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Lactobacilli Count, Morphology Analysis and Biochemical Identification of Lactic Acid Bacteria Isolates from Top Yogurt Brands

Galina Satchanska*, Gulesme Mustapha, Valeria Ahad, Daphina Ilin, Ana Doycheva
Biolab, Department of Natural Sciences, New Bulgarian University, Montevideo St. 21, 1618 Sofia

Abstract

Lactic acid bacteria (LAB) are pronounced probiotics. They strongly improve the human immune system and produce beneficial for the human health vitamins. Lactobacilli show a strong bactericidal and antifungal activity. Due to the synthesis of many enzymes involved in the degradation of toxic substances, yogurt is known as a food favorable to longevity. Six top brands of 3.6% cow's milk yogurt available on the Bulgarian market were examined for viable lactobacilli count, and both morphological and biochemical analysis of LAB isolates was carried out. Lactobacilli were enumerated in 1 g of yogurt *via* the Koch method. API 50-CH kit with 50 different metabolites was applied to examine the biochemical activity of LAB isolates and their identification. The morphology of LAB isolates was studied through light microscopy and Scanning Electron Microscopy (SEM). Our results revealed that the number of lactobacilli per g in all studied yogurt samples conformed to the Bulgarian and EU standards: in Elena yogurt - $0{,}7x10^6$, in Vereia yogurt – $2.6x10^6$, in Na baba yogurt - $3.4x10^6$, in Rodopeia yogurt - $4x10^6$, in Parshevitza yogurt - $2.8x10^7$, in LB yogurt - $7.1x10^7$. Slightly under the standard was the number of lactobacilli in Elena yogurt. 12 LAB isolates were cultured and identified as *Lactobacillus delbrueckii* ssp. *bulgaricus,* being positive in the degradation of only four out of totally 50 metabolites: D-glucose, D-fructose, D-mannose and D-lactose. In three out of six samples, the score of affiliation reached 99% while for the remaining three samples it was lower. The morphology analysis of the isolates *via* light microscopy and SEM showed characteristics typical of lactobacilli. The examined six top yogurt brands available on the Bulgarian market are safe for consumption and contain *Lactobacillus delbruekii.* subsp. *bulgaricus* at the standard concentrations recommended by the EU.

Key words: Lactobacilli, commercial yogurt, biochemistry, scanning electronic microscopy

Резюме

Млечно-киселите бактерии са утвърдени пробиотици, които укрепват имунната система на човека и синтезират важни за неговото здраве витамини. Благодарение на синтезата на много ензими, включени в разграждането на токсични вещества, киселото мляко е храна, която повлиява положително дълголетието. Шест (3.6%), най-често употребявани от българите марки краве кисело мляко бяха изследвани за брой жизнеспособни лактобацили и беше проведен анализ на тяхната морфология, а изолирани от пробите млечно-кисели бактерии бяха идентифицирани с биохимични методи. Броят бактерии беше анализиран посредством метода на Кох в 1 грам кисело мляко. За изследване биохимиячната активност на изолатите и тяхното идентифицирeане беше приложен API 50-CH кит, чрез който се доказва разграждането на 50 различни въглехидрати. Морфологията на изолираните млечно-кисели бактерии беше изучена както чрез светлинна, така и чрез електронна микроскопия. Получените резултати показаха достатъчен брой лактобацили в отделните проби кисело мляко, съответстващ на българския и европейски стандарт: „Елена“ - $0{,}7x10^6$, Верея – $2.6x10^6$, „На баба“ - $3.4x10^6$, Родопея - $4x10^6$, „Пършевица“ - $2.8x10^7$, „LB” - $7.1x10^7$. Близо до стандарта, но с недостатъчен брой млечно-кисели бактерии се оказа кисело мляко „Елена“. Изолирани и култивирани бяха 12 изолати на лактобацили, която бяха идентифицирани като *Lactobacillus*

* Corresponding author:
E-mail: gstachanska@nbu.bg

delbrueckii ssp. *bulgaricus*, показвайки разграждане на 4 от общи 50 метаболити: D-глюкоза, D-фруктоза, D-маноза и D-лактоза. При три от шестте проби, идентичността достигна 99%, а при останалите три беше по-ниска. Морфологичният анализ на изолатите чрез светлинна и електронна микроскопия показа типични за лактобацилите характеристики. В заключение, изследваните топ-марки кисело мляко са здравословни за консумиране, тъй като съдържат *Lactobacillus delbruekii* subsp. *bulgaricus* в концентрации, препоръчани от българския и европейски стандарти.

Introduction

Lactic acid bacteria (LAB), a part of human microbiota, are pronounced probiotics (probios, for life) with century-long proven health benefits to the host. They maintain a healthy and equilibrated intestinal microbiota (Hoque *et al*, 2010; Morelli, 2014). The beneficial effect of lactobacteria in Bulgarian yogurt was first studied by the Nobel Prize winner and Pasteur Institute's Director Elie Metchnikoff. He found a correlation between longevity (over 100 years) of rural Bulgarian people and the high daily consumption of yogurt (Metchnikoff, 1907; Gasbarrini *at al*., 2016). It is well known that the Bulgarian medical student in Geneve, 27 year-old Stamen Grigorov, was the first to isolate and describe the bacterium responsible for yogurt fermentation. He named the bacterium after his home country – *Bacillus bulgaricus,* and in 1905 was invited by Metchnkoff to present his results at a conference at the Pasteur Institute in Paris (Grigoroff's biography, Kingsley and Reid, 2007).

Briefly, genus *Lactobacillus* unifies more than 120 species. LAB produce lactic acid due to lactose fermentation. Lactic acid lowers pH in yogurt, thus favouring the growth of acidophilic lactobacilli and conserving this food in a natural way. Additionally, lactic acid inhibits the growth of other microorganisms in the intestinal tract, most of which are pathogens. LAB are generally facultative anaerobes, microaerophilic, non-spore forming and non-respiring rods or cocci. Grampositive, non- or rarely motile bacteria and acid tolerant ones. They are rod-shaped, 0.5–1.2 /1.0-10 micrometers in size, colonies on agar are 2-5 mm, without pigment and grow on rich, complex media. LAB possess saccharoclastic metabolism, do not reduce nitrates, do not liquify gelatin and are catalase-negative. Lactobacteria possess *cis*-fatty acids and grow optimally at 37-41^{o}C.

One of the valuable properties of lactobacteria is their detoxification ability, producing enzymes involved in toxicant decomposition. LAB improve the human immune system and produce beneficial to the human health B-group vitamins (LeBlanc *et al.*, 2011). They display a pronounced antagonistic effect on *Helicobacter pillory* (Fujimura *et al*., 2012) and inhibit biofilm formation of other pathogenic bacteria like *Staphylococcus aureus* (Agrawal and Prakas, 2013), *Clostridium dificile* (Naaber *et al*., 2004), *Listeria monocitogenes* and *Escherichia coli* (Cleusix *et al*., 2007). Antimicrobial substances, such as low-molecular weight bacteriocins, produced by LAB during fermentation play a crucial role in LAB pathogen antagonism (Stoianova *et al*., 2012). Despite their antibacterial activity, LAB also exhibit antifungal activity to a variety of molds such as *Aspergillus, Penicillium, Fusarioum, Rhizopus* and *Mucor* (Batish et *al.,* 2007). The cell wall of LAB has been proven to be associated with antitumor activity (Shiby and Mishra, 2013). Decrease in serum cholesterol is another beneficial effect of lactic acid bacteria (Shiby and Mishra, 2013). During the last decade, LAB have been reported to synthesize neurotransmitters (Yunes *et al*., 2015), show resistance to antibiotics like tetracycline, vancomycin and erythromycin (Qin *et al.,* 2013), act against allergens (de Vreese and Schrezenmeyer, 2008), influence positively the lipid metabolism and stimulate the mineral absorption in indirect pattern (Gasbarrini G *et al*., 2016), display antiviral effect *via* their extracellular polysaccharides (Kanmani *et al.,* 2017).

For probiotic and beneficial effect to human health, yogurt should contain above 1x10 ^{6} cells of *Lactobacullus delbruekii* subsp. *bulgaricus* (Bulgarian State Standard, 2010; EU Codex Standard for fermented milk, 2003; Ashraf *et al*., 2011; Bandiera *et al*., 2013).

The aim of this study was to evaluate qualitatively and quantitatively the microbial quality of six yogurt top brands *via*: enumeration of viable lactic acid bacteria, lactic acid content, isolation of LAB from yogurt samples and morphological and biochemical identification of the isolates.

This study is a first comparative analysis of microbial quality of commercial yogurts available on the Bulgarian market.

Materials and Methods

Samples collection

Six top cow's milk yogurt brands were selected on the basis of popularity: "most frequently consumed among the Bulgarian population". Samples were purchased from a big food retail store. All manufacturers declared yogurt does not contain any milk powder or preservatives (Fig. 1). The fat content of all samples was 3.6 %.

Morphology study of LAB isolates

Lactobacteria isolated from yogurt were studied morphologically using light microscope Olympus CX-21 (Japan) and Scanning Electron Microscope JSM 5510 (Japan). Preparation for the SEM analysis included triple washing of the liquid cultures of isolates with 0.9 % NaCl and consequent triple centrifugation with Eppendorf centrifuge (3000 rpm/ 5 min). Dehydration of the samples

Fig. 1. Yogurt samples: 1 - Vereya, 2 - Na baba, 3 - Elena, 4 - Parshevitza, 5 - Rodopeya and 6 - LB

Lactobacilli count in yogurt samples

Lactobacilli were enumerated in 1 g of yogurt *via* the Koch method with serial dilutions and plating on MRS agar (Oxoid, UK). Incubation was performed at 45° C for 24 h.

Isolation and cultivation of lactobacilli from yogurt

Lactobacilli were initially enriched in skim milk for 48 h at 45 °C and further LAB cultures of each yogurt sample were isolated on MRS plates (Oxoid, UK).

pH of yogurt samples

pH of yogurt samples was evaluated using pH indicator strips 0-14 (Merck) and pH-meter Hanna (Germany)

*Lactic acid analysis after Toerner (oT***)**

10 mL of each yogurt sample were placed in a Becher cup and 20 mL of distilled water together with 3 drops of phenolphthalein were added. The mixture was vigorously stirred on a magnetic shaker to complete homogenization. Further, under continuous stirring, a titration with 0.1 N NaOH was accomplished. Titration lasted until the sample color became pale pink and was stable for at least 1 min. Calculation of acidic degrees of Toerner went *via* multiplication of milliliters NaOH spent to 10. Liquid acidity can be also presented as % of lactic acid by multiplying the obtained oT by 0.009 (1 mL 0.1 N of NaOH corresponds to 0.009 g of lactic acid).

with rising EtOH solutions (70-90%) was conducted. Samples were gold filmed for 45 sec prior to microscoping and beamed thereafter with 10 kV. Objects were observed at various magnifications – from x1000 to x20 000.

Biochemical identification of LAB isolates

Isolated LAB cultures were examined for their capability to degrade carbohydrates using specific for lactobacilli API 50 CH test kit (bioMerieux, France) consisting of 50 different metabolites: glycerol, erythrol, D-and L-arabinose, D-ribose, D- and L-xylose, D-adonitol, methyl-beta-D-xylopiranoside, D-galactose, D-glucose, D-fructose, D-mannose, D-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, methyl-alpha D-mannopyranoside, methyl-alpha D-glucopyranoside, N-acetylglucosamin, amygdalin, arbutin, esculin/ ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, inulin, D-melizitose, D-rafinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D- and L-fucose, D- and L-arabitol, potassium guconate, potassium 2-ketoguconate, potassium 5-ketoguconate ribose was used for examining the biochemical activity of isolates. API software 4.0 identification was used for data handling.

Results and Discussion

The content of the examined six yogurt samples (g/100 g) was described by the producers and

appeared similar in all investigated samples: milk protein - 3.2, milk fat – 3.6 and carbohydrates – 4.2. pH of samples was found to vary between 4.4 (4 samples) and 4.5 (2 samples) (Table 1).

As the optimal pH for the members of genus *Lactobacillus* is 4.4- 4.6, the studied samples showed appropriate pH values. Hoque et al. (2010) have reported LAB can grow within a wide pH range - from 2.2 to 8.5, which allows them to survive in acidic gastric conditions.

The highest concentration of lactic acid (see Table 2) was observed in LB yogurt (16.47%), and the lowest one in Parshevitsa yogurt (10.53%). Overall, in all samples studied the lactic acid content was above 10%.

The analysis of lactobacilli count revealed a sufficient amount of viable lactobacilli per g of yogurt in compliance with the Bulgarian and EU standards ($1x10^6$ - $1x10^7$ CFU *Lactobacillus bulgaricus*/g yogurt): Elena yogurt - $0,7x10^6$, Vereia yogurt – $2.6x10^6$, Na baba yogurt - $3.4x10^6$, Rodopeia yogurt - $4x10^6$, Parshevitza yogurt - $2.8x10^7$, LB yogurt - $7.1x10^7$. The highest colonization of viable lactobacilli was found in LB yogurt ($7.1x10^7$), exceeding the standard by 700 000 LAB per g. The lowest, but still close to the standard, number of viable LAB was recorded in Elena yogurt with 300 000 LAB under the recommended level. (Table 3).

Figure 2 shows colonies of LAB with typical *Lactobacillus* morphology – small, white, oval colonies, opalescent, with no ridged edge.

Twelve LAB isolates (2 cultures per sample)

Table 1. pH value of yogurt samples

No	Yogurt sample	pH value
1	Vereya	4.4
2	Na baba	4.4
3	Elena	4.5
4	LB	4.4
5	Parshevitza	4.5
6	Rodopeia	4.5

Table 2. Lactic acid content

No	Yogurt sample	oT	% lactic acid
1	Vereya	142	12.78
2	Na baba	137	12.33
3	Elena	142	12.78
4	LB	183	16.47
5	Parshevitza	117	10.53
6	Rodopeia	127	11.43

Table 3. Count of lactobacilli in yogurt samples

No	Yogurt sample	Lactobacilli CFU/g
1	Vereya	2.6×10^6
2	Na baba	3.4×10^6
3	Elena	0.7×10^6
4	LB	7.1×10^7
5	Parshevitza	2.8×10^7
6	Rodopeia	4.0×10^6

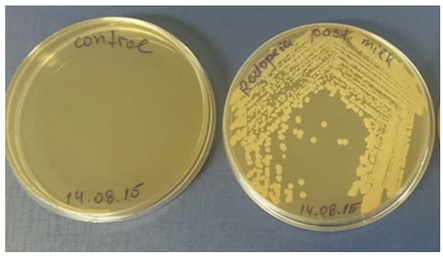

Fig. 2. Colonies of LAB

Fig. 3. API-CH 50 for *L. delbruekii* subsp. *bulgaricus* identification (11, 12 and 13 – fermented glucose, fructose, mannose; 29 – fermented lactose)

were identified using API-CH50 specific for lactobacilli test. As lactobacilli are known to degrade only four carbohydrates: glucose (No 11), fructose (No 12), mannose (No 13) and lactose (No 29), the obtained results and software analysis showed that the isolates of Na baba, Elena and LB yogurt were 98.8% identical with *L. delbrueckii* subsp. *bulgaricus*. Parshevitsa-, Rodopeya- and Vereya yogurt isolates showed lower identity. The results are shown in Table 4 and Fig. 3.

Klayruang *et al.* (2008) described Lab LAB isolates from traditional Thai food and also showed bile tolerance and gastric juice resistance. A small number of isolates have been reported to show antibiotic resistance (to gentamicin and linezolid), a larger number were susceptible mainly to protein synthesis inhibitors - chloramphenicol, erythromycin, kanamycin, rifampicin, streptomycin and tetracycline. Another report (Hoque *et al*., 2010) presented data about LAB isolates, resistant to nalidixic acid, metronidazole and cefradine, and sensitive to amoxicillin, clindamycin. In the last decade, LAB from yogurt as well as from other fermented food could act as reservoirs of antimicrobial/resistance genes (*Flores et al.*, 2005). On the other hand, LAB resistance genes could be transferred to pathogenic bacteria (Mathur and Singh, 2005).

The morphology of the six yogurt samples was initially observed with a light microscope. Figure 4 displays the diversity in form and shape of bacteria at magnification x400.

Table 4. API test of LAB isolated from yogurt samples

		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49
		0	GLY	ERY	DARA	LARA	RIB	DXYL	LXYL	ADO	MDX	GAL	GLU	FRU	MNE	SBE	RHA	DUL	INO	MAN	SOR	MDM	MDG	NAG	AMY	ARB	ESC	SAL	CEL	MAL	LAC	MEL	SAC	TRE	INU	MLZ	RAF	AMD	GLYG	XLT	GEN	TUR	LYX	TAG	DFUG	LFUC	DARL	LARL	GNT	2KG	5KG
NA BABA 1	24 h												+	+	+															+																					
	48 h												+	+	+															+																					
NA BABA 2	24 h												+	+	+															+																					
	48 h												+	+	+															+																					
LB	24 h												+	+	+															+																					
	48 h												+	+	+															+																					
ELENA	24 h												+	+	+															+																					
	48 h												+	+	+															+																					
PARSHEVITSA	24 h											+	+	+	+					+				+			+			+	+		+	+									+								
	48 h											+	+	+	+					+				+			+			+	+		+	+									+								
VEREIA	24 h						+					+	+	+	+					+				+			+	+	+	+	+		+	+	±	+						+		+				±		
	48 h						+					+	+	+	+					+	+			+			+	+	+	+	+		+	+	±	+					±	+		+				±		

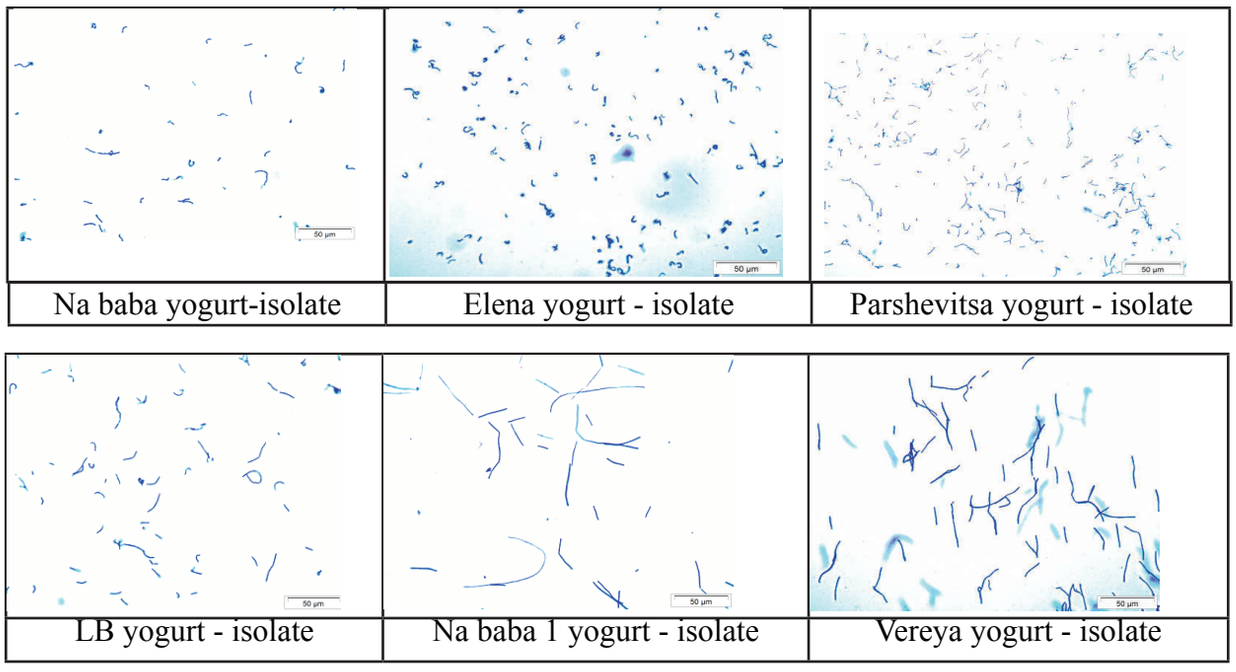

Fig. 4. LAB isolates study using light microscope (x400)

Further, a scanning electron microscopy was conducted in order to study in detail the morphology of the *L. delbrueckii* subsp. *bulgaricus* isolates. Figure 5 presents SEM pictures of the cultured isolate of Na baba-1 yogurt (1-4), isolate of Na baba-2 yogurt (5,6), isolate of LB yogurt (7) and isolate of Elena yogurt (8,9).

SEM analysis, at magnifications x 1000, x 1800, x 2000, x3000, x5000, x7000 and x10 000, undoubtedly shows that the examined bacteria possess distinctive *L. bulgaricus* morphology - rod, oval edges shape and size (3, 4 to 10 mm length and 1 mm width).

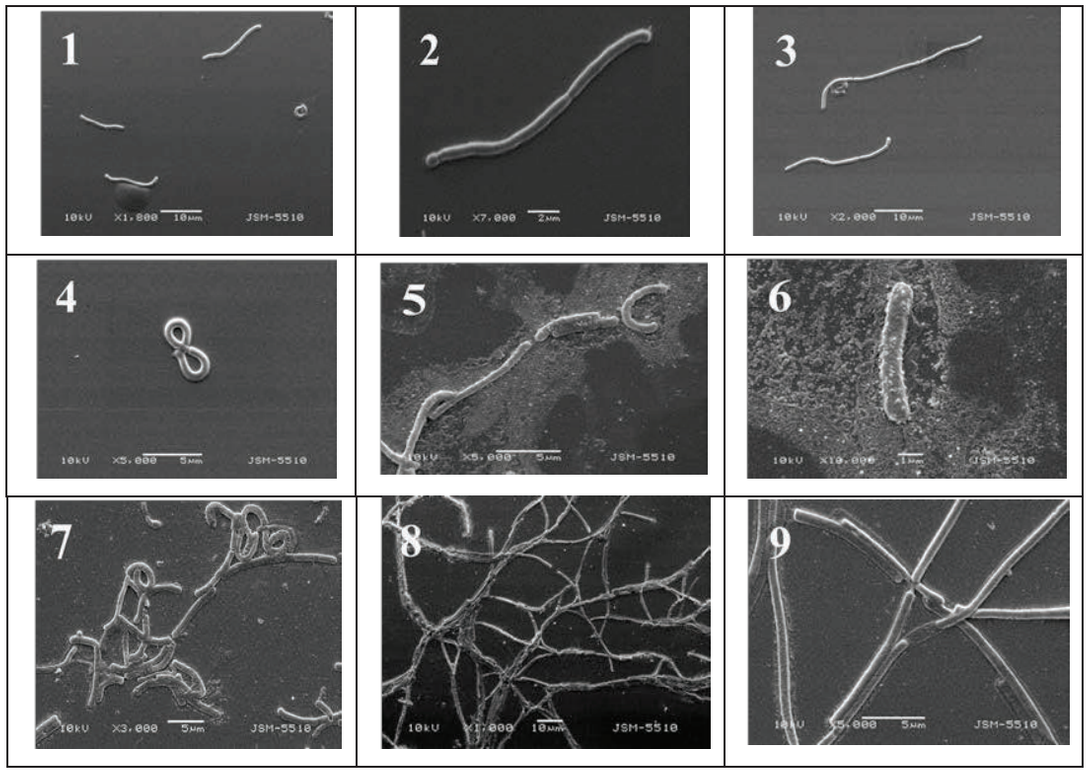

Fig. 5. Scanning electron microscopy of LAB isolates. Na baba-1 yogurt (1-4), Na baba-2 yogurt (5, 6), LB yogurt (7) and Elena yogurt (8, 9). Magnification x 1000, x 1800, x 2000, x3000, x5000, x7000 and x10 000

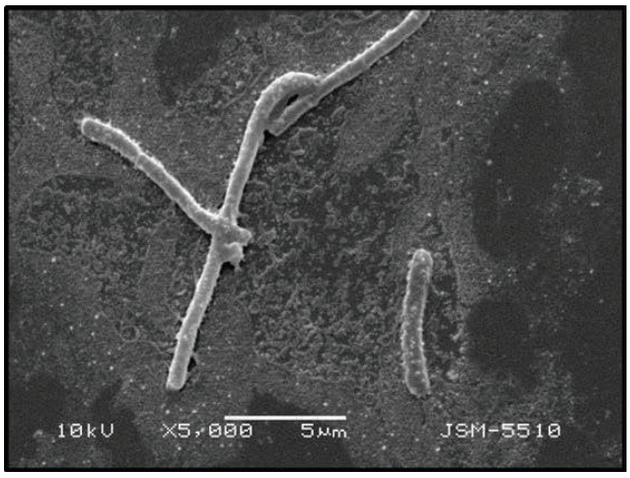

Fig. 6. Scanning electron microscopy of LAB isolate (Na baba -2 yogurt) with typical Y-shape (magnification x5 000)

Figure 6 demonstrates the peculiar Y-shape morphology of the isolate from Na baba -2 yogurt sample.

Conclusions

All six investigated commercial yogurt brands, known to be the most popular with Bulgarian consumers, comply with the Bulgarian state and EU standards for yogurt production containing between $1x10^6$ to $7x10^7$ CFU/g lactobacteria. The highest number of lactobacilli was enumerated in LB yogurt, exceeding both standards. The same brand of yogurt was also found to contain the highest lactic acid concentration. LAB isolates were identified as *L. delbrueckii* subsp. *bulgaricus*. Light microscopy and SEM analysis have revealed that the isolated LAB possesses morphology distinctive of *L. bulgaricus*. All results obtained definitively demonstrate that the examined yogurt brands comply with Bulgarian and EU standards and are safe for consumption. This study is the first comparative analysis of yogurt microbial quality in Bulgaria.

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