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Impact of fresh alfalfa silage exposition on piglet health and gut microbiome and resistome

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Title: Impact of fresh alfalfa silage exposition on piglet health and gut microbiome and resistome

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Abstract

The agricultural sector accounts for approximately 82% of antibiotics sold in Canada, with the swine industry as the top consumer. Natural alternatives aimed at enhancing pig health and reducing reliance on antibiotics are being explored to mitigate the negative consequences of current piglet weaning practices.

This study examined the effects of exposing sows and piglets to alfalfa silage or chlortetracycline on piglets general health, gut microbiome, and antimicrobial resistance (AMR). Gut microbiomes were assessed by 16S rRNA amplicon sequencing (ileum and colon), measuring intestinal concentrations of volatile fatty acids (cecum and colon), and counting viable *Lactobacillaceae,* beneficial bacteria, and potentially pathogenic *Enterobacterales* populations (feces). Resistomes were evaluated by quantifying the proportions of *Enterobacterales* resistant to seven antibiotics and using High-Throughput qPCR to determine the relative abundance of hundreds of AMR genes and mobile genetic element markers in feces.

The innocuity of both treatments was validated as sows and piglets remained healthy throughout the course of the experiment. Alfalfa silage increased bacterial diversity in piglet feces, as well as the proportion of beneficial bacteria and the cecal concentration of volatile fatty acids, important nutrients generated by microbial activity. Alfalfa silage only had mild effects on the frequencies of viable *Lactobacillaceae* and *Enterobacterales*. Nevertheless, silage influenced the resistomes of sows and piglets, reducing the frequency of some AMR genes, while favoring others.

Alfalfa silage is a sustainable and resilient farming practice adapted to agricultural contexts in which it is accessible to swine producers. This intervention promoted the piglets' gut microbial diversity, suggesting enhanced resilience when encountering enteric pathogens. AMR bacteria of clinical relevance are commonly encountered in livestock, urging the need to develop alternatives to antibiotic use and mitigation approaches. This study provides findable, accessible, interoperable, and reusable data reflecting the spatiotemporal nature of gut microbiomes and AMR that appear relatively stable at the animal population scale for a given life stage and herd, yet exhibit dynamic AMR arrangements at the microbial population scale.

Keywords

Antimicrobial resistance, swine, pig, alfalfa silage, gut microbiome, chlortetracycline

Background

The agricultural sector is responsible for roughly 82% of all antibiotics sold in Canada, with swine production representing the largest share of annual purchases [1]. To mitigate the adverse effects of conventional weaning practices, natural alternatives to antibiotics are being investigated as a mean to boost pig health. At this stage, piglets are separated from their mothers while their immune systems are still immature, making this transition a period of increased vulnerability to multiple stressors [2, 3]. Furthermore, the growing impact of climate change and global socioeconomic issues on grain availability calls for locally sourced feed supplements and enrichment strategies [4, 5]. This substantiates the importance of implementing sustainable and resilient farming practices to address these challenges.

This study focuses on post-weaning piglets, a critical stage in swine production where antibiotics are commonly administered to prevent and control enterotoxigenic *E. coli* and cooccurring bacterial pathogens responsible for post-weaning diarrhea. However, sows' health, microbiomes and resistomes were also monitored, given their pivotal role in shaping the early gut environment of piglets [6, 7]. Healthy sows are more likely to produce robust offspring and supply high-quality colostrum and milk in sufficient quantities, both essential for supporting the early growth and immune development of piglets.

Alfalfa silage, a fermented product commonly used in ruminant farms (bovine, goat and sheep) was one of the two treatments investigated in this study. During the ensiling process, a microbial subgroup of the alfalfa holobiont (including epiphytic, endophytic, and soil-borne microorganisms) drives and/or survives lactic fermentation. This process leads to the enrichment of beneficial microorganisms, such as members of the *Lactobacillaceae* family [8]. Alfalfa silage is a source of fibre, protein and other nutrients essential for animal health and growth [9]. Silage was also recognized as an enrichment strategy in pig production [10]. Including alfalfa silage in pig feed enhanced meat quality by increasing the water-holding capacity and marbling, reducing the drip loss, and changing the profile of fatty acids and amino acids in muscle [11].

In this study, we explored the effects of early exposure to alfalfa silage (SIL) or post-weaning administration of chlortetracycline (CTC) on the pig gut microbiome and resistome. Additionally, we provide comprehensive data regarding the primary factors influencing microbiome composition, such as animal health status and growth stage. The main hypotheses

driving this research were: 1) SIL is safe for the pigs and offers locally-sourced beneficial microorganisms; 2) sow and piglet exposition to SIL during lactation increases the proportion after weaning of *Lactobacillaceae* in the piglet gut microbiome, as SIL is a product of lactic acid fermentation and this bacterial family is known for its tolerance to gastric acidity; 3) SIL increases gut microbiome diversity, disfavouring AMR pathogens and supporting beneficial bacteria, using *Enterobacterales* and *Lactobacillaceae* as indicators, respectively. Ultimately, we investigated whether SIL could serve as an alternative to antibiotics, specifically the commonly used CTC.

Methods

Animals

For the primary experiment, conducted from August 2021 to June 2022, twenty-four multiparous Yorkshire–Landrace sows and their litters were utilised in a generalised, randomised complete block design with four blocks. All animal procedures were approved by the Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines [12], protocol #583. Each block comprised six sows and their litters, randomly assigned to three treatments (n = 8 sows/litters per treatment). Within two days after birth, litters were standardised to 12 piglets. At 22 days of age, piglets were vaccinated against circovirus (Circovac, Med'Vet), weaned by removing the lactating sows from the pens, and transitioned to solid diets (Tables S1, S2, S3). Littermates remained together in the farrowing crates until the end of the experiment at D42. Feed intake, piglet and sow body weight, and sow backfat thickness were recorded at specific time points throughout the experiment (Figure 1). Additional details can be found in the Supplementary File.

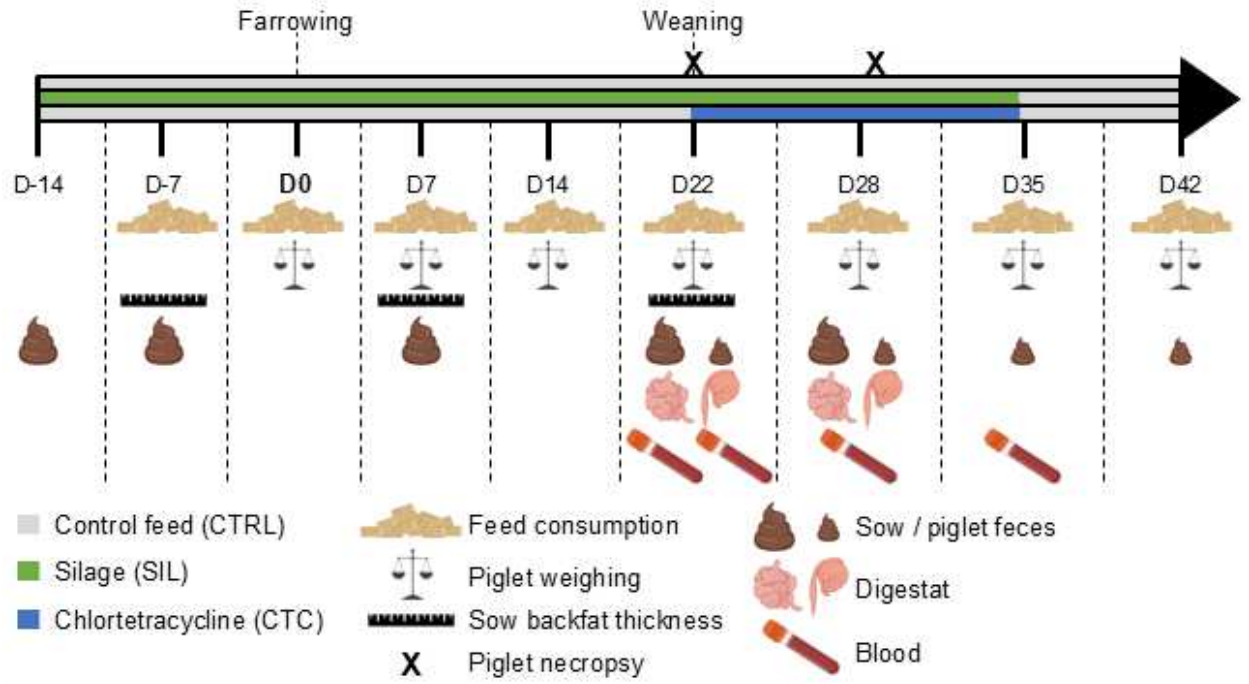


Figure 1. Schematic representation of the animal trial showing key steps (farrowing, weaning and piglet slaughter), treatment periods and data and samples collection.

Diets and treatments

The treatments administered to sows and piglets were 1) the control diets (CTRL); 2) the control diets in conjunction with early exposure to alfalfa silage (SIL); and 3) the control diet for sows and supplementation of the piglet control diet with 440 ppm of chlortetracycline from weaning to D35 (CTC, Chlor100) (Figure 1). All diets served to sows and piglets met the nutrient requirements recommended by the National Research Council [13], with minimal copper and zinc concentrations (Tables S1, S2 and S3). All piglets returned to the control diet from D35 to the end of the experiment on D42.

Preliminary assays were conducted to optimise the silage type and handling procedures to ensure innocuousness, preserve microbial integrity, and maintain practical feasibility. The Harvxtra® alfalfa was selected for its reduced lignin content and improved digestibility. The second cutting of the season was harvested at the pre-flower bud stage. SiloSolve MC (DLF) was used as the inoculant to initiate alfalfa fermentation. Every two weeks, fermented silage was transported to the Sherbrooke Research and Development Center and vacuum-packed in 10 kg bags before storage at 4°C until use. Silage was provided by a local dairy farmer who stored it in fully enclosed and rodent- and bird-proof concrete silos. Prior to use, silage was tested for mycotoxins, *Salmonella*, yeast and mold content by a commercial laboratory (Agri-Analyse, Sherbrooke, QC, Canada) to ensure its safety.

At 100 days of gestation, sows were distributed in two rooms of the maternity ward. One room housed animals assigned to CTRL and SIL, while the other housed the CTC group. In the shared room, pens for different treatments were separated by a central alley. Measures were taken to limit cross-contamination between treatments. Each employee had dedicated sets of boots for each room, and wearing gloves when handling animals was mandatory. Gloves were disinfected using 70% ethanol between each pen. At 101 days of gestation (D-14), sows in the SIL group began receiving the treatment, which continued for five weeks until weaning (Figure 1). Farrowing occurred (D0), and adoptions were conducted within 48 h to standardise litters to 12 piglets. Adoptions were limited to exchanges between CTRL and CTC sows within a block.

To ensure consistent consumption and exposure to silage within and across litters, a preliminary behaviour trial was conducted with four sows and their litters to optimise the timing and quantity

of silage per serving. Observations from video recordings focused on silage-directed feeding and exploratory behaviour by sows and piglets during daylight at D20. Detailed methods and results of the behavioural observations are provided in the supplementary file.

In the main experiment, SIL sows received 4 kg of silage daily, divided into two servings in addition to their gestation or lactation concentrate feed. During gestation (D-14 to D0), 2 kg of silage was provided at 9:00 a.m. on a rubber mat placed beneath the sow's head, avoiding the water bowl area (Figure S1A). During lactation (D0 to D21), silage was served at 7:30 a.m. alongside the sow's morning meal. At 11:00 a.m., any remaining silage was gathered and repositioned in front of the sow. The second 2 kg serving was provided after the second meal, between 2:00 p.m. and 3:00 p.m. Any leftover silage was removed before the next morning's feed service at 7:30 a.m. SIL piglets were exposed to silage from birth until D35 (Figure S1A). From D9 to D35, 2 kg of silage was provided daily in two feed servings (7:30 a.m. and 2:00 to 3:00 p.m.), on a separate rubber mat located in a piglet-only zone (Figure S1B and S1C).

Sampling and processing

Fresh fecal samples from sows and litters were collected either directly from the rectum or from the ground within 2 minutes after defecation, ensuring the sample was taken from the center of the fecal mass to avoid contact with the ground. Samples were transferred into sterile Whirl-Pak bags (Nasco) and kept on ice until processing. Fecal samples from sows were stored individually. For piglets, feces from three individuals of varying sizes per litter were collected at four timepoints and pooled per litter (Figure 1). Within 4 h of collection, fecal samples were aliquoted and processed as follows: stored at -20°C for subsequent quantifications of volatile fatty acids;

diluted 1:2 in 0.9% saline and stored at -80°C for molecular biology methods; diluted 1:4 in Cary-Blair medium (CB; Dalynn) with 20% glycerol to preserve viable bacterial cells and stored at -80°C for future microbiology analyses. Blood samples from two piglets per litter were collected in Vacutainer® EDTA tubes (Becton, Dickinson and Company). Euthanasia of two piglets per litter was carried out at weaning (D21/D22) and one week later (D29/D30) by exsanguination following tranquillisation with an intramuscular injection of Stresnil (2.5 mg/kg) and stunning with a matador-type slaughter gun. After euthanasia, the pH of the cecal and colonic digesta was measured, and digesta from the ileum and colon were collected and preserved in liquid nitrogen, then stored at -80°C.

Quantification of piglets' immune markers

Endotoxin units per mL were evaluated at day 35 using the Pierce Chromogenic Endotoxin Quant Kit (ThermoFisher Scientific). Superoxide dismutase (U/mL) and TNF-alpha levels (pg/mL) were measured at days 20, 23, 28 and 35 using the Superoxide Dismutase Assay kit (Cayman Chemicals) and the Porcine TNF-alpha DuoSet ELISA (R&D Systems Inc.), respectively. All these specific proteins were quantified in piglet plasma according to the kit manufacturer's instructions.

Analysis of volatile fatty acids

The concentrations of acetate, propionate, butyrate, isobutyrate, valerate and isovalerate were quantified in the cecum and colon digesta of piglets just before weaning and a week later. Samples were diluted 1:4 in bidistilled water, then vortexed and centrifuged at 12,000 × g for 15 min. A weight of 1.3 g of supernatant was homogenised with 250 µl of 0.5 M H_2SO_4 and

subsequently centrifuged at 12,000 × g for 15 min. Next, 0.5 mL of the supernatant was combined 1:1 with the internal standard 2-ethylbutyric acid at a concentration of 2 g/L, and mixed with 0.15 g of Dowex 50W X8 beads (Millipore Sigma, QC, Canada). The mixture was vortexed and incubated for 10 min at room temperature to allow the beads to settle before passing through a 0.45 µm Minisart filter (Sartorius, Germany). Analyses were performed using a Perkin-Elmer gas chromatograph, model Clarus 580 (Perkin-Elmer Corporation, Shelton, CT, USA), coupled to a high-resolution DB-FFAP column connected to a flame ionization detector. VFA concentrations, expressed in mg/L, were deduced from measurements obtained for a standard solution containing acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic acids at 2 g/L in reverse-osmosed deionised water with a resistivity > 10 megohm/cm.

Bacterial counts

Enterobacterales and *Lactobacilli* were enumerated in the feces of individual sow samples and composite samples of piglets by plating using the easySpiral automatic plater (Interscience). Bacterial colonies were then counted using the Scan 4000 (Interscience). MacConkey II Agar (MAC, BD Biosciences) ± antibiotics was used to quantify sub-populations of *Enterobacterales*. The antibiotics were supplemented at half the minimum inhibitory concentration (MIC) and consisted of cefotaxime (CTX; 2 µg/ml), ciprofloxacin (CIP; 0.5 µg/ml), co-trimoxazole (SXT; 2 µg/ml trimethoprim, 38 µg/ml sulfamethoxazole), gentamicin (GEN; 8 µg/ml), tetracycline (TET; 8 µg/ml) and meropenem (MER; 2 µg/ml) according to the CLSI guideline [14]. All MAC plates were then incubated at 37°C for 16 to 24 hours. *Lactobacilli* were enumerated using LAMVAB media consisting of *Lactobacillus* anaerobic MRS agar (Merck) supplemented with 2 mg/ml vancomycin and bromocresol green [15]. The LAMVAB plates were incubated at 37°C

for 48 hours. The colony-forming units (CFU) were expressed as CFU per gram of the primary sample. The limit of detection was 10 CFU/ml.

Preparation of total DNA from primary samples

Total DNA from digesta and feces samples was extracted as described by Zaheer R, Noyes N, Ortega Polo R, Cook SR, Marinier E, Van Domselaar G, Belk KE, Morley PS and McAllister TA [16] with the following modifications. The thawed, diluted fecal samples and digesta (350 mg) were transferred to a tube containing sterile zirconia beads and resuspension buffer with β-mercaptoethanol and pre-heated 70% SDS. A Bead Ruptor Elite (OMNI International) at 4000 rpm for 3 min was used for the homogenization step. Dried pellets were dissolved in 200 µL of TE 1X instead of 400 µL before overnight incubation at 4°C. Purification steps were done with the QIAamp Fast DNA stool mini kit (Qiagen). The elution step was repeated twice for a final volume of 100 µL of DNA. Samples were diluted to 75 ng/µl in order to be passed through the OneStep PCR inhibitor removal kit (Zymo Research) according to the manufacturer's instructions. DNA concentrations and OD ratios (A260/A280 and A260/A230) were determined with a NanoDropTM 8000 spectrophotometer (ThermoFisher Scientific). Integrity was verified by agarose (1%) gel electrophoresis. The presence of PCR inhibitors was also evaluated by amplifying 100 ng of DNA with universal 16S rRNA gene primers 27F and 355R (Table 1).

Table 1. Primers and probes used in this study[1].

Primers	Sequence (5' - 3')	Target	Reference
27F[2]	AGAGTTTGATCMTGGCTCAG	V1-V2 16s rRNA	[43]
355R[2]	GCTGCCTCCCGTAGGAGT	V1-V2 16s rRNA	[44]
515F[3]	GTGCCAGCMGCCGCGGTAA	V4 16s rRNA	[17]
806R[3]	GGACTACHVGGGTWTCTAAT	V4 16s rRNA	[17]

[1]The primers used by Resistomap for conducting HT-qPCR are not included in this table but can be found in Stedtfeld RD, Guo X, Stedtfeld TM, Sheng H, Williams MR, Hauschild K, Gunturu S, Tift L, Wang F, Howe A, Chai B, Yin D, Cole JR, Tiedje JM and Hashsham SA [45].

[2]To test the presence of PCR-inhibitors in DNA preparations.

[3]To generate amplicons for sequencing.

16S rRNA amplicon sequencing and data analysis

To characterise the sow and piglet gut microbiomes, DNA samples were transferred to the *Centre d'expertise et de services Génome Québec* (Montreal, Canada), where amplicon library preparations and DNA sequencing were performed using Illumina MiSeq technology in paired-end format for a read length of 250 bp. The bacterial V4 hypervariable region of 16S rRNA genes was amplified using the primer set 515F and 806R (Table 1). Based on a previous study, this prokaryotic primer pair produced the highest estimates of species richness and diversity for all sample categories, as well as the highest diversity coverage of reference databases in *in silico* primer analysis [17]. An average of 69,017, 73,136, and 28,585 reads, for the ileum, colon, and feces samples, respectively, was used as input for the nf-core/ampliseq v2.3.1 workflow, which uses DADA2 to generate amplicon sequence variants (ASVs) [18]. The Silva 138 SSU database was used to infer taxonomy [19]. Rarefaction curves showed that all samples were sequenced to a depth sufficient to reach a plateau of ASV discovery, consistent with adequate coverage of the microbiota composition.

Resistome and mobilome assessment using HT-qPCR

HT-qPCR assays were conducted using the SmartChip™ Real-Time PCR system (Takara Bio) operated by Resistomap (Helsinki, Finland). Further details are available in the Supplementary File. In a first round, DNA extracted from primary fecal samples of sows at D-14 and D21

(pooled by treatments and blocks, n=8 sows represented per sample), and of piglets at D35 (pooled by litters, treatments and blocks, n=24 piglets represented per sample) was subjected to HT-qPCR for assessing the abundance of resistance genes, markers of mobile genetic elements and 16S rRNA genes. In a second round, a set of the 54 most abundant targets in all samples, and contrasting with CTRL and CTC, was selected from post-treatment animals only (chip #4), for which DNA was pooled by treatment only, keeping the four blocks separate as biological replicates (n=2 sows represented per sample; n=6 piglets represented per sample).

Statistics

The experimental setup was designed as generalised random blocks, consisting of four blocks of eight litters (two litters per treatment per block, n = 8 sows per treatment, total of 32 sows or litters). The fixed effects of the models were set to treatment and block. The experimental unit to test the effect of the treatments was the sow/litter. To represent the litters, blood and digesta samples were analysed from pools of two piglets per litter. For fecal analyses, three piglets per litter were pooled. The day effect was considered as repeated measures on the litter with the appropriate variance-covariance matrix selected based on fit statistics. The analyses of variance were computed using SAS [20] on sows' weight, backfat thickness and daily feed intake, on piglets' weight, average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR), the concentration of endotoxins, superoxide dismutase and TNF-α in piglets' blood, on bacterial counts of sows and piglets feces samples, piglet digesta' pH and volatile fatty acids concentrations, and alpha diversity indices of digesta and fecal samples. Assumptions of normality and homoscedasticity were assessed for all ANOVA models. An area under the curve

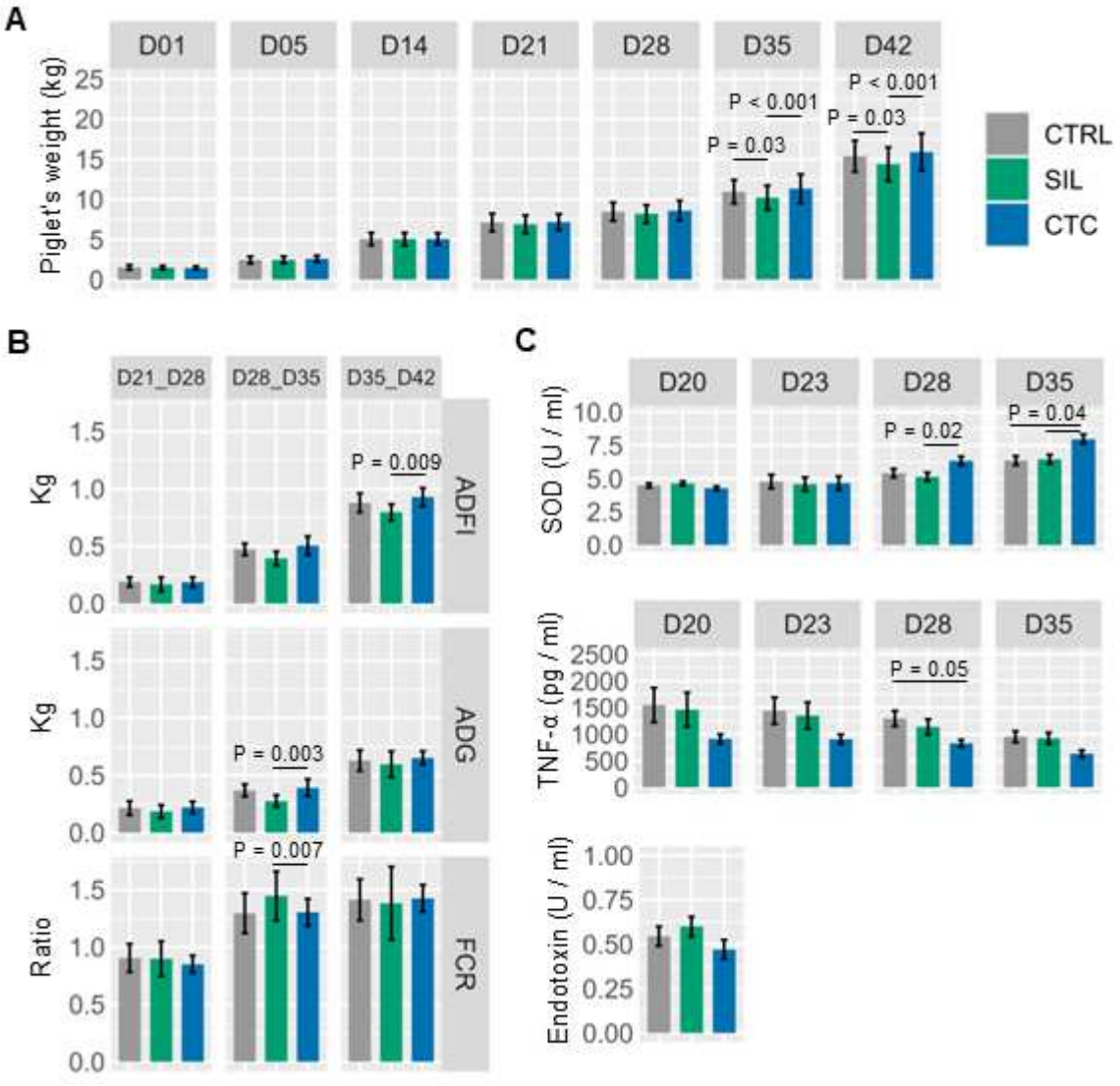
analysis of sows' feed intake was conducted for each block-sow-treatment combination. ADFI, ADG and FCR were calculated for three periods: D21 to D28, D28 to D35 and D35 to D42.

R packages phyloseq version 1.44.0 [21] and vegan version 2.6.4 [22] and their dependencies were used to compute and plot alpha diversity indices, abundance plots, and non-metric multidimensional scaling (NMDS) ordinations. We used permutest and adonis to assess data dispersion and conducted PERMANOVA, respectively. Alpha diversity indices were computed on unrarefied data. Correlation matrices were generated to assess redundancy between the seven alpha indices calculated. The number of observed ASVs and Shannon and Inverse Simpson alpha diversity indices were complementary in capturing the effects of the days and treatments on microbial diversity. Multiple models using the Microbiome Multivariable Association with Linear Models (MaAsLin2) version 1.14.1 [23] were fitted to conduct multivariable associations relying on general linear models using the default parameters to identify ASVs that were differentially abundant under given conditions specified in the figure legend. Analyses of variance and pairwise comparisons were computed on the relative abundances of the detected genes from Resistomap using the package emmeans 1.10.1 [24]. Assumptions of normality and homogeneity were confirmed on the residuals using the Shapiro-Wilk and Levene tests. Figure content was generated by ggplot2 and BioRender.

Results

Animal behaviour towards alfalfa silage

312 The use of SIL in swine production is unusual. Therefore, a preliminary trial was conducted to
313 determine the appropriate dosage and administration methods for exposing sows and piglets to
314 fresh alfalfa silage, aiming to minimize waste and ensure biosecurity. Sows readily accepted this
315 addition, and piglets' interest in silage was synchronised with the sow's behaviour and consistent
316 across the two crate zones where SIL was served. More piglets were observed exploring silage in
317 the front area of the crate, near the sow, compared to the back area. Additional details are
318 provided in the supplementary material and Figure S2.
319
320 **Impact of silage on weaning piglet's health and gut microbiome**
321 Apart from a slight decrease in SIL piglets' weight at D35 and D42, the weight, average daily
322 feed intake, average daily gain, feed conversion ratios, and blood concentrations of superoxide
323 dismutase, TNF-α, and endotoxin of SIL piglets were comparable to those of CTRL piglets
324 (Figure 2). SIL piglets tended to display lower average daily feed intake and average daily gain
325 than CTC piglets during the D35 to D42 and D28 to D35 periods, respectively (Figure 2B).
326 However, the feed conversion ratio was higher for SIL piglets than for CTC piglets.
327



328

329 **Figure 2.** Modulation of the growth performance and immunity markers following treatment
330 with silage or chlortetracycline. The bars represent A) the average piglets' weight by litter, B) the
331 average daily feed intake (ADFI), average daily weight gain (ADG), and feed conversion rate
332 (FCR) of piglets calculated for the three weeks following weaning, C) the concentrations in
333 blood of superoxide dismutase (SOD) in units per milliliter, TNF-α in picograms per milliliter,
334 and endotoxins in units per milliliter. All bars represent the adjusted means plus or minus the
335 standard error of the mean of values. Two-way analysis of variance with Tukey's multiple

comparisons test was used to compare the means for the day and treatment variables. P values beyond the 0.05 threshold are indicated.

At D28, the concentration of acetate in the cecum was significantly higher in SIL than in CTC animals, with a trend towards increased levels of propionate and butyrate as well (Figure 3A). Alpha diversity was generally greater in the feces of SIL piglets compared to the CTRL and CTC groups, especially at weaning and a week later (Figure 3B). Consistently, NMDS ordination revealed a modest shift in the microbiome composition of SIL digesta (Figure 3C).

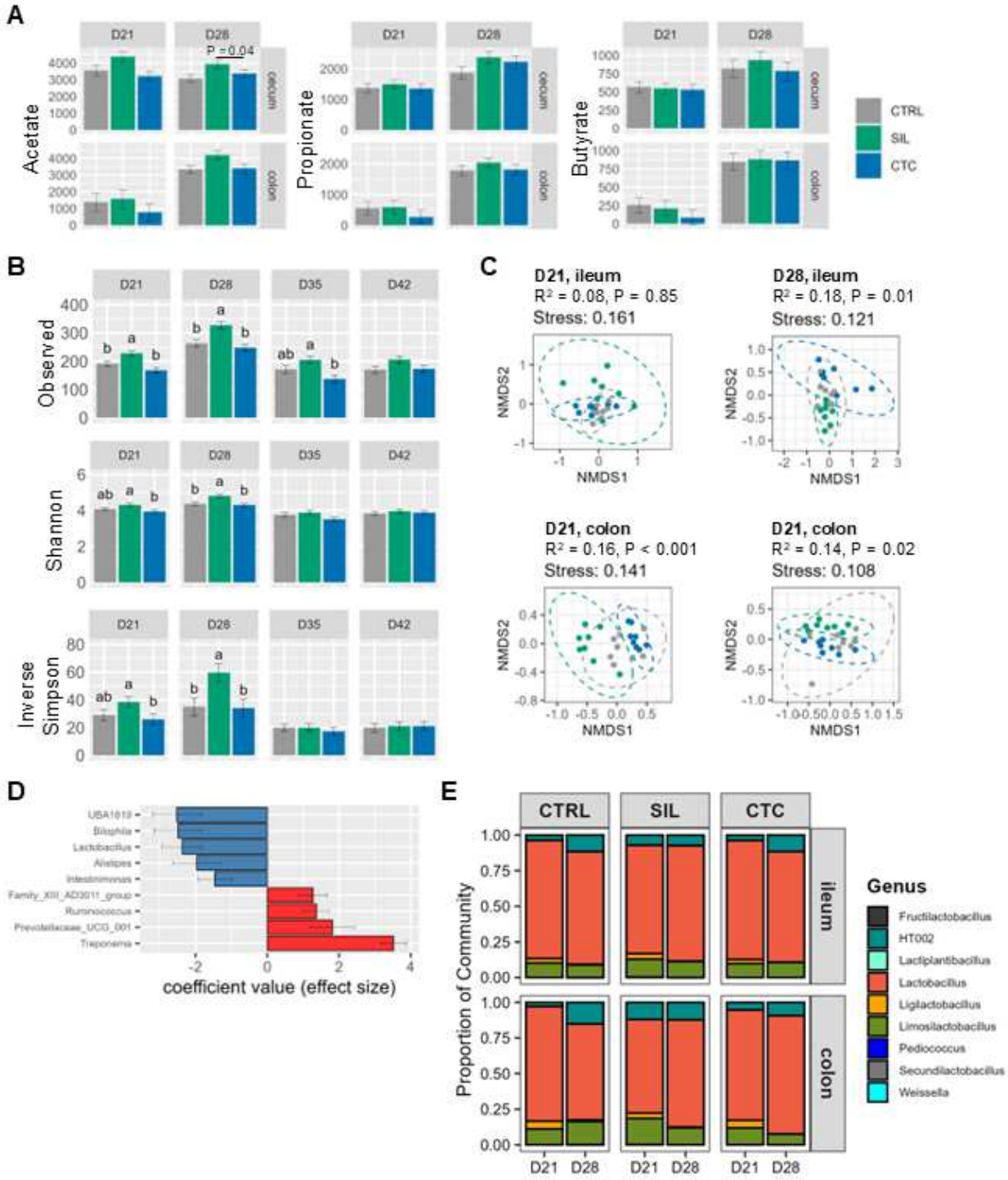
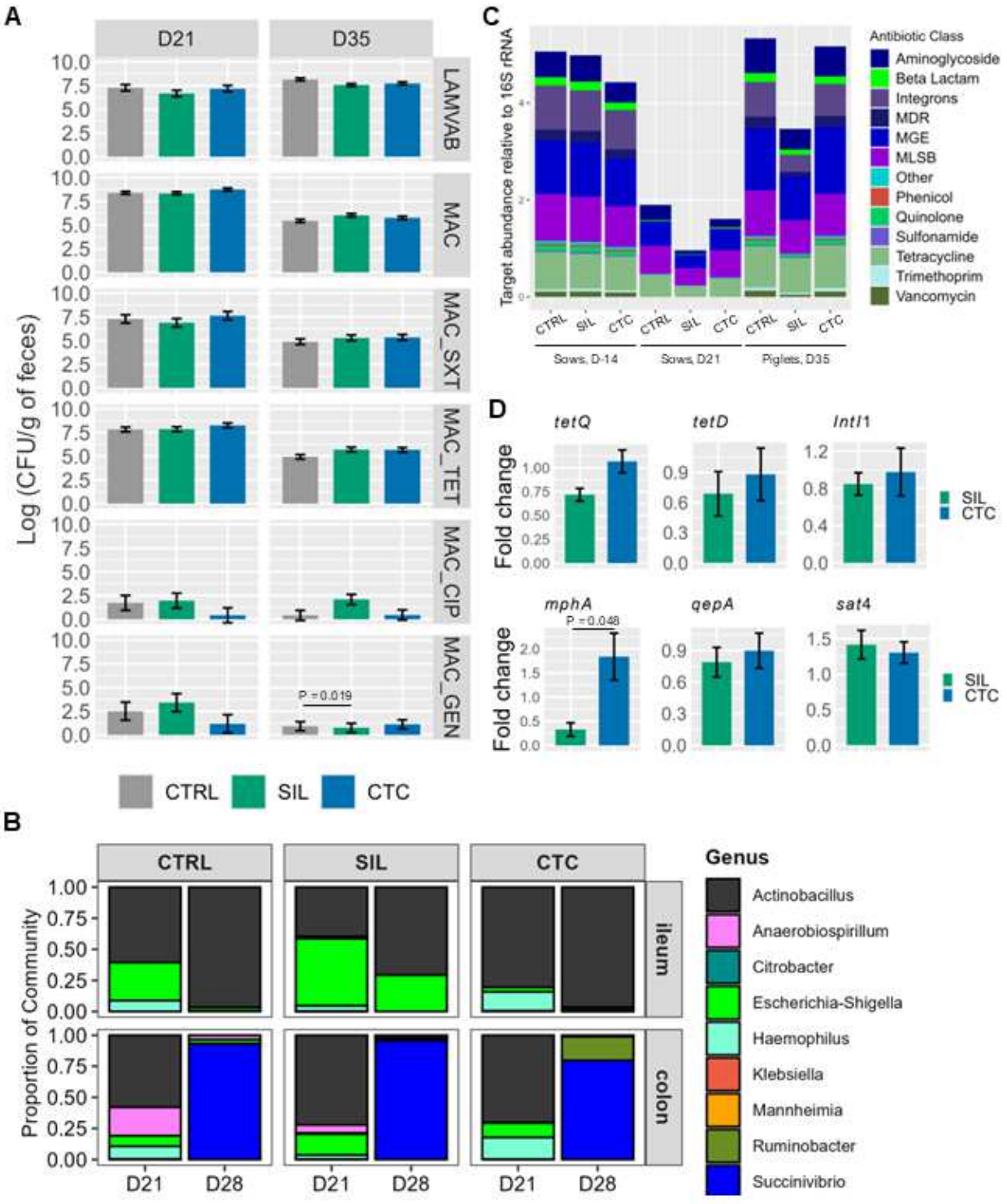


Figure 3. Distinct features of piglet gut microbiome subjected to silage or chlortetracycline. A) The concentrations of volatile fatty acids in the cecal and colonic digesta of piglets expressed as mg / L. Two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test was

used to compare the means of the treatment groups. B) Alpha diversity analysis of the piglets' feces. The bars represent the adjusted means plus or minus standard error of the mean for the observed ASVs, and the Shannon and Inverse Simpson indices. Different letters on top of bars indicate significantly different results ($P < 0.01$). C) Non-metric multidimensional scaling (NMDS) based on Bray-Curtis distances of ASV relative abundances and results from the corresponding permutest and PERMANOVA for the treatment variable. D) A Microbiome Multivariable Association with Linear Model [6] was fitted to compare the abundances of taxa at the genus phylogenetic level in the feces of 21-day-old piglets subjected to CTRL versus SIL. E) Abundance plot showing the proportion of genera from the *Lactobacillaceae* family in ileal and colonic digesta of piglets at weaning (D21) and a week later (D28) according to the treatments.

A Microbiome Multivariable Association with Linear Model analysis revealed differences in taxa enrichment at D28 between the ileum and colon of SIL piglets compared to CTRL. *Bifidobacterium* (coefficient value = 6.58, standard error = 1.43), *Romboutsia* (coefficient value = 3.31, standard error = 0.94), and *Lactiplantibacillus* (coefficient value = 1.71, standard error = 0.43) were more frequent in the ileum, whereas *Terrisporobacter* (coefficient value = 4.55, standard error = 1.09), *Turicibacter* (coefficient value = 4.12, standard error = 0.98), *Coriobacteriales* (coefficient value = 2.38, standard error = 0.66), *Oribacterium* (coefficient value = 1.83, standard error = 0.51), *Lachnospiraceae* (coefficient value = 1.43, standard error = 0.43), and *Corynebacterium* (coefficient value = 0.75, standard error = 0.22) were enriched in the colon. Unexpectedly, at weaning, the relative abundance of *Lactobacillus* was lower in SIL piglets feces compared to the CTRL group (Figure 3D). This finding was supported by the proportions of this genus within the *Lactobacillaceae* family (Figure 3E) and by a lower, yet

371 statistically non-significant, colony count on LAMVAB in the SIL group (Figure 4A). While the
372 proportion of viable enterobacteria in feces was comparable across treatments (Figure 4A), the
373 proportion of *Escherichia-Shigella* at D21 was higher in the ileum and colon of SIL piglets
374 compared to the two other groups (Figure 4B).



375

376 **Figure 4.** Dynamics of culturable beneficial and pathogenic bacterial populations according to
377 the silage and chlortetracycline treatments, and their AMR profiles. A) Bar plots of culturable
378 populations of *Lactobacillaceae* and antibiotic-resistant *Enterobacterales* from pre- and post-

379 treatment piglet fecal samples. Results are presented as mean bacterial counts plus or minus
380 standard error of the mean for samples enumerated on *Lactobacillus* anaerobic MRS agar
381 (LAMVAB), as well as MacConkey agar plates (MAC), with or without supplementation of co-
382 trimoxazole (SXT), tetracycline (TET), ciprofloxacin (CIP), gentamicin (GEN). Growth on
383 cefotaxime plates was scarce. No colonies were obtained on MAC agar supplemented with
384 meropenem. B) Abundance plot showing the proportion of genera belonging to the
385 *Enterobacterales* family in colonic and ileal digesta of piglets at weaning (D21) and a week later
386 (D28). C) The resistome in sows' and piglets' feces is presented as stacked bars of relative
387 abundance of 374 targets detected in pre- and post-treatment feces from sows, and post-treatment
388 feces from piglets, as quantified by HT-qPCR. D) Specific gene abundance was detected by HT-
389 qPCR in post-treatment feces from piglets, standardized using the 16S rRNA genes (ΔCt), and
390 then fold changes compared to CTRL were calculated ($2^{-\Delta\Delta Ct}$). A Welch's t-test was performed
391 for each gene to compare treatment means. P-values above the 5% threshold are indicated.
392
393 The summation of 374 antimicrobial resistance genes and mobile genetic element markers was
394 lower in SIL sows and piglets compared to the CTRL and CTC groups (Figure 4C). Notably, SIL
395 piglets exhibited a lower relative abundance of *mphA* compared to CTRL animals (Welch's t-test,
396 $t = 2.99$, $df = 3.49$, $P = 0.048$) (Figure 4D). The magnitude of the difference was large (Cohen's d
397 $= 2.11$).
398
399 **Impact of CTC on piglets' health and microbiome**
400 CTC piglets tended to display higher average daily feed intake and average daily gain than SIL
401 piglets (Figure 2B). Consequently, CTC piglets were heavier than all other groups from D35

onward (final weight in kg at D42 – CTRL: 15.36, SIL: 14.38, CTC: 15.93, sem = 0.422). Superoxide Dismutase remained stable three days after weaning, but increased at D28 and D35 in piglets consuming CTC (Figure 2C). At all time points, CTC piglets had lower circulating TNF-α concentrations (Figure 2C). CTC had no significant effect on VFA concentrations. The microbial diversity in the piglets' gut was similar between CTC and CTRL animals (Figure 3B and 3C).

Discussion

For successful implementation in commercial operations, feed additives and environmental enrichment strategies must be accessible and accepted by pigs, either through good palatability or by promoting natural behaviours. It is also paramount that these additives are safe for the animals. Our first hypothesis was verified: SIL is safe for both sows and piglets under the conditions of this study. Both sows and piglets actively interacted with the alfalfa silage through play and chewing, leading to the ingestion of its microorganisms and fermentation products. However, losses due to evaporation and through the slatted floor prevented the quantitative assessment of silage consumption. SIL slightly reduced piglet feed intake during the D35 to D42 window, which was reflected in their weight, although the differences observed in ADG or FCR were not significant at D42. This may be because these measurements coincided with the gut microbiome transition period from lactation to a solid diet. Therefore, we hypothesise that the control diet, consisting mostly of grains, allows for an easier transition for immature, milk-adapted microbiomes than silage, which requires adaptation of the intestinal track and slow-growing microbes capable of degrading complex fibres. Had an additional time point been

investigated a week or two later, we might have captured the stabilization of a gut microbiome adapted to assimilating nutrients from alfalfa silage. Previous studies have shown that including alfalfa in the diet of growing or fattening pigs can increase ADFI or have only a modest impact on ADG and FCR [11, 25, 26]. Ideally, the early-life halted weight gain in SIL piglets would need to fade over time, allowing them to reach market weight as efficiently as pigs raised without antibiotics. This presents a valuable opportunity for producers to supplement the animal concentrated feed with beneficial microorganisms, especially during years of abundant alfalfa harvest.

Since silage is the product of lactic acid fermentation partly driven by *Lactobacillaceae*, a bacterial family recognised for its beneficial properties and tolerance to gastric acidity [27], we investigated whether SIL could increase the proportion of *Lactobacillaceae* in the gut microbiome. Following weaning, the proportions of *Lactobacillaceae* increased, as confirmed by amplicon sequencing and bacterial counts, and this family dominated the ileum microbiome. At D21, however, the proportion of *Lactobacillaceae* was lower for SIL piglets than for CTC piglets. However, differential abundances indicated that bacteria contributing to silage fermentation (i.e. *Lactiplantibacillus*) [28] and indigenous fibre-degrading beneficial bacteria (i.e. *Bifidobacterium* and *Romboutsia*) [29, 30] were favoured in the ileum of SIL piglets. SIL was prepared using a commercial silage inoculant. Although the manufacturer did not disclose the specific strains, the inoculant was described as containing ‘three highly-selected strains of lactic acid bacteria that direct and control the overall fermentation’. Although not performed in this study, amplicon sequencing of the microbial populations in the SIL could have enabled source tracking of the observed taxa.

448

449 We then assessed whether SIL could increase gut microbiome diversity, disfavouring AMR
450 pathogens and supporting beneficial bacteria, using *Enterobacterales* and *Lactobacillaceae* as
451 indicators. SIL promoted a healthier gut microbiome by increasing diversity and the relative
452 abundance of beneficial bacteria. Piglets exposed to SIL exhibited a more diverse gut microbiota
453 at weaning and throughout the following two weeks. This increased diversity is considered
454 advantageous for microbial resilience when piglets are particularly vulnerable and prone to post-
455 weaning diarrhea. The SIL composition and induced microbial diversity also supported the
456 metabolism of polysaccharides and other substrates, leading to higher concentrations of acetate,
457 propionate, and butyrate in the cecal digesta of SIL piglets. These findings align with previous
458 studies showing that alfalfa supported some VFA-producing bacteria [31, 32]. Likewise, another
459 study reported higher proportions of taxa, such as *Terrisporobacter* and *Turicibacter*, in the colon
460 of SIL piglets compared to controls [11]. Both are Gram-positive anaerobes known to produce
461 VFAs [33, 34]. These VFAs serve not only as an energy source but also promote epithelial
462 integrity, reduce inflammation, and have regenerative properties [35, 36]. Additionally,
463 fermented alfalfa meal has been shown to improve the villus height and crypt depth of the
464 jejunum and duodenum of pigs and to improve systemic immune function [25]. However, the
465 proportion of *Escherichia-Shigella* at D21 was higher in the digesta of SIL piglets compared to
466 the three other groups.

467

468 A large proportion of clinically relevant AMR genes are associated with the *Enterobacteriaceae*
469 family. However, their relative abundance within the microbial communities did not reflect the
470 overall decrease in ARGs observed in piglets subjected to SIL treatment. Bacterial enumerations

revealed particularly high frequencies of resistance to tetracycline and cotrimoxazole across all animals, with 97% and 92% of the viable *Enterobacterales* population, respectively. These levels are significantly higher than the temporal trends in Canada for antimicrobial-resistant *E. coli* in grower-finisher pigs, which are approximately 70% resistant to TET^R and 15% to SXT^R [37]. These antibiotic classes are amongst the most commonly used in Canadian agriculture [38], likely contributing to the high resistance levels observed. The detected tetracycline resistance genes were associated with at least two bacterial orders: the commensal *Bacteroidales* and the fermentative and opportunistic pathogens of the *Enterobacterales*. *tetQ* was the most frequently detected TET^R gene by HT-qPCR but was less prevalent in SIL piglets. It is mainly associated with members of the *Bacteroidales* order, which are dominant in the gut. The *mphA* gene was also significantly reduced in SIL piglets. It encodes a macrolide 2′-phosphotransferase I and is commonly found in Gram-negative bacteria, particularly members of the *Enterobacteriaceae*. HT-qPCR also detected *tetD*, which is typically associated with Gram-negative bacteria. Sulfonamides have been used for nearly 90 years, and their systematic use during World War II contributed to the development of widespread resistance. Today, they are usually administered in combination with trimethoprim [39]. In this study, HT-qPCR detected the *sul1*, *sul2*, and *sul3* genes. β-lactams are also widely used to treat infections in humans and are considered last-resort treatments for pigs in Canada. Third-generation cephalosporins (e.g., CTX) are classified as critically important antimicrobials in human medicine, and their veterinary use is scrutinised [40]. Resistance to CTX was rarely observed in the bacterial counts. However, we detected the genes $bla_{SHV\text{-}11}$, bla_{ACT} and $bla_{\text{TEM-1}}$ by HT-qPCR, which may have clinical implications. Fluoroquinolones (e.g. CIP) and aminoglycosides (e.g. GEN) are classified as antibiotics of very high and high importance in human medicine, respectively [40]. *Enterobacteriaceae* resistant to

these compounds were frequently detected via bacterial enumeration, and multiple genes conferring resistance to these antibiotics were detected. Taken together, these findings suggest that while AMR remains stable at the phenotypic level among viable bacteria regardless of treatment, it is dynamic at the molecular level, showing shifts in both the abundance and distribution of resistance genes.

Conclusions

Ultimately, we investigated whether SIL could serve as a tool to modulate the gut microbiome, thereby facilitating the weaning transition and mitigating the selective pressure exerted by antibiotics, for example the commonly used CTC. The physiological and microbiological impacts of CTC differ markedly from those associated with SIL. Piglets that consumed CTC were heavier than others, which was primarily due to increased feed intake. SIL did not induce the growth-promoting effect observed with CTC. Additionally, CTC piglets exhibited higher levels of circulating superoxide dismutase, an important antioxidant defense against oxidative stress, and lower concentrations of circulating TNF-α. These health-related impacts were not observed in SIL piglets. The level of gentamicin resistance remained stable in the gut microbiota of CTC piglets, likely due to the selective pressure exerted by its ingestion. Overall, SIL enhanced bacterial diversity, presumably increasing the piglets' resilience to pathogens. These findings warrant further investigation into the optimal SIL exposure rate and timing, and ensure compatibility with concentrate feed composition to maximize animal performance and gut microbiome responses.

Pigs have been good models for studying the human gut [41]. Given the vast similarities between humans and pigs, it is expected that health is not only a consequence of meeting nutritional requirements, but that a pig experiencing good welfare is more likely to be healthy. Silage use encourages natural foraging behaviour in sows and piglets, presumably increasing welfare. Further investigations could evaluate the impact of SIL usage on the efficiency of rearing piglets to reach market weight, considering meat quality, sustainability, and profitability.

Declarations

Ethics approval

The study was conducted at the Swine Complex of the Sherbrooke Research and Development Centre of Agriculture and Agri-Food Canada. The animals were cared for according to the guidelines of the National Farm Animal Care Council [42]. All animal procedures were approved by the Institutional Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines, protocol #583 [12].

Data availability

Sequencing data from this study were deposited into the National Center for Biotechnology Information (NCBI) BioProject PRJNA1143549. The raw sequence reads of 16S amplicon sequencing for 384 samples of intestinal digesta and feces were deposited under accession numbers SRR30724181 to SRR30724564 in the NCBI Sequence Reads Archive (SRA) public database.

Competing interests

The authors declare that there are no conflicts of financial, general or institutional competing interests.

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Authors' contributions

Conceptualization: M.B., F.G., G.T. and D.P.L.; investigation: J.S., M.B., N.D., V.B., G.T. and D.P.L.; funding acquisition: M.B., G.T. and D.P.L.; formal analysis: J.S., N.D. and D.P.L.; wet-lab experiments: J.S., M.B., K.L. and N.G.; data analysis: J.S., J.-S.B and D.P.L.; data curation: J.S., K.L., N.G. and D.P.L.; writing (original draft preparation): D.P.L. and J.S.; writing (review and editing): J.S., M.B., K.L., N.G., N.D., F.G., V.B., G.T. and D.P.L. All authors have read and agreed to the published version of the manuscript.

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