Development of a competitive exclusion product for poultry meeting the regulatory requirements for registration in the European Union

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Competitive exclusion treatment is able to increase the pathogen colonization resistance of day-old chicks by applying probiotic bacteria stabilizing the indigenous microflora. In order to develop a safe microbial feed additive, various bacterial strains were isolated out of the gastrointestinal tract of healthy chickens. One hundred twenty-one representatives were selected based on differences in whole-cell protein patterns and screened for antagonistic properties. Five effective strains (*Pediococcus acidilactici*, *Enterococcus faecium*, *Bifidobacterium animalis* ssp. *animalis*, *Lactobacillus reuteri*, and *Lactobacillus salivarius* ssp. *salivarius*) exhibited *in vitro* the ability to inhibit a range of common pathogens and were evaluated with regard to the risks associated with genetic transfer of antibiotic resistances from animals to humans *via* the food chain. The probiotic strains were sensitive to several clinically effective antibiotics, though some of them showed single resistances. None of the vancomycin-resistant (R) strains carried the enterococcal *vanA* gene. Two tetracycline R strains were shown to harbor a *tet*(M)-associated resistance. The strains contained no extrachromosomal DNA and were not able to transfer the resistance by means of conjugation. On basis of the collected data the presence of easy transferable resistances was excluded and the chicken strains were considered to be suitable for the use as feed additive.

Keywords: Competitive exclusion / Defined CE product / European Registration / Risk assessment / *Salmonella*

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1 Introduction

For many decades antibiotic growth promoters have been used in the feed for farm animals. Because of the general problem of increased resistance of bacteria and the decreasing acceptance of the consumers for this type of additive, antibiotics have been banned already in some countries and will not have a future in the European Union [1]. Due to the foreseen problems associated with the legislative ban of antibiotic growth promoters in the European Union by 2006 (*e.g.*, performance losses in animal husbandry, food borne disease in humans, and increased use of therapeutic antibiotics), the demand for alternative feed ingredients in the EU has increased. Especially the poultry industry deals with the problem of transfer of pathogens (*e.g.*, *Salmonella*, *Campylobacter*) from chickens to humans. Newly hatched broiler chickens of the modern poultry husbandry do not come into contact with the mother hens. This lack of contact is believed to result in a delayed development of the intestinal microflora, and as a consequence, broilers at very young age are particularly susceptible (S) to pathogen colonization. In this respect, probiotics from the animals' gut are of current interest because they offer biological alternatives which should find acceptance by both the producers and consumers. The protective ability of probiotic bacteria, nowadays known as competitive exclusion (CE), was first described by Nurmi and Rantala [2] who demonstrated that introduction of mixed bacterial preparations from caecal contents of healthy adult chickens can protect young birds against *Salmonella* infection. Research in the field of CE has led to the manufacture of several commercially available CE products [3–6]. However, most of the products

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Abbreviations: BHI, brain heart infusion; **CE,** competitive exclusion; **LAB,** lactic acid bacteria; **MIC,** minimal inhibitory concentration; **MRS,** deMan-Rogosa-Sharpe; **MTPY,** modified trypticase-phytone-yeast extract; **SCAN,** Scientific Committee on Animal Nutrition; **R,** resistant

being developed so far are preparations of unknown bacterial composition posing the risk of containing pathogenic bacteria or viruses [6, 7]. In viewpoint of the risks associated with undefined preparations, the CEX project (C-EX QLK-CT-2002-71662) was initiated to develop a defined CE product consisting of various well-characterized bacterial strains that meet the European regulatory demands [8]. In an attempt to gain a subset of potential probiotics various bacteria from the chicken intestinal tract were isolated, characterized, and strains showing antagonistic activity against common poultry pathogens *in vitro* were carefully evaluated with regard to the safety of use. With the recently awakened awareness of the risks of agricultural sources for antimicrobial resistance in food [9], EU experts propose as safety criteria for probiotics in feed additives the exclusion of resistances or the lack of transferability. In 2001 (updated 2003), the Scientific Committee on Animal Nutrition (SCAN) has adopted an opinion on this matter which provides guidance to study the potential of feed additive strains to bear resistances and to transfer them [10]. Following the European guidelines, a safety evaluation was carried out to investigate the presence of the main risk factors related to the use of micro-organisms as feed additive (*e.g.*, spread of antibiotic resistances, virulence activity). Particular interest was aimed at five representatives of the group of lactic acid bacteria (LAB) and the genus *Bifidobacterium* because of their antagonistic properties and their frequent application in numerous feed and food supplements [11]. Insights obtained in this study are reported and discussed in context of the major safety aspects.

2 Materials and methods

2.1 Isolation of bacterial strains from the gastrointestinal tract of broilers

Isolation of strains was performed out of fresh gastrointestinal contents from healthy chickens of various ages. Two 14-day-old broilers (C1, C2) and two 28-day-old broilers (C3, C4) from a local commercial poultry farm and one adult, homegrown chicken in the age of 12 wk (C5) were the source of the bacterial isolates. The broilers were sacrificed by cervical dislocation, and crop, jejunum, ileum, and caeca were prepared for isolating bacteria. Intestinal bacteria were isolated by using standard cultivation techniques at 37°C under aerobic, facultative anaerobic, and strict anaerobic conditions using a range of nonselective and selective media. Nutrient medium (Merck, Austria) was chosen for isolating aerobes, and deMan-Rogosa-Sharpe (MRS) medium (Oxoid, Hampshire, UK), azide glucose bouillon (Merck) and kanamycin aesculin azide agar base (Merck), *Enterobacteriaceae* enrichment broth (Oxoid), and violet red bile dextrose (VRBD) agar (Merck) for isolating facultative anaerobes. Modified trypticase-phytone-yeast extract (MTPY) broth and agar supplemented with raftiline (10 g/L), nalidixic acid (0.15 g/L), lithiumchloride (3 g/L), and natriumproprionate (3 g/L) was chosen for isolating bifidobacteria, as described previously [12]. The nonselective Viande Levure medium [13] was applied for isolating anaerobes. Inoculated media were incubated for 48 h at 37°C, and appropriate dilutions were plated onto the respective agar plates (see above). Anaerobic conditions were achieved using gas-tight tubes for broth cultures and anaerobic jars for agar plates. Sterile enzyme additives (Oxyrase®, Oxyrase, Mansfield, Ohio) were applied according to the manufacturers' instructions to generate strict anaerobic conditions in agar and broth.

2.2 Classification of bacterial isolates

A polyphasic approach was carried out combining morphological, physiological, and genotypic methods. Isolates were examined for cell morphology and spore formation using phase contrast microscopy (Olympus VANOX AHBT-3). Catalase activity was studied by dropping 3% hydrogen peroxide on well-grown colonies on agar plates and observing the gas bubbles. The presence of fructose-6-phosphate phosphoketolase activity was detected by using a microtiterplate assay and reagents as described by Scardovi [14]. Protein patterns were investigated by whole-cell extract SDS-PAGE as described formerly [15] using the Hoefer SE 600 unit (Amersham Biosciences, Austria). In agreement with the previously collected experience, isolates with more than 90% similarity were clustered and their assignment to the same species was considered. Analysis of the metabolic end products was done after clarification of the cell-free broth according to a standard method [16] with a high-performance liquid chromatograph (Hewlett Packard HP1100) equipped with a refractive index detector (HP 1047A) and a polyspher OA KC RT 300–700 column (Merck) using 0.01 N sulfuric acid at a flow rate of 0.4 mL/min as a mobile phase. For 16S rDNA sequence analysis of relevant strains the genomic DNA was extracted according to the protocol of Chan *et al.* [17]. Amplification of the 16S rDNA was performed with a TPersonal Cycler (Biometra, Göttingen, Germany) using the eubacterial primer set 27f and 1492r [18] corresponding to *Escherichia coli* 16S rRNA positions 8–27 and 1492–1513, respectively. A standard 50 μL reaction mixture contained 0.2 mM each of dATP, dCTP, dGTP, dTTP (Boehringer Mannheim), 1 unit Dynazyme Polymerase (Finnzymes), 1 × standard PCR-buffer (Finnzymes), autoclaved distilled water, 16S-primers (1 μmol/L), and template DNA. The PCR program started with an initial denaturing step at 95°C for 5 min which was followed by a hot start and 30 cycles of DNA denaturation at 94°C for 2 min, primer annealing at 50°C for 1.5 min, and DNA extension at 72°C for 5 min. Amplification pro-

ducts were purified with the QIAquick PCR Purification Kit (Qiagen). Purified PCR products were directly sequenced at the Service Department at the Vienna Biocenter (VBC-GENOMICS Bioscience Research, Vienna, Austria). 16S rRNA gene sequence similarity studies were carried out by using FASTA3.

2.3 *In vitro*-evaluation of antagonistic properties

A representative number of chicken strains ($n = 121$) was screened for antagonistic activity against a variety of indicator pathogens by using a cocultivation agar plate assay. *Salmonella* serotypes (*Salmonella enteritidis* strain Bio59, *Salmonella choleraesuis* ssp. *choleraesuis* strain Bio554) were provided by the company Biomin (Herzogenburg, Austria). Strains of *E. coli* O147:H19 (CCUG 11447), *Clostridium perfringens* (CCUG 47895), and *Campylobacter jejuni* (CCUG 25903) were obtained from the Culture Collection University Göteborg (Sweden), whereas the *E. coli* O157:H7 strain (USDA71) derived from the United States Department of Agriculture (Washington, D.C., USA). One drop of a pure culture of the respective test strain (corresponding to approximately 1.0×10^5 cells) was streaked out in the middle of an agar plate. After growth of the test strain the plate was overlaid with 10 mL semisolid agar containing approximately 1.0×10^8 cells of the pathogen strain. MRS agar (Oxoid) and anaerobic conditions were used for all cocultivation approaches, except for *C. jejuni* which was cultivated in brain heart infusion (BHI) (Oxoid) under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) generated by using a gas package (Campygen, Oxoid) in anaerobic jars for 48 h. After 24 h of cocultivation at 37°C the agar plate was evaluated for an inhibition zone around the test strain. The diameter of the inhibition zone and the growth zone of the test strain was measured and recorded. The test for each isolate against each pathogen strain was carried out at least three times with duplicates each time.

2.4 Antibiotic susceptibility testing

Susceptibility of representative strains to the main therapeutic antibiotic agents was examined by determination of the minimal inhibitory concentration (MIC) with a microbroth dilution method that conforms to National Committee for Clinical Laboratory Standards (NCCLS) specifications with modifications [19]. In preliminary experiments, several broth media were evaluated for their ability to support growth of the strains: MRS broth, BHI broth (both from Oxoid), cation-adjusted Mueller-Hinton (CAMHB; Becton Dickinson, Austria), isosensitest (Becton Dickinson), and supplemented folic acid casei medium (FACM, Difco/BD) as described previously [20]. Susceptibilities to antibiotics were investigated as defined by the SCAN [10]. All antibiotics except linezolid (Pfizer, Austria) and quinupristin/dalfopristin (Sanofi-Aventis Germany) were purchased from Sigma. For the final susceptibility testing, the enterococci were grown aerobically in CAMHB, the pediococci anaerobically in BHI broth at 37°C, while the lactobacilli were grown facultative anaerobically in MRS broth and bifidobacteria anaerobically (AnaeroGen; Oxoid) in MTPY broth at 37°C, respectively. Following incubation for 24 h under the defined conditions growth was evaluated by visual inspection of the turbidity. The MIC was read as the lowest concentration of a given antibiotic at which bacterial inhibition was apparent. One control strain (*E. faecalis* ATCC 29212) was included in the tests, as recommended in the National Committee for Clinical Laboratory standard procedures for testing therapeutic antibiotics [21]. For the interpretation of results the breakpoints defined by the SCAN [10] were used. Interpretative charts were not available for categorizing members of the genus *Bifidobacterium*. The MIC values obtained for the bifidobacterial strain were therefore compared with the lowest breakpoints defined by SCAN for probiotic feed additive strains.

2.5 Conjugation assays

Conjugative transferabilities of vancomycin and tetracycline resistances were studied by direct plate colony (DPC) mating using the enterococcal recipient strains DSM 13589 (*Enterococcus faecium*) and LMG 19456 (*Enterococcus faecalis*) obtained from the BCCM™/LMG Bacteria collection (Ghent, Belgium) and DSMZ collection (Braunschweig, Germany). The procedure was performed as described previously [22] by using mid-exponential growth phase cultures and a 1:1 donor-recipient mixture. Antibiotic pressure was achieved prior to conjugation by subcultivating the strains in medium containing the respective antibiotic agent. Rifampin (Sigma) was chosen as counterselection marker to select against donor cells. The transfer was examined at least two times by using an average of three platings.

2.6 PCR assays

The strains showing resistance to vancomycin and tetracycline were tested for the presence of the *vanA* gene and the *tet*(M) gene by PCR as published previously [23, 24]. Evaluation of the PCR was done by using *vanA*- and *tet*(M)-positive reference strains (DSM 13590, LMG 21677) obtained from the DSMZ collection (Braunschweig, Germany) and the BCCM™/LMG Bacteria collection (Ghent). The incidence of enterococcal virulence factors such as *esp*, *cylA*, and *gelE* was examined by PCR using oligonucleotide primers as published by Eaton and Gasson [25]. Control strains were two *E. faecalis* strains, strain ATCC 29212

(*gelE*+, *cylA*+) from the American Type Culture Collection (University Boulevard, Manassas, USA) and one clinical isolate designated EF-4 (*esp*+, *gelE*+) kindly provided by the Life Sciences Department of the ARC Seibersdorf research (Seibersdorf, Austria).

2.7 Plasmid analysis

Plasmid-DNA was isolated from 4 h-subcultures of the finally selected strains supplemented with the relevant antibiotic by using the Nucleo Spin Plasmid DNA Purification Kit (Machery-Nagel) according to the manufacturers' instructions. Extrachromosomal DNA from resistant (R) strains was additionally isolated on a large scale by the alkaline lysis method as described by Anderson and McKay [26] and subsequent separation by the cesium chloride gradient method [27]. Strain LMG 21677 (*Lactobacillus plantarum*) harboring a *tet*(M) encoding plasmid was included as reference.

3 Results and discussion

3.1 Composition of collection of chicken intestinal bacteria

In an attempt to gain a diverse spectrum of potential probiotic bacteria deriving from the chickens' gastrointestinal habitat, contents of different intestinal compartments from several broilers were used for enrichment of gut bacteria under various oxygen conditions. Among the numerous intestinal microbes that do normally inhabit the intestinal tract, those that are expected to beneficially affect the host by improving the intestinal microbial balance include species of the genera *Lactobacillus*, *Bifidobacterium*, and *Enterococcus* [11]. In our study, these specific key bacteria were targeted by using a set of semiselective and selective media. Out of five broilers, 477 pure cultures were obtained under aerobic, facultative anaerobic, and strict anaerobic conditions and included in the project culture collection. Taxonomic grouping by comparison of whole-cell protein patterns enabled a grouping of the isolated strains into similarity clusters in order to reduce the large number of isolates. In agreement with previously collected experience, isolates with more than 90% similarity were clustered and their assignment to the same species was considered. The clusters of aerobically and facultative anaerobically growing strains from the 14-day- and 28-day-old industrial broilers (C1 – C4) were dominated by members of the *Enterobacteriaceae* representing a percentage of 56 and 75%, respectively. As shown in Fig. 1A, clusters of anaerobically isolated strains contained more than 92% LAB, mainly bacilli of the genus *Lactobacillus* sp. (85%), cocco-bacilli of uncertain identity (8%) and cocci of the genera *Enterococcus* (2%) and *Pediococcus* (5%). About 47% of the *Lactobacillus*-assigned isolates were clustered, but remained unidentified on a species level. Members of the assigned clusters were identified on a species level as *Lactobacillus salivarius*, *Lactobacillus johnsonii*, and *Lactobacillus reuteri*. Protein-similarity clusters consisting of chicken C5 isolates harbored mainly facultative anaerobes originally deriving from various compartments of the chickens' intestine. As shown in Fig. 1B, a percentage of around 60% was dominated by several genera of LAB, mainly by *Lactobacillus* sp. (45%) and *Enterococcus* sp. (15%). The LAB clusters were *L. reuteri*- and *L. salivarius*-assigned clusters, genus-assigned *Lactobacillus*-clusters (9%), and nonidentified clusters (4%), respectively. One main cluster was formed by strict anaerobic Gram-positive bacteria representing a percentage of around 15%. Representatives from this cluster were identified on a genus level as members of the genus *Bifidobacterium*. Bifidobacteria were detected only in samples deriving from the jejunum, ileum, and caeca of the 12-wk-old homegrown chicken C5. The phosphoketolase reaction identified 17 Gram-positive isolates ascribable to *Bifidobacterium* spp., six strains originally isolated from the jejunum, four strains from the ileum, and seven strains from the caecum.

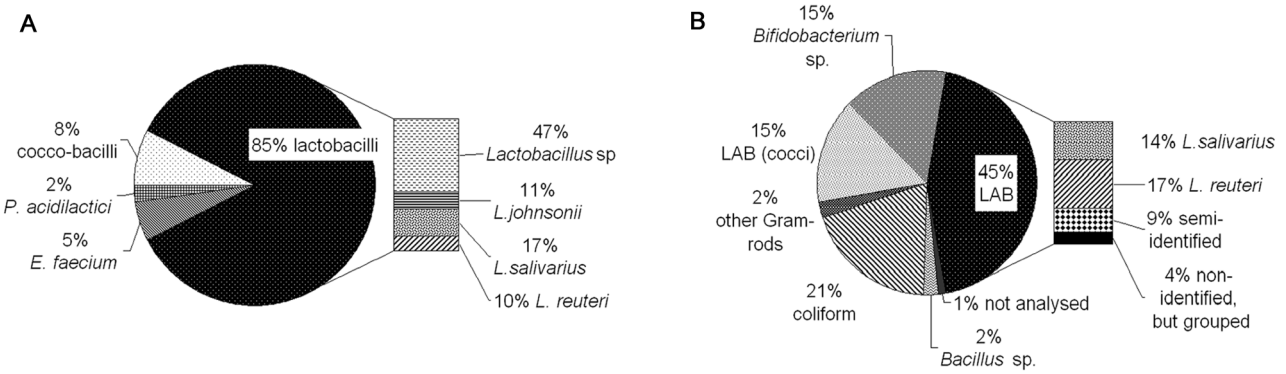

Figure 1. Composition of the intestinal strain collection of anaerobically isolated bacteria deriving from 14-day and 28-day-old industrial broilers (A) and a 12-week-old homegrown broiler (B).

Further assuming that identical profiles represented identical strains the number of isolates could be reduced and out of 477 original isolates 121 strains were selected as representatives of different similarity clusters for investigations in order to identify potential probiotic strains. A desirable property of probiotic bacteria is the ability to produce certain antimicrobial substances. All representatives were screened for the production of metabolic end products such as organic acids and volatile fatty acids which are very likely to contribute to the CE of pathogens in the gastrointestinal tract of young broilers. Differentiation with regard to the type of fermentative metabolism revealed three major groups of lactic acid producers among the representatives: 71 representative strains were strict homofermentative LAB generating lactate as the sole product of fermentation, 21 representatives were strict heterofermentative LAB producing a mixture of ethanol, acetate and lactate, and three representatives were fermenting hexose through the "bifid shunt" producing acetate and lactate. However, the results reveal that in our study mainly lactic acid-producing bacteria have been isolated and selected as potential probiotic bacteria from different parts of the intestines of chickens. On the basis of the differences in whole-cell protein patterns all of the isolated strains could be grouped and out of 121 representatives only four strains could not be assigned to a genus. Due to the selectivity of the media the obtained spectrum of bacterial strains though isolated from various gastrointestinal compartments was of low diversity when compared to the original intestinal habitat that represents an ecosystem of the highest complexity. In particular anaerobic, fastidious organisms like *Bifidobacterium* spp. were difficult to isolate showing the need for more efficient isolation media. As a result, a high percentage of *Enterobacteriaceae* and *Lactobacillaceae* were found to dominate our strain collection. Because of the selectivity of our culture approach for readily cultivated bacteria, we are aware of the fact that we cannot compare our results with data from other investigators studying the composition of the intestinal microbiota of chickens [28, 29]. Anyhow we could not detect other key bacteria which are commonly postulated as dominant inhabitants of the chickens' GI tract (*e.g.*, *Bacteroides*, *Clostridium*, *Eubacterium*, and *Fusobacterium*). The presence of *Eubacterium* spp. was excluded because no other major end product than lactic acid was found among the anaerobes. Members of the genus *Fusobacterium* were excluded, since no Gram-negative bacilli were detected which produced butyric acid without iso-acids.

3.2 *In vitro*-evaluation of probiotic strains

In total, 121 representative chicken strains were screened for *S. enteritidis* inhibition using the cocultivation agar plate assay. The screening efforts resulted in a further reduction of strains since 68 of these strains showed no inhibitory activity, *in vitro*, and 53 of these strains exhibited the ability to inhibit the *S. enteritidis* strain. The ability to produce certain metabolic end products like volatile fatty acids and organic acids correlated with the inhibitory capability in the cocultivation agar plate assay. Strains that produced high amounts of organic acids were able to inhibit pathogens *in vitro* suggesting a pH-related CE effect, mainly by the production of lactate and acetate. Inhibitory activity caused by the production of organic acids and volatile fatty acids (in particular lactate, acetate, propionate, and butyrate) has been documented by several researchers [30–32]. However, as the inhibition test was carried out *in vitro* the mode of action of the probiotic candidates in the chickens' GIT environment remains unclear. Based on 16S rDNA sequence comparison the positive isolates were phylogenetically assigned to members of the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Bifidobacterium*, respectively. Effective strains were tested again with good reproducibility of results. The 20 most effective strains were further shown to inhibit a range of common pathogenic species including strains of *S. choleraesuis*, *E. coli* O147:H19, *E. coli* O157:H7, *C. perfringens*, and *C. jejuni.* The probiotic strains were affiliated to *E. faecium*, *E. faecalis*, *Pediococcus acidilactici*, *L. johnsonii*, *L. salivarius* ssp. *salivarius*, *L. reuteri*, and *Bifidobacterium animalis* ssp. *animalis*. Five representatives of protein-similarity clusters deriving from a different intestinal source were selected on the basis of their good antagonistic properties against several indicator pathogens (Fig. 2) and subjected to 16S rDNA sequence analysis. Analysis of more than 1000 bases of the 16S rRNA gene sequence of the strains revealed that they are phylogenetically affiliated to different species of well-known lactic acid-producing bacteria: CE1 (*P. acidilactici*), CE2 (*E. faecium*), CE3 (*B. animalis* ssp. *animalis*), CE4 (*L. reuteri*), and CE5 (*L. salivarius* ssp. *salivarius*) (Table 1).

Table 1. Percent similarity of 16S rDNA sequences of preselected probiotic strains to sequences of their closest bacterial relatives available in the EMBL nucleotide sequence database

Strain code	Intestinal origin	Sequence (nt)	Similarity (%)	Phylogenetic assignment
CE1	Caecum	1094	99.6	*P. acidilactici*
CE2	Jejunum	1033	100.0	*E. faecium*
CE3	Ileum	1139	98.9	*B. animalis* ssp. *animalis*
CE4	Crop	1083	99.7	*L. reuteri*
CE5	Caecum	1066	99.5	*L. salivarius* ssp. *salivarius*

Abbreviations: nt (nucleotides).

3.3 Risk assessment of potential CE strains

The results of the antibiotic susceptibility studies are summarized in Table 2. Due to insufficient growth in recommended standard test media (CAMBH, isosensitest) these

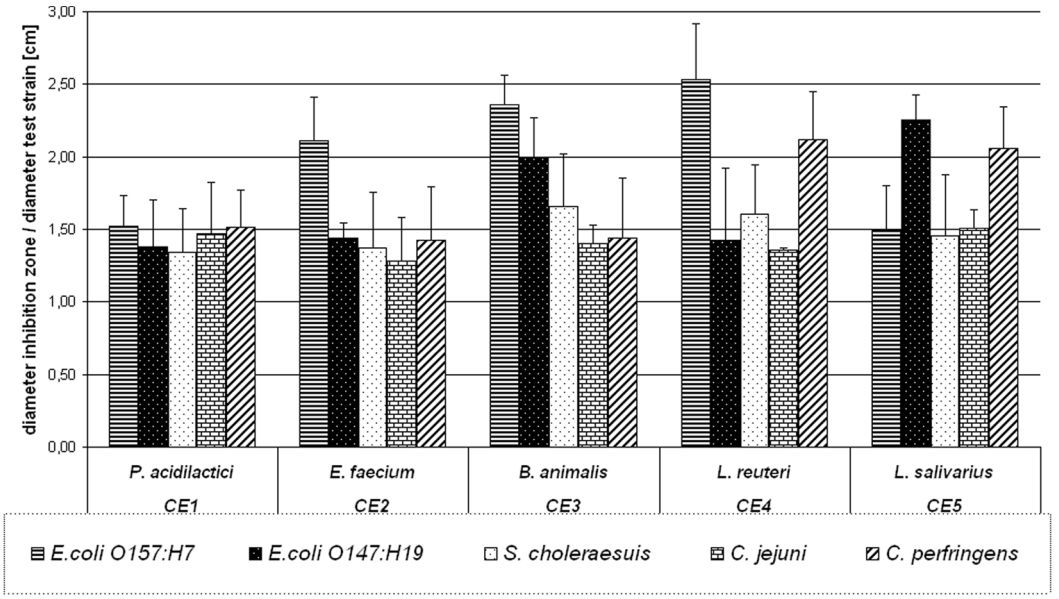

Figure 2. *In vitro* inhibitory activities of five potential probiotic bacterial strains against common poultry pathogens based on inhibition zones obtained after cocultivation on agar plate.

Table 2. Categorization of potential CE strains originating from the gastrointestinal tract of broilers as S or R based on the MICs (μg/mL) for selected antibiotic agents according to SCAN (2003)

Antibiotic	*P. acidilactici* strain #CE1		*E. faecium* strain #CE2		*B. animalis* strain #CE3		*L. reuteri* strain #CE4		*L. salivarius* strain #CE5	
β-Lactams										
Ampicillin	≤0.5	S	≤2	S	≤1	(S)	1	S	≤0.5	S
Aminoglycosides										
Streptomycin	≤32	S	≤128	S	≤64	(R)	64	(R)	>64	(R)
Kanamycin	16	S	≤128	S	128	(R)	>64	(R)	>64	(R)
Neomycin	≤32	S	≤128	S	128	(R)	32	(R)	≤64	(R)
Gentamicin	≤4	S	≤63	S	63	(R)	>4	(R)	>4	(R)
Amphenicole										
Chloramphenicol	4	S	≤2	S	4	(S)	≤4	S	≤4	S
Tetracycline										
Tetracycline	32	R	≤16	R	≤1	(S)	≤32	R	≤8	S
Macrolide										
Erythromycin	≤0.5	S	≤0.5	S	≤1	(S)	0.5	S	≤2	S
Ansamycine										
Rifampin	≤0.5	S	≤0.5	S	≤1	(S)	≤4	S	≤4	S
Streptogramin										
Quinu/dalfopristin	≤0.5	S	≤1	S	≤1	(S)	1	S	≤1	S
Fluoroquinolone										
Enrofloxacin	4	S	8	R	≤4	(S)	≥16	R	16	R
Oxazolidione										
Linezolid	2	S	2	S	≤2	(S)	2	S	≤4	S
Folate inhibitor										
Trimethoprim	32	R	≤1	S	≤0.25	(S)	>64	(R)	≤32	(R)
Glycopeptide										
Vancomycin	>16	R	≤4	S	≤1	(S)	>16	R	16	R

Uncertain categorizations due to media interference or missing breakpoints are indicated by parentheses.

media were not suitable for susceptibility testing of the nonenterococcal strains, namely CE1, CE3, CE4, and CE5. Following the evaluation of the media, the BHI broth was used for testing the pediococcal strain CE1, the CAMBH broth for testing the enterococcal strain CE2, the MTPY broth for the bifidobacterial strain CE3, and the MRS broth for the two lactobacilli strains CE4 and CE5. In some cases the categorization of strains as R remained to be questioned

because it was difficult to interpret the MIC values due to media interference or missing interpretative charts. According to the obtained MIC values, both lactobacilli displayed a resistance toward the aminoglycosides and trimethoprim. Since it is known that the activity of these antibiotics can be affected by ingredients of the MRS broth, the influence of the medium might be responsible for the observed high aminoglycoside and trimethoprim MIC values. Media interference has been reported by other investigators especially for testing LAB [20, 33]. Danielsen *et al.* [20] showed increased resistance levels in lactobacilli to trimethoprim due to uptake of thymidine, when MRS was used. However, due to their inability to sufficiently grow in suitable test media (CAMBH, isosensitest, FACM) in our study the categorization of the two lactobacilli on the basis of the trimethoprim and aminoglycoside MICs remained uncertain. In case of the bifidobacterial strain, the observed levels of aminoglycoside resistances were in accordance with the profiles recorded by other investigators [34 – 36] for members of this genus showing resistances – some being most likely intrinsic – to neomycin, gentamycin, kanamycin, and streptomycin. As described in these reports the reduced susceptibility to aminoglycosides might be a naturally occurring characteristic of bifidobacteria. Anyhow the clear mechanism behind the resistance is still unknown showing the need for elucidation of the genetic basis of resistances in the genus *Bifidobacterium*. In addition, contrarily to other prominent probiotic genera (*Enterococcus*, *Lactobacillus*, and *Pediococcus*) there are still no established breakpoints available to interpret the MICs on bifidobacteria.

The finding that all strains except the pediococcal and the bifidobacterial strain showed a resistance to enrofloxacin was considered to be not of a serious concern for the use as feed additive since quinolone antibiotics are known to possess only a weak effect on LAB strains in general [37]. The *P. acidilactici* chicken strain and the two lactobacilli strains showed additionally resistances to vancomycin. These results are in accordance with previous findings in *Lactobacillus* and *Pediococcus* species, which have shown that the vancomycin resistance in these species is intrinsic, chromosomally coded, and nontransferable [38 – 40]. Intrinsic resistances or resistances by mutation of chromosomal genes in feed additive strains are generally acceptable because the risk of horizontal dissemination is considered as minimal. Nevertheless, as recommended by the SCAN further investigations were carried out which demonstrated that the intrinsic vancomycin resistance of the chicken strains did not mask a *vanA*-mediated resistance. VanA is an acquired resistance type which is transferable by conjugation [41]. The results of the plasmid preparation and the conjugation experiments revealed the absence of plasmids and nontransferability of the vancomycin resistance in the R strains. Testing these strains for the enterococcal *vanA* gene by PCR gave also a negative result which supported our assumption that the vancomycin resistance in these strains is not acquired. Since the resistance was not associated with plasmids, the risk of transfer to other organisms can be considered as minimal. Besides this, the MIC values displayed a low level-resistance to tetracycline for the *P. acidilactici* strain (CE1), the *E. faecium* strain (CE2), and the *L. reuteri* strain (CE4). The presence of tetracycline R bacteria in CE products is a cause of concern because the tetracycline resistance genes can be mobilized by means of conjugative elements [23, 42, 43]. The presence of added genes coding for antibiotic resistance particularly when carried by mobile genetic elements presents the greatest risk for horizontal dissemination of resistance. According to the results of the PCR a *tet*(M) specific PCR product was detected in both the *E. faecium* strain and the *P. acidilactici* strain, while it could not be obtained for the *L. reuteri* strain. *Tet*(M) confers resistance by ribosomal protection and has been reported in 42 genera, some of which were located both on the chromosome and on plasmids [43]. As a further consequence, the tetracycline R strains were rigorously examined for the presence of plasmids. In an effort to isolate plasmid-DNA in large scale, a cesium chloride density gradient was generated showing that the chicken strains were devoid of plasmid-DNA. Our finding that their tetracycline resistance is not plasmid-linked was confirmed by conjugation studies which demonstrated that the resistance was not transmissible to the enterococcal recipient.

Another key requirement for strains intended for the use in feed additives is that they should not harbor virulence determinants. Since members of the genus *Enterococcus* can carry some pathogenic potential [44], the *E. faecium* strain CE2 was additionally examined for the presence of genes associated with pathogenic strains of this genus [45 – 47]. The strain was screened for *esp*, a typical virulence determinant associated with epidemic enterococcal strains of nosocomial origin, the cytolysin activator *cylA* playing a role in the progression of enterococcal infections and the gelatinase gene *gelE* coding a protease that hydrolyses gelatin, collagen, casein, hemoglobin, and other bioactive peptides. The results of the PCR study indicated that none of the described virulence genes are present in the enterococcal chicken strain, giving a further support for the safety of the strain.

Together the collected data obtained in this study encouraged us to proceed with a more detailed study of the five chicken strains which are intended for the combined use as a feed additive for the broiler production. The chicken strains were found to exert inhibition against several indicator pathogens *in vitro*. Since the mode of inhibition is not exactly known, further work needs to be done in feeding experiments to evaluate the protective ability of the strains *in vivo*. Single antibiotic resistance characteristics of some strains, *e.g.*, those of the pediococcal and the lactobacilli

strains toward trimethoprim and/or the aminoglycosides are still under examination due to the uncertainty of the obtained results. Susceptibility testing will be optimized by using validated broth formulations which do not interfere with antimicrobials or growth medium components. As reported, easy transferable resistances or potential virulence traits were excluded mainly on basis of the absence of plasmids and nontransferability by conjugation. Anyhow horizontal dissemination of the *tet*(M)-associated resistance in the tetracycline R strains cannot be excluded *per se*. According to the actual guidelines [10, 48] acquired antibiotic resistance traits are not acceptable unless it can be shown that the genetic basis of the resistance is due to chromosomal mutation. Therefore some work is in progress to localize the *tet*(M) gene in the chromosome of the R strains. This future work will fill the gaps of uncertainty regarding their potential for transmission of the tetracycline resistance.

4 Concluding remarks

The current situation in the European Union opens possibilities to implement new strategies to control pathogens in feed and meat on basis of natural resources. Isolation, characterization, and risk assessment of intestinal strains are essential parts in the development of a safe probiotic feed additive. Selection of probiotic strains for the use in feed additives requires a critical evaluation, especially in the light of the EU requirements. Our work showed that fundamental research is still rather limited relating to the antibiotic resistance of LAB and bifidobacteria and their potential for transmission of genetic elements to other intestinal micro-organisms. Harmonized standard methods and breakpoints for susceptibility testing of probiotic strains have to be established in order to enable an effective safety assessment. The methods involved in assessing resistance in probiotics are currently under evaluation in the EU-funded project - named "Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain" (ACE-ART, CT-2003-506214). The scientific outcome is expected to support our work in future.

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