# Research Note: Cecal microbiota harbored by free-range chickens may influence the reduction of *Helicobacter pullorum* relative abundance

Luca Borrelli ,[*,†,1] Lorena Varriale,[*,2] Lorena Coretti ,[†,‡] Antonino Pace ,[*] Tamara Pasqualina Russo ,[*] Antonio Santaniello ,[*] Luigi Gavazzi,[§] Alessandro Fioretti,[*] and Ludovico Dipineto[*,†]

[*]*Department of Veterinary Medicine and Animal Productions, University of Naples Federico II, Naples, Italy;* [†]*Task Force on Microbiome Studies, University of Naples Federico II, Naples, Italy;* [‡]*Department of Pharmacy, School of Medicine and Surgery, University of Naples Federico II, Naples, Italy; and* [§]*Veterinary Practice of Poultry Sector, 25100 Brescia, Italy*

**ABSTRACT** *Helicobacter pullorum* is recognized as an emerging food-borne pathogen that may colonize the intestinal tract and the liver of avian species and humans causing several gastrointestinal and liver diseases. However, not all strains are reported to be capable of causing clinical disease, thus making poultry as reservoir for the zoonotic transmission of the infection through carcass contamination of broilers at slaughter. In poultry, the prevalence of this bacterium could be underestimated and the available data mainly refer to conventional rearing systems, whereas free-range and organic breedings have been poorly investigated. Therefore, this study was aimed to characterize the caecal microbiota community of free-range grown chickens and determine the presence and the relative abundance of *H. pullorum* by using NGS-based 16S rDNA sequencing. A total of 18 chickens reared for 56 d on a semi-extensive management system were euthanized at two time points: 9 birds at 28 d of age (before have access to outdoor; I = Indoor) and other 9 birds at 56 d of age (before slaughter; O = Outdoor). Cecal contents were collected for microbiota analyses. *H. pullorum* was detected in the cecum of 16/18 samples and its proportion in indoor was significantly higher than outdoor chickens (2.46 and 0.52%, respectively; $P < 0.05$), showing 78.8% of decrease with the outdoor access of the chickens. Therefore, it may be assumed that the potential for zoonotic infection is less likely. Moreover, *H. pullorum* was negatively correlated with 17 bacterial species as significantly more abundant in Outdoor microbial caecal communities. Among these, we highlighted the presence of *Mucispirillium schaedleri* and *Oscillospira,* already previously associated with a healthy gut and thus representing promising gut bacterial markers for host health. Our findings suggest that alternative production systems with outdoor access, may play a crucial role in the establishment of a healthy gut microbiota, which in turn might prevent colonization of harmful bacteria such as *Helicobacter pullorum.*

**Key words:** *Helicobacter pullorum*, free-range chicken, ceacal microbiota, zoonoses

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## INTRODUCTION

*Helicobacter pullorum* is an enterohepatic *Helicobacter* species (**EHS**) recently recognized as an emerging human food-borne pathogen. Since the first report in 1994 by Stanley et al., *H. pullorum* has been described in poultry, turkeys, guinea fowls, psittacine birds and, more recently, rats and mice (Abd El-Ghany, 2020). This Gram-Negative bacterium has been isolated from the intestine and the hepatobiliary system of both asymptomatic poultry and the livers and cecal contents of hens with vibrionic hepatitis (Ceelen et al., 2007). Moreover, *H. pullorum* has been associated with cases of diarrhea, gastroenteritis, inflammatory bowel disease, hepatobiliary disease, and hepatic cancer in both immunocompetent and immunocompromised human patients. The zoonotic potential of *H. pullorum* emerged after its isolation from chicken meat, as surface of broiler chickens' carcasses can be contaminated with the cecal contents during slaughtering, processing and handling, thus undercooked chicken meat products may constitute a major source of infection for humans



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[2]Co-author.

[1]Corresponding author: luca.borrelli@unina.it

(Abd El-Ghany, 2020). So far, few pathogenic mechanisms and the underlying molecular determinants have been characterized, however their role in *H. pullorum* infection has not been fully understood. In vitro studies highlighted *H. pullorum* proinflammatory properties involving cytolethal distending toxin (**CDT**) and lipopolysaccharide (**LPS**) by inducing IL-8 through the NF-$\kappa\beta$ pathway of epithelial cells (Manfreda et al., 2011). Furthermore, it has been shown that *H. pullorum* infection activates the host's macrophages and secretion of cytokines (TNF-$\alpha$, IL-1$\beta$, IL-6, and murine MIP-2), as well as production of nitric oxide in murine macrophages. Recent findings also identified in *H. pullorum* a Type VI secretion system as an important virulence factor involved in the pathogenesis that may interact with endocytic vesicles and may trigger adherence to intestinal epithelial cells and invasion (Abd El-Ghany, 2020).

Despite the increasing number of reported clinical cases involving this pathogen, prevalence may have been underestimated due to several key phenotypic traits shared with *Campylobacter* spp. and the fastidious growth requirements that hamper its isolation. In poultry, variable prevalence rates have been reported from various regions with a range from 4 to 100% depending on detection methods, kind of sample, geographical area and farming practices (Abd El-Ghany, 2020). Based on the current scientific literature, the available data mainly refer to conventional housing systems, whereas free-range and organic breedings have been poorly investigated. In a study conducted in Italy by Manfreda et al. (2011), chickens reared on free-range system (i.e., 57%) showed a significant less contamination than birds reared on conventional (i.e., 84%) or organic (i.e., 97.4%) production systems, although remain unclear how these findings are related to environmental factors, host age and diet. Since farming practices, particularly antibiotic use, may affect the cecal microbiota composition of broiler chickens, this study was aimed to characterize the cecal microbiota community of free-range grown chickens and determine the presence and the relative abundance of *H. pullorum* by using NGS-based 16S rDNA sequencing in order to gain a better understanding of the influence of the gut microbiota on the carriage of this pathogen. In addition, the identification of microbial taxa that may be related to the absence or the increasing presence of this bacterium could provide new insights in the development of competitive exclusion strategies to reduce the prevalence of *H. pullorum* in the key reservoirs of this emerging food-borne disease.

## MATERIALS AND METHODS

### Ethic Statement

All chickens were treated in accordance with Directive of the European Parliament of the Council on the Protection of Animals Used for Scientific Purpose and in agreement with the Institutional Animal Care and Use Committee of the University of Naples Federico II, D.lgs n.26 04/03/2014. All experiments involving chickens were approved by the Bioethical Committee of the University of Naples Federico II, under number of protocol: 2018/0056762.

### Animals and Housing Conditions

Hubbard broiler crossbreeds (ISA 956) were provided by a certified brand breeding located southern Italy. Chickens were reared for 56 d on a semi-extensive management system (from d 29 birds had free access to outdoor areas and vegetation, seeds, fruits, soil particles, and insects become part of their diet) and slaughtered with average body weight of 2 Kg. Feed, provided ad libitum, consisted of 50 to 60% cereals and different proportions of wheat and soya according to age requirements (1−28d 5% wheat and 30% soya, 29−56d 11% wheat and 24% soya), supplemented with calcium carbonate, dicalcium phosphate, sodium chloride and sodium bicarbonate. The vaccines administered were those against Newcastle disease, infectious bronchitis, and Gumboro disease. No antibiotics were used.

### Sample Collection

During the whole production period (February −March 2019), a total of 18 chickens were randomly selected at 2 time points: 9 birds at 28 d of age (before have access to outdoor; I = Indoor) and other 9 birds at 56 d of age (before slaughter; O = Outdoor). All chickens were euthanized by cervical dislocation and dissected under sterile condition. From each carcass the ceca were tied at both ends, separated by sterile instruments from the rest of the gastrointestinal tract, placed in a sterile 15 mL falcon and stored at −80°C.

### Gut Microbiota Sequencing and Data Analysis

Bacterial genomic DNA was extracted from approximately 0.18 g of cecal content using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction, quantified with NanoDrop (ThermoFisher Scientific Inc. Waltham, MA) and checked for integrity by 0.8% agarose gel electrophoresis. DNA samples were stored at −20°C until processed for amplification. Libraries for the V3−V4 16S rDNA sequencing were prepared according to the protocol 16S Metagenomic Sequencing Library Preparation for Illumina Miseq System and sequenced as reported by Borrelli et al. (2017).

Demultiplexed sequence data were imported into QIIME2 software (v2021.4) for analysis (Bolyen et al., 2019). Reads were denoised, filtered, trimmed, and checked for chimeras with the DADA2 plugin (Callahan et al., 2016), which generated unique amplicon sequence variants (**ASVs**). Subsequently, ASVs were classified within QIIME2 using the SILVA v138 database, with a classifier trained on the amplified regions (Quast et al., 2013). Species level differences

between groups were determined by Student's *t* test with a Benjamini-Hochberg false discovery rate (**FDR**) correction to adjust *P*-values for multiple testing. Correlations between *H. pullorum* and all the other identified bacterial species were calculated by Spearman correlation analysis. Statistics, bar-plots and heatmap were obtained using GraphPad Prism version 9.3.1 for Windows, GraphPad Software, San Diego, CA, www.graphpad.com".

## RESULTS AND DISCUSSION

Eighteen samples were sequenced for microbiota data analysis, containing a total of 189,473 sequences assigned in 3,104 features, with an average of 10,526.28 sequences per sample (the lowest value observed for a sample was 5,766 sequences and the highest was 14,787 sequences). Rarefaction was conducted and showed that after subsampling each sample at 5,766 sequences a good coverage was obtained for all the samples. Most of the sequences were bacterial sequences with 103,650 sequences (99.87 %) followed by archaeal sequences with 137 sequences (0.13 %) and 1 unclassifiable sequence (0.001%).

*H. pullorum* was detected in the cecum of 16/18 samples of the chickens that were sampled. Proportion of *H. pullorum* in indoor was significantly higher than outdoor chickens (2.46% and 0.52%, respectively; $P < 0.05$), showing 78.8% of decrease at the end of chickens' outdoor access period (Figure 1A), as obtained by calculating the mean percentage of *H. pullorum* reduction in outdoor with respect to indoor group. The significant lower abundance of *H. pullorum* following the period of outdoor access, is a result of considerable interest and, at our knowledge, this is the first report documenting this finding. *Helicobacter pullorum* is recognized as a food-borne pathogen that may colonize the intestinal tract and the liver of avian species and humans causing several gastrointestinal and liver diseases. In poultry, the prevalence of this bacterium could be underestimated due to its fastidious growth requirements and the phenotypic similarity with *Campylobacter* species, in particular *C. coli* and *C. lari* (Ceelen et al., 2007). Given the paucity and the fragmentary nature of data in the scientific literature regarding the occurence of *H. pullorum* in poultry, the present study was intended to take a global picture of a semi-extensive production system assessing 2 fundamental time points of the chicken life cycle, with respect to the presence of this emerging zoonotic pathogen. We chose 28 d of age as the first time point since this stage represents the transition from indoor to outdoor, when the microbiota has reached a point of maturity, although we are aware that the microbial community development is a dynamic succession based both on age related processes and environmental factors (feed changes, immune system development, and exposure to external microbes). The second time point (56 d of age) represents the final stage of this production system, when chickens are slaughtered, allowing to assess the cecal microbiota composition after the entire outdoor access period. Although *H. pullorum* has been associated with cases of enteritis or suspected vibrionic hepatitis in poultry, not all strains are reported to be capable of causing clinical disease, thus acting as harmless flora of intestinal tract, and making poultry as reservoir for the zoonotic transmission of the infection. The mechanisms underlying this trait are not well known, however, the presence of strains with differing virulence could explain the different scenarios. Indeed, in contrast to what has been observed in *Campylobacter* species, not all the EHSs express cytolethal distending toxin (CDT) activity and genes, which is argued to play an etiological role in the development of illness signs and diarrhea (Ceelen et al., 2007). Based on these considerations, our study documented that at the end of their productive period, all birds were clinically healthy, and no illness signs, diarrhea or mortality were pointed out. Necropsy showed no signs of typhlitis, neither macroscopic lesions in the gastrointestinal tract were observed. Genomic and molecular characterization would be recommended in order to deeply understand the pathogenicity of *H. pullorum* and the most prevalent virulence profiles, as well as to elucidate its zoonotic potential. So far, it has been associated with approximately 12% of human zoonotic cases (Abd El-Ghany, 2020), due to carcass contamination of broilers at slaughter, since this agent has been detected in high numbers of cecal samples demonstrating that it is able to persist in broilers until the age of slaughter.

Therefore, it may be assumed that the potential for zoonotic infection (through broiler carcass contamination) is less likely. Moreover, *H. pullorum* was negatively correlated with 45 bacterial species, of these 22/45 showed a Spearman correlation coefficient <-0.6. Subsequently, comparison of mean relative abundance identified 17 bacterial species with relative abundance >0.5% as significantly more abundant in Outdoor microbial cecal communities (Figure 1B). These results suggest that these bacterial species may represent specific competitors of *H. pullorum* acquired when chickens are breaded outdoor.

Based on the time points addressed, the above-described stages of the entire broiler lifespan plainly documented the evolution of microbial communities as a continuous process in which new taxa replaced others after outdoor access, when grass and other environmental sources were introduced in the diet. Consequently, we could hypothesize that the higher diversity in microbial community and the expansion of other taxa associated with a healthy gut could reduce the likelihood of colonization by *H. pullorum*. Interestingly, one month after outdoor pasture, new phyla emerged including Deferribacteres, and, at species level, we highlighted the occurence of *Mucispirillium schaedleri* (0% and 1.34% in Indoor and Outdoor, respectively) This observation is in line with other studies describing the microbial community of free-range chickens. This bacterium is known to

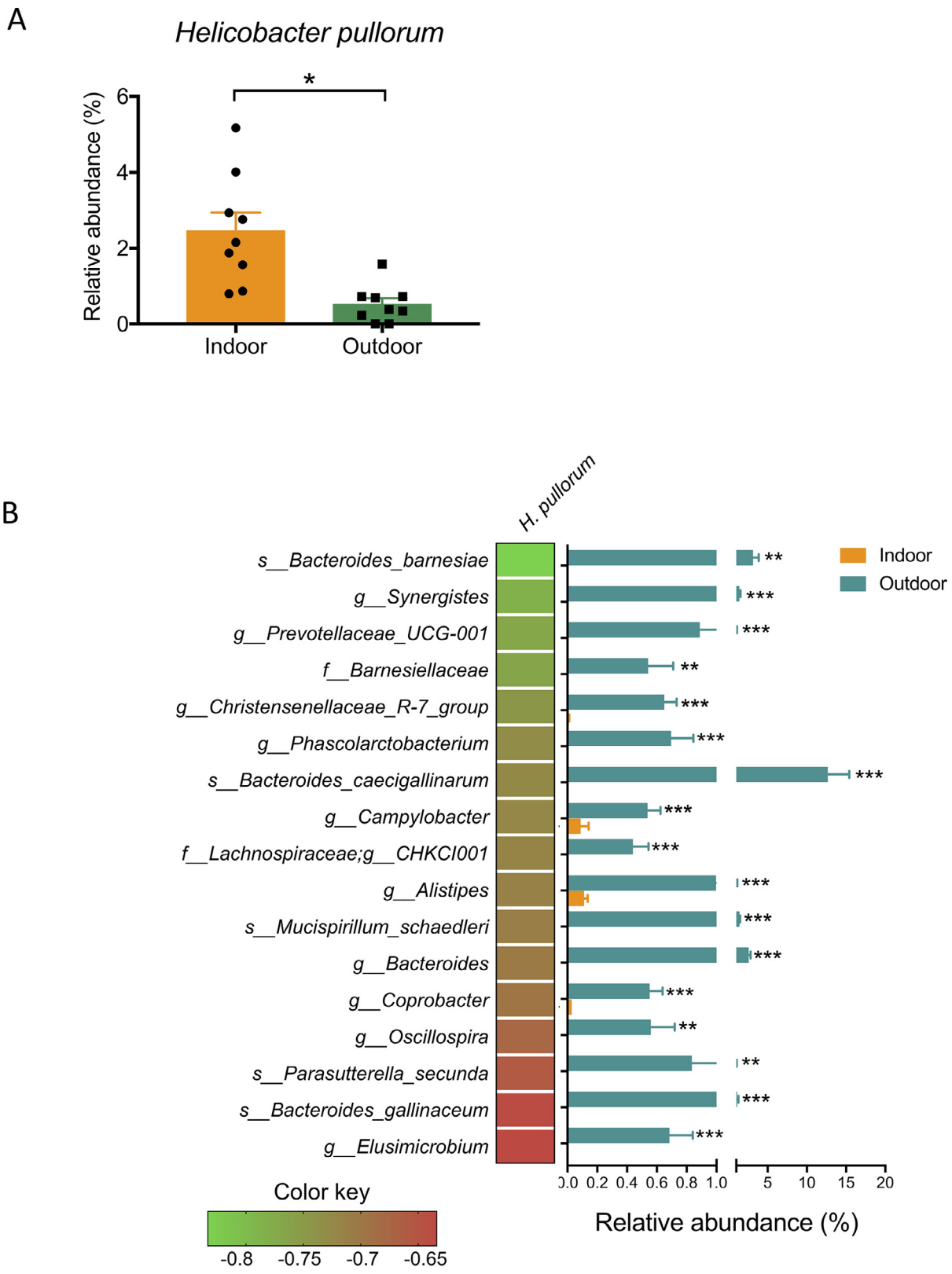


**Figure 1.** (A) Relative abundance of *H. pullorum* in Indoor and Outdoor chickens (2.46% and 0.52%, respectively; $P < 0.05$) showing 78.8% of decrease with the Outdoor access of the chickens. (B) Significant bacterial species negatively correlated with *H. pullorum* (Spearman correlation coefficient <-0.6). On the left: Heatmap showing the highest (green) and lowest (red) correlation of significant bacterial species with *H. pullorum*. On the right: mean relative abundance of significant bacterial species in Indoor and Outdoor chickens based on linear discriminant analysis (LDA) combined with effect size (LEfSe) algorithm ($P > 0.05$ for both Kruskal−Wallis and pairwise Wilcoxon tests and a cutoff value of LDA score above 2.0; only species with relative abundances >0.5% in at least one group are listed).

have a mucus associated niche in the gut and, thus, it can be considered a marker for health of the surface mucus layer in distal intestinal tract (Ocejo et al., 2019). *M. schaedleri* has also been reported to promote health in the immunocompetent host and protect from *Salmonella Typhimurium*-induced colitis in mice by interfering with the expression of the pathogen's invasion mechanism, thus having a potential for therapeutic application against human *Salmonella* infection (Herp et al., 2021).

Outdoor chicken group also showed a significant increase of *Oscillospira,* a genus considered a candidate for the next generation probiotics with great potential for health applications. *Oscillospira* plays an important role in the gut microbiota and its abundance is closely related to host health. In poultry farming, external interventions with probiotics and polyphenols, significantly influenced the abundance of *Oscillospira* in the gut, improving broilers weight gain and enhancing their gut health status. Furthermore, the abundance of *Oscillospira* significantly reduced the horizontal transmission of pathogenic *E. coli*, alleviated the severity of the infection in neonatal broiler chicken, and prevented *Clostridium diffcile*-associated infectious disease (**CDAD**) in humans, of which poultry represents a possible reservoir (Borrelli et al., 2017; Yang et al., 2021).

Chicken gut microbiota is known to play a crucial role in preserving host health and our study suggests that production system, and in particular, alternative production systems with outdoor access can be considered relevant factors for the establishment of a healthy gut microbiota. In particular, both *Mucispirillium schaedleri* and *Oscillospira* are recognized to exhibit beneficial microbial traits and might represent interesting gut bacterial markers for host health. These bacteria also might regulate the susceptibility to infectious diseases such as *Helicobacter pullorum* infection, mainly through competitive exclusion and thus preventing colonization. A healthy chicken gut microbiota may have therefore a strong influence both on host health, productivity and consequently for consumer health.

## DISCLOSURES

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

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