***Capsicum annuum* enhances L-lactate production by *Lactobacillus acidophilus*: Implication in curd formation**

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ABSTRACT

Lactobacillus acidophilus is commonly used lactic acid bacteria for producing fermented milk products. In general household practice, curdling is known to occur faster in the presence of red chili. Herein we analyzed the enhanced effect of red chili (*Capsicum annuum*) and its major component, capsaicin, on *Lactobacillus acidophilus* (ATCC 4356) in the production of L-lactate in de Man, Rogosa, and Sharpe medium at various temperatures (15, 20, 25, 30, and 37°C). The addition of red chili showed significant increase in the amount of L-lactate produced by *L. acidophilus* compared with the control at all temperatures. Similar results were observed with addition of capsaicin alone. This was accompanied by an increase in the consumption of D-glucose. Capsazepine, a known antagonist of capsaicin, inhibited the production of L-lactate by *L. acidophilus* in the presence of both capsaicin and red chili. Because no increase occurred in the growth of *L. acidophilus* in the presence of red chili, the enhanced production of L-lactate in the presence of red chili or capsaicin is due to increased metabolic activity.

Key words: *Lactobacillus acidophilus*, capsaicin, L-lactate, curdling

INTRODUCTION

Lactic acid bacteria are among the most important groups of acid-producing bacteria used in the food industry for the production of fermented food products such as cheese, yogurt, and sausages (Rhee et al., 2011). One of the most commonly used lactic acid bacteria is *Lactobacillus acidophilus*, which is preferably used as starter culture for fermentation along with *Streptococcus thermophilus* (Ahmed and Kanwal, 2004). Bacterial fermentation reduces the pH of milk by conversion of milk sugar lactose into lactic acid (Lee and Lucey, 2010). This acidification of milk (decrease in pH from 6.6 to 6) leads to the disruption of casein micelles, which reduces the net negative charge on casein and,

hence, the electrostatic repulsion. Further decrease in the pH to 5 or lower causes casein to coagulate, leading to curd formation (Lucey, 2004).

In a conventional household setup, curdling of milk may be induced by adding several different curdling agents or adding a small amount of preformed curd, with subsequent incubation at warm temperature. Red chili pepper is also used to induce curdling of milk, particularly in the colder regions or during winters or when starter cultures run out (Katz, 2011). Red chili is a common spice used in many food items and also in various pharmaceutical preparations (Barceloux, 2008). Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), one of the active ingredients in red chili, is known to have several medicinal properties, such as its potential to attenuate hypertension (Yang et al., 2010) and to suppress tumor growth by inducing apoptosis (Pramanik and Srivastava, 2012). The present study was designed to elucidate the effect of red chili and its main component capsaicin in the enhanced production of L-lactate by *L. acidophilus*.

MATERIALS AND METHODS

Chemicals, Biochemicals, and Reagents

Capsaicin, capsazepine, and glycerol were purchased from Sigma Chemical Co. (St. Louis, MO). Red chili and toned milk containing 3% fat and 8.5% SNF were purchased from a local retail outlet and Mother Dairy (New Delhi, India), respectively. de Man, Rogosa, and Sharpe (MRS) medium, customized Columbia colistin nalidixic acid (CNA) plates with 5% sheep blood and carbohydrate estimation kit were purchased from HiMedia Laboratories (Mumbai, India). D-Lactate and L-lactate colorimetric assay kits were procured from BioVision Inc. (Milpitas, CA). Absolute ethanol, methanol, and sodium hypochlorite used in the study were of Excelsa R grade and purchased from Qualigens Fine Chemicals Pvt. Ltd. (Mumbai, India).

Bacterial Strain Used, Its Revival, and Maintenance of Bacterial Culture

Lyophilized pellets of *L. acidophilus* were purchased from American Type Culture Collection (ATCC; Manas-

Received October 9, 2012.

Accepted March 20, 2013.

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sas, VA). The bacterial culture was revived following the ATCC guidelines. Briefly, a single bacterial pellet was suspended in 1 mL of sterile water. The bacterial suspension was evenly spread using sterile cotton swabs on nonselective MRS agar. The plates were incubated at 37°C for 48 h under aerobic conditions. The colonies obtained were later transferred to CNA agar containing 5% sheep blood and incubated anaerobically at 37°C for 48 h. An isolated colony was inoculated in MRS broth and grown at 37°C with constant shaking at 200 rpm, until the optical density at a wavelength of 600 nm (OD_{600}) reached about 0.7 (mid-logarithmic phase; Kerr and McHale, 2001). The bacterial culture, at this stage, was stored at 4°C until further use (Rooj et al., 2010). Pure culture was maintained by adding 30% glycerol with immediate freezing at -80°C, whereas growing culture was maintained by weekly transfer in MRS broth (de Man et al., 1960).

Chili Samples and Treatments

Capsicum annuum (red chili) was surface sterilized using 2% sodium hypochlorite solution. Chili was kept immersed in sodium hypochlorite solution for 2 to 3 min, rinsed thoroughly with sterile water 3 to 4 times, and dried in laminar flow hood. For experiments with aqueous extract of chili, the appropriate amount (400 mg) of chili was weighed, mashed, and mixed thoroughly with sterile water by vortexing. The mixture was centrifuged at $6,700 \times g$ for 10 min. The supernatant was collected and filter sterilized using a 0.2- μ m filter. Capsaicin and capsazepine were dissolved in absolute ethanol and methanol, respectively, for preparing stock solutions of 500 mg/mL. Toned milk with 3% fat and 8.5% SNF was used in all experiments. Milk was heated to 75°C and then cooled to 35°C before adding the starter culture. Bacterial culture ($OD_{600} = 0.6$ – 0.7) was added to milk in the ratio of 1:5 (vol/vol). In control samples, no chili was added, whereas test samples contained 400 mg of whole chili per 10 mL of milk. The samples were incubated at 5 temperatures (15, 20, 25, 30, and 37°C) without shaking and the time required for curdling was monitored. We noted the time of separation of whey from curd as the endpoint of curdling. Samples were analyzed in duplicate and triplicate.

Analysis

pH Measurement and Viable Cell Count. The pH of milk was measured at all 5 incubation temperatures (15, 20, 25, 30, and 37°C) at initial and final time points in the control and test samples using a digital pH meter. The final time point was considered as the time of complete curd formation. Direct microscopic

count was used to estimate the number of viable bacterial cells at initial and final time points. The samples were diluted 10-fold and mixed in a 1:1 ratio (vol/vol) with 0.04% trypan blue. Viable cells were counted using a hemocytometer under 40 $\times$ magnification of a compound microscope (Nikon Eclipse 80i; Nikon Corp., Tokyo, Japan).

Determination of Lactate Production and D-Glucose Consumption in MRS Broth. Initially, we determined the concentration of D-lactate and L-lactate produced by *L. acidophilus* at 37°C. A significant increase in the concentration of L-lactate was observed. However, no significant difference in the concentration of D-lactate was produced at the initial and final time points. The results were in accordance with a previous study, which reported that L-lactate is produced in higher amounts by *L. acidophilus* in MRS medium compared with D-lactate (Juárez Tomás et al., 2003). Therefore, the amount of L-lactate produced by *L. acidophilus* was determined because we investigated the effects of red chili and its components on bacterial culture grown in MRS media.

Different test samples containing *L. acidophilus*, with any of the following combinations of crude red chili, filtered aqueous extract of red chili, pure capsaicin, pure capsazepine, filtered aqueous chili extract with capsazepine, and capsaicin with capsazepine, were analyzed for L-lactate production. Each sample had bacterial culture and MRS broth in the ratio of 1:5 (vol/vol). In samples with capsaicin and capsazepine, both components were added to an equal working concentration of 5 mg/mL. The effect of capsazepine was examined by adding it before addition of chili extract or capsaicin and incubated for 20 to 30 min at the given temperature. All incubations with bacteria, after initial mixing, were carried out without shaking at all temperatures to emulate the incubation of milk inoculated with bacterial culture for curd formation.

Control and test samples were assayed to determine the amount of L-lactate produced using a colorimetric L-lactate assay kit (BioVision Inc.) as per the manufacturer's instructions. Briefly, 50 μ L of test sample at 1:10 dilution in MRS broth was added to a microtiter plate. An equal volume of reaction mixture (46 μ L of lactate assay buffer, 2 μ L of lactate probe, and 2 μ L of enzyme mix) was added to each sample. The samples were incubated in the dark for 30 min at room temperature and absorbance was measured at 570 nm. Sterile MRS broth was used as blank. L(+)-Lactate standards (BioVision Inc.), in the range of 2 to 10 nmol, were used to generate the standard curve.

The same control and test samples were also analyzed for D-glucose consumption using a carbohydrate estimation kit (HiMedia Laboratories, Mumbai, India) as per

the manufacturer's instructions. Briefly, 200 μ L of test sample at 1:100 dilution in sterile water was added to the reaction mixture containing 500 μ L each of 3,5-dinitrosalicylic acid (DNSA) reagent and 40% potassium sodium tartrate. The absorbance was measured at 540 nm. Glucose standards (HiMedia Laboratories), diluted to concentrations of 0.2 to 1.0 mg/mL, were used to generate the standard curve. The differences in absorption of initial and final values were taken for calculating the amount of D-glucose consumed in the samples. The amount of L-lactate produced and D-glucose consumed were reported in picomoles per microliter and nanomoles per microliter of the samples, respectively.

Statistical Analysis

Experiments were performed either in duplicate or triplicate. Data are reported as mean $\pm$ 1 standard deviation. In Figure 1, the error bars represent 95% confidence intervals, which can be used for direct comparative assessment. Welch's Student's *t*-test was performed to test the statistical significance in all the experiments, except in the last case, where the Tukey honestly significant difference test was applied, to determine the statistical significance of differences in D-glucose consumption and L-lactate production in different treatments.

RESULTS AND DISCUSSION

The time required for curdling was monitored over a variable range of temperatures, starting from 15 to 37°C. At 15, 20, and 25°C, both the control and the sample containing red chili took a long time to curdle, which was about 10 to 11 h. The time taken for curdling in samples with red chili was about an hour less compared with the control. At 30 and 37°C, the time taken for curdling, without chili, was 9 and 6.5 h, respectively. Addition of red chili decreased the curdling time to 8 and 6 h at 30 and 37°C, respectively. The results showed that the time required for curdling was decreased in the presence of red chili and this effect was observed even at lower incubation temperatures. These results indicate that addition of red chili had a greater influence on the time taken for curdling at lower temperatures compared with that at 37°C. It is possible that because the optimal growth temperature range for *L. acidophilus* is 35 to 38°C (Zamfir and Grosu-Tudor, 2009), the difference in time taken for curdling at 37°C in the presence versus absence of chili is small compared with the bacteria grown at lower temperatures.

Because curdling is dependent on the reduction in the pH of milk (Lee and Lucey, 2010), we measured the pH of samples before and after curdling. Incubation

of milk with bacterial culture at higher temperatures showed a sharp decrease in the pH of milk compared with incubation at lower temperatures in both controls and test samples with red chili (Figure 1). The initial average pH of milk was 6.22. We observed a significant difference in pH in the presence of chili compared with controls at all temperatures ($P < 0.01$). The significant difference was also apparent in the 95% confidence intervals at all temperatures between control and test samples.

Because the increased acidity of milk during curd formation is the result of lactic acid produced by bacterial fermentation (Lee and Lucey, 2010), we determined the amount of lactic acid produced in our samples. Under normal growth conditions, *L. acidophilus* is known to produce D-lactate. However, when *L. acidophilus* is grown in MRS medium, L-lactate is mainly produced (Juárez Tomás et al., 2003). To confirm, we assayed the samples in initial experiments for the amount of D-lactate and L-lactate produced at 37°C. No detectable amount of D-lactate was produced in the control samples, whereas a very small amount of D-lactate was produced in test samples with red chili (Appendix Table A1).

We therefore initially determined the amount of L-lactate produced in control and test samples using red chili at different incubation temperatures (15, 20, 25, 30, and 37°C) in MRS broth. The results are shown in Figure 2. We observed a significant increase in the amount of L-lactate produced in test samples contain-

Figure 1. pH measurement in control (without red chili; ○) and test (with red chili; △) samples after complete curdling of milk over a variable temperature range of 15, 20, 25, 30, and 37°C. Each point is the mean of triplicate analyses. The bars at each point represent 95% CI.

Figure 2. Amount of L-lactate produced in control (without red chili) and test (with red chili) samples, determined in de Man, Rogosa, and Sharpe (MRS) broth at different temperatures. The error bars at each point represent 1 SD. We observed the final time point at 11 h for 15°C, 10 h for 20°C, 9 h for 25°C, 8 h for 30°C, and 6 h for 37°C.

ing red chili compared with controls at all incubation temperatures ($P < 0.01$). However, the enhanced effect of red chili on L-lactate production was more profound at incubation temperatures of 25°C and above.

The increase in the amount of L-lactate produced by *L. acidophilus* could be attributed to 1 of the 2 hypotheses: either addition of chili enhances the growth of bacteria, resulting in an increased number of viable bacterial cells, with consequent higher production of L-lactate or red chili influences bacterial metabolism, leading to increased release of L-lactate. In an attempt to examine the former hypothesis, we directly counted the number of viable bacterial cells in control and test samples at initial and final time points incubated at 25, 30, and 37°C for the same time periods, using trypan blue stained samples under the microscope. The viable

cells were visible as transparent rods, whereas the dead cells absorbed trypan blue stain and appeared blue in color. The results are shown in Table 1. Except in the case of the control at 37°C, we did not observe a significant increase in the number of viable bacteria either in the presence or in the absence of chili at different temperatures. This is perhaps due to the lower-than-optimum growth temperature for *L. acidophilus*. Even the increased growth observed at 37°C in one set of experiments was not consistent. This trend may be attributed to the fact that in our experiments, incubation with bacteria after initial mixing was carried out without shaking at all temperatures to emulate the standing incubation of milk inoculated with bacterial culture for curd formation. Furthermore, we did not observe any morphological difference in bacterial cell shapes between control and test sets of samples. Therefore, the increased production of L-lactate by *L. acidophilus* does not appear to be due to increased growth rate in the presence of red chili.

Next, we investigated the effect filtered aqueous extract of red chili on L-lactate production by *L. acidophilus* at temperatures of 25, 30, and 37°C, as the enhancement effect was prominently observable from 25°C onwards. The results are shown in Appendix Figure A1. The enhancement effect was consistent, as observed using red chili itself. Subsequently, we chose to investigate the enhancement effects of filtered aqueous extract of red chili and capsaicin on L-lactate production at 30°C, which is 5°C less than the optimal growth temperature range in the present context.

Capsaicin, an active ingredient of red chili (responsible for its pungency), is a naturally occurring vanilloid. Its consumption is linked with increased metabolic rate and core body temperature (Kobayashi et al., 1998; Mahmmoud, 2008). Capsazepine is a synthetic analog of capsaicin, which is known to competitively inhibit the effects of capsaicin (Szallasi and Blumberg, 1999). Therefore, we next investigated the effect of capsaicin

Table 1. Number of viable cells counted in control (without chili) and test samples (with red chili) at initial and final time points in de Man, Rogosa, and Sharpe (MRS) broth, incubated at different temperatures¹

Temperature (°C)	Sample	No. of viable cells ($\times 10^6$ cells/mL)					
		Experiment 1 (in duplicate)			Experiment 2 (in triplicate)		
		Initial	Final	P-value	Initial	Final	P-value
25	Control	1.9, 2.1	1.2, 1.8	0.33	2.5 $\pm$ 0.35	2.6 $\pm$ 0.31	0.36
	Test	1.7, 1.9	1, 1.1	0.04	2.4 $\pm$ 0.25	2.3 $\pm$ 0.2	0.26
30	Control	2.3, 2.1	1.8, 2	0.17	2.4 $\pm$ 0.27	2.2 $\pm$ 0.15	0.2
	Test	1.9, 2.1	1.8, 1.5	0.21	2.3 $\pm$ 0.2	1.9 $\pm$ 0.2	0.04
37	Control	2.3, 2.1	2.5, 2.3	0.3	2.5 $\pm$ 0.35	3.7 $\pm$ 0.25	<0.01
	Test	1.8, 2.4	1.2, 1.4	0.2	2.2 $\pm$ 0.15	2.1 $\pm$ 0.42	0.45

¹In experiment 1, the duplicate values are represented, whereas in experiment 2, the values represent mean $\pm$ 1 SD of triplicate experiments.

on L-lactate production by *L. acidophilus* and whether the enhanced effects of capsaicin could be annulled by capsazepine. The amount of L-lactate produced in the control and test samples using filtered aqueous extract of red chili, pure capsaicin, pure capsazepine, a combination of filtered aqueous extract of red chili and capsazepine, and a combination of capsaicin and capsazepine were determined. The results of these experiments are shown in Figure 3.

We observed enhancement of L-lactate production by *L. acidophilus* in the presence of capsaicin, which was similar to that observed from filtered aqueous extract of red chili (Figure 3). This enhancement effect was inhibited in the presence of capsazepine, where the amount of L-lactate produced was lower than that of the control. The effect of capsazepine alone on L-lactate production was closer to that of the control, showing slightly enhanced effect but far less than either filtered aqueous extract of red chili or capsaicin. The data for L-lactate production and D-glucose consumption were analyzed for statistically significant differences using the Tukey honestly significant difference test. A significant increase was observed in the amount of L-lactate produced in both cases in the presence of filtered aqueous extract of red chili and capsaicin compared with the control (adjusted $P < 0.01$; Appendix Table A2). Also, in the presence of capsazepine alone, a significant amount of L-lactate was produced (adjusted $P < 0.01$), indicating that capsazepine alone does not in-

hibit L-lactate production by *L. acidophilus*. In parallel, consumption of D-glucose was significantly enhanced in both cases in the presence of filtered aqueous extract of red chili and capsaicin (Figure 3). L-Lactate production was severely inhibited in samples containing capsazepine and either capsaicin or filtered aqueous extract of red chili (adjusted $P < 0.01$; Appendix Table A2). However, D-glucose was still consumed, similar to consumption in the control, indicating the ongoing metabolic activity. It has been reported that capsaicin activates ion channels of plasma membrane and capsazepine is a competitive antagonist (Bevan et al., 1992). These observations suggest that the enhancement of L-lactate production due to capsaicin is likely at the level of L-lactate export from the bacteria.

Therefore, it is evident that, in the presence of filtered aqueous extract of red chili or its active ingredient capsaicin, the enhancement in L-lactate production is due to the enhanced metabolic activity of *L. acidophilus*. We were intrigued by the effect of capsaicin on enhancement of L-lactate production with accompanied increase in D-glucose consumption by *L. acidophilus*, considering that capsaicin is insoluble in water. However, the effect we observed was reproducible and this may perhaps be due to the interaction of surface receptors of *L. acidophilus* with capsaicin. In the presence of red chili, capsaicin may be present as an emulsion allowing favorable interaction with bacterial cell membranes.

Capsaicin has also been shown to stimulate uncoupled ATP hydrolysis, which is likely of significance to modulate thermogenesis (Mahmoud, 2008). Many studies in the medical literature have linked the consumption of chili-enriched meals with increased energy expenditure and oxidation of fats (Diepvens et al., 2007). In humans, capsaicin and its nonpungent analogs have been shown to act through surface transient receptor potential vanilloid type 1 (TrpV1) receptors, which consequently affect the basal metabolic rate by increasing energy metabolism (Inoue et al., 2007). Our efforts in identifying homologs of TRPV1 receptors in *L. acidophilus* through BLAST (Altschul et al., 1990) searches revealed ABC transporters with weak similarity. In the future, the detailed mechanistic action of capsaicin on *L. acidophilus* receptors remains to be unraveled.

Figure 3. Amount of D-glucose consumed (nmol/μL) and L-lactate produced (pmol/μL) at 30°C in control and test samples with different treatments, determined in de Man, Rogosa, and Sharpe (MRS) broth. The histograms (y-axis scale on the left) show the amount of D-glucose consumed. The curves (y-axis scale on the right) indicate the amount of L-lactate produced. The error bars at each point represent 1 SD. C = control without chili; Chl = aqueous extract of red chili alone; Cap = capsaicin alone; Czp = capsazepine alone; Chl+Czp = combination of aqueous extract of red chili with capsazepine; Cap+Czp = combination of capsaicin with capsazepine. The final time point was observed at 8 h.

ACKNOWLEDGMENTS

The authors acknowledge the Council of Scientific and Industrial Research (CSIR, New Delhi, India) for funding. We also thank Rakesh Sharma (CSIR) for the stimulating discussions, Bhanwar Lal Puniya (CSIR) for helping with the statistical analysis, and A. Ram-

achandran (International Institute of Health Management Research, Delhi, India) for sharing the details of household practice of enhanced curdling using red chili during winters. The present study was financially supported by the Council for Scientific and Industrial Research (project code EXP 004). The funding source had no role in the study design; collection, analysis, and interpretation of data; writing of the manuscript; or the decision to submit the article for publication.

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APPENDIX

Table A1. Amount of D-lactate produced in control and test samples (mean $\pm$ SD), using red chili, when grown in de Man, Rogosa, and Sharpe (MRS) broth at 37°C

Sample	D-Lactate produced during incubation (pmol/ μ L)
Control (without red chili)	0
Test (with red chili)	14 $\pm$ 2.24

Figure A1. Amount of L-lactate produced in control (without filtered aqueous extract of red chili) and test (with filtered aqueous extract of red chili) samples during incubation at different temperatures determined in de Man, Rogosa, and Sharpe broth. The error bars at each point represent 1 SD. We observed the final time point at 9 h for 25°C, 8 h for 30°C and 6 h for 37°C.

Table A2. The results of the Tukey honestly significant difference test used for the amount of L-lactate produced and D-glucose consumed at 30°C in different test samples as compared with control

Sample	Adjusted <i>P</i> -value	
	L-Lactate produced	D-Glucose consumed
Aqueous extract of red chili (Chl)	<0.01	<0.01
Capsaicin (Cap)	<0.01	<0.01
Capsazepine (Czp)	<0.01	0.21
Aqueous extract of red chili + capsazepine (Chl + Czp)	<0.01	0.999
Capsaicin + capsazepine (Cap + Czp)	<0.01	0.054