Journal of Applied Microbiology 2005, **99,** 1383–1391 doi:10.1111/j.1365-2672.2005.02726.x

Effects of culture conditions on the growth and auto-aggregation ability of vaginal *Lactobacillus johnsonii* CRL 1294

M.S. Juárez Tomás[1], B. Wiese[2] and M.E. Nader-Macías[1]

[1]CERELA, Chacabuco 145, Tucumán, Argentina, and [2]Institut fuer Biometrie, Medizinische Hochschule Hannover, Hannover, Germany

2004/1490: received 22 December 2004, revised 8 April 2005 and accepted 9 June 2005

ABSTRACT

M.S. JUÁREZ TOMÁS, B. WIESE AND M.E. NADER-MACÍAS. 2005.

Aims: To evaluate the effects of different physico-chemical factors on the growth and auto-aggregating ability of vaginal *Lactobacillus johnsonii* CRL 1294.

Methods and Results: *L. johnsonii* CRL 1294 was cultivated in different culture media, initial pH and temperature of incubation. The growth parameters were estimated by the Gompertz model, being optimal (higher final biomass and growth rate, and shorter lag phase) at an initial pH of 6·5 and at a temperature of 37°C, both in LAPTg and MRS. The auto-aggregation ability, which was assessed by a model of exponential association, was evidenced in all the growth phases, being higher at pH 5 or 6·5.

Conclusions: The growth of *L. johnsonii* CRL 1294 was affected in different way by all the physico-chemical factors tested. However, the auto-aggregation ability increased mainly at low initial pH of growth media.

Significance and Impact of the Study: The auto-aggregation ability under different culture conditions of a vaginal *Lactobacillus* strain was systematically and statistically evaluated for the first time. The higher cellular aggregation evidenced at low pH could be a fundamental characteristic in the acidic vaginal environment to promote the protective role of lactobacilli.

Keywords: auto-aggregation ability, nonlinear regression, vaginal *Lactobacillus*.

INTRODUCTION

The auto-aggregation ability, or formation of multicellular clumps between micro-organisms of the same strain, is one of the proposed mechanisms to explain the protective role of lactobacilli in the human vagina (Boris *et al.* 1997). This property, related to the adhesion ability to epithelial vaginal cells, could cause the lactobacilli to produce a biofilm on the vaginal epithelia, which prevents the entry of pathogens (Lepargneur and Rousseau 2002). However, the coaggregation (aggregation between genetically different strains) between lactobacilli and pathogens could prevent the access of the latter to the tissues and their adhesion to epithelia, avoiding the establishment of a vaginal infection (Boris *et al.* 1997).

The phenomena of auto-aggregation and coaggregation are important to maintain the microbial populations on different mucosal surfaces (Jankovic *et al.* 2003). In order to know the interaction between micro-organisms of the normal microflora or with pathogenic micro-organisms, several authors studied the aggregation of micro-organisms isolated from different ecological niches, such as the oral cavity (Kolenbrander and London 1993; Kolenbrander 2000); the human (Boris *et al.* 1997; Kmet and Lucchini 1997) or bovine (Bujnakova and Kmet 2002) urogenital

Correspondence to: María Elena Nader-Macías, CERELA, Chacabuco 145, 4000 Tucumán, Argentina (e-mail: fnader@cerela.org.ar).

tract; the gastrointestinal tract of chickens (Vandevoorde *et al.* 1992; Bujnakova and Kmet 2002), pigs (Kmet *et al.* 1995; Roos *et al.* 1999) and humans (Cesena *et al.* 2001).

As demonstrated in studies by numerous groups (Drago *et al.* 1997; Mastromarino *et al.* 2002; Kos *et al.* 2003; Castagliuolo *et al.* 2005), microbial aggregation is a desirable property of probiotic bacteria. In those studies, auto-aggregation ability (Kos *et al.* 2003) and coaggregation ability with pathogenic *Escherichia coli* (Drago *et al.* 1997) of potentially probiotic gastrointestinal lactobacilli were determined. Mastromarino *et al.* (2002) studied the coaggregation of vaginal lactobacilli with *Candida albicans* and *Gardnerella vaginalis*. The experimental results reported by Castagliuolo *et al.* (2005) indicated for the first time that the aggregation property (both auto-aggregation and coaggregation) of *Lactobacillus crispatus* M247 is a relevant probiotic characteristic to exert protective effects on colitis in mice.

The biochemical characterization of the aggregation-promoting factors of lactobacilli from different origins was performed by other scientists, subjecting the microbial cells or the culture supernatant to different treatments (pH, temperatures, enzymes, etc.) (Reniero *et al.* 1992; Vandevoorde *et al.* 1992; Boris *et al.* 1997; Roos *et al.* 1999). However, the effect of several culture conditions on the auto-aggregation phenomenon was not deeply evaluated until now (Boris *et al.* 1997; Ventura *et al.* 2002). The potentially probiotic lactobacilli would be exposed to vaginal environment with fluctuating conditions, such as different pH values (pH 4·0–4·5 in normal women; pH 5·0–6·0 in women with bacterial vaginosis, pH close to 7·0 around the menstruation) (Larsen 1993). Therefore, the aggregating micro-organisms will survive and proliferate under conditions that promote the approach of partner cells (Rickard *et al.* 2003).

The auto-aggregation of different vaginal lactobacilli isolated from women of Tucumán, Argentina, was reported previously (Ocaña and Nader-Macías 2002). *Lactobacillus johnsonii* CRL 1294 (previously classified as *Lactobacillus acidophilus* CRL 1294 by using phenotypic tests) presents the following beneficial properties: a remarkable auto-aggregating pattern associated to a protein factor of the bacterial cellular surface; coaggregates with *Candida* sp., yeast isolated from a vaginal swab (Ocaña and Nader-Macías 2002); is a moderately H_2O_2 producer and reaches low pH values (indirectly related to lactic acid levels) after 12 h at 37°C in LAPTg broth (Raibaud *et al.* 1973; M.S. Juárez Tomás, D. Zonenschain, L. Morelli and M.E. Nader-Macías unpublished data).

Lactobacillus johnsonii CRL 1294 is a candidate micro-organism to be included in a vaginal probiotic formula, therefore an objective of the present paper was to determine the optimal growth conditions for biomass production. The growth parameters were estimated by the model of Gompertz, a mathematical function used to describe sigmoidal growth curves (Zwietering *et al.* 1990), and largely applied to the growth data (measured both as optical density or as log of the colony-forming units) of different micro-organisms (Dalgaard *et al.* 1994; McClure *et al.* 1994; Zaika *et al.* 1994; Dengremon and Membré 1995; Neumeyer *et al.* 1997; Dalgaard and Koutsoumanis 2001; Dufossé *et al.* 2001; Juárez Tomás *et al.* 2002a). However, in order to know the factors which affect the auto-aggregating ability of *L. johnsonii* CRL 1294, the extent of auto-aggregation under different culture conditions was assessed.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus johnsonii CRL 1294 (from the CERELA Culture Collection) was originally isolated from the human vagina (Ocaña *et al.* 1999), and genetically identified by applying the amplified ribosomal DNA restriction analysis. This micro-organism was stored in milk–yeast extract (13% nonfat milk, 0·5% yeast extract and 1% glucose) at −70°C.

Before the growth experiments *L. johnsonii* CRL 1294 was subcultivated in either MRS broth (Biokar Diagnostics, Beauvais, France) (De Man *et al.* 1960) or LAPTg broth (1·5% peptone, 1% tryptone, 1% glucose, 1% yeast extract, 0·1% Tween 80) (Raibaud *et al.* 1973) at a temperature of 37°C for 24 h, and subcultured twice at 37°C for 12 h in the same media. To prepare the inoculum for the growth experiments, the third culture was centrifuged (6000 *g*, 10 min), washed with saline solution (0·85% NaCl), and the pellet was resuspended in the same solution to a final optical density (OD) of 1·4 at 540 nm.

Lactobacillus johnsonii CRL 1294 was inoculated (2%, v/v) into 100 ml of each medium (LAPTg or MRS, initial pH adjusted to 5, 6·5 or 8), and then incubated in a water-batch without agitation (Masson Model; Vicking S.R.L., Buenos Aires, Argentina) at constant temperatures (30, 37 or 44°C).

Analytical procedures

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

The extent of auto-aggregation was assessed according to the technique described by Vandevoorde *et al.* (1992). Briefly, cultures of *L. johnsonii* CRL 1294, grown for different times and under various growth conditions, were centrifuged (6000 *g*, 15 min), washed with PBS buffer (g l^{-1}: NaCl, 8; KH_2PO_4, 0·34; K_2HPO_4, 1·21; pH 7), and resuspended in the same buffer to an OD of 0·6 ± 0·05 at 600 nm. Variation of OD at 600 nm of cellular suspensions was monitored every 1 h during 4 h, without agitation of

these suspensions during the spectrophotometric determinations. The percentage of auto-aggregation was calculated by the following expression:

$$\text{Auto-aggregation } (\%) = \frac{\text{OD}_{\text{initial}} - \text{OD}_{\text{final}}}{\text{OD}_{\text{initial}}} \times 100, \quad (1)$$

where $\text{OD}_{\text{initial}}$ is the OD at initial time ($t = 0$) of auto-aggregation assay, and OD_{final} is the OD at each time after beginning this assay ($t = 1$, 2, 3 or 4 h).

Because of the difficulty in determining the auto-aggregation quantitatively during the growth curves under 10 simultaneous cultures conditions of the experimental design, the influence of the growth phase on the auto-aggregating ability of *L. johnsonii* CRL 1294 was determined only at initial pH 6·5, and at 37°C (both in LAPTg and MRS). Samples were taken at 3, 6, 9, 12 and 24 h of culture. The effects of the growth media, pH and temperatures of incubation on this property were evaluated in all the culture conditions assayed, from the samples taken only at one time of growth: at 12 h for cultures performed at 37 or 44°C, and at 24 h for cultures at 30°C.

Experimental design

During the growth experiments, all the possible combinations of three factors at two levels were evaluated ($2^3 = 8$): growth media (LAPTg and MRS), temperatures of incubation (30 and 44°C) and initial pH (5 and 8). The condition 37°C and initial pH 6·5 was also assayed with each growth medium. The growth experiments were repeated at least twice in different days.

Estimation of the growth parameters

The bacterial growth parameters were estimated from the OD data by the modified four-parameter Gompertz model (Zwietering *et al.* 1990), according to the following function:

$$D_t = D_0 + A\exp\{-\exp[(\mu e/A)(\lambda - t) + 1]\}, \quad (2)$$

where D_t is the OD at time t, t the time of growth in hours, D_0 the OD at $t = 0$, A the difference between the final and initial ODs, μ the maximum specific growth rate (h^{-1}), λ the duration time of lag phase in hours and e the base of the Napierian logarithm (2.718281828). Each one of the four parameters included in the mathematical Gompertz model [see eqn (2)] has a particular meaning within the bacterial growth dynamic because it reflects the behaviour of the micro-organisms in each one of the assayed conditions (Zwietering *et al.* 1990; Juárez Tomás *et al.* 2002a).

The estimation of the growth parameters was performed using a constrained nonlinear regression with a sequential quadratic programming algorithm (Zwietering *et al.* 1990; Juárez Tomás *et al.* 2002b).

Estimation of the auto-aggregation parameters

The auto-aggregation percentages obtained for each sample of bacterial culture were plotted as a function of the time of duration of auto-aggregation assay (0–4 h). The resulting curves of experimental data were adjusted to a model of exponential association (a model from the Box Lucas family) that allows to estimate two auto-aggregating parameters, according to the following mathematical expression:

$$\text{Auto-aggregation } (\%) = A_a(1 - e^{-Ct}), \quad (3)$$

where A_a is the maximal auto-aggregation percentage, and C is a factor of increment or constant auto-aggregation rate (h^{-1}). From A_a and C, the maximum auto-aggregation rate (μ_a, expressed in h^{-1}) can be calculated as follows: $\mu_a = A_a C$ (data not shown). Therefore, the values of C are directly related to the values of maximum auto-aggregation rate.

Statistical analysis

To calculate the standard errors of the growth and the auto-aggregation parameters estimated, the bootstrapping technique was applied, using repeated samples from the original data set (Efron 1982; Huet *et al.* 1996; Juárez Tomás *et al.* 2002a). The number of bootstrap samples was chosen as 1000 and 100 for each growth curve and auto-aggregation curve respectively.

To determine the statistical significance of the effects of each growth medium (LAPTg and MRS) on the growth and auto-aggregation parameters, the differences between the parameters were included directly in the equation of the model, in order to estimate the confidence intervals for them (data not shown). An assumption was made that the effect of growth media on the growth or auto-aggregation parameters is statistically significant when the zero value is not included within the confidence interval estimated for the difference of each parameter between MRS and LAPTg.

To evaluate the multivariate effects of the different conditions (temperature, initial pH and culture media) on the growth and auto-aggregation parameters, the nonlinear mixed-effects model was applied using restricted maximum likelihood (Lindstrom and Bates 1990). SAS 8.2 (SAS Institute, Cary, NC, USA), SPSS 12 (SPSS Inc., Chicago, IL, USA) and S-Plus 6·0 (Insightful, Seattle, WA, USA) were used for the statistical analyses and to generate the corresponding graphics.

RESULTS

Optimal growth conditions

Figure 1 shows the $\text{OD}_{540\text{nm}}$ and pH values of *L. johnsonii* CRL 1294 grown in LAPTg and MRS broths under

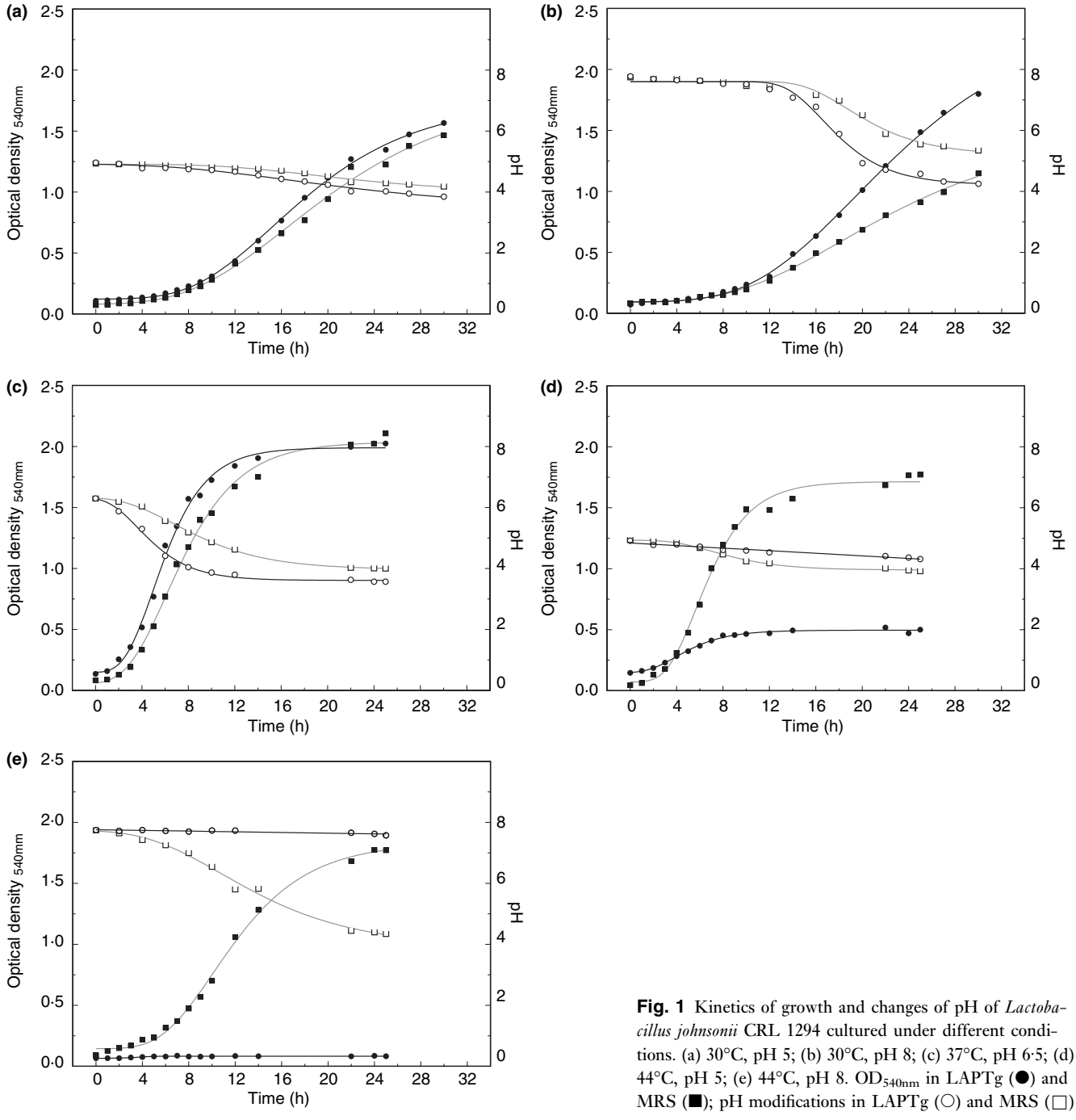

Fig. 1 Kinetics of growth and changes of pH of *Lactobacillus johnsonii* CRL 1294 cultured under different conditions. (a) 30°C, pH 5; (b) 30°C, pH 8; (c) 37°C, pH 6·5; (d) 44°C, pH 5; (e) 44°C, pH 8. OD_{540nm} in LAPTg (●) and MRS (■); pH modifications in LAPTg (○) and MRS (□)

different culture conditions of initial pH and temperature of incubation. The growth parameters obtained from absorbance data by applying the Gompertz model are summarized in Table 1.

The values of the growth parameters of *L. johnsonii* CRL 1294 changed according to the culture condition tested (Table 1). The increase of biomass (A) and the growth rate (μ) were significantly affected by the culture medium. Only at 44°C, the final biomass was significantly lower in LAPTg (pH 5 or 8) than in MRS. On the contrary, at 30 or 37°C and at a specific pH, the growth rate was higher in LAPTg than in MRS (Table 1 and Fig. 1).

The temperature and the initial pH of the growth media significantly affected the lag phase (λ). In LAPTg broth, an inverse relation between the lag phase duration and the temperature of incubation was observed. On the

Table 1 Estimation of the growth parameters of *Lactobacillus johnsonii* CRL 1294 obtained from optical density measurements using the Gompertz model

T (°C)	pH	Growth media	D_0*	±SE†	A*	±SE†	μ*	±SE†	λ*	±SE†	N‡
30	5	LAPTg	0·12	0·01	1·62	0·13	0·09	0·00	8·35	0·24	40
30	5	MRS	0·08	0·01	1·71	0·21	0·07	0·00	8·03	0·43	40
30	8	LAPTg	0·09	0·01	2·35	0·60	0·10	0·01	10·46	0·62	40
30	8	MRS	0·09	0·01	1·37	0·13	0·05	0·00	8·90	0·33	40
37	**6·5**	**LAPTg**	**0·15**	**0·04**	**1·84**	**0·05**	**0·27**	**0·02**	**2·48**	**0·30**	**32**
37	**6·5**	**MRS**	**0·05**	**0·02**	**1·98**	**0·06**	**0·21**	**0·01**	**2·65**	**0·26**	**32**
44	5	LAPTg	0·15	0·02	0·36	0·02	0·05	0·00	1·47	0·41	32
44	5	MRS	0·07	0·02	1·64	0·04	0·24	0·01	3·14	0·31	32
44	8	LAPTg	ND		ND		ND		ND		
44	8	MRS	0·14	0·03	1·69	0·22	0·14	0·01	5·63	0·41	32

ND: not determined; Bold letters: Optimal growth conditions.
*Parameters of the Gompertz model: D_0, initial optical density; A, increase of biomass; μ, maximum specific growth rate (h^{-1}); λ, lag phase (h).
†Standard errors.
‡Sample size.

contrary, in MRS the shorter lag phase was obtained at 37°C. In cultures performed in the same growth medium and at the same temperature, the lag phase was longer at pH 8.

Among all the conditions evaluated, the optimal growth conditions for *L. johnsonii* CRL 1294 were MRS and LAPTg broths at pH 6·5 and temperature of 37°C. Under these conditions, the higher final biomass (A values close to 2·0) and growth rate (0·21–0·27 h^{-1}) close to a shorter lag phase (2·5 h) were reached. In the majority of the culture conditions evaluated, the final pH was higher in LAPTg than MRS, except at 44°C because of the small growth of *L. johnsonii* in the first medium. The optimal conditions for a decrease of pH were coincident with the optimum growth conditions.

Auto-aggregation at different growth phases

Table 2 shows the auto-aggregation parameters estimated by the model of exponential association, from the experimental aggregation data obtained from cellular suspensions at different culture times in LAPTg and MRS (37°C, pH 6·5). The maximum auto-aggregation percentages (A_a) estimated from 3 to 24 h of culture in LAPTg or MRS broths presented no statistically significant differences, in spite of the higher dispersion of values observed in the last medium. However, both the growth time and the culture medium affected significantly the constant auto-aggregation rate (C).

Auto-aggregation under different growth conditions

According to the auto-aggregation results obtained at different growth times, the comparison of auto-aggregation ability of *L. johnsonii* CRL 1294 under a series of different culture conditions was performed at one point of the growth curve: 12 h for cultures at 37 and 44°C, and 24 h for cultures at 30°C were selected. Both times corresponded to the beginning of the stationary phase of growth at those temperatures. Figure 2 summarizes the experimental auto-aggregation data obtained after the growth in two growth media, at different pH and temperatures. Table 3 shows the auto-aggregation parameters estimated by the model of exponential association.

The growth media significantly affected the auto-aggregation ability mainly at 44°C and initial pH 5, obtaining the higher auto-aggregation percentage and constant auto-

Table 2 Estimation of the auto-aggregation parameters of *Lactobacillus johnsonii* CRL 1294 at different times of cultures in LAPTg and MRS (initial pH 6·5 and 37°C), using the model of exponential association

Growth media	Growth time (h)	A_a (%)*	±SE†	C*	±SE†
LAPTg	3	79·49	1·57	2·35	0·49
LAPTg	6	77·65	0·23	3·55	2·81
LAPTg	9	78·68	0·91	2·42	1·25
LAPTg	12	76·22	1·52	1·72	0·23
LAPTg	24	77·44	4·46	1·49	0·45
MRS	3	81·57	2·42	1·63	0·30
MRS	6	68·84	0·99	1·97	0·30
MRS	9	65·38	0·73	1·36	1·51
MRS	12	80·38	0·75	1·36	0·10
MRS	24	71·65	3·29	1·16	0·21

*Parameters of model of exponential association: A_a, maximal auto-aggregation percentage; C, factor of auto-aggregation increase (h^{-1}).
†Standard errors.

aggregation rate in LAPTg ($A_a = 88\%$ and $C = 2{\cdot}86\ h^{-1}$) (Fig. 2 and Table 3).

When the two broths were incubated at the same temperature, the higher auto-aggregation percentages were obtained at pH 5 (Fig. 2 and Table 3). The optimal auto-aggregation patterns were observed after the growth of *L. johnsonii* CRL 1294 in LAPTg, at pH 5 and 44°C. Similar results were also obtained both in LAPTg and MRS broth, with an initial pH of 6·5 and a temperature of 37°C, or at 30°C and pH 5·0.

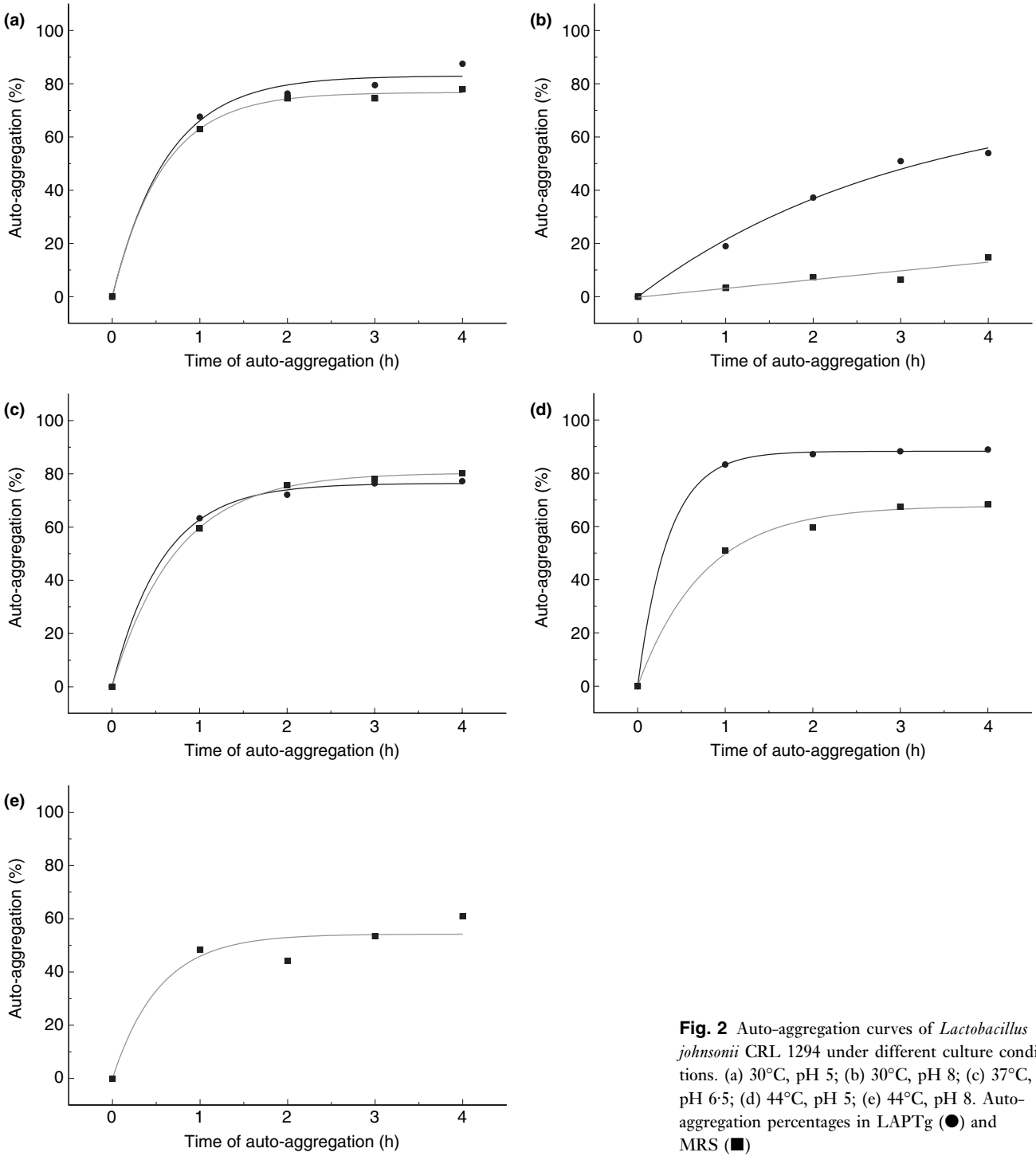

Fig. 2 Auto-aggregation curves of *Lactobacillus johnsonii* CRL 1294 under different culture conditions. (a) 30°C, pH 5; (b) 30°C, pH 8; (c) 37°C, pH 6·5; (d) 44°C, pH 5; (e) 44°C, pH 8. Auto-aggregation percentages in LAPTg (●) and MRS (■)

Table 3 Estimation of the auto-aggregation parameters of *Lactobacillus johnsonii* CRL 1294 under different culture conditions, by the model of exponential association

T (°C)	pH	Growth media	A_a (%)*	±SE†	*C**	±SE†
30	5	LAPTg	82·97	3·62	1·59	0·32
30	5	MRS	76·73	1·62	1·72	0·87
30	8	LAPTg	76·52	14·73	0·33	0·14
30	8	MRS	100·00	30·97	0·03	0·20
37	6·5	LAPTg	76·44	0·97	1·72	0·20
37	6·5	MRS	80·38	0·67	1·36	0·10
44	5	LAPTg	88·25	0·41	2·86	0·46
44	5	MRS	67·76	2·66	1·32	0·25
44	8	LAPTg	ND	ND	ND	ND
44	8	MRS	54·23	7·68	1·87	2·70

ND: not determined.

*Parameters of model of exponential association: A_a, maximal auto-aggregation percentage; *C*, factor of auto-aggregation increase (h^{-1}).

†Standard errors.

DISCUSSION

The cellular aggregation between micro-organisms of the same strain (auto-aggregation) or between genetically different strains (coaggregation) is of considerable importance in several ecological niches (Jankovic *et al.* 2003). The coaggregation phenomenon has been observed between bacteria from the human oral cavity and urogenital tract, the mammalian gut and potable water supply systems (Rickard *et al.* 2003). In the oral cavity, the coaggregation occurs between bacteria from dental plaque from healthy individuals as well as between bacteria that can cause disease (Kolenbrander 2000). However, different authors had suggested that the cellular aggregation could be positive to promote the colonization of beneficial micro-organisms, as suggested for lactobacilli in the gastrointestinal or vaginal tract (Vandevoorde *et al.* 1992; Kmet and Lucchini 1997; Cesena *et al.* 2001; Jankovic *et al.* 2003).

In this paper, the auto-aggregation ability of a vaginal *Lactobacillus* strain grown under several culture conditions was systematically and statistically evaluated for the first time. Our results indicate that the physico-chemical factors tested (growth media, initial pH and temperature) affected in a different way the growth and the auto-aggregation ability of *L. johnsonii* CRL 1294. The growth of this micro-organism was significantly higher at 37°C, initial pH 6·5, both in LAPTg and MRS. At a lower temperature (30°C), the lag phase was longer and the growth rate was lower in both growth media. However, at 44°C the growth was only evidenced in MRS. These results suggest that in extreme conditions, such as unfavourable high temperature, *L. johnsonii* could utilize the nutrients of MRS but not of LAPTg. The first medium is supplemented with salts and mineral ions stimulating the biomass production (De Man *et al.* 1960).

The auto-aggregation of *L. johnsonii* CRL 1294 was evidenced in all the growth phases, already from 3 h of culture in LAPTg or MRS (pH 6·5 and at 37°C), time corresponding to the end of the lag phase and the beginning of the exponential phase. Only in certain culture conditions the auto-aggregation percentages were significantly higher in LAPTg than in MRS (mainly at pH 5 and 44°C). Different to our results, Boris *et al.* (1997) reported that the diffusible aggregation-promoting factor of vaginal *Lactobacillus gasseri* 2459 [a diffusible peptide of 2 kDa, resistant to treatments at elevated temperatures (121°C) and active only at pH 3–4] was only detected in the stationary growth phase. The production of that factor was maximal in LAPTg compared with MRS, both in aerobiosis or in a 5% CO_2 atmosphere.

However, Ventura *et al.* (2002) reported that the genes responsible of the synthesis of an auto-aggregating factor of 32 kDa of *L. gasseri* 4B2 are expressed more remarkably in the exponential growth phase rather than in the stationary phase. In a former work, the aggregation-promoting factor of *L. gasseri* 4B2 was able to mediate a high frequency of conjugation between *Lactobacillus* strains that could allow the acquisition of new phenotypic characteristics (Reniero *et al.* 1992). Recent genetic and physiological studies suggested that this factor presents certain similarities with the proteins of S-layer, having an essential function to maintain the cellular shape (Ventura *et al.* 2002; Jankovic *et al.* 2003).

The results obtained in the present work indicate that the auto-aggregation ability of *L. johnsonii* CRL 1294 was expressed under different initial pH of growth media and incubation temperatures. The effect of pH on the auto-aggregation percentages was more significant than those of temperature, obtaining the higher values at pH 5 or 6·5. A higher temperature of incubation (44°C) did not inhibit the auto-aggregation ability of *L. johnsonii* CRL 1294. Similarly to our results, the expression of a protein of 56 kDa responsible for the auto-aggregation of *Lactobacillus reuteri* 1063 (micro-organism isolated from the intestine of pig) was evidenced after the growth at 46°C (Roos *et al.* 1999). However, the coaggregation among chicken lactobacilli, which was promoted by proteinaceous structures of cell surface, increased when monitored in buffer at pH 4 compared with buffers at higher pH (Vandevoorde *et al.* 1992).

The higher aggregation obtained at low pH could be explained by modifications of the bacterial surface charge, such as a decreasing of Coulomb repulsive forces, which could promote the approach of the cells (Vandevoorde *et al.* 1992). This fact could be relevant in the vaginal ecosystem, where a normal pH < 4·5 could favour the cellular interaction between lactobacilli to form a protective biofilm

on the vaginal mucosae. Later studies are required to elucidate this hypothesis, as the biofilm establishment and development is a complex process affected by multiple factors (Kjelleberg and Molin 2002; Rickard *et al.* 2003). The environmental conditions, the cellular functions and activities influenced by regulator systems operating under high-cell density conditions, as the quorum sensing signals, are included between those factors (Kjelleberg and Molin 2002; McNeill and Hamilton 2003).

ACKNOWLEDGEMENTS

This paper was supported by CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina) (PID 0359-1998-2000). Birgitt Wiese was supported by BMBF, Germany and CONICET (bilateral co-operation project ARG 99/025). We thank Elena Bru for their collaboration in the experimental design. The strain *L. johnsonii* CRL 1294 was licensed to ANIDRAL, an Italian company.

REFERENCES

Boris, S., Suárez, J.E. and Barbés, C. (1997) Characterization of the aggregation promoting factor from *Lactobacillus gasseri*, a vaginal isolate. *J Appl Microbiol* **83**, 413–420.

Bujnakova, D. and Kmet, V. (2002) Aggregation of animal lactobacilli with O157 enterohemorrhagic *Escherichia coli*. *J Vet Med* **49**, 152–154.

Castagliuolo, I., Galeazzi, F., Ferrari, S., Elli, M., Brun, P., Cavaggioni, A., Tormen, D., Sturniolo, G. *et al.* (2005) Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimental induced colitis in mice. *FEMS Immunol Med Microbiol* **43**, 197–204.

Cesena, C., Morelli, L., Alander, M., Siljander, T., Tuomola, E., Salminen, S., Mattila-Sandholm, T., Vilpponen-Salmela, T. *et al.* (2001) *Lactobacillus crispatus* and its nonaggregating mutant in human colonization trials. *J Dairy Sci* **84**, 1001–1010.

Dalgaard, P. and Koutsoumanis, K. (2001) Comparison of maximum specific growth rates and lag times estimated from absorbance and viable count data by different mathematical models. *J Microbiol Methods* **43**, 183–196.

Dalgaard, P., Ross, T., Kamperman, L., Neumeyer, K. and McMeekin, T.A. (1994) Estimation of bacterial growth rates from turbidimetric and viable count data. *Int J Food Microbiol* **23**, 391–404.

De Man, J.C., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of lactobacilli. *J Appl Bacteriol* **23**, 130–135.

Dengremon, E. and J. M. Membré (1995) Statistical approach for comparison of the growth rates of five strains of *Staphylococcus aureus*. *Appl Environ Microbiol* **61**, 4389–4395.

Drago, L., Gismondo, M.R., Lombardi, A., de Haen, C. and Gozzini, L. (1997) Inhibition of *in vitro* growth of enteropathogens by new *Lactobacillus* isolates of human intestinal origin. *FEMS Microbiol Lett* **153**, 455–463.

Dufossé, L., De La Broise, D. and Guerard, F. (2001) Evaluation of nitrogenous substrates such as peptones from fish: a new method based on Gompertz modeling of microbial growth. *Curr Microbiol* **42**, 32–38.

Efron, B. (1982) The bootstrap. In *The Jackknife, the Bootstrap and Other Resampling Plans*. pp. 27–36. Philadelphia, PA: SIAM (Society for Industrial and Applied Mathematics).

Huet, S., Bouvier, A., Gruet, M.A. and Jolivet, E. (1996) Accuracy of estimators, confidence intervals and tests. In *Statistical Tools for Nonlinear Regression* ed. S. Huet, A. Bouvier, N.A. Gruet and E. Jolivet. pp. 29–59. New York: Springer-Verlag.

Jankovic, I., Ventura, M., Meylan, V., Rouvet, M., Elli, M. and Zink, R. (2003) Contribution of aggregation-promoting factor to maintenance of cell shape in *Lactobacillus gasseri* 4B2. *J Bacteriol* **185**, 3288–3296.

Juárez Tomás, M.S., Bru, E., Wiese, B., de Ruiz Holgado, A.A.P. and Nader-Macías, M.E. (2002a). Influence of pH, temperature and culture media on the growth and bacteriocin production of vaginal *Lactobacillus salivarius* CRL 1328. *J Appl Microbiol* **93**, 714–724.

Juárez Tomás, M.S., Bru de Labanda, E., Ruiz Holgado, A.P. and Nader-Macías, M.E. (2002b) Estimation of vaginal probiotic lactobacilli growth parameters with the application of the Gompertz model. *Can J Microbiol* **48**, 82–92.

Kjelleberg, S. and Molin, S. (2002) Is there a role for quorum sensing signals in bacterial biofilms? *Curr Opin Microbiol* **5**, 254–258.

Kmet, V. and Lucchini, F. (1997) Aggregation-promoting factor in human vaginal *Lactobacillus* strains. *FEMS Immunol Med Microbiol* **19**, 111–114.

Kmet, V., Callegari, M.L., Bottazzi, V. and Morelli, L. (1995) Aggregation-promoting factor in pig intestinal *Lactobacillus* strain. *Lett Appl Microbiol* **21**, 351–353.

Kolenbrander, P.E. (2000) Oral microbial communities: biofilms, interactions, and genetic systems. *Annu Rev Microbiol* **54**, 413–437.

Kolenbrander, P.E. and London, J. (1993). Ecological significance of coaggreggation among oral bacteria. In *Advances in Microbial Ecology* ed. Marshal, K.C. pp. 183–217. New York: Plenum Press.

Kos, B., Suskovic, J., Vukovic, S., Simpraga, M., Frece, J. and Matosic, S. (2003) Adhesion and aggregation ability of probiotic strain *Lactobacillus acidophilus* M92. *J Appl Microbiol* **94**, 981–987.

Larsen, B. (1993) Vaginal flora in health and disease. *Clin Obstet Gynecol* **36**, 107–121.

Lepargneur, J.P. and Rousseau, V. (2002) Protective role of the Döderlein flora. *J Gynecol Obstet Biol Reprod* **31**, 485–494.

Lindstrom, M.J. and Bates, D.M. (1990) Nonlinear mixed effects models for repeated measures data. *Biometrics* **46**, 673–687.

Mastromarino, P., Brigidi, P., Macchia, S., Maggi, L., Pirovano, F., Trinchieri, V., Conte, U. and Mateuzzi, D. (2002) Characterization and selection of vaginal *Lactobacillus* for the preparation of vaginal tablets. *J Appl Microbiol* **93**, 884–893.

McClure, P. J., Cole, M. B. and Davies, K. W. 1994. An example of stages in the development of a predictive mathematical model for microbial growth: the effects of NaCl, pH and temperature on the growth of *Aeromonas hydrophila*. *Int J Food Microbiol* **23**, 359–375.

McNeill, K. and Hamilton, I.R. (2003) Acid response of biofilm cells of *Streptococcus mutans*. *FEMS Microbiol Lett* **221**, 25–30.

Neumeyer, K., Ross, T. and McMeekin, T. A. (1997) Development of a predictive model to describe the effects of temperature and water activity on the growth of spoilage pseudomonas. *Int J Food Microbiol* **38**, 45–54.

Ocaña, V. and Nader-Macías, M.E. (2002) Vaginal lactobacilli: self and coaggregation ability. *Br J Biomed Sci* **59**, 183–190.

Ocaña, V.S., Bru, E., de Ruiz Holgado, A.P. and Nader-Macías, M.E. (1999) Surface characteristics of lactobacilli isolated from human vagina. *J Gen Appl Microbiol* **45**, 203–212.

Raibaud, P., Galpin, J. V., Duclezeau, R., Mocquot, G. and Oliver, G. (1973) Le Genre Lactobacillus dans le tube digestif du rat. II. Caracters de souches heterofermentaires isolates de rats. "holo" et "gnotoxeniques". *Ann Microbiol (Annales de L'Institute Pasteur)* **124**, 2223–2235.

Reniero, R., Cocconcelli, P., Bottazzi, V. and Morelli, L. (1992) High frequency of conjugation in *Lactobacillus* mediated by an aggregation-promoting factor. *J Gen Microbiol* **138**, 763–768.

Rickard, A.H., Gilbert, P., High, N.J., Kolenbrander, P.E. and Handley, P.S. (2003) Bacterial coaggregation: an integral process in the development of multi-species biofilms. *Trends Microbiol* **11**, 94–100.

Roos, S., Lindgren, S. and Jonsson, H. (1999) Autoaggregation of *Lactobacillus reuteri* is mediated by a putative DEAD-box helicase. *Mol Microbiol* **32**, 427–436.

Vandevoorde, L., Christiaens, H. and Verstraete, W. (1992) Prevalence of coaggregation among chicken lactobacilli. *J Appl Bacteriol* **72**, 214–219.

Ventura, M., Jankovic, I., Walker, D.C., Pridmore, R.D. and Zink, R. (2002) Identification and characterization of novel surface proteins in *Lactobacillus johnsonii* and *Lactobacillus gasseri*. *Appl Environ Microbiol* **68**, 6172–6181.

Zaika, L.L., Moulden, E., Weimer, L., Phillips, J.G. and Buchanan, R.L. (1994) Model for the combined effects of temperature, initial pH, sodium chloride and sodium nitrite concentrations on anaerobic growth of *Shigella flexneri*. *Int J Food Microbiol* **23**, 345–358.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. and Van't Riet K. (1990) Modeling of the bacterial growth curve. *Appl Environ Microbiol* **56**, 1875–1881.