Anaerobe 29 (2014) 118–127

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

In vitro evaluation of the safety and probiotic properties of *Lactobacilli* isolated from chicken and calves

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ARTICLE INFO

Article history:
Received 15 May 2013
Received in revised form
16 September 2013
Accepted 25 October 2013
Available online 27 November 2013

Keywords:
Lactobacilli
Escherichia coli
Klebsiella
Maldi-Tof MS
β-Glucuronidase
Antibiotic resistance

ABSTRACT

A total of 73 chicken and calves isolates were diagnosed using matrix-assisted laser desorption ionization-time-of flight mass spectrometry (Maldi-Tof MS). After a preliminary subtractive screening based on the high acid tolerance at pH 2.5 and bile resistance at 0.3% oxgall, twenty isolates belonging to the species *Lactobacillus salivarius, Lactobacillus agilis*, *Lactobacillus reuteri*, *Lactobacillus murinus* and *Lactobacillus amylovorus* were *in vitro* screened for the safety assessment and probiotic properties, including antibiotics susceptibility patterns, biochemical activity and potential for competitive exclusion of biofilm producing pathogens determined by crystal violet and/or quantitative Fluorescent *in situ* Hybridisation (FISH) assays utilizing 5′Cy 3 labelled probe *Enter1432* for enteric group.

Antibiotic susceptibility testing was performed according to the ISO norm 10932. The sixteen strains were susceptible to certain antimicrobial agents, except for two chicken (*L. salivarius* 12K, *L. agilis* 13K) and two calves (*L. reuteri* L10/1, *L. murinus* L9) isolates with the presence non wild-type ECOFFs (epidemiological cut-off) for gentamicin (≥256 μg ml^{-1}), tetracycline (≥128 μg ml^{-1}), kanamycin (≥256 μg ml^{-1}) and streptomycin (≥96 μg ml^{-1}). The two referenced chicken isolates gave positive *aac(6′)Ie-aph(2″)Ia* and *tet(L)* PCR results.

The wild-type ECOFFs isolates were subjected to the apiZYM analysis for enzyme profile evaluation and amino acid decarboxylase activities determined by qualitative plate method and multiplex PCR for the detection of four genes involved in the production of histamine (histidine decarboxylase, *hdc*), tyramine (tyrosine decarboxylase, *tyrdc*) and putrescine (via eithers ornithine decarboxylase, *odc*, or agmatine deiminase, *agdi*). From examined strains only two chicken isolates (*L. reuteri* 14K; *L. salivarius* 15K) had no harmful β-glucuronidase, β-glucosidase activities connected with detrimental effects in the gastrointestinal tract and together no amino acid decarboxylase activities and no genes associated with biogenic amines production though only chicken *L. salivarius* 15K whole cells and acid supernatants shown strong suppressive potential against biofilm-forming *Klebsiella* and *Escherichia coli*. Our results highlight that above-mentioned isolate *L. salivarius* 15K fulfils the principle requirements of a qualified probiotic and may be seen as a reliable candidate for further validation studies in chicken.

1. Introduction

Antibiotics used as growth promoters in animal feeds have been banned and nowadays, the possibility of using alternative additives instead of antibiotics is being researched. One such opportunity is probiotics. In recent years, worldwide interest in the use of probiotic bacteria for health promotion and disease prevention has increased significantly in scientific community, consumers and food producers. This interest is based on the knowledge that the targeted use of microorganisms with suitable properties may have beneficial effect on animal and human health. Bacterial strains most commonly used as probiotics are *Lactobacillus, Lactococcus, Enterococcus and Bifidobacterium* [1] which belong to the lactic acid bacteria.

Before a screening of promising probiotic functional efficacy it is necessary to provide of preliminary *in vitro* screening to ensure the safety aspects of tested strains. Probiotic microorganisms should be free of undesirable traits, such as transmissible antibiotic resistance [2,3] (to avoid spreading resistance determinants in intestinal pathogenic or opportunistic bacteria), specific amino acid decarboxylase activities and thus, the possibility to synthesize biogenic amines with potential health risks to consumers [4] and harmful biochemical activities such as α-chymotrypsin, β-glucosidase, β-glucuronidase, and N-acetyl-ß-glucosaminidase activities, which are often associated with intestinal diseases and involved in

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http://dx.doi.org/10.1016/j.anaerobe.2013.10.009

generating carcinogens and tumour promoters [5]. The next important criterion is survival under gastrointestinal condition (acid pH and bile salt). Though the safety and efficacy of probiotics needs to be demonstrated in controlled clinical trials [6], their primary selection is based on a series of well-defined *in vitro* tests [7,8].

With the aim of selecting new promising probiotic candidates with minimized undesirable properties, twenty chicken and calves *lactobacilli* isolates were subjected to comprehensive *in vitro* analyses to assess their key safety and functionality.

2. Materials and methods

2.1. Bacterial isolates and growth condition

From 73 chicken and calves isolates identified using Maldi-Tof MS, twenty selected *lactobacilli* belonged to the five following species: *Lactobacillus salivarius* ($n = 7$), *Lactobacillus agilis* ($n = 2$), *Lactobacillus reuteri* ($n = 9$) and *Lactobacillus amylovorus* ($n = 1$), *Lactobacillus murinus* ($n = 1$). All *lactobacilli* isolates were routinely grown in MRS medium (Oxoid, England) by anaerobic incubation at 37 °C for 48 h.

The pathogens used in the experiments were *Klebsiella pneumonii* KPC (carbapenemase positive strain); uropathogenic *Escherichia coli* K188 and *E. coli DH5a/pCIB10B* (*ibeA* positive strain), which was obtained from VA Medical Center, Minneapolis, United States. The strains of *E. coli and K. pneumonii* were grown in MacConkey agar (Oxoid, England) overnight at 37 °C.

2.2. Bacterial identification

2.2.1. Maldi-Tof MS bacterial identification

Maldi-Tof MS was performed on a Microflex LT instrument (Bruker Daltonik GmbH, Leipzig, Germany) as described Bessede et al. [9]. To identify microorganisms, the raw spectra obtained for each isolate were imported into BioTyper software, version 2.0 (Bruker Daltonik). When the obtained score was >2.00, identification was considered correct at the species level; in the range 1.7–1.999, the identification was considered correct at the genus level; and <1.7, the identification was not sufficient. Merely *lactobacilli* strains with obtained scores higher than 2.3 were selected for further experiments.

2.2.2. Genotypic bacterial identification

Amplification of DNA was carried out using genus-specific primers *LBLMA 1-rev* and *R16-1*. The PCR mixture and cycle parameters were set according to Dubernet et al. [10] and PCR amplification was carried out in a C-1000 Thermal Cycler (Biorad). For the species identification, *lactobacilli* were firstly separated by a multiplex PCR (with primers *Ldel-7; LU-1; LU-3; LU-5; Lac-2*) into four groups based on the nucleotide sequences of the 16S–23S rRNA intergenic spacer region and adjacent 23S rRNA gene [11] and then merely *L. salivarius* strains together with finally selected *L. salivarius* 15K and *L. salivarius* 12K (*aac(6′)-aph(2′)-Ia* positive strain) were identified with species-specific primers *(For-Sal-3; Rev-Sal-1)* for confirmation of our Maldi-Tof results as described Harrow et al. [12].

2.3. Tolerance to acid and bile

Tolerance to low pH and bile content was assessed as described by Anderson et al. [13], with minor modifications. Briefly, *lactobacilli* were grown overnight at 37 °C in MRS broth in 5% CO_2. Each culture was vortexed and separated 10-ml aliquots were collected by centrifugation (3800 *g* for 20 min). Cell pellets were suspended to an approximate cell concentration of 10^6 CFU ml^{-1} in the following test solutions: MRS broth control, MRS broth adjusted to pH 2.5, MRS broth containing 0.3% oxgall (Difco). As a control, broth without inoculation was used. For survival under the different conditions, the samples were taken at 0 h and 4 h and then 100 μl cultures were plated after appropriate dilution on MRS agar plates. Enumeration was done following 48 h incubation at 37 °C.

2.4. Antibiotic susceptibility testing and MIC determinations

Antibiotic susceptibility testing was performed according to the ISO 10932/IDF 223 standard [14]. The minimal inhibitory concentrations (MICs) of the *lactobacilli* strains towards gentamicin, kanamycin, streptomycin, neomycin, tetracycline, erythromycin, clindamycin, chloramphenicol, ampicillin, penicillin, vancomycin, virginiamycin, linezolid, trimethoprim, ciprofloxacin and rifampicin was determined by the microdilution method using the microtiter VetMIC Lact-1 and Lact-2 panels for susceptibility testing of bacteria (Statens Veterinarmedicinska Anstalt, Uppsala, Sweden). In brief, strains were grown in MRS agar, colonies were resuspended in LSM medium consisted from 90% Isosensitest medium (Oxoid, England) and 10% MRS medium (Oxoid, England). The suspension was diluted 1000 times in the same medium, 100 μl (approximately 3×10^4 CFU/well) were then added to each well of the microdilution plate and incubated at 28 °C under anaerobic conditions for 48 h. Growth within each well was determined visually after incubation for each antibiotics by comparing with the positive control as recommended ISO 10932/IDF 223 standard [14]. MIC values surpassing the microbiological breakpoints were filled and confirmed moreover by using MIC strip tests (Liofilchem, Italy).

The MICs (μg ml^{-1}) were interpreted according to the recent FEEDAP document of the European Food Safety Authority (EFSA) on the update of the criteria used in the assessment of antibiotics bacterial resistance of human or veterinary importance [15] as well as epidemiological cut-off values defined by the ACE – ART Project results, ISO 10932 (2010) [14].

2.4.1. PCR Detection of resistance genes

Total DNA was extracted from bacterial cultures with the Dneasy Tissue Kit (Qiagen, France) according to manufacturer's instructions. Purified DNA samples were then stored at −20 °C.

PCR-based detection of genes responsible for resistance to gentamicin and other aminoglycosides, but not streptomycin [*aac(6′)-aph(2′)-Ia*], tetracycline [*tet(L), tet(M), tet(S)*], using primers and protocols described by Refs. [16,17], kanamycin [aph(3′)-IIIa] according Vakulenko et al. [18] and streptomycin/spectinomycin [aadA] according Clark et al. [19], was applied to strains suspected to carry acquired resistance.

2.5. Determination of ability to produce biogenic amines

The biogenic amines production (tyramine, histamine and putrescine) was assessed using the decarboxylase agar previously described by Bover-Cid and Holzapfel [20]. The precursor amino acids (tyrosine, histidine and ornithine, respectively) were purchased from Sigma. *Lactobacilli* strains were inoculated onto the decarboxylase plates and incubated for 4 days at 37 °C under aerobic and anaerobic conditions. The positive results were indicated by a change of the colour from yellow to purple in response to the pH shift caused by the production of the alkaline biogenic amines from the amino acids included in the medium.

2.5.1. The multiplex PCR for detection of genes responsible for biogenic amines production

DNA was extracted from bacterial cultures as described previously. For simultaneous detection of four biogenic amines genes: histamine (histidine decarboxylase, *hdc*), tyramine (tyrosine