Determination of Growth Parameters of Lactococci in Milk and Ultrafiltered Milk

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ABSTRACT

Fresh skim milk and a highly standardized skim milk powder (Nilac) were ultrafiltrated at 50°C to concentration factors of approximately 2, 2.5, 3.5, and 4.5, based on total protein. Growth characteristics of *Lactococcus lactis* ssp. *cremoris* E8 and the mesophilic mixed-strain Bos starter culture were investigated in the UF retentates and non-UF skim milk under conditions of regulated pH. The maximum specific growth rate and the maximum specific acidification rate showed extreme variation and, therefore, could not be used to measure the effect of UF on the growth characteristics of *L. lactis* ssp. *cremoris*. The mean total growth of strain E8 and the Bos mixed-strain starter after 22 h decreased by 25 and 40%, respectively, in UF retentate with a concentration factor of 3.6 compared with growth reached in regular skim milk. Part of this effect could be explained as growth inhibition from the higher concentration of whey proteins in the UF retentates.

(**Key words**: growth parameters, lactococci, ultrafiltered milk)

Abbreviation key: μ_{acid} = maximum acidification rate, μ_{max} = maximum specific growth rate, **WPC** = whey protein concentrate.

INTRODUCTION

Ultrafiltration is increasingly used in the dairy industry to concentrate the milk proteins (casein and whey proteins) and minerals in dairy products (13). By use of UF, skim milk can be concentrated to a retentate with a composition similar to that of drained soft cheese. An economic advantage of this process, compared with traditionally made soft cheeses, is the incorporation of whey proteins into the cheese, which increases yield by up to 20% (10). Sutherland and Jameson (16) used a similar technique to make hard cheese. They demonstrated that concentrated retentates obtained by UF can be converted into Cheddar cheese using a fairly conventional Cheddar manufacturing process.

Despite the potential economic advantages, the production of semi-hard and hard cheeses from more than 2-fold concentrated milk is not used extensively in practice because of several biophysical and microbiological difficulties: less acceptable flavor characteristics, problems with consistency (4), and poor growth of the starter bacteria in UF milk. The reports dealing with the effects of the concentration factor of the milk on the growth and activity of lactic acid bacteria are somewhat contradictory. Some (3) indicate a stimulating effect, but others (12) report an inhibitory effect on growth.

The microbiological problems of making semi-hard cheese from UF retentates and the conflicting nature of the literature prompted us to undertake this study. Our objectives were to select parameters that were appropriate to define the growth of lactic acid bacteria in milk and to study the growth of *Lactococcus lactis* ssp. *cremoris* E8 and the Bos mixed-strain starter in UF retentates compared with growth in milk.

MATERIALS AND METHODS

Microorganisms

Lactococcus lactis ssp. *cremoris* strain E8, a proteolytic strain, and the Bos mixed-strain

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starter culture were used. Strain E8 was routinely stored in litmus milk with $CaCO_3$ and .1% yeast extract, and the Bos starter was maintained as a starter concentrate (15). Both were kept at –40°C.

Preparation of Milk Retentate

The UF retentate was prepared from skim milk or Nilac milk powder [a highly standardized low heat spray powder; Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands]. The milk was ultrafiltered to a concentration factor of approximately 5:1 according to the method reported by Sutherland and Jameson (16). The one-stage UF system contained a spirally wound membrane (Abcor S4, HFK 131 VSV; KOCH Membrane Systems Inc., Wilmington, DE) with a total surface of 11 m^2 and a cutoff of 5 kDa. The temperature during the UF process was 50°C, and the inlet and outlet pressures were approximately 500 and 100 kPa, respectively. The maximum volume flow rate over the membrane was 8 m^3/h. The retentate was used to prepare subsequent dilutions, with permeate, for use in the experiments. The non-UF skim milk and reconstituted Nilac milk powder were also kept at 50°C during the UF process.

Preparation of the Whey Protein Concentrate

The whey protein concentrate (WPC) was prepared from mixed cheese whey. The mixed cheese whey was acidified with HCl to a pH of 3.0 and concentrated by a three-stage UF system. Each UF stage contained a spirally wound membrane (Abcor S4, HFK 131 VSV). The total membrane surface of the three-stage UF system was 135 m^2. The UF conditions were the same as during the preparation of the milk retentate. After UF, the retentate was neutralized with 33% NaOH to pH 7.0 and diluted 6-fold with demineralized water. The diluted WPC was concentrated again by the three-stage UF system. The retentate was concentrated to a DM >93% by spray-drying at a temperature of 50°C. The Ca content of the WPC was <.1%. The functional properties of the WPC have been described by de Wit and de Boer (5).

Media

Lactococcus lactis ssp. *cremoris* strain E8 and the Bos starter were grown in various milk media. In initial experiments, the following media were used: fresh skim milk, UF retentates of the skim milk in the concentrations of approximately 1 to 5, and concentrated reconstituted skim milk with a DM content comparable with the DM of the UF retentates. In further experiments, reconstituted skim milk was made by dissolving 10% (wt/vol) Nilac low heat milk powder in water (14). Dissolved Nilac milk powder was chosen because its composition is constant, and it contains the same milk protein concentration as fresh skim milk. The UF retentates were also made from reconstituted Nilac skim milk. The compositions of the fresh and Nilac skim milk concentrates are shown in Table 1. In UF milk, the protein concentration and the amount of Ca^{2+} increased as concentration factor increased. The increase in Ca^{2+} correlated with the higher ash content. The lactose content decreased slightly as the concentration factor increased.

Reconstituted Nilac skim milk, with whey proteins added to a concentration corresponding to the concentration factor of the retentate, was also used. All media were pasteurized at 63°C for 30 min before the growth experiments.

Media used for the viable count determination contained 1% tryptone, .3% meat extract, .5% yeast extract, 4.0% tomato juice, 2.0% glucose, .1% Tween 80, .2% K_2HPO_4, and 1.5% bactoagar. After sterilization, pH was adjusted at 6.8 to 7.0. The viable count of the full-grown precultures was determined after 2 to 3 d of incubation at 30°C.

Batch Cultivation

Precultures of the Bos starter were made by 1% inoculation of the starter concentrate in high temperature pasteurized milk (skim milk pasteurized for 30 min at 100°C). The precultures were incubated for 20 h at 20°C. Precultures of strain E8 were obtained by inoculation of .2% in high temperature pasteurized milk. The precultures were incubated for 16 h at 30°C. The inoculum used for these precultures was frozen 1-ml fractions, which were identical for all of the described experiments. The full-grown precultures were only used under standardized conditions. The characteristics for the Bos starter and strain E8 full-grown preculture were 1×10^9 cfu/ml and 7×10^8 cfu/ml,

TABLE 1. Composition of UF retentates prepared from fresh skim milk (SM) and Nilac SM and of the reconstituted SM (RSM) with a DM content comparable to the UF retentates.

Medium[1] and CF	DM	Protein	Lactose	Ash	Ca
	(%)				
SM	9.14	3.62	ND[2]	.77	ND
UF SM					
.9	8.77	3.36	4.60	.74	.115
1.8	12.01	6.38	4.52	.98	.201
2.6	15.27	9.42	4.50	1.25	.284
3.5	18.50	12.58	4.39	1.50	.380
4.3	21.71	15.46	4.36	1.76	.467
Nilac SM	10.2	3.66	4.88	ND	.131
UF Nilac					
1.0	10.1	3.81	ND	ND	.135
1.9	13.9	7.13	4.99	ND	.231
2.9	17.8	10.56	4.80	ND	.339
3.6	21.6	13.23	4.69	ND	.443
4.7	25.7	17.03	4.63	ND	.534
RSM					
.9	8.84	3.48	4.59	.74	.120
1.8	12.02	4.67	6.29	1.00	.159
2.6	15.37	5.99	8.06	1.26	.203
3.5	18.60	7.24	9.89	1.55	.243
4.3	21.70	8.42	11.4	1.82	.292

[1]CF = Concentration factor.

[2]ND = Not determined.

the pH was 4.60 ± .05 and 4.50 ± .02, and the amount of lactic acid produced was 111 ± 6 and 108 ± 4 m*M*, respectively. The Bos starter and strain E8 were grown in 1-L Erlenmeyer flasks containing .6 L of medium. An inoculum of 2% (vol/vol) was used. The pH of the growth medium was maintained at 6.3, in all the experiments, by the addition of 10% $NaHCO_3$ and 7.5% NH_4OH automatically by an Impulsomat 614 and a Dosimat 655 (both from Metrohm, Applicon, Herisan, Switzerland). Incubation was at 30°C. Anaerobic conditions were achieved by passing N_2 gas over the culture. The contents of the fermenter were mechanically stirred at 250 rpm.

Growth Parameters

The maximum specific growth rate and the total growth of the cultures were determined spectrophotometrically. For the measurement of the optical density, the milk and retentate samples were clarified by mixing .5 ml of sample with 4.5 ml of 50 m*M* EDTA and .5 *M* NaH_3BO_3 (pH 8). After 5 min, the absorbance was measured at 578 nm (Biotron Atom Data Test 366 photometer; Meyvis, Bergen op Zoom, The Netherlands). The measured absorbances at 578 nm were between .2 and 1.0. Each measurement was in triplicate. No cell lysis occurred during this treatment, and the absorbance of the diluted cultures remained stable for >30 min. In all cases, the uninoculated medium was used as a blank. The absorbance at 578 nm of the culture was in all cases linearly correlated with the viable count and the amount of cell protein. An absorbance at 578 nm of one corresponded to 225 μg of cell protein/ml and approximately 10^8 cfu/ml of the strain E8.

Maximum specific growth rates (μ_{max}) were calculated during exponential growth according to the equation:

$$\ln X = \ln X_0 + \mu \times t$$

where X_0 = biomass produced at initiation of the experiment, time t_0, and X = biomass produced at time t. The slope of the linear part of the curve of ln x versus t is μ_{max}. The μ_{max}

was calculated from slopes with a correlation coefficient higher than .99; μ_{max} is expressed per hour.

Total growth is defined as the total amount of biomass that is formed after 22 h of growth at regulated pH. The amount of biomass was determined spectrophotometrically as described.

Maximum acidification rates (μ_{acid}) were calculated in the same manner as the μ_{max} using the following equation:

$$\ln Y = \ln Y_0 + \mu_{acid} \times t$$

where Y_0 = quantity of acid produced at initiation of the experiment, time t_0, and Y = acid produced at time t. The acidity of the culture was calculated from the amount of base that was added to the pH-regulated culture and is expressed as millimolar per hour.

Analytical Methods

Protein concentrations and the amounts of nonprotein N of the milk were determined with the micro-N method as described by Koops et al. (9). The lactose concentration was determined according to the method of Schoorl (11). Ash was determined according to International Dairy Federation standard 27 (8). The Ca^{2+} content was measured according to the method of Evenhuis and de Vries (6).

Cell protein was assayed according to Bradford (1), using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Specific Growth Rates

Initial experiments showed clearly the large variation in the specific growth rate of lactococci grown on milk between experiments performed in duplicate on different days. This result, in combination with the conflicting data in the literature (3, 12), prompted us to study this variation in more detail.

The μ_{max}, the specific biomass increase per time interval, of strain E8 was determined in fresh skim milk and in UF retentate prepared from the same milk and concentrated 4.3-fold. The mean maximum values for specific growth rate on those substrates were .60 (SD = ± .17; n = 18) and .62 (SD = ± .10; n = 11), respectively. The μ_{max} on skim milk varied between .85 and .22/h. These high variations were unexpected because of the standardization of the storage procedure for the bacterial stock, the thawing procedure, the normalized preparation of the inoculum, and the use of completely automated equipment. This variation was not seen with duplicate cultures grown on the same day or when the cultures were inoculated with cultures prepared from different bacterial stocks. Duplicate E8 cultures grown on M17 medium on different days with identical growth conditions showed a mean μ_{max} of .82 (SD = .02; n = 10). Thus, possible variations in growth, except those induced by the use of different milk preparations, were apparently excluded.

To diminish the variability introduced by the use of fresh skim milk, this medium was replaced by highly standardized Nilac skim milk powder, which is similar in composition to fresh skim milk (14). However, variation in growth rates was the same, using the same number of experiments, as variation on fresh skim milk (data not shown). These findings may explain the conflicting results reported in the literature (3, 12). Srilaorkul et al. (12) found that μ_{max} decreased as concentration of the retentate increased. However, an opposite effect was reported by Hickey et al. (7) and Christopherson and Zottola (3). The observed variation of the μ_{max} suggests that this parameter is not suitable to describe the growth of lactic acid bacteria in milk and not suitable to determine the effects of UF of milk on growth of *L. lactis* ssp. *cremoris*.

Acidification Rate

To obtain a more reproducible parameter to describe the growth of lactococci in milk and UF retentates, μ_{acid} was studied in more detail. The μ_{acid} was defined as the specific increase in acidity of the culture per time interval. The effect of the concentration factor of the Nilac milk on μ_{acid} was investigated under conditions in which the pH was controlled at pH 6.3 (Table 2). The μ_{acid} increased by about 40 to 25% for a 2.8-fold increase of the concentration factor of the retentate in Experiments 5, 10, and 11, which is consistent with the results of Hickey et al. (7). In the other experiments,

TABLE 2. Maximum specific acidification rates (μ_{acid}) per hour of *Lactococcus lactis* ssp. *cremoris* strain E8 (protease positive) and a Bos mixed-strain starter grown in Nilac skim milk (SM) and UF-concentrated Nilac milk.[1]

Culture and experiment[2]	Nilac SM	UF			
		1.0	1.9	2.8	3.6
E8					
1	.62	. . .[3]	. . .	. . .	. . .
2	.60	. . .	. . .	. . .	. . .
3	.78	. . .	.66	.43	. .
4	. . .	.55	.69	. . .	.58
5	.58	. . .	. . .	.95	. . .
6	. . .	.85	.94	. . .	. . .
Bos					
7	. .	. . .	.44	.48	.76
8	.58	. . .	.41	. . .	.81
9	.58	.55	. . .	.51	. . .
10	.44	. . .	. . .	.69	. . .
11	.69	. . .	. . .	.85	. . .
12	.9	. . .	. . .	. . .	. . .
13	. . .	. . .	.51	. . .	.60
14	.79	.78	. . .	.60	. .

[1]Maintained at pH 6.3.

[2]Experiment numbers indicate cultures performed on the same day under identical conditions.

[3]Not done.

μ_{acid} did not show an effect or was up to 45% lower in the case of UF retentate concentrated 2.8- or 3.6-fold. These variations in μ_{acid} were similar to those with the μ_{max} measurements, demonstrating that neither parameter can be used to define growth of *L. lactis* ssp. *cremoris* on UF milk.

Total Growth

The effect of concentration of Nilac milk by UF on the total growth of the culture, reached after 22 h of pH-regulated (pH 6.3) growth, was investigated. Total growth was defined as the total amount of biomass, expressed in optical density at 578 nm, which is formed after 22 h of growth under conditions of regulated pH. Strain E8 and the Bos starter were studied. The total growth of strain E8 in UF retentate of Nilac milk concentrated 3.6-fold decreased by 40% (SD = 4.2) compared with the total growth in Nilac skim milk (Figure 1). For Bos culture, the decrease in growth was 25% (SD = 6.7). In all cases, lactose was the growth-limiting compound. Therefore, the small decrease of lactose in the UF retentate concentrated 3.6-fold (Table 1), could be responsible for 4% of the decrease in total growth compared with the total growth in milk that had not been concentrated.

Differences in total growth on Nilac skim milk and UF retentates could not be explained by the inoculum size. The inoculum size for

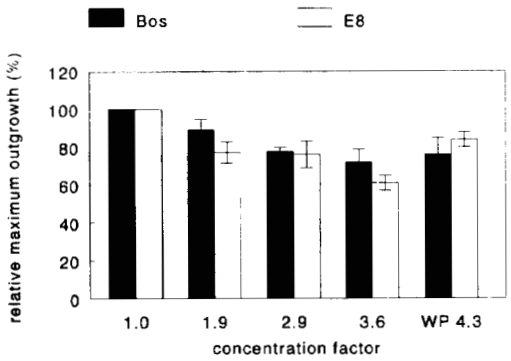

Figure 1. Relative total growth of the Bos mixed-strain starter and *Lactococcus lactis* ssp. *cremoris* E8 (protease positive) on Nilac skim milk (1.0) its UF retentates and Nilac skim milk (SM) with added whey proteins (WP) in a concentration equal to those in UF retentate concentrated 4.3-fold. Growth was regulated at pH 6.3. The total growth on skim milk was used as control for each experiment and set at 100%. Each bar represents the mean of three experiments.

strain E8 and the Bos starter was highly standardized at 2% of 7×10^8 cfu/ml and 2% of 1×10^9 cfu/ml, respectively. In milk that had not been concentrated, the absolute absorption at 578 nm, after correction for dilutions, varied from 9.0 to 13.0. The corresponding colony-forming units per milliliter were 1×10^9 and 6×10^9, respectively. Despite the variation in absolute values, in all cases, the concentration factor of the milk correlated with the decrease in the total growth. The same result was found when the number of colony-forming units or the amount of cellular protein was measured (data not shown). These results show that the total growth can be used as a parameter to define growth of *L. lactis* in milk.

Effect of Whey Proteins

The total growth of strain E8 and the Bos starter decreased in UF retentates compared with the total growth reached in regular skim milk. The concentration of whey proteins is increased specifically by the UF process. To study the effect of the increased WPC on the total growth, purified whey proteins were added to Nilac skim milk in concentrations similar to those found in UF retentates concentrated 4.3-fold. This addition caused a mean decrease of 15% in the total growth of the culture E8 and 20% of the Bos culture in 10 independent experiments compared with the decrease in total growth of 40% and 25% on UF retentates (Figure 1). This result indicates that the decrease in total growth, observed in cultures grown on UF retentates, can be partly explained by the increase in concentration of whey proteins in UF retentates. An opposite effect has been described by Broome et al. (2). They suggested that addition of WPC to milk stimulated the specific growth rate and the acid production of *Streptococcus salivarius* ssp. *thermophilus* and *Lactobacillus helveticus*.

Further research should focus on the possible mechanisms that are involved in the inhibiting effect of whey proteins on the growth of lactic acid bacteria and on which whey proteins are involved in this process. In addition, more attention should be directed to the other factors responsible for the growth inhibition of lactic acid bacteria on UF retentates.

CONCLUSIONS

Despite the use of highly standardized conditions, μ_{max} (the specific biomass increase per time interval) and μ_{acid} (the specific increase in acidity of the culture per time interval) could not be used to characterize the growth of lactic acid bacteria on milk and UF retentates because of the huge variations in the measurements. Thus, growth experiments in milk that have been described in the literature have to be interpreted with much care.

Concentration of milk by UF clearly inhibited the total growth (the total amount of biomass, expressed in optical density at 578 nm, which is formed after 22 h of growth at pH-regulated conditions) of *L. lactis* ssp. *cremoris* E8 and the Bos starter. Part of the inhibition could be explained by the increased concentration of whey proteins in UF retentates.

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