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Not Everything is a Question of Reputation: Safety of Bacteriocinogenic LAB Isolated from Smoked Salmon

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Total DNA extracted from bacteriocinogenic and potential probiotic lactic acid bacteria (LAB) (*Lactobacillus curvatus* ET06, ET30 and ET31, *Lactobacillus fermentum* ET35, *Lactobacillus delbrueckii* ET32, *Pediococcus acidilactici* ET34 and *Enterococcus faecium* ET05, ET12 and ET88), have been screened for presence of more than 50 genes related to production of biogenic amines (histidine decarboxylase, tyrosine decarboxylase and ornithine decarboxylase), virulence factors (sex pheromones, gelatinase, cytolisin, hyaluronidase, aggregation substance, enterococcal surface protein, endocarditis antigen, adhesion of collagen, integration factors) and antibiotic resistance (vancomycin, tetracycline, erythromycin, gentamicin, chloramphenicol, bacitracin). Tested strains presented low frequencies of presence for virulence genes. Only few genes were detected in some strains, indicating their safety for application in fermented food products. Besides all beneficial properties studied for various LAB, most considered as GRAS, a special attention need to be addressed on the possible presence of virulence factors, production of biogenic amines and antibiotic resistance. Results from appropriate biochemical tests and detected main genes associated to virulence factors and antibiotic resistance in LAB strains, could be considered as potential hazard of application of this organisms in food products. Moreover, additional *in vitro* and *in vivo* experiments must be designed in the system simulating GIT conditions in order to investigate deeper the possible expression of the present virulence genes.

1. Introduction

Since establishing beneficial potential of lactic acid bacteria (LAB) in fermented products, intensive research in different aspects for application of these microorganisms, including investigation of probiotic potential, re-establish of the gastrointestinal (GIT) microbiota balance after antibiotics treatment, prevention and handling of Gastro-Intestinal Tract (GIT) disorders, stimulation of immune system, treatment of skin diseases, control of some pathogens, involvement in prevention of some type of cancer and role in oral health been carried (Martinez et al., 2012). However, despite a high numbers of studies were reported on beneficial properties of probiotic LAB, only a limited number of publications focus on the safety aspects of these strains. We can clearly state, that based on the fact that majority of the *Lactobacillus* spp. have well accepted Generally Recognized as Safe (GRAS) status and are considered as safe for human and other animal application based on their historical safety record and application in different fermented food products from plant, meat and dairy origin. However, scanning the existing literature can be scarily realized that some infection clinical cases were linked to some *Lactobacillus* spp. strains (Goldstein et al., 2015). One of the essential points in evaluation of safety of the future beneficial LAB is presence of antibiotic resistance. To have or to be free of antibiotic resistance genes is a very important question, when related to safety of LAB. From one point, probiotics frequently are applied as parallel treatment with antibiotics or in prevention of diseases. In these cases, resistance to antibiotics can be considered as only way of combined application of probiotics and antibiotics. However, from other point, if probiotic bacteria carries antibiotics resistance genes, especially on easy transferable genetic material, these can be a hazard of spread of these genes via horizontal genes transfer to

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other bacteria, including to some relevant human and other animals pathogens. Related to previous, it is important to know antibiotic resistance profile for beneficial LAB, as they can act as potential reservoirs of resistance genes that can result in generation of multidrug resistant strains (Dicks et al., 2011). In addition, probiotics are frequently prescribed to the consumers under treatment for a variety of illnesses as an accompanying therapy. However, we need to be aware that the beneficial effects of the probiotic strain may be reduced by possible interactions with the medicaments used by these patience/consumers. Moreover, other very important point is that the interaction between medicaments and/or antibiotics from one side and probiotic bacteria from other side in the GIT will depend on their concentration in this specific environment (Todorov et al., 2009). Minimal Inhibitory Concentration (MIC) values for different drugs in the interaction between probiotic LAB and medicaments needs to be carefully evaluated in order to ensure efficacy of application for probiotics. Special attention need to be considered to drugs for treatment of chronic diseases, since due to their long-term application they may have accumulate in the GIT and affect the viability of probiotic LAB (de Carvalhon et al., 2009).

LABs were applied in the bio-preservation process since early stages of human civilizations. Preparation procedure of different fermented food products applied empirically antimicrobial metabolites of different LAB. In addition, traditional medicine recommended these fermented food products for the treatment of different diseases (Jacome et al., 2014; Bogsan et al., 2015). In fact, most of these traditional fermented food products on point of view of 21 century science can be considered as a probiotic, beneficial, functional products and some of biopreservation properties can be addressed to the production of antimicrobial peptides (bacteriocins) by LAB (Bogsan et al., 2015). However, even bacteriocins are described as generally safe, cytotoxicity of the bacteriocins it is a point that needs to receive more attention. Toxicological studies showed that nisin intake does not cause toxic effects to the human body with an estimated lethal dose (LD50) of 6950 mg/kg, which is similar to that of salt, when administered orally (Jozala et al., 2017).

In this work we explore safety aspects of *Lactobacillus curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *Pediococcus acidilactici* ET34 and *Enterococcus faecium* ET05, ET12 and ET88, bacteriocinogenic (Tome et al., 2008) and potentially probiotic strains (Todorov et al., 2011) isolated from smoked salmon, related to presence of genes associated to virulence, antibiotic resistance and production of biogenic amines and physiological test on expression of some virulence factors.

2. Material and Methods

2.1 Strains and media: *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88, bacteriocinogenic strains isolated from smoked salmon previously identified by physiological, biochemical and biomolecular approaches (Tome et al., 2008) were cultured in MRS broth (Difco, Detroit, MI, USA), at 37 ^{o}C and stored at −80 ^{o}C, in presence of 20% glycerol.

2.2 Detection of virulence genes: Total DNA from studied selected strains was isolated using the ZR Fungal/Bacterial DNA Kit (Zymo Research, Irvine, CA, USA) following the instructions of the manufacturer. DNA concentration was determined on NanoDrop (TermoFisher, Waltham, MA, USA). All PCR reactions were performed using the GeneAmp® PCR Instrument System 9700 (Applied Biosystems, Foster City, USA). All strains were tested for presence of virulence genes: production of gelatinase (*gel*E), hyaluronidase (*hyl*), aggregation substance (*asa*1), enterococcal surface protein (*esp*), cytolisin (*cyl*A), endocarditis antigen (*efa*A), adhesion of collagen (*ace*), vancomycin resistance (*van*A, *van*B, *van*C1, *van*C2, *van*C2/C3), erythromycin resistance (*erm*A, *erm*B, *erm*C), tetracycline resistance (*tet*K, *tet*L, *tet*M, *tet*O, *tet*S), related to gentamycin resistance (*aac*(6')-Ie-*aph*(2")-Ia), aminoglycosides resistance (*aph*(3')-IIIa, *ant*(4')-Ia, *aph*(2")-Id, *aph*(2")-Ic, *aph*(2")-Ib, *ant*(6)-Ia), chloraphenicol resistance (*cat*A), bacitracin resistance (*bcr*B, *bcr*D, *bcr*R), production of sex pheromones (*ccf*, *cob*, *cpd*), serine protease (*spr*E), transposom related (*int*, *int*Tn) and genes for amino acid decarboxylases: *hdc*1 and *hdc*2 (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase), using PCR protocols of Moraes et al. (2012) and Fortuna et al. (2008).

2.3 Virulence activity: Cultures of the nine studied strains were subjected to phenotypical tests to identify expression of virulence activity, as previously described by Moraes et al. (2012). Tests screening for expression of virulence potential (hemolytic activity, gelatinase, lipase and DNAse production), by studied LAB were performed with different combinations of time and incubation temperature with aim to verify the production of the virulence factors in diverse conditions related to production, storage and application of studied strains, detailed in the following, in three independent trials.

3. Results and Discussion

Based on the performed PCR screening for presence of 50 virulence genes, it can be observed that *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88 carry at least few virulence genes. However, no evidences for presence of *hyl*, *ace*, *aph*(3′)-IIIa, *aph*(2″)-Ib, *ant*(6)-Ia, *cat*A, *bcr*D, *bcr*R, *ddl*E, *mur*2ed, *mur*2ef, *spr*E and *odc* in all tested 9 strains were recorded (Table 1). Moreover, the observed frequency of possible presence of the virulence factors in *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88 was lower than that reported in other studies on *Lactobacillus* spp. and *Enterococcus* spp. isolated from foods (Todorov et al., 2014) and also in comparison to studies with clinical isolates (De Sousa, 2003). 10, 10 and 18 from 50 tested virulence genes were detected in *Lb. curvatus* ET 06, ET30 and ET31, respectively; 15, 13 and 14 from 50 tested virulence genes in *E. faecium* ET05, ET12 and ET88, respectively; 15, 17 and 14 from 50 tested virulence genes in *Lb. delbruekii* ET32, *P. acidilacticii* ET35 and *Lb. fermentum* ET35, respectively. In previous study (Todorov et al., 2014) *Lb. plantarum* ST16Pa isolated from papaya was described to be positive for presence of *gel*E (gelatinase), *hyl* (hyaluronidase), *asa*1 (aggregation substance), *ace* (adhesion of collagen) and *tdc* (tyrosine decarboxylase), a high virulence profile when compared to the results obtained in the present study for *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88.

Table 1. Presence/absence for virulence factors, antibiotic resistance and biogenic amine production

	ET06	ET30	ET31	ET05	ET12	ET88	ET32	ET34	ET35
gelE	-	-	+	-	-	-	+	-	-
asa1	-	-	-	-	-	+	-	+	-
esp	+	+	+	+	-	+	+	+	-
cytA	-	-	+	+	+	+	-	-	+
efaA	-	-	+	-	-	-	-	+	+
ermA	+	+	+	+	-	-	+	-	+
ermB	-	-	-	-	+	+	+	-	+
ermC	+	+	+	+	+	+	+	+	+
tetK	-	-	+	-	+	-	+	+	-
tetL	-	-	-	-	-	+	-	-	-
tetM	+	+	+	-	-	-	-	-	-
tetO	+	+	+	-	-	-	-	+	+
tetS	-	-	-	+	+	+	-	-	-
aac(6')-Ie-aph(2")-Ia	-	-	-	-	+	-	+	-	-
ant(4')-Ia	-	-	-	-	-	-	+	-	-
aph(2")-Id	-	-	-	-	-	-	-	+	-
aph(2")-Ic	+	-	+	-	-	-	+	+	+
vatE	-	+	+	+	-	-	-	+	+
bcrB	+	+	+	+	-	-	+	+	+
aac(6')-Ii	+	-	+	-	-	-	-	-	-
ccf	-	-	-	-	+	-	-	+	+
cob	-	-	+	+	-	-	-	+	+
cpd	-	-	-	-	-	-	-	+	-
int	-	-	-	+	+	-	-	+	-
fsrA	-	-	-	+	-	-	-	-	-
fsrB	-	-	-	-	-	+	-	-	-
fsrC	-	-	-	-	-	-	+	-	-
int-Tn	+	+	+	+	-	-	+	+	+
vanA	-	-	+	-	-	-	-	-	+
vanB	-	-	+	-	+	+	+	-	-
vanC1	-	-	-	-	+	+	-	+	-
vanC2	+	+	+	+	-	+	+	+	+
vanC1(2)	-	-	-	+	+	+	+	-	-
vanC1/C2	-	+	-	+	+	+	-	-	-
hdc1	-	+	-	-	-	-	-	-	-
hdc2	-	-	-	-	+	-	-	-	-
tdc	-	-	-	+	-	+	-	-	-
odc, sprE, mur2ef, mur2ed, ddlE, bcrD, bcrR, catA, ant(6)-Ia, aph(2")-Ib, aph(3')-IIIa, ace, hyl	-	-	-	-	-	-	-	-	-

Positive results (+) for genes for virulence, biogenic amines and antibiotic resistance in *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88

Presence of virulence factors was always been subject of investigation for pathogenic bacteria related to clinical isolates. Special attention was given to *Enterococcus* spp. as some of them can be described as opportunistic pathogens; however, some of other LAB can carry virulence factors as well and even they cannot be expressing them, need to consider this LAB as potential health hazard since at some specific conditions they may express them, or can be transferring this genetic information to other bacterial species. Moreover, the determination of virulence factors in LAB by molecular and phenotypic procedures needs to be considering as an important point, due to the risk of genetic transfer, since these genes are usually encoded by genes located in conjugative plasmids. Most LAB have remarkable long history as beneficial organisms, used in different fermentation processes and applied as probiotics (Martinez et al., 2012); however, some of them, including several species from genera *Enterococcus* and *Streptococcus* are considered as opportunistic pathogens and can be related with some clinical cases (Goldstein et al., 2015). Some *Enterococcus* spp. were described as carrying different determinants of pathogenicity, such as colonisation factors that promote the adhesion of bacteria to host cells and invasion factors that promote the invasion of epithelial cells disordering the immune system (De Sousa, 2003). Cell wall anchored surface proteins can be related to the bacterial virulence involved in the enterococcal pathogenicity, aggregation processes, including enterococcal surface protein, and collagen binding components. Some of these proteins (Enterococcal surface proteins, aggregation substance, adhesins and other adhesive molecules, such as Enterococcus endocarditis antigen) have important role and may facilitate close contact between cells and in this way facilitate conjugation processes and subsequent transfer of virulence plasmids (Hendrickx et al., 2009). However, on other side, some of the same proteins can be involved in the better adhesion and colonisation of the GIT. As negative factor, the aggregation substance protein may have a role in translocation of enterococci into epithelial cells (Hendrickx et al., 2009) and to be involved into the pathogenicity of these bacteria. A cell wall-anchored protein characterised by its ability to form biofilms, as Enterococcus surface protein are, they may be implicated in enterococcal infections associated with biofilms (Hendrickx et al., 2009).

Pheromones are Quorum sensing (QS) related molecules (Cook and Federle, 2014) involve in the bacterial interaction. They are produced by different bacteria and are normally small peptides, having a key role in regulatory processes in bacterial cells, including different bacterial basic processes, such as conjugation, a natural competence for transformation, biofilm development, and regulation of virulence factor processes (Cook and Federle, 2014). Even if there are not many works focusing on *Lactobacillus* spp. and *Pediococcus* spp. peptide pheromones, it is important to remind that these processes are relevant to all bacterial groups. Several genes related to production and expression of peptide pheromones can be a natural part of the genome of different bacterial species, however their presence can be result of the horizontal gene transfer within and between species via conjugative plasmids. Generally, conjugation processes, well studied in *Enterococcus* spp., are controlled via peptide pheromones (Cook and Federle, 2014). However, based on performed biochemical test, *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88 were not expressing any of the tested virulence factors (gelatinase, lipase and DNAse production and hemolytic activity). Not detecting expression of these factors can be related with the fact that expression may be related to the specific growth condition for the tested strains, or may be related to the more complex condition/interaction with different factors, or simply most probably the genes are partially inactivated or parts of the entire operon are damaged or not present.

As previously observed, the majority of the tested antibiotic inhibited the growth of *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88 to some extent, pointing that strains are not supposed to be hosting numerous genes related to antibiotic resistance (Todorov et al., 2011). Only five of tested antibiotics by Todorov *et al.* (2011) (amikacin, metronidasole, oxacillin, vancomycin and nalidixic acid) had no inhibitory effect on the tested selected strains. The fluoroquinolone ciprofloxacin presented an inhibitory effect only on strains *E. faecium* ET05 and ET12, *Lb. curvatus* ET31, *Lb. fermentum* ET35 and *P. acidilactici* ET34. The β-lactamic antibiotic Furazolidone inhibited the growth only of *E. faecium* ET05. The aminoglycoside kanamicin was active against strains *E. faecium* ET05 and ET12, *Lb. curvatus* ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32 and *P. acidilactici* ET34. Nitrofurantion inhibited only the growth of strain *Lb. curvatus* ET06. Tobramycin, another aminoglycoside antibiotic, inhibited the growth of strains *E. faecium* ET05, *Lb. curvatus* ET30, *Lb. delbrueckii* ET32 and *P. acidilactici* ET34, while trimethoprim was active against strains *E. faecium* ET05, *Lb. curvatus* ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32 and *P. acidilactici* ET34 (Todorov et al., 2011). However, in previous study (Todorov et al., 2011) only phenotypic evidences for antibiotic resistance were reported. This study was focused on presence of genes related to resistance of different antibiotics. Conflict between results related to antibiotic resistance based on the phenotypic studies and presence of appropriate genes is very common and needs to be understood as a fact, that we don't know yet all genes involved in this resistance processes from one side and that more complex processes can be involved in the physiological antibiotic resistance in different LAB. Based on our study *Lb. curvatus* ET06, ET30 and ET31 generated

positive results for some of evaluated genes related to erythromycin, tetracycline, aminoglicosides, bactitracin, gentamycin and vancomycin; *E. faecium* ET05, ET12 and ET88 for erythromycin, tetracycline, gentamycin and vancomycin; *Lb. deldrueckii* ET32 was positive for presence of erythromycin, tetracycline, amynoglicosides, bacitracin, gentamycin and vancomycin; *P. acidilactici* ET34 for erythromycin, tetracycline, amynoglicosides, bacitracin and vancomycin; and *Lb. fermentum* ET35 for erythromycin, tetracycline, amynoglicosides, gentamycin and vancomycin (Table 1). However, spread of the antibiotic resistance genes was a strain and gene specific (Table 1). Resistant strains to different antibiotics have been also identified, and several genes providing such resistance have been identified and studied; e.g., a chloramphenicol resistance gene (*cat*) has been detected in *Lactobacillus* spp.; different erythromycin-resistance genes (*erm*) have been found in many species of LAB, as well as a number of tetracycline resistance genes – *tet*(K, M, O, Q, S, W) (Dicks et al., 2011). Rivals *et al.* (2007) reported on presence and function of different antibiotic resistance genes in *Lb. delbrueckii* subsp. *bulgaricus,* including bacitracin and vancomycin (*bac*R, *van*R, acting on wall synthesis), novobiocin (*nov*R, acting on DNA replication), and kanamycin, spiramycin, streptomycin (*kan*R, *spi*R, *str*R, acting on RNA translation). Rojo-Bezares *et al.* (2006) reported, that *ant*(6), *aph* (3′)-IIIa and *tet*L genes were found in *Lactobacillus* and *Pediococcus* strains.

The aminoglycoside resistance gene *aac*(6')-Ie-*aph*(2'')-Ia has been reported in some *Pediococcus* species from animal and wine origin, including *P. pentosaceus* and *P. parvulus* (Tenorio et al., 2001). Genetic determinants for macrolides resistance [*erm*(AM) genes] have been analyzed in *P. acidilactici* strains (Tankovic et al., 1993), and one of these genes was found to be encoded on a 46-kbp non-transferable plasmid (Tankovic et al., 1993). An *erm*B gene has been associated to a plasmid in a *P. acidilactici* strain (Danielsen et al., 2007). Bacterial cells can use a variety of mechanisms to share and spread resistance determinants. In natural environment conditions the main mechanisms for horizontal gene transfer in bacteria are conjugation and transduction via bacteriophages (Kleinschmidt et al., 1993). In conjugation, plasmids may be important in spreading of antibiotic resistance (Kleinschmidt et al., 1993). Even, to be only limited information available regarding the lactobacilli and native conjugation system, this scenario is not impossible. Has been reported the transfer of genetic material - plasmid and transposoms from LAB to LAB and from LAB to other Gram-positive or Gram-negative bacteria (Dicks et al., 2011). The bacteria belong to Genera *Enterococcus* are well known for their ability as natural acceptor during conjugation process (Clewell and Weaver, 1989). Moreover, Enterococci may be also very good donors for the antibiotic resistance gene transfer to lactiobacilli (Shrago and Dobrogosz, 1988) and other enterococci (Rice et al., 1998). Gevers *et al.* (2003) was showing the possibility for an *in vitro* transfer of resistance to tetracycline between donor of *Lactobacillus* spp. and *E. faecalis* as receptor.

4. Conclusions

The studied *Lb. curvatus* ET06, ET30 and ET31, *Lb. fermentum* ET35, *Lb. delbrueckii* ET32, *P. acidilactici* ET34 and *E. faecium* ET05, ET12 and ET88 have been previously described as a bacteriocinogenic and potential probiotic LAB. However, priority needs to be given to safety aspects for any specific strain in order to be recommend/liberate for human or other animals application. Among previously determined positive features, studied strains presented their inability to produce histamine from the amino-acid histidine, and their lack of haemolytic activity. Low level of presence of virulence factors related genes can be considered as positive feature for studied LAB, moreover, additional *in vitro* and *in vivo* experiments must be designed in the system simulating GIT conditions in order to investigate deeper the possible expression of the few present virulence genes.

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