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MINI-REVIEW

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Nutrient requirements of lactococci in defined growth media

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Abstract Many attempts have been made for the last six decades to design defined media for species of the lactococcus group. The general outcome of the studies suggests that this group is heterogeneous with respect to specific requirements for nutrients. Lactococcal species are limited in various metabolic pathways. Early attempts to trace the required nutrients were not always successful because of the poor quality of analysis and the presence of impurities in the medium components.

Introduction

Organisms belonging to the species *Lactococcus lactis* have been and still are studied intensively because of their commercial potential. Research focuses on strains originating from the dairy industry, because of the major role they play in the production of sour milk, sour cream and cheese, for example. Currently, special attention is paid to improving the aroma, ropiness and probiotic properties of dairy food products. Less regard is given to lactococci that have been isolated from other sources such as plant material and decaying meat. The lactococci belong to the group of lactic acid bacteria (LAB). The occurrence of these gram-positive microorganisms is restricted to habitats that are rich in nutrients. Lac-

tococci lack various biosynthetic pathways so that they require nutrients, especially amino acids and vitamins. *Lactococcus* is represented by two species, namely *L. lactis* and *L. cremoris*. Among both species there are strains that differ widely in their specific nutrient requirements. Many genes coding for anabolic enzymes in LAB are present on plasmids, which may be easily lost during growth of a culture in the absence of appropriate pressure. Therefore, in general, the amount of nutrients required by a strain depends on (i) the source from which the strain was isolated, (ii) how long it has been kept in a culture collection, and (iii) what medium was used to maintain the strain. This adds to the tediousness of developing a generally applicable defined medium for these organisms.

For several purposes a defined medium is not required, e.g. for most genetic studies rich, undefined media, such as MRS (De Man et al. 1960) or M17 (Terzaghi and Sandine 1975), are used. For isolation and enumeration of lactococci the medium proposed by Elikker et al. (1956) is widely used. For physiological studies, however, one prefers to use a medium that is chemically defined so that metabolism and its regulation can be observed. This defined medium should preferably support the growth of all strains of *Lactococcus*, for the purpose of enhancing the reliability of comparative interspecific studies (Ledesma et al. 1977). Since the late 1930s many attempts have been made to design a defined medium. Numerous strains from both public and private culture collections have been tested. One of the conclusions from these investigations is that every strain has its own particular set of growth requirements. As a result, the information on this subject is scattered throughout the literature.

This review aims to catalogue most of this rather complex material and to rationalise the basic problems encountered with the growth of lactococci. Especially in view of modern metabolic engineering and metabolic flux studies, chemically defined media are required and should meet the following demands: (i) only one or none of the components should be present in flux-limiting

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amounts (see, e.g., Egli 1991), (ii) any component required for the desired catabolic and anabolic pathways to occur should be present, (iii) any component that could obscure the interpretation of the results should be avoided.

We will focus here on media designed for both *L. lactis* ssp. *lactis* (*L. lactis*) and *L. lactis* ssp. *cremoris* (*L. cremoris*). Nevertheless, the rationale behind the rich chemically defined media treated in this review is applicable to most of the LAB since the overall requirements are alike among this group (Porubcan and Sellars 1979; Teuber 1995). This is reflected in the general defined and semi-defined media that have been formulated for other LAB such as lactobacilli and enterococci (Guirard et al. 1946; Deibel and Niven 1964; Ledesma et al. 1977). Therefore, we will consider the special requirements of LAB in general when no specific study has been done for lactococci. First, an overview of all the defined media will be given and discussed critically. Second, each of the components required or stimulatory for growth will be treated in a separate section.

Organisms

Undertaking the design of a chemically defined medium for strains of lactococci started in the early 1940s. Since we mention these investigations on various occasions, the strains studied and their origin are listed in Table 1. When we refer to literature that uses the old name, *Streptococcus*, for the lactococci group we replaced it by the new accepted name, *Lactococcus* (Schleifer et al. 1985), for the sake of convenience.

Defined and semi-defined media

Lactococci require several amino acids and B vitamins besides carbohydrates, phosphate, potassium and magnesium. The amino acids can either be supplied in pure chemical form (Poolman and Konings 1988; Jensen and Hammer 1993; Cocaign-Bousquet et al. 1995) or as a hydrolyzed casein product (Niven 1944; Otto et al. 1983). When pure amino acids are used, minimal media can be devised by applying the single-omission technique (Niven 1944). Recently two such minimal media have been designed, containing 8 amino acids (BL medium, Jensen and Hammer 1993) and 6 amino acids (MS13 medium, Cocaign-Bousquet et al. 1995), allowing maximal growth rates of 0.3 h⁻¹ and 0.23 h⁻¹ respectively. Neither medium contains any nucleic acid bases and MS13 contains no trace elements. Therefore heed should be given to the possibility that minimal media tend to limit growth in ways other than the one that is desired. Successful sustained growth for at least 50 generations (Cocaign-Bousquet et al. 1995) is therefore not a good criterion to test whether the medium is adequate for physiological studies. An appropriate method would be to step-up the concentration of the limiting substrate. A linear response of the biomass concentration to the inflow substrate concentration is proof that all nutrients other than the limiting substrate are present in excess. Supplementing the minimal media with other amino acids or vitamins always has a stimulating effect on the growth rate. Good growth was obtained with media containing 18–19 amino acids, e.g. medium SA (Table 2), so that the maximum specific growth rate

Table 1 Strains of *Lactococcus lactis* and *Lactococcus cremoris* used and their origin in most of the studies described here

Strain	Origin	Reference
<i>L. cremoris</i>	Dairy	Bibal et al. (1989)
<i>L. cremoris</i> (22 strains)	Dairy	Collins et al. (1950)
<i>L. cremoris</i> (8 strains)	Dairy	Law (1977)
<i>L. cremoris</i> (2 strains)	Dairy	Law et al. (1976)
<i>L. cremoris</i> Wg 2	Dairy	Otto et al. (1983)
		Smid and Konings (1990)
<i>L. cremoris</i> (18 strains)	Dairy	Reiter and Oram (1962)
<i>L. diacetalis</i>	Dairy	Law et al. (1976)
<i>L. diacetalis</i> (3 strains)	Dairy	Reiter and Oram (1962)
<i>L. lactis</i> IL 1403	Dairy	Bardowski et al. (1992)
		Cocaign-Bousquet et al. (1995)
<i>L. lactis</i> NCDO 2118	Plant material	Cocaign-Bousquet et al. (1995)
<i>L. lactis</i> (31 strains)	Dairy	Collins et al. (1950)
<i>L. lactis</i> 125	Dairy	Guirard et al. (1946)
<i>L. lactis</i> MG 1363	Plasma cured strain NCDO 712	Jensen and Hammer (1993)
<i>L. lactis</i> MG 611 (mutant)	Dairy	Juillard et al. (1995a)
<i>L. lactis</i> CNR 1076	Dairy	Juillard et al. (1995b)
<i>L. lactis</i> NCDO 763		
<i>L. lactis</i>	Dairy	Law et al. (1976)
<i>L. lactis</i> (21 strains)	Dairy	Niven (1944)
<i>L. lactis</i> (6 strains)	Dairy	Reiter and Oram (1962)
<i>L. lactis</i> C10	Dairy	Selby Smith and Lees (1975)
<i>L. lactis</i> ML3	Dairy	Smid and Konings (1990)
<i>L. lactis</i> IO-1 (JCM)	Dairy	Tanaka et al. (1995)
<i>L. lactis</i> ATCC 19435	Plant material	van Niel et al. (submitted)
<i>L. lactis</i> 65.1	Dairy	

Table 2 Composition of three (semi)-defined media (g l^{-1}). SA (Jensen & Hammer 1993), MS10 (Cocaign-Bousquet et al. 1995), and SD3 (van Niel et al., submitted). In SD3, all the amino acids except glutamine and asparagine originate from casamino acids (Difco) 5–10 g l^{-1}

Chemicals	SA	MS10	SD3
Alanine	0.3	0.24	–
Arginine	0.18	0.12	0.2–0.4
Aspartic acid	–	–	0.025–0.05
Cysteine (HCl)	0.1	0.17	–
Glutamic acid	0.31	–	0.25–0.5
Glycine	0.2	0.17	0.05–0.1
Histidine	0.05	0.11	0.1–0.2
Isoleucine	0.1	0.2	0.22–0.45
Lysine HCl	0.2	0.35	0.35–0.7
Methionine	0.1	0.12	0.12–0.22
Phenylalanine	0.2	0.28	0.2–0.4
Proline	0.3	0.68	–
Serine	0.3	0.34	–
Threonine	0.2	0.23	0.2–0.4
Tryptophan	0.1	0.05	0.015
Tyrosine	0.05	0.29	0.1–0.2
Valine	0.11	0.33	0.35–0.7
Leucine	0.1	0.47	0.5–1.0
Asparagine	0.11	0.34	0.4
Glutamine	0.1	0.51	0.4
Thiamine HCl	0.0001	–	0.001
Riboflavin	0.001	–	0.001
Niacin	0.001	0.001	0.0013
Calcium pantothenate	0.001	0.001	0.0012
Pyridoxal HCl ^a	0.002	0.005	0.0023
<i>p</i> -Aminobenzoic acid	–	–	0.0001
Folic acid	0.001	–	0.001
Biotin	0.0001	0.01	0.0001
Inositol	–	–	0.0015
Acetate	1.2	–	–
Adenine	–	–	0.03
Guanine	–	–	0.03
Uracil	–	–	0.06
NH ₄ Cl/(NH ₄) ₂ SO ₄	0.48	0.18	3.74
NaCl	2.9	–	0.525
KH ₂ PO ₄	–	9	2.5
K ₂ HPO ₄	0.18	7.5	3.25
MgSO ₄ ·7H ₂ O/MgCl ₂	0.106	0.2	0.87
(NH ₄) ₆ Mo ₇ O ₂₄	0.0000037	–	0.0004
MnSO ₄ ·4H ₂ O/MnCl ₂	0.000013	–	0.001
CaCl ₂ ·2H ₂ O	0.055	–	0.08
ZnSO ₄ ·7H ₂ O	0.000003	–	0.0048
CoCl ₂ ·6H ₂ O	0.000007	–	0.0003
CuSO ₄ ·5H ₂ O	0.00002	–	0.0003
FeSO ₄ ·7H ₂ O	0.0015	–	0.003
H ₃ BO ₃	0.000025	–	0.001
K ₂ SO ₄	0.05	–	–
KI	–	–	0.00018
MOPS	8.36	–	–
Tricine	0.72	–	–
EDTA	–	–	0.015
Glutathione	–	–	0.01

^a Or pyridoxamine

increased from 0.3 h^{-1} to 0.7 h^{-1} (Jensen and Hammer 1993). However, the media mentioned above were tailor-made for particular strains, as shown by the failure of our strains *L. lactis* ATCC 19435 and strain 65.1 to grow on these media (Fig. 1).

In general, preparing a medium containing pure amino acids is considered tedious. Therefore a compro-

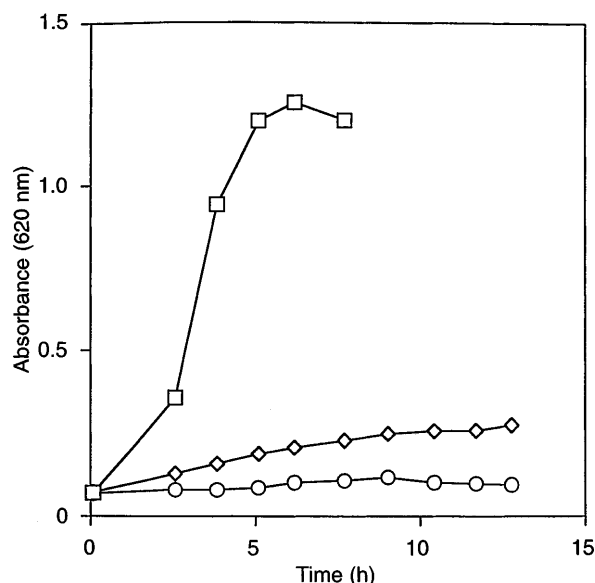


Fig. 1 Comparison of growth of *Lactococcus lactis* ATCC 19435 on three different media in anaerobic batch cultures on glucose: two defined mineral media, SA (\diamond) and MS10 (\circ) and our semi-defined medium SD3 ($\mu_{\max} = 1 \text{ h}^{-1}$) (\square). For a list of medium components see Table 2. No growth was observed after 48 h on either SA or MS10. The same result was obtained with strain 65.1

mise is to use acid-hydrolysed casein instead. Since the amino acid composition of this product, casamino acids, is prone to fluctuations, media based on this amino acid source are referred to as semi-defined media. For significant growth, these media have to be supplemented with glutamine and asparagine, which are absent from casamino acids (Niven 1944). Both types of media are often further supplemented with nucleic acid bases, trace elements, and special chemicals (see below) that are required for growth or for its stimulation. In addition, yeast nitrogen base (YNB), a defined mixture of compounds commercially available for carbon assimilation tests, appears to be essential for the organisms, and stimulates growth by 30% when glutamine and asparagine were present (medium SD3, Table 2). The reason why YNB has this effect remains to be clarified.

Temperature and pH

The effect of temperature on growth and product formation in LAB has been studied in a few cases only. On the other hand, the influence of pH on various characteristics of LAB has been extensively studied. The maximum lactic acid formation on glucose was observed at 33.5 °C for *L. lactis* ATCC 19435 (Åkerberg et al. 1998). The optimal pH for growth and product formation has been established to be around 6 for lactococci (Bibal et al. 1989; Parente et al. 1994; Åkerberg et al. 1998). The proteolytic activity of *L. lactis* was found to be highest at temperatures above 45 °C and at around pH 5.5 (De Giori et al. 1985).

According to Poolman and Konings (1988) one of the growth-rate-determining steps could be amino acid or peptide transport, which is a function of the pH. Usually the optimum pH varies between 6.0 and 6.5 and decreases rapidly at higher and lower values. Since *L. cremoris* and *L. lactis* can regulate their internal pH between 7.0 and 7.5 when the external pH ranges from 5.5 to 7.5, the failure to grow at alkaline and acidic pH is unlikely to be caused by a limitation of cytoplasmic processes (Poolman and Konings 1988). The growth rates of *L. cremoris* and *L. lactis* were limited at alkaline pH by their capacity to accumulate glutamate. Glutamic acid uptake rate and μ_{\max} decreased logarithmically when the pH was above 6.5, and because glutamic acid is an essential amino acid this could be one of the reasons why LAB do not grow at higher pH. With glutamine instead of glutamate, the μ_{\max} was high up to pH 8. At pH below 6.0 no difference between glutamate and glutamine was observed.

Amino acids

Free amino acids

In general, biosynthesis of amino acids results in a substantial energy demand and should therefore be repressed when the purpose of the fermentation is production of cell mass. On the other hand, LAB are incapable of growing at the expense of mineral nitrogen in the absence of exogenous amino acids. The most inclusive studies on amino acid requirements for the various subspecies of *L. lactis* strains employed the single-omission technique (Niven 1944; Reiter and Oram 1962; Coccagn-Bousquet et al. 1995). The essential amino acids could be traced accordingly, but it is not the appropriate procedure for constructing a minimal medium. This was proven by Jensen and Hammer (1993) who showed that *L. lactis* could not be cultivated in a defined medium containing the 6 essential amino acids determined by Reiter and Oram (1962). Similarly, Niven (1944) found that for growth of *L. lactis* L103, comparable to that in a complete amino acid mixture, 7 amino acids were needed in addition to the essential 6.

Over the years several research groups have determined the essential amino acids and the ones that stimulate growth for various *L. lactis* strains (Table 3). Similar data for other strains can be found in a paper by Chopin (1993). Reiter and Oram (1962) defined nutrients as essential if, in their absence, there was less than half-maximum growth after 48 h of incubation; likewise, stimulatory conditions were defined as producing more than half-maximum growth by the time the control in the complete medium was fully grown (within 18–24 h). Coccagn-Bousquet et al. (1995) applied a more quantitative method to the single-omission technique by determining μ_{\max} values and lag times. Nearly all of the strains of *L. lactis* that were investigated shared the same 6 essential amino acids: glutamic acid, valine, methio-

nine, histidine, leucine, and isoleucine. Besides the 6 essential amino acids that *L. cremoris* has in common with *L. lactis*, they also required proline and phenylalanine (Anderson and Elliker 1953; Reiter and Oram 1962). Several strains of *L. cremoris* also needed serine, tyrosine, lysine or alanine. However, in a study by Law et al. (1976) using the defined medium of Ford (1962), the greater requirement of *L. cremoris* strains for amino acids was apparently not present.

This apparent contrast is explained either by the absence of functional specific biosynthetic genes or by the presence of specific regulatory mechanisms (Chopin 1993; Raya et al. 1998). For example, some *L. lactis* strains isolated from non-dairy environments did require valine but not isoleucine nor leucine. This is not a defect in a structural gene because all the genes required for valine synthesis are also required for leucine and isoleucine synthesis (Godon et al. 1993). It rather suggests a coordinated expression of these genes, e.g. leucine or isoleucine inhibiting valine biosynthesis. Up till now, four clusters of genes involved in amino acid synthesis in LAB have been characterized. Regulation mechanisms for these clusters are similar to those found for other microorganisms, except for the branched-chain amino acids. Preliminary evidence suggests that a new type of regulatory mechanism, involving a metabolic shunt, might also control the branched-chain amino acid biosynthesis in *L. lactis* (Godon et al. 1992). Imbalances were observed early-on between several amino acids, such as aspartic acid and glutamic acid (Baumgartner et al. 1945), valine and leucine, phenylalanine and tyrosine (Shankman et al. 1947) and serine and threonine (Horn et al. 1947). Therefore the relative amounts of the amino acids are probably more important than their actual concentrations. This is also valid for certain amino acids that enter the cell via the same transport system (Poolman and Konings 1988). Especially at higher growth rates, active transport of certain amino acids might become the growth-limiting factor because of competitive inhibition by other amino acids. On the other hand, limitation of growth as a result of biosynthesis of non-essential amino acids, such as tryptophan, has also been observed (Law and Kolstad 1983; Poolman and Konings 1988). The growth of one strain at least was stimulated by tryptophan (Selby Smith et al. 1975). The inactivation of several of the biosynthetic pathways of amino acids in dairy lactococci seems to be a consequence of their adaptation to milk.

More variation exists among the strains in their requirements for the other, non-essential amino acids (Table 3). For the initiation of growth, *L. lactis* L103 needed both glutamine and asparagine at concentrations of at least $10 \mu\text{g l}^{-1}$ (Niven 1944). Higher concentrations (at least $100 \mu\text{g l}^{-1}$) also assured a shorter lag phase. Because only these small quantities were necessary, it was concluded that the remaining need for these two amino acids was supplied by their biosynthesis from other constituents in the medium. In a few strains, requirements for both glutamine and glutamate were

Table 3 Essential amino acids (*E*) and amino acids that stimulate growth (*s*) of *Lactococcus* strains as cited in the literature

Amino acid	Niven 1944	Reiter and Oram 1962		Cocaign-Bousquet et al. 1995		Law et al. 1976			
		<i>L. lactis</i> strains	<i>L. cremoris</i> strains	NCDO 2118	IL 1403	<i>L. lactis</i>	<i>L. lactis diacetalis</i>	<i>L. cremoris</i>	
								1	2
Glycine			(E)	s					
Alanine			s/E	s					
Valine	E	E	E	E	E	E	E	E	E
Leucine	E	E	E	s	E	E	E	E	E
Isoleucine	E	E	E	s	E	E	E	E	E
Serine			s/E	s	s				
Threonine			s		s				
Cysteine				s	s	s	s	E	s
Methionine	E	E	E	s	E	E	E	E	E
Phenylalanine		(E)	(E)			E		E	
Tyrosine			s/E					s	
Aspartate									
Glutamate		E	E	s		E	E	E	E
Glutamine	(E)				E				
Asparagine	(E)				E	E	E	E	E
Arginine	E		(E)		E				
Lysine			s/E						
Proline			E				E	E	
Histidine		E	E	s	E	E	E	E	E
Tryptophan			(E)						

detected that could be due to lack of proper regulation of the balance between concentrations of ammonium, glutamate and glutamine (Chopin 1993). Addition of glutamine alone or together with asparagine was also observed to shorten the lag phase of other *L. lactis* and *L. cremoris* strains (Poolman and Konings 1988; Jensen and Hammer 1993).

Most strains do not need arginine in the medium, since they are able to synthesize it from glutamic acid. However, several strains lack the whole or part of the pathway, and therefore they required arginine, citrulline or ornithine (Reiter and Oram 1962). Other strains are also able to grow with homocysteine instead of methionine, but not on intermediates preceding homocysteine in the biosynthetic pathway (Reiter and Oram 1962). If tryptophan is required, it could be replaced by serine plus either indole or anthranilic acid.

Poolman and Konings (1988) determined the amino acid composition of *L. lactis* ML3. Asparagine plus aspartate, glutamine plus glutamate, glycine and alanine were present at the highest concentrations (10–13 mol%). Next came the branched-chain amino acids valine, leucine and isoleucine (6–8 mol%). Cysteine (0.1 mol%), tyrosine (2 mol%) and methionine and histidine (each 2.2 mol%) were in the lowest concentrations. The concentration of essential amino acids below which growth was no longer optimal for *L. lactis* and *L. cremoris* was 10–80 mg l⁻¹ at a lactose concentration of 12 g l⁻¹ (Law et al. 1976). Reiter and Oram (1962) determined that, for growth on 10 g lactose l⁻¹, the amino acids had to be present in concentrations between 0.2 g l⁻¹ and 1 g l⁻¹. Each amino acid was required at a certain concentration, glutamic acid being

needed in the largest amounts (77 mg l⁻¹). The other amino acids were required in concentrations of 20–40 mg l⁻¹. The optimal glutamate concentrations in synthetic medium at pH 6.5 has been estimated to be 0.3–0.45 g l⁻¹.

Quite a different picture was obtained with *L. lactis* NCDO 2118 growing on four defined media, each composed of different numbers and concentrations of amino acids (Novak et al. 1997). The values for biomass yield and maximum growth rate increased with the complexity of the medium, the amino acid composition having the most pronounced effect. Glucose strongly contributes as a carbon source, while amino acids are not only involved in anabolism, but also give various fermentation products. Some amino acids lead to the production of unknown nitrogen-containing carbon compounds. In minimal media, containing only 6 amino acids, high concentrations of serine were deaminated to pyruvate. Part of the ammonium produced was reconsumed after exhaustion of serine. This phenomenon did not occur in defined media containing more amino acids, suggesting that the deamination was regulated by an amino acid. It shows yet again that these compounds should be supplied to the medium in a balanced manner.

Peptides

On the requirement for peptides there exists some controversy. Strains were found to be either totally dependent on peptides (Tanaka et al. 1995) or their growth was stimulated by peptides to different degrees (Selby

Smith et al. 1975; Juillard et al. 1995b; Law et al. 1976), although, as seen above, many strains can grow very well without them. Again the common dominator for the different observations is the huge strain variation among the lactococci. The advantage of direct transport of peptides into the cell prior to hydrolysis lies in the reduction of the amount of metabolic energy used for amino acid uptake (van Boven and Konings 1986). Peptides that are taken up come in different sizes (2–8 units) and sequences (in total 2.7×10^{10} possibilities). Studies with many different *Lactococcus* strains have shown that exo-enzymes and transport systems involved in the peptide uptake are highly substrate-specific, with respect to the length of peptides and the amino acid sequence (Law 1977; Juillard et al. 1995a, b). Three types of transport of amino acids were distinguished: (i) oligopeptides (4–8 units); (ii) di- and tripeptides; (iii) free amino acids (Konings et al. 1989).

Peptides can be derived from different sources, such as papain-digested skim milk, yeast extract, tryptone (trypsin-treated casein), soy peptones, peptones of animal origin, corn steep liquor, liver extracts, whey protein hydrolysates, etc. (Porubcan and Sellars 1979). It is obvious that, since each source contains different peptides, each strain will respond differently to each of them because of the specificity of the enzymes involved. For example, yeast extract in which most amino acids are in the free form is a poor source for peptides (Benthin and Villadsen 1996). Speck et al. (1958) found that fractions of yeast extract containing polypeptides stimulated growth of LAB since, upon hydrolysis of these fractions, the stimulating activity was lost. By contrast, the growth of *L. lactis* C10 was best stimulated by a fraction of yeast extract containing 75% free amino acids and 25% peptides. After hydrolysis of the peptides this stimulation was a bit lower, indicating that the peptides did only contribute marginally to the stimulation (Selby Smith et al. 1975). In tryptone most amino acids are peptide-bound and some are virtually only present in this form (Benthin and Villadsen 1996). Of tryptone fractionated over a gel-filtration column, only a small fraction, containing peptides of 6–9 units, stimulated growth of *Streptococcus equisimilis* (Phillips and Gibbs 1961). We obtained similar results with our *L. lactis* strains. In a comparative study on growth of *L. cremoris* FD1 on glucose, more peptides were consumed in a tryptone-based medium than in a YE-based one. This may well be due to the structure of peptides in tryptone, which may be similar to those to which *Lactococcus* is adapted (Benthin and Villadsen 1996). Most (90%) of the different N sources contributing to growth of *L. lactis* in milk are oligopeptides released from caseins by the action of the proteinase located in the cell envelope; the remainder come from oligopeptides (7%–8%) and free amino acids (2%–3%) initially present in milk.

Hydrolysis of β -casein by a cell-envelope proteinase resulted in more than 100 different oligopeptides, of which most were 4–8 amino acids long (Juillard et al. 1995a). One-fifth of the oligopeptides identified were

taken up by the oligopeptide transport system of *L. lactis* strains. Uptake of these peptides could supply the organism with all amino acids, including the essential ones. The di/tripeptide transport system was not essential for the use of peptides from β -casein in milk (Juillard et al. 1995b). However, Marugg et al. (1995) described for *L. lactis* SK11 a medium-dependent regulation of proteinase synthesis. The expression of the proteinase was repressed by extracellular peptides and free amino acids or by specific intracellular dipeptides (or derivatives). Active uptake of dipeptides via the specific transport system for di- or tripeptides was essential for control of the proteinase promoter. The consumption of free amino acids was very low (approx. 5 mg l^{-1}), suggesting that these N sources play only a minor role in growth on milk. Overproduction of proteinase resulted in an increased growth rate in milk. The addition of a mixture of 20 amino acids to milk (0.1 g l^{-1} each) significantly stimulated the growth rate of a strain lacking oligopeptide transport, but not that of a strain where oligopeptide transport was functional. Growth in milk suggests that its initial pool of amino acids allows growth of lactococci (Juillard et al. 1995b).

In a study by Law (1977) 8 strains of *L. lactis* were tested for growth on defined media. Five of them grew almost as well or better in media containing essential amino acids supplied as dipeptides as they did in media containing the equivalent free amino acids. The other three strains grew only poorly or not at all in these dipeptide-containing media. When these dipeptides were added to a complete defined medium the growth of the latter strains was not affected. Peptide-utilizing strains grew in media with dipeptides consisting either of histidine, isoleucine or glutamic acid. However, growth of these strains was inhibited by also including structurally related dipeptides in the medium, because of competition for uptake by the transport systems. Both peptide-utilizing and non-peptide-utilizing strains excreted dipeptidases in the culture during the exponential phase. Addition of partly purified extracellular dipeptidases to media with dipeptides initiated growth of strains that were unable to use peptides. The specificity of the dipeptidases was constrained to only a few dipeptides. Other dipeptides that were structure-related were converted at lower rates or not at all.

Proline, being the most abundant residue in β -casein, stimulated the growth of *L. cremoris* W92 (a proline-requiring strain) and *L. lactis* ML₃ (a proline-prototrophic strain) (Smid and Konings 1990). Both strains lack a proline-specific uptake system and free proline entered the cell only by passive diffusion. In other *L. cremoris* strains active uptake of proline-containing peptides proceeds via a di/tripeptide transport system. Lactococcal growth on amino-acid-based media was highly stimulated by the addition of proline-containing di- and tripeptides. The specific growth rate was determined by a balanced supply of different di- or tripeptides that competed for the same di- and tripeptide transport system.

Vitamins

Besides amino acids, several vitamins of the B group are essential for growth of LAB. Many studies have looked into which of them were vital or stimulatory. Most studies agree upon that nicotinate, pantothenate and biotin are essential for growth (Cocaign-Bousquet et al. 1995; Ledesma et al. 1977; Niven 1944; Reiter and Oram 1962). Nicotinate is necessary for the synthesis of NAD(P) and pantothenate is the cofactor of coenzyme A.

In one study it was noted that biotin was not required when Tween 80 was present in the medium (Ledesma et al. 1977), but this was in contrast with the earlier study by Reiter and Oram (1962). The latter observed that carbon dioxide and especially aspartate markedly stimulated growth in the absence of Tween 80 and hence could elevate the requirement of biotin to a certain level. Carbon dioxide had a stimulating effect on growth of *Lactobacillus arabinosus* in the absence of oleate. Biotin was required for the synthesis of both oleic acid and aspartic acid (Williams and Fieger 1946). However, Broquist and Snell (1951) showed that some LAB have a different pathway, not involving biotin, for aspartic acid synthesis and that some species require biotin even in the presence of unsaturated fatty acids. When the synthesis of aspartate and oleic acid was rendered unnecessary, biotin became either completely non-essential for growth or was required in such greatly reduced amounts that the necessary quantity could be supplied by synthesis in several *Lactobacillus* strains and an *Enterococcus faecalis* strain (Broquist and Snell 1951). In conclusion, some amino acids or other components can become essential when other essential components are not included in the medium.

Pyridoxal or pyridoxine, which is involved in the synthesis of amino acids, was not essential. With alanine in the medium pyridoxal was seen to stimulate growth, but without both compounds no growth occurred (Reiter and Oram 1962). The strains used by Niven (1944) were found to be having a limited ability to synthesize pyridoxine. Glycine at a concentration of 2 g l⁻¹ could completely destroy the stimulatory effect of 5 µg pyridoxine l⁻¹. For several strains of *L. lactis* and *L. cremoris* thiamine and riboflavin were found to be either essential or stimulatory (Niven 1944; Reiter and Oram 1962). Riboflavin is necessary for the cofactor FAD, e.g. in NADH oxidase. Indeed, it was found that the NADH oxidase activity increased when the medium was supplied with higher concentrations of riboflavin (van Niel et al., submitted). Thiamine has its function in the oxidation of pyruvate by pyruvate dehydrogenase, which is necessary for the production of acetoin. Folic acid or folinic acid and vitamin B12 were not required for growth (Niven 1944; Ledesma et al. 1977; Cocaign-Bousquet et al. 1995). These vitamins function as cofactors in the synthesis of purines and pyrimidines and their presence in the medium can therefore be stimulatory or essential in media without nucleic acid bases. Indeed, Ledesma et al. (1977) observed that these vita-

mins were not necessary when thymine or thymidine was present.

Fatty acids

Acetate

Acetate is the building block for fatty acids consisting of an even number of carbons, the average length being 18 carbons. The fatty acids are normally saturated and linear, but some are branched or unsaturated. The synthesis of these compounds requires CO₂, which is temporarily incorporated and subsequently released, hence only a minor quantity is necessary. Thus it is not surprising that the requirement of LAB for acetate arises from its function in the production of cellular lipoidal materials. This conclusion was reached by Guirard et al. (1946) from the finding that, with *Lb. arabinosus*, acetate could be replaced by, among others, saturated fatty acids (capric acid, lauric acid and myristic acid), unsaturated fatty acids (oleic acid, lineic acid and linoleic acid), sterols (cholesterol and ergosterol), fat-soluble vitamins, terpenes and carotenoids.

In a study with 22 strains of *L. cremoris* the growth of most of these strains was found to be dependent on both acetate and oleic acid (sorbitan monooleate) (Collins et al. 1950), despite the presence of biotin in the medium. The same was found with 9 of 31 strains of *L. lactis*. Sorbitan monooleate without acetate permitted growth of only 1 strain of *L. cremoris*. Acetate seemed either to perform an additional function in the metabolism or to be transformed into some compound that is essential for growth.

Snell and Broquist (1949) showed that acetate could be replaced by α-lipoic acid (see below). As later found for *L. lactis* ML3 (Reiter and Oram 1962), Shockman (1956) showed that *E. faecalis* required acetate or lipoic acid and thiamine only in the presence of oxygen and without reducing substances. Addition of 5% carbon dioxide to the aerobic gas phase had no effect on the growth. No growth was seen in the presence of oxygen with only lipoic acid or thiamine. In the presence of a maximal 25% air in the gas phase, good growth was possible in unsupplemented media. Above 60% air in the gas phase no growth was observed. For growth under aerobic conditions both good quantities of inocula and reducing agents such as cysteine, ascorbic acid or thioglycollate were necessary, even in unsupplemented media. Under aerobic conditions with the sodium acetate concentrations increasing to 130 mg l⁻¹, both the growth rate and biomass yield increased. But growth stimulation by acetate, as observed by Snell et al. (1937) and Guirard et al. (1946), was not found under truly anaerobic conditions.

Lipoic acid

Lipoic acid, or 6,8-thioctic acid, can be a growth factor for some LAB. It functions in the acyl-generation, acyl-

transfer and oxidative reactions of 2-oxo acids (Gunsalus 1954). The biological role arises from the presence of two sulfur atoms in a single molecule and the reactions made possible by this disulfide linkage. Arsenite is inhibitory for this compound even at concentration of 20–50 μM , meaning that it is more sensitive than glutathione- or cysteine-activated systems.

Like thiamine, lipoic acid functions, as a cofactor in the pyruvate dehydrogenase complex. This complex oxidizes pyruvate to acetate and carbon dioxide and takes part in the pathway of acetoin production.

A *L. lactis* strain was first noticed to require lipoic acid and thiamine when growing in an acetate-free medium (Reed and DeBusk 1954). Growth of *E. faecalis* on pyruvate required lipoic acid at a minimum concentration of 1 mg l^{-1} (Deibel and Niven 1964). The compound was not needed when hexoses, pentoses, hexitols, glycerol or gluconic acid were used as energy sources. This indicated that lipoic acid could be synthesized from these compounds. Several forms of lipoic acid have been found in yeast extract (Reed and DeBusk 1954), which might explain part of its growth-promoting effect.

Nucleic acid bases

So far each study that has looked into the requirements of nucleic acid components has come to the conclusion that these compounds are not required for growth, but that they have a stimulatory effect. Increases of growth rates of up to 35% have been found when nucleic acid bases were added to rich or defined media lacking these components (Cocaign-Bousquet et al. 1995). Inclusion of nucleic acid bases as well as several of their precursors (inosine, deoxythymidine, xanthine, orotic acid) have been studied. Of the precursors, orotic acid is a compound that is present in milk in high quantities (50–80 mg l^{-1}). In these high quantities it is more stimulatory than is required for the synthesis of pyrimidines, suggesting that it may have yet another function in the metabolism of *L. lactis*. In a specific study on pyrimidine metabolism of several *L. lactis* and *L. cremoris* strains, Martinussen et al. (1994) showed that cytosine, thymine and thymidine could not be used as sole pyrimidine sources, because of a lack of the proper enzymes. However, uracil, uridine, deoxyuridine, cytidine and deoxycytidine could support growth. Selby-Smith et al. (1975) observed that addition of purines to the medium had a greater stimulatory effect than pyrimidines. Generally, the purine riboside was found to be more stimulatory than the respective free base. Niven (1944) noticed that any one of the nucleic acid bases could be omitted without significant differences in the growth. With a simplified medium, growth of *L. lactis* NCDO 2118 was not possible unless nucleic acid bases were included (Cocaign-Bousquet et al. 1995). This was attributed to the omission of one or more vitamins. Pyridoxamine restored growth in a medium containing

the vital vitamins nicotinate, pantotenate and biotin, but lacking nucleic acid bases.

Minerals and buffers

Minerals and especially trace elements enter the media as impurities in other medium components making the requirements for these elements hard to define. Rich media are provided with adequate amounts of minerals through the use of yeast extract. They contain especially (in $\mu\text{g g}$ dry weight of yeast extract⁻¹) Fe (150), Cu (71), Mg (1270), Zn (74) and V (44). For a detailed analysis see Grant and Pramer (1962).

Comprehensive contradictory observations have been published on the requirements for minerals. For example, Olsen and Quteb (1970) saw stimulatory growth with an *L. lactis* and a *L. cremoris* strain when a pasteurized skim-milk medium was provided with either 2 ppm Fe, Mg or Mo or 1 ppm Se. Only a slight stimulation was found following the addition of 2–4 ppm Zn or 2 ppm Co. In the presence of 2 ppm Fe, 4 ppm Mg and 0.5 ppm Se the rate of acid production was significantly enhanced. B, Pb and Li were not or only slightly inhibitory at concentrations higher than 4 ppm. In the much-cited medium of Otto et al. (1983) no salts of Ni, Mn, Cu, Mo, Se, B or I were used. In a recent study Cocaign-Bousquet et al. (1995) showed that salts of Mn, Mo, B, Cu and Li were not necessary for growth of *L. lactis* NCDO 2118 in a defined medium; even Ca, Zn, Co or Fe could be removed from this medium. From a study on iron requirements of 23 strains of lactic acid bacteria, including *L. lactis* MG 1363, it was concluded that LAB did not require iron (Pandey et al. 1994). No iron-uptake capacity or siderophore production, to enhance uptake of Fe, could be detected. For growth under aerobic conditions, superoxide dismutase activity should be present, therefore the medium should contain at least about 60 $\mu\text{g Mn}^{2+} \text{l}^{-1}$ (Hansson and Häggström 1984) since this enzyme requires this metal ion.

Mg is an important component essential in many enzymatic reactions. Loubiere et al. (1997) found that Mg concentrations lower than 15 μmol (0.36 mg l^{-1}) affected the growth rate, though final biomass levels were dependent on the initial Mg concentration of the cultures containing less than 100 μmol (2.4 mg l^{-1}). About 2.8 $\text{mg Mg g biomass}^{-1}$ was necessary. However, when citrate is present in the medium, e.g. as a buffer, it forms complexes with Mn^{2+} and Mg^{2+} . This might decrease the growth rate, but that can be overcome by increasing the concentrations of both or one of the metal ions. An increase of one of them exerts a sparing effect on the requirement for the other (MacLeod and Snell 1947).

Phage infection has been observed in lactococci. Absorption of these phages to the cell wall is mediated by Ca^{2+} . Therefore Ca^{2+} -free media for starter propagation are necessary (Reiter 1973).

Inorganic orthophosphates of K, Na or ammonium are normally used as buffers. As an alternative, morpholinepropanesulfonic acid (MOPS) was shown to be a suitable buffer (Jensen and Hammer 1993). Optimum yields were reached at 0.12 M MOPS (when the pH was not adjusted). When MOPS was replaced by phosphate a lower growth rate was obtained with one of the strains at phosphate concentrations above about 40 mM. From this it was concluded that phosphate possibly causes partial dissipation of the proton gradient across the cytoplasmic membrane. Other buffers successfully used for lactic starter cultures include K, Na or ammonium salts of citrate, glycerol phosphate, and acetate (Porubcan and Sellars 1979), but these compounds are obviously less suitable for physiological studies.

Reducing agents

Lactococci do not possess a citric acid cycle or a respiratory chain and, in that sense, are true anaerobic bacteria, but they are able to grow in the presence of oxygen partially because of certain oxygen-metabolizing enzymes like superoxide dismutase, NADH oxidases and NADH peroxidases. In principle, therefore, the lactococci do not require a completely oxygen-free environment. However, hydrogen peroxide ($10\text{--}20\text{ mg l}^{-1}$) can be generated by superoxide dismutase and/or NADH oxidase and may accumulate in the medium since no catalase or no or low NADH peroxidase activities are present in these bacteria (Smart and Thomas 1987). Inhibitory levels of hydrogen peroxide (H_2O_2) in starter culture production can be reduced by addition of catalase to the growth medium. Addition of reduced glutathione (GSH), pyruvate, ferrous sulfate and catalase to the medium indeed completely or largely prevented accumulation of H_2O_2 in *L. lactis* cultures (Gilliland and Speck 1969).

One report mentioned that *L. lactis* was able to produce GSH since the total amount associated with the cells was much greater than what was available in the growth medium (Fahey et al. 1978). In contrast, Fernandez and Steele (1993) and Wiederholt and Steele (1994) observed no production of this compound in their *L. lactis* and *L. cremoris* strains. However, several strains were able to transport GSH into the cell. In this way, GSH can protect these strains more efficiently since oxygen metabolites are generated inside the cell. Several studies indicated that GSH addition to the medium resulted in accelerated growth (Kristoffersen et al. 1967) and production of volatile sulfhydryl compounds (Samples 1985), possibly due to GSH functioning as a contributor to low redox potential.

Lately it was shown that GSH was necessary for *L. lactis* ATCC 19435 to grow on a poor growth medium (SD1 = SD3 without asparagine and glutamine, Table 2) under aerobic conditions (van Niel et al., submitted). Other reducing agents that have been used in

media for lactococci are ascorbic acid, thioglycolate, dithiothreitol, and other thiols, although it has been observed that the latter at concentrations of $0.1\text{ to }1\text{ g l}^{-1}$ could inhibit growth (Kulshrestha and Marth 1970).

Selby Smith et al. (1975) concluded that YE contains a component that decomposes hydrogen peroxide under aerobic conditions and inhibits growth. Their results indicated that 1 g YE could decompose $38.7\text{ }\mu\text{M}$ peroxide. No oxygen was liberated from the medium, therefore water would be the end-product. It is known that YE contains several compounds that may react with H_2O_2 , such as glutathione, methionine, cysteine and pyruvate.

Concluding remarks

Lactic acid bacteria grow only in complex media and are therefore considered fastidious in nutrient requirements. For the lactococci this fastidiousness can be brought down to the requirement for at least 6 amino acids and 3 B vitamins. Nevertheless, for optimal growth, the medium should be enriched with more amino acids and vitamins, preferably in a balanced formulation. However, each of the resulting few minimal growth media is designed for a particular strain. As the lactococci consist of a heterogeneous group with respect to specific growth requirements, this restricts the general use of minimal media. Especially for microbial engineering or physiological investigations where comparison between different strains is compulsory, one requires an identical growth medium for all. It is therefore advisable to apply a rich, defined medium, which might look like the medium described by Poolman and Konings (1988), or its derivative MCD (Cocaign-Bousquet et al. 1995) or medium SD3, but in the latter case the casamino acids should be replaced by the proper amino acids to make it a defined medium.

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