Development of a Chemically Defined Medium for the Growth of *Leuconostoc mesenteroides*

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A chemically defined medium for the growth of *Leuconostoc mesenteroides* was developed. This medium contained lactose, Mn^{2+} , Mg^{2+} , 12 amino acids, eight vitamins, adenine, uracil, and Tween 80. We showed the beneficial effect of aerobic conditions on growth and that potassium phosphate (135 mM) is a suitable buffer. The growth rate in this medium was $0.85 \pm 0.10 \text{ h}^{-1}$ for the six strains examined, and cell densities up to $3.5 \times 10^9 \text{ CFU/ml}$ were attained.

Leuconostoc mesenteroides subsp. *mesenteroides* and *Leuconostoc mesenteroides* subsp. *dextranicum* are commonly found in starter cultures used in the dairy industry, where they are involved in texture and flavor production (5). For metabolic investigations, it is desirable to have a defined growth medium for these bacteria which (i) provides reproducibility of chemical composition; (ii) avoids an unnecessary excess of the nutrients, facilitating the adjustment of their levels; (iii) meets the experimentally determined nutritional requirements of several strains; and (iv) supports growth at a reasonably high rate. Comprehensive reviews of *Leuconostoc* differential and selective media have been published recently (12), but no attempt to formulate a defined medium has been made. In this paper we describe a new medium, which we designate *L. mesenteroides* chemically defined medium (LMCDM), that fulfills the nutritional requirements of *L. mesenteroides* and supports good growth of several strains.

The strains used for development of the medium were originally isolated from different French raw milk cheeses and are as follows: *L. mesenteroides* subsp. *mesenteroides* CNRZ1463, CNRZ1697, and CNRZ1750 and *L. mesenteroides* subsp. *dextranicum* CNRZ1694, CNRZ1742, and CNRZ1744 (8). They were stored in MRS (6) containing 1% (vol/vol) lactose and 10% (vol/vol) glycerol at -20°C . As a basis of our medium, we used the chemically defined medium (CDM) described by Rogosa et al. (17) and modified by Poolman and Konings (15) as listed in Table 1. The final pH was adjusted to 6.8 ± 0.1 (mean $\pm$ standard deviation) and experiments were performed at 30°C . All chemicals were of analytical grade and were obtained from commercial sources. Medium components were prepared separately as follows: (i) carbohydrate, (ii) basal salts, (iii) amino acids, (iv) vitamins, (v) metals, and (vi) purines and pyrimidines. CDM were sterilized by membrane filtration (Millex-GV filter; Millipore Corp., Bedford, Mass.). Working cultures of *Leuconostoc* were propagated in CDM. To ensure elimination of carryover nutrients, cultures were centrifuged and washed twice in 50 mM potassium phosphate, pH 6.5. Inocula consisted of 5% (vol/vol) exponentially growing cells. Growth was measured as the A_{620} with enzyme-linked immunosorbent assay plates in a Ceres 900 microplate reader (Bio-Tek Instruments, Inc., Winooski, Vt.) or a DU-68 spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Cell counts were determined on MRS, CDM, or LMCDM contain-

ing 12 g of agar per liter with a spiral plater (Spiral Systems DS, Cincinnati, Ohio).

L. mesenteroides metabolized various carbohydrates (lactose, glucose, fructose, saccharose, mannose, galactose, maltose, arabinose, xylose, ribose, and raffinose), each at a concentration of 5 g/liter, as described by Garvie (11); the lowest maximal A_{620} ($A_{620\text{max}}$) was obtained with galactose, arabinose, and mannose. In CDM, 10 g of lactose per liter was sufficient to sustain exponential growth up to an A_{620} of 1.26 ± 0.08 (mean $\pm$ standard deviation for six strains). The growth rate ranged from $0.51 \pm 0.13 \text{ h}^{-1}$ (mean $\pm$ standard deviation of three independent experiments) for CNRZ1694 to $0.81 \pm 0.15 \text{ h}^{-1}$ for CNRZ1750. Higher concentrations of lactose (20 to 50 g/liter) were slightly inhibitory.

The addition to CDM of the 10 amino acids (Gln, Leu, Ile, Val, Met, His, Trp, Arg, Pro, and Phe) previously shown to be essential for growth (8) did not allow growth of most leuconostoc strains (Table 2). The addition of cysteine together with threonine to the essential amino acids was more effective than the addition of serine and threonine to sustain growth (Table 2). Therefore, the combination of the essential amino acids with cysteine and threonine is a simple composition of amino acids that allows for the growth of six *L. mesenteroides* strains. It is worth noting that a twofold increase of the amino acid concentration did not affect the $A_{620\text{max}}$, whereas a twofold decrease reduced it by 10 to 20%.

The metal ion requirement of *L. mesenteroides* strains in CDM was also determined by omitting each metal ion in turn. We confirmed that Mn^{2+} was stimulatory (1, 24), whereas Mg^{2+} , Ca^{2+} , Fe^{2+} , Zn^{2+} , Co^{2+} , and Cu^{2+} were not essential (data not shown). However, in the absence of metal ions no growth was detected (Table 3), indicating the absolute requirement for metal ions. The addition of Mn^{2+} , and to a lesser extent that of Mg^{2+} , to CDM sustained significant growth of all leuconostoc strains examined. The effect on growth can be explained by the effect on enzymatic functions of the bacteria (4). A CDM mixture produced by the simultaneous addition of optimal concentrations of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (10 mg/liter) and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (200 mg/liter) was as effective as the complete metal mixture of CDM to promote growth (data not shown).

Deletion of individual vitamins from CDM confirmed the requirement for pantothenic and nicotinic acids (9–11, 18, 19). Thiamine, riboflavin, thymidine, folic acid, and inosine were stimulatory (10, 11, 23, 24); other vitamins were unnecessary (10) (Table 4 and data not shown). Biotin was not essential for growth; the requirement is apparently a function of the carbohydrate used (10, 23). Addition of thiamine with pantothenic

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TABLE 1. Composition of initial CDM and newly formulated LMCDM

Component	Concn in ^a :	
	CDM	LMCDM
Lactose	10 g/liter	10 g/liter
KH ₂ PO ₄	2.5 g/liter	2.5 g/liter
K ₂ HPO ₄	3.0 g/liter	3.0 g/liter
(NH ₄) ₃ -citrate	0.6 g/liter	0.6 g/liter
Na-acetate	1.0 g/liter	1.0 g/liter
Gln	390	390
Ile	213	213
Met	125	125
Val	325	325
His	150	150
Leu	475	475
Trp	50	50
Thr	225	225
Arg	125	125
Pro	675	675
Cys	250	250
Phe	275	275
Tyr	290	
Asp	300	
Asn	346	
Ala	238	
Lys	438	
Ser	336	
Gly	173	
Pyridoxal chloride	2	2
Nicotinic acid	1	1
Thiamine dichloride	1	1
Riboflavin	1	1
Calcium pantothenate	1	1
Folic acid	1	1
2-Deoxythymidine	5	5
Inosine	5	5
DL-6,8-Thioctic acid	2.5	
Pyridoxamine dichloride	5	
Sodium <i>p</i> -aminobenzoate	10	
Biotin	10	
Vitamin B ₁₂	1	
Orotic acid	5	
MnSO ₄ · H ₂ O	28	10
MgCl ₂ · 6H ₂ O	200	200
CaCl ₂ · 6H ₂ O	50	
FeCl ₂ · 4H ₂ O	5	
ZnSO ₄ · 7H ₂ O	5	
CoCl ₂ · 6H ₂ O	2.5	
CuSO ₄ · 5H ₂ O	0.1	
Adenine	10	10
Uracil	10	10
Xanthine	10	
Guanine	10	
Tween 80		1 ml/liter

^a Concentration in milligrams per liter unless otherwise indicated.

and nicotinic acids was necessary for growth of four of six strains (Table 5). The subsequent addition of riboflavin, folic acid, inosine, or thymidine stimulated growth to a variable extent as a function of the strain examined. All four vitamins were necessary for most strains to reach the A_{620} max, while CNRZ1750 and CNRZ1742 required the addition of pyridoxal to reach an A_{620} max comparable to that in CDM (2, 9).

FIG. 1. Growth of *L. mesenteroides* CNRZ1463 in CDM (●, A_{620}) and LMCDM (▽, A_{620} ; □, cell count).

As previously reported (10, 24), deletion of the nucleotides did not affect leuconostoc growth in CDM. However, in CDM containing lactose, 12 amino acids, Mn^{2+} , Mg^{2+} , and eight vitamins, we observed the requirement for adenine and uracil (10, 20).

The effect of ascorbic acid (16, 17, 21, 22) and oleate (as Tween 80, 1 ml/liter) (3, 6) on growth was also examined. Ascorbic acid did not affect growth. In contrast, the addition of Tween 80 stimulated growth of all strains except CNRZ1750. The A_{620} max increased from 1.26 ± 0.08 to 1.47 ± 0.05 in the presence of Tween 80.

Because *L. mesenteroides* converts a large part of the carbohydrate substrate into acid(s) (5), we anticipated that the buffer capacity of the medium would be important, particularly for high cell yield. Addition of potassium phosphate (135 mM) was suitable to buffer CDM, as the final pH remained high (5.79 ± 0.08 versus 3.88 ± 0.08) and growth was improved (the A_{620} max ranged from 2.49 to 3.25 versus 1.12 to 1.43). Addition of sodium β -glycerophosphate (62 mM) (6, 7) or morpholinepropanesulfonic acid (MOPS; 40 mM) (13, 14) was less effective than addition of potassium phosphate in buffering CDM. The final pH was higher in the presence of sodium β -glycerophosphate (5.58 ± 0.13) than in that of MOPS (4.07 ± 0.06). However, the A_{620} max increased only 10 to 20%, suggesting that the beneficial effect of potassium phosphate could also be correlated to the potassium or phosphate ions serving as a nutrient in the medium.

When the effect of O_2 was increased by constant stirring (150 rpm) (1), the A_{620} max was 10 to 20% higher than under static conditions, indicating the beneficial effect of aeration on growth (5).

Finally, we compared growth parameters of leuconostoc strains in CDM and in the new CDM we proposed (LMCDM). The composition of the latter met the nutritional requirements of the strains and also took advantage of the beneficial effects of Tween 80, potassium phosphate, and aeration conditions (Table 1). For all strains, growth rates in LMCDM and CDM were comparable (0.85 ± 0.10 and 0.73 ± 0.11 h⁻¹, respectively). A higher A_{620} max was attained in LMCDM than in CDM [1.67 ± 0.18 versus 1.26 ± 0.08 , respectively, corresponding to cell densities of $(3.5 \pm 0.3) \times 10^9$ and $(1.6 \pm 0.8) \times 10^9$ CFU/ml, respectively] for *L. mesenteroides* CNRZ1463 (Fig. 1). On LMCDM containing agar, bacterial colonies were

TABLE 2. Influence of addition of amino acids on *L. mesenteroides* growth in CDM containing only Gln, Leu, Ile, Val, Met, His, Trp, Arg, Pro, and Phe

Additional amino acid(s) ^a	<i>A</i> ₆₂₀ max of <i>L. mesenteroides</i> :					
	subsp. <i>mesenteroides</i>			subsp. <i>dextranum</i>		
	CNRZ1463	CNRZ1697	CNRZ1750	CNRZ1694	CNRZ1742	CNRZ1744
None	0.05	0.05	0.04	0.04	0.44	0.05
Ser	0.06	0.07	0.04	0.07	0.40	0.06
Thr	0.62	0.39	0.28	0.04	0.57	0.39
Cys	0.08	0.11	0.56	0.47	0.35	0.10
Cys + Ser	0.06	0.08	0.51	0.44	0.32	0.07
Cys + Thr	0.70	0.62	0.58	0.75	0.54	0.60
Ser + Thr	0.65	0.60	0.43	0.22	0.62	0.59
Thr + Cys + Ser + Tyr + Asp + Asn + Ala + Lys + Gly	0.85	0.79	1.09	0.77	0.77	0.88

^a Concentrations as in Table 1 (CDM).TABLE 3. Growth of *L. mesenteroides* in CDM containing metal ion(s)

Metal ion(s) ^a	<i>A</i> ₆₂₀ max of <i>L. mesenteroides</i> :					
	subsp. <i>mesenteroides</i>			subsp. <i>dextranum</i>		
	CNRZ1463	CNRZ1697	CNRZ1750	CNRZ1694	CNRZ1742	CNRZ1744
None	0.06	0.10	0.07	0.05	0.05	0.07
Mn ²⁺	0.73	0.69	0.87	0.72	0.79	0.77
Mg ²⁺	0.27	0.45	0.53	0.49	0.34	0.38
Mn ²⁺ + Mg ²⁺	0.83	0.75	0.97	0.76	0.92	0.78
Ca ²⁺	0.11	0.12	0.18	0.21	0.12	0.12
Fe ²⁺	0.13	0.15	0.25	0.21	0.16	0.14
Zn ²⁺	0.06	0.09	0.11	0.13	0.11	0.09
Co ²⁺	0.19	0.21	0.32	0.33	0.23	0.22
Cu ²⁺	0.23	0.31	0.43	0.43	0.31	0.32

^a Concentrations as in Table 1 (CDM).TABLE 4. Vitamin requirements of *L. mesenteroides* for growth

Vitamin	Importance for <i>L. mesenteroides</i> ^a :					
	subsp. <i>mesenteroides</i>			subsp. <i>dextranum</i>		
	CNRZ1463	CNRZ1697	CNRZ1750	CNRZ1694	CNRZ1742	CNRZ1744
Nicotinic acid	+	+	+	+	s	+
Calcium pantothenate	s	+	+	+	+	+
Thiamine Cl ₂	s	s	s	s	s	s
Riboflavin	s	—	—	s	s	—
2-Deoxythymidine	—	—	s	—	—	—
Folic acid	—	—	s	—	—	—
Inosine	—	—	s	—	—	—

^a +, essential for growth; —, nonessential; s, stimulatory.TABLE 5. Effect of addition of vitamin(s) on *L. mesenteroides* growth in CDM containing only nicotinic acid and calcium pantothenate

Addition(s) with thiamine ^a	<i>A</i> ₆₂₀ max of <i>L. mesenteroides</i> :					
	subsp. <i>mesenteroides</i>			subsp. <i>dextranum</i>		
	CNRZ1463	CNRZ1697	CNRZ1750	CNRZ1694	CNRZ1742	CNRZ1744
None (thiamine alone)	0.27	0.44	0.07	0.58	0.09	0.55
Riboflavin	0.63	0.46	0.06	0.67	0.29	0.69
Folic acid	0.27	0.55	0.21	0.57	0.18	0.60
Inosine	0.28	0.43	0.05	0.61	0.06	0.55
2-Deoxythymidine	0.29	0.49	0.29	0.63	0.11	0.54
All four	0.80	0.61	0.26	0.65	0.35	0.69

^a Concentrations as in Table 1 (CDM).

detected after 24 h of incubation at 30°C and counts were similar to those on MRS or CDM. The pH decrease was smaller in LMCDM than in CDM (0.78 ± 0.10 and 2.92 ± 0.10 , respectively). Finally, growth in LMCDM of 22 *L. mes-*

enteroides strains originally isolated from dairy products and belonging to the CNRZ collection was estimated. All strains grew in LMCDM, attaining an *A*₆₂₀max of 1.60 ± 0.12 .

The defined medium reported here, LMCDM, is of the

minimal composition needed to fulfill the nutritional requirements of a representative number of *L. mesenteroides* strains and to produce a rapid and heavy growth comparable to that in a complex medium. Therefore, it will be useful in conducting physiological, nutritional, and biochemical studies on *L. mesenteroides*.

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