

Short Communication: Effects of dietary inulin and animal density on stress in pigs^{1,2}

Pawel Siegien[†], Margot Gillis[†], Jiaheng Li[†], Sandrine Dufourny[‡], Luke Comer[§], Marc Vandenheede[#], José Wavreille[‡], Jérôme Bindelle^{†*}, Martine Schroyen^{†*}

[†] Animal Sciences, TERRA Teaching and Research Centre, Gembloux Agro-Bio Tech, University of Liège, 5030 Gembloux, Belgium

[‡] Animal production unit, Walloon Agricultural Research Centre, 5030 Gembloux, Belgium

[§] Animal and Human Health Engineering, KU Leuven, 3001 Leuven, Belgium

[#] Fundamental and Applied Research for Animal & Health (FARAH), Department of Veterinary Management of Animal

d Resources, Faculty of Veterinary Medicine, University of Liège, 4000 Liège, Belgium

* Both authors contributed equally to this study

Abstract

Cortisol is a key glucocorticoid hormone involved in the physiological stress response of animals. Its secretion, largely regulated by the hypothalamic–pituitary–adrenal (HPA) axis, is essential for animal welfare and growth. Recent studies have shown that this stress-regulatory system operates in coordination with the gut microbiota, communicating with the brain through the gut-brain axis. Given that the diet can modulate microbial composition, dietary interventions thus offer potential in modulating HPA activity. Inulin is a prebiotic that promotes beneficial gut microbiota such as *Lactobacilli* and enhances intestinal health. Microbial fermentation exerts anti-inflammatory effects and strengthens the gut barrier through short-chain fatty acids, potentially influencing stress physiology via the gut-brain axis. In this study, we focused on the effects of inulin on cortisol in saliva and hair samples of pigs as indicators of acute and chronic stress responses, respectively. Animals were housed at low or high density and received inulin supplementation or none. Mixing at weaning and at the start of fattening was used as an acute social stressor. Saliva samples were collected from 3 pens per experimental group, from 84 animals before and after animal mixing. Due to contamination, only 153 samples were retained for analysis. Hair samples were collected from 48 animals at the end of the post-weaning phase and 5 weeks into the fattening phase. Due to insufficient hair material, only 53 samples were suitable for analysis. Cortisol quantification was done by ELISA. Data were analysed using a Four Parameter Logistic (4PL) calibration curve, and linear mixed-effects models (LLMs) were performed on the obtained results to verify significance. Inulin reduced basal salivary cortisol levels before the second animal mixing ($P < 0.05$) but

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pre- and post-mixing cortisol levels did not differ significantly at either phase, nor did cortisol elevation differ between groups. For chronic stress, a significant interaction between animal density and diet was observed at the end of the post-weaning period ($P < 0.05$), where inulin mitigated the effect of high density. Overall, environmental and dietary factors jointly influenced stress responses. To conclude, beyond its well-established benefits for intestinal health, inulin may serve as a promising dietary strategy to enhance animal welfare by modulating circulating cortisol levels and mitigating stress responses in pigs.

Key words

pig density, cortisol, inulin, welfare, gut-brain axis

Lay summary

Cortisol is a key hormone released when animals experience stress. When cortisol rises too high or too often, negative consequences for welfare and development may occur. Because the gut and the brain are tightly connected, an animal's diet can influence how it responds to stress.

This study examined whether supplementing pig diets with inulin could influence cortisol levels. Salivary cortisol was measured to assess short-term stress and hair cortisol evaluated longer-term stress. Pigs were kept in either high or low housing density and were mixed at weaning and at the start of the fattening phase to induce stress.

The results indicated that pigs receiving inulin showed lower baseline salivary cortisol before the second mixing event. With respect to longer-term stress, hair cortisol levels were elevated by the housing density, but this effect was reduced by the inulin supplementation when measured during the post-weaning phase.

Overall, these findings suggest that inulin supplementation can help reduce cortisol levels in pigs and may contribute to improved animal welfare.

Teaser text

Commonly used prebiotics such as inulin can reduce stress levels in pigs, illustrated by a reduction in cortisol levels.

List of Abbreviations

HPA, hypothalamic–pituitary–adrenal; 4PL, Four Parameter Logistic

INTRODUCTION

Weaning is widely regarded as one of the most stressful events in the life of a pig, and can have profound consequences, including death (Gresse *et al.*, 2017). Separation from the sow, dietary change, a new environment and mixing with unfamiliar pen mates often lead to anorexia and post-weaning diarrhoea, thereby impairing nutrient absorption and reducing growth (Campbell *et al.*, 2013). While occurring early in life, weaning stress can negatively shape growth trajectories and long-term development, making effective stress management critically important (Faccin *et al.*, 2020). Together, these factors highlight the need for appropriate measures to mitigate stress.

Post-weaning diarrhoea can be alleviated by prebiotics such as inulin, which improve intestinal integrity and modulate gut microbiota composition (Davani-Davari *et al.*, 2019). Beyond gut health, changes in microbiota can influence well-being through the gut-brain axis, notably via regulation of the HPA axis and cortisol secretion (Cryan & Dinan, 2012, Gur & Bailey, 2016). Based on this microbiota–gut–brain connection, this study investigated whether inulin supplementation could modulate cortisol levels in pigs, assessing both acute and chronic stress responses. Through saliva measures, we hypothesized that dietary inulin would attenuate cortisol levels and modulate stress reactivity in weaned pigs. As for hair measures, we further hypothesized that high stocking density would induce a state of chronic stress, and that this effect would be mitigated by inulin supplementation.

MATERIAL AND METHODS

Animal Trial Design

All animal procedures were reviewed and approved by the Institutional Animal Ethics Committee of the University of Liège (ULiège), Belgium (ethical approval dossier no. 23-2573). The experiment was conducted in accordance with Directive 2010/63/EU of the European Parliament and of the Council on the protection of animals used for scientific purposes, as implemented in Belgian law, and followed internationally recognized standards for the care and use of agricultural animals in research and teaching. Animal care and experimental procedures were consistent with internationally accepted guidelines, including principles comparable to those described in the ADSA–ASAS–PSA Guide for the Care and Use of Agricultural Animals in Research and Teaching. This study assessed the effects of inulin on pig welfare, focusing on its potential to mitigate social stress as reflected by reduced cortisol levels. Indeed, regular animal mixing in pig production disturbed social structures and induced social stress. To reach this objective, pigs (Landrace x Piétrain) were raised under different conditions using a 2 x 2 factorial design, incorporating two factors: (1) the animal density and (2) the diet. The trial spanned the entire life of a pig in production, starting from the pre-weaning phase through the post-weaning phase, and concluding with the fattening phase. Piglets remained in the same experimental

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group throughout the study. Twenty-one sows were farrowed and the piglets were weaned at 28 days of age. A total of 144 piglets, with both sexes pooled, entered the post-weaning pens while only 96 pigs remained for the fattening pens at 9 weeks of age. Excluded animals were maintained under standard rearing conditions outside the experimental framework. Within the study pens, they served to increase stocking density and social interactions, acting as social partners. Before animal mixing, at the pre-weaning phase, all the piglets from the same mother remained together with no contact with piglets from other pens. All piglets received an iron injection and had their teeth and tails clipped, and the males were castrated.

The first factor, animal density, was tested in two modalities defined by differing numbers of animals per pen while simultaneously respecting the European directive regarding space availability for rearing pig (Directive 2008/120/EC). These different modalities were applied after animal mixing at weaning, with 0.36 m² per pig at high density (14 pigs/pen, 6 pens) and 0.51 m² per pig at low density (10 pigs/pen, 6 pens) where the total size of a post-weaning pen was 5.1 m². Five weeks later, after another animal mixing at the beginning of the fattening phase, pigs were put in pens with 0.96 m² per pig at high density (10 pigs/pen, 6 pens) and 1.60 m² per pig at low density (6 pigs/pen, 6 pens) where the total size of the fattening pen was 9.6 m². Throughout all experimental phases, pigs were housed in straw-bedded, solid-floored pens equipped with a feeder and a water dispenser. Pen size was consistent within each phase, although layouts differed. During the post-weaning phase, 4 pens were arranged side by side in 3 rows, each measuring 3.4 m in length and including a 1.4 m manure-scraper area, with 1.45 m corridors separating rows. During the fattening phase, six pens were arranged side by side in two rows. The manure scraper zone was shared between the two rows and occupied 0.8 m per row of the total 4.8 m.

The second factor was the diet, with commercial, concentrated feed either supplemented or not with inulin (Orafti SIPX, Beneo, Belgium) at a 1 % dosage. As the pigs grew, the diet was adjusted to meet their evolving nutritional requirement. The diet switch was implemented simultaneously for both treatments, with and without inulin supplementation. The piglets destined to join the supplemented groups at weaning had been pre-exposed to inulin through creep feed starting at 2 weeks of age during the pre-weaning phase. Inulin supplementation was maintained in the feed of the inulin group throughout all subsequent phases.

Sampling

Saliva samples were collected from different animals during two production phases: at weaning (4 weeks of age) and at the beginning of the fattening phase (9 weeks of age). Within each phase, samples were obtained from the same animals at two time points (moment factor): before and after exposure to the stressor, namely animal mixing. At weaning, 3 animals per post-weaning pen were selected for saliva sampling. Before mixing, these piglets originated from all 21 pre-weaning pens, resulting in approximately one sampled piglet per pre-weaning pen. During the fattening phase, 4

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animals per pen were selected for sampling. However, some samples were discarded due to blood contamination, resulting in 31 and 46 usable samples for the weaning and fattening phases, respectively.

The first sampling point served as a means to establish basal cortisol levels. A second set of saliva samples was collected two hours after stress exposure. This was to allow piglets enough time for interactions while remaining in the elevated salivary cortisol temporal window as demonstrated by Merlot *et al.* (2004). Saliva was collected by handling piglets individually above their pens and by using oral swabs. Per collection time point, saliva sampling could take up to 1 hour. Swabs were centrifuged at 1500 RPM for 15 minutes to eliminate feed particles and mucus. Clear supernatant was frozen at -20°C until further analysis. With regard to the hair samples, 48 animals (4 per pen) were randomly selected at weaning for the hair collection. To ensure sampled hair represented only the targeted phase, animals were shaved at weaning on the collection zone at the lower back. Hair samples were subsequently collected from the same pigs at five weeks post-weaning, marking the end of the post-weaning phase, and five weeks later, during the fattening phase. Hair samples were taken as described by Heimbürge *et al.* (2020). In line with their recommendations, hair samples were systematically collected from the lower back zone, which is less prone to scratching or injuries during fights and is associated with higher cortisol concentrations. Before cortisol quantification in hair, cleaning, extraction and concentration steps were required. The protocol used was adjusted according to protocols proposed by Davenport *et al.* (2006), Casal *et al.* (2017), Prims *et al.* (2019), Heimbürge *et al.* (2020), and Levallois *et al.* (2024). In short, 70 mg of hair was washed twice with isopropanol, after which it was dried for 20 hours in the dark. Then, the washed hair was crushed in liquid nitrogen with a pestle and mortar. One mL of methanol was added to 50 mg of powdered hair and shaken vigorously for 24 hours. The cortisol-rich methanol was transferred into new tubes, which were concentrated using a vacuum concentrator (Eppendorf, Germany) at 45°C until completely dry. The obtained powder was rehydrated using 0.2 mL of phosphate buffered saline (PBS) for cortisol dosage.

Cortisol Quantification

Cortisol concentrations were determined in both saliva and hair samples using the salivary cortisol ELISA kit following the manufacturer’s guidelines (Salimetrics, cat n° 1-3002, USA) as illustrated by Davenport *et al.* (2006) and Casal *et al.* (2017). Measurements were done in duplicate. A Four Parameter Logistic (4PL) calibration curve was established using standardized cortisol solutions of known concentrations to convert absorbance values into concentrations micrograms per deciliter (µg/dL). The concentrations obtained from hair samples were then further converted to nanograms per gram (ng/g) to express the amount per unit weight of the analyzed hairs. Data points falling outside the quantification range were diluted and remeasured.

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Due to occasional saliva sample contamination by blood, some samples were excluded before analysis, resulting in an unbalanced dataset. Given the presence of repeated measures and minor missing data for saliva samples, linear mixed-effects models (LLMs) were preferred over repeated-measures ANOVA to avoid loss of observations and so account for unbalanced data structure.

The calculations were performed in Rstudio using the lme4, lmer.test and emmeans packages with animalID and pen as random factors and feed, density, moment and phase as fixed factors while statistical significance was set at $P < 0.05$. Given the strong phase $\times$ moment interaction observed in the global model for salivary cortisol, this interaction was further decomposed by analysing the data separately for each phase $\times$ moment combination. Within each subset, the effects of feed and density were evaluated while accounting for pen as a random effect. Estimated marginal means (EMMs) were subsequently compared using pairwise contrasts with Tukey-adjusted p-values. The moment factor is only applicable to the saliva samples due to the nature of the sampling.

Although hair cortisol sampling was initially planned as a repeated-measures design, incomplete longitudinal overlap between phases resulted in only a partially overlapping dataset. Of the 96 expected samples (48 pigs sampled at both phases), only 53 samples were ultimately collected from 41 pigs, with only 11 pigs successfully sampled at both time points. Similarly to the saliva cortisol analysis, feed and density were included as fixed effects, while pen was included as a random effect. Given the effect of phase observed in the global model, this factor was further decomposed by fitting separate models within each phase. Estimated marginal means (EMMs) were subsequently compared using pairwise contrasts with Tukey-adjusted p-values.

RESULTS

A significant difference was observed for the interaction between phase and moment ($P < 0.05$). When this interaction was further decomposed, we observed that salivary cortisol levels at weaning showed no significant differences between experimental groups, either before or after animal mixing. However, a difference in basal salivary cortisol was observed in inulin-fed piglets at the beginning of the fattening phase showing $0.18 \pm 0.18 \mu\text{g/dL}$ versus $0.47 \pm 0.58 \mu\text{g/dL}$ of the control group ($P = 0.02$). Indeed, in the low-density groups, piglets receiving inulin exhibited lower cortisol levels ($0.16 \pm 0.13 \mu\text{g/dL}$) compared to the control group ($0.51 \pm 0.60 \mu\text{g/dL}$) (Table 1). A similar pattern was observed in the high-density groups, with inulin-fed piglets showing reduced concentrations ($0.20 \pm 0.24 \mu\text{g/dL}$) relative to control animals ($0.44 \pm 0.59 \mu\text{g/dL}$). Two hours after stressor exposure, the cortisol levels rose to similar levels across all experiment modalities with low-density groups showing an average $0.50 \pm 0.37 \mu\text{g/dL}$ ($0.49 \pm 0.36 \mu\text{g/dL}$ in control groups and $0.51 \pm 0.41 \mu\text{g/dL}$ in inulin group) and high-density groups showing an average $0.34 \pm 0.31 \mu\text{g/dL}$.

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(0.36 ± 0.34 µg/dL in control groups and 0.32 ± 0.30 µg/dL in inulin group), indicating no marked effect of density or feed at this moment. Additionally, when statistical analyses were performed on the difference between cortisol levels at the two sampling moments (before and after the event) for each animalID, no significant effects were observed.

Hair cortisol levels measured at the end of the post-weaning phase were affected by the interaction between the animal density and the diet (P = 0.03) (Table 2). Control piglets in the high-density pens exhibited the highest concentrations (24.75 ± 6.89 ng/g) and inulin supplementation effectively mitigated this effect, reducing hair cortisol to 12.50 ± 8.59 ng/g. This attenuating property of inulin was not observed in low animal density pens where cortisol levels were lower overall, ranging from 15.37 ± 12.15 ng/g in inulin-fed piglets to 13.83 ± 10.68 ng/g in controls. However, pairwise comparisons between diet and animal density combinations did not remain significant after Tukey correction. After five weeks of hair growth during the fattening phase, neither animal density, nor inulin supplementation, independently or in interaction, showed any significant effect. A difference was observed between hair cortisol levels measured, regardless of feed or animal density parameters, during the post-weaning phase (16.28 ± 10.60 ng/g) and the fattening phase (10.25 ± 8.09 ng/g) (P = 0.084).

DISCUSSION

The scope of this animal trial was to characterize both acute and chronic stress based on cortisol measurements. Acutely elevated cortisol levels can be observed after animal mixing, and in this study the animals were mixed twice, at weaning and at the beginning of the fattening phase. This mixing procedure disrupts the social hierarchy, requiring the newly constituted group to establish a new one (Tong *et al.*, 2019). Our hypothesis was that inulin had an attenuating effect on cortisol elevation and could impact stress reactivity resulting in contrasting cortisol levels after stressor exposure. Through the saliva collection procedure, the study aimed to measure cortisol elevation while expecting distinct patterns of stress reactivity between the trial modalities. However, cortisol elevation was not significantly different between trial modalities at either time point suggesting that inulin was not able to rewrite the fundamental functioning of the HPA axis and change the perceived intensity of the stressor. Similar results were observed with probiotic studies conducted by Kelly *et al.* (2017) where *Lactobacillus rhamnosus* (JB-1) did not change the HPA axis response in healthy males. However, in our study, basal salivary cortisol levels were significantly altered due to inulin supplementation at the beginning of the fattening phase and thus, as suggested by Bus *et al.* (2023) and Ekel *et al.* (1996), systemic cortisol levels were probably altered as well. However, in our study, the effect was only visible at the beginning of the fattening stage, and not at weaning. This may be attributed to a longer exposure to inulin, as for the first collection period, the piglets had only limited exposure to inulin through not fully controlled access to creep feed. Interestingly,

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despite sampling occurring after animal mixing, salivary cortisol was not significantly elevated. This is likely due to circadian fluctuations rather than the stressor being insufficient. In fact, Merlot *et al.* (2004) distinguished the effects of weaning from animal mixing and observed elevated salivary cortisol levels up to 3 hours post-mixing even with a group size of only 8 pigs. Cortisol typically follows a circadian rhythm, characterized by higher concentrations in the early morning followed by a progressive decline throughout the day (Ruis *et al.*, 1997). Without measuring salivary cortisol at the same time of day on a rest day, it is difficult to evaluate to what extent animal mixing influenced the measured cortisol levels. Nevertheless, the natural diurnal decline in cortisol does not preclude an effect of the stressor on cortisol elevation. It is possible that the increase induced by animal mixing was counterbalanced by the physiological decline in cortisol occurring over the course of the day. Nonetheless, because salivary cortisol was measured repeatedly in the same animals before and after mixing, our results suggest that inulin supplementation did not significantly alter cortisol secretion in response to an acute stressor.

Differing chronic stress levels can be observed by changing animal density per pen (Siemińska & Pejsaki, 2022). As a group's size increases, associated in our case with an increase in animal density, the number of social interactions rises, and opportunities to escape diminish due to crowding. Although group size, group composition, and stocking density are all known to influence stress-related parameters (Spooler *et al.*, 1999, Merlot *et al.*, 2004, Schodl *et al.*, 2021, Siemińska & Pejsak, 2022;), the fixed dimensions of the pens prevented independent manipulation of stocking density in our trial. Consequently, group size and stocking density could not be differentiated and therefore remained confounded throughout the experiment. Our hypothesis was that higher density would lead to chronic stress, and again, this effect could be attenuated by supplementation with inulin. By looking at cortisol levels in hair, we assessed the average stress experienced over two separate five-week periods. We observed a significant effect of feed and animal density interaction over the post-weaning period. However, post-hoc pairwise analysis did not significantly distinguish the groups, likely due to reduced statistical power. Yet, we observed that, in the high-density group, the animals receiving the inulin supplementation indeed showed reduced cortisol levels, similar as those in the low-density group. However, this situation was not observed in animals from low-density pens. Consistent with Kelly *et al.* (2017), our findings show that the impact in healthy individuals under lower stocking density is limited but becomes pronounced under high stress environments with higher stocking density. In a study conducted by Casertano *et al.* (2024), where probiotics were administered to enhance mood and cognitive performance, a ceiling effect has been observed after 12 weeks of probiotic exposure, limiting the improvement curve.

Another interesting observation was that, overall, cortisol levels, whether measured in hair or saliva, decreased as animals aged. Heimbürge *et al.* (2020) studied parameters that can influence cortisol levels in the hair of cattle and pigs. They observed an evolution of cortisol levels in pigs with high levels of cortisol in hair at 2 weeks of age,

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reduced levels on the next shave at 10 weeks of age, followed by a steady increase at 27 and 71 weeks of age. They suggest this might be due to lower corticosteroid binding globulin in blood, resulting in higher circulating free cortisol at a young age. Ekkel *et al.* (1997) reported similar results, observing that the daily salivary cortisol peak gradually diminished over the course of the growing period. In their study, Bus *et al.* (2023) agreed that the HPA axis is not fully developed at 3 weeks of age regardless of the rhythmicity of cortisol secretion appearing before 10 days of age. At that age, it is thus expected that the HPA axis is immature and that cortisol levels will still fluctuate highly with age. In our study, we observed that the average cortisol concentrations were indeed decreased over time.

In conclusion, animal density influenced chronic stress, with higher density associated with elevated cortisol levels in hair samples. Inulin supplementation attenuated this density-related stress, lowering chronic cortisol levels in post-weaning phase in high-density pens to those observed under low-density conditions, while having no effect in low-density environments. Inulin supplementation was also associated with lower basal salivary cortisol levels around the beginning of the fattening period, regardless of the animal density but does not seem to influence cortisol elevation after an acute stressor. This confirms that using a prebiotic such as inulin could be a stress-mitigating nutritional strategy, possibly acting through modulation of gut microbiota, reduction of inflammation, and gut–brain axis signalling.

DISCLOSURE

All authors declare no conflict-of-interest for this publication.

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TABLES AND FIGURES

Table 1: Mean salivary cortisol concentrations per pen around animal mixing at weaning and fattening

Phase	Moment	Treatment				P-value		Variance parameter estimates		
		LD Ctrl	LD Inul	HD Ctrl	HD Inul	Animal density* Diet	Animal density	Diet	Pen	Residuals
Weaning	Before	Mean ± SD, µg/dL	Mean ± SD, µg/dL	Mean ± SD, µg/dL	Mean ± SD, µg/dL					
	After									
Fattening	Before									
	After									

Salivary cortisol values are expressed in µg/dL and are reported as mean ± standard deviation (SD). Salivary cortisol was measured 2 hours before and 2 hours after animal mixing, both at weaning and again 5 weeks later at the beginning of the fattening phase. The pen was considered the experimental unit (n = 3), and 3–4 pigs were sampled within each pen. P-values are presented per timepoint for the factors followed by the variance estimates for random factors and residuals. Superscript letters (a, b) denote significant differences identified by Tukey's post hoc pairwise comparisons; values sharing the same letter are not significantly different (P > 0.05). Abbreviations: LD Ctrl = low-density control diet; LD Inul = low-density inulin-enriched diet; HD Ctrl = high-density control diet; HD Inul = high-density inulin-enriched diet; SD = standard deviation

Running title: Inulin and Density Effects on Pig Cortisol

421 Table 2: Mean hair cortisol across post-weaning and fattening phases

Phase	Treatment								P-value		Variance parameter estimates		
	LD Ctrl		LD Inul		HD Ctrl		HD Inul		Animal density*	Animal density	Diet	Pen	Residuals
	n	Mean ± SD, ng/g	n	Mean ± SD, ng/g	n	Mean ± SD, ng/g	n	Mean ± SD, ng/g					
Post-weaning	3	13.83 ± 10.68	3	15.37 ± 12.15	3	24.75 ± 6.89	3	12.50 ± 8.59	0.03	0.22	0.1	<0.001	<0.001
Fattening	3	8.16 ± 4.56	2	8.78 ± 7.51	1	12.25	3	13.4 ± 12.1	0.91	0.54	0.78	<0.001	<0.001

422 Hair cortisol values are expressed in ng/g and are reported as mean ± standard deviation (SD). Hair cortisol was measured in samples collected at the end of post-weaning (5
423 weeks after weaning) and 5 weeks after the start of the fattening phase. Hair growth covered a 5-week period for each sampling point. The pen was considered the
424 experimental unit (n) and 1-5 pigs were sampled within each pen. P-values are presented per phase for each factor followed by the variance estimates for random factors and
425 residuals. Abbreviations: LD Ctrl = low-density control diet; LD Inul = low-density inulin-enriched diet; HD Ctrl = high-density control diet; HD Inul = high-density inulin-enriched
426 diet; SD = standard deviation