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Antibacterial and Plasmid Curing Activity of Lactic Acid Bacteria against Multidrug Resistant Bacteria Strains

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ABSTRACT

Multiple Drug Resistance (MDR) is a serious health problem and major challenge to global drug discovery programs. Most of the genetic determinants that confer resistance to antibiotics are located on plasmids in bacteria. The present investigation was undertaken to investigate the antibacterial effect and the ability of extra- and intra-cellular extracts of Lactic Acid Bacteria (LAB) to cure plasmid acquiring resistance in certain clinical antibiotic-resistant bacterial isolates (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae* and *Shigella* sp.). Transformation experiments were carried out using clinical isolates as plasmid donor and *Escherichia coli* strain HB101 (sensitive to the tested antibiotic), as recipient. Minimal Inhibitory Concentration (MIC) of LAB extracts was determined using the microtiter plate method. Plasmid curing activity of LAB extracts was determined by evaluating the inability of bacterial colonies (pre-treated with LAB extract for 18 h) to grow in the presence of antibiotics. The physical loss of plasmid DNA in the cured derivatives was further confirmed by agarose gel electrophoresis. The LAB showed antibacterial effect, inhibited up to 90% of bacterial biofilm formation and cured the pathogenic bacteria from plasmids. The presence of plasmid in transformants was confirmed through electrophoresis and the transformants were also tested for each antibiotic resistance already recorded for the donor isolates. Both extracts (extra- and intra-cellular extracts) inhibited the growth of the clinical isolates. Extracellular extracts exceeded 90% inhibition on some isolates. The LAB extract mediated plasmid curing resulted in the subsequent loss of antibiotic (Chl, Dox, Ery, Gm, Kaf, Lin and Pen) resistance encoded in the plasmids as revealed by antibiotic resistance profile of cured strains. The extracellular extract of LAB may be a source of anti-plasmid (plasmid borne multiple antibiotic resistance) agents of natural origin.

Key words: Plasmid curing, LAB, multiple antibiotic resistance

INTRODUCTION

The effectiveness of currently available antibiotics is decreasing due to the increasing number of resistant strains causing infections. The emergence of antibiotic resistance in pathogenic bacteria, both in hospital and community acquired infections, represents a significant public health problem (Lipsitch, 2001; Lipsitch and Samore, 2002). Bacterial cells are capable of transferring genes horizontally. This DNA

transfer can take place in three ways, through plasmids, phages, or uptake of naked DNA (Thomas and Nielsen, 2005). Most of the genetic determinants that confer resistance to antibiotics are located on plasmids (Schelz *et al.*, 2006). Plasmids are extra-chromosomal pieces of DNA which are capable of replicating independently of the genome and have been directly implicated in the acquisition of resistance to many antibiotics (Clewell *et al.*, 1975; Weigel *et al.*, 2003). This is particularly problematic since plasmids can cross many

species and genus barriers and the rate of plasmid transfer has even been shown to increase in more heterogeneous communities (Dionisio *et al.*, 2002). Plasmids thus allow resistance to spread and persist in niches that are not necessarily subject to antibiotics (Hughes and Datta, 1983).

The use of antiplasmid agents in combination with antibiotics may serve as a possible way to combat this resistance encoded by plasmids (Molnar *et al.*, 2003). However, majority of the known plasmid curing agents including acridine orange, ethidium bromide and sodium dodecyl sulphate which are toxic or mutagenic and hence unsuitable for therapeutic applications. Also, no single curing agent can cure all plasmids from different hosts. Thus, there is a constant need to develop new curing agents with high efficacy and safety. Traditional natural products have always been a rich source of drug discovery programs (Newman *et al.*, 2000). There is evidence for the health properties that are attributable to exopolysaccharides from LAB.

The present study was planned to investigate the antibacterial and antiplasmid activities of extra and intra-cellular fractions purified from the LAB extract against multidrug resistant bacterial strains.

MATERIALS AND METHODS

Test organisms: The antibiotic resistant bacterial strains, three isolates of *Pseudomonas aeruginosa*, two isolates of *Staphylococcus aureus*, four isolates of *Klebsiella pneumoniae* and one *Shigella* isolate were acquired from the culture collection of both faculties of Science and Medicine, Tanta University, Egypt. The isolates were subcultured in Luria Bertani (LB) broth and stored at -80°C in LB broth with 50% glycerol.

LAB strains: *Streptococcus thermophilus*, *Streptococcus lactis* ssp. *cremoris*, *Lactobacillus casei*, *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. delbrueckii* ssp. *bulgaricus* DSMZ 20080 and 20081 T, *Lactobacillus fermentum* DSMZ 20049, *Lactobacillus acidophilus* DSMZ 20079 T, *Lactobacillus rhamnosus*, *Lactobacillus plantarum* ssp. *plantarum* DSMZ 20174 and *Bifidobacterium longum* ssp. *longum* DSMZ 200707, were collected from culture collection of the Faculty of Agriculture, Ain Shams University, Faculty of Agriculture, Kafr El-Sheikh University and the Faculty of Science Tanta University.

Cell lines: Caco-2 cell line (human epithelial colorectal adenocarcinoma cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM L-glutamine, 10% Fetal Calf Serum (FCS) and 1% penicillin-streptomycin solution in 50 and 100 cm² cell culture flasks, incubated at 37°C in a humidified atmosphere of 5% CO₂, 95% air for 24 h.

Preparation of intracellular and extracellular extracts of LAB: The LAB were cultured in De Man-Rogosa-Sharpe (MRS) (Biokar Diagnostics, Beauvais, France), *Streptococcus thermophilus*, Reinforced Clostridial (RCM) and modified

MRS agar media and incubated at 30°C under aerobic and anaerobic conditions; cultures were collected at the late lag phase of incubation and centrifuged at 10,000 rpm at 20°C for cells re-suspended in PBS followed by ultrasonic disruption in five 1 min intervals in an ice bath. Cell debris were removed by centrifugation at 10,000 rpm at 20°C for 10 min and the resulting supernatant was used as the intracellular extract, both extracellular and intracellular extracts stocks were filtered through a 0.22 µm pore-size filter (Millipore, Bedford, Mass.) and stored frozen at -80°C till used. The total cell numbers were adjusted to 10⁹ CFU mL⁻¹ for the preparation of both extracellular and intracellular extracts.

Cytotoxicity assay: For the determination of treatments concentrations that are non-toxic to Caco-2 cells, the cytotoxic assay was performed according to Schnitzler *et al.* (2001). Briefly, a cell suspension of 6×10⁴ cell mL⁻¹ was collected and seeded in 96-well plates (100 µL cell suspension per well). The plates were incubated at 37°C in humidified 5% CO₂ for 24 h. After obtaining a semi confluent cell layer, the exhausted media was discarded and 100 µL of different treatment concentrations prepared in DMEM were added. Wells containing only media were used as a negative control. The cell plates were incubated at the same growth conditions for 3 days. After 3 days, 100 µL of neutral red stain was added to each well and incubated for 3 h at 37°C in humidified 5% CO₂. Excessive dye was discarded and the stained cells were fixed with 100 µL fixing solution (0.5% formalin with 1% CaCl₂) for 1 min then cells were destained in 100 µL destaining solution (50% ethanol with 1% glacial acetic acid) by shaking for 5 min. The stain intensity was assayed using automated ELISA microplate reader adjusted at 540 nm (reference filters 620 nm).

Antibiotic susceptibility test: Antimicrobial susceptibility was performed by Bauer *et al.* (1966). The antibiotics discs of amoxicillin, nalidixic acid, co-trimoxazole, furazolidone, amikacin, cefotaxime, ciprofloxacin, norfloxacin, ofloxacin, chloramphenicol and gentamicin.

Antibacterial activities of lactic acid bacterial strains extracts against resistant pathogenic bacteria: In order to select the most potent LAB strains against the resistant bacterial strains, the susceptibility of the resistant bacterial growth to LAB extracts was evaluated by a microplate reader assay. The inhibition percentage of LAB extracts was calculated according to the following equation:

$$\text{Inhibition (\%)} = \frac{A - A_1}{A_0} \times 100$$

where, A is absorbance of the treated group, A₁ is absorbance of the blank, A₀ is absorbance of the control group.

Inhibitory effects of lactic acid bacterial extracts on biofilm formation of resistant pathogenic bacteria: Biofilm formation assay was performed by the Tissue Culture Plate

(TCP) method. Each of the strain suspensions was diluted to 10^6 CFU mL⁻¹ in fresh LB media. Then 200 μ L aliquots of the diluted cultures were added to 96-well flat-bottom tissue culture plates. Broth alone served as the control. Wells containing only broth and nontoxic concentrations of LAB extracts (prepared in LB broth) were incubated for 24 h at 37°C. After incubation, the content of each well was gently removed by tapping of the plates. The wells were washed twice with 200 μ L of deionized water to remove free-floating planktonic bacteria. Biofilms in plates were dried at 60°C for 1 h and adherent bacteria were stained at room temperature with 200 μ L of a 0.1% (w/v) aqueous solution of Crystal Violet (CV) for 5 min. The plates were rinsed twice with deionized water to remove excess stain. After the plates were dried at 37°C for 2 h, biofilm formation was quantified by solubilization of the CV stain in 200 μ L of 30% (w/v) glacial acetic acid for 10 min with shaking at 300 rpm. The concentration of CV was determined using a Synergy HT multidetection microplate reader at a wavelength of 492 nm. The mean absorbance obtained from the medium control well was deducted from the test absorbance values (Mathur *et al.*, 2006).

Plasmid analysis: Plasmid isolation was carried out using the large-scale alkaline lysis method as described by Davis *et al.* (1980). Plasmid samples (25 μ L) were electrophoresed through 0.8% agarose (type 1; Sigma) in TBE buffer at 150 V, 60 mA for 5.5 h by the method of Portnoy *et al.* (1981).

Transformation: In order to ensure that the antibiotic resistance to the above mentioned antibiotics was plasmid encoded, bacterial conjugation experiments were performed. Bacterial conjugation was done by combining equal volumes (3 mL) of overnight Luria-Bertani cultures of donor and recipient strains. The donor strains were the resistant bacterial strains and the recipient was *E. coli* J53 (negatively plasmid carrying strain). Bacteria were conjugated for 12 h at 37°C and transconjugants were selected on Luria-Bertani media containing the previous antibiotics. Potential transconjugants were verified by plasmid extraction (Carlson *et al.*, 2002).

Plasmid-curing experiments: The method described by Guerry and Colwell (1977) was used with slight modifications. An overnight culture of each strain was standardized to 1×10^8 CFU mL⁻¹ with phosphate buffer saline (pH 7.6) containing 1.25% ethidium bromide as positive curing agent and the non-toxic doses of LAB extracts as determined by El-Adawi *et al.* (2012) as test agent. Incubated for 24 h was applied with continuous shaking (120 rpm). Cell pellets were obtained by centrifugation at 5000 rpm for 10 min at 4°C and plated on LB agar to test their antibiotic susceptibility. In addition, Plasmid curing was checked through plasmid analysis by electrophoresis.

RESULTS

Antibacterial effect of lactic acid bacterial extracts: Extracellular LAB extracts were shown to have a high

antibacterial effect, whereas intracellular extracts didn't show any significant inhibitory effect. Extracellular extracts of the eleven above mentioned LAB strains caused 60-90% inhibition of three *P. aeruginosa* strains (Fig. 1), four *Klebsiella* sp. (Fig. 2) and two *S. aureus* strains (Fig. 3). Whereas *Shigella* spp. growth was only inhibited by two extracellular LAB extracts of *B. longum* and *S. thermophilus* causing 65 and 85% growth inhibition respectively (Fig. 4), observing that *S. thermophilus* extract was ineffective on all other bacterial strains except *Shigella* sp.

Inhibitory effects of different lactic acid bacteria on biofilm formation of resistant pathogenic bacteria: It was shown that the extracellular extracts of *L. plantarum* caused 77.38% inhibition of *P. aeruginosa* biofilm (Fig. 5). The extracellular extracts of *L. bulgaricus* DSMZ 20081T inhibited upto 69.23 and 68.3% of the biofilm formation of *Klebsiella* isolates and *S. aureus*, respectively (Fig. 6 and 7). The extracellular extracts of *L. plantarum* caused 62.23% inhibition of *Shigella* sp. biofilm formation (Fig. 8).

Plasmid profile screening: The results showed that all the isolates conferred resistance to one or more of the various antibiotics used. The plasmid screening profile showed that all isolates harbored plasmids, *P. aeruginosa* strains contained three to five plasmids (200-170 bp), *Klebsiella* isolates showed two plasmid bands (200-150 bp), *S. aureus* showed 3 plasmid bands (200-70 bp), *Shigella* isolate showed three plasmids

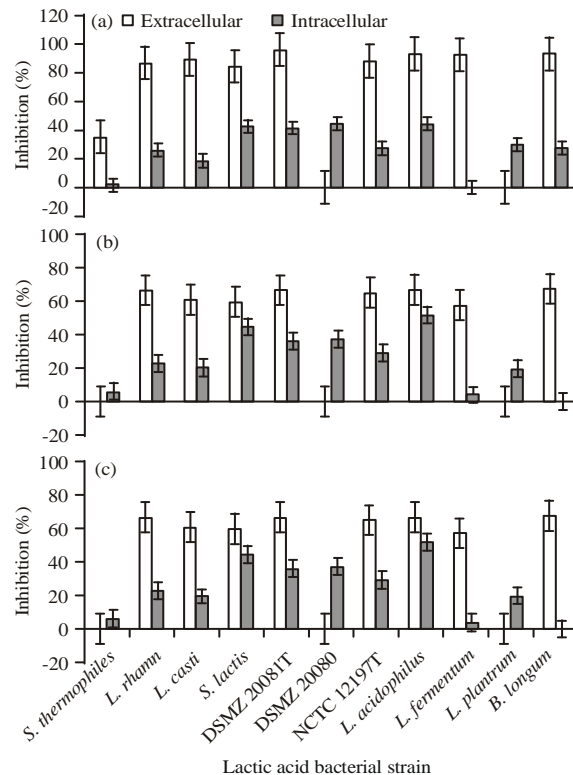


Fig. 1(a-c): Antibacterial effect of LAB extract on resistant *Pseudomonas aeruginosa* isolates, (a) I, (b) II and (c) III

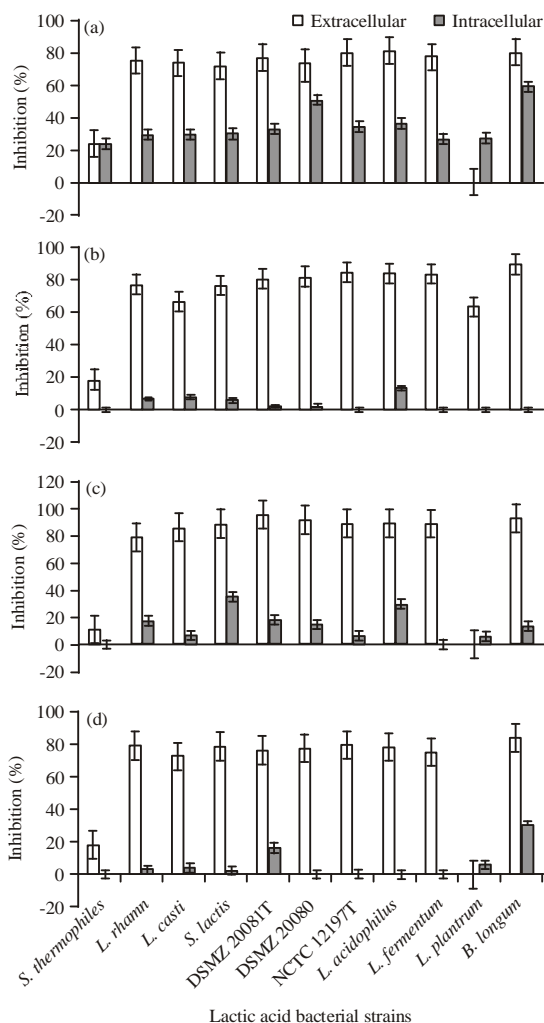


Fig. 2(a-d): Antibacterial effect of LAB extract on resistant *Klebsiella* isolates, (a) I, (b) II, (c) III and (d) IV

(200-70bp). After treatment with non-toxic concentration of LAB extracts and 24 h incubation at 37°C, a complete plasmid curing of all above mentioned pathogenic bacteria was observed (Fig. 9).

DISCUSSION

In the present study, extracellular and intracellular extracts of eleven LAB strains, were tested for antibacterial effect, inhibition effect on biofilm formation and on plasmid curing of ten antibiotic resistant bacterial strains.

Antibacterial effect: The extracellular extract of *Bifidobacterium longum* (*B. longum*) was found to have the broadest bactericidal effect, causing growth inhibition of all above mentioned tested bacterial strains, even *Shigella* sp. which was only sensitive to two extracts. On the other hand, *S. thermophilus* extract only inhibited the growth of

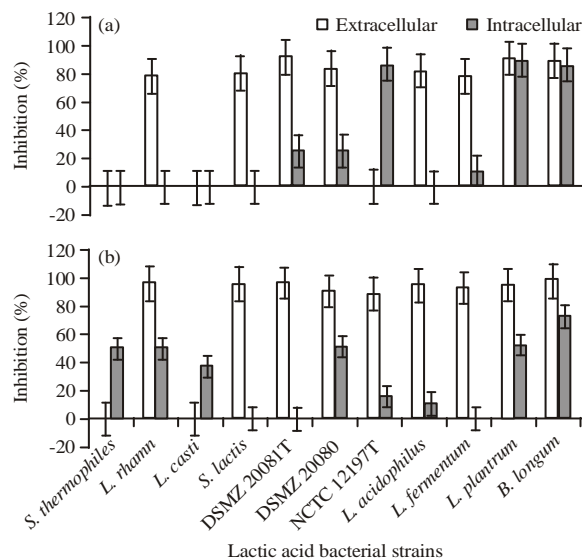


Fig. 3(a-b): Antibacterial effect of LAB extract on resistant *Staphylococcus aureus* isolates, (a) I and (b) II

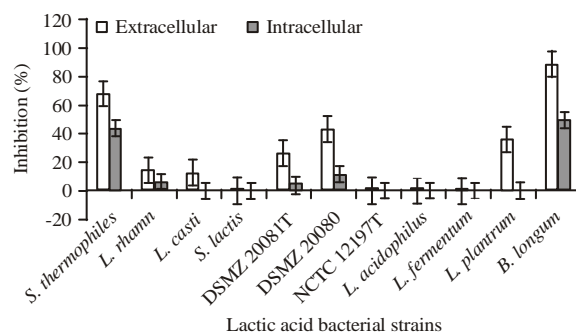


Fig. 4: Antibacterial effect of LAB extract on resistant *Shigella* isolates

growth of *Shigella* spp. which is the least sensitive strain. It was also observed that *Lactobacillus plantarum* (*L. plantarum*) extract only inhibited both *S. aureus* strains. The observed broad spectrum and variability in antibacterial effectiveness indicates the presence of a number of bacteriocins in each LAB and indicates variability between the different LAB.

Bacteriocins of LAB extracts were reported to inhibit protein synthesis (De Graaf *et al.*, 1968) and in other reports they were suggested to cause depletion of the Proton Motive Force (PMF) (Bruno and Montville, 1993). Later they were identified by Parada *et al.* (2007), as peptides. It should also be considered that the variability in effect could be partially due to changes in pH value since cell permeability to bacteriocides needs acidic medium (Yang *et al.*, 2012).

Bacteriocins play a role in the natural survival competition against surrounding microbes. Therefore, bacteriocins are concentrated in extracellular and not in intracellular extracts. Intracellular extracts generally didn't show any significant antibacterial effect (0-30% inhibition). Only the extracts of *B. longum*, *L. plantarum* and NCTC 12197T caused 83%

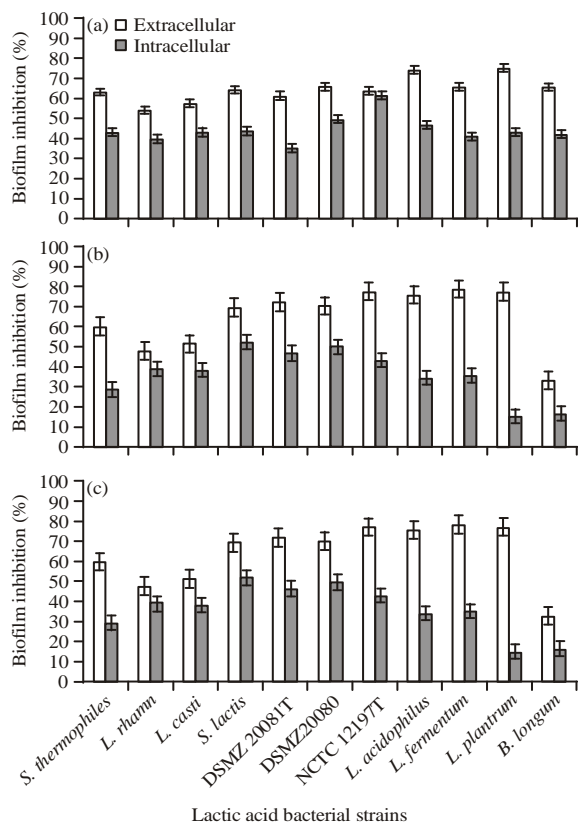


Fig. 5(a-c): Effect of LAB extract on biofilm of resistant *Pseudomonas aeruginosa* isolates, (a), I (b) II and (c) III

growth inhibition of only one strain of *S. aureus* and caused 50, 70 and 15% growth inhibition, respectively of the second strain of *S. aureus*.

Biofilm formation: The effect of intracellular and extracellular extracts of eleven different LAB were tested for anti-biofilm formation of three *P. aeruginosa* isolates. Three *Klebsiella* sp., two *S. aureus* and one *Shigella* sp. All of them are resistant to the five antibiotics mentioned above.

Intracellular extracts of LAB inhibited 40-60% biofilm formation of only one *P. aeruginosa* isolate and 30-50% biofilm formation of *P. aeruginosa* isolate 2 (Fig. 5a and b), whereas they didn't show any significant effect on the other eight bacterial isolates (Fig. 5c, 6, 7 and 8).

Most of the extracellular LAB extracts showed (50-60%) inhibition in biofilm formation of two *P. aeruginosa* isolates (Fig. 5a, b). A great variability was observed in the anti-biofilm effect of LAB extracts on the rest of the eight bacterial strains (Fig. 5c, 6, 7 and 8). Each bacterial isolate was affected by extracts of only some of the LAB strains which differed in each bacterial pathogen. Only biofilm formation of *Klebsiella* isolate 4 was not affected by any LAB extracts (Fig. 6c).

Biofilms consist of multispecies microbial cells encased in a self-synthesized polymeric matrix and attached to a tissue or surface (Costerton *et al.*, 1987; Stoodley *et al.*, 2002). Structural components of the EPS matrix include cell-surface

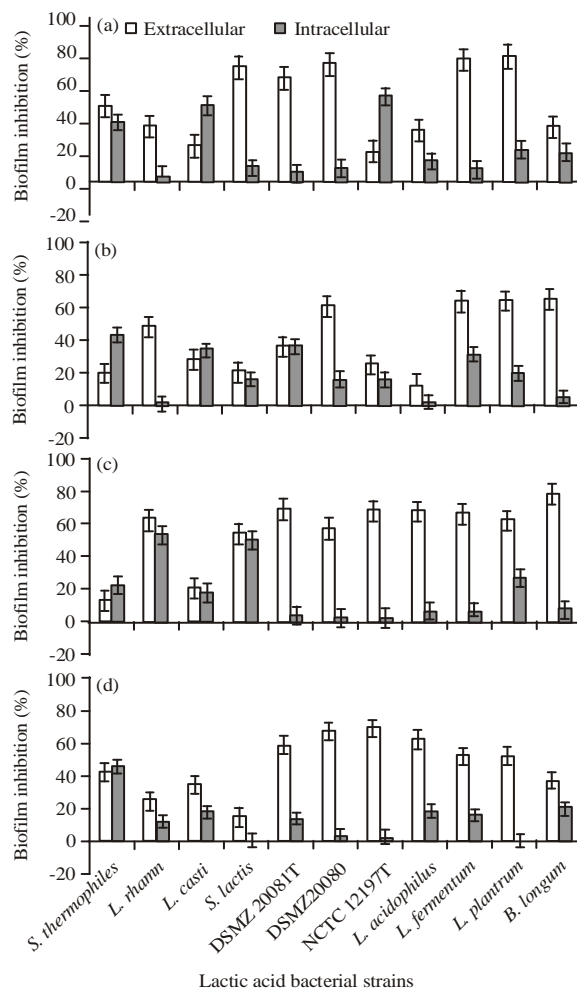


Fig. 6(a-d): Effect of LAB extract on biofilm formation of resistant *Klebsiella* isolates, (a) I, (b) II, (c) III and (d) IV

proteins, proteinaceous pili, DNA, RNA lipids and polysaccharides (Flemming and Wingender, 2010). Therefore, the effective extracts presented in this study could contain compounds affecting any of these bonds. It is obvious that the tested bacterial strains presented here have different forms of biofilm matrix. There is even a great variability in the biofilm matrix between the three *P. aeruginosa* isolates (Fig. 5), *Klebsiella* sp. (Fig. 6) and *S. aureus* isolates (Fig. 7). This also indicates that each of the different LAB strains is excreting different compounds, each of them assumed to have a different mode of action. The EPS matrix of one of the *Klebsiella* sp. seems to be composed of a material which is not affected by any of the compounds produced by the LAB strains.

LAB was reported to produce arabinose and a number of unknown polysaccharides which inhibit biofilm formation but don't show any bacteriostatic or bactericidal effect (Rendueles *et al.*, 2013; Kanmani *et al.*, 2011). It is assumed that most antibiofilm polysaccharides act as surfactant molecules that modify the physical characteristics of bacterial cells and abiotic surfaces (Rendueles *et al.*, 2013). It was also

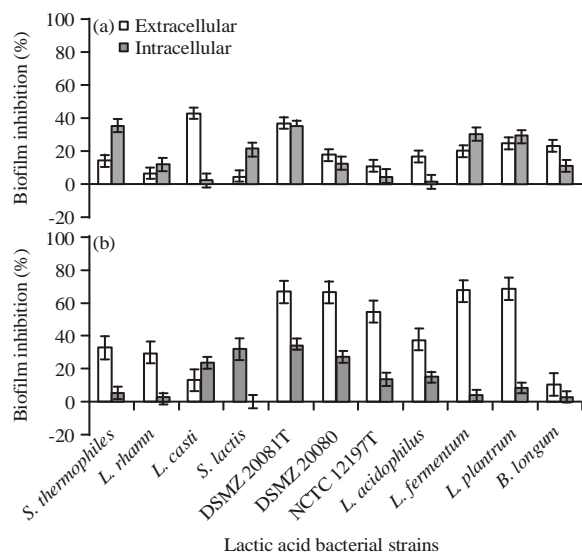


Fig. 7(a-b): Effect of LAB extract on biofilm formation of resistant *Staphylococcus aureus* isolates, (a) I, (b) II

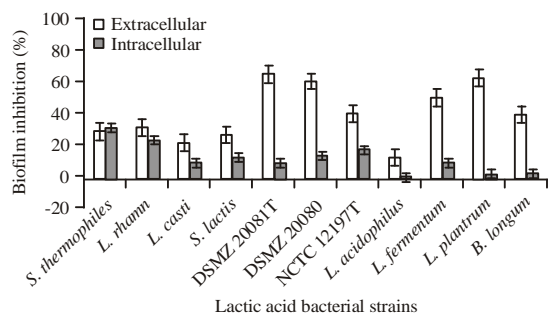


Fig. 8: Effect of LAB extract on biofilm formation of resistant *Shigella* isolates

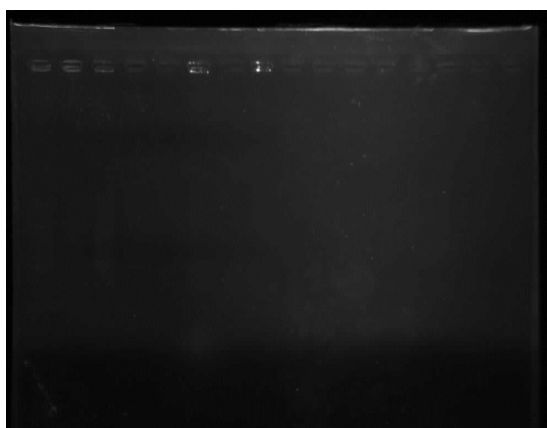


Fig. 9: Plasmid profile on gel electrophoresis showing the absence of plasmids in the rested bacterial strains after treatment with LAB extracts

suggested that they act as signaling molecules that modulate gene expression of recipient bacteria (Kim and Kim, 2009) or as competitive inhibitors of multivalent carbohydrate-protein interactions (Wittschier *et al.*, 2007), by blocking lectins or sugar binding proteins present on the surface of bacteria, or by blocking adhesions of fimbriae and pili. (Rendueles *et al.*, 2013; Zinger-Yosovich *et al.*, 2009).

Extracellular LAB extracts presented in this study contain various effective compounds, each having its specific effect on the biofilm of the different bacteria. The difference is not only in the strength of the effect but also in the mode of action, since there are extracts having no effect at all on some bacteria whereas being very effective against other bacteria. As an example, *Lactobacillus acidophilus* inhibited 59% biofilm formation of *Shigella* sp. and one *Klebsiella* sp. isolate 3 (Fig. 6c), whereas it was not effective against *Klebsiella* isolate 2 and 4 (Fig. 6b, d). In addition it caused 65% inhibition of biofilm formation of two *P. aeruginosa* isolates and was not able to affect *P. aeruginosa* isolate c. In the present study any of the suggested mode of actions could be presented. Further analyses of biofilm matrices and LAB extracts could give further indications about the mode of action.

Plasmid curing: The LAB extracts were tested for their effect on plasmids of the ten antibiotic resistant bacteria mentioned above. After plasmid profile screening it was observed that the extracts were able to stop 100% plasmid replication (Fig. 9) which means that they reversed antibiotic resistance of the pathogens. Thus these extracts offer a natural safe therapy if combined with antibiotic treatment.

There are thirty one phenothiazine tricyclic compounds and thirty non-phenothiazine tricyclic compounds which are known to cure plasmids. In the case of phenothiazines, the substitution in the aromatic ring is expected to prevent an interaction between the π -electrons of the phenothiazine ring and target molecules essential to plasmid replication. In general it is assumed that chemicals curing plasmids have receptors to enter cell membranes which can be opened or blocked (Spengler *et al.*, 2006). Further analyses of the present LAB extracts should be performed to indicate whether the extract contains one or more of these known substances. It should be noted that, in literature, only one plasmid curing agent (2-chloro-10-(2-diethylaminoethyl) is known to cause 90% plasmid curing, whereas the other known compounds don't exceed 38% plasmid curing effect (Spengler *et al.*, 2006). In the present study the extracts of all tested LAB caused 100% plasmid curing to all pathogenic resistant bacteria under investigation (Fig. 9).

CONCLUSION

The present observations indicate the significant effect of extracellular LAB extracts on bacteria inhibition through direct bactericidal effect, through inhibition of bio-film formation and through increasing antibiotic sensitivity by plasmid curing.

LAB, generally, excrete a very high number of various compounds. A number of these compounds are assumed to be different peptides which have antibacterial effects. They are also known to produce a number of polysaccharides which inhibit biofilm formation of pathogenic bacteria. In addition, they excrete one or more products which cause 100% plasmid curing.

Since these effects could not be observed in intracellular extracts, the antibacterial effect in the human body could only be achieved either by continuous treatment with extracellular extracts or by maintaining living flora of LAB in the body. These extracts could also be used as natural food preservatives and for safe plasmid curing.

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