EFFECT OF METHIONINE AND CYSTEINE DEPRIVATION ON GROWTH OF DIFFERENT NATURAL ISOLATES OF *LACTOBACILLUS* SPP. IN CHEMICALLY DEFINED MEDIA

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Abstract — The purpose of this study was to determine the ability of natural isolates of lactobacilli from different ecological niches to grow in a chemically defined medium in the presence or absence of sulphur-containing amino acids, methionine and/or cysteine. The obtained results indicate that cysteine is essential for growth of *L. paracasei* subsp. *paracasei* BGHN14 and BGSJ2-8, while methionine is essential for isolates BGHN40, BGCG31, and BGHV54T of the species *L. plantarum*. Methionine is also essential for growth of *L. rhamnosus* BGHV58T. Other analyzed strains, such as *L. plantarum* BGSJ3-18, BGZB19, BGHV52Ta, and BGHV43T, require the presence of both amino acids for their growth.

Key words: Chemically defined medium, lactobacilli, methionine, cysteine

INTRODUCTION

Lactobacilli and other lactic acid bacteria (LAB) are widely distributed in nature and have been isolated from various sources, including humans, animals, plants, dairy products, wines, ensilages, etc. However, many individual species appear to have adapted to specific environments and the growth rate of LAB depends on their ability to use the substrates available in the surroundings in which they grow. Lactic acid bacteria have numerous nutritional requirements for their growth, including the presence of amino acids, vitamins, purines, and pyrimidines (A u d i s i o et al., 2001., A v o n t s et al., 2004). Among LAB, members of the *Lactobacillus* genus have complex nutritional requirements that can be satisfied only by their culturing in a medium containing energy sources, precursors for cell growth and division, and growth-stimulatory substances. Lactic acid bacteria are widely used in a variety of dairy fermentation processes in which they contribute to flavour development as a result of a series of (bio)chemical processes during fermentation. As a part of starter cultures or non-starter lactic acid bacteria (NSLAB), LAB provide enzymes for these processes. Conversion of amino acids, especially the

aromatic, branched-chain, and sulfur-containing residues, is assumed to be essential for the formation of volatile aroma compounds (E n g e l s and V i s s e r, 1996).

Various LAB strains differ in amino acid converting abilities, and these activities are in fact linked to the ability to synthesize amino acids. It has been shown that natural isolates have unique and diverse properties compared to commercially available starter strains. For instance, it was found that these strains have a much greater potential ability to synthesize their own amino acids when compared to industrial strains. Natural isolates are not normally associated with a rich environment such as milk, which makes them more dependent on their own biosynthesis of amino acids (S m i t et al., 2005). Relatively little information is available regarding amino acid catabolism in LAB. The past few years have witnessed increased interest in elucidating in the pathways of amino acid catabolism by LAB and their production of sensory compounds and biogenic amines.

Sulfur is a constituent of many indispensable components of the cell such as cysteine, methionine, biotin, lipoic acid, coenzyme A, etc. Among these

compounds, cysteine has a central role, since its *de novo* synthesis represents the main pathway of sulphur acquisition in bacteria. The second sulphur amino acid, methionine, is a key compound controlling the initiation of translation and is crucial to a variety of methyltransferase reactions.

Sulfur metabolism in Gram-positive bacteria has been poorly characterized. One way to elucidate the nature of cheese flavor development and the contribution of amino acid catabolism to this process is to study LAB growth in the presence/absence of sulfur-containing amino acids. Lactococci have been described as prototrophic for cysteine and auxotrophic for methionine. Nevertheless, it was expected that the genes coding for the enzymes involved in methionine biosynthesis are present in lactococci (C h o p i n , 1993). Results obtained for lactobacilli were less clear, and it appears that methionine and cysteine requirements are frequently strain-dependant (A m a r i t a et al., 2001).

The aim of this work was to investigate the influence exerted by the presence of methionine and/or cysteine in a chemically defined medium (CDM) on the growth of natural isolates of lactobacilli from different sources. Bearing in mind that the lactobacilli were of different origin, we investigated the effect of methionine and/or cysteine on their growth within the same species as well among different species.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study and their origins are listed in Table 1. Lactobacilli were isolated using standard microbiological procedures and identified with the API 50 CHS system (Api System S.A., Bio-Merieux, Montelieu–Vercieu, France) and by using the rep-PCR fingerprinting method (V e r s a l o v i c et al., 1994).

Media and growth conditions. Strains were grown in MRS medium (Merck, GmbH Darmstadt, Germany), and agar plates were prepared by the addition of agar (1.5%, w/v) (Torlak, Belgrade) to MRS broth. To test the influence exerted by the presence of methionine and cysteine in the medium,

strains were grown in a chemically defined medium (CDM): glucose, 10 gl^{-1}; sodium acetate, 6 gl^{-1}; ammonium citrate, 1 gl^{-1}; K_2HPO_4, 3 gl^{-1}; KH_2PO_4, 3 gl^{-1}; $MgSO_4x7H_2O$, 0.5 gl^{-1}; $MnSO_4xH_2O$, 0.05 gl^{-1}; $FeSO_4x7H_2O$, 0.02 gl^{-1}; Tween 80, 1 gl^{-1}; p-aminobenzoic acid, 0.0002 gl^{-1}; biotin, 0.00001 gl^{-1}; folic acid, 0.0001 gl^{-1}; riboflavin, 0.001 gl^{-1}; nicotinic acid, 0.001 gl^{-1}; pyridoxal, 0.002 gl^{-1}; pantothenic acid, 0.001 gl^{-1}; arginine, isoleucine, leucine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine, 0.1 gl^{-1}; and aspartic and glutamic acids, 0.2 gl^{-1} (M o r i s h i t a et al., 1981). To test the effect of the presence of methionine (Met) and/or cysteine (Cys) in the CDM on strain growth, strains were grown in four different combinations: CDM I (Met⁻Cys⁻), CDM II (Met⁺Cys⁻), CDM III (Met⁻Cys⁺), and CDM IV (Met⁺Cys⁺). In each combination, the final concentration of methionine was 0.1 gl^{-1}, while that of cysteine was 0.2 gl^{-1}. All components were filter-sterilized or autoclaved (121°C for 15 min) prior to use.

Growth experiments. Cells were grown overnight in MRS medium, washed twice in sterile saline solution (0.9% NaCl) to avoid transfer of essential nutrients, and resuspended to the original volume with sterile saline solution to an optical density (OD) at 600 nm of 1.0. This suspension was used to inoculate (1%) a set of liquid CDM media. Cultures were incubated at 30 or 37°C, depending on the strain, for 72 h. Bacterial growth was monitored at the times indicated by measuring the OD_{600nm} of culture using an Ultrospec 500pro spectrophotometer (Amersham Biosciences), and experiments were repeated three times.

RESULTS AND DISCUSION

Lactic acid bacteria have become the focus of a rapidly increasing number of genetic studies, mostly because of their industrial significance. In particular, several clusters of amino acid biosynthetic genes have been studied, the results providing information about gene organization and also on how these bacteria control expression of their genes. The study of amino acid biosynthetic pathways may help us understand the reasons for the fastidiousness of lactic acid bacteria, which makes them unable to

Table 1. Bacterial strains.

Bacterial strain	Origin	Source or references
Lactobacillus paracasei subsp. *paracasei*		
BGHN14	Natural isolate from home-made cheese kept in olive oil, Herceg Novi, Montenegro	Kojic et al., 1991
BGSJ2-8	Natural isolate from home-made semi-hard cheese, Sjenica Serbia	Lozo et al., (2007)
Lactobacillus plantarum		
BGHN40	Natural isolate from home-made cheese kept in olive oil, Herceg Novi, Montenegro	Laboratory collection
BGCG31	Natural isolate from home-made semi-hard cheese, Adrovici, Montenegro	Laboratory collection
BGSJ3-18	Natural isolate from home-made semi-hard cheese, Sjenica, Serbia	Laboratory collection
BGZB19	Natural isolate from home-made semi hard cheese, Žabljak, Montenegro	Laboratory collection
BGHV43T	Vaginal isolate	Laboratory collection
BGHV54T	Vaginal isolate	Laboratory collection
BGHV52Ta	Vaginal isolate	Laboratory collection
Lactobacillus rhamnosus		
BGT10	Vaginal isolate	Pastar et al., 2003.
BGHV58T	Vaginal isolate	Laboratory collection

grow unless certain amino acids are supplied in the medium.

In this paper, we analyze the ability of 11 *Lactobacillus* strains to grow in CDM with or without methionine and/or cysteine. The analyzed lactobacilli were human vaginal isolates and natural isolates from home-made cheeses collected from different ecological niches. According to results of the API test and rep-PCR identification, isolates belonged to three different species, *L. paracasei* subsp. *paracasei, L. plantarum*, and *L. rhamnosus* (data not shown). The growth capacity of the analyzed strains was monitored by measuring culture OD values every 24 h over 72 h of growth.

The obtained results showed that growth of *L. paracasei* subsp. *paracasei* BGHN14 and BGSJ2-8 strains depended on the presence of cysteine in the CDM medium. In contrast, the absence of methionine had no significant influence on the growth

of these strains (Figs. 1A and 1B). Previous results showed that sulfur-containing amino acids can be used by *L. casei* IFPL731 to produce the typical cheese flavor (A m a r i t a et al., 2001). According to M o r i s h i t a et al. (1981) cysteine is an essential amino acid for *L. casei* ATCC 7469, while methionine is not required for growth in liquid media, but stimulates growth on agar media. Nevertheless, in a study involving both *L. casei* subsp. *casei* and *L. paracasei* subsp. *paracasei* strains, it was reported that they require methionine and cysteine for their growth (S e e f e l d t and W e i m e r, 2000).

L. plantarum strains isolated from different home-made cheeses showed some differences in growth requirements. A methionine requirement was demonstrated for *L. plantarum* strains BGHN40 and BGCG31, while the influence of cysteine on the growth of these strains was less significant (Figs. 2A and 2B). The influence of methionine on growth of *L. plantarum* BGSJ3-18 and BGZB19 was not as dis-
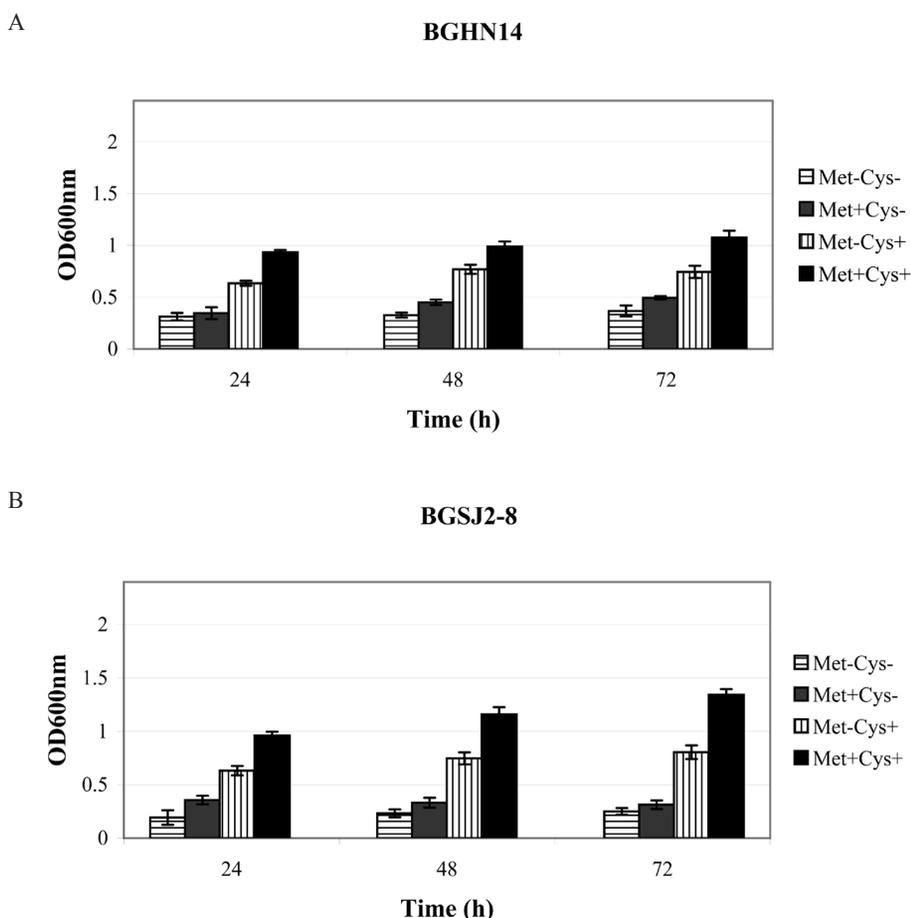
A

BGHN14

B

BGSJ2-8

Fig. 1. Growth of *L. paracasei* subsp. *paracasei* BGHN14 (A) and BGSJ2-8 (B) in the presence/ absence of cysteine (Cys) and/or methionine (Met). Error bars indicate the standard deviation of three independent measurements.

cernible as for the previous strains. In the case of *L. plantarum* BGSJ3-18, there is no considerable difference between its growth in the presence of methionine or cysteine in CDM. The effect of methionine on growth of *L. plantarum* BGZB19 was less pronounced than that of cysteine (Figs. 2C and 2D).

Human vaginal isolate *L. plantarum* BGHV54T showed the same pattern of growth as *L. plantarum* strains BGHN40 and BGCG31 isolated from cheese. Methionine was essential for its growth (Fig. 3A). Unlike human vaginal isolate BGHV54T, *L. plantarum* BGHV52Ta and BGHV43T required the presence of both methionine and cysteine for growth in CDM (Figs. 3B and 3C).

A slow growth rate was observed for human vaginal isolate *L. rhamnosus* BGT10. After 72 h of cultivation in CDM IV, this strain attained an OD value corresponding to the early exponential phase of growth (OD=0.494) (Fig. 4A). The growth of human vaginal isolate *L. rhamnosus* BGHV58T was dependent on the presence of methionine in the medium (Fig. 4B). The obtained pattern of BGHV58T growth was very similar to those obtained for *L. plantarum* strains BGHN40 and BGCG31 (Figs. 2A and 2B).

Little is known about organization and regulation of the sulphur assimilation and methionine biosynthesis pathways in Gram-positive bacteria. In
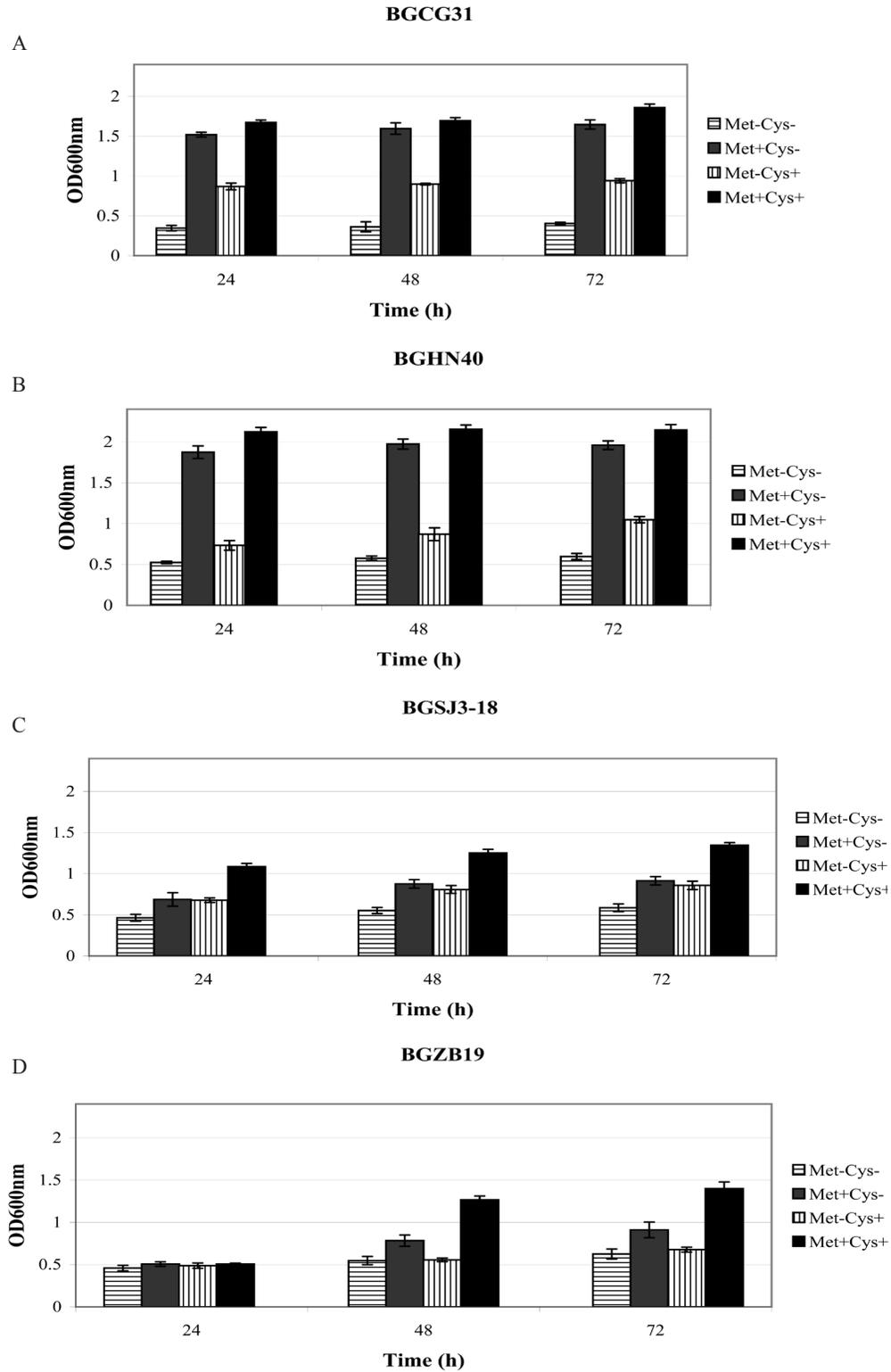
BGCG31

A

BGHN40

B

BGSJ3-18

C

BGZB19

D

Fig. 2. Growth of *L. plantarum* BGHN40 (A), BGCG31 (B), BGSJ3-18 (C), and BGZB19 (D) in the presence/absence of cysteine (Cys) and/or methionine (Met). Error bars indicate the standard deviation of three independent measurements.
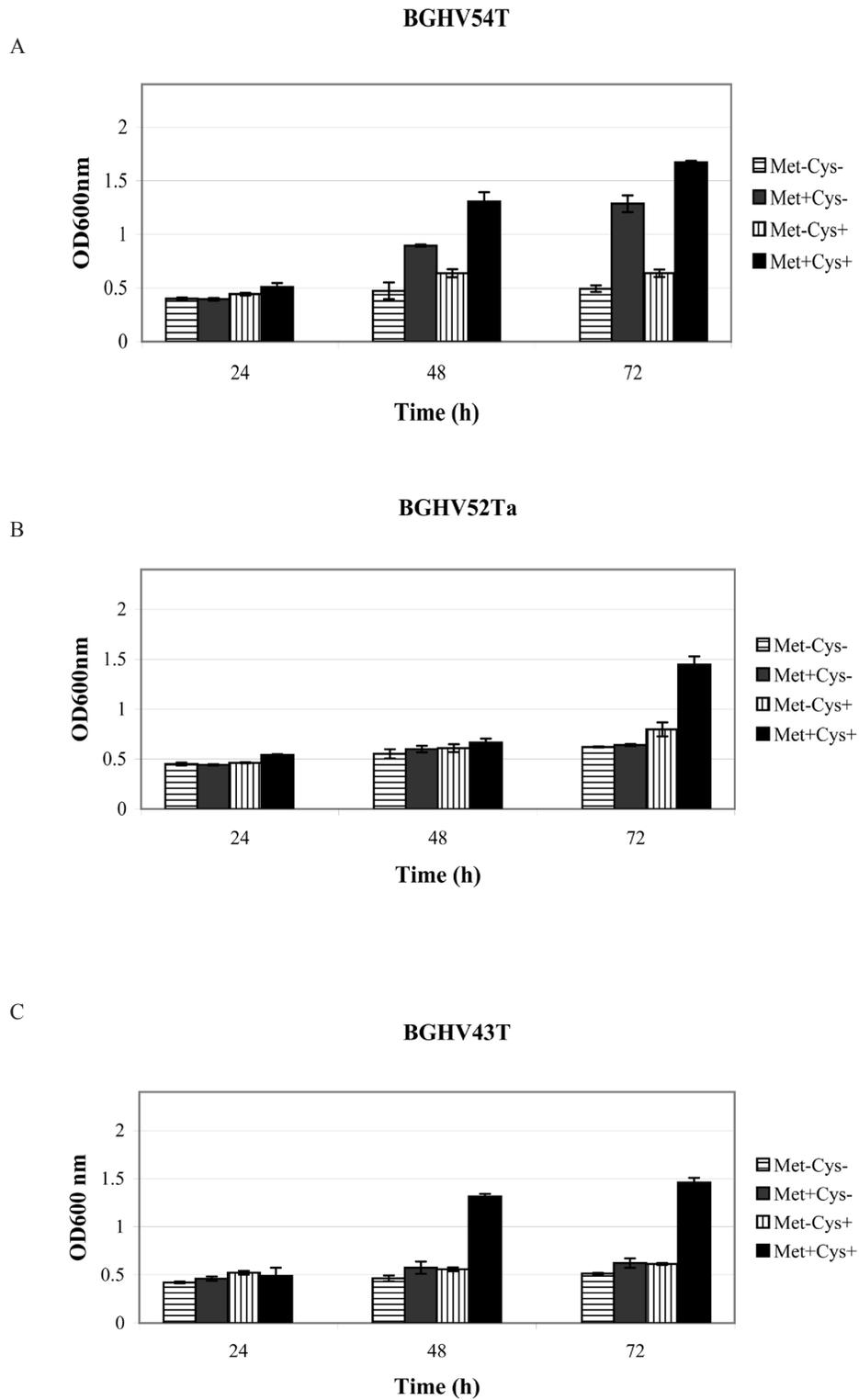
BGHV54T

A

BGHV52Ta

B

C

BGHV43T

Fig. 3. Growth of *L. plantarum* BGHV54T (A), BGHV52Ta (B), and BGHV43T (C) in the presence/absence of cysteine (Cys) and/or methionine (Met). Error bars indicate the standard deviation of three independent measurements.

BGT10

A

BG58T

B

Fig. 4. Growth of *L. rhamnosus* BGT10 (A) and BGHV58T (B) in the presence/absence of cysteine (Cys) and/or methionine (Met). Error bars indicate the standard deviation of three independent measurements.
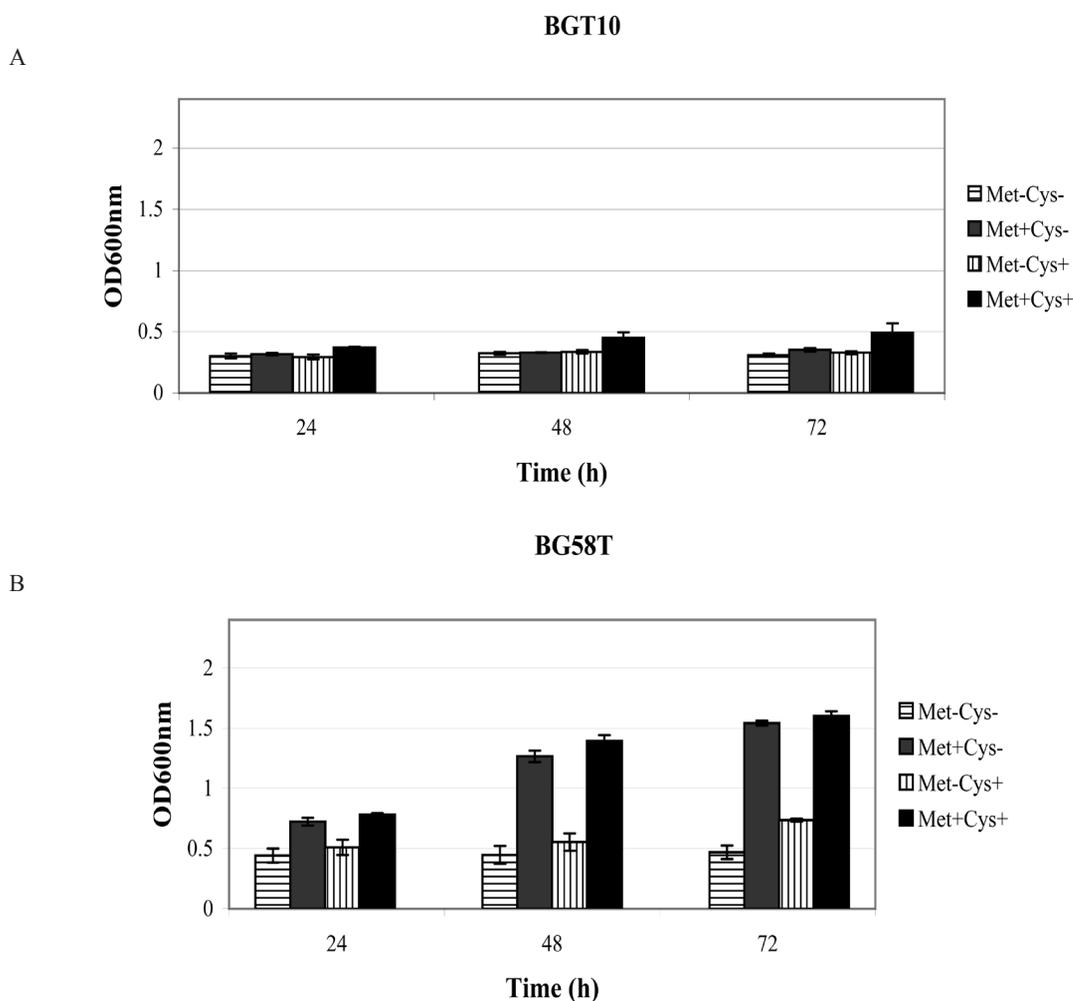
Lactococcus lactis, the gene coding for cystathionine β-lyase (*metC*) is clustered together with the gene encoding cysteine synthase (*cysK*) (F e r n á n d e z et al., 2002), indicating that biosynthesis pathways are genetically linked and under the same regulation. The expression of the *metC-cysK* gene cluster is strongly influenced by the amounts of methionine and cysteine in the culture medium (F e r n á n d e z et al., 2000). High concentrations of these two amino acids completely abolish transcription of this gene cluster and result in *L. lactis* cells almost bereft of cystathionine β-lyase activity.

According to previous reports, lactobacilli are auxotrophic for both methionine and cysteine, meaning they either lack the enzymes needed for biosynthesis of these amino acids or these pathways are interrupted (S e e f e l d t and W e i m e r, 2000). The results obtained for the *L. rhamnosus* BGT10 and *L. plantarum* BGSJ3-18 and BGZB19 strains are in correlation with these findings. The *L. rhamnosus* BGHV58T and *L. plantarum* BGHN40, BGCG31, and BGHV54T strains showed the same pattern as previously reported for lactococci (C h o p i n, 1993). Furthermore, analysis of an additional four *L. plantarum* strains of human or dairy origin showed

the same pattern of growth in CDM containing or lacking methionine and/or cysteine as was observed for *L. plantarum* BGHN40 and BGCG31 (data not shown).

Comparative analysis of genomes of the *Lactococcus lactis* subsp. *lactis* IL1403 (B o l o t i n et al., 2001) and *Lactobacillus plantarum* WCFS1 (K l e e r e b e z e m et al., 2003) strains for the presence of genes involved in cysteine and methionine biosynthetic pathways revealed that the same putative genes are present in both genomes. On the other hand, analysis of the sequenced genomes of *Lactobacillus* strains revealed a significantly lower number of putative genes involved in these biosynthetic pathways. Previous results showed that seven amino acids are essential for growth of the *L. plantarum* 8014 strain (the parent strain of *L. plantarum* WCFS1) (M o r i s h i t a et al., 1981). *In silico* analysis of the *L. plantarum* WCFS1 strain revealed that three additional amino acids - arginine, methionine, and phenylalanine - are essential for its growth (T e u s i n k et al., 2005).

In conclusion, the results of the present study indicate that different growth requirements exist in strains belonging to the same species. These differences are possibly due to the different origin of strains adapted to the nutrient source available in their specific ecological niches. In addition, it is likely that requirements for sulfur-containing amino acids, methionine and cysteine, are strain-dependent regardless of their origin. Taken together, these results show that natural *Lactobacillus* natural isolates have different amino acids requirements, which may result from either regulatory mechanisms or the absence of functional biosynthetic genes.

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ЕФЕКАТ НЕДОСТАТКА МЕТИОНИНА И ЦИСТЕИНА НА РАСТ РАЗЛИЧИТИХ ПРИРОДНИХ ИЗОЛАТА ЛАКТОБАЦИЛА У ХЕМИЈСКИ ДЕФИНИСАНИМ МЕДИЈУМИМА

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Циљ овог рада је био да се утврди способност природних изолата лактобацила, изолованих из различитих еколошких ниша да расту у хемијски дефинисаном медијуму са или без присуства аминокиселина које садрже сумпор, метионин и/или цистеин. Добијени резултати су показали да је есенцијална аминокиселина за раст изолата *L. paracasei* subsp. *paracasei* BGHN14 и BGSJ2-8 цистеин, док је за изолате BGHN40, BGCG31, BGHV54T, који припадају врсти *L. plantarum* -метионин. Метионин је есенцијална аминокиселина за раст соја *L. rhamnosus* BGHV58T. Остали анализирани сојеви, као што су *L. plantarum* BGSJ3-18, BGZB19, BGHV52Ta и BGHV43T за свој раст захтевају присуство обе аминокиселине у медијуму.