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MICROBIAL CONTAMINATION OF POULTRY LITTER DURING FATTENING PERIOD

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Abstract: The results of the research into the microbiological contamination of litter used by broiler chickens are presented. Litter samples were taken prior to the introduction of chicks (day 0) and in 7-day intervals until the end of the fattening period. The total numbers of aerobic mesophilic microorganisms, yeasts, moulds and *Clostridium perfringens* spores, and the presence of bacteria of the *Salmonella* genus were determined. The total microbial count in newly laid litter was 7 $\log_{10}$ CFU/g, which increased to 9 $\log_{10}$ CFU/g by the 4th week. However, at the end of the 5th week, it was at the same level as in newly laid litter. *C. perfringens* spores, presumably originating from chicks' faeces, were first detected on day 7. In the next 7 days their number increased, reaching 3-4 $\log_{10}$ CFU/g, and remained at approximately same levels until the end of the research. The initial mould contamination was 5-6 $\log_{10}$ CFU/g. However, from day 21 moulds were not isolated, but only yeasts of the *Saccharomyces* genus. It is supposed that these were deposited with chicks' faeces, due to their presence in complete broiler feed. No bacteria of the *Salmonella* genus were ever isolated from the litter. In conclusion, the total numbers of microorganisms in deep litter reach their peak in approximately a month, which is followed by their decrease. Deep litter is a favourable environment for probiotic yeast cultures. Added to feed intended for broilers, they can positively influence the microbial composition of litter, providing healthier environment to fattening broilers.

Key words: poultry litter, broiler fattening, total number of microorganisms, yeast, mould, *Clostridium perfringens*

Introduction

Bedding material for animal housing is of organic or inorganic origin, primarily made from various by-products of agriculture and wood industry: baled hay, chopped straw, peanut or rice hulls, wood shavings and chips, sawdust, shredded paper etc. (*Torok et al., 2009*). When used in intensive broiler production it is expected to absorb moisture, dilute chicken manure and protect the birds from the thermal effects of the floor. Litter is defined as the combination of bedding material, wasted feed and water, manure, feathers, and other detritus from the chicken (*Tabler et al., 2009; Ritz et al., 2005: Torok et al., 2009*). Owing to the close link between chicken welfare and bedding, it should be of adequate quality and chosen carefully. The birds are continuously in close contact with litter, which may heavily affect their health and influence production performance owing to ammonia, dust particles, high humidity, pH, temperature (*Torok et al., 2009*) and, primarily, to microorganisms originating from the environment and the chicken's guts.

Microbial species which can be detected in bedding are hypothesized, rather than known. Certain data originate from research using standard culture methods, which enable the isolation and identification of cultivable, known microbial species, but not of some fastidious ones existing in the environment (*Lu et al., 2003*). In spite of the logical expectation that enteric bacteria are prevalent in litter, enteric bacteria such as enterococci and colifoms account only for the minority of the total bacteria (0.1% and 0.11%, respectively), which suggests that they can barely survive in poultry litter (*Lu et al., 2003*). Research on 340 bacterial clones originating from broiler litter revealed that the microbiota consisted predominantly of gram-positive bacteria (87%), members of *Lactobacillaceae, Bacillus, Staphylococcus, Clostridiaceae, Enterococcaceae, Corynebacteriaceae, Micrococcaceae, Microcinaceae* and *Proteobacteria* groups. Staphylococci made up for the majority of aerobic bacteria (*Martin and McCann 1998; Lu et al., 2003*). Certain microbes in bedding material are not only potential causative agents of bird diseases, but are also human pathogens threatening people involved in poultry production. For example, carried by dust particles into the air fungal spores may reach concentrations 10^6-$10^9/m^3$ of air, which may lead to respiratory diseases in birds and farmers, such as allergic alveolitis or hypersensitivity pneumonitis (*Kotimaa et al., 1991*). *C. perfringens* is the causative agent of necrotic enteritis and gangrenous dermatitis of broiler chickens (*Lu et al., 2003; Tan et al., 2013*).

The assessment of the total number of microorganisms in poultry litter revealed neither increase with time (resulting from increased faecal content), nor that it was directly proportional to the number of flocks successively grown in the building (*Schefferle, 1965; Thaxton et al., 2003*): the average number of aerobics plunged after four flocks were kept on the bedding, after which it remained similar, whilst

anaerobic bacteria number was approximately the same until as many as 28 flocks were raised (*Thaxton et al., 2003*). In some other research, the populations of bacteria and fungi reached maximum about a month after the bedding has been laid, after which they declined and remained similar until the end of the investigation (*Martin and McCann, 1998; Terzich et al., 2000*).

Broiler litter may be utilized as a fertilizer (*Terzich et al., 2000*), owing to potentially valuable nutrients, almost 30% of crude protein content and high levels of minerals (*Lu et al., 2003*). Adequately used, chicken litter is a precious resource, which improves soil fertility, aeration and its water-holding capacity (*Omeira et al., 2006*). However, when used as a fertilizer, it poses a serious risk of introducing pathogenic bacteria into soil (*Trawinska et al., 2016*), including zoonotic agents. In addition, non-pathogenic, animal staphylococci and enterococci, part of microbiota, may contribute to the transmission of antibiotic resistance to human commensals (Lu et al., 2003). Poultry litter can also be used as feed supplemented to cattle (*Smith et al. 1974; Terzich et al., 2000; Lanyasunya et al., 2006; Ghaly and MacDonald 2012; Rankin, 2018*), in spite of the risk of possibly present microorganisms such as *Campylobacter*, *Salmonella*, *Listeria monocytogenes* etc. (*Lu et al. 2003*). However, bacteria loads can significantly be reduced at high temperature (60^oC), which is easily achievable by compressing and wrapping manure to prevent oxygen entry and preserve nitrogen content (*Lanyasuny et al., 2006*). Knowing the microbial composition of animal waste, including poultry litter, restricts its impact on the environment and human and animal health, contributing to the management of animal diseases (*Lu et al., 2003*).

This research was aimed at the assessment of microbial contamination levels, that is the total aerobic mesophilic bacteria, yeasts and moulds in bedding material and litter sampled from a broiler farm in Vojvodina. In addition, the numbers of *Clostridium perfringens* spores were determined, given that necrotic enteritis is one of the leading health problems on poultry farms in Serbia.

Materials and Methods

Bedding material subjected to this research was made of peat, cellulose pellet, wood chips and a pH stabiliser. It was laid in two broiler houses with 6,550 chicks in each. The specific weight of the fresh bedding was 320-340 kg/m^3. The chicks were of *Gallus gallus domesticus* species, ROSS 308 hybrid. They were fed on standard complete feed for fattening chickens, in compliance with the technological normative for the hybrid.

For microbial examination the bedding material was sampled at the beginning of the experiment and litter in 7-day intervals during 5 weeks, in April and May 2018. Litter was sampled randomly, from under the nipple-drinkers and

along the length of the flock house, and pooled. Two pooled samples were taken from both houses at each timepoint.

For microbiological analysis, 20 g of each sample was put into a sterile 500-mL BagPage (Interscience, France) and 180 g of Buffered Peptone Water (BPW) was added. The suspension was homogenized in Stomacher BagMixer® 400 (Interscience, France) set to the maximum speed, after which a series of ten-fold dilutions were made by transferring 1 mL of suspension into 9 mL of BPW. The analyses were done in compliance with the procedures described in the following standards: Horizontal method for the enumeration of microorganisms, Part 1: Colony count at 30°C by the pour plate technique (*ISO 4833-1:2014*), Horizontal method for the enumeration of yeast and moulds (*ISO 21527-2:2011*) and Horizontal method for the detection, enumeration and serotyping of *Salmonella* (*ISO 6579:2017*). To eliminate the sulphite-reducing bacteria which are non-sporogenic, in order to more precisely determine the numbers of *C. perfringens* on sulphite cycloserine agar (Biokar Diagnostics, France), the ten-fold dilutions of samples were thermally treated at 80°C for 15 minutes. This is how the total number of *C. perfringens* spores were determined using Horizontal method for the enumeration of *Clostridium perfringens* - Colony-count technique (*ISO 7937:2010*).

Results and Discussion

The levels of microbial contamination of the bedding material in two broiler houses were determined at the beginning of the broilers' fattening period – prior the introduction of the flocks, and in seven-day intervals throughout the 35 days of fattening. The results referring to the total number of microorganisms, the numbers of *C. perfringens* spores, yeast and mould were expressed quantitatively, as $\log_{10}$ colony-forming units per gram of material (CFU/g), and are shown in Tables 1 and 2.

Table 1. Average total aerobic microorganisms, *Clostridium perfringens*, yeast and mould counts in bedding material (day 0) and litter (day 7-35) in broiler house No.1

Day	Sample No.	Counts ($\log_{10}$ CFU/g)			
		Total microorganisms	*Clostridium perfringens* spores	Yeasts	Moulds
0	1	7.48	<10	<100	5.40
	2	7.40	<10	<100	5.30
7	1	8.18	2.90	8.20	5.48
	2	8.20	2.78	8.43	6.51
14	1	8.65	4.30	8.18	<3
	2	8.70	4.18	8.43	<3
21	1	9.23	4.70	8.18	<3
	2	9.28	4	8.30	<3
28	1	9.30	4.30	7.40	<3
	2	9.48	3.70	7.30	<3
35	1	7.48	4.78	<4	<3
	2	7.30	5.48	4.90	<3

Table 2. Average total aerobic microorganisms, *Clostridium perfringens*, yeast and mould counts in bedding material (day 0) and litter (day 7-35) in broiler house No. 2

Day	Sample No.	Counts ($\log_{10}$ CFU/g)			
		Total microorganisms	*Clostridium perfringens* spores	Yeast	Mould
0	1	7.397	<10	<100	6
	2	7.30	<10	<100	6.18
7	1	8.20	2.70	<100	7.18
	2	8.18	2.81	<100	7.38
14	1	8.60	3.30	7.15	6.53
	2	8.78	3.60	7.15	7.13
21	1	9.26	4.17	8.30	5.30
	2	9.18	4.30	8.48	6.70
28	1	9.48	4.30	8.18	<3
	2	9.48	3.78	8.30	<3
35	1	7.70	3.45	5	<3
	2	7.60	4.85	5.85	<3

The total number of microorganisms in bedding materials was 7 $\log_{10}$ CFU/g. This included the total number of aerobic, mesophilic bacteria, yeast and moulds which form visible colonies at 30°C on Plate count agar (Biokar Diagnostics, France) during 72h of incubation in aerobic conditions. Given that a number of microbial species from the environment is non-cultivable in laboratory conditions and that strict anaerobes cannot be detected with this method, the real number of microorganisms was certainly higher. An additional hindrance for the precise determination of total microbial count were unsedimented particles of bedding

material which were transferred from the sample dilutions onto Petri plates, and may have been erroneously considered to be microbial colonies. In litter samples, from the end of the 1st to the end of the 4th week of research, the total number of microorganisms increased by 1-2 $\log_{10}$ CFU/g. The values of total microbial counts in broiler litter detected in the current research (7-9 $\log_{10}$ CFU/g) are in accordance with the results of investigations published previously. For example, by cultivation on brain heart infusion (BHI) agar *Lu et al. (2003)* detected 10^9 aerobic bacteria per g of poultry litter, and *Tan et al. (2013)* approximately 5-7 $\log_{10}$ CFU/g. *Martin and McCann (1998)* found total bacterial counts ranging between 1.2 x 10^3 and 8.4 x 10^7, and *Terzich et al. (2000)* from 1.72 x 10^7 to 8.80 x 10^{11} on Triptic soy agar plates after 24 h incubation at 37°C.

From litter samples no bacteria of the *Salmonella* genus were isolated. *Salmonella* spp. counts are usually very low due to competition with other bacteria (*Omeira et al., 2006*), and since they are not part of the intestinal microbiome, they are highly unlikely to be found in litter (*Martin and McCann., 1998; Lu et al., 2003; Omeira et al., 2006; Wang et al., 2016*).

Clostridium perfringens was not detected in any of the 4 bedding material samples taken at the beginning of the research. *C. perfringens* spores were detected on day 7 (concentration 2 $\log_{10}$ CFU/g) in litter samples from both houses, which suggests that they are deposited to litter from the broilers' faeces. Litter may be an environment in which *C. perfringens* grows on condition anaerobic conditions are achieved, that is, if its porosity declines, which happens when the moistness and the compactness of the litter increase. The number of *C. perfringens* spores increased on day 14 to reach 3-4 $\log_{10}$ CFU/g and remained similar until the end of the experiment. *Tan et al. (2013)* detected similar numbers of *C. perfringens* in poultry litter in both gangrenous dermatitis (GD)-positive and GD-negative farms (from 1 to 3 $\log_{10}$ CFU/mL and 2 to 4 $\log_{10}$ CFU/g, respectively), but witnessed its tendency to decrease in the period from the 3rd to the 7th week. Decreasing numbers of *C. perfringens* has been explained by the its lower eliminated with chicken faeces (*Craven et al., 2001*). In addition, the enumeration pattern of *C. perfringens* in bedding does not always follow its occurrence pattern in faecal samples originated from chicks which are grown on the deep litter.

In the current research, the contamination level of the bedding material with moulds was 5-6 $\log_{10}$ CFU/g (Figure 1).

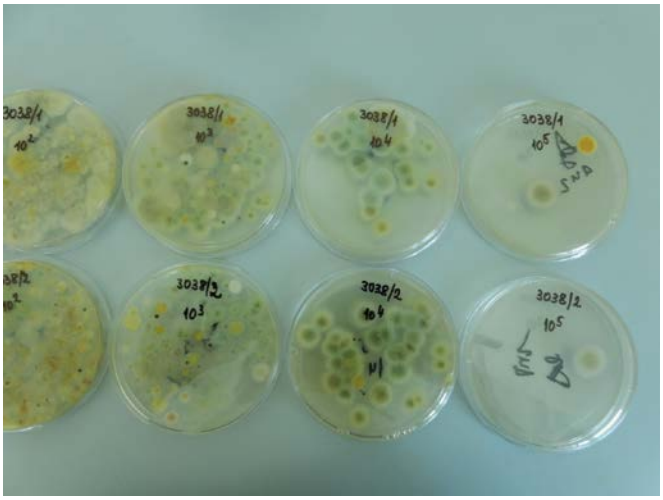

Figure 1. Mould colonies on DG18 agar isolated from ten-fold dilutions of bedding material (at the beginning of the experiment).

On day 14 from litter samples taken from house No. 2 moulds were isolated (Figure 2, plates in the top row), but from litter samples originating from house No. 1. on DG18 agar only yeasts were isolated (Figure 2, plates in the bottom row). Mould colonies were not noticeable, which is why their number was expressed as fewer than 3 $\log_{10}$ CFU/g (less than 1,000/g), because 100 μL of the first dilution ($1:10^{-2}$) was streaked on DG18 agar.

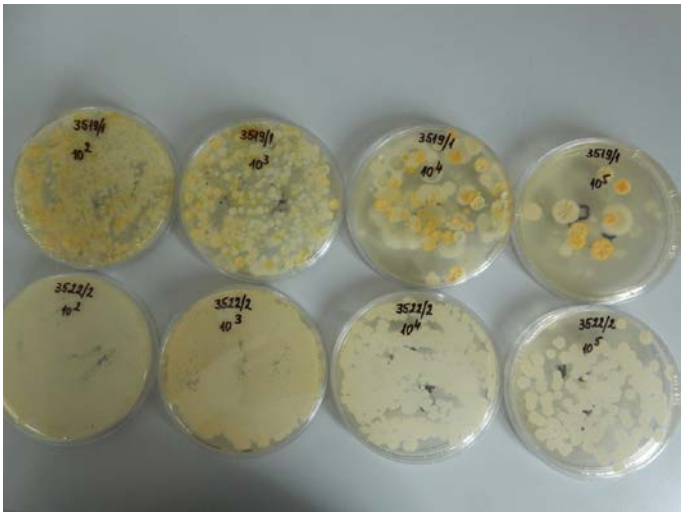

Figure 2. Mould colonies on DG18 agar isolated on day 14 from litter samples taken from house No. 2 (plates in the top row) and yeast colonies isolated from litter samples taken from house No. 1 (plates in the bottom row).

In litter samples taken from house No. 1 until the end of the experiment only yeast grow was detected, but not mould. With litter samples taken from house No. 2, an identical phenomenon occurred on days 28 and 35 (Figure 3). Only few investigations were conducted to detect the fungal contamination of poultry litter. The most frequent genus found was *Penicillium*, followed by *Alternaria*, *Cladosporium* and *Aspergillus* (*Viegas et al., 2012*). Fungal contamination of poultry litter (CFU/g) is in direct correlation with fungal contamination of the air (CFU/m^3), which poses health risks to exposed workers and animals. If inhaled, dust particles containing fungal spores may cause irritation, or even allergic and/or toxic respiratory diseases (*Viegas et al., 2012*). Moreover, spreading of poultry litter as fertiliser on agricultural land is a potential public health concern due to the possible dissemination of keratinophilic (*Scopulariopsi*s and *Fusarium* genus) and toxigenic fungi (*Aspergillus*, *Fusarium* and *Penicillium* genus).

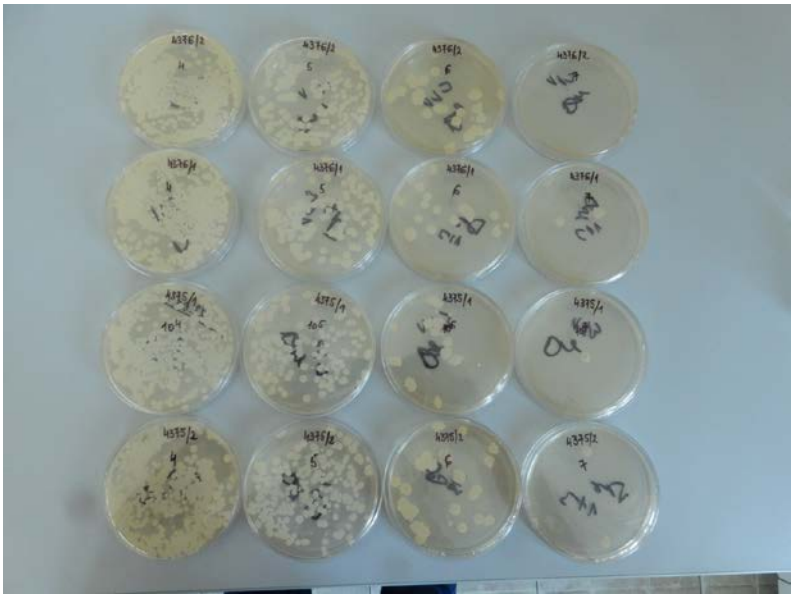

Figure 3. Mould colonies on DG18 agar isolated on day 28 from litter samples taken from both broiler houses

Based on cultural and microscopic characteristics (Figures 4A and 4B), the yeast isolate was identified as a species of the *Saccharomyces* genus. It was supposed to have ended up in the litter from the chicks' digestive tract, owing to the fact that it has been used as a probiotic culture in complete diet intended for fattening chickens. It is obvious that in litter the conditions are favourable for the survival of these yeasts, which account for the considerable part of total microbial

counts. These results point to the influence of probiotic cultures in complete broiler diets on the microbial composition in litter, which provides beneficial environment to chicken health.

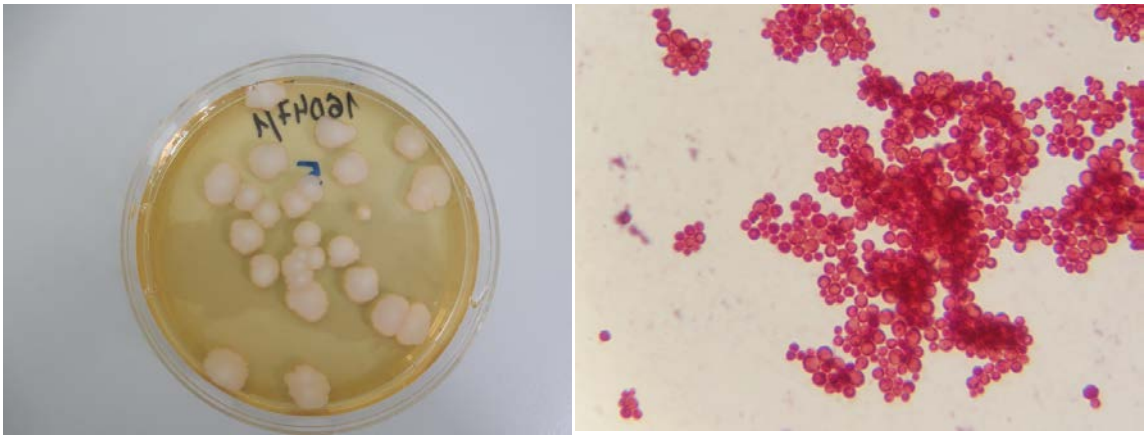

Figure 4. A. Yeast colonies on DG-18 agar; B. Microscopic view of yeast in a Gram-stain preparation (100x oil immersion objective)

Microbes normally present in the chicks' gastrointestinal tract will end up in litter. It is expected that the increase in the quantity of fresh faecal material (as it is being deposited) leads to an increase in the total number of microbes in litter. However, in the last taken samples (day 35), the total number of microorganisms was similar to that detected in bedding material before it was inhabited by the chicks. This result is in line with some others reported previously which claimed that the bacteria numbers in litter after a while remain similar, not depending on the number of flocks that have been grown on it (*Thaxton et al., 2003*). It was recognised earlier that the numbers of bacteria and fungi reach a peak in a months' time, when they decrease and remain roughly unchanged (*Schefferle, 1965*), which was confirmed in the current research. Microbial species vary in conditions they need for growth regarding nutrition and atmosphere conditions. The number of enteric bacteria decreases with time because litter is not an appropriate environment for the survival of coliform bacteria (*Thaxton et al., 2003; Omeira et al., 2006*). In litter some species involved in composting organic material were identified, which also explains the absence of species pathogenic to people and animals (*Lu et al., 2003*). Thus, it is not obligatory to lay new bedding material, but the same may be reused, lacing fresh litter shavings on top of the old litter. Repeated use of the same deep litter even several times does not pose particular

risk to the chicks' health, but influences the composition of their gastrointestinal microbiome (*Wang et al., 2016*). The digestive tract harbours a complex microbiota which is of immense importance to the functions of the immune system, defence against enteric pathogens and food digestion.

Conclusion

The total number of microorganisms in poultry litter reaches a peak in about a month from the beginning of the fattening period, which is followed by its decrease and maintenance at the levels established. Litter is not a favourable environment for coliform bacteria and moulds, and if sufficiently loose, does not enable the multiplication of *Clostridium perfringens*. Yeasts of the *Saccharomyces* genus survive successfully in litter, which is why their addition in the form of probiotic cultures to complete feed for fattening chicks influences the composition of the litter microbial composition, and thus indirectly provides more favourable environment conditions for chicks' health in a deep litter rearing system.

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Mikrobiološka kontaminacija prostirke tokom tova brojlera

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Rezime

U radu su prikazani rezultati ispitivanja mikrobiološke kontaminacije prostirke korišćene u tovu pilića. Prostirka je uzorkovana pre naseljavanja objekata i u nedeljnim intervalima tokom 35 dana tova pilića. Uzorci su ispitani na ukupan broj aerobnih mezofilnih mikroorganizama, kvasaca, plesni i spora *Clostridium perfringens*, kao i prisustvo bakterija roda *Salmonella*. Ukupan broj mikroorganizama u svežoj prostirci iznosio je 7 $\log_{10}$ CFU/g, a do 4 nedelje tova pilića povećao se do 9 $\log_{10}$ CFU/g. Međutim, na kraju pete nedelje tova, ukupan

broj mikroorganizama u prostirci bio je na nivou vrednosti ustanovljenih u svežoj prostirci. Spore *C. perfringens* su ustanovljene tek sedmog dana od naseljavanja objekta, što ukazuje da u prostirku dospevaju fecesom pilića. Za 14 dana broj spora *C. perfringens* se povećao do 3-4 $\log_{10}$ CFU/g i na približno istim vrednostima zadržao do kraja ispitivanja. Sveža prostirka bila je kontaminirana plesnima u nivou od 5-6 $\log_{10}$ CFU/g, ali od 21. dana iz uzoraka prostirke nisu izolovane plesni, već samo kvasci roda *Saccharomyces*. Pretpostavka je da su kvasci u prostirku dospeli fecesom pilića, jer se koriste kao probiotske kulture u smešama za njihov tov. Bakterije roda *Salmonella* nisu izolovane iz prostirke. Rezultati ispitivanja pokazuju da ukupan broj mikroorganizama u prostirci dostiže svoj pik za oko mesec dana, nakon čega se smanjuje. Prostirka je pogodna sredina za život probiotskih kultura kvasaca i njihovo dodavanje u smeše za tov brojlera može imati povoljan uticaj na sastav mikroorganizama u prostirci, a time i obezbeđenje zdravije životne sredine u podnom sistemu uzgoja brojlera.

Ključne reči: prostirka za piliće, tovni pilići, ukupan broj mikroorganizama, kvasci, plesni, *Clostridium perfringens*

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