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Nitrogen source: an effective component for the growth and viability of *Lactobacillus delbrueckii* subsp. *bulgaricus*

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Research Article

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Abstract

In this study, we developed and optimized a growth media by evaluating various nitrogen sources for the cultivation of *Lactobacillus bulgaricus*, a probiotic and an important dairy starter culture. We modified the composition of deMan, Rogosa and Sharpe (MRS) culture media and substituted the nitrogen content with alternative nitrogen sources X-Seed KAT, X-Seed Carbo Max and X-Seed Nucleo Max in various blends of 5 g/l and 10 g/l respectively. Results showed that bacterial growth was significantly higher when the nitrogen source blend KCMax (10/10) was used. The optical density ($OD_{610\ nm}$) of the *Lactobacillus bulgaricus* strains were higher (1.34 and 1.79) in the KCMax (10/10) medium than in the MRS medium (0.89 and 1.42) ($P < 0.05$). There was no significant difference in the bacterial counts for both the MRS medium and the KCMax (10/10) medium, and all bacterial counts were estimated at 8 log CFU/ml. The buffering capacity of KCMax (10/10) was also tested and supplemented with L-histidine and was significantly different ($P < 0.05$) than that of the MRS control medium. Calcium supplemented in the KCMax (10/10) also served as a cryoprotectant for the cells during freezing and freeze-drying. Bacterial counts of the recovered calcium-treated freeze-dried cells were statistically significant ($P < 0.05$). We hypothesized that alternative nitrogen sources such as selected yeast extracts from the X-Seed brand of complex nitrogen sources could efficiently support the viability of *Lb. bulgaricus*. Our results thus suggested the growth of *Lb. bulgaricus* was efficiently supported by the X-Seed KAT, X-Seed Nucleo Max and X-Seed Carbo Max nitrogen sources. Consequently, these alternative nitrogen sources could potentially be recommended for dairy starter culture fermentations.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the two bacteria required for commercial yogurt production and is used synergistically with *Streptococcus thermophilus* on an industrial scale. *Lactobacillus delbrueckii* subsp. *bulgaricus* is also an important member of the uniquely diverse group of lactic acid bacteria (LAB) (Gyawali *et al.*, 2020) that has many industrial applications. 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 potential human health benefits (Aponte *et al.*, 2020). In addition, *Lactobacillus bulgaricus* plays a vital role in the development of the organoleptic (Petry *et al.*, 2000) and probiotic properties of yogurt. Fermented functional foods containing *Lb. bulgaricus* are believed to enhance human health and promote wellness (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 *Streptococcus thermophilus*. Consequently, an imbalance in these two bacteria will affect the sensorial characteristics and qualities of yogurt as a fermented dairy product (Sieuwerts, 2016).

Lb. bulgaricus as a starter culture is a vital component of many fermentation processes. Consequently, their fermentation performance and functionality are enhanced based on their nutritional requirements and the overall enrichment of the fermentation medium (Ayivi *et al.*, 2020). The food or dairy industry is constantly exploring and in search of various probiotic bacteria for many food product applications with cost-effective fermentation processes including growth media requirements (Akabanda *et al.*, 2013; Hayek and Ibrahim, 2013; Mani-López *et al.*, 2014). Generally, the nutritional quality of the fermentation medium is enhanced by the supplementation of casein hydrolysates and yeast extract, which are key nitrogen sources that act as growth-promoters boosting high bacterial cell growth (Norton *et al.*, 1994; Hayek and Ibrahim, 2013).

Growth media supplementation with hydrolyzed proteins essentially releases more nitrogen for uptake by LAB, thereby enhancing the bacteria's biofunctionality (Dąbrowska *et al*., 2017; Ponomarova *et al*., 2017; Berisvil *et al*., 2020).

Significant biomass levels and high volumes of lactic acid production by LAB species have also been attributed to the presence of amino acids, vitamins and yeast extract in the fermentation medium (Alazzeh *et al*., 2009; Manzoor *et al*., 2017; Ayad *et al*., 2020). Researchers have recommended calcium supplementation for growth media development as it helps to promote bacterial cell division which impacts high cell counts (Wright and Klaenhammer, 1983). Calcium or skim-milk powder is also typically used as a cryoprotectant in the dairy industry for probiotic cells during freeze-drying operations in order to significantly limit cell viability losses (Wright and Klaenhammer, 1983; Chervaux *et al*., 2000; Fenster *et al*., 2019).

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 was also confirmed by a study by Chen *et al*. (2017) who reported how a supplemented MRS medium significantly impacted 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).

However, the limiting factor for supplementing fermentation media with high amounts of nitrogen source is linked to its exorbitant cost (Manzoor *et al*., 2017; Hayek *et al*., 2019). Moreover, the standard LAB fermentation medium deMann, Rogosa and Sharpe (MRS) is also expensive due to its nitrogen source (meat, peptone and yeast extract) and does not support the growth of all LAB and probiotic cultures. Available literature shows that significant research is warranted in order to find alternative cost-effective ingredients that can help to lower the cost of LAB growth media as well as obtain higher cell densities. Thus, the objective of this study was to investigate the growth and viability of *Lb. bulgaricus* cultivated with alternative nitrogen sources and to assess its impact on viability after freeze-drying. We hypothesized that alternative nitrogen sources such as selected yeast extracts from the X-Seed brand of complex nitrogen sources could efficiently support the fermentation performance and viability of *Lactobacillus delbrueckii* subsp. *bulgaricus*.

Materials and methods

Source of Lactobacillus bulgaricus *and pre-culture fermentation medium*

Ten *Lb. bulgaricus* strains (online Supplementary Table S1) were used for the preliminary study (all data not shown). Two strains (S9 and LB6) were then selected for the main study based on their growth rates. The origin of all strains is elucidated in the online Supplementary File. We used deMan, Rogosa Sharpe (MRS, Neogen Co, Michigan, USA), as the pre-culture fermentation medium. The detailed pre-culture fermentation procedure is shown in the online Supplementary File.

Optimized lactobacilli growth medium and validation of nitrogen sources

The optimized lactobacilli growth medium was prepared after various nitrogen sources (online Supplementary Table S3) and their total nitrogen content (online Supplementary Table S2) were validated. The optimized growth medium had a similar composition to that of MRS (online Supplementary Table S4). However, the nitrogen sources were replaced by X-Seed Nucleo Max, and optimized proportions of blends of X-Seed KAT, and X-Seed Carbo Max as alternative nitrogen sources. Online Supplementary Table S5 shows the summarized composition of MRS and the optimized medium.

Determination of pH and buffering capacity with L*-arginine and* L*-histidine supplementation*

The pH values of each medium were measured in duplicate at the start and end of fermentation. Buffering capacity of all media were evaluated with 4 g/l of L-arginine and L-histidine respectively. The online Supplementary File highlights the pH and the buffer capacity determination.

Bacterial enumeration in growth media supplemented with and without L*-histidine*

Bacterial enumeration for the KCMax media blends with L-histidine supplementation is detailed in the online Supplementary File. All plates were anaerobically incubated for 48 h at 42°C and plates with 25–250 colonies were counted. Bacterial populations were expressed in log CFU/ml.

Impact of calcium on the growth, freeze-stability and viability of Lb. bulgaricus

The optimized KCMax (10/10) medium was supplemented with different concentrations of calcium and inoculated with the S9 *bulgaricus* strain as detailed in the online Supplementary File. Freeze-dried cells from calcium supplemented KCMax (10/10) medium were enumerated by the bacterial count method.

Statistical analyses

SAS version 9.4 (Cary, NC, USA) was used for data analysis. One-way Analysis of Variance (ANOVA) was used to determine significant differences between the values obtained 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 as log 10 before analyses were made.

Results and discussion

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. Some evaluated strains such as the S9 and LB6 were fast-growing ($OD_{610\ nm}$), thus explaining their selection for the main study. The optimized medium had a basal formula with 2 g/l of Nucleo-Max (N-Max) in conjunction with various blends of KAT (K) and Carbo-Max (C-Max) (see results under online Supplementary File).

Growth and cell density in the final optimized growth media

The S9 and LB6 *bulgaricus* strains demonstrated varied growth patterns during twelve hours of fermentation at 42°C with

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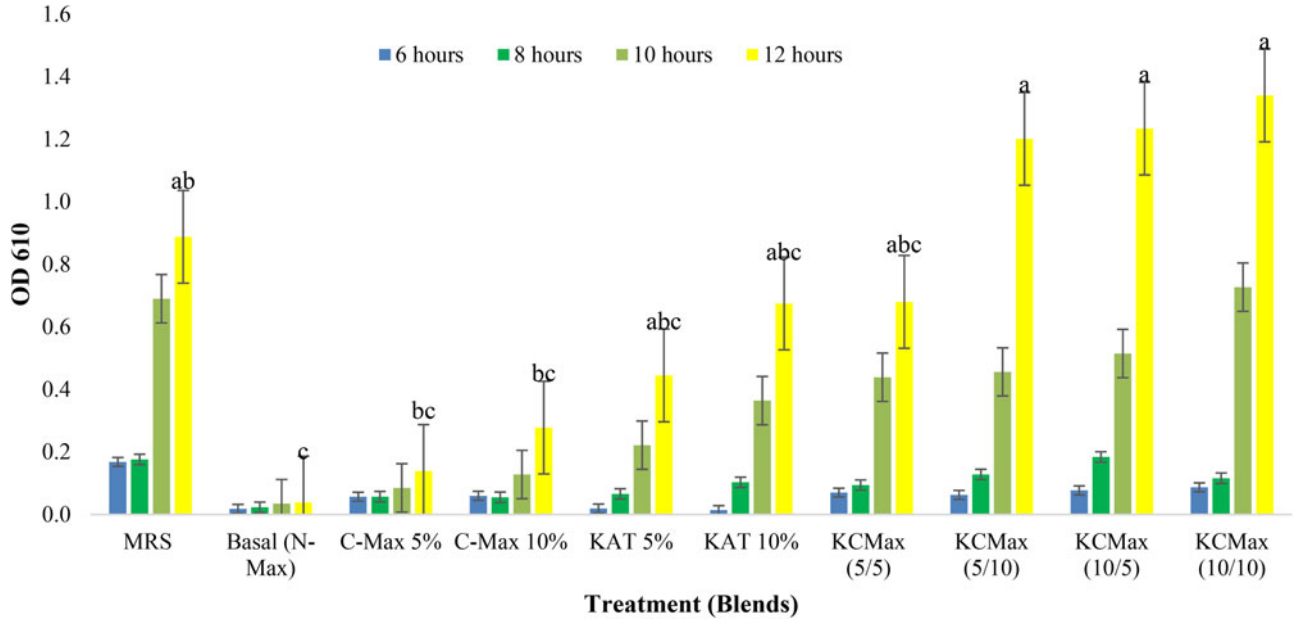

Fig. 1. (a) Changes in the growth of S9 *Lb. bulgaricus* strain at $OD_{610\ nm}$ in the different final growth media. The media included the following: MRS, basal medium (N-Max), and the basal medium supplemented with different nitrogen sources (KAT: K, and Carbo-Max: C-Max) at different proportions and blends after 12 h of fermentation at 42 °C. (b) Changes in the growth of LB6 *Lb. bulgaricus* strain at $OD_{610\ nm}$ in the different final growth media. The media included the following: MRS, basal medium (N-Max), and the basal medium supplemented with different nitrogen sources (KAT: K, and Carbo-Max: C-Max) at different proportions and blends after 12 h of fermentation at 42 °C.

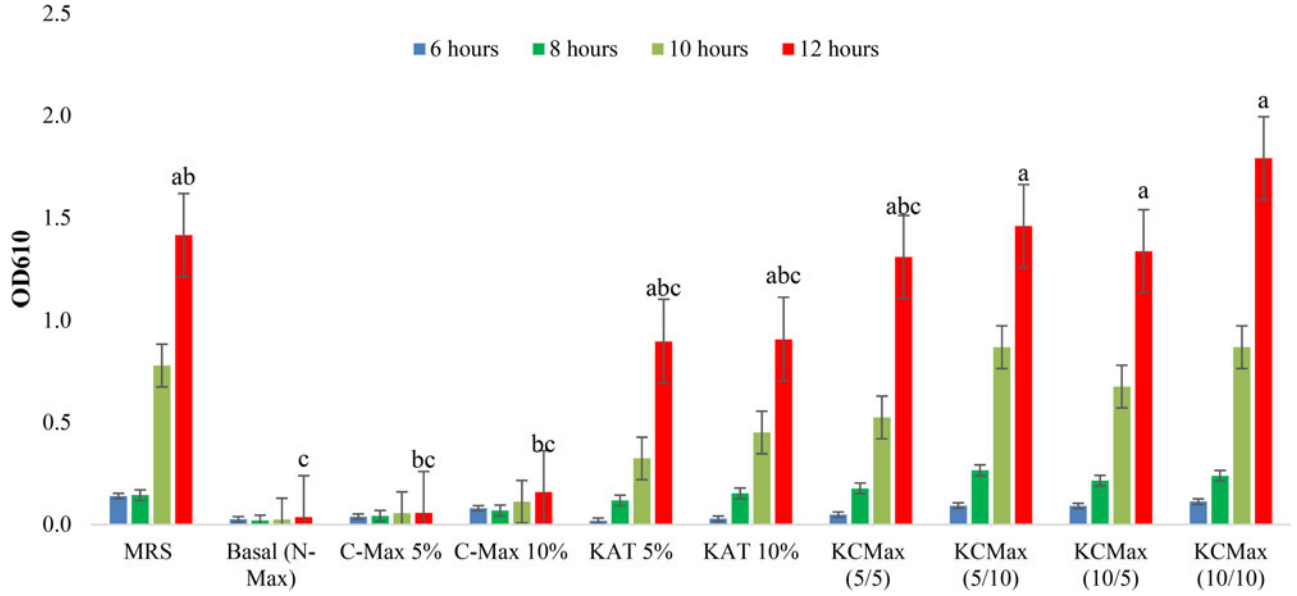

Fig. 1. Continued.

confirmation of their viability in MRS. There was a significant difference ($P < 0.05$) in the $OD_{610\ nm}$ results for both strains in the KAT and Carbo-Max media blends, with growth in the KCMax (10/10) medium outperforming that in MRS (Figs. 1a and 1b). The auxotrophic and fastidious nature of *Lb. bulgaricus* warrants supplementing the growth media with amino acids, peptides and vitamins, hence, yeast extracts are recommended as complex nitrogen sources for growth promotion (Atilola *et al.*, 2015; Curk *et al.*, 1993; Klotz *et al.*, 2017). Bacterial growth in the MRS and KCMax media blends suggested their nitrogen content impacted bacterial growth (Hossain *et al.*, 2020; Papizadeh *et al.*, 2020). Generally, nutritional requirements for LAB growth are strain-dependent (Degeest and De Vuyst 1999; Saguir and de Nadra, 2007), thus, alternative yeast extracts (X-Seed Nucleo Max, KAT, and Carbo Max) in the optimized medium demonstrated promising results as compared to conventional MRS. A basal medium (2 g/l of X-Seed Nucleo Max: N-Max) with KCMax (10/10) i.e. 10 g/l each of X-Seed KAT (K) and X-Seed Carbo Max (C-Max) yielded

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Table 1. Bacterial count (mean ± SD; $n = 3$) of *Lactobacillus bulgaricus* strains expressed as log CFU/ml in different growth media after incubation at 42°C for 48 h

	Strain	
Growth medium	S9	LB6
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,b,c,d}Means with different superscripts within a row differ significantly ($P < 0.05$).

superior results to those of MRS. To obtain superior LAB growth (1.34 and 1.79 at $OD_{610\ nm}$) for S9 and LB6 strains respectively, the KCMax (10/10) medium is highly recommended, as compared to MRS (0.89 and 1.42 at $OD_{610\ nm}$) respectively.

Enumeration of Lb. bulgaricus strains

There was a significant difference ($P < 0.05$) in the bacterial counts between the basal medium (N-Max), and all evaluated media blends for both evaluated strains. However, no significant difference ($P > 0.05$) in bacterial counts for the MRS and the KCMax media blends was recorded. Moreover, the bacterial count reported by the KCMax (10/10) medium for both strains were 7.2 and 6.5 log CFU/ml (S9 and LB6 respectively) as compared to MRS (7.6 and 7.1 log CFU/ml respectively). This suggested both media were efficient and promoted the viability of the *bulgaricus* strains (Savijoki *et al.*, 2006). Thus, the KCMax media blend could be an alternative to MRS as a growth medium (Polak-Berecka *et al.*, 2010) and will potentially support probiotic growth (Grześkowiak *et al.*, 2013). Table 1 shows the bacterial counts of the S9 and LB6 strains.

pH value changes in the different growth media

The initial pH values were adjusted to 6.5 before fermentation commenced. However, at the end of fermentation, the S9 strain exhibited a rapid decline in pH (between 5.33–5.62) for all evaluated media (MRS, KAT, and blends of KAT and Carbo-Max). This pH decline was also observed for the LB6 strain with post-fermentation pH values between 5.06–5.72. There was a significant difference ($P < 0.05$) for the optimized medium in comparison to MRS (Liu *et al.*, 2016). There was no significant difference ($P > 0.05$) for the N-Max and C-Max medium after fermentation. The observed pH changes for the various media (online Supplementary Fig. S3) could be attributed to the nitrogen sources in the media that impacted the media's buffering capacity.

Buffering capacity of growth media with L-arginine and L-histidine supplementation

Buffering capacity of a growth medium is essential, as it provides a stable pH for enhanced LAB metabolic activity. This stable pH promotes high cell density (biomass), as well as limiting the production of organic acids such as lactic acid, which could cause cell injury thereby decreasing growth (Hayek and Ibrahim, 2013). The buffering capacity was thus evaluated with two essential amino acids (L-arginine and L-histidine). Significant changes in buffering capacity were only observed with L-histidine supplementation. Generally, the three non-supplemented KCMax media blends had low buffering values (15.24, 15.24, and 15.50 respectively) as compared to non-supplemented MRS (21.57). The L-histidine supplemented blends; KCMax (5/10), KCMax (10/5), and KCMax (10/10), had the highest significant buffering values of 36, 36.5, and 39 respectively ($P < 0.05$) as compared to the L-histidine supplemented MRS (35.22). It was evident that the KCMax (10/10) medium with L-histidine had the highest value (39) and thus promoted enhanced biomass production during fermentation, thus is recommended for LAB fermentations. Online Supplementary Table S6 summarizes the different buffering capacities for the L-arginine and L-histidine supplemented media. In addition, online Supplementary Figs S4 to S13 inclusive report the different pH buffering of the evaluated amino acids.

Impact of L-histidine supplementation on the growth of S9 Lb. bulgaricus *strain*

Supplementing the growth media KCMax (5/10), KCMax (10/5), and KCMax (10/10) with varying L-histidine concentrations (4 g/l or 0.4% and 0.5 g/l or 0.005%) impacted cell growth during fermentation (Aumiller *et al.*, 2021). The bacterial count for the buffered MRS and the KCMax blends (0.4% and 0.005% L-histidine) elucidated its impact on strain viability. There was no significant difference ($P > 0.05$) for bacterial counts for the 0.4% L-histidine supplemented and the non-supplemented media. There was, however, a significant difference ($P < 0.05$) for the bacterial count for the 0.005% L-histidine supplementation (online Supplementary Fig. S13). In general, the unbuffered control media (MRS and the KCMax blends) had higher counts than the L-histidine supplemented media. The KCMax (10/10) medium did, however, have the same count (8.1 log CFU/ml) for both 0.4% and 0.005% L-histidine treatments. This infers a higher nitrogen blend such as KCMax (10/10) could mask L-histidine's impact. Also, cell morphologies were larger and more aggregated (Fig. 2) after L-histidine treatment than in the control sample. Zeidan et al. (2014) also confirmed that L-histidine modified the cell wall of yeast bacteria. Furthermore, the reduced cell viability could be attributed to the inhibitory effect of L-histidine (Lamberti *et al.*, 2011; Suzuki *et al.*, 2014; Zeidan *et al.*, 2014).

Calcium effect on the growth and viability of Lactobacillus delbrueckii subsp. bulgaricus

The KCMax 10/10 blend with calcium supplementation had a significant impact on the growth of *Lb. bulgaricus* (Chervaux *et al.*, 2000). The cell-wash treatment after fermentation decreased the total calcium absorbed by the cell. It was thus observed that the control sample had significant ($P < 0.05$) bacterial counts (8.2 and 8.6 log CFU/ml, respectively) for the unwashed and washed S9 *bulgaricus* cells. Calcium supplementation (0.4%) for both unwashed and washed cell treatments had bacterial counts of 8.3 and 8.4 log CFU/ml respectively. There was higher calcium content in the unwashed cells (256.4 ppm) than in the washed cells (125.8 ppm). Furthermore, the 0.8% calcium supplementation for both unwashed and washed cells had bacterial counts

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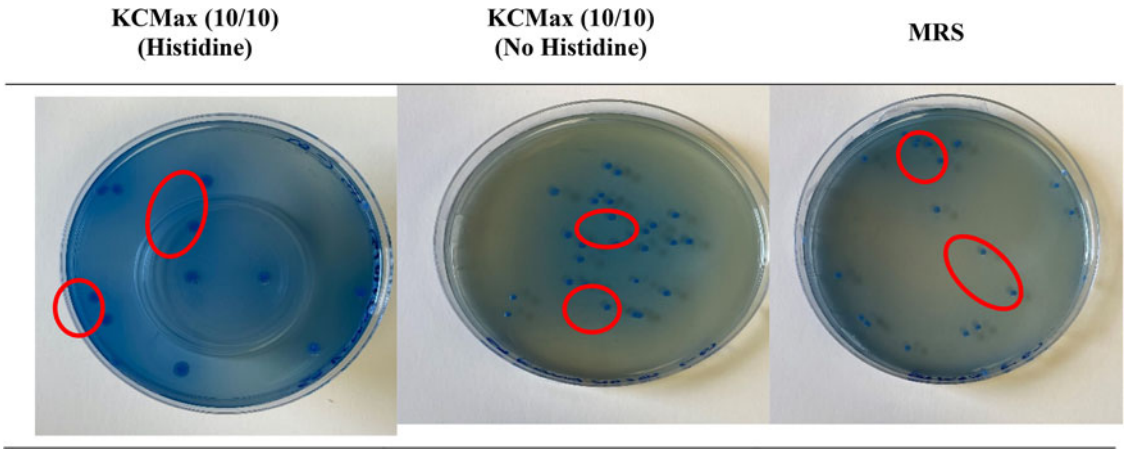

Fig. 2. Morphology of *Lb. bulgaricus* colonies (encircled in red) on the growth media plates: KCMax (10/10) supplemented with L- histidine, KCMax (10/10) without L-histidine supplementation and (MRS).

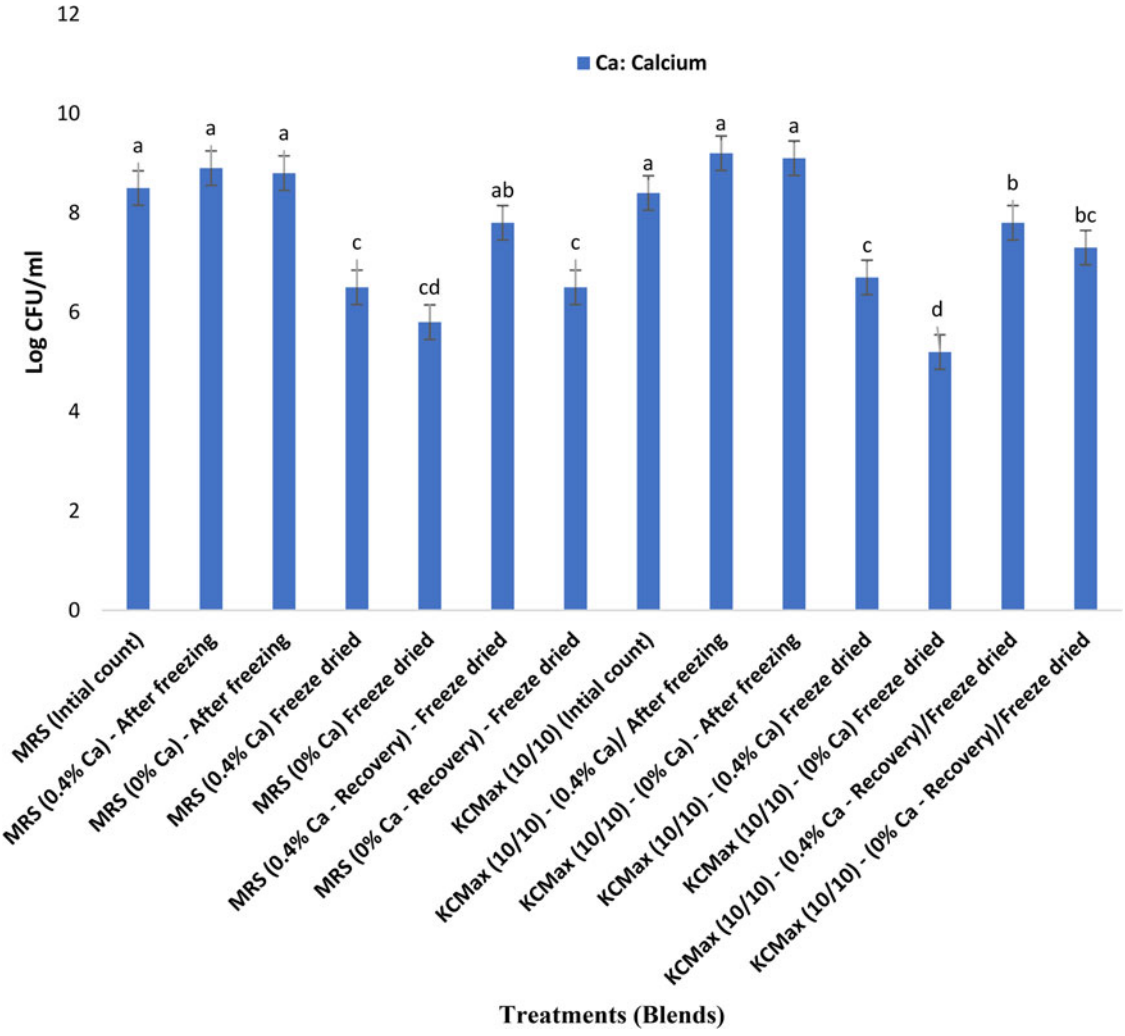

Fig. 3. Viability and bacterial count of S9 *Lb. bulgaricus* strain before and after freezing and freeze-drying with and without 0.4% calcium supplementation after fermentation in MRS and KCMax (10/10) medium.

of 7.9 and 8.2 log CFU/ml respectively. Calcium content was thus directly proportional to the cell treatment, hence, calcium supplementation (1.2%) corresponded to the calcium content of 616.6 ppm (unwashed) and 585.5 ppm of (washed) treatments (online Supplementary Fig. S15). However, this concentration (1.2%), decreased bacterial count significantly ($P < 0.05$) for both

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unwashed (7.5 log CFU/ml) and washed cell (7.7 log CFU/ml) treatments (online Supplementary Fig. S16). Thus, excessive calcium content is detrimental to the viability of *Lb. bulgaricus* strains (Isshiki and Azuma, 1995). Dixon *et al.* (2018), also confirmed that the excess addition of 0.5 M calcium inhibited biofilm formation in dairy wastewater.

The effect of calcium on the freeze-stability and viability of Lb. bulgaricus after freeze-drying

Calcium as a cryoprotectant aids in minimizing probiotic cell viability after freeze-drying (Wright and Klaenhammer, 1983; Fenster *et al.*, 2019). Initial *bulgaricus* counts for both media (KCMax (10/10) and MRS) was 8.5 log CFU/ml. The population counts for the treatments with and without (0.4%) calcium supplementation in MRS after overnight freezing (−80°C) were 8.9 and 8.8 log CFU/ml respectively. Furthermore, the KCMax (10/10) medium under the same treatments, had higher population counts (9.2 and 9.1 log CFU/ml respectively). There was, however, no significant difference ($P > 0.05$) between counts for both media after freezing. It was noteworthy that the freeze-dried cell counts with and without calcium supplementation in MRS decreased to 6.5 and 5.8 log CFU/ml respectively. The same trend was observed for the KCMax (10/10) freeze-dried cells and counts were 6.7 and 5.2 log CFU/ml respectively (Fig. 3). Thus calcium-treated cells had slightly higher counts than non-calcium-treated cells for both media. The freeze-dried cell counts, however, increased after recovery in skimmed milk which could be attributed to the resuscitation of stressed and injured cells after freeze-drying (Celik and O'Sullivan, 2013). The MRS recovered freeze-dried cell counts were 7.8 and 6.5 log CFU/ml respectively with and without calcium treatment. Consequently, the KCMax (10/10) recovered freeze-dried cell counts were also 7.8 and 7.3 log CFU/ml respectively. The effect of calcium and milk recovery on probiotic viability has been confirmed by Wright and Klaenhammer (1981, 1983).

In conclusion, our results demonstrated that X-Seed Nucleo Max, KAT, and Carbo Max efficiently supported the growth and viability of *Lb. bulgaricus*. L-Histidine enhanced the buffering capacity of the KCMax 10/10 medium. Consequently, 0.4% L-histidine supported higher bacterial growth than 0.005% and caused cell aggregation and larger cell morphology than that observed in MRS. Research is warranted to understand L-histidine's mechanism on the growth of *Lactobacillus* species as only a few studies are available. The calcium supplemented KCMax (10/10) medium enhanced cell viability after freezing and freeze-drying, resulting in high bacterial counts and more rounded cell morphologies than in MRS. A future study is thus needed to examine the morphological change due to calcium. Our optimized medium has cryoprotectant properties and can potentially support the growth and viability of *Lb. bulgaricus* and is thus recommended for LAB fermentations.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000541

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