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Natural Betaine Maintains Intestinal Epithelial Cell Integrity and Energy Levels and Does Not Induce Immune Reactions in an *in vitro* Caco-2 Model Compared with Synthetic Betaine-HCL

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Abstract: Betaine is commonly used in feed, improving feed conversion ratios and performance in animals. Betaine can be supplemented as natural betaine, isolated from natural sources; synthetic anhydrous betaine or synthetic betaine-HCl. Scientific data on the comparison between synthetic betaine-HCl and natural betaine are limited, prompting us to examine whether the 2 forms of betaine have disparate cellular effects. Natural betaine or betaine-HCl was mixed with feed in equimolar amounts and subjected to *in vitro* digestion, mimicking the conditions in the upper gastrointestinal tract of poultry. After digestion, the soluble component was separated and Caco-2 intestinal epithelial cells were used to examine how soluble factors affect inflammatory markers, ATP and tight junction integrity at 1, 6 and 24 h after treatment. Soluble factors from *in vitro*-digested feed that was supplemented with betaine-HCl changed significantly compared with those from control feed or feed to which natural betaine was added. Tight junction integrity at 1 and 6 h decreased, recovering to basal levels at 24 h. ATP levels declined after 1 h and were restored to baseline values after 6 and 24 h. IL-8 rose after 6 and 24 h as did IL-6 after 24 h, indicating that betaine-HCl induces inflammatory responses in Caco-2 cells compared with natural betaine and the control. Our results show that despite possessing the same core betaine molecule, synthetic betaine-HCl induced cellular changes that were indicative of transient damage in cells. Thus, the supplementation of feed with various sources of betaine is not equal at the cellular level which can influence animal performance and welfare, especially during stress.

Key words: ATP, transepithelial electrical resistance, IL-8, *in vitro* digestion, Caco-2, influence animal

INTRODUCTION

Chemically, betaine, also known as trimethylglycine is a trimethyl derivative of the amino acid glycine. Betaine is a naturally occurring molecule in various plant and animal species (Eklund *et al*., 2005; Ross *et al*., 2014; Amerah and Ravindran, 2015). For animals receive a sustained and controlled source of betaine, it is used as a supplement in feed. In poultry and pigs, betaine enhances the digestion and absorption of nutrients; stimulates bacterial fermentation of fiber; improves feed conversion ratios (Metzler-Zebeli *et al*., 2009; Ratriyanto *et al*., 2010), breast meat yield and meat quality and reduces carcass fat (Eklund *et al*., 2005).

Physiologically, betaine acts as a methyl-group donor and an osmolyte. The intestinal luminal content is hyperosmotic and in its capacity as an osmolyte, betaine is particularly important regarding cellular dehydration, minimizing water loss against the prevailing osmotic gradient (Kettunen *et al*., 2001a). Betaine has been suggested to be especially useful at high environmental temperatures (Cronj'e, 2006) and in coccidiosis by positively influencing water balance which is disrupted during the infection due to malabsorption and diarrhea and thus, improving the performance of animals (Fetterer *et al*., 2003). During this infection, betaine affects intestinal cells by increasing their water-binding capacity (Kettunen *et al*., 2001a, b), stabilizes the structure of the mucosa (Kettunen *et al*., 2001b) and reduces lesion scores (Amerah and Ravindran, 2015). Betaine also increases total cell activity as indicated by the higher activity of proteolytic enzymes in weaned pigs following supplementation of the diet with betaine (Xu and Yu, 2000).

As a methyl donor, betaine alters protein synthesis by enhancing the availability of methionine and influences several physiological functions such as membrane synthesis and acetylcholine formation by increasing the availability of choline (Eklund *et al*., 2005). It also upregulates the synthesis of methylated compounds such as carnitine which is required for the transport of fatty acids across the inner mitochondrial membrane where fatty acid oxidation occurs (Eklund *et al*., 2005).

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Betaine typically exists in animal feeds in its anhydrous form, extracted from sugar beet or as synthetic betaine-HCl. Despite the lack of evidence on synthetic betaine-HCl, it is considered to be used equally as natural betaine. Two animal trials have compared these betaines in broilers, showing that natural betaine improves their performance and nutrient digestibility better than synthetic betaine, especially under stress (Amerah, 2014).

MATERIALS AND METHODS

***In vitro* digestion of feed supplemented with betaine using simulated upper gastrointestinal tract:** *In vitro* digestion of feed with or without natural or synthetic betaine was performed by mimicking the chicken proximal digestive tract, using hydrochloric acid, pepsin and pancreatin. Ten grams of corn/soy-based feed was crushed with a mortar and supplemented with Betafin®S1 natural betaine (Danisco Animal Nutrition, Naantali, Finland) or commercially available synthetic betaine-HCl in amounts that corresponded to a betaine content of 2 kg/ton feed. Feed without betaine served as a control.

Two replicates of each digest were prepared. The conditions in the crop were mimicked by hydrating the feed with water at a 3:1 ratio. The pH of digests with betaine-HCl was adjusted to pH 5.5 using 1 M NaOH; the pH in the other digests was already approximately 5.5. The samples were incubated at 39°C for 20 min, after which the digestion in the proventriculus and gizzard was modeled by adding 5 mL of water, containing 9 mg pepsin (Sigma Aldrich, St. Louis, US). The pH was measured and adjusted to pH 2.5 with 1.5 M HCl. The resulting suspension was incubated at 39°C for 40 min, after which the digestive conditions in the small intestine were modeled by adjusting the pH to 6.3-6.5 with 1 M $NaHCO_3$ (Sigma Aldrich, St. Louis, US), containing 37 mg pancreatin (Sigma Aldrich, St. Louis, US) and providing constant stirring at 39°C for 60 min. The supernatant was collected by centrifugation at 10.000×g for 10 min and the digestive enzymes were inactivated by boiling the sample for 4 min. The supernatants were cooled on ice, aliquoted and stored at -20°C until use in the cell culture experiments.

Measurement of betaine and Trimethylamine (TMA) content from betaine sources and supplemented feed samples: The betaine content in the betaine preparations and in feed prior to and after the *in vitro* digestion was measured. Samples were diluted with water and cleaned by filtering by reverse-phase chromatography on a C18 column (Waters, Milford, MA, US). Then, they were analyzed by liquid chromatography on an HPX-87C cation exchange column (Bio-Rad, Helsinki, Finland), 0.001M with dilute $Ca(NO_3)_2$ used as the eluent with flow rate 0.8 ml/min, column temperature +85°C and refractive index as detector. The quantification was based on an external standard, using peak heights to calculate the amount of betaine in the sample. Betaine has a retention time of 23 min.

Trimethylamine (TMA) content was also measured in the natural betaine and synthetic betaine-HCl samples. TMA was determined by static headspace gas chromatography-mass spectrometry (Agilent Technologies, Santa Clara, CA, US). In this method, the sample is made basic with Potassium hydroxide and salted out with 5% NaCl and is analyzed with gas chromatography consisting of CP-SIL 8CB column, helium as carrier gas with constant flow 1.1 mL/min, temperature program from 33-170°C (25°C/min) and mass spectrometer as detector. The identification of TMA is made with specific ion m/e = 58 and quantitation is made with inner standard. This method is semi quantitative, due to the lack of repeatability of headspace injections.

Solubility assay for natural and synthetic betaine: The dissolving rate and solubility of the 2 betaine products were tested at 25 and 40°C. A water bath and underwater magnetic stirrer were used to maintain identical temperatures and mixing conditions. The assays were performed in 250 mL decanters with 100 mL water. A specified quantity (g) of betaine (calculated from the sample purity) was placed in 100 mL water and mixed with a magnetic stirrer at a constant rate. The apparent concentration of the liquid phase (Brix, sucrose Dry substance % W/W) was measured after 1, 5, 10, 15 and 60 min on a refractometer (Automatic Refractometer GPR 11-37, Index Instruments, Huntingdon, UK). If any crystalline material remained, water was added (and weighed) until the entire product was dissolved. Apparent concentration was measured to confirm the limit of solubility.

Caco-2 cell culture and differentiation: Caco-2 cells (ATCC, LGC Standards, Boras, Sweden) were maintained in basal media, comprising Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, MA USA) that was supplemented with 20% fetal bovine serum (Life Technologies), 1×nonessential amino acids (Thermo Fisher Scientific), 1×sodium pyruvate (Thermo Fisher Scientific), 20 U/mL penicillin, 20 mg/mL streptomycin and 0.5 mg/mL amphotericin (Thermo Fisher Scientific), at 37°C in a 5% CO_2 atmosphere.

Caco-2 cells were differentiated for 21 days under asymmetric conditions (Ferruzza *et al*., 2012). Briefly, 3.5×10^5 cells/cm^2 were plated on cell culture inserts with 0.4 μm pores (Corning, NY, US) that were coated with rat

type 1 collagen (Corning, NY, US) using serum-containing basal medium on the apical and basolateral compartments. On Day 3, the cell culture medium was shifted to asymmetric conditions, wherein the apical compartment was supplemented with basal medium without serum and the basal compartment was supplied with serum-containing basal medium. The medium was changed 3 times/week and the cells were used on day 21 and the Transepithelial Electrical Resistance (TEER) of the cells was verified to exceed 200 ohm×cm^2.

Treatment of differentiated Caco-2 cells with soluble factors from *in vitro*-digested feed supplemented with natural or synthetic betaine: The basolateral medium was replenished with fresh basal medium, whereas the apical medium was replaced with test medium that contained supernatant from the *in vitro*-digested feed that was prepared above. The *in vitro*-digested feed was diluted 1:1 in bicarbonate-free DMEM (Thermo Fisher Scientific) that was supplemented with 1×nonessential amino acids (Life Technologies), 1×sodium pyruvate (Thermo Fisher Scientific), 20 U/mL penicillin, 20 mg/mL streptomycin and 0.5 mg/mL amphotericin (Thermo Fisher Scientific). The pH in all samples was adjusted to 6.8 which was the pH in the control digestion without betaine supplementation, using 1 M $NaHCO_3$, after which the media was passed through 0.2 μm sterile syringe filter units (Sartorius, Goettingen, Germany). The cells were incubated with the *in vitro*-digested feed for 1, 6 and 24 h.

Measurement of Transepithelial Electrical Resistance (TEER) in Caco-2 cells: The integrity of the monolayer was verified by measuring the TEER before applying on the cells the 1:1 diluted supernatant from *in vitro* digested feed and after 1 and 6 h of incubation using an EVOM 2 vΩm (World Precision Instruments, Hitchin, UK). The background TEER of the insert was subtracted from the TEER in the monolayer and insert to yield the monolayer resistance and was multiplied by the area of the insert to generate values in ohm×cm^2.

Measurement of ATP content in Caco-2 cells: The ATP content in the cells was measured by ATP Lite luminescence assay (Perkin Elmer, Waltham, US) with modifications. The cells were lysed in 150 μL of lysis buffer (provided in the kit) and transferred to Eppendorf tubes. The lysate was then diluted 1:30 with PBS (Life Technologies) from which 50 μL was transferred to white microplates for luminometric measurements (Perkin Elmer). Then, 50 μL of substrate solution was pipetted into each well and the plate was shaken for 5 min after 10 min in the dark, the luminescence was measured on a 1450 Microbeta scintillation counter (Perkin Elmer).

Measurement of cytokine response by Caco-2 cells: The cytokine response of Caco-2 cells to *in vitro*-digested feed was measured in samples of basolateral medium from the experiment above. The samples were stored at -80°C and thawed before analysis. Cytokine levels were measure by multiplex sandwich ELISA (Aushon Biosystems, Billerica, MA, US) using array 84619B which contained spotted primary antibodies against IL-1α, IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12p70, IFNγ and TNF-α. In brief, triplicate samples were diluted 1:2 in Assay Buffer, applied to a single 96-well plate in duplicate and incubated at room temperature for 1 h on a shaker (200 rpm). The plate was washed 3 times on a BioTek ELx50™ plate washer (BioTek, Winooski, US) with wash buffer provided by Aushon (Aushon Biosystems, Billerica, MA, US). Biotin-conjugated secondary antibody and streptavidin-horseradish peroxidase were added sequentially to the wells, the addition of each reagent was followed by three washes with same wash buffer as above. Luminol was added to the plate and signals were measured on a signature plus analyzer (Aushon biosystems). The data were analyzed in ProArray (Aushon biosystems).

Statistical analyses: The significance of differences between treatments in the Caco-2 assays was determined by one-way ANOVA with Tukey's post hoc test in GraphPad Prism, Version 7. Probability (p)-values of 0.05 or less were considered to be significant. All comparisons were made against the respective control treatment.

RESULTS AND DISCUSSION

Betaine and trimethylamine content and solubility of natural and synthetic betaine. Equimolar amounts of synthetic and natural betaine were included in the *in vitro* gastrointestinal digestion model, based on the betaine content in the original betaine samples. The betaine content in the synthetic betaine that was used in the cell culture studies was 73% whereas natural betaine was 97% betaine. The betaine content in the *in vitro* digestions was adjusted according to these values to correspond to a betaine level of 2 kg/ton feed. After *in vitro* digestion of the corn/soy feed, the betaine content was redetermined to be 2.38 and 2.39 mg/mL of digestion supernatant in the two digestions with natural betaine, versus 2.30 and 2.25 mg/mL digestion supernatant with synthetic betaine. The control had a betaine content of <0.05 mg/mL. These results confirm that the *in vitro*-digested feed samples with synthetic or natural betaine had comparable amounts of betaine whereas the control digestion had negligible levels.

We examined whether there were any traces of precursors in the sources of betaine. The TMA content in betaine-HCl was 5594 mg/kg and no TMA was detected in natural betaine sample.

The solubility of natural betaine and synthetic betaine-HCl was measured at 25°C and 40°C. Natural betaine (160 g/100 g water) was 3.2 times more soluble than synthetic betaine (50 g/100 g water) at 25°C. Natural betaine also dissolved faster than synthetic betaine (3 min vs. 8 min). The solubility of natural betaine increased at 40°C-172 g/100 g water whereas that of synthetic betaine was 60 g/100 g or 2.8 times less soluble. The complete dissolution of natural betaine took 3 min at 40°C while synthetic betaine dissolved in 7 min.

Changes in transepithelial electrical resistance in Caco-2 cells by *in vitro* digested feed amended with betaine or betaine-HCl: The effect of soluble factors from *in vitro*-digested feed with betaine or betaine-HCl on intestinal epithelial cell tight junction integrity was examined with asymmetrically differentiated Caco-2 cells after 1, 6 and 24 h. Treatment with soluble factors from *in vitro*-digested feed that was supplemented with synthetic betaine decreased TEER values compared with natural betaine after 1 h ($p<0.0005$, Fig. 1), remaining lower after 6 h ($p<0.001$). Although natural betaine increased TEER at 1 and 6 h these changes did not reach significance ($p = 0.106$ and $p = 0.096$, respectively). The decrease in TEER due to soluble factors from *in vitro*-digested feed with betaine-HCl reverted to basal levels at 24 h at which time no difference between treatments was visible.

Changes in ATP levels in Caco-2 cells by *in vitro* digested feed amended with betaine or betaine-HCl: The influence of soluble factors from betaine and betaine-HCl-supplemented *in vitro*-digested feed on intestinal epithelial cell metabolism was determined, based on intracellular ATP content in asymmetrically differentiated Caco-2 cells after 1, 6 and 24 h of treatment. ATP levels declined significantly after a 1 h incubation with soluble factors from *in vitro*-digested feed that was supplemented with synthetic betaine (Fig. 2) compared with control ($p<0.0001$) and natural betaine-supplemented *in vitro*-digested feed ($p<0.0001$). ATP content returned to basal levels at 6 h and was comparable with the amounts in the control and betaine conditions at 24 h.

Production of cytokines in Caco-2 cells by *in vitro* digested feed amended with betaine or betaine-HCl: The cytokine response of asymmetrically differentiated Caco-2 cells to soluble factors from *in vitro*-digested feed that was supplemented with betaine or betaine-HCl was monitored for 1, 6 and 24 h in culture. IL-1α, IL-1β, IL-2, IL-10, IL-12p70, IFNγ and TNF-a were undetectable.

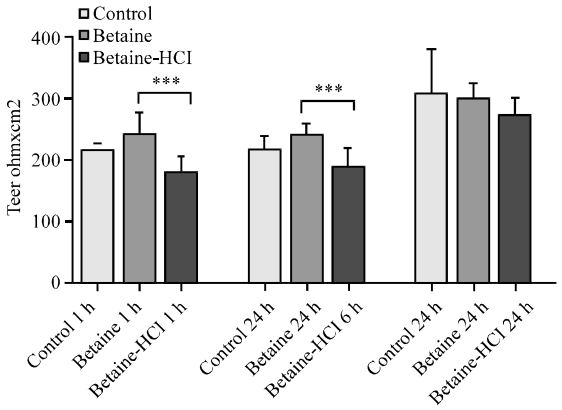

Fig. 1: Tight junction integrity (TEER, ohm×cm^2) measured in Caco-2 cells treated with soluble factors from *in vitro*-digested feed supplemented with betaine or betaine-HCl after 1, 6 and 24 h. Mean+SD is shown ***$p<0.001$

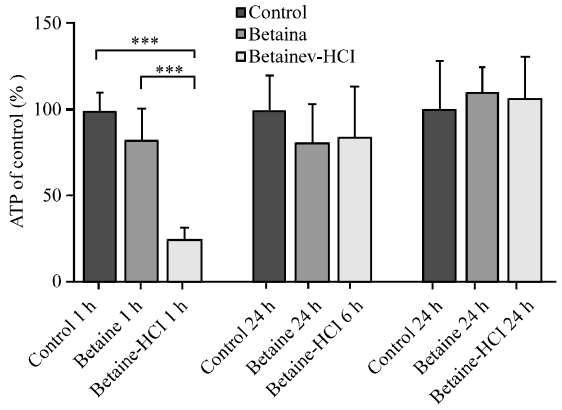

Fig. 2: ATP levels (ATP % of control) in Caco-2 cells treated with soluble factors from *in vitro*-digested feed supplemented with betaine or betaine-HCl after 1, 6 and 24 h. Mean+SD shown ***$p<0.001$

After 24 h, Caco-2 cells produced, however, lower IL-6 levels when the cells were treated with soluble factors from natural betaine-containing feed versus cells treated with betaine-HCl and the control ($p<0.05$) (Fig. 3a). At 1 and 6 h there was no difference in IL-6 production between the three treatments. Additionally, Caco-2 produced higher level of IL-8 after 6 and 24 h when the cells were treated with soluble factors from betaine-HCl-supplemented feed versus the control and natural betaine ($p<0.05$) (Fig. 3b).

There is a lack of comparative research between synthetic and natural betaine products in animal feed-only 2 animal trials have been conducted (Amerah, 2014). This study is the first to compare the biological effects of natural and synthetic betaine-HCl products at the cellular level. Synthetic betaine-HCl induced rapid declines in

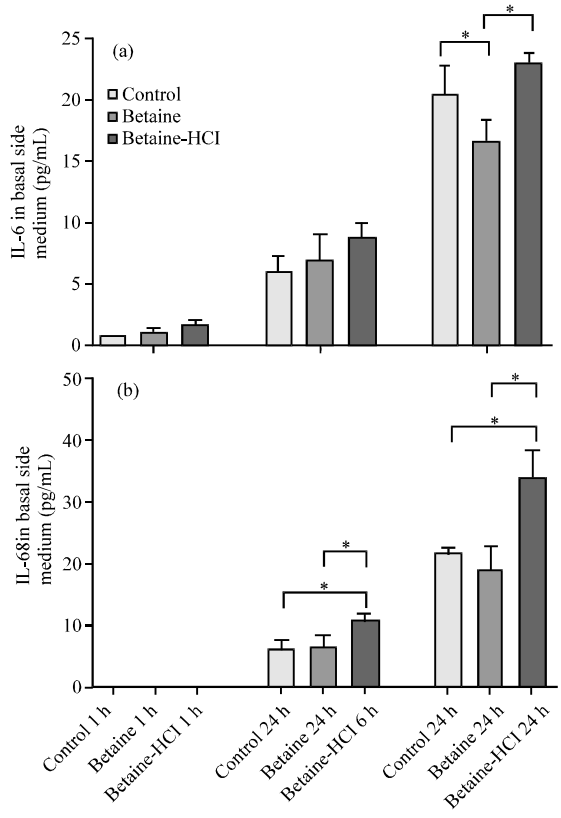

Fig. 3: IL-6 and IL-8 response by $Caco_2$ cells to *in vitro*-digested feed supplemented with betaine or betaine-HCl after 1, 6 and 24 h. Mean+SD shown, *p<0.05

tight junction integrity and cellular ATP content after 1 and 6 h and upregulated the inflammatory cytokines IL-6 and IL-8 after 24 h.

Tight junctions between intestinal epithelial cells are dynamic multiprotein complexes that form a selective semipermeable paracellular barrier that facilitates the passage of ions and other solutes through the intracellular space while preventing the translocation of luminal antigens, micro-organisms, and their toxins (Ulluwishewa *et al*., 2011). The decrease in TEER that was induced by betaine-HCl indicates that there are components in synthetic betaine-HCl that increase paracellular permeability which can affect the leakage of harmful luminal or apical substances to the basolateral side thus eliciting immune responses (Suzuki, 2013). In the *in vitro* digestion supernatants, the betaine content was comparable between synthetic and natural betaine. Thus, the difference in betaine content cannot explain the cellular effects that were caused by betaine-HCl. We hypothesize that the rise in IL-6 and IL-8 in betaine-HCl-treated cells at 24 h was secondary to the increase in paracellular permeability.

This study lacked a control in which natural betaine was combined with hydrochloric acid for the *in vitro* digestion to simulate betaine-HCl. However when hydrochloric acid from betaine-HCl dissolves, it provides only 0.01-0.02% of chloride ions when supplementing feed at similar amounts as natural betaine-this amount is insignificant, considering that the optimal dietary requirement for chloride is 0.25% for a young broiler chicken (Oviedo-Rondon *et al*., 2001). Thus, the amount of chloride ions that are provided by betaine-HCl is negligible in the *in vitro* digestion samples. Further, we adjusted the pH in each sample to correspond to values that are normally observed in corresponding regions of the chicken gastrointestinal tract (Dono *et al*., 2014).

Two benefits of using natural betaine in feed are its higher solubility compared with betaine-HCl and faster dissolution rate. The lower solubility of betaine-HCl reduces its osmolytic capacity (Eklund *et al*., 2005). We also found that betaine-HCl contains TMA at 6 g/kg. Synthetic betaine is generated from a reaction between TMA and chloroacetic acid in the presence of sodium hydroxide (Vassel, 1957; Nagy, 1969). TMA is a volatile tertiary aliphatic amine that smells of rotting fish and is readily absorbed from the gastrointestinal tract, the majority of which is excreted in the urine (Al-Waiz *et al*., 1987). Various toxic effects have been ascribed to TMA by *in vitro* and *in vivo* studies, ranging from abnormal neurological symptoms (Simenhoff *et al*., 1978) to teratogenic effects (Guest and Varma, 1993) and disruptions in rRNA synthesis (Shiokawa *et al*., 1986), and it is potentially carcinogenic (Lijinsky *et al*., 1972). TMA is corrosive to the eyes and skin and is a known irritant to the respiratory tract (Kenyon *et al*., 2004).

Based on these properties, it is possible that residual TMA weakened the tight junction integrity and decreased ATP levels thus upregulating inflammatory markers. The mucus layer that covers the intestinal wall provides a physical barrier that protects the underlying epithelium (Allen and Flemstrom, 2005). Small molecules can penetrate this barrier, causing exfoliation of the epithelial layer and a concomitant increase in mucosal permeability (Allen and Flemstrom, 2005). In poultry, the digestive process is quick and in pigs, gastric ulceration is common (Melnichouk, 2002), causing significant losses to pig farms (Millet *et al*., 2012). This type of ulceration might exacerbate the effects of TMA or other trace elements in betaine-HCl. When TMA was administered orally to rats, 75% of the dose was excreted in the urine within 24 h, indicating that trimethylamine is absorbed from the gastrointestinal tract (Al-Waiz and Mitchell, 1991), thus, TMA has possible systemic toxic effects.

At the cellular level, ATP levels fell in cells that were treated with betaine-HCl-supplemented *in vitro*-digested feed after 1 h compared with control feed and betaine-supplemented feed. This decrease reflects impaired efficiency of the respiratory chain enzymes in mitochondria, the machinery that generates cellular energy-an effect that can be caused by for instance, Reactive Oxygen Species (ROS) (Taha *et al*., 2010) and is a hallmark of apoptosis (Skulachev, 2006). Notably, ATP levels were restored in the betaine-HCl-treated cells after 6 h which might be attributed to the betaine component of the molecule, betaine stimulates mitochondrial and cellular respiration, increases the membrane potential of mitochondria and elevates ATP levels (Lee, 2015). We did not observe an increase in ATP after betaine treatment alone as reported by Lee (2015) which might have been due to differences in how betaine content was normalized. Lee (2015) normalized ATP content to the total cell protein concentration whereas we compared it directly with the amount of ATP in the control digestion.

Besides TMA of the compounds that are used to synthesize betaine-HCl, chloroacetic acid-also, called monochloroacetic acid-is another explanation of the cellular effects. Chloroacetic acid is absorbed rapidly after ingestion (Kaphalia *et al*., 1992) and damages the blood-brain barrier (Berardi *et al*., 1987). In Neuro-2a cells, chloroacetic acid from 0.1-3.0 mM dose-dependently induces the formation of ROS and effects mitochondrial dysfunction which can trigger apoptosis (Chen *et al*., 2013; Lu *et al*., 2015). ROS increase paracellular permeability (Katsube *et al*., 2007) and are associated with inflammatory responses that are mediated by macrophages, neutrophils, cytokines and chemokines (Zhu and Kaunitz, 2008). IL-8 is a proinflammatory cytokine that is stimulated by ROS and as a chemoattractant, activates neutrophils, further generating more ROS (Paszti-Gere *et al*., 2012). IL-6 in turn is a pleiotropic cytokine and is upregulated in inflamed intestinal mucosa (Maloy and Powrie, 2011; Suzuki, 2013). IL-6 increases paracellular permeability directly by elevating pore-forming claudin-2 levels in intestinal cells (Yang *et al*., 2003; Suzuki, 2013; Al-Sadi *et al*., 2014). This upregulation then increases the permeability of small molecules below 4 Å *in vivo* and *in vitro* (Al-Sadi *et al*., 2014).

Notably, heat stress, often encountered by farm animals, elevates ROS (Yu *et al*., 2013). Thus, it would be interesting to determine whether heat augments these effects on permeability and the cytokine response with betaine-HCl treatment and whether betaine-HCL treatment upregulates ROS in the intestinal epithelial cell model that was used in our study. The inflammatory response and paracellular permeability were restored at 6 h possibly due to the betaine component of the samples. Betaine has anti-inflammatory effects, downregulates IL-6 (Olli *et al*., 2013) and improves tight junction integrity in intestinal epithelial cells when co-administered with ethanol which increases permeability (Thomes *et al*., 2015).

The difference between natural betaine and synthetic betaine-HCl has been examined previously in 2 broiler trials (Amerah, 2014). When broilers were exposed to heat stress, natural betaine was superior over synthetic betaine-HCl in improving carcass breast meat yield, bodyweight gain and feed conversion ratio. In another study that used the Eimeria challenge model, ileal digestibility was enhanced by both betaines but only broilers that were fed natural betaine recovered from the impairments in nutrient digestibility that were caused by the coccidia challenge. Gastrointestinal tract challenges, which involve immune system activation can divert nutrients and energy away from growth (Korver, 2006). It is possible that by increasing paracellular permeability and luminal antigen flow inside of the body, synthetic betaine could induce *in vivo* unnecessary inflammatory responses,, that could reduce the amount of available energy for broiler growth and thus limiting performance (Yegani and Korver, 2008). Our observation on the reduction in ATP, the cellular energy molecule by betaine-HCl is significant in this respect as well. Thus, the immune response of broilers that are fed either with natural betaine or synthetic betaine-HCl should be measured and these observations warrant further *in vivo* study.

Synthetic betaine-HCl when digested with feed in an *in vitro* digestion model and applied to intestinal epithelial cells, decreases paracellular permeability, upregulates proinflammatory cytokines and lowers cellular levels of ATP. The gastrointestinal tract is constantly exposed to luminal substances that are prevented from entering the body. Thus, the increase in permeability of intestinal epithelial cells by betaine-HCl even transiently could affect inflammation of the intestine and consequently decrease the performance of animals.

CONCLUSION

To examine the biological equivalence of these forms of betaine and explain the differences between feeding trials at the cellular level we conducted a series of experiments using a well-defined intestinal epithelial cell line, Caco-2. We supplemented soy/corn-based feed with natural betaine or synthetic betaine-HCl in equal amounts and simulated the chicken upper gastrointestinal tract to digest the feed *in vitro*. We then applied soluble factors

from the digested feed to Caco-2 cells and monitored the resulting changes in the intestinal barrier, energy metabolism and inflammatory profile.

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