Impact of a probiotic-based cleaning product on the microbiological profile of broiler litters and chicken caeca microbiota

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ABSTRACT This study investigated for the first time the decontamination efficacy of a probiotic-based cleaning product containing *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus megaterium* spores on fresh and reused broiler litters during 3 rearing cycles of 6 wk each. Moreover, the impact of reused litters treated with the cleaning product on the chicken caeca microbiota was assessed at the end of the rearing cycles in comparison to untreated litter. The *Bacillus* spores provided with the cleaning treatment were able to successfully colonize the reused poultry litters, decreasing the mean counts of total aerobic bacteria, Enterobacteriaceae, and coagulase positive *Staphylococci*. The decrease of Enterobacteriaceae, mainly represented by the genus *Escherichia*, was also observed in the caeca of broilers reared on reused litters treated with the cleaning product. Moreover, the treatment retained the caeca content of Ruminococcaceae and *Faecalibacterium* as well as the level of biodiversity among the bacteria genera colonizing the caeca of animals reared on reused litter. Overall, the results of this study highlight a positive effect of the probiotic-based cleaning strategy on the microbial decontamination of reused litters and on broiler caeca stability, thereby enhancing animal health and prevention of poultry diseases.

Key words: *Bacillus*, probiotics, litter, broilers, microbiota

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INTRODUCTION

Meat chickens are commonly raised in open-style houses with a litter covered floor. Litter is composed of the basic substrate (wood shavings, sawdust, straw, rice husk, sand or other alternative materials) with the addition of excreta, feathers, wasted feed and water, and other residues that continually accumulate throughout the broiler rearing cycle (Torok et al., 2009; Ritz et al., 2014). Litter is used to provide a cushioning and insulating barrier between the birds and the ground (Dunlop et al., 2015). It needs to absorb moisture, dry readily and allows birds to display natural behaviour, such as scratching and dust-bathing (Shepherd and Fairchild, 2010; Collett, 2012).

According to the European rules for the protection of broiler chickens, the broiler litter should be changed for each flock (Council of the European Union, 2007). However, the reuse of broiler litter is a common practice in the Brazilian and US poultry industry for flocks of healthy chickens primarily because it lowers production costs and enhances environmental sustainability. In terms of litter volume produced, between 1.0 and 1.6 tons are generated for every 1,000 broilers (Coufal et al., 2006). Thaxton et al. (2003) have observed that the practice of reusing caked litter from the broiler house has become a very common practice in the North American poultry industry. Wang et al. (2016) reported that in the US, broiler chicken litter (primarily pine shavings) is commonly reused for six or more consecutive growth cycles before a thorough cleanout. This practice reduces the cost of purchasing fresh litter material and disposal of reused litter (Coufal et al., 2006). In Europe, poultry manure has long been recognized as the most desirable natural fertilizer because of its high nitrogen content (Delgado et al., 2012). In addition, manures supply essential plant nutrients and serve as a soil amendment by adding organic matter (Cooperband, 2002).

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In order to assess a possible litter decontamination strategy to promote the option of reusing poultry litters in Europe, we examined the effects of a microbial cleaning product containing spores of *Bacillus*. Out of all known *Bacillus* spp., only a few are commonly used as probiotics in humans and animals, including *B. coagulans*, *B. clausii*, *B. cereus*, *B. subtilis*, and *B. licheniformis* (Cutting 2011; Fijan 2014). Members of the genus *Bacillus* are known to produce a number of antimicrobial compounds including lipopeptides, surfactin, bacteriocins, and bacteriocin-like inhibitory substances. These antimicrobial agents are typically active against Gram-positive bacterial pathogens, but some display activity against Gram-negative bacterial pathogens as well as fungal pathogens (Kerr 1999; Teo and Tan, 2005; Khochamit et al., 2015). The antagonistic activities of probiotics also include the production of organic acids that lower pH and exert a competitive exclusion effect on pathogens.

Based on previous results showing the ability of probiotic *Bacilli* to replace pathogens on surfaces (Vandini et al., 2014), Caselli et al. (2016a) applied a cleanser supplemented with spores of non-pathogenic probiotic *Bacilli* to hospital surfaces using a sanitation system named PCHS (Copma srl, Ferrara, Italy). This treatment demonstrated that *Bacilli* spores can germinate on dry inanimate surfaces, generating active vegetative cells capable of killing pathogens and effectively exchanging them on the treated surfaces. Interestingly, this treatment did not select for antibiotic resistant species but caused a loss of antibiotic resistance genes in the contaminating microbial population. This microbiota modulation was associated with a significant decrease in the incidence of hospital infections (Caselli et al., 2016b; Caselli et al., 2018). Based on these observations, we wanted to test the impact of a similar system on treating poultry litter. Therefore, we examined the effects of a microbial cleaning product containing spores of *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus megaterium* on mean microbial counts of fresh and reused broiler litters tested within and between three consecutive rearing cycles. Moreover, the impact of different litter ecosystems on caeca microbiota of chickens was investigated at the end of rearing on fresh or reused litters that had been treated or not with the probiotic. Currently, there have been only 2 studies that have examined the microbiota of both the broiler gastro intestinal tract and poultry litter using Sanger sequencing (Cressman et al., 2010) and pyrosequencing (Wang et al., 2016), respectively. Their focus was on the interaction between the two ecosystems.

MATERIALS AND METHODS

Cleaning Probiotic Product

The cleaning probiotic product (**PB**) used in the field trials was supplied by Copma srl, Ferrara, Italy and consists of an eco-friendly detergent solution supplemented with spores of three species of probiotic non-pathogenic bacteria, namely *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus megaterium*, at the concentration of 5×10^8 colony forming unit (**CFU**) per ml of product (Chrisal, Lommel, Belgium). A working product concentration was obtained following the manufacturer's instructions, by making a 1:10 dilution of the concentrate in water. PB was applied at an average bacterial spore concentration of 9×10^9 CFU/m^2 by direct spraying onto the litter and avoiding contact with the chickens and trying to minimize an increase of the average litter relative humidity. The relative humidity ranged from approximately 10.5% at the placement of the chickens on the litter to 36% after 24 d of rearing and 62% at the end of the rearing period (42 d) (data not shown).

Experimental Design

Three groups of 1-day-old male Ross-308 chicks each, obtained from the same breeder flock and hatching session, were reared during three consecutive rearing cycles at the experimental broiler housing facility located at the University of Bologna, resulting in nine broiler groups tested. The trials were authorized by the ethical committee of the University of Bologna with protocol number 74,816. The 3 groups reared in each cycle were housed in three separate rooms. Each room, labelled as A, B, and C, housed 180 birds within three 5.5 m^2 pens (60 birds per pen) resulting in a stocking density of 10 chicks/m^2. Pens were equipped with pan feeders, to assure at least 2 cm/bird of front space and an independent drinking system containing 1 nipple/5 birds. Feed and water were provided *ad libitum*. Photoperiod and temperature programs were set up according to the European welfare regulation 43/2007. All housed chicks were vaccinated against infectious bronchitis virus, Marek's disease virus, Newcastle and Gumboro diseases and coccidiosis at the hatchery.

Each rearing cycle lasted 6 wk. At the beginning of the first cycle all rooms (A, B, and C) contained fresh litter. During the first rearing cycle the litter in room A was not treated; the litter in room B was treated with saline (placebo) (0.9% sodium chloride in sterile water, pH 7.0) by direct spraying onto the litter; and the litter in room C was treated by direct spraying with the PB product (Table 1). The treatments with the saline and PB were applied to the litter of each pen twice a week, the day before the litter was sampled (Monday and Wednesday). In the second cycle, the litter of room A was replaced with fresh litter, while the litters of both rooms B and C were reused from the first rearing cycle and supplemented with PB twice a week as described above (Table 1). Finally, in the third cycle the litter of room A was reused from the previous cycle, while those of rooms B and C were reused for a second time and each treated with PB twice a week, as previously described.

Table 1. Experimental design. Trials were performed within separate rooms (A, B, C) in 3 consecutive rearing cycles. The litter used in each cycle was either fresh or reused from the previous group and treated or not treated with the probiotic product (PB) or saline solution (0.85% NaCl in sterile water) (S).

	Room A (group label)	Room B (group label)	Room C (group label)
Cycle 1	Fresh litter (A1)	Fresh litter + S (B1)	Fresh litter + PB (C1)
Cycle 2	Fresh litter (A2)	Reused litter + PB (B2)	Reused litter + PB (C2)
Cycle 3	Reused litter (A3)	Reused litter + PB (B3)	Reused litter + PB (C3)

Microbiological Analysis of the Litter

The litter of each tested group was sampled twice a week on Tuesday and Thursday for 6 consecutive weeks. Three composite samples of 25 gr of litter were collected from each room at each sampling time. The composite samples were obtained from five different areas across a single pen. The 5 areas tested were the same for all samplings and included litter from the 4 corners of each pen and from the centre of the pen. Each composite sample was divided into 2 aliquots of 10 g each. One sample aliquot was serially diluted in 90 ml of physiological saline solution and then the suspension further serially diluted in physiological saline solution to enumerate total aerobic bacteria, Enterobacteriaceae, and coagulase-positive *Staphylococci*. Total aerobic bacteria were enumerated on Plate Count Agar (Oxoid, Milan, Italy), according to the ISO method 4833–1. Enterobacteriaceae were enumerated on Violet Red Bile Glucose Agar (VRBGA) (Oxoid), according to the ISO method ISO 21,528–2:2004. Coagulase-positive *Staphylococci* were enumerated on Baird-Parker Agar supplemented with egg yolk tellurite emulsion (BPA) (Oxoid), according to ISO method 6888–1:1999. Moreover, 5 ml of the suspension were treated at 80°C for 30 min, immediately chilled and serially diluted in 9 ml of physiological solution for the enumeration of *Bacillus* spp. on Polymyxin Pyruvate Egg Yolk Bromothymol Blue Agar (PEMBA). All plates were incubated at 37°C for 24 to 48 h and the number of CFU enumerated multiplying the number of colonies detected by the dilution factor and the population expressed as the number of CFU per gram of litter composite sample. The second aliquot of composite sample was diluted 1:10 in a potassium chloride solution (0.1 mol/l) to measure the litter pH according to the ISO method 2917:1999 using a Crison 507 pH meter. All listed microbiological analyses were conducted on litter samples collected from all treatment groups with the exception of the coagulase-positive *Staphylococci* which were only tested in the litter samples collected from the second and third cycles.

Molecular Analysis

Bacillus spp presence and concentration in the treated and untreated litter were also evaluated by real time quantitative PCR (**qPCR**) which allowed for the evaluation of both spores and vegetative cells. Briefly, 1 ml of litter homogenate was centrifuged at 6500 x g at 4°C for 20 min to concentrate the cells and then the pellet stored at −80°C until further testing. Total DNA was extracted from the pelleted cells by the UCP-Pathogen mini kit (Qiagen, Germany) and analysed by two different qPCRs, as previously described (Caselli et al., 2016a). A *panB* qPCR served to quantify the total amount on bacterial DNA, whereas *spo0A* qPCR detected and quantitated only the fraction of *Bacillus* bacteria (Caselli et al., 2016a).

Sequencing Analysis

To characterize the impact of each treatment on broiler caeca microbiota, at the slaughterhouse nine chickens per room were randomly selected among those processed at the end of the second and third cycle. The entire gastrointestinal tract of each individual selected bird was dissected and a small portion (i.e., 0.5 to 2 g) of caecum content was collected from both caeca and transferred to 2 ml sterile plastic tubes. The 54 collected samples were flash frozen in liquid nitrogen, transported to the laboratory and then stored at −80°C until DNA extraction. The DNA was extracted from each sample as previously described (De Cesare et al., 2017).

The libraries were prepared following the Illumina 16S Library preparation protocol, amplifying the variable V3 and V4 regions of the 16S rRNA in order to obtain a single amplicon of approximately 460 bp. Sequencing was performed in paired-end in the Illumina MiSeq with the MiSeq Reagent kit v2 500 cycles, characterised by a maximum output of 8.5 Gb. All sequences were analyzed using MG- RAST (http://metagenomics.anl.gov/). After applying the quality control procedure described in the instructions of the MG-RAST manual, the taxonomic classification of the sequencing data was performed by applying the Best Hit Classification method and using the M5RNA database. All metagenomes deposited in MG-RAST are public under the project labelled as HYCHIFA and detailed in Supplementary Table S1.

Statistical Analysis

The microbiological counts were analyzed with the Statgraphics package (version 5.1). Arithmetic counts of CFU/g were transformed into $\log_{10}$ values and analyzed with the ANOVA procedure using a bifactorial

Table 2. *Bacillus* spp. counts in litter samples (PEMBA medium). Values are expressed as mean ± standard deviation of $\log_{10}$ CFU/g. Statistically different counts are indicated by different letters within the same column ($P \leq 0.01$).

	Mean ± standard deviation ($\log_{10}$ CFU/g)						
Group	At placement	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
A1	5.83 ± 0.12^{b}	$5.98 \pm 0.17^{b,c}$	5.72 ± 0.18^{c}	5.77 ± 0.24^{c}	5.65 ± 0.17^{b}	5.45 ± 0.13^{c}	5.41 ± 0.12^{b}
B1	5.78 ± 0.20^{b}	6.04 ± 0.20^{c}	$5.47 \pm 0.29^{b,c}$	5.78 ± 0.20^{c}	5.7 ± 0.12^{b}	5.39 ± 0.39^{c}	5.22 ± 0.27^{b}
C1	5.64 ± 0.09^{b}	$5.86 \pm 0.28^{b,c}$	5.76 ± 0.22^{c}	5.86 ± 0.07^{c}	5.78 ± 0.11^{b}	5.57 ± 0.25^{c}	5.28 ± 0.24^{b}
A2	$5.34 \pm 0.28^{a,b}$	$5.40 \pm 0.19^{b,c}$	5.01 ± 0.17^{b}	$5.06 \pm 0.18^{a,b}$	4.87 ± 0.20^{a}	$4.64 \pm 0.24^{a,b}$	4.54 ± 0.16^{a}
B2	$5.14 \pm 0.30^{a,b}$	$5.35 \pm 0.20^{b,c}$	$5.38 \pm 0.11^{b,c}$	$5.49 \pm 0.13^{b,c}$	5.43 ± 0.21^{b}	$5.24 \pm 0.15^{b,c}$	5.35 ± 0.26^{b}
C2	$5.17 \pm 0.12^{a,b}$	5.32 ± 0.17^{b}	$5.46 \pm 0.22^{b,c}$	5.72 ± 0.10^{c}	5.56 ± 0.11^{b}	$5.26 \pm 0.17^{b,c}$	5.27 ± 0.28^{b}
A3	4.59 ± 0.23^{a}	4.52 ± 0.17^{a}	4.45 ± 0.21^{a}	4.53 ± 0.18^{a}	4.54 ± 0.18^{a}	4.23 ± 0.21^{a}	4.47 ± 0.36^{a}
B3	$5.45 \pm 0.25^{a,b}$	$5.63 \pm 0.29^{b,c}$	$5.24 \pm 0.11^{b,c}$	$5.40 \pm 0.26^{b,c}$	5.66 ± 0.13^{b}	5.46 ± 0.15^{c}	5.43 ± 0.17^{b}
C3	$5.37 \pm 0.08^{a,b}$	$5.50 \pm 0.13^{b,c}$	$5.39 \pm 0.29^{b,c}$	$5.42 \pm 0.13^{b,c}$	5.76 ± 0.07^{b}	5.36 ± 0.26^{c}	5.51 ± 0.17^{b}

Table 3. Total aerobic microbial contamination in litter. Values are expressed as mean ± standard deviation of $\log_{10}$ CFU/g. Statistically different counts are indicated by different letters within the same column ($P \leq 0.01$).

	Mean ± standard deviation ($\log_{10}$ CFU/g)						
Group	At placement	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
A1	6.44 ± 0.16^{a}	7.62 ± 0.36^{a}	8.64 ± 0.38	9.40 ± 0.24	$9.65 \pm 0.18^{a,b}$	$9.49 \pm 0.13^{a,b}$	9.86 ± 0.35^{d}
B1	6.18 ± 0.06^{a}	7.86 ± 0.45^{a}	8.47 ± 0.40	9.50 ± 0.20	$9.64 \pm 0.09^{a,b}$	$9.38 \pm 0.10^{a,b}$	$9.72 \pm 0.19^{b-d}$
C1	6.12 ± 0.11^{a}	8.08 ± 0.65^{a}	8.70 ± 0.49	9.49 ± 0.13	$9.45 \pm 0.11^{a,b}$	$9.48 \pm 0.11^{a,b}$	$9.82 \pm 0.30^{c,d}$
A2	5.84 ± 0.18^{a}	8.05 ± 0.17^{a}	8.55 ± 0.38	9.42 ± 0.29	9.81 ± 0.15^{b}	9.75 ± 0.20^{b}	9.85 ± 0.20^{d}
B2	9.73 ± 0.01^{c}	9.52 ± 0.13^{b}	9.04 ± 0.22	9.48 ± 0.33	$9.23 \pm 0.30^{a,b}$	9.14 ± 0.27^{a}	$9.41 \pm 0.33^{a-d}$
C2	$9.49 \pm 0.18^{b,c}$	9.37 ± 0.17^{b}	8.98 ± 0.12	9.46 ± 0.23	$9.51 \pm 0.12^{a,b}$	$9.27 \pm 0.21^{a,b}$	$9.26 \pm 0.27^{a-c}$
A3	$9.67 \pm 0.23^{b,c}$	9.80 ± 0.14^{b}	9.49 ± 0.13	9.64 ± 0.18	$9.01 \pm 0.57^{a,b}$	$9.57 \pm 0.29^{a,b}$	9.82 ± 0.22^{c}
B3	9.04 ± 0.19^{b}	9.32 ± 0.16^{b}	8.92 ± 0.27	9.16 ± 0.15	$9.22 \pm 0.24^{a,b}$	9.13 ± 0.17^{a}	$9.16 \pm 0.15^{a,b}$
C3	$9.10 \pm 0.05^{b,c}$	9.20 ± 0.34^{b}	8.94 ± 0.16	9.06 ± 0.19	8.78 ± 0.61^{a}	9.17 ± 0.21^{a}	9.01 ± 0.13^{a}

model. The mean counts/g were calculated across the three replicates collected in each room (i.e., one for each pen). Mean counts between samplings were compared using the Scheffe's test and $P \leq 0.05$ was considered statistically significant.

The reads obtained for each sample were analysed using MG-RAST (https://www.mg-rast.org/) and the mean values for the relative abundance of each taxonomic level within each caeca were compared using the Turkey–Kramer t test found in the Statistical Analysis of Metagenomic Profile (STAMP) v 2.0.9 software. $P < 0.05$ were considered statistically significant.

Alpha and beta diversity analyses were performed using python 3.6.3. Alpha diversity was computed using an in-house pipeline that calculates the indices from the normalized read counts. Alpha diversities of different groups were compared using the Student's *t*-test. *P*-values were adjusted for multiple testing using the Benjamini–Hochberg procedure. Bray−Curtis beta diversity and Principal Coordinate analysis were computed using scikit-bio 0.4.2. Heat maps and complete linkage clustering dendrograms based on beta diversity were obtained with scipy 1.1.0.

RESULTS

Microbiological Results

The *Bacillus* mean counts enumerated on the litter samples from all tested rooms are detailed in Table 2. Overall, at the end of the rearing period, the litter samples collected in the groups where the initial litter was represented by fresh litter (group A1) or fresh litter supplemented with PB (group C1) or physiological solution (group B1) as well as litter reused, once or twice, treated with PB (groups B2, C2, B3, C3) did not show significant differences in *Bacillus* mean counts. However, those counts were significantly higher than those quantified in the litter samples collected from groups A2, where the initial litter was fresh, and A3, where the initial litter was reused. *Bacillus* presence and relative percentage in litters were also evaluated by qPCR which allows to quantify both spores and vegetative cells. Total DNA was extracted from litter samples and subjected to 2 real-time quantitative PCR analyses which yields both total bacterial DNA (*panB*) and *Bacillus* DNA (*spo0A*). The results showed that the percentage of *Bacillus* present was significantly higher in the treated litters compared to the untreated ones. In particular, *Bacillus* concentrations were maintained about 2 logs higher in PB-treated recycled litters (1.5×10^9 genomes per gram of litter, at 42 d) compared to the untreated ones (9.5×10^6 genomes per gram of litter, at 42 d). Comparing the *Bacillus* concentrations to total bacterial concentrations, PB-treated litters had an increasing percentage of *Bacillus* DNA over time, whereas in untreated litters the *Bacillus* ratio to total bacterial DNA decreased.

Table 3 summarises the total bacteria counts enumerated on the litter samples from all tested rooms. Starting from week 4 up to the end of the rearing period, the total aerobic mean counts of fresh litter samples

Table 4. Enterobacteriaceae contamination in litter. Values are expressed as mean ± standard deviation of $\log_{10}$ CFU/g. Statistically different counts are indicated by different letters within the same column ($P \leq 0.01$).

	Mean ± standard deviation ($\log_{10}$ CFU/g)						
Group	At placement	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
A1	0.00^{a}	4.26 ± 1.26	$7.22 \pm 0.54^{a,b}$	7.02 ± 0.63	7.19 ± 0.39	6.93 ± 0.33^{b}	$6.55 \pm 0.58^{b,c}$
B1	0.00^{a}	4.98 ± 0.50	$7.23 \pm 0.57^{a,b}$	7.35 ± 0.69	7.12 ± 0.60	$6.67 \pm 0.23^{a,b}$	$6.43 \pm 0.91^{a-c}$
C1	0.00^{a}	4.82 ± 1.27	7.44 ± 0.50^{b}	7.36 ± 0.23	6.75 ± 0.43	$5.88 \pm 0.48^{a,b}$	$6.59 \pm 0.33^{b,c}$
A2	0.00^{a}	6.57 ± 0.24	7.37 ± 0.37^{b}	7.05 ± 0.15	6.79 ± 0.67	$6.34 \pm 0.68^{a,b}$	$5.41 \pm 0.98^{a-c}$
B2	4.34 ± 0.27^{b}	5.79 ± 0.91	$6.02 \pm 0.58^{a,b}$	6.80 ± 0.65	5.52 ± 0.26	5.13 ± 0.85^{a}	4.84 ± 0.78a
C2	4.07 ± 0.43^{b}	5.90 ± 0.89	$5.72 \pm 0.58^{a,b}$	5.64 ± 0.70	6.00 ± 0.66	$5.47 \pm 0.51^{a,b}$	$5.01 \pm 0.88^{a,b}$
A3	4.80 ± 0.31^{b}	5.81 ± 0.69	$5.98 \pm 0.56^{a,b}$	6.35 ± 0.91	6.87 ± 0.84	$6.56 \pm 0.87^{a,b}$	6.67 ± 0.56^{c}
B3	4.63 ± 0.26^{b}	5.06 ± 0.94	5.30 ± 0.70^{a}	6.24 ± 0.52	6.68 ± 0.56	$6.20 \pm 0.50^{a,b}$	$5.93 \pm 0.19^{a-c}$
C3	3.44 ± 1.01^{b}	4.08 ± 1.88	5.51 ± 0.87^{a}	6.10 ± 0.60	6.69 ± 1.08	$6.27 \pm 0.75^{a,b}$	$5.08 \pm 0.32^{a,b}$

Table 5. Staphylococcal contamination in litter. Values are expressed as mean ± standard deviation of $\log_{10}$ CFU/g. Statistically different counts are indicated by different letters within the same column ($P \leq 0.01$).

	Mean ± standard deviation ($\log_{10}$ CFU/g)						
Group	At placement	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6
A2	4.40 ± 0.05^{a}	7.18 ± 0.78^{a}	7.95 ± 0.11^{a}	$9.19 \pm 0.36^{a,b}$	9.75 ± 0.21^{b}	9.91 ± 0.18^{c}	9.77 ± 0.21^{c}
B2	7.19 ± 0.21^{b}	8.99 ± 0.71^{b}	9.44 ± 0.23^{c}	9.55 ± 0.27^{b}	$9.36 \pm 0.36^{a,b}$	$9.38 \pm 0.27^{a-c}$	9.25 ± 0.34^{b}
C2	6.88 ± 0.17^{b}	$8.43 \pm 0.18^{a,b}$	9.41 ± 0.06^{c}	9.51 ± 0.21^{b}	$9.66 \pm 0.12^{a,b}$	$9.55 \pm 0.21^{b,c}$	9.28 ± 0.25^{b}
A3	9.64 ± 0.36^{d}	9.58 ± 0.24^{b}	9.41 ± 0.11^{c}	$9.34 \pm 0.23^{a,b}$	$9.00 \pm 0.41^{a,b}$	$9.33 \pm 0.18^{a,b}$	9.26 ± 0.08^{b}
B3	8.97 ± 0.03^{c}	9.11 ± 0.22^{b}	8.83 ± 0.26^{b}	$8.99 \pm 0.16^{a,b}$	$9.24 \pm 0.29^{a,b}$	8.91 ± 0.19^{a}	8.75 ± 0.13^{a}
C3	9.18 ± 0.04^{c}	8.94 ± 0.22^{b}	8.79 ± 0.25^{b}	8.84 ± 0.20^{a}	8.61 ± 0.77^{a}	$9.03 \pm 0.29^{a,b}$	8.66 ± 0.15^{a}

(groups A1, B1, C1, A2) were significantly higher than the groups where the initial litter was reused twice and treated with PB for 2 (group B3) or 3 cycles (group C3).

Concerning Enterobacteriaceae, at placement of the chicks they were not detected in the fresh litter samples from groups A1, B1, C1, and A2. However, they were detected in litters that were reused once or twice including those supplemented with PB (groups B2, C2, A3, B3, C3) (Table 4). Starting from week 2, the Enterobacteriaceae mean counts were nearly 2 logs lower in the litter samples that were reused twice and supplemented with PB (groups B3 and C3) compared to fresh litter samples treated (group C1) or not treated (group A2) with PB (Table 4). After 6 wk, the B2 litter samples showed the lowest Enterobacteriaceae mean count. In contrast, the litters sampled from groups A1, A3, and C1 exhibited Enterobacteriaceae mean counts nearly two logs higher than group B2 (Table 4).

Coagulase-positive *Staphylococci* were only enumerated in the litter samples collected in the second and third cycles. At the chicks placement, the fresh litter samples from group A2 exhibited a mean population of coagulase-positive *Staphylococci* significantly lower than litter samples that were either reused once and not treated with PB (group A3) or reused once or twice and treated with PB (groups B2, C2, B3, C3) (Table 5). Starting from week 4, the samples with the lowest *Staphylococci* mean counts were from group C3 (housed on litter reused twice and treated with PB for 3 consecutive cycles). This trend was repeated in the following weeks and at the end of 6 wk the samples with the lowest *Staphylococci* mean counts, ranging from 8.66 to 8.75 log10 CFU/g, were collected from groups B3 and C3. In contrast, the samples with the highest *Staphylococci* count were collected from group A2 (Table 5).

At chicks placement the pH values of all fresh litter samples (groups A1, B1, C1, A2) ranged between 5.69 and 5.94 and they were significantly lower than those of reused litters treated or not treated with PB, ranging from 7.32 to 8.99 (Supplementary Table S2). However, at the end of 6 wk the litter pH values were comparable among all groups, except for group C1, initially housed on fresh litter and treated with PB. Indeed, samples collected from C1 showed a pH mean value significantly higher than samples collected in groups A3 and B3 (8.40 vs. 7.62 and 7.64) (Supplementary Table S2).

Caeca Microbiota Results

Firmicutes was the most abundant phylum in the caeca collected from all tested groups, followed by Bacteroidetes and Proteobacteria (Supplementary Table S3). Firmicutes significantly decreased between the second and third cycle in groups A ($P < 0.000$), B ($P < 0.000$), and C ($P = 0.005$). In contrast, Bacteroidetes significantly increased in groups A ($P < 0.000$), B ($P < 0.000$), and C ($P = 0.035$). At the class level (Supplementary Table S4) in the third cycle Negativicutes was significantly higher in the caeca of birds belonging to group C in comparison to groups A and B. Moreover, between the second and third cycle, Bacteroidia, significantly increased in all groups ($P < 0.000$; $P < 0.000$; $P = 0.033$), while Clostridia significantly decreased in both groups

B ($P = 0.001$) and C ($P = 0.032$). At the order level (Supplementary Table S5), following the second cycle Lactobacillales was significantly higher in the caeca of chickens belonging to group A compared to both groups B and C, while in the third cycle this order significantly decreased in group C compared to groups A and B ($P < 0.000$). In the third cycle Selenomonadales was significantly higher in the caeca of birds belonging to group C compared to groups A and B ($P = 0.017$). Furthermore, between the second and third cycle the order Clostridiales significantly decreased in the caeca of birds belonging to groups B ($P = 0.001$) and C ($P = 0.036$). At level of the most abundant families (Supplementary Table S6) in the second cycle the family Ruminococcaceae was significantly lower in the caeca of chickens belonging to group A in comparison to groups B and C ($P = 0.003$) and the same trend was observed for Lachnospiraceae ($P = 0.047$). Concerning differences between abundances quantified in the caeca of birds belonging to the same group and tested between the second and third cycle, Ruminococcaceae significantly increased in group A ($P = 0.004$), although in the group C it significantly decreased ($P = 0.041$); Lactobacillaceae and Lachnospiraceae significantly decreased in group A ($P = 0.014$; $P < 0.000$); Bacillaceae and Eubacteriaceae significantly decreased in group B ($P = 0.033$; $P = 0.021$); Clostridiaceae significantly increased in group C ($P = 0.039$). At level of the most abundant genera (Supplementary Table S7), *Lactobacillus* in the second cycle was significantly higher in the caeca of chickens belonging to group A compared to both groups B and C ($P < 0.000$), while in the third cycle showed comparable results in the caeca of broilers belonging to groups A and B, although it was significantly lower in group C ($P = 0.024$). In the second cycle the genus *Faecalibacterium* was significantly lower in the caeca of chickens belonging to group A compared to groups B and C ($P = 0.015$) (Supplementary Table S7). On the contrary, in the third cycle it was significantly higher in the caeca of chickens belonging to group A compared to groups B and C ($P = 0.002$). In the third cycle, the genus *Clostridium* was significantly higher in the caeca of chickens belonging to group C compared to groups A and B ($P = 0.004$), while *Bacteroides* was largely lower in the caeca of chickens belonging to group C in comparison to groups A and B. The genus *Robinosniella* significantly decreased in the caeca of birds belonging to groups A ($P = 0.001$), B ($P = 0.001$) and C ($P = 0.002$) between the second and the third cycles, while *Bacteroides* significantly increased in both groups A ($P < 0.000$) and B ($P < 0.000$). Moreover, *Lactobacillus* significantly decreased in the group A ($P = 0.013$), where *Faecalibacterium* significantly increased ($P < 0.000$). Moreover, *Eubacterium* ($P = 0.021$), *Bacillus* ($P = 0.042$), and *Subdoligranulum* ($P = 0.005$) significantly decreased in the group B. Finally, *Blautia* decreased in the group C ($P = 0.003$), where *Clostridium* ($P = 0.006$) and *Roseburia* ($P = 0.001$) significantly increased.

Table 6. Mean values of the Simpson, Shannon, and Pielou indexes quantified for the genera identified in the caeca sampled at the end of rearing within groups A2 (fresh litter), A3 (reused litter), B2 (litter reused once and treated with PB for 1 cycle), B3 (litter reused twice and treated with PB for 2 cycles), C2 (litter reused once and treated with PB for 2 cycles), and C3 (litter reused twice and treated with PB for 3 cycles).

	A2	A3	B2	B3	C2	C3
Simpson	0.087	0.101	0.082	0.089	0.078	0.084
Shannon	2.974	2.817	3.045	2.651	2.403	2.600
Pielou	0.574	0.552	0.600	0.587	0.601	0.601

The alpha diversity values for the genera identified in the caeca of broilers tested in each group following the second and third rearing cycle were calculated with the Pielou, Shannon and Simpson indexes (Table 6). The results clearly show that at genus level the higher caeca biodiversity indexes were observed at the end of the second cycle (Figure 1). Overall, the alpha diversity indexes confirm that there was significantly lower biodiversity among the genera colonizing the caeca of birds belonging to group A3 reared on reused litter, in comparison to groups B2, C2, and C3 reared on reused litters treated once or twice with PB and for 3 consecutive cycles, respectively (Table 7). In regard to the beta diversity index, Figures 2 and Supplementary Figure S1 show a very good separation between the genera identified in the caeca of birds sampled at the end of the second rearing cycle (groups A2, B2, and C2) compared to those sampled at the end of the third rearing cycle (A3, B3, and C3).

DISCUSSION

Council Directive 2007/43/EC of 28 June 2007, layed down the minimum rules for the protection of chickens raised for meat production. This directive states that those parts of buildings, equipment, or utensils which are in contact with the chickens shall be thoroughly cleaned and disinfected every time after the house is depopulated and before a new flock is introduced into the house. Moreover, after the final depopulation of a house, all litter must be removed and clean litter must be added to the house. The justification for not reusing litter is based exclusively on the aspects of health and welfare of the birds (Roll et al., 2011). However, several studies have shown that the use of substances or treatment methods that promote decontamination of materials are viable alternatives to be applied in the reuse of litter for several subsequent flocks (Roll et al., 2011). Indeed, the litter reuse has been used in poultry production for many years, yielding performance results that do not differ from chickens reared on fresh litter (Roll et al., 2011).

Two recent studies have shown that reused litter can affect the immune system of chickens (Lee et al., 2011; Shanmugasundaram et al., 2012), which suggests that litter conditions can also affect the gastro-intestinal (**GI**) microbiota of chickens indirectly through their

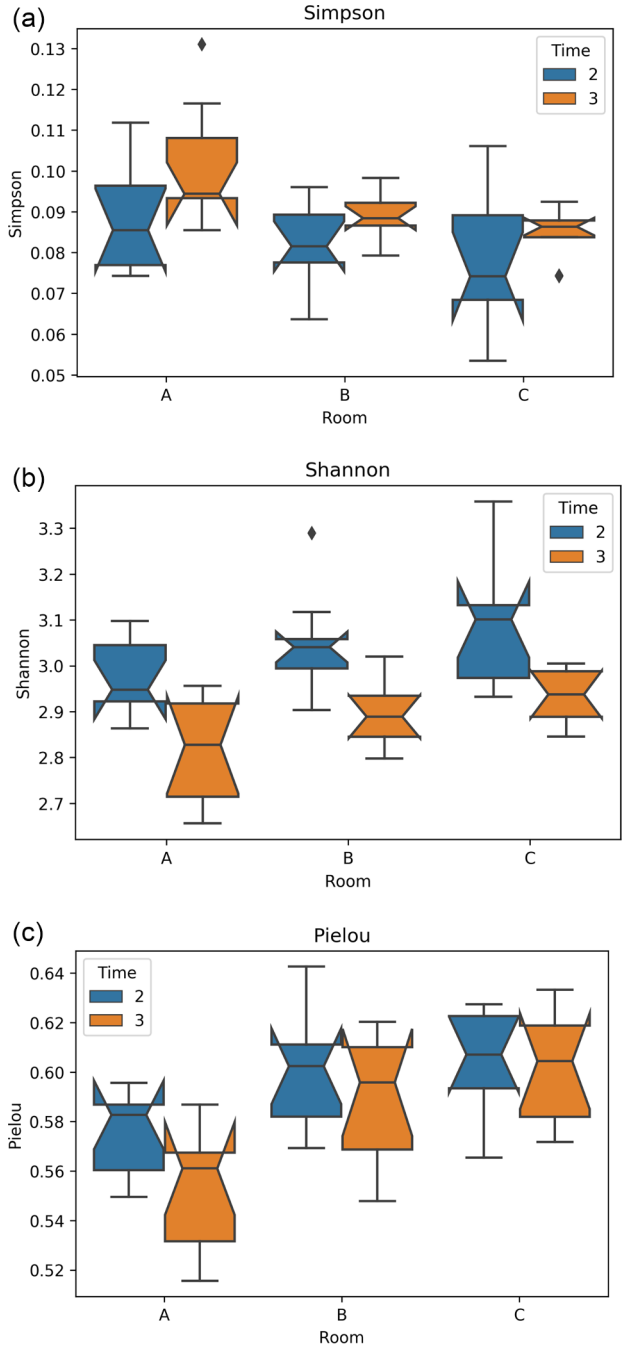

Figure 1. Box plot of the Simpson (a), Shannon (b), and Pielou (c) indexes quantified for the genera identified in the caeca sampled at the end of rearing within groups A2 (fresh litter), A3 (reused litter), B2 (litter reused once and treated with PB for 1 cycle), B3 (litter reused twice and treated with PB for 2 cycles), C2 (litter reused once and treated with PB for 2 cycles), and C3 (litter reused twice and treated with PB for 3 cycles).

immune system (Wang et al., 2016). Cressman et al. (2010) showed that the litter and GI microbiota affect each other in a reciprocal manner and that using fresh litter increase the diversity and predominance of environmental bacteria in the GI tract of young chicks, while using reused litter increases the bacteria of gut origin. Beginning from approximately day 1, chicks begin pecking at and consuming litter materials, thereby inoculating their GI tract with bacteria present in the litter. Therefore, litter can have a significant effect on the development of the GI microbiota and its composition in chickens (Torok et al., 2009; Wang et al., 2016).

Bacillus spp. have been extensively studied and developed as commercial PB for animal use but the available studies refer only to the supplementation of *Bacillus* spp. in the feed (Mingmongkolchai and Panbangred, 2018). On the contrary, in our study we investigated for the first time the impact of a cleaning product containing *Bacillus subtilis*, *Bacillus pumilus*, and *Bacillus megaterium*, labelled as PB and supplemented in the litter, on the microbial population of fresh and reused litters and on the caeca microbiota of chickens reared on *Bacillus spp.* supplemented litters. At the placement of chicks, *Bacillus* spp. was enumerated in the sample from fresh litter but it decreased during the rearing cycle. However, treatment with PB successfully supported the colonization of *Bacillus* in the litter. Thus, besides the detectable increase of *Bacillus* populations in the PB treated groups, the overall decontamination effect of the cleaning product was supported by the significant decrease of total aerobic counts detected in the litter sample groups that had received the PB treatment. A similar reduction in population was observed for the Enterobacteriaceae, including relevant zoonotic bacteria, such as *Salmonella enterica* and *Escherichia coli,* and coagulase-positive *Staphylococci.* Roll et al., 2011 verified the presence of *Salmonella* in broiler litters that had been reused up to 14 times on Brazilian poultry farms and further revealed that after the fifth reuse the incidence of *Salmonella* positive samples was much lower than expected. The findings of the present study demonstrate that the treatment with PB can enhance natural process of decreasing populations of Enterobacteriaceae, which primarily included the genus *Escherichia.*

The decrease in Enterobacteriaceae populations observed in the different litter group samples agreed with the observed reduction of this same family of bacteria in the caeca of the birds at the end of the second and third rearing cycles. Indeed, the mean relative abundance of Enterobacteriaceae decreased in the caeca of birds belonging to the groups A2 and A3 (Supplementary Table S6) and in the treated groups the reduction was much higher. It is important to highlight that different abundances were not always assessed as significant by the statistic test due to the high standard deviations associated with mean values. Such variations are attributed to the high intrinsic variability found in the caeca samples and also to the different number of reads obtained as sequencing output for each tested sample. A decreasing trend was observed for the genus *Escherichia* which was the most abundant within the family Enterobacteriaceae and which completely disappeared in the caeca of birds reared within the group C3, treated with PB for 3 consecutive cycles (Supplementary Table S7). Considering *Salmonella*, its mean relative abundance was less than 0.001% in all groups with no *Salmonella* reads detected in the caeca of broilers reared in groups

Table 7. P values calculated for the Simpson, Shannon, and Pielou indexes quantified for the genera identified in the caeca sampled at the end of rearing between groups, including groups A2 (fresh litter), A3 (reused litter), B2 (litter reused once and treated with PB for 1 cycle), B3 (litter reused twice and treated with PB for 2 cycles), C2 (litter reused once and treated with PB for 2 cycles), and C3 (litter reused twice and treated with PB for 3 cycles). Values in bold are show statistically significant difference between the groups indicated in the first line of the table.

	B3-C3	B3-A2	B3-A3	B3-B2	B3-C2	C3-A2	C3-A3	C3-B2	C3-C2	A2-A3	A2-B2	A2-C2	A3-B2	A3-C2	B2-C2
Simpson	0.192	0.816	**0.033**	0.114	0.097	0.590	**0.009**	0.508	0.299	0.053	0.344	0.241	**0.005**	**0.005**	0.602
Shannon	0.256	0.051	0.098	**0.006**	**0.003**	0.257	**0.020**	**0.020**	**0.025**	**0.005**	0.163	0.163	**0.001**	**0.000**	0.498
Pielou	0.245	0.266	**0.012**	0.301	0.257	**0.010**	**0.000**	**0.000**	0.930	**0.035**	**0.021**	**0.021**	**0.001**	**0.000**	0.940

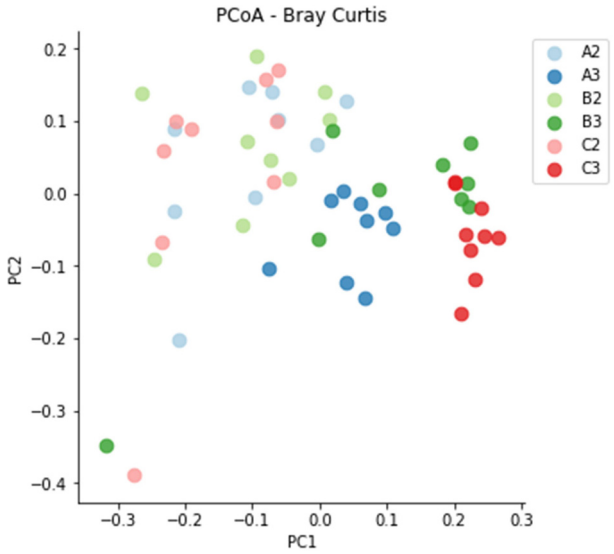

Figure 2. PCoA of beta diversity for the genera identified in the caeca sampled at the end of rearing within chickens belonging to groups A2 (fresh litter), A3 (reused litter), B2 (litter reused once and treated with PB for 1 cycle), B3 (litter reused twice and treated with PB for 2 cycles), C2 (litter reused once and treated with PB for 2 cycles), and C3 (litter reused twice and treated with PB for 3 cycles).

B2 and C3. These low levels of abundances have no biological meaning as those quantified for *Staphylococcus* lower than 0.01% in all tested groups.

In regards to the other genera, *Lactobacillus* decreased in the caeca of birds reared on reused litters and was negatively impact by treatment with PB. Indeed, at the end of the second cycle this genus was significantly higher in the caeca of chickens belonging to group A (reared on fresh litter) while at the end of the third cycle it was significantly lower ($P = 0.024$) in the caeca of broilers collected from group C, receiving three consecutive doses of PB (Supplementary Table S7). According to the results achieved by Cressman et al., 2010, Lachnospiraceae was significantly higher in the caeca of birds reared on fresh litter (group A2) ($P < 0.000$) while Ruminococcaceae was significantly higher in the caeca of birds reared on reused litter (group A3) ($P = 0.004$). Furthermore, in agreement with Lu et al. (2003) who observed an increase in *Clostridium* spp. in the broiler ileum from days 3 to 49, in our study *Clostridium* spp significantly increased between the second and the third cycles in the caeca of broilers reared on litter reused twice and treated with PB for three consecutive cycles (group C). Finally, Wang et al. (2016) investigated how litter management regimens affect the chicken GI microbiota and reported that *Faecalibacterium* was more predominant in the caecal digesta of reused-litter chickens. In our study, we observed the same finding for *Faecalibacterium*. Its relative abundance in the caeca of birds housed on fresh litter (group A2) was significantly lower than that found in the caeca of birds reared on reused litter (group A3) (9.214 vs. 17.328%, $P < 0.000$). Concerning *Faecalibacterium,* over the past 5 yr, an increasing number of studies have clearly described the importance of this highly metabolically active commensal bacterium as a component of the healthy human microbiota (Miquel et al., 2013) and changes in the abundance of *Faecalibacterium prausnitzii* have been linked to dysbiosis in several human disorders.

The alpha diversity values indicated a statistically significant lower biodiversity among the genera colonizing the caeca of birds belonging to group A3 (reared on reused litter) in comparison to groups B2, C2, and C3 which were reared on reused litters treated with PB once, twice and for 3 consecutive cycles, respectively. This finding confirms that PB support bacterial richness and diversity which is presumed to reflect caeca stability and resilience (Lozupone et al., 2012). The impact of PB was confirmed by the PCoA of beta diversity for the genera identified in the caeca of birds sampled at the end of the second and third rearing cycle which were very well separated. These results also confirm that both the reuse of litter and treatment with PB for consecutive cycles has a continuing influence on the litter microbiota which subsequently influences the bacteria genera that chickens acquire from the litter and then colonize the gastrointestinal tract up to the caeca.

The findings of this study demonstrate a positive effect of the probiotic-based cleaning product derived from the PCHS sanification system for the hospital surfaces on the decontamination of reused litters and its beneficial effect on the caeca of broilers reared on treated litters.

SUPPLEMENTARY DATA

Supplementary data are available at *Poultary Science* online.

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