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Effects of delayed feeding, sodium butyrate and glutamine on intestinal permeability in newly-hatched broiler chickens

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ABSTRACT

The aim of the current study was to investigate the effects of delayed feeding, and supplementation with sodium butyrate or glutamine in drinking water, on intestinal permeability (IP) in young broiler chickens. Newly-hatched male chickens (Ross 308) were allocated to four groups comprising Control, 24 h delayed fed (DF), DF supplemented with sodium butyrate (0.1%) in the drinking water and DF supplemented with glutamine (1%) in the drinking water. On days 2, 4 and 7, twelve birds per group were randomly selected, weighed and orally gavaged with fluorescein isothiocyanate dextran (FITC-d) at 2.2 mg / ml / chicken. Serum FITC-d concentration was analysed by spectrophotometry while serum diamine oxidase and D-lactic acid concentrations were analysed by microplate reader. FITC-d concentrations in the Control and DF groups were not statistically different on any day, suggesting that delayed feeding did not affect IP. Additionally, sodium butyrate increased IP compared to DF and Control on day 2 only ($p < 0.05$), while glutamine increased IP on all days, compared to DF and Control ($p < 0.05$). Diamine oxidase and D-lactic acid concentrations of all groups were not statistically different.

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Fluorescein isothiocyanate dextran; diamine oxidase; D-lactic acid; leaky gut and feed withdrawal

Introduction

Timing of the first feed in newly hatched chickens influences development of the intestinal tract and subsequent productivity. In practise, access to their first feed may be delayed by 36–72 h (Noy and Uni 2010). Geyra et al. (2001a) have shown that the intestine grows exponentially in chickens during the first 24 h and the surface area continues to grow more slowly afterwards. Delayed feeding after hatch has been shown to affect intestinal growth by decreasing villi height (Mahmoud and Edens 2012), crypt proliferation, enterocyte migration (Geyra et al. 2001b) and weight to length ratios of jejunum and ileum until day 4 of age (Lamot et al. 2014). Mahmoud and Edens (2012) have also shown that the adverse effects (shortened villi and decreased surface area) of delayed feeding continued till 14 days of age. Conversely, Shinde et al. (2014) showed that intestinal morphology and growth performance were affected by 24 h, rather than 12 h, post-hatch fasting. Fasting has been known to increase intestinal permeability (IP) in growing chickens (Gilani et al. 2016a). Increased IP can increase bacterial translocation and activate the immune response leading to reduced performance (Gilani et al. 2016). Recently, chicken antibody titers against human serum albumin were shown to be raised in delayed fed (DF)

chickens (Simon et al. 2015). To date, studies on increased IP in chickens have been limited and there is no published literature on whether IP is affected by delayed feeding.

Butyrate is the primary source of energy for intestinal epithelial cells and has been shown to increase villus height and crypt depth in chickens (Abdelqader and Al-Fataftah 2016). Sodium butyrate has been utilized to improve gut health in chickens as reviewed by Ahsan et al. (2016). However, the effects of sodium butyrate on IP have yet to be investigated. Recently, sodium butyrate decreased IP in mice (Han et al. 2015) and piglets (Huang et al. 2015). Glutamine, which is a non-essential amino acid, has also been shown to decrease IP in *in vitro* cell cultures (Le Bacquer et al. 2003), pigs (Wang et al. 2015) and rats (Beutheu et al. 2014). However, glutamine did not improve intestinal barrier function in chickens in *in vitro* studies after a mycotoxin challenge (Awad and Zentek 2015). We have recently shown that glutamine supplementation prior to fasting challenge did not ameliorate increased IP (Gilani et al. 2017). However, its effects on improving IP after a post-hatch delayed feeding challenge have yet to be explored in chickens. The aims of this study were to investigate whether IP was increased by post-hatch delayed feeding, and also whether sodium butyrate or glutamine supplementation could ameliorate increased IP following a post-hatch delayed

feeding challenge in chicks. Additionally, three biomarkers fluorescein isothiocyanate dextran (FITC-d, D-lac, diamine oxidase) were compared for evaluating IP. These biomarkers have been utilized previously (Gilani et al. 2016a).

Materials and methods

All procedures were approved by the Animal Ethics Committees of the University of Adelaide and the Primary Industries and Regions South Australia. All animal studies were performed in compliance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. Newly-hatched male chickens ($n = 144$, Ross 308) were obtained from a local hatchery (Baiada, Willaston, Australia). Birds were randomly allocated to four groups and kept on paper (without saw dust) with 16 h day and 8 h night cycle reared under infrared heater lamps (25°C–30°C). The Control group had *ad-libitum* access to feed and water, while the remaining groups received feed and water after a delay of 24 h. Two of the three DF groups were supplemented with sodium butyrate or glutamine in water at 0.1% and 1% concentrations, respectively (Sigma Aldrich, New South Wales, Australia) for 7 days. All birds were provided commercial starter crumbles (Ridley Agri Products Pty., Murray Bridge, South Australia). Each bird was considered as a single experimental unit. On days 2, 4 and 7, chickens ($n = 12$ per group per day) were randomly selected, weighed and gavaged with FITC-d (4000 mol weight; Sigma Aldrich, New South Wales, Australia). FITC-d was stored at 4°C, wrapped in aluminium foil to avoid light exposure. FITC-d was gavaged at 1 ml / bird (2.2 mg FITC-d / ml of water) at three days of age. Blood was collected from the jugular vein 150 min after gavage. Standards and serum samples were prepared and analysed in triplicate as previously described (Gilani et al. 2016b). Standards were spiked with FITC-d at 0, 0.0001, 0.001, 0.01, 0.1, 1.0 and 10 µg/ml to obtain a standard curve utilizing a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK) at the excitation and emission wavelengths of 485 and 530 nm, respectively. Chicken specific antibodies of diamine oxidase and D-lactic acid Enzyme Linked Immunosorbent Assays (ELISA) were obtained from MyBioSource, (San Diego, USA). The procedure was performed as described previously (Gilani et al. 2016b). Standards and plasma samples were measured in duplicate in a microplate reader at 450 nm wavelength (Bio-Rad laboratories, California, USA) following manufacturer's instructions.

Statistical analysis

All observations on day 2, 4 and 7 were normally distributed and were included in the statistical analysis using SPSS 22 (IBM SPSS; IBM Corp., Armonk, New York, USA). Significance (p value) was measured at 0.05. Body weight and FITC-d were analysed using the general linear model multivariate procedure and means were compared by Tamhane analysis. No significant interaction between body weight and FITC-d was found. Diamine oxidase and D-lactic acid ELISA concentrations were analysed by one way ANOVA and means were separated by Tamhane analysis.

Results

Body weights

Body weights were measured on days 2, 4 and 7 before oral gavage with FITC-d (Table 1). Body weight of the DF group was significantly lower at day 2 and 4 than Control. Although the mean body weight remained lower at day 7, it was not statistically significant ($p > 0.05$). Body weights of the sodium butyrate and glutamine supplemented groups were not significantly different on each day compared with DF. However, sodium butyrate improved body weight on day 7 and was comparable to the Control ($p > 0.05$), while glutamine supplementation failed to improve body weights compared with Control on each day ($p < 0.05$).

Fluorescein isothiocyanate dextran (FITC-d), diamine oxidase (DAO) and D-lactic acid ELISA

FITC-d concentrations of the DF group were not statistically different compared to Control on all days (Table 1). FITC-d concentrations within Control and DF groups increased on day 7 compared to day 4 ($p < 0.05$). The concentration of FITC-d in the sodium butyrate supplemented group was significantly higher on day 2 compared to Control and the DF group. Although, mean FITC-d concentrations were higher in the sodium butyrate group compared to the Control and DF groups on day 4 and 7, these were not statistically significant ($p > 0.05$). FITC-d concentrations of glutamine supplemented group were significantly higher than those of the Control and DF group on all days. Additionally, FITC-d concentration increased significantly compared to the sodium butyrate supplemented group on day 7. D-lactic acid and DAO levels of all groups were not statistically different compared to Control (Table 1).

Discussion

Lower body weights in the DF group up to seven days of age were comparable with previous studies (Geyra et al. 2001b; Lamot et al. 2014), while the finding that there was no significant increment in body weight in the sodium butyrate treated group compared to the DF group was contrary to the literature (Ahsan et al. 2016). In these earlier studies, delayed feeding was not investigated. Also, body weight was measured over a time period of two and four weeks compared to the current study in which body weight was measured in the first week post-hatch. However, in the current study, body weights of the sodium butyrate supplemented group were not statistically different compared to Control. This is comparable to previous studies (Ahsan et al. 2016) which suggested that sodium butyrate demonstrated positive effects. Lower body weights in the glutamine supplemented group compared to DF alone are contrary to a previous study (Murakami et al. 2007). However, decreased body weights compared to DF and the Control are comparable to another study in which glutamine supplementation decreased body weight in chickens (Bartell and Batal 2007). The differences in previous studies could have been due to different crude protein in the diets.

Table 1. Body weights (BW), fluorescein isothiocyanate dextran (FITC-d), diamine oxidase (DAO) and D-lactic acid concentrations in serum of newly-hatched chickens measured on days 2, 4, and 7.

Treatments	N	Control	DF	DF + Sodium butyrate	DF + Glutamine	S.E.M	P values					
							Con v. DF	Con v.DF + SB	Con v. DF + G	DF v.DF + SB	DF v.DF + G	DF + SB v. DF + G
BW at day 2	12	66.8 ^a	60.0 ^b	61.2 ^{ab}	56.9 ^b	0.17	0.01	0.09	0.00	0.94	0.48	0.20
BW at day 4	12	110.6 ^a	97.5 ^b	96.5 ^b	94.0 ^b	0.93	0.01	0.01	0.00	0.99	0.69	0.86
BW at day 7	12	186.2 ^a	163.9 ^{ab}	179.9 ^a	148.9 ^b	1.46	0.09	0.89	0.00	0.30	0.35	0.01
FITC-d at day 2	12	0.62 ^a	0.68 ^a	1.20 ^b	1.28 ^b	0.05	0.99	0.00	0.00	0.00	0.00	0.34
FITC-d at day 4	12	0.58 ^{aA}	0.59 ^{aA}	0.76 ^{ac}	0.73 ^{bc}	0.02	1.00	0.08	0.01	0.11	0.02	0.89
FITC-d at day 7	12	0.78 ^{aB}	0.79 ^{aB}	0.86 ^a	1.06 ^b	0.03	1.00	0.21	0.00	0.35	0.00	0.02
DAO at day 4	10	0.39 ^a	0.44 ^a	0.40 ^a	0.40 ^a	0.02	0.86	1.00	1.00	0.91	0.91	1.00
D-lactic acid at day 7	10	6.28 ^a	5.70 ^a	5.03 ^a	5.23 ^a	0.12	0.73	0.13	0.25	0.61	0.82	0.98

Means with the same superscript within a row are not significantly different ($p > 0.05$). S.E.M is standard error mean. Con is control, DF is delayed fed, SB is sodium butyrate and G is glutamine.

^{A,B}Values within a column with different superscripts differ significantly ($p < 0.05$).

Higher crude protein diets (22% in the previous and current study) with the added glutamine decreased body weight. However, in these earlier studies, delayed feeding was not investigated. Finally, glutamine supplementation could not reverse reduced body weight due to delayed feeding, consistent with the recent study in delayed-fed chickens (Zulkifli et al. 2016).

In terms of IP, no previous study has been conducted in post-hatch DF chickens. Fasting in older chickens has been shown to increase IP (Gilani et al. 2016a, 2017). The current study is the first to report that delayed feeding did not increase IP as indicated by FITC-d concentrations on day 2, 4 and 7. There are two potential explanations for this. Firstly, delayed feeding may not have increased IP in very young chickens, possibly due to some yolk being absorbed into the small intestine (Noy and Sklan 2001), potentially reducing the effect of fasting on IP. Secondly, it is also possible that feeding after 24 h restored the permeability to a healthy state. In an earlier study it was shown that microscopic changes in the enterocytes of fasted chickens were reversed after refeeding for 24 h (Yamauchi and Tarachai 2000). However, IP was not measured in that study. Nonetheless, the current study shows for the first time that 24 – hour delayed feeding did not increase IP on days 2, 4 and 7. Additionally, the current study has shown that FITC-d permeation in Control birds on day 2 and 4 was 0.6 µg/ml, which increased to 0.8 µg/ml on day 7 ($p < 0.05$), suggesting that IP can change quickly with age, even in normal healthy chickens. These concentrations are numerically lower than our previous studies (Gilani et al. 2016a) in which Control birds were shown to have FITC-d permeation at 1.91 µg/ml. This could have been due to age differences in birds, 21 days of age compared to seven days of age in the current study. Vicuna et al. (2015) have shown FITC-d concentration of 0.2 µg/ml in young chickens. Finally, when compared to the Control and DF groups, supplementation with glutamine increased IP on each day ($p < 0.05$), whereas supplementation with sodium butyrate increased IP on day 2 ($p < 0.05$) and on day 4 and 7 only numerically ($p > 0.05$). These results are incongruent with previous studies conducted in other species (Le Bacquer et al. 2003; Beutheu et al. 2014; Han et al. 2015; Huang et al. 2015). However, this is the first time the effects of these additives on IP have been investigated in newly-hatched chickens *in vivo*, and require further investigation.

Possible reasons why these feed additives showed different results are summarized as follows. Sodium butyrate has been shown to increase IP in rat colon in Ussing chambers (Mariadason et al. 1999). In a recent study it was reported that over production of short chain fatty acids led to increased IP in rats (Ten Bruggencate et al. 2005). Additionally, in another study, sodium butyrate decreased IP at 2 mM concentration and to increase IP at 8 mM concentration in human Caco-2 cells (Peng et al. 2007). This suggests that the effects of sodium butyrate on IP may be dose dependent and hence further research is required to find the optimal dose for improving IP in chickens.

The European Food Safety Authority (EFSA) has concluded in their report of scientific opinion that there was insufficient evidence for glutamine in improving IP in humans (EFSA 2011). In a recent study in humans, mortality was increased in a glutamine supplemented group in critically ill patients (Mundi et al. 2016). Similarly, IP was not different in glutamine supplemented subjects in Crohn's disease (Den Hond et al. 1999) and in children following digestive tract surgery (Albers et al. 2005). These studies, along with the limited literature in chickens, suggest that further investigations are required for utilizing glutamine to improve IP in chickens. Potentially, glutamine could be converted to nitric oxide (Santos et al. 2014) which has been known to increase intestinal damage at higher concentrations (Mercer et al. 1996). Since glutamine was supplemented through water in addition to the normal diet, the excess nitrogen may have caused detrimental effects on IP in chickens as reflected in the body weight results in the current study.

DAO and D-lactic acid tests have been utilized in chickens as biomarkers of increased IP (Gilani et al. 2016). The results, however, were not different significantly and the probable reason could be that sodium butyrate or glutamine at the tested levels did not cause severe intestinal inflammation. This may have led to FITC-d permeation but not permeation of DAO and D-lactic acid, as the molecular weights of DAO and D-lactic acid are much higher (250 k Dalton(Da)) compared to FITC-d (4 k Da) (Gilani et al. 2016). We conclude that delayed feeding did not impact on IP as measured on days 2, 4 and 7 of age in chickens. Additionally, sodium butyrate and glutamine increased IP *in vivo*. Further studies are required to investigate the optimal dose of sodium butyrate and glutamine for their potential to improve IP in chickens.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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