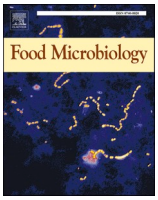
Food Microbiology 97 (2021) 103754

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm



Comparison between cage and free-range egg production on microbial composition, diversity and the presence of *Salmonella enterica*

Annaleise Wilson [a,c,*], P. Scott Chandry [b], Mark S. Turner [a], Jodi M. Courtice [d], Narelle Fegan [c]

[a] School of Agriculture and Food Sciences, University of Queensland, St Lucia, Queensland, Australia
[b] CSIRO Agriculture and Food, Werribee, Victoria, Australia
[c] CSIRO Agriculture and Food, Coopers Plains, Queensland, Australia
[d] Division of Research and Innovation, University of Southern Queensland, Toowoomba, Queensland, Australia

ARTICLE INFO

Keywords:
Culture-independent
Bacterial microbiota
Salmonella enterica
Eggs
Food safety
Farm environmental bacteria

ABSTRACT

The microbial composition of the food production environment plays an important role in food safety and quality. This study employed both 16 S rRNA gene sequencing technology and culture-based techniques to investigate the bacterial microbiota of an egg production facility comprising of both free-range and conventional cage housing systems. The study also aimed to detect the presence of *Salmonella enterica* and determine whether its presence was positively or negatively associated with other taxa. Our findings revealed that microbiota profiles of free-range and cage houses differ considerably in relation to the relative abundance and diversity with a number of taxa unique to each system and to individual sampling sites within sheds. Core to each housing system were known inhabitants of the poultry gastrointestinal tracts, *Romboutsia* and *Turicibacter*, as well as common spoilage bacteria. Generally, free-range samples contained fewer taxa and were dominated by *Staphylococcus equorum*, differentiating them from the cage samples. *Salmonella enterica* was significantly associated with the presence of a taxa belonging to the *Carnobacteriaceae* family. The results of this study demonstrate that the diversity and composition of the microbiota is highly variable across egg layer housing systems, which could have implications for productivity, food safety and spoilage.

1. Introduction

Eggs are one of the most widely consumed protein sources worldwide. The market for eggs produced in alternative production systems, including cage-free housing, has increased in Australia in recent decades and now makes up a considerable market share with 61% of retail sales in the 2019–20 financial year (Australian Eggs Limited, 2020). Despite mitigation efforts, both cage and free-range produced egg products are reservoirs for *Salmonella enterica* and are frequently implicated in salmonellosis outbreaks and sporadic cases (Glass et al., 2016; Sloan--Gardner et al., 2019; Threlfall et al., 2014). Horizontal transfer via direct contact between the eggshell surface and contaminated faeces is the main route of contamination of *S. enterica* serovar Typhimurium, the serovar most frequently implicated in egg-related foodborne disease in Australia (Pande et al., 2016).

The microbiota of the poultry farm environment, including pathogenic bacteria, play an important role in the microbial colonization and development of the hen's gastrointestinal tract during production (Cressman et al., 2010; Wang et al., 2016). This is especially true in commercial single-age production systems where chicks do not develop their microbiota through contact with the adult hen after hatching (Rychlik, 2020). Bacteria in the environment can also provide a direct source of contamination of eggs after lay. There is also evidence to suggest certain mixed-species cultures, particularly within biofilms, can promote prolonged survival, or exclusion, of pathogens (Indikova et al., 2015). There are a number of physically and functionally distinct areas of the poultry house, each providing potential ecological niches for bacterial life, including cages, nest boxes, feeders and egg collection machinery. As such, multiple sampling of the various sites throughout the farm is necessary to comprehensively characterize the farm microbiota (Locatelli et al., 2017).

It is believed that less than 2% of bacterial taxa are cultivatable, and as a consequence, using culture-based methods to characterize a microbiota may understate the depth of its microbial composition and diversity present within samples (Wade, 2002). The use of culture-independent methods, including next generation sequencing

* Corresponding author. School of Agriculture and Food Sciences, University of Queensland, St Lucia, Queensland, Australia.
E-mail address: annaleise.wilson@csiro.au (A. Wilson).

https://doi.org/10.1016/j.fm.2021.103754
Received 20 October 2020; Received in revised form 21 January 2021; Accepted 28 January 2021
Available online 3 February 2021

technology, evades the issues associated with traditional culture-based methods. Using this technology, the fecal microbiota of layer hens in alternate and conventional production houses have been previously characterized (Rothrock and Locatelli, 2019; Seidlerova et al., 2020; Videnska et al., 2014). However, with the exception of the poultry litter (Torok et al., 2009), and bioaerosol microbiota in broiler houses (Chinivasagam et al., 2010), previous studies that have characterized the microbial populations of poultry environments have focused on culturable bacteria and human pathogens (McWhorter and Chousalkar, 2019, 2020). To our knowledge, there have been no studies that have investigated the differences in the environmental microbiota between cage and free-range housing systems within a single commercial egg production farm using 16 S rRNA amplicon sequencing techniques.

The overall aim of this study was to use culture-dependent and culture-independent techniques to compare the composition and diversity of bacteria present across a dual system egg production facility with a history of *S. enterica* isolation. We wished to investigate which taxa, if any, were differentially abundant between cage and free-range housing systems and across environmental sites (egg conveyer belts, manure conveyer belts, cages, nest boxes, litter samples and cage floors). The study also sought to investigate what taxa were associated with the presence or absence of *S. enterica*.

2. Materials and methods

2.1. Sample collection

Collection of environmental samples occurred in July 2019 from an Australian egg production facility that produces free-range and cage eggs. Samples were collected from single-age housing facilities containing layer hens in mid to late egg production, aged between 34 and 77 weeks. Thirty-five samples were collected from four sites within five cage houses ($n = 20$) and from three sites within five free-range houses ($n = 15$). Within each cage house, a sample was collected from the manure conveyer belt, egg conveyer belt, steel cage and concrete floor. Within each free-range house, a nest box, and egg conveyer belt was swabbed, and a litter sample was collected. Ambient temperature at the time of collection was approximately 26 °C. Samples were obtained from 1 m^2 surfaces using Whirl-Pak® sponges (Nasco) pre-soaked in 25 ml of Buffered Peptone Water (BPW, Oxoid). Following collection, samples were transported to the laboratory at 4 °C and processed on the same day.

2.2. Bacterial enumeration and identification

In order to assess total plate counts of bacteria with different growth capabilities, four culture conditions were used. Samples were diluted with 75 ml of BPW and homogenised with a Stomacher for 1 min (Bag Mixer®, InterScience, Markham, Ontario, Canada). Homogenised suspensions were serially diluted in physiological saline (0.85%). For each dilution 100 μl aliquots were spread onto three Plate Count Agar (PCA; Oxoid) plates and one of each plate was incubated either aerobically at 30 °C, aerobically at 20 °C or anaerobically at 30 °C. Dilutions were also spread on de Man, Rogosa, and Sharpe (MRS; Oxoid) plates and incubated anaerobically at 30 °C. All plates were incubated for 72 h prior to enumeration.

Eight colonies, two from each of the four culture conditions, were randomly chosen for each sample for purification on fresh agar and identification using Sanger sequencing of the 16 S rRNA gene. DNA was extracted by boiling lysis of cultures grown for 18 h ± 2 h in Tryptic Soy Broth. Amplification of the 16 S rRNA gene by PCR was performed as previously described (Magnusson et al., 2003). PCR products were Sanger sequenced by Macrogen (South Korea). Sequence reads were quality trimmed manually in Geneious 11.1.5 (https://www.geneious.com) and analysed using the Greengenes database (https://greengenes.secondgenome.com/). Taxa identified as lactobacilli using the Greengenes database were reclassified using the emended nomenclature of the genus proposed by Zheng et al. (2020).

2.3. Salmonella enterica enrichment and confirmation

The presence of *S. enterica* in samples was determined using enrichment methods outlined in ISO 6579:2017 (International Organization for Standardization, 2017). Presumptive *S. enterica* colonies were subsequently confirmed using a *Salmonella* Latex test kit (Oxoid). Genomic DNA was isolated from sero-positive isolates using the DNeasy Blood and Tissue kit following the manufacturer's instructions (Qiagen, Hilden, Germany). Whole genome sequencing was performed by the Public Health Microbiology laboratory at Queensland Health using the NextSeq 500 platform (Illumina, San Diego, California, USA). Raw sequence reads were quality and adapter trimmed using Trimmomatic v0.36.6 prior to assembly into contiguous sequences using SPAdes v3.12.0 (Bankevich et al., 2012; Bolger et al., 2014). In silico serotyping was performed using online *Salmonella* In Silico Typing Resource (SISTR) (Yoshida et al., 2016).

2.4. Microbiota DNA extraction and 16 S rRNA amplicon sequencing

DNA was extracted from environmental samples using the QIAamp PowerFecal Pro DNA kit as per the manufacturer's instructions (Qiagen, Hilden, Germany). The DNA quantity was determined using the Qubit® 2.1 Fluorometer (Life Technology, Singapore) and then each sample was standardised to 10 ng/ul. The PCR methodology and duel-index sequencing strategy developed by Kozich et al. (2013) was used for amplification and sequencing of the V4 region of the bacterial 16 S ribosomal RNA.

An amplicon library was prepared by pooling 50 ng of PCR product of each sample and purified by AMpure magnetic beads, as per the manufacturer's instructions (Agencourt Bioscience Corporation, Beverly, MA). Purified libraries were quantified using Qubit® 2.1 Fluorometer and sample quality was determined using a Nanophotometer (IMPLEN 1298, United Kingdom). Sequencing was performed using the MiSeq v2 platform producing 2 × 250 paired-end reads by the Ramaciotti Center for Genomics (University of New South Wales, Australia).

2.5. Bioinformatic analysis

Sequence files were analysed using the QIIME2 v2019.7 pipeline (Bolyen et al., 2019). The cutadapt (Martin, 2011) and demux plugins were used to remove adapter sequences and generate sequence read quality profiles, respectively. Denoising, chimera removal and amplicon sequence variant (ASVs) table generation was performed using DADA2 (Callahan et al., 2016). ASVs were aligned with mafft (Katoh et al., 2002) and approximately-maximum-likelihood phylogenetic tree from the generated representative sequences were generated with fasttree2 (Price et al., 2010). Taxonomy was assigned to ASVs using the feature-classifier plugin (Bokulich et al., 2018) against the Greengenes gg_13_8_99% database (McDonald et al., 2012).

For alpha diversity analysis of microbial richness and evenness, sequence reads were rarefied to 11.0 k reads per sample and Chao1, Shannon-Wiener diversity (H) and the Inverse Simpson indexes were computed using the phyloseq v1.26.1 package in R v1.2.1335 (McMurdie and Holmes, 2013; Shannon, 1948; Simpson, 1949). A compositional biplot was created using Principal Component Analysis (PCA) on centred log-ratio transformed sequence counts with the zCompositions R package v1.3.4 (Palarea-Albaladejo and Martín-Fernández, 2015). The identification of differentially abundant taxa between hen housing systems (cage and free-range) and *S. enterica* status (positive and negative) was performed using ANCOM-II code (Kaul et al., 2017) implemented in R (R Core Team, 2014).

Raw sequence data were submitted to the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/) under

BioProject ID PRJNA648795.

3. Results

3.1. Enumeration of culturable bacterial microbiota

Bacterial counts were enumerated using four culture conditions involving PCA plates incubated aerobically at 30 °C, PCA plates incubated aerobically at 20 °C, PCA plates incubated anaerobically at 30 °C and MRS plates incubated anaerobically at 30 °C (Fig. 1). The highest mean counts were observed in manure conveyer belt and floor samples isolated in the cage houses and litter samples isolated in the free-range system. Significantly lower counts ($p > 0.001$) were observed in samples from the cage walls, nest boxes, free-range egg conveyers and cage egg conveyers. There were no significant differences in CFU/cm^2 when comparing the egg conveyer belts found in cage housing to egg conveyer belts in the free-range housing ($p > 0.05$). Similarly, there was no significant difference observed between swabs taken from free-range nest boxes and the walls of cages sampled in the cage housing.

3.2. Identification of culturable microbiota

For each sample, two colonies were selected at random for identification from each of the enrichment conditions. Purification, DNA extraction, 16 S rRNA Sanger sequencing and analysis was possible for 240 of the 280 selected colonies (Table 1). Forty-three Gram-positive taxa dominated the microbial population of both the cage (77 colonies) and free-range systems (83 colonies). Amongst isolated strains, *Staphylococcus equorum* ($n = 27$), *Enterococcus faecium* ($n = 22$) and *Kurthia populi* ($n = 11$) were the most prevalent. Only 21 Gram-negative taxa were identified, primarily of the Proteobacteria phyla. Psychrotrophic bacteria, including *Pseudomonas* sp. ($n = 17$) and *Psychrobacter pulmonis* ($n = 11$), were frequently identified in both housing systems using culture-based techniques.

There were notable differences in the abundance of taxa between different layer housing systems. *S. equorum* was disproportionally more prevalent in free-range samples with more than three times the number of isolations compared to cage samples. The opposite was observed with the prevalence of Gram-negative *Proteus vulgaris* and *Shigella flexneri* which were higher in cage samples. All 11 isolations of the Gram-positive, spore-forming bacteria *K. populi*, were from free-range samples.

3.3. Recovery of Salmonella enterica from environmental samples

Of the 35 samples collected, 13 were positive for *S. enterica*. All five cage houses had at least one *Salmonella*-positive sample. Sampling sites where *S. enterica* were detected included the manure belt (5/5), cage walls (1/5), floor (5/5) and egg conveyer belt (2/5). Despite using the same sampling and testing methodology, no samples collected from free-range sites were positive for *S. enterica*. Whole genome sequencing and *in silico* typing determined that *S. enterica* isolates belonged to one of three serotypes; Singapore ($n = 8$), Agona ($n = 4$) and Liverpool ($n = 1$).

3.4. Microbial composition of cage and free-range housing systems based on 16 S rRNA amplicon sequencing

A total of 1.47 million sequence reads was produced with a mean read count of 40,971 reads per sample prior to processing. Following amplicon denoising and filtering there were 501 ASVs identified, 181 of which were shared by all sample types. The cage system manure conveyer belt samples possessed the most unique ASVs ($n = 70$), followed by other cage house samples ($n = 17$), free-range litter samples ($n = 3$) and other free-range samples ($n = 14$). As shown in Fig. 2A, the manure belt samples, other cage house samples and free-range samples were dominated by three bacterial phyla; Firmicutes (41% ± 9, 72% ± 10 and 85% ± 5, respectively), Proteobacteria (43% ± 7, 7.2% ± 6 and 6.2% ± 4, respectively), and Actinobacteria (6% ± 4, 18% ± 7 and 6.8% ± 3, respectively). Other phyla present included Deinococcus-Thermus, Eremiobacteraeota, Euryarchaeota, Chloroflexi, Cyanobacteria, Synergistetes, Tenericutes, Fusobacteria and Verrucomicrobia, each occurring at a relative abundance below 1%.

Analysis of the bacterial community by the lowest taxonomical classification revealed high diversity and variation between sampling sites (Fig. 2B). Within manure belt samples, the most abundant taxa were *Acinetobacter lwoffi* (7.2%), *Arcobacter cryaerophilus* (6.8%), *Acinenetobacter johnsonii* (5.7%), *Romboutsia timonensis* (2.6%) and *Comamonas composti* (2.4%). *A. cryaerophilis* and *A. johnsonii* were present at less than 0.1% relative abundance across all other cage and free-range samples. In other cage sample sites, *R. timonensis*, *Turicibacter* sp., *Jeotgalicoccus psychrophilis*, *Corynebacterium stationis* and *P. pulmonis* dominated in addition to *A. lwoffi*. These samples also contain a higher relative abundance of lactobacilli compared to manure samples.

The taxon *S. equorum* was the most prevalent in litter (33.7%), nest box (25.1%) and egg conveyer belt (35.0%) samples isolated from the free-range system. This species was found in low abundance in all cage house samples with all sampling sites recording a mean relative abundance less than 1.2%. As with cage samples, *R. timonensis* appears as a significant feature within free-range samples. *A. lwoffi* is also present at high relative abundance in samples collected from the free-range nest boxes (7.4%) and egg conveyer belts (10.9%) but is found at 0.1% relative abundance in litter samples. Similarly, *A. johnsonii* is present in less than 0.1% relative abundance across all free-range samples.

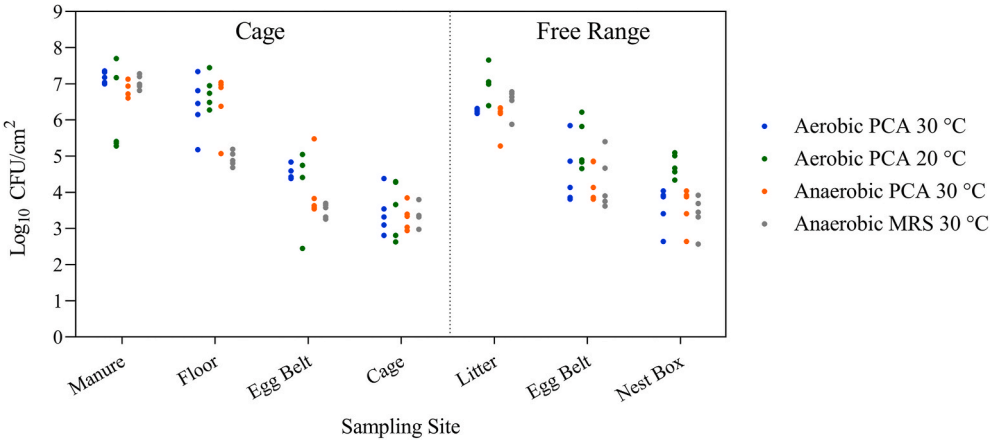


Fig. 1. Viable counts of culturable bacteria isolated from 35 samples within cage and free-range housing systems. Litter samples counts are expressed in Log10 CFU/g.

Table 1
The number of individual isolations of bacterial taxa from cage and free-range houses ($n = 240$). The relative abundance of a taxon by housing system is given in brackets.

Gram-positive taxa	Cage	Free Range	Gram-negative taxa	Cage	Free-range
Aerococcus urinaequi	3 (2.4)	–	*Acinetobacter johnsonii*	1 (0.8)	6 (5.3)
Aerococcus viridans	3 (2.4)	3 (2.8)	*Acinetobacter lwoffi*	3 (2.4)	5 (4.4)
Bacillus safensis	1 (0.8)	–	*Acinetobacter* sp.	1 (0.8)	–
Bacillus sp.	3 (2.4)	5 (4.4)	*Brevundimonas* sp.	1 (0.8)	1 (0.9)
Brachybacterium sp.	1 (0.8)	–	*Chryseobacterium* sp.	–	3 (2.6)
Carnobacterium mobile	2 (1.6)	2 (1.7)	*Comamonas* sp.	4 (3.2)	–
Clostridium perfringens	1 (0.8)	–	*Empedobacter brevis*	1 (0.8)	–
Corynebacterium glutamicum	2 (1.6)	–	*Moraxella osloensis*	–	1 (0.9)
Corynebacterium stationis	3 (2.4)	–	*Myroides odoratimimus*	1 (0.8)	–
Desemzia incerta	–	1 (0.9)	*Pantoea ananatis*	3 (2.4)	–
Enterococcus cassiflavus	1 (0.8)	1 (0.9)	*Pantoea gaviniae*	–	1 (0.9)
Enterococcus cecorium	2 (1.6)	1 (0.9)	*Pantoea stewartia*	–	1 (0.9)
Enterococcus durans	3 (2.4)	2 (1.7)	*Proteus vulgaris*	9 (7.1)	–
Enterococcus faecalis	1 (0.8)	3 (2.6)	*Pseudomonas fragi*	3 (2.4)	–
Enterococcus faecium	7 (5.6)	15 (13.2)	*Pseudomonas fulva*	1 (0.8)	1 (0.9)
Enterococcus gallinarium	1 (0.8)	–	*Pseudomonas putida*	5 (4.0)	3 (2.8)
Enterococcus sp.	6 (4.8)	–	*Pseudomonas stutzeri*	1 (0.8)	1 (0.9)
Glutamicibacter creatinolyticus	3 (2.4)	2 (1.7)	*Pseudomonas* sp.	–	2 (1.7)
Kocuria palustris	1 (0.8)	–	*Psychrobacter pulmonis*	7 (6.4)	4 (3.5)
Kurthia gibsonii	1 (0.8)	–	*Shigella flexneri*	7 (5.6)	2 (1.7)
Kurthia populi	–	11 (9.6)	*Sphingobacterium* sp.	1 (0.8)	–
Kurthia zopfii	1 (0.8)	1 (0.9)	***Total Gram-negative***	**49 (38.9)**	**31 (27.2)**
Ligilactobacillus agilis	2 (1.6)	1 (0.9)			
Lactobacillus crispatus	–	1 (0.9)			
Latilactobacillus curvatus	3 (2.4)	1 (0.9)			
Lactobacillus gallinarum	1 (0.8)	–			
Latilactobacillus graminis	1 (0.8)	–			
Lactobacillus johnsonii	–	1 (0.9)			
Lactobacillus kitasatonis	–	1 (0.9)			
Latilactobacillus sakei	1 (0.8)	1 (0.9)			
Ligilactobacillus salivarius	2 (1.6)	3 (2.6)			
Lactobacillus sp.	3 (2.4)	–			
Lactococcus garvieae	1 (0.8)	–			
Lysinibacillus sp.	1 (0.8)	–			
Macrococcus caseolyticus	–	1 (0.9)			
Microbacterium sp.	1 (0.8)	1 (0.9)			
Rothia amarae	1 (0.8)	–			
Sporasarcina aquimarina	–	1 (0.9)			
Staphylococcus equorum	6 (4.8)	21 (18.4)			
Staphylococcus saphrophyticus	3 (2.4)	–			
Streptococcus sp.	–	2 (1.7)			
Trichococcus sp.	2 (1.6)	–			
Weissella hellenica	3 (2.4)	–			
Total Gram-positive	**77 (61.1)**	**83 (72.8)**			

3.5. Alpha and beta diversity metrics

To determine the alpha diversity of samples, the Chao1 richness estimate, Shannon-Wiener index (H) and Simpson Reciprocal indices (1/D) were measured. Chao1 measured species richness, and the differentially weighted Shannon-Wiener (H) and Simpson Reciprocal (1/D) indices measured both richness and evenness of the distribution of species abundances. Considerable variability was observed when comparing free-range and cage house samples using these three alpha diversity indices (Fig. 3), with significantly higher Chao1 ($p < 0.0001$), Shannon-Wiener ($p < 0.0005$), and the Simpson reciprocal index ($p < 0.0001$) scores observed in cage shed samples. The observed species richness in cage samples, which ranged from 172 to 270 taxa, was on average higher than the free-range system which ranged from 73 to 204 taxa. On a whole, free-range system samples showed lower microbial richness and evenness than cage samples.

The beta-diversity, a measure of variance in taxa composition between sampling sites, was visualized by plotting distances between samples on a PCA biplot (Fig. 4). The first and second plot coordinates represented 17.1% and 42.2% of the variation in beta-diversity, respectively. It is clear from the biplot that cage samples distinctly cluster separately from free-range samples. However, manure belt samples, which contain a higher number of unique ASVs, did not cluster directly with other samples isolated from the cage house environment. Three free-range egg belt samples also varied considerably from other free-range samples. This variation could be accounted by fewer ASVs contained within these samples and a mean lower taxa evenness being observed in these samples.

An ANCOM-II analysis was performed on ASV counts to calculate the pairwise log ratios between all ASVs and test for significant differences in ASV ratios between sampling sites. The analysis revealed that 11 ASVs were differentially abundant between the microbial compositions of cage and free-range samples (Table 2). Not surprisingly, *S. equorum* was found to be significantly more abundant in free-range samples. Three lactic acid bacteria, *Limosilactobacillus pontis*, *Limosilactobacillus pontis* and *Enterococcus faecalis*, were also found to be more abundant in free-range samples (Table 2). Seven taxa were differentially abundant in cage samples, predominantly aerobic cocci and anaerobic gastrointestinal bacteria. An ANCOM analysis was also performed comparing *Salmonella*-positive and *Salmonella*-negative samples. This analysis revealed that only one taxa, *Isobaculum melis,* was differentially more abundant in samples positive for *S. enterica* ($0.43\% \pm 0.41$) compared to *Salmonella*-negative samples ($0.02\% \pm 0.06$). There were no taxa differentially less abundant in *Salmonella*-positive samples compared to *Salmonella*-negative samples.

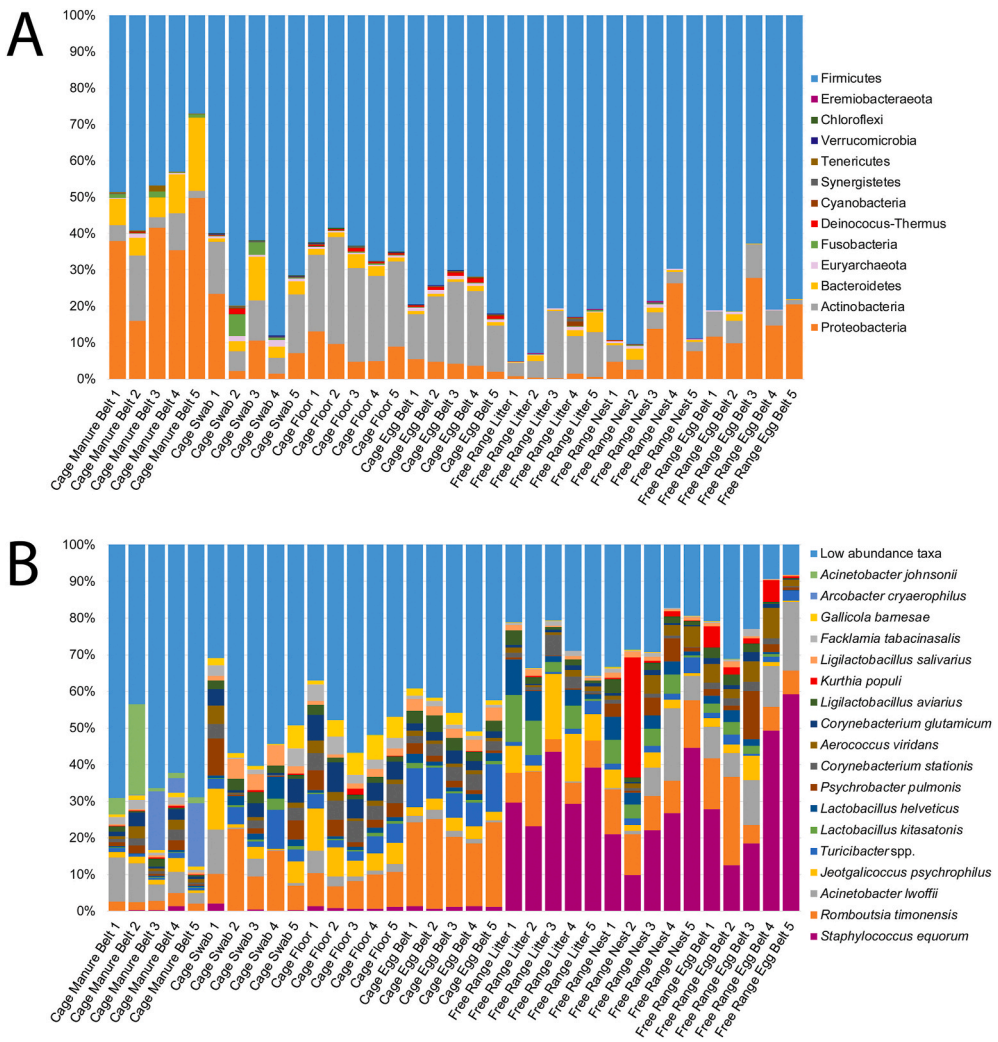


Fig. 2. Relative abundance taxaplot of taxa present in samples isolated from four locations within five cage houses and three locations within five free-range houses. A) Relative abundance of taxa by phylum. B) Relative abundance of taxa by species. Taxa occurring at less than 1% relative abundance were combined and labelled "Low abundance taxa".

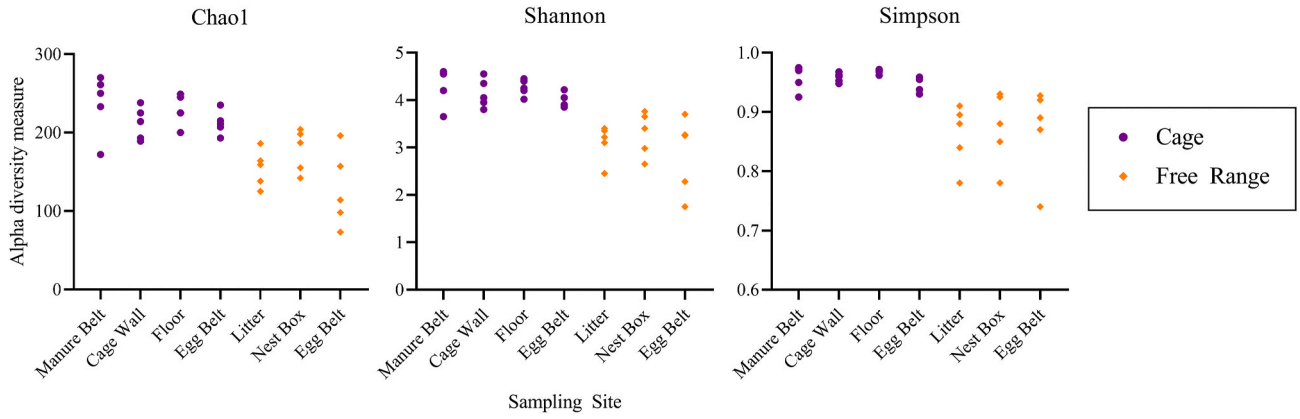


Fig. 3. Alpha diversity metrics Chao1, Shannon Diversity and Simpson Diversity by sampling site.

4. Discussion

Various studies have highlighted the importance of the resident bacteria in food processing environments due to their potential to promote adhesion, biofilm formation and persistence of pathogens (Fox et al., 2014; Kostaki et al., 2012; Makovcova et al., 2017). With the exception of fecal matter (Elokil et al., 2020; Videnska et al., 2014), little is known about the microbiota of the egg production environment or the variation in microbial composition and diversity between cage and free-range housing systems. An understanding of the composition of

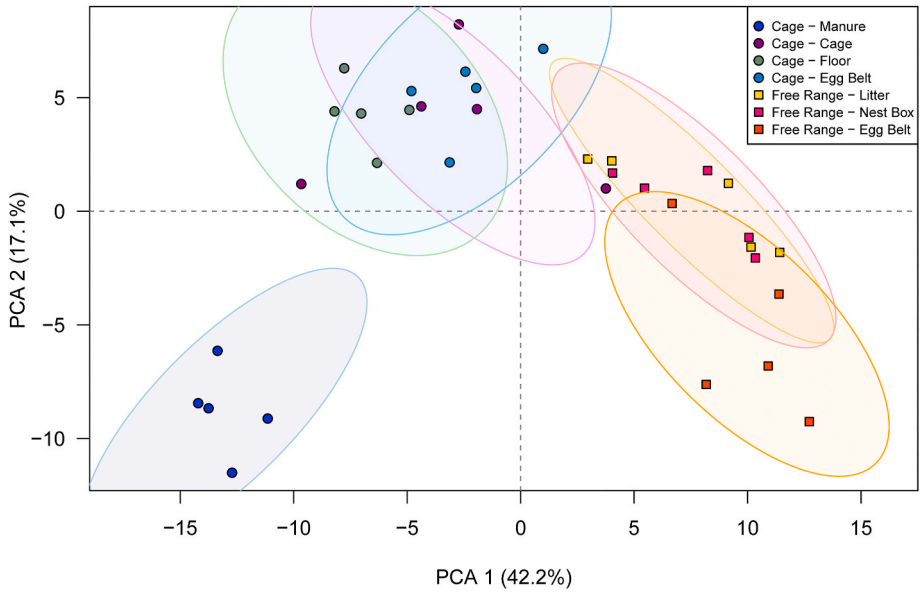


Fig. 4. Compositional biplot of bacterial community composition of cage (circles) and free range (square) housing samples. The proportion of variation is stated in the axis titles. Coloured ellipses represent 95% confidence intervals for each sampling site.

Table 2
Differentially abundant taxa between cage and free-range housing systems as per ANCOM-II analysis.

Amplicon Sequence Variant	Relative abundance (% ± SD)	
	Cage	Free Range
Corynebacterium humireducens	**1.05 ± 0.99**	0.01 ± 0.04
Asaccharospora sp.	**0.48 ± 0.46**	<0.01 ± 0.00
Tissierella sp.	**0.42 ± 0.41**	<0.01 + 0.00
Oceanisphaera avium	**0.51 ± 0.34**	0.01 ± 0.03
Isobaculum melis	**0.25 ± 0.34**	0.01 ± 0.05
Lysinibacillus saudimassiliensis	**0.66 ± 0.34**	<0.01 ± 0.01
Staphylococcus saprophyticus	**1.34 ± 1.18**	0.08 ± 0.22
Staphylococcus equorum	0.79 ± 0.59	**30.48 ± 13.51**
Limosilactobacillus reuteri	0.05 ± 0.11	**0.54 ± 0.37**
Limosilactobacillus pontis	0.02 ± 0.03	**0.42 ± 0.30**
Enterococcus faecalis	0.03 ± 0.09	**0.22 ± 0.15**

Note: The housing system in which taxa were found to be differentially more abundant are highlighted in bold text.

resident bacteria in egg production, and of how these bacteria interact with *S. enterica*, could lead to a better understanding of the persistence of this important pathogen. As microbiotas can be highly diverse and possess highly variable compositions dependent on environmental factors, nutrient and water availability, it is important to sample multiple sites across the production environment to accurately characterize the microbial community structure and composition (Locatelli et al., 2017).

The aim of this study was to understand the variation in microbial composition and diversity between cage and free-range housing in an egg production facility. We also investigated the presence of *S. enterica* in hen houses in order to determine whether the presence of this pathogen was significantly associated with the presence or absence of other bacterial taxa in the environment. As there are vast differences in airflow, flock density and flooring between the two housing systems, we hypothesized that there would be considerable variation in microbial composition and diversity observed. Indeed, analysis of alpha and beta diversity metrics demonstrated the bacterial composition and diversity of cage and free-range environments varied significantly. Comparisons of phyla and species-level relative abundances were also highly variable, not only between housing systems, but between different sampling sites within houses. Amongst core genera, *Turicibacter* and *Romboutsia* have also been previously reported as major constituents of the eggshell microbiota and have been recognized for their important role as a regulator of energy metabolism and hormonal regulation of host organisms (Gao et al., 2019; Maki et al., 2020; Martinez-Guryn et al., 2018). The findings also suggest that spoilage bacteria including lactobacilli, *Acinetobacter, Psychrobacter and Pseudomonas* are core to both housing systems and present in high proportions. These genera have been associated with spoilage of eggs products (Sparks, 2014). Several significant differences in microbial composition were detected; namely the high relative abundance (>25%) of *S. equorum* in free-range sheds. *Staphylococci* are natural inhabitants on the skin and mucous membranes of warm-blooded animals. *S. equorum* has previously been isolated from the skin of horses (Karakulska et al., 2012), and recently in wild house mice (Belheouane et al., 2020). It is also notable for its role as a starter culture in fermented sausages food products (Leroy et al., 2010). Using culture-based techniques, researchers have previously determined that *Staphylococcus* is found in high concentrations in the litter and aerosols of mechanically ventilated broiler sheds in Australia (Chinivasagam et al., 2010) and in the egg shell microbiota (Suwannarach et al., 2017), although *S. equorum* has not been reported as a dominant species in either study. Further research is required to determine whether this species is endemic within free-range production facilities. While the relative abundance of *S. equorum* in cage samples was low, other aerobic, halophilic genera including *Jeotgalicoccus* and *Corynebacterium* are significantly more abundant in these samples than in free-range samples. These taxa have also been previously detected in bioaerosols of poultry houses (Chinivasagam et al., 2010; Martin et al., 2010). Further investigations would be required to determine if these differences in environmental microbiota between productions systems translates to variations to egg microbiota, safety and shelf-life profiles.

This study also sought to investigate whether certain taxa were differentially abundant in cage or free-range houses or among *S. enterica* positive or negative samples. We found that *S. equorum* and *J. psychrophilus* were differentially abundant in the free-range and cage housing, respectively. In addition, three lactic acid bacteria were found to be differentially more abundant in free-range samples. Lactic acid bacteria are known for their efficacy as a probiotic in poultry as a prevention against *S. enterica* (Reuben et al., 2019). Nonetheless, these taxa were not found to be more abundant in *S. enterica* negative samples in this study. According to the ANCOM analysis, only one ASV was differentially abundant between *S. enterica* positive and negative samples. An ASV similar to the relatively uncharacterized lactic-acid

bacteria, *I. melis*, was found to be differentially more abundant in *S. enterica* positive samples. This bacterium has not been reported in publications since its discovery in the small intestine of a badger in 2002 (Collins et al., 2002). Previous studies have shown that *S. enterica* can form synergistic interactions with bacteria isolated from animal feed factories, including *Staphylococcus* and *Pseudomonas*, through increased biofilm formation and tolerance to disinfectants (Habimana et al., 2010). Investigation of the potential interactions between *S. enterica* and *Isobaculum melis* could provide better understanding of the persistence of *S. enterica* in egg production.

In the past ten years there has been an increasing shift in the methods that are used to investigate foodborne pathogens and monitor supply chains (Kovac et al., 2017). The increased accessibility of next generation sequencing platforms due to reduced costs have led to the rapid adoption of 16 S amplicon sequencing as a tool for profiling microbial populations with the benefit of not requiring colony isolation (Jagadeesan et al., 2019). It has been noted however that this methodology may have a limited ability to assign some enteric pathogens, such as *S. enterica*, if they are present at low abundance (Ceuppens et al., 2017; Haley et al., 2016; Jarvis et al., 2015; Peruzy et al., 2019). In this study, we detected *S. enterica* in 13 of the 35 samples tested using enrichment and culture-based techniques. However, *S. enterica* was not detected using the 16 S amplicon sequencing methodology employed, even in samples determined to be positive using culture-based techniques. This result suggests that the use of 16 S rRNA amplicon sequencing alone may be not be suitable for the detection of *S. enterica* in environmental samples. Culture-based techniques or alternate molecular based approaches, such as shotgun metagenomics, may be more appropriate for the detection of this pathogen (Grützke et al., 2019). On the other hand, other potential pathogens of clinical importance, *A. lwoffi*, *Corynebacterium* sp., *Trichococcus* sp., *Facklamia* sp., and *Clostridium perfringens* were detected in this study using 16 S rRNA amplicon sequencing. Importantly, the taxon *A.* cryaerophilus, although not detected by the culture-based techniques used, was found to be a major constituent of manure belt sample amplicon sequences. A. cryaerophilus is an emerging pathogen and has previously been implicated in cases of gastroenteritis and septicemia (Barboza et al., 2017). Previous studies have found it to contaminate food products including poultry meat, pork and beef (Zacharow et al., 2015), however this pathogen has not historically been associated with layer hens or been implicated in illness associated with egg products. Despite its limited ability to detect *S. enterica*, this study demonstrates that 16 S amplicon sequencing can provide a greater understanding of the microbial composition and diversity of samples collected from the food production environment than culture-based techniques. The combined use of both culture-dependent and -independent techniques can provide a more comprehensive method for the detection of pathogens and spoilage bacteria than either method when used alone.

As this research only collected samples once from each sampling site, the results are a limited representation of the microbiota of the cage and free-range housing systems at the time of sampling. Further research, with repeated sampling of the same sites, may provide insights into how microbial communities shift through production cycles and seasonal changes. Such information may elucidate the transmission pathways of key pathogens, such as *S. enterica*, throughout egg layer systems which can be used to develop better control and more focused risk management strategies. The study of microbial communities found in egg production facilities might also provide insights into how microbial communities influence the presence and persistence of *S. enterica* which, in turn, could lead to manipulation of communities to control *S. enterica.* Although this wasn't a topic investigated in this publication, the approaches and methods used could support such an application.

Funding

This research was supported by an Australian Government Research Training Program (RTP) Scholarship and the Commonwealth Scientific and Industrial Research Organisation.

Declarations

No declarations.

Conflicts of interest/Competing interests

No conflicts to declare.

Ethics approval

Not applicable.

Code availability

Not applicable.

Availability of data and material

Raw sequence data were submitted to the National Center for Biotechnology Information https://www.ncbi.nlm.nih.gov/) under Biosample accession numbers SAMN15648731 to SAMN15648765.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank the egg farm that helped with sampling the environments and for allowing access to these samples.

References

Australian Eggs Limited, 2020. Australian Eggs Annual Report 2020. https://www.australianeggs.org.au/who-we-are/dmsdocument/1197-annual-report-2020/. (Accessed 11 January 2021).

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V. M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A.V., Sirotkin, A.V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J. Comput. Biol. 19, 455–477. https://doi.org/10.1089/cmb.2012.0021.

Barboza, K., Cubillo, Z., Castro, E., Redondo-Solano, M., Fernández-Jaramillo, H., Echandi, M.L.A., 2017. First isolation report of *Arcobacter cryaerophilus* from a human diarrhea sample in Costa Rica. Rev. Inst. Med. Trop. Sao Paulo 59, 72. https://doi.org/10.1590/s1678-9946201759072.

Belheouane, M., Vallier, M., Čepić, A., Chung, C.J., Ibrahim, S., Baines, J.F., 2020. Assessing similarities and disparities in the skin microbiota between wild and laboratory populations of house mice. ISME J. 14, 2367–2380. https://doi.org/10.1038/s41396-020-0690-7.

Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Gregory Caporaso, J., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. Microbiome 17, 90. https://doi.org/10.1186/s40168-018-0470-z.

Bolger, A.M., Lohse, M., Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics 30, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K.B., Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.-X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A.,

Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K. C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583. https://doi.org/10.1038/nmeth.3869.

Ceuppens, S., De Coninck, D., Bottledoorn, N., Van Nieuwerburgh, F., Uyttendaele, M., 2017. Microbial community profiling of fresh basil and pitfalls in taxonomic assignment of enterobacterial pathogenic species based upon 16S rRNA amplicon sequencing. Int. J. Food Microbiol. 257, 148–156. https://doi.org/10.1016/j.ijfoodmicro.2017.06.016.

Chinivasagam, H.N., Tran, T., Maddock, L., Gale, A., Blackall, P.J., 2010. The aerobiology of the environment around mechanically ventilated broiler sheds. J. Appl. Microbiol. 108, 1657–1667. https://doi.org/10.1111/j.1365-2672.2009.04571.x.

Collins, M.D., Hutson, R.A., Foster, G., Falsen, E., Weiss, N., 2002. *Isobaculum melis* gen. nov., sp. nov., a *Carnobacterium*-like organism isolated from the intestine of a badger. Int. J. Syst. Evol. Microbiol. 52, 207–210. https://doi.org/10.1099/00207713-52-1-207.

Cressman, M.D., Yu, Z., Nelson, M.C., Moeller, S.J., Lilburn, M.S., Zerby, H.N., 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Appl. Environ. Microbiol. 76, 6572–6582. https://doi.org/10.1128/AEM.00180-10.

Elokil, A.A., Magdy, M., Melak, S., Ishfaq, H., Bhuiyan, A., Cui, L., Jamil, M., Zhao, S., Li, S., 2020. Faecal microbiome sequences in relation to the egg-laying performance of hens using amplicon-based metagenomic association analysis. Animal 14, 706–715. https://doi.org/10.1017/s1751731119002428.

Fox, E.M., Solomon, K., Moore, J.E., Wall, P.G., Fanning, S., 2014. Phylogenetic profiles of in-house microflora in drains at a food production facility: comparison and biocontrol implications of *Listeria*-positive and -negative bacterial populations. Appl. Environ. Microbiol. 80, 3369–3374. https://doi.org/10.1128/AEM.00468-14.

Gao, H., Jiang, Q., Ji, H., Ning, J., Li, C., Zheng, H., 2019. Type 1 diabetes induces cognitive dysfunction in rats associated with alterations of the gut microbiome and metabolomes in serum and hippocampus. Mol. Basis. Dis. 1865, 165541. https://doi.org/10.1016/j.bbadis.2019.165541.

Glass, K., Fearnley, E., Hocking, H., Raupach, J., Veitch, M., Ford, L., Kirk, M.D., 2016. Bayesian source attribution of salmonellosis in South Australia. Risk Anal. 36, 561–570. https://doi.org/10.1111/risa.12444.

Grützke, J., Malorny, B., Hammerl, J.A., Busch, A., Tausch, S.H., Tomaso, H., Deneke, C., 2019. Fishing in the Soup – pathogen detection in food safety using metabarcoding and metagenomic sequencing. Front. Microbiol. 10, 1805. https://doi.org/10.3389/fmicb.2019.01805.

Habimana, O., Møretrø, T., Langsrud, S., Vestby, L.K., Nesse, L.L., Heir, E., 2010. Micro ecosystems from feed industry surfaces: a survival and biofilm study of *Salmonella* versus host resident flora strains. BMC Vet. Res. 6 https://doi.org/10.1186/1746-6148-6-48, 48-48.

Haley, B.J., Pettengill, J., Gorham, S., Ottesen, A., Karns, J.S., Van Kessel, J.A.S., 2016. Comparison of microbial communities isolated from feces of asymptomatic *Salmonella*-shedding and non-*Salmonella* shedding dairy cows. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00691, 691-691.

Indikova, I., Humphrey, T.J., Hilbert, F., 2015. Survival with a helping hand: *Campylobacter* and microbiota. Front. Microbiol. 6, 1266. https://doi.org/10.3389/fmicb.2015.01266.

International Organization for Standardization, 2017. ISO 6579-1:2017 - Microbiology of the Food Chain - Horizontal Method for the Detection, Enumeration and Serotyping of *Salmonella* - Part 1 Detection of *Salmonella* Spp. International Organization for Standardization, Geneva, Switzerland.

Jagadeesan, B., Gerner-Smidt, P., Allard, M.W., Leuillet, S., Winkler, A., Xiao, Y., Chaffron, S., Van Der Vossen, J., Tang, S., Katase, M., McClure, P., Kimura, B., Ching Chai, L., Chapman, J., Grant, K., 2019. The use of next generation sequencing for improving food safety: translation into practice. Food Microbiol. 79, 96–115. https://doi.org/10.1016/j.fm.2018.11.005.

Jarvis, K.G., White, J.R., Grim, C.J., Ewing, L., Ottesen, A.R., Beaubrun, J.J.-G., Pettengill, J.B., Brown, E., Hanes, D.E., 2015. Cilantro microbiome before and after nonselective pre-enrichment for *Salmonella* using 16S rRNA and metagenomic sequencing. BMC Microbiol. 15 https://doi.org/10.1186/s12866-015-0497-2, 160-160.

Karakulska, J., Fijałkowski, K., Nawrotek, P., Pobucewicz, A., Poszumski, F., Czernomysy-Furowicz, D., 2012. Identification and methicillin resistance of coagulase-negative *Staphylococci* isolated from nasal cavity of healthy horses. J. Microbiol. 50, 444–451. https://doi.org/10.1007/s12275-012-1550-6.

Katoh, K., Misawa, K., Kuma, K., Miyata, T., 2002. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 30, 3059–3066. https://doi.org/10.1093/nar/gkf436.

Kaul, A., Mandal, S., Davidov, O., Peddada, S.D., 2017. Analysis of microbiome data in the presence of excess zeros. Front. Microbiol. 8 https://doi.org/10.3389/fmicb.2017.02114, 2114-2114.

Kostaki, M., Chorianopoulos, N., Braxou, E., Nychas, G.-J., Giaouris, E., 2012. Differential biofilm formation and chemical disinfection resistance of sessile cells of *Listeria monocytogenes* strains under monospecies and dual-species (with *Salmonella enterica*) conditions. Appl. Environ. Microbiol. 78, 2586–2595. https://doi.org/10.1128/AEM.07099-11.

Kovac, J., Bakker, H.d., Carroll, L.M., Wiedmann, M., 2017. Precision food safety: a systems approach to food safety facilitated by genomics tools. Trends Anal. Chem. 96, 52–61. https://doi.org/10.1016/j.trac.2017.06.001.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. https://doi.org/10.1128/AEM.01043-13.

Leroy, S., Giammarinaro, P., Chacornac, J.-P., Lebert, I., Talon, R., 2010. Biodiversity of indigenous *Staphylococci* of naturally fermented dry sausages and manufacturing environments of small-scale processing units. Food Microbiol. 27, 294–301. https://doi.org/10.1016/j.fm.2009.11.005.

Locatelli, A., Hiett, K.L., Caudill, A.C., Rothrock, M.J., 2017. Do fecal and litter microbiomes vary within the major areas of a commercial poultry house, and does this affect sampling strategies for whole-house microbiomic studies? J. Appl. Poultry Res. 26, 325–336. https://doi.org/10.3382/japr/pfw076.

Magnusson, J., Ström, K., Roos, S., Sjögren, J., Schnürer, J., 2003. Broad and complex antifungal activity among environmental isolates of lactic acid bacteria. FEMS Microbiol. Lett. 219, 129–135. https://doi.org/10.1016/S0378-1097(02)01207-7.

Maki, J.J., Bobeck, E.A., Sylte, M.J., Looft, T., 2020. Eggshell and environmental bacteria contribute to the intestinal microbiota of growing chickens. J. Anim. Sci. Biotechnol. 11, 60. https://doi.org/10.1186/s40104-020-00459-w.

Makovcova, J., Babak, V., Kulich, P., Masek, J., Slany, M., Cincarova, L., 2017. Dynamics of mono- and dual-species biofilm formation and interactions between *Staphylococcus aureus* and Gram-negative bacteria. Microb. Biotechnol. 10, 819–832. https://doi.org/10.1111/1751-7915.12705.

Martin, E., Fallschissel, K., Kämpfer, P., Jäckel, U., 2010. Detection of *Jeotgalicoccus* spp. in poultry house air. Syst. Appl. Microbiol. 33, 188–192. https://doi.org/10.1016/j.syapm.2010.03.008.

Martin, M., 2011. Cutadapt removes adapter sequences from high-throughput sequencing reads. EMBnet J 17, 10–12. https://doi.org/10.14806/ej.17.1.200.

Martinez-Guryn, K., Hubert, N., Frazier, K., Urlass, S., Musch, M.W., Ojeda, P., Pierre, J. F., Miyoshi, J., Sontag, T.J., Cham, C.M., Reardon, C.A., Leone, V., Chang, E.B., 2018. Small intestine microbiota regulate host digestive and absorptive adaptive responses to dietary lipids. Cell Host Microbe 23, 458–469. https://doi.org/10.1016/j.chom.2018.03.011.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R., Hugenholtz, P., 2012. An improved greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J. 6, 610–618. https://doi.org/10.1038/ismej.2011.139.

McMurdie, P.J., Holmes, S., 2013. phyloseq: an R Package for reproducible interactive analysis and graphics of microbiome census data. PloS One 8, e61217. https://doi.org/10.1371/journal.pone.0061217.

McWhorter, A.R., Chousalkar, K.K., 2019. From hatch to egg grading: monitoring of *Salmonella* shedding in free-range egg production systems. Vet. Res. 50, 58. https://doi.org/10.1186/s13567-019-0677-4.

McWhorter, A.R., Chousalkar, K.K., 2020. *Salmonella* on Australian cage egg farms: observations from hatching to end of lay. Food Microbiol. 87, 103384. https://doi.org/10.1016/j.fm.2019.103384.

Palarea-Albaladejo, J., Martín-Fernández, J.A., 2015. zCompositions — R package for multivariate imputation of left-censored data under a compositional approach. Chemometr. Intell. Lab. Syst. 143, 85–96. https://doi.org/10.1016/j.chemolab.2015.02.019.

Pande, V.V., Devon, R.L., Sharma, P., McWhorter, A.R., Chousalkar, K.K., 2016. Study of *Salmonella* Typhimurium infection in laying hens. Front. Microbiol. 7, 203. https://doi.org/10.3389/fmicb.2016.00203.

Peruzy, M.F., Murru, N., Yu, Z., Kerkhof, P.J., Neola, B., Joossens, M., Proroga, Y.T.R., Houf, K., 2019. Assessment of microbial communities on freshly killed wild boar meat by MALDI-TOF MS and 16S rRNA amplicon sequencing. Int. J. Food Microbiol. 301, 51–60. https://doi.org/10.1016/j.ijfoodmicro.2019.05.005.

Price, M.N., Dehal, P.S., Arkin, A.P., 2010. FastTree 2 – approximately maximum-likelihood trees for large alignments. PloS One 5, e9490. https://doi.org/10.1371/journal.pone.0009490.

R Core Team, 2014. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.

Reuben, R.C., Roy, P.C., Sarkar, S.L., Alam, R.U., Jahid, I.K., 2019. Isolation, characterization, and assessment of lactic acid bacteria toward their selection as poultry probiotics. BMC Microbiol. 19, 253. https://doi.org/10.1186/s12866-019-1626-0.

Rothrock, M.J., Locatelli, A., 2019. Importance of farm environment to shape poultry-related microbiomes throughout the farm-to-fork continuum of pasture-raised broiler flocks. Front. Sustain. Food Syst. 3, 48. https://doi.org/10.3389/fsufs.2019.00048.

Rychlik, I., 2020. Composition and function of chicken gut microbiota. Animals 10, 103. https://doi.org/10.3390/ani10010103.

Seidlerova, Z., Kubasova, T., Faldynova, M., Crhanova, M., Karasova, D., Babak, V., Rychlik, I., 2020. Environmental impact on differential composition of gut microbiota in indoor chickens in commercial production and outdoor, backyard chickens. Microorganisms 8, 767. https://doi.org/10.3390/microorganisms8050767.

Shannon, C.E., 1948. A mathematical theory of communication. Bell Syst. Tech. J. 27, 379–423. https://doi.org/10.1002/j.1538-7305.1948.tb01338.x.

Simpson, E.H., 1949. Measurement of diversity. Nature 163. https://doi.org/10.1038/163688a0, 688-688.

Sloan-Gardner, T.S., Waters, N., Marmor, A., Mude, W., 2019. Free range eggs does not mean safe eggs: an outbreak of *Salmonella* Typhimurium linked to free range eggs. Comm. Dis. Intell. 2018, 43. https://doi.org/10.33321/cdi.2019.43.52.

Sparks, N.H.C., 2014. Eggs: Microbiology of fresh eggs. In: Batt, C.A., Tortorello, M.L. (Eds.), Encyclopedia of Food Microbiology, second ed. Elselvier, Amsterdam, pp. 617–621.

Suwannarach, N., Kaewyana, C., Yodmeeklin, A., Kumla, J., Matsui, K., Lumyong, S., 2017. Evaluation of *Muscodor cinnamomi* as an egg biofumigant for the reduction of microorganisms on eggshell surfaces and its effect on egg quality. Int. J. Food Microbiol. 244, 52–61. https://doi.org/10.1016/j.ijfoodmicro.2016.12.021.

Threlfall, E.J., Wain, J., Peters, T., Lane, C., De Pinna, E., Little, C.L., Wales, A.D., Davies, R.H., 2014. Egg-borne infections of humans with *Salmonella*: not only an *S.* Enteritidis problem. World's Poult. Sci. J. 70, 15–26. https://doi.org/10.1017/S0043933914000026.

Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., MacAlpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poultry Sci. 88, 2474–2481. https://doi.org/10.3382/ps.2008-00381.

Videnska, P., Rahman, M.M., Faldynova, M., Babak, V., Matulova, M.E., Prukner-Radovcic, E., Krizek, I., Smole-Mozina, S., Kovac, J., Szmolka, A., Nagy, B., Sedlar, K., Cejkova, D., Rychlik, I., 2014. Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. PloS One 9. https://doi.org/10.1371/journal.pone.0110076 e110076-e110076.

Wade, W., 2002. Unculturable bacteria-the uncharacterized organisms that cause oral infections. J. Roy. Soc. Med. 95, 81–83. https://doi.org/10.1258/jrsm.95.2.81.

Wang, L., Lilburn, M., Yu, Z., 2016. Intestinal microbiota of broiler chickens as affected by litter management regimens. Front. Microbiol. 7 https://doi.org/10.3389/fmicb.2016.00593, 593-593.

Yoshida, C.E., Kruczkiewicz, P., Laing, C.R., Lingohr, E.J., Gannon, V.P.J., Nash, J.H.E., Taboada, E.N., 2016. The *Salmonella* in Silico Typing Resource (SISTR): an open web-accessible tool for rapidly typing and subtyping draft *Salmonella* genome assemblies. PloS One 11, e0147101. https://doi.org/10.1371/journal.pone.0147101.

Zacharow, I., Bystroń, J., Wałecka-Zacharska, E., Podkowik, M., Bania, J., 2015. Genetic diversity and incidence of virulence-associated genes of *Arcobacter butzleri* and *Arcobacter cryaerophilus* isolates from pork, beef, and chicken meat in Poland. BioMed Res. Int. 2015, 956507. https://doi.org/10.1155/2015/956507.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P, Harris, H., Mattarelli, P., O'Toole, P. W., Vandamme, P., Walter, J., Watanabe, K., Wuyts, S., Felis, G.E., Gänzle, M.G., Lebeer, S., 2020. A taxonomic note on the genus *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70 (4), 2782–2858. https://doi.org/10.1099/ijsem.0.004107. In press.