Association between intestinal microbiota composition and tail-biting in pigs

Nassima Rabhi
Universite de Montreal Faculte de medecine veterinaire
Alexandre Thibodeau
Universite de Montreal Faculte de medecine veterinaire
Jean-Charles Côté
Universite de Montreal Faculte de medecine veterinaire
Nicolas Devillers
Agriculture and Agri-Food Canada
Benoit Laplante
GNU Institut eksperimental'noj botaniki im V F Kuprevica Nacional'noj akademii nauk Belarusi
Philippe Fravalo
Universite de Montreal Faculte de medecine veterinaire
Guillaume Larivière-Gauthier
Universite de Montreal Faculte de medecine veterinaire
William P. Thériault
Universite de Montreal Faculte de medecine veterinaire
Luigi Faucitano
Agriculture and Agri-Food Canada
Guy Beauchamp
Universite de Montreal Faculte de medecine veterinaire
Sylvain Quessy (sylvain.quessy@umontreal.ca)
Universite de Montreal Faculte de medecine veterinaire https://orcid.org/0000-0003-4726-1278

Research

Keywords: Behavioral disorder, Intestinal microbiota, Pig, Stress, Tail-biting

Posted Date: April 14th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-21643/v1

Abstract

Background: Tail-biting (TB) is a serious behavioral disorder in pigs. It is defined as a pig chewing the tail of another pig. It is an important challenge in swine production as it impacts the animal welfare, its health and the economics and safety of the pork meat supply chain. Some treatments have been proposed but have not proven optimal. Nutrition, feed type and composition, appears to be an important factor in TB behavior, perhaps by modulating the intestinal microbiota (IM). In humans, IM is increasingly recognized as a modulator of behavior. Our aim was to assess an association between TB behavior and IM in pigs through comparisons of IM in groups of biter or bitten pigs to the IM in a non-biter and non-bitten negative control group, and through comparisons of IM between the biter and bitten groups. Each group, composed of 12 pigs, was formed at the beginning of the growing/finishing phase based on a target behavior analysis centered on TB behavior for the biter group and a score of damages caused to the tail for the bitten group. Fecal and blood samples were collected from each pig during a TB episode, at time 0, t0, and after a TB episode, four weeks later, at time 1, t1. Serum cortisol level was determined by ELISA and used as an indicator of stress. The pig's fecal microbiota was analyzed from DNA extracted from freshly collected fecal matter using amplicon sequencing of the V4 hypervariable region of the 16S rRNA gene.

Results: Serum cortisol levels were significantly higher in either the biter or bitten pig groups compared to the negative control group (p =0.02 and p =0.01, respectively). Interestingly, no significant difference was revealed between the biter and bitten groups. The microbiota alpha-diversity was not significantly different between all groups, biter, bitten and negative control. Analyses of beta-diversity, however, revealed a significant difference between either the biter or the bitten group in comparison to the negative control group in terms of structure and composition of the microbiota. Lactobacillus were significantly more abundant in the negative control group compared to the two other groups (p =0.001). No significant difference was revealed between the biter and bitten groups. Quantitative real-time PCR (qPCR) confirmed that lactobacilli were more abundant in the negative control group.

Conclusions: This study demonstrates that TB behavior is associated to the IM composition in pigs.

Background

Tail-biting (TB) is a behavior often observed on pig farms and is expressed by a pig nibbling the tail of a pen mate. Tail-biting is considered a severe behavioral disorder related to cannibalism (1, 2), and causes pain, infection, stress (3–5) and reduced growth performance (6) in bitten pigs.

The average incidence of TB behavior reported by several studies varies between 1.3 and 7.2% (1, 6). These results are, however, based on the assessment of *ante-* and *post-mortem* lesions and not based on observation of TB behavior at the farm. At slaughter, the degree of lesions on the carcass varies from detectable (70%) to severe (1 to 3%), resulting in partial or total loss of the tail (7–9). Tail-biting does not only have consequences on animal welfare but also on the economics of the pork meat supply chain due

to veterinary treatment (9), reduction of growth performances and total or partial carcass condemnation at the abattoir (8, 9).

Several risk factors have been associated with TB, most linked to a stressful farm environment such as housing on slatted floor and indoors (10), lower space allowance in the pen (11) and poor air quality (7, 12–14). A strong correlation between the prevalence of diseases, such as respiratory disorders and TB has also been reported (11, 15). Individual and genetic characteristics appear to play a significant role in the expression of TB behavior in pigs, with for example, the Yorkshire pigs being more likely to be victims of TB compared to the Landrace pigs (16). Age and sex have been reported as risk factors. Tail-biting is more frequently reported at the fattening stage, and females bite more than males (17). However, insufficient information is available about the possible correlation between these different risk factors, and it is difficult to clearly identify the triggering cause of TB (18). Nutrition is also considered a risk factor for TB (12). Attraction to blood from a bleeding tail may increase TB when diet is low in proteins, minerals, or fibres (18–20). In addition, studies have found that the dietary supplementation with tryptophan decreased the general activity of pigs and their attraction to blood in farms where TB is present (19, 21, 22). A link has been shown between serotonin, a precursor of tryptophan, and TB in biter pigs (6, 23). Pigs are also more active during daylight, and it has been shown that TB is more frequent before midday (12, 24).

Biter, bitten and control pigs exhibit different jejunal morphology, blood metabolites, activity of the immune system and stress levels (4, 25, 26). It is tempting to speculate that biter, bitten and control pigs may harbor different intestinal microbiota (IM) composition (18) which can be regulated by nutrition, feed composition and stress conditions (18, 27). Studies on mice have shown that a modification of IM composition may influence the hypothalamic-pituitary-adrenal (HPA) response to stress (28, 29), resulting in anxiety and depression (30, 31). Furthermore, some bacteria in the IM may regulate the pathophysiology of stress-related disorders through the synthesis of serotonin (e.g. *Candida* spp., *Streptococcus* spp., *Escherichia* spp. and *Enterococcus* spp.) (32, 33) and gamma-aminobutyric acid (some *Lactobacillus* spp. strains and *Bifidobacterium* spp.) (34). Cortisol secretion is regulated by the brain and increases with an animal's coping strategy to stress (35). It affects some permeability parameters of the intestinal epithelial barrier which leads to a modification of the IM (35, 36).

The aim of this study was to assess the association between TB behavior and IM in pigs by characterizing the animal stress response as measured by its serum cortisol level and by comparing the IM structure and composition between biters, bitten and control pigs, during and after a TB episode at the farm.

Results

Animal selection

Tail-biting behavior was observed in ten pens (10/32; 31%) after the first 5 weeks of the growing/finishing phase at a commercial farm. About 40 biter pigs were pre-selected within these pens using the “behavior sampling method” (two observations of 2 h/24 h for 7 days). Initial pig selection was determined based on total TB behavior frequency ranging from 4 to 39 bites over the one-week pre-selection period (Fig. 1). To obtain the targeted sample size of 12 pigs per group, the biters with the highest biting frequency were selected (> 20 bites) in the different observation periods within the same week (Fig. 1). All biter pigs came from seven pens.

Within the ten pens where TB behavior was observed, 70% of pigs presented no tail damage (0 score), 13% and 17% showed some scratches (score 1) and a small bleeding lesion (score 2) on the tail. Twelve pigs with a score 2 tail damage and from the same seven pens that housed the biters were selected to form the bitten group. In addition, 12 pigs randomly selected from three pens with no TB (0 score) formed the control group.

Behavior And Tails Condition

In biter pigs, TB behavior was at its highest peak at the time of selection (time 0, week 1; t0, w1) and decreased over the following weeks until it became almost non-observed, at the onset of the fourth week (t1, w4) (Fig. 2).

Tails of all bitten pigs healed between 2 and 4 weeks after selection and remained intact until the end of the finishing period. Biters were never bitten; they were true biters, and the negative control pigs never became biters or bitten pigs.

Variation Of Serum Cortisol Concentration

Serum cortisol levels were higher in biter and bitten pigs compared to negative control pigs at t0 ($p = 0.02$ and $p < 0.01$, respectively; Fig. 3). No significant difference in cortisol level was observed between the biters and bitten groups at t0 and t1 or between all three groups, biter, bitten, negative control, at t1 ($p > 0.05$).

Microbiota Description And Analysis

A total of 6 434 624 sequences were obtained by the sequencing of 47 samples at t0 and 47 samples from the same pigs at t1. The sequences were grouped into 12 810 OTUs using 97% similarity between sequences. The lowest value observed for a sample was 32 374 sequences and 252 OTUs and the highest value was 92 843 sequences and 1 257 OTUs. Most of the sequences were bacterial (6 297 598 sequences, 97.01% of all sequences) and a small fraction was Archean (193 567 sequences, 2.98%).

The negative controls for DNA extraction and PCR showed 139 and 4 807 sequences, respectively. Based on the composition of the microbial community positive sequencing control, an acceptable error rate of 0.094% was calculated.

No significant difference in the alpha-diversity indices, OTUs, Shannon-even and inverse Simpson, was revealed between all three groups, biter, bitten and negative control, at t0 and t1 (Table 2).

Table 1
Ethogram of pig behaviors recorded during the study
(Adapted from Quent (2015) (69))[1].

Behavior Classes	Codes	Behavior Components	Description
Rest	R	Rest	Eyes closed (= not awaken) or opened (= awaken)
Intake	N B	Eating Drinking	Food intake from the feeder Drinking from the watering trough
Explorative behavior towards the enclosure	FL	Sniffing, licking of the enclosure, nosing	Orientation of the head towards a substrate (floor, walls) and sniffs, smells or licks
Harmless social behavior	CQ CQ^{-} DQ^{-} DQ	Contact with the tail (with reaction -) Contact with the tail (without reaction) Moves the tail (with reaction -) Moves the tail (without reaction)	Touches the tail with a reaction from the receiver, who is startled or moves away. Touches the tail without a reaction from the receiver, who doesn't move. Moves the tail with a reaction from the receiver, who is startled or moves away. Moves the tail without a reaction from the receiver, who doesn't move.
Harmful social behavior (biting of the tail)	PQG^{-} PQG	Takes the tail in its mouth (with a reaction -) Takes the tail in its mouth (without reaction)	Takes the tail in its mouth with a reaction from the receiver, who is startled or moves away. Takes the tail in its mouth without a reaction from the receiver, who doesn't move.

[1] Ethogram elaborated from pre-observations of a 48 h video recording from four pens where TB behavior was present.

Table 2

Comparison of alpha-diversity indices of the intestinal microbiota of three groups of pigs, biter, bitten and the negative control pigs.

	Periods[1]					
Indices	Groups at t0			Groups at t1		
	Biter pigs	Bitten pigs	Negative controls	Biter pigs	Bitten pigs	Negative controls
OTUs	777.85	784.38	791.55	769.28	815.56	834.31
Shannon-even	0.72	0.73	0.711	0.70	0.734	0.73
Inverse Simpson	50.31	55.63	44.22	44.26	53.57	55.21

[1] t0: immediately following selection of the pig groups; and t1: four weeks later.

Beta-diversity was compared between the three pig groups at t0 and t1 (Table 3). A significant difference in microbiota structure was observed between the biter and the negative control, and between the bitten and the negative control group, both at t0 with the Yue & Clayton index (Fig. 4).

Table 3

Comparison of the intestinal microbiota structure of pig groups[1]

Time[2]	Compared pig groups		AMOVA (*p*-value)	
			Yue & Clayton index	Jaccard index
t0	Biter	Negative	0.001*	0.246
	Bitten	Negative	0.001*	0.306
	Biter	Bitten	0.476	0.439
t1	Biter	Negative	0.244	0.318
	Bitten	Negative	0.295	0.55
	Biter	Bitten	0.866	0.46

[1] Based on 1000 subsampling of 32 374 sequences.

[2] t0: immediately following selection of the pig groups; and t1: four weeks later.

* Statistically significant differences after adjusting the alpha threshold downwards with the sequential Benjamini and Hochberg procedure (1995) (68).

A LEfSe was performed to identify bacterial taxa indicators of the different groups at each sampling time, t0 and t1. When the biter group was compared to the negative control group at t0, two different genera and 22 different OTUs were significantly ($p \leq 0.05$) associated with the biter group and three genera and 19 OTUs were associated with the negative control group. In the biter group, the genera *Coprococcus* and *Clostridium* IV showed the highest LDA scores (LDA=3.3 and 2.69, respectively), while in the negative control group, the genus *Lactobacillus* (LDA=4.47) and a *Lactobacillus* OTU (LDA=4.51) showed the highest LDA scores. At t1, two genera, *Roseburia* and *Anaeroplasma* (LDA=3.23 and 2.91, respectively), and nine OTUs were associated with the negative control group (Supplementary file, Tables S1, S2).

Likewise, when the bitten group was compared to the negative control group at t0, two genera, *Sphaerochaeta* and *Blautia* (LDA = 3.21 and 2,96, respectively) and 17 different OTUs, most notably *Phascolarctobacterium* (LDA = 3.61) were associated with the bitten group, and two genera, *Lactobacillus* and *Intestinimonas* (LDA = 4.57 and 3.38, respectively) and nine OTUs were associated with the negative control group. At t1, a single genus, *Alistipes* (LDA = 2.91) and five OTUs were associated to the bitten group and five OTUs were associated with the negative control group (Supplementary file, Tables S3-S4).

The composition and diversity of the intestinal microbiota was not significantly different between the biter and the bitten groups ($p > 0.05$).

As expected, when the positive control group (non-biter and non-bitten-antibiotic-treated) was compared with the negative control group (non-biter and non-bitten), significant differences were found in alpha-diversity indices at t0 (Shannon-even $p = 0.002$ and inverse Simpson $p = 0.002$), and beta-diversities at t0 (Yue & Clayton $p = 0.001$; Jaccard $p < 0.001$) and t1 (Yue & Clayton $p = 0.028$; Jaccard $p < 0.003$) (Supplementary file, Figure S2). This validates the experimental model and subsequent bioinformatics analysis abilities to measure and identify microbiota modification in the studied animals.

Real-time Quantitative Polymerase Chain Reaction

A qPCR assay was performed to quantify and validate the results obtained from LEfSe for the genus *Lactobacillus*. A significant difference was observed when comparing either the biter or the bitten pig groups to the negative control pig group at t0 (during TB), with respective averages of 1.15 and 1.11 log of gene copies per ng of DNA. However, no difference was observed at t1 (four weeks after TB) (Fig. 5).

Discussion

Our aim was to study a possible association between IM composition and tail-biting. First, animal stress was assessed by serum cortisol level, second, IM structure and composition were compared between either biters or bitten pigs and control pigs, during and after a TB episode at the farm. Four pig groups, biter, bitten, a non-biter, non-bitten negative control, and an antibiotic-treated positive control for the IM composition, were formed in a commercial fattening farm during a TB behavior episode that was video-recorded. Tail-biting appeared two weeks after the beginning of the growing period and initially only in two non-contiguous pens. This is in agreement with similar studies on pig TB behavior (7, 37–39). Tail-

biting within the farm appeared variable over time, as previously described (40). Once present, TB behavior frequency decreased gradually in the 12 selected biter pigs four weeks after pig recruitment (40).

Cortisol levels were compared among groups. The levels were higher in both biter and bitten pigs at t0, when TB behavior was at its peak in selected animals, than in the negative control group, presumably a result of increased response to acute stress in pigs (41, 42). Our results for the biter and bitten pigs are in agreement with those of Smulders et al. (2006) (43, 44). Four weeks after the animal selection, at t1, cortisol levels were high in all three groups, biter, bitten and control. The cortisol levels between biter and bitten pig groups, showed no difference either at t0 or t1, indicative that both groups contained stressed animals.

Additionally, we also studied a possible association between TB and IM and we generated novel information on the structure and composition of the IM in these stressed animals. Numerous studies have used high throughput sequencing methods to explore the composition of animal's IM (45–48). Here, the structure and the composition of the IM in feces samples of biter and bitten pigs were compared to negative controls, using the V4 region nucleotide sequence of the 16S rRNA gene. In our study, a group of pigs was treated with antibiotics in order to modify their intestinal microbiota composition and structure. This group served as a positive control to validate the ability of our experimental design to detect changes in the animal's microbiota. As expected, the intestinal microbiota of the animals included in this positive control group was different than the animal's microbiota from the negative control group. This is in agreement with Jernberg et al. (2007) (49). We showed that biter or bitten pig groups have different IM between each other and when each is compared to the negative control pig group. To the best of our knowledge, the bacterial genera and OTUs associated respectively to the biter, the genus *Coprococcus*, and bitten pig groups, the genus *Sphaerochaeta* and the *Phascolarctobacterium* OTU, both stressed groups, had not been previously associated with behavior disorders.

We also showed that the relative abundance determined by qPCR of *Lactobacillus*, could distinguish biter and bitten groups from the negative control group. It has been reported that some *Lactobacillus* species are associated with behavior disorders such as anxiety and depression in both humans (31, 50, 51) and mice (52, 53). Conversely, different *Lactobacillus* species are used in probiotics to reduce behavior disorders, such as *Lactobacillus rhamnosus* JB-1 for anxiety and stress (54) or *Lactobacillus helveticus* R0052 for depressive behaviors (55). It would be interesting to test whether a manipulation of the IM of pigs exhibiting TB behavior through the introduction of either or both *Lactobacillus* species could shorten the disorder.

Because this study was carried out on a commercial farm, it is important to emphasize that several risk factors, most of which are also stress factors associated with TB behavior, were present, such as intact tails, slatted floors pens without enrichment and a relatively high number of pigs per pen (11 pigs per pen) (7, 10). However, it is noteworthy that biter and bitten pigs were in the same pens and all subjected to the same environment. In this study, no specific event could be recorded as a trigger for TB behavior.

With respect to the relationship between IM and TB, at least two hypotheses can be proposed. First, stress can increase the permeability of the gastro-intestinal mucosa (56) and cause dysbiosis, a microbial imbalance, and modify the bacterial populations that play a role in the regulation of animal behavior, leading to TB. Alternatively, dysbiosis can cause stress in pigs, resulting in pig's predisposition to TB. In our study, as the microbiota of all pigs was not assessed at the time of their allocation in the fattening unit, it was not possible to determine the association between the IM initial composition and the risk for the expression of TB or to observe modifications of the IM before the onset of TB. Such analysis would contribute to a better understanding of the relationship between IM and TB risk factors. Additionally, it would be interesting to study the effects of various feed modifications, the addition of plants extracts, essential oils, probiotics on IM, and whether they lead to changes in TB behavior.

Conclusion

To the best of our knowledge, our results provide the first evidence of a relationship between the occurrence of a behavior problem, TB, in biters and bitten pigs and IM. We showed that biter and bitten pigs were stressed in comparison to the negative control group, as revealed by higher cortisol levels. Pigs in the negative control group had more *Lactobacillus* in their IM than those expressing TB, which is consistent with human and mice studies on the relationship between behavioral disorders and microbiota composition. However, the mechanisms underlying the association between TB and IM are still unknown. Further studies are needed to gain a better understanding of the cause-effect relationship between both.

Methods

Animals and housing

All pigs were handled and treated in accordance with the guidelines of the Canadian Council on Animal Care (57). The protocol was approved by the Animal Use Ethics Committee of the *Faculté de médecine vétérinaire* of the *Université de Montréal* (certificate number 17-Rech-1858).

A total of 352 individually ear-tagged pigs (Landrace x Large White hybrid sows sired with Duroc x synthetic hybrid boar), with undocked tail and of 8 weeks of age, were randomly distributed into 32 growing-finishing pens (2.06 × 3.35 m) located in two separate rooms (16 pens/room) at a commercial farm in Ange-Gardien, Quebec, Canada (Additional file 1). Each pen housed 11 pigs (6 gilts and 5 barrows). To facilitate video identification, in each pen, a 30 cm number ranging from 0 to 10 was painted on each pig back. Each number was associated to each pig ear-tag identifier.

In accordance with the feeding practices in place at the commercial farm, pigs were fed a corn- and soy-based granulated diet. The feeding protocol consisted of a pre-fattening feed (supplemented with 0.5 kg/ton of salinomycin) for 2.5 weeks, a fattening feed (supplemented with 0.5 kg/ton of salinomycin) for 3.5 weeks, a first growth feed (supplemented with 0.21 kg/ton of salinomycin) for 3.5 weeks, a second growth feed (supplemented 0.21 kg/ton of salinomycin) for 10 days, and a finishing feed (supplemented

with 0.15 kg/ton of narasin) (F. Ménard, Inc., Ange-Gardien, QC, Canada) until slaughter. Animals had free access to food and water at all times. Ambient conditions, ventilation and temperature, were established according to standard housing recommendations.

Behavior Assessment

Tail-biting behaviors were recorded on video cameras (1080p resolution, day/night) with a recording capacity of 7 days. A total of 16 cameras were installed above the 32 growing-finishing pens under study in a position that allowed the recording of two pens simultaneously. Recordings were done 24 h/24 h and transferred to a computer every 48 h for analysis of pig behavior. A 2-minute interval sampling on a 24 h video in four pens from which animals presented TB behavior was done as a preliminary test to determine the periods when TB occurred as recommended (58).

Subsequently, five pens where TB behavior was present were selected for developing an ethogram (Table 1) based on a pre-observation period of 48 h video recordings. The main behaviors presented in the ethogram included tail accessibility, nibbling of the tail by the biter, duration of the nibbling behavior, frequency of tail nibbling, time when the nibbling occurred and the receiver's response.

In order to further define the groups of animals to be studied, the "target behavior sampling" method (58), centered on observations of the behavior of interest, here TB, was used on recordings of the 10 pens where TB behavior was present, based on the ethogram. Sampling observations were distributed among two periods: from 10:00 am to noon and from 8:00 pm to 10:00 pm, which corresponded to the peaks of TB behavior. Sampling survey lasted for eight consecutive days until the study groups included 12 biter pigs (distributed in 8 pens), 12 bitten pigs (distributed in 7 pen) and 12 negative control pigs (distributed in 3 pens). The control pigs (non-bitten and non-biter) were selected in pens where TB behavior was absent according to the criteria defined in the ethogram. For the group of bitten pigs, all animals were examined based on a growing scale of tail damage scores from 0 to 3, where 0: no lesion; 1: presence of scratches; 2: presence of a moderate bleeding lesion; and 3: significant lesion with sometimes loss of a tail Sect. (58). The bitten and biter pigs were selected by video according to the ethogram. For confirmation of the pig tail status, an examination was made on-farm, every two days in the pens where TB behavior was present among the 32 pens in the study. All selected pigs were kept in their respective pens to continue their monitoring in their original and controlled environment. The behavior of all groups was studied simultaneously throughout a TB behavior episode that lasted four weeks, during which blood and fresh feces samples were taken for cortisol and IM analysis, respectively.

A positive control group consisting of 12 non-biter/non-bitten antibiotic-treated pigs was also formed. Blood samples for serum cortisol analysis were not taken. Chlortetracycline at 1210 ppm was added to their diet seven days prior to each sampling date to induce conformational changes in their IM. This group served as a positive control in our later 16S rRNA gene amplification, sequencing and analysis.

The average bite number of biter pigs was plotted on a weekly basis, w1, w2, w3 and w4.

Blood And Feces Sampling

Blood and feces were sampled in the biter, bitten and negative control groups, during two different periods, t0 and t1. No sampling was done on the AT group. At t0, pigs were 12 weeks of age, and TB behavior episode was beginning. Four weeks later, at t1, the TB behavior episode was considered finished.

Blood (approx. 5 ml) was collected from the jugular vein of snared pigs by a single experienced animal technician in the pen. Blood samples were kept at room temperature for 2 h to allow blood to clot prior to centrifugation (15 min at 1,000 x *g*). The serum was transferred to 1.5-mL Eppendorf tubes and stored at -80ºC pending cortisol concentration analysis. Serum cortisol concentration was determined using the Cortisol ELISA Kit (Pig) (Abnova, Taiwan) according to the manufacturer's recommendation. The minimum detectable concentration of cortisol was 0.2 ng/ml.

Fresh fecal material was collected directly from the rectum of animals. A one-gram fraction (from five 200 mg subsamples from the same animal) was frozen immediately in liquid nitrogen and subsequently stored at -80ºC until DNA extraction.

DNA extraction, 16S ribosomal RNA gene amplification, sequencing and analysis

Total DNA was extracted from 500 µg of each feces sample according to Thibodeau *et al.*, (2016) (59). Briefly, samples were put in tubes containing 0.1 mm glass beads. Bacteria were lysed with 500 ml of lysis buffer (500 mM Tris-HCl, 200 mM EDTA, 1% SDS; Fisher Scientific, Ottawa, ON, Canada) and a FastPrep-24 5G™ High Speed Homogenizer (mpbio, Santa Ana, CA, USA) for 2 cycles of 40 seconds at 6 m/s. Samples were kept on ice between cycles. DNA was purified using phenol: chloroform: isoamyl alcohol 25:24:1 (Sigma-Aldrich, St. Louis, MO, USA). The phenol traces were removed using chloroform: isoamyl alcohol 24:1 (Sigma-Aldrich). The DNA was precipitated in 90% ethanol for 24 h at -20 °C and resuspended in 1 mM Tris-HCl:0.1 M EDTA, pH 8.0. Negative controls without feces and positive controls with a known bacterial community (ZymoBIOMICS ™ Microbial Community Standard; Zymo Research, Irvine, CA, USA) were processed in parallel with the fecal samples. The purified DNAs were quantified using a QFX Fluorometer (DeNovix, Wilmington, DE, USA) with Qubit BR reagents (Fisher Scientific). DNA extracts were stored at -80ºC.

The hypervariable V4 region of the 16S rRNA gene was amplified for each sample by PCR using the primer pair 515FP1-CS1F ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA and 806RP1-CS2R TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) [57]. The Platinum SuperFi PCR Master Mix (Invitrogen, Thermo Fisher Scientific) was used with 12.5 ng of DNA in a total reaction volume of 25 µl. The amplification was carried out with an initial denaturation step at 95ºC for 5 min, followed by 23 cycles at 98ºC for 30 sec, 55ºC for 30 sec and

72ºC for 180 sec, and a final elongation step at 72ºC for 10 min (60). Amplification was confirmed by gel electrophoresis and amplicons sent to the McGill University and Génome Québec Innovation Centre (Montreal, QC, Canada) for barcoding and subsequent Illumina Miseq sequencing (250 paired-ends).

All sequences were analyzed using Mothur software version 1.35.5 (61) according to Larivière-Gauthier *et al.*, (2017) (62). Taxonomic assignment of the sequences was made using the Ribosomal Database Project (RDP; https://rdp.cme.msu.edu) (63). The sequences with 97% similarity (equivalent to species level) were grouped into operational taxonomic units (OTUs). Alpha-diversity (number of OTUs per sample, Shannon-even and inverse Simpson indices) of fecal samples from pigs of different groups, at t0 and t1were calculated in Mothur, using a subsample of 32,374 sequences, the lowest number of samples returned in all samples. For beta-diversity analysis, the distance between all samples was measured by the Yue & Clayton and the Jaccard indices using the same subsampling. Structure of the bacterial communities were visualized by a non-metric multidimensional scaling (NMDS) graph, and each combination of two pig groups, biter/negative, bitten/negative and biter/bitten, was compared at t0 and t1 by the analysis of molecular variance (AMOVA) (64). In addition, a Linear discriminant analysis (LDA) effect size (LEfSe) (65) was used to discover bacterial taxa significantly associated with each group at each sampling time.

Real-time Quantitative Pcr Of Specific Bacterial Populations

To validate and quantify results obtained from sequencing, a quantitative PCR (qPCR) targeting lactobacilli was performed on all samples (66). Standard curves were made from amplicons derived from the control strain *Lactobacillus acidophilus* ATCC 314. Each well contained 4 µl of Evagreen (MBI Montreal Biotech, Kirkland, QC, Canada), 0.6 µl of forward primer, 0.6 µl of reverse primer, 12.8 µl of water and 20 ng of DNA. The amplification was done in a LightCycler 96 real-time PCR (Roche Diagnostics, Mannheim, Germany) using the following program: 50ºC for 120 sec, 95ºC for ten minutes, 45 amplification cycles of 95ºC for 15 seconds and 60ºC for 60 seconds, and a final high-resolution melt analysis. The results, the number of gene copies, were expressed in log per ng of DNA.

Statistical analysis

A linear model for repeated measures with time as within-subject factor (SAS v9.3, Cary, NC, USA) was used to compare variations in TB across time. For the comparison of the different alpha-diversity indices, a linear model for repeated measures with time as within-subject factor and group as between-subject factor was used. For quantitative PCR results and serum cortisol levels, a linear model was used with group as factor. The AMOVA test was used to compare the beta-diversity between the different groups (comparison of two groups at a time) using Mothur (67). Statistical tests were made on comparisons between the following pig groups: biter/negative, bitten/negative and biter/bitten, at t0 and t1. The alpha level for these comparisons was adjusted downwards with the sequential method of Benjamini and Hochberg (1995) (68).

Declarations

Ethics approval and consent to participate

The protocol was approved by the *Comité d'éthique de l'utilisation des animaux* (Animal Use Ethics Committee) of the *Faculté de médecine vétérinaire* of the *Université de Montréal*, certificate number 17-Rech-1858, in accordance with the Canadian Council on Animal Care guidelines (57).

Consent for publication

All co-authors have read and approved the final version of the manuscript and consent to its publication.

Availability of data and material

All data and material will be made freely available upon acceptance of the manuscript for publication.

Competing interests

The authors declare that they have no competing interests.

Fundings

This work was supported by a grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) RDCPJ 520873–17, including a scholarship to NR.

Authors' contributions

SQ, NR, AT, BL and PF planned the overall design of the experiments; NR, BL, LF and ND planned the animal behavior assessment and blood analysis; AT, NR, PF planned the intestinal microbiota analysis; NR carried out the experimental work; NR, AT, PF, SQ and JCC analyzed the results with contributions from all co-authors; NR, JCC, AT and SQ wrote the manuscript with contributions from all co-authors. AT and SQ supervised the project.

References

1. Taylor NR, Main DCJ, Mendl M, Edwards SA. Tail-biting: A new perspective. Vet J. 2010;186(2):137–47.
2. Temple D, Mainau E, X. M. Tail biting in pigs. Farm Animal Welfare Education Centre, Barcelona, Spain; 2014.
3. Sihvo HK, Simola O, Munsterhjelm C, Syrja P. Systemic spread of infection in tail-bitten pigs. J Comp Pathol. 2012;146(1):73.
4. Munsterhjelm C, Brunberg E, Heinonen M, Keeling L, Valros A. Stress measures in tail biters and bitten pigs in a matched case-control study. Anim Welf. 2013;22(3):331–8.
5. Di Giminiani P, Edwards SA, Malcolm EM, Leach MC, Herskin MS, Sandercock DA. Characterization of short- and long-term mechanical sensitisation following surgical tail amputation in pigs. Sci Rep. 2017;7(1):4827.
6. Valros A, Heinonen M. Save the pig tail. Porcine Health Management. 2015;1(1):2.

7. European Food Safety Authority. The risks associated with tail biting in pigs and possible means to reduce the need for tail docking considering the different housing and husbandry systems - Scientific Opinion of the Panel on Animal Health and Welfare. EFSA Journal. 2007;5(12):611.
8. Harley S, More SJ, Connell NE, Hanlon A, Teixeira D, Boyle L. Evaluating the prevalence of tail biting and carcase condemnations in slaughter pigs in the Republic and Northern Ireland, and the potential of abattoir meat inspection as a welfare surveillance tool. Vet Rec. 2012;171(24):621.
9. Harley S, Boyle LA, O'Connell NE, More SJ, Teixeira DL, Hanlon A. Docking the value of pigmeat? Prevalence and financial implications of welfare lesions in Irish slaughter pigs. Anim Welf. 2014;23(3):275–85.
10. Richards D. Tail Biting. Ontario Ministry of Agriculture, Food and Rural Affairs, Guelph, ON, Canada; 2013.
11. Moinard C, Mendl M, Nicol CJ, Green LE. A case control study of on-farm risk factors for tail biting in pigs. Appl Anim Behav Sci. 2003;81(4):333–55.
12. Sonoda LT, Fels M, Oczak M, Vranken E, Ismayilova G, Guarino M, et al. Tail biting in pigs-causes and management intervention strategies to reduce the behavioural disorder. A review Berliner Münchener tierärztliche Wochenschrift. 2013;126(3–4):104–12.
13. D'Eath RB, Niemi JK, Vosough Ahmadi B, Rutherford KMD, Ison SH, Turner SP, et al. Why are most EU pigs tail docked? Economic and ethical analysis of four pig housing and management scenarios in the light of EU legislation and animal welfare outcomes. Animal. 2016;10(4):687–99.
14. Scollo A, Gottardo F, Contiero B, Edwards SA. A cross-sectional study for predicting tail biting risk in pig farms using classification and regression tree analysis. Prev Vet Med. 2017;146:114–20.
15. Nannoni E, Valsami T, Sardi L, Martelli G. Tail docking in pigs: A review on its short- and long-term consequences and effectiveness in preventing tail biting. Italian Journal of Animal Science. 2014;13(1):3095.
16. Sinisalo A, Niemi JK, Heinonen M, Valros A. Tail biting and production performance in fattening pigs. Livestock Science. 2012;143(2):220–5.
17. Keeling LJ, Wallenbeck A, Larsen A, Holmgren N. Scoring tail damage in pigs: an evaluation based on recordings at Swedish slaughterhouses. Acta Vet Scand. 2012;54(1):32.
18. Brunberg EI, Rodenburg TB, Rydhmer L, Kjaer JB, Jensen P, Keeling LJ. Omnivores going astray: A review and new synthesis of abnormal behavior in pigs and laying hens. Frontiers in Veterinary Science. 2016;3:57.
19. Fraser D. Mineral-deficient diets and the pig's attraction to blood: implications for tail-biting. Canadian Journal of Animal Science. 1987;67(4):909–18.
20. Steenfeldt S, Kjaer JB, Engberg RM. Effect of feeding silages or carrots as supplements to laying hens on production performance, nutrient digestibility, gut structure, gut microflora and feather pecking behaviour. Br Poult Sci. 2007;48(4):454–68.
21. Fraser D. Attraction to blood as a factor in tail-biting by pigs. Appl Anim Behav Sci. 1987;17(1):61–8.

22. Poletto R, Meisel RL, Richert BT, Cheng H-W, Marchant-Forde JN. Aggression in replacement grower and finisher gilts fed a short-term high-tryptophan diet and the effect of long-term human–animal interaction. Appl Anim Behav Sci. 2010;122(2):98–110.
23. Ursinus WW, Bolhuis JE, Zonderland JJ, Rodenburg TB, de Souza AS, Koopmanschap RE, et al. Relations between peripheral and brain serotonin measures and behavioural responses in a novelty test in pigs. Physiol Behav. 2013;118:88–96.
24. Haske C, Bogner H, Peschke W. Investigations on the behaviour of fattening pigs in different stall-housing systems in relation to tail and ear biting. Bayerische Landwirtschaftliches Jahrbuch. 1979;56:162–200.
25. Palander PA, Heinonen M, Simpura I, Edwards SA, Valros AE. Jejunal morphology and blood metabolites in tail biting, victim and control pigs. Animal. 2013;7(9):1523–31.
26. Munsterhjelm C, Simola O, Keeling L, Valros A, Heinonen M. Health parameters in tail biters and bitten pigs in a case–control study. Animal. 2013;7(5):814–21.
27. Longpré J, Fairbrother JM, Fravalo P, Arsenault J, LeBel P, Laplante B, et al. Impact of mash feeding versus pellets on propionic/butyric acid levels and on total Escherichia coli load in the gastrointestinal tract of growing pigs1. J Anim Sci. 2016;94(3):1053–63.
28. Sudo N, Chida Y, Aiba Y, Sonoda J, Oyama N, Yu X-N, et al. Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. The Journal of Physiology. 2004;558(Pt 1):263–75.
29. Neufeld KM, Kang N, Bienenstock J, Foster JA. Reduced anxiety-like behavior and central neurochemical change in germ-free mice. Neurogastroenterology Motility. 2011;23(3):255-e119.
30. Diaz Heijtz R, Wang S, Anuar F, Qian Y, Björkholm B, Samuelsson A, et al. Normal gut microbiota modulates brain development and behavior. Proc Natl Acad Sci USA. 2011;108(7):3047–52.
31. Cepeda MS, Katz EG, Blacketer C. Microbiome-gut-brain axis: probiotics and their association with depression. The Journal of Neuropsychiatry Clinical Neurosciences. 2016;29(1):39–44.
32. Lyte M. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. BioEssays. 2011;33(8):574–81.
33. Bonaz BL, Bernstein CN. Brain-gut interactions in inflammatory bowel disease. Gastroenterology. 2013;144(1):36–49.
34. Barrett E, Ross RP, O'Toole PW, Fitzgerald GF, Stanton C. γ-Aminobutyric acid production by culturable bacteria from the human intestine. J Appl Microbiol. 2012;113(2):411–7.
35. Di Mauro A, Neu J, Riezzo G, Raimondi F, Martinelli D, Francavilla R, et al. Gastrointestinal function development and microbiota. Italian Journal of Pediatrics. 2013;39:15.
36. Montiel-Castro AJ, González-Cervantes RM, Bravo-Ruiseco G, Pacheco-López G. The microbiota-gut-brain axis: neurobehavioral correlates, health and sociality. Frontiers in Integrative Neuroscience. 2013;7:70-.

37. Taylor NR, Parker RMA, Mendl M, Edwards SA, Main DCJ. Prevalence of risk factors for tail biting on commercial farms and intervention strategies. Vet J. 2012;194(1):77–83.
38. D'Eath RB, Arnott G, Turner SP, Jensen T, Lahrmann HP, Busch ME, et al. Injurious tail biting in pigs: how can it be controlled in existing systems without tail docking? Animal. 2014;8(9):1479–97.
39. Marchese A, Barbieri R, Coppo E, Orhan IE, Daglia M, Nabavi SF, et al. Antimicrobial activity of eugenol and essential oils containing eugenol: A mechanistic viewpoint. Crit Rev Microbiol. 2017;43(6):668–89.
40. Ursinus WW, Van Reenen CG, Kemp B, Bolhuis JE. Tail biting behaviour and tail damage in pigs and the relationship with general behaviour: Predicting the inevitable? Appl Anim Behav Sci. 2014;156:22–36.
41. Ruis MAW, te Brake JHA, Engel B, Buist WG, Blokhuis HJ, Koolhaas JM. Adaptation to social isolation: Acute and long-term stress responses of growing gilts with different coping characteristics. Physiol Behav. 2001;73(4):541–51.
42. Valros A, Munsterhjelm C, Puolanne E, Ruusunen M, Heinonen M, Peltoniemi OAT, et al. Physiological indicators of stress and meat and carcass characteristics in tail bitten slaughter pigs. Acta Vet Scand. 2013;55(1):75.
43. Smulders D, Verbeke G, Mormède P, Geers R. Validation of a behavioral observation tool to assess pig welfare. Physiol Behav. 2006;89(3):438–47.
44. Ursinus WW, Van Reenen CG, Reimert I, Bolhuis JE. Tail biting in pigs: blood serotonin and fearfulness as pieces of the puzzle? PloS One. 2014;9(9):e107040-e.
45. Lepage P, Leclerc MC, Joossens M, Mondot S, Blottière HM, Raes J, et al. A metagenomic insight into our gut's microbiome. Gut. 2013;62(1):146.
46. Zeng B, Han S, Wang P, Wen B, Jian W, Guo W, et al. The bacterial communities associated with fecal types and body weight of rex rabbits. Sci Rep. 2015;5:9342.
47. Wu X, Zhang H, Chen J, Shang S, Wei Q, Yan J, et al. Comparison of the fecal microbiota of dholes high-throughput Illumina sequencing of the V3–V4 region of the 16S rRNA gene. Appl Microbiol Biotechnol. 2016;100(8):3577–86.
48. Zhao Y, Li B, Bai D, Huang J, Shiraigo W, Yang L, et al. Comparison of fecal microbiota of Mongolian and thoroughbred horses by high-throughput sequencing of the V4 region of the 16S rRNA gene. Asian-Australasian Journal of Animal Sciences. 2016;29(9):1345–52.
49. Jernberg C, Löfmark S, Edlund C, Jansson JK. Long-term ecological impacts of antibiotic administration on the human intestinal microbiota. The ISME Journal. 2007;1(1):56–66.
50. Maes M, Kubera M, Leunis J-C, Berk M. Increased IgA and IgM responses against gut commensals in chronic depression: Further evidence for increased bacterial translocation or leaky gut. J Affect Disord. 2012;141(1):55–62.
51. McKean J, Naug H, Nikbakht E, Amiet B, Colson N. Probiotics and subclinical psychological symptoms in healthy participants: a systematic review and meta-analysis. The Journal of Alternative Complementary Medicine. 2016;23(4):249–58.

52. Wikoff WR, Anfora AT, Liu J, Schultz PG, Lesley SA, Peters EC, et al. Metabolomics analysis reveals large effects of gut microflora on mammalian blood metabolites. Proc Natl Acad Sci USA. 2009;106(10):3698–703.
53. Valladares R, Bojilova L, Potts AH, Cameron E, Gardner C, Lorca G, et al. Lactobacillus johnsonii inhibits indoleamine 2,3-dioxygenase and alters tryptophan metabolite levels in BioBreeding rats. FASEB J. 2013;27(4):1711–20.
54. Bravo JA, Forsythe P, Chew MV, Escaravage E, Savignac HM, Dinan TG, et al. Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. Proceedings of the National Academy of Sciences USA. 2011;108(38):16050.
55. Messaoudi M, Lalonde R, Violle N, Javelot H, Desor D, Nejdi A, et al. Assessment of psychotropic-like properties of a probiotic formulation (Lactobacillus helveticus R0052 and Bifidobacterium longum R0175) in rats and human subjects. Br J Nutr. 2011;105(5):755–64.
56. Melanie GG, Manuel AS, Mary HP. Pathophysiological Mechanisms of Stress-Induced Intestina Damage. Curr Mol Med. 2008;8(4):274–81.
57. Canadian Council on Animal Care. CCAC guidelines on: The care and use of farm animals in research, teaching and testing. Ottawa, ON, Canada; 2009.
58. Anonymous. Prévenir la douleur chez le porc. IFIP-Institut du porc, INRA-Institut national de la recherche agronomique, Agricultures & Territoires – Chambres d'Agriculture, RMT Bien-Être Animal; 2016.
59. Thibodeau AFP, Yergeau É, Arsenault J, Lahaye L, Letellier A. Chicken caecal microbiome modifications induced by Campylobacter jejuni colonization and by a non-antibiotic feed additive. PLoS ONE. 2015;10(7):e0131978.
60. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME Journal. 2012;6(8):1621–4.
61. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, et al. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009;75(23):7537.
62. Larivière-Gauthier G, Thibodeau A, Letellier A, Yergeau É, Fravalo P. Reduction of Salmonella shedding by sows during gestation in relation to its fecal microbiome. Front Microbiol. 2017;8:2219-.
63. Cole JR, Wang Q, Fish JA, Chai B, McGarrell DM, Sun Y, et al. Ribosomal Database Project: data and tools for high throughput rRNA analysis. Nucleic Acids Res. 2014;42(Database issue):D633-D42.
64. Martin AP. Phylogenetic approaches for describing and comparing the diversity of microbial communities. Appl Environ Microbiol. 2002;68(8):3673.
65. Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol. 2011;12(6):R60-R.
66. Castillo M, Martín-Orúe SM, Manzanilla EG, Badiola I, Martín M, Gasa J. Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet Microbiol.

2006;114(1):165–70.
67. Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl Environ Microbiol. 2013;79(17):5112.
68. Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B (Methodological). 1995;57(1):289–300.
69. Quent D. Mise au point d'un modèle de déclenchement du cannibalisme en élevage porcin. INRA – UMR PEGASE 1348; Université Paris 13 – UFR lettres, sciences de l'homme et des sociétés; 2015.

Figures

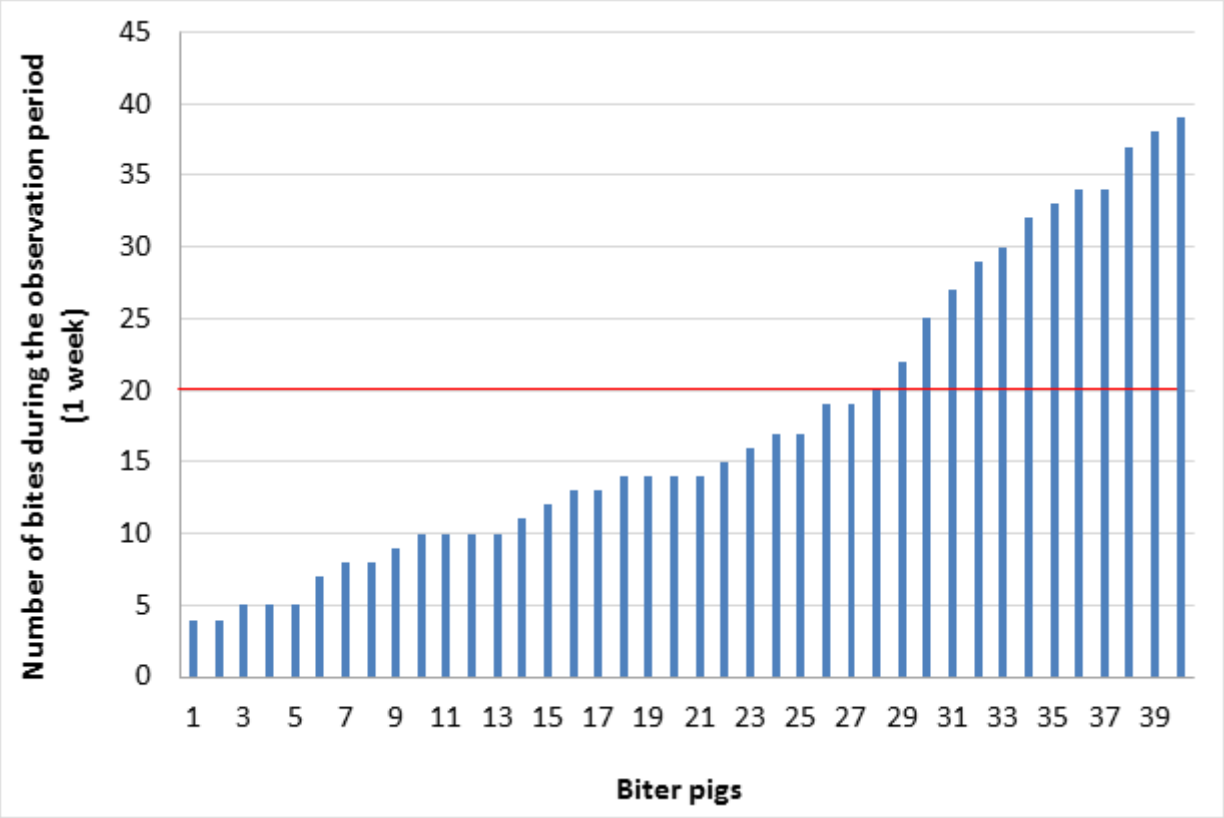

Figure 1

Number of bites for each biter pig over a one-week observation period and the threshold retained to select biter pigs. This threshold (20) was selected based on the highest tail-bite number and according to the required number of pigs (12) for the study.

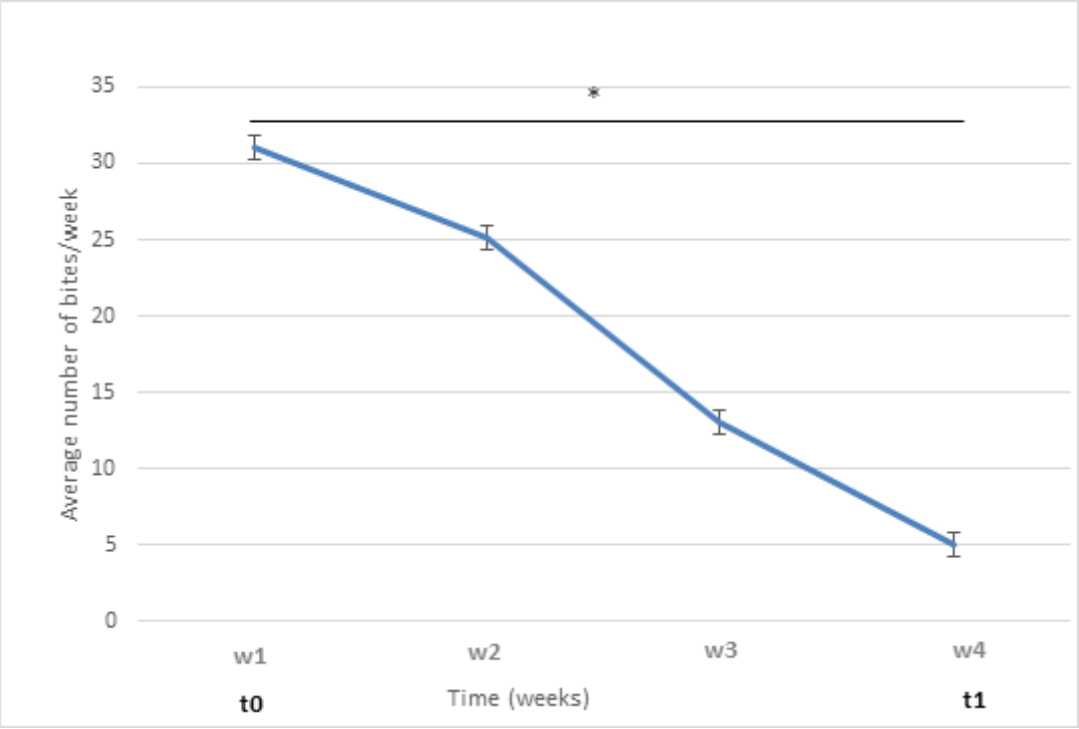

Figure 2

Variation of tail-biting (TB) behavior in biters over time. The average bite numbers of 12 biter pigs/week are shown according to time, w1 to w4 (week 1 to week 4) during the TB episode. t0: time of biter pig group selection; t1: 4 weeks following selection. The vertical lines represent the standard error. The horizontal line indicates statistically significant differences between groups (*p < 0.0001).

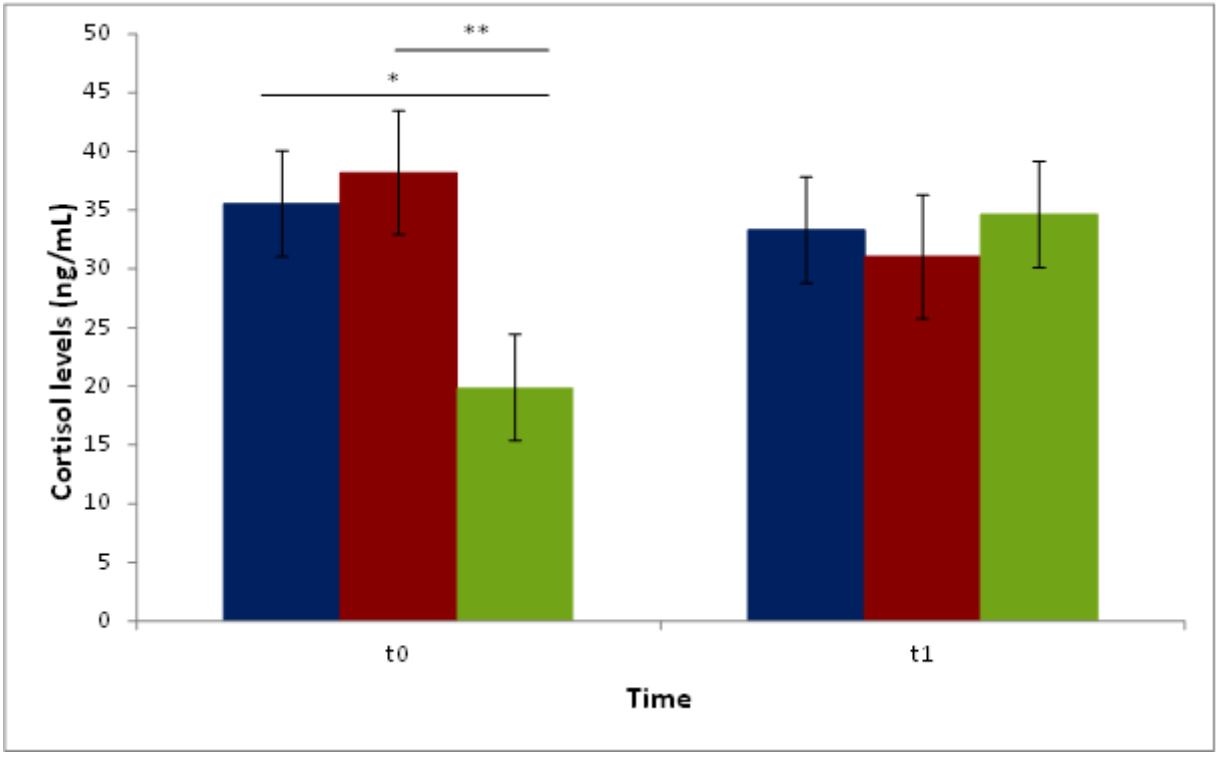

Figure 3

Average cortisol serum levels (ng/mL) of biter and bitten pigs vs. the negative control pigs during a tail-biting episode. t0: time of pig group selection; t1: four weeks following selection. Blue: biter pigs; Red: bitten pigs; Green: negative control pigs. The vertical lines represent the standard error. The horizontal lines indicate statistically significant differences between groups (* p = 0.02; ** p = 0.0076). No difference in blood cortisol levels within the t1 period (p > 0.05).

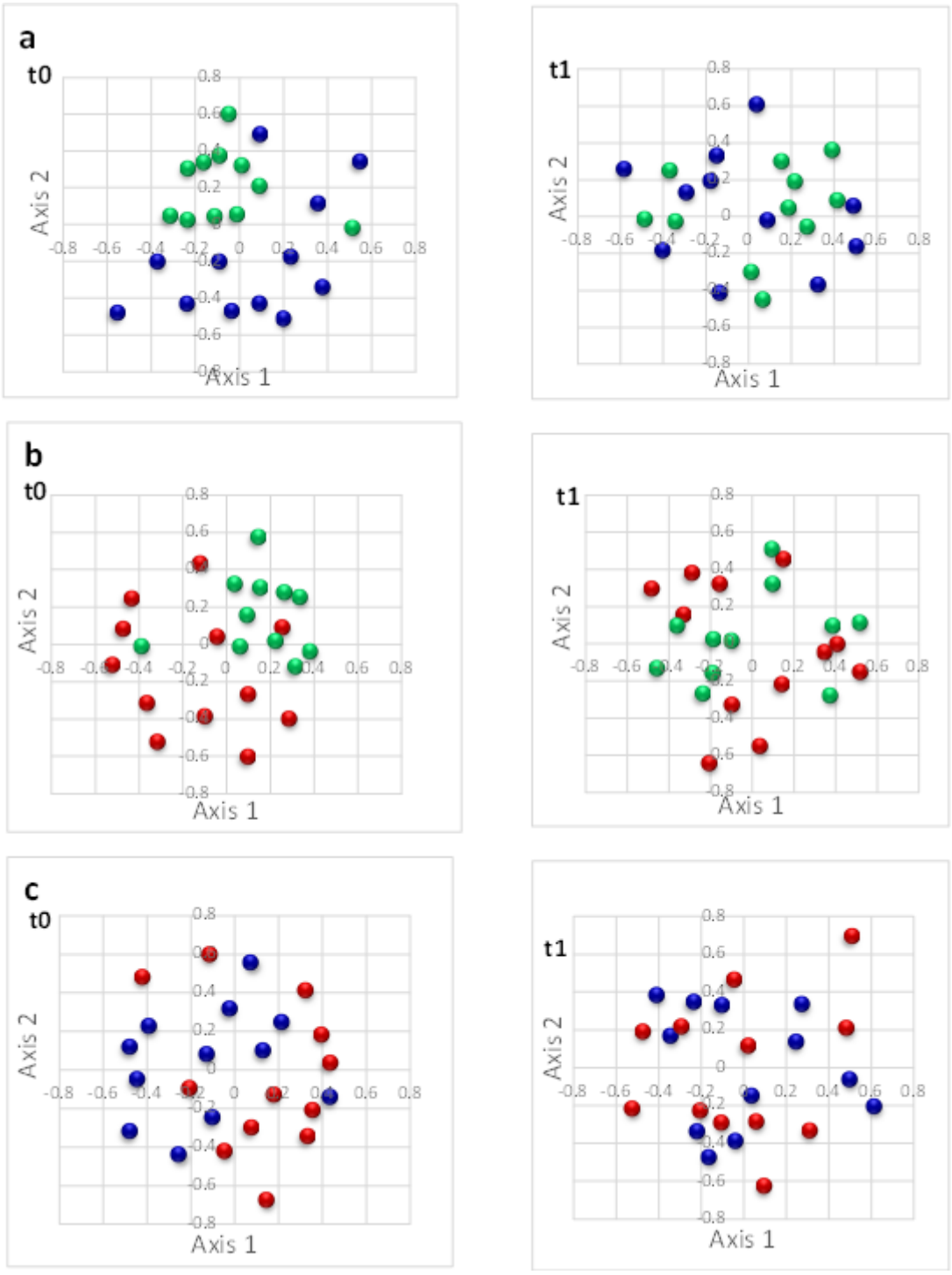

Figure 4

Non-metric multidimensional scaling (NMDS) plot of Yue & Clayton illustrating the comparison of intestinal microbiota of studied pigs. a: the comparison of intestinal microbiota of biter pigs vs. the

negative control pigs. b: the comparison of intestinal microbiota of bitten pigs vs. the negative control pigs. c: the comparison of intestinal microbiota of biter pigs vs. bitten pigs. t0: time of pig group selection; t1: four weeks following selection. Green: negative control pigs; Blue: biter pigs; Red: bitten pigs.

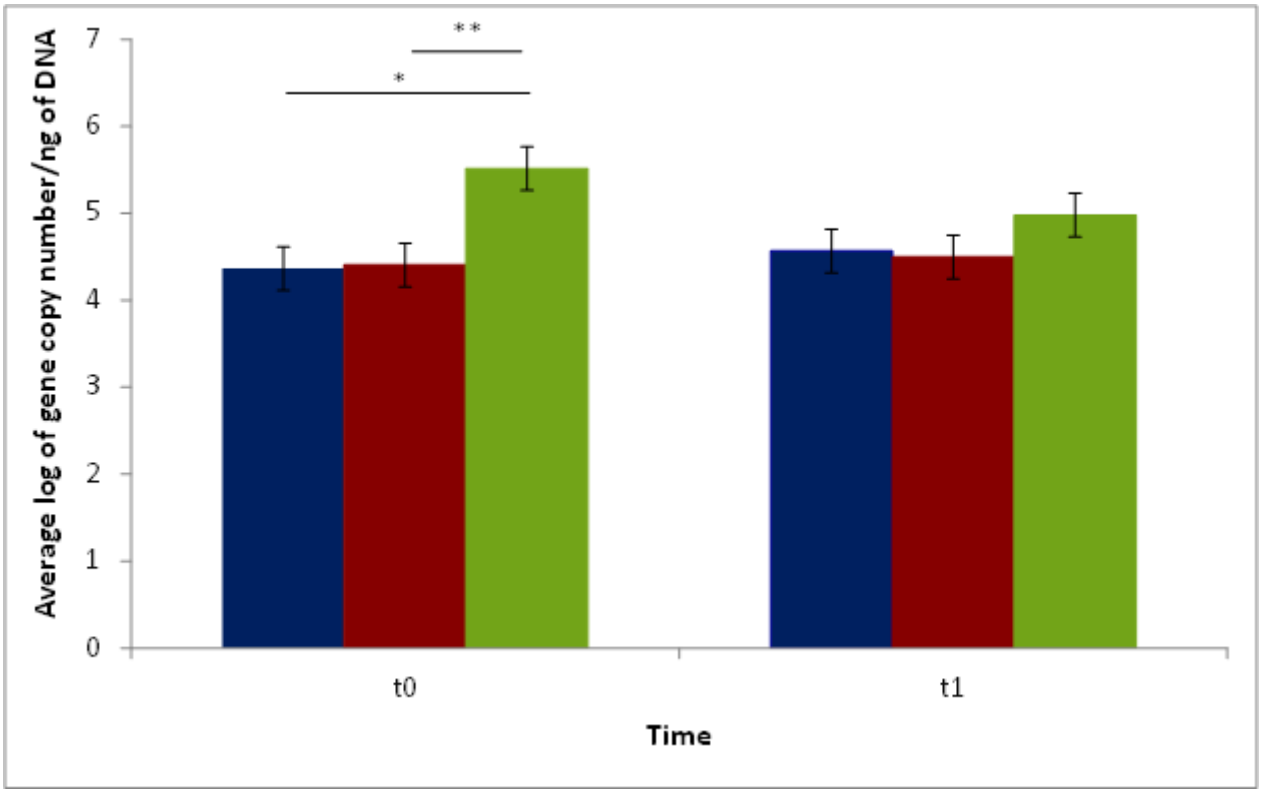

Figure 5

Average log of gene copy numbers of lactobacilli in biter and bitten pig fecal matter samples vs. the negative control pigs during the tail-biting episode. t0: time of pig group selection. t1: four weeks following selection. Blue: biter pigs; Red: bitten pigs; Green: negative control pigs. The vertical lines represent the standard error. The horizontal lines indicate statistically significant differences between groups (* $p < 0.003$; ** $p < 0.003$). NS: No difference in average log of gene copy number/ng of DNA within the t1 period ($p > 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfile2020031110h00.docx