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The impact of alternative nitrogen sources on the growth and viability of *Lactobacillus delbrueckii* ssp. *bulgaricus*

Raphael D. Ayivi,[1,2] Salam A. Ibrahim,[1]* Albert Krastanov,[3] Abishek Somani,[4] and Shahida A. Siddiqui[5,6]
[1]Department of Food and Nutritional Sciences, North Carolina Agricultural and Technical State University, Greensboro 27411
[2]Department of Nanoscience, Joint School of Nanoscience and Nanoengineering, University of North Carolina, Greensboro 27412
[3]Department of Biotechnology, University of Food Technologies, Plovdiv, Bulgaria
[4]Ohly GmbH, Wandsbeker Zollstrasse 59, 22041 Hamburg, Germany
[5]Technical University of Munich Campus Straubing for Biotechnology and Sustainability, Essigberg 3, 94315 Straubing, Germany
[6]German Institute of Food Technologies (DIL e.V.), Prof.-von-Klitzing Str. 7, 49610 D-Quakenbrück, Germany

ABSTRACT

In this study, we developed and optimized a growth medium using various nitrogen sources for the cultivation of *Lactobacillus delbrueckii* ssp. *bulgaricus*, a probiotic and essential dairy starter culture. The composition of de Man, Rogosa, and Sharpe (MRS) culture medium was modified, and the nitrogen content was replaced by alternative nitrogen sources X-Seed Nucleo Max, X-Seed KAT, and X-Seed Carbo Max (Ohly GmbH) in various blends of 5 and 10 g/L. Results showed that bacterial growth was significantly higher when the nitrogen source blend of 10 g/L of KAT and 10 g/L of Carbo Max [KCMax (10/10)] was used. The optical densities of the *Lb. bulgaricus* strains were significantly higher in the KCMax (10/10) medium than in the MRS medium. There was no significance in bacterial counts for both the MRS and the KCMax (10/10) medium, and all bacterial counts were estimated at 8 log cfu/mL. The buffering capacity of the KCMax (10/10) medium was also tested and supplemented with L-histidine and was significantly higher than that of the MRS control medium. KCMax (10/10) also supported the freeze-stability and viability of the *Lb. bulgaricus* cells during freezing and freeze-drying operations. Our results suggest that the alternative nitrogen sources X-Seed Nucleo Max, X-Seed KAT and X-Seed Carbo Max can substantially support the growth of lactic acid bacteria as demonstrated with *Lb. bulgaricus*. These alternative nitrogen sources could thus be recommended for lactic acid bacteria fermentation and for the cultivation of dairy starter cultures.

Key words: *Lactobacillus bulgaricus*, growth media, nitrogen source, starter culture, bacterial enumeration

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* is an important member of the uniquely diverse group of lactic acid bacteria (**LAB**; Gyawali et al., 2020) that has many industrial applications. The LAB are generally used as starter cultures for the production of fermented dairy products such as yogurt and are also considered as probiotics due to their significant human health benefits (Aponte et al., 2020). *Lactobacillus delbrueckii* ssp. *bulgaricus* is one of the 2 bacteria required for commercial yogurt production and is used synergistically with *Streptococcus thermophilus* on an industrial scale for the production of yogurt. This yogurt bacterium plays a vital role in the development of the organoleptic (Petry et al., 2000) and probiotic properties of yogurt (Hayek et al., 2019; Ayivi et al., 2020). Moreover, it has been reported to be a safe probiotic with several health benefits when administered in adequate amounts at an effective dose (Adolfsson et al., 2004). The flavor, texture, and organoleptic properties of yogurt are generally a result of the symbiotic interaction of *Lb. bulgaricus* and *Strep. thermophilus*. Consequently, an imbalance in these 2 bacteria will affect the sensorial characteristics and qualities of yogurt as a fermented dairy product (Sieuwerts, 2016).

Lactobacillus delbrueckii ssp. *bulgaricus* as a starter culture is very vital in many fermentation and bioprocessing operations. The fermentation performance and functionality of most strains of *Lb. bulgaricus* is enhanced by nutritional requirements and the overall enrichment of the fermentation medium (Ayivi et al., 2020). The food and dairy industries are continually in search of various probiotic bacteria for applications with cost-effective fermentation processes including growth medium requirements (Akabanda et al., 2013; Hayek and Ibrahim, 2013; Mani-López et al., 2014; Atilola et al., 2015). Generally, the nutritional quality of the fermentation medium is enhanced by the supplementation of casein hydrolysates and yeast extract, which are

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*Corresponding author: ibrah001@ncat.edu

key nitrogen sources that act as growth-promoters for boosting high bacterial cell growth (Norton et al., 1994; Hayek and Ibrahim, 2013). Significant biomass levels and high volumes of lactic acid production by LAB species have also been attributed to the presence of AA, vitamins and, yeast extract in the fermentation medium (Alazzeh et al., 2009; Manzoor et al., 2017; Ayad et al., 2020). The freeze-drying of LAB cells is also highly dependent on the growth medium, fermentation pH, cryoprotectant and other intrinsic factors (Carvalho et al., 2004). This characteristic has been confirmed by a study by Chen et al. (2017) in which a supplemented MRS medium significantly affected the growth and freeze-drying viability of *Lb. bulgaricus* strains. An enriched growth medium has also been credited with improving the freeze-drying survival rate of LAB due to the medium's composition (carbon, nitrogen, and lipids such as Tween 80), which efficiently helps to reduce bacterial stress during the freeze-drying process (Li et al., 2012). Chervaux et al. (2000) confirmed that *Lb. bulgaricus* cultivated in a growth medium highly supplemented with different carbon sources enhanced the stress resistance of the bacteria during various treatments. Interestingly, Tween 80 supplemented in a growth medium transformed the fatty acid composition of the LAB cells after fermentation, significantly enhancing the resistance of the cells to freezing and freeze-drying (Carvalho et al., 2004).

However, the limiting factor for supplementing fermentation media with high levels of nitrogen sources stems from the astronomical cost (Manzoor et al., 2017; Hayek et al., 2019). Moreover, the standard LAB fermentation medium, de Man, Rogosa, and Sharpe (**MRS**), is expensive due to its nitrogen sources (meat, peptone, and yeast extract) and does not support the growth of all LAB and probiotic cultures. The available literature also suggests that significant research is aimed at finding alternative cost-effective ingredients for LAB growth media, as well as obtaining higher cell densities and biomass levels. Therefore, the objectives of the present study were to investigate the growth and viability of *Lb. bulgaricus* cultivated with alternative nitrogen sources and to assess the impact on the viability of *Lb. bulgaricus* after freeze-drying.

MATERIALS AND METHODS

Source of Lb. bulgaricus and Preparation of Preculture Fermentation Medium

A total of 10 *Lb. bulgaricus* strains were used for the preliminary study (not all data are shown) as elucidated in Table 1 with the original source. Strains (S9 and LB6) were selected in this study based on different growth rates (Georgiev et al., 2021). Strains were activated in MRS as the prefermentative medium for the *Lb. bulgaricus* strains as detailed in the Supplemental File S1 (https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022). Enumeration of the evaluated strains was done using MRS agar and modified reinforced clostridial medium-pyruvate (mRCM-PYR) with details of their preparation described in the Supplemental File S1. No animals were used in this study, and ethical approval for the use of animals was thus deemed unnecessary.

Validation of Nitrogen Sources, Optimization of Growth Medium, and Culture Propagation in Optimized Growth Medium

Five yeast samples [X-Seed Nucleo Max (**N-Max**), X-Seed KAT (**KAT**), X-Seed Peptone (**PEP**), X-Seed Carbo Peptone (**C-Pep**), and X-Seed Carbo Max (**C-Max**)] provided by Ohly GmbH were evaluated in different blends and proportions during the preliminary study. Both C-Pep and C-Max are developmental products, and as such their description is proprietary and not available. The preliminary work thus confirmed N-Max, KAT, and C-Max yeast samples as superior alternative complex nitrogen sources for use in the study. Supplemental Table S1 (https://doi.org/

Table 1. Probiotic strains used in the preliminary study

Product code	Sample	Original source	Bacterial composition as labeled
S9	Pure industrial strain	Bulgaria	*Lactobacillus bulgaricus*
LB6	Pure industrial strain	Bulgaria	*Lb. bulgaricus,*
THT	Pure industrial strain	Belgium	*Lb. bulgaricus*
ATCC 11842	Pure industrial strain	ATCC	*Lb. bulgaricus*
DAW	Yogurt	USA	*Lb. bulgaricus,* other live culture
S8	Pure industrial strain	Bulgaria	*Lb. bulgaricus*
E22	Yogurt	USA	*Lb. bulgaricus,* other live culture
S5	Pure industrial strain	Bulgaria	*Lb. bulgaricus*
S19	Pure industrial strain	Bulgaria	*Lb. bulgaricus*
Genesis	Probiotic supplement	Bulgaria	*Lb. bulgaricus*

10.17632/hzx7t8htdp.1; Ayivi et al., 2022) highlights the yeast description, total percentage of protein, and total percentage of nitrogen in the N-Max, KAT, and C-Max, respectively. The optimized growth medium was prepared similarly to the composition of MRS (Supplemental Table S2; https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022); however, its major nitrogen sources were replaced with N-Max, and other optimized proportions of blends of KAT, and C-Max as alternative nitrogen sources (Supplemental Table S3; https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022). The 2 validated strains (S9 and LB6) were then propagated in the optimized growth medium per the formulations in Supplemental Table S4 (https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022). Details of the culture propagation procedure are highlighted in the supplemental file.

Determination of pH Values and Buffering Capacity of Growth Medium with L-Arginine and L-Histidine Supplementation

The pH values of each medium were measured in duplicates at the start and end of fermentation. The buffering capacity of all media was also evaluated with 4 g/L of L-arginine and L-histidine, respectively. Supplemental File S1 highlights the pH and the buffer capacity determination.

Bacterial Enumeration and Effect of Growth Media on the Freeze-Stability and Viability of Lb. bulgaricus.

The bacterial growth of the 2 strains (LB6 and S9) was monitored via spectrophotometric measurement of their optical densities (OD_{610nm}) at different time intervals (0, 6, and 12 h) in the optimized media. All strains were enumerated at the end of the fermentation period (12 h), by the surface-plated method as detailed in the supplemental file. The S9 *Lb. bulgaricus* strain demonstrated higher cell growth than the LB6 strain and as such was chosen and evaluated for viability after freezing and freeze-drying with and without recovery treatment as detailed in the Supplemental File S1. All plates were then anaerobically incubated for 48 h at 42°C and plates with 25 to 250 colonies were counted. Bacterial populations were expressed in log colony-forming units per mililiter (Table 2).

Statistical Analyses

SAS version 9.4 (SAS Institute Inc.) was used to analyze the data obtained in the study, and ANOVA was used to determine significant differences between obtained values for the final optimized growth media blends but not for the preliminary study. Significant differences ($P < 0.05$) between treatment means were compared using Tukey's test. Bacterial population counts were expressed in $\log_{10}$ before analyses were made.

RESULTS

Preliminary Study (Validation of the Nitrogen Source on the Growth of Lb. bulgaricus Strains)

Evaluation of the nitrogen source on the growth of the different strains of *Lb. bulgaricus* was done in the preliminary study. Although it was known that bacterial growth in the fermentation medium was mostly strain-dependent, it was generally observed that some *Lb. bulgaricus* strains possessed higher growth rates (OD_{610nm}) than others, and therefore, their growth was significant ($P < 0.05$). Consequently, S9 and LB6 *bulgaricus* strains were selected for the study. In validating the different nitrogen sources, N-Max, KAT, PEP, C-Pep, and C-Max were tested in different blends and proportions (Supplemental Table S4) thereby confirming which complex nitrogen source could best support the growth of the *Lb. bulgaricus* strains. Our findings also showed that blends containing PEP and C-Pep inhibited the growth of the *Lb. bulgaricus* strains and were therefore not selected for the main study. As a result, N-Max, KAT, and C-Max were considered superior complex nitrogen sources and were therefore

Table 2. Bacterial count (mean ± SD; n = 3) of *Lactobacillus bulgaricus* strains expressed as log cfu/mL after 12 h in different growth media

Growth medium[1]	S9 strain	LB6 strain
MRS	7.63 ± 0^{a}	7.06 ± 0^{a}
N-Max (basal medium)	5.0 ± 0^{d}	5.0 ± 0.14^{d}
C-Max 5%	5.0 ± 0^{d}	5.15 ± 0.21^{bc}
C-Max 10%	5.24 ± 0.34^{b}	5.0 ± 0^{d}
KAT 5%	6.04 ± 0.06^{c}	7.08 ± 0.0^{abc}
KAT 10%	6.74 ± 0.06^{bc}	7.16 ± 0.02^{ab}
KCMax (5/5)	6.49 ± 0.04^{bc}	6.53 ± 0.04^{ab}
KCMax (5/10)	7.32 ± 0.01^{ab}	6.31 ± 0.01^{a}
KCMax (10/5)	6.97 ± 0.02^{ab}	6.64 ± 0^{a}
KCMax (10/10)	7.22 ± 0^{ab}	6.49 ± 0.02^{a}

[a–d]Means with different superscripts within a row differ significantly ($P < 0.05$).

[1]MRS = de Man, Rogosa, and Sharpe medium (Neogen Corporation). Ohly GmbH is manufacturer of all of the nitrogen source blends: N-Max = X-Seed Nucleo Max; C-Max = X-Seed Carbo Max; KAT = X-Seed KAT; KCMax = a blend of both KAT and C-Max. The nitrogen source blends KCMax (5/5) represented 5 g/L of KAT and 5 g/L of C-Max, and KCMax (5/10) was a combination of 5 g/L of KAT and 10 g/L of C-Max. The blend KCMax (10/5) consisted of 10 g/L of KAT and 5 g/L of C-Max, and the final blend KCMax (10/10) was a combination of 10 g/L of KAT and 10 g/L of C-Max.

blended in different proportions for use as the final growth medium. The selected complex nitrogen sources were not unexpected. Their total nitrogen percentages were 71, 75, and 30% for N-Max, KAT, and C-Max, respectively, compared with the total nitrogen percentages of 67 and 45% of PEP, and C-Pep, respectively.

Various blends of the different nitrogen sources were used in formulating the growth media, and these formulations were inspired based on the study design of Somani et al. (2020b). Several trials were done with various strains of *Lb. bulgaricus* until a final formulation with comparable results to MRS was obtained. The first formulation had a blend of 1% (wt/vol) of the different nitrogen sources, whereby 5 strains (E22, S9, LB6, RDA, and ATCC 11842) were used. MRS was also used as a control medium in all of the preliminary formulations. The growth of the bacteria was monitored for 8 h at an incubation temperature of 42°C. Although the optical density (**OD**; OD_{610nm}) results of the strains in the first formulated growth medium were not promising, MRS (the standard control) had good growth confirming the viability of the strains as shown in Supplemental Figure S1 (https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022).

It was also observed that the *Lb. bulgaricus* strains S9 and LB6 were fast growing, especially in the MRS medium, hence their selection for the fermentation study. Formulations 2, and 4 (data not shown) did not yield promising growth results; however, formulation 3 (Supplemental Figure S2; https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022) included a basal medium with 2 g/L of N-Max in conjunction with various blends of KAT and C-Max yielded exceptional results.

Based on the positive outcome of formulation 3, formulation 5 was developed and consequently chosen as the final optimized recipe for the study. The fifth and final growth medium formulation was based on 5% (5 g/L) and 10% (10 g/L) of the validated nitrogen sources (KAT and C-Max, respectively), and a blend of both KAT and C-Max (**KCMax**) in a supplemented basal medium containing 2 g/L of N-Max. Thus, the various formulations employed were C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, KCMax (5/5), KCMax (5/10), KCMax (10/5), and KCMax (10/10), all of which were supplemented with a basal medium (2 g/L of N-Max). The MRS was also used as the control medium in all cases for the fermentation study. The nitrogen source blends KCMax (5/5) represented 5 g/L of KAT and 5 g/L of C-Max, and KCMax (5/10) was a combination of 5 g/L of KAT and 10 g/L of C-Max. The blend KCMax (10/5) consisted of 10 g/L of KAT and 5 g/L of C-Max, and the final blend KCMax (10/10) was a combination of 10 g/L of KAT and 10 g/L of C-Max.

Growth and Cell Density in the Final Optimized Growth Medium

The *Lb. bulgaricus* strains (S9 and LB6) used in the study demonstrated varied growth patterns in the final medium during the 12-h incubation period at 42°C. The growth medium MRS also confirmed that the strains were viable as the strains adequately utilized the nutrients inherent in the MRS medium for their cellular activity. There was a significant difference ($P < 0.05$) in the OD results for the blends of KAT and C-Max, with KCMax (10/10) performing better than the MRS medium after 12 h of fermentation. Figure 1 shows the growth pattern (OD_{610nm}) of the S9 and LB6 strains in the complex nitrogen-supplemented media during 12 h of fermentation at 42°C.

There was also a significant difference ($P < 0.05$) in the OD results of the blends of KAT and C-Max media in reference to the other growth media. The observed growth of S9 and LB6 in the different media suggests the availability of complex nitrogen in the formulated medium was vital for microbial growth. The basal medium alone for both S9 and LB6 strains was not adequately enriched to support the metabolic activities of the *Lb. bulgaricus* strains; hence, the observed poor growth result. The bacterial growth in MRS and KAT and KCMax media thus suggested that bacterial growth was affected by the nitrogen content in the media.

Enumeration of Lb. bulgaricus Strains

There was a significant difference ($P < 0.05$) in the bacterial counts between the basal medium (N-Max), C-Max 5%, C-Max 10%, KAT 5%, KAT 10%, and the different blends of KCMax media. However, no significant difference in the bacterial counts of the MRS medium and the KCMax (5/10), KCMax (10/5), and the KCMax (10/10) were recorded. Although bacterial growth performance is mostly strain-dependent, the observed bacterial counts especially from the blends of nitrogen sources KAT and C-Max presupposes that these media can be compared with the performance of MRS as a growth medium and could be selected as alternative media for supporting the growth of *Lb. bulgaricus*. The bacterial counts of the strains (S9 and LB6) are presented in Table 1.

pH Value Changes in the Different Growth Media

The initial pH value of all of the media was adjusted to 6.5 before the start of the fermentation process using the S9 and LB6 strains. However, at the end of the 12-h fermentation period for the S9 strain, there was

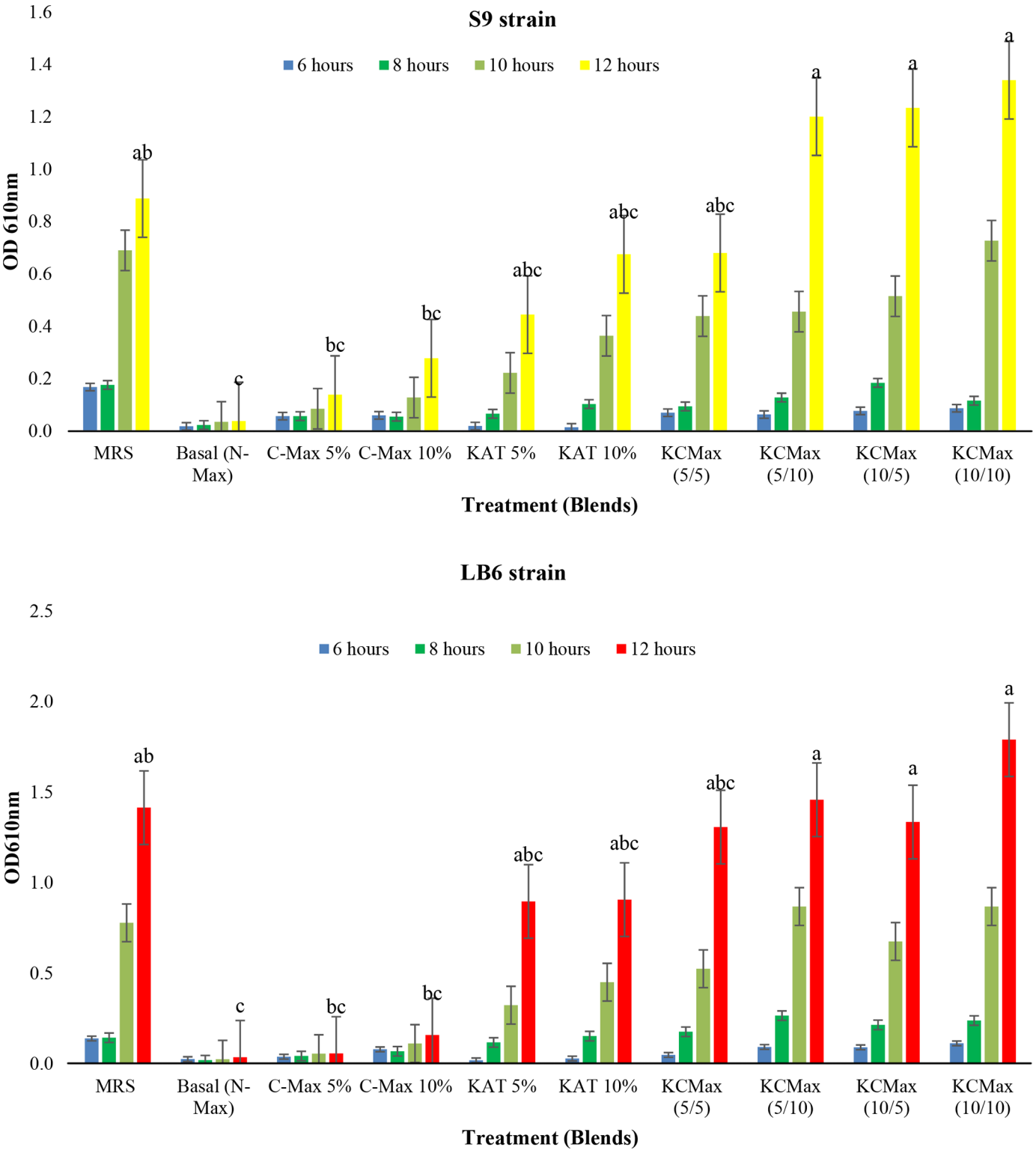

Figure 1. Changes in the growth of S9 and LB6 *Lactobacillus bulgaricus* strains at optical density at 610 nm (OD_{610nm}) in the different final growth media at different proportions and blends after 12 h of fermentation at 42°C. MRS = de Man, Rogosa, and Sharpe. Basal medium: N-Max = X-Seed Nucleo Max. The basal medium supplemented with different nitrogen sources: C-Max = X-Seed Carbo Max; KAT = X-Seed KAT; KCMax = blend of both KAT and C-Max. Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation. Error bars indicate SD for an experiment performed in duplicate; different lowercase letters indicate statistical differences between the various growth media.

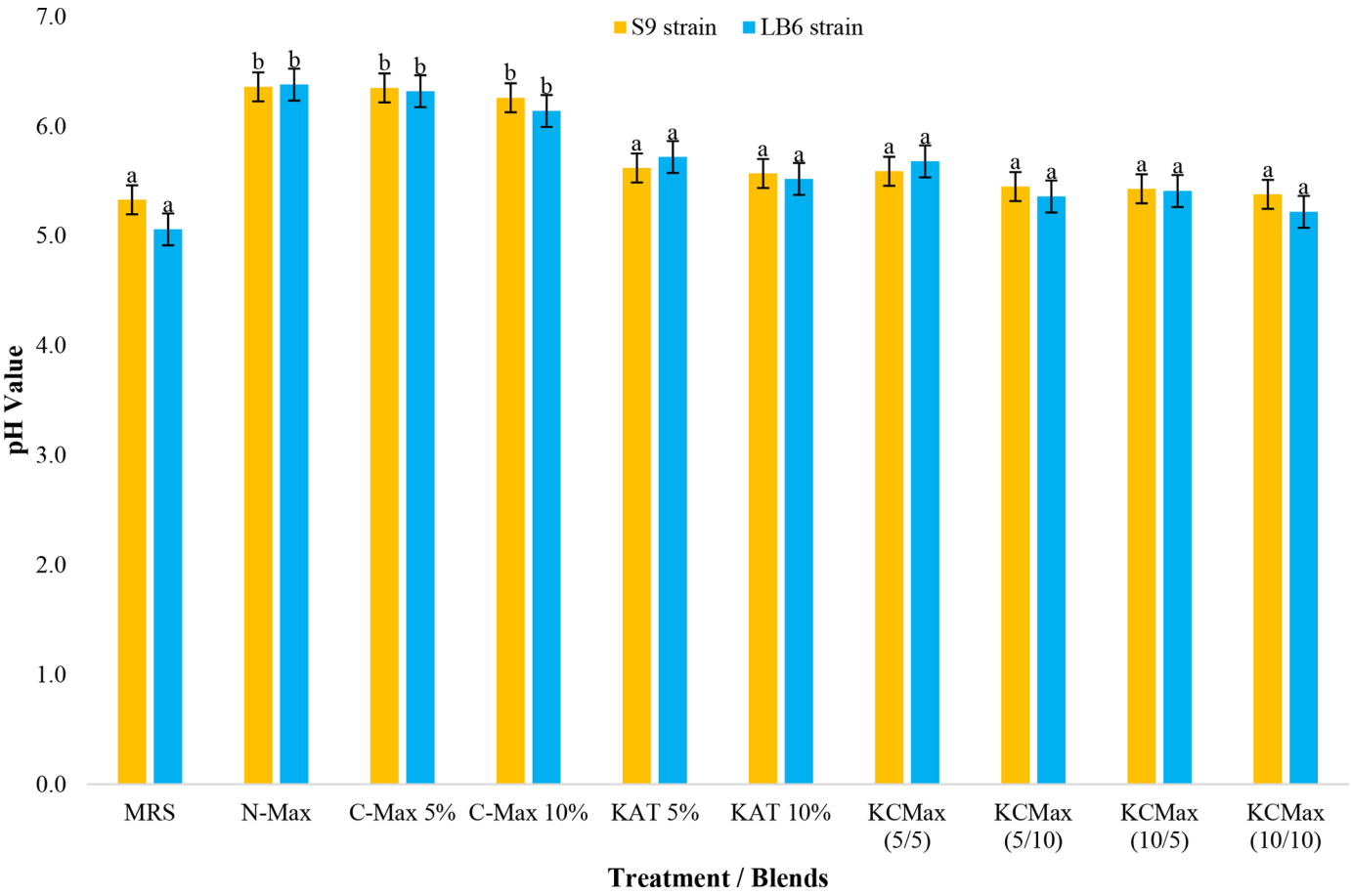

Figure 2. Changes in pH of the *Lactobacillus bulgaricus* strains (S9 and LB6) in the control medium de Man, Rogosa, and Sharpe (MRS), basal medium X-Seed Nucleo Max (N-Max), and the basal medium supplemented with different nitrogen sources X-Seed KAT (KAT) X-Seed Carbo Max (C-Max) at different proportions and blends after 12 h of fermentation. Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation. Error bars indicate SD for an experiment performed in duplicate; different lowercase letters indicate statistical differences between the various growth media.

a rapid decline in pH from the initial value of 6.5 to a final value between 5.33 and 5.62 for MRS, KAT, and blends of KAT and C-Max. This rapid decline in pH was similarly observed for the LB6 strain whereby the final pH values after fermentation were in the range of 5.06 to 5.72 for the MRS control medium, KAT, and the blends of KAT and C-Max media. Therefore, a significant difference ($P < 0.05$) was observed for the formulated media with different complex nitrogen sources in comparison to the MRS (control) medium. Interestingly, the observed pH results for the strains (S9 and LBS) for the N-Max and C-Max media following the fermentation period were in contrast to that observed in the MRS, KAT, and the blends of KAT and C-Max. Thus, the final pH values of the S9 and LB6 strains for N-Max and C-Max were in the range of 6.26 to 6.36 and 6.14 to 6.38, respectively. Changes in pH of the various media are depicted in Figure 2. The observed differences in pH values could be attributed to the presence of the alternative protein sources in the various media, which resulted in a buffering potential and thus restricted changes in pH. To determine this effect, an additional study related to the buffering capacity of the different protein sources was performed.

Buffering Capacity of Growth Media with L-Arginine and L-Histidine Supplementation

Buffering capacity of a growth media is an important fermentation parameter, as it provides a stable pH range for the metabolic activity of LAB. This stable pH, promoted high cell density of bacterial growth, as well as limiting the production of organic acid (lactic acid), which could cause cell injury thereby decreasing the growth of the strains. The buffering capacity of the growth medium was evaluated with 2 essential AA (L-arginine and L-histidine). The change in buffering capacity was highly observed with the supplementation

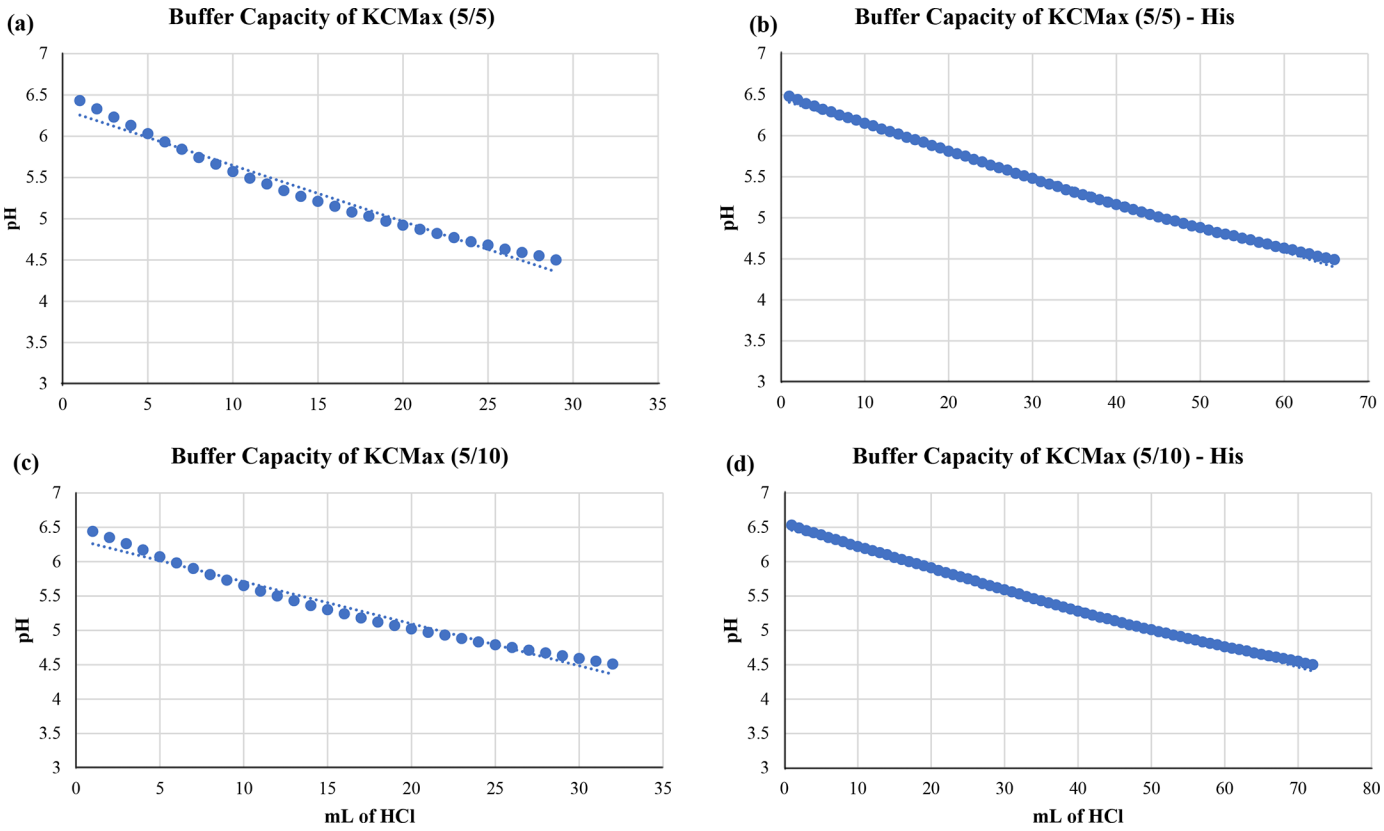

Figure 3. The different buffering capacities of (a) KCMax (5/5) and (b) KCMax (5/5) supplemented with L-histidine (His), and (c) KCMax (5/10) and (d) KCMax (5/10) medium supplemented with His. KCMax = a blend of both X-Seed KAT (KAT) and X-Seed Carbo Max (C-Max). The nitrogen source blends KCMax (5/5) represented 5 g/L of KAT and 5 g/L of C-Max, and KCMax (5/10) was a combination of 5 g/L of KAT and 10 g/L of C-Max. The blend KCMax (10/5) consisted of 10 g/L of KAT and 5 g/L of C-Max and the final blend KCMax (10/10) was a combination of 10 g/L of KAT and 10 g/L of C-Max. Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation.

of L-histidine than with L-arginine. Three growth media blends, KCMax (5/10), KCMax (10/5), and KCMax (10/10), had the highest significant buffering capacity values of 36, 36.5, and 39, respectively (Figures 3 and 4), with the L-histidine supplementation ($P < 0.05$) as compared with the buffering capacity of the standard L-histidine supplemented MRS medium (35.22; Table 3). Similarly, these 3 KCMax media blends also reported high buffering capacity values (15.24, 15.24, and 15.50, respectively) without any L-arginine or L-histidine supplementation but were, however; lower than the value reported in the standard MRS medium (21.57; Table 3). When the growth media was supplemented with L-arginine, no significant difference ($P > 0.05$) was observed in all cases for the blends, as the buffering capacity values were almost the same as that of the normal media without any AA supplementation. Therefore, L-arginine supplementation in the growth media did not have any significant effect on the media's buffering capacity. It was, therefore, evident that the KCMax (10/10) medium had the best buffering capacity (39) with the supplementation of L-histidine as compared with the standard MRS medium (35.22). A higher buffering capacity value, therefore, suggested the medium was well suited to promote higher cell density of LAB growth during fermentation operations. Consequently, bacterial growth and metabolic activity are sustained to a high level, therefore promoting better fermentation processes. Generally, for LAB fermentation, it is recommended to consider a growth medium that provides a high pH buffering. Table 3 summarizes the buffering capacity for the different growth media with the supplementation of L-arginine and L-histidine.

The L-histidine supplementation provided a better indication of the pH buffering capacity of the media and was thus very promising, having a stable resistance to pH change over a longer period with higher consumption of the 0.1 N HCl solution. There was a significant difference ($P < 0.05$) in the buffering capacity values of the L-histidine supplemented media compared with that of the L-arginine supplemented and the nonsupplemented growth media. The use of a minimal amount of

Table 3. Buffering capacity of the different growth media with and without L-arginine and L-histidine supplementation

Growth medium[1]	Buffer capacity value (no supplementation)	Buffer capacity value (arginine supplementation)	Buffer capacity value (histidine supplementation)
MRS	21.57	21.84	35.22
N-Max (Basal)	13.05	13	30
C-Max 5%	12.91	12.17	31
C-Max 10%	13.15	13.25	34
KAT 5%	12.97	13.04	32
KAT 10%	14.07	14.33	34.5
KCMax (5/5)	15.05	14.03	33
KCMax (5/10)	15.24	15.0	36
KCMax (10/5)	15.24	14.5	36.5
KCMax (10/10)	15.50	16.25	39

[1]MRS = de Man, Rogosa, and Sharpe; N-Max = X-Seed Nucleo Max; C-Max = X-Seed Carbo Max; KAT = X-Seed KAT; KCMax = a blend of both KAT and C-Max. Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation. The nitrogen source blends KCMax (5/5) represented 5 g/L of KAT and 5 g/L of C-Max, and KCMax (5/10) was a combination of 5 g/L of KAT and 10 g/L of C-Max. The blend KCMax (10/5) consisted of 10 g/L of KAT and 5 g/L of C-Max, and the final blend KCMax (10/10) was a combination of 10 g/L of KAT and 10 g/L of C-Max.

this EAA proved effective in maintaining a stable pH over some time, ensuring a good buffering for higher cell growth (cfu/mL). The observed results confirmed L-histidine as a potential natural alternative that could be used for maintaining a stable fermentation pH in fermentation cultures for pH buffering of growth media.

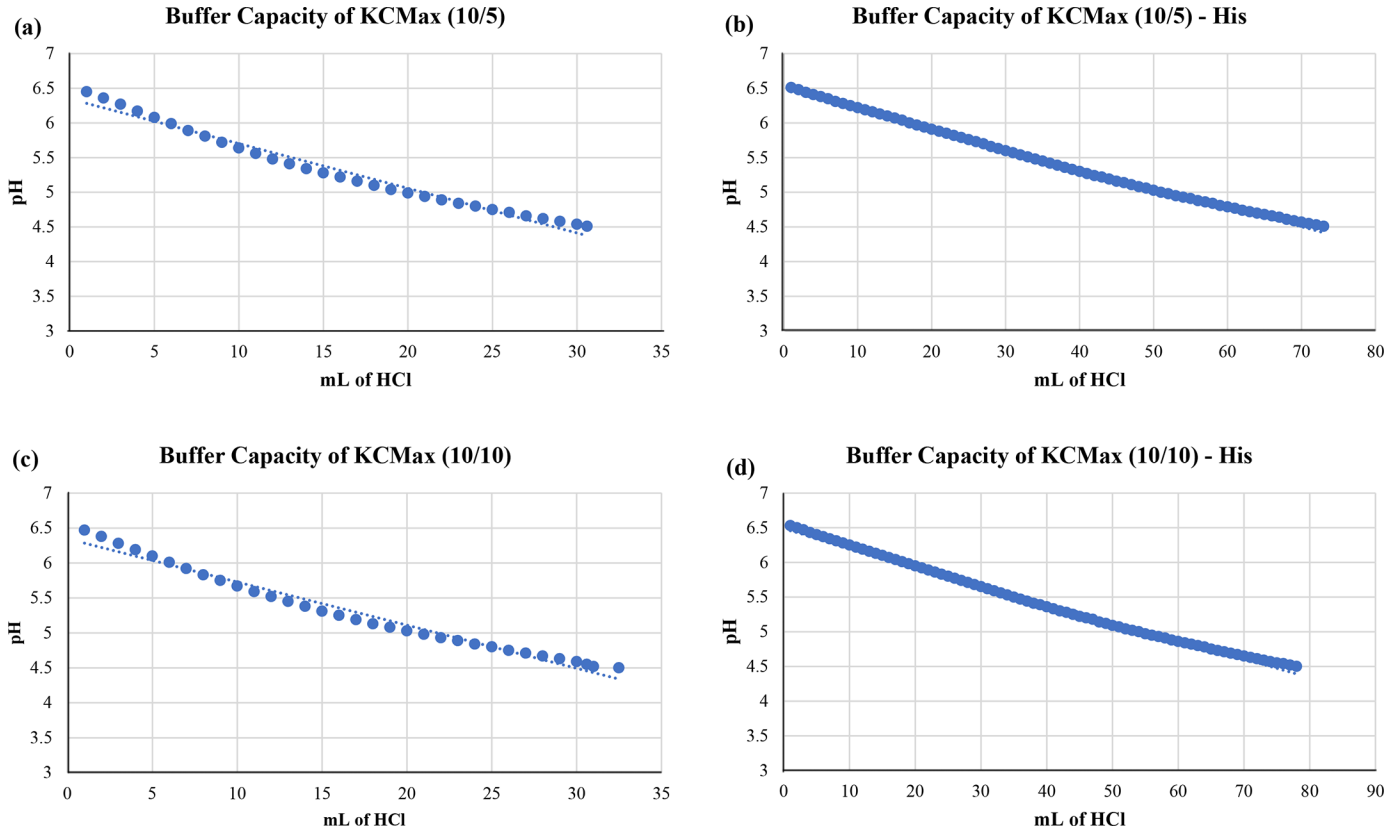

Figure 4. The varying buffering capacities of (a) KCMax (10/5) and (b) KCMax (10/5) supplemented with L-histidine (His), and (c) KCMax (10/10) and (d) KCMax (10/10) medium supplemented with His. KCMax = a blend of both X-Seed KAT (KAT) and X-Seed Carbo Max (C-Max). The nitrogen source blends KCMax (5/5) represented 5 g/L of KAT and 5 g/L of C-Max, and KCMax (5/10) was a combination of 5 g/L of KAT and 10 g/L of C-Max. The blend KCMax (10/5) consisted of 10 g/L of KAT and 5 g/L of C-Max, and the final blend KCMax (10/10) was a combination of 10 g/L of KAT and 10 g/L of C-Max. Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation.

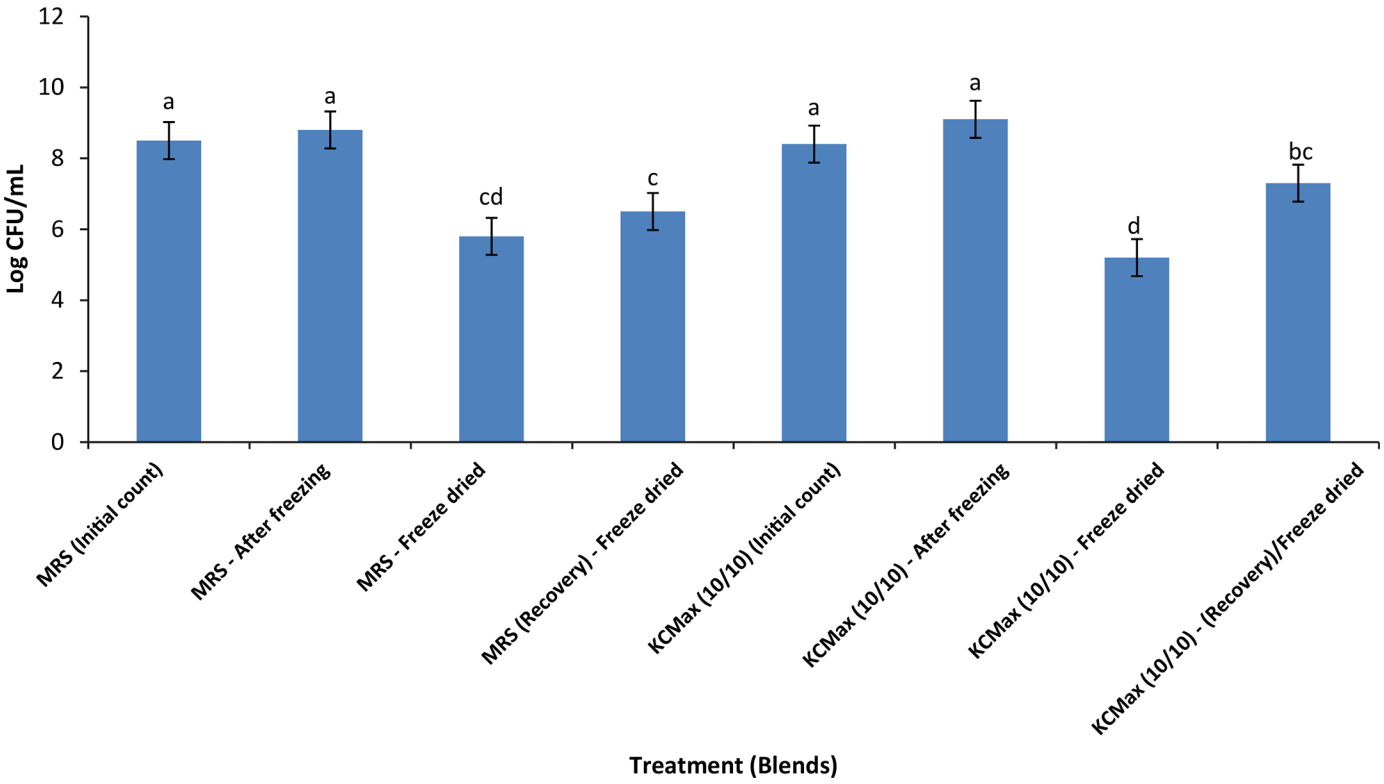

Figure 5. Viability and bacterial count of S9 *Lactobacillus bulgaricus* strain before and after freezing and freeze-drying with and without recovery after fermentation in de Man, Rogosa, and Sharpe (MRS) and KCMax (10/10) medium. KCMax (10/10) = a blend of 10 g/L X-Seed KAT (KAT) and 10 g/L X-Seed Carbo Max (C-Max). Ohly GmbH is the manufacturer of all the nitrogen sources; MRS is from Neogen Corporation. Error bars indicate SD for an experiment performed in duplicate; different lowercase letters indicate statistical differences between the various growth media.

Supplemental Figures S3 to S10 (https://doi.org/10.17632/hzx7t8htdp.1; Ayivi et al., 2022) highlight the different pH buffering of the growth media with and without the supplementation of the 2 AA (L-arginine and L-histidine), respectively.

Effect of the Optimized Growth Media (KCMax 10/10) on the Freeze-Stability and Viability of Lb. delbrueckii ssp. bulgaricus

The initial count of the MRS medium strain was 8.5 log cfu/mL and that for the KCMax (10/10) medium was 8.4 log cfu/mL. The population count for the MRS cultivated strain after overnight freezing at −80°C was 8.8 log cfu/mL. Interestingly, the population count for the KCMax (10/10) cultivated strain after overnight freezing was a bit higher (9.1 log cfu/mL) than the MRS cultivated strain. There was, however, no significant difference ($P > 0.05$) between the counts of the MRS and the KCMax (10/10) medium after the freezing process. The count of the freeze-dried *bulgaricus* cells cultivated from the MRS medium decreased compared with the initial count and was 5.8 log cfu/mL. A similar observation of reduced viability (5.2 log cfu/mL) was reported for the freeze-dried *bulgaricus* cells cultivated from the KCMax (10/10) medium. However, there was a much higher increase in cell counts for the freeze-dried cells after the cells were recovered in skim milk which resulted in the resuscitation and repair of the stressed and injured cells after the freeze-drying process. The population count for the MRS recovered freeze-dried cells was 6.5 log cfu/mL. Consequently, the population count for the KCMax (10/10) recovered freeze-dried cells was higher than what was observed in the MRS (standard) at 7.3 log cfu/mL. Figure 5 elucidates the viability of *Lb. bulgaricus* in both the standard (MRS) and the KCMax (10/10) media after the freeze-drying process.

DISCUSSION

Several studies have evaluated the effect of commercial nitrogen sources in various selective growth media for the enhanced growth promotion of LAB cultures (Aeschlimann and Von Stockar, 1990; Champagne et al., 1999; Gaudreau et al., 1999; Atilola et al., 2015). In

the present study, we modified the composition of MRS and evaluated the effect of different nitrogen sources (N-Max, KAT, and C-Max) in an optimized medium on the growth and performance of *Lb. bulgaricus* strains. Yeast extracts are excellent sources of vitamins, AA, and peptides and have been credited with the enhanced stimulatory growth of LAB cultures when employed in culture medium (Juillard et al., 1995; Champagne et al., 1999). Furthermore, the major criteria for enhanced viability and high yields of dairy starter cultures industrially have been characterized by the nitrogen component of the culture medium utilized for their production (Proust et al., 2019).

The strain-dependent characteristics of *Lactobacillus* species for nutritional requirements warrant several optimization techniques to promote bacterial growth and achieve high cell density (Ren et al., 2019). The alternative yeast extracts (N-Max, KAT, and C-Max) in the modified growth media were highly efficient and showed promising results in comparison to the standard and conventional MRS medium. The effect of yeast extract on the growth of LAB cultures was also corroborated by Zhang et al. (2020) in their study when a modified MRS medium was enhanced with yeast extract to promote bacteriocin production. Another study by Abbasiliasi et al. (2011) also confirmed the effect of yeast extract as a nitrogen source that significantly enhanced the production of bacteriocin-like inhibitory substances in a developed growth medium for *Lactobacillus paracasei* LA07. The blends of KAT and C-Max in the basal medium containing N-Max yielded superior results than the MRS medium and this could be attributed to the bioavailability of free nucleotides, amino acids and peptides in the optimized medium (Somani et al., 2020b). We also observed that the combination of these alternative yeast extracts was responsible for the enhanced growth of the LAB strains, as the optimized medium in the absence of these nitrogen sources confirmed poor or no growth of the evaluated LAB strains. Consequently, blending and selection of appropriate nutrient-rich yeast extracts for growth medium supplementation potentially enhances the cellular activity and the growth performance of LAB cultures (Somani et al., 2020b; Xiao et al., 2020). The enhanced superior growth at OD_{610nm} and the cell counts for both S9 and LB6 strains in the optimized medium were also confirmed by Somani et al. (2020b) in their study when the same yeast extracts had a significant effect on the growth performance of LAB cultures. The pH and buffering capacity for the growth medium were evaluated with 2 AA (L-arginine and L-histidine); however, L-histidine was confirmed as a better alternative for enhancing the growth medium's buffering capacity, which promoted enhanced LAB growth. Buffering capacity and pH are thus very critical factors in media development as they ensure a stable pH without comprising the enhanced cellular activities of LAB cultures (Hayek et al., 2019). This was also confirmed by Azatian et al. (2019) whereby increasing a growth medium's buffering capacity led to higher protein yields in *Escherichia coli*. The growth medium's composition plays a vital role in minimizing viability losses during freeze-drying processes and also enhances the freeze-stability of LAB cell cultures (Chervaux et al., 2000; Carvalho et al., 2004; Fenster et al., 2019). We also observed that the optimized growth medium (KCMax 10/10) had a significant effect on the viability of LAB after freezing and freeze-drying processes with and without cell recovery in milk. This observation could be explained due to the rich nitrogen composition, free AA, vitamins, minerals, and peptides of the yeast extract that could significantly enhance cell survival during freeze-drying (Somani et al., 2020a). In addition, these yeast extracts as alternative nitrogen sources have been confirmed as not only affecting the growth of LAB cultures but also enhancing the viability and affecting cell survival during freeze-drying (Somani et al., 2020a). According to a study by Chervaux et al. (2000), a growth medium supplemented with all known AA, vitamins, and micronutrients was able to efficiently support the growth and viability of *Lb. bulgaricus* CNRZ397 during fermentation and freeze-drying processes. Carvalho et al. (2004) also confirmed the effect of AA on reinforcing the intrinsic protective properties in bacterial cell walls. This uniquely shields the cell from stress and enhances its resistance during freeze-drying processes. For the freeze-dried cells, viability was also enhanced after cell recovery in milk. The successful resuscitation of freeze-dried dairy LAB cultures in milk has been widely reported and employed for enhancing the viability of freeze-dried cells (Celik, and O'Sullivan, 2013). Thus, the high bacterial count observed for the recovered freeze-dried strains cultivated in the KCMax (10/10) medium could be attributed to both the nitrogen-rich profile of the yeast extracts as well as the effect of the milk nutrients (Somani et al., 2020a). The KCMax (10/10) medium could thus adequately support LAB viability and enhance stability during freezing and freeze-drying processes and could potentially be better than the conventional MRS medium.

CONCLUSIONS

In the present study, a modified growth medium was developed using alternative nitrogen sources (Ohly yeast extracts) to evaluate the fermentation capability of some strains of *Lb. bulgaricus*. Our results demon-

strated that the nitrogen sources N-Max, KAT, and C-Max can efficiently support the growth and viability of *Lactobacillus delbrueckii* ssp. b*ulgaricus* and could thus be used for the fermentation of LAB or probiotic cultures. In addition, these nitrogen sources could potentially act as cryoprotectants to ensure the freeze-stability and viability of freeze-dried probiotic cells. The AA L-histidine could also be supplemented in growth medium to enhance the medium's buffering capacity. Consequently, N-Max, KAT, and C-Max yeast extracts are superior nitrogen sources that have promising potential for bioprocessing operations and could be employed for dairy starter fermentation processes due to their enhanced growth-promoting factors.

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ORCIDS

Raphael D. Ayivi https://orcid.org/0000-0001-6671-7715
Salam A. Ibrahim https://orcid.org/0000-0001-5395-9993