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Differences in fungal contamination of broiler litter between summer and winter fattening periods

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This study aimed to compare fungal contamination of poultry litter between warm and cold seasons. It was carried out in commercial production conditions over two five-week fattening periods: one in the summer (July-August) and one in the winter (December-January). Broilers were reared on a litter composed of chopped straw and sawdust. Litter fungal concentration and composition were investigated weekly, along with litter temperature, moisture, and pH. Litter concentration of total fungi increased over both fattening periods, with no differences in median concentrations between them. Season also had no effect on yeast, *Aspergillus* section *Nigri,* and *Cladosporium*, *Fusarium*, and *Rhizopus* spp. concentrations, while the *Aspergillus* section *Flavi* and *Aspergillus* spp. combined showed higher concentrations in the summer, and *Mucor* and *Penicillium* spp. in the winter. Total fungal concentration highly correlated with litter temperature, moisture, and pH, regardless of the season. Our findings can be useful in the assessment and control of potential harmful effect of fungi on the health of poultry and poultry farm workers.

KEY WORDS: cold season; moisture; mycoflora; pH; poultry; temperature; warm season

Poultry/broiler litter is a bedding material, usually straw in Europe, mixed with excreta, spilled feed/water, and feathers, whose quality is important for broiler welfare and productivity as it absorbs moisture, dilutes faecal content, and serves as a thermal insulator and protective cushion between broilers and the floor (1–4).

However, it is also known to favour fungal growth (5), which is why its quality should be monitored continuously, mostly by moisture assessment. In the initial fattening period, the level of broiler litter moisture is 10–15 %, which by the end of this period rises to 25–50 %. Recommended levels span between 30 % and 40 % (6–8). Other recommended conditions include neutral pH, low ammonia production, and loose, crust-free litter. High moisture leads to litter caking that favours ammonia release, whereas low moisture favours respiratory problems due to high levels of dust that carries microorganisms, including fungal spores (3, 9). Another crucial condition for the biochemical process of litter ripening is litter temperature, which depends on air temperature in a broiler house (7, 10).

Compared to bacterial and viral diseases, fungal diseases are less prevalent in poultry but often devastating when they break out (11, 12). Fungi can cause disease directly or with mycotoxins they produce, which usually enter the body by ingestion and cause poisoning and immunosuppression. This can lead to great economic losses through loss of meat and egg production (13, 14).

Fungal diseases in poultry have come into focus all over the world due to overuse of antibiotics, which eliminate innate bacterial microflora and give way to infections with opportunistic pathogens (14). In addition, fungal infections in poultry are very difficult and expensive to treat, as vaccines are not available, and resistance to drugs is increasing, which makes prognosis uncertain. Therefore, the best way to fighting these diseases is prevention (5, 12, 15). Prevention is also important as a way to minimise zoonosis implications (16–19).

One of the first steps in that direction is to determine the composition and concentration of mycoflora in poultry litter as a way to assess the risks, especially if poultry litter is later to be used to fertilise open fields, as it can get airborne and affect neighbouring rural areas (9, 20).

Yet, only a few studies have investigated fungal contamination of poultry litter (e.g., 9, 21–26) and reports

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on seasonal concentrations and incidence of diseases are inconsistent and mainly focused on *Aspergillus* spp. as the most important pathogen from the health and economic viewpoint (23, 26–32).

The aim of our study was to get a broader picture of fungal contamination (than limiting ourselves to *Aspergillus* spp.) by investigating seasonal fungal flora in poultry litter and test the following hypotheses: (i) the season will influence total fungal concentrations and composition in the litter, and (ii) fungal concentrations will depend on other parameters of litter quality, such as temperature, moisture, and pH.

MATERIALS AND METHODS

The study protocol was approved by the Ethics Committee in Veterinary Medicine of the University of Zagreb Faculty of Veterinary Medicine, Zagreb, Croatia (class: 640-01/16-17/43; record no.: 251-61-01/139-16-2; 21 April 2016).

Study design, animals, and farm management

The study was conducted in commercial farming conditions in a broiler house over the five-week production cycles in the summer (July-August 2016) and winter (December-January 2016/2017). During each production cycle, 18,000 Ross hybrid broilers were kept in controlled conditions on a 10-cm thick litter made of a 2-cm bottom pine sawdust layer and a 8-cm wheat straw layer (up to 33 kg/m^2 stocking density). The broiler house used straw from its own farming fields (chopped to the length of 5 cm) and sawdust from a sawmill (neither dedusted nor disinfected). Litter was neither turned over nor additional amount of litter was added during either production cycle. The broiler house has an oil heater, negative pressure mechanical ventilation, and lighting as recommended for Ross hybrids (33). Broilers were fed complete feed mix (Biodar, Varaždin, Croatia) from round pan feeders and watered from nipple drinkers with cups. Feed and water were provided *ad libitum*.

The broiler house is cleaned and disinfected with high-pressure cleaners between production cycles, which are separated by a two-week house rest. Floors are disinfected with caustic soda (PCC Rokita SA, Brzeg Dolny, Poland) and the rest of the house, including equipment, with Ecocid® S (Krka d.d., Novo Mesto, Slovenia). Once the litter is spread, the house is fumigated with a Formaster G tablet (Formaster di Emanuela Magnani & C.s.a.s., Piacenza, Italy).

Litter fungal contamination, temperature, moisture, and pH were determined once a week over the five-week production cycles.

Litter sampling and analytical methods

Litter temperature (°C) was measured with a Testo 925 thermocouple thermometer (Testo SE & Co. KGaA, Lenzkirch, Germany) at 3 cm below litter surface at five points of the broiler house (four corners and one in the centre). At these points we also took one litter sample per point into sterile bags (Aptaca Spa, Canelli, Italy) and transported them to the laboratory for analysis on the same day. Litter moisture (%) was determined using the gravimetric method by calculating weight loss after drying, whereas pH was determined electrometrically using a WTW inoLab pH 720 pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany). More detailed descriptions are available elsewhere (8, 34).

Litter fungal contamination was determined as follows: 10 g of each sample was first diluted 10-fold with sterile distilled water and then 100 µL of the dilution plated on Sabouraud dextrose agar (Biolife, Milan, Italy) and incubated at 25 °C for 5–7 days. The fungi were identified based on macroscopic appearance of grown colonies and on microscopic examination of spores stained with lactophenol blue solution (Sigma-Aldrich, St. Louis, MO, USA) (35, 36). The concentration was expressed as colony forming units per gram of litter (CFU/g). Analytical procedures were performed in triplicate.

Statistical analysis

All data were analysed with Statistica v. 13.5 (TIBCO Software Inc., Palo Alto, CA, USA, 2018). The normality of data distribution was tested with the Kolmogorov-Smirnov test. The significance of differences in median litter fungal concentrations between summer and winter measurements was tested with the Mann-Whitney *U*-test. Differences in mean litter temperature, moisture, and pH and differences between weekly measurements of all parameters between the seasons were compared with Student's *t*-test. Differences between weekly measurements within one season were tested with the repeated-measures ANOVA and *post-hoc* Tukey HSD test. Correlations between fungal concentrations and litter temperature, moisture, and pH were assessed with Spearman rank-order correlation. In all tests, the level of statistical significance was set at $P<0.05$.

RESULTS AND DISCUSSION

Litter fungal concentrations ranged from 10^2 to 10^5 CFU/g, which is consistent with previous reports (9, 21, 37–39), and increased with time (fattening weeks), as expected (39). We found no significant seasonal differences at the beginning and the end of the fattening periods (Figure 1) or in the median fungal concentrations for either period (Table 1). This is because fungal concentrations highly correlated with litter moisture and pH throughout the study

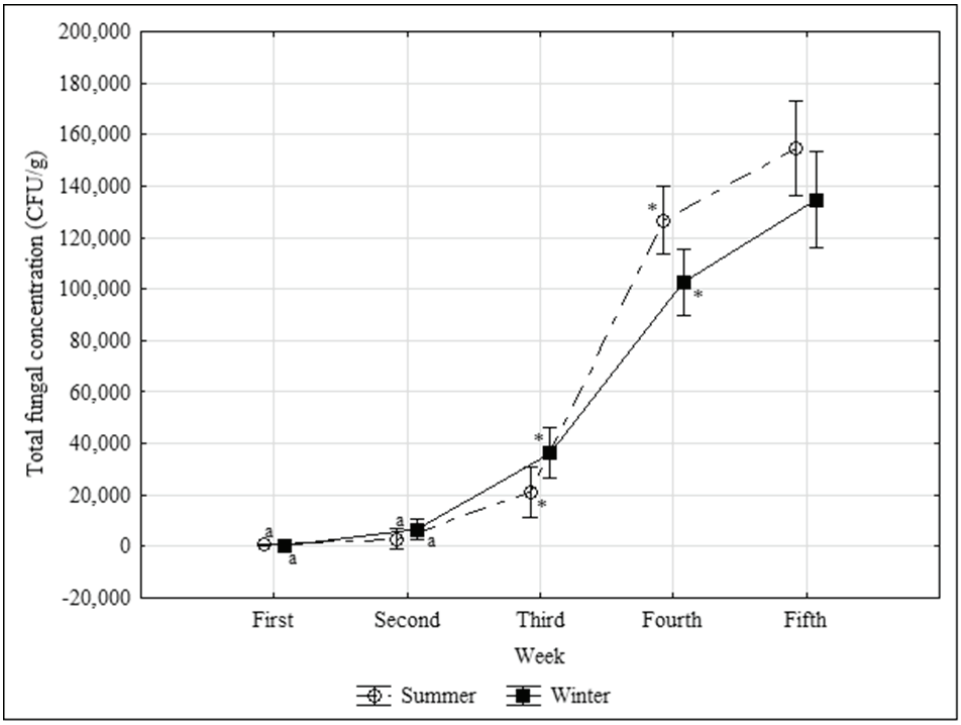

Figure 1 Comparison of weekly total fungal concentrations in broiler litter between the summer and winter fattening periods (values are expressed as means ± 95 % confidence intervals). [a] marks weekly values of the same season that did not differ significantly; other values differed significantly ($P<0.05$). * weekly values that differed significantly between the seasons ($P<0.05$). CFU – colony forming units

(Table 2), which did not significantly differ between the seasons (Table 1). The correlation between fungal growth and litter moisture and pH was also reported by Hutson (40) and Schefferle (41), but there are reports to the contrary as well (38). According to Arné et al. (5), the impact of variations in litter moisture and pH on fungal population density remains a controversial issue, although wet and soiled areas can intensify fungal growth.

Even though the five-week fattening periods in our study were shorter than in the above studies, and higher litter moisture and pH were recorded in the last week of both periods, the comparison makes sense, as according to Milanov et al. (25) microorganism growth (including fungal) in broiler litter reaches its peak in about one month of fattening, after which it declines and reaches steady levels.

We also found a highly significant correlation between fungal concentrations and litter temperature (Table 2), which increased with time in both seasons (Figure 2), yielding values consistent with the reports by Spindler and Hartung (42) and Knížatová et al. (43). However, the latter study found no major differences in litter temperature between summer and winter fattening periods, whereas we did (Table 1, Figure 2). Despite controlled conditions in the broiler house, this was likely related to seasonal differences in outdoor temperature. Even so, it seems that the five-week fattening periods in our study were too short for the differences in litter temperature to cause seasonal differences in fungal concentrations or to affect litter moisture and/or pH.

Litter moisture and pH in our study increased over both seasons as weeks went by (Figures 3 and 4) and were consistent with other studies of straw as a litter material (44–46). Their mean values did not significantly differ between the seasons (Table 1), save for the higher moisture level in the last fattening week of the winter fattening

Table 1 Comparison of total litter fungal concentrations, temperature, moisture, and pH between the summer and winter broiler fattening periods

Parameter		Summer	Winter	*P*
Fungi (CFU/g)	Median (range)	1.82×10^4 (3.00×10^2–1.65×10^5)	3.32×10^4 (2.00×10^2–1.58×10^5)	0.62
Temperature (°C)	Mean±SD (range)	28.80±1.74 (25.60–31.80)	26.34±1.75 (22.90–29.80)	**<0.001**
Moisture (%)	Mean±SD (range)	28.18±10.12 (7.90–42.30)	32.17±11.07 (12.60–49.00)	0.19
pH	Mean±SD (range)	7.06±1.18 (4.95–8.71)	6.83±1.23 (5.42–8.61)	0.50

CFU – colony forming units. Significant difference is marked in bold type

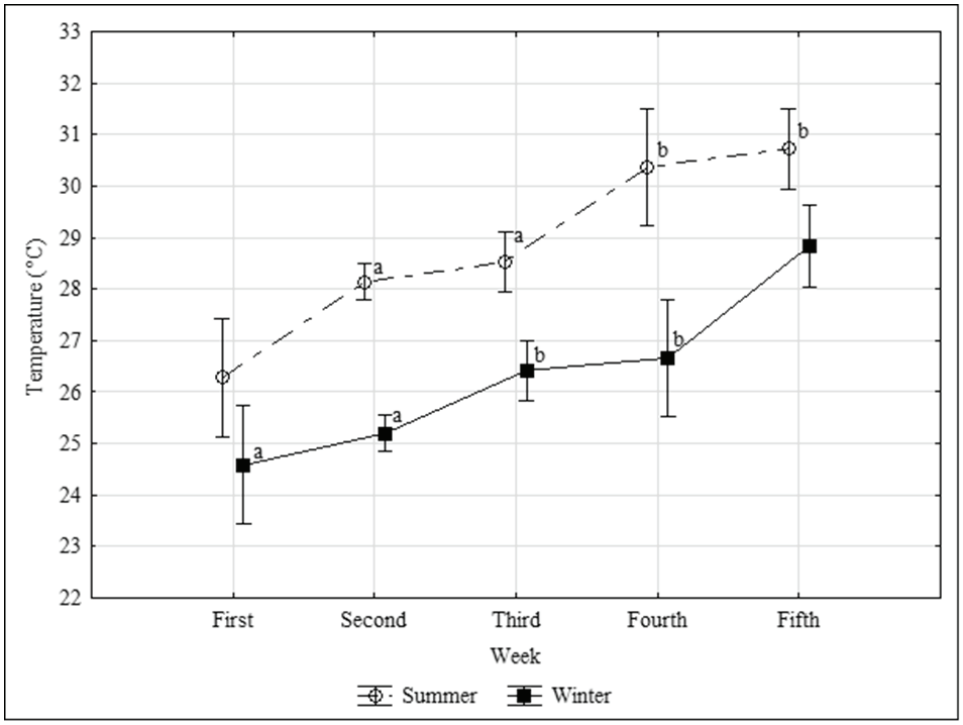

Figure 2 Comparison of weekly broiler litter temperatures between the summer and winter fattening periods (values are expressed as means ± 95 % confidence intervals). [a,b] same letters mark weekly values of the same season that did not differ significantly; other values differed significantly ($P<0.05$). All weekly values differed significantly between the seasons ($P<0.05$)

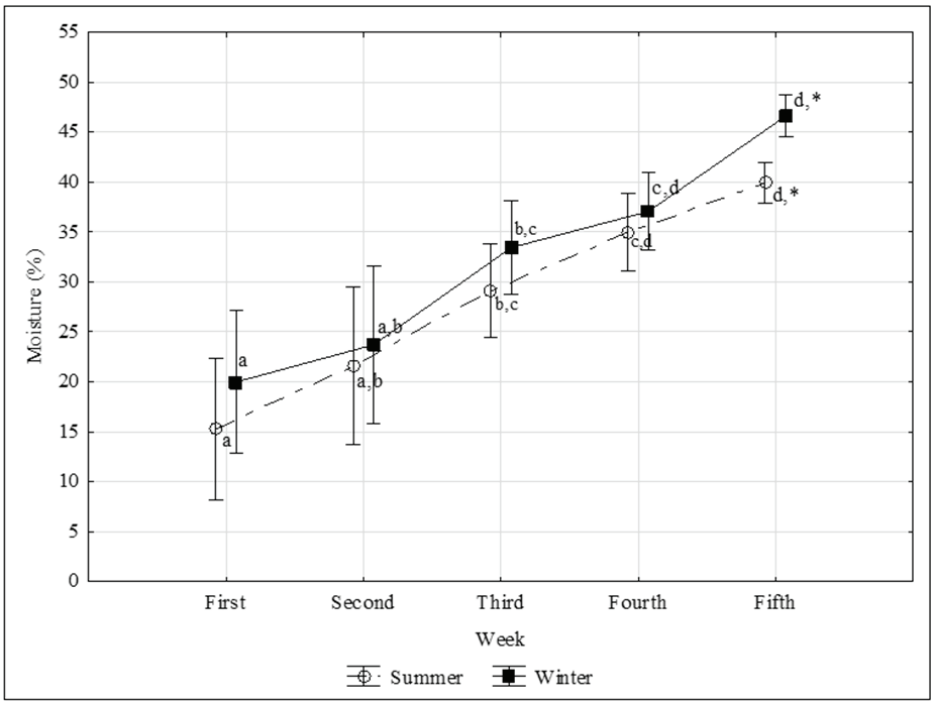

Figure 3 Comparison of weekly broiler litter moisture between the summer and winter fattening periods (values are expressed as means ± 95 % confidence intervals). [a,b,c,d] same letters mark weekly values of the same season that did not differ significantly; other values differed significantly ($P<0.05$). [*] weekly values that differed significantly between the seasons ($P<0.05$)

Table 2 Correlations between total litter fungal concentrations and litter temperature, moisture, and pH

Parameter	Temperature (°C)	Moisture (%)	pH
Fungi (CFU/g)	0.710*	0.791*	0.918*

CFU – colony forming units. * $P<0.05$

Table 3 Comparison of litter fungal composition (with concentrations by genera/sections) between the summer and winter broiler fattening periods

Fungal genera	CFU/g Median (range)		*P*
	Summer	Winter	
Aspergillus spp.	2.00×10^2 ($0–1.40\times10^3$)	0 ($0–5.00\times10^2$)	**<0.01**
A. section *Flavi*	1.00×10^2 ($0–1.00\times10^3$)	Not detected	**<0.001**
A. section *Nigri*	1.00×10^2 ($0–1.40\times10^3$)	0 ($0–5.00\times10^2$)	0.18
Cladosporium spp.	0 ($0–1.00\times10^2$)	Not detected	0.82
Fusarium spp.	Not detected	0 ($0–1.00\times10^2$)	0.82
Mucor spp.	Not detected	1.00×10^2 ($0–2.90\times10^4$)	**<0.001**
Penicillium spp.	0 ($0–4.00\times10^2$)	1.50×10^3 ($0–2.85\times10^4$)	**<0.001**
Rhizopus spp.	0 ($0–1.00\times10^2$)	0 ($0–2.00\times10^2$)	0.83
Yeasts	1.71×10^4 ($0–1.64\times10^5$)	9.20×10^3 ($0–1.35\times10^5$)	0.19
Unidentified	0 ($0–1.00\times10^3$)	0 (0–50)	**0.03**

CFU – colony forming units. Significant differences are marked in bold type

(Figure 3). Comparing different materials for broiler litter between summer and winter fattening periods, Garcia et al. (47) reported higher moisture for straw in the summer and higher pH in the winter, which may be owed to different farm management in their study. Previous studies have revealed that litter moisture also depends on litter quantity, stocking density, watering system, ventilation, indoor microclimate, broiler age, nutrition, and health (enteritis) (48, 49). Even though we did not keep track of broiler health, no enteritis outbreaks were recorded in either season.

Our findings have shown a prevalence of yeasts in broiler litter, with no significant seasonal difference (Table 3), as also reported by other studies (23, 25, 26, 37).

Besides yeasts, the most prevalent fungal species detected in our study were *Aspergillus* (encompassing the *Flavi* and *Nigri* sections), *Mucor*, and *Penicillium* spp. (Table 3), which is in line with previous reports (9, 22, 37–39, 50, 51). These three also showed significant seasonal differences. *Aspergillus* spp. combined, including *A.* section *Flavi*, were more frequent in the summer, and *Mucor* and *Penicillium* spp. in the winter. Other species showed no seasonal differences (Table 3). Soliman et al. (23) also found *Aspergillus* spp. to prevail in closed broiler houses in the summer and *Penicillium* spp. in the winter, but unlike our study, theirs reported *Mucor* spp. to be more prevalent in the summer.

Some studies using same methodology for fungal identification detected more mould genera, including *Aspergillus* sections, than we did (9, 37, 38), most likely because of different litter material. This suggests that straw litter, known to have higher moisture, pH, and temperature than other materials (44, 52–54), favours the development of more homogeneous mycoflora.

Even though we used the same methods for fungal identification as Chollom et al. (24), we did not find any dermatophytes in the litter, which are of particular health concern. Their finding of high *Trichophyton* spp. prevalence in poultry litter may be owed to higher incubation temperature than in our study, but may also point to our less precise fungal identification.

Nevertheless, several fungal genera isolated in our study pose a health risk both as pathogens or producers of mycotoxins, which can cause severe poisoning in humans and animals (55), *Aspergillus* spp. in particular.

Aspergillus fumigatus (section *Fumigati*) is the most pathogenic fungus affecting poultry, accounting for 95 % of all cases of aspergillosis, but other species, such as *A. flavus* (section *Flavi*), *A. niger* (section *Nigri*), *A. nidulans* (section *Nidulantes*), and *A. terreus* (section *Terrei*), alone or combined, also present a health risk (14, 56, 57). Besides birds, *Aspergillus* species are known opportunistic pathogens in humans, immunocompromised patients in particular. *Aspergillus fumigatus* accounts for over 80 % of diseases, including invasive pulmonary aspergillosis, aspergilloma, and different forms of hypersensitivity disorders such as allergic asthma, allergic sinusitis, pneumonitis, and allergic bronchopulmonary aspergillosis (58–60). Another species with emerging evidence of a broad spectrum of infections that are difficult to treat are the *Fusarium* spp., which can cause onychomycosis, skin infections, and keratitis (61). We did not find any species from the *Aspergillus* section *Fumigati* in our litter samples, regardless of the season, while *Fusarium* spp. was detected in the winter (Table 3).

As regards the risk of mycotoxin poisoning, *A.* sections *Flavi* and *Nigri*, and *Fusarium* and *Penicillium* spp. we

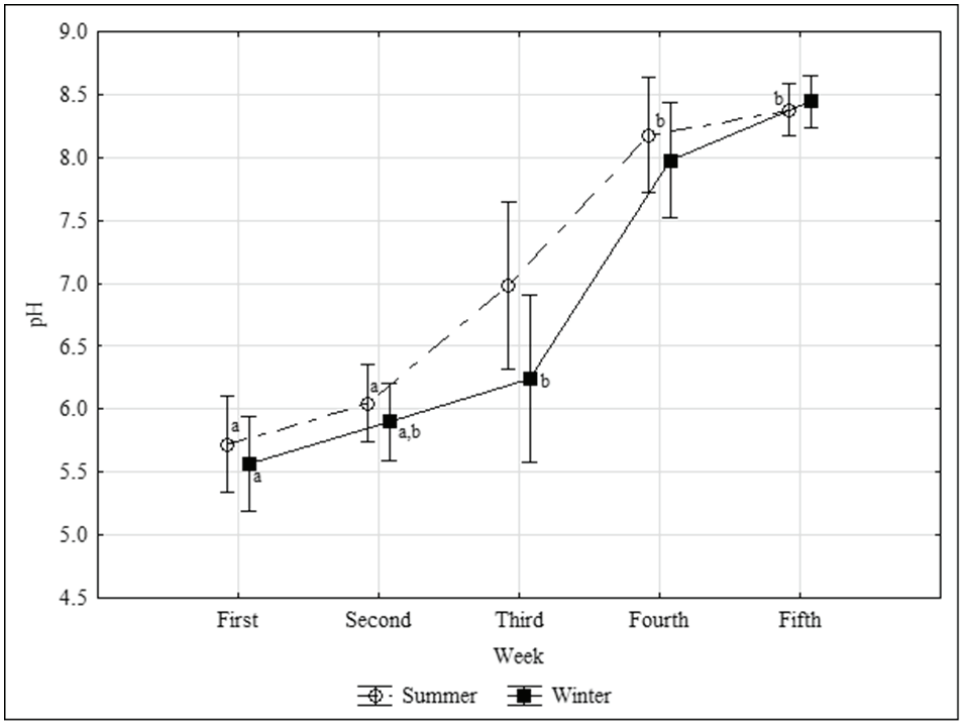

Figure 4 Comparison of weekly broiler litter pH between the summer and winter fattening periods (values are expressed as means ± 95 % confidence intervals). [a,b] same letters mark weekly values of the same season that did not differ significantly; other values differed significantly (P<0.05). No weekly values differed significantly between the seasons

found in our litter samples (Table 3) are known mycotoxin producers (62, 63).

CONCLUSIONS

Our findings suggest that season does not influence total fungal concentrations as much as fungal composition in broiler litter. They have also confirmed that fungal concentrations much depend on litter temperature, moisture, and pH and can be useful in the assessment and control of potential adverse effects on poultry and poultry farm workers.

To the best of our knowledge, other studies did not compare fungal contamination of broiler litter on the same farm between seasons. Furthermore, our study contributes to the scarce data available on straw as litter material in broiler rearing. What limits the interpretation of our findings is that they do not distinguish enough fungal isolates at the species level, as we used methods specific enough to allow comparison with the results of other studies investigating poultry litter mycoflora. Future studies should observe various litter materials in the same production conditions across seasons with more specific identification of fungi, including the use of sensitive methods of genotyping.

Conflicts of interest

None to declare.

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Razlike u onečišćenju stelje gljivicama tijekom tova pilića između ljetnog i zimskog razdoblja

Cilj ovoga rada bio je usporediti onečišćenje stelje za perad gljivicama između toplog i hladnog razdoblja godine. Istraživanje je provedeno u komercijalnim uvjetima proizvodnje tijekom petotjednog tova pilića ljeti (srpanj – kolovoz) i zimi (prosinac – siječanj). Pilići su držani na stelji od sjeckane slame i piljevine. Koncentracija i sastav gljivica u stelji istraživani su tjedno, zajedno s temperaturom, vlagom i pH stelje. Ukupna koncentracija gljivica u stelji povećavala se tijekom tova u oba godišnja razdoblja, bez utvrđenih razlika u prosječnim koncentracijama između razdoblja. Razdoblje godine također nije imalo utjecaja na koncentracije kvasaca, aspergila iz sekcije *Nigri* te *Cladosporium*, *Fusarium* i *Rhizopus* spp., a koncentracije aspergila iz sekcije *Flavi*, kao i ukupnih aspergila u stelji bile su veće u ljetnom, a koncentracije *Mucor* i *Penicillium* spp. u zimskom razdoblju. Utvrđena je visoka pozitivna povezanost ukupne koncentracije gljivica s temperaturom, vlagom i pH stelje, neovisno o razdoblju godine. Dobiveni rezultati mogu biti korisni u procjeni i kontroli potencijalnoga štetnog učinka gljivica na zdravlje peradi i radnika na peradarskim farmama.

KLJUČNE RIJEČI: hladno razdoblje; mikoflora; perad; pH; temperatura; toplo razdoblje; vlaga