

Article

## Effect of Different Feed Structures and Bedding on the Horizontal Spread of *Campylobacter jejuni* within Broiler Flocks

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**Abstract:** In this study, we investigated the effects of different feed structures and beddings on the spread of *C. jejuni* in broiler flocks, and the effect on the cecal microbiota. Broiler chickens raised in 24 eight-bird group cages on either rubber mat or wood shavings were fed either a wheat-based control diet (Control), a diet where 50% of the ground wheat was replaced by whole wheat prior to pelleting (Wheat), or a wheat-based diet, such as the control diet diluted with 12% oat hulls (Oat). Samples from the cloacal mucosa of all birds were taken daily for *C. jejuni* quantification and cecum samples were collected at the end of the experiment for *C. jejuni* quantification and microbiota analyses. We have shown a statistically significant effect of increased feed structure on the reduced spread of *C. jejuni* in chicken flocks, but no significant differences were detected between types of structure included in the feed. No significant changes in the dominating microbiota in the lower lower gastrointestinal (GI) tract were observed, which indicates that feed structure only has an effect on the upper GI tract. Delaying the spread of *C. jejuni* in broiler flocks could, at time of slaughter, result in fewer *C. jejuni*-positive broilers.

**Keywords:** *Campylobacter jejuni*; poultry; whole wheat; oat hulls; litter; microbiota

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## 1. Introduction

*Campylobacter* spp. is a leading cause of bacterial food-borne gastroenteritis in humans in the developed world [1,2]. Most cases of campylobacteriosis are caused by *Campylobacter jejuni* [3,4]. *C. jejuni* is zoonotic, with a low infection dose needed for disease, and poultry is an important source for human infections [4–7]. *C. jejuni* spreads rapidly within broiler flocks through horizontal transmission. The prevalence within the flock may increase from <5% to >95% in a week [8–10]. The principal site of colonization is the lower gastrointestinal (GI) tract, especially in the cecum [5,11–13]. The *C. jejuni* positive broiler flocks can cause carcass contamination during slaughter [14,15] with a high risk of cross-contamination to other food products at the consumer level [9]. In order to reduce the colonization of *C. jejuni* in poultry, there have been major intervention efforts targeting the lower GI tract [16]. However, few efforts have been on modifying the upper GI tract in order to combat *C. jejuni* colonization in lower GI tract. The chicken has several natural barriers in the upper GI track to kill pathogens. The crop contains lactic acid bacteria [17], and the gizzard contains hydrochloric acid to aid digestion of the feed and may also have a sterilizing effect where food-pathogens might get killed time dependently in the acid environment [18]. Huang *et al.* [19] proposed that the gizzard may be a critical control point for reducing *Salmonella* contamination in growing broilers, and we have recently shown that a stimulated gizzard delays the horizontal spread of *C. jejuni* in broiler flocks [20]. Inclusions of hulls or whole cereals in feed have been shown to modify the upper GI tract of broilers, with increased gizzard weights and lower pH levels [21]. It has also been shown that birds eat or may eat litter, and that the extent to which birds eats litter is dependent on the amount of structural components in the diet [22,23]. Structural components in the diet may therefore also affect horizontal spread of pathogens through a smaller consumption of litter material.

The aim of this work was to investigate the effects of different feed structures and beddings (with or without litter) on the spread of *C. jejuni* in broiler flocks and the effect on the cecal microbiota. Our hypothesis is that modification of the upper GI tract increases the killing of pathogens entering the gizzard, without influencing the dominating microbiota in the lower digestive track. To investigate the effect on the total microbiota in the lower GI track we performed in depth 16S rRNA gene sequencing (pyrosequencing) on selected cecum samples as well as real-time PCR to quantify some well-known gut-bacteria.

## 2. Materials and Methods

### 2.1. Experimental Design

One-day-old broiler male chickens (Ross 308) were raised on commercial starter feed. At 7 days of age, the chickens were divided into three different groups of feed structure. They were given control diet (ground wheat, Control), a diet where 50% ground wheat was replaced by whole wheat (Wheat) prior to pelleting, or the control diet diluted with 12% oat hulls (Oat). All feeds were pelleted through a

3 mm pellet press, and produced at Centre for Feed Technology (Ås, Norway). Diet composition is shown in Table S1. In addition, four cages for each diet were covered by a rubber mat (non-litter floor), while the other four cages were covered with wood shavings (litter floor). Rubber mats were washed every day, by the use of a cleaning brush, to reduce the chickens' contact with manure. There were a total of six treatments with four parallels of each treatment, in total 24 cages with 8 chickens per cage, in a  $3 \times 2$  factorial experiment with diet and bedding as factors. All *in vivo* experiments were in accordance with guidelines approved by the Norwegian governmental committee for experimental animals [24].

Chickens and feed were weighed on a weekly basis during the experimental period. Analysis of variance (ANOVA) was performed using the GLM procedure of SAS 9.2 software [25] for detection of significant changes in weight gain, feed consumption, and feed/weight gain ratio.

## 2.2. Challenge Strains

*C. jejuni* strains used for infection in these experiments were strain C484 (isolated from poultry leg [26]), G109 (isolated from cecal dropping [27]), and G125 (isolated from dog feces [27]). All three strains have earlier shown to be able to colonize chickens [28]. Preparation of challenge strains and inoculation of chickens were as described by Moen *et al.* [20]. Briefly, the strains were grown micro-aerobically at 42 °C for 48 h in Mueller-Hinton broth (Oxoid Ltd., Basingstoke, UK), and then diluted into buffered peptone water (BPW) and incubated at 38 °C for 24 h. Over-night cultures of all strains were mixed 1:1:1. One chicken per cage, called the infected chicken, was inoculated orally with the *Campylobacter* mix at 32 days of age as described by Moen *et al.* [20]. The mixture of challenge strains contained approximately  $10^4$  cfu mL<sup>-1</sup>. Chickens challenged with *C. jejuni* appeared healthy and showed no signs of disease.

## 2.3. Sample Preparation

Cloacal swabs were used for pre-inoculation control of birds and for post-inoculation detection of *C. jejuni*. The cloacal mucosa of all birds in each cage was swabbed on days 1, 2, 3, 4, and 5 post-inoculation (pi) and once immediately before inoculation. Cloacal swabs were put into separate tubes with 5 mL of *Campylobacter* growth broth and incubated under micro aerobic conditions at 42 °C for 48 h. This incubation step was performed in order to increase the detection limit of *C. jejuni*. 500 mL of *Campylobacter* growth broth consisted of 475 mL Nutrient broth no. 2 (CM0067; Oxoid) supplemented with 25 mL Laked Horse Blood (SR0048C, Oxoid), one ampule of *Campylobacter* Growth Supplement (SR0232, Oxoid), and one ampule of CCDA Selective Supplement (SR0155, Oxoid).

Four random (the infected chicken not included) cecum samples per cage were collected the day of slaughter (Day 7 pi), and frozen immediately after sampling. The pH in gizzard was measured at Day 7 pi.

## 2.4. DNA Isolation

For cecum samples, swabs with cecal lumen contents were separately mixed with 1 mL of Solution 1 (25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0). For pre-inoculation control and detection of *C. jejuni* post-inoculation from cloacal swabs, 150 µL of *Campylobacter* growth broth was diluted 1:4

in 4 M guanidinium thiocyanate (GTC), and all samples were lysed by mechanical lysis (FastPrep<sup>®</sup>, Qbiogene Inc., Carlsbad, CA, USA). Isolation and purification of DNA was further performed using an automated procedure with silica particles (Bioclone Inc., San Diego, CA, USA) as described earlier by Skånseng *et al.* [28].

Bacterial cultures (see Table S2) were homogenized with use of FastPrep<sup>®</sup>, and DNA was isolated with use of DNeasy Blood & Tissue Kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's protocol. DNA from these cultures was used for testing of specificity for *Lactobacillus* spp. primers and probe designed for use in this experiment (see Section 2.7.2).

### 2.5. *C. jejuni* Detection

Quantification of *C. jejuni* was performed relative to the total microbiota [29] in cecum samples (Day 7 pi) using real-time PCR. For the cloacal swabs (after enrichment) the detection of *C. jejuni* was performed using only the *C. jejuni*-specific primer/probe set.

Universal 16S rDNA primers and probe [30] was used for quantification of the total microbiota. *C. jejuni*-specific real-time PCR was performed using the primer/probe set described by Nogva *et al.* [31]. The real-time PCR reaction mixture contained 1× Hot Start Buffer (Finnzymes Oy, Espoo, Finland), 0.5 μM ROX reference dye (Invitrogen, Carlsbad, CA, USA), and 200 μM dNTP mix. Universal 16S rDNA real-time PCR contained 0.2 μM of each primer, 0.1 μM probe, 1 U DyNAzyme<sup>™</sup> II Hot Start DNA Polymerase, and 0.5 μL DNA in a 25 μL PCR reaction. *C. jejuni*-specific real-time PCR reactions contained 0.3 μM of each primer, 0.02 μM probe, 1 U DyNAzyme<sup>™</sup> II Hot Start DNA Polymerase (Finnzymes) and 2 μL DNA in a 25 μL reaction. The amplification profile was 40 cycles of 95 °C for 30 s and 60 °C for 1 min, with an initial heating step of 94 °C for 10 min. The reactions were performed in an ABI PRISM<sup>®</sup> 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the data were analyzed using the SDS 2.2 Software [32].

In six of 24 cages, the infected chicken did not test positive for *C. jejuni*. Results from these six cages were not used in the analysis on spread of *C. jejuni*. Chi-square tests (SYSTAT 12 [33]) were performed for each day to see whether there were differences with respect to *C. jejuni*-positive and *C. jejuni*-negative birds between the feeding regimes. The *p*-values were computed for the homogeneity tests (chi-square tests). There are 3 models: first an “unfolded design” with 6 experimental levels (Control/Wood, Control/Rubber, Oat/Wood, Oat/Rubber, Wheat/Wood, Wheat/Rubber) and the 2 levels of the microbiological test (Positive/Negative) (Model 1). In the second model the results for Wood and Rubber have been summed together, leaving 3 experimental levels (Control, Oat, Wheat) (Model 2). In the third model the results for Control, Oat, and Wheat have been summed together, leaving 2 experimental levels (Wood, Rubber) (Model 3). The second and third models are conceptually related to testing the main effects in an ANOVA (the first model, however, is not related to ANOVA-interactions). All three models were analyzed separately for each day (Day 2–5 pi).

### 2.6. Typing of Colonizing *C. jejuni* Strain

Amplification of the *C. jejuni* *gltA* genes in the cecum samples from the experimental infection was performed using *glt1F* and *glt1R* [34]. The PCR amplification reactions contained 1× Hot Start Buffer (Finnzymes), 200 μM dNTP mix, 1U DyNAzyme<sup>™</sup> II Hot Start DNA Polymerase (Finnzymes),

0.2  $\mu$ M of each primer, and 1  $\mu$ L DNA in a 25  $\mu$ L reaction. The amplification profile was an initial step of 94 °C for 10 min, then 35 cycles of 94 °C for 30 s, 50 °C for 2 min, and 72 °C for 30 s, and a final extension at 72 °C for 7 min.

The PCR products were purified before sequencing, using 0.4  $\mu$ L of ExoSap-IT (USB Corp., Cleveland, OH, USA) to 5  $\mu$ L of PCR product. Thermal profile was 37 °C for 30 min and 80 °C for 15 min. The sequencing reaction contained 0.75 $\times$  BigDye<sup>®</sup> v1.1/3.1 Sequencing Buffer (Applied Biosystems, Foster City, CA, USA), 1  $\mu$ L BigDye<sup>®</sup> Terminator v1.1 Cycle Sequencing Kit, 0.25  $\mu$ M of primer glt1F, and 1  $\mu$ L of purified PCR product in a 10  $\mu$ L reaction. The sequencing reactions were carried out in 25 cycles of 96 °C for 15 s, 50 °C for 10 s, and 60 °C for 4 min. A BigDye XTerminator Purification Kit (Applied Biosystems) was used according to the manufacturer's recommendations to clean up the sequencing reactions. Sequencing was performed on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems).

The relative proportions of *C. jejuni* strains were determined by multivariate decomposition of mixed gltA gene sequence electropherograms according to the direct PLSR method, as previously described [35].

## 2.7. Microbiota Analyses in Cecum

### 2.7.1. Pyrosequencing

Two cecum samples from each cage (in total 48 samples) were submitted for pyrosequencing. Amount of purified DNA was measured by NanoDrop ND-1000 (NanoDrop Technologies Inc., Wilmington, DE, USA) and diluted to the appropriate concentration of 10–20 ng/ $\mu$ L. Two  $\mu$ L of DNA was amplified by PCR using 16S rRNA gene primers, forward primer (5'-AYTGGGYDTAAAGNG-3') and reverse primer (5'-TACNVGGGTATCTAATCC-3') (RDP (Ribosomal Database Project [36])), producing a 240 bp fragment covering the variable region V4 in 16S rRNA genes. PCR reactions were performed using 50  $\mu$ L (final volume) mixtures containing 1 $\times$  FastStart Buffer #2 (Roche Ltd., Basel, Switzerland), 0.2 mM dNTP mix, 0.4  $\mu$ M of each primer and 2.5 U FastStart HiFi Polymerase (Roche). The amplification protocol was 94 °C for 4 min, followed by 35 cycles of 94 °C for 50 s, 40 °C for 30 s and 72 °C for 1 min, and a final elongation step at 72 °C for 5 min. Purification of PCR products were performed using Agencourt AMPure PCR purification (Beckman Coulter Inc., Danvers, MA, USA). Concentrations of DNA were measured with use of Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen), and the samples were pooled before running an emulsion-based clonal amplification (emPCR amplification, Roche). All samples were run as multiplex on the same picotiter plate in the GS Junior System (Roche) using nucleotide barcodes on primers as described on the RDP website.

The output sequences and the quality score file was processed together with the mapping file using the QIIME 1.4.0 (Quantitative Insights Into Microbial Ecology) pipeline. QIIME is an open source software package for comparison and analysis of microbial communities, primarily based on high-throughput amplicon sequencing data (such as SSU rRNA) [37]. The multiplexed reads were assigned to starting samples based on their nucleotide barcode, key tag, and primers were trimmed and sequences of low quality were removed. The sequences were clustered into Operational Taxonomic Units (OTUs) based on their sequence similarity using a 97% similarity threshold. Representative

sequences for each OTU was identified and assigned to taxonomic identities using the RDP classifier. The representative sequence set was aligned using Python Nearest Alignment Space Termination (PyNAST) (default in QIIME), filtered and a phylogenetic tree and OUT table (abundance in each sample) was generated. Beta diversity (the change in species composition across geographical space) between samples were calculated by weighted (quantitative) and unweighted (qualitative) UniFrac [38,39]. UniFrac is a method to calculate a distance measure between organismal communities using phylogenetic information, and is widely used in metagenomics. The resulting distance matrices were visualized in 2-dimensional PCoA plots [40].

### 2.7.2. Real-Time Quantification of Specific Bacterial Groups

TaqMan PCR was used for detection of changes in relative amounts of specific bacteria in cecum samples (Day 7 pi). Samples were tested for *Bifidobacterium* spp. [41], *Cl. perfringens* [29], *Enterococcus* spp., and *E. coli* [42]. *Lactobacillus* spp. specific probe was designed for this study using Primer Express v3.0 based on 16S rRNA gene of lactobacilli, the FAM-TAMRA probe was (5'-FAM-CGGCTAACTACGTGCCAGCAGC-TAMRA-3'). The sequence of forward primer was (5'-AGCAGTAGGGAATCTTCCA-3' [43]), and reverse primer (5'-CAC CGC TAC ACA TGG AG-3'; [44]). Primer and probe were tested for specificity to *Lactobacillus* spp., see Table S1 in Supporting Materials for results.

All real-time PCR reactions were performed as for *C. jejuni* (see Section 2.5). ANOVA of the quantitative data for all bacterial groups tested, were performed using the GLM procedure of SAS 9.2 software [25]. Tukey's Studentized Range Test was used for grouping the treatments that were not significantly ( $\alpha = 0.05$ ) different from each other.

## 3. Results

### 3.1. Horizontal Spread of *C. jejuni* within Chicken Flocks

Increased feed structure (inclusion of oat hulls and whole wheat) delayed the spread of *C. jejuni* in broiler flocks (Tables 1 and 2). This effect was particularly strong in birds kept on rubber mats, as indicated by a significant interaction effect. At Day 3–5 pi, there was a statistically significant difference in the spread of *C. jejuni* between chickens given different feeds, with the highest spread in flocks given control diet. The effect of bedding was only statistically significant at Day 5 pi, where the spread was lower in birds on rubber mats. Only at Day 4 pi, with rubber mat as bedding, a statistically significant difference ( $p = 0.004$ ) between diets oat hulls and whole wheat was revealed by the chi-square model.

**Table 1.** Spread of *C. jejuni* in broiler flocks by time-points (Day 2–5 post-inoculation) for the different treatment groups based on cloacal swabs. The infected chicken is not included in the table.

Treatment		Cage <sup>a</sup>	Day 2 pi		Day 3 pi		Day 4 pi		Day 5 pi	
Feed	Bedding		Positive	Total	Positive	Total	Positive	Total	Positive	Total
Control	Wood	2	0	7	2	7	3	7	4	7
		4	0	7	2	7	4	7	6	7
		Sum	0	14	4	14	7	14	10	14
Oat	Wood	1	0	7	2	7	5	7	7	7
		3	0	7	1	7	2	7	4	7
		4	0	7	0	7	1	7	3	7
		Sum	0	21 <sup>a</sup>	3	21	8	21	14	21
Wheat	Wood	1	0	7	0	7	0	7	2	7
		2	0	7	0	7	2	7	5	6
		3	0	7	0	7	4	7	6	7
		Sum	0	21 <sup>a</sup>	0	21	6	21	13	20 <sup>b</sup>
Control	Rubber	1	0	7	1	7	6	7	7	7
		2	2	7	3	7	6	7	6	7
		3	0	7	1	7	2	6	2	6
		4	0	7	2	7	7	7	7	7
		Sum	2	28	7	28	21	27 <sup>b</sup>	22	27
Oat	Rubber	1	0	7	0	7	0	7	2	7
		3	0	7	0	7	0	7	0	7
		4	0	7	0	7	0	7	1	7
		Sum	0	21 <sup>a</sup>	0	21	0 <sup>c</sup>	21	3	21
Wheat	Rubber	1	0	7	0	7	3	7	4	7
		2	0	7	0	7	0	7	0	7
		3	0	7	0	7	4	7	4	7
		Sum	0	21 <sup>a</sup>	0	21	7 <sup>c</sup>	21	8	21
Total			2	126	14	126	49	125	70	124

<sup>a</sup> Originally there were 4 cages included for each combination of feed and bedding. Due to that the infected chicken in some of the cages did not test positive for *C. jejuni* after being experimentally infected, these cages were further excluded from the study; <sup>b</sup> The total number of chickens are reduced because of death of chickens in two cages; <sup>c</sup> There were found statistically significant ( $p = 0.004$ ) differences in spread of *C. jejuni* between chickens fed oat hulls and whole wheat on rubber mat at Day 4 pi.

**Table 2.** *P*-values for the effect of feed and bedding for the spread of *C. jejuni* in broiler flocks, using chi-square test.

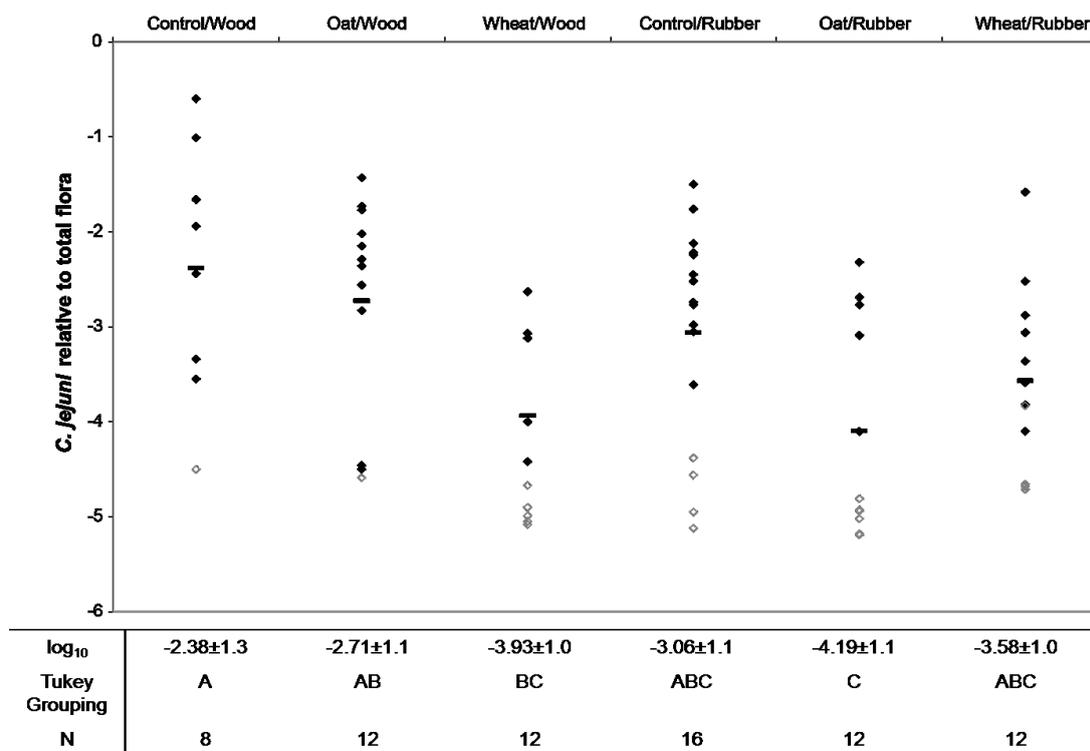
Days Post-Inoculation	<i>P</i> -values		
	Feed × Bedding <sup>a</sup>	Feed <sup>b</sup>	Bedding <sup>c</sup>
Day 2 pi	0.212	0.131	0.202
Day 3 pi	0.007	0.001	0.896
Day 4 pi	<0.001	<0.001	0.578
Day 5 pi	<0.001	0.002	0.030

The *p*-values were calculated as described in Materials and Methods using three different models: <sup>a</sup> Model 1; <sup>b</sup> Model 2 and <sup>c</sup> Model 3.

3.2. Colonization Levels of *C. jejuni* in Cecum

Colonization level of *C. jejuni* in cecum at the end of the study (Day 7 pi) is shown in Figure 1. There was a statistically significant ( $p = 0.009$ ) effect of diet (control, oat hulls and whole wheat) on the mean colonization level of *C. jejuni* relative to the total microbiota, with the highest levels in chickens given control feed. Comparing the two types of feed structure (oat hulls and whole wheat), statistically significant ( $p = 0.01$ ) differences in colonization levels were only found for chickens on wood shavings, where chickens fed oat hulls had the highest number ( $\lg (-2.71 \pm 1.1)$  relative to total microbiota). There was a lower amount of *C. jejuni* relative to total microbiota in chicken cecum in birds raised on rubber mat than on wood shavings (mean colonization level of  $\lg (-3.55 \pm 1.2)$  vs.  $\lg (-3.08 \pm 1.3)$ ,  $p = 0.03$ ).

**Figure 1.** *C. jejuni* relative to the total microbiota in cecum Day 7 pi. Mean colonization value in graph is marked with (-), samples with no detected *C. jejuni* has the detection limit marked in grey ( $C_T$ -value are set to 40 for calculation of the detection limit of the specific sample). Table below the graph shows mean colonization value with standard deviation, Tukey Grouping for treatment groups with the same letter are not significantly different, and number of samples ( $N$ ) in each treatment group. Results are based on four cecum samples per cage, and cages where the infected chicken tested negative for *C. jejuni* during the study are not included in this figure.

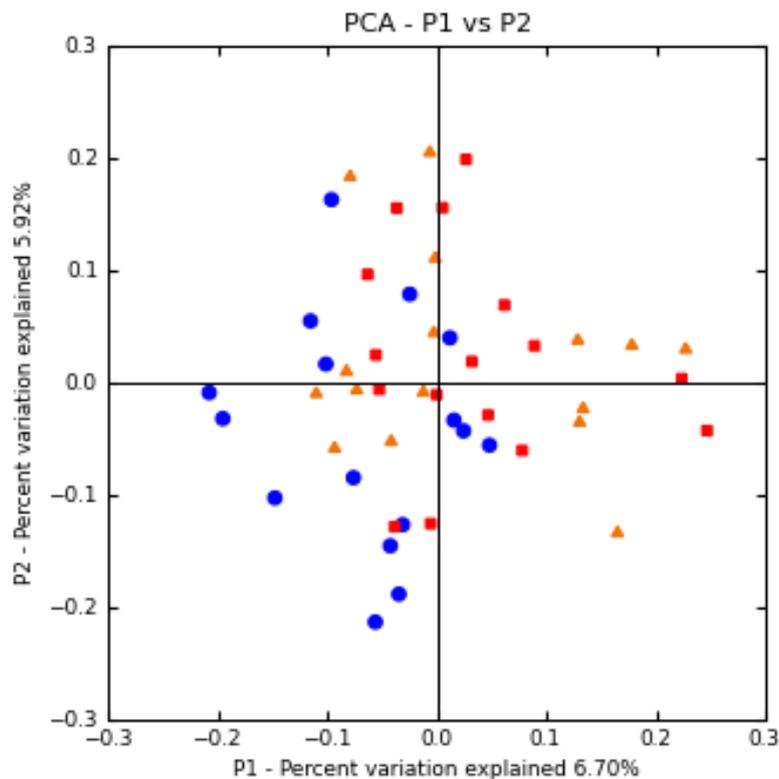


The distribution of colonizing *C. jejuni* strains in cecum of positive chickens at Day 7 pi is shown in Supplementary Figure S1. We found that *C. jejuni* strain C484 dominated the *Campylobacter* flora in cecum independent of diet and bedding, with a relative abundance of approximately 90% in all chickens.

### 3.3. Effect of Treatment on the Microbiota in Cecum

A total of 100,532 raw sequences was obtained by pyrosequencing. Filtering in QIIME resulted in 42,480 sequences that were distributed on 48 samples (number of sequences per sample ranging from 404 to 1454, with an average of 885). QIIME analyses identified 1373 OUT's, 188 OUT's were represented by more the 20 sequences across all samples. Analyzing the 188 OUT's, all samples were dominated by the order Clostridiales (class Clostridia, phylum Firmicutes) representing 86% of all sequences in the dataset (Supplementary Figure S2A). The most dominating genus was *Faecalibacterium* (order Clostridiales; Family Ruminococcaceae) represented by 47.9% of all sequences in the dataset (Supplementary Figure S2B). Analysis of beta diversity of the microbiota in cecum is shown in the unweighted PCoA plot in Figure 2, where samples are marked according to type of feed structure. There were no significant effects of different treatment groups, feed or bedding on the microbiota, analyzed by both unweighted and weighted UniFrac. Unweighted UniFrac is a qualitative measure, which compares communities due to presence or absence of organisms, while weighted UniFrac is a quantitative measure, which takes relative abundances of specific organisms into account [39].

**Figure 2.** The microbiota in cecum analyzed by pyrosequencing. PCoA plots show the  $\beta$  diversity analyzed by unweighted UniFrac. Samples are colored due to type of feed structure; red = control, blue = oat hulls, orange = whole wheat.



In-depth analyses of specific bacterial groups were performed by real-time PCR (Table 3). The results showed statistically significant lower amount of *Lactobacillus* spp. in chickens given control feed than in chickens given feed with structure ( $p = 0.001$ ); however, bedding had no effect on this bacterial group. There was a tendency of higher amounts of *Enterococcus* spp. in chickens living on wood shavings, but no effects of bedding was seen on levels of neither *E. coli* nor *Cl. perfringens*.

Diets had no significant effects on the relative levels of *Enterococcus* spp., *E. coli* and *Cl. perfringens*. However, there was observed a tendency of higher levels of *E. coli* and lower levels of *Cl. perfringens* in birds given control feed. Effect of type of feed structure (oat hulls vs. whole wheat) was found for *E. coli* on rubber mat, with a higher level of *E. coli* in oat hull fed chickens ( $\lg (-4.48 \pm 0.5)$  vs.  $\lg (-5.10 \pm 0.7)$ ,  $p = 0.01$ ). *Bifidobacterium* spp. was not detected in any of the cecal samples with use of real-time PCR or pyrosequencing.

#### 3.4. Slaughter Weight and pH in Gizzard

Slaughter weight was significantly affected by different treatments. Inclusion of oat hulls in the feed gave reduced slaughter weight (Table 4). Chickens raised on wood shavings had a slightly higher slaughter weight than chickens raised on rubber mat.

The pH measured in gizzard (Table 4) was significantly lower in both whole wheat- and oat hull-fed chickens than in chickens given control feed ( $p < 0.001$ ). Oat hull-fed chickens had the lowest measured pH-values. pH in gizzard was not influenced by the type of floor bedding.

## 4. Discussion

We have shown a statistically significant effect of increased feed structure on the reduced spread of *C. jejuni* in chicken flocks. However, the form in which the structure was presented, oat hulls or whole wheat, did not significantly alter the effect. Feed with increased structure has been shown to stimulate the gizzard [21]. Huang *et al.* [19] proposed that the gizzard may be a critical control point for reducing *Salmonella* contamination in growing broilers. They found that chickens with the largest gizzard had the lowest *Salmonella* concentrations in cecum. We have also previously shown a correlation between increased gizzard and lower relative abundance of *C. jejuni* in the cecum [20]. Bjerrum *et al.* [45] suggested that the reduction of *Salmonella* was probably due to lowered pH in gizzard and longer retention time in gizzard in chickens fed whole wheat. Svihus *et al.* [46], however, did not observe an increased retention time in the gizzard when broiler chickens were fed whole wheat. Optimum pH for *C. jejuni* growth is between 6.5 and 7.5, and the cell numbers significantly decrease when the pH is below 4.0 [47]. A reduction in pH, from approximately pH 4.0 in control chickens to below pH 3.0 in chickens fed oat hulls, would clearly have an effect on the survival of *C. jejuni* in passing through the gizzard and upper GI tract. We observed that both whole wheat and oat hull fed chickens had a reduced pH in gizzard compared to chickens fed fine diet, which is in accordance with the findings of Bjerrum *et al.* [45], and Amerah and Ravindran [48].

Litter, shown to be eaten by the birds [22,23], can be a source of insoluble fiber but could also increase the horizontal spread of pathogens. Access to litter has been shown to have an impact on the relative gizzard weight [49,50]. In addition to increased gizzard weight, Santos *et al.* [50] also observed a tendency to lower pH in gizzard for chickens raised on litter. Our results, however, did not reveal significant differences in gizzard pH between chickens raised on different beddings. The effect of bedding on spread of *C. jejuni* was only significant at Day 5 pi, when the highest number of positive chickens were found on wood shavings. Wood shavings could have an impact on the relative gizzard weight, but this type of bedding does also have a higher probability of *C. jejuni*-spread due to litter

consumption. This in fact indicates that the beneficial effect of structural components is mainly due to the sterilizing properties of a well-functioning gizzard.

The amount of *C. jejuni* in cecum was significantly lower in chickens fed whole wheat on wood shavings than in chickens given control feed. However, we could not detect significant differences between the two types of structure on the effect of spread and colonization levels of *C. jejuni* in chickens. The treatment groups with the lowest colonization levels of *C. jejuni* in cecum were the same groups that had a delay in the spread of *C. jejuni*. There had probably not been sufficient time to establish a full colonization level in the lower GI tract at the time of sampling in these chickens. A delay in the spread of *C. jejuni* can lead to a reduced level of *C. jejuni* in cecum at time of slaughter. It has been reported that a 2-log reduction in *C. jejuni* numbers on chicken carcasses can lead to a 30 times lower risk of human campylobacteriosis [51].

We also observed that only one strain, C484, was dominating the colonization of *C. jejuni* positive chickens, even though three challenge strains were used. In our previous study by Moen *et al.* [20], the same three strains were used but here the chickens were mainly colonized by strain G125 and some chickens dominated by strain C484. Studies of natural colonized chickens have shown that broiler flocks often are colonized by multiple strains [52], and that the dominating strain can change during an infection period [27], which shows the importance of using multiple strains when performing infection trials.

The effect of feed structure and bedding on the microbiota in the lower GI tract was also investigated. Pyrosequencing did not reveal any major changes in the dominating microbiota in cecum due to the inclusion of increased feed structure. This lack of changes in lower GI tract microbiota is in accordance with observations by Gabriel *et al.* [53], who found effects mainly in the upper part of the digestive tract (increased relative weights of gizzard and pancreas) and no effects on the microbial counts in cecum when feeding whole wheat. To get more information about specific bacterial groups, we chose to quantify some well-known gut bacteria [54,55], together with two human pathogens *Cl. perfringens* [56] and *C. jejuni* [5] by real-time PCR. Except from *C. jejuni*, *Lactobacillus* spp. were the only group with significant different levels between treatment groups, with the lowest relative level of this bacterium in chickens given control feed. There was a tendency of lower levels of *E. coli* and *Cl. perfringens* in chickens fed coarse feed. Others have hypothesized that chickens fed coarse feed (Brewer's spent grain) had a stronger barrier for releasing two phylogroups related to *E. coli/Shigella* and *Lactobacillus* through the digestive system than those fed fine Brewer's spent grain [57]. That study was based on direct sequencing of 16S rDNA in feces. Our study, using cecum samples and more in depth techniques, identified the opposite effect for *Lactobacillus* spp., but for *E. coli* the effect was the same (although the *p*-value was just above 0.05). Sekelja *et al.* [57], also found that the cecum/colon was dominated by a phylogroup related to unclassified *Clostridiales* and unclassified *Lachnospiraceae*. This is in accordance with our study, which identified the *Clostridiales* family dominating the ceca. *Lactobacillus* spp. and other lactic acid bacteria are usually considered to be beneficial for the host [58]. Culture-based methods have reported relatively high numbers of bifidobacteria in chicken cecum [54]. Bifidobacteria were not detected in any of the samples by use of pyrosequencing or specific real-time PCR in our study. This concurs with previous studies that DNA-based methods have reported low frequencies of bifidobacteria in chickens [59,60].

**Table 3.** Quantification of specific groups of the total microbiota in cecum at Day 7 pi, measured by real-time PCR. Mean colonization values are given relative to the total microbiota (lg) with standard deviation. Samples with target bacteria below the detection limit were given a  $C_T$ -value of 40. *P*-values are estimated for the effect of feed, bedding and feed  $\times$  bedding.

Strains	Control/Wood ( <i>n</i> = 16)	Oat/Wood ( <i>n</i> = 16)	Wheat/Wood ( <i>n</i> = 16)	Control/Rubber ( <i>n</i> = 16)	Oat/Rubber ( <i>n</i> = 15)	Wheat/Rubber ( <i>n</i> = 15)	<i>P</i> -value		
							Feed	Bedding	F $\times$ B
<i>Lactobacillus</i> spp.	-2.73 $\pm$ 0.6	-2.21 $\pm$ 0.6	-2.10 $\pm$ 0.5	-2.40 $\pm$ 0.3	-2.25 $\pm$ 0.4	-2.18 $\pm$ 0.4	0.001	0.470	0.160
<i>Enterococcus</i> spp.	-3.63 $\pm$ 0.8	-4.11 $\pm$ 0.8	-3.98 $\pm$ 0.7	-4.28 $\pm$ 0.9	-4.19 $\pm$ 1.0	-4.11 $\pm$ 0.5	0.629	0.084	0.304
<i>E. coli</i>	-4.45 $\pm$ 0.9	-4.98 $\pm$ 0.9	-4.93 $\pm$ 0.7	-4.63 $\pm$ 0.8	-4.48 $\pm$ 0.5	-5.10 $\pm$ 0.7	0.052	0.759	0.138
<i>Cl. Perfringens</i>	-4.65 $\pm$ 0.6	-4.44 $\pm$ 0.7	-4.19 $\pm$ 0.7	-4.49 $\pm$ 0.8	-4.40 $\pm$ 0.9	-4.06 $\pm$ 0.9	0.068	0.480	0.946

**Table 4.** Mean and standard deviation for the slaughter weight of chickens and pH measured in gizzard according to different treatment groups (at Day 7 pi, *n* = 16 for all treatment groups).

Gizzard Weight/pH	Control/Wood	Oat/Wood	Wheat/Wood	Control/Rubber	Oat/Rubber	Wheat/Rubber	<i>P</i> -value		
							Feed	Bedding	F $\times$ B
Weight (g)	2981 $\pm$ 172	2853 $\pm$ 197	3009 $\pm$ 202	2921 $\pm$ 221	2745 $\pm$ 191	2920 $\pm$ 234	0.002	0.043	0.893
pH in gizzard	3.89 $\pm$ 0.4	2.98 $\pm$ 0.7	3.63 $\pm$ 0.4	3.79 $\pm$ 0.3	2.98 $\pm$ 0.6	3.37 $\pm$ 0.6	<0.001	0.258	0.563

## 5. Conclusions

In this study, we have reported that the spread of *C. jejuni* is delayed due to increased feed structure, while the microbiota of the lower GI tract is mostly unchanged. There were no significant differences detected between types of structure included in the feed. These findings support our theory that the modification of the upper GI tract is essential for preventing *C. jejuni* colonization of the lower GI tract. A stimulated gizzard with a low pH will create a barrier for pathogens to reach the lower GI tract in chickens. If the pathogens manage to pass the upper GI tract, there will not be significant effect on the colonization levels of the pathogen in the lower GI tract. Our results show that increased feed structure in general is a promising intervention strategy in order to reduce the occurrence of *C. jejuni* in poultry products and to obtain safer food.

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## Conflicts of Interest

The authors declare no conflict of interest.

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### Supplementary Information

**Table S1.** Composition of experimental diets.

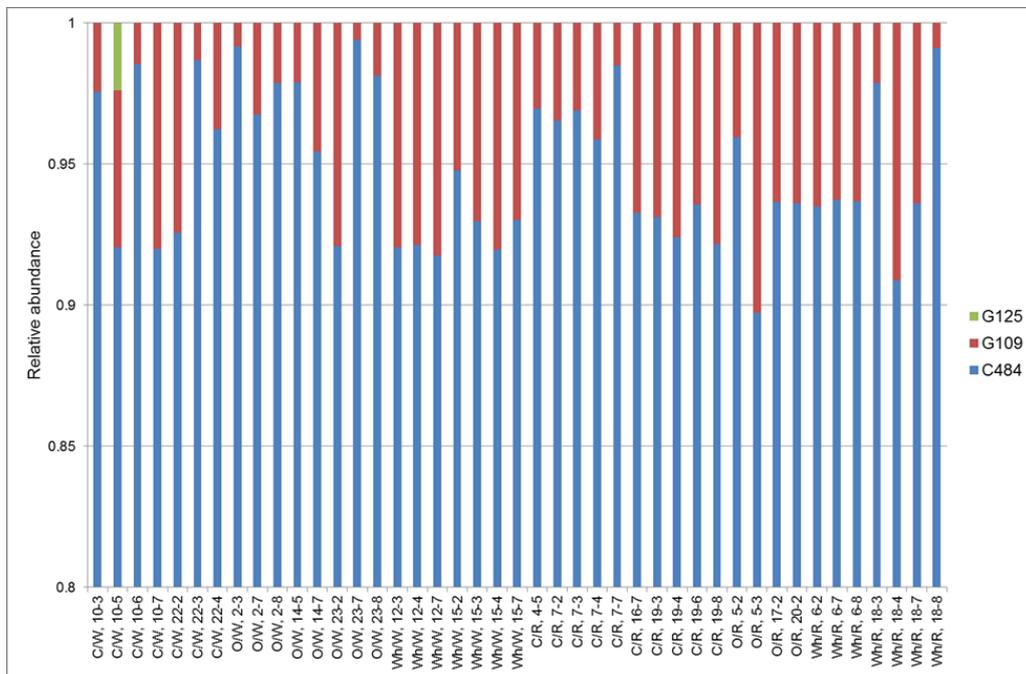
	Control/Whole Wheat Diet	Oat Hulls Diet
<b>Corn, %</b>	25.34	22.30
<b>Ground/whole wheat, %</b>	42.00	36.96
<b>Soybean meal, %</b>	17.34	15.26
<b>Fish meal, %</b>	7.00	6.16
<b>Coarse oat hulls, %</b>	0.00	12.00
<b>Soybean oil, %</b>	4.00	3.52
<b>Limestone, %</b>	1.30	1.14
<b>Monocalcium phosphate, %</b>	1.00	0.88
<b>L-lysine-HCl, %</b>	0.50	0.44
<b>DL-methionine, %</b>	0.30	0.26
<b>L-threonine, %</b>	0.10	0.09
<b>Salt, %</b>	0.20	0.18
<b>Sodium bicarbonate, %</b>	0.30	0.26
<b>Choline chlorate, %</b>	0.15	0.13
<b>Microminerals/vitamins, %</b>	0.44	0.39

**Table S2.** Bacterial strains used for validation of *Lactobacillus* spp. primers and probe.

Species	Strain <sup>a</sup>	Medium <sup>b</sup>	TaqMan PCR <sup>c</sup>	Relative to total DNA <sup>d</sup>	Std <sup>e</sup>
Positive Controls					
<i>Lb. acidophilus</i>	ATCC 4356	MRS	++	-0.65	0.03
<i>Lb. brevis</i>	DSM 20556	MRS	++	-0.61	0.09
<i>Lb. casei</i>	ATCC 393	MRS	++	-0.72	0.04
<i>Lb. curvatus</i>	DSM 20019	MRS	++	-0.67	0.11
<i>Lb. delbrueckii subsp. lactis</i>	ATCC 12315	MRS	++	-0.62	0.01
<i>Lb. gasseri</i>	ATCC 33323	MRS	++	-0.66	0.02
<i>Lb. helveticus</i>	ATCC 15009	MRS	++	-0.61	0.08
<i>Lb. pentosus</i>	ATCC 8041	MRS	++	-0.64	0.04
<i>Lb. plantarum</i>	NCIMB 8826	MRS	++	-0.73	0.07
<i>Lb. reuteri</i>	DSM 17938	MRS	++	-0.59	0.04
<i>Lb. rhamnosus (GG)</i>	ATCC 53103	MRS	++	-0.66	0.10
<i>Lb. sakei</i>	DSM 20017	MRS	++	-0.51	0.30
<i>Lb. salivarius</i>	DSM 20555	MRS	++	-0.55	0.14
Other LAB					
<i>Lactococcus cremoris</i>	ATCC 19257	MRS	-	-5.64	0.06
<i>Lc. Lactis</i>	ATCC 15346	MRS	-	-5.41	0.07
<i>Leuconostoc mesenteroides</i> <sup>f</sup>	ATCC 19255	MRS	++	-0.61	0.05
<i>Carnobacterium divergens</i> <sup>f</sup>	NCDO 2306	MRS	+	-3.85	0.09
<i>Enterococcus faecalis</i>	DSM 12956	MRS	-	-6.04	0.02
<i>Streptococcus thermophilus</i>	MF 2403	MRS	-	-5.47	0.07
Negative controls					
<i>Bifidobacterium longum</i>	DSM 20219	W-C	-	-5.08	0.09
<i>Staphylococcus aureus</i> <sup>f</sup>	ATCC 12600	BHI	+	-3.96	0.02
<i>Bacillus cereus</i>	ATCC 4516	BHI	-	-6.61	0.08
<i>Clostridium perfringens</i>	ATCC 13124	BHI	-	-6.25	0.14
<i>Escherichia coli</i>	ATCC 47076	BHI	-	-6.86	0.13
<i>Campylobacter jejuni</i>	DSM 4688	M-H	-	-6.29	0.19

<sup>a</sup> ATCC, American Type Culture Collection, Manassas, VA, USA; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; NCIMB, National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland, UK; NCFB/NCDO, National Collection of Food Bacteria, c/o NCIMB Ltd., Aberdeen, Scotland, UK; MF, strain located at Nofima, Ås, Norway; <sup>b</sup> MRS, de man, Rogosa, Sharpe Agar (Oxoid, CM0361); W-C, Wilkins-Chalgren Anaerobe Agar (Oxoid, CM0619); BHI, Brain Heart Infusion Agar (Oxoid, CM1136); M-H, Mueller-Hinton Agar (Oxoid, CM0337); <sup>c</sup> Specificity of *Lactobacillus*-primers; positive, ++ ( $\geq -2$ ); low detection, + ( $\geq -4$ ); not detected, - ( $\leq -4$ ); <sup>d</sup> Quantified amount of *Lactobacillus* spp. relative to total microbial DNA; <sup>e</sup> Standard deviation based on three technical replicates; <sup>f</sup> Except from *Lactobacillus* spp., the primer- and probe set did also detect *Leuc. mesenteroides* and had a low detection of *Carnob. divergens* and *S. aureus*.

**Figure S1.** Quantification of the colonizing *C. jejuni* strains in chicken cecum at Day 7 pi. Y-axis starts at 0.8, and the relative abundances are given for the three *C. jejuni* strains; C484 in light greyblue, G109 in red, and G125 in green. Samples are marked with C = control, O = oat hull, Wh = whole wheat, W = wood, R = rubber. Numbers indicates cage number and chicken individual.



**Figure S2.** Relative abundance (%) of the microbiota in the chicken cecum. (A) Relative abundance of the class, the Y-axis start at 75%; (B) Relative abundance of groups under the class Clostridiales.

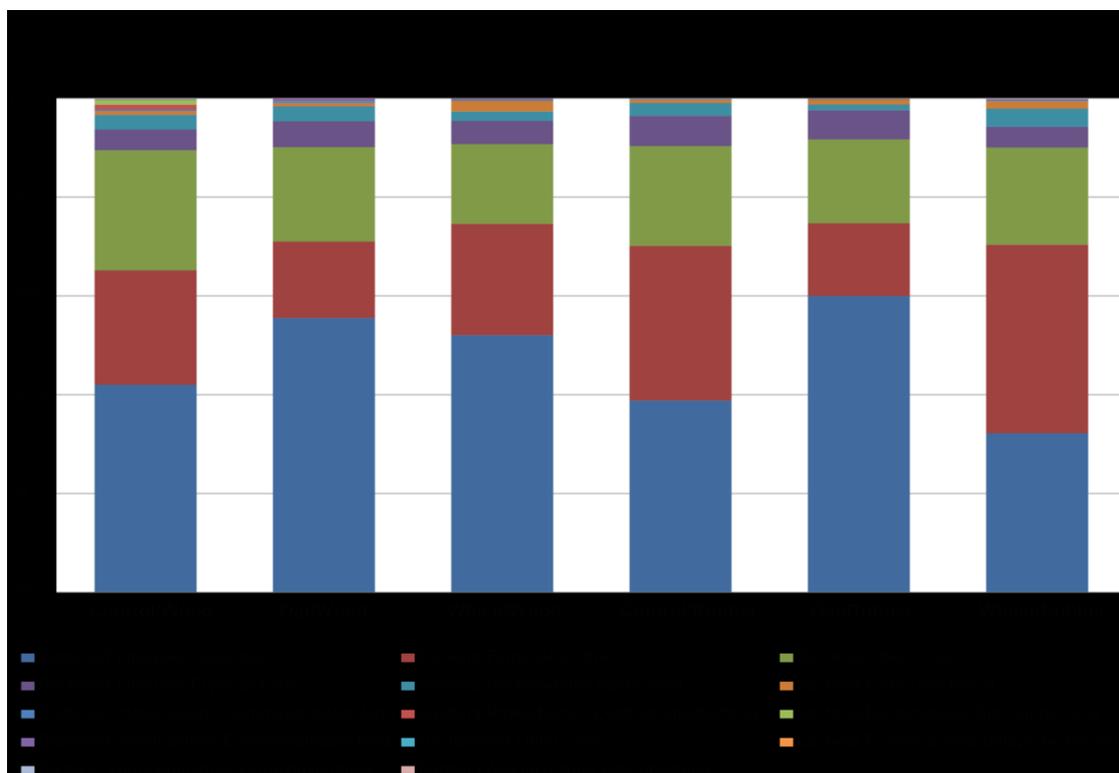
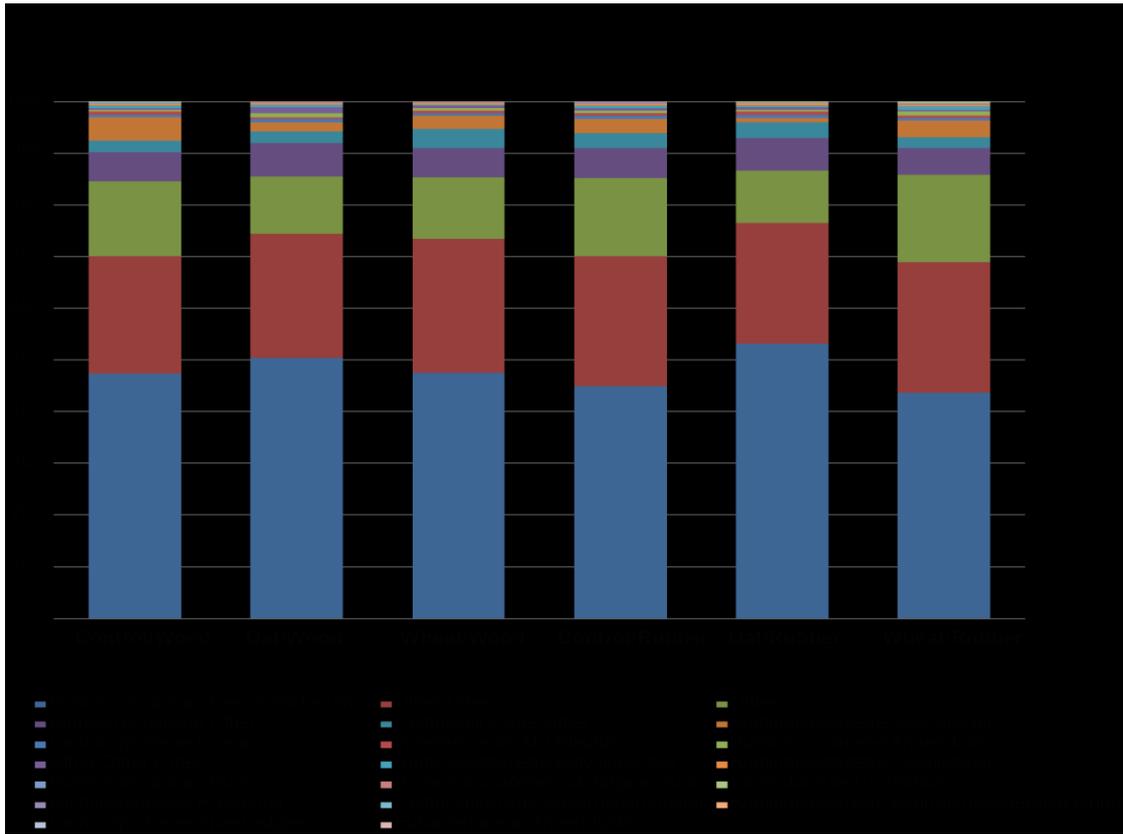


Figure S2. Cont.



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