Detection of *Campylobacter* in air samples from poultry houses using shot-gun metagenomics – a pilot study

Thomas H.A. Haverkamp[1*], Bjørn Spilsberg[1], Gro S. Johannessen[1], Mona Torp[1], Camilla Sekse[1]

[1.] Norwegian Veterinary institute, Oslo, Norway

*Corresponding author: thomas.haverkamp@vetinst.no

bjorn.spilsberg@vetinst.no; gro.johannessen@vetinst.no; mona.torp@yahoo.no; camilla.sekse@vetinst.no

Abstract

Background:

Foodborne pathogens such as *Campylobacter jejuni* are responsible for a large fraction of the gastrointestinal infections worldwide associated with poultry meat. *Campylobacter spp*. can be found in the chicken fecal microbiome and can contaminate poultry meat during the slaughter process. The current standard methods to detect these pathogens at poultry farms use fecal dropping or boot swaps in combination with cultivation / PCR. In this study, we have used air filters in combination

with shotgun metagenomics for the detection of *Campylobacter* in poultry houses and MOCK communities to test the applicability of this approach for the detection of foodborne pathogens.

Results:

The spiked MOCK communities showed that we could detect as little as 200 CFU *Campylobacter* per sample using our protocols. Since we were interested in detecting *Campylobacter,* a DNA extraction protocol for Gram negative bacteria was chosen, and as expected, we found that the DNA extraction protocol created a substantial bias affecting the community composition of the MOCK communities. It can be expected that the same bias is present for poultry house samples analyzed. We observed significant amounts of *Campylobacter* on the air filters using both real-time PCR as well as shotgun metagenomics, irrespective of the amount of spiked in *Campylobacter* cells, suggesting that the flocks in both houses harboured *Campylobacter* spp.. Interestingly, in both houses we find diverse microbial communities present in the indoor air. In addition, have we tested the *Campylobacter* detection rate using shotgun metagenomics by spiking with different levels of *C. jejuni* cells in both the mock and the house samples. This showed that even with limited sequencing *Campylobacter* is detectable in samples with low abundance.

Conclusions:

These results show that air sampling of poultry houses in combination with shotgun metagenomics can detect and identify *Campylobacter* spp. present at low levels. This is important since early detection of *Campylobacter* in food production can help to decrease the number of food-borne infections.

Keyword: Microbial communities, metagenomics, Poultry, mock communities, spike controls, gelatine air filter

Background

Campylobacter spp. infection is one of the most frequently reported gastrointestinal infections of bacterial origin in Europe and worldwide [1, 2]. They cause campylobacteriosis with symptoms ranging from mild gastroenteritis to severe diarrhea. Complications can lead to a variety of diseases such as inflammatory bowel disease, reactive arthritis and Guillain-Barré syndrome [2]. For the genus *Campylobacter* more than 30 species and subspecies from several sources have been described and for many of these species the role as a human pathogenic bacteria is unclear [3]. Thermotolerant *Campylobacter* species, such as *C. jejuni* and *C. coli*, are most commonly associated with human infection and are often isolated from poultry and poultry products [1, 4]. *Campylobacter* has been isolated from the environment and a range of wild and domesticated animals, but poultry, especially broilers and laying hens, is considered as the main reservoir [5, 6]. In the EU, monitoring of *Campylobacter* is mandatory [7], and from 2018 monitoring of *Campylobacter* in broiler carcasses after chilling has been implemented in the member states [8]. In addition, to ensure a whole chain approach as recommended by EFSA, control measures should also be implemented at the farm level [9]. At present, on-farm sampling of poultry is carried out by sampling faecal droppings or using boot swabs, which are also widely used for *Salmonella* monitoring [10]. Interestingly, after *Campylobacter* colonize a flock, it is not only detected in faecal droppings and the litter, but also in the air inside the house. This knowledge was recently used to show that air sampling can be used as an alternative strategy for screening of *Campylobacter* in broiler flocks [11, 12]. Air sampling was demonstrated to detect the presence of *Campylobacter* and in some cases even earlier than the current conventional methods [11, 13, 14].

The air filters used, collect airborne material on a gelatin matrix, which is a product obtained from bovine or porcine skins and bones. The extraction process includes the use of extreme temperatures, pH and drying, which should create a sterile product. However, literature on contaminated gelatin indicates that common contaminants belong to thermotolerant, aerobic, endospore-forming bacteria. For instance, varieties of *Bacillus* species might be present, that are more resistant to the

71 processes used in gelatin production and can therefore survive [15]. Thus, gelatin membrane air
72 filters are sterilized using gamma irradiation before use, which should kill all organisms present [13].
73 Nonetheless, DNA of dead bacteria is likely still present in the gelatin matrix of the air filter.

74 In many air filter experiments, the collected airborne material is used for cultivation or specific PCR-
75 based methods such as denaturing gradient gel electrophoresis (DGGE) or real-time PCR [11, 13, 16,
76 17]. The efficiency of cultivation is however suboptimal for specific organisms. A study by
77 Johannessen et al., indicated that cultivation of air filters often failed to detect *Campylobacter* spp. as
78 compared to boot swabs [11]. A follow-up study by Hoorfar et al supported this observation. This is
79 in contrast to a higher *Campylobacter* detection rate when real-time PCR is used directly on the air
80 filters compared to the same assay used material collected from boot swabs [11, 12]. The reason for
81 the latter difference might be that the material on air filter is "cleaner" and therefore contain less
82 inhibitors affecting PCR reactions.

83 Air filtering can collect all the microorganisms present in the enclosed air of buildings, which makes it
84 suitable for diversity studies applying either amplicon or shotgun sequencing. Several studies
85 describe poultry farm air samples indicating presence of a diverse community present in the poultry
86 house air. For instance, Dai et al., showed that a range of potentially pathogenic bacterial genera,
87 such as *Corynebacterium*, *Escherichia* and *Staphylococcus* are present in Chinese poultry house air
88 [9]. Most of these organisms are associated to particulate matter particles present in the air with a
89 particle size diameter smaller than 2.5 µm fraction [18]. Other studies using 16s rRNA DGGE profiling
90 [16], shotgun sequencing [19], or cultivation [17] from material collected on air filters showed similar
91 organisms being present in Canadian or Polish poultry houses. All these studies indicate a dominant
92 airborne community dominated by Firmicute and Actinobacterial species, which are both phyla
93 containing sporeformers [19]. In addition, it is also shown that these communities have a higher
94 abundance of antibiotic resistance genes than found in the fecal material communities from which

the farm air dust is originating [19]. As such, the presence of potential pathogens in the air of poultry houses therefore possess a possible health risk for both animals and workers.

The chicken fecal material deposited on the farm floor is partly responsible, via aerosol formation, for the bacterial community present in the poultry farm air. Therefor the chicken fecal microbiome has an influence on the air microbiome. The chicken fecal microbiome consist of a variety of species belonging to the Actinobacteria, Bacteroidetes, Firmicutes and Proteobacteria [20–23]. At a finer taxonomic scale, the dominant taxa are *Lactobacillaceae*, *Ruminicoccaceae* and *Enterobacteriaceae* in broiler fecal samples [23–25].

Pathogenic bacterial species such as *Acinetobacter*, *Campylobacter*, *Listeria*, *Proteus* and *Salmonella*, seem only present at low abundances in microbiomes from fecal material of healthy broiler flocks or are not present [23]. Interestingly, those taxa can be present on shells of eggs from the same animals that had a negative fecal sample [23]. Although fecal fluid can contaminate the eggshell, it is also possible that other sources, such as animal feathers, parental care, or even the environment could be responsible for the presence of pathogenic microbes. As such, sampling of fecal material might not give an adequate view of the presence of pathogens.

Here we tested the application of shotgun metagenomics on air filter samples spiked with mock communities and different numbers of *C. jejuni* to study the usefulness of the specific DNA extraction protocol as well as sequence depth in this study. In addition, air filter samples from two Norwegian poultry houses were tested as a proof of concept.

Results

Four gelatin air filters (“MOCK” samples) were split into halves and spiked with either 75 or 125 µl of a mock community consisting of cells from 10 different species in a log abundance distribution (Figure 1, Table 1). Two of the half filters spiked with mock community were further spiked with 200

CFU (colony forming units) of *C. jejuni* CCUG 11284T and another two with 20.000 CFU, while the remaining four halves were not spiked and used as negative controls. One air sample from each of the two poultry houses on the same farm were collected ("HOUSE"). Both filters were split in four pieces. One piece from each filter was not spiked, while the other three were spiked with three different levels of *C. jejuni* 927 (Figure 1, Table 1). All filter pieces were treated as separate samples.

Real-time PCR was used to detect *Campylobacter* in all samples. Campylobacter was detected at approximately Cq 29 in the samples spiked with 20.000 CFU and at approximately 36 in the samples spiked with 200 CFU (Table 1). The MOCK samples that were not spiked with *Campylobacter* were all negative (two "No Cq" and two above our standard hard cutoff Cq value of 40, Table 1). *Campylobacter* was detected in all HOUSE samples including the non-spiked samples, demonstrating that both poultry houses harboured *Campylobacter*.

Microbial community composition

Microbial community composition of the air filter communities was determined using metagenomic shotgun sequencing (Table 2). In order to assess which fraction of the microbial communities was captured using shotgun sequencing we used Nonpareil3 [26]. This indicated that for the MOCK community samples we had reached sufficient coverage (> 0.97), while the HOUSE samples with a chicken house background had an average coverage of 0.76. This result was expected as the MOCK communities only consisted of 10 species, while the HOUSE communities were natural samples with many more. Thus, in line with this, we find that the HOUSE samples showed a higher Nonpareil index of sequence diversity (N_d) (19.3 ± 0.1) than the MOCK samples (17.6 ± 0.2) [26]. This index describes the community diversity in sequence space using a natural logarithm. The N_d found for our MOCK samples falls in the same range as Mock communities tested by the authors of Nonpareil3. Most host-associated communities tested in Rodriquez-R et al., show a N_d range between 17 and 22, and our HOUSE samples show similar N_d values [26].

The taxonomic community composition of the air filter communities was analyzed using Kraken [27].

For the MOCK communities we found that on average 9 % of the reads were unclassified, while for the HOUSE communities this was 48.8 %. At the phylum level we detect 10 taxa with a relative abundance >= 0.01 % in most of the samples (Supplementary materials figure S1). Firmicutes and Proteobacteria dominated the MOCK samples, while in the HOUSE samples we found the same together with the phyla Actinobacteria and Bacteroidetes. The other eight phyla were all present in the HOUSE samples at relative abundances below 0.02 % except for Deinococcus-Thermus (average relative abundance = 0.023 ± 0.003%). The later thermophilic phylum was present in all our samples and was likely a contaminant from the air filter gelatin matrix.

The main bacterial genera found in the MOCK samples were *Listeria* and *Pseudomonas* and they showed relative abundances deviating from the expected abundance (Figure 2). Both taxa were predicted to have abundances of 89.9% and 8.9%, but instead show relative abundances of 37.9% (±13.5) vs 42.7% (±14.8). This indicates that our DNA extraction protocol, aimed to extract DNA from Gram-negative bacteria, caused an underestimation of Gram-positive bacteria in our samples.

In the HOUSE samples we found a diverse group of different genera with some known to contain pathogenic species (Figure 2). The dominant taxa were *Brevibacterium*, *Brachybacterium*, *Bacteroides*, *Corynebacterium*, *Lactobacillus*, *Staphylococcus*, *Faecalibacterium*, and *Helicobacter*. Most taxa were found with similar abundances in both houses, but a few had higher abundances in the HOUSE 2 samples (Figure 2). Those taxa were *Bacteriodes*, *Alistipes* and *Megamonas*. Overall, there is a high reproducibility between the samples from a single house.

A few genera are especially notable, since they were detected in both HOUSE and MOCK samples (Figure 2). A more detailed analysis showed that all samples contain several thermophilic bacteria, e.g. *Aeribacillus, Meiothermus* and the Betaproteobacterial genus *Cupriavidus*, while the MOCK samples also contained the Betaproteobacterial genus *Burkholderia*. Since these genera are not part of the MOCK community, it is likely that they are contaminants present in the gelatin matrix of the air filter. Interestingly, *Cupriavidus* was present with more than 1 million reads (max 4 Million) in the

HOUSE samples, while in the MOCK communities they only showed up with on average 0.5 million reads. Although the MOCK communities were created using only 10 distinct species, we detected many more species although most were in low abundance (< 0.01%). These classifications are likely due to misclassifications. For instance, in the MOCK communities that were not spiked with *Campylobacter*, we consistently detected this genus using Kraken with an average classified read count of 43 (Figure 3A). A similar number was identified when we mapped the metagenomics reads against a collection of *Campylobacter* genomes and the two genomes of our isolates used for spiking (Figure 3B). This indicates a consistent detection irrespective of the method used for detection. For the MOCK communities spiked with 200 or 20000 CFUs we could classify on average 100 or 6825 reads respectively. These results indicate that species detected using Kraken with close to 50 reads or less are likely not present in our samples, and should be ignored. Nonetheless, even with the restriction that a species has to be present in all MOCK samples with more than 100 reads, we still find 121 genera present.

Campylobacter detection of communities spiked with *C. jejuni*

We used both Kraken taxonomic classification as well as mapping with BBsplit to isolate genomes to identify *Campylobacter* spiked into MOCK and HOUSE samples. Both methods identified reads matching to *C. jejuni*, but since the genomes were not available, neither Kraken not BBsplit could differentiate at the strain level for the two strains used for spiking. We thus sequenced the two isolates *C. jejuni* 927 and *C. jejuni* CCUG 11284T with Illumina HiSeq 3000 and used the resulting assemblies to map our metagenomics reads.

The draft assemblies for *C. jejuni* 927 and *C. jejuni* CCUG 11284T were submitted to ENA (accession numbers: CAJPVE01; CAJQFQ01) and have a genome size of 1.59 Mbp and 1.73 Mbp respectively (Supplementary table 1). We used the Tetra correlation search at the JspeciesWS webserver [28] to identify the taxonomically closest known isolates for our strains. These isolates are *C. jejuni* subsp. *jejuni* 327 for 927 (Average Nucleotide Identity (ANI): 100.00) and *C. jejuni* subsp. *jejuni* ATCC33560

196 for CCUG 11284T (ANI: 99.66). The match for CCUG 11284T indicates the same strain but with a
197 different culture collection identifier. The ANI value between 927 and CCUG 11284T is 97.5%.

198 With the mapping approach, we find a clear difference between the samples on how abundant either
199 spike isolate was (Figure 3B). In the MOCK samples that was spiked with CCUG 11284T we find reads
200 uniquely matching C. *jejuni* 927 (Figure 3B). Likewise, reads with a unique match to CCUG 11284T
201 were found in the HOUSE samples that were spiked with C. *jejuni* 927. In addition, do we find many
202 more *Campylobacter* species in the HOUSE samples (Figure 3A, Supplemental figure S2B).

203

204 Discussion

205 *Campylobacter* detection using metagenomics

206 The prevention of gastrointestinal disease due to foodborne pathogens requires early and sensitive
207 detection of pathogens along the food production chain. For pathogens such as *Campylobacter* spp.,
208 it is important to identify the presence at the farm to prevent (or limit) further contamination. The
209 current method for on-farm sampling of poultry is by boot swabs of the floor environment, including
210 fecal droppings, analyzed by either cultivation or real-time PCR. These methods may have limitations
211 in their application due to cultivation bias or PCRs limitation to one target per assay. In recent years,
212 shotgun metagenomics has been shown to be able to detect a large variety of microbes without
213 cultivation and as such, it can be used to identify multiple pathogens simultaneously. Furthermore,
214 air sampling at poultry farms combined with PCR detection indicated earlier detection of pathogens
215 that conventional sampling [11, 12]. In addition, the DNA isolates from the air samples did contain
216 less PCR inhibitors. Thus, combining metagenomics with air sampling could help to identify a variety
217 of pathogens without being affected by cultivation biases.

218 Here, we used real-time PCR and metagenomic shotgun sequencing to analyze microbial
219 communities present in MOCK and HOUSE samples with a specific focus on the detection of
220 *Campylobacter*. With real-time PCR we were able to detect *Campylobacter* in the MOCK communities

when spiked with two different levels (Table 1). For the HOUSE samples we found Cq values between 29 and 33, with the lower values in the non-spiked samples (Table 1). The detection of *Campylobacter* in the non-spiked sair filters demonstrated that both poultry houses were infected with *Campylobacter*. These results were also confirmed by cultivation of boot swabs sampled simultaneously as the air samples from the two houses [11].

With the shotgun data we used two different approaches to identify *Campylobacter* spp. in our samples (Figure 3). The classification approach with Kraken showed that the HOUSE samples had between 100 and 300 reads / million reads that could be classified to *Campylobacter spp*. Interestingly, BBsplit mapped between 40 and 170 reads / million reads to both genomes of the spiked isolates, which is a slightly smaller fraction of the metagenomics datasets. BBsplit was used with a with a variety of Campylobacter spp genome, and it only counts reads mapping unambiguously to a single genome. When a read would map equally well to multiple genomes it was not counted. Interestingly, the mapping showed that in the HOUSE samples strains *C. jejuni* CCUG 11284T and 927 were found. The latter was spiked into these samples, while the former was used in the MOCK communities as a spike. This suggests the presence of *C. jejuni* strains sharing genomic similarity with CCUG 11284T in the HOUSE samples. An additional 0.03 % of reads mapped to a variety of other *Campylobacter* spp. including a third *C. jejuni* (NCTC 11168) reference genome (Supplementary materials Figure S2). In contrast, we only find few reads (<1 read / million reads) in the MOCK samples that were assigned to *C. jejuni* 927, while 3 (200 CFU) to 300 reads / million reads (20.000 CFU) were assigned to *C. jejuni* CCUG 11284T in the spiked samples.

Our results suggest that both Kraken and BBsplit can be used to identify *Campylobacter spp.* in the air of Poultry houses as does real-time-PCR [11]. The added value of using shotgun metagenomics for detection is the possibility to identify a variety of *Campylobacter* species and other bacteria of interest present at a farm, which is not possible with single 16s rRNA based real-time PCR. However, our metagenomic results also indicate that interpretation of such data should be done with care. For instance, the BBsplit results suggests the presence of a large number of different species

(supplementary materials Figure S2), while Kraken predominantly identified *C. jejuni* (Figure 3). Some of the species genomes used in the BBsplit mapping approach have very distinct host ranges. For instance, the species *C. fetus*, *C. hyointestinalis* and *C. iguaniorum* are associated with other hosts [29–31] and because of that it seems unlikely that they are present in our samples. Others like *C. jejuni* are known to be genomically versatile with broad ecological/ host ranges, including wild birds, livestock and humans [32, 33]. In addition, *Campylobacter* spp. *are* known for their high rate of horizontal gene transfer making it likely that environmental isolates from different species might contain genome regions shared by multiple species [34, 35]. That makes it difficult to identify these different species unambiguously and caution is needed for the interpretation. Nonetheless, by using two different approaches we show that multiple *Campylobacter* species are likely present in the air of our HOUSE samples and that most are at low abundance.

Air sample microbiomes vs broiler fecal microbiomes

The microbiome composition of the air in poultry houses is related, but not similar, to the fecal microbiome composition. After deposition of the feces on the litter layer in the house the microbiome composition likely changes in community composition and only a part of the community becomes airborne. In our analysis, we find a highly diverse community present in the air, with several genera also known to be present in the broiler feces. Typical genera found in broiler feces are *Bacteriodes*, *Brevibacterium*, *Corynebacterium*, *Enterococcus*, *Lactobacillus and Staphylococcus* [22–24, 36, 37]. These taxa were also identified in our air sample microbiomes of the poultry houses as well as in other studies using different detection methods [16–18]. That suggests that the air microbiome in poultry houses can be used as a proxy for the fecal microbiome. It also implies that the poultry house air contains many of the microbes associated with the animals and as such gives a good overview of the microbiome of the entire flock. Interestingly, many of the genera identified in our analysis are known gut microbiome species, but not all of them. *Brachybacterium* spp. for instance can be isolated from a wide range of sources including poultry litter, Gouda cheese, oil brine

or via air sampling [38, 39]. Not unexpected, *Brachybacterium* spp. have also been identified in the dust from poultry farms [18]. They have been rarely identified as pathogenic agents in humans [40]. The same is true for the genera *Dietzia and Pseudomonas*, which are both widely distributed bacteria that can be opportunistic pathogens [41]. Interestingly, *Pseudomonas* spp. can regularly be found in chicken fecal material as well as the air of poultry houses and are of concern for food production [17, 23, 42, 43]. These results therefore indicate that shotgun metagenomics of air samples provide a suitable approach to monitor poultry farms for a wide variety of pathogens.

Sensitivity of air sample metagenomics

The use of air sampling combined with real-time PCR or sequencing to detect pathogens at poultry farms has clear benefits over the gold standard with boot swabs with respect to the higher sensitivity and faster throughput [11, 12]. An additional advantage is that the potential to use the same samples to study all the microbial species present with metagenomic approaches. However, there is some ptifalls for these approaches. The first factor is the low input amount of DNA collected by this technique. Such samples are highly sensitive to contaminants from kits and reagents themselves, in addition to laboratory contamination [44, 45].

In addition, the DNA extraction method can introduce bias. Here, we used a method aimed at Gram-negative bacteria and specifically at lysing the cells from *Campylobacter* spp.. Using MOCK communities, as expected we find a large difference between the expected and observed MOCK community composition of the samples. This was especially clear for the abundances of *L. monocytogenes* and *P. aeruginosa*, which had a predicted relative abundance of 89 vs 8.9 %, but instead show an almost equal abundance in this experiment (Figure 2, supplementary figure S3). This was most likely due to the gram positive *L. monocytogenes* DNA being extracted with lower efficiency that the gram negative *P. aeruginosa*. Nonetheless, in the HOUSE samples, we do find many gram-positive bacterial genera, but the relative abundance of these taxa is likely underestimated relative

298 to Gram-negative bacteria. This underscores the importance of including mock communities in
299 microbiome studies, in order to understand the DNA extraction bias for particular communities.

300 Our results suggest that the gelatine filters contain DNA despite the fact that these filters are gamma
301 sterilized [15]. Gelatine production is a harsh, but not a sterile process, which requires sterilization
302 before shipment of such filters. By using mock communities we were able to identify several
303 contaminating genera likely present in the gelatin matrix of the air filter. The taxa *Meiothermus*,
304 *Aeribacillus* and *Burkholderia* could be found in all MOCK samples and with similar abundances in
305 some of the HOUSE samples. More interestingly, we also find a high abundance of *Cupriavidus* in all
306 samples, which strongly suggests this is a contaminant genus. This *Burkholderiales* genus, together
307 with *Burkholderia* and the sister genus *Ralstonia* can be found as contaminants in microbiome
308 studies, as shown previously [44, 46]. This genus has also been found in another air sampling study of
309 poultry houses where polyvinyl chloride filters were used [16], but there is no indication that the
310 authors of that study controlled for contamination by analyzing clean filters. Thus, it is unclear if this
311 genus is part of the poultry house microbiome as indicated by Just et al [16], or that it is also present
312 as a contaminant in their non-gelatine filters. Since we observe *Cupriavidus* in all our air filter
313 samples it is unlikely that this genus is present in the poultry house, but rather introduced into the
314 samples via the gelatin matrix or through laboratory handling. What is clear from our analysis is that
315 contaminating species in air filter experiments are present and easily detected when using mock
316 communities or even negative samples. This indicates a need to include such sample types in all
317 microbiome experiments using air sampling, to be able to filter out contamination from the filters.

318

319 ## Application of air sampling

320 Our study showed that air sampling indoor air of poultry houses together with shotgun metagenomic
321 sequencing can be used to detect pathogenic microorganisms. This approach can be used to study a
322 various of pathogens, moulds, antimicrobial resistence in environments where a high hygiene

standard is required [19, 47]. These findings indicate that in l.e. animal production facilities, slaughter houses or in the food industry airborne pathogens and their resistome might cause health concerns for the humans working in such spaces [17, 48]. Likewise, the animals in these facilities are also exposed to the same potential pathogens that are present in the air [18, 49]. With the increasing availability of shotgun metagenomics, it becomes possible to monitor animal house facilities for a wide variety of pathogens without the limits in detection when using cultivation or dedicated PCR approaches. This enables the industry not only to monitor for pathogens that can cause foodborne outbreaks at an early stage, but it could also stimulate the development of intervention methods to reduce the proliferation of such pathogens at the farm level. In addition, these approaches can help to improve the animal health by developing measures to reduce the exposure to a wide variety of potential pathogens. Such measures could include better regulation of the indoor climate of poultry houses. The extra benefit of such developments is that the working conditions for humans at those farms might improve as well.

Nonetheless, to really make use of the power of metagenomics in an industrial monitoring set-up, it will be needed to further reduce the cost of this method, and to develop analysis approaches that are both user friendly and easy to use for non-bioinformaticians. Finding potential pathogens in the air of poultry farms might cause concern, but since many of the organisms found are also part of the healthy fecal microbiome of chicken it is difficult to identify at what level pathogen detection might require intervention. These difficulties require further investigations in order to improve the sensitivity and specificity of metagenomic detection methods.

Conclusions

Here we used shotgun metagenomics and real-time PCR to study the microbial community present in the air of poultry houses. Our results indicate the presence of a diverse community that contains many genera that are also found in the chicken fecal microbiome. This shows that air sampling of

poultry house air combined with shotgun metagenomics can be used as a proxy for the study of the fecal microbiome of the flock present in a farm house. The shotgun metagenomics results allowed us to identify *Campylobacter* spp. present at low levels, as well as other pathogenic microbes that could be of health concern. By using mock communities, we address some technical limitations that can help data analysis and interpretation of data generated with the air sampling method. Those include DNA extraction biases due to the extraction method chosen, as well as the background contamination of the samples due to laboratory handling and/or background DNA present in the gelatin matrix of the filters.

By using air sampling methods with shotgun metagenomics it will be possible to study the microbial communities associated with animal production and food industry with emphasis on the presence of microbes considered as food-borne pathogens. Understanding the dynamics of microbial communities present in poultry houses will help to identify processes to reduce to the introduction of pathogens in the poultry production as well to improve poultry and human worker health.

Methods

Sample collection

Air filters from two Norwegian broiler houses on the same farm were collected using an AirPort MD8 device (Sartorious Stedim Biotech, France) with disposable gelatin filters (80 mm diameter; Sartorius, 17528-80ACD) as described by Johannessen and coworkers [11], one from each house. The filters collected were used for spiking as described below.

Preparation of artificially contaminated air filters

Four gelatin membrane filters were divided into two pieces each (Table 1, Figure 1). Six half filters were spiked with 75 µl of microbial community standard (MOCK), and two half filters were spiked with 125 µl of the same standard. The microbial community standard (MOCK) used in this study has a log abundance distribution of different bacterial species and fungi ranging from 10^8 to 10^2 cells

373 (ZymoBIOMICS Microbial Community Standard II, ZYMO RESEARCH EUROPE GMBH, Freiburg,
374 Germany) and is well suited for assessing the accuracy of composition and ideal for the quality
375 control of microbiome measurements. In addition, we spiked four of the half filters already spiked
376 with mock communities, with two different concentrations of *C. jejuni subsp. jejuni CCUG 11284T*
377 (Accession number CAJQFQ01) (200 and 20.000 cfu) (Table 1). One air filter collected from each of
378 two broiler houses (see above) were divided into quarters and spiked with three different
379 concentrations of *C. jejuni* 927 (Accession number CAJPVE01) (5600, 560 and 56 cfu) (Table 1). One
380 quarter of each filter was not inoculated with *C. jejuni* 927 and will reflect the content of the real
381 sample (Figure 1).

382 The DNA extraction is performed as previously described in Hoorfar et al., 2020 [12]. Briefly, air filters
383 were pretreated for DNA extraction as follows; half a filter was dissolved in 3.5 ml ddH_2O and 100 µl
384 alkaline protease (Protex 6L, Genencor International BA, The Netherlands) was added. The samples
385 were mixed by vortexing until the filters were dissolved. The samples were then incubated at 37°C in
386 a thermal mixer for 6 min at 1000 rpm, centrifuged at 4°C for 5 min at 8000 x g and the supernatant
387 discarded. The pellet was used for DNA extraction using the Gram-negative protocol for DNeasy
388 Blood and Tissue kit (Qiagen) with some minor modifications described briefly below. The incubation
389 period at 56°C was set to one hour for all samples and all samples were treated with 4 µl RNase A
390 (100 mg/ml, Qiagen). DNA was eluted in 100 µl TE buffer with 0.1 mM EDTA. The DNA quantity was
391 determined by using the high sensitivity DNA Qubit assay (ThermoFisher Scientific) and DNA quality
392 was assessed, if enough DNA, by using Nanodrop spectrophotometer (ThermoFisher Scientific).

393 DNA from the two *C. jejuni* strains used for spiking were extracted using QIAamp DNA Mini
394 kit (Qiagen) according to the manufacturer's protocol. DNA quantity and quality was determined by
395 using the broad range DNA Qubit assay and Nanodrop spectrophotometer. The DNA from the two *C.*
396 *jejuni* isolates was whole genome sequenced using the same protocols as for the metagenomic
397 samples, for details see below under metagenomic library preparation and sequencing.

Real-time PCR for Campylobacter

Real-time PCR was performed with forward primer 5'-CTGCTTAACACAAGTTGAGTAGG-3' and reverse primer TTCCTTAGGTACCGTCAGAA as combined and optimized by Lübeck *et al* (xx) with LNA probe 5'-6FAM CA[T]CC[T]CCACGCGGCG[T]TGC BHQ1-3' [50–53]. Shortly, 500 nM primerers and 300 nM probe were added to Brilliant III Ultra-Fast QPCR mastermix (Agilent) or Tth DNA polymerase mastermix (containing: 1x PCR buffer for Tth DNA polymerase, 25 mM MgCl2, 7% glycerol, 0.6 mM dNTPs, 0,2 mg BSA and 1 U Tth polymerase). For the Brilliant mastermix was the cycling conditions were 95° degrees for 3 minutes followed by 45 cycles of 95° degrees for 5 seconds and 60° degrees for 15 seconds. For the Tth DNA polymerase mastermix was the cycling conditions were 95° degrees for 3 minutes followed by 45 cycles of 95° degrees for 15 seconds, 60° degrees for 60 seconds and 72° degrees for 30 seconds. In both cases a CFX96 instrument (Bio-Rrad) was used.

Metagenomic library preparation & metagenomics shotgun sequencing

Metagenomic shotgun libraries were prepared at the Norwegian Sequencing Centre (Oslo, Norway) from the air filter DNA extracts and the two *C. jejuni* isolates. DNA was mechanically fragmented using sonication to 350 bp (Covaris) and Dual indexed libraries were generated using SMARTer ThruPLEX (Takara) adapted to low-input DNA. The libraries were sequenced on a HiSeq 3000 (Illumina) generating 150 bp Paired end reads.

Genome assembly of Campylobacter genomes.

The *Campylobacter* sequence data was processed with the Bifrost pipeline [54] (https://github.com/NorwegianVeterinaryInstitute/Bifrost) with modifications. In brief, PhiX sequences were removed using BBmap (v 38.76) [55], adapter sequences and low quality bases were removed using Trimmomatic (v 0.39) [56]. Assembly was performed with Skesa (v 2.3.0) [57], using default settings. Due to the very high coverage (> 2000 X, we screened the contigs for contamination using Kraken2 (v 2.0.8_beta) [58] and a Minikraken database containing Refseq microbial genomes. We identified a contaminant *Staphylococcus epidermis* genome (accession number: CAJPVF01) in the *C. jejuni 927* assembly that was present as ≈ 1% of the reads. All non-Campylobacter contigs were removed from the *C. jejuni* 927 assembly. Next, we

425 use Pilon (v 1.23) [59] to correct the assemblies of both isolates and Prokka (v 1.14.5) [60] was used
426 to annotate both genomes.

427 ### *Metagenomic data quality control and classification*

428 Metagenome sequence data quality control was performed using the Sunbeam pipeline (v 2.1.0) [61]
429 (Supplementary materials figure S4). In brief, the pipeline runs Cutadapt (v 2.8) and Trimmomatic (v
430 0.36) to remove adapters and low quality bases. Next, low-complexity regions are masked using
431 Komplexity (v 0.3.6) and subsequently host contamination filtering was performed to remove read
432 matching both the PhiX (NC_001422.1) and a masked version of the human genome (Hg19,
433 https://drive.google.com/file/d/0B3llHR93L14wd0pSSnFULUlhcUk/edit?usp=sharing).
434 The sequence depth for the individual samples was estimated using Nonpareil (version3) [26].
435 Taxonomic classification was performed using Kraken (v 1.0) [27]. Species abundances were
436 subsequently estimated using Bracken (v2.6.0) [62].

437 ### *Mapping of metagenome sequences*

438 Species abundances based on reads were also determine in the following way: a collection of fasta
439 sequences was downloaded that included the mock community species genomes
440 (https://s3.amazonaws.com/zymo-files/BioPool/ZymoBIOMICS.STD.refseq.v2.zip), 29 Complete
441 *Campylobacter* spp. reference genomes from Refseq (downloaded 25-11-2020, Supplementary table
442 S1), and the *Gallus gallus* reference genome version 6 (GCA_000002315.5). We also added the
443 genome of *C. coli* NTICC13 (GCF_009756375.1). All genomes were masked using BBmask (BBTools
444 version 38.86), with entropy set to 0.7 [55]. Metagenomic reads were mapped against the masked
445 genomes using BBsplit with default settings and discarding reads with ambigious mapping within a
446 genome or between genomes. A summary of the mapped reads per genome was used for
447 visualization.

448

Visualization of the results

The figures were created using R-studio (version 1.3.959) with R (version 4.0.0). The following R-packages were used to process data tables and generate figures: dplyr (v1.0.2), forcats (v 0.5.0), ggplot2 (v3.3.2), purr (v 0.3.4) , readr (v1.4.0), tidyr (v1.1.2).

References

1. European Food Safety Authority, European Centre for Disease Prevention and Control (ECDC). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2014. EFSA Journal. 2015;13. doi:10.2903/j.efsa.2015.4329.

2. The global view of campylobacteriosis. Expert consulation. Geneva: World Health Organization; 2013.

3. Costa D, Iraola G. Pathogenomics of emerging *Campylobacter* species. Clin Microbiol Rev. 2019;32. doi:10.1128/CMR.00072-18.

4. EFSA Panel on Biological Hazards (BIOHAZ). Scientific Opinion on Quantification of the risk posed by broiler meat to human campylobacteriosis in the EU. EFSA Journal. 2010;8:1437. doi:10.2903/j.efsa.2010.1437.

5. Bailey RA, Kranis A, Psifidi A, Watson KA, Rothwell L, Hocking PM, et al. Colonization of a commercial broiler line by *Campylobacter* is under limited genetic control and does not significantly impair performance or intestinal health. Poult Sci. 2018;97:4167–4176. doi:10.3382/ps/pey295.

6. European Food Safety Authority. Technical specifications on harmonised epidemiological indicators for biological hazards to be covered by meat inspection of poultry. EFSA Journal. 2012;10. doi:10.2903/j.efsa.2012.2764.

7. European Parliament and the Council. Directive 2003/99/EC of the European Parliament and of the Council of 17 November 2003 on the monitoring of zoonoses and zoonotic agents, amending Council Decision 90/424/EEC and repealing Council Directive 92/117/EEC. OJ. 2003;L325:31–40.

8. European Commission. COMMISSION REGULATION (EU) 2017/1495-of 23 August 2017-amending Regulation (EC) No 2073/2005 as regards *Campylobacter* in broiler carcases. European Journal of the European Union. 2017.

9. EFSA Panel on Biological Hazards (BIOHAZ), Koutsoumanis K, Allende A, Alvarez-Ordóñez A, Bolton D, Bover-Cid S, et al. Update and review of control options for *Campylobacter* in broilers at primary production. EFSA J. 2020;18:e06090. doi:10.2903/j.efsa.2020.6090.

10. International Organization for Standardization. ISO - ISO 10272-1:2017 - Microbiology of the food chain — Horizontal method for detection and enumeration of *Campylobacter* spp. — Part 1: Detection method. 2017. https://www.iso.org/standard/63225.html. Accessed 14 Oct 2020.

11. Johannessen GS, Garofolo G, Di Serafino G, Koláčková I, Karpíšková R, Wieczorek K, et al. *Campylobacter* in chicken - Critical parameters for international, multicentre evaluation of air sampling and detection methods. Food Microbiol. 2020;90:103455. doi:10.1016/j.fm.2020.103455.

12. Hoorfar J, Koláčková I, Johannessen GS, Garofolo G, Marotta F, Wieczorek K, et al. A multicenter proposal for a fast tool to screen biosecure chicken flocks for the foodborne pathogen *Campylobacter*. Appl Environ Microbiol. 2020;86. doi:10.1128/AEM.01051-20.

13. Søndergaard MSR, Josefsen MH, Löfström C, Christensen LS, Wieczorek K, Osek J, et al. Low-cost monitoring of *Campylobacter* in poultry houses by air sampling and quantitative PCR. J Food Prot. 2014;77:325–330. doi:10.4315/0362-028X.JFP-13-268.

14. Olsen KN, Lund M, Skov J, Christensen LS, Hoorfar J. Detection of *Campylobacter* bacteria in air samples for continuous real-time monitoring of *Campylobacter* colonization in broiler flocks. Appl Environ Microbiol. 2009;75:2074–2078. doi:10.1128/AEM.02182-08.

15. De Clerck E, Vanhoutte T, Hebb T, Geerinck J, Devos J, De Vos P. Isolation, characterization, and identification of bacterial contaminants in semifinal gelatin extracts. Appl Environ Microbiol. 2004;70:3664–3672. doi:10.1128/AEM.70.6.3664-3672.2004.

16. Just N, Kirychuk S, Gilbert Y, Létourneau V, Veillette M, Singh B, et al. Bacterial diversity characterization of bioaerosols from cage-housed and floor-housed poultry operations. Environ Res. 2011;111:492–498. doi:10.1016/j.envres.2011.01.009.

17. Bródka K, Kozajda A, Buczyńska A, Szadkowska-Stańczyk I. The variability of bacterial aerosol in poultry houses depending on selected factors. Int J Occup Med Environ Health. 2012;25:281–293. doi:10.2478/S13382-012-0032-8.

18. Dai P, Shen D, Tang Q, Huang K, Li C. PM2.5 from a broiler breeding production system: The characteristics and microbial community analysis. Environ Pollut. 2020;256:113368. doi:10.1016/j.envpol.2019.113368.

19. Luiken REC, Van Gompel L, Bossers A, Munk P, Joosten P, Hansen RB, et al. Farm dust resistomes and bacterial microbiomes in European poultry and pig farms. Environ Int. 2020;143:105971. doi:10.1016/j.envint.2020.105971.

20. Skarżyńska M, Leekitcharoenphon P, Hendriksen RS, Aarestrup FM, Wasyl D. A metagenomic glimpse into the gut of wild and domestic animals: Quantification of antimicrobial resistance and more. PLoS One. 2020;15:e0242987. doi:10.1371/journal.pone.0242987.

21. Locatelli A, Hiett KL, Caudill AC, Rothrock MJ. 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? Journal of Applied Poultry Research. 2017;26:325–336. doi:10.3382/japr/pfw076.

22. Videnska P, Rahman MM, Faldynova M, Babak V, Matulova ME, Prukner-Radovcic E, et al. Characterization of egg laying hen and broiler fecal microbiota in poultry farms in Croatia, Czech Republic, Hungary and Slovenia. PLoS One. 2014;9:e110076. doi:10.1371/journal.pone.0110076.

23. Trudeau S, Thibodeau A, Côté J-C, Gaucher M-L, Fravalo P. Contribution of the broiler breeders' fecal microbiota to the establishment of the eggshell microbiota. Front Microbiol. 2020;11:666. doi:10.3389/fmicb.2020.00666.

24. Siegerstetter S-C, Petri RM, Magowan E, Lawlor PG, Zebeli Q, O'Connell NE, et al. Feed restriction modulates the fecal microbiota composition, nutrient retention, and feed efficiency in chickens divergent in residual feed intake. Front Microbiol. 2018;9:2698. doi:10.3389/fmicb.2018.02698.

25. Bauer BW, Radovanovic A, Willson N-L, Bajagai YS, Hao Van TT, Moore RJ, et al. Oregano: A potential prophylactic treatment for the intestinal microbiota. Heliyon. 2019;5:e02625. doi:10.1016/j.heliyon.2019.e02625.

26. Rodriguez-R LM, Gunturu S, Tiedje JM, Cole JR, Konstantinidis KT. Nonpareil 3: fast estimation of metagenomic coverage and sequence diversity. mSystems. 2018;3. doi:10.1128/mSystems.00039-18.

27. Wood DE, Salzberg SL. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome Biol. 2014;15:R46. doi:10.1186/gb-2014-15-3-r46.

28. Richter M, Rosselló-Móra R, Oliver Glöckner F, Peplies J. JSpeciesWS: a web server for prokaryotic species circumscription based on pairwise genome comparison. Bioinformatics. 2016;32:929–931. doi:10.1093/bioinformatics/btv681.

29. Gilbert MJ, Duim B, Zomer AL, Wagenaar JA. Living in cold blood: *Arcobacter*, *Campylobacter*, and *Helicobacter* in reptiles. Front Microbiol. 2019;10:1086. doi:10.3389/fmicb.2019.01086.

30. Nadin-Davis SA, Chmara J, Carrillo CD, Amoako K, Goji N, Duceppe M-O, et al. A comparison of fourteen fully characterized mammalian-associated *Campylobacter fetus* isolates suggests that loss of defense mechanisms contribute to high genomic plasticity and subspecies evolution. PeerJ. 2021;9:e10586. doi:10.7717/peerj.10586.

31. Wilkinson DA, O'Donnell AJ, Akhter RN, Fayaz A, Mack HJ, Rogers LE, et al. Updating the genomic taxonomy and epidemiology of *Campylobacter hyointestinalis*. Sci Rep. 2018;8:2393. doi:10.1038/s41598-018-20889-x.

32. Epping L, Antão E-M, Semmler T. Population biology and comparative genomics of *Campylobacter* species. Curr Top Microbiol Immunol. 2021;431:59–78. doi:10.1007/978-3-030-65481-8_3.

33. Weis AM, Storey DB, Taff CC, Townsend AK, Huang BC, Kong NT, et al. Genomic comparison of *Campylobacter* spp. and their potential for zoonotic transmission between birds, primates, and livestock. Appl Environ Microbiol. 2016;82:7165–7175. doi:10.1128/AEM.01746-16.

34. Golz JC, Stingl K. Natural competence and horizontal gene transfer in *Campylobacter*. Curr Top Microbiol Immunol. 2021;431:265–292. doi:10.1007/978-3-030-65481-8_10.

35. Iraola G, Pérez R, Naya H, Paolicchi F, Pastor E, Valenzuela S, et al. Genomic evidence for the emergence and evolution of pathogenicity and niche preferences in the genus *Campylobacter*. Genome Biol Evol. 2014;6:2392–2405. doi:10.1093/gbe/evu195.

36. Díaz-Sánchez S, Perrotta AR, Rockafellow I, Alm EJ, Okimoto R, Hawken R, et al. Using fecal microbiota as biomarkers for predictions of performance in the selective breeding process of pedigree broiler breeders. PLoS One. 2019;14:e0216080. doi:10.1371/journal.pone.0216080.

37. Xiao Y, Xiang Y, Zhou W, Chen J, Li K, Yang H. Microbial community mapping in intestinal tract of broiler chicken. Poult Sci. 2017;96:1387–1393. doi:10.3382/ps/pew372.

38. Takeuchi M, Fang CX, Yokota A. Taxonomic study of the genus *Brachybacterium*: proposal of *Brachybacterium conglomeratum* sp. nov., nom. rev., *Brachybacterium paraconglomeratum* sp. nov., and *Brachybacterium rhamnosum* sp. nov. Int J Syst Bacteriol. 1995;45:160–168. doi:10.1099/00207713-45-1-160.

39. Hlaing PPT, Junqueira ACM, Uchida A, Purbojati RW, Houghton JNI, Chénard C, et al. Complete genome sequence of *Brachybacterium* sp. Strain SGAir0954, isolated from Singapore air. Microbiol Resour Announc. 2019;8. doi:10.1128/MRA.00619-19.

40. Brummaier T, Hinfothong P, Soe NL, Tongmanakit J, Watthanaworawit W, Ling C. *Brachybacterium nesterenkovii* isolated from a human blood culture-a first report. New Microbes New Infect. 2020;36:100699. doi:10.1016/j.nmni.2020.100699.

41. Koerner RJ, Goodfellow M, Jones AL. The genus *Dietzia*: a new home for some known and emerging opportunist pathogens. FEMS Immunol Med Microbiol. 2009;55:296–305. doi:10.1111/j.1574-695X.2008.00513.x.

42. Rothrock MJ, Locatelli A, Feye KM, Caudill AJ, Guard J, Hiett K, et al. A microbiomic analysis of a pasture-raised broiler glock elucidates foodborne pathogen ecology along the farm-to-fork continuum. Front Vet Sci. 2019;6:260. doi:10.3389/fvets.2019.00260.

43. Oakley BB, Morales CA, Line J, Berrang ME, Meinersmann RJ, Tillman GE, et al. The poultry-associated microbiome: network analysis and farm-to-fork characterizations. PLoS One. 2013;8:e57190. doi:10.1371/journal.pone.0057190.

44. Salter SJ, Cox MJ, Turek EM, Calus ST, Cookson WO, Moffatt MF, et al. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. BMC Biol. 2014;12:87. doi:10.1186/s12915-014-0087-z.

45. Minich JJ, Sanders JG, Amir A, Humphrey G, Gilbert JA, Knight R. Quantifying and understanding well-to-well contamination in microbiome research. mSystems. 2019;4. doi:10.1128/mSystems.00186-19.

46. Vandamme P, Coenye T. Taxonomy of the genus *Cupriavidus*: a tale of lost and found. Int J Syst Evol Microbiol. 2004;54 Pt 6:2285–2289. doi:10.1099/ijs.0.63247-0.

47. Just NA, Létourneau V, Kirychuk SP, Singh B, Duchaine C. Potentially pathogenic bacteria and antimicrobial resistance in bioaerosols from cage-housed and floor-housed poultry operations. Ann Occup Hyg. 2012;56:440–449. doi:10.1093/annhyg/mer105.

589 48. Kaur G, Sethi RS. Multiple exposures to poultry barn air and lipopolysaccharide synergistically
590 increase the pulmonary expression of TLR-4 and IL-1β. J Occup Health. 2020;62:e12094.
591 doi:10.1002/1348-9585.12094.
592 49. Zhang J, Li Y, Xu E, Jiang L, Tang J, Li M, et al. Bacterial communities in PM2.5 and PM10 in broiler
593 houses at different broiler growth stages in spring. Pol J Vet Sci. 2019;22:495–504.
594 doi:10.24425/pjvs.2019.129957.
595 50. Josefsen MH, Löfström C, Hansen TB, Christensen LS, Olsen JE, Hoorfar J. Rapid quantification of
596 viable *Campylobacter* bacteria on chicken carcasses, using real-time PCR and propidium monoazide
597 treatment, as a tool for quantitative risk assessment. Appl Environ Microbiol. 2010;76:5097–5104.
598 doi:10.1128/AEM.00411-10.
599 51. Uyttendaele M, Schukkink R, van Gemen B, Debevere J. Identification of *Campylobacter jejuni*,
600 *Campylobacter coli* and *Campylobacter lari* by the nucleic acid amplification system NASBAR. J Appl
601 Bacteriol. 1994;77:694–701. doi:10.1111/j.1365-2672.1994.tb02821.x.
602 52. Docherty L, Adams MR, Patel P, McFadden J. The magnetic immuno-polymerase chain reaction
603 assay for the detection of *Campylobacter* in milk and poultry. Lett Appl Microbiol. 1996;22:288–292.
604 doi:10.1111/j.1472-765x.1996.tb01163.x.
605 53. Lübeck PS, Wolffs P, On SLW, Ahrens P, Rådström P, Hoorfar J. Toward an international standard
606 for PCR-based detection of food-borne thermotolerant *Campylobacters*: assay development and
607 analytical validation. Appl Environ Microbiol. 2003;69:5664–5669. doi:10.1128/aem.69.9.5664-
608 5669.2003.
609 54. Lagesen K. The Bifrost genomic epidemiology pipeline. Computer software.
610 DOI:10.5281/zenodo.3984659: Norwegian Veterinary Institute; 2020.
611 55. Bushnell B. BBMap: A Fast, Accurate, Splice-Aware Aligner. Computer software.
612 https://sourceforge.net/projects/bbmap/: JGI; 2014.
613 56. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data.
614 Bioinformatics. 2014;30:2114–2120. doi:10.1093/bioinformatics/btu170.
615 57. Souvorov A, Agarwala R, Lipman DJ. SKESA: strategic k-mer extension for scrupulous assemblies.
616 Genome Biol. 2018;19:153. doi:10.1186/s13059-018-1540-z.
617 58. Wood DE, Lu J, Langmead B. Improved metagenomic analysis with Kraken 2. Genome Biol.
618 2019;20:257. doi:10.1186/s13059-019-1891-0.
619 59. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al. Pilon: an integrated tool
620 for comprehensive microbial variant detection and genome assembly improvement. PLoS One.
621 2014;9:e112963. doi:10.1371/journal.pone.0112963.
622 60. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics. 2014;30:2068–2069.
623 doi:10.1093/bioinformatics/btu153.
624 61. Clarke EL, Taylor LJ, Zhao C, Connell A, Lee J-J, Fett B, et al. Sunbeam: an extensible pipeline for
625 analyzing metagenomic sequencing experiments. Microbiome. 2019;7:46. doi:10.1186/s40168-019-
626 0658-x.
627 62. Lu J, Breitwieser FP, Thielen P, Salzberg SL. Bracken: estimating species abundance in
628 metagenomics data. PeerJ Computer Science. 2017;3:e104. doi:10.7717/peerj-cs.104.
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631 List of abbreviations

Declarations

Ethics declarations

"Not applicable"

Availability of data and Materials

All sequence data was submitted to the ENA database and is publically available under the umbrella project PRJEB44024. The individual project numbers are: Genome assemblies - PRJEB43619; Genome fastq files - PRJEB43621; Metagenome fastq files - PRJEB43623. For sample specific accession numbers, please see Supplementary materials table S1 and S3.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conception and design: GJ,CS,MT. Drafting of manuscript: TH, CS, GJ, BS. Review and revision of manuscript: TH, CS, GJ, BS, MT. Sample collection: CS, GJ, MT. Analysis and data interpretation: TH, CS, GJ, BS. The authors read and approved the final manuscript.

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660

661 Electronic supplementary material

662 Supplementary materials.pdf

Tables

Table 1. Description of samples and *Campylobacter* 16S rDNA real-time PCR results. Samples are listed by levels of spiking.

Sample ID	Sample - *C. jejuni* spike	Spike (CFU)	Mock amount (µl)	*Campylobacter*, Cq
5-MOCK1-S2a	Mock - CCUG 11284T	20000	75	29.1
6-MOCK1-S2b	Mock - CCUG 11284T	20000	75	29.2
3-MOCK1-S1a	Mock - CCUG 11284T	200	75	37.2
4-MOCK1-S1b	Mock - CCUG 11284T	200	75	35.2
1-MOCK1-Ba	Mock – no spike	0	75	43.1
2-MOCK1-Bb	Mock – no spike	0	75	No Cq
7-MOCK2-Ba	Mock – no spike	0	125	41.1
8-MOCK2-Bb	Mock – no spike	0	125	No Cq
9-H1-3	House 1 - 927	5650	-	29.4
10-H1-4	House 1 - 927	565	-	30.4
11-H1-5	House 1 - 927	56,5	-	30.3
12-H1-B	House 1 – no spike	0	-	31.1
13-H2-3	House 2 - 927	5650	-	30.6
14-H2-4	House 2 - 927	565	-	31.3
15-H2-5	House 2 - 927	56,5	-	31.3
16-H2-B	House 2 – no spike	0	-	32.6

669 **Table 2**. Overview of the sequencing effort for each of the samples in this study.

Sample ID	*C. jejuni* cfu	Raw Paired-end sequences	After quality trimming /filtering (%)	PhiX sequences	Human sequences	Final PE sequences	Metagenome Coverage	Sequence diversity index (Nd)	Average genome size (Mbp)
1-MOCK1-B-a		24675209	93.8	12	16022	21444439	0.99	16.0	4.10
2-MOCK1-B-b		18611989	92.6	11	6553	16267510	0.98	16.1	3.80
3-MOCK1-S1-a	200	21636112	93.2	16	6197	18775078	0.98	16.1	3.95
4-MOCK1-S1-b	200	25799040	93.7	18	44918	22326211	0.99	15.9	4.30
5-MOCK1-S2-a	20000	25760599	92.3	16	33337	22067796	0.98	16.0	4.02
6-MOCK1-S2-b	20000	19029552	93.9	17	16598	16649467	0.98	16.0	4.08
7-MOCK2-B-a		16256676	92.7	25	52135	14515655	0.99	15.3	3.38
8-MOCK2-B-b		17247065	90.9	11	8124	14417819	0.99	15.9	4.21
9-H1-3	5650	16183867	96.4	10	4236	14063204	0.74	19.4	3.24
10-H1-4	565	15842872	96.2	7	3936	13728192	0.74	19.3	3.20
11-H1-5	56	26674096	96.2	15	7839	23171336	0.78	19.4	3.20
12-H1-B		31645569	97.0	10	9333	27869436	0.79	19.4	3.24
13-H2-3	5650	23296681	96.1	14	20021	20297450	0.79	19.2	3.14
14-H2-4	565	20578293	95.9	15	4504	17927138	0.78	19.2	3.12
15-H2-5	56	13873052	95.9	6	2728	12152054	0.75	19.2	3.14
16-H2-B		15071970	96.2	11	3183	13215124	0.76	19.2	3.19

670

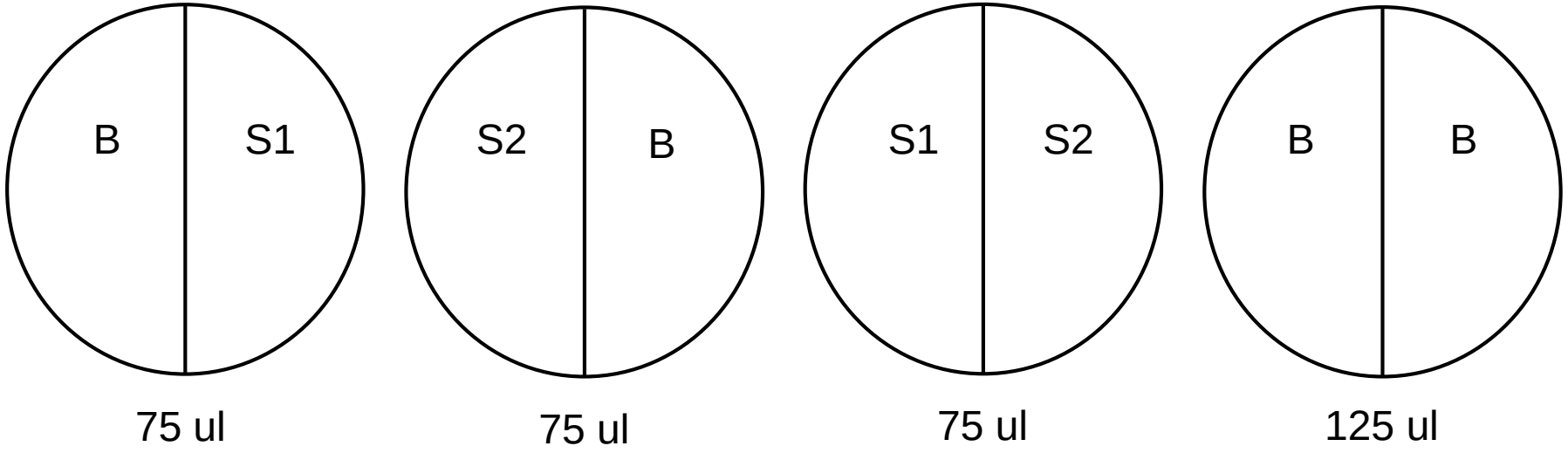
Figure legends

Figure 1. Design of the experiment using different air filters. The MOCK samples were spiked with 200 (S1) or 20.000 (S2) CFU of *C. jejuni* CCUG 11284T. The HOUSE samples were spiked with three different levels of *C. jejuni* 927.

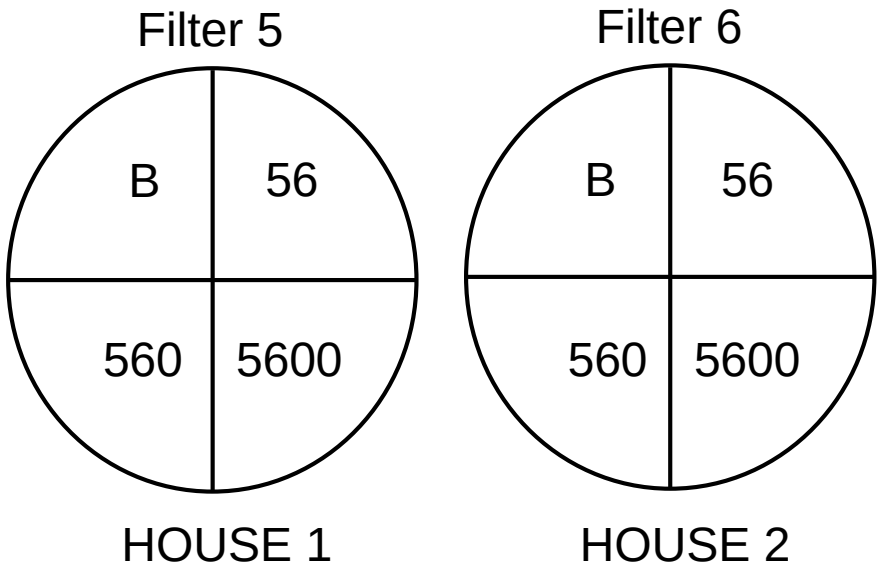
Figure 2. Relative abundances of bacterial genera found in MOCK and HOUSE samples. The first column shows the expected abundances of MOCK community taxa as provided by the manufacturer. Genera with a relative abundance equal or higher than 0.1% in at least one sample were kept for visualization. Taxa relative abundances below 0.01% are not shown.

Figure 3. *Campylobacter* spp. abundances in MOCK and HOUSE samples using two different methods. **A**) Normalized abundances of *Campylobacter* spp. as found with Kraken read classification. Read abundances for the different taxa are shown as classified reads per million reads. **B**) Normalized abundances of reads mapped by BBsplit to the genome sequences of *C. jejuni* CCUG 11284T (MOCK sample spike) or *C. jejuni* 927 (HOUSE sample spike). Note that in the HOUSE samples we find reads mapping to *C. jejuni* CCUG 11284T, indicating the presence of strains closely related to this isolate.

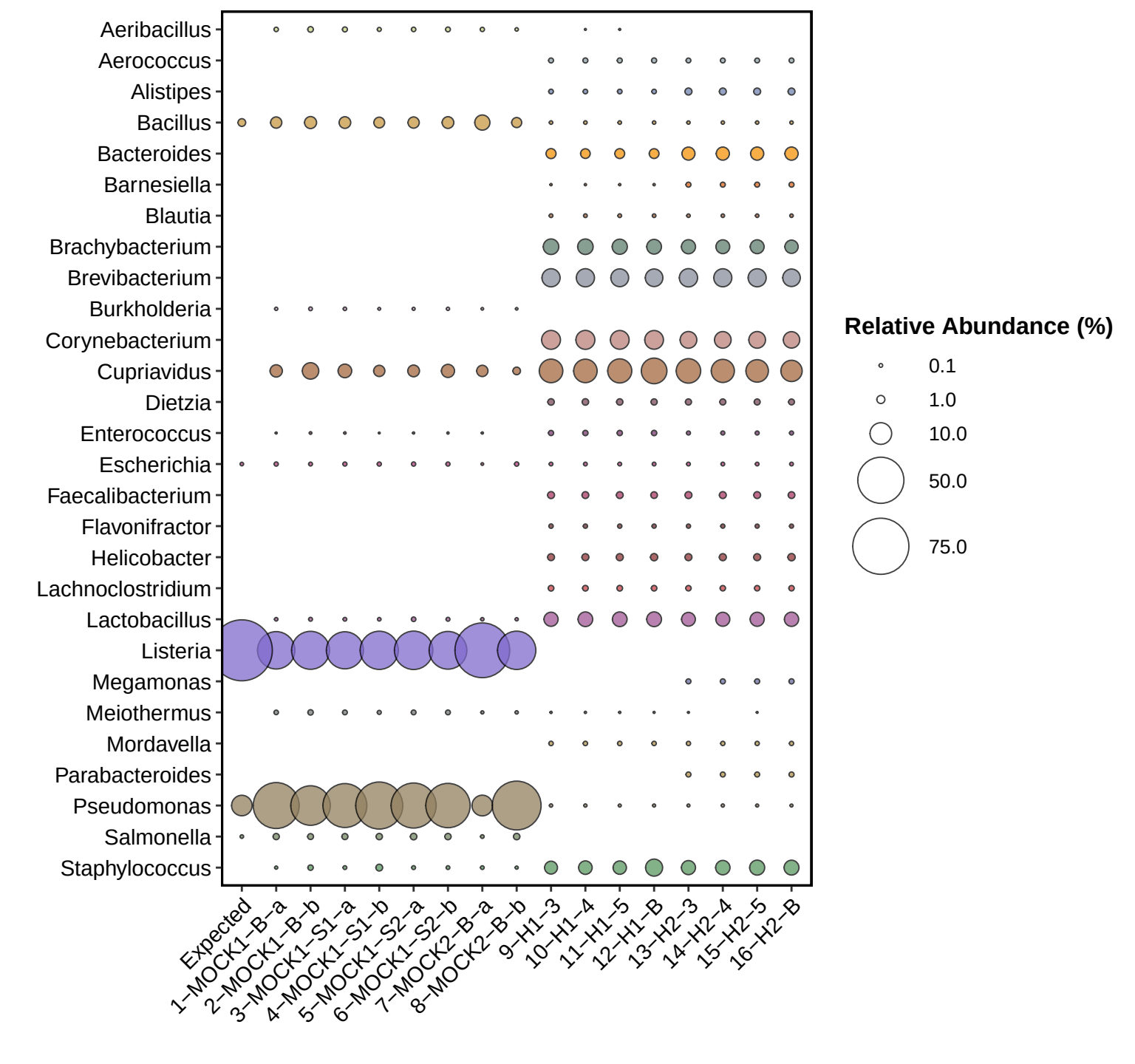
Mock communities

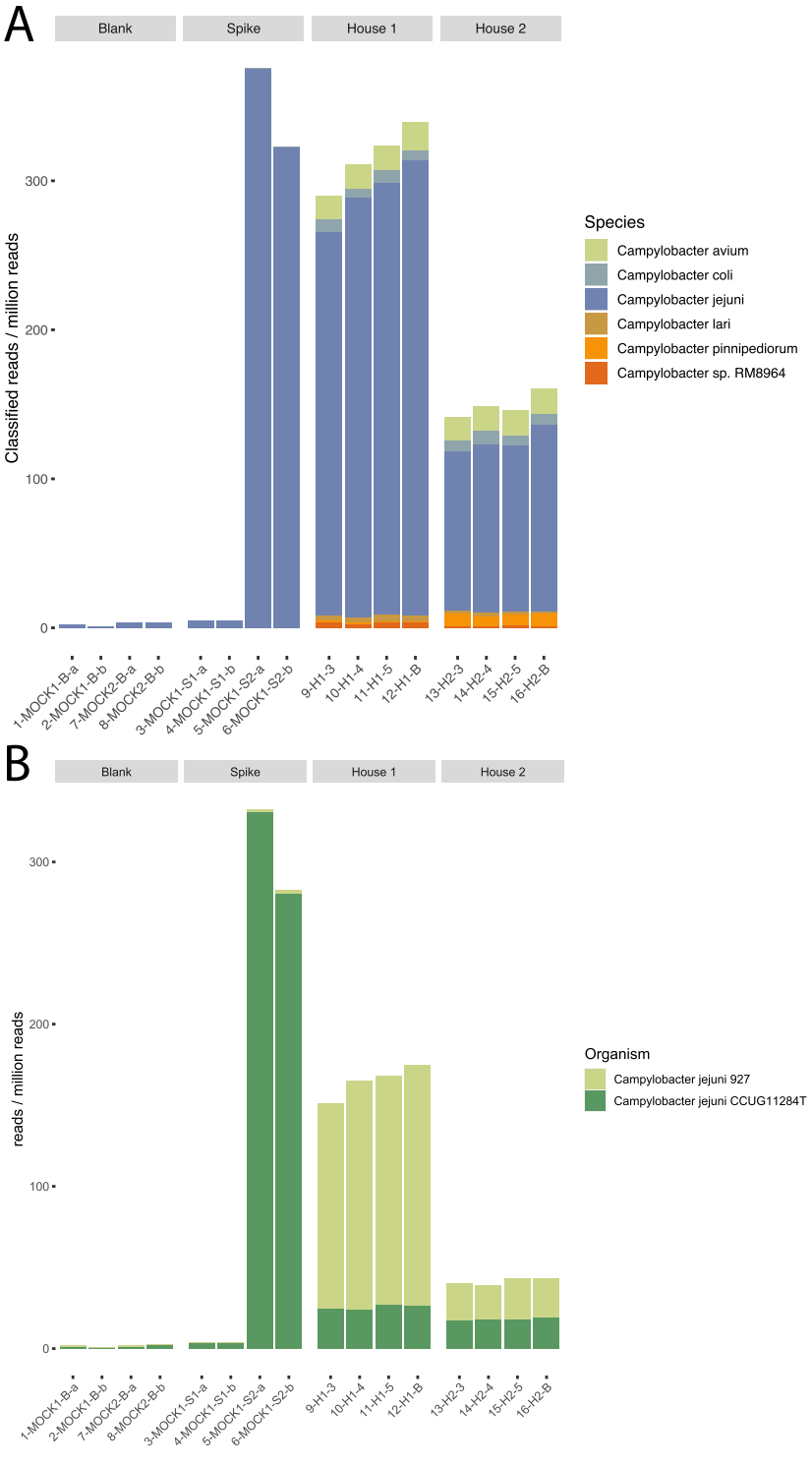


House samples



B – Blank
S1 – Sample 1 with low spiked *Campylobacter* level
S2 – Sample 2 with higher spiked *Campylobacter* level
Number – *Campylobacter* CFU amount added to filter.


Aeribacillus
Aerococcus
Alistipes
Bacillus
Bacteroides
Barnesiella
Blautia
Brachybacterium
Brevibacterium
Burkholderia
Corynebacterium
Cupriavidus
Dietzia
Enterococcus
Escherichia
Faecalibacterium
Flavonifractor
Helicobacter
Lachnoclostridium
Lactobacillus
Listeria
Megamonas
Meiothermus
Mordavella
Parabacteroides
Pseudomonas
Salmonella
Staphylococcus
Expected
1−MOCK1−B−a
2−MOCK1−B−b
3−MOCK1−S1−a
4−MOCK1−S1−b
5−MOCK1−S2−a
6−MOCK1−S2−b
7−MOCK2−B−a
8−MOCK2−B−b
9−H1−3
10−H1−4
11−H1−5
12−H1−B
13−H2−3
14−H2−4
15−H2−5
16−H2−B
Relative Abundance (%)
0.1
1.0
10.0
50.0
75.0


A
Blank
Spike
House 1
House 2
Classified reads / million reads
300
200
100
0
1-MOCK1-B-a
2-MOCK1-B-b
7-MOCK2-B-a
8-MOCK2-B-b
3-MOCK1-S1-a
4-MOCK1-S1-b
5-MOCK1-S2-a
6-MOCK1-S2-b
9-H1-3
10-H1-4
11-H1-5
12-H1-B
13-H2-3
14-H2-4
15-H2-5
16-H2-B
Species
Campylobacter avium
Campylobacter coli
Campylobacter jejuni
Campylobacter lari
Campylobacter pinnipediorum
Campylobacter sp. RM8964
B
Blank
Spike
House 1
House 2
reads / million reads
300
200
100
0
1-MOCK1-B-a
2-MOCK1-B-b
7-MOCK2-B-a
8-MOCK2-B-b
3-MOCK1-S1-a
4-MOCK1-S1-b
5-MOCK1-S2-a
6-MOCK1-S2-b
9-H1-3
10-H1-4
11-H1-5
12-H1-B
13-H2-3
14-H2-4
15-H2-5
16-H2-B
Organism
Campylobacter jejuni 927
Campylobacter jejuni CCUG11284T