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Conditions required for citrate utilization during growth of *Lactobacillus casei* ATCC334 in chemically defined medium and Cheddar Cheese extract

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Abstract Conditions required for citrate utilization by *Lactobacillus casei* ATCC334 were identified. Citrate was utilized by this microorganism in modified Chemically Defined Media (mCDM) as an energy source, solely in the presence of limiting concentrations of galactose. The presence of glucose inhibited citrate utilization by this microorganism even when added in limiting concentrations. Utilization of citrate occurred at pH 6.0 ± 0.2 and 5.1 ± 0.2. Together these observations suggest that citrate is an energy source for *L. casei* in ripening cheese only when the residual levels of carbohydrate post-fermentation are limiting (< 2.5 mM), and lactose or glucose are absent. However, citrate utilization by this organism was observed in Cheddar cheese extract (CCE), which naturally contains both lactose and galactose, at the beginning of late-logarithmic phase and regardless of the galactose concentration present in the media.

Keywords Citrate utilization · Energy sources · Lactobacilli · Cheese

Abbreviations

CCE	Cheddar cheese extract
LAB	Lactic acid bacteria
NSLAB	Non-starter lactic acid bacteria
mCDM	Modified chemically defined media
SLAB	Starter lactic acid bacteria

Introduction

Lactic acid bacteria (LAB) metabolize lactose primarily to lactic acid, which is essential for the manufacture of most cheese varieties. Selected strains of LAB referred to as starter culture (SLAB) are intentionally added to milk during cheese manufacture. In contrast, nonstarter LAB (NSLAB), present in cheese as the result of contamination, increase from approximately 10^2 CFU/g to greater than 10^8 CFU/g within the first 2–3 months of ripening (Naylor and Sharpe 1958; Perry and Sharpe 1960; Peterson and Marshall 1990). Growth of NSLAB in cheese is important

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due to their contribution to cheese flavor development (Kieronczyk et al. 2001; Kristoffersen 1956; Law et al. 1976; Reiter et al. 1967). The SLAB cultures are thought to deplete residual lactose in the curd during the first 2 weeks of ripening. Consequently, the NSLAB are excluded from utilizing lactose as an energy source. Potential energy sources for NSLAB in ripening cheese include nucleic acids derived from the autolysis of SLAB (Thomas 1986), amino acids (Kieronczyk et al. 2001; Kristoffersen 1956), sugars liberated from glycoproteins or glycolipids present in milk (Fox et al. 1998; Williams and Banks 1997), and citrate (Branen and Keenan 1977; Campbell and Gunsalus 1944; Fryer 1970; Fryer et al. 1970; Starrenburg and Hugenholtz 1991).

Although citrate is present in young Cheddar cheese at concentrations up to 9 mmol/kg (Faulkner et al. 1986), its utilization as an energy source by NSLAB, more specifically lactobacilli, is restricted to those cheeses in which the SLAB are unable to metabolize the organic acid. Citrate utilization by NSLAB has been the subject of numerous publications. These research efforts have focused on determining the influence of factors such as pH, the presence of monovalent and divalent cations, the presence and absence of a carbohydrate source, the carbohydrate type and the presence of mixed cultures on citrate utilization by NSLAB (Branen and Keenan 1977; Campbell and Gunsalus 1944; Fryer 1970; Fryer et al. 1970; Perry and Sharpe 1960; Starrenburg and Hugenholtz 1991; Williams et al. 2000). Most of these studies were conducted in complex media such as milk or MRS. The utilization of undefined media and variability among the different experimental designs has resulted in contradictory observations.

Publications from the 1940s to 1980s suggest that citrate is an energy source for lactobacilli. Growth stimulation of lactobacilli was reported upon addition of citric acid to tryptone broth (Campbell and Gunsalus 1944) and other complex culture media (Branen and Keenan 1977; Thomas 1986). Under conditions of pH and carbohydrate concentration simulating those in cheese, some *Lactobacillus casei* strains utilized between 64 and 94% of the citrate present (Fryer et al. 1970). The presence of excess fermentable carbohydrate was identified as a regulator of the extent of citrate utilization by some *L. casei* strains (Fryer 1970). However, more recent publications suggest that citrate may not be an energy source for lactobacilli. It has been shown that citrate was not used as an energy source by either *L. casei* ATCC393 or *Lactobacillus plantarum* 1919 (Palles et al. 1998). Co-metabolism of citrate and glucose or galactose did not influence growth rate or yield under the conditions studied (Palles et al. 1998). Additionally, Williams et al. (2000) observed that citrate was not catabolized by a group of 60 NSLAB, representing five strains of *L. casei*, 10 additional lactobacilli species and two *Weisella* species.

A putative citrate cluster and citric acid cycle have been identified in the NSLAB, *L. casei* ATCC334 genome sequence (Díaz-Muñiz et al. 2006; Klaenhammer et al. 2002). The putative citrate cluster contains the coding genes for citrate lyase, a membrane bound oxaloacetate decarboxylase, and a regulatory gene. The genome sequence analysis suggested that the *L. casei* oxaloacetate decarboxylase is a three-subunit protein complex, comprised of a dicarboxylic acid decarboxylase (OadA), a membrane-spanning carboxybiotin-carrier protein decarboxylase (OadB), and a biotin-binding protein (OadC) (Dudley and Steele 2000). The OadABC complex seems to belong to a family of membrane-bound decarboxylases found in fermentative bacteria (Dimroth and Schink 1998), which use the energy released during decarboxylation to generate a membrane chemical gradient. Identification of a putative citric acid cycle for *L. casei* was possible due to the presence of the putative fumarase and malic enzyme coding genes.

The purpose of this research was to characterize citrate utilization by growing cells of *L. casei* ATCC334 under conditions similar to those found in ripening cheese (4% sodium chloride, limiting carbohydrate and pH 5.1) and in Cheddar Cheese extract (CCE). It was found that the concentrations of some carbohydrates act as triggers for citrate utilization by this NSLAB in mCDM and CCE. Additionally, it was observed that under appropriate culture conditions, *L. casei* ATCC334 is able to utilize citrate as an energy

source. Such information is essential for a better understanding of energy sources available for growth of NSLAB in ripening cheese.

Materials and methods

Bacterial strain

L. casei ATCC334 was obtained from the American Type Culture Collection (Rockville, MD). *L. casei* ATCC334 is a cheese isolate and the proposed neotype for the *L. casei* cluster (Dellaglio et al. 2002; Dicks et al. 1996). This culture was maintained at –80°C in lactobacilli MRS broth (Difco Laboratories, Detroit, MI) containing 10% glycerol. *L. casei* was transferred to MRS broth from frozen stocks. Incubation was performed in a water bath at 37°C for 16–18 h. Cultures were transferred twice in MRS broth prior to inoculation of the experimental media. Growth was observed in either mCDM or CCE.

mCDM and growth conditions

mCDM was prepared as described by Christensen and Steele (2003) with the following modifications: (1) all individual amino acids were replaced by 10.0 g of Bacto Casitone (Becton Dickinson and Company, Sparks, Maryland), and (2) the medium was supplemented with 2.5 mg pyridoxamine dihydrochloride (Sigma-Aldrich Co., St. Louis, MO) and 1 mg resazurin sodium salt (Sigma-Aldrich Co.) per liter. Substitution of individual amino acids by Bacto Casitone was essential for growth of *L. casei* ATCC334 in mCDM. The mCDM carbohydrate, sodium chloride, diammonium citrate content and pH were modified as indicated in Table 1. Growth data were collected from 1.5 l batch cultures. Cells were grown under a nitrogen headspace in a 2 l fermentor vessel (New Brunswick Scientific, Co., Edison, NJ) at 37°C. The agitation rate was 40 rpm. Growth was initiated with a 0.1% washed inoculum. Bacterial cells were washed twice using Dulbecco's Phosphate Buffered Saline (Invitrogen Corporation, Carlsbad, CA) at 5930 × *g* for 10 min at 25°C using an induction drive centrifuge (Beckman Coulter) prior to their transfer to the experimental media. pH was kept constant using a VirTis Omni Culture Fermenter (The VirTis Co., Inc., Gardiner, NY) connected to a Chemtrix Type 45AR pH Controller (Whatman Lab Sales, Inc., Hillsboro, OR), a MasterFlex Speed Controller (Barnant Co., Barrington, IL) and a MasterFlex Pump Model No. 701400 (Cole-Parmer Instruments Co., Chicago, IL). pH was automatically adjusted with 1 M sodium hydroxide solution unless otherwise indicated. pH was monitored using an autoclavable pH electrode with refillable liquid electrolyte (Mettler-Toledo, Columbus, OH). All the experiments conducted in mCDM were performed in duplicate.

CCE and growth conditions

CCE was obtained from 3 kg of a 2.5-months-old Cheddar cheese and prepared as described by Díaz-Muñiz et al. 2006. In brief, the Cheddar cheese block was cut, lyophilized, and powdered. The lyophilized, and powdered Cheddar cheese was extracted using a ratio of four parts of water to six parts of cheese. Fine solids were removed from the CCE by centrifugation and the supernatants were filter sterilized. The filtered sterilized CCE was stored at –20°C until needed. The CCE was filtered sterilized once more after thawing, and prior to its utilization. The CCE naturally contained 0.58 ± 0.01 mM lactose, 1.40 ± 0.09 mM galactose and 2.3 ± 0.13 mM citrate.

A 300 ml aliquot of the CCE was supplemented with 12.7 mM diammonium citrate to obtain a final concentration of 15 mM citrate prior to inoculation and the pH readjusted to 5.1 ± 0.1 with 0.1 M sodium hydroxide. Additionally, a 150 ml sub-sample of the CCE containing 15 mM citrate was supplemented with 47.6 ± 0.9 mM galactose to achieve a final concentration of hexose equivalents of 50 mM. The 150 ml supplemented CCE aliquots were individually filter-sterilized using 0.2 μm cellulose nitrate filter units (Nalge Nunc International, Rochester, NY). Four 36 ml aliquots of the filter-sterilized CCEs supplemented with either diammonium citrate or diammonium citrate and

Table 1 Summary of growth conditions tested: modified Chemically Defined Media (mCDM) was supplemented, and pH adjusted and maintained as described

Observations	Carbohydrate concentrations (mM)	Diammonium citrate concentrations (mM)	Sodium chloride concentrations (mM)	pH
Effect of carbohydrate concentration	2.4 ± 0.3 galactose	0.0 ± 0.1	3.4 ± 0.1	5.1 ± 0.2
	2.4 ± 0.2 galactose	14.9 ± 0.6	3.4 ± 0.1	5.1 ± 0.2
	50 ± 1.0 galactose	14.5 ± 0.9	3.4 ± 0.1	5.1 ± 0.2
Effect of carbohydrate	1.3 ± 0.2 lactose	14.8 ± 0.2	3.4 ± 0.1	5.1 ± 0.2
	2.4 ± 0.2 galactose	14.9 ± 0.6	3.4 ± 0.1	5.1 ± 0.2
	2.4 ± 0.1 glucose	14.5 ± 0.7	3.4 ± 0.1	5.1 ± 0.2
Effect of pH	2.3 ± 0.1 galactose	14.2 ± 0.1	3.4 ± 0.1	6.0 ± 0.2
	2.4 ± 0.2 galactose	14.9 ± 0.6	3.4 ± 0.1	5.1 ± 0.2
	2.5 ± 0.1 galactose	14.9 ± 0.4	3.4 ± 0.1	4.5 ± 0.2
*Effect of sodium chloride	2.4 ± 0.2 galactose	14.9 ± 0.6	3.4 ± 0.1	5.1 ± 0.2
	2.5 ± 0.1 galactose	0.1 ± 0.1	340 ± 1.0	5.1 ± 0.2
	2.5 ± 0.1 galactose	15.2 ± 0.6	340 ± 1.0	5.1 ± 0.2

*pH of the mCDM containing 340 mM (2%) sodium chloride was automatically adjusted with a 10 mM ammonium hydroxide solution instead of 1 M sodium hydroxide solution

galactose were aseptically transferred to 40 ml GC vials, fitted with teflon septa and screw caps (I-Chem Brand Products, Rockwood, TN) and incubated in an anaerobic jar for 48 h prior to inoculation. Growth was initiated with a 0.1% washed inocula. Bacterial cells were washed twice using Dulbecco's Phosphate Buffered Saline (Invitrogen Corporation, Carlsbad, CA) at 5930 × *g* for 10 min at 25°C prior to their transfer to the experimental media. Cultures were incubated at 37°C in anaerobic jars. All the experiments performed in CCE were done in triplicate.

Growth assessment

Growth in mCDM was monitored by OD_{600} using a DU-65 Spectrophotometer (Beckman Coulter, Fullerton, CA). Culture samples in which the OD_{600} was higher than 0.3 were diluted with fresh media before measurement. Growth in CCE was monitored by plating serial dilutions on lactobacilli MRS Agar (Becton Dickinson and Company). Dilutions for plating were made in Dulbecco's Phosphate Buffered Saline (Invitrogen Corporation). Plates were incubated at 37°C under anaerobic conditions.

Citrate and carbohydrate detection

Citrate, lactose and galactose disappearance were monitored enzymatically (R-Biopharm, Inc. Marshall, MI). Samples were collected over time and spun at 16,000 × *g* for 5 min. mCDM and CCE supernatants were stored at –20°C until analyzed. Substrates were determined from duplicate analyses of duplicate samples.

Results and discussion

Effect of carbohydrate concentration

The present study demonstrated that a growth-limiting level of galactose was required for citrate to serve as an energy source during growth for *L. casei* ATCC334 in mCDM. Citrate utilization by *L. casei* in the presence of excess galactose was only observed after the culture reached late stationary phase and the initial carbohydrate concentration was depleted to less than ca. 40% of the initial amount (Fig. 1c). However, citrate enhanced *L. casei* growth in the presence of limiting galactose (Fig. 1a, b). Final optical density of the *L. casei* culture in mCDM containing limiting galactose and citrate (Fig. 1b) was 0.8-fold higher than the final optical density observed for the culture in mCDM containing limiting galactose alone (Fig. 1a). Initial citrate concentrations decreased to less than 0.5 mM over time (Fig. 1b). These results are in agreement with Fryer's (1970) previous observation regarding the influence of fermentable carbohydrate on citrate

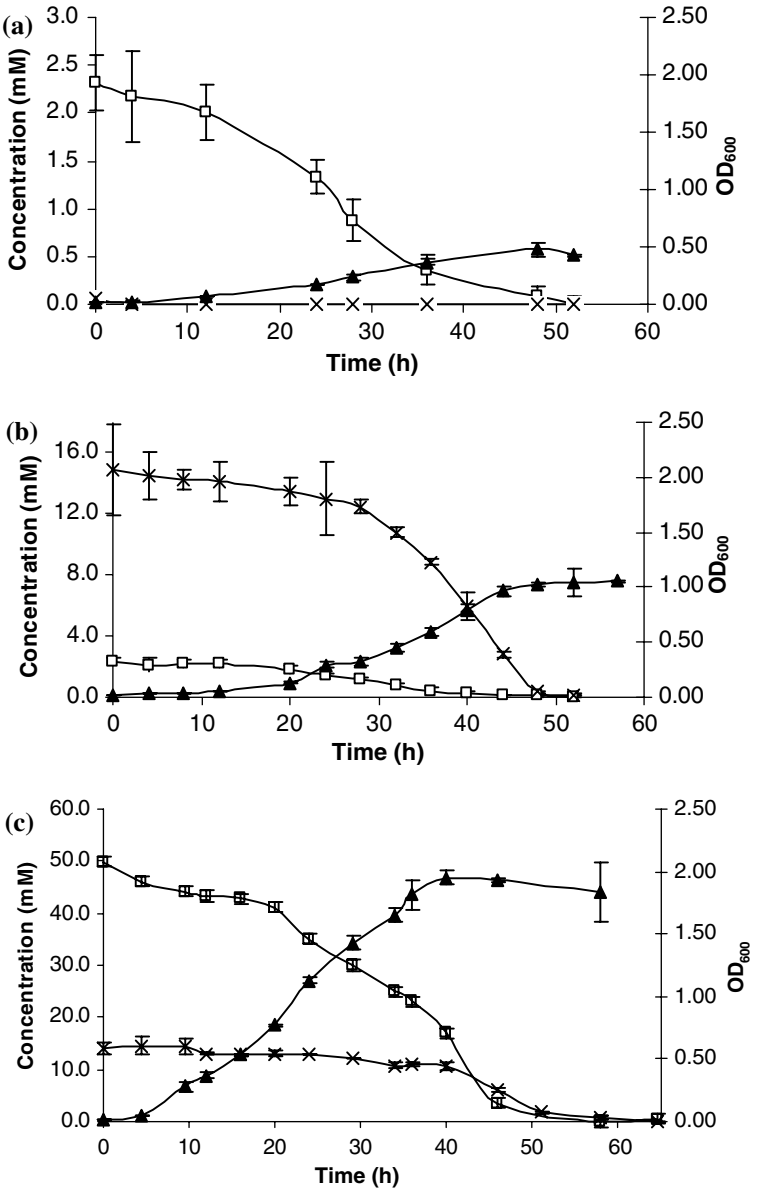

Fig. 1 Citrate utilization by *Lactobacillus casei* ATCC334 in the presence of excess and limiting carbohydrate: Growth (▲), and galactose (□), and citrate (×) utilization in modified-Chemically Defined Media containing (**a**) 2.4 ± 0.3 mM galactose (limiting), (**b**) 2.4 ± 0.2 mM galactose (limiting) and 14.9 ± 0.6 diammonium citrate, and (**c**) 50 ± 0.6 galactose (excess) and 14.5 ± 0.9 mM diammonium citrate at pH 5.1 ± 0.2

utilization by some strains of *L. casei*. Additionally, this result is consistent with the previously observed growth stimulation by citrate in the absence of excess carbohydrate by Thomas (1986) and lack of *L. casei* growth stimulation by citrate in the presence of 20 mM galactose and 20 mM glucose by Palles et al. (1998). Growth of NSLAB in ripening cheese occurs under conditions of severe carbohydrate limitation. Therefore, carbohydrate content should not restrict citrate utilization by *L. casei* in ripening cheese.

Effect of carbohydrates on citrate utilization by *L. casei*

The most abundant carbohydrate source for NSLAB in ripening cheese is lactose. Therefore, *L. casei* growth in mCDM containing limiting (1.25 mM) lactose and 15 mM diammonium citrate was examined. Additionally, the effect of the lactose components, glucose and galactose, on citrate utilization by this microorganism was studied. As mentioned above, citrate enhanced

L. casei growth and was utilized in the presence of limiting galactose (Figs. 1b and 2c). However, *L. casei* growth was not enhanced by citrate in the presence of either limiting glucose or lactose (Fig. 2a, b). Moreover, citrate utilization in the presence of these two carbohydrates was not observed (Fig. 2a, b). These results indicated that citrate utilization by *L. casei* is inhibited in the presence of glucose and lactose. Such observations suggest that the expression of the *L. casei* citrate utilization genes are subject to catabolite repression by glucose or an inhibitory effect of glucose metabolism intermediaries on the regulation of enzymes involve in citrate utilization. Palles et al. (1998) reported that citrate utilization by *L. casei* growing cells was greater in cells co-metabolizing galactose than in those co-metabolizing glucose or lactose. Inhibition of citrate utilization by glucose was also observed by De Figueroa et al. (1996) in *Lactobacillus rhamnosus*. Specifically, they observed catabolite repression by glucose on citrate permease expression. Although, De Figueroa et al. (1996) observed citrate utilization in the presence of glucose after media supplementation with cAMP, the same outcome was not obtained with growing

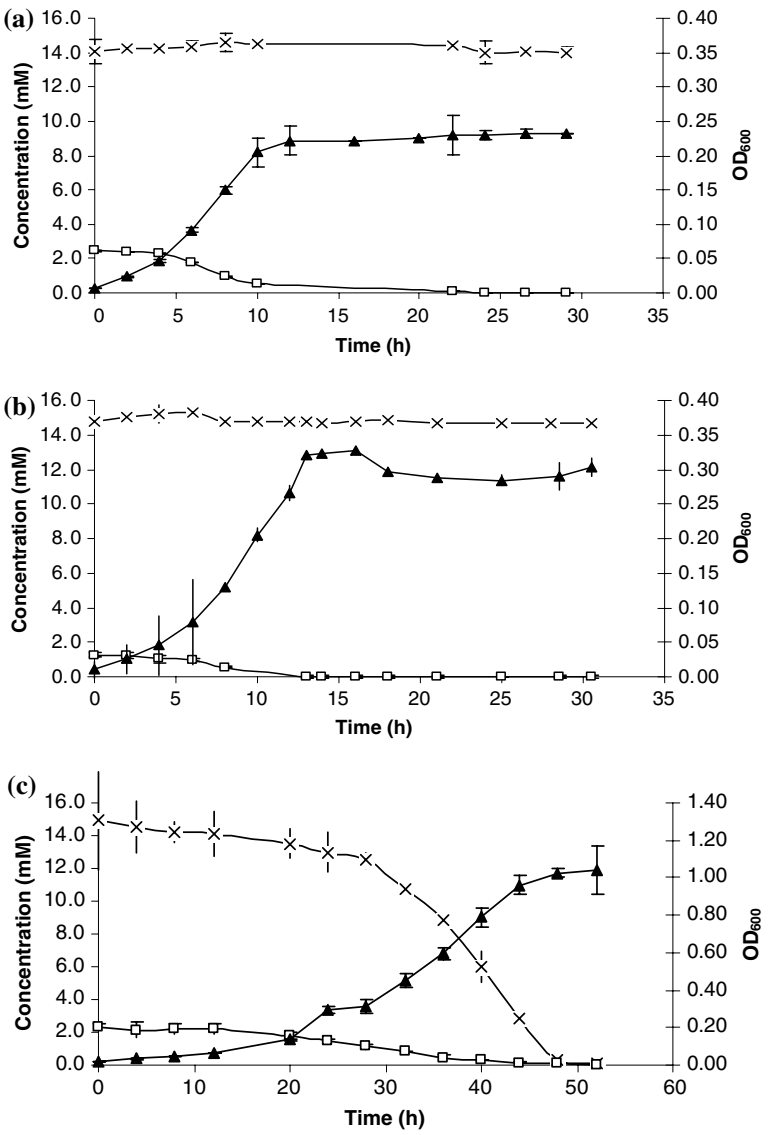

Fig. 2 Citrate utilization by *Lactobacillus casei* ATCC334 in the presence of glucose, lactose, and galactose: Growth (▲), and carbohydrate (□) and citrate (×) in modified-Chemically Defined Media containing (**a**) limiting glucose (2.4 ± 0.1 mM), (**b**) limiting lactose (1.3 ± 0.2 mM) or (**c**) limiting galactose (2.4 ± 0.2 mM) at pH 5.1 ± 0.2

cells of *L. casei* ATCC334 in the studies reported herein (data not shown).

A potential role of slow growth rate in citrate utilization by *L. casei* is also suggested by the data herein. Growth rate of *L. casei* in mCDM containing either lactose or glucose and citrate was 0.15 and 0.25-fold faster than growth rate in the presence of galactose and citrate, respectively (Table 2). As mentioned above, citrate utilization was not observed in the presence of lactose or glucose.

Effect of pH on citrate utilization by *L. casei*

A pH effect on citrate utilization by *Lactococcus* and *Leuconostoc* spp. has been previously observed (Starrenburg and Hugenholtz 1991). Therefore, *L. casei* growth was followed in mCDM containing limiting (2.5 mM) galactose and 15 mM diammonium citrate at different pH values (6.0 ± 0.2, 5.1 ± 0.2, and 4.5 ± 0.2). Citrate enhanced *L. casei* growth yield in the presence of limiting galactose at pH 6.0 ± 0.2 (Fig. 3a), and pH 5.1 ± 0.2 (Fig. 3b) but not at pH 4.5 ± 0.2 (Fig. 3c). The cultures at pH 6.0 ± 0.2 and 5.1 ± 0.2, reached stationary phase once both the initial galactose and citrate concentrations were depleted (Fig. 3). *L. casei* ATCC334 was able to utilize citrate at a higher rate at pH 6.0 ± 0.2 than at pH 5.1 ± 0.2 whereas citrate was not utilized to a significant extent by *L. casei* ATCC334 at pH 4.5 ± 0.2 (Fig. 3). Although, galactose was utilized to a significant extent at pH 4.5 ± 0.2 in mCDM, the specific growth rate of this microorganism was lower than at pH 6.0 ± 0.2 and 5.1 ± 0.2 (Table 2). Together these observations suggest that *L. casei* ATCC334 cells were stressed at pH 4.5 and therefore significant citrate utilization was prevented. However, the possibility of a pH effect on citrate utilization by *L. casei* ATCC334 cannot be excluded. At pH 4.5 ± 0.2 the ionic species balance is favored towards monovalent citrate, which may not be compatible with the citrate transport system(s) present in this bacterium. The fact, that *L. casei* ATCC334 was able to utilize citrate at pH 5.1 ± 0.2 suggests that the pH of ripening cheese does not restrict citrate utilization as an energy source by this microorganism.

Effect of 2% sodium chloride on citrate utilization by *L. casei*

Ripening cheese is characterized by a high salt content. Therefore, we tested the ability of *L. casei* ATCC334 to utilize citrate in the presence of excess sodium chloride. Sodium chloride is present in Cheddar cheese moisture at levels up to 4.5% (Morris et al. 1988; Nair et al. 2004). The growth rate of *L. casei* in mCDM supplemented with 4% sodium chloride decreased eight-folds compared to growth in the presence of 0.04% sodium chloride (data not shown).

Table 2 Growth rates for *Lactobacillus casei* ATCC334 in modified-Chemically Defined Media and Cheddar cheese extract under the conditions tested

Observations	Conditions tested	Growth rates
Effect of carbohydrate concentration	Limiting galactose/w/o citrate	0.09/h
	Limiting galactose/with citrate	0.09/h
	Excess galactose/with citrate	0.18/h
Effect of carbohydrate	1.3 ± 0.2 lactose/with citrate	0.24/h
	2.4 ± 0.2 galactose/with citrate	0.09/h
	2.4 ± 0.1 glucose/with citrate	0.34/h
Effect of pH	Limiting galactose/with citrate/pH 6.0 ± 0.2	0.14/h
	Limiting galactose/with citrate/pH 5.1 ± 0.2	0.09/h
	Limiting galactose/with citrate/pH 4.5 ± 0.2	0.04/h
Effect of sodium chloride	Limiting galactose/with citrate/3.4 ± 0.1 mM sodium chloride	0.09/h
	Limiting galactose/w/o citrate/340 ± 0.9 mM sodium chloride	0.03/h
	Limiting galactose/with citrate/340 ± 0.1 Mm sodium chloride	0.03/h
CCE	Limiting carbohydrate/with citrate	0.07/h
	Excess carbohydrate/with citrate	0.11/h

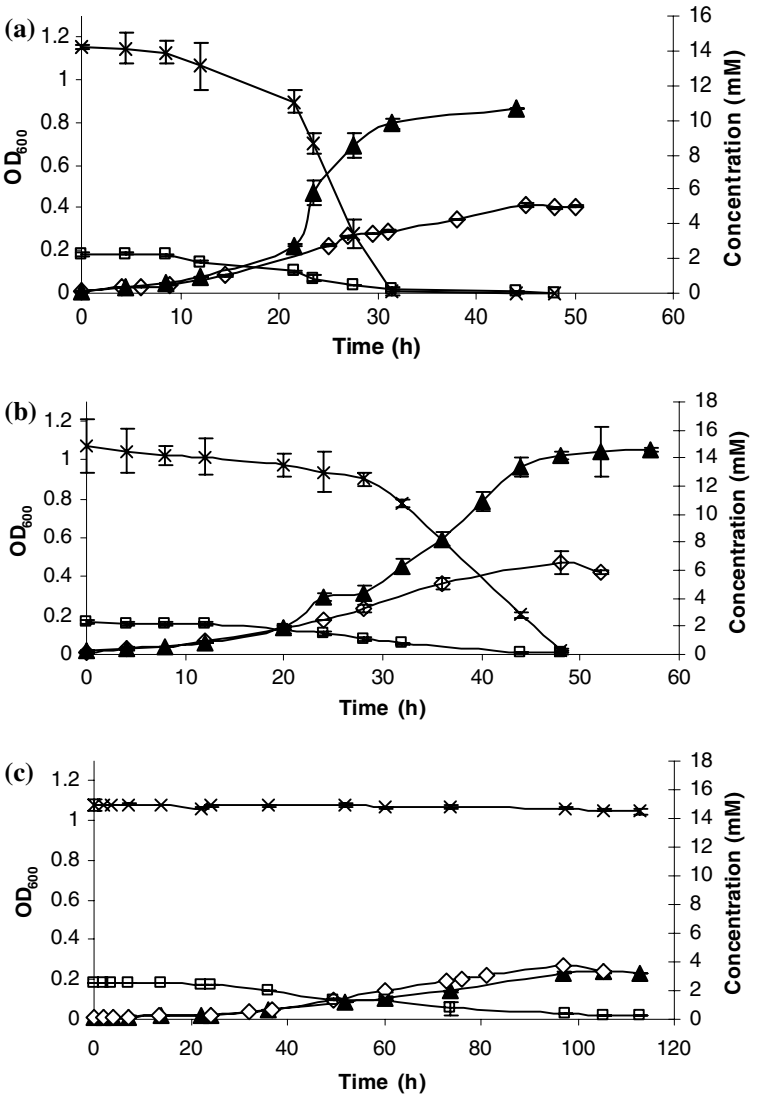

Fig. 3 *Lactobacillus casei* ATCC334 growth at pH 6.0 ± 0.2, 5.1 ± 0.2, and 4.5 ± 0.2: Growth in modified-Chemically Defined Media containing 2.4 ± 0.2 mM galactose, and 0.05 ± 0.1 mM (◇) or 14.9 ± 0.6 mM (▲) diammonium citrate at (**a**) pH 6.0 ± 0.2, (**b**) 5.1 ± 0.2, and (**c**) 4.5 ± 0.2. Citrate (×) and galactose (□) utilization in modified-Chemically Defined Media

Therefore, sodium chloride concentration in mCDM was adjusted to 2%. Culture medium pH was controlled at 5.1 ± 0.1 with 10 mM ammonium hydroxide instead of 1 M sodium hydroxide. Replacement of sodium hydroxide by ammoniun hydroxide to control pH in the presence of excess sodium chloride was necessary to prevent detrimental effects on the growth of *L. casei* (data not shown). Although, *L. casei* was able to grow in the presence of 2% (340 mM) sodium chloride, growth rate decreased six-fold compared to growth rate in the presence of 0.04% (3.4 mM) sodium chloride (Table 2). Citrate was utilized in the presence of 3.4 mM sodium chloride but not in the presence of 340 mM sodium chloride (Fig. 4). These results suggest that the presence of 2% sodium chloride restricted growth and directly or indirectly affected citrate utilization by *L. casei* ATCC334 in mCDM.

Citrate utilization by *L. casei* in CCE

The results obtained with mCDM suggested that citrate can serve as an energy source for *L. casei* in the presence of limiting galactose at pH

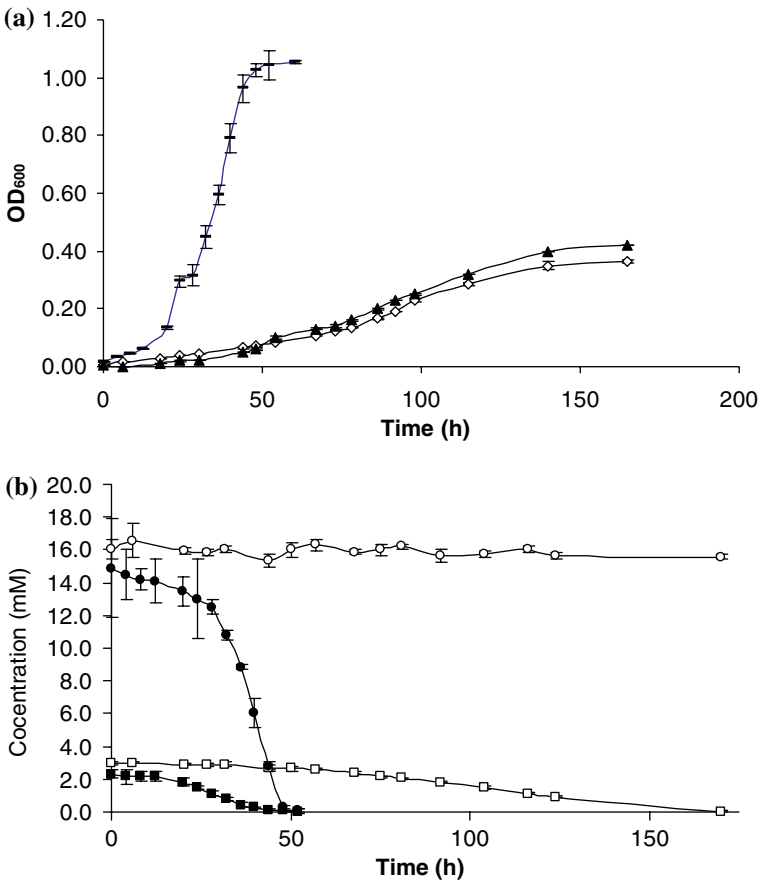

Fig. 4 *Lactobacillus casei* ATCC334 growth and citrate utilization in the presence of 2% NaCl: (**a**) Growth in modified Chemically Defined Media (mCDM) containing 2.4 ± 0.2 mM galactose (limiting), 14.9 ± 0.6 diammonium citrate and 3.4 ± 0.1 mM NaCl (–), 2.5 ± 0.1 mM galactose, and 340 ± 0.9 mM NaCl (◇), and 2.5 ± 0.1 mM galactose, 15.2 ± 0.6 mM diammonium citrate and 340 ± 0.9 mM NaCl (▲) at pH 5.1 ± 0.2. (**b**) Galactose (■,□), and citrate utilization (●,○) in the presence of 3.4 ± 0.1 mM NaCl (0.04%), 2.5 ± 0.1 mM galactose, and 15.2 ± 0.6 mM diammonium citrate (■, ●), and 340 ± 0.9 mM NaCl (2%), 2.5 ± 0.1 mM galactose, and 15.2 ± 0.6 mM diammonium citrate (○,□) at pH 5.1 ± 0.2 in mCDM

5.1 ± 0.2. However, the ability of this bacterium to utilized citrate may be restricted by excess sodium chloride. To further evaluate this hypothesis, growth studies were conducted in CCE prepared from 2.5-months-old Cheddar cheese. *L. casei* ATCC334 reached almost 10^9 CFU/ml of CCE in the presence of both excess and limiting carbohydrate (Fig. 5). Citrate utilization in CCE containing either excess or limiting carbohydrate began when the cultures reached late-logarithmic phase, by which time more than 70% of the initial lactose concentration was depleted (Fig. 5). In the presence of excess and limiting carbohydrate, *L. casei* utilized ca. 60 and 90% of the initial citrate concentration, respectively (Fig. 5a, b). Inhibition of citrate catabolism in the presence of excess galactose was relieved in CCE when the cultures reached late logarithmic phase and the galactose concentration had fallen to less than ca. 40% of the initial concentration (Fig. 5). Additionally, these results suggest that the sodium chloride concentration present in CCE (4.5% salt: moisture content) was not inhibitory for citrate catabolism by *L. casei*. These observations may be an indication that CCE is a more appropriate culture media to grow *L. casei* than the commonly used culture media.

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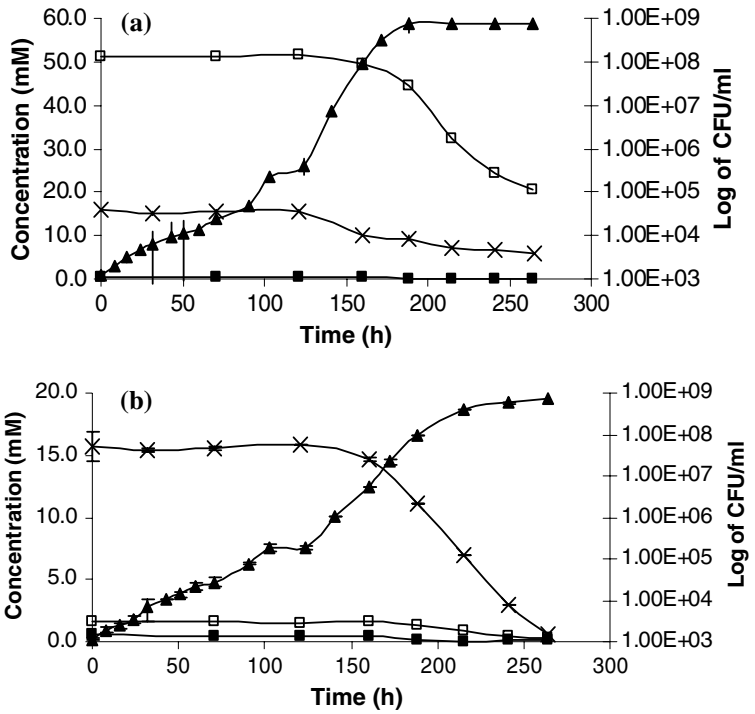

Fig. 5 Citrate utilization by *Lactobacillus casei* ATCC334 in the presence of excess and limiting carbohydrate in Cheddar cheese extract prepared from 2-months-old Cheddar cheese. Growth (▲), and citrate (×), galactose (□), and lactose (■) utilization in Cheddar cheese extract (CCE) containing 15.0 ± 1 mM citrate, and (**a**) excess carbohydrate (52 ± 0.9 mM total hexose) or (**b**) limiting carbohydrate (2.6 ± 0.1 mM total hexose)

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