

*Full Length Research Paper*

# The influence of prebiotics on bacteriocin synthesis using the strain *Lactobacillus paracasei* CMGB16

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Accepted 25 February, 2010

The aim of this study is to determine the effect of certain prebiotics on the synthesis of bacteriocins. The *Lactobacillus paracasei* CMGB16 strain producing bacteriocins was used. *Escherichia coli* was used as the sensitive strain. In the nutritive environment (MRS); the carbon source (glucose) was supplemented with inulin from chicory and Dahlia, raffinose and lactulose. The cells were eliminated using centrifuge at 5,000 rpm for 10 min. The pH of the resulted supernatant was adjusted to the value of 5.5 with NaOH 0.2N and the inhibitory activity was determined by agar well diffusion method. The resistance to various inhibitory substances (pepsin, trypsin, pronase E, subtilisin, catalase) was also determined in concentration of 0.5 mg/ml. These tests were also performed with the fluid concentrated up to 1:3, at 48°C, 200 rpm and 100 mbar. The strains were cropped in these environments for 96 h. Thus, the witnessed strain is sensitive to the bacteriocin produced by the *L. paracasei*. A significant increase of the activity of the bacteriocin was noticed when supplementing the cropping environment with inulin, lactulose and raffinose, within the time range of 25 - 96 h. The diameter of the inhibition area was at least 2 cm visible in the use of all prebiotics. The largest inhibitory area was visible after 48 and 72 h of fermentation.

**Key words:** Bacteriocin, prebiotic, *Escherichia coli*, lactulose.

## INTRODUCTION

The strains of lactic bacteria have been in used for long due to their ability to act as food preservative. This feature is used in food industry to preserve vegetal products, milk or meat. The antimicrobial activity is shown by the synthesis of organic acids (mainly, lactic acid) and proteic molecules, named bacteriocins. The antimicrobial activity occurs against the strains of *Escherichia coli*, *Bacillus cereus*, *Clostridium perfringens*, etc. (Vinod et al., 2006).

The producing strains appertain to the types; *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Enterococcus*, *Lactococcus*, etc. can produce a great variety of antimicrobial substances. Bacteriocins represent peptides or proteins with specific action on bacterial strains. Bacteriocins produced by the strains of lactic bacteria have an average size of 3 - 6 kDa. These

substances are divided into three classes on the basis of their chemical structure and genetic properties and are represented by lantibiotics, small thermo-stable bacteriocins and thermo-sensitive bacteriocins respectively (Oh et al., 2000).

Bacteriocins are synthesized as pro-peptides. Their production is strictly associated with the phases of microbial growth of the synthesized strain. Generally, they are synthesized during the growing phase and at the end of the exponential growing phase. The synthesis of bacteriocins is directly influenced by the source of carbon and nitrogen in the culture medium. The most frequent source of carbon is glucose, but there are some studies which also imply lactose, galactose or sucrose. (Savadogo et al., 2006)

## MATERIALS AND METHODS

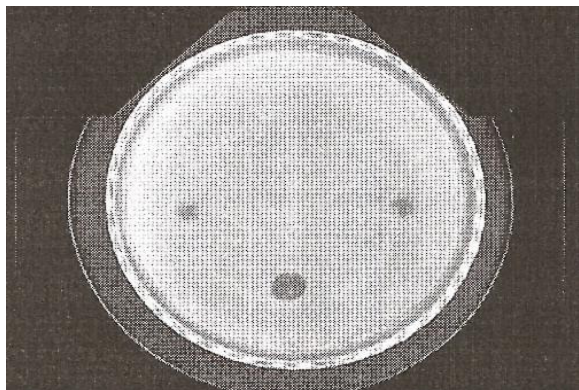
### Strains used and culture media

*Lactobacillus paracasei* CMGB16 was used as a bacteriocin

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**Table 1.** The inhibitory activity on the strain *Lactobacillus paracasei*.

|                          |           |
|--------------------------|-----------|
| Supernatant (MRS medium) | 400 AU/ml |
| Supernatant Concentrate  | 400 AU/ml |
| P1 medium                | 400 AU/ml |
| P2 medium                | 400 AU/ml |
| P3 medium                | 600 AU/ml |
| P4 medium                | 600 AU/ml |



**Figure 1.** The aspect of the inhibitory area of supernatant (up - 2 spots) and of the concentrated supernatant (down-1 spot) during the analysis with Colony Quant.

producing strain. *E. coli* 2 were used as a sensitive strain. Both strains were kept at  $-80^{\circ}\text{C}$ , in 20% glycerol. Lactic bacterium was revived through cultivation in MRS. The sensitive strain through cultivation in LB medium was also revived.

#### Bacteriocin activity

After the cultivation of *L. paracasei*, the cells were removed through centrifuging at 5,000 rpm for 10'. The antimicrobial activity of the supernatant of lactic bacteria as well as of the isolated bacteriocins was developed through serial dilutions (1/2) in phosphate buffer pH 6.5. The pH of the resulted supernatant was corrected to the value of 5.5 with NaOH 0.2 N. 10  $\mu\text{l}$  of supernatant were placed on solid LB medium within a Petri plate and were sown with the sensitive strain of *E. coli*. After 30 min., the plates are introduced at  $28^{\circ}\text{C}$ , for 24 h. The diameter of the inhibition area was measured using Colony Quant software. (Sengul et al., 2003; Strompfova et al., 2003).

The same test was also made after the concentration of supernatant in a Buchi rotavapor up to 1:3, at  $48^{\circ}\text{C}$ , 200 rpm and 75 mbar. Moreover, the inhibitory area was also determined through the cultivation of the producing strain in MRS and supplemented with 1% inulin from chicory (P1 medium), *Dahlia imperialis* (P2 medium), lactulosis (P3medium), raffinose (P4 medium).

#### Bacteriocin characterization

##### Stability to temperature

A volume of 2 ml of supernatant was placed in each of the 3

Eppendorf tubes and they were introduced in the drying stove at different temperatures: at  $75$  and  $100^{\circ}\text{C}$  for 20 min. and at  $120^{\circ}\text{C}$  for 15 min. Bacteriocin activity was determined through the method previously described.

##### The pH effect

A volume of 2 ml supernatant was placed in each of the 8 Eppendorf tubes and pH was adjusted in each of them at values ranging from 2 to 9 with HCl 1M and NaOH 1 M. After 1 - 2 h, the bacteriocin activity from each tube was determined through the method previously described

##### The effect of enzymes

Pepsin, trypsin, proteinase K, papain, lipase were used in a concentration of 1 mg/ml each. An Eppendorf tube was prepared for each enzyme and was left at  $37^{\circ}\text{C}$  for 2 h. The activity of bacteriocin from each tube was determined in relation to a control sample through the method previously described. (Savadogo et al., 2004; Vinod et al., 2006; Narcisa et al., 2008)

##### Partial purification of bacteriocin

The supernatant was got after the concentration was treated with different concentrations of ammonia sulphate. Each mixture was kept over-night in the refrigerator. The precipitate formed was removed through centrifuge at 10,000 rpm, and it was re-dissolved in sodium phosphate buffer 20 mM after 10 min. The bacteriocin activity was determined in relation to a control sample from each tube through the method previously described (Vinod et al., 2006).

## RESULTS AND DISCUSSION

The inhibitory activity of the supernatant of *Lactobacillus paracasei* culture on *Escherichia coli* culture was presented in Table 1. The determination was made in parallel with the supernatant concentrated in Buchi rotavapor as well as in MRS supplemented with prebiotic, at 24 h of fermentation. It should be noticed that, though at the concentration of supernatant the inhibitory area increased with 0.3 cm, the inhibitory activity was unchanged (Figure 1).

With the use of prebiotic the activity increased in the presence of lactulose and raffinose. In this case, it was noticed that the diameter of the inhibitory area can be maintained for up to 72 h of fermentation when increased to 50% compared to 24 h of fermentation.

Also, the optical density and the production of lactic acid were determined during their respective 96 h of fermentation. The data were presented in Figures 2 and 3. It was noticed that the synthesis of lactic acid as well as the increase of the cellular density were correlated with the synthesis of bacteriocins. The greatest quantity of lactic acid was synthesized in the case of P4 medium, which contained raffinose. The maximum value of O.D. was found when using lactulose.

From Figure 4, it was noticed that the synthesis of bacteriocin was closely connected with cellular

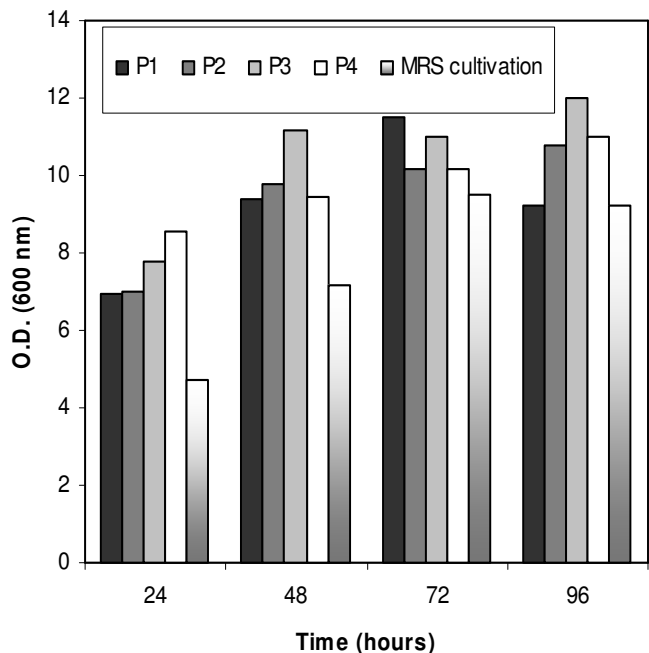


Figure 2. O.D. at 600 nm at the cultivation of the *Lactobacillus paracasei* strain.

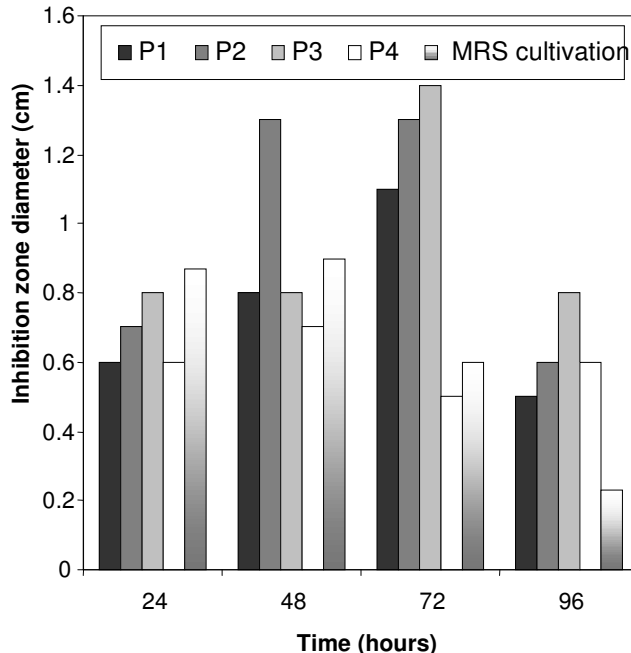


Figure 4. The diameter of the inhibitory area for the *Lactobacillus paracasei* strain.

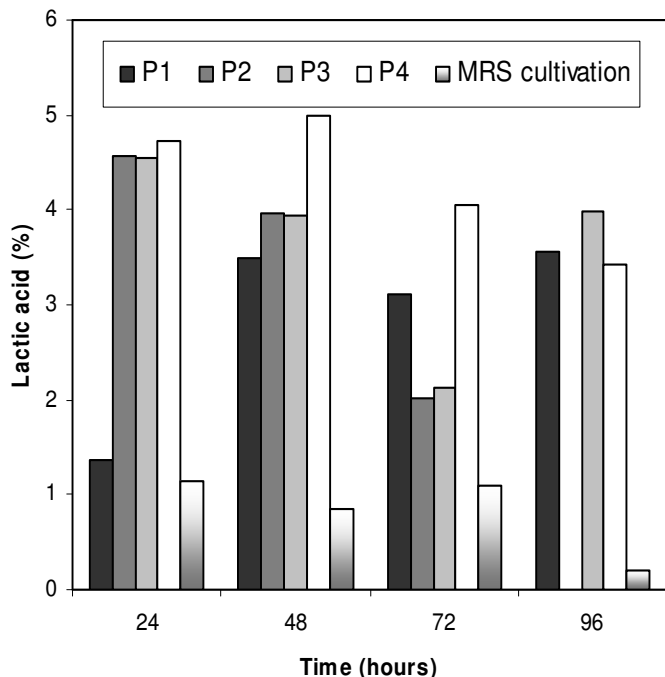


Figure 3. The quantity of lactic acid produced by the *Lactobacillus paracasei* strain.

productivity at the *L. paracasei* strain and pH reduction does not have a direct role in the negative influence on the bacteriocin activity. The decrease of the diameter was only made due to the long period of fermentation because O.D. was maintained at a high value for the 96 h

Table 2. Antimicrobial activity depending on T and pH.

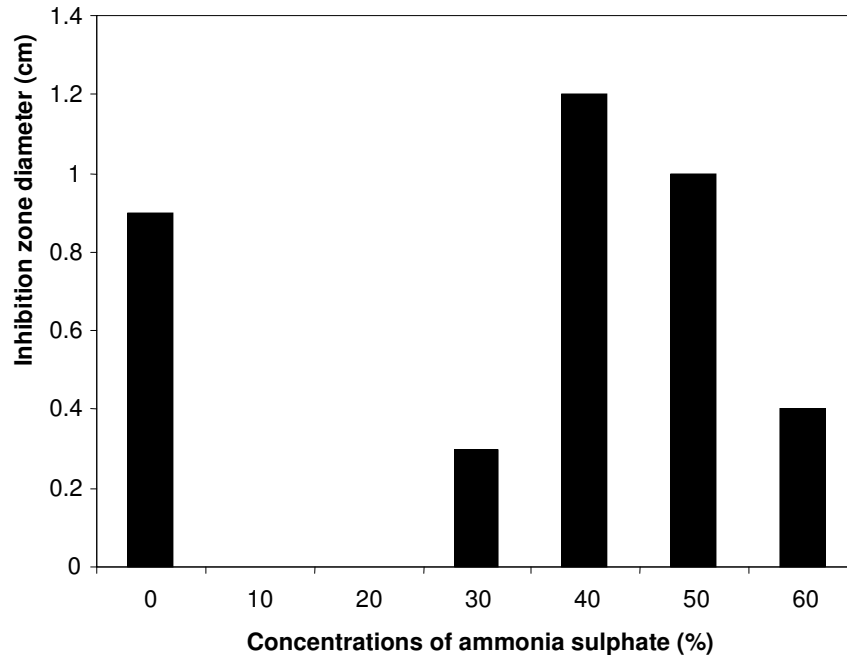
| Treatment      | Antimicrobial activity |   |
|----------------|------------------------|---|
| Temperature °C | 75                     | + |
|                | 100                    | + |
|                | 120                    | + |
| pH             | 2                      | - |
|                | 3                      | + |
|                | 4                      | + |
|                | 5                      | + |
|                | 6                      | + |
|                | 7                      | + |
|                | 8                      | + |
| 9              | -/+                    |   |

for lactulose.

In order to characterize the bacteriocin, the supernatant was subjected to treatment with enzymes and different values of pH and T. Bacteriocin was resistant to trypsin, papain and lipase, and was inhibited by the presence of proteinase K and pepsin. The results for the influence of T and pH were presented in Table 2.

From the tests developed bacteriocin was thermo-resistant, its diameter maintained at a minimum of 0.7 cm. The same effect was observed at the different values of pH, except for the extreme values of 2 and over 9.

During the test for pH effect, the diameter can significantly vary, even decreasing by 50% for extreme



**Figure 5.** The antimicrobial activity through precipitation with ammonia sulphate.

basic values. The tests made with the concentrated supernatant presented the same variations, and the differences could be included in the margin for error.

Following partial purification, a concentration of 40% ammonia sulphate was sufficient for the increase of the inhibitory activity against *E. coli* strain (Figure 5). The increase of antimicrobial activity was of 30% compared to the bacteriocin within the untreated supernatant. The increase of concentration of ammonia sulphate also determined the gradual reduction of antimicrobial activity. Smaller concentrations had no effect, because there were no precipitated bacteriocin results. It should also be noticed that, at a concentration of over 60%, the solution becomes saturated.

## Conclusion

Following the experiments developed, some observations were formulated. The *L. paracasei* CMGB16 strain was capable of synthesizing bacteriocins with direct effect on *E. coli*. The synthesized bacteriocin was thermo-stable, and also resistant to a pH that varied from 3 to 9, as well as been active to enzymes such as trypsin, papain and lipase, inhibited by proteinase K and pepsin. The supplementation of the culture medium with prebiotics determined an increase of the inhibitory activity on the *E. coli* strain. The optimum inhibitory activity resulted from the supplementation of the medium with lactulose and raffinose. Due to the antimicrobial activity, the probiotic strain can be successfully used as natural preservative or in products which act on human intestinal micro-flora.

## ACKNOWLEDGMENT

This research work was financed through a project PNCDI II no. 62-050/2008 (<http://proiectprobac.emanuelvamanu.ro/>).

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