Synthesis and Partial Biochemical Characterization of Bacteriocin Produced by Lactobacillus Paracasei YR Strain

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The Lactobacillus paracasei YR strain, producing bacteriocins, was used against three bacterial strains, namely Bacillus cereus CMGB 215, Listeria innocua CMGB 218, and Escherichia coli CBAB2. The bacteriocin synthesis was tested by using the MRS medium, thus resulting in a maximum inhibiting effect against all three sensitive strains, even if the carbon source is replaced by lactose, sucrose, sorbitol, sorbose, arabinose, maltose, galactose and trehalose. To mark out the prebiotics effect on the bacteriocin synthesis, MRS is supplemented with 1% chicory inulin, dahlia inulin, lactulose, raffinose, stachyose, xylose. It results that the maximum inhibiting effect is determined when using lactulose with a concentration of 1%. Furthermore, the bacteriocin was characterized from the chemical point of view, resulting high thermal stability, resistance to acidic pH, resistance to organic solvents and to the used enzymes. The inhibiting effect of the precipitated bacteriocin is obtained by using ammonium sulphate with a concentration of 60%, with an average particle diameter of 1 cm against sensitive strains.

Key words: lactulose, Listeria innocua, Bacillus cereus, Escherichia coli, ammonium sulphate

Many lactic bacteria strains produce antimicrobial peptides, bacteriocins which are used against pathogenic strains. In the food industry, they are used intensively as a natural preservative, since eliminate the use of traditional preservatives and have antioxidant effects. By this property, they increase the nutritional value of the product [1]. The probiotic cultures synthesizing bacteriocins are mainly used to obtain fermented milk products [2] These peptides are important because they are thermally stable, maintaining their capacity at low concentrations.

This property was outlined in the past decades, being one of the most intensely studied properties of the probiotic strains of lactic bacteria [3, 4]. These bacteriocins may act against certain related probiotic strains. There have been searched those strains synthesizing bacteriocins which are efficient against pathogenic strains, such as: Staphylococcus aureus, Clostridium difficile, Listeria monocytogenes, Escherichia coli, Bacillus cereus. In order to be considered efficient, in addition to the capacity of releasing bacteriocins, the lactic bacteria strains must be able to survive in the gastrointestinal system, to adhere and to colonize the intestinal tract [5].

Since they can inhibit pathogenic strains, they are used to obtain freeze-dried nutritional supplements operating at the level of the intestinal tract. The freeze-dried probiotic strains may be improved by adding prebiotic substances stimulating their multiplication. In addition to the classic prebiotics, such as inulin and lactulose, the aim was to add strains synthesizing exopolysaccharides with prebiotic effect. The bacteriocin synthesis may be increased by adding carbohydrates, oligosaccharides and vitamins in the medium, or by using selected strains resistant to low p*H values [6].*

Experimental part

Microorganisms and culture media.

Within the studies, the *Lactobacillus paracasei* YR strain was used for the bacteriocin synthesis. The following sensitive strains were used: *Bacillus cereus* CMGB 215, *Listeria innocua* CMGB 218, *Escherichia coli* CBAB2. All strains are kept at a temperature of -82^{0}C, in glycerol 20%. The revival was made in MRS medium for the *Lactobacillus* strain and in LB medium for the three sensitive strains.

The bacteriocin synthesis was marked out by growth at 37^{0}C, for a maximum of 72 h, in MRS medium (M1). Furthermore, the glucose (carbon source in its composition) was replaced with lactose (M2), sucrose (M3), sorbitol (M4), sorbose (M5), arabinose (M6), maltose (M7), galactose (M8), trehalose (M9). The minimum inhibitory concentration (AU/mL) was established.

The prebiotics effect on the bacteriocin synthesis was performed by supplementing the MRS with 1% of each of the following: chicory inulin, dahlia inulin, lactulose, raffinose, stachyose, xylose. Once the prebiotic was selected, there was also determined the influence of various concentrations on the bacteriocin's capacity of synthesis.

The partial biochemical characterization of the bacteriocin was performed by determining the resistance to temperature, *p*H, enzymes and organic solvents. After the cells are removed, the supernatant is submitted for 15 minutes to temperatures of 60, 80, 100 and 121^{0}C, in order to check its resistance to temperature. After incubation, the liquid is cooled in an ice bath and the inhibiting diameter is determined [7].

In order to determine the *p*H effect, it is corrected at the values of 2, 5, 7, 9 and 11, using NaOH 1N or HCl 1N sterilized by filtration. The samples were kept at 30^{0}C for an hour [7, 8].

The effect of the proteolytic enzymes (pepsin, trypsin, chymotrypsin) and nonproteolytic enzymes (lipase, pronase E) was tested by adding them to the supernatant sample, with a concentration of 1 mg/mL. The samples were kept at 30^{0}C, for two hours, and afterwards the inactivation was performed by immersing them in a water bath at 95^{0}C, for 3 – 5 min. The cooling is made by an ice-bath and, thus, the inhibiting effect is determined by

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measuring the diameter obtained against the sensitive strains [7-9].

The effect of organic solvents (methyl alcohol, ethyl alcohol, acetone, ethyl acetate, acetonitrile, benzene, chloroform), with a concentration of 10 %, was observed by keeping the samples at a temperature of 30^0C for an hour. The solvent is removed by evaporation and afterwards the inhibiting diameter is determined [7- 10].

The partial purification of the bacteriocin was performed by adding ammonium sulphate with concentrations of 10, 20, 30, 40, 50, and 60% to the supernatant. The obtained precipitates were re-dissolved in 10 mL of phosphate buffer with *p*H 7 and the inhibiting effect was determined [9].

Results and discussion

The first phase consisted of determining the synthesis capacity of the bacteriocin in the presence of various carbon sources. The tests were performed in parallel, by using all three pathogenic strains. The results of the antimicrobial activity of the bacteriocin, provided in AU/mL, are presented in figures 1, 2 and 3.

From the presented data, it results that the *Lactobacillus paracasei* YR strain has the most significant activity against the *Listeria innocua* CMGB 218 strain. The weakest activity is observed to be that against *Escherichia coli* CBAB2. The maximum inhibiting activity has a value of 6400 AU/mL, after 48 h of fermentation, using the M1 medium, against the three sensitive strains. By changing the carbon source with sorbitol and sorbose, it results that the strain is active, with a peak value against *Bacillus cereus* CMGB 215. For *Listeria innocua* CMGB 218, the maximum inhibiting value is present even when the carbon source consists of lactose, sucrose, sorbose, arabinose and maltose.

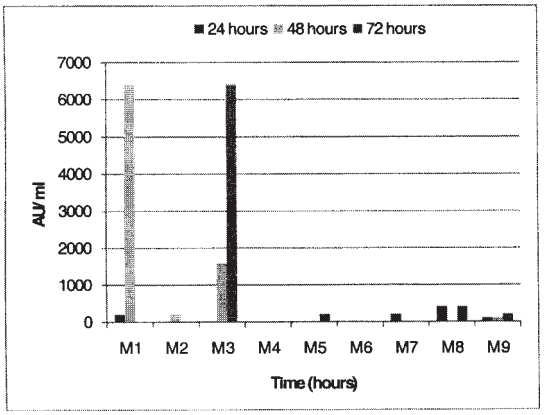

Fig. 1. Inhibiting activity of the *Lactobacillus paracasei* YR strain against *Escherichia coli* CBAB2 in the presence of various carbon sources

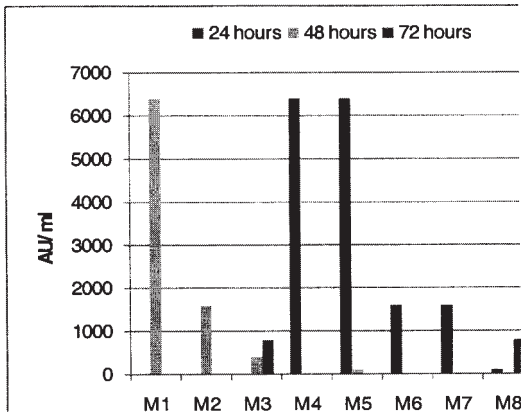

Fig. 2. Inhibiting activity of the *Lactobacillus paracasei* YR strain against *Bacillus cereus* CMGB 215 in the presence of various carbon sources

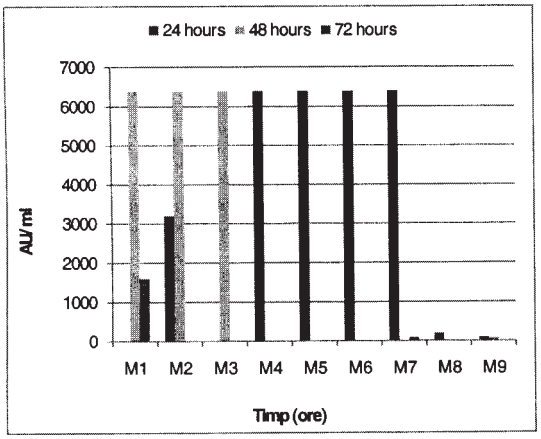

Fig. 3. Inhibiting activity of the *Lactobacillus paracasei* YR strain against *Listeria innocua* CMGB 218 in the presence of various carbon sources

There must be outlined the fact that the *Lactobacillus paracasei* YR strain has no inhibiting activity within 72 h. Except for the use of sucrose against *Escherichia coli* CBAB2, the rest of the strains barely exceeds a maximum of1000 AU/mL. It results that a maximum of 48 h of fermentation is enough to obtain the peak antimicrobial activity against all sensitive strains which have been used.

Further on, there was determined which probiotic causes the maximum inhibiting activity by growing the strain in MRS medium supplemented by 1% prebiotic. The growth was made in Duran tubes and the samples were taken through the septum of the autoclavable cap. There was determined the diameter of the inhibiting area for each sensitive strain.

From the data provided in relation to the inhibition of the three sensitive strains, there results that a concentration of 1% of lactulose determines a maximum inhibition area. Furthermore, within 48 h from the fermentation, the maximum inhibiting area is obtained for all three strains which have been used (fig. 4, 5 and 6). It was observed that the maximum inhibiting effect was obtained against *Bacillus cereus* CMGB 215, resulting in a diameter of 1.8 cm. For the other two strains, the diameter of the inhibiting area is of 1 cm against *Escherichia coli* CBAB2 and of 0.9 cm against *Listeria innocua* CMGB 218.

As regards the other prebiotics, a high inhibiting activity against *Escherichia coli* CBAB2 was observed, with an average diameter of 0.8 cm. When dahlia inulin is used, a constant inhibiting area is maintained, with a diameter of 0.8 cm. This result is valid as well for the use of raffinose and the variation of 0.05 cm within 48 h may be explained by the differences arising from the determinations.

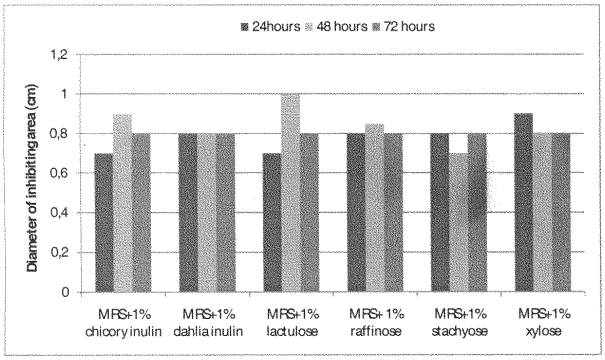

Fig. 4. Inhibition of *Escherichia coli* CBAB2 by *Lactobacillus paracasei* YR cultivation in MRS medium supplemented by prebiotic of 1%

Once there has been determined the prebiotic generating the maximum inhibiting effect, namely the lactulose, there was proceeded by establishing the minimum concentration. The tests were performed in Duran tubes, provided with a septum for taking samples, against the same three sensitive strains. The maximum antimicrobial effect is obtained at a concentration of 1% of lactulose. Although the concentration is doubled to 2%, the diameter of the inhibiting area does not increase proportionally. It continues to have the same value of 1 cm for 72 h of fermentation. For the *Bacillus cereus* CMGB 215 and *Listeria innocua* CMGB 218 strains, the inhibiting activity is not noticeable at a concentration below 0.6% of the lactulose, for up to 48 h of fermentation. For the *Escherichia coli* CBAB2 strain, the inhibiting activity is observed at concentrations of 0.1 – 2%, during all three tested intervals, every 24 h. The maximum inhibiting area has a diameter of 1 cm, against the three tested sensitive strains, for 24-72 h of fermentation.

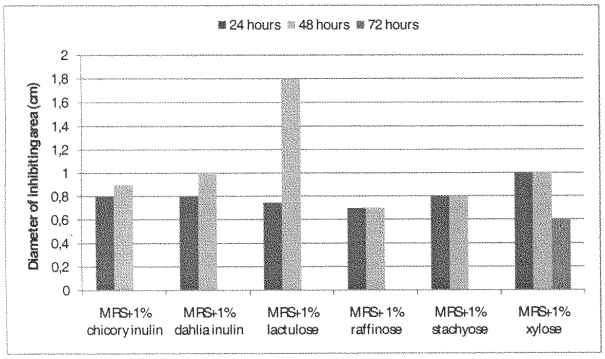

Fig. 5. Inhibition of *Bacillus cereus* CMGB 215 by *Lactobacillus paracasei* YR cultivation in MRS medium supplemented by prebiotic of 1%

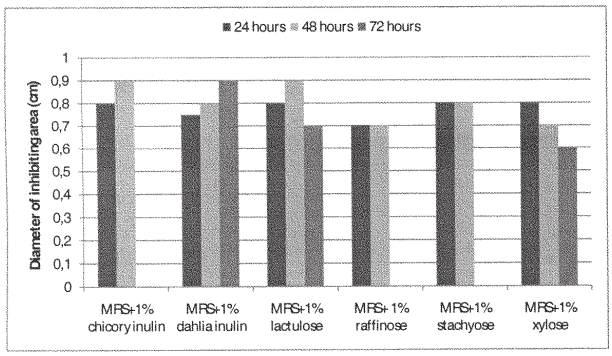

Fig. 6. Inhibition of *Listeria innocua* CMGB 218 by *Lactobacillus paracasei* YR cultivation in MRS medium supplemented by prebiotic of 1%

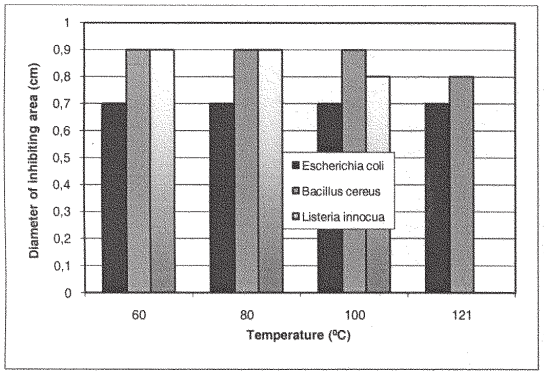

Fig. 7. The effect of temperature on the bacteriocin produced by *Lactobacillus paracasei* YR

For the physical and chemical characterization of the bacteriocin produced against the same three sensitive strains, there has been determined the effect of temperature, pH, enzymes, solvents and of the precipitation at various concentrations of the ammonium sulphate. *Lactobacillus paracasei* YR (fig. 7) produces a thermally resistant bacteriocin, its effect continuing to be constant, except for the incubation at 121^{0}C against *Listeria innocua* CMGB 218. The inhibiting diameter against *Bacillus cereus* CMGB 215 decreases at the same temperature. However, *Escherichia coli* CBAB2 is sensitive notwithstanding the incubation temperature of the bacteriocin.

The bacteriocin produced by *Lactobacillus paracasei* YR is resistant particularly to acidic *p*H (fig. 8). As the *p*H value increases, it is only active against *Listeria innocua*, up to a *p*H value of 9.

In case of precipitation with ammonium sulphate (fig. 9), there is observed that the maximum inhibiting area is obtained at a concentration of 60%. For *Escherichia coli* CBAB2, the inhibiting diameter is constant up to a concentration of 50% ammonium sulphate, with a diameter of 0.8 cm. For *Bacillus cereus* CMGB 215, the obtained precipitate is not efficient, since there is no inhibition area at a concentration of ammonium sulphate below 40%. The obtained precipitate is efficient especially against *Listeria innocua* CMGB 218, determining a diameter of 0.9 cm starting from a concentration of 10% ammonium sulphate and a diameter of 1 cm starting from a concentration of 40% ammonium sulphate.

Furthermore, there has been tested the resistance of the bacteriocin produced by *Lactobacillus paracasei* YR against various enzymes, resulting that it continues to be active in the presence of pronase E, pepsin, trypsin, chymotrypsin and lipase against the three sensitive strains.

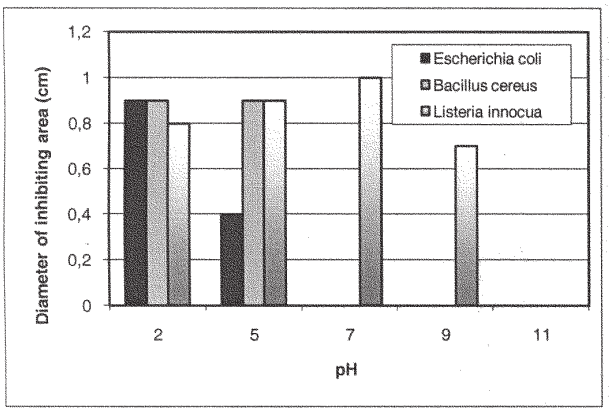

Fig. 8. The effect of *p*H on the bacteriocin produced by *Lactobacillus paracasei* YR

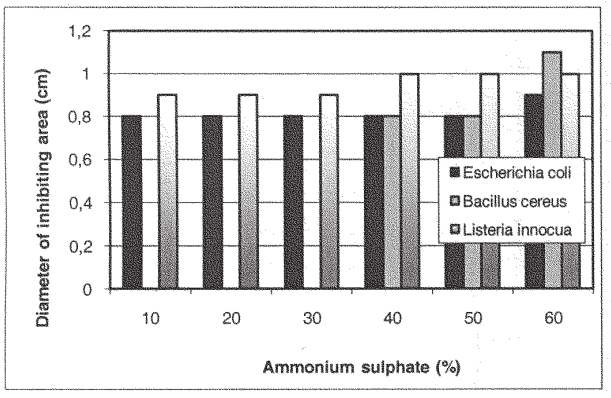

Fig. 9. The effect of precipitation with ammonium sulphate on the bacteriocin produced by *Lactobacillus paracasei* YR

The single exception is the effect of the lipase against *Listeria innocua* CMGB 218. Related to the treatment with organic solvents in a concentration of 10%, it results that the produced bacteriocin is resistant, creating an inhibition area, even if it has a smaller diameter, against the three sensitive strains which have been tested.

Conclusions

The characterization studies of the bacteriocin synthesized by the strain *Lactobacillus paracasei* YR have generated significant results. Due to the presented resistance, the bacteriocin may be used for obtaining products acting for the biological control of the human gut flora. The studies indicate that the type of the culture medium and the used prebiotic influence directly the inhibiting effect. It was established that lactulose, with a concentration of 1%, determines the maximum inhibiting capacity. Therefore, the combination between the lactulose and the probiotic strain may generate synbiotic products, with an effect in modulating the human gut flora.

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