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Bacteriocin production of *Leuconostoc carnosum* LA54A at different combinations of pH and temperature

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SUMMARY

Leuconostoc carnosum LA54A produces a bacteriocin which is active against *Listeria monocytogenes* and *Listeria innocua*. The ability of *Lc. carnosum* to produce the bacteriocin at various combinations of the growth parameters pH and temperature was analyzed. In the case of this strain bacteriocin production seems to be coupled to growth rate. This fact enables the prediction if *Lc. carnosum* can produce the bacteriocin at a given set of growth parameters, simply by predicting the growth rate of this organism. In addition we have analyzed the growth behavior of the target organism *L. innocua* WS2258 at the same set of growth parameters.

INTRODUCTION

Vegetable-type convenience foods are increasingly preferred by consumers in industrialized countries. During recent years especially, shredded fresh salad mixes, prepacked in polythene bags, have earned a special place in the European market. In spite of modern technological advances and adherence to 'Good Manufacturing Practice' (GMP), the initial microbial load of the prepackaged product frequently exceeds 10^6 g^{-1} [4]. This explains the relatively limited shelf life of practically all salad mixes, even under refrigeration at 4 °C. The initial microbial population is dominated by Gram-negative bacteria, mainly *Enterobacteriaceae* and *Pseudomonas* sp. Gram-positive bacteria are present in lower numbers; generally saprophytic ones are encountered, but also *Listeria monocytogenes* [9] may be present and thus constitute a health risk. For our approach towards the development of novel concepts for food safety assurance, the pathogenic Gram-positive bacterium, *Listeria* has been chosen as model target organism for the analysis of bacteriocinogenic lactic acid bacteria, which may be used as protective cultures to control *Listeria* in these food systems.

Lactic acid bacteria which produce bacteriocins are regarded as potential controlling factors for improvement of the microbiological stability of different food products [2,3,7,10,11]. Bacteriocins are produced by a number of lactic acid bacteria [3,11]. By definition, bacteriocins are small proteins with bactericidal activity against closely related species [6,12].

The growth parameters usually used for analyzing bacteriocin production by lactic acid bacteria often ensure optimal growth of the bacteriocinogenic culture. These optimal growth conditions however are not given in the processed and stored food product. Moreover in the concept of stabilizing foods by protective cultures, the use of additional controlling factors, like pH and temperature, to suppress the growth of undesired pathogenic or spoilage microbes, but not the growth and metabolism of the protective culture, is suggested [8]. However the metabolic activity of lactic acid bacteria is certainly influenced by these growth parameters, so information about bacteriocin production at different environmental conditions is required. Protective cultures should actively produce bacteriocins at combinations of growth parameters where growth of the pathogenic target organism is possible.

MATERIALS AND METHODS

Strains and culture conditions

Leuconostoc carnosum LA54A [13] and *Listeria innocua* WS2258 (culture collection of the Federal Research Centre for Nutrition) were used throughout the study. *Lc. carnosum* was grown in MRS broth (Merck, Darmstadt, Germany) at pH values and temperatures as indicated in Table 1. *L. innocua* was grown in standard I broth (Merck). The pH of the medium was adjusted before inoculation either with 0.1 M HCl or NaOH. During growth of the cultures no further pH adjustment was carried out. Determinations of the viable counts were carried out on MRS agar plates in the case of *Lc. carnosum* and on standard I agar plates in the case of *L. innocua*.

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TABLE 1

Combination of the growth parameters temperature and pH

L. innocua:	*Growth* at combination of: temperature: 10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 37 °C pH: 4, 5, 6, 7
Lc. carnosum:	*Growth* at combination of: temperature: 4 °C, 10 °C, 15 °C, 25 °C, 30 °C pH: 4, 5, 5.8, 6.5, 7.1, 7.9
Lc. carnosum:	*Bacteriocin production* at combinations of: temperature: 4 °C, 10 °C, 15 °C, 25 °C, 30 °C pH: 4, 5, 5.8, 6.5, 7.1, 7.9

Determination of bacteriocin activity

For quantitative determination of the produced bacteriocin, *Lc. carnosum* LA54A was grown at different combinations of pH and temperature. At certain time intervals 2 ml samples were withdrawn; 1 ml was used for determining the viable counts of the cultures. The other part of the same samples was centrifuged and the supernatants were transferred to a new tube. The supernatants were sterilized by filtration (Schleicher and Schüll, disposable filter, FP 030/3) and diluted serially in a ratio of 1:1. 10 μl of each dilution were applied in drops on standard I soft agar plates containing 10^4 *L. innocua* cells ml^{-1}. The plates were incubated overnight at 25 °C. The reciprocal value of the highest dilution where bacteriocin activity (e.g. formation of a clearing zone) was detectable multiplied by 100 was taken as activity units per ml (AU ml^{-1}).

Growth rate predictions

For predicting the growth rate of *Lc. carnosum* the collected growth data were analyzed with the method of Baranyi et al. [1]. Predictions were made at the same combinations of controlling factors where the measured data were collected.

RESULTS

Leuconostoc carnosum LA54A produces a bacteriocin which is active against *Listeria monocytogenes* and also against *Listeria innocua*. As *L. innocua* shows very similar growth characteristics to *L. monocytogenes* the apathogenic strain was used for the experiments. The growth data of *L. innocua* and the growth data and bacteriocin production data of *Lc. carnosum* were collected from pure broth cultures of both organisms. The growth curves for each combination of pH and temperature were determined by plotting the logarithmic values of the cell numbers against incubation time (data not shown). From these curves the specific growth rates of the different cultures were determined. Fig. 1 shows a three-dimensional plot of the measured specific growth rates of *L. innocua* WS 2258 in relation to the growth parameters pH and temperature. The growth rates at each temperature at pH 4 were very low. Negligible growth rates

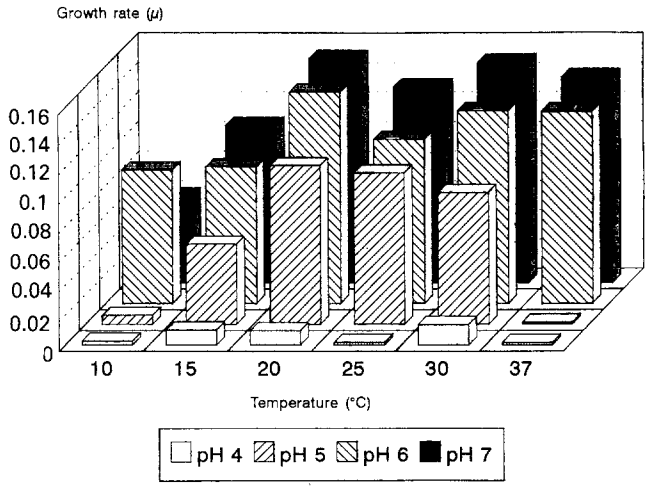

Fig. 1. Three-dimensional plot of the growth rate (μ) of *Listeria innocua* WS 2258 in relation to the growth parameters temperature and pH.

of *L. innocua* were also found at the combinations pH 5/10 °C and pH 5/37 °C. All other analyzed combinations of pH and temperature allow the growth of *Listeria* with a relatively high specific growth rate. A potential protective culture for controlling the growth of *Listeria* should actively produce the bacteriocin at the same conditions.

To analyze the bacteriocin production ability of *Lc. carnosum* LA54A, growth behavior and bacteriocin production of this strain were determined at the same sets of growth parameters. Fig. 2(a) shows the growth curves and Fig. 2(b) the bacteriocin production of *Lc. carnosum* at the optimum temperature of 25 °C and at different pH values (growth curves and bacteriocin production curves at the other combinations of pH and temperature are not shown). Growth at pH 5 was delayed; however after extended incubation, numbers of 10^9 cells ml^{-1}, comparable to those at more optimal conditions, were reached. Despite these high cell numbers only minor amounts of the bacteriocin were produced at this pH. At higher pH values (pH 6.4) the amount of bacteriocin produced was more than one order of magnitude higher compared to pH 5.0. Similar results could be found at other temperatures. These results indicate that a correlation between the cell number of a culture and the amount of bacteriocin produced does not necessarily exist.

The growth rate of *Lc. carnosum* LA54A in relation to the controlling factors, temperature and pH is illustrated in a three-dimensional plot in Fig. 3(a). The growth of this strain at pH 4 at all combinations with the temperatures is very poor. Higher growth rates could be observed at combinations of pH and 4 °C, except at pH 4. At all other combinations where growth of *L. innocua* is possible, the bacteriocinogenic *Lc. carnosum* strain is also able to grow. From these results however it cannot be concluded that *Lc. carnosum* is able to produce an inhibitory amount of the bacteriocin at all combinations of growth parameters. To clarify this question we have analyzed the bacteriocin

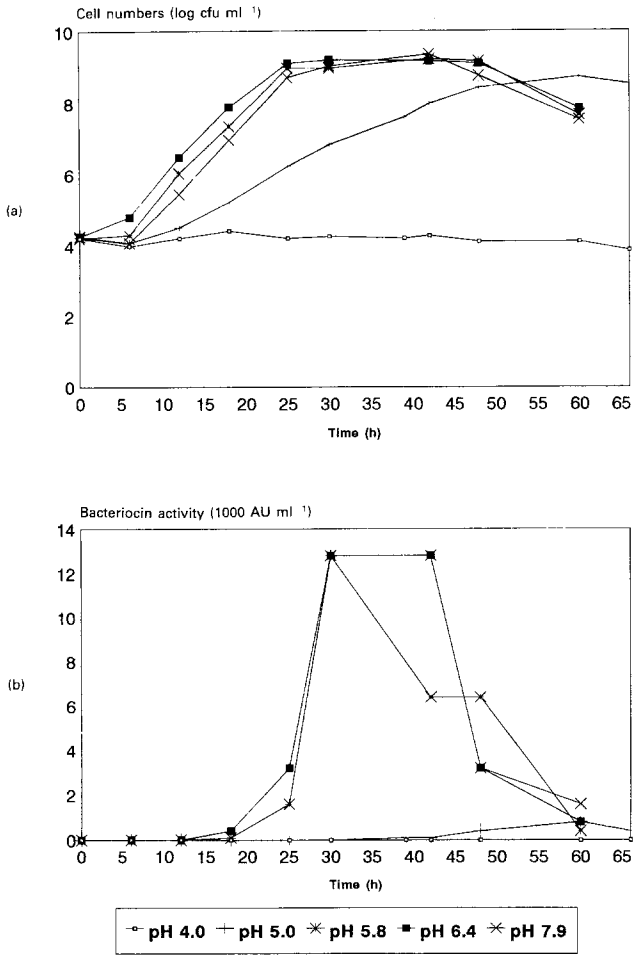

Fig. 2. Growth curves of *Leuconostoc carnosum* LA54A at the optimum temperature of 25 °C and different pH values (a) and the activity units of bacteriocin produced at the same set of the growth parameters pH and temperature (b). The values were plotted against growth time. Bacteriocin activity units were determined as described in Materials and Methods.

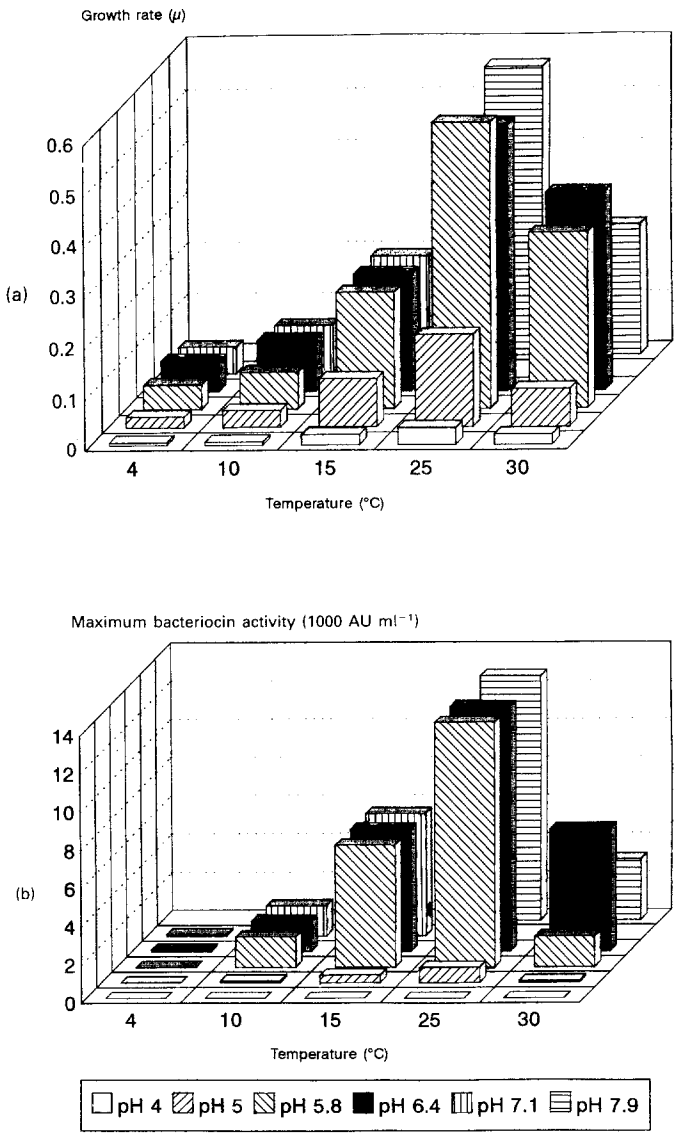

Fig. 3. Comparison of the determined growth rate of *Leuconostoc carnosum* LA54A (a) to the maximum amount of bacteriocin produced at the same combination of growth parameters pH and temperature (b). The determination of the bacteriocin activity units is described in Materials and Methods.

production of *Lc. carnosum*. Fig. 3(b) shows the maximum bacteriocin production of each culture (in terms of AU ml^{-1}) in relation to the controlling factors, temperature and pH. The bacteriocin production data coincide with the growth rate, which suggests that in the case of *Lc. carnosum* LA54A a correlation between growth rate and bacteriocin production exists. This correlation enables the prediction if bacteriocin production is possible at a given set of growth parameters. For this purpose a lower limit for bacteriocin units produced should be chosen, for example 5000 AU ml^{-1}. This amount of bacteriocin produced by *Lc. carnosum* should be high enough to act as an additional controlling factor against the growth of *Listeria*. The growth rates at those points will represent the limiting growth rates for a bacteriocin producing culture at reasonable amounts, which is the growth rate of 0.22 in the described experiments. All growth rates higher than those at these specific points should indicate a bacteriocin production above that limit. So prediction of bacteriocin production by *Lc. carnosum* should be possible simply by predicting the growth rate of this strain. We have used our collected growth data from *Lc. carnosum* LA54A to generate predictions of the growth rate of this organism by the use of the method described by Baranyi et al. [1]. This method allows predictions of the growth rate of a microorganism at different combinations of controlling factors if enough measured growth data were analyzed. Fig. 4(a) shows the predicted growth rates of *Lc. carnosum* LA54A. Fig. 4(b) shows the predicted bacteriocin production of this strain by using a minimal growth rate for a reasonable bacteriocin production of 0.22. All indicated cultures should produce bacteriocin above 5000 AU ml^{-1}. The predicted values (Fig. 4(b)) fit very well with the measured values (Fig. 3(b)). The only inaccurate predictions are at the combinations of pH 6/30 °C and pH 8/30°C where the predicted values are higher than the measured values (e.g., 1600 AU ml^{-1}

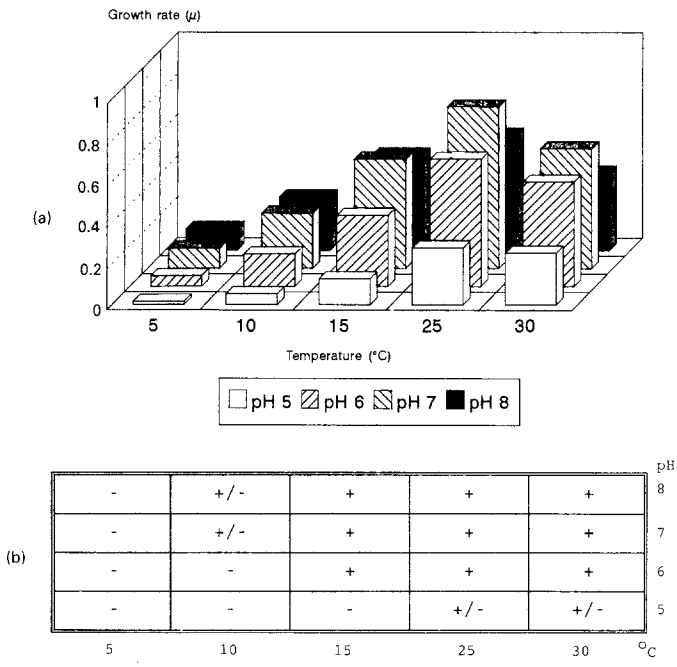

Fig. 4. The predicted growth rate of *Leuconostoc carnosum* LA54A (a) and the predicted bacteriocin production of that strain (b), if a minimal growth rate of 0.22 is used.

respectively 3200 AU ml^{-1} instead of predicted 5000 AU ml^{-1} or higher) despite the fact that relatively high growth rates could be observed at these points.

DISCUSSION

Leuconostoc carnosum LA54A is able to produce a bacteriocin against *Listeria* at a wide range of combinations of temperature and pH. Optimum bacteriocin production could be observed at 25 °C and pH 5.8–7.9. Hastings and Stiles [5] found optimum conditions for bacteriocin production of *Leuconostoc gelidum* at a combination of 25 °C and pH 6.5. *Lc. carnosum* is not able to produce the bacteriocin at different combinations of pH and 4 °C or at different combinations of temperature and pH 4, despite the fact that at some combinations slow growth is possible (Fig. 3(a)). This strain shows no obvious correlation between the cell number of a culture and bacteriocin production. Cultures with slow growth produce more than one order of magnitude less bacteriocin, than cultures with higher growth rates, despite the fact that the same cell numbers are reached after extended incubation. These results indicate that the production of the bacteriocin is regulated depending on environmental conditions.

The bacteriocin production of *Lc. carnosum* seems to be tightly coupled to the growth rate. High growth rates resulted in high maximum bacteriocin activity units. The same behavior is described for *Lc. gelidum* [5] which showed also reduced bacteriocin production at slower growth rates. The coupling of bacteriocin production to growth rate enables the prediction if *Lc. carnosum* is able to produce the bacteriocin at inhibitory amounts at a given set of growth parameters, simply by predicting the growth rate of this organism. The only fact which has to be known is the minimal growth rate at which a reasonable bacteriocin production takes place. At two growth points (pH 5.8/30 °C and pH 7.9/30 °C) the relatively high growth rates indicate a bacteriocin production above 5000 AU ml^{-1}. The measured values however are below the predicted values. Further experiments with improved methods for measuring bacteriocin activity should clarify this discrepancy. *Lc. carnosum* LA54A is not able to produce the bacteriocin at reasonable amounts at all combinations of pH and temperature, where growth of *Listeria* is possible (Figs 1 and 3(b)). These results indicate that *Lc. carnosum* LA54A might not be able to control the growth of *Listeria* at all combinations of pH and temperature and additional controlling factors have to be used at those points.

The described method allows the assessment of the bacteriocin producing ability of lactic acid bacteria and their potential to act as protective cultures in relation to formulation, processing or storage conditions of foods.

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