

## *In vitro* Study of Some Safety and Beneficial Properties of Bacteriocinogenic *Lactococcus lactis* subsp. *lactis* MK02R

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*Lactococcus lactis* subsp. *lactis* MK02R, isolated from rocket salad and previously characterized as a bacteriocin producer strain, was evaluated for its beneficial and safety properties. Genetic and physiological tests indicated that this strain has some probiotic properties such as tolerance to low pH and high levels of ox-bile, production of  $\beta$ -galactosidase, deconjugation of bile salts, high level of hydrophobicity, functional auto- and co- aggregation properties, few virulence factors and absence of genes related to production of biogenic amines and antibiotic resistance. Tests performed with thirty-one antibiotic disks indicated that *Lc. lactis* subsp. *lactis* MK02R is resistant to metronidazol, trimetoprim and nalidixic acid, but all other tested antibiotics inhibited its growth to some extent.

The strain grew well in the presence of various commercial medicaments (34 tested) spotted on the surface on of MRS agar inoculated with *Lc. lactis* subsp. *lactis* MK02R ( $10^6$  CFU/mL), being inhibited only by those containing amoxicillin ( $\beta$ -lactam antibiotic, MIC = 0.39 mg/mL), amiodarone, (antiarrhythmic, MIC = 5.0 mg/mL), diclofenac (non-steroidal anti-inflammatory, MIC = 0.62 mg/mL and 1.25 mg/mL, depending on the manufacturer), dimenhydrinate (antiemetic, MIC = 20.0 mg/mL), ibuprofen arginine (non-steroidal anti-inflammatory, MIC = 15.0 mg/mL) and norfloxacin (antibiotic, MIC = 5.0 mg/mL). PCR analysis evidenced that DNA of *Lc. lactis* subsp. *lactis* MK02R lacks the majority of the tested virulence genes, except *esp* (enterococcal surface protein), *tdc* (tyrosine decarboxilase) and *vanA* (vancomycin resistance) genes. These results suggest that bacteriocinogenic *Lc. lactis* subsp. *lactis* MK02R is a promising probiotic candidate, with low virulence features, complementing its antimicrobial activity.

### 1. Introduction

Lactic acid bacteria (LAB) have been subjected to intensive research aiming at exploring their potential applications as beneficial probiotic microorganisms. Normally granted with a GRAS (generally recognized as safe) status (Nishie et al., 2012), recent alarming communications on possible presence of virulence factors (Todorov et al. 2017; Moraes et al. 2017) indicate that LAB need a special attention concerning their safe and easy application as food preservatives.

*Lactococcus lactis* subsp. *lactis* MK02R is a LAB strain capable to produce a bacteriocin active against *Listeria innocua* and *L. monocytogenes* from different serological groups (Kruger et al. 2013). This strain was isolated from rocket salad and identified based on PCR-ARDRA approach. The bacteriocin MK02R is heat-stable (one hour at 60 °C and 100 °C), sensitive to proteolytic enzymes, stable at pH between 2.0 and 9.0 and the production at 37 °C is stimulated by cysteine added to the MRS broth. Adsorption of bacteriocin MK02R to the producer cells surface is low. The bacteriocin remains bound to the outer surface of the producer cells and is released when the pH of the environment increases. Chromatographic and genetic tests using appropriate primers indicated that bacteriocin MK02R is a natural variant of nisin. Partial sequencing of the purified peptide showed that bacteriocin MK02R has a change in the amino acid sequence of the leader peptide, when compared to nisin A, Z, Q, U and F, but the structure of the mature bacteriocin is homologous to that of nisin F (Kruger et al. 2013).

Although many studies have evaluated a wide range of bacteriocins produced by *Lc. lactis* strains, few have evaluated the safety aspects of the bacteriocins and the producer strains, which is essential for their

successful application in foods. The aim of this work was to characterize the beneficial properties and safety of bacteriocinogenic *Lc. lactis* subsp. *lactis* MK02R in terms of behavior in a simulated gastrointestinal tract environment, production of  $\beta$ -galactosidase, deconjugation of bile salts, hydrophobicity, aggregation properties, presence of genes encoding virulence factors and interaction with commercial medicaments.

## 2. Material and methods

### 2.1 Strains and media

*Lactococcus lactis* subsp. *lactis* MK02R, isolated from rocket salad (Kruger et al. 2013) and the bacteriocin activity indicators *Listeria monocytogenes* 603 and *Enterococcus faecalis* ATCC 19443 were cultured at 37 °C in MRS broth and BHI (Difco, Detroit, MI, USA), respectively, and stored at -80 °C, in presence of 20 % glycerol.

### 2.2 Growth at different pH and bile concentrations

*Lactococcus lactis* subsp. *lactis* MK02R was grown in MRS broth (Difco) with pH adjusted to 3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0 and 13.0 adding 1 mol/L HCl or 1 mol/L NaOH before sterilization. If needed, pH was re-adjusted after sterilization adding sterile 1 mol/L HCl or 1 mol/L NaOH. *Lc.* subsp. *lactis* MK02R was also grown in MRS broth containing 0.2 %, 0.4 %, 0.6 %, 0.8 %, 1.0 %, 2.0 % and 3.0 % (w/v) oxbile (Sigma). All tests were conducted in sterile flat-bottom 96-well microtiter plates (TPP, Switzerland). Optical density readings were recorded at 600 nm every hour for 12 h, using a microtiter plate reader (TPP, Switzerland). Cultures grown in MRS broth at pH 6.5 and without bile served as controls.

### 2.3 $\beta$ -galactosidase activity

$\beta$ -galactosidase activity was assessed employing sterile filter paper disks impregnated with *o*-nitrophenyl- $\beta$ -D-galactopyranoside (ONPG Disks, Fluka, Buchs, Switzerland), according to the manufacturer instructions.

### 2.4 Bile salts deconjugation

Bile salts deconjugation was tested streaking overnight cultures of *Lc. lactis* subsp. *lactis* MK02R on MRS agar plates containing 0.5 % (w/v) of sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC) or glycodeoxycholic acid (GDC) (Sigma-Aldrich) according to de Moraes et al. (2017).

### 2.5 Cell surface hydrophobicity

Cell surface hydrophobicity (%H) was tested according to de Moraes et al. (2017), and calculated as Eq(1)

$$\%H = (A_0 - A)/A_0 \times 100 \quad (1)$$

where  $A_0$  and  $A$  were the absorbance values before and after extraction with the organic solvent, respectively.

### 2.6 Auto-aggregation and co-aggregation

Aggregation properties were recorded according to Todorov et al. (2008). Percentage of auto-aggregation was calculated as Eq(2)

$$\% \text{ Auto-aggregation} = [(OD_0 - OD_{60})/OD_0] \times 100 \quad (2)$$

$OD_0$ , where  $OD$  and  $OD_{60}$  refer to the OD determined after 60 min. For evaluation of co-aggregation, *Lc. lactis* subsp. *lactis* MK02R and *L. monocytogenes* 603 and *E. faecalis* ATCC 19443 were grown on MRS or BHI, at 37 °C. Cells suspension were prepared and combined as described by Todorov et al. (2008) and co-aggregation was calculated as Eq(3)

$$\% \text{ Co-aggregation} = [(OD_{tot} - OD_s)/OD_{tot}] \times 100 \quad (3)$$

$OD_{tot}$  refers to the initial OD, taken immediately after the relevant strains were paired.  $OD_s$  refers to the OD of the supernatant after 60 min.

### 2.7 Interaction of *Lc. lactis* subsp. *lactis* MK02R with commercial drugs

*Lactococcus lactis* subsp. *lactis* MK02R was tested for growth in the presence of medicaments listed in Table 1. Culture of *Lc. lactis* subsp. *lactis* MK02R grown in MRS broth was added to MRS soft agar (1.0 %, w/v, Difco) to achieve a population of  $10^6$  CFU/mL. The solidified agar plates were spotted with 10  $\mu$ L of the medicaments solutions, incubated at 37 °C for 24 h, and examined for growth inhibition zones around the spotted medicaments. Those that resulted in inhibition zones larger than 2 mm diameter were subjected to the determination of the minimal inhibition concentration (MIC).

## 2.8 Antimicrobial resistance

The susceptibility to selected antimicrobials was assessed according to Charteris et al. (1998), using test discs (Oxoid, Basingstoke, UK) from different antibiotics groups.

## 2.9 Detection of virulence genes

Genomic DNA was extracted from *Lc. lactis* subsp. *lactis* MK02R using a DNA extraction kit (Zymo Research, USA) and tested for the virulence genes *gelE* (gelatinase), *hyl* (hyaluronidase), *asa1* (aggregation substance), *esp* (enterococcal surface protein), *cylA* (cytolysin), *efaA* (endocarditis antigen), *ace* (adhesion of collagen), *vanA* and *vanB* (both related to vancomycin resistance), and genes for amino acid decarboxylases: *hdc1* and *hdc2* (both related to histidine decarboxylase), *tdc* (tyrosine decarboxylase), and *odc* (ornithine decarboxylase), using PCR protocols of de Moraes et al. (2017).

## 3. Results and discussion

Good growth of *Lc. lactis* subsp. *lactis* MK02R was recorded in MRS broth (Difco) at pH 5.0, 6.0, 7.0, 9.0 and 11.0 (Fig. 1A), but no growth was observed at pH of 3.0, 4.0 or 13.0. Supplementation of MRS with 2.0 % or 3.0 % oxbile inhibited growth of *Lc. lactis* subsp. *lactis* MK02R (Fig. 1B). The effect of pH and oxbile on growth of LAB is species and strain specific. In general *Enterococcus* spp. and *Lactococcus* spp. are more resistant to low pH and oxbile than *Lactobacillus* spp. and *Leuconostoc* spp., which can be explained by the specificity of the ecological niches for these groups of LAB. Previous studies have shown that the growth of several strains of LAB is suppressed at pH 3.0 and 4.0, whereas variable results were recorded for pH 11.0 and 13.0 (Todorov et al. 2008). Growth of some LAB was affected by oxbile levels as low as 0.6 % (w/v) (Todorov et al. 2008). Haller et al. (2001) reported variable results for LAB when exposed to HCl (pH 2.0) and bile salts, observing that 10.0 % of the *Lb. plantarum* strains but less than 0.001 % of *Lb. sakei* and *Lb. paracasei* strains survived these conditions.

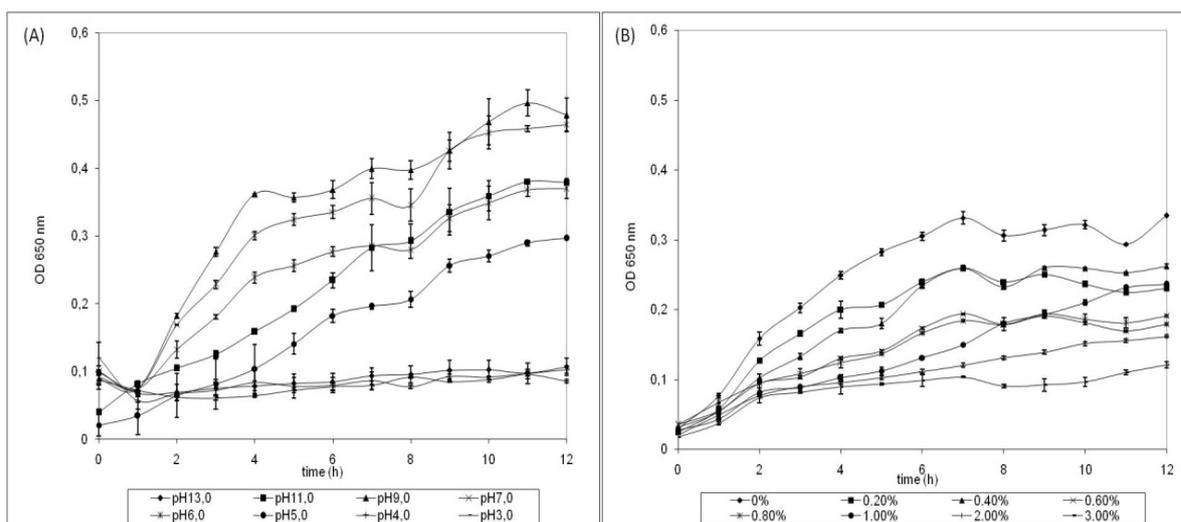


Figure 1. Effect of pH (A) and oxbile concentration (B) on growth of *Lactococcus lactis* subsp. *lactis* MK02R in MRS broth (Difco). Average of three experiments  $\pm$  SD.

*Lactococcus lactis* subsp. *lactis* MK02R produced  $\beta$ -galactosidase and was able to survive in the presence of oxbile, which can be considered positive characteristics.  $\beta$ -galactosidase contributes to the alleviation of the symptoms of lactose intolerance due to lactase deficiency (Charteris et al., 1998), while survival to oxbile favors the viability at the intestinal level, necessary for good probiotic activity, as deconjugation of bile salts protects the bacteria against the toxicity of these compounds (Vizoso-Pinto et al. 2006). Moreover, this reaction contributes to lowering serum cholesterol levels (De Smet et al. 1995).

The recorded hydrophobicity value for *Lc. lactis* subsp. *lactis* MK02R was 29.8 %. Cell surface hydrophobicity is considered an important factor for the interaction between the microbial cells and the host. Bacterial cells with a high hydrophobicity usually present strong interactions with mucosal cells. Hydrophobicity may assist in adhesion, but is not a prerequisite for strong adherence. Hydrophobicity varies among genetically closely related species and even among strains of the same species (Schar-Zammaretti and Ubbink, 2003). Todorov et al. (2008) have reported 75 % - 80 % hydrophobicity values for *Lb. rhamnosus* and *Lb.*

*plantarum* strains, which are much higher than that recorded for *Lb. rhamnosus* GG (55 %), a model probiotic strain.

Auto-aggregation for *Lc. lactis* subsp. *lactis* MK02R was 19.6 %, and co-aggregation with *L. monocytogenes* 603 and *E. faecalis* ATCC 19443 was 32.4 % and 29.8 %, respectively. Aggregation is a strain-specific characteristic, related to interaction with the same strain (auto-aggregation) or with other microorganisms (co-aggregation). The detected high levels of co-aggregation can be interpreted as a positive feature, considering that *Lc. lactis* subsp. *lactis* MK02R produces a bacteriocin that is active against these two test microorganisms.

Patients taking probiotics are often treated for other illnesses. It is thus important to determine the effect of medicaments on the survival of probiotic strains. As shown in Table 1, growth of *Lc. lactis* subsp. *lactis* MK02R was inhibited not only by the two tested antibiotics (amoxil and urotrobel) but also by non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine, and by antiemetic (dimenhydrinate) and antiarrhythmic (amiodarone) drugs.

Table 1. Effect of commercial medicaments on growth of *Lactococcus lactis* subsp. *lactis* MK02R

Commercial name	Concentration (mg/mL)	Active substance	Medicament class	Inhibition halo diameter (mm)	MIC (mg/mL)
Amoxil	100	Amoxicillin	$\beta$ -Lactam antibiotic (Penicillin)	40	< 0.39
Atlansil	40	Amiodarone	Antiarrhythmic	15	5.0
Cataflam	10	Potassium diclofenac	Non-steroidal anti-inflammatory drug (NSAID)	10	0.62
Diclofenac	10	Potassium diclofenac	NSAID	18	1.25
Dramin	20	Dimenhydrinate	Antiemetic	9	20.0
Spidufen	120	Ibuprofen arginine	NSAID	25	15.0
Urotrobel	80	Norfloxacin	Antibiotic	10	5.0

AAS (20 mg/mL), Antak (30 mg/mL), Arotin (4 mg/mL), Aspirin (100 mg/mL), Celebra (40 mg/mL), Clorana (5 mg/mL), Coristina R (10 mg/mL), Dorflex (10 mg/mL), Doxuran (0.8 mg/mL), Fenergan (5 mg/mL), Flumucil (8 mg/mL), Flutec (30 mg/mL), Higroton (10 mg/mL), Medley (4 mg/mL), Neosaldina (60 mg/mL), Nimesulida (20 mg/mL), Nisulid (20 mg/mL), Redulip (3 mg/mL), Seki (3.54 mg/mL), Superhist (80 mg/mL), Tylenol (150 mg/mL), Tylex (6 mg/mL), Yasmin (0.6 mg/mL), Zestril (4 mg/mL), Zocor (2 mg/mL) and Zyrtec (2 mg/mL) had no effect on growth of *Lc. lactis* MK02R.

The interference of anti-inflammatory drugs containing diclofenac on growth of certain LAB (*Lb. plantarum*, *E. faecium*, *L. mesenteroides* subsp. *mesenteroides* and *Lc. lactis* subsp. *lactis*) was also reported previously (Todorov and Dicks, 2008, Todorov et al., 2017). It is, however, important to underline that the activity of these medicaments against the probiotic and other beneficial bacteria in the gastrointestinal tract (GIT) depends on the concentration of the active substances in the GIT of these medicaments, so the correct evaluation of possible interactions between them depends on the determination of MIC of these medicaments (Todorov et al. 2007; Todorov et al. 2008) as the relationship between the daily dose and the MIC determines the possible effect on probiotic bacteria. Special attention needs to be given to medicaments used for long course treatment of chronic diseases, such as Atlansil, as their long-term application causes accumulation in the GIT and MIC be reached, affecting viability of the probiotics and other beneficial cultures.

As indicated in Table 1, the anti-inflammatory medicaments were the ones that affected the tested *Lc. lactis* subsp. *lactis* MK02R strain more significantly. These results agree with other studies, performed using other probiotic LAB and gastrointestinal tract related bacteria (Todorov et al. 2007; Todorov and Dicks, 2008; Todorov et al. 2008). Their inhibitory activity may be a consequence of the increased concentration of potassium ions in the gastric content as a result of the dissolution of potassium diclofenac in the stomach. The excess of potassium ions in the environment is incompatible with microbial cell viability. Other potassium-based medicaments may cause a similar negative effect and individuals under permanent therapy should be aware that the beneficial effects of the probiotic bacteria may be reduced.

*Lactococcus lactis* subsp. *lactis* MK02R was resistant to metronidazol, trimetoprim and nalidixic acid, but all other tested antibiotics inhibited its growth to some extent. The antibiotics were rifampicin (treatment of

*Mycobacterium* infections, including tuberculosis and leprosy), cefazolin, cefotaxime, ceftazidime, ceftriaxone and cefepime (cephalosporins /  $\beta$ -lactams that interfere with cell wall synthesis), amikacin, gentamicin, kanamycin, neomycin, streptomycin and tobramycin (aminoglycosides, inhibitors of protein synthesis), furazolidone (antiparasitic), imipenem (carbapenem,  $\beta$ -lactam that interferes with bacteria cell wall synthesis), ampicillin and penicillin G (penicillins,  $\beta$ -lactams that interfere with bacteria cell wall synthesis), cefuroxime and oxacillin ( $\beta$ -lactams that interfere with bacteria cell wall synthesis), clindamycin, ofloxacin (floxacin,  $\beta$ -lactam that inhibits protein synthesis), erythromycin (macrolide that inhibits protein synthesis), tetracycline, nitrofurantoin and chloramphenicol (inhibit protein synthesis), bacitracin (cyclic polypeptide that inhibits bacteria cell wall synthesis), ciprofloxacin (fluoroquinolone that inhibits bacterial topoisomerase II) and vancomycin (glycopeptide that inhibits bacteria cell wall synthesis).

Resistance of probiotic LAB to antibiotics is a delicate subject, as these bacteria may be reservoirs of antibiotic resistance genes (Salyers et al., 2004), and horizontal gene transfer to other bacteria present in the human GIT is possible (Dicks et al. 2011). Bacterial resistance to antibiotics is a result of misuse of antibiotics in veterinary and human medicine, however, it is also a natural evolutionary process in defense of LAB. Antibiotic resistance may be inherent to a bacterial genus or species, but may also be acquired through exchange of genetic material, mutations and incorporation of new genes (Ammoret al. 2007).

Among the virulence genes tested, PCR analysis gave evidence that DNA of *Lc. lactis* subsp. *lactis* MK02R contains the genes *esp* (enterococcal surface protein), *tdc* (tyrosine decarboxylase) and *vanA* (vancomycin A). The *esp* gene encodes for the production of extracellular surface protein in enterococci, which contributes to intestinal adhesion, favouring colonization and permanence in the host organism (Shankar et al. 2001). In this sense, the *esp* gene is a relevant virulence factor for intestinal pathogens, although not necessarily for commensal lactobacilli. Most probably the presence of this gene in *Lc. lactis* subsp. *lactis* MK02R results from horizontal transfer, a possible scenario in the GIT. However, the presence of a gene is not equal to expression of this gene, as gene expression is a complex process related to integrity and function of entire genome and to environmental factors. Positivity for gene *tdc* (tyrosine decarboxylase) in *Lc. lactis* subsp. *lactis* MK02R is also a potential problem, as production of the biogenic amine tyramine by this strain is possible, if growth and environmental conditions are favourable. Search for gene *van A* resulted positive in the PCR analysis, but the test with vancomycin disk resulted negative. A possible explanation is that the gene is deactivated or lacked expression in the tested conditions.

#### 4. Conclusions

The observed characteristics for *Lc. lactis* subsp. *lactis* MK02R suggest that this bacteriocinogenic LAB strain has probiotic properties. Apart from the recorded beneficial properties, the most common medicaments did not affect the growth of the strain, suggesting that these medicaments will hardly affect the beneficial properties of *Lc. lactis* subsp. *lactis* MK02R. The presence of virulence genes is a relevant criterion to evaluate the safety of probiotic candidates. Although the importance of specific virulence factors depends on the conditions for its expression, and should be related to the pathogenicity of the microorganism, it is necessary to consider the risk of their transfer to other opportunistic microorganisms.

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