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Influence of pH, temperature and culture media on the growth and bacteriocin production by vaginal *Lactobacillus salivarius* CRL 1328

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Aims: To study the influence of pH, temperature and culture medium on the growth and bacteriocin production by vaginal *Lactobacillus salivarius* subsp. *salivarius* CRL 1328.

Methods and Results: The study was performed using a complete factorial experimental design. *Lactobacillus salivarius* was cultivated in LAPTg and MRS broths, adjusted to specific initial pH, and at different temperatures of incubation. The growth, which was evaluated by the Gompertz model, was higher in MRS broth than in LAPTg broth. The initial pH of the culture medium and the temperature had a dramatic effect on the production of bacteriocin. The optimal conditions for bacteriocin production were different to those for optimal growth. The decrease in the pH of the culture medium was parallel to the growth; pH had similar final values in both the MRS and the LAPTg broths.

Conclusions: The optimal growth conditions were recorded in MRS broth, with an initial pH of 6·5 and a temperature of 37°C. The maximum bacteriocin activity was obtained in LAPTg after 6 h at 37°C, and at an initial pH of 6·5 or 8·0.

Significance and Impact of the Study: The application of a complete factorial design, and the evaluation of the growth parameters through the Gompertz model, enabled a rapid and simultaneous exploration of the influence of pH, temperature and growth medium on both growth and bacteriocin production by vaginal *Lact. salivarius* CRL 1328.

INTRODUCTION

Lactobacilli, as well as some species of the genus *Bifidobacteria* and Streptococci, are micro-organisms which are classified in the GRAS (Generally Regarded as Safe) group. They have also been proposed as probiotics for both the gastrointestinal and urogenital tracts (Redondo López *et al.* 1990). This proposition is based on the fact that they where isolated in high numbers from human faeces and the vaginas of healthy pre-menopausal women (Larsen 1993) where they can protect against pathogenic micro-organisms.

One of the main mechanisms used by the lactic acid bacteria to interfere with the colonization of pathogens and avoid proliferation of those potential pathogens is production of antimicrobial agents, such as organic acids, hydrogen peroxide and bacteriocins or related substances (Redondo López *et al.* 1990; Klebanoff *et al.* 1991; McGroarty *et al.* 1992; McGroarty 1993; Baerheim *et al.* 1994). Bacteriocins are proteinaceous, bactericidal substances synthesized by bacteria. They usually have a narrow spectrum of activity, meaning that they only inhibit strains of the same or closely-related species (Jack *et al.* 1995). The term 'bacteriocin-like substance' is applied to any antagonistic substance which does not fit the typical criteria of bacteriocins. These bacteriocin-like substances inhibit wider ranges of bacteria, both Gram-positive and Gram-negative, and fungi (McGroarty 1993).

The lactic acid bacteria which produce bacteriocin are widely used in probiotic products for human and animal consumption to prevent pathogen growth in the gastrointestinal tract (Guillilan 1979; Nader-Macías *et al.* 1993;

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Audisio *et al.* 2001). Lactic acid bacteria and their bacteriocins are also used as food preservatives.

Bacteriocins are usually produced in complex media (Biswas *et al.* 1991; Parente and Hill 1992; De Vuyst and Vandamme 1993; Parente and Ricciardi 1994; De Vuyst *et al.* 1996; Lejeune *et al.* 1998; Leroy and De Vuyst 1999), although some have also been produced at high concentrations in relatively simple broths (Biswas *et al.* 1991; Yang *et al.* 1992; Yang and Ray 1994). Only a few bacteriocins have been studied in a defined medium (De Vuyst 1995; Møretrø *et al.* 2000). Physicochemical factors, for example pH or temperature, have a dramatic effect on the production of bacteriocins; the optimal pH values and temperatures for maximum bacteriocin production rarely coincide with the best conditions for the growth of the bacteria (Biswas *et al.* 1991; Geisen *et al.* 1993; Parente and Ricciardi 1994; Parente *et al.* 1994; Lejeune *et al.* 1998; Leroy and De Vuyst 1999; Møretrø *et al.* 2000).

The isolation, identification, and certain characteristics such as the adhesion abilities and production of hydrogen peroxide, of vaginal lactobacilli isolated from women from Tucumán, Argentina, has been reported (Ocaña *et al.* 1999a, b, c), along with the description of the characteristics and mode of action of a bacteriocin produced by vaginal *Lactobacillus salivarius* subsp. *salivarius* CRL 1328 (Ocaña *et al.* 1999d). Characteristics of *Lact. salivarius*, such as optimization of biomass production, have been investigated in the light of the possibility of including it in a pharmaceutical product with probiotic properties for local application in the vaginal tract (Juarez Tomás *et al.* 2002). The purified bacteriocin might also be included in a probiotic formula to be used directly as an antagonistic substance.

The objective of the present work was to study the influences of initial pH, temperature and culture medium on the kinetics of growth, the decrease of pH and the production of bacteriocin by *Lact. salivarius* CRL 1328, in order to ascertain (a) the most favourable growth conditions to obtain the highest biomass in the shortest possible time, and (b) the optimal culture conditions for bacteriocin production *in vitro* for subsequent purification. This was undertaken using a complete factorial experimental design. To estimate the growth parameters, the Gompertz model (Zwietering *et al.* 1990) was adjusted to the experimental data.

MATERIALS AND METHODS

Bacterial strains

Bacteriocin-producing *Lactobacillus salivarius* subsp. *salivarius* CRL 1328 (from the CERELA Culture Collection) was originally isolated from the human vagina (Ocaña *et al.* 1999a,d). *Enterococcus faecalis*, which was also isolated from a vaginal sample and obtained from the Instituto de Microbiología of the Universidad Nacional de Tucumán, Tucumán, Argentina, was the indicator strain used. The microorganisms were stored in a milk–yeast extract (13% non-fat milk, 0·5% yeast extract and 1% glucose) at −70°C.

Inoculum preparation

Before the growth experiments were performed, *Lact. salivarius* CRL 1328 was propagated in either MRS (Biokar Diagnostics, Beauvais, France) (De Man *et al.* 1960) or LAPTg broths (1·5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0·1% Tween 80) (Raibaud *et al.* 1963) at a temperature of 37°C for 24 h, and subcultured twice at 37°C for 12 h in the same media. The third culture was centrifuged (6000 *g*, 10 min), washed with saline solution (0·85% NaCl), and the pellet resuspended in the same solution to a final optical density (O.D.) of 1·4 at 540 nm. This cell suspension was used as the inoculum for the growth experiments. Before inhibition assays, *Ent. faecalis* was propagated twice in LAPTg broth at 37°C for 12 h; the $O.D._{540\ nm}$ of the last culture was adjusted to 0·6.

Growth conditions

The initial pH of the LAPTg and MRS broths was adjusted to 5·0, 6·5 or 8·0 with either 1 N HCl or NaOH before inoculation. *Lactobacillus salivarius* CRL 1328 was inoculated (2% v/v) into 100 ml of each medium in 250 ml Erlenmeyer flasks and incubated in a water-bath (Vicking S.R.L. Masson Model, Buenos Aires Industria Argentina) at constant temperatures of 30°C, 37°C or 44°C, as appropriate.

Analytical procedures

Samples were taken at specific time intervals and the O.D. measured at 540 nm in glass cuvettes with a 10 mm light path (Spectrophotometer Model 250; Gilford Instrument Laboratory, Oberlin, OH, USA). The pH was determined with a pH meter (Digimeter IV; Luftman, Buenos Aires, Argentina).

Determination of colony-forming units (cfu). The number of cfu ml^{-1} was quantified using the plate dilution method, with peptone water (0·1% peptone) as the dilution medium and MRS or LAPTg agar as the culture medium. The relationship between O.D. and cfu ml^{-1} was obtained by application of the exponential function: log (cfu ml^{-1}) = $a(O.D.)^b$, where a and b were estimated using the least squares method.

Bacteriocin activity. The plate diffusion method was used (Jack *et al.* 1995) to detect the inhibitory activity of the supernatant fluid of *Lact. salivarius* CRL 1328 culture. The

supernatant fluids were separated by centrifugation (6000 *g*, 5 min, 20°C), neutralized with 2 N NaOH, sterilized through a filter (Millipore, 0·22 μm) and then serially diluted in each medium. Aliquots (25 μl) of each dilution were poured into the 4 mm holes of the LAPTg agar plates (LAPTg 1% agar) which contained vaginal *Ent. faecalis* (10^6–10^7 cfu ml^{-1}). The plates were incubated for 5 h at room temperature and then for 24 h at 37°C. The highest dilution that produced a distinct inhibition zone was referred to as Arbitrary Units per milliliter (AU ml^{-1}). The bacteriocin concentration was also expressed as Arbitrary Units per log cfu [AU (log cfu)$^{-1}$].

Experimental design

The growth experiments were repeated twice following a complete factorial design of 2×3^2. Two levels of culture medium in both LAPTg and MRS broth, three levels of temperature (30, 37 and 44°C) and three initial pH levels of 5·0, 6·5 and 8·0, were evaluated.

Estimation of the growth curves

The bacterial growth curves were described using the modified 4-parameter-Gompertz model (Zwietering *et al.* 1990), which included the independent term, D0, representing the initial O.D.

At time t, the reparametrized Gompertz model is expressed by the following function:

$$D_t = D_0 + A \exp\{-\exp[(\mu \cdot e/A)(\lambda - t) + 1]\} \qquad (1)$$

where D_t is the O.D. at time t, t the time of growth in hours, D_0 the O.D. at $t = 0$, A the difference between the final and initial O.D.s, μ the maximum specific growth rate (h^{-1}), λ the duration time of lag phase in hours and e the base of the neperian logarithm (2·718281828).

Using the A and μ values, the exponential phase time, ε_g (h), of the micro-organism was calculated. This parameter is given by the following expression: $\varepsilon_g = A/\mu$ (Zwietering 1993; Juárez Tomás *et al.* 2002).

The same model was applied to analyse the decrease of pH having both parameters negative signs (named A the difference between the final and initial pH) and μ (pH decrease rate) (data not shown).

The growth parameters from the viable count data were also calculated using the Gompertz model, according to the above equation (1), by replacing D_t with log (cfu cfu_0^{-1}), where cfu_0 is the cfu number in $t = 0$, therefore without the independent term D_0. As the latency phase was not observed in any of the growth curves where log cfu ml^{-1} was determined, λ was considered 0, estimating only the parameters A and μ.

The estimation of the Gompertz model parameters was performed using the constrained non-linear regression with a sequential quadratic programming method. To calculate the standard errors from parameter estimations, the bootstrapping technique was applied using repeated samples from the original data by sampling with replacement (Efron 1982; Huet *et al.* 1996). The number of bootstrap samples was chosen as 1000 and 100 for each growth curve of O.D. and cfu ml^{-1}, respectively. SPSS 10 for Windows was used for statistical analysis, and S-PLUS 2000 was used to generate the corresponding graphics.

RESULTS

Growth parameters from absorbance measurements

Figure 1 summarizes the O.D. $_{540\ nm}$ and pH values of *Lact. salivarius* CRL 1328 grown in LAPTg and MRS broths under varying culture conditions, and subsequent adjustment of the data by application of the Gompertz model. The growth parameters obtained from absorbance data are summarized in Table 1. These values varied depending on the culture medium, temperature and pH tested.

For all the conditions evaluated, results show that the MRS broth with a pH of 6·5 and a temperature of 37°C yield the highest growth and are therefore the optimal conditions (higher final O.D. and growth rate, as well as shorter lag phase and exponential phase time). Similar growth conditions were also obtained in MRS broth with a temperature of 37°C and with an initial pH of either 5·0 or 8·0. At the relatively lower temperature of 30°C and pH values of 6·5 and 8·0, higher final O.D. values were obtained but longer lag phases and lower growth rates were observed.

For all the pH and growth media assayed, the difference between the final and initial O.D. values was found to be at a maximum at 37°C (Table 1). The same behaviour was observed with regard to growth rates. However, when the LAPTg and MRS broths had a pH of 6·5, growth rate increased with temperature. Both the lag phase and exponential phase times were inversely related to the growth temperature. Only in LAPTg did the lag phase increase with the pH, and the exponential phase time showed the inverse behaviour.

Relationship between absorbance measurements and viable count data

Figure 2 shows the viable count data (cfu ml^{-1}) plotted with the O.D. determinations. For each growth medium, the correlation between both growth measurements showed the same behaviour, independent of the different initial pH and temperatures assayed. The plots fitted to the proposed exponential models (see Materials and methods). The a and b parameters had statistically different values for each

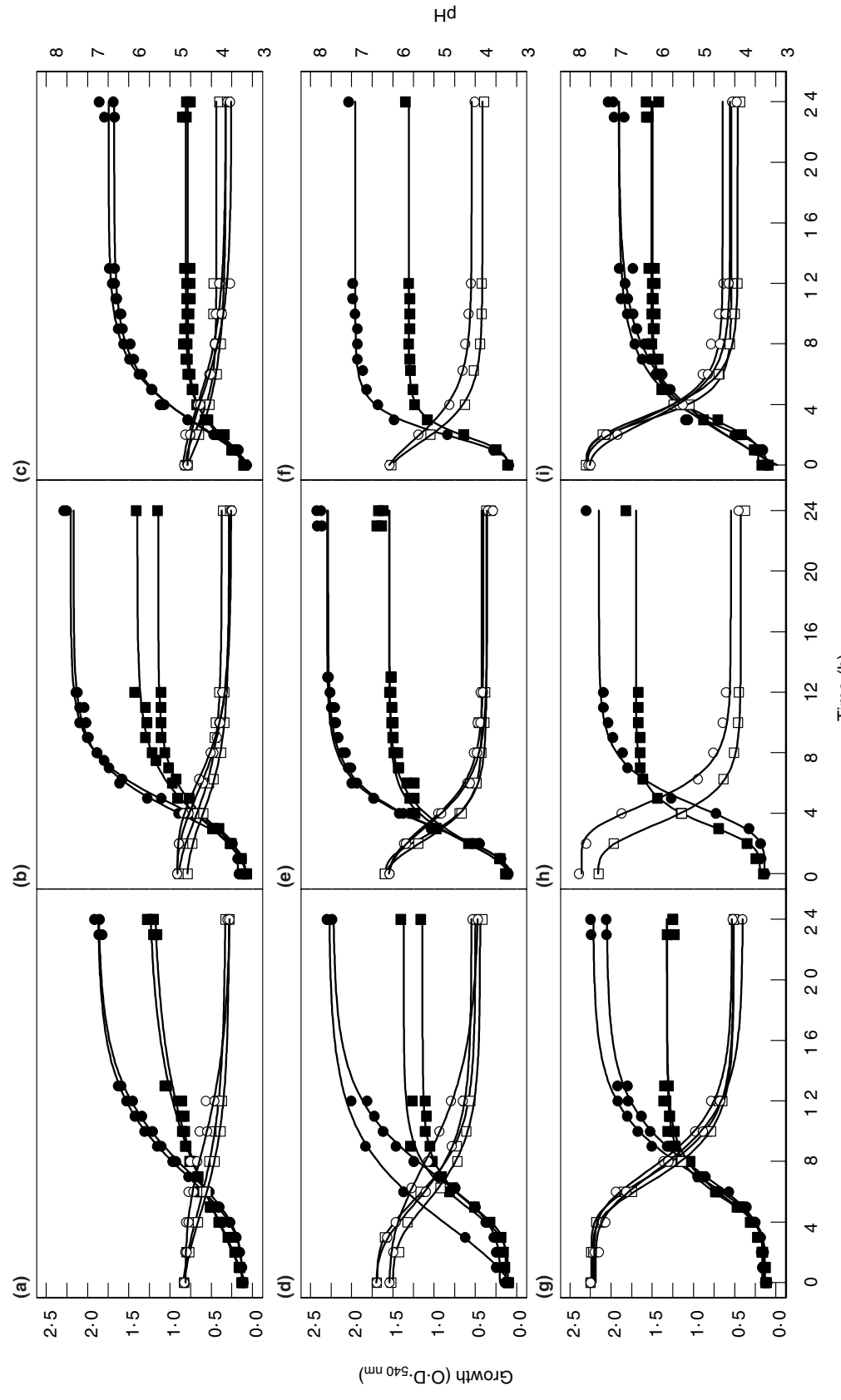

Fig. 1 Kinetics of growth and decrease of pH of *Lactobacillus salivarius* CRL 1328 under different culture conditions. (a) 30°C, pH 5·0; (b) 37°C, pH 5·0; (c) 44°C, pH 5·0; (d) 30°C, pH 6·5; (e) 37°C, pH 6·5; (f) 44°C, pH 6·5; (g) 30°C, pH 8·0; (h) 37°C, pH 8·0; (i) 44°C, pH 8·0. $OD_{540\ nm}$ in LAPTg (■) and MRS (●); pH modifications in LAPTg (□) and MRS (○). The lines represent the subsequent adjustment of the experimental data by the application of the Gompertz model

Table 1 Estimation of the growth parameters from optical density measurements by the Gompertz model with 95% confidence intervals

Growth medium	pH	T (°C)	D_0*	±95%†	A*	±95%†	μ*	±95%†	λ*	±95%†	ε_g‡	±95%‡	N§
LAPTg	5·0	30	0·04	±0·03	1·19	±0·05	0·09	±0·01	0·01	±0·15	13·82	±1·70	34
LAPTg	5·0	37	0·06	±0·06	1·21	±0·16	0·20	±0·04	0·99	±0·70	6·09	±1·70	28
LAPTg	5·0	44	0·09	±0·09	0·71	±0·09	0·18	±0·02	0·36	±0·68	3·85	±0·73	34
LAPTg	6·5	30	0·12	±0·05	1·12	±0·15	0·21	±0·07	3·02	±0·94	5·40	±2·13	20
LAPTg	6·5	37	0·08	±0·07	1·49	±0·09	0·35	±0·06	0·69	±0·55	4·24	±0·90	48
LAPTg	6·5	44	0·12	±0·27	1·18	±0·27	0·51	±0·17	0·93	±0·80	2·38	±0·93	14
LAPTg	8·0	30	0·16	±0·03	1·15	±0·05	0·26	±0·04	3·81	±0·36	4·36	±0·73	34
LAPTg	8·0	37	0·20	±0·17	1·50	±0·18	0·46	±0·09	1·86	±0·68	3·32	±0·82	14
LAPTg	8·0	44	0·16	±0·09	1·35	±0·10	0·41	±0·06	1·39	±0·50	3·30	±0·59	34
MRS	5·0	30	0·12	±0·01	1·77	±0·04	0·19	±0·01	3·62	±0·28	9·56	±1·24	34
MRS	5·0	37	0·13	±0·04	2·06	±0·09	0·37	±0·03	2·14	±0·36	5·56	±0·74	28
MRS	50·	44	0·01	±0·02	1·69	±0·05	0·29	±0·03	0·40	±0·28	5·91	±0·83	34
MRS	6·5	30	0·15	±0·18	2·13	±0·53	0·22	±0·13	2·56	±2·32	10·71	±6·87	20
MRS	6·5	37	0·10	±0·06	2·22	±0·09	0·50	±0·06	1·26	±0·33	4·44	±0·70	48
MRS	6·5	44	0·07	±0·32	1·88	±0·32	0·64	±0·19	0·77	±0·45	3·01	±1·04	14
MRS	8·0	30	0·14	±0·02	2·02	±0·11	0·26	±0·03	4·14	±0·35	7·67	±1·03	34
MRS	8·0	37	0·14	±0·08	2·01	±0·17	0·43	±0·09	2·58	±0·75	4·69	±1·26	14
MRS	8·0	44	0·01	±0·08	1·88	±0·10	0·29	±0·05	0·05	±0·30	6·62	±1·48	34

*Parameters of the Gompertz model: D_0, initial optical density; A, difference between the final and initial optical densities; μ, maximum specific growth rate (h^{-1}); λ, lag phase (h).
†95% confidence intervals.
‡Exponential phase time (h).
§Sample size.

growth medium (data not shown). Although the correlation seems weaker at higher O.D.s, the relationship between those two growth measurements had an increasing function, indicating that the maximum and minimum values of O.D. and log cfu ml^{-1} were simultaneously obtained under the same growth conditions. The values of growth parameters calculated from the viable count data (Table 2) using different media, temperature and pH were numerically different to those estimated from absorbance data. However, they showed the same type of tendency with respect to the determination of the optimal conditions.

pH modifications

The decrease in culture medium pH was parallel to growth. When the LAPTg and MRS broth shared the same temperatures of incubation and initial pH, at the time of the lactobacilli growth phase, the final pH values were very similar. The difference between initial and final pH was dependent on the initial pH of the broth in both growth media at different temperatures. For all the conditions tested, the pH rate decreased more in LAPTg than in MRS. When the two broths were incubated at the same temperature, the decrease in pH was directly related to the initial pH (data not shown). The optimal conditions for a decrease in pH coincided with the optimum growth conditions, thus indicating that the decrease in pH is directly related to the growth of the lactobacilli.

Bacteriocin production

Bacteriocin production during the growth period of *Lact. salivarius* CRL 1328 was higher in LAPTg than in MRS. However, cell mass and growth rates were higher in MRS medium (Fig. 3). Optimum conditions for the production of bacteriocin are as follows: temperature of 37°C, with an initial pH of either 6·5 or 8·0 and LAPTg culture medium, which gave the highest production of 1280 AU ml^{-1} after 6 h of incubation. In LAPTg with an initial pH of 6·5 and at 30°C, high levels of bacteriocin were also produced (640 AU ml^{-1}) after 9 h, remaining stable up to 24 h. Respectively, 320 AU ml^{-1} was the maximum bacteriocin activity obtained in the MRS broth at 30 and 37°C with an initial pH of 6·5 and 8·0. At an initial pH of 5·0, bacteriocin production in LAPTg was higher at 30°C than at 37°C and in MRS, bacteriocin activity was minimal (40–80 AU ml^{-1}) at all temperatures tested. At a relatively high temperature, such as 44°C, there was no production of bacteriocin at any of the three pH levels assayed in either medium.

The maximum activity was always detected during the second part of the exponential phase, indicating that this bacteriocin is produced while the micro-organisms are

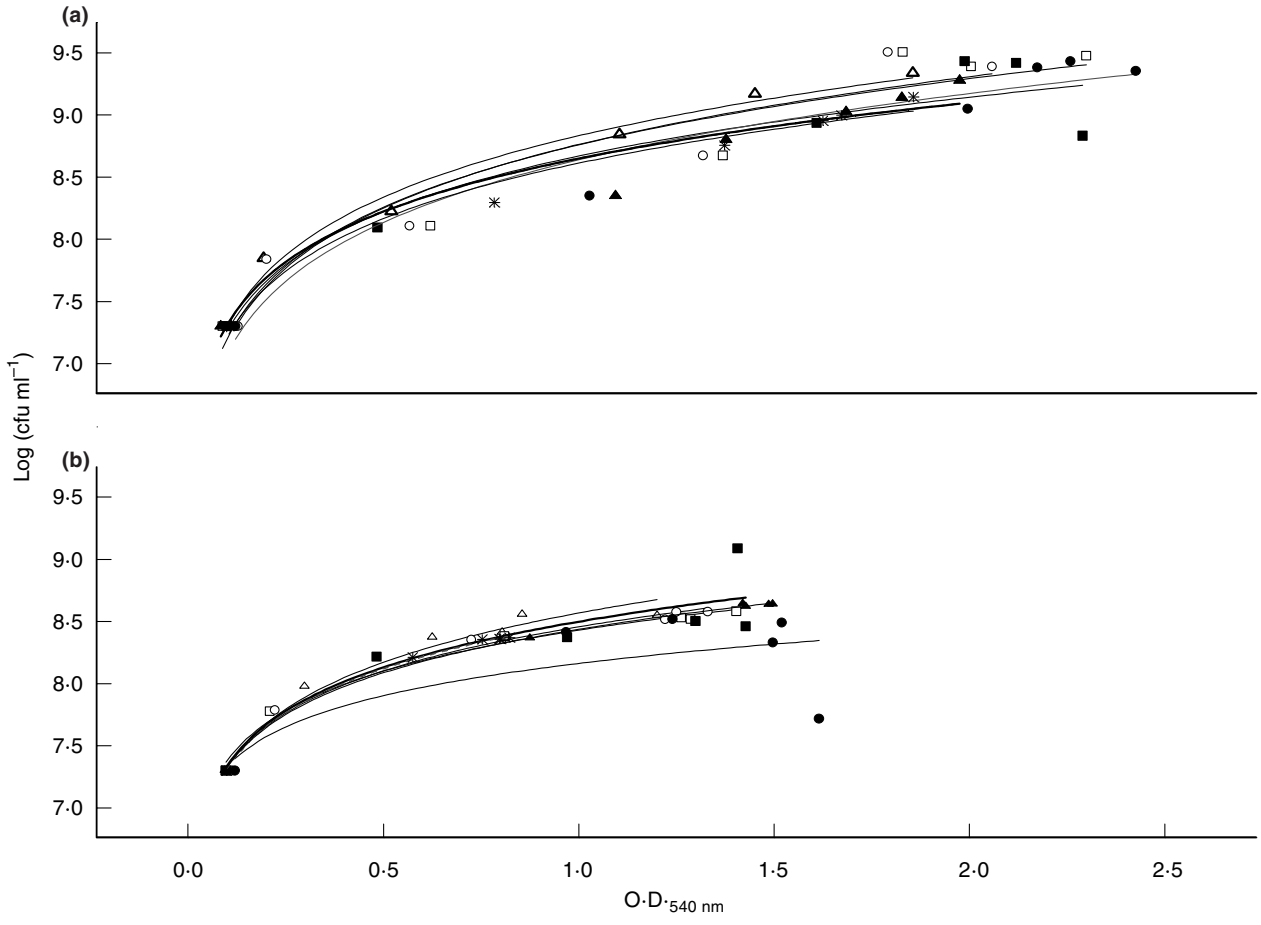

Fig. 2 Relationship between viable count data [Log (cfu ml^{-1})] and absorbance measurements (O.D.$_{540\ nm}$) during the growth of *Lactobacillus salivarius* CRL 1328 in MRS (a) and LAPTg (b) at 30°C [initial pH of 5·0 (○), 6·5 (□) and 8·0 (△)], 37°C [initial pH of 5·0 (■) and 6·5 (●)] and 44°C [initial pH of 5·0 (✳) and 8·0 (▲)]. The plots were fitted to the proposed exponential model (see Materials and methods)

actively dividing but before they enter the stationary phase. The bacteriocin activity in the stationary phase varied between the growth conditions. There was a strong decrease in bacteriocin concentration in LAPTg at 37°C with a pH of 6·5 and 8·0. For all other conditions, however, the activity decreased only slightly or remained stable.

The same general behaviour was observed when the growth data were expressed as Log cfu $(cfu_0)^{-1}$ and bacteriocin concentration was expressed as [AU (log cfu)$^{-1}$] (Fig. 4).

DISCUSSION

Probiotics have been defined as viable cultures of micro-organisms which can be administered to improve the ecological balance of the microflora of the host (Freter *et al.* 1983; Havenaar *et al.* 1992). In recent years, there has been a tendency to consume natural products which promote the health of man and animals. In this area of health improvement, probiotics and prebiotics (Gibson and Roberfroid 1995) have been increasingly recognized. Probiotics which contain bacteria of the genus *Lactobacillus* are widely used to prevent infections in a variety of animal host situations. One of the main concerns is the organism's ability to colonize the tract under study. Another approach which can be adopted is to administer, along with the live bacteria, metabolites or by-products that have an antagonistic effect on pathogens.

Bacteriocins or bacteriocin-like substances have been described as being produced by lactic acid bacteria isolated from a wide variety of environments, mainly from foods. Bacteriocin-producing bacteria as well as bacteriocins *per se* are of growing interest to scientists because they can be used as biological controls against pathogens in the manufacture of beverages and fermented products, primarily in the area of dairy products (Khedkar *et al.* 1990; Okereke and Montville 1991). Production of these types of substances probably occurs *in vivo* and has been suggested as one of the antagonistic mechanisms that bacteria of the normal flora

Table 2 Estimation of the growth parameters from viable count data by the Gompertz model with 95% confidence intervals

Growth medium	pH	T (°C)	A*	± 95%†	μ*	± 95%†	ε_g‡	± 95%†	N§
LAPTg	5·0	30	1·22	± 0·07	0·22	± 0·28	5·50	± 1·92	6
LAPTg	5·0	37	1·38	± 0·49	0·26	± 0·65	7·10	± 9·94	6
LAPTg	5·0	44	1·06	± 0·03	0·38	± 0·92	2·85	± 0·96	6
LAPTg	6·5	30	1·29	± 0·12	0·19	± 0·30	6·90	± 2·46	6
LAPTg	6·5	37	1·14	± 0·12	0·66	± 2·94	2·87	± 3·58	6
LAPTg	8·0	30	1·30	± 0·09	0·19	± 0·35	7·05	± 2·15	6
LAPTg	8·0	44	1·34	± 0·01	0·41	± 0·15	3·31	± 0·92	6
MRS	5·0	30	2·10	± 0·75	0·18	± 0·32	11·89	± 4·06	6
MRS	5·0	37	2·28	± 0·37	0·29	± 1·85	7·94	± 4·25	6
MRS	5·0	44	1·75	± 0·12	0·32	± 0·19	5·58	± 2·14	6
MRS	6·5	30	2·24	± 0·65	0·27	± 3·76	8·39	± 3·96	6
MRS	6·5	37	2·12	± 0·08	0·35	± 137	6·01	± 1·60	6
MRS	8·0	30	2·25	± 1·25	0·17	± 0·42	13·25	± 8·74	6
MRS	8·0	44	1·87	± 0·14	0·32	± 0·23	5·90	± 2·63	6

*Parameters of the Gompertz model: D_0, initial optical density; A, difference between the final and initial optical densities; μ, maximum specific growth rate (h^{-1}).
†95% confidence intervals.
‡Exponential phase time (h).
§Sample size.

utilize to prevent the colonization by undesirable micro-organisms (Hudault *et al*. 1997). Only a few bacteriocins of vaginal origin have been analysed (Klaenhammer 1988; Piard and Desmazeaud 1992; Jack *et al*. 1995; Ocaña *et al*. 1999d; Okkers *et al*. 1999). Until now, there have been no reports of bacteriocins produced *in vivo* by vaginal *Lactobacillus*, but some authors have raised the possibility of the use of bacteriocins to prevent urogenital infections (Reid *et al*. 1992; McGroarty 1993). Okkers *et al*. (1999) reported a bacteriocin-like peptide with anti-*Candida* activity which was produced by a strain of *Lact. pentosus* that had been isolated from the human vagina.

The use of potentially effective probiotic *Lact. salivarius* strains have been studied by several authors (Rada and Rychly 1995; Aiba *et al*. 1998). Hughes and Hillier (1990) reported its presence in 'Acidophillus powders' which were used for colonization of the vagina. In these studies and other reports (Huey *et al*. 1984), the host specificity of the strain used for probiotic purpose is noted. Only two reports have mentioned bacteriocin production in *Lact. salivarius* isolates obtained from Japanese grass leaves (Arihara *et al*. 1996) and from animal faecal samples (Robredo and Torres 2000). Although *Lact. salivarius* is not the predominant species isolated from the vaginal tract, there are several reports of its existence in this environment (Giori *et al*. 1987).

Because our research group is interested in the development of a probiotic to prevent urogenital infections, *Lactobacillus* strains with beneficial properties were selected, and we are attempting to determine the optimum conditions in order to obtain both the highest number of viable micro-organisms and the highest level of bacteriocin production. This paper describes an investigation of the conditions in which the highest number of cells are obtained, the growth parameters obtained, and bacteriocin production in different media, temperature and pH conditions. The study was performed and analysed through a factorial experimental design and the application of the Gompertz model to absorbance and viable count data. Determining bacterial growth rates in broth systems using turbidimetric methods provides a rapid and inexpensive means of obtaining numerical values which can be used for modelling. The results showed a correlation between both types of growth determinations, and although the growth parameters estimated from absorbance measurements were different in value from those which were estimated by the classical method (cfu ml^{-1}), they can reliably be obtained by applying both methodologies because they show the same trend of results. This agrees with the conclusions drawn by others (Dalgaard *et al*. 1994; Dalgaard and Koutsoumanis 2001). These authors have suggested that, although different numerical values for the generation times or growth rates may be obtained using O.D. determinations (indirect method) rather than viable counts, the absorbance measurements may be used for accurate and rapid estimation of such parameters.

Lactobacillus salivarius CRL 1328 grows to a greater extent in MRS than in LAPTg, as evidenced by the higher cell mass and growth rates obtained. The optimal conditions producing these rates were found to be a temperature of 37°C and an initial pH of 6·5. Despite the higher growth in

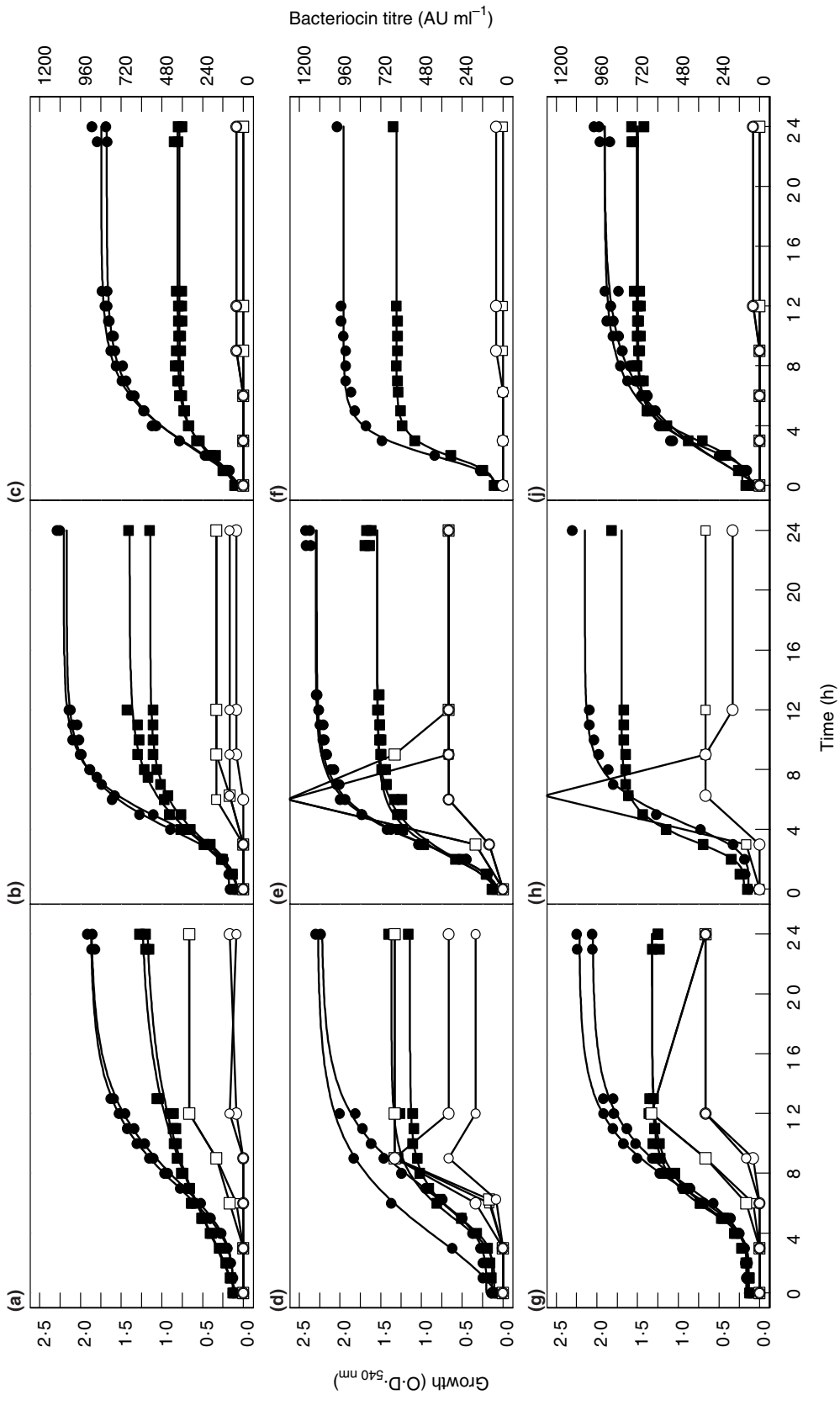

Fig. 3 Kinetics of growth and bacteriocin production of *Lactobacillus salivarius* CRL 1328 under different culture conditions. (a) 30°C, pH 5·0; (b) 37°C, pH 5·0; (c) 44°C, pH 5·0; (d) 30°C, pH 6·5; (e) 37°C, pH 6·5; (f) 44°C, pH 6·5; (g) 30°C, pH 8·0; (h) 37°C, pH 8·0; (i) 44°C, pH 8·0. O.D.$_{540\ nm}$ in LAPTg (■) and MRS (●). Bacteriocin production in LAPTg (□) and MRS (○). The lines represent the subsequent adjustment of the growth data by the application of the Gompertz model

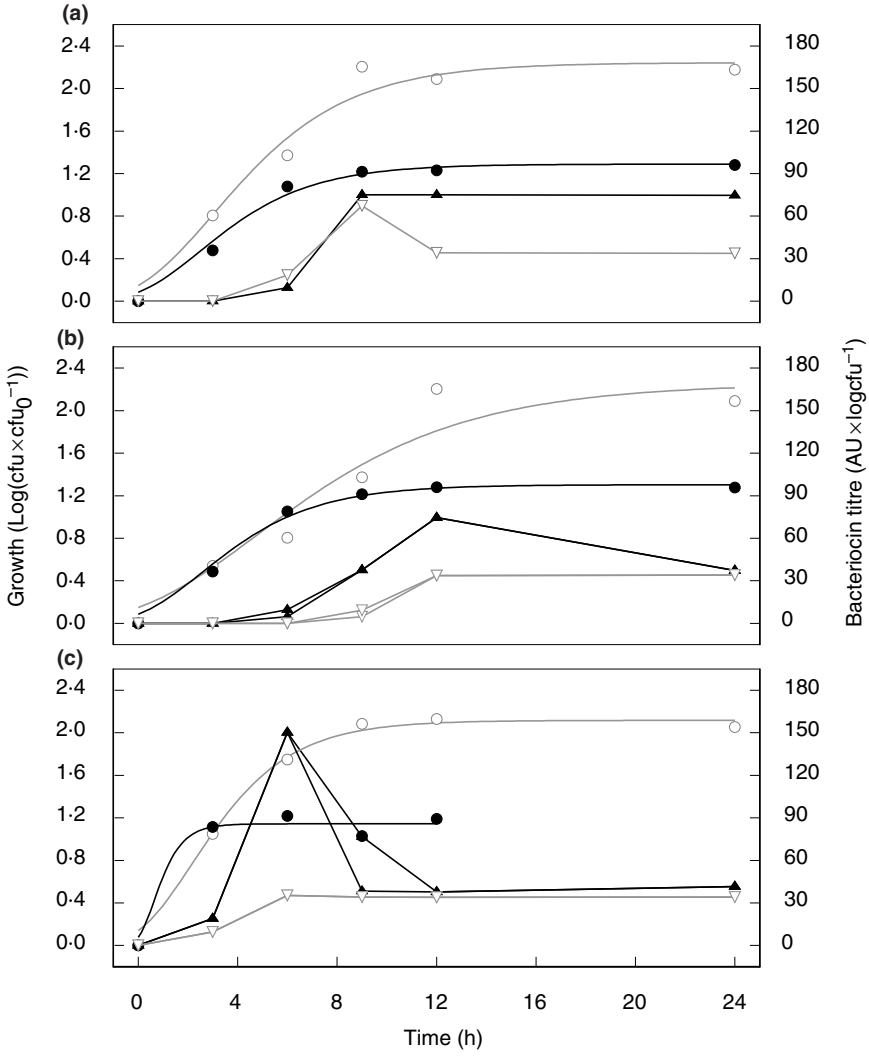

Fig. 4 Kinetics of growth and bacteriocin production of *Lactobacillus salivarius* CRL 1328 under the conditions (a) 30°C and pH 6·5, (b) 30°C and pH 8 and (c) 37°C and pH 6·5. Growth expressed as Log cfu $(cfu_0)^{-1}$ in LAPTg (●) and MRS (○). Bacteriocin concentration [AU (log cfu)$^{-1}$] in LAPTg (▲) and MRS (▽)

MRS, the final pH was similar to that in LAPTg because of the buffering capacity of medium. The optimum bacteriocin production was observed in LAPTg at 37°C with an initial pH of 6·5 and 8·0. There was no obvious correlation between the cell number of a culture and bacteriocin production. The same behaviour was described for *Leuconostoc carnosum* LA54A (Geisen *et al*. 1993), *Pediococcus acidilactici* H in MRS and TGE broths (Biswas *et al*. 1991), and in six producer strains of sakacina P in MRS and a completely defined medium (Møretrø *et al*. 2000). These results indicate that bacteriocin production is regulated by environmental conditions, and that low growth rates (like those obtained in LAPTg) increase production. *Lactobacillus salivarius* CRL 1328 did not produce bacteriocin at different combinations of pH at 44°C in either growth medium, despite the fact that growth was possible under those conditions. At high temperatures (44°C), the micro-organisms were unable to synthesize or secrete the bacteriocin, and degradation/inactivation could not be increased (Leroy and De Vuyst 1999). It appears that *Lact. salivarius* CRL 1328 makes better use of the necessary medium components at 37°C and an initial of pH 6·5–8·0, which were the optimal temperature and pH for growth and bacteriocin production. However, for other bacteriocins, the optimum pH and temperature for cell growth did not correspond with the pH or temperature requirements for maximum bacteriocin activity (Parente and Ricciardi 1994; Parente *et al*. 1994; Lejeune *et al*. 1998; Møretrø *et al*. 2000).

The heat-resistant and proteinaceous nature of the bacteriocin reported previously (Ocaña *et al*. 1999d), along with its production in a pH range between 6·5 and 8·0 in LAPTg (a relatively simple and economical medium) and the maintenance of the activity even within a large pH range, are characteristics that support the possibility of using it as an additive in probiotic products. The properties of bacteriocin-producing *Lact. salivarius* CRL 1328 will allow further study of its probable application as a probiotic to prevent urogenital infections.

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