A modified reinforced clostridial medium for the isolation and enumeration of *Lactobacillus delbrueckii* ssp. *bulgaricus* in a mixed culture

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ABSTRACT

In this study, we modified reinforced clostridial medium (RCM) to selectively enumerate and isolate *Lactobacillus delbrueckii* ssp. *bulgaricus*, a probiotic and important starter culture in the dairy industry. The disparity in the reported carbohydrate fermentation pattern of *L. delbrueckii* ssp. *bulgaricus* was used to develop a growth medium not only selective for *L. delbrueckii* ssp. *bulgaricus* but significantly inhibitory to the growth of other lactic acid bacteria. A recently modified RCM (mRCM) was optimized for this study by the addition of 0.5% fructose, 0.5% dextrose, 1% maltose, and 0.25% sodium pyruvate while replacing lactose as a carbohydrate source. The cell recovery and bacterial counts of *L. delbrueckii* ssp. *bulgaricus* in tested products (pure *L. delbrueckii* ssp. *bulgaricus* strains, starter culture, probiotic supplements, and yogurt) using our mRCM with sodium pyruvate (mRCM-PYR) were significantly higher than in the recently modified RCM and the common de Man, Rogosa, and Sharpe (MRS) culture medium. The growth of other lactic acid bacteria (*Streptococcus thermophilus*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, and *Lactobacillus reuteri*) and *Bifidobacteria* was retarded in this modified medium compared with their growth in MRS and mRCM. This result is a significant improvement in the enumeration and differentiation of *L. delbrueckii* ssp. *bulgaricus* in mRCM-PYR compared with the results in MRS and mRCM where the high background growth of similar species interferes with the accuracy of bacterial population counts. Our results thus suggest that mRCM-PYR could be recommended as a reliable alternative growth medium for the selective enumeration and isolation of *L. delbrueckii* ssp. *bulgaricus* in a mixed culture.

Key words: *Lactobacillus bulgaricus*, isolation, mixed culture, selective media, bacterial enumeration

INTRODUCTION

Lactobacillus delbrueckii ssp. *bulgaricus* is an important and highly regarded species of lactic acid bacteria due to its application in the production of several dairy products including yogurt, a fermented milk product that is well known for its health benefits (Zhu et al., 2010). Yogurt starter cultures require a symbiotic blend of *L. delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* in approximately equal amounts to obtain the desired organoleptic properties that result from the metabolic activity of both microorganisms during their growth in milk. Consequently, the final quality of yogurt can suffer with regard to texture, acidity, and flavor if this proper bacterial balance is not achieved (Lourens-Hattingh and Viljoen, 2001).

The probiotic potential of *L. delbrueckii* ssp. *bulgaricus* has been mentioned in several studies (Singh et al., 1979; Mahmood, et al., 2015; Pescuma et al., 2015), and as with any probiotic, it confers various health benefits to the host when administered at an effective dose. Consequently, in order for *L. delbrueckii* ssp. *bulgaricus* to play its role as a probiotic, the requisite bacterial population levels are critical. To support the quality of yogurt, many countries have adopted standards for these viable bacteria numbers and the ratio of *L. delbrueckii* ssp. *bulgaricus* to *Streptococcus thermophilus*. For example, the Codex Alimentarius, a collection of internationally recognized standards for food production, and most other national regulatory agencies in the US have established a minimum level of 1×10^7 cfu/g of yogurt starter microorganisms. Other countries such as France, Switzerland, Italy, and Spain have also agreed to follow the Codex Alimentarius recommendation of a minimum level of yogurt starter microorganisms at the time of consumption. However, in the United Kingdom, a minimum requirement of 10 million viable lactobacilli per mL of yogurt at the time of sale was established

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(Davis and McLachlan, 1974; Robinson and Tamime, 1976; Hamann and Marth, 1984).

The current standard medium, de Man, Rogosa, and Sharpe (**MRS**), is unreliable as a selective method for enumerating *L. delbrueckii* ssp. *bulgaricus* species in mixed bacterial cultures as true counts are often imprecise due to the presence of high background colonies of similar species. Other media proposed for the selective enumeration of specific lactic acid bacteria (Matalon and Sandine, 1986; Ghoddusi and Robinson, 1996; Yamani and Ibrahim, 1996; Tabasco et al., 2007; Galat et al., 2016; Hayek et al., 2019) also struggle to selectively enhance the growth of *L. delbrueckii* ssp. *bulgaricus* in the presence of other lactic acid bacteria. A modified reinforced clostridial medium (**mRCM**) by Nwamaioha and Ibrahim (2018) recently showed remarkable results in the differentiation and enumeration of 2 strains of *L. delbrueckii* ssp. *bulgaricus* in a mixed bacterial culture by giving distinct and large colonies. This culture medium supports the growth of lactic acid bacteria other than *L. delbrueckii* ssp. *bulgaricus* but still makes the isolation and enumeration steps required

for *L. delbrueckii* ssp. *bulgaricus* very challenging. Nevertheless, preliminary studies have demonstrated that mRCM can be optimized to improve the recovery of stressed bacterial cells and select for a broader range of *L. delbrueckii* ssp. *bulgaricus* strains. Therefore, the objective of this study was to examine the modification of reinforced clostridial medium (**RCM**) for the isolation and enumeration of *L. delbrueckii* ssp. *bulgaricus* from a mixed culture.

MATERIALS AND METHODS

Source of *L. delbrueckii* ssp. *bulgaricus*

A total of 32 sources of *L. delbrueckii* ssp. *bulgaricus* strains were used in the study (Table 1). Eleven strains of freeze-dried *L. delbrueckii* ssp. *bulgaricus* cultures intended for industrial production of fermented milk products were supplied by Albert Krastanov (Department of Biotechnology, University of Food Technologies, Plovdiv, Bulgaria). Six yogurt starter cultures and 3 probiotic supplements from Europe were selected. All

Table 1. Description of samples used

No.	Product code	Sample	Source	Bacterial composition as labeled
1	S28	Pure industrial strain	Bulgaria	<i>Lactobacillus bulgaricus</i>
2	S6	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
3	S19	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
4	S8	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
5	LB6	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
6	S1	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
7	LB9	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
8	S22	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
9	S9	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
10	S7	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
11	S5	Pure industrial strain	Bulgaria	<i>L. bulgaricus</i>
12	ST11	Starter culture	Bulgaria	<i>Streptococcus thermophilus</i> , <i>L. bulgaricus</i> , <i>Lactobacillus rhamnosus</i>
13	ST12	Starter culture	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i>
14	ST13	Starter culture	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i>
15	ST14	Starter culture	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i>
16	ST15	Starter culture	Canada	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>Lactobacillus acidophilus</i>
17	ST16	Starter culture	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i>
18	SP17	Probiotic supplement	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>Lactobacillus reuteri</i>
19	SP18	Probiotic supplement	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i>
20	SP19	Probiotic supplement	Bulgaria	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>L. rhamnosus</i>
21	SP8	Yogurt	Spain	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>Bifidobacterium</i> , <i>L. reuteri</i>
22	SP9	Yogurt	Spain	Live and active cultures
23	SP10	Yogurt	Spain	Live and active cultures
24	E1A	Yogurt	The Netherlands	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>L. acidophilus</i> , <i>Bifidobacterium</i>
25	E2Z	Yogurt	The Netherlands	Live and active culture
26	E3D	Yogurt	Spain	Live and active culture
27	E4P	Yogurt	Bulgaria	Live and active culture
28	L6N	Yogurt	United States	<i>S. thermophilus</i> , <i>L. bulgaricus</i> , <i>Bifidobacterium lactis</i> , <i>L. acidophilus</i> , <i>L. rhamnosus</i>
29	BY2	Yogurt	Bulgaria	<i>L. bulgaricus</i> , other live culture
30	BY4	Yogurt	Bulgaria	<i>L. bulgaricus</i> , other live culture
31	BY5	Yogurt	Bulgaria	<i>L. bulgaricus</i> , other live culture
32	BY6	Yogurt	Bulgaria	<i>L. bulgaricus</i> , other live culture

samples were maintained at -20°C until further use. In addition, 12 yogurt samples were obtained from Europe and stored under refrigeration until further use.

Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Fisher Scientific (Pittsburgh, PA).

Lactobacilli MRS Agar Medium

A lactobacilli MRS agar medium was prepared by dissolving 55 g of MRS broth (Neogen Co., Lansing, MI) and 0.5 g of L-cysteine in 1 L of deionized distilled water (DDW), and the resultant solution was stirred well until all particles were completely dissolved. Agar powder (15 g; Neogen) was added, and the agar medium was sterilized at 121°C for 15 min and then cooled in a water bath.

Modified Reinforced Clostridial Medium

Modified RCM was prepared according to the method of Nwamaioha and Ibrahim (2018) by completely dissolving 10 g of proteose peptone #3, 10 g of beef extract, 5 g of yeast extract, 10 g of lactose, 5 g of sodium chloride, 3 g of sodium acetate, 2 g of K_2HPO_4 , 0.1 g of uracil, 0.25 g of calcium chloride, 0.2% Tween 80, and 0.5 g of L-cysteine in 1 L of DDW. This solution was adjusted to a final pH of 6.0 ± 0.2 using 6 M HCl before the addition of 0.01% aniline blue and 15 g of agar. This medium was then autoclaved at 121°C for 15 min and cooled in a water bath.

Modified Reinforced Clostridial Medium-Pyruvate

A reinforced clostridial medium was optimized for selectivity and accurate enumeration of *L. delbrueckii* ssp. *bulgaricus* by dissolving 10 g of peptone #3, 10 g of beef extract, 5 g of yeast extract, 5 g of sodium chloride, 3 g of sodium acetate, 2 g of K_2HPO_4 , 0.1 g of uracil, 0.25 g of calcium chloride, 5 g of dextrose, 5 g of fructose, 10 g of maltose, 2 g of sodium pyruvate, 0.2% Tween 80, and 0.5 g of L-cysteine in 1 L of DDW. This solution was adjusted to a final pH of 6.0 ± 0.2 using 6 M HCl before the addition of 0.008% aniline blue and 15 g of agar. The medium was autoclaved at 121°C for 15 min and then cooled in a water bath. All freshly prepared media in this study were poured into sterile Petri dishes and stored at 4°C until needed. The difference in the carbohydrate sources of mRCM and modified reinforced clostridial medium-pyruvate (mRCM-PYR) is highlighted in Table 2.

Enumeration of *L. delbrueckii* ssp. *bulgaricus* in Pure Industrial Strains, Yogurt Starter Culture, and Probiotic Supplements

For the enumeration of *L. delbrueckii* ssp. *bulgaricus* in these products, 0.1 g of freeze-dried samples was measured and transferred into 3 mL of skim milk with the addition of 10 μL of Tween 80 and 0.1 mL of yeast extract to enhance the recovery of stressed bacterial cells. This mix was fermented at 44°C for 7 h, at which point the skim milk curdled. The curdled milk was shaken, and 1 mL was transferred for serial dilution into a 9 mL of peptone solution. Aliquots (100 μL) of appropriate dilutions were plated in duplicates onto the 3 culture media (MRS, mRCM, and optimized RCM) for the enumeration of *L. delbrueckii* ssp. *bulgaricus*. The inoculated plates were incubated for 72 h at 44°C in anaerobe chambers, and the colonies were counted using a Quebec colony counter (Fisher Scientific).

Enumeration of *L. delbrueckii* ssp. *bulgaricus* in Commercial Yogurt Samples

Dilutions of yogurt samples were prepared by adding 10 g of each sample to a screw-capped bottle containing 90 mL of deionized water and then properly shaking the mixture. This mixture was placed in an incubator at 44°C for 15 min to repair weak and damaged cells. Subsequent dilutions of up to 10^{-7} were made in a 0.1% peptone solution and vortexed well between transfers. Aliquots (100 μL) of appropriate dilutions were then plated in duplicate onto the 3 culture media (MRS, mRCM, and optimized RCM) for the enumeration of

Table 2. Composition of modified reinforced clostridial medium (mRCM) and modified reinforced clostridial medium-pyruvate (mRCM-PYR)

mRCM	mRCM-PYR
10 g of proteose peptone #3	10 g of proteose peptone #3
10 g of beef extract	10 g of beef extract
5 g of yeast extract	5 g of yeast extract
5 g of sodium chloride	5 g of sodium chloride
0.25 g of calcium chloride	0.25 g of calcium chloride
3 g of sodium acetate	3 g of sodium acetate
2 g of ammonium phosphate dibasic	2 g of ammonium phosphate dibasic
0.5 g of L-cysteine	0.5 g of L-cysteine
0.1 g of uracil	0.1 g of uracil
0.01% aniline blue	0.008% aniline blue
0.2% Tween 80	0.2% Tween 80
10 g of lactose ¹	5 g of dextrose ¹
	5 g of fructose ¹
	10 g of maltose ¹
	2 g of sodium pyruvate ¹

¹Carbohydrate sources.

L. delbrueckii ssp. *bulgaricus*. The inoculated plates were incubated for 72 h at 44°C in anaerobe (CO₂/N₂) chambers (Labconco, Kansas City, MO), and the colonies were counted using a Quebec colony counter (Fisher Scientific).

Enumeration of *L. delbrueckii* ssp. *bulgaricus* in Traditional Bulgarian Yogurt

To enumerate *L. delbrueckii* ssp. *bulgaricus* in traditional Bulgarian yogurt, 5 mL of samples was measured and transferred into 45 mL of skim milk with 2 mL of yeast extract (20%) added to repair damaged and stressed bacterial cells. This mix was fermented at 44°C for 7 h, at which point the skim milk curdled. The curdled milk was stirred, and 10 mL was sampled for the enumeration of bacterial cells following the method outlined for the enumeration of *L. delbrueckii* ssp. *bulgaricus* in yogurt samples.

Statistical Analyses

The SAS version 9.4 (SAS Institute Inc., Cary, NC) was used to analyze the experiment data obtained in this study. One-way ANOVA was used to determine significant differences between the values. Significant differences ($P < 0.05$) between means of bacterial counts (cfu/g) were compared using Tukey's test. Bacterial population counts were converted to log₁₀ transformation before analysis.

RESULTS

Preliminary Study

In preliminary studies, the effect of different carbon sources on the morphology of *L. delbrueckii* ssp. *bulgaricus* colonies was evaluated. We observed that the use of 2% sodium citrate and 1% sodium pyruvate as alternatives to a proteose peptone diluent solution not only produced more prominent and distinguishable colonies in the previously modified RCM by Nwamaioha and Ibrahim (2018) but also improved the bacterial count (data not shown). Our findings also showed that sodium pyruvate retarded the growth of lactic acid bacteria other than *L. delbrueckii* ssp. *bulgaricus*, which otherwise grew when 2% sodium citrate and 0.1% proteose peptone were used as diluent media. These observations would suggest that the inclusion of sodium pyruvate as a component in a growth medium could improve the recovery of injured or damaged bacterial cells. Further tests employed various concentrations of individual and combined treatments of fructose, maltose, dextrose, lactose, sodium citrate, and sodium pyruvate as components of mRCM. Initially, a combined treatment of 0.5% dextrose, 0.5% fructose, 1% maltose, and 1% sodium pyruvate replacing 1% lactose as a carbon source in mRCM appeared to be the most effective at selecting only for *L. delbrueckii* ssp. *bulgaricus* in the mixed bacterial culture. However, it became evident during the screening that a reduced concentration of sodium pyruvate would be needed to be effective in improving the selectivity of the modified medium. A 1%

Figure 1. The red arrow shows a rough-edged *Lactobacillus bulgaricus* colony in modified reinforced clostridial medium (mRCM; left image) morph into a round and smooth colony in modified reinforced clostridial medium-pyruvate (mRCM-PYR) having a concentration of 1% sodium pyruvate.

sodium pyruvate concentration in this growth medium composition not only retarded other bacterial growth but also converted the characteristic rough-edge morphology of the surviving *L. delbrueckii* ssp. *bulgaricus* colonies to a smooth one (Figure 1). Thus, a combination of 0.5% dextrose, 0.5% fructose, 1% maltose, and 0.2% sodium pyruvate as carbon sources in our growth medium proved to be the most effective at improving the morphology and cell recovery of *L. delbrueckii* ssp. *bulgaricus* colonies.

Cell Repair and Recovery

The *L. delbrueckii* ssp. *bulgaricus* colonies that were isolated from freeze-dried probiotic supplements on MRS, mRCM, and mRCM-PYR growth media are shown in Figure 2. The SP18 cell colonies (*L. delbrueckii* ssp. *bulgaricus* circled in red) appear small and fragile in both the MRS and mRCM, suggesting that the cells were stressed or injured; however, this was not the case in mRCM-PYR where the *L. delbrueckii* ssp.

Figure 2. Morphology of *Lactobacillus bulgaricus* colonies (circled in red) on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR). See Table 1 for sample description.

bulgaricus cells appeared as large colonies. The result for SP19 further highlighted the advantage of using the mRCM-PYR growth media over the previously modified RCM with regard to the repair of damaged or stressed microbial cells. Nonlethal injury to bacterial cells can occur for several reasons. For example, the destruction of bacterial cells as a result of exposure to low temperatures in the form of freeze drying, freezing/thawing, and prolonged storage at low temperatures is common in the industrial production of probiotic supplements and starter cultures.

The cell colonies in mRCM were the smallest while the mRCM-PYR culture media facilitated the optimum growth and repair of *L. delbrueckii* ssp. *bulgaricus* cells, resulting in larger colonies. Similar results were noted with other starter cultures that were evaluated for the viability of *L. delbrueckii* ssp. *bulgaricus*. For instance, in the previously modified RCM growth media, the colonies of *L. delbrueckii* ssp. *bulgaricus* in samples ST 11 and ST 14 were tiny and very difficult to identify in the presence of other bacteria colonies. In contrast to the mRCM, our optimized RCM produced visible and large colonies for the same samples, and the growth of other bacteria was significantly inhibited (Figure 2).

Selective Growth and Enumeration of Pure *L. delbrueckii* ssp. *bulgaricus* Strains

The bacterial counts of 11 pure *L. delbrueckii* ssp. *bulgaricus* strains are presented in Figure 3. The

mRCM-PYR performed significantly better than MRS and mRCM ($P < 0.05$) in 6 of the strains (S6, S19, LB9, S9, S7, and S5) as measured by the bacterial populations. No significant difference was observed in the bacterial counts recorded in mRCM and mRCM-PYR in 4 *L. delbrueckii* ssp. *bulgaricus* strains (S28, S8, S1, and S22). However, both growth media performed significantly better than MRS ($P < 0.05$) in these strains. The only case in which MRS performed noticeably better ($P < 0.05$) than mRCM-PYR and mRCM was with *L. delbrueckii* ssp. *bulgaricus* strain LB6. The effect of the 3 media on the morphology of *L. delbrueckii* ssp. *bulgaricus* colonies is shown in Figure 4.

Selective Enumeration and Differentiation of *L. delbrueckii* ssp. *bulgaricus* in Mixed Cultures

The bacterial counts of *L. delbrueckii* ssp. *bulgaricus* recovered from 21 products using MRS, mRCM, and mRCM-PYR are presented in Figure 5. The 21 products consisted of a starter culture, probiotic supplements, and yogurt and contained mixed bacterial cultures. The 3 media had similar effects on the bacterial counts of 4 products (E1A, BY2, BY4, and SP8). No significant difference was observed in the bacterial counts in mRCM and mRCM-PYR for 6 products (E2Z, L6N, BY5, ST12, SP17, and SP19). However, both growth media performed significantly better than MRS ($P < 0.05$) in these products. The mRCM-PYR performed better than MRS and mRCM ($P < 0.05$) in 10 prod-

Figure 3. Bacterial count of 11 pure *Lactobacillus bulgaricus* strains on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) at 44°C after 72 h of anaerobic incubation. Error bars indicate SD for an experiment performed in duplicate.

Figure 4. Morphology of pure *Lactobacillus bulgaricus* strains on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) after anaerobic incubation at 44°C for 72 h.

ucts (E3D, BY6, SP9, ST11, ST14, SP10, ST13, ST15, ST16, and SP18). The bacterial counts of *L. delbrueckii* ssp. *bulgaricus* in E4P were similar in mRCM-PYR and MRS but considerably higher than those in mRCM ($P < 0.05$). The results thus confirmed the effectiveness of our growth medium, mRCM-PYR, as a suitable alternative to MRS and mRCM in the selective enumeration and isolation of *L. delbrueckii* ssp. *bulgaricus* in mixed bacterial cultures. Figure 6 shows the morphology of *L. delbrueckii* ssp. *bulgaricus* in mixed cultures. The colonies of *L. delbrueckii* ssp. *bulgaricus* in mRCM-PYR were easy to identify and enumerate as the growth of other lactic acid bacteria was highly retarded. The identification of colonies was confirmed using direct microscopic observation and the PCR method.

DISCUSSION

In a previous study by Nwamaioha and Ibrahim (2018), a growth medium was modified to selectively enhance the isolation and enumeration of *L. delbrueckii* ssp. *bulgaricus* in dairy products. In the present study, we further modified this medium for the superior selectivity and enumeration of *L. delbrueckii* ssp. *bulgaricus* by replacing lactose with 0.5% dextrose, 0.5% fructose, 1% maltose, and 0.2% sodium pyruvate. Various carbohydrates that are the primary energy source for bacteria in growth media have been used to selectively enumerate lactic acid bacteria. For example, Tabasco et al. (2007) supplemented MRS with fructose to enumerate *L. delbrueckii* ssp. *bulgaricus*. Another justification

Figure 4 (Continued). Morphology of pure *Lactobacillus bulgaricus* strains on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) after anaerobic incubation at 44°C for 72 h.

for the use of different carbon sources is the disparity in the reported carbohydrate use in *L. delbrueckii* ssp. *bulgaricus*. The inconsistencies recorded in the use of fructose, mannose, and maltose among other sugars in *L. delbrueckii* ssp. *bulgaricus* have been attributed to strain type and composition of the growth medium (Zourari et al., 1992; Chervaux et al., 2000; Yuksekdağ and Aslim, 2008; Li et al., 2016). The mRCM-PYR was developed to optimally enumerate and select for *L. delbrueckii* ssp. *bulgaricus* regardless of the source and strain type. We observed that the combination of carbon sources was responsible for the selectivity and high bacterial counts in mRCM-PYR. However, the substitution or elimination of any of the carbon sources, particularly sodium pyruvate, did not yield the same result. Pyruvate is an essential intermediate in the metabolism of lactic acid bacteria and can be converted into several end products such as acetic acid, formic acid, and lactic acid. Higashio et al. (1977) attributed the stimulatory effect that *S. thermophilus* had on the growth of *L. delbrueckii* ssp. *bulgaricus* to the production of formic and pyruvic acids. Polak-Berecka et al. (2010) recorded a better yield of cell biomass when sodium pyruvate was combined with glucose as carbon sources. These results might help to explain the importance of pyruvate in the cell repair and recovery observed in this study.

Many of the medium available in the literature such as MRS (de Man et al., 1960), Lee's agar (Lee et al., 1974), yogurt lactic agar (Matalon and Sandine, 1986),

reinforced clostridial Prussian blue (Ghoddusi and Robinson, 1996), and bromocresol green whey agar (Yamani and Ibrahim, 1996) have not been able to selectively enhance the growth of *L. delbrueckii* ssp. *bulgaricus* in a mixed culture. The mRCM-PYR performed well in the enumeration of *L. delbrueckii* ssp. *bulgaricus* in a mixed culture while retarding the growth of other lactic acid bacteria. This is an improvement over the previous study by Nwamaioha and Ibrahim (2018) where a lack of significant differences in bacterial counts coupled with the growth of other lactic acid bacteria was recorded in the tested products.

Further study may subject *L. delbrueckii* ssp. *bulgaricus* strains isolated in this study using mRCM-PYR to genotypic methods of identification with a view to discovering relationships between the origins of various strains. The enzyme activity, secreted proteins, aroma production, and stress resistance of the various strains should also be tested and compared. In addition, understanding of the pyruvate metabolism is critical to demonstrate the applications and health benefits of *L. delbrueckii* ssp. *bulgaricus*. This additional exploration could lead to the identification and isolation of superior probiotic and industrial strains.

CONCLUSIONS

Our results demonstrated that mRCM-PYR modified by the addition of sodium pyruvate, fructose, and maltose was effective in selectively isolating and

Figure 5. Bacterial count of *Lactobacillus bulgaricus* in mixed culture on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) at 44°C after 72 h of anaerobic incubation. Error bars indicate SD for an experiment performed in duplicate.

Figure 6. Morphology of *Lactobacillus bulgaricus* (circled) in mixed culture on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) after anaerobic incubation at 44°C for 72 h.

Figure 6 (Continued). Morphology of *Lactobacillus bulgaricus* (circled) in mixed culture on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) after anaerobic incubation at 44°C for 72 h.

enumerating *L. delbrueckii* ssp. *bulgaricus* in a mixed culture. The versatility of mRCM-PYR also makes it highly suitable for the study and rapid cultivation of

L. delbrueckii ssp. *bulgaricus*. Thus, mRCM-PYR is recommended as a growth medium for the isolation and enumeration of *L. delbrueckii* ssp. *bulgaricus* from

Figure 6 (Continued). Morphology of *Lactobacillus bulgaricus* (circled) in mixed culture on de Man, Rogosa, and Sharpe (MRS) medium, modified reinforced clostridial medium (mRCM), and modified reinforced clostridial medium-pyruvate (mRCM-PYR) after anaerobic incubation at 44°C for 72 h.

different sources due to this medium's inhibitory effect on the growth of other LAB.

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