ORIGINAL ARTICLE

Comparative Analysis on Antibiotic Resistance Characteristics of *Listeria* spp. and *Enterococcus* spp. Isolated From Laying Hens and Eggs in Conventional and Organic Keeping Systems in Bavaria, Germany

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Impacts

- Structural changes in the laying hen husbandry raise numerous questions both for supporters and opponents of organic keeping systems.
- Arguments of opponents concerning a higher contamination with zoonotic bacteria could not be endorsed, as in this study, prevalence of Listeria [1.3% in organic (org) and 1.8% in conventional keepings (conv)] and Enterococcus [$95.5\%_{(org)}$ and $97.5\%_{(conv)}$] was much the same in both management systems.
- Almost all isolated Listeria were susceptible to all tested antibiotics. In contrast, antimicrobial resistance rates of Enterococci isolated from organic keeping systems tended to be lower than from conventional ones ($P < 0.05$) which confirms the theory of the supporters of organic farming. Besides animal welfare, these results demonstrate that organic farming contributes to further efficacy of antibiotics.

Keywords:
Laying hens; antibiotic resistance; minimal inhibitory concentration (MIC); conventional keeping; organic keeping; Gram-positive bacteria

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Summary

By investigating the prevalence and antimicrobial resistance characteristics of Gram-positive bacteria from organic and conventional keeping systems of laying hens, it was to be determined to what extent these properties are influenced by the different systems. For this purpose, a total of 799 cloacal swabs and 800 egg samples were examined. Prevalences for all selected bacteria from cloacal swabs were much the same for both organic and caged birds: *Listeria* spp. $1.3\%_{[org]}$ versus $1.6\%_{[con]}$; *Enterococcus* spp. $95.5\%_{[org]}$ versus $97.5\%_{[con]}$. Egg contents and eggshells were generally contaminated to a lesser extent, primarily with *Enterococcus* spp. *Listeria* isolates were susceptible to almost all tested antibiotics, only three *Listeria innocua* from conventional keepings were resistant to clindamycin; one isolate additionally to imipenem. High percentages of *Enterococcus faecalis* were resistant to doxycycline and macrolides. *Enterococcus faecium* proved to have high resistance rates to clindamycin, fosfomycin and erythromycin; 9.1% were even resistant to the reserve antibiotic synercid. Further, *Enterococcus* spp. showed higher resistance rates to doxycycline, erythromycin, fosfomycin and rifampicin. No glycopeptide resistant enterococci were detected. A correlation between keeping system and resistance/susceptibility rates could be demonstrated. In detail, *E. faecalis* from organic laying hen husbandries showed significant lower resistance prevalences to tylosin, streptomycin and doxycycline; susceptibility rates were higher for enrofloxacin and ciprofloxacin. Rifampicin and imipenem were more effective in isolates from conventional keepings ($P < 0.05$). The amounts of resistant isolates of the *Enterococcus raffinosus* from organic farms were significantly lower, the amounts of sensitive isolates were significantly higher than from conventional

farms concerning eight antibiotics ($P < 0.05$). When comparing the susceptibility/resistance rates, as well as the mean minimum inhibitory concentrations values, the consistent tendency is that bacteria from organic layer flocks are more susceptible to antimicrobials. These results show that organic livestock farming plays a part in contributing to reduced antibiotic resistance.

Introduction

Structural changes in the laying hen husbandry in Germany, as well as in the European Union, raise numerous questions for contracting parties. The EU-Eco-regulation (EEC N° 2092/91, supplemented by regulation (EC) N° 1804/1999), defines and regulates the term 'organic production'. Important matters are among other things the exclusive usage of in-house produced feeding stuff, fertilization with antibiotic-free manure, as well as a total ban on growth promoters and prophylactic antibiotic application. There are various controversial subjects from both supporters of conventional and organic farming concepts. The mean point of criticism for opponents of organic keeping systems states that free-ranging animals are rumoured to be more highly contaminated with zoonotic bacteria, which could represent a health risk for the end-user (Methner, 2004). Counter-arguments of proponents are animal welfare on the one side and beyond that, the strictly limited drugs application is supposed to have a positive effect for the consumer, such as reduced antibiotic residues (Lund, 2006). This study should clarify, if organic laying hen husbandries are more highly contaminated with Gram-positive zoonotic and commensal bacteria than conventionally reared flocks. Furthermore, a subsequent investigation of the resistance properties should reveal if the different keeping concepts exert influence on the effectiveness of antibiotics. For these purposes, selected Gram-positive bacteria from both systems were isolated, differentiated and tested for their phenotypic resistance to various antibiotics.

Materials and Methods

Samples

A total of 799 cloacal swabs and 800 eggs were examined from 10 organic and 10 conventional randomly selected Bavarian laying hen farms. Periodical randomized sampling (four times each) was accomplished in quarterly periods from January 2004 to April 2005. For the cloacal swabs, 10 laying hens were randomly selected and a sterile swab was directly inserted into the cloaca. After that, the swab was immediately preserved in tubes containing 1 ml of Amies transport medium (COPAN; Sarstedt, Nürnbrecht, Germany). Eggs were randomly chosen directly from the collecting conveyer and stored in new egg cartons. All samples were transported in a cooling box and kept cool at 4°C until preparation (maximum 72 h after sampling). Eggs were cracked with a sterile laboratory glass after disinfection of the predetermined breaking point with ethanol 90% to avoid cross contaminations. Further procedures were carried out as previously described (Schwaiger et al., 2008).

Selection of Gram-positive bacteria

Enterococci are generally classified as low pathogenic. However, they are also considered as emerging pathogens associated with nosocomial infections (Peters et al., 2003). Furthermore, Enterococci have a strong tendency to develop new antimicrobial resistances. Therefore, *Enterococcus* spp. were chosen as a spectrum of Gram-positive microorganisms. This meets the requirements of the World Health Organisation (WHO, 1997) and the Office International des Epizooties (OIE; Franklin et al., 2001), that recommend inclusion of so-called indicator and reservoir bacteria generally into resistance surveillances. Following further recommendations of the OIE, *Listeria* spp. were also monitored in this study because of their potential role as pathogenic and zoonotic Gram-positive bacteria. This is in accordance to a previous study, where we selected the pathogenic Gram-negative bacteria *Campylobacter* spp. and *Salmonella* spp. (Schwaiger et al., 2008).

Listeria spp.

According to DIN EN 11290-1 (1996), 1 ml of the swab suspension, 25 g of the pooled egg samples and 10 g of the eggshell samples were incubated (24 h, 30°C) at a ratio of 1 : 10 in Demi Fraser Broth (containing one tube of Fraser Listeria Selective Enrichment Broth Base and Fraser Listeria Supplement in 500 ml total volume; Merck, Darmstadt, Germany). After that, 0.1 ml of this pre-enriched sample was transferred into 10 ml of Fraser Broth (like Demi Fraser, but containing two tubes of Fraser Listeria Supplement in 500 ml total volume; 24 h, 37°C). Of the enriched broths, 100 μl (egg/eggshell) and 300 μl (swabs) were plated on both PALCAM and Oxford Listeria Selective Agar (Merck) and incubated (37°C; 24–48 h). Suspicous colonies were subcultivated on Standard I Nutrient Agar (Merck) and differentiated (Gram-staining, motility testing, catalase-reaction). The final

identification was given by API® Listeria (Biomèrieux, Nürtingen, Germany).

Enterococcus spp.
Swab suspensions, pooled egg samples and eggshells, respectively, were enriched in buffered peptone water (Merck; 37°C, 24 h). Each one loop of the pre-enriched samples was smeared out on Citrate Azide Tween Carbonate Agar (Merck) and incubated (37°C; 24 h). After subcultivation of the red colonies, Gram-staining and catalase-reaction were carried out. Phenol red containing solutions with sodium pyruvate (Pyr), arabinose (Ara), xylose (Xyl), mannite (Man), respectively, were inoculated with the strains. According to Bejuk et al. (2000), colour change reactions after incubation (37°C, 24 h) were basis for classification into the species groups *Enterococcus faecalis* (Pyr+, Ara−, Xyl−, Man+), *Enterococcus faecium* (Pyr−, Ara+, Xyl−, Man+), *Enterococcus raffinosus* (Pyr+, Ara+, Xyl+, Man+), *Enterococcus avium* (Pyr+, Ara+, Xyl−, Man+), *Enterococcus hirae/durans* (Pyr−, Ara−, Xyl−, Man−) and *Enterococcus gallinarum/flavescens/mundti/casseliflavus* (*E. gfmc* group; Pyr−, Ara+, Xyl+, Man+).

Determination of the phenotypic antibiotic resistance, data processing and statistical analysis

Phenotypic resistance was determined according to DIN 58940-4/1 (2004, 2002) and as previously described (Schwaiger et al., 2008), with a few modifications: Adjusting of overnight cultures to McFarland 0.5 was controlled photometrically. Hereof, 100 μl were suspended in 13-ml Mueller–Hinton (MH) Bouillon (Becton–Dickinson, Heidelberg, Germany), which was supplemented with one vial of Haemophilus test medium (Oxoid, Wesel, Germany) per 500 ml for a better growth of *Listeria* spp. and *Enterococcus* nonfaecalis/nonfaecium. One-hundred microlitre was applied into the antibiotic-containing wells of microtitreplates (MERLIN; Table 1) with the aid of a Micronaut® automatic dispenser (MERLIN, Bornheim-Hersel, Germany). *Listeria monocytogenes* DSM 20600 and ATCC 2482, *Listeria innocua* ATCC 14298, *Listeria welshimeri* SL 5324, *Enterococcus faecalis* DSM 2570 and *Enterococcus casseliflavus* DSM 20680 were used as reference strains. The minimal inhibitory concentration (MIC) were read photometrically via Micronaut®-scan-system. A comparison of the MIC with certain breakpoints of the

Table 1. Investigated antibiotics with ranges of concentration and breakpoints

Names of antibacterials in group	Abbreviation	Concentration range (mg/l)	*S* (≤)	*R* (>)	Source of breakpoint*
Amoxicillin/clavulanic acid	AMC	0.125/2–8/2	2/2	8/2	DIN
Ampicillin	AMP	0.125–16	2	8	DIN
Mezlocillin	MZL	2–256	4	16	DIN
Oxazillin	OXA	0.25–32	1	1	DIN
Imipenem	IMP	0.125–16	2	4	DIN
Chloramphenicol	CMP	2–64	16	16	DANMAP 2004
Florfenicol	FLL	2–32	16	16	DANMAP 2004
Ciprofloxacin	CIP	0.25–32	1	2	DIN
Enrofloxacin	ENR	0.0625–8	0.25	2	NCCLS
Moxifloxacin	MOX	0.0625–8	1	2	DIN
Teicoplanin	TPL	0.25–32	8	16	NCCLS
Vancomycin	VAN	0.5–64	4	8	DIN
Gentamicin high level	GNH	512	512	512	DIN
Kanamycin	KAN	8–64	32	32	DANMAP 2004
Neomycin	NEO	2–32	8	8	DANMAP 2004
Streptomycin high level	SNH	256–2048	1024	1024	DANMAP 2004
Erythromycin	ERY	0.0625–8	1	4	DIN
Tylosin	TLS	0.5–8	4	4	DANMAP 1997
Clindamycin	CLI	0.0625–8	1	4	DIN
Linezolid	LIZ	0.125–16	2	4	Manufacturer
Quinupristin/dalfopristin	SYN	0.125–16	1	2	NCCLS
Doxycycline	DOX	0.125–16	1	4	DIN
Fosfomycin	FOS	8–64	32	32	SFM
Nitrofurantoin	NFT	32–256	64	256	DIN
Rifampicin	RAM	0.5–4	1	2	NCCLS

*DIN, Deutsches Institut für Normung; DANMAP, Danish Integrated Antimicrobial Resistance Monitoring and Research Programme; NCCLS, National Committee on Clinical Laboratory Standards; SFM, Comité de l'Antibiogramme de la Société Française de Microbiologie.

concerning antibiotic allows a classification into susceptible, intermediate or resistant (Table 1). Data were collected and statistically evaluated as previously described (Schwaiger et al., 2008).

Results

Prevalence of the selected bacteria

Cloacal swabs were considerable more frequently positive for the selected bacteria than egg/eggshell samples in both keeping systems. For example, more than 95% of the swabs were positive for *Enterococcus* spp., but only in 20% of the pooled egg samples enterococci were detected. The prevalence of the considered bacteria was in the same order of magnitude for the different keeping systems (Table 2). Prevalence of *Listeria* spp. was generally low. In detail, *Listeria innocua* were isolated from five cloacal swabs (1.3%) of organic kept laying hens and from seven conventional hens (1.8%) respectively. Only one pooled egg sample from a conventional keeping system was positive for *Listeria seeligeri*. In contrast, enterococci could be found in most (>96%) of the cloacal swabs. In more than 30%, the presence of various *Enterococcus* spp. in one single swab was detectable. Enterococci were also detected in pooled egg (21%) and eggshell samples (53%).

Phenotypic antibiotic resistance of the selected bacteria

For the presentation of the results of the phenotypic resistance analysis there are only antibiotics displayed where no intrinsic resistances of the concerning genera or species are known. For statistical analyses only bacteria from cloacal swabs were evaluated, as the minor quantity of isolates from eggs could not provide significant results.

Listeria spp.

Almost all isolated *Listeria* spp. were susceptible to all tested antibiotics. Only three *L. innocua* from conventional keepings were resistant against clindamycin. Hereof, one isolate was additionally resistant to imipenem.

Enterococcus faecalis

The highest resistance rates of *E. faecalis* were observed for doxycycline (54.8%), streptomycin high level (18.0%), erythromycin (28.0%) and tylosin (26.2%). All further agents were more potent (resistance rates 0.3–11.3%). Ampicillin, chloramphenicol, mezlocillin, nitrofurantoin, florfenicol, teicoplanin and vancomycin were fully efficient in all tested isolates (Table 3). *Enterococcus faecalis* of organically raised hens had significantly lower resistance rates ($P < 0.05$) for three of 20 antibiotics, namely doxycycline, streptomycin high level and tylosin. In contrast, the resistance rate for rifampicin was lower in isolates from conventional keepings ($P < 0.05$). Considering the susceptibility, the rates in organic isolates resulted to be higher for ciprofloxacin, doxycycline and enrofloxacin, whereas for imipenem the susceptibility rates of isolates from conventionally raised hens were significantly higher ($P < 0.05$). Noticeable, resistance rates were not higher in conventional isolates for fluoroquinolones, but there is rather a drift into the intermediate range.

Comparison of mean MIC showed statistically significant differences ($P < 0.05$) for 13 of 20 antibiotics. Hereof, for eight antibiotics the values from organic farms were lower, with the highest differences for doxycycline. A converse situation was observed for amoxicillin/clavulanic acid, imipenem, chloramphenicol, linezolide and rifampicin (Fig. 1).

Table 2. Number of selected Listeria and Enterococci isolated from cloacal swabs and eggs from organic or conventional kept laying hens

		Cloacal swabs (n = 799)		Egg content (n = 80†)		Eggshell (n = 80†)	
Genus	Species	Organic (n = 399)	Conv. (n = 400)	Organic (n = 40)	Conv. (n = 40)	Organic (n = 40)	Conv. (n = 40)
Listeria	*innocua*	5	7	0	0	0	0
	seeligeri	0	0	0	1	0	0
Total *Listeria* spp.		5	7	0	1	0	0
Enterococcus	*faecalis*	164	164	4	6	12	11
	faecium	26	29	1	1	1	1
	raffinosus	219	226	2	4	8	7
	avium	20	34	1	0	1	4
	gfmc group‡	10	25	0	0	2	1
	durans/hirae	3	3	0	0	0	0
Total *Enterococcus* spp.		442	481	8	11	24	24

†Pooled egg/eggshell samples contained 10 samples each.

‡*gfmc* group contains *E. gallinarum*, *E. flavescens*, *E. mundti* and *E. casseliflavus*.

Table 3. Percent distribution of susceptible, intermediate and resistant *Enterococcus faecalis* from cloacal swabs of organically and conventionally kept laying hens

	Organic keeping (n = 164)			Conventional keeping (n = 164)		
Antibiotic*	*S*	*I*	*R*	*S*	*I*	*R*
AMC	99.4	0.0	0.6	100.0	0.0	0.0
AMP	100.0	0.0	0.0	100.0	0.0	0.0
CMP	100.0	0.0	0.0	100.0	0.0	0.0
CIP	**96.4**‡	**3.0**	**0.6**	**85.3**‡	**14.0**	**0.6**
DOX	**45.1**‡	**15.2**	**39.7**†	**18.3**‡	**11.6**	**70.1**†
ENR	**98.2**‡	**1.2**	**0.6**	**91.4**‡	**8.5**	**0.6**
ERY	21.4	51.0	27.7	21.9	40.2	37.8
FLL	100.0	0.0	0.0	100.0	0.0	0.0
FOS	95.7	0.0	4.2	94.5	0.0	5.5
GNH	100.0	0.0	0.0	98.2	0.0	1.8
IMP	**86.7**‡	**9.1**	**4.3**	**93.8**‡	**0.6**	**5.5**
LIZ	98.8	0.6	0.6	100.0	0.0	0.0
MZL	98.1	1.2	0.0	99.4	0.6	0.0
MOX	99.3	0.0	0.6	98.1	1.8	0.0
NFT	99.4	0.6	0.0	99.4	0.6	0.0
RAM	**26.8**‡	**55.5**	**17.7**†	**65.3**‡	**29.9**	**4.9**†
SNH	**89.6**‡	**0.0**	**10.4**†	**74.4**‡	**0.0**	**25.6**†
TPL	100.0	0.0	0.0	100.0	0.0	0.0
TLS	**86.0**‡	**0.0**	**14.0**†	**61.6**‡	**0.0**	**38.4**†
VAN	100.0	0.0	0.0	99.4	0.6	0.0

*Antibiotic codes: see Table 1.
†Significant difference between the resistance rates from organic and conventional origin ($P < 0.05$) in terms of the antibiotic.
‡Significant difference between the susceptibility rates from organic and conventional origin ($P < 0.05$) in terms of the antibiotic.
Bold: Distribution of susceptible, intermediate and resistant isolates from organic and conventional keepings varies signifcantly ($P < 0.05$) in terms of the appointed antibiotic.

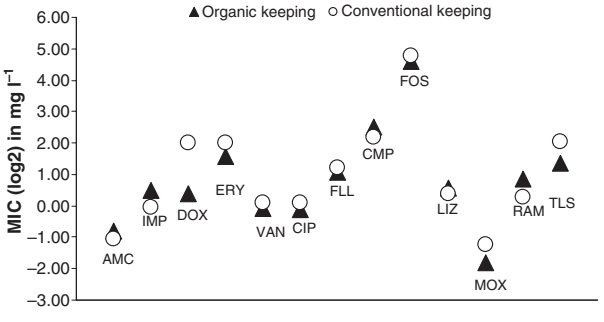

Fig. 1. Comparison of significantly different mean minimal inhibitory concentration of *Enterococcus faecalis* from cloacae of organically or conventionally kept laying hens ($P < 0.05$). Antibiotic codes: see Table 1.

Of 328 *E. faecalis* isolates, 36.3% were resistant to more than one antibiotic. Multiple resistances are statistically significant more frequent in isolates from conventional keepings. According to this, *E. faecalis* isolated from

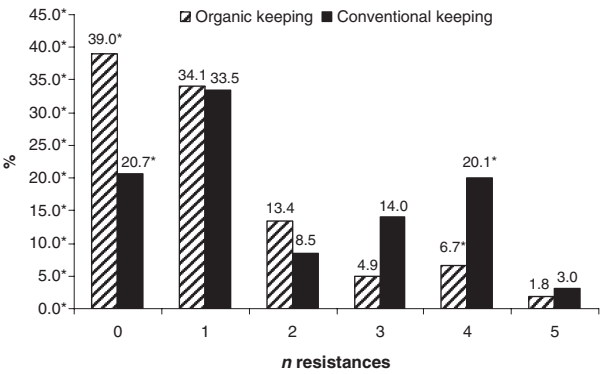

Fig. 2. Distribution of susceptible, single- and multiple resistant *Enterococcus faecalis* from cloacae of organically or conventionally kept laying hens (n_{org} = 164 and n_{con} = 164). *Statistical significance ($P < 0.05$).

organic keepings were more frequently susceptible to all tested antibiotics ($P < 0.05$; Fig. 2).

Isolates with multiple resistances mostly expressed resistance to doxycycline and rifampicin or streptomycin high level. Multiple resistances affected a maximum of four therapeutic groups, mainly tetracyclines, macrolides and aminoglycosides.

Enterococcus faecium

The investigated isolates proved to have high resistance rates to clindamycin, fosfomycin and erythromycin. A high percentage [3.8%$_{(org)}$ and 13.8%$_{(conv)}$] of *E. feacium* had already been classified as resistant to the reserve antibiotic synercid.

Due to the low number of isolated *E. faecium*, a statistical analysis was not carried out for comparison of the resistance rates. However, certain tendencies are clearly recognizable: For ciprofloxacin, clindamycin, doxycycline, imipenem and quinupristin/dalfopristin (synercid) the values were consistently higher in isolates from conventional keepings. The reverse was true for fosfomycin, enrofloxacin and erythromycin, even if *E. faecium* from organic keeping systems had higher susceptibility rates for enrofloxacin (Table 4).

Considering the mean MIC, significant differences ($P < 0.05$) were shown for four antibiotics. Hereof, organic isolates seemed to have higher MIC for chloramphenicol, whereas conventional isolates had higher MIC for clindamycin, synercid and doxycycline (Fig. 3).

Only very few isolates of both keeping systems were totally susceptible to all tested substances (<4%). Pronounced differences could be observed in view of 1-, 2- and 3-fold resistances: Isolates from organic keepings were up to 2-fold resistant in 76.9% of cases, from conventional ones only in 51.7%. In contrast, 3-fold resistances were

Table 4. Percent distribution of susceptible, intermediate and resistant *Enterococcus faecium* from cloacal swabs of organically and conventionally kept laying hens

	Organic keeping (n = 55)			Conventional keeping (n = 29)		
Antibiotic*	*S*	*I*	*R*	*S*	*I*	*R*
AMC	100.0	0.0	0.0	100.0	0.0	0.0
AMP	96.1	3.8	0.0	93.0	0.0	6.9
CMP	100.0	0.0	0.0	100.0	0.0	0.0
CIP	57.6	38.5	3.8	44.8	37.9	17.2
CLI	80.7	3.8	15.3	34.3	3.4	62.0
DOX	84.6	11.5	3.8	58.6	20.7	20.7
ENR	53.8	15.4	30.8	41.3	37.9	20.7
ERY	23.1	19.2	57.7	34.3	51.7	13.8
FLL	100.0	0.0	0.0	100.0	0.0	0.0
FOS	65.4	0.0	34.6	75.8	0.0	24.1
GNH	100.0	0.0	0.0	100.0	0.0	0.0
IMP	26.9	65.4	7.6	68.9	6.9	24.1
LIZ	96.1	0.0	3.8	100.0	0.0	0.0
MZL	73.0	23.1	3.8	72.4	27.6	0.0
MOX	69.3	30.8	0.0	51.6	44.8	3.4
NFT	96.2	3.8	0.0	93.1	6.9	0.0
SYN	92.3	3.8	3.8	82.7	3.4	13.8
RAM	53.9	11.5	34.6	58.6	6.9	34.4
SNH	100.0	0.0	0.0	93.1	0.0	6.9
TPL	100.0	0.0	0.0	100.0	0.0	0.0
TLS	96.1	0.0	3.8	96.5	0.0	3.4
VAN	100.0	0.0	0.0	100.0	0.0	0.0

*Antibiotic codes: see Table 1.

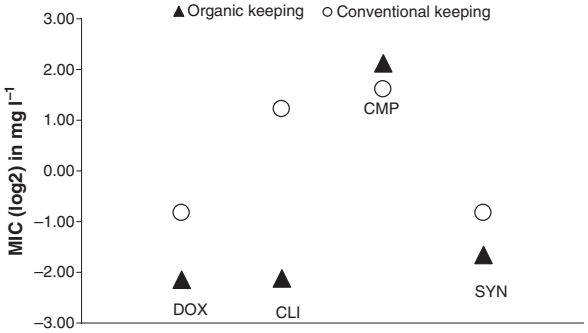

Fig. 3. Comparison of significantly different mean minimal inhibitory concentration of *Enterococcus faecium* from cloacae of organically or conventionally kept laying hens ($P < 0.05$). Antibiotic codes: see Table 1.

detected only in 11.5% of organic, but in 34.6% of conventional *E. faecium*. Higher multiple resistant isolates (up to 6-fold) occurred only in single cases (Fig. 4).

Enterococcus nonfaecalis/nonfaecium

Entercooccus raffinosus showed high resistance rates to rifampicin (48.1%), erythromycin (28.5%), doxycycline

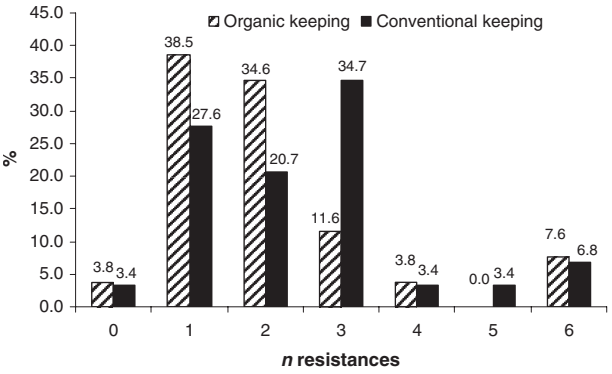

Fig. 4. Distribution of susceptible, single- and multiple resistant *Enterococcus faecium* from cloacae of organically or conventionally kept laying hens (n_{org} = 26 and n_{con} = 29).

(21.3%) and fosfomycin (20.0%). There were no glycopeptide-resistant enterococci.

Significant higher resistance rates of *E. raffinosus* from conventional keepings were detected for fosfomycin, rifampicin and tylosin. In accordance with these results, susceptibility rates of *E. raffinosus* from organic keepings were significantly higher for ciprofloxacin, doxycycline, fosfomycin, nitrofurantoin, synercid, rifampicin and tylosin ($P < 0.05$). In the majority of cases, this phenomon is explainable through a drift of conventional isolates into the intermediate range (Table 5).

Statistically significant differences of the mean MIC were detectable for 12 of the tested antibiotics. Hereof, 11 values were higher for isolates from conventional keeping systems. The maximum deviation of 1.0 $\log_2$ was demonstrable for doxycycline. Only choramphenicol was more effective in lower doses in conventional isolates (Fig. 5; $P < 0.05$).

Multiple resistant *E. raffinosus* were mainly resistant to two antibiotics, predominantly to rifampicin in combination with fosfomycin. Higher multiple resistances (up to 7-fold) were also detectable, if only to a very slight extent (Fig. 6). Resistance combinations with doxycycline, erythromycin and tylosin could be observed frequently. In high multiple resistant isolates, the predominant resistances were detectable for antibiotics of the macrolides, tetracyclines, streptogramins, together with further therapeutic groups. *Enterococcus raffinosus* from organic keepings were significant more frequent totally susceptible or resistant to only one antibiotic, whereas in conventional isolates resistances against two substances occurred more frequently ($P < 0.05$).

Due to the low isolation rate of *E. avium* [$n = 20_{(org)}/34_{(conv)}$], *E. gfmc* group [$n = 10_{(org)}/25_{(conv)}$] and *E. durans/hirae* [$n = 3_{(org)}/3_{(conv)}$] in swab samples, statistical analysis was not carried out. *Enterocccus avium* was up to

Table 5. Percent distribution of susceptible, intermediate and resistant *Enterococcus raffinosus* from cloacal swabs of organically and conventionally kept laying hens

	Organic keeping (n = 213)			Conventional keeping (n = 226)		
Antibiotic*	*S*	*I*	*R*	*S*	*I*	*R*
AMC	100.0	0.0	0.0	100.0	0.0	0.0
AMP	99.5	0.5	0.0	99.6	0.4	0.0
CMP	99.1	0.0	0.9	100.0	0.0	0.0
CIP	**75.3**‡	**22.4**	**2.3**	**52.7**‡	**46.0**	**1.3**
DOX	**44.7**‡	**35.2**	**20.1**	**27.9**‡	**49.6**	**22.6**
ENR	83.1	15.5	1.4	88.5	10.6	0.9
ERY	25.6	46.6	27.9	21.7	49.1	29.2
FLL	100.0	0.0	0.0	100.0	0.0	0.0
FOS	**85.8**‡	**0.0**	**14.2**†	**74.3**‡	**0.0**	**25.7**†
GNH	98.6	0.0	1.4	98.7	0.0	1.3
IMP	90.9	5.9	3.2	88.5	4.4	7.1
LIZ	98.6	0.5	0.9	99.6	0.4	0.0
MZL	85.8	13.2	0.9	89.4	10.2	0.4
MOX	98.2	1.8	0.0	99.1	0.9	0.0
NFT	**92.7**‡	**7.3**	**0.0**	**98.2**‡	**1.8**	**0.0**
SYN	**44.7**‡	**46.1**	**9.1**	**30.5**‡	**56.2**	**13.3**
RAM	**44.7**‡	**16.9**	**38.4**†	**22.1**‡	**20.4**	**57.5**†
SNH	98.2	0.0	1.8	97.3	0.0	2.7
TPL	100.0	0.0	0.0	100.0	0.0	0.0
TLS	**97.7**‡	**0.0**	**2.3**†	**88.5**‡	**0.0**	**11.5**†
VAN	99.5	0.5	0.0	98.2	1.8	0.0

*Antibiotic codes: see Table 1.
†Significant difference between the resistance rates from organic and conventional origin ($P < 0.05$) in terms of the antibiotic.
‡Significant difference between the susceptibility rates from organic and conventional origin ($P < 0.05$) in terms of the antibiotic.
Bold: Distribution of susceptible, intermediate and resistant isolates from organic and conventional keepings varies signifcantly ($P < 0.05$) in terms of the appointed antibiotic.

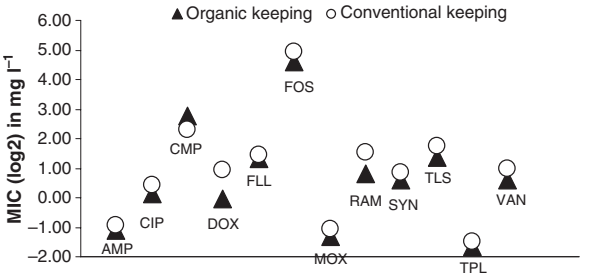

Fig. 5. Comparison of significantly different mean minimal inhibitory concentration of *Enterococcus raffinosus* from cloacae of organically or conventionally kept laying hens ($P < 0.05$). Antibiotic codes: see Table 1.

5-fold resistant when isolated from organic keepings and up to 7-fold resistant when isolated from conventional keepings, in the majority of cases to fosfomycin, erythromycin, doxycycline, synercid, rifampicin and tylosin. *Enterococcus*

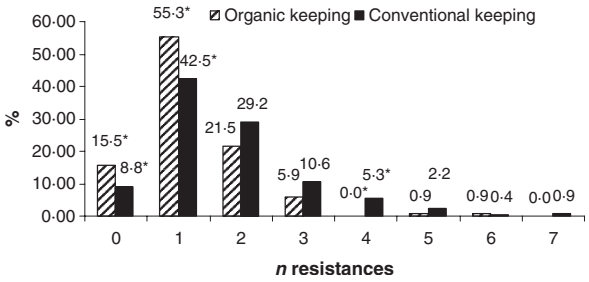

Fig. 6. Distribution of susceptible, single- and multiple resistant *Enterococcus raffinosus* from cloacae of organically or conventionally kept laying hens (n_{org} = 219 and n_{con} = 229). *Statistical significance ($P < 0.05$).

gfmc of both keeping types were resistant to up to six antibiotics, mostly to rifampicin, distantly followed by erythromycin, fosfomycin and doxycycline. Only one *E. durans/hirae* isolate of organic keeping was resistant to one antibiotic, namely fosfomycin. One conventional isolate was also susceptible to all tested agents, whereas one was resistant to doxycycline, erythromycin, synercid and tylosin and the third one additionally to fosfomycin and nitrofurantoin.

Discussion

Prevalence of Gram-positive bacteria in the sample material

Prevalence of *Listeria* spp. was very low, both in cloacal swabs (n = 12) and in egg/eggshell samples (n = 1). Farber et al. (1992) also isolated only two *L. innocua* from eggshells, but not from egg contents. These low contamination rates could be because of bactericidal activity of lysozyme in the egg albumen, as stated by Hughey and Johnson (1987), Hughey et al. (1989).

Enterococci are commensals of the intestinal flora of man and animals, but in some cases they may also cause so-called nosocomial infections (Murray, 1990). In this study, enterococci could be detected from most of the cloacal swabs (>95%) from both keeping systems. Isolation rates from eggshells were 52.5% and ca. 20% of the pooled egg content samples respectively. The high contamination rates are most probable referable to the direct contact of the eggs with dust, soil and faeces in the chicken-coop or to a cross-contamination with feculent packaging (Board et al., 1964).

There was no evidence that organic keeping systems were more highly contaminated with zoonotic or commensal Gram-positive bacteria. These results are in concordance with a previous study where the zoonotic Gram-negative bacteria *Salmonella* spp. and *Campylobacter* spp., as well as *Escherichia coli* and coliforms were screened (Schwaiger et al., 2008). The widespread

presumption that animals from free-range husbandries are more highly contaminated with zoonotic bacteria because of their frequent contact with wild animals, ground-dwellers and birds, proved not to be true. To attempt an explanation, it is necessary to consider that in chicken coops, as well as in all stables without run for animals, there is much more technical equipment than outdoors, for example ventilators and artificial lighting. These tools are frequently overlooked in the terminal disinfection and may therefore function as 'hiding places' for bacteria (Blaha, 2008). In contrast, microbes in the free range system are exposed to ultraviolet rays of the sunlight, which have a natural disinfectant effect (Chang et al., 1985). Furthermore, lower stocking densities in organic farming systems may decelerate the transmission of bacteria from animal to animal (Blaha, 2008).

Influence of the keeping system on phenotypic resistances

A sufficient number of isolates is necessary to appraise their resistance properties. As only very few bacteria were detected in egg samples, and furthermore a small number of *Listeria* spp. was identified in all, only results of *E. faecalis* and *E. raffinosus* from cloacal swabs are discussed hereafter.

Enterococcus faecalis

Isolates from organic laying hen husbandries were more susceptible or less resistant to antibiotics in most cases (Table 3). Resistances against doxycycline (39.7%), tylosin (14.0%) and erythromycin (27.7%) could also be found in enterococci from organic keepings, but yet to a lesser extent than in conventional ones. This might be through a less frequent application of antibiotics under severe restrictions in organic farming. Aarestrup et al. (2002) also recognized a causal relationship between the ban on macrolides and lower resistance rates of porcine enterococci in Sweden and Denmark. Coexistence of resistance to antibiotics and heavy metals on the same plasmid may explain the selection of antibiotic-resistant *E. faecalis* in an antibiotic-free environment (Aarestrup et al., 2002). The high resistance rates to rifampicin in organic isolates (17.7% versus 4.9%; Table 3) seem astonishing. Busani et al. (2004), as well as Johnson et al., 2000; also found high rates in *E. faecalis* from poultry, but this cannot explain the lower values in conventional keepings. A spontaneous mutation of the *rpo*-gene is reported for rifampicin-resistant *E. faecium* (Enne et al., 2004), however, existence of similar mechanisms in *E. faecalis* remains unknown.

Mean MIC of eight antibiotics, namely doxycycline, erythromycin, vancomycin, ciprofloxacin, florfenicol, fosfomycin, moxifloxacin and tylosin, were significantly lower in *E. faecalis* from organic keepings ($P < 0.05$). In contrast, four antibiotics required lower MIC in conventional isolates (Fig. 4). In the cases of amoxicillin/clavulanic acid, imipenem and linezolid, this was merely attributable to a drift of a small number of organic isolates into the intermediate resistance range; higher MIC of chloramphenicol was because of the displacements of some organic isolates within the susceptible range. Of note, rifampicin, an antibiotic that is not used in veterinary medicine, but only in human medicine, seemed to be less effective in organic *E. faecalis*, as the modal value was in the intermediate range – the reason for this phenomenon remains unknown.

Multiple resistances were found in 26.8% of *E. faecalis* from organic keepings and even in 43.6% of isolates from conventional keepings. This difference was statistically significant ($P < 0.05$). A correlation between antibiotic application and a higher prevalence of multiple resistances, as shown by van den Bogaard et al. (2001) for *E. coli*, can be presumed. Resistance combinations with streptomycin high level were significant more frequent in *E. faecalis* from conventional keepings ($P < 0.05$). Numerous studies document that various transposons often act as transmitter of single but also combined antibiotic resistances, e.g. to erythromycin, streptomycin and tetracycline (Bonafede et al., 1997; Rice, 1998; Rice and Caria, 1998; Aarestrup et al., 2000; De Leener et al., 2005).

Enterococcusss raffinosus

Isolates from organic keepings had significantly lower resistance rates for fosfomycin, rifampicin and tylosin ($P < 0.05$). Furthermore, susceptibility rates were higher for ciprofloxacin, doxycycline, fosfomycin, nitrofurantoin, synercid, rifampicin and tylosin ($P < 0.05$). Even if no data from the literature are available concerning the influence of the keeping system on resistance properties of *E. raffinosus*, similar findings are recorded for various bacteria species, for example *Staphylococcus aureus*, *Enterococcus* spp. and Gram-negative enteric bacteria (Gellin et al., 1989; Heuer et al., 2002; Tikofsky et al., 2003;. Husbandry specific factors like indoor or outdoor housing may have an effect on diversified resistance behaviour of the microbial intestinal flora of pigs (Langlois et al., 1988). A similar effect in the free-range laying hen husbandry is therefore also conceivable. Admittedly, resistant isolates were also detectable in organic keepings. As an explanation, it seems possible that some antibiotic resistance properties lead additionally to a better biological fitness, which advances a natural selection of resistant strains, as known for *Campylobacter jejuni* (Luo et al., 2005). Some authors also demonstrated persistence of antibiotic resistances in *E. coli* even in complete absence

of antibiotics, which may be because of resistance transfers, either within one or between different species. Additionally, resistant microorganisms can spread from hen to hen, from animal to man and vice versa (Walton, 1966; Levy et al., 1976; Guillot et al., 1977; Chaslus-Dancla et al., 1987; Frei et al., 2001).

Generelly speaking, it is surprising that a significant favourable influence of organic systems to antibiotic resistance could be shown, even if antibiotics are applied very sparsely in conventional laying hen husbandries as well. This leads to the assumption that in keeping types with higher drug consumption rates, such as in fattening husbandries, the positive effect of organic farming may be even considerably larger. First comparative in-house analyses indicate that this proposition may be true (data not shown).

Conclusions

Considering the occurrence of pathogenic or commensal bacteria, there were no noteworthy differences in the two keeping systems. Doubts of opponents of the organic husbandry, who persist in saying that products of these keeping systems should be more highly contaminated with zoonotic bacteria, can therefore be allayed. A previous study, where the Gram-negative zoonotic bacteria *Salmonella* spp. and *Campylobacter* spp. were screened, came to the same result (Schwaiger et al., 2008). On the contrary, resistance rates, as well as mean MIC and multiple resistances varied considerably in the different keeping systems. Comparison of statistically significant different resistance rates ($P < 0.05$) makes obvious that significant lower resistant rates and coincidentally higher susceptibility rates of Gram-positive bacteria, respectively, occur in organic laying hen husbandries. These findings are in concordance with a previous study, where Gram-negative bacteria were investigated in the same way (Schwaiger et al., 2008). Hence, the prior thesis that organic husbandries may contribute to further effectiveness of antibiotics, could be reaffirmed.

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