Ruminococcaceae 1	.	.	.	1.33	1.02	1.24	0.19	.	.	.
Eubacterium	.	.	.	1.15	0.91	1.02	0.22	.	.	.
Ruminococcaceae 2	.	.	.	1.72	0.88	0.67	0.70	.	.	.
Escherichia-coli	.	.	.	1.64	1.18	0.60	0.80	.	.	.
Others	39.16	39.89	33.55	39.11	42.09	36.08	2.98	0.228	0.419	0.833

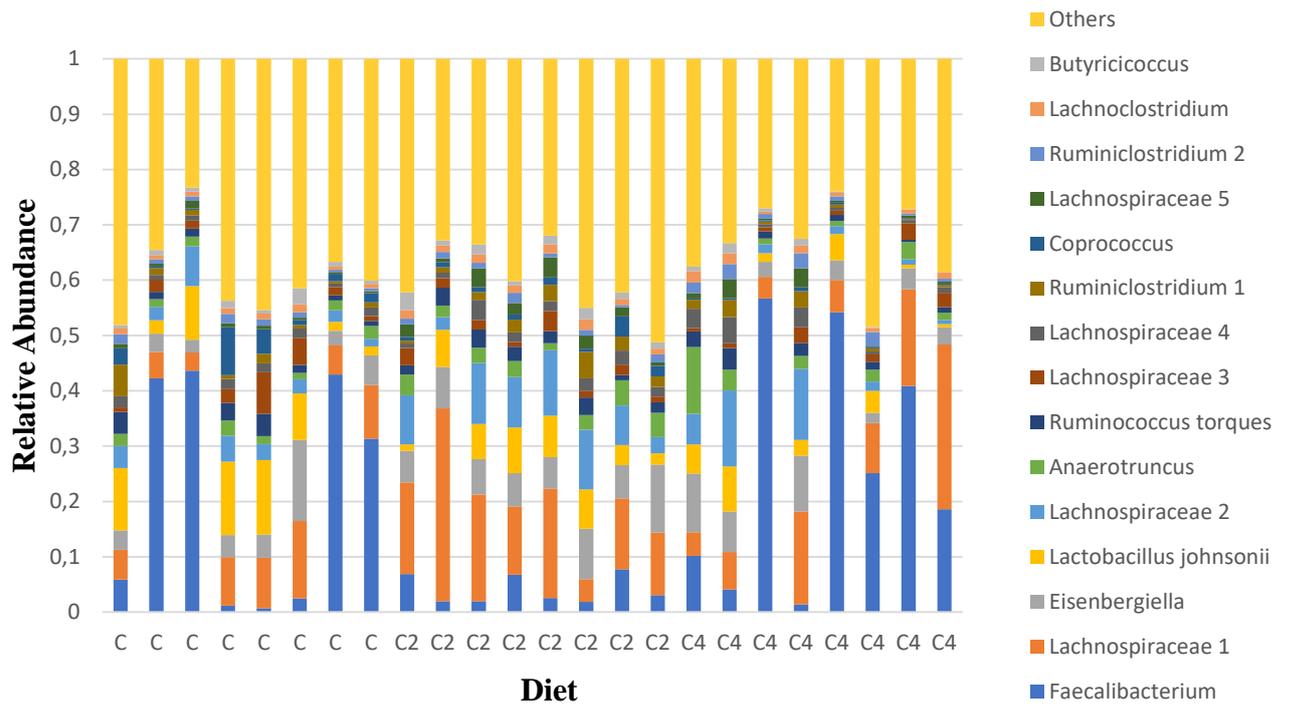*Different letters (ab, a, b) within row shows difference at p > 0,05*

Figure 1. Mean relative abundance of bacterial OTUs in caeca of broiler fed control diet (C), 2% (C2) and 4% (C4) inclusion of *C. intestinalis* at 12 days of age.
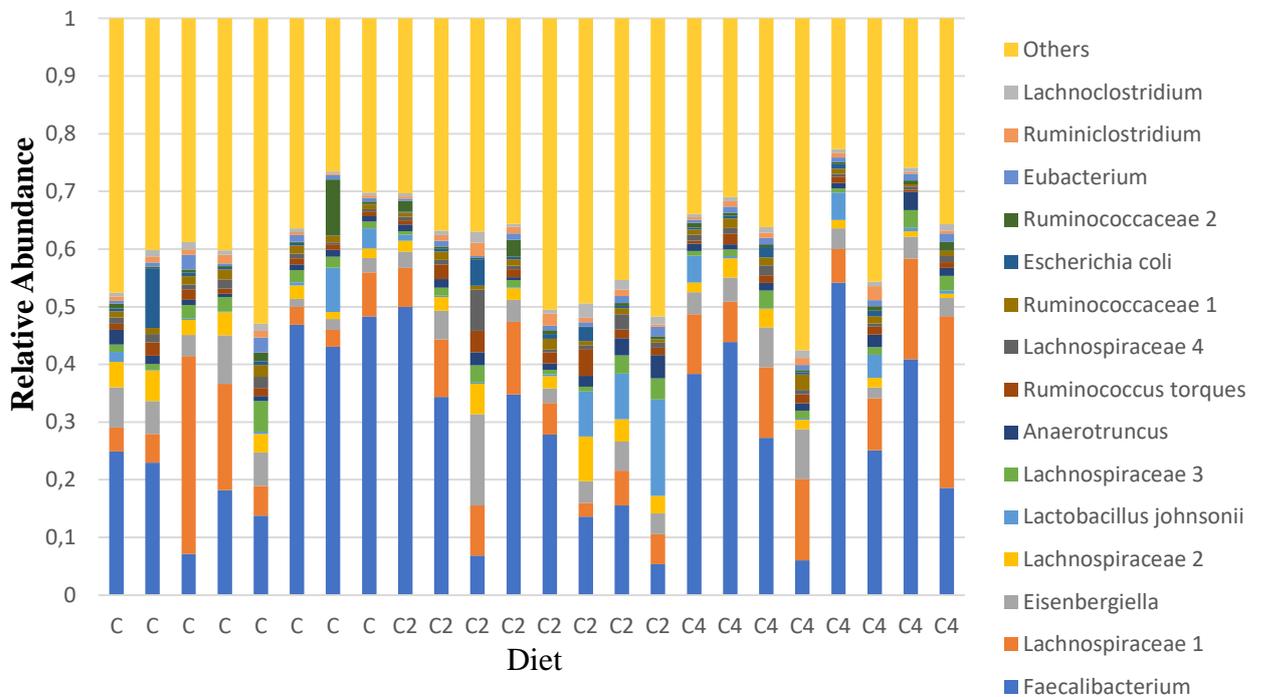

Figure 1.1. Mean relative abundance of bacterial OTUs in caeca of broiler fed control diet (C), 2% (C2) and 4% (C4) inclusion of *C. intestinalis* at 32 days of age.

5. Discussion

Body weight gain and feed intake were not significantly different (P > 0.05) among any of the dietary treatment and these results were consistent with the findings of Cao et al. (2003) where inclusion of 3.5 % pure cellulose resulted in no effects on body weight. The reason for having non-significant effect could be a low inclusion of dietary fibre source in our experimental diets and formulation of experimental diets to have the same nutritional values as control diet. FCR was higher with inclusion of 4% *C. intestinalis* compared to 2% ciona. Birds fed with C4 also had a lower DM of excreta which might explain the higher FCR. The lower DM of excreta might be because of a high content of sodium in the C4 diet and thereby a higher water intake. According to NRC (1994) the content of sodium for broilers should be 1.5g/kg diet, and in the C4 diet it was 1.97g/kg. Borges (2001) observed that increasing dietary electrolyte balance results in increased water intake to overcome the osmotic imbalance caused by higher Na^+ and K^+ levels. Ravindran et al. (2008) evaluated the effects of dietary electrolyte levels on dry matter content of excreta and observed that the excreta of broilers fed diets with 300 and 375 mEq/kg were more humid (76.9 and 81.2%, respectively) compared with broilers fed 150 and 225 mEq/kg (73.1 and 72.3%, respectively). Decrease DM of excreta lead to wet litter problem that may induce foot pad dermatitis (Mayne, 2005). This could be a problem especially in commercial settings. However, we did not observe any effects on litter quality in our experiment, which could be due to small animal groups, low density and high standard of the environmental conditions.

Relative weight of different organs like heart, liver, spleen and bursa were measured to see the relation between body weight and organs development but there was no significant effect of any treatment on relative weight of different organs. This could be explained by a similar content of total dietary fibre in the control and ciona diets (Table 5). The total dietary fibre content was only 1% higher in C4 than the control, and the largest difference between the fibre compositions in the diets was an increased amount of glucose in the ciona diets where the glucose builds up the cellulose. The results from this study relate to the findings of Jiménez-Moreno et al. (2010) where inclusion of 3% microcrystalline cellulose resulted in no stimulation of gizzard functioning. Hetland and Svihus (2007) also reported that intake of cellulose from litter bath in layers at the age of 35 weeks is not expected to produce any increase in size of digestive organs. Generally, the relative weight of different organs decreased with the age in agreements with findings reported by the Ravindran et al. (2006) and by Gracia et al. (2009).

Bursa of fabricious is the primary lymphoid organ, necessary for B-cell differentiation, development and maturation in avian species (Wu et al., 2013). It is also responsible for establishment and maintenance of B-cell apartment (Li et al., 2015). According to Cazaban et al. (2015) the bursa to body weight ratio for broilers under minimal stress and no disease conditions should be 0.11 or above from 7 to 42 days of age which are in accordance to our findings. In our results relative weight of bursa is not decreasing with age; and this confirms its continuous development with age. Bursa development begins during incubation and reaches maximum size between 8 to 10 weeks of age (Oláh et al., 2014).

Diversity and density of bacterial population in different parts of GIT significantly change with age. The result of the present study showed that there was no effect of any dietary treatment except for Lachnoclostridium but there was clear effect of age. According to Corrigan et al. (2015) the main predominant phyla detected at early age (day 7) were firmicutes followed by proteobacteria and main families were Lachnospiraceae,

Ruminococcaceae and Lactobacillacea which are similar to our findings. At later stages (32 days) firmicutes remained most predominant phylum in caeca and main detected families were Ruminococcaceae, Lachnospiraceae, Enterobacteriaceae and Lactobacillaceae which are consistent with the findings of Pourabedin et al. (2015). One reason for having no significant effect could be the low inclusion of level of ciona. The major findings of our study closely relate to the general pattern of microbial diversity in the broilers at different age as seen in other studies (Gong et al., 2007; Yin et al., 2010; Danzeisen et al., 2011). According to Torok et al. (2011) unclassified Clostridiales, Clostridia, Lachnospiraceae and Enterobacteriaceae were related to improved performance, while *L. salivarius, L. aviaries and L. crispatus* were related to poor growth performance. Large individual variation in microbial composition within the treatments were observed, which are in agreement with the findings of previous studies (Torok et al., 2011; Sergeant et al., 2014; Ocejo et al., 2019) and can be decreased by taking the pooled samples from the same module. Continuous filling and emptying of caeca could also be another reason for individual variation in microbiota. Furthermore, chick gut development has been shown to alter due to maternal nutrition (Rebel et al., 2006), which has been indirectly linked to microbiota development and biological variation between birds (Lumpkins et al., 2010). Interestingly, compared to the control, inclusion of 2% ciona stimulated Lachnospiraceae1 and Lachnoclostridium at day 12. Both OTU belongs to the most abundant phylum Firmicutes and moreover, some authors have suggested that these microorganisms could be developed as poultry probiotics (Torok et al., 2011; Stanley et al., 2016), which indicate some beneficial effects of including 2% ciona at early age .

In accordance with the microbiota data no clear effect on concentration or proportion of SCFA were observed. This might be due to the similar level of dietary fibre in the different diets and due to that cellulose is a water insoluble fibre. Moreover, nutrient analysis of ciona showed that it contains 3.1g/kg of lignin. Indigestible components of dietary fibre (indigestible cellulose and lignin) will have insignificant fermentation and may not affect SCFA concentration in caeca of chickens (Angkanaporn et al., 1994). However, there is a lack of information regarding the SCFA profile in birds fed diets supplemented with cellulose. Possible future changes in an experimental setup to see the effects of *C. intestinalis* could be an extended pre-treatment of *C. intestinalis* to lower the sodium content further, before its inclusion in the experimental diets. Inclusion of higher-level of *C. intestinalis* in the experimental diets could then be interesting. Moreover, *C. intestinalis* possess a high biological value of protein which makes it interesting as a protein source in animals' diet and to see its effects on animals' health and production.

6. Conclusion

The findings of the present study showed that the inclusion of 4% ciona in the diet resulted in lower DM in the excreta, which can be related to high sodium content. However, ciona as a source of fibre showed no clear effects. Since, there are not many studies on the effect of ciona in chickens, further investigations are necessary to confirm the results of the present experiment.

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8. Acknowledgement