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Original Research

A natural carbohydrate fraction Actigen™ from *Saccharomyces cerevisiae* cell wall: effects on goblet cells, gut morphology and performance of broiler chickens

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Summary

A study was conducted to evaluate a natural carbohydrate fraction Actigen™ (NCF), derived from mannanoligosaccharide, in feed on growth performance, intestinal morphology and goblet cell number and area of male broilers'. Dietary treatments included: 1) control diet (antibiotic and NCF free), 2) NCF at 200 g/t, 3) NCF at 400 g/t, and 4) NCF 800 g/t. Two hundred and forty birds were placed into 12 replicate pens per treatment (5 birds/pen), sixty birds per treatment. Body weight and feed intake were recorded weekly up to day 42. At this time a 2.5cm section of jejunum and duodenum were excised post mortem for morphological analysis. Birds fed 200 g/t and 800 g/t NCF were significantly ($P < 0.01$) heavier from day 14 onwards than the control birds. Feed intake was significantly higher in birds fed 200 g/t NCF compared to those fed the control at 21 and 35 days ($P < 0.05$). Diets containing 200 g/t and 800 g/t of NCF significantly decreased broiler feed conversion ratio (FCR) compared to the control in the first phase (1–14 days) ($P < 0.01$) and levels of NCF decreased FCR ($P < 0.05$) in the second phase (15–28 days). NCF had no significant effect on villus height, villus width, crypt depth or villus to crypt ratio in either duodenum or jejunum. NCF did not significantly affect goblet cell area or goblet cell number in the duodenum, however, in the jejunum, 800 g/t NCF significantly ($P < 0.05$) increased goblet cell area over the control. In conclusion, NCF showed a positive effect on broiler performance in the starter and grower phases, and increased goblet cell area in the jejunum, suggesting higher levels of mucin production. This indicated that the performance benefit of NCF could be age-dependent, with younger birds responding more than the older ones. There were no additional benefits to performance when feeding NCF for a longer period (after 28 d of age), however it is postulated that birds fed NCF would have greater defence to pathogenic challenge through increased storage capacity of mucin.

Keywords: **Actigen™: prebiotic: villi: broiler: goblet cell**

Introduction

Over the past few years there has been increased concern about antibiotic resistant bacteria and the inclusion of antibiotics in animal diets for growth promotion. This led to the ban of a number of antibiotic growth promoters (Dibner and Richards, 2005). Since the EU ban on using antibiotics as growth promoters in 2006 (Huff *et al.,* 2006), there has been an increase in the incidence of endemic diseases in poultry (Chee, 2008), in addition to reports of slower growth and higher disease challenges causing significant economic losses (Thomke and Elwinger, 1998) and negative welfare implications for poultry. In particular, gut health has been affected, and without a healthy intestinal tract a broiler cannot reach its full performance potential. Due to this, there has been a drive in the market for feed supplements that will improve health and production of poultry, but remain safe for humans. The morphological structure of the gastrointestinal tract (GIT) offers key information to evaluate gut health. Longer, thinner villi are considered to indicate

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that the bird will have a better ability to absorb nutrients, due to increased surface area (Gao *et al.,* 2008). Shorter villi height (VH) and deeper crypt depth (CD) are associated with decreased digestibility of nutrients (Zhang *et al.*, 2005). Deeper CD are considered a negative indication of gut health because new epithelial cells are produced in the crypts and migrate along the villi to the tip (Gao *et al.,* 2008), therefore a deeper CD indicates that there is a higher tissue turnover of epithelium cells. It is thought that the faster turnover of tissue is due to the host compensating for villus atrophy, due to inflammation resulting from pathogens and their toxins (Gao *et al.,* 2008).

Mannanoligosaccharides (MOS) are mannose-based carbohydrates that can be derived from yeast cell walls and have a prebiotic function (Chee, 2008). MOS has been shown to have a positive effect on gut heath, by binding to enteropathogens, inhibiting their proliferation and stimulating specific microbial populations in the GIT (Spring *et al.,* 2000; Kocher *et al.*, 2004). This leads to increased VH and decreased CD, which may in turn improve nutrient absorption (Santin *et al.,* 2001; Sims, 2004; Mourão *et al.*, 2006).

Goblet cells are found in the epithelial layer along the villi of the bird's GIT. Goblet cells produce and secrete mucin glycoproteins that make up part of the mucus layer, which protects the intestinal surface against damage by bacterial and environmental toxins, microorganisms and some coarse dietary components (Santos *et al.,* 2007). Several studies have found that feeding MOS increases the number and/or the volume of goblet cells found in the small intestinal tract (Baurhoo *et al.,* 2009; Brummer *et al.,* 2010). It has not only been found that MOS increased the number of goblet cells, but also their size (Uni and Smirnov, 2006). Mucin is thought to be beneficial to developing the innate immune system (Koutsos and Arias, 2006).

Actigen™ (Alltech Inc., Nicholasville, Kentucky, USA), is a specific natural carbohydrate fraction (NCF) that has been derived from the cell wall of *Saccharomyces cerevisiae*. NCF contains a high affinity for the mannose- specific type-1 fimbriae of pathogenic bacteria such as *Escherichia coli* (Ofek *et al.*, 1977) and *Salmonellae* (Spring *et al.*, 2000; Miguel *et al.*, 2004). The objective of this study was to evaluate the effect of this specific natural carbohydrate fraction isolated from yeast cell wall oligosaccharides, on VH, CD and the goblet cell profiles of broilers.

Materials and methods

Two hundred and forty one-day-old male Ross 308 broilers were placed in 48 pens, each containing five birds and fed one of four dietary treatments (12 replicates for each treatment), which were allocated as a randomised block design. The room temperature was initially adjusted to 32°C and then gradually lowered to reach approximately 21°C by day 21. Temperature was monitored on a daily basis and light was 23L:1D for the duration of the experiment. Birds were kept on fine sand litter, with a calculated stocking density of 30 kg per m^2 on day 42. Birds were provided with *ad libitum* access to water and feed. Feed was a wheat/soya based mash containing no enzymes or coccidiostats. The trial lasted 42 days with a three phase feeding programme, starter (1 to 14 d); grower (15 to 28 d) and finisher (29 to 42 d). The basal diet was formulated and made by Target feeds (Coton, Whitchurch, Shropshire, UK) and the formulations are shown in Table 1.

The four dietary treatments used were; 1) control (antibiotic and NCF free), 2) NCF 200 g/t, 3) NCF 400 g/t, and 4) NCF 800 g/t. Weekly and overall feed intakes and weight gains were recorded and FCR was calculated and corrected for mortality. At the end of the 42 day trial, a 2.5cm section of jejunum and duodenum were removed and immediately rinsed with PBS solution. The tissue was then placed in Bouin's fixative for eight hours and then stored in 70% industrial methylated spirits. The tissue samples were then embedded in paraffin and cut at 8 µm intervals using a rotary microtome (Leitz Wetzlar 1512 microtome Leitz, Milton Keynes, Bucks, UK). The sections were

Table 1. Composition of basal diet and calculated analysis of the basal diet

Item	Starter	Grower	Finisher
Ingredients (%)			
Barley	10.60	8.46	7.23
Wheat	50.00	55.00	60.00
Soybean meal, 48% CP	26.00	23.00	19.00
Full-fat soybean meal	5.00	5.00	0.50
L lysine HCL	0.31	0.26	0.25
DL methionine	0.38	0.35	0.33
L threonine	0.14	0.13	0.14
Soya oil	4.00	4.50	4.75
Limestone	1.25	1.25	1.25
Monocalcium phosphate	1.50	1.25	1.25
Salt	0.25	0.25	0.25
Sodium bicarbonate	0.15	0.15	0.15
Premix*	0.40	0.40	0.40
Calculated analysis			
ME MJ/kg	12.80	13.00	13.20
CP %	21.80	20.60	19.10
Lys %	1.37	1.16	1.13
Met + Cys %	1.01	0.95	0.89
Ca	0.96	0.90	0.90
Total P	0.73	0.66	0.65

*Premix supplied per kg diet: Mn 100 mg, Zn 80 mg, Fe 20 mg, Cu 10 mg, I 1 mg, Mb 0.48 mg, Se 0.2 mg, retinol 13.5 mg, cholecalciferol, 3 mg, tocopherol 25 mg, menadione 5.0 mg, thiamine 3 mg, riboflavin 10.0 mg, pantothenic acid 15 mg, pyroxidine 3.0 mg, niacin 60 mg, cobalamin 30 µg, folic acid 1.5 mg, biotin 125 mg

stained with a combination of 1% alcian blue (pH 2.5) and periodic acid-Schiff's reagent. The following measurements were taken using a light microscope; CD, VH, villus width (VW), villus/crypt ratio (VCR), and goblet cell area and number in the jejunum. VH was measured as the length between the villus-crypt axis and the tip of the villus (20 villi per sample, 240 per treatment).

The VW was measured at the midpoint between the villus-crypt axis and the tip of the villus. CD was measured from the villus-crypt axis to the base of the specific crypt. Goblet cell area was measured as the 'cup' area of the goblet cells (μm^2). Ten measurements on 20 villi were made for each intestinal sample. Goblet cell density was determined as the number of goblet cells per 165 μm, with samples obtained from nine pens per treatment. Gut morphology was analysed using an Olympus BX51 microscope fitted with an Olympus DP71 camera (Olympus Microscopy, Essex, UK) and Cell F software (Olympus Europa GmbH, Hamburg) used for all measurements. Gut morphology measurements and performance data were analysed using SPSS software version 12 for Windows, with the data being initially analysed for normality. If the data was normally distributed a one-way ANOVA was used, if the data was not normally distributed a Kruskal-Wallis test was performed. Treatment means were separated using the Bonferroni's post hoc test, and statistical significance was declared at $P < 0.05$. Institutional and national guidelines for the care and use of animals were followed, and all experimental procedures involving animals were approved by the Nottingham Trent University College of Science Ethical Review Committee.

Results and discussion

Live body weight and FCR results are presented in Table 2.

Body weight and FCR means were derived from 12 pens per treatment, except for between 15–28 days for the 200 g/t and 800 g/t treatments, where 11 pens per treatment were used due to mortality. FCR means at 29–42 days for NCF 200 g/t were derived from 11 pens per treatment due to data loss from accidental feed spillage in one pen.

Significant ($P < 0.01$) differences between treatments were observed for body weight on days 14, 21, 28, 35 and 42. Over the whole trial period, the feeds containing either 200 g/t or 800 g/t NCF increased live weight compared to the control. Feed intake was significantly increased for birds fed 200 g/t of NCF over the control at days 21 and 35 (respectively $P = 0.021$, $P = 0.025$, data not shown). For FCR, significant differences were observed in the first (1–14 d) and second phase (15–28 d) of feeding. Diets containing 200 g/t and 800 g/t NCF resulted in significantly lower FCR compared to the control in the first phase ($P < 0.01$), whereas in the second phase all inclusion levels of NCF decreased FCR ($P < 0.05$). However the FCR seen in week one for the control and 400 g/t NCF was abnormally high for nutritionally complete diets, indicating excessive spillage. Mortality for the treatment groups was not significantly different from the control.

Morphometric measurements from the stained slides are shown in Table 3. It was observed that NCF had no significant effect on VH, VW, CD or VCR in either the jejunum or duodenum.

When examining the goblet cell numbers and area in the duodenum (Table 4), it was concluded that the diets including NCF did not significantly affect goblet cell area, goblet cell number per 165 μm or goblet cell measurements as a ratio, however, in the jejunum, birds fed 800 g/t NCF showed significantly ($P < 0.05$) increased goblet cell area compared to the control.

Table 2. Effect of NCF in broiler diets on the average body weight (g/bird) and FCR

Parameter	Age d	Control	Dietary NCF treatment			
			200 g/t	400 g/t	800 g/t	SEM
Body wt (g)	1	43	44	44	43	0.34
	7	102	114	107	107	2.02
	14	217^{a}	303^{c}	250^{ab}	291^{bc}	8.15
	21	525^{a}	715^{b}	600^{ab}	680^{b}	20.42
	28	1026^{a}	1335^{b}	1198^{ab}	1287^{b}	31.60
	35	1765^{a}	2119^{b}	1943^{ab}	2112^{b}	39.76
	42	2516^{a}	2867^{b}	2655^{ab}	2842^{b}	42.16
FCR	0–14	2.03^{a}	1.59^{b}	1.91^{ab}	1.61^{b}	0.05
	15–28	1.85^{a}	1.61^{b}	1.63^{b}	1.63^{b}	0.03
	29–42	1.76	1.81	1.84	1.82	0.02
	0–42	1.80	1.73	1.76	1.74	0.01

[a–c]Differing superscripts in a row denote means are significantly different at $P < 0.05$

Table 3. Effect of NCF on the gut morphology of the duodenum and jejunum (μm)

Parameter	Control	NCF 200 g/t	NCF 400 g/t	NCF 800 g/t	SEM
Duodenum					
Villus Height (μm)	2819	2791	2896	2704	53.3
Villus Width (μm)	274	265	262	282	5.5
Crypt Depth (μm)	202	202	210	213	5.9
Villus/Crypt Ratio	15.1	13.6	14.2	13.2	0.47
Jejunum					
Villus Height (μm)	1284	1285	1379	1315	25.5
Villus Width (μm)	247	235	234	249	5.9
Crypt Depth (μm)	159	151	155	158	2.7
Villus/Crypt Ratio	8.1	8.6	8.9	8.4	0.17

Differences were not statistically significant ($P > 0.05$)

Duodenum and jejunum goblet cell means are from nine pens per treatment.

Duodenum gut morphology samples were taken from birds selected from 12 pens per treatment, and jejunum gut morphology samples from 12 pens for NCF 200 g/t and 11 pens for the control, 400 g/t and 800 g/t due to the loss of one sample per treatment from histological processing.

Supplementation of diets with 200 g/t and 800 g/t NCF significantly increased broiler weekly body weights compared to the control diet from 14 days, but the inclusion level of 400 g/t NCF did not significantly change body weight from the control. This indicated that there may be different mechanisms behind the observed response to supplementation; one occurring at the lowest inclusion and one occurring at the highest inclusion, with apparently antagonist effects at the median level. This contradicts both Reisinger *et al.* (2012) and Gao *et al.* (2008), who found a positive quadratic response in broiler body weight to both a yeast derivative and yeast cell culture. From these responses, it was considered that the higher inclusion levels were interacting with the immune system, causing energy to be partitioned towards the immune system, rather than supporting growth. Whilst this mechanism may explain the observed response at 400 g/t NCF in the current trial, it does not support the improved performance at 800 g/t NCF.

Increased body weight following supplementation with other yeast cell wall material MOS (such as that observed at 200 g/t and 800 g/t in the current study) has been attributed to the reduced effects of pathogenic bacteria in the intestinal tract as the binding of pathogenic bacteria to MOS results in their evacuation from the intestine with other non-digested feedstuffs (Spring *et al.,* 2000). This may have reduced sloughing of villi in birds fed MOS, thereby contributing to increased performance compared to the control, due to an increased capacity to absorb nutrients (Sun *et al.,* 2005). When feeding NCF to broilers it could be postulated that birds will also have longer VH and shorter CD via a similar reduction in sloughing. This is because Spring *et al.* (2000) reported that pathogens with the mannose-specific type-1 fimbriae, such as some strains of *Escherichia coli*, *Salmonella typhimurium* and *Salmonella enteritidis,* are attracted to mannans, which are reported to be present in NCF (Che *et al.*, 2012), and preferentially bound to them instead of attaching to intestinal epithelial cells (Castillo *et al.,* 2008). Therefore these pathogenic bacteria cannot colonise the GIT and release toxins. These bacterium and their toxins can cause inflammation that in turn cause atrophy of the epithelial cells of the villi

Table 4. Effect of NCF on the goblet cell (GC) of the duodenum and jejunum

Parameter	Control	NCF 200 g/t	NCF 400 g/t	NCF 800 g/t	SEM
Duodenum					
GC Area (μm^2)	59.2	69.4	58.3	62.6	2.5
N° of GC per 165μm	11.8	11.4	11.7	11.9	0.2
GC Area per 165μm	683.1	747.0	664.4	719.3	23.6
Jejunum					
GC Area (μm^2)	67.6^{b}	68.3^{b}	73.8^{ab}	82.8^{a}	2.2
N° of GC per 165μm	12.4	12.2	12.0	11.8	0.2
GC Area per 165μm	847.0	868.0	882.1	988.4	21.9

$^{a–c}$Differing superscripts in a row means are significantly different at $P < 0.05$

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(Gao *et al.,* 2008), thus reducing the absorptive function of the gut through shorter VH and deeper CD (Yason *et al.,* 1987). If the NCF bind the pathogenic bacteria and reduce their levels in the GIT, there will be less villi damage in the gut, therefore improving the gut health of the bird. To compensate for this atrophy, the bird has to increase its tissue turn over, and as epithelial cells are produced in the crypts and migrate along the villi to the tip, it is thought that the higher turnover in the crypt cell cause it to become deeper (Gao *et al.,* 2008). Therefore shallower CD can be considered a good indicator of gut health. MOS are also thought to increase the number of beneficial bacteria in the gut.

Morphometric analysis at six weeks of age revealed that NCF had no significant effect on the gut morphology of the birds at this time point. A possible reason for this may be due to the birds not being challenged with pathogenic microbes at this point. This trial supports the VH findings of White, *et al.* (2002) in pigs and Yitbarek *et al.* (2012) and Sohail *et al.* (2012) in broilers when feeding MOS. However, Iji *et al.* (2001) and Zhang *et al.* (2005) found that birds fed yeast cell wall fractions had longer VH than control birds at 21 days. Similarly, Baurhoo *et al.* (2007) measured VH and found that MOS improved VH at 28 days but not at 42 days. A similar, early age response may have occurred in this present study, but as histological measures were not taken at 21 or 28 days this cannot be verified.

When looking at CD, Zhang *et al.* (2005), Yitbarek *et al.* (2012) and Sohail *et al.* (2012) found feeding MOS had no effect on CD in birds, as seen in this study. Santin *et al.* (2001) also saw no effect of MOS at 42 days, however decreased CD was observed at seven days of age in broilers fed MOS. In addition, the present study found no effect on VCR, which can be used as a marker of overall intestinal health, as it takes into account both CD and VH. This trial included a total of 240 measures for each treatment, which is a considerable amount, and it should be noted that taking more measures to try and reduce associated error would have major time and cost implications. The inherent variability both in this study and other studies suggests that measuring VH and CD may not be an optimal approach to quantifying gut health.

This study showed that the greatest benefit of feeding NCF on FCR occurred at the beginning of the trial, indicating there may be an optimum time for the supplementation of NCF to increase feeding efficiency of the birds. This may be due to the fact that the gut microflora of younger birds is more transient in nature and less established than in older birds, and hence more susceptible to colonisation by pathogenic bacteria. Therefore prebiotic intervention may shorten the time required to create a beneficial microflora population if it is offered early in life. However, similar studies have found variable early performance effects (Iji *et al.*, 2001; Sun *et al.*, 2005; Zhang *et al.,* 2005; Midilli *et al.*, 2008). In this study it was generally shown that NCF improved FCR over the starter and grower feeding phases. This concurs with the findings of Zhang *et al.* (2005), however it must be mentioned that other studies have shown no early response (Iji *et al.*, 2001; Sun *et al.*, 2005; Midilli *et al.*, 2008).

It could be hypothesised that the improvements in FCR in younger birds fed NCF may have been due to improved absorption, due to an increase in lactobacilli and bifidobacterial populations and a reduction in pathogenic bacteria. This has been seen in other studies where improvements in gut morphology were associated with increased lactobacilli and bifidobacterial populations (Baurhoo *et al.,* 2009). However, improvements in gut health were not observed in this present study, as histological measurements were only recorded at 42 d of age, when the microflora was probably already established with a stable population of beneficial bacteria. This means that birds on treatment diets were not under any challenge, which would explain why NCF had no effect on the performance in the last phase of the trial, which is consistent with the histological measurements at 42 d.

There is no consensus on whether an increase in goblet cell numbers and area is considered an improvement in bird health. Increasing the number and area of goblet cells is thought to improve the volume of mucin stored in the GIT and, possibly, its production (Brümmer *et al.*, 2010). Mucin is essential for a number of brush border processes, including facilitating absorption of nutrients, enzyme production, lubrication and decreasing the binding and colonisation ability of pathogenic bacteria to the intestine (Blomberg *et al.,* 1993; Smirnov *et al.,* 2004). An increase in the level of mucin could have a beneficial effect on the first line of defence of the immune system (Baurhoo *et al.,* 2009) and the absorptive function of the gut. Contrarily, overproduction of mucin may result in an negative effect, by increasing the mucus thickness on the GIT wall to a level that might negatively affect the absorption of nutrients through the gut epithelium (Smirnov *et al.,* 2004; Brummer *et al.,* 2010).

In this study, the number and area of goblet cells in the duodenum and jejunum were not affected by supplementation with any levels of NCF at 42 days. This would suggest that NCF has no effect on the mucin profile of broilers in the duodenum. This absence of response was also reported by Castillo *et al.* (2008) and Yitbarek *et al.* (2012). Conversely, Baurhoo *et al.* (2007; 2009), Chee *et al.* (2010), Morales-lopez *et al.* (2010) and Muthusamy *et al.* (2012) reported that goblet cell numbers were increased by yeast cell wall product supplementation. In the jejunum NCF increased the area of goblet cells and there was a trend for larger goblet cell area per 165 µm of villi, which suggested that NCF at 800 g/t affected the mucin profile of broilers. Published data on goblet cell area is limited. Brummer *et al.* (2010) found that area of goblet cells increased with MOS supplementation. Insight into the mechanisms behind this goblet cell response would be beneficial to understanding the effect of NFC supplementation on gut health.

An increase in goblet cell area is thought to show that its mucin storage capacity has increased (Smirnov *et al.*, 2005). The increase in mucin storage suggests that the bird is more capable of forming a protective layer on the villi, thereby helping protect the intestine from damage caused by enteropathogens in the event of a challenge from pathogenic bacteria (Smirnov *et al.*, 2006; Brümmer *et al.*, 2010). One suggested mechanism of MOS on mucin production is through changing the gene expression of key genes through direct crosstalk between beneficial intestinal microbes and goblet cells (Mack *et al.*, 1999; Freitas *et al.*, 2003; Smirnov *et al.*, 2005; Uni and Smirnov 2006; Chee, 2008). The effects of NCF on the goblet cell area observed in this trial agree with this suggestion in that goblet cell area was increased with higher supplementation levels, but it is also possible that NCF has a direct effect on mucin production and subsequently increasing bifidobacteria due to the increase in mucin production, as these microbes can produce enzymes allowing them to utilise and proliferate on mucin glycoproteins (Katayama *et al.*, 2005; Jung *et al.*, 2008; Ruas-Madiedo *et al.*, 2008).

Conclusions

Published research in this field appears to be highly variable, which may be due to differences in the type of MOS product, experimental conditions, diet formulation, or health status of the birds. This means that the mechanism of action of yeast cell wall carbohydrates, like MOS and NCF, and interactions with other nutritional and production parameters are still not fully understood. Under the conditions of this trial, NCF had a positive effect on performance in the starter and grower phases, indicating that the performance benefit of NCF could be age-dependent, with younger birds responding more than the older ones. Although there were no additional performance benefits to feeding NCF for a longer period, goblet cell area in the jejunum was increased, suggesting that birds fed NCF would have better defences to pathogenic challenges due to higher levels of mucin.

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Declaration of interest

This study was part of a PhD project that is part-funded by Alltech. Peter Spring holds the role of a consultant to Alltech and Jules Taylor-Pickard is currently employed by Alltech.

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