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Detection of *Chlamydia ibidis* in the neck skin microbiome of broiler carcasses at the end of slaughter

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Abstract
Chlamydia is the etiological agent of chlamydiosis in wild and domestic birds, mammals, and humans. In this study, *Chlamydia* reads were detected in the microbiome of the neck skin of 76 broiler carcasses collected in the same slaughterhouse at the end of the chilling tunnel. The carcasses originated from four different flocks of female Ross 308, reared in two broiler houses located in Northern Italy. One flock from each poultry house was sampled in 2019 and one flock in 2023. The carcass neck skin microbiome was investigated by shotgun metagenomic sequencing. *Chlamydia* reads displayed a mean relative abundance of 7.38%, with significant differences between carcasses obtained from the two poultry houses, sampled at both sampling times. *Chlamydia ibidis* was the prevalent species among time points and poultry houses. The zoonotic potential of *C. ibidis* and foodborne transmission have never been demonstrated. However, it is known that the genus *Chlamydia* has "spore"-like extracellular forms able to survive for months outside the host. Therefore, the presence of *C. ibidis* reads on broiler carcasses at the end of the chilling tunnel deserves further investigation. The results of this study highlight the feasibility of microbiome investigations to detect unexpected biological hazards in foods.

Introduction
Chlamydia is an obligate, intracellular Gram-negative bacterium with a unique biphasic developmental cycle. It causes avian chlamydiosis in domestic and wild birds, mammals, and humans. Among the bird's species, chicken, turkey, Pekin duck, Muscovy duck, goose, and pigeon are frequently identified as *Chlamydia*-positive (Kaleta and Taday, 2003). Most *Chlamydia* species cause respiratory diseases, which might be asymptomatic in poultry, turkeys and ducks, thus preventing the quarantine of sick animals during the *ante-mortem* inspection (De Meyst *et al*., 2024; Lee *et al*., 2023; Zaręba-Marchewka *et al*., 2020).
Transport of broilers from the farm to the slaughterhouse can act as a significant stressor that enhances pathogen shedding. This increased shedding can result in carcass contamination during slaughtering and processing, ultimately influencing meat safety (EFSA Panel on Biological Hazards, *et al*., 2022, 2021; Marmion *et al*., 2021; Park *et al*., 2023).
In humans, *Chlamydia* can cause atypical pneumonia. Different studies describe patients infected with *Chlamydia* after contact with pigeon and chicken manures (Zhang *et al*., 2025) as well as wild birds (Rehn *et al.*, 2013; Wallensten *et al*., 2014). Moreover, *Chlamydia* spp. have been reported as the cause of severe respiratory illness among poultry slaughter plant workers (Shaw *et al*., 2019).
Various studies acknowledge the complex epidemiological patterns of avian Chlamydiosis. Besides the commonly reported *Chlamydia psittaci,* other species, such as *C. gallinacea, C. ibidis, C. avium, C. abortus, C. pecorum,* and *C. trachomatis,* have been involved in the aetiology of the disease in birds and humans (Marchino *et al*., 2022; Lee *et al*., 2023; De Meyst *et al*., 2024). Stokes *et al*. (2021) reported that *Chlamydia* spp. was identified in more than 70 species of wild birds. Zoonotic transmission to humans occurs through inhalation of aerosolized fecal droppings or respiratory secretions from diseased birds or asymptomatic carriers infected with *Chlamydia* spp. (Laroucau *et al*., 2015).
The United States of America and the European Union (EU) classify *Chlamydia* related outbreaks as notifiable diseases. In 2023, in the EU, *Chlamydia* has been reported in various animal species in Austria, Denmark, and North Macedonia (EFSA, 2024). However, to date, there are no data on the presence of *Chlamydia spp.* in foods of animal origin.
This study was not initially designed to detect *Chlamydia* in broiler carcasses; rather, its primary objective was to characterize the carcass microbiome and to detect *Salmonella*. Unexpectedly, the microbiome analysis revealed a high relative abundance (RA) of *Chlamydia* reads in the neck-skin samples, as detailed in this manuscript.

Materials and Methods

Description of the study setting

This study was conducted on four batches of broiler carcasses, originating from four broiler flocks of female Ross 308 raised up to 33-35 days, in two commercial poultry houses (designated as CM and ZR) located in Northern Italy. Both poultry houses had a capacity of over 30,000 birds each. Two batches of carcasses, each originating from one poultry house, were collected at the same slaughterhouse, at the end of the chilling tunnel, between November and December 2019 (*i.e.*, T1). The other two batches were collected in the same slaughterhouse, between February and March 2023 (*i.e.*, T2).

Sample collection

A total of 76 carcasses were picked up at the end of the chilling tunnel. In particular, 15 carcasses were randomly selected from each of the two flocks slaughtered in 2019 and 23 from each of the two flocks slaughtered in 2023. Each carcass was placed in a sterile plastic bag kept at 0-4°C in a refrigerated box with an ice pack. All samples were transported to the University of Bologna and processed immediately after arrival. In the laboratory, approximately 25 g of neck skin was removed from the carcass by cutting around the furcula or wishbone alongside the keel bone by using sterile forceps and single-use scalpels, and then weighed. The neck skin was diluted 1:9 in sterile physiological solution, homogenized in the stomacher (MAYO HG 400V, Italy) at normal speed for 1 min, and centrifuged at 4°C for 20 min at 9980 x g. The obtained pellet was stored at -80°C until DNA extraction.

Microbiome analysis

For the microbiome analysis, total DNA was extracted from the pellets obtained from the neck skin by using the DNeasy PowerFood Microbial kit (Qiagen, Milan, Italy) as previously described (De Cesare *et al*., 2019). The quality and quantity of the extracted DNA were assessed using Eppendorf BioSpectrometer® Fluorescence (Eppendorf, Milan, Italy). All samples containing at least 4 ng/µL of extracted DNA, with a 260/280 nm absorbance ratio between 1.8 and 2.00 were submitted to library preparation. For the library preparation, the extracted DNA was fragmented and tagged with sequencing indexes and adapters using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA). Shotgun metagenomic sequencing was performed using the NextSeq500 (Illumina) 2 X 150bp in paired-end mode, with an average yield per sample of 9.18 GB.

Bioinformatics and biostatistics analysis

The raw data were obtained by the bcl2fastq conversion software (RRID:SCR_015058) that was adopted to perform both the demultiplexing and the adapter trimming procedures. Then, the cleaned reads were processed with *kaiju* program (Menzel *et al*., 2016) to obtain the taxonomic classification by comparing each sequence to the *nr_euk* reference protein databases, containing bacteria, archaea, viruses, fungi, and other microbial eukaryotes. The abundance of each taxon was calculated as RA. R environment was adopted for all the downstream analysis: the package rstatix to compare the RA among the group of interest and the package ggplot2 to build the barplots and boxplots. The significance of differences among groups was checked by the Kruskal-Wallis test followed by a t-test for multiple comparisons, and values of $p<0.05$ were considered significant.

Results

In this study, 76 neck skin microbiomes were characterized. Figure 1 shows the phyla identified in the neck skin microbiome of each carcass. Unexpectedly, the phylum Chlamydiota is consistently quantified among carcasses, despite differences between single carcasses of the same flock. Since in previous investigations we did not identify this phylum, we looked at the corresponding genera and species.

At the genus level, reads of *Chlamydia* showed a mean RA of 7.3803%, ranging between a minimum of 0.0520% and a maximum of 49.8637% (Figure 2). In the poultry house CM, at T1 reads of *Chlamydia* showed a mean RA of 10.2173%, ranging between a minimum of 0.9699% and a maximum of 34.9007%. In the same poultry house, at T2, reads of *Chlamydia* showed a mean RA of 11.8931%, ranging between a minimum of 3.2594% and a maximum of 22.6998% (Figure 2). In the poultry house ZM, at T1, reads of *Chlamydia* showed a mean RA of 4.8974%, ranging between a minimum of 0.0520% and a maximum of 49.8637%. In the same poultry house, at T2, reads of *Chlamydia* showed a mean RA of 2.8213%, ranging between a minimum of 0.1161% and a maximum of 16.1042% (Figure 2).
The comparison between carcasses grouped by poultry house and time points showed a significant difference in the RA of *Chlamydia* reads (p=1e-07) between the four groups. In particular, the multiple comparison analysis showed a higher abundance of *Chlamydia* reads on carcasses obtained from the poultry house CM (T1=10.2173%; T2=11.8931%) compared to ZR (T1=4.6144%; T2=2.8213%) at both time points (p=5.8e-04 and p=1.2e-07, respectively) (Figure 3).
Table 1 summarizes all the reads of *Chlamydia* species quantified in the tested carcass neck skin microbiomes. Besides very few reads of *C. psittaci, C. trachomatis*, and *C. gallinacea,* identified on carcasses from flocks reared in the poultry house CM, at both sampling times, the only species consistently identified on carcasses from both poultry houses, at both sampling times was *C. ibidis.*

Discussion

Shotgun metagenomics is a culture-independent approach with considerable potential for detecting biological hazards in foods. However, the lack of standardized wet-laboratory protocols, prior to sequencing, as well as the lack of harmonized bioinformatic pipelines for analyzing sequencing data, currently limits the full exploitation of this methodology to improve food safety. Moreover, there is a need to clearly define a threshold for sequencing reads that determines whether a biological hazard should be considered present in a food sample and possibly cause a human disease (Sala *et al*., 2020).
This study was not specifically designed to investigate *Chlamydia* but rather to screen pathogens, such as *Salmonella,* in poultry carcasses. However, during the microbiome analysis, an unexpectedly high RA of reads of *Chlamydia* was detected.
The only *Chlamydia* species quantified at RA>1% in the neck skin microbiomes from all carcasses and time points was *C. ibidis.* The quantified RA is likely to indicate the presence of live cells. However, this study was not planned to detect *Chlamydia,* and at the time when the microbiome results were available, it was not possible to verify the presence of live cells anymore. However, ad hoc samplings to detect *C. ibidis* will be organized in the near future because *C. ibidis* is considered an emerging *Chlamydia* species (Long *et al*., 2025). Such a species was detected for the first time in France in feral birds collected between March 2009 and July 2010 (Vorimore *et al*., 2015). Feral birds originated from African Sacred Ibises which escaped in 1990 from a zoological park and then colonized the wetlands of the French Atlantic coast (Vorimore *et al*., 2015).
In samples from poultry house CM, only very few reads corresponding to species associated with avian chlamydiosis (*i.e*., *C. psittaci*, *C. gallinacean*, and *C. trachomatis*) were detected. However, given their very low RA, it is highly unlikely that these reads originated from viable cells. In the literature, *C. psittaci* is primarily associated with occupational exposure and zoonotic transmission (Vorimore *et al*., 2015; Ossa-Giraldo *et al*., 2023) and with a possible human to human transmission (Dembek *et al*., 2023). The occurrence of *C. psittaci* has been reported in slaughterhouse environments and among slaughterhouse workers involved in chicken evisceration, where aerosol transmission has been implicated as a potential route for human infections (Laroucau *et al*., 2015). Kowalczyk and Wójcik-Fatla (2022) reported the presence of *Chlamydia psittaci* in fresh and dried fecal samples obtained from feral pigeons in urban areas in Poland. A recent case of a patient with atypical pneumonia disclosed the association of the Psittacosis pneumonia with a history of contact with pigeon and chicken manures (Zhang *et al*., 2025). A study in the Netherlands showed significant as-

sociation between the presence of chicken processing plants in a province and a high occurrence of human Chlamydiosis (Hogerwerf *et al*., 2017). During an outbreak of *Chlamydia psittaci* infection that occurred in poultry slaughterhouse workers in the USA, 53% of affected workers were employed at the chicken evisceration sites (Shaw *et al*., 2019). A study conducted in Belgium, to investigate the zoonotic potential of different avian *Chlamydia* spp. in chickens, demonstrated a significant correlation between the presence of *Chlamydia* DNA in humans and *Chlamydia* infections in chickens (p=0.028).

C. gallinacea is widely distributed in Europe, the USA, Australia and South America (Marchino *et al.*, 2022). In Italy has been detected since 2008 in the Piedmont and Liguria regions. Moreover, free-range chicken farms have been detected as positive by PCR in other Italian regions (Marchino *et al*., 2022). The zoonotic potential of *C. gallinacea* has been linked to atypical cases of pneumonia in French slaughterhouses (Laroucau et al., 2009). Heijne *et al*. compared the pathogenicity of *C. psittaci* and *C. gallinacea* by performing experimental infections in embryonated chicken eggs and compared the genomes of the two species. The results revealed the presence of putative virulence factors in *C. gallinacea,* also causing mortality in embryonated eggs (Heijne *et al*., 2021). Therefore, *C. gallinacea* should be regarded as an opportunistic pathogen rather than an innocuous commensal.

C. trachomatis is primarily associated with human and other mammalian hosts (Sharma and Khan, 2025). It has also been identified in wild birds (Krawiec *et al*., 2015) but is generally not considered among the infective species for birds, such as *C. psittaci* and *C. gallinacea*.

Regulation (EC) 178/2002 requires European Member States to implement measures ensuring the presence of systems capable of identifying and responding to food safety issues (European Parliament and Council of the European Union, 2002). The detection of such issues within the food system relies primarily on official food controls, carried out by competent authorities, complemented by internal monitoring performed by food business operators. While targeted food controls are valuable for tracking known biological hazards, such as those listed in Commission Regulation (EC) 2073/2005 (Commission of the European Communities, 2005), the use of untargeted monitoring methods allows for the identification of previously unrecognized potential pathogens circulating within the food system, including emerging agents. The findings of our study demonstrate, for the first time, the feasibility of shotgun metagenomics to detect unexpected *C. ibidis* reads on broiler carcasses at the exit of the chilling tunnel. Although the zoonotic potential or the foodborne transmission of *C. ibidis* have not been demonstrated, it is well established that *Chlamydia* species exhibit two morphological forms: elementary bodies, which are extracellular, infectious particles, and reticulate bodies, which are intracellular, non-infectious, yet metabolically active (Zaręba-Marchewka *et al.*, 2020). Because elementary bodies are considered “spore-like” extracellular forms capable of surviving for months outside the host (Essig and Longbottom, 2015), the detection of *Chlamydia* reads on broiler carcasses at the end of the chilling tunnel warrants further investigation.

Conclusions

This study revealed the unexpected presence of *C. ibidis* reads on broiler carcasses at the end of the chilling tunnel. Unfortunately, the potential presence of viable cells was not assessed. However, ad hoc samplings to detected *C. ibidis* will be organized because *C. ibidis* is considered an emerging *Chlamydia* species.

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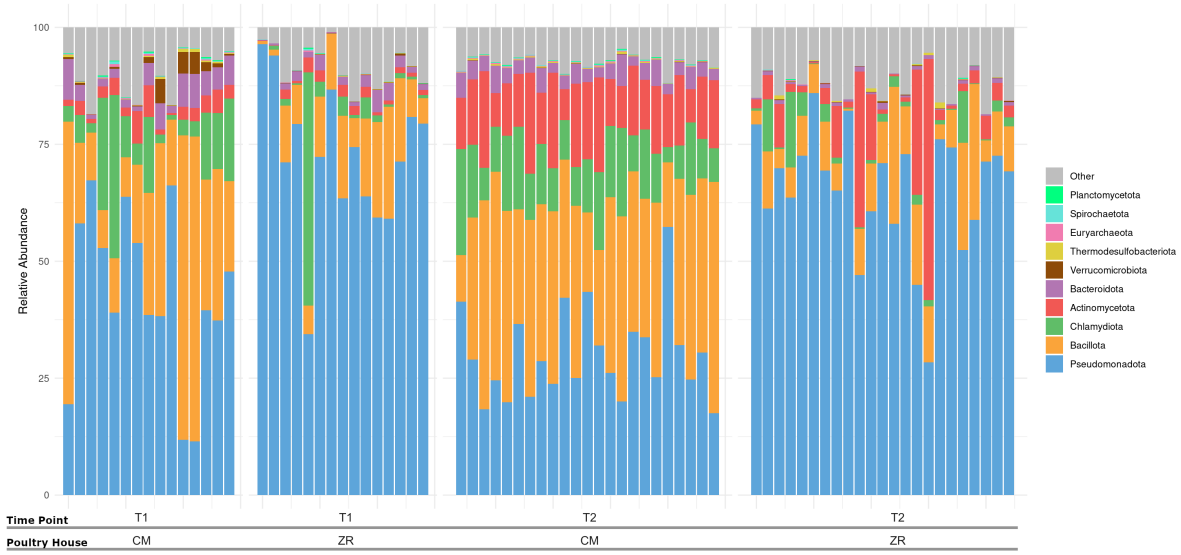

Figure 1. Phyla identified in the carcass neck skin microbiomes. Carcasses originated from the poultry houses CM and ZR and were tested in 2019 (T1) and 2023 (T2).

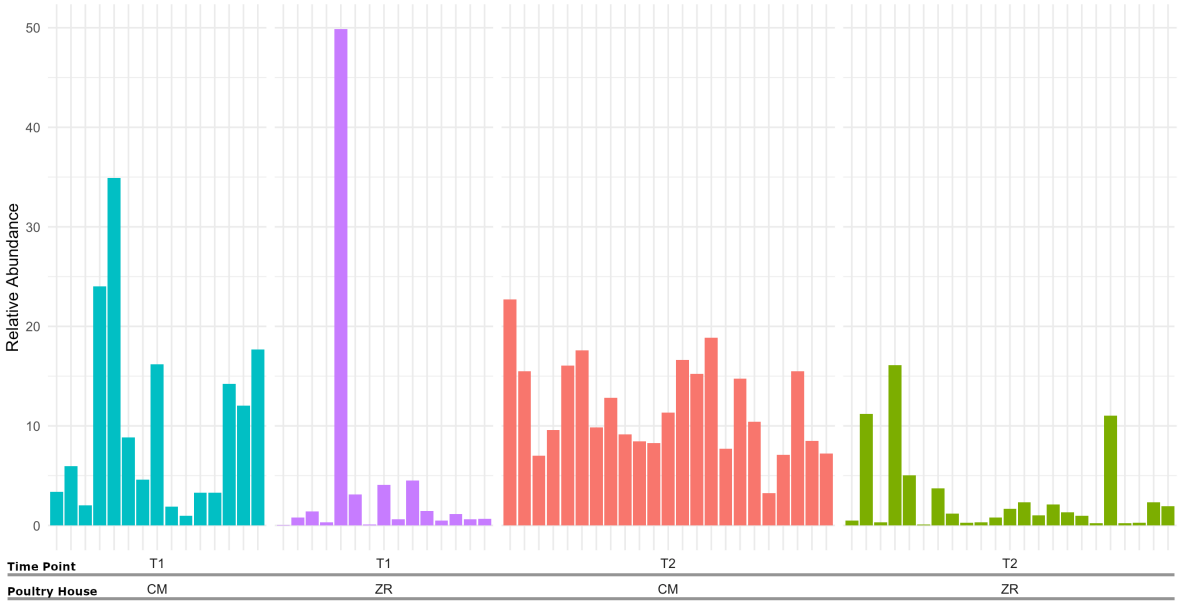

Figure 2. Relative abundance of *Chlamydia* reads quantified in the carcass neck skin microbiomes. Carcasses originated from the poultry houses CM and ZR and were tested in 2019 (T1) and 2023 (T2). Each bar of the plot corresponds to a neck skin sample (n=76).

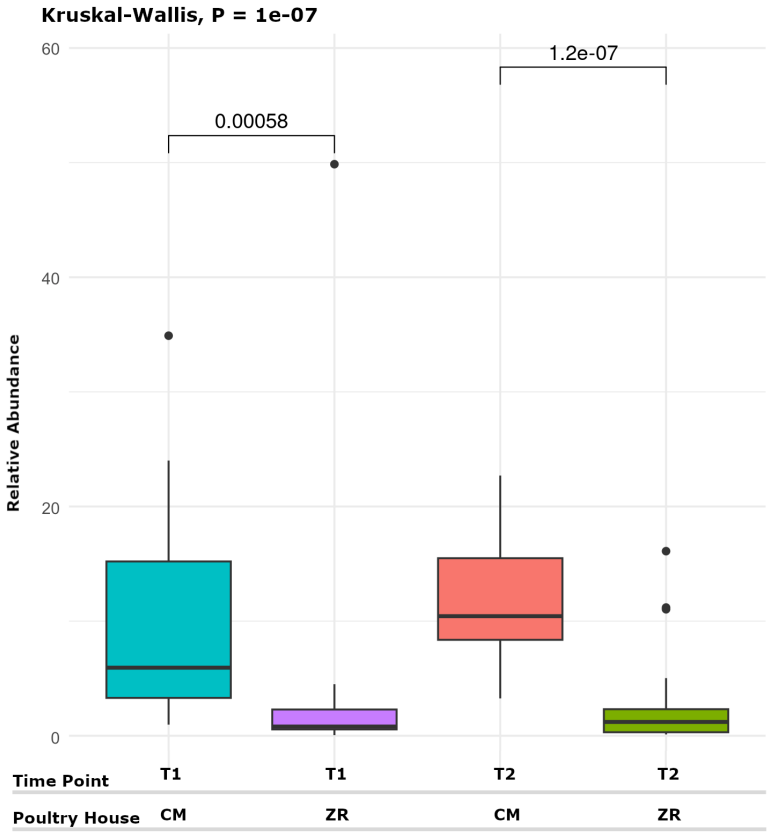

Figure 3. Mean relative abundance of *Chlamydia* reads quantified in the carcass neck skin microbiomes. Carcasses originated from the poultry houses CM and ZR and were tested in 2019 (T1) and 2023 (T2). Each box shows the distribution of *Chlamydia* reads relative abundance (i.e., median, Q1, Q3 and outliers).

Table 1. Mean relative abundance of reads of *Chlamydia* species quantified in carcass neck skin microbiomes. Carcasses originated from the poultry houses CM and ZR and were tested in 2019 (T1) and 2023 (T2).

***Chlamydia* species**	**T1**	**T1**	**T2**	**T2**
	CM	**ZR**	**CM**	**ZR**
C. ibidis	10.2147	4.6143	11.8922	2.8212
C. psittaci	0.0017	-	-	-
C. trachomatis	0.0007	-	0.0007	-
C. gallinacea	-	-	0.0002	-