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TITLE

Identification and characterization of tetracycline resistance in *Lactococcus lactis* isolated from Polish raw milk and fermented artisanal products.

Joanna Zycka-Krzesinska[1#], Joanna Boguslawska[1,2,#], Tamara Aleksandrzak-Piekarczyk[1], Jakub Jopek[1^] and Jacek K. Bardowski[1*]

[1] Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warszawa, Poland

[2]: Department of Biochemistry and Molecular Biology, The Centre of Postgraduate Medical Education, Marymoncka 99, 01-813 Warsaw, Poland

^ Present address: Department of Bacterial Genetics, Institute of Microbiology, Warsaw Uniwersity, Miecznikowa 1, 02-096 Warsaw, Poland

# Both first authors equally contributed to this paper.

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[*]Corresponding author: Mailing address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawinskiego 5a, 02-106 Warszawa, Poland

Phone: +48 22 / 592 12 23; Secretariat: +48 22 / 592 21 45; Fax: +48 22 / 592 21 90

Email: jacek@ibb.waw.pl

ABSTRACT

To assess the occurrence of antibiotic-resistant Lactic Acid Bacteria (LAB) in Polish raw milk and fermented artisanal products, a collection comprising 500 isolates from these products was screened. Among these isolates, six strains (IBB28, IBB160, IBB161, IBB224, IBB477 and IBB487) resistant to tetracycline were identified. The strains showing atypical tetracycline resistance were classified as *L. lactis*: three of them were identified as *L. lactis* ssp. *cremoris* (IBB224, IBB477 and IBB487) and the other three (IBB28, IBB160, IBB161) were identified as *L. lactis* ssp. *lactis*. The mechanism involving Ribosomal Protection Proteins (RPP) was identified as responsible for tetracycline resistance. Three of the tested strains (IBB28, IBB160 and IBB224) had genes encoding the TetS protein, whereas the remaining three (IBB161, IBB477 and IBB487) expressed TetM. The results also demonstrated that the genes encoding these proteins were located on genetic mobile elements. The *tet*(S) gene was found to be located on plasmids, whereas *tet*(M) was found within the Tn*916* transposon.

1. Introduction

Lactococcus lactis is one of the best known and characterized species of Lactic Acid Bacteria (LAB). These bacteria are present in the natural environment, including products of spontaneous milk or plant fermentation. They play an important role in the production of various dairy products but are also used as food additives and animal feed. In addition, lactococci are also responsible for flavour formation through their proteolytic and amino acid conversion pathways (Rattanachaikunsopon and Phumkhachorn, 2010).

The common use of antibiotics in medicine and as "growth promoters" in animal breeding has caused a significant increase in the number of strains resistant to antibiotics, including among LAB. Tetracyclines are one example of such extensively used antibiotics. They are broad-spectrum antibiotics active against both Gram-positive and Gram-negative bacteria that act at the ribosomal level to interfere with bacterial protein synthesis. Tetracyclines have been widely used in livestock farming as well as in the prophylactic and therapeutic treatment of human and animal infections. Unfortunately, this prevalent use of tetracyclines has led to an increase in antibiotic resistance. The major mechanisms of tetracycline resistance involve efflux pumps, ribosomal protection proteins, and direct enzymatic drug inactivation. To date, more than 40 tetracycline-resistance genes have been identified and characterized, and the best known are *tet*(M), *tet*(S) and *tet*(O) (van Hoek et al., 2011). These genes are often located on mobile elements, such as plasmids and transposons, and may therefore be easily transferred between bacteria (Clewell et al., 1995; Rice, 1998).

The concomitant presence of antibiotics and bacteria growing at high densities enables the spread of antibiotic resistance among microorganisms. LAB, which are an element of the gastrointestinal microbiota, are potentially vulnerable to acquired antibiotic resistance. Furthermore, genes conferring antibiotic resistance can be easily transferred between

pathogenic, potentially pathogenic and commensal bacteria (Delgado et al., 2005; Devirgiliis et al., 2011; Mathur and Singh, 2005). Based on the above-described observations, it has been hypothesized that the gastrointestinal bacteria, including commensals, may be reservoirs of antibiotic-resistance genes (Salyers et al., 2004).

The increase in antibiotic resistance among microorganisms has been recognized as one of the most serious public health problems in the European Union (ECDC/EMEA, 2009). Therefore, it is crucial to estimate the level of antibiotic resistance of lactic acid bacteria and assess the role of LAB as a source of antibiotic-resistance genes. Thus, the aim of this study was to evaluate the presence of LAB strains resistant to antibiotics in samples isolated from Polish raw milk and fermented artisanal products as well as to molecularly characterize the *L. lactis* strains resistant to tetracycline.

2. **Materials and methods**

2.1. Bacterial strains and growth conditions

Five-hundred LAB isolates were recovered at the turn of the century from samples of Polish artisanal dairy products and raw milk from cows, sheep and goats collected from individual farms and local food markets were used in this study. The isolates were grown in M17 broth (Difco, Detroit, MI, USA) supplemented with 0.5% (wt/vol) glucose (POCH, Gliwice, Poland) (GM17) at 30°C for 24 to 48 h. Agar plates were prepared by adding 1.5% (wt/vol) agar (Merck, Darmstadt, Germany) to the liquid medium. The Tet^R (tetracycline-resistant; Tet-resistant) strains, were grown in GM17 supplemented with 10 μg ml^{-1} tetracycline (Sigma, St. Louis, MO, USA).

2.2. Antibiotic resistance screening

Ten microliters of overnight cultures of examined LAB isolates were dropped onto GM17 plates supplemented or not supplemented with antibiotics (purchased from Sigma, St. Louis, MO, USA) at the following concentrations: 2 μg ml^{-1} tetracycline (Tet), 6 μg ml^{-1} vancomycin (Van), 5 μg ml^{-1} erythromycin (Ery) and 200 μg ml^{-1} streptomycin (Str). The plates were incubated for 48 h for assessing the Tet-, Van-, Str- and Ery-resistant phenotypes. The concentrations of the antibiotics used in this study were chosen according to the published antibiotic breakpoints (Ammor et al., 2007; Florez et al., 2007). A bacterial isolates was assessed as antibiotic resistant if it was able to grow on GM17 with the given antibiotic.

2.3. Isolation of total and plasmid DNA

The total DNA from lactococcal cells was extracted according to a method reported by Anderson and McKay (1983). Plasmid DNA isolation was performed following the procedure reported by Birnboim (1979). The DNA preparations were stored at -20°C until use.

2.4. Species identification of antibiotics resistant LAB strains.

PCR assays were used for the taxonomic identification of the analysed isolates. To this end, the 16S rRNA gene fragments were amplified using two pairs of primers, namely 212-F/1406-R and 68F/1406-R to evaluate a isolate as *Lactococcus* species (Salama et al., 1991). All of the primers used in this study are described in Table 1. LacF/LacreR primers were used to discriminate *L. lactis* subsp. *lactis*, whereas the 68-F/1406-R and Cre-F/LacreR primers were used to distinguish *L. lactis* subsp. *cremoris* from other lactococci (Table 1) (Salama et al., 1991; Pu et al., 2002). To identify IBB11, IBB62 and IBB64 isolates, the G1 and L2 primers were used (Barangou et al., 2002).

PCR amplifications were performed with a MJ Research PTC-200 apparatus (Bio-Rad, Hercules, CA, USA) in a final volume of 20 µl containing 10 pmol of each primers 1x PCR buffer, 1x $MgCl_2$, 0.25 mM of each dNTP, 1U (Taq)-polymerase (Fermentas, Vilnius, Lithuania) and 10 ng of DNA template. The primer sequences, annealing temperatures and expected amplicon sizes are listed in Table 1. The PCR program consisted of denaturation at 94°C for 5 min followed by 30 cycles of 94°C for 30 s, annealing (temperature shown in Table 1) for 1 min, and elongation at 72°C for 2 min and a final extension at 72°C for 7 min. The PCR products were separated by electrophoresis on a 0.8% agarose gel (BioShop, Ontario, Canada) and visualized after ethidium bromide (Merck, Darmstadt, Germany) staining under UV light. The PCR products were then purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and sequenced by the DNA Sequencing and Oligonucleotide Synthesis Service at IBB PAS in Warsaw, using an ABI377 Sequencer. Homology searches were performed by BLAST (Basic Local Alignment Search Tool) against the protein database in the NCBI website.

2.5. Pulsed-Field Gel Electrophoresis (PFGE)

Genomic DNA was prepared *in situ* in agarose blocks (InCert Agarose, Lonza, Walkersville, MD, USA) as described previously (Hung and Bandziulis, 1990) and was digested overnight with the restriction enzyme SmaI (Fermentas, Vilnius, Lithuania) at 30°C (Howard et al., 1992). The SmaI-generated DNA fragments were resolved by PFGE in 1% agarose (PFGE-certified, Bio-Rad, Hercules, CA, USA) and 0.5x TBE buffer (Merck, Darmstadt, Germany) using a CHEF DR III (Bio-Rad, Hercules, CA, USA) apparatus. Electrophoresis was performed at a constant voltage of 6 V/cm and a temperature of 14°C for 23 h with a 120° angle and a pulse time of 1 to 25 s. The Lambda DNA ladder (BioLabs, Hitchin, UK) was used as a molecular size marker. The PFGE DNA patterns were compared, and a dendrogram was constructed using SynGene GeneTools, File version 4.00.00 (SynGene, Cambridge, UK).

2.6. Level of antibiotic resistance – Etest and disc diffusion methods

Using the Etest method, the levels of antibiotic resistance of the six Tet^R lactococcal strains and *L. lactis* IL1403 (as a control) were assessed. Individual colonies from GM17 plates (Klare et al., 2005) were suspended in 2 ml of sterile saline (Oxoid, Hampshire, UK) to a density corresponding to that of McFarland standard 1 (approximately $3x10^8$ cfu ml^{-1}). This suspension was used to inoculate the Iso-Sensitest (Oxoid, Hampshire, UK) agar plates or the Mueller-Hinton (Oxoid, Basingstoke, UK) agar plates (15 cm of diameter) by striking with a sterile cotton swab, and the plates were them dried for approximately 15 min at room temperature. Seven Etest strips (for seven different antibiotics: tetracycline, streptomycin, ampicillin, erythromycin, chloramphenicol, vancomycin and clindamycin; AB Biodisk, Solna, Sweden) were then applied to the Iso-Sensitest agar plates. This medium is recommended for determining the susceptibility of *Lactococcus* to antibiotics (ISO standard 10932:2010 (IDF

223:2010)). Antibiotic discs (39 different antibiotics tested from 14 groups of antibiotics, Oxoid, Hampshire, UK) were applied to the Mueller-Hinton agar plates (Huys et al., 2002). The zone of inhibition for each antibiotic were recorded after 48 h of incubation at 30°C, following the appropriate recommendations for the Etest (Florez et al., 2007; Klare et al., 2005; Mayrhofer et al., 2008) and disc diffusion methods (Charteris et al., 1998). The experiment was repeated twice for each bacterial strain.

2.7. Identification of tetracycline resistance genes.

In the Tet^R strains, *tet* genes encoding RPP were probed using the universal and degenerated primers DI and DII, as described by Gevers et al. (2003) (Table 1). In cases of positive results for the presence of RPP genes, additional PCR assays were performed with primers specific for the *tet*(M), *tet*(S), *tet*(O), *tet*(W), *tet*(Q) and *tet*(T) genes (Table 1) (Aminov et al., 2001; Gevers et al., 2003; Ng et al., 2001; Villedieu et al., 2003). The Tet^R strains were also tested for the presence of the tetracycline efflux genes *tet*(K) and *tet*(L) (Table 1) (Gevers et al., 2003;Ng et al., 2001).

Genes conferring antibiotic resistance are often located on transposons. Therefore, the detection of Tn916, which is the most common transposon in Gram-positive bacteria, was performed by PCR assays using previously published primers ReversTet(M)–2/Tn916-2 (Table 1) (Agerso et al., 2002). The PCR conditions used are described above. The amplified PCR products were purified using the Wizard SV Gel and PCR Clean-Up System and sequenced. Sequence alignments were performed using the BLAST search with the GenBank database (Altschul et al., 1990).

2.8. DNA hybridization

Both genomic DNA and plasmid DNA were used in this experiment. The genomic DNA from the tetracycline-resistant strains was digested with the EcoRI restriction enzyme (Fermentas, Vilnius, Lithuania) and was run on a 1% agarose gel (Sigma, St. Louis, MO, USA) with non-digested samples of plasmid DNA. The DNA was then transferred and blotted onto a nylon membrane (Hybond N+; Amersham Pharmacia, Little Chalfont, UK) using standard protocols (Sambrook, 2001). DNA fragments of the *tet*(M) and *tet*(S) genes obtained by PCR using specific primers (Table 1) were used as probes after labelling with α^{32}P-dATP (Hartmann Analytic, Braunschweig, Germany) and the HexaLabel^{TM} DNA Labelling Kit (Fermentas, Vilnius, Lithuania). The labelling, hybridization and detection steps were performed following the manufacturer's recommendations.

3. Results

3.1. Isolation and taxonomic identification of antibiotic-resistant strains

Tetracycline-resistant (Tet^R) and vancomycin-resistant (Van^R) isolates were recovered from various regions of Poland (data not shown). Of the 500 LAB isolates that were tested in the study, only seven (IBB11, IBB28, IBB160, IBB161, IBB224, IBB477, and IBB487) were able to grow on plates with tetracycline, whereas two isolates (IBB62 and IBB64) grew on plates with vancomycin. None of the isolates grew on media with the other tested antibiotics.

Six Tet^R isolates namely IBB28, IBB160, IBB161, IBB224, IBB477 and IBB487, were identified as *Lactococcus* species because a 1200-bp DNA fragment (coding 16S rRNA gene) was amplified using the primers 212-F and 1406-R (Salama et al., 1991). Three of the six isolates (IBB224, IBB477 and IBB487) were found to belong to the subspecies of *cremoris* (*L. lactis* subsp. *cremoris*) because PCR products of the expected lengths of approximately 1300-bp (68-F/1406-R primers) and 160-bp (Cre-F/LacreR) specific for *L. lactis* subsp. *cremoris* were obtained. A 160-bp fragment of DNA from the IBB28, IBB160 and IBB161 isolates was amplified using the primers LacF and LacreR indicating that these bacteria are *Lactococcus lactis* subsp. *lactis* (Fig. 1A).

Using the primers 212-F and 1406-R, no PCR product (fragment of 16S rRNA gene) was obtained from three of the studied isolates: IBB11, IBB62 and IBB64. Further taxonomic identification of these isolates was performed by amplification and subsequent sequencing of the 550-bp DNA fragment located between the 16S and 23S rRNA genes using the G1 and L2 primers (Fig. 1B). The DNA sequences of the amplified fragments were compared with those reported in the GenBank database. The results revealed that two Van^R isolates (IBB62 and IBB64) belong to *Leuconostoc citreum*, whereas IBB11 was identified as *Streptococcus parauberis*. Because *Leuconostoc citreum* bacteria are intrinsically resistant to vancomycin

and *Streptococcus parauberis* is known as a bacterial pathogen associated with bovine mastitis, they were not subjected to further investigations.

To examine the degree of mutual similarity of the tested Tet^R isolates, the PFGE technique was used. The SmaI PFGE profiles obtained for the six tetracycline-resistant strains displayed specific patterns for all of the tested samples (Fig. 1C). These patterns were then compared using the SynGene GeneTools program (File version 4.00.00). This analysis showed some differences and similarities between the Tet^R strains. The patterns of the bands for the IBB28, IBB160, IBB161 and IBB224 strains were significantly different. Additionally, it was observed that two of these (IBB477 and IBB487) exhibited very similar (but not identical) profiles that differed from each other in just a few bands (marked by arrows on Fig. 1C).

In conclusion, these results suggested that all six Tet^R isolates are different strains belonging to species *L. lactis*.

3.2. Level of antibiotic resistance

The susceptibility of six Tet^R strains to seven antibiotics essential for the treatment of animals and humans (tetracycline, streptomycin, ampicillin, erythromycin, chloramphenicol, vancomycin and clindamycin) was assessed by the Etest method. *L. lactis* IL1403 was used as the control strain. All of the tested strains were susceptible to chloramphenicol (MIC range = 1.5 – 3 μg ml^{-1}), vancomycin (MIC range = 0.25 – 1 μg ml^{-1}), streptomycin (MIC range = 8 – 32 μg ml^{-1}), erythromycin (MIC range = 0.047 – 0.094 μg ml^{-1}), ampicillin (MIC range = 0.125 – 0.25 μg ml^{-1}) and clindamycin (MIC range = 0.016 – 0.19 μg ml^{-1}). However, six Tet^R strains displayed resistance to tetracycline with MIC ≥ 256 μg ml^{-1} compared with the control strain, which, as expected, was susceptible to this antibiotic (MIC = 3 μg ml^{-1}; Table 2).

To examine whether the tetracycline resistance of the six Tet^R strains is correlated with their resistance to other antibiotics, the susceptibility to 39 antibiotics from 14 different chemical groups was tested using the disk diffusion method. Significant differences in the growth inhibition zones between the six Tet^R strains and the control strain were observed only in the case of doxycycline, another antibiotic from the tetracycline group. The diameters of the growth inhibition zones obtained for the IBB28, IBB160, IBB477 and IBB487 strains were smaller by approximately 52% in comparison with the control strain, whereas that obtained for IBB161 was approximately 56% smaller (Table 3).

Thus, there was no correlation between the resistance to tetracycline and the resistance to antibiotics from other classes in the tested strains.

3.3. Identification of tetracycline-resistance genes

The genetic basis of the observed phenotypic resistance was investigated by PCR with the primers DI and DII specific for the genes encoding RPP or primers specific for tetracycline efflux proteins (primers TetK-F, TetK-R, TetL-F, and TetL-R, presented in Table 1). In all of the tested strains, the RPP genes were identified (Fig. 2A), but neither *tet*(K) nor *tet*(L) was detected (data not shown). To identify specific genes involved in the RPP resistance mechanism, primers specific for the *tet*(M), *tet*(S), *tet*(O), *tet*(T), *tet*(Q) and *tet*(W) genes were used. The presence of the *tet*(M) gene was detected in the IBB161, IBB477, and IBB487 strains, whereas *tet*(S) was found in IBB28, IBB160 and IBB224 (Figs. 2B and 2C). None of the strains harboured the other analysed *tet*-resistance determinants (data not shown).

To further characterize the identified genes (*tet*(M) and *tet*(S)), their PCR-amplified fragments were sequenced. The obtained nucleotide sequences were analyzed using bioinformatic programs Clone Manager and BLAST. The results showed that the nucleotide sequence of the *tet*(M) gene from all three strains had highest homology to the *tet*(M) gene of

Enterococcus faecalis (Altschul et al., 1990; Su et al., 1992). The sequence of the *tet*(S) gene present in the IBB160 and IBB224 strains had maximal homology to the gene of *Listeria monocytogenes*, whereas the *tet*(S) gene from IBB28 was almost identical (99%) to the *tet*(S) gene from the lactococcal plasmid pK214 (Altschul et al., 1990; Perreten et al., 1997).

The results of these experiments demonstrated that the RPP mechanism of tetracycline resistance is present in all six examined Tet^R strains. The *tet*(S) gene is present in the IBB28, IBB160 and IBB224 strains, whereas *tet*(M) is present in IBB161, IBB477 and IBB487.

3.4. Presence of mobile elements in Tet^R strains

Many bacterial antibiotic-resistance genes are present on mobile genetic elements, such as plasmids and transposons. For this reason, the presence of plasmids in six Tet^R *L. lactis* strains was examined. The results demonstrated that most strains contain more than one plasmid. The analysis of the electrophoretic plasmid profiles showed the significant similarity between two pairs of strains, namely IBB28/IBB161 and IBB477/IBB487, whereas IBB160 and IBB224 were found to have their own unique plasmid profiles (Fig. 1S A).

The *tet*(M) gene is frequently associated with the *Tn*916 transposon. Therefore, to verify the presence of this mobile element in Tet^R *L. lactis* strains, the RevTet(M)-2 and Tn916-2 primers were used to amplify the *tet*(M) gene in connection with Tn*916* (2865-bp). In three Tet^R *L. lactis* strains, namely IBB161, IBB477 and IBB487, the appropriate PCR products were obtained, proving that these strains contain the *Tn*916 transposon (Fig. 1S B).

3.5. Location of the tetracycline-resistance gene

To identify the location of the *tet*(M) and *tet*(S) genes in the Tet^R *L. lactis* strains, the Southern hybridization technique was used. The 2256-bp DNA fragment (coding *tet*(M) gene with promotor fragment) and the 589-bp internal fragment of *tet*(S) gene obtained by PCR

were radiolabeled and hybridized to the total DNA digested with the EcoRI restriction enzyme and to the undigested plasmid DNA. In three strains (IBB161, IBB477 and IBB487), the *tet*(M) probe hybridized to the digested total DNA, whereas in two strains (IBB477 and IBB487), the probe hybridized to the plasmid. Regarding the *tet*(S) probe, it hybridized to both the digested genomic DNA and the plasmids in three strains (IBB28, IBB160 and IBB224; Figs. 2S A and B).

These results indicated that the *tet*(M) gene is present in IBB161 on the chromosomal DNA. In IBB477 and IBB487, the concomitant labelling of the plasmid and genomic DNA indicated that *tet*(M) was located on the plasmid excised from the chromosomal DNA. In contrast to the variable location of *tet*(M), the *tet*(S) gene from IBB28, IBB477 and IBB487 was located only on plasmid elements.

4. Discussion

Antibiotics play an important role in decreasing morbidity and mortality associated with bacterial infections and have a significant impact on the success of medicine. Additionally, they are also used as therapeutic agents and animal growth promoters and in agriculture for the control of plant diseases (Ammor et al., 2007; Wegener, 2003). This huge amount of used antibiotics has significantly affected the bacterial environment and has led to the selection of new antibiotic-resistant strains (Bronzwaer et al., 2002; EFSA, 2005), including lactic acid bacteria. These observations raise the importance of the regular screening of antibiotic-resistance gene transfer in these species. *L. lactis* have a long history of apparent safe use as food starter cultures. Based on their habitat and their extensive application in the food and feed sector *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* have been included in the QPS list, provided that the presence of acquired antibiotic resistance determinants has been excluded (EFSA, 2013; Leuschner et al., 2010). Because of these reasons our study aimed to identify the prevalence of antibiotic resistance in *L. lactis* strains isolated from Polish raw milk and fermented artisanal products and to characterize the molecular features of these isolates.

From a large collection of 500 LAB isolates from natural environments, only six *L. lactis* strains were revealed to be resistant to tetracycline, with markedly high MIC values greater than 256 μg ml^{-1} (Table 2) (EFSA, 2012). No *L. lactis* strains resistant to the other tested antibiotics (vancomycin, erythromycin and streptomycin) were detected. These results overlap with the results reported by other research groups, indicating that most of the antibiotic-resistant LAB strains are resistant to tetracycline and erythromycin (Devirgiliis et al., 2010). Similar results were obtained for both the bacteria isolated from the human digestive system (Mitsou et al., 2010) and as the bacteria isolated from dairy products (Ammor et al., 2008; Florez et al., 2005; Florez et al., 2007; Gad et al., 2014; Nawaz et al.,

2011). The reason for this may be the huge amount of tetracyclines that have been used for years in agriculture livestock farming as "growth promoters". However, it should be noted that the Tet^R genes were present in only 1% of the screened *L. lactis*, which is rare in comparison with the rate of approximately 20% demonstrated by other groups working within the ACE-ART Project. This discrepancy indicates that there is a markedly lower number of antibiotic-resistant bacteria present in the natural environment in Poland. However, it should be taken into account that a total ban on the use of antibiotics as growth promoters in Poland was introduced in 2006 and that the samples were collected at approximately 2000. A number of strains resistant to tetracycline and other antibiotics were therefore able to grow over the next few years.

The detailed molecular characterization of six Tet^R *L. lactis* strains revealed the presence of *tet*(M) and *tet*(S) genes belonging to the ribosomal protection protein mechanism (Aminov et al., 2001; Chopra and Roberts, 2001). The presence of RPP genes has been described in a number of bacterial genera, and it is the most popular mechanism among LAB (Ammor et al., 2008; Florez et al., 2008; Gevers et al., 2003; van Hoek et al., 2008; Roberts, 2005).

The main threat associated with antibiotic resistance in commensal bacteria is the risk of the horizontal transfer of its genetic elements to other bacteria, particularly to pathogens. Guglielmetti et al. (2009) demonstrated the transfer of *tet*(S) gene by conjugation from *Lactococcus garvieae* (fish pathogen) to the human pathogen *L. monocytogenes*, indicating the possibility of the transfer of *tet* genes from lactococci to human bacteria. The analysis of the presence of plasmids and the Tn*916* transposon in six Tet^R *L. lactis* strains supported these assumptions because most of the *tet* genes investigated in this study were also identified on mobile elements. The *tet*(S) gene was located on plasmids in all of the strains containing this gene, whereas the *tet*(M) gene was present on the Tn*916*, a highly infective transposon.

Similarly, Florez et al. (2008) demonstrated the association of the *tet*(M) gene in *L. lactis* with Tn*916*, which participates in the transfer of tetracycline resistance. This observation was previously confirmed by other researchers (Rice, 1998; Roberts and Mullany, 2009).

The result of hybridization experiments in case of IBB161 strain revealed no signal in a pool of plasmid DNA, suggesting the presence of the Tn*916* transposon on the chromosome. However, this does not preclude the possibility of the transfer of *tet* genes. The Southern hybridization results revealed that in two strains, IBB477 and IBB487, the *tet*(M) gene was located on the plasmid DNA. These results may indicate the location of the Tn*916* transposon either on the bacterial plasmid (a very rare situation) or it is situation when a transposon is present in the bacterial cell in the form a covalently closed circular intermediate ready for transfer to the recipient cells. In the IBB477 strain, the presence of the Tn*916* transposon on a large plasmid (60 kb) was confirmed by the results obtained from the sequencing of the entire genome of this bacterium (Kowalczyk, unpublished data; Radziwill-Bienkowska et al., 2014).

The transferability of Tn*916* to other bacteria was proven in our laboratory by Boguslawska (2009) and was confirmed by others research groups (Agerso et al., 2002; Poyart et al., 2000). Moreover, Boguslawska et al. showed that the *tet*(S) gene can be transferred between bacteria strains, which was likely connected with the presence of the plasmid. Therefore, we assume that all of the identified Tet^R genes are potentially transferable, which suggests that *L. lactis* may be a biological reservoir of tetracycline-resistant genes.

To summarize, the results of this study showed that the possibility of transferable resistance to tetracycline among Polish LAB strains exists, but that it is not as serious a problem as was suggested by other researchers (EFSA, 2012; Florez et al., 2005). The observation that multi-resistance is uncommon among *L. lactis* is in accordance with other published reports (Ammor et al., 2007; Florez et al., 2005). The results obtained both by

Roberts (2005) and our group showed that the risk of transfer of antibiotic-resistance genes to pathogens is low (Boguslawska et al., 2009; Roberts, 2005). Thus, the use of LAB species as probiotics in medicine and industry appears to be not only justifiable but also safe. However, the increasing number of antibiotic-resistant bacteria and the potency of transferring the resistance genes between pathogens should be under regular genetic monitoring, particularly in environments with high risk of mutations. The careful screening of LAB intended for use in food systems should allow the avoidance of the spread of antibiotic-resistance determinants in the gastrointestinal environment (Teuber et al., 1999; Teuber and Perreten, 2000).

Such studies involving the critical evaluation of antibiotic resistance in natural environmental and food chain have been published recently (Ammor et al., 2007; Devirgiliis et al., 2010; Florez et al., 2005; Florez et al., 2008; Hummel et al., 2007; Klare et al., 2007; Nawaz et al., 2011). The results of our study are part of an international project, ACE-ART (Assessment and Critical Evaluation of Antibiotic Resistance Transferability in Food Chain). ACE-ART was intended to investigate the antibiotic resistance in LAB strains from 11 countries. The low frequency of antibiotic resistance in LAB strains described in our study reflects the general tendency in other countries participating in ACE-ART. The successful cooperation in ACE-ART encourages the idea of international microbiological monitoring. Such strategy should be continued and supported to guarantee the safety of the use of probiotics in medicine, agriculture and industry.

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Figures

Figure legends:

Figure 1. Taxonomic identification of six tetracycline-resistant strains and analysis of the genetic similarity of the tetracycline-resistant *Lactococcus* strains.

A. Electrophoretic analysis of the PCR products obtained using primers for the detection of *Lactococcus* species. The names of the tested strains are provided on top of the figures. Lac: amplification of the fragment of the 16S rRNA gene using the LacF and LacreR primers; Cre: amplification of the fragment of the 16S rRNA gene using the CreF and LacreR primers; Lc: amplification of the fragment of the 16S rRNA gene using the: 212-F and 1406-R primers; Lcre: amplification of the fragment of the 16S rRNA gene using the 68-F and 1406-R primers. M, 1-kb DNA Ladder; M2, DNA Ladder Mix.

B. Electrophoretic analysis of the PCR products using the L1 and G1 primers, which are specific for *Lactobacillales*. The names of the tested strains are provided on top of the figures. Lane M, 1-kb DNA Ladder.

C. Pulsed-field gel PFGE profiles of Tet^R. Lane M, Lambda Ladder PFG Marker (BioLabs, New England). The names of the tested strains are provided in the upper part of the gel. The differences in the pattern of the bands between the IBB477 and IBB487 strains are marked with white arrows.

Figure 2. PCR detection of tetracycline-resistance determinants in all Tet^R *L. lactis* strains. The panels show that electrophoretic analysis of the PCR products amplified using the following specific primers:

A: DI and DII, **B**: DI and tetM-R, **C**: TetS-F and TetS-R.

Tables

Table 1.

Primers used in this study.

Table 2.

Level of antibiotic resistance assessed by the Etest method.

Table 3.

Level of antibiotic resistance assessed by the disc diffusion method.

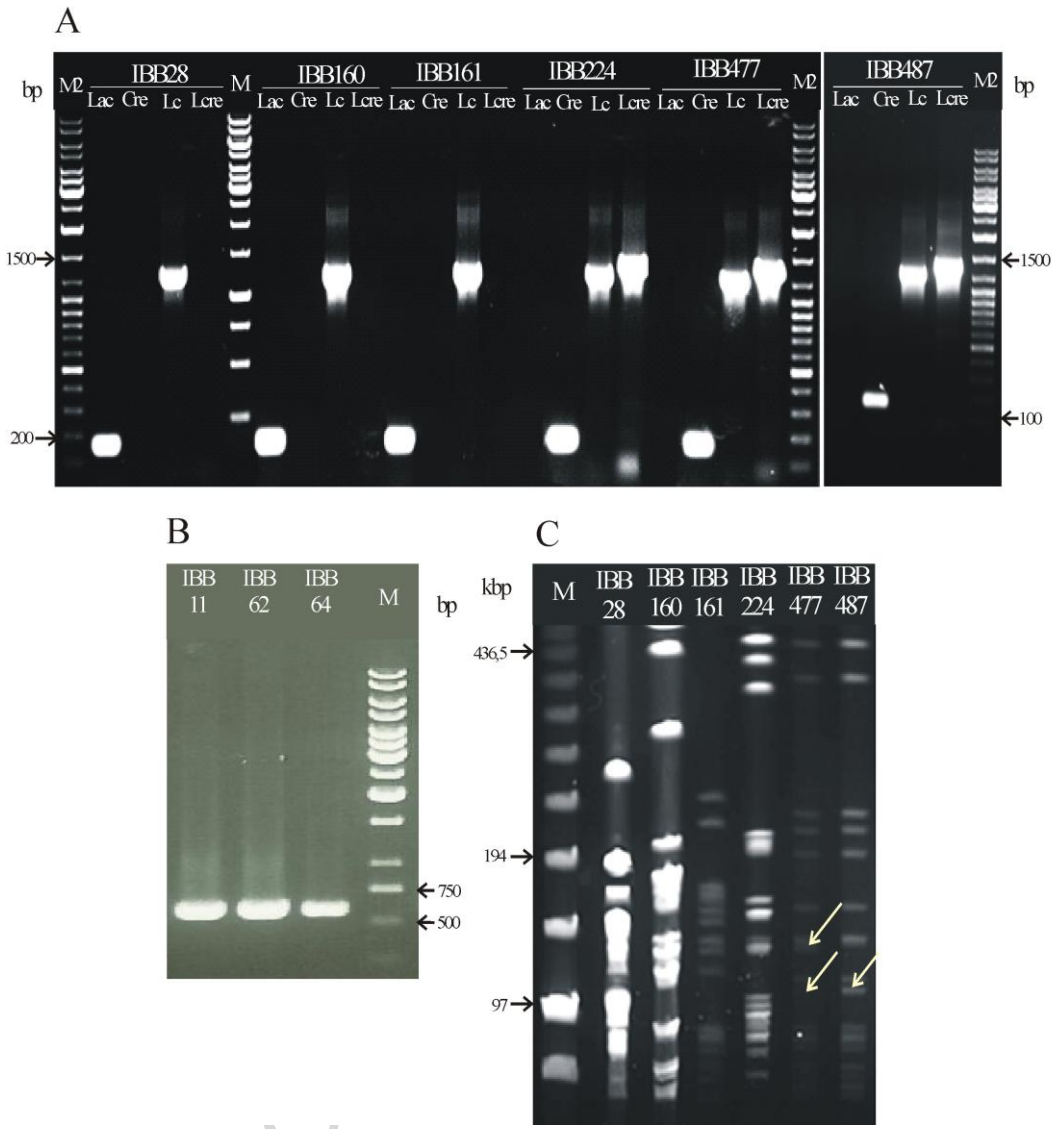

Figure 1

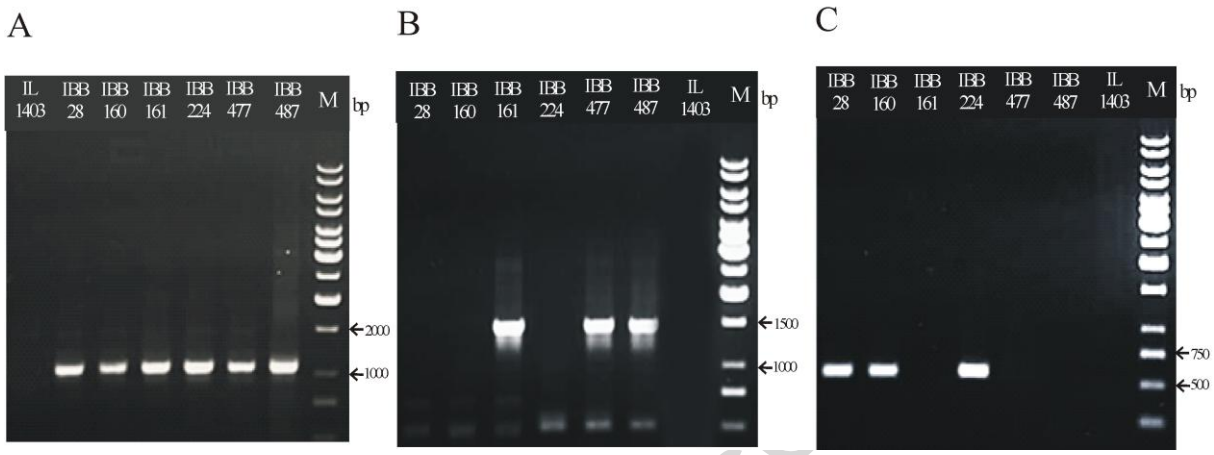

Figure 2

Table 1

Target	Primers	Sequence	Annealing temp (°C)	Product size (bp)	Reference
fragment of 16S rRNA	68-F 1406-R	5'- GATGAAGATTGGTGCTTGCA -3' 5'- ACGGGCGGTGTGTRC -3'	55	1338	Salama et al., 1991
fragment of 16S rRNA	212-F 1406-R	5'- GATGCAATTGCATCACTCAAAG -3' 5'- ACGGGCGGTGTGTRC -3'	55	1194	Salama et al., 1991
fragment between 16S and 23S rRNA	G1 L2	5'- GAAGTCGTAACAAGG -3' 5'- GGGTTTCCCCATTCGGA -3'	55	550	Barrangou et al., 2002
fragment of 16S rRNA	LacF LacreR	5'- GTACTTGTACCGACTGGAT -3' 5'- GGGATCATCTTTGAGTGAT -3'	45	160	Pu et al., 2002
fragment of 16S rRNA	CreF LacreR	5'-GTGCTTGCACCGATTTGAA -3' 5'- GGGATCATCTTTGAGTGAT -3'	45	160	Pu et al., 2002
RPP	DI DII	5'-GAYACNCCNGGNCAYRTNGAYTT-3' 5'-GCCCARWANGGRTTNGGNGGNACYTC -3'	45	1,083	Gevers et al., 2003
tet(M)	DI	5'-	55	1,513	Gevers et

		GAYACNCCNGGNCAYRTNGAYTT -3’			al., 2003
	TetM	5’- CACCGAGCAGGGATTTCTCCAC -3’			
tet(S)	TetS-F TetS-R	5’- ATCAAGATATTAAGGAC -3’ 5’- TTCTCTATGTGGTAATC -3’	50	573	Gevers et al., 2003
tet(O)	TetO-F	5’- AATGAAGATTCCGACAATTT -3’	55	781	Gevers et al., 2003
	TetO-R	5’- CTCATGCGTTGTAGTATTCAA -3’			
tet(W)	TetW-F	5’- GAGAGCCTGCTATATGCCAGC -3’	64	168	Villedieu et al., 2003
	TetW-R	5’- GGGCGTATCCACAATGTTAAC -3’			
tet(Q)	TetQ-F	5’- AGAATCTGCTGTTTGCCAGTG -3’	50	169	Aminov et al., 2001
	TetQ-R	5’- CGGAGTGTCAATGATATTGCA -3’			
tet(T)	TetT-F	5’- AAGGTTTATTATATAAAAGTG -3’	45	169	Villedieu et al., 2003
	TetT-R	5’- AGGTGTATCTATGATATTTAC -3’			
tet(K)	TetK-F	5’- TTATGGTGGTTGTAGCTAGAAA -3’	55	348	Gevers et al., 2003
	TetK-R	5’- AAAGGGTTAGAAACTCTTGAAA -3’			
tet(L)	TetL-F	5’- GTMGTTGCGCGCTATATTCC -3’	55	696	Gevers et al., 2003
	TetL-R	5’- GTGAAMGRWAGCCCACCTAA -3’			

fragment of Tn916	RevTet(M)–2 Tn916-2	5’- TTGTTAGAGCCATATCTTAG -3’ 5’- CTAGATTGCGTCCAA -3’	45	2835	Agerso et al., 2002
tet(M) gene and promotor fragment	TetM-FP TetM-RK	5’-GCGGGAACAAATAATTGGATGTCC -3’ 5’-CAGGACACAATATCCACTTG -3’	50	2256	This work

N = A, C, G or T; R = A or G; W = A or T; Y = C or T; M = A or C

Table 2

ANTIBIOTICS	MINIMUM INHIBITORY CONCENTRATION, MIC, FOR ANTIBIOTICS [µg/ml]						
	IL 1403	**IB B28**	**IB B160**	**IB B161**	**IB B224**	**IB B477**	**IB B487**
Tetracycline	3	**≥ 256**	**≥ 256**	**≥ 256**	**≥ 256**	**≥ 256**	**≥ 256**
Streptomycin	16	8	8	32	12	24	16
Ampicillin	0,125	0,125	0,25	0,25	0,125	0,25	0,25
Erythromycin	0,064	0,07	0,094	0,094	0,064	0,094	0,094
Chloramphenicol	3	2	2	1,5	2	3	1,5
Vancomycin	0,25	0,38	0,38	0,75	1	0,75	0,75
Clindamycin	0,047	0,016	0,094	0,19	0,125	0,125	0,125

Table 3

ANTIBIOTIC			THE ZONE OF INHIBITION [mm]						
GROUPS	**NAME**	**Concentrations [µg/dysk]**	**IL 1403**	**IBB 28**	**IBB 160**	**IBB 161**	**IBB 224**	**IBB 477**	**IBB 487**
Penicillins,/ Penicillins with inhibition of β-lactamas,	**Penicillin G**	10	26	24	20	21	24	22	21
	Ampicillin	10	25	26	20	23	25	22	21
	Amoxycillin	25	28	28	22	25	27	24	26
	Piperacillin	100	27	27	20	21	24	21	21
	Azlocillin	75	27	30	22	27	26	23	24
	Cloxacillin	1	6	6	6	6	6	6	6
	Carbenicillin	100	28	27	21	25	23	24	23
	Amoxycillin/Clavulanic acid	30	26	28	21	25	25	23	23
Cephalosporins	**Cephalothin**	30	23	25	19	22	21	22	20
	Cephradine	30	20	19	16	16	14	15	13
	Cefuroxime	30	30	31	26	28	27	29	27
	Cefepime	30	32	34	31	32	30	32	31
	Cefotaxime	30	30	32	26	28	24	30	25
	Ceftazidime	30	18	20	15	17	17	16	17
	Cefoperazone	75	27	27	24	24	25	25	25
Carbapenemases	**Imipenem**	10	36	38	30	33	33	33	32
Monobactams	**Aztreonam**	30	6	6	6	6	6	6	6
Glycopeptides	**Teicoplaina**	30	19	21	16	17	18	20	27
Aminoglycosides	**Amikacin**	30	16	21	15	17	17	14	14
	Gentamycin	120	22	24	21	21	23	22	20
	Kanamycin	30	18	22	17	17	20	16	16
	Neomycin	30	15	20	16	16	16	15	13
	Netilmicin	30	20	23	19	20	20	20	17
	Streptomycin	10	12	15	11	12	16	11	11
	Tobramycin	10	15	17	14	16	17	16	14
	Telithromycin	15	26	30	26	28	27	27	25
Tetracyclines	**Doxycycline**	30	25	12	12	11	12	12	12
Macrolides	**Linezolid**	30	28	30	26	28	26	26	26
Lincosamides	**Clindamycin**	2	26	27	20	19	21	21	20
Other inhibitor of	**Chloramphenicol**	30	24	30	25	24	25	25	23

protein synthesis									
Quinolones	**Nalidixic acid**	30	6	6	6	6	6	6	6
	Moxifloxacin	5	27	27	25	24	22	26	24
	Levofloxacin	5	22	22	20	20	21	23	24
	Pefloxacin	5	19	16	16	14	15	20	20
Polymyxins	**Colistin**	10	6	6	6	6	6	6	6