A centrifugation-based clearing method allows high-throughput acidification and growth-rate measurements in milk

Sieze Douwenga,^{1,2} Patrick Janssen,^{1,3} Bas Teusink,^{1,2} and Herwig Bachmann^{1,2,3*}

¹Ti Food and Nutrition, 6709 PA, Wageningen, the Netherlands

²Systems Biology Lab, Vrije Universiteit Amsterdam, 1081 HZ, Amsterdam, the Netherlands

³Health Department, NIZO Food Research, 6718 ZB, Ede, the Netherlands

ABSTRACT

The turbidity of milk prohibits the use of optical density measurements for strain characterizations. This often limits research to laboratory media. Here, we cleared milk through centrifugation to remove insoluble milk solids. This resulted in a clear liquid phase, termed milk serum, in which optical density measurements can be used to track microbial growth until a pH of 5.2 is reached. At pH 5.2 coagulation of the soluble protein occurs, making the medium opaque again. We found that behavior in milk serum was predictive of that in milk for 39 *Lactococcus lactis* ($R^2 = 0.81$) and to a lesser extent for 42 *Lactiplantibacillus plantarum* (formerly *Lactobacillus plantarum*; $R^2 = 0.49$) strains. Hence, milk serum can be used as an optically clear alternative to milk for comparison of microbial growth and metabolic characteristics. Characterization of the growth rate, specific acidification rate for optical density at a wavelength of 600 nm, and the amount of acid produced per unit of biomass for all these strains in milk serum, showed that almost all strains could grow in milk, with higher specific acidification and growth rates of *Lc. lactis* strains compared with *Lb. plantarum* strains. Nondairy *Lc. lactis* isolates had a lower growth and specific acidification rate than dairy isolates. The amount of acid produced per unit biomass was relatively high and similar for *Lc. lactis* dairy and nondairy isolates, as opposed to *Lb. plantarum* isolates. *Lactococcus lactis* ssp. *lactis* showed slightly lower growth and acidification rates when compared with ssp. *cremoris*. For *Lc. lactis* strains a doubling of the specific acidification rate occurred with a doubling of the maximum growth rate. This relation was not found for *Lb. plantarum* strains, where the acidification rate remained relatively constant across 39 strains with growth rates ranging from 0.2 h⁻¹ to 0.3 h⁻¹. We conclude that milk serum

is a valuable alternative to milk for high-throughput strain characterization during milk fermentation.

Key words: milk serum, milk clearing, growth rate, optical density, acidification rate

INTRODUCTION

Microbes play a key role in the dairy industry, with 37.7% of the produced milk (172.2 billion kg) being fermented to cheese in the European Union alone in 2018 (Silva et al., 2019). The starter cultures used during fermentation are often composed of numerous strains; they are responsible for the conversion of lactose into lactic acid and production of flavor compounds, and they affect the texture of the final product. There are big genotypic and phenotypic differences between microorganisms in starter cultures (Siezen et al., 2011; Wels et al., 2019), which causes differences in specific acidification rates, growth rates, and lag phases during industrial fermentations of milk. Quantifying these traits for different strains in milk can be challenging as the turbidity of milk does not allow for online monitoring of cell growth, which is typically done through optical density (OD) measurements.

Multiple solutions to determine the cell concentration in milk exist, one of which is the standard plate count. However, this process is laborious and typically takes more than a day, and counts can be imprecise. Fowler et al. (1978) report 18% cell count variation between analysts, and 7% variation for repetitions by one analyst. However, precision can improve with experience and training of the analyst (Fruin et al., 1977). Similar findings were also reported in (Jarvis et al., 2007). A faster (≤ 1 h) method was developed by (Gunasekera et al., 2000). Casein micelles are enzymatically digested, allowing them to dissolve. Cells are then separated from the milk by centrifugation. This allows cell staining and subsequent cell counting using flow cytometry. The digestive enzyme must be carefully selected, however, as not all cells remain culturable when savinase is used (Smith et al., 2003; Meisel et al., 2011). In an even faster protocol (~ 5 min), the milk is cleared by

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*Corresponding author: Herwig.Bachmann@nizo.com

mixing it with a solution of 0.2% sodium hydroxide and 0.2% EDTA, dissolving the suspended casein micelles. This allows OD measurements as an indirect measure for cell density (Bachmann et al., 2012). Meisel et al. (2011) separate cells from milk using buoyant density centrifugation. Their method is relatively quick (≤ 1 h), and does not require chemicals, but it recovers only 35% of microorganisms in the milk. Hence, although cell densities can be determined in milk, current methods can be labor intensive, inaccurate, give the readout with long delays, are destructive to the sample, and are therefore unsuited for continuous online monitoring.

Although continuous growth monitoring of the cell count is currently difficult, acidification of the environment can be measured continuously and is used to detect metabolic activity of lactic acid bacteria. Acidification can be monitored either directly with pH probes or indirectly through the use of fluorescent pH-sensitive dyes. The dyes allow acidification tracking of many cultures in parallel using multiwell plates (John et al., 2003; Wegkamp and Reemst, 2018; Nugroho et al., 2020). Acidification is a function of the cell density and biomass specific acidification rate. Therefore, acidification is only a proxy for growth and cannot be used to determine the exact growth rate, lag phase, lactic acid production per unit biomass, or specific acidification rate of the culture being analyzed.

We developed a protocol to clear milk and use the obtained serum for the simultaneous high-throughput online analysis of both the growth rate through OD and the pH through the use of a fluorescent probe. This facilitated accurate analysis of growth rate, lag phase, and specific acidification rates in milk. The protocol was validated for 39 *Lactococcus lactis* and 42 *Lactiplantibacillus plantarum* strains (the latter being formerly known as *Lactobacillus plantarum*; Zheng et al., 2020), showing that acidification profiles in milk and milk serum were similar.

MATERIALS AND METHODS

Strains and Media

The strains used in this study are listed in Table 1. All strains were taken from the NIZO culture collection (NIZO). The majority of *Lc. lactis* strains are based on a set described by Rademaker et al. (2007) and Brooijmans (2008). The *Lb. plantarum* strains are described in (Molenaar et al., 2005; Smits et al., 2005; Siezen et al., 2010). Skimmed milk (Tritium Microbiologie) from the same batch was used for all growth experiments. This milk was reconstituted from powdered skim milk and autoclaved at 115°C for 10 min. The term milk

throughout the paper refers to skim milk prepared this way.

Lactococcus lactis and *Lb. plantarum* strains were precultured in a 96-well plate (Greiner Bio-One) at 30°C and 37°C for 16 h, in 0.5% glucose M17 (Tritium Microbiologie; Oxoid) and MRS broth (Tritium Microbiologie; Merck), respectively. For acidification measurements, milk and milk serum were inoculated with 0.2% vol/vol of the stationary preculture.

Milk Clearing and Acidification Measurements

Milk serum was prepared by centrifuging a total of 650 mL milk divided over 16 sterile metal centrifuge tubes, with 40 mL of milk in each tube. A Beckman Coulter Avanti J26XP centrifuge was run with a Beckman Coulter JA-14.50 rotor at $35,000 \times g$ at 10°C for 1 h, maximum acceleration, and slow deceleration. This results in solid precipitate below and a thin layer of solids on top of the supernatant. The top 30 mL of the supernatant was harvested immediately upon stopping of the centrifuge. Care was taken to transfer as little as possible of the top layer of solids by sticking the pipet through this surface before aspiration, although this has little effect on the turbidity of the milk serum. The harvested supernatant was then again divided over sterile centrifuge tubes, 40 mL/tube, and centrifuged with the same settings. This time 25 mL supernatant was removed and pooled in a sterile bottle. The pooled milk serum (supernatant) was stored at 4°C until further use. For all experiments milk serum from the same batch was used.

To be able to measure pH through fluorescence, both milk serum and milk were supplemented with 1:1,000 of 0.4% wt/vol 5(6)-Carboxyfluorescein BioReagent (Sigma-Aldrich) suspended in 10% vol/vol ethanol in demineralized water [final 5(6)-carboxyfluorescein concentration of 0.0004% wt/vol; Bachmann et al., 2013]. Carboxyfluorescein fluorescence positively correlates with the pH. Milk and milk serum from the same batch were used for all experiments.

After preculture, strains were diluted 20× in phosphate buffer (100 mM, pH 6.5) and subsequently another 26× in milk or milk serum (520× final dilution), in 2 new 96-well (300 μ L/well) plates (Greiner Bio-One). The inoculated milk and milk serum were transferred to a black 384-well plate (100 μ L/well) with a transparent bottom (Greiner Bio-One). The plate was placed in a plate reader (Tecan Safire II) at 30°C without shaking, where fluorescence (excitation: 485/20 nm; emission: 520/20 nm) and OD at a wavelength of 600 nm (**OD₆₀₀**) were measured every 6 min for 42 h. The milk and milk serum pH and proton production

Table 1. *Lactococcus* and *Lactiplantibacillus* strains used throughout this study, including their phenotypic characterization and origin¹

Genotype	Phenotype	Strain	Origin
<i>Lactococcus lactis</i> ssp. <i>cremoris</i>	<i>lactis</i>	NCDO763	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	R1	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	ML8	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	ATCC19435	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	SK11	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>lactis</i>	AM2	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	LMG8526	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>		M20	Nondairy
<i>Lc. lactis</i>	Unknown	M9	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E7	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	Li-1	Nondairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	P2	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	HP	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	AM1	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	KH	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	E8	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	Wg2	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E30	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E33	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E34	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E32	Nondairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>lactis</i>	N41	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	N42	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>		ZK	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	UC317	Dairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>lactis</i>	MG1363	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	E10	Nondairy
<i>Lc. lactis</i>	Unknown	E27	Nondairy
<i>Lc. lactis</i> ssp. <i>cremoris</i>	<i>cremoris</i>	E9	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	LMG8528	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	LMG9446	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>		C17	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i> biovar <i>diacetylactis</i>		B35	Dairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	D53	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	K231	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	K335	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	K336	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	K337	Nondairy
<i>Lc. lactis</i> ssp. <i>lactis</i>	<i>lactis</i>	K338	Nondairy
<i>Lactiplantibacillus plantarum</i>		WCFS1	Nondairy
<i>Lb. plantarum</i>		ATCC14917	Nondairy
<i>Lb. plantarum</i>		MLC43	Dairy
<i>Lb. plantarum</i>		CHEO3	Nondairy
<i>Lb. plantarum</i>		NCTH19-1	Nondairy
<i>Lb. plantarum</i>		NCTH19-2	Nondairy
<i>Lb. plantarum</i>		NCTH27	Nondairy
<i>Lb. plantarum</i>		LD2	Nondairy
<i>Lb. plantarum</i>		NOS140	Nondairy
<i>Lb. plantarum</i>		Q2	Nondairy
<i>Lb. plantarum</i>		H4	Nondairy
<i>Lb. plantarum</i>		H14	Nondairy
<i>Lb. plantarum</i>		L9	Dairy
<i>Lb. plantarum</i>		KOG18	Nondairy
<i>Lb. plantarum</i>		KOG24	Dairy
<i>Lb. plantarum</i>		LMG9208	Nondairy
<i>Lb. plantarum</i>		Lp95	Nondairy
<i>Lb. plantarum</i>		BLL	Nondairy
<i>Lb. plantarum</i>		CECT221	Nondairy
<i>Lb. plantarum</i>		N58	Nondairy
<i>Lb. plantarum</i>		X17	Nondairy
<i>Lb. plantarum</i>		LAC7	Nondairy
<i>Lb. plantarum</i>		LD3	Nondairy
<i>Lb. plantarum</i>		DKO22	Nondairy
<i>Lb. plantarum</i>		299	Nondairy
<i>Lb. plantarum</i>		CIP104440	Nondairy

Continued

Table 1 (Continued). *Lactococcus* and *Lactiplantibacillus* strains used throughout this study, including their phenotypic characterization and origin¹

Genotype	Phenotype	Strain	Origin
<i>Lb. plantarum</i>		SF2A35B	Nondairy
<i>Lb. plantarum</i>		NCIMB12120	Nondairy
<i>Lb. plantarum</i>		CIP104441	Nondairy
<i>Lb. plantarum</i>		CIP1044550	Nondairy
<i>Lb. plantarum</i>		CIP104451	Nondairy
<i>Lb. plantarum</i>		CIP104452	Nondairy
<i>Lb. plantarum</i>		299v	Nondairy
<i>Lb. plantarum</i>		NC8	Nondairy
<i>Lb. plantarum</i>		LM3	Nondairy
<i>Lb. plantarum</i>		LP80	Nondairy
<i>Lb. plantarum</i>		LP85-2	Nondairy
<i>Lb. plantarum</i>		ATCC8014	Nondairy
<i>Lb. plantarum</i>		NCDO1193	Nondairy
<i>Lb. plantarum</i>		CIP102359	Nondairy
<i>Lb. plantarum</i>		CIP104448	Nondairy
<i>Lb. plantarum</i>		Unknown	Dairy

¹All strains were obtained from the NIZO culture collection.

were determined from fluorescence measurements in combination with calibration curves.

Calibration Curves

We determined the relation between fluorescence, pH, proton production, and turbidity (as a cause of increasing casein precipitation at decreasing pH). To this end milk and milk serum [both with 5(6)-carboxy-fluorescein] at varying concentrations of lactic acid were prepared by titrating known amounts of 0.484 M lactic acid into 10 mL of milk and milk serum. The pH was determined using a pH probe (Unitrode, Metrohm AG). The different solutions were added to a black 384-well plate and fluorescence and OD₆₀₀ were measured in a plate reader.

Data Analysis

Data analysis was done using Python v3.7.4. Packages used were: pandas v1.0.3 (McKinney, 2010), numpy v1.18.1 (van der Walt et al., 2011), matplotlib v3.1.2 (Hunter, 2007), seaborn v0.10.1 (Waskom et al., 2020), and scipy v1.4.1 (Virtanen et al., 2020).

The relation of fluorescence to pH and proton production was determined by fitting least squares second order polynomials to the calibration curves for milk and milk serum in each plate (adapted from Nugroho et al., 2020). Numpy's polyfit algorithm was used.

In all cases where OD₆₀₀ was measured in milk serum, before further data analysis the background OD₆₀₀ was subtracted. The background was determined in wells containing sterile milk serum.

Continuous growth rates [$\mu(t)$] were determined using a savitsky-golay filter (Savitzky and Golay, 1964)

on the natural logarithm of the OD₆₀₀ data versus time. The filter was set to fit a first order polynomial over a measurement window of 2 h, returning the first derivative as the growth rate. The maximal growth rate ($\mu_{\max}$) was determined from the continuous growth rates at OD₆₀₀ > 0.024 and at lactic acid concentrations <9.2 mM (to rule out the effect background noise and casein precipitation, respectively). Additionally, at these lactic acid concentrations the pH was higher than 5.5, meaning almost all lactic acid dissociates [pK_a (acid dissociation constant) = 3.8], making lactic acid addition equal to H⁺ addition.

Acid production per unit produced OD₆₀₀ ($Y_{H/OD}$) was determined by fitting least squared first order polynomials to H⁺ versus OD₆₀₀ curves where their relation is linear (at: 0.02 < OD₆₀₀ < 0.08). Numpy's polyfit algorithm was used. Maximum specific acidification rates (qH^+) were determined by multiplying $\mu_{\max}$ with $Y_{H/OD}$. When relevant the squared Pearson correlation coefficient (R^2) and corresponding 2-tailed *P*-value were calculated with the scipy stats pearsonr function, which assumes normally distributed data.

RESULTS

Removing the Insoluble Fraction of Milk Drastically Reduces the Background OD₆₀₀

Milk is too turbid to measure cell growth using OD₆₀₀. We centrifuged the milk at 35,000 × *g* at 10°C for 1 h, took the supernatant and centrifuged this again at the same settings to separate the colloidal milk particles from the serum. This reduced the OD₆₀₀ from >4 (outside the detection range) to 0.38. This OD₆₀₀ is low enough to allow tracking of microbial growth

through periodic OD₆₀₀ measurements. In milk serum, OD₆₀₀ measurements increased linearly with increasing cell density up to an OD₆₀₀ of 0.66. The pelleted particles are likely to contain a lot of micellar casein, and perhaps some other nutrients.

Removal of the Insoluble Fraction of Milk Slightly Reduces the Growth Rate

We wondered if the removal of the colloidal particles affected growth of microorganisms in milk. In addition to OD₆₀₀ measurements, which are only useful for milk serum, we also indirectly monitored pH over time. This was done by adding a pH-sensitive fluorescent probe to milk and milk serum. Calibration curves (Supplemental Figure S1, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021) allowed the conversion of the fluorescence signal to pH profiles and the total amount of protons produced. Normally, the slope of a logarithmic OD₆₀₀ curve can be used to determine the growth rate. In milk, this is not possible, but the H⁺ concentration should also increase logarithmically as long as the H⁺ production per OD₆₀₀ increase is constant in time. Hence, if the insoluble fraction of milk does not contribute to growth, the slopes of logarithmic H⁺ curves in milk and milk serum of each strain are expected to be equal.

Using the methods described above, we determined the difference between acidification in milk and milk serum for 40 *Lc. lactis* and 42 *Lb. plantarum* strains. The lactococcal strains used include dairy and nondairy isolates of subspecies *cremoris* and subspecies *lactis*, to validate the method for a broad range of strains. A comparison of the measurements in milk and milk serum for *Lc. lactis* NCDO763 shows that the pH profiles are very similar (Figure 1A). pH profiles of other strains show a comparable similarity between milk and milk serum, although in some cases the final pH in milk serum is slightly lower (<0.3 pH points) (Supplemental Figure S2, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021). For *Lb. plantarum* strains, the final pH in milk serum is always slightly lower than that in milk (roughly 0.3 pH points; Supplemental Figure S3, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021). As carboxyfluorescein becomes less sensitive to pH changes below pH 5, further pH decreases are difficult to determine (Supplemental Figure S4, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021). The H⁺ production profiles show some differences (Figure 1B), but considering that growth is exponential, these differences can be the result from only minor growth-rate differences. The slopes of the log H⁺ production rates (Figure 1C) differ by around 24% (0.96 h⁻¹ in milk vs. 0.76 h⁻¹ in milk serum). The

Figure 1. pH (A), H⁺ produced (B), log H⁺ (C), optical density at a wavelength of 600 nm (OD₆₀₀) (D), and OD₆₀₀ at pH > 5.4 (E, same data as D, but zoomed in on the y-axis 50 times) versus time for *Lactococcus lactis* NCDO763 grown in milk (orange, n = 4) and milk serum (blue, n = 4). A.U. = absorbance units. Measurement frequency = 10 h⁻¹.

higher cumulative proton production with similar pH profiles indicates that milk has a higher buffer capacity than milk serum. This is consistent with the calibration curve pH versus lactic acid (Supplemental Figure S5, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021).

Growth rates were determined from OD₆₀₀ measurements in the milk serum. For this, only OD₆₀₀ values were used where the amount of H⁺ produced was less than 9 mM (Figure 1D, E). At higher H⁺ levels, casein starts precipitating, resulting in a sharp OD₆₀₀ increase (observed after approximately 7 h for *Lc. lactis* NCD0763; Figure 1D and Supplemental Figure S6, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021).

Based on log H⁺ production rates, there appears to be an effect on growth when the insoluble fraction of milk is removed; behavior in milk serum is predictive of behavior in milk for *Lc. lactis* ($R^2 = 0.81$, $P < 0.001$) and to a lesser extent for *Lactiplantibacillus plantarum* ($R^2 = 0.49$, $P < 0.001$) strains (see Figure 2 and Supplemental Figures S7 and S8, <https://doi.org/10.5281/zenodo.4694160>; Douwenga et al., 2021).

Figure 2. Relations between logH⁺ rates, i.e., the slopes of a logH⁺ curve, in milk (x-axis) and milk serum (y-axis), for *Lactiplantibacillus* (blue) and *Lactococcus* (orange). *Lactiplantibacillus* $R^2 = 0.49$; *Lactococcus* $R^2 = 0.81$. Each point represents a strain average of 4 replicates (in some cases 2–3 replicates when $R^2 < 0.9$ of the linear fit to the logH⁺ data).

Strain Characterization Through OD₆₀₀ and H⁺ Measurements

The OD measurements act as a rough estimate of cell density, as the relation between these measurements and cell density can vary between strains and growth environment. However, OD measurements are useful for rapid screening of large numbers of strains or growth environments. Slower, more accurate methods can be used to further characterize the strains of interest. To illustrate the rapid screening capacity of milk serum, we determined the biological parameters: $\mu_{\max}$, OD₆₀₀-specific acidification rate (qH⁺), and acid produced per unit OD₆₀₀ (i.e., the acid on biomass yield). We studied these parameters for different lactic acid bacteria and different strain origins (dairy and nondairy). All the *Lb. plantarum* strains could grow in milk with low specific acidification rates, regardless of their origin (Figure 3A, B). For *Lc. lactis* ssp *lactis*, their origin did have a large influence in most cases. For most of the nondairy isolates, the growth rate and acidification rate were lower than that of the average dairy isolate (Figure 3A, B). The amount of acid produced per unit biomass, however, remained relatively high for *Lc. lactis* nondairy isolates. This indicates that a major metabolic by-product is still acidic, in contrast to *Lb. plantarum* strains, which produce less acid per unit biomass (Figure 3C). The specific acidification rate increases with the growth rate only for *Lc. lactis*.

The specific acidification and maximal growth rates allowed us to determine whether these 2 parameters correlated for *Lc. lactis* and *Lb. plantarum* strains. Figure 4A shows this relation for all strains tested. For *Lc. lactis*, the specific acidification rate doubles with a doubling in the growth rate, $R^2 = 0.89$, $P = 4E^{-15}$ (Figure 4B). This relation is not present for *Lb. plantarum*, $R^2 = 0.02$, $P = 0.34$ (Figure 4C). Differentiating based on dairy or nondairy origin (Figure 4A), or *cremoris* or *lactis* genotype (Figure 4B) does not appear to play a role in this relationship. Together this data suggests a coupling between growth and acid production in *Lc. lactis*, which is not present in *Lb. plantarum*.

DISCUSSION

We used centrifugation to mechanically remove casein micelles from milk to facilitate direct measurements of microbial growth. We conclude that casein micelle (and other sediment) removal does have a limited effect on *Lc. lactis* and *Lb. plantarum* growth rates or specific acidification rates. The effect is consistently found over many strains but the results indicate that the method is predictive for growth and acidification in milk. This

Figure 3. Boxplots indicating the maximum growth rate (μ_{max}) (A), specific acidification rate (qH^+) (B), and acid on biomass yield (H^+ produced/ OD_{600} produced, where OD_{600} = optical density at a wavelength of 600 nm) (C) for the different species studied. A.U. = absorbance units. The x-axis labels indicate the genotype, where *plantarum* = *Lactiplantibacillus plantarum*, *cremoris* = *Lactococcus lactis* ssp. *cremoris*, and *lactis* = *Lactococcus lactis* ssp. *lactis*. Strains were isolated from dairy (orange) or nondairy (blue) environments. Each point represents a strain average of 4 replicates (in some cases 2–3 replicates when $R^2 < 0.9$ of the linear fit to the log OD_{600} data). Whiskers extend to the first and last data point not defined as an outlier. Outliers are data points that are outside the first and third quartile (box edges) by 1.5 times the interquartile range (distance between box edges).

makes it valuable for high-throughput screening (see Figure 1C and Figure 2).

The effect of our clearing protocol on microbial behavior cannot be fully explained by insoluble casein

removal. Milk serum contains roughly 30% of the total casein at a concentration of 0.8% wt/vol (Rose, 1968). Additionally, Rose (1968) showed that no extra micellar casein dissolves when milk is diluted with protein-

Figure 4. (A) Specific acidification rate (qH^+) versus maximum growth rate (μ_{max}) for all strains consisting of: *Lactiplantibacillus plantarum* (blue) and *Lactococcus lactis* (orange) both nondairy (plus sign) and dairy (circle) isolates. A.U. = absorbance units. (B) The same data as in panel A for *Lactococcus lactis*, with shape annotation indicating the subspecies, and with a linear model (solid lines), the model R^2 and a 2-tailed P -value. (C) The same data as in panel A for *Lactiplantibacillus plantarum*, with a linear model (solid lines). Each point represents a strain average of 4 replicates (in some cases 2–3 replicates when $R^2 < 0.9$ of the linear fit to the log OD₆₀₀ data; OD₆₀₀ = optical density at a wavelength of 600 nm).

free milk dialysate. This suggests that if the amount of casein dissolved in milk were to limit growth, the micellar undissolved casein could not replace the casein consumed from the aqueous phase. Furthermore, (Mistry and Kosikowski, 1985) showed that increasing the amount of dissolved casein does not affect the specific lactic acid production rates or the growth rates of multiple *Lc. lactis* and *L. cremoris* starter cultures (still called *Streptococcus lactis* and *Streptococcus cremoris* at the time; Schleifer et al., 1985). Higher casein concentrations do increase the buffer capacity of milk, allowing a higher growth-arresting lactic acid concentration to be reached, in addition to higher final cell density and higher final pH (Mistry and Kosikowski, 1985). This is consistent with our observations between milk serum (low buffer capacity) and milk (high buffer capacity). However, the decreased buffer capacity should only affect cell behavior near the end of the fermentation, when the pH drops. We cannot exclude that other relevant insoluble compounds were removed during milk clearing. One fraction that was likely reduced during milk clearing and which could affect bacterial growth is milk fat. The role of fatty acids in growth medium is demonstrated in MRS, a standard growth medium for many lactobacilli where Tween is added as a source of fatty acids. However, Tween was also shown to negatively affect growth of *Lactobacillus delbrueckii* in MRS (Partanen et al., 2001).

To exclude effects of unknown interactions between the carboxyfluorescein with milk solids we based our conversions from fluorescence signals to produced protons on calibration curves that were obtained in either milk or milk serum. Overall, our data shows good correlation between growth and acidification in milk serum and milk.

Literature describes several studies that assessed growth of lactic acid bacteria in milk (Siezen et al., 2010; Bayjanov et al., 2013). In Bayjanov et al. (2013) a qualitative growth score of 0, 1, or 2 was given after an unknown incubation time in milk at 30°C. In Siezen et al. (2010), a single growth measurement was determined once after 48 h of incubation. Although these methods provide valuable information for genotype-phenotype matching, they do not accurately assess how well different strains grow on milk. Ma et al. (2016) determined cell densities every 4 h by standard plate count for a subset of strains and conditions. The lack of more precise growth data in milk, such as quantitative rather than qualitative data, with more time points and not just endpoint measurements, is possibly due to a lack of appropriate measurement methods, emphasizing the value of the method presented here.

Our finding that all *Lb. plantarum* strains we tested were able to grow in milk is in contrast with results

reported by (Siezen et al., 2010). However, a more detailed study tested *Lb. plantarum* WCFS1, ST-III, and LPHS and found growth and stationary culture pH values consistent with our study (Ma et al., 2016). Furthermore, the levels of free amino acids can vary between milk batches and increase by drying, resuspension, and sterilization of the milk (Menefee et al., 1941; Berg, 1993), which could also cause differences between various studies.

Dairy isolates are suggested to be more specialized for growth on milk than nondairy isolates (Kelly et al., 2010; Bachmann et al., 2012; Wels et al., 2019). Similarly, we see higher growth and specific acidification rates for *Lc. lactis* dairy isolates.

We found that the acidification rate doubles with a doubling in the growth rate for *Lc. lactis*. This makes sense when considering that both hetero- and homofermentative catabolic routes generate acid, and the ATP demand will likely double with a doubling of the growth rate. However, we did not find this relation for *Lb. plantarum*. Considering the difference with *Lc. lactis* this might suggest that *Lb. plantarum* strains produce only limited amounts of acidic catabolic end products in milk serum. Potentially, these strains generate part of the energy required for growth from amino acid catabolism as opposed to lactose catabolism (Konings, 2002; Teusink et al., 2006; Goffin et al., 2010). It was also suggested that *Lb. plantarum* does not degrade casein but instead consumes the available free amino acids for the limited growth observed (Ma et al., 2016). This could explain us not finding a correlation between growth and acidification for this species. ATP generation through increased amino acid catabolism could be beneficial for flavor formation (Smit et al., 2009) which has been described for some *Lb. plantarum* strains (Di Renzo et al., 2018; Wang et al., 2020).

We conclude that milk serum is a useful alternative to milk for high-throughput strain characterization. Additionally, removing casein micelles before inoculation might benefit other bacterial characterization methods that were originally made difficult by casein interference, such as proteomics, dry weight measurements, or flow cytometry.

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ORCIDS

Bas Teusink <https://orcid.org/0000-0003-3929-0423>
Herwig Bachmann <https://orcid.org/0000-0002-8224-0993>