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NOTE

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Susceptibility of chicken *Lactobacillus* bacteria to coccidiostats

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ABSTRACT. The aim of this study was to determine the susceptibility of *Lactobacillus* bacteria to selected coccidiostats. Seventy-five *Lactobacillus* isolates obtained from chickens were classified by MALDI-TOF mass spectrometry and 16S rDNA restriction analysis into seven species, among which *L. salivarius* (33%) and *L. johnsonii* (24%) were dominant. Susceptibility of lactobacilli to coccidiostats was determined by broth microdilution method. The ranges of minimum inhibitory concentrations (MICs) were 0.5–≥128 μg/ml for monensin, 0.125–8 μg/ml for salinomycin, ≤0.03–2 μg/ml for lasalocid A, and 4–16 μg/ml for robenidine. Coccidiostats in low concentrations inhibited *in vitro* growth of most lactobacilli and therefore there is a high probability that administration of this drugs to chickens would reduce the number of lactobacilli in the gut.

KEY WORDS: antibacterial activity, chicken, coccidiostats, ionophores, *Lactobacillus*

Lactobacillus bacteria are components of the natural intestinal microflora of animals, including birds. They have recently gained considerable attention due to their health-promoting effects. Lactobacilli maintain the microbiological balance of mucous membranes, improve digestion and nutrient assimilation, remove toxic substances, and enhance immunity. They prevent colonization of the intestine by enteropathogens via competition with other microorganisms for nutrients and for sites of adhesion to epithelial cells, as well as through production of bactericidal and bacteriostatic substances. This beneficial effect of lactobacilli translates into better feed utilization, faster weight gain, and increased production efficiency. The health-promoting effect of lactobacilli depends not only on the probiotic properties of the strains, but also on factors influencing the colonization and survival of these bacteria in the bird's intestine [7]. These factors may include antimicrobial substances, including coccidiostats [19].

Coccidiostats are drugs commonly used in the prevention and treatment of coccidiosis, a severe disease found in poultry worldwide, caused by a protozoan parasite of the genus *Eimeria*. Two groups of anticoccidials are generally considered– ionophorous antibiotics, or 'ionophores', and non-ionophore coccidiostats, also referred to as 'chemicals'. Ionophores are natural substances obtained by fermentation with *Streptomyces* spp. or *Actinomadura* spp., while chemicals are synthetically produced drugs [25]. The list of coccidiostatic and histomonostatic feed additives approved for use in poultry in the EU includes ionophores such as monensin, narasin, salinomycin, lasalocid, maduramicin, and semduramicin, and non-ionophore coccidiostats such as robenidine, halofuginone, diclazuril, nicarbazin and decoquinate [22].

Coccidiostats show antimicrobial activity not only against protozoa, but also against bacteria, mainly Gram-positive, including clostridia, lactobacilli, enterococci, staphylococci, and streptococci [10, 13, 16, 17]. Gram-negative bacteria are usually resistant to coccidiostats due to the protective role of their outer membrane [24]. Ionophores are highly lipophilic polyethers, which accumulate in cell membranes and catalyze rapid ion movement, principally sodium, potassium, and calcium [23]. The increased concentration of Na^+ ion inhibits certain mitochondrial functions, such as substrate oxidation and ATP hydrolysis. Moreover, the exchange of intracellular Na^+ ion for extracellular Ca^{++} and the consequent increase in the intracellular concentration of calcium ions lead to cytotoxicity [14]. The antimicrobial activity of ionophores is influenced by cation concentrations and pH in the medium. Lasalocid and monensin have been shown to be more inhibitory to *Streptococcus bovis* at pH 5.7 than at pH 6.7 [5]. The mode of action of non-ionophore coccidiostats is not precisely understood, although the activity of some anticoccidial quinolones and robenidine is known to depend on the disruption of electron transport in the cytochrome system of the mitochondria in coccidia (causing inhibition of sporozoite development), while decoquinate blocks DNA synthesis by inhibiting DNA gyrase [14].

The aim of our research was to assess the susceptibility of bacteria of the genus *Lactobacillus* isolated from poultry to selected coccidiostats. Existing literature data on this subject mainly date back to the 1990s and provide a summary of information obtained from studies carried out on a small number of strains. It is also significant that recent years have seen substantial changes in the

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systematics of *Lactobacillus* spp. as well as in bacterial resistance, so that research from the 20th century must be updated.

The *Lactobacillus* bacteria used in this study were isolated from fresh feces or cloacae of 20 healthy chickens (14 broilers and 6 Green-legged Partridge hens) from 6 large-scale poultry farms located in different parts of Poland. According to information from veterinarians supervising the flocks, the birds were vaccinated against coccidiosis and were not administered coccidiostats. Lactobacilli were cultured on deMan Rogosa Sharpe medium (MRS, BTL, Łódź, Poland) at 37°C for 48 hr in 5% CO_2. Pure cultures supplemented with 20% glycerol were stored at −80°C until further analysis.

Lactobacilli were identified to species level by MALDI-TOF mass spectrometry (MS) using a direct on-plate extraction method and an UltrafleXtreme MALDI TOF mass spectrometer (Bruker, Germany) [7]. In the case of ambiguous results, 16S rDNA restriction analysis was additionally used, as previously described [8].

The antibiotic susceptibility of all bacterial isolates was determined by the broth microdilution method using LSM medium in accordance with ISO 10932|IDF 223:2010 [12]. Coccidiostats, i.e. monensin, salinomycin, lasalocid A and robenidine, were obtained from Sigma-Aldrich (Poland). Stock solutions (10 mg/m*l*) were prepared by dissolving the drugs in methanol (monensin and salinomycin) or in DMSO (robenidine). The ready-to-use lasalocid A solution had a concentration of 100 μg/m*l*.

Prior to antimicrobial susceptibility testing, cultures were streaked on LSM agar and incubated for about 20 hr at 37°C in 5% CO_2. Inocula were prepared by suspending bacteria in 0.85% NaCl to the turbidity of a 0.8 McFarland standard using DENSI-LA-METER II (Erba, Czech Republic). Microdilution plates were inoculated with 50 μl of a 1:500-diluted (in LSM broth) inoculum and 50 μl of the appropriate antibiotic concentration. After the plates had been incubated at 37°C in 5% CO_2 for 48 hr, the minimum inhibitory concentration (MIC) values were read as the lowest concentration of the antimicrobial agent at which visible growth was inhibited.

A total of 75 isolates were classified by MALDI-TOF MS as bacteria of the genus *Lactobacillus*, with a Biotyper log (score) equal to or greater than 1.70. Eighteen isolates for which MALDI-TOF MS identification results were ambiguous, i.e. *L. johnsonii*/*L. gasseri*, were further identified by 16S rDNA restriction analysis using the *Mse*I restriction enzyme. The analysis showed that all these isolates belonged to the species *L. johnsonii*. Finally, the *Lactobacillus* isolates were classified into seven species: *L. salivarius* (n=25), *L. johnsonii* (n=18), *L. ingluviei* (n=8), *L. agilis* (n=3), *L. reuteri* (n=8), *L. crispatus* (n=10), and *L. saerimneri* (n=3).

The range of MIC values was different for individual coccidiostats, i.e.: 0.5–≥128 μg/m*l* for monensin, 0.125–8 μg/m*l* for salinomycin, 4–16 μg/m*l* for robenidine and ≤0.03–2 μg/m*l* for lasalocid A. In contrast to the ionophores, robenidine had a narrow range of MIC values, which in 99% of the isolates were 4 or 8 μg/m*l*. The widest range of MIC values was recorded for monensin; the results may indicate that *L. reuteri* isolates are less sensitive to this ionophore antibiotic (MIC 8–32 μg/m*l*) than other *Lactobacillus* species (Table 1).

The coccidiostat MIC values obtained are partly consistent with the results reported by other authors. Rada *et al*. [18] noted growth inhibition of all *Lactobacillus* isolates used in their study (mainly *L. fermentum*, n=14) at a 10 μg/m*l* concentration of monensin, lasalocid, salinomycin and narasin. In a later study, involving four *Lactobacillus* isolates, the same research team determined the MICs for the ionophores using MRS broth. They ranged from 0.4 to 5 μg/m*l* for monensin, narasin, lasalocid, salinomycin and maduramicin [16]. We obtained similar results for salinomycin and lasalocide, where MICs reached 8 and 2 μg/m*l*, respectively. The range of MIC values for monensin in the present study was much wider (0.5–≥128 μg/m*l*) than that observed by the Czech team, but reliable comparison of the results is not possible due to the very small number of strains used in the Czech study. A wide range of monensin MICs, i.e. 0.5–32 μg/m*l*, has also been noted by Aarestrup *et al*. [1] for *E. faecium* isolates from Scandinavian broiler and pig farms. According to the cut-off point proposed by these authors (16 μg/m*l*), as much as 34.7% of the *Lactobacillus* isolates tested could be classifies as monensin resistant. Increased monensin MICs observed in this work may be due to bacterial extracellular polysaccharides (the glycocalyx) that exclude ionophores from the cell membrane [23]. As the production of extracellular polysaccharide by lactobacilli is strain-specific, some strains may be less susceptible to monensin than others [15]. The sensitivity of Gram-positive bacteria colonizing the rumen to monensin has been shown to decrease following repeated exposure to this ionophore (change in MIC value from 4 to 128 μg/m*l*). Moreover, this effect was found to be reversible, as upon consecutive subculturing, monensin MICs returned to the baseline, or one dilution above, for the monensin-adapted strains [4, 26]. Monensin in low concentrations, i.e. 1–4 μg/m*l*, inhibited also lactic acid bacteria used as dairy starter cultures and the strain found to be most susceptible to monensin was *Lactobacillus acidophilus* La-5 (MIC=1 μg/m*l*) [11]. Dennis *et al*. [9] have shown that even such low doses of monensin and lasalocid as 0.38–3.0 μg/m*l* inhibit most lactate-producing rumen bacteria, including *Lactobacillus ruminis*.

The robenidine MIC values presented in this study (4–16 μg/m*l*) deviate significantly from the results reported by Rada *et al*. [21], who observed growth inhibition of two *Lactobacillus* isolates (*L. casei* and *L. salivarius* from chicken) at much higher concentrations of this non-ionophore coccidiostat, i.e. 90–110 μg/m*l*. For monensin, low MIC values of 2 μg/m*l* were noted for the same strains. Our results are more consistent with the data provided by the Panel on Additives and Products or Substances in Animal Feed [10], according to which lactobacilli are inhibited by robenidine at concentrations of 8–32 μg/m*l*. Similar MIC ranges of this drug were obtained for other Gram-positive bacteria, including streptococci (8–32 μg/m*l*), enterococci and staphylococci (8–16 μg/m*l*), and clostridia (1–8 μg/m*l*) [10].

No clear bimodal distribution of MIC values that might indicate acquired resistance in the lactobacilli was observed for any antimicrobial substance used in this study. This is in line with the results of many studies confirming the high susceptibility of Gram-positive bacteria (*Enterococcus* spp., *C. perfringens*, *P. acidilactici* and *S. aureus*) to coccidiostats [1, 2, 6, 17, 27]. Genes responsible for ionophore resistance in bacteria have not been identified [23]. However, some studies report the presence of

Table 1. Distribution of minimum inhibitory concentrations (MICs) of coccidiostats among various *Lactobacillus* species of chicken origin

		≤0.03	0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	≥128
Monensin	*L. salivarius* (n=25)						1	12	6	2	3	1		
	L. agilis (n=3)					1	1		1					
	L. johnsonii (n=18)						1	6	4	1	3	2	1	
	L. crispatus (n=10)							2		1	2	1	3	1
	L. reuteri (n=8)									1	1	6		
	L. ingluviei (n=8)					1	2	2		1	2			
	L. saerimneri (n=3)					2	1							
	All (n=75)					4	6	22	11	6	11	10	4	1
Salinomycin	*L. salivarius* (n=25)				1	5	7	8	4					
	L. agilis (n=3)					1	1	1						
	L. johnsonii (n=18)					1	11	5	1					
	L. crispatus (n=10)			1		1		3	5					
	L. reuteri (n=8)					1	2	2	1	2				
	L. ingluviei (n=8)				1	4		3						
	L. saerimneri (n=3)				3									
	All (n=75)			1	5	13	21	22	11	2				
Lasalocid A	*L. salivarius* (n=25)		2	3	2	13	5							
	L. agilis (n=3)		1	2										
	L. johnsonii (n=18)			1	3	9	5							
	L. crispatus (n=10)			*2*		1	3	4						
	L. reuteri (n=8)				2	1	3	2						
	L. ingluviei (n=8)		3	1	1		3							
	L. saerimneri (n=3)	1		2										
	All (n=75)	1	6	11	8	24	19	6						
Robenidine	*L. salivarius* (n=25)								3	21	1			
	L. agilis (n=3)								1	2				
	L. johnsonii (n=18)								7	11				
	L. crispatus (n=10)								7	3				
	L. reuteri (n=8)									8				
	L. ingluviei (n=8)								2	6				
	L. saerimneri (n=3)									3				
	All (n=75)								20	54	1			

ionophore resistance (mainly to salinomycin, less often to narasin and monensin) in lactic acid bacteria other that *Lactobacillus* spp. [1, 3, 28, 29]. Butaye *et al*. [3] noted bimodal distribution of narasin MICs indicating acquired resistance and cross-resistance to salinomycin and narasin in enterococci from Belgian farms.

It is worth noting that the *in vitro* inhibitory effect of coccidiostats on the growth of *Lactobacillus* bacteria has not always been reflected *in vivo*. Rada and Mauronek [20] showed that administration of maduramicin and monensin to newly hatched chickens at 5 and 100 mg per kg of diet, respectively, did not eliminate lactobacilli. They were still present in high numbers in the crop and caecal contents of chickens 5 days after inoculation with *L. salivarius* strain 51R. In another experiment, the same authors [19] found that the addition of monensin to the feed (100 mg/m*l*) significantly lowered the number of lactobacilli and decreased the concentrations of organic acids in the crop contents. Increased intestinal pH was accompanied by proliferation of coliforms and enterococci. The lactobacilli counts returned to the pretreatment level within 5 days of monensin administration, but the counts of coliforms and enterococci remained elevated. Changes in the composition of chicken microflora have also been observed following the administration of robenidine at 44 mg/kg. The coccidiostat delayed colonization with lactobacilli and depression of *E. coli* counts during the first two weeks of life [10].

In summary, coccidiostats such as monensin, salinomycin, lasalocid and robenidine in low concentrations inhibit *in vitro* growth of most *Lactobacillus* bacteria from chickens. There is therefore a probability that administration of this type of drugs to chickens would reduce the number of lactobacilli in the gut, which could translate into a microbial imbalance of the mucous membranes. Therefore, it seems advisable to correlate coccidiostatic therapy in poultry with the administration of microbial feed additives, i.e. probiotic *Lactobacillus* strains. However, it should be emphasized that *in vitro* results do not always translate into *in vivo* effects, and confirmation of the inhibitory effect of coccidiostats on intestinal lactobacilli in chickens requires further testing using animal models. Data derived from this study may contribute to the establishment of standards for categorization of susceptible and resistant strains of *Lactobacillus* genus in reference to coccidiostats.

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