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Nutritional Requirements of *Lactobacillus delbrueckii* subsp. *lactis* in a Chemically Defined Medium

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Abstract. This study was undertaken to determine the nutritional requirements of *Lactobacillus delbrueckii* subsp. *lactis* and to develop a minimal chemically defined medium that supports sustained growth of these microorganisms. The single-omission technique was applied to each component of complete chemically defined medium in order to determine the nutritional requirements. *L. delbrueckii* subsp. *lactis* was prototrophic for alanine, glycine, aspartic acid, asparagine, glutamine, threonine, and proline. The lysine requirement was strain-dependent. Magnesium was the only essential oligoelement. These microorganisms also required uracil and guanine and adenine as pyrimidine and purine sources, respectively. In view of the nutritional requirements we designed a new minimal defined medium which supports sustained growth of *L. delbrueckii* subsp. *lactis.* This medium is simple and well defined, and should be preferable to complex media for conducting future biochemical, physiological, and genetic studies on *L. delbrueckii* subsp. *lactis.*

The lactic acid bacteria (LAB) are important industrial microorganisms because of their role in food fermentations, especially dairy products. In addition, it has been shown that LAB exhibit a range of physiological and therapeutic effects in their consumers, including immune stimulation, pathogen exclusion, production of bioactive peptides, and health-related products [6, 12, 19]. *Lactobacillus delbrueckii* subsp. *lactis (L. lactis)* is an important species of LAB currently used in the industrial production of hard cheeses, such as Grana, Emmenthal and Provolone. Despite the industrial interest in *L. lactis* little is known about the physiology and genetics of this microorganism. Lactobacilli are extremely fastidious organisms, adapted to complex organic substrates. They require not only carbohydrates as energy and carbon source, but also nucleotides, amino acids, and vitamins for their growth in a defined medium [8, 11, 17]. Their complex nutrient requirements are usually satisfied in natural or complex growth media by the addition of undefined compounds such as peptone, meat and yeast extract [4]. A chemically defined medium that supports the growth of *L. lactis* is essential for the design of reproducible biochemical, physiological, and genetic studies. The use of a chemically defined medium is also important to enhance the proteinase activity by thermophilic lactobacilli [8] and to study the regulation of this enzyme.

Chemically defined media have been developed for several LAB species [2, 3, 7, 8, 17]. Chervaux et al. [2] formulated a synthetic medium which allows the growth of several LAB, including *L. delbrueckii* subsp. *bulgaricus,* but *L. lactis* grew poorly in this medium. Recently, a synthetic culture medium for *L. johnsonii* has been formulated [5]. This medium was able to support the development of *L. lactis* ATCC 7830. However, several strains of *L. lactis* were unable to grow in this defined medium [5]. Moreover, the nutritional requirements of *L. lactis* have never been elucidated. This study was undertaken to determine the minimal requirements of *L. lactis* by first using a complete defined medium from which minimal requirements could be determined, ultimately leading to the development of a minimal defined medium that supports growth of these microorganisms.

Materials and Methods

Bacterial strains. Two strains of *L. delbrueckii* subsp. *lactis,* CRL 581 and CRL 654, which displayed a high caseinolytic activity able to hydrolyze α- and β-casein into smaller peptides were used throughout

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this study [9]. These strains were obtained from CERELA (Centro de Referencia para Lactobacilos, Argentina) and they were originally isolated from Argentine hard cheeses [9]. Cultures were stored at −70°C in 10% sterile reconstituted skim milk (RSM) containing 0.5% yeast extract and 10% glycerol, and were activated in MRS [4] broth at 40°C for 16 h.

Chemicals, media, and growth conditions. All chemicals were of analytical grade unless stated otherwise. Amino acids, vitamins, purines and pyrimidines, sugars, and inorganic salts were purchased in the highest grade available from Sigma Chemical Co. (St. Louis, MO). The complete chemically defined medium (CDM, Table 1) was adapted from that described by Morishita *et al.* [17]: CDM (pH 6.5) was prepared from concentrated individual stock solutions, which were stored at −4°C after filtration, except for the cysteine solution that was freshly prepared. Media and stock solutions were sterilized by filtration through a cellulose acetate membrane (0.20 μm pore size; Sartorius, Göttingen, Germany).

CDM was used in a series of deletion and add-back experiments to determine the minimal essential nutrients required for growth of *L. lactis.* Initial deletion of entire nutrient groups, including 11 vitamins, 6 minerals, 5 bases, or 20 amino acids, was followed by deletions of individual components when the group was determined to be necessary for growth. Subsequently, each nutrient that yielded no growth when omitted was individually added to the medium to confirm that it was essential for growth. A CDM without bases was called CDMWB (chemically defined medium without bases).

Working cultures of lactobacilli were propagated in MRS at 40°C for 16 h. To eliminate carryover nutrients, the cells were harvested by centrifugation at 8,000 *g* for 15 min, washed twice in sterile saline, and resuspended in this solution to the original volume. This cell suspension was used to inoculate the different media at an initial optical density (Spectronic 2000, Bausch and Lomb, Rochester, NY) at 560 nm (OD_{560}) of 0.07. Bacterial growth was monitored by measuring the OD_{560}, and these measurements were correlated with the cell dry weight determinations. Cells were harvested by filtration (0.2 μm), washed once with deionized water, and dried to a constant weight at 60°C under partial vacuum (200 mmHg). A change of 1 unit of optical density was shown to be equivalent to 0.50 g dry matter. In all growth experiments, complete CDM and CDM without any amino acids was inoculated as a positive and negative control, respectively.

The following terms are used to describe the relationship between medium components and growth as determined by the single-omission technique. A constituent was considered as essential (E) if its omission caused less than half maximum growth rate of the positive control; stimulatory (S) when the growth rate was between 50% and 80% of that observed in complete CDM; and non-essential (N) when the growth rate was 80% (or more) of that obtained in the complete CDM.

Table 1. Composition of the complete chemically defined medium (CDM) and the new formulated minimal defined medium (MDM)

Constituent	Concentration (g/L)	
	CDM	MDM
Glucose	10	10
Sodium acetate	5	5
KH_2PO_4	3	3
K_2HPO_4	3	3
$MgSO_4 \cdot 7H_2O$	0.2	0.2
$MnSO_4 \cdot 4H_2$	0.05	
$FeSO_4 \cdot 7H_2O$	0.02	
Tween 80	1	
L-Alanine	0.10	0.10
L-Arginine	0.10	0.10
L-Asparagine	0.20	
L-Aspartic acid	0.20	0.20
L-Cysteine	0.20	0.20
L-Glutamine	0.20	
L-Glutamic acid	0.20	0.20
Glycine	0.10	
L-Histidine	0.10	0.10
L-Isoleucine	0.10	0.10
L-Leucine	0.10	0.10
L-Lysine	0.10	0.10
L-Methionine	0.10	0.10
L-Phenylalanine	0.10	0.10
L-Proline	0.10	
L-Serine	0.10	0.10
L-Threonine	0.10	
L-Tryptophan	0.10	0.10
L-Tyrosine	0.10	0.10
L-Valine	0.10	0.10
Nicotinic acid	0.001	0.001
Pantothenic acid	0.001	0.001
Pyridoxal	0.002	0.002
Riboflavin	0.001	0.001
p-Aminobenzoic acid	0.01	
Folic acid	0.001	
Cyanocobalamin	0.001	0.001
D-Biotin	0.01	
Thiamine	0.001	
Adenine	0.01	0.01
Guanine	0.01	0.01
Inosine	0.01	
Xanthine	0.01	
Orotic acid	0.01	
Uracil	0.01	0.01
Thymine	0.01	

Results

To determine the absolute nutritional requirements of *L. lactis* CRL 581 and CRL 654 the single- or multiple-omission technique was applied to each component of the complete CDM. Initial determination of vitamin, purine, pyrimidine, mineral, and amino acid requirements utilized a defined medium that was made deficient in the individual nutrient groups. Results indicated that deletion of the nucleotides, vitamins, minerals, or amino acids completely abrogated growth; thus they were evaluated separately.

The growth rate of the two strains was not affected when $MnSO_4$, $FeSO_4$, or ammonium citrate were omitted individually or together. In contrast, $MgSO_4$ and phosphate appeared to be essential for growth. The omission of sodium acetate did not affect the growth rate, whereas a slightly lower final OD_{560} (approximately 8%) was observed in the absence of this compound. Addition of Tween 80 (0.1%) to the growth medium had no effect.

Table 2. Growth of *L. delbrueckii subsp. lactis* CRL 581 and CRL 654 in a CDM deficient in a specific vitamin

Deleted vitamin	Specific growth rate μ (h^{-1})[a]		Cell density (OD_{560})[b]	
	CRL 581	CRL 654	CRL 581	CRL 654
Pantothenic acid	0.07 ± 0.02	0.09 ± 0.02	0.28 ± 0.02	0.47 ± 0.03
Pyridoxal	0.14 ± 0.02	0.28 ± 0.03	0.73 ± 0.05	1.26 ± 0.06
Thiamine	0.36 ± 0.04	0.33 ± 0.03	1.55 ± 0.09	1.52 ± 0.07
Niacin	0.16 ± 0.01	0.17 ± 0.02	1.18 ± 0.06	1.08 ± 0.04
D-Biotin	0.35 ± 0.03	0.36 ± 0.03	1.48 ± 0.08	1.43 ± 0.07
Riboflavin	0.19 ± 0.02	0.28 ± 0.02	0.68 ± 0.04	0.95 ± 0.05
Cyanocobalamin	0.28 ± 0.02	0.27 ± 0.02	1.21 ± 0.06	0.84 ± 0.04
Folic acid	0.37 ± 0.03	0.34 ± 0.03	1.59 ± 0.07	1.45 ± 0.06
p-Aminobenzoic acid	0.36 ± 0.03	0.35 ± 0.02	1.55 ± 0.07	1.51 ± 0.08
None	0.37 ± 0.04	0.35 ± 0.03	1.58 ± 0.08	1.49 ± 0.07

[a] Growth rate, μ = ln 2/doubling time (h).
[b] OD measurements were performed after 12 h of incubation. Values are the mean of triplicate measurements ± standard deviation.

Subsequent deletion of individual vitamins indicated that niacin, pantothenic acid, and pyridoxal were essential for *L. lactis* CRL 581 (Table 2). The essential vitamins for strain CRL 654 were niacin and pantothenic acid, while pyridoxal was stimulatory for this microorganism. Riboflavin and cyanocobalamin showed a stimulatory effect for both bacteria (Table 2). d- Biotin, thiamine, folic acid, and *p*-aminobenzoate could be omitted without any effect on the growth rate or final cell density of the analyzed strains (Table 2).

The effect of bases on the growth rate of *L. lactis* CRL 581 and CRL 654 was determined in CDM, CDMWB, and CDMWB supplemented with different purine and pyrimidine combinations. With the exception of uracil, which proved to be an essential compound, none of the nucleic acid bases and their assayed precursors (orotic acid, inosine, adenine, guanine, xanthine, thymine) were found to be required by *L. lactis* using the single-omission approach. However, the strains could not grow when all purine bases (A, G, I) were removed at once. The growth rate observed in CDMWB supplemented with uracil, adenine, and guanine was similar than that obtained in complete CDM.

Amino acid requirements were tested by a single- or multiple-omission technique. No growth was observed for any strains when arginine, cysteine, histidine, isoleucine, leucine, methionine, phenylalanine, tryptophan, tyrosine, or valine was omitted, suggesting that these amino acids are essential (Table 3). *L. lactis* CRL 654 showed a requirement for lysine in addition to the amino acids necessary for the growth of strain CRL 581. The single omission of aspartic acid, asparagine, glycine, glutamic acid, glutamine, proline, or threonine did not affect the growth of either strain. However, when glutamic acid and glutamine were removed together, no growth was possible, indicating that glutamic acid is essential for *L. lactis* strains (Table 3). The omission of aspartic acid and asparagine diminished the growth rate of *L. lactis* CRL 581 by 29% but had no effect on the growth of CRL 654. The absence of alanine alone reduced the growth rate by 21%, and therefore this amino acid was considered growth stimulating. The removal of serine resulted in an important decrease in the growth rate (62% and 45% for strains CRL 581 and CRL 654, respectively) and in an extended lag phase (5 h).

On the basis of these nutritional requirements, a minimal defined medium (MDM) for *L. lactis* was formulated (Table 1). The specific growth rate of *L. lactis* CRL 581 and CRL 654 in MDM was 0.32 h^{-1} and 0.30 h^{-1}, respectively.

Discussion

This report describes a complete chemically defined medium (CDM) and a minimal defined medium (MDM) that support sustained growth of *L. lactis.* Chemically defined media have been developed for several *Lactobacillus* species [2, 5, 7, 8, 14, 17], including *L. lactis* [5]. However, the nutritional requirements of *L. lactis* have never been elucidated. CDM was used to determine the nutritional requirements of *L. lactis* strains and to define a minimal medium. The designed MDM supported growth at a reasonably high rate for the *L. lactis* strains tested. The positive effect of acetate on final OD of the strains could be attributed to the effect of acetate on the size of the cell [14] or to a buffering effect; this compound is necessary in phosphate-buffered media because of their low buffering capacity [16]. The omission of ammonium salt from the CDM did not affect the growth rate, indicating that the amino acid and nucleotide con-

Table 3. The effect of the single and multiple omission of amino acids from the complete CDM on the growth of *L. delbrueckii subsp. lactis* CRL 581 and CRL 654

Deleted amino acid	Specific growth rate μ (h^{-1})[a]		Cell density (OD_{560})[b]	
	CRL 581	CRL 654	CRL 581	CRL 654
Ala	0.29 ± 0.03	0.27 ± 0.02	1.44 ± 0.10 (S)	1.41 ± 0.11 (S)
Arg	nd	nd	0.09 ± 0.01 (E)	0.10 ± 0.02 (E)
Asp	0.36 ± 0.03	0.35 ± 0.02	1.48 ± 0.12 (N)	1.53 ± 0.11 (N)
Asn	0.37 ± 0.03	0.37 ± 0.03	1.50 ± 0.10 (N)	1.46 ± 0.09 (N)
Asp-Asn	0.27 ± 0.02	0.33 ± 0.03	1.19 ± 0.05 (S)	1.30 ± 0.08 (N)
Cys	nd	nd	0.10 ± 0.01 (E)	0.09 ± 0.02 (E)
Gln	0.36 ± 0.03	0.35 ± 0.03	1.48 ± 0.13 (N)	1.45 ± 0.12 (N)
Glu	0.38 ± 0.03	0.36 ± 0.03	1.40 ± 0.09 (N)	1.43 ± 0.08 (N)
Gln-Glu	nd	nd	0.08 ± 0.02 (E)	0.08 ± 0.01 (E)
Gly	0.35 ± 0.03	0.36 ± 0.02	1.43 ± 0.07 (N)	1.45 ± 0.09 (N)
His	nd	nd	0.11 ± 0.02 (E)	0.12 ± 0.03 (E)
Ile	nd	nd	0.09 ± 0.02 (E)	0.11 ± 0.02 (E)
Leu	nd	nd	0.08 ± 0.03 (E)	0.09 ± 0.04 (E)
Lys	0.40 ± 0.04	nd	1.59 ± 0.08 (N)	0.13 ± 0.04 (E)
Met	nd	nd	0.11 ± 0.03 (E)	0.09 ± 0.02 (E)
Phe	nd	nd	0.13 ± 0.02 (E)	0.08 ± 0.01 (E)
Pro	0.35 ± 0.02	0.37 ± 0.03	1.48 ± 0.07 (N)	1.41 ± 0.05 (N)
Ser	0.09 ± 0.01	0.15 ± 0.01	0.42 ± 0.02 (E)	0.71 ± 0.03 (E)
Thr	0.36 ± 0.03	0.35 ± 0.02	1.41 ± 0.08 (N)	1.47 ± 0.06 (N)
Trp	nd	nd	0.09 ± 0.01 (E)	0.08 ± 0.01 (E)
Tyr	nd	nd	0.10 ± 0.02 (E)	0.09 ± 0.02 (E)
Val	nd	nd	0.11 ± 0.02 (E)	0.10 ± 0.01 (E)
None	0.38 ± 0.03	0.39 ± 0.04	1.58 ± 0.11	1.49 ± 0.11

nd, not determined; E, essential for growth; S, stimulating; N, not essential.

[a] Growth rate, μ = In 2/doubling time (h).

[b] OD measurements were performed after 12 h of incubation. Values are the mean of triplicate measurements ± standard deviation.

tent of the medium satisfies the nitrogen requirement for biomass synthesis. This result is in accordance with those observed for most LAB [3, 6]. The removal of Tween 80 did not affect the growth rate of the strains. Tween 80 had differing effects on several LAB, stimulating *L. delbrueckii* subsp. *bulgaricus* NCFB 2772, *L. plantarum* and *L. sake* Lb16, and inhibiting *L. sake* NCFB 2714 [7, 16]. This compound was essential for *L. curvatus* [16] and *L. reuteri* CRL 1098 (unpublished data). In contrast, Tween 80 was not included in different defined media for *L. delbrueckii* subsp. *bulgaricus, Lactococcus lactis,* and *Streptococcus thermophilus* [3, 15, 18].

L. lactis CRL 581 and CRL 654 required 12 and 13 essential amino acids for growth in CDM, respectively. Like other thermophilic lactobacilli, *L. lactis* appears more demanding than other LAB such as *L. sake, L. plantarum, L. curvatus, L. casei* [7, 8, 14, 16, 17], and *Lactococcus* [3]. *L. lactis* is an obligately homofermentative lactobacillus [10]; the phosphoketolase enzyme is absent in this group. This fermentative pattern could explain the degree of amino acid auxotrophy in the species. Thus, D-erythrose-4-phosphate and ribose-5-phosphate, the precursors for the aromatic amino acid family and histidine, respectively, can not be synthesized in these strains. The *L. lactis* strains assayed had an absolute requirement for the branched amino acids isoleucine, leucine, and valine, suggesting that genetic lesions affecting these amino acid biosynthetic pathways have occurred [17]. Genome sequencing of *L. johnsonii, L. rhamnosus, L. casei,* and *L. plantarum* revealed a lack of the branched-chain amino acid biosynthesis pathway [13]. Argininine was an essential amino acid for *L. lactis.* Bringel and Hubert [1] suggested that LAB evolve by progressively losing unnecessary genes upon adaptation to specific habitats, with genome evolution toward cumulative DNA degeneration. Thus, association with dairy products might favor amino acid auxotrophies [1]. The vitamins niacin, pantothenic acid, and pyridoxal were essential. These vitamin auxotrophies appear to be common to most lactobacilli [2, 7, 8, 14, 17]. These vitamins are involved in coenzyme biosynthesis by *Lactococcus* and *Lactobacillus* [3].

L. lactis requires uracil, adenine, and guanine for sustained growth in a defined medium. The essential role of uracil in a CDM containing orotic acid indicated that this compound was unable to replace uracil as a pyrim-

idine precursor. When all purine bases (adenine, guanine, inosine) were removed at once, no growth was observed, confirming the inability of these microorganisms to synthesize purine bases de novo. Moreover, the biosynthesis of these bases was not possible in a medium containing pyridoxamine, which mainly acts as a catalytic agent of transaminases and might therefore be involved in the biosynthesis of inosinic acid from 5′-phosphoribosylpyrophosphate.

L. lactis generally requires the use of complex or enriched media for growth. The MDM reported in this study is a simple and well-defined medium to be used in preference to complex media for conducting future biochemical, physiological, and genetic studies on *L. lactis.*

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