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INFLUENCE OF THE CULTIVATION CONDITION ON CARBOHYDRATE UTILIZATION BY *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS*

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Abstract

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One of the main phenotypic identification characteristics is carbohydrate utilization. It is generally accepted that most of the strains of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) utilize few carbohydrates. In this study carbohydrate utilization of *L. bulgaricus* strains under different cultivation conditions specific to two different identification kits - Api 50 CHL (bioMerieux) and Biolog's AN MicroPlate were compared. The cultivation conditions in Biolog's AN MicroPlate are giving indications of a more complete expression of the metabolic phenotype of the *L. bulgaricus* strains in this study. It could reflect on the probiotic and technological properties of *L. bulgaricus* cultivated in the presence of different carbohydrates.

Key words:Lactobacillus delbrueckii subsp. *bulgaricus*; identification; carbohydrate utilization; phenotype

Introduction

The first bacteriological study of yogurt originating from Bulgaria was done by the Bulgarian researcher Dr. Stamen Grigoroff (1905) in the laboratory of Prof. Leon Massole at the Geneva Medical University. However, the popularity of the Bulgarian yogurt, recently popular as yogurt, can be attributed to the Nobel Prize winner Ilya Metchnikoff. He first introduced the probiotic concept, observing the long life of Bulgarian peasants who consumed fermented milk products (Metchnikoff 1908). One of the isolates is nowadays classified as *L. delbrueskii* ssp. *Bulgaricus.* It plays a major role in the yogurt industry (Codex Stan 243-2003; Serror, 2003; Guchte, 2006) and as a dietary supplement due to its significant beneficial influence on the human host.

One of the main phenotypic identification characteristics is carbohydrate utilization. It is generally accepted that most of the strains of *L. bulgaricus* utilize few carbohydrates (Bergey's Manual, 2009); glucose, fructose, mannose and lactose (Chervaux, 2000; Germond, 2003; IDF 146:2003,). According to Zourari et al. (1992), galactose and mannose are fermented only in some strains of *L. bulgaricus*. Although *L. bulgaricus* is one of the most consumed lactic acid bacterium, with beneficial impact on human nutrition, surprisingly little is known about the physiology or the biochemical and regulatory pathways of this microorganism (Serror, 2003). Information on the carbohydrate utilization of *L. bulgaricus* has not been updated for many years.

The aim of this study was to compare carbohydrate utilization of *L. bulgaricus* strains under different cultivation conditions specific to two different identification kits - Api 50 CHL (bioMerieux) and Biolog's AN MicroPlate.

Materials and Methods

300 *L. bulgaricus* strains were examined isolated from different sources in the period from 1960 until

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today. The origins and the distribution of the examined strains are presented in Table 1. Approximately 90% of the studied strains originated from Bulgaria, in particular, isolated from homemade products and green plants. All of the cultures are maintained at LB Bulgaricum's PLC Microbial Collection. The type strain ATCC 11842 (DSM 20081) also was isolated from Bulgarian yogurt (Van de Guchte et al., 2006) (Table 1).

Actively growing bacterial cultures were examined according to the instructions for use of Api 50 CHL (Api 50CH Strip and Api 50 CHL Medium, bioMerieux) and AN MicroPlate (Biolog) kits. In brief – prior to examination with Api 50 CHL *L. bulgaricus* strains were cultivated anaerobically (Anaero*Gen*™, OXOID) on MRS agar (OXOID) at 37°C for 24-32 hours; the strips were inoculated with cell suspension with a turbidity equivalent to Mc Farland Standard point 2 and cultivated aerobically covered with mineral oil at 37°C for 48 hours. On the other hand, prior to examination with AN MicroPlate the strains were cultivated anaerobically (Anaero*Gen*™, OXOID) on Biolog Universal Anaerobe agar at 37°C for 24-32 hours; cell density in the inoculating fluid was 65% transmittanse; the inoculated microplates were incubated in a hydrogen free anaerobic atmosphere (Anaero*Gen*™, OXOID) at 37°C for 20-24h. MicroLog M5.1.1 and api*web*™ identification software were used. All 300 strains were initially tested with the Api 50 CHL kit. Twenty-nine of these strains were then selected for analysis with the AN MicroPlate. Strains whose carbohydrate metabolism was compared under different conditions of cultivation (i.e. Api 50 CHL and Biolog AN MicroPlate) were selected so as to have minimum similarity according to their macrorestriction profiles obtained with *XhoI*, *ApaI* and *NotI* and phenotype technological and probiotic characteristics (acidification profile, viscosity, growth rate, resistance to freezing and freeze-drying, survivability in simulated gastric and intestinal juice, *β*-galactosidase activity, proteinase activity). The only *L. bulgaricus* strain among 300, which were able to utilize N-acetyl-glucosamine growing in Api 50 CHL, were also included in the group to be analyzed with the Biolog AN MicroPlate.

The two kits have a different set of carbohydrates. Glucose, fructose, mannose, rhamnose, N-acetyl-glucosamine, salicin, cellobiose, maltose, lactose, trehalose, gentiobiose, turanose, L-fucose and gluconate are in common for both kits.

Table 1
Distribution of *L. bulgaricus* strains from different origins studies by commersially avalable kits for identification

Origins of *L. bulgaricus*	Api 50 CHL		Biolog AN MicroPlate	
	Number of strains	%	Number of strains	%
Home-made yogurt	231	77.0	13	41.4
Home-made cheese	10	3.3	1	3.4
Green plants	30	10.0	8	27.6
Market isolates from yogurt	25	10.0	5	17.2
Market isolates from cheese	3	1.0	1	3.4
Type strain (DSM 20081/ATTC 11842)	1	0.3	1	3.4
Total	300		29	

Results

Following the recommendations of the IDF Standard 146:91(146:2003) carbohydrate utilization of more than 300 *L. bulgaricus* strains had been confirmed by Api 50 CHL identification kit (Table 2).

Interestingly, only one of 300 strains was utilizing N-acetyl-glucosamine growing in Api 50 CHL. After this finding, the strain was identified as *L. bulgaricus* also by ARDRA, 16S sequencing and SDS-PAGE of

Table 2
Percent distribution of 300 *L. bulgaricus* strains according to carbohydrate utilization in Api 50 CHL

Carbohydrate	bioMerieux database, %	LB Bulgaricum's data, %
Galactose	25	0
Glucose	98	99
Fructose	98	99
Mannose	25	99
N-Acetyl-D-Glucosamine	0	0.33
Lactose	98	100
Trehalose	2	1
Gluconate	1	0

total cell proteins (data not shown). That strain does not differ significantly from others in the group studied by Biolog's AN MicroPlate. On the other hand, all of 29 *L. bulgaricus* strains tested with both kits are utilizing N-acetyl-glucosamine in the Biolog AN MicroPlate (Table 3).

Data from colorimetric measurement for carbohydrates utilized from 80-100% of examined *L. bulgaricus* strains showed that in the AN MicroPlate lactose, mannose, fructose, glucose and lactulose are most intensively utilized, followed by N-acetyl-glucosamine, turanose, 3-methyl-D-glucose, dextrin and palatinose. From Table 3 it can be seen that there is a significant difference between Biolog's database and the results from this study regarding maltose and trehalose utilization by *L. bulgaricus* strains in the AN MicroPlate. These AN MicroPlate and Api 50CHL data concerning mannose utilization differs also from Bergey's Manual (2009) where more than 90% of the strains are reported as mannose negative. In addition, there are some differences between Biolog's database and the results from LB Bulgaricum's strains in utilization, for example, of glyoxylic acid, á-ketobutiric acid, DL-lactic acid and D-lactic acid methyl ester (Table 3).

Table 3
Percent distribution of 29 L. bulgaricus strains based on carbohydrate and acids utilization in Biolog AN MicroPlate

Substrate	Biolog's database, %	LB Bulgaricum's data, %
N-Acetyl-D-Glucosamine	100	100
D-Cellobiose	30	25*
Dextrin	80	82
D-Fructose	100	100
L-Fucose	40	36*
D-Galactose	0	0
Gentiobiose	40	18*
D-Gluconic Acid/Gluconate	0	0
α-D-Glucose	100	100
D-Glucose-6-Phosphate	20	18*
α-D-Lactose	100	100
Lactulose	100	100
Maltose	60	0
D-Mannose	100	100
3-Methyl-D-Glucose	100	96
Palatinose	90	89
L-Rhamnose	10	7*
Salicin	30	30*
D-Trehalose	0	2
Turanose	100	93
Fumaric Acid	0	11*
Glyoxylic Acid	70	37
α- Hydroxybutyric Acid	100	85
α-Ketobutyric Acid	0	43
D,L-Lactic Acid	60	100
D-Lactic Acid Methyl Ester	20	89
L-Malic Acid	0	26*
Pyruvic Acid	100	78
Pyruvic Acid Methyl Ester	60	54
m-Tartaric Acid	10	0
Inosine	30	25*
Uridine	10	36*

*Weak utilization

Discussion

As could be seen from Table 2 there is a significant difference in the percent of strains utilizing galactose and mannose in the Api 50 CHL kit. The cultivation conditions in Biolog's AN MicroPlate are giving indications of a more complete expression of the metabolic phenotype of the *L. bulgaricus* strains under study, as can be seen from Table 3. Some of the available in both kits carbohydrates are utilized in AN MicroPlate but not in Api 50 CHL. Tetrazolium-based redox assays can be used to measure the active *in vivo* energy-producing pathways of a very wide range of microbial cells (Bochner, 1989; Bochner, 2006). According to Bochner (2009), the colorimetric measurement of cell respiration, based on reduction of tetrazolium, responds to the process of metabolism (oxidation of substrates) rather than to metabolic by-products (e.g. acid).

Based on the phenotypic profiles from the AN MicroPlates the strains were not clustered according to the original habitat. We observed a tendency for weaker utilization of palatinose by plant isolates of *L. bulgaricus*. According to Germond et al. (2003), the analysis of the genetic basis of the expression of the galT gene for lactose metabolism shows specific gene variation in relation to habitat colonization capability. However, in our data, there was no difference between yogurt and plant isolates of *L. bulgaricus* in lactose utilization. The

data from this study also showed higher lactose utilization in comparison with other carbohydrates.

Other knowledge about the physiology of *L. bulgaricus*, which can be obtained using Biolog AN MicroPlate, is concerning utilization of acids (Table 3).

Conclusions

Phenotypic identification/characterization by both kits was useful to find out the phenotypic manifestations of environmental adaptation. It could reflect on the probiotic properties of *L. bulgaricus* cultivated in the presence of different carbohydrates and on the technological processes, for example, in the production of freeze-dried yogurt starter cultures or supplements by increasing survival and activity of the cultures.

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