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Influence of nutrient availability on *in vitro* growth of major bovine mastitis pathogens

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Research Article

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Abstract

The aim of the present study was to investigate the effects of milk composition changes on the *in vitro* growth of bovine mastitis pathogens. Nutritional requirements of three major bovine mastitis pathogens *Escherichia coli* (*E. coli*), *Staphylococcus aureus* (*S. aureus*), and *Streptococcus uberis* (*S. uberis*) were investigated *in vitro*. We used ultra-high temperature (UHT) treated milk with different contents of fat, protein, and carbohydrates to test the influence of the availability of various milk constituents on pathogen growth characteristics. Additionally, the bacterial growth was investigated under experimentally modified nutrient availability by dilution and subsequent supplementation with individual nutrients (carbohydrates, different nitrogen sources, minerals, and different types of B vitamins) either to milk or to a conventional medium (thioglycolate broth, TB). Varying contents of fat, protein or lactose did not affect bacterial growth with the exception of growth of *S. uberis* being promoted in protein-enriched milk. The addition of nutrients to diluted whole milk and TB partly revealed different effects, indicating that there are media-specific growth limiting factors after dilution. Supplementation of minerals to diluted milk did not affect growth rates of all studied bacteria. Bacterial growth in diluted whole milk was decreased by the addition of high concentrations of amino acids in *S. aureus*, and by urea and additional B vitamins in *E. coli* and *S. aureus*. The growth rate of *S. uberis* was increased by the addition of B vitamins to diluted whole milk. The present results demonstrate that growth-limiting nutrients differ among pathogen types. Because reduced bacterial growth was only shown in diluted milk or TB, it is unlikely that alterations in nutrient availability occurring as a consequence of physiological changes of milk composition in the cow's udder would directly affect the susceptibility or course of bovine mastitis.

Bacteria require various nutrients for growth, and it is obvious that the milk in the lactating mammary gland provides an ideal nutrient source for mastitis pathogens. In addition to an energy source, bacteria need a carbon source, which is provided in milk by lactose and free glucose but to a lower extent also by protein and fat (Frank, 2007). Amino acids from caseins, whey proteins or non-protein nitrogen compounds such as urea serve as nitrogen sources. Bulk and trace elements are utilized to synthesize and activate various microbial enzymes and vitamins (Frank, 2007). Former studies revealed that the stage of lactation seems to affect the susceptibility to intramammary infections (IMI), peaking in early lactation and around dry-off (reviewed by Burton and Erskine, 2003). These stages are accompanied by considerable changes in milk composition relative to the rather constant composition during lactational stages with lower IMI incidence (Silvestre *et al.*, 2009; Gross *et al.*, 2011). Therefore, besides the immune status of the cow, the milk composition may contribute to a changing risk of IMI (Dutt *et al.*, 1986; Kornalijnslijper *et al.*, 2003; Boerhout *et al.*, 2016). Furthermore, milk composition differs among different locations within the udder with lower concentrations of fat and lactose in the cisternal compartment compared to alveolar milk fractions (Ontsouka *et al.*, 2003). This could have an additional influence on bacterial growth and distribution within the udder as bacteria usually invade through the teat canal where their growth starts in milk with low fat content before they colonize the parenchyma of the infected quarter.

The aim of the present work was to investigate the impact of milk composition on pathogen growth independent of any physical, biochemical or cellular defense mechanisms of the mammary gland. Therefore, we studied the *in vitro* growth of mastitis pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Streptococcus uberis*) in ultra-high temperature (UHT) treated milk and thioglycolate broth (TB) with changed contents of individual constituents.

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Materials and methods

Mastitis pathogens

In vitro growth assays were conducted with milk strains isolated from bovine mastitis cases of *Escherichia coli* (*E. coli* IVB M2279 from the collection of the Institute of Veterinary Bacteriology, Vetsuisse Faculty, University of Bern, Bern, Switzerland), *Staphylococcus aureus* (*S. aureus* BL28, Roesch *et al*., 2006) and *Streptococcus uberis* (*S. uberis* UB6, Wellnitz *et al*., 2012).

Preparation of bacterial suspensions

The bacterial suspensions were prepared and cultured following common bacteriological procedures which are described in detail in the online Supplementary File. The bacterial suspension was centrifuged (2000 × ***g***, 5 min, 25°C (Rotina 35R, Hettich, Kirchlengern, Germany)) and washed twice with 10 ml sterile phosphate buffered saline (PBS; 137 mM NaCl, 10 mM phosphate, 2.7 mM KCl; pH 7.4). Subsequently, the bacterial suspension was diluted to reach a bacterial density of 10^6 bacteria/ml using a spectrometer (BioSpectrometer® basic, Eppendorf, Hamburg, Germany) where an optical absorption of 0.6 at a wavelength of 600 nm was considered as reference value for a bacterial density of approximately 10^9 bacteria/ml. Finally, 10 µl of the prepared bacteria suspension was inoculated into 10 ml of the experimental growth medium, reaching a final density of approximately 10^3 bacteria/ml.

Bacterial growth in different types of commercial cow milk

To investigate the effect of the different nutrients on the growth of our target pathogens and to prevent potential growth of other bacteria, UHT milk was used instead of raw milk. At first, each of the investigated pathogens was grown simultaneously in triplicates in milk and TB (Thioglycollate Broth U.S.P. Alternative, cat. no. CM0391, Oxoid Ltd, Basingstoke, England; prepared according to the manufacturer's protocol) for 24 h at 37°C under gentle shaking (180 rpm). Bacteria were plated at time points 0 h (to check quantity of inoculated bacteria), 4 h, and 8 h, followed by 2 h intervals until 24 h to compare growth characteristics and to determine the duration until the stationary phase was reached. Therefore, samples were first mixed homogenously (Vortex-Genie 2; Scientific Industries Inc., Bohemia, NY, USA), diluted serially in 96-well plates (microplate, 96 well, PS, U-bottom, cat. no. 650101, Greiner Bio-One International GmbH, Kremsmuenster, Austria), streaked on BHI agar plates, and incubated overnight at 37 °C. Finally, the colony forming units (CFU) were enumerated and CFU/ml was calculated.

Based on the first results, microbial growth in milk was only determined for 8 h, which included both lag and log phase. Microbial growth was determined in milk of divergent compositions to investigate nutritional requirements of the pathogens. The compositions and further details of the selected types of milk are summarized in Supplementary Table S1. To test the impact of milk fat, bacterial growth in UHT treated milk with varying fat contents was examined. Bacteria were grown simultaneously in commercially available skim milk (0.1% milk fat), two variants of fat-reduced milk (1.5 and 2.5% milk fat), whole milk (3.5% milk fat) and cream (25% milk fat). CFU were enumerated after 0, 4, and 8 h of incubation. Finally, the mean growth rate during 8 h of growth was calculated using the difference of log 10-transformed CFU/ml between 0 and 8 h measurements.

The same procedure was conducted to investigate effects of higher protein availability using a protein-enriched skim milk (7% milk protein) and effects of previous cleavage of lactose into glucose and galactose using a lactose-free whole milk (5% carbohydrates), which has been enriched by the lactase enzyme during manufacturing process.

Preparation of experimental additives for milk and TB

To investigate more specifically the importance of various nutrients for microbial growth characteristics, we enriched whole milk and TB with different specific nutrients. Details about these nutrients can be retrieved from Supplementary Table S2. Within the carbohydrate fraction as potential carbon and energy sources, lactose (Lactose-Monohydrate) and glucose (D-(+)-Glucose) were tested. Tested nitrogen sources were urea, ammonium sulphate, L-glutamine and two different amino acid mixtures (detailed compositions are shown in Supplementary Table S3). For testing the impact of minerals on bacterial growth, mixtures of trace elements and bulk elements were used either individually or in combination. The detailed compositions of the mineral mixtures are shown in Supplementary Table S4. To test the importance of B vitamins for bacterial growth, we used a vitamin mixture. Detailed composition of this mixture of B vitamins are shown in Supplementary Table S5.

For experimental use, additives that were purchased as powders (lactose, glucose, urea, ammonium sulphate, L-glutamine) were dissolved in PBS followed by sterile filtration (Fisherbrand™ Disposable PES Filter Units, cat. no. FB12566502, Thermo Fisher Scientific, Waltham, MA, USA) to prepare stock solutions at the required concentrations. Vitamins were dissolved individually in sterile PBS to get solutions containing 100 mg/ml. In addition, a vitamin B mixture solution with equal proportions (9.09 mg/ml) of each vitamin was prepared.

Bacterial growth with components added to diluted TB and milk

Preliminary experiments showed a similar bacterial growth in milk and TB. Growth recording in TB can be performed using optical density in contrast to milk. This enables automated generation of highly accurate growth curves with reliable repeatability and precise calculation of additional kinetic parameters such as maximal growth rate (vmax) and time at maximal growth rate (t at vmax). Therefore, part of the experiments were conducted in TB instead of milk as basic medium.

Since it was assumed that bacterial growth in TB is already optimal, it was diluted using sterile PBS to decrease bacterial growth and enable improvements by additives. TB had to be diluted more for *E. coli* (1 : 10) than for *S. aureus* (1 : 4) to achieve a comparable growth limitation for both pathogens. The various additives were added to the diluted TB to study their effects on bacterial growth. After preparing the modified nutrient media, bacterial inoculation was performed as described before in *Preparation of bacterial suspensions*. Then, 200 µl of the inoculated experimental suspensions were transferred to 96-well plates (Corning® CellBIND® 96-well Clear Flat Bottom Polystyrene Microplate, cat. no. 3300, Corning, Inc., Corning, NY, USA) in triplicates to measure bacterial growth by optical density using an imaging reader (Cytation 5 Cell Imaging Multi-Mode Reader, BioTek Instruments, Inc., Winooski, VT, USA). To ensure the absence of contamination, nutrient medium without any inoculated bacteria was used as control. Optical density was

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Table 1. Growth rates *[mean ± SD]* of *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus uberis* during 8 h of growth in thioglycolate broth and different types of cow milk

	Growth rate [log_{10} CFU/h]		
Growth medium	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus uberis*
Milk and Thioglycolate Broth			
Whole milk	0.75 ± 0.01[a]	0.64 ± 0.01[a]	0.52 ± 0.01[a]
Thioglycolate broth	0.80 ± 0.01[b]	0.61 ± 0.01[a]	0.50 ± 0.01[a]
Milk with varying Fat Content			
Skim milk (0.1% fat)	0.80 ± 0.02[a]	0.69 ± 0.01[a]	0.65 ± 0.02[a]
Fat-reduced milk (1.5% fat)	0.78 ± 0.01[a]	0.70 ± 0.00[a]	0.64 ± 0.01[a]
Fat-reduced milk (2.5% fat)	0.79 ± 0.01[a]	0.67 ± 0.00[a]	0.58 ± 0.02[a]
Whole milk (3.5% fat)	0.79 ± 0.00[a]	0.68 ± 0.01[a]	0.59 ± 0.01[a]
Cream (25% fat)	0.79 ± 0.03[a]	0.69 ± 0.00[a]	0.63 ± 0.02[a]
Different Types of Milk			
Whole milk	0.79 ± 0.01[a]	0.71 ± 0.00[a]	0.64 ± 0.01[a]
High protein milk	0.80 ± 0.01[a]	0.76 ± 0.02[a]	0.75 ± 0.02[b]
Lactose-free milk	0.82 ± 0.00[a]	0.73 ± 0.01[a]	0.69 ± 0.01[a]

[a–b]Growth rates within group of growth media and pathogen with different superscripts differ ($P < 0.05$).

automatically measured at a wavelength of 600 nm during 16 h at 37 °C in 20 min intervals after each 30 s of double orbital shaking. Bacterial growth rates, vmax, and t at vmax were calculated by the data analysis software (Gen5 Software Features for Detection, release 3.04; BioTek Instruments, Inc., Winooski, VT, USA).

To confirm if stimulatory and inhibitory effects of the various nutrients added to TB can be achieved in milk, part of the experiments were also performed in milk. Therefore, diluted whole milk (1 : 20 for *E. coli*, 1 : 10 for *S. aureus* and *S. uberis*) was enriched with lactose, amino acid solution, urea, bulk and trace element solutions and vitamin B mixture solution (details and composition of solutions are described in *Preparation of experimental additives for milk and TB*). Bacterial growth was determined by quantitative plating as described before.

Statistical analysis

Statistical analysis was performed using the SAS software package (release 9.4; SAS Institute, Cary, NC, USA), using the mixed models procedure (PROC MIXED). Evaluations were carried out individually for each bacteria type. The respective model included the type of medium (e.g., milk, TB, diluted media, additives) as fixed factor. Individual replicates were considered as random subjects. Dependent variables included (1) for automatic measurements of optical density by the imaging reader: the growth rate (mOD/h), vmax, and t at vmax, and (2) growth rate (log 10 CFU/h) for the manual preparation of smears after incubation with the respective pathogens and media. Differences between different media were localized by Tukey's *t*-test ($P < 0.05$).

Results

Bacterial growth in different types of cow milk

Mean growth rates (log 10 CFU/h) of *E. coli*, *S. aureus* and *S. uberis* during 8 h of growth in TB and different types of UHT milk are presented in Table 1. Mean growth rate of *E. coli* was higher ($P < 0.05$) in TB compared to whole milk, whereas growth of *S. aureus* and *S. uberis* did not differ between whole milk and TB. Growth rates in skim milk, fat-reduced milk, whole milk and cream did not differ for all three bacteria. There was a numerical increase in *S. aureus* growth in milk with 1.5% fat compared to 2.5% fat but this was not statistically significant ($P = 0.057$). Growth of *E. coli* did not differ in whole milk and lactose-free milk compared to high-protein milk, respectively, but there was a non-significant ($P = 0.058$) numerical increase in growth in lactose-free milk compared to whole milk. Growth of *S. aureus* did not differ in whole milk, high-protein milk, and lactose-free milk. Growth of *S. uberis* was increased in high-protein milk if compared to whole milk ($P < 0.01$) or lactose-free milk ($P < 0.01$) but did not differ between whole milk and lactose-free milk.

Bacterial growth in diluted and supplemented TB

These experiments were only performed with *E. coli* and *S. aureus*, because the growth of *S. uberis* could not be determined by optical density due to aggregation. Mean growth rates (mOD/h) in native and modified TB during 16 h of growth are shown in Table 2. Maximal growth rates (data not shown) highly correlated with mean growth rates ($r = 0.97$). Tenfold and fourfold dilutions of TB decreased growth rates ($P < 0.001$) of *E. coli* and *S. aureus* compared to pure TB, respectively.

The supplementation of diluted TB with various concentrations of carbohydrates (lactose, glucose) did not affect mean growth rates, or t at vmax (data not shown), of both bacteria compared to growth in diluted TB alone. Thus, the growth rates of *E. coli* and *S. aureus* in diluted TB enriched with various carbohydrates were still reduced ($P < 0.001$) as compared to the growth in pure TB. The addition of amino acids to diluted TB in low concentrations did not affect growth rates in both bacteria. However,

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Table 2. Growth rates *[mean ± SD]* of *Escherichia coli* and *Staphylococcus aureus* during 16 h of growth in pure thioglycolate broth (TB) and in diluted TB enriched with specific nutrient additives

	Growth Rate [mOD/h]	
Growth medium	*Escherichia coli*	*Staphylococcus aureus*
Carbohydrates		
TB pure	68.67 ± 0.33[a]	66.33 ± 1.45[a]
TB diluted*	37.00 ± 2.52	34.67 ± 2.85
TB diluted + lactose [1%]	37.33 ± 2.60	32.33 ± 3.84
TB diluted + lactose [5%]	34.00 ± 1.73	39.00 ± 1.15
TB diluted + glucose [1%]	30.00 ± 1.00	39.00 ± 2.31
TB diluted + glucose [5%]	30.33 ± 0.33	38.33 ± 0.33
Nitrogen sources I		
TB pure	65.67 ± 1.57[a]	55.17 ± 2.99[a]
TB diluted	28.78 ± 1.27	28.83 ± 1.08
TB diluted + amino acid standard solution [0.5%]	33.33 ± 3.18	30.00 ± 2.08
TB diluted + amino acid standard solution [1%]	33.67 ± 3.38	26.33 ± 1.20
TB diluted + amino acid standard solution [3%]	33.00 ± 3.00	15.67 ± 0.88[b]
TB diluted + amino acid standard solution [5%]	36.67 ± 2.96	12.00 ± 1.53[b]
TB diluted + amino acid standard solution [10%]	06.33 ± 4.48[b]	*No data*
Nitrogen sources II		
TB pure	58.83 ± 0.98[a]	63.00 ± 0.93[a]
TB diluted	36.00 ± 1.63	33.67 ± 0.92
TB diluted + urea [0.5%]	37.83 ± 1.56	33.17 ± 1.45
TB diluted + urea [1%]	38.50 ± 1.45	28.83 ± 1.19
TB diluted + urea [3%]	32.17 ± 1.99	14.50 ± 0.92[b]
TB diluted + ammonium sulphate [0.5%]	39.00 ± 2.24	01.83 ± 0.17[b]
TB diluted + ammonium sulphate [1%]	39.33 ± 1.78	<0.01 ± 0.00[b]
TB diluted + ammonium sulphate [3%]	32.17 ± 0.95	<0.01 ± 0.00[b]
TB diluted + L-glutamine [0.5%]	42.67 ± 1.02	31.33 ± 1.12
TB diluted + L-glutamine [1%]	45.00 ± 0.45[b]	24.50 ± 2.70[b]
TB diluted + L-glutamine [1.5%]	45.17 ± 0.60[b]	25.00 ± 4.49[b]
Minerals		
TB pure	63.71 ± 1.07[a]	57.33 ± 1.74
TB diluted	31.57 ± 1.05	27.92 ± 0.89
TB diluted + trace element solution [10%]	32.83 ± 2.36	48.50 ± 0.81[b]
TB diluted + bulk element solution [10%]	45.33 ± 1.36[b]	59.17 ± 0.95[bc]
TB diluted + bulk and trace element solutions [5% of each]	50.67 ± 1.86[b]	49.67 ± 1.33[b]
TB diluted + bulk and trace element solutions [7.5% of each]	49.00 ± 2.08[b]	47.67 ± 5.36[b]
B vitamins		
TB pure	62.75 ± 0.86[a]	58.47 ± 1.64[a]
TB diluted	31.80 ± 1.16	25.73 ± 1.67
TB diluted + aminobenzoic acid [10%]	30.67 ± 2.43	24.00 ± 0.52
TB diluted + biotin [10%]	34.33 ± 1.93	30.83 ± 1.70
TB diluted + folic acid [10%]	34.00 ± 1.48	32.00 ± 1.00
TB diluted + niacinamide [10%]	34.11 ± 2.23	31.33 ± 0.33

(*Continued*)

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Table 2. (*Continued.*)

Growth medium	Growth Rate [mOD/h]	
	Escherichia coli	*Staphylococcus aureus*
TB diluted + pantothenic acid [10%]	34.33 ± 2.04	42.78 ± 1.38[b]
TB diluted + pyridoxal hydrochloride [10%]	31.00 ± 3.51	28.67 ± 2.06
TB diluted + pyridoxamine dihydrochloride [10%]	33.33 ± 3.18	18.67 ± 0.67
TB diluted + pyridoxine hydrochloride [10%]	33.00 ± 2.07	18.50 ± 0.81
TB diluted + riboflavin [10%]	31.50 ± 2.09	18.83 ± 1.28
TB diluted + thiamine hydrochloride [10%]	33.17 ± 2.01	30.17 ± 1.30
TB diluted + lipoic acid [10%]	34.11 ± 2.23	33.56 ± 3.98

*TB was diluted to 1 : 10 in case of E. coli, and 1 : 4 in S. aureus, respectively.
[a]Significant differences to all variants of diluted TB, with or without additives ($P < 0.05$).
[b]Significant differences of diluted TB with additive to diluted TB without additives ($P < 0.05$).
[c]Only variant which is not significantly different to pure TB ($P > 0.05$).

the addition of 10% amino acid standard solution to diluted TB decreased growth rate ($P < 0.001$) and increased t at vmax ($P < 0.001$) of *E. coli* compared to growth in diluted TB without additives. Growth of *S. aureus* was decreased by the addition of 3% ($P < 0.01$) and 5% ($P < 0.001$) of amino acid standard solution, while t at vmax was increased ($P < 0.05$) when amino acid standard solution was added at a concentration of 1% or more. The addition of urea and ammonium sulphate to diluted TB did not affect growth rate of *E. coli*, whereas both additives decreased t at vmax ($P < 0.001$) when added at a concentration of 3%. The addition of 0.5% L-glutamine caused resulted in a non-significant ($P = 0.068$) numerical increase in growth of *E. coli*, while higher concentrations [1% and 1.5%] increased the growth rates significantly ($P < 0.01$). Time at vmax of *E. coli* was not affected by the addition of L-glutamine. Growth of *S. aureus* was decreased when diluted TB was enriched by urea [3%] ($P < 0.001$), ammonium sulphate [0.5%, 1% and 1.5%] ($P < 0.001$), and L-glutamine [1% and 1.5%] ($P < 0.05$). The addition of urea increased t at vmax of *S. aureus* non-significantly ($P = 0.057$) when added in high concentration [3%] to diluted TB compared to diluted TB without additives. As no nitrogen containing additive increased the growth rate of *E. coli* or *S. aureus* in diluted TB, the growth of both bacteria in diluted TB with any kind of nitrogen sources remained decreased ($P < 0.001$) as compared to growth in pure TB.

Growth rate of *E. coli* was not affected by the addition of trace elements only to diluted TB compared to diluted TB without additives. However, growth rate of *E. coli* was increased by the addition of bulk element solution [10%] ($P < 0.001$), as well as by the addition of combined bulk and trace element solutions [5% and 7.5% of each, respectively] ($P < 0.001$). Addition of the bulk element solution to diluted TB increased t at vmax of *E. coli* ($P < 0.001$) compared to growth in diluted TB without additives. Despite growth promotion of *E. coli* in diluted TB by added bulk element solution or by combined bulk and trace element solutions, growth in diluted TB with any kind of mineral supplementation remained decreased ($P < 0.001$) as compared to growth in pure TB. Growth curves of *E. coli* in TB, diluted TB, and diluted TB enriched with minerals are shown in Figure 1a. The growth rate of *S. aureus* in diluted TB was increased when trace element solution [10%] ($P < 0.001$), bulk element solution [10%] ($P < 0.001$), or combined bulk and trace element solutions in both concentrations [5% and 7.5% of each] ($P < 0.001$) were added. When combined bulk and trace element solutions were added to diluted TB at a concentration of 7.5% each, t at vmax was increased ($P < 0.001$) compared to diluted TB without additives. After supplementation of diluted TB with trace element solution [10%] ($P < 0.001$), or combined bulk and trace element solutions in both concentrations [5 and 7.5% of each, respectively] ($P < 0.05$), growth of *S. aureus* remained decreased compared to growth in pure TB. However, only the addition of bulk element solution [10%] to diluted TB resolved the decreased growth of *S. aureus*, resulting in non-differing growth compared to growth in pure TB. Growth curves of *S. aureus* in TB, diluted TB, and diluted TB enriched with minerals are shown in Figure 1b.

None of the vitamin B additives affected growth rate of *E. coli* in comparison to diluted TB without additives. However, added aminobenzoic acid and pyridoxal hydrochloride solutions [10%] increased t at vmax ($P < 0.001$). By the addition of pantothenic acid solution [10%] to diluted TB, growth rate ($P < 0.001$), and t at vmax ($P < 0.001$) of *S. aureus* increased. Additionally, there was a non-significant ($P = 0.052$) numerical increase in growth rate of *S. aureus* if diluted TB was enriched with lipoic acid solution [10%], while the addition of pyridoxine hydrochloride solution [10%] or riboflavin solution [10%] resulted in small (non-significant: $P = 0.053$ and $P = 0.063$ respectively) decreases in growth if compared to diluted TB without additives. Growth of *E. coli* and *S. aureus* in diluted TB with any kind of vitamin B additions was still decreased ($P < 0.001$) as compared to growth in pure TB.

Bacterial growth in diluted and supplemented milk

Mean growth rates (log 10 CFU/h) during 8 h of growth in milk and diluted whole milk with additives of *E. coli*, *S. aureus* and *S. uberis* are presented in Table 3. The growth of *E. coli*, *S. aureus* and *S. uberis* in diluted whole milk (1 : 20 for *E. coli*, 1 : 10 for *S. aureus* and *S. uberis*) was decreased ($P < 0.001$) compared to the growth in whole milk, regardless whether with or without nutrient additives. The addition of lactose [4%] or bulk and trace element solution [5% of each] to diluted whole milk did not affect growth rates of all three bacteria. The addition of MEM amino acid solution [3%] decreased growth rate of *S. aureus* ($P < 0.001$) but did not affect growth rates of *E. coli* and *S. uberis*. The addition of urea [3%] decreased growth rates of *E. coli*

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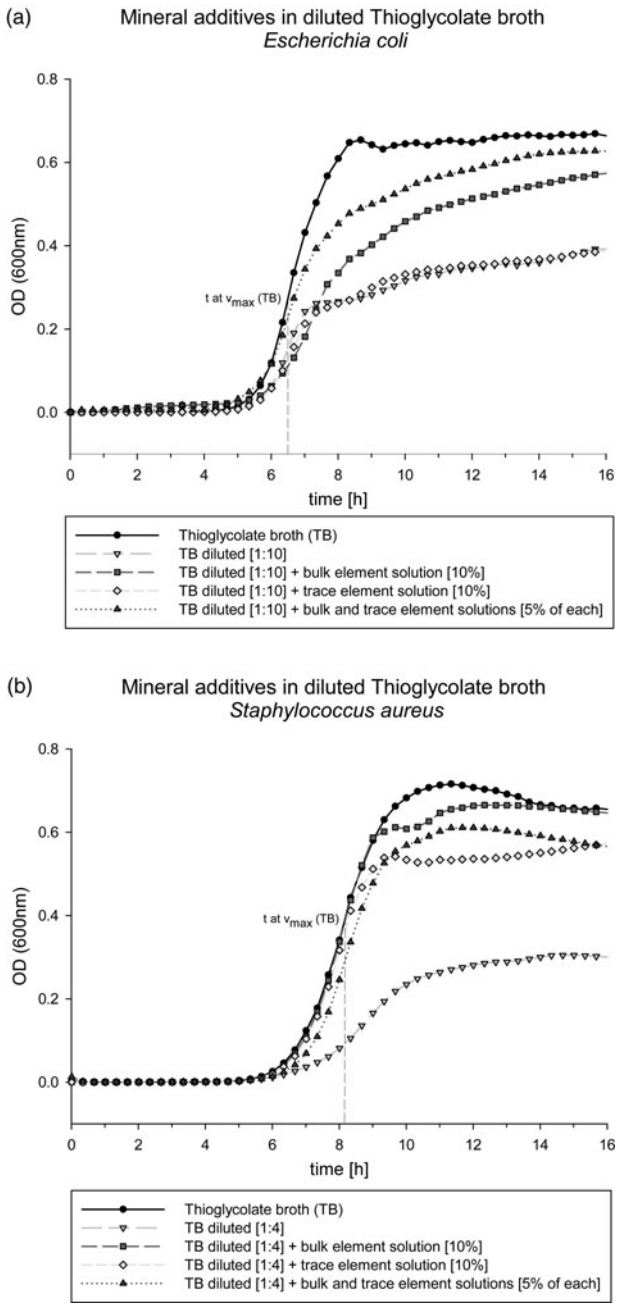

Fig. 1. Growth (increase of OD measured at 600 nm) of *Escherichia coli* and *Staphylococcus aureus* in Thioglycolate broth (TB), diluted TB, and diluted TB with mineral additives. The variable 'time at maximal growth rate' (t at vmax) is visualized in an exemplary growth curve of *Escherichia coli* and *Staphylococcus aureus* in non-diluted TB.

($P < 0.001$) and *S. aureus* ($P < 0.001$) but did not affect growth rate of *S. uberis*. The addition of vitamin B mixture solution [10%] to diluted whole milk revealed decreased growth rates of *E. coli* ($P < 0.001$) and *S. aureus* ($P < 0.001$) but an increased growth rate of *S. uberis* ($P < 0.05$).

Discussion

Only a few reports are available that indicate a relationship between raw milk components and pathogen growth (Kornalijnslijper *et al.*, 2003; Boerhout *et al.*, 2016; Eisenberg *et al.*, 2016; Fijałkowski *et al.*, 2017). To investigate the importance of compositional differences of milk for bacterial growth, independent of the contribution of the immune system, we followed bacterial growth in UHT treated milk and TB supplemented with various nutrients *in vitro* under controlled experimental settings. Using commercially available UHT treated milk instead of raw milk ensured the absence of an accompanying bacterial flora and enabled the repeatability of experiments due to constant milk composition. Furthermore, heat treatment of milk can reduce the function of inhibiting factors of bacterial growth, as demonstrated in raw milk for *S. uberis* (Kliem and Hillerton, 2002). Additionally, the influence of extreme nutrient shifts in various commercially available dairy products on bacterial growth could be investigated.

We selected three pathogens that were isolated from mastitis cases and that belong to the most important bacteria causing mastitis in dairy cows. They represent both Gram-positive and Gram-negative bacteria as well as cow- and environment-associated pathogens.

General aspects of pathogen growth in milk and TB

Due to aggregation, investigations of *S. uberis* were limited to experiments using quantitative plating. To achieve a similar growth limitation between pathogens before adding the experimental nutrients, we had to dilute the TB more for *E. coli* [1 : 10] compared to *S. aureus* [1 : 4], as well as we diluted the milk more for *E. coli* [1 : 20] than for *S. aureus* and *S. uberis* [1 : 10]. This could indicate that *E. coli* can better adapt to a nutrient-poor environment than *S. aureus* and *S. uberis*. Pathogen growth in diluted milk was more limited for *S. uberis* compared to *S. aureus*, indicating that out of the three investigated pathogens *S. uberis* is the most demanding pathogen in terms of nutrient requirements. Although different technical approaches and durations for bacterial growth were used among experiments with milk and TB, one could speculate that milk better meets the nutritional requirements of mastitis pathogens, as a higher dilution was necessary for milk than for TB to achieve a similar growth limitation. Although the growth in undiluted whole milk and TB seemed similar at first, the addition of the same amount of exogenous nutrients to diluted milk and TB finally resulted in different growth rates for the same pathogen. This implies that dilution leads to different growth-limiting components in milk and TB.

Contribution of milk fat to pathogen growth

Milk fat content is subject to the greatest fluctuations during the course of lactation in dairy cows. The content of milk fat is highest immediately after parturition, decreases likely due to dilution effects concomitantly with increasing milk yield, and increases again with decreasing milk yield towards the end of lactation (Stanton *et al.*, 1992; Silvestre *et al.*, 2009). Elevated milk fat in early lactation is a consequence of abundant lipolysis from fat depots due to the transient energy deficiency at simultaneously low lactose and protein synthesis (Gross *et al.*, 2011). For drying-off high-yielding cows, it is a common practice to limit milk yield by feed restriction. Similar to the energy deficiency in early lactation, the nutrient withdrawal prior to dry-off can additionally increase the milk fat content as a consequence of adipose tissue mobilization as shown by elevated NEFA concentrations in a study by Ollier *et al.* (2015). High-risk periods of mastitis are closely overlapping with periods of elevated milk fat contents.

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Table 3. Growth rates *[mean ± SD]* of *Escherichia coli, Staphylococcus aureus* and *Streptococcus uberis* during 8 h of growth in pure milk and in diluted milk enriched with specific nutrient additives

	Growth rate [log_{10} CFU/h]		
Growth medium	*Escherichia coli*	*Staphylococcus aureus*	*Streptococcus uberis*
Whole milk	0.79 ± 0.01[a]	0.71 ± 0.00[a]	0.64 ± 0.01
Whole milk diluted*	0.65 ± 0.00	0.48 ± 0.00	0.23 ± 0.02
Whole milk diluted + lactose [4%]	0.62 ± 0.01	0.48 ± 0.01	0.21 ± 0.01
Whole milk diluted + MEM amino acid solution [3%]	0.68 ± 0.01	0.38 ± 0.01[b]	0.38 ± 0.08
Whole milk diluted + urea [3%]	0.51 ± 0.01[b]	0.35 ± 0.01[b]	0.12 ± 0.01
Whole milk diluted + bulk and trace element solution [5% of each]	0.66 ± 0.01	0.46 ± 0.01	0.23 ± 0.09
Whole milk diluted + vitamin mixture solution [10%]	0.55 ± 0.01[b]	0.35 ± 0.01[b]	0.49 ± 0.01[bc]

*Whole milk was diluted to 1 : 20 in case of E. coli, and 1 : 10 in S. aureus and S. uberis, respectively.
[a]Significant differences to all variants of diluted milk, with or without additives ($P < 0.05$)
[b]Significant differences of diluted whole milk with additive to diluted whole milk without additives ($P < 0.05$).
[c]Only variant which is not significantly different to whole milk ($P > 0.05$).

Based on that observation, we rather expected a positive association between increased milk fat and bacterial growth, even if the presence of free fatty acids and monoglycerides were described to have antibacterial effects (Isaacs *et al.*, 1995; van Hooijdonk *et al.*, 2000). It was, therefore, surprising that bacterial growth was not altered despite fat contents ranging from skim milk (0.1% fat) to cream (25% fat). Our findings are consistent with results from other *in vitro* studies (Kornalijnslijper *et al.*, 2003, Fijałkowski *et al.*, 2017 for *E. coli* and *S. aureus*, respectively) that did not find significant associations between fat content of raw milk and *in vitro* growth of mastitis pathogens. Thus, the elevated risk of IMI coinciding with elevated milk fat content cannot be attributed to effects of fat on bacterial growth. The results also indicate that different bacterial growth in different areas of the mammary gland due to different fat content is unlikely.

Contribution of carbohydrates to pathogen growth

Microbial growth depends on both nitrogen and carbon sources. Based on the glucose-lactose diauxie described by Monod (1942), we supposed an accelerated growth in lactose-free milk, as the lactose is already cleaved into the monosaccharides glucose and galactose. Although bacterial growth was not increased in lactose-free milk in our study, the early phase of growth could have been accelerated until glucose supply had been used up, followed by the switch to galactose as energy source with a slower growth, which finally resulted in a similar mean growth rate over 8 h compared to the growth in whole milk. Similarly, the addition of lactose and glucose to diluted milk and TB, respectively, did not increase microbial growth. Apparently, carbohydrates were not growth-limiting factors. An earlier study of Fijałkowski *et al.* (2017) confirms our finding on the lack of lactose effects for growth of *S. aureus*. Considering the range of lactose concentrations added *in vitro*, the rather constant milk lactose concentrations *in vivo* can be excluded as a cause of different pathogen growth rates.

Contribution of nitrogen sources to pathogen growth

The high protein milk, a lactose-free skim milk with doubled protein content compared to whole milk, promoted growth of *S. uberis* in comparison to growth in whole milk. As our results revealed a negligible influence of fat and carbohydrate contents on bacterial growth, we assume that the higher protein availability in the high protein milk promoted the growth of *S. uberis*. Besides milk fat, also the protein content in milk is highest during the two marked high-risk periods for IMI (at the start and end of lactation; reviewed by Burton and Erskine, 2003; Silvestre *et al.*, 2009). A positive association between milk protein content and bacterial growth might be assumed. However, the protein-enriched milk for our *in vitro* experiments contained twice as much protein compared to regular milk. With the exception of colostrum, milk protein content of dairy cows is only subject to minor fluctuations (Silvestre *et al.*, 2009). The *in vitro* growth of *E. coli* and *S. aureus* in protein-enriched milk did not differ from growth in whole milk. Possibly, the slightly lower content of carbohydrates in the high protein milk (45 g/l *v.* 50 g/l) could be limiting for microbial growth and, therefore, conceal a potential promoting effect of milk protein as nitrogen source. However, pathogen growth in cream, which contained less lactose (40 g/l) and protein (25 g/l), did not differ from growth in whole milk. This finding confirms that neither lactose nor milk protein are limiting for bacterial growth in the range we investigated. Based on our results, we speculate that growth of *E. coli* and *S. aureus* in milk is not affected by possible fluctuations of the milk protein content in dairy cows. Further assumptions in this direction were recently made by Fijałkowski *et al.* (2017), who found no association between *in vitro* growth of *S. aureus* and the protein content of raw milk. Nevertheless, potential growth promoting effects of *S. uberis* by a greater availability of milk protein warrants further investigation.

The addition of amino acids to diluted TB decreased growth of *E. coli* and *S. aureus* dependent on concentration and pathogen. Growth of *S. aureus* was also reduced in diluted milk by the addition of amino acids. Although we did not assess the pH value before and after the addition of amino acids, we assume that the pH value might have declined and affected bacterial growth.

Urea is described to have bactericidal effects (Weinstein and McDonald, 1945; Schlegel *et al.*, 1961). Our own results showed that bacterial growth is particularly limited for *S. aureus* at high urea concentrations. Commonly, milk urea content in dairy cows ranges within 20–30 mg/dl. Even greater urea concentrations *in vivo* do not reach the level where pathogen growth is restricted.

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Other nitrogen sources such as ammonium sulphate were described to be utilized by *E. coli* (Bandary *et al*., 2016), though our results on varying ammonium sulphate and L-glutamine concentrations revealed contradictory findings for the different pathogens. It can only be speculated that nitrogen sources are abundantly available, and that experimental addition exceeded nutritional requirements of bacteria.

Contribution of minerals and vitamins to pathogen growth

The addition of bulk and trace elements promoted growth in *E. coli* and *S. aureus*. In particular, bulk elements in *S. aureus* turned out to be growth limiting, as growth retardation after initial limitation in diluted media was fully abolished by the re-addition of bulk elements. Sodium, calcium, magnesium, and phosphorus contents in milk are described to vary contrariwise to the milk yield during lactation. Potassium content is closely related to milk yield (Manuelian *et al*., 2018; Visentin *et al*., 2018). Thereby, the mineral content seems to rise especially towards the end of the lactation and could potentially contribute to the greater IMI risk around dry-off.

Out of the different B vitamins, only pantothenic acid (also known as vitamin B5) was stimulatory for growth of *S. aureus* when added to diluted TB. Sevag and Green (1944) described pantothenic acid as an essential vitamin in the absence of tryptophan in two strains of *S. aureus*. The content of pantothenic acid in cow milk is rather high with about 3.5 mg/l (Schlimme and Buchheim, 1996). However, the vitamin mixture consisting of eleven types of B vitamins decreased growth rates of *E. coli* and *S. aureus* in diluted milk, whereas growth rate in *S. uberis* was increased. Our result of the stimulatory effects of vitamins in *S. uberis* is in agreement with an earlier study where the addition of vitamins intensified the growth of *S. uberis* in skimmed milk whereas the addition of minerals had no effect (Kliem and Hillerton, 2002). The contradictory results for the growth of different pathogens in diluted milk and other media types confirms the different nutrient requirements of different bacteria. Therefore, depending on the pathogen, different nutrients may be the first to be limiting. Likewise, interactions among imbalances of vitamins and minerals could further explain our divergent findings. Unfortunately, only a few reports about the vitamin B requirements of bacteria, especially of mastitis pathogens are available.

In conclusion, results of the present study indicate that only fundamental experimental changes of milk composition affect bacterial growth, but not physiologically relevant fluctuations during the course of lactation. The increased susceptibility to IMI at different stages of lactation is, therefore, rather the consequence of the interaction of pathogens with metabolic and immunological systems of the animal than of changes in milk composition.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029921000133.

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