UDC 579.864.1:663.12:637.146
ISSN 1330-9862

preliminary communication

Interactions Between *Lactococcus lactis* subsp. *lactis* and *Issatchenkia orientalis* at Milk Fermentation

*Elise Borregaard and Nils Arneborg**

Department of Dairy and Food Science, The Royal Veterinary and Agricultural University, Rolighedsvej 30, DK-1958 Frederiksberg C, Denmark

Received: September 10, 1997
Accepted: January 15, 1998

Summary

Growth, substrate utilization, and product formation during growth of Lactococcus lactis subsp. lactis and Issatchenkia orientalis as single cultures and as a mixed culture in reconstituted skim milk were measured in order to detect interactions between the two microorganisms in fermented milk. As to substrate utilization, no interactions could be detected. L. lactis slightly stimulated the growth of I. orientalis. I. orientalis slightly decreased the acidification of the growth medium by L. lactis. In addition, the lactic acid, acetic acid, acetaldehyde, and acetoin concentrations in the growth medium were significantly decreased, when L. lactis was grown in the presence of I. orientalis.

Keywords: interactions, fermentation, *Lactococcus lactis* subsp. *lactis*, *Issatchenkia orientalis*, milk

Introduction

It is widely accepted that the successful fermentation of milk relies not only on good growth of the introduced microorganisms, but also on their metabolic activities. As a result of the activity of lactic acid bacteria, fermented milk products are dominated by lactic acid, but the contribution of other end metabolites is equally important. *Lactococcus lactis* subsp. *lactis*, a homofermentative lactic acid bacterium, is widely used as a starter culture in fermented dairy products (*1*). It is now well documented that *L. lactis*, although homofermentative, is able to produce end metabolites other than lactic acid, *e.g.* acetic acid, acetaldehyde, diacetyl, and acetoin, when grown under specific conditions (*2*). When grown as single culture in milk substrate, the growth and metabolic activity of *L. lactis* are well known (*3-5*). When grown as mixed culture in milk substrate, however, knowledge on the growth and metabolic activity of *L. lactis* is very limited. The yeast *Issatchenkia orientalis* has been isolated from fermented milk (*6,7*) and cheese (*6,8*) products. Very little is reported on the growth and metabolic activity of *I. orientalis* when grown either as single or mixed culture in milk substrate. In this study we determine growth, substrate utilization, and product formation during growth of *L. lactis* and *I. orientalis* as single cultures and as a mixed culture in reconstituted skim milk (RSM) in order to detect interactions between the two microorganisms in fermented milk.

Materials and Methods

Microorganisms. *L. lactis* subsp. *lactis* ATCC 19435 was maintained on plates with LM17 agar (containing per liter: 5 g lactose and 37.25 g M17 agar) at 4 °C. *I. orientalis* CBS 1914 was maintained on plates with MYGP agar (containing per liter: 25 g glucose, 3 g malt extract, 3 g yeast extract, 5 g peptone, and 15 g agar, pH = 5.6) at 4 °C. Inoculation cultures of *L. lactis* were prepared using over-night cultures grown in LM17 broth (containing per liter: 5 g lactose and 48.25 g M17 broth) at 30 °C. Before inoculation, 0.3 mL over-night culture was suspended in 0.3 mL 50% (volume fraction) glycerol and 1.0 mL 9.5% reconstituted skim milk (RSM). Inoculation cultures of *I. orientalis* were prepared using over-night cultures grown in MYGP broth (containing per liter: 25 g glucose, 3 g malt extract, 3 g yeast extract, and 5 g peptone, pH = 5.6) at 25 °C. Before inoculation, cells were suspended in 10 mL 9.5% RSM.

* Corresponding author; Phone: +45/3528 3266, Fax: +45/3528 3214, E-mail: na@kvl.dk

Fermentations. Fermentations with single and mixed cultures were performed in 9.5% RSM (pH = 6.5) in a 1-L Applikon fermentor using an Applikon ADI 1020 control unit. *L. lactis* and *I. orientalis* were inoculated to concentrations of $10^{6.2}$ and $10^{5.9}$ colony forming units (CFU) per mL, respectively. In all experiments, the effective fermentor volume was 750 mL, the stirring rate was 200 rpm, and the temperature was 30 °C. The fermentations were carried out without aeration.

Growth analyses. Growth of bacteria and yeast in single and mixed cultures was determined by plate counting. Harvested cells were diluted appropriately using dilution medium (containing per liter: 15 g caseine peptone, 9 g NaCl, and 1.14 mL antifoam agent, pH = 7.0). Plate counting of *L. lactis* in single and mixed cultures was performed using Leesment agar with cycloheximide (containing per liter: 10 g lactose, 20 g tryptone, 5 g yeast extract, 2.5 g gelatine, 4 g NaCl, 2 g tri-natrium citrate · $2H_2O$, 8 g calcium lactate · $5H_2O$, 0.1 g cycloheximide, and 15 g agar). Plate counting of *I. orientalis* in single and mixed cultures was performed using Ø agar with chloramphenicol (containing per liter: 20 g glucose, 5 g yeast extract, 0.1 g chloramphenicol, and 15 g agar, pH = 6.6). Plates with *L. lactis* were incubated at 30 °C for three days before counting, whereas plates with *I. orientalis* were incubated at 25 °C for two days before counting. The data presented are mean values from two independent batch fermentations. Maximum variations in the data were ±5%.

Analysis of substrate components and metabolites. Cell-free samples were obtained by centrifuging the growth medium at 3000 g for 4 min at 4 °C. Samples were subsequently stored at –40 °C. The concentrations of lactose, galactose, glucose, citric acid, lactic acid, and acetic acid in the growth medium were determined by HPLC using a Shodex SH1011 column. The column was eluted at 60 °C with a mobile phase consisting of 8 mM H_2SO_4 and 0.3 mM EDTA at a flow rate of 1 mL/min. Lactose, galactose, and glucose were determined with a Merck RI-71 refractive index detector, whereas citric acid, lactic acid, and acetic acid were determined with a Merck L-4000 UV detector at 220 nm. The concentrations of acetaldehyde, diacetyl, and acetoin in the growth medium were determined by Head Space Gas Chromatography using a Perkin Elmer 8500 Gas Chromatograph equipped with a Perkin Elmer HS101 Head Space Sampler, a flame ionization detector, and a 25 m × 0.20 mm i.d. (0.30 μm film thickness) Hewlett-Packard HP-FFAP capillary column under the following conditions: injector temperature 180 °C, detector temperature 200 °C, column head pressure (helium as carrier gas) 30 psi, and the oven programme: initial temperature 80 °C for 1.5 min, rate 30 °C/min to 140 °C, which was maintained for 1.5 min. The data presented are mean values from two independent batch fermentations. Maximum variations in the data were ±2% for lactose, galactose, glucose, citric acid, lactic acid, and acetic acid, and ±5% for acetaldehyde, diacetyl, and acetoin.

Results

L. lactis grown as single culture

During growth of *L. lactis* as single culture, the concentration of lactose decreased from 45.3 g/L to 42.4 g/L, and the concentration of galactose decreased from 580 mg/L to 355 mg/L in the growth medium (Fig. 1). The glucose concentration decreased from 110 mg/L to 40 mg/L during the first 6 h of fermentation, after which it increased, probably due to hydrolysis of lactose, reaching a final concentration of 70 mg/L (Fig. 1). The RSM contained 2.1 g/L citric acid which was not utilized by *L. lactis* (data not shown). Exponential growth of *L. lactis* occurred during the first 6 h of fermentation, reaching 8.6 log CFU/mL, after which the cells entered the stationary growth phase (Fig. 2). Production of lactic acid and acetic acid commenced after 3 h of fermentation and continued throughout the fermentation reaching concentrations of 5.3 g/L and 230 mg/L, respectively (Fig. 3). Hereby the pH of the growth medium was lowered from 6.5 to 4.7 (Fig. 2). In addition, after 3 h of fermentation the cells started to produce acetaldehyde and acetoin, and this production continued throughout the fermentation reaching concentrations of 4.6 mg/L and 110 mg/L,

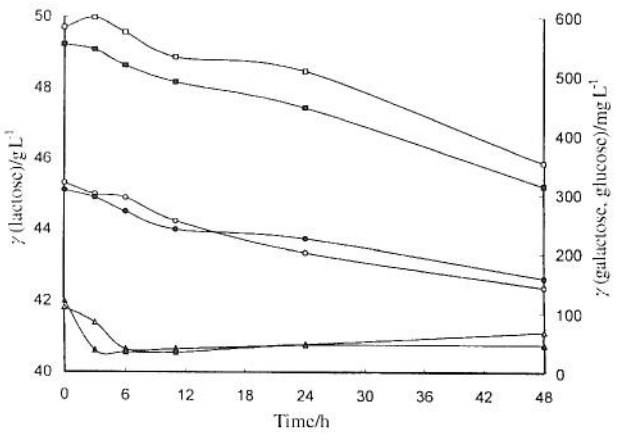

Fig. 1. Lactose (O, ●), galactose (□, ■), and glucose (Δ, ▲) concentrations in the growth medium during batch fermentation of *Lactococcus lactis* alone (open symbols) or with *Issatchenkia orientalis* (closed symbols) in 9.5% RSM.

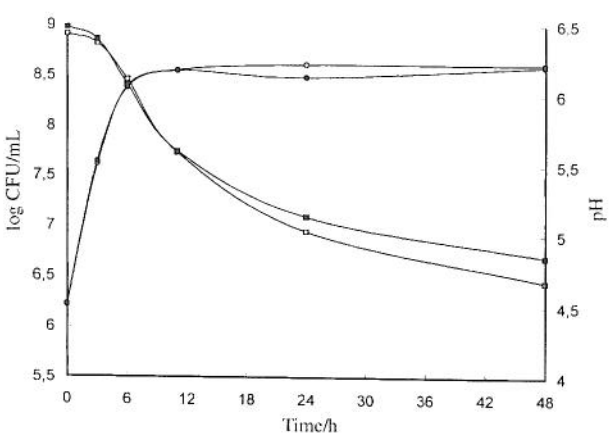

Fig. 2. Growth (O, ●) and acidification of the growth medium (□, ■) during batch fermentation of *Lactococccus lactis* alone (open symbols) or with *Issatchenkia orientalis* (closed symbols) in 9.5% RSM.

respectively (Fig. 3). Diacetyl was not detectable in the growth medium (data not shown).

I. orientalis grown as single culture

During growth of *I. orientalis* as single culture, the lactose and galactose concentrations in the growth medium remained constant (data not shown). These results agree with the fact that *I. orientalis* is not able to utilize lactose and galactose as carbon and energy sources (*9*). Although *I. orientalis* is able to utilize citric acid as a carbon and energy source (*9*), no change in the citric acid concentration in the growth medium was observed during the fermentation (data not shown). Exponential growth of the yeast cells occurred during the first 6 h of fermentation, reaching 6.6 log CFU/mL, after which the cells entered the stationary growth phase (Fig. 4). This growth of *I. orientalis* was found to be due to utilization of glucose and acetic acid by the yeast (Fig. 4). During the first 3 h of fermentation the glucose concentration in the growth medium decreased from 115 mg/L to 35 mg/L resulting in an increase of the acetic acid concentration from 15 mg/L to 30 mg/L (Fig. 4). The acetic acid concentration decreased from 30 mg/L to 5 mg/L between 3 h and 6 h of fermentation, after which it remained constant (Fig. 4). The pH of the growth medium remained constant at 6.5 throughout the fermentation (Fig. 4). Acetaldehyde, diacetyl, and acetoin were not detectable in the growth medium (data not shown).

L. lactis and I. orientalis grown as mixed culture

In the presence of *L. lactis*, the growth of *I. orientalis* was slightly increased, whereas the concentration of glucose in the growth medium was not changed (Fig. 5). In the presence of *I. orientalis*, the acidification of the growth medium by *L. lactis* was slightly decreased, whereas the growth of *L. lactis* was not significantly affected (Fig. 2). The lactic acid, acetic acid, acetaldehyde, and acetoin concentrations in the growth medium were significantly decreased (Fig. 3), whereas the concentrations of lactose, galactose, and glucose in the growth medium were not affected (Fig. 1), when *L. lactis* was grown in the presence of *I. orientalis*.

Discussion

In this study, the single culture of *L. lactis* produces significant amounts of acetic acid, acetaldehyde, and acetoin, besides producing lactic acid (Fig. 3). Growth of *L. lactis* on excess galactose induces production of acetic acid and acetaldehyde besides production of lactic acid; *i.e.* mixed acid fermentation (2). The RSM used in this work contains galactose, and *L. lactis* utilizes this substrate component during the fermentation (Fig. 1). Therefore, the observed production of acetic acid and acetaldehyde by *L. lactis* may be due to the presence of excess galactose in the growth medium. In addition, the presence of oxygen has been reported by various authors to induce both mixed acid fermentation and production of diacetyl and acetoin by *L. lactis* (*2,10,11*). The fermentations in this study were carried out without aeration but also without flushing with nitrogen. Hereby small amounts of oxygen may have diffused into the fer-

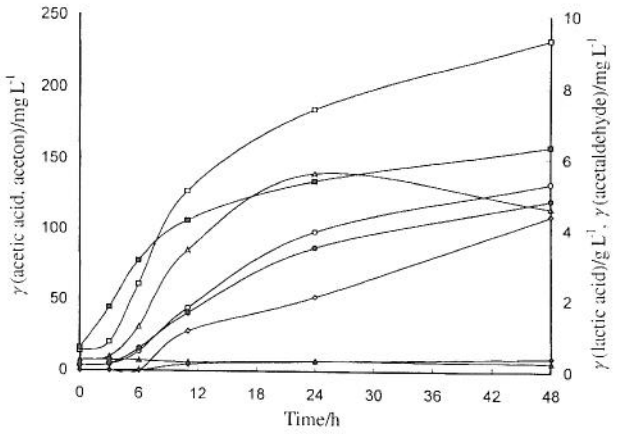

Fig. 3. Lactic acid (○, ●), acetic acid (□, ■), acetaldehyde (Δ, ▲), and acetoin (◊, ♦) concentrations in the growth medium during batch fermentation of *Lactococcus lactis* alone (open symbols) or with *Issatchenkia orientalis* (closed symbols) in 9.5% RSM.

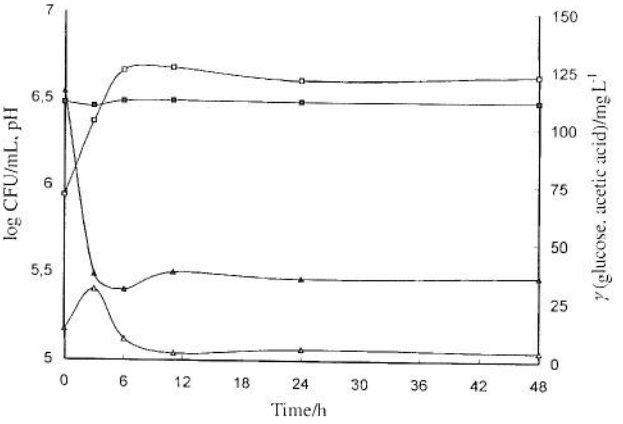

Fig. 4. Growth (□), pH (■), and concentrations of acetic acid (Δ) and glucose (▲) in the growth medium during batch fermentation of *Issatchenkia orientalis* in 9.5% RSM.

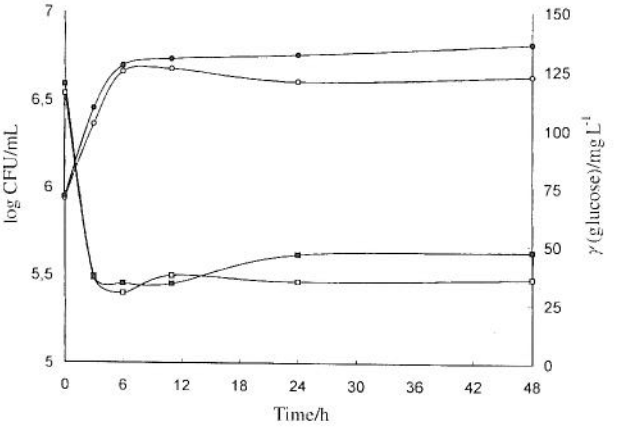

Fig. 5. Growth (○, ●) and glucose concentration (□, ■) in the growth medium during batch fermentation of *Issatchenkia orientalis* alone (open symbols) or with *Lactococcus lactis* (closed symbols) in 9.5% RSM.

mentor during the experiments. It is therefore possible that the observed production of acetic acid, acetaldehyde, and acetoin by *L. lactis* is due to the presence of oxygen in the growth medium. Probably the production of end metabolites other than lactic acid by *L. lactis* observed in this work is a result of the presence of both galactose and oxygen in the growth medium. As shown in Fig. 4, the single culture of *I. orientalis* utilizes glucose and acetic acid for growth. Yeast requires oxygen for the oxidation of acetic acid (*12*). These results hereby support that oxygen has been present in the growth medium during the experiments.

Although the lactose, galactose, and glucose uptake of *L. lactis* (Fig. 1) and the glucose uptake of *I. orientalis* (Fig. 5) are not affected in the mixed culture, several interactions between the two microorganisms can be detected. In the mixed culture, growth of *I. orientalis* is slightly stimulated by the presence of *L. lactis* (Fig. 5). *I. orientalis* is able to utilize acetic acid and lactic acid as carbon and energy sources (*9*), and the yeast cells may therefore utilize the acetic acid and lactic acid produced by *L. lactis* for growth. This explanation is supported by the observation that the concentrations of lactic acid and acetic acid are lower in the mixed culture than in the single culture of *L. lactis* (Fig. 3). Besides the concentrations of lactic acid and acetic acid, the concentrations of acetaldehyde and acetoin are lower in the mixed culture as compared with the single culture of *L. lactis* (Fig. 3). Assuming oxygen limiting conditions in the fermentor, these decreases in acetaldehyde and acetoin concentrations may be explained by the fact that oxygen is needed by the yeast for the oxidation of organic acids (*12*). Hereby less oxygen will be available for *L. lactis* to produce acetaldehyde and acetoin.

In conclusion, the results reported in this study demonstrate that *L. lactis* slightly stimulates growth of *I. orientalis* in RSM. Moreover, the lactic acid, acetic acid, acetaldehyde, and acetoin concentrations are significantly decreased, when *L. lactis* is grown in the presence of *I. orientalis*. Future experiments in our laboratory will attempt to elucidate the mechanisms underlying these interactions.

Acknowledgements

Financial support for this work was provided by Chr. Hansen A/S, Hørsholm, Denmark. We wish to thank Hans Holm and Ulf Houlberg at Chr. Hansen A/S for valuable discussions and suggestions.

References

1. M. E. Sanders, Mixed Cultures in Dairy Fermentations. In: *Mixed Cultures in Biotechnology*, J. G. Zeikus, E. A. Johnson (Eds.), McGraw-Hill Inc., New York (1991) pp. 105-133.
2. M. Cocaign-Bousquet, C. Garrigues, P. Loubiere, N. D. Lindley, *Ant. van Leeuwenhoek, 70* (1996) 253-267.
3. A. T. Christopherson, E. A. Zottola, *J. Dairy Sci. 72* (1989) 2856-2861.
4. K. N. Jordan, T. M. Cogan, *J. Dairy Res. 55* (1988) 227-238.
5. K. N. Jordan, T. M. Cogan, *Irish J. Agricult. Food Res. 34* (1995) 39-47.
6. H. Rohm, F. Eliskases-Lechner, M. Bräuer, *J. Appl. Bacteriol. 72* (1992) 370-376.
7. H. Seiler, M. Kümmerle, *Deutsche Milchwirtschaft, 48* (1997) 438-440.
8. H. Seiler, M. Busse, *Int. J. Food Microbiol. 11* (1990) 289-304.
9. C.P. Kurtzman, *Issatchenkia* Kudriavzev. In: *The Yeasts, a Taxonomic Study*, N. J. W. Kreger-van Rij (Ed.), Elsevier Science Publishers B.V., Amsterdam (1984) pp. 214-223.
10. S. Condon, *FEMS Microbiol. Rev. 46* (1987) 269-280.
11. J. F. Cogan, D. Walsh, S. Condon, *J. Appl. Bacteriol. 66* (1989) 77-84.
12. C. Gancedo, R. Serrano, Energy-Yielding Metabolism. In: *The Yeasts, vol. 3*, A. H. Rose, J. S. Harrison (Eds.), Academic Press, London (1989) pp. 205-259.

Međudjelovanje *Lactococcus lactis* subsp. *lactis* i *Issatchenkia orientalis* tijekom fermentacije mlijeka

Sažetak

Izmjeren je rast, iskorištenje supstrata i nastanak proizvoda tijekom uzgoja Lactococcus lactis i Issatchenkia orientalis kao zasebnih i miješanih kultura u rekonstituiranom obranom mlijeku, kako bi se utvrdilo međudjelovanje obaju mikroorganizama u fermentiranom mlijeku. Pri korištenju supstrata nije se moglo utvrditi neko međudjelovanje bakterija. L. lactis je djelomično stimulirao rast I. orientalis. Bakterija I. orientalis malo je smanjila kiselost podloge uzrokovane djelovanjem L. lactis. Osim toga, znatno su bile smanjene koncentracije mliječne i octene kiseline, te acetaldehida i acetoina u podlozi kada je L. lactis rastao u prisutnosti I. orientalis.