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Antibiotic Resistance in Commensal Intestinal Microflora

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ABSTRACT. The susceptibility toward antibiotics was determined by disc and MIC methods in *Lactobacillus* and, for comparison, in *Escherichia coli* strains isolated from cloacal swabs of broiler chickens derived from various farms in Slovakia. The occurrence of acquired tetracycline resistance in *E. coli* and lactobacilli isolated from the same sample was similar. The presence of *tet*(M), *tet*(S), *tet*(L) and *ermB* genes was demonstrated in lactobacilli while the *tet*(M) gene was not detected in *E. coli*.

Abbrevations

GIT gastrointestinal tract MIC minimum inhibitory concentration MICxG geometric mean of MIC

Bacteria of *Lactobacillus* species are capable of colonizing habitats as diverse as fresh and fermented plant materials, meat and meat products, fish, dairy, sourdoughs, fermented beverages and the human and animal GIT (Bernardeau *et al.* 2006). The majority of studies, including national monitoring programs, focused on selection and dissemination of antibiotic resistance in indicator intestinal bacteria, *e.g.*, *Escherichia coli* and enterococci, not taking into account that lactobacilli may also act as reservoirs of antibiotic resistance genes.

Intrinsic resistance involves the absence of the target or the presence of low-affinity targets, low cell permeability, antibiotic inactivation of the antibiotics and the presence of efflux mechanisms. The acquisition of antibiotic resistance occurs *via* the mutation of pre-existing genes or by horizontal transmission (*FEEDAP Panel* 2005). Intrinsic resistance is generally accepted to be nontransferable while acquired resistance may be more easily transferred to other bacterial species (Rosander *et al.* 2008). Distinguishing between intrinsic and acquired resistance is therefore essential.

As a general rule, lactobacilli have a high intrinsic resistance to cefoxitin, ciprofloxacin, kanamycin, gentamicin, streptomycin, teicoplanin, cotrimoxazol, and vancomycin (Danielsen and Wind 2003). Cefoxitin resistance was observed in most *Lactobacillus* isolates with MIC levels 4–64 mg/L and penicillin susceptibility with MIC levels 0.06–0.25 mg/L or penicillin resistance with MIC 4 mg/L (Delgado *et al*. 2007).

Multiplex PCR analyses revealed that the most common genes found in *E. coli* (from pigs and chickens) were *tet*(B) (63 %) and *tet*(A) (35 %); however, *tet*(C), *tet*(D), and *tet*(M) were also found (Bryan *et al.* 2004). The *tet*(M) gene is widely dispersed among various Gram-positive organisms (including lactobacilli); it has been only rarely documented in Gram-negative bacteria.

The aim of our study was to determine the susceptibility of *Lactobacillus* and *E. coli* isolated from the same sample and the detection of antibiotic resistance genes, especially *tet*(M) in both species.

MATERIAL AND METHODS

A total of 64 isolates lactobacilli and 61 isolates *E. coli* from cloacal swabs of broiler chickens from various farms in Slovakia were investigated; the samples from the same farm were compared. Suspect *Lactobacillus* strains from Rogosa agar were confirmed by species specific PCR (Dubernet *et al*. 2002). Diagnostics of *E. coli* was performed by inoculation in Triple Sugar Agar (*ImunaPharm*, Slovakia) and by Enterotest 24 (*Pliva-Lachema*, Czech Republic).

Six antibiotic discs (*Oxoid*), tetracycline (TTC; 30 μg), erythromycin (ERY; 15 μg), gentamicin (GEN; 10 μg), streptomycin (STM; 30 μg), cefoxitin (FOX; 30 μg) and penicillin (PNC; 10 IU) were used for determination of antibiotic resistance. Cell suspensions of lactobacilli (1.0 McFarland) were inoculated on the surface of MRS agar and incubated in CO_2 atmosphere for 24 h. Susceptibility testing of lactobacilli was conducted by disc-method diffusion according to the guidelines of *CLSI* (2008) for *Staphylococcus aureus*.

Interpretative zone diameter for resistance to streptomycin, which is not stated in the *CLSI* (2008) guidelines, was considered as ≤14 mm (Kim *et al.* 2004).

The minimum inhibitory concentrations of ampicillin (AMP), ampicillin with sulbactam (A+IB), ceftiofur (CFF), cefquinom (CFQ), STM, GEN, ciprofloxacin (CIP), enrofloxacin (ENR), TTC, chloramphenicol (CMP), florfenicol (FLO), cotrimoxazol (COT) were determined in each *E. coli* strain using colorimetric microdilution test. The test is a modification of standard *CLSI* (2008) microdilution method that uses 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye for detecting the viable bacteria (Gatringer *et al*. 2002).

The determination of the genetic background for *Lactobacillus* antimicrobial resistance was done using PCR reactions and specific primers for antimicrobial resistance determinants (Table I).

Table I. Primers used

Gene	Sequence (5′→3′)	Product size bp	Annealing temperature, °C	Reference
Genus specific Lactobacillus	CTC AAA ACT AAA CAA AGT TTC CTT GTA CAC ACC GCC CGT CA	250	55	Dubernet *et al.* 2002
ermB	CTA TCT GAT GTT GAA GAA GGA TT GTT TAC TCT TGG TTT AGG ATG AAA	142	55	Martineau *et al.* 2000
tetM	GTG GAC AAA GGT ACA ACG AG CGG TAA AGT TCG TCA CAC AC	406	55	Ng *et al.* 2001
tetL	TCG TTA GCG TGC TGT CAT TC GTA TCC CAC CAA TGT AGC CG	267	55	
tetS	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	667	55	
aac(6′)-Ie-aph(2″)-Ia	CAG AGC CTT GGG AAG ATG AAG CCT CGT GTA ATT CAT GTT CTG GC	348	55	Vakulenko *et al.* 2003

RESULTS AND DISCUSSION

Among tested *E. coli* isolates high resistance rates (largely more than 30 %) were recorded to AMP, TTC, STM, CIF, ENR and COT (Table II). Resistance to gentamicin (6.5 %) and chloramphenicol (3.2 %) was lower. All of the tested *E. coli* isolates were sensitive to A+IB, CFF, CFQ and FLO. The high level MICs of CIP and ENR were probably observed as a consequence of the frequent use the floroquinolones in the therapy of broilers. ESBLs were not found.

Table II. Antibiotic (ATB) resistance (R) and minimum inhibitory concentrations in *Escherichia coli*

ATB	R, %	MICxG	MIC_{90}
AMP	47	10.9	128
A+IB	0	3.1	8
CFF	0	0.3	1
CFQ	0	0.4	2
GEN	6.5	0.5	4
STM	34	14.1	256
CIP	42	0.9	8
ENR	31	2.1	32
TTC	31	3.2	32
CMP	3.2	2.4	8
FLO	0	2	4
COT	27	9.6	128

Table III. Comparison of antibiotic (ATB) resistance in 64 lactobacilli and 61 *Escherichia coli* strains

ATB class	ATB	*Lactobacillus* resistance, %	*E. coli* resistance, %
β-Lactams	PNC	0	nt[a]
	AMP	0	47
	FOX	3.1	nt[a]
Aminoglycosides	STM	60	34
	GEN	43	6.5
Tetracyclines	TTC	28	31
Macrolides	ERY	29	nt[a]

[a]Not tested.

Our effort in the investigation of antibiotic resistance in lactobacilli isolated from the same swab samples as *E. coli* was oriented to TTC and ERY, whose resistances are most frequently reported in the intes-

tinal and probiotic *Lactobacillus* strains. The rate of TTC resistance in lactobacilli (Table III) was very similar to TTC resistance in *E. coli* (28 *vs*. 31 %). A high rate of ERY resistance (31 %) was also observed in fecal lactobacilli from chicken broilers. Both (TTC and ERY resistance) are considered to be an acquired resistance in lactobacilli. Moreover, genes identical to those described in other bacteria, *e.g*., staphylococci and enterococci, from the same environments [*tet*(M), *tet*(O/W), *tet*(W), *erm*(B)] were identified in dairy and intestinal lactic acid and bifidobacterial strains (Ammor *et al*. 2008). In *E. coli* we found *tetA* (50 %) or *tetB* (10 %) (*data not shown*); on the other hand, *tetM* was not detected.

The presence of *tet*(M), *tet*(S), *tet*(L) and *erm*B genes in our group of lactobacilli was detected by PCR. Partial sequencing revealed that the *ermB* genes found in chicken *Lactobacillus* strains belong to *ermB* sequence homology, previously found in enterococci (*GenBank* EU595407.1).

Lactobacilli were sensitive to β-lactams, including FOX, with only 3.1 % resistance (Table III). Because there are no data concerning the presence of β-lactamase genes in lactobacilli they seem to be sensitive to penicillins and to AMP with MICs from 0.12 to 2 mg/L (Danielsen *et al.* 2003).

A high rate of GEN resistance (47 %) was observed in lactobacilli, however, the *aac(6′)-Ie-aph(2″)-Ia* gene was not detected by PCR. Similar negative results with the detection of *Lactobacillus* GEN- and STM-resistance genes were described also by Ammor *et al.* (2008). However, Tenorio *et al.* (2001) described these bifunctional enzymes in seven GEN-resistant lactobacilli and one *Pediococcus* strain of animal origin.

Further more detailed investigations should better distinguish between acquired and intrinsic resistance in probiotic lactobacilli and enterococci. When the resistance has been acquired by a strain belonging to a taxonomic group naturally susceptible to an antimicrobial, then the degree of transfer risk is generally considered to be substantially greater than that associated with intrinsic resistance (*FEEDAP Panel* 2005). DNA sequencing of PCR products showed that a *Lactobacillus rhamnosus* strain of human origin is resistant to macrolides but no resistance determinants have been detected by specific PCR and/or microarray screening; the strains were shown to contain a heterozygous A→G transition mutation at position 2058 of its 23S rRNA genes (Florez *et al*. 2007). Lauková *et al*. (2008) described a potential probiotic *E. faecium* strain EE3, which was resistant to ERY and TTC; on the other hand, lactobacilli isolated from milk products on Slovak market (yogurt, soft cheese bryndza, fermented milk) were sensitive to TTC and ERY (Demankova and Kmet 2008).

Determination of the molecular mechanisms of antibiotic resistance in commensal bacteria, especially probiotic lactic acid bacteria, would be essential for the control of the resistance spread *via* the food chain.

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