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Review article

Lactic acid bacteria and metal ions

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Summary — While substantial literature has accumulated on the utilization of major nutrients such as carbon and nitrogen by lactic acid bacteria, little is known about the effects of «minor» nutrients such as metal ions. An attempt has been made to summarize the available information on transport, nutrition, enzymatic implications and importance of metal ions to lactic acid bacteria. Mg^{++}, Mn^{++}, Fe^{++}, Ca^{++}, K^{+} and Na^{+} have been considered in detail.

Knowledge of the relationships between lactic acid bacteria and heavy metals is very fragmentary. Generally, they have an inhibitory effect (Cd, Cs, Cu, Zn, Ur, Th) or no effect (Mo); however, they sometimes have a positive effect (Co, Zr).

lactic acid bacteria – metal ions – nutrition – transport – calcium – magnesium – potassium – iron – manganese – sodium – heavy metals

Résumé — Les minéraux et les bactéries lactiques. *Si la nutrition azotée et glucidique des bactéries lactiques est maintenant relativement bien connue, il n'en est pas de même de leurs besoins en minéraux. La complexité des milieux utilisés pour satisfaire les besoins nutritionnels de ces micro-organismes rend cette étude particulièrement difficile. En effet, les milieux complexes couvrant l'ensemble de leurs besoins contiennent généralement, comme contaminants, des quantités suffisantes d'éléments minéraux essentiels au développement d'une croissance plus ou moins importante. De plus, la présence de nombreux composés organiques rend inefficaces différentes méthodes destinées à enlever certains minéraux en vue d'étudier ensuite l'ajout d'une supplémentation minérale. Les variations des teneurs minérales dans le lait sont probablement l'une des causes, non négligeables, responsables des problèmes d'acidification rencontrés dans l'industrie laitière.*

Une supplémentation en magnésium du lait permet une stimulation de la croissance de S. thermophilus, S. lactis, L. acidophilus *et beaucoup d'autres bactéries. De nombreux procédés de production d'acide lactique ont été améliorés, au niveau de la productivité, par l'apport de cet ion. Le rôle d'activateur enzymatique joué par le magnésium ainsi que son intervention dans de nombreuses étapes de la vie cellulaire (division, stabilisation des acides nucléiques, synthèse de la paroi, métabolisme du pyruvate, hydrolyse de peptides, survie, etc.) rendent sa présence dans le milieu indispensable. Le manganèse fut mis en évidence dans les jus de tomate, dont l'apport dans les milieux de culture stimulait la croissance des bactéries lactiques. Le rôle particulier joué par les ions manganèse, liés à des polyphosphates, dans la résistance des lactobacilles à l'oxygène a été mis en évidence. Son rôle dans certaines activités enzymatiques est discuté (LDH, ARN polymérase, xylose isomérase, manganocatalase, superoxide dismutase...). Le problème de la toxicité de H_2O_2 est relié aux très faibles teneurs en fer intracellulaire. La présence chez les bactéries lactiques de sidérochromes n'a pas été mise en évidence. Les besoins en fer semblent*

très variables d'une espèce à l'autre, voire d'une souche à l'autre. Le fer est impliqué dans différentes activités enzymatiques chez les bactéries lactiques. Les streptocoques lactiques semblent requérir le calcium pour leur croissance. L'effet du Ca^{++} sur la croissance des lactobacilles semble moins net. Certains transporteurs membranaires du calcium ont été étudiés et des hypothèses formulées sur leurs natures et leurs modes d'action. Le calcium intervient dans la lyse cellulaire causée par les phages, mais pas au niveau de leur adsorption. Le calcium joue un rôle dans la liaison de certaines enzymes avec la paroi cellulaire. L'ATPase membranaire stimulée par le calcium et le magnésium exerce un effet crucial dans le métabolisme énergétique des bactéries lactiques. Le potassium joue un rôle important dans le contrôle du pH intracellulaire. Une addition de KCl modifie peu le gradient électrochimique d'ions hydrogène. Cet ion est nécessaire à la croissance de nombreuses bactéries lactiques. Le sodium exerce un effet sélectif sur les différentes espèces de bactéries lactiques. Il peut stimuler la production d'acide de certains streptocoques lactiques et en inhiber totalement d'autres. Les informations relatives aux relations des bactéries lactiques avec les métaux lourds sont très fragmentaires. Ils présentent, à des degrés divers, un profil inhibiteur vis-à-vis des bactéries lactiques. C'est le cas du cadmium, du césium, du cuivre, du zinc, de l'uranium, du thorium et du thallium. Le molybdène semble ne pas avoir d'effet, tandis que le cobalt est nécessaire pour certaines bactéries lactiques.

De nouvelles techniques de dosages des métaux permettront une étude plus fine des relations métaux-bactéries lactiques.

bactéries lactiques – minéraux – nutrition – transport – calcium – magnésium – potassium – fer – manganèse – sodium – métaux lourds

Introduction

Pasteur clearly pointed out the importance of minerals in microbial nutrition when he found that addition of ash was necessary for yeast growth. Studies on the role of minerals in microbial growth lagged because procedures for purification and detection were not sensitive enough to measure the small amounts of ions ordinarily required by bacteria.

In addition to these technical difficulties, comprehensive studies on the mineral requirements for the growth of organisms are complicated because of the following (Knight, 1951; Brulé, 1981) :
– metals replace each other;
– some metals adsorb others;
– some metals interact differently in the presence of others;
– many organic substances can combine with metals and render them unavailable for growth;
– colloidal suspensions of metals can precipitate because of shifts in pH before or during growth.

Interactions between metals and microorganisms are diverse, but can be divided into 3 major categories :
– metals essential for metabolism. Toxic metals can stop metabolic reactions;
– metals which accumulate : intracellular uptake and binding;
– metals which undergo biochemical transformation (including leaching). The third point will not be covered in this review (see Brierley, 1978; 1982).

The role of metal ions on the action of various types of metalloenzymes has been reviewed by Lehninger (1950). Three individual functions (not necessarily mutually exclusive) were presented :
– metal ions act as catalytic centers of enzymes;
– metal ions, not primarily involved in the catalysis, act as binding groups to bring enzyme and substrate together;

N.B. : The term «leaching» is used to describe the solubilization of metals (industrially copper and uranium) from low grade ores and their subsequent recovery.

– metal ions maintain physiological control (antagonism with other metals).

More recently, other aspects of the role of metal ions in metabolism have been investigated, *e.g.* the involvement of metal ions in the reactivation of EDTA inhibited proteolytic enzymes from lactic acid bacteria (Table I) and the narrow tolerance for specific metals in the synthesis of secondary metabolites (Weinberg, 1970, 1978).

The ionic environment may interfere with bacterial cell walls, especially in Gram-positive bacteria such as *Lactobacillus* and *Streptococcus* which contain teichoic and teichuronic acids (Ellwood and Tempest, 1972). The relative affinities of various cations for Gram-positive bacterial cell walls have been reported by Marquis *et al.* (1976).

Determination of mineral requirements : technical problems

In order to determine the mineral requirements of microorganisms, media free of metallic ions should be used. Many media components and water are contaminated with traces of metallic ions. Therefore, successful medium preparation is the first step in determining the significance of a mineral in metabolism. Different methods have been used (Guirard and Snell, 1962) :

— Coprecipitation of metals with phosphate (satisfactory for yeast and mold growth) has not been found satisfactory for bacteria, probably because bacteria require smaller amonts of ions (Knight, 1951).

— Chelation of metals with organic compounds and extraction of the complexes with anionic solvents. This method is useful, but is not effective when the metal is tightly combined or adsorbed on organic materials present in the medium. McLeod and Snell (1947) used citrate to remove magnesium and manganese from a medium for lactic acid bacteria. Near neutrality, the bivalent ions form stable complexes with the anion and the medium is likely to be deficient. Bentley *et al.* (1947) and McLeod and Snell (1948) removed traces of Mn^{++}, Mg^{++}, Fe^{++} and K^{+} by first growing *L. arabinosus* in the medium for 24 h. The cells were subsequently removed and the medium refortified with different compounds with the exception of the metal to be tested.

This method was used by Molish (1892) but would probably not be applicable to organisms which are feed back-inhibited or sensitive to metabolic products released in the medium.

Hutner *et al.* (1950) listed purification methods for major nutrients, trace elements and water. They described several procedures for preparing well defined media to study the role of metal ions. In fact, in chemically defined media, the mineral mixture most commonly used is that of Speakman (1923), which contains K^{+}, Na^{+}, Mg^{++}, Mn^{++}, Fe^{++}, PO_4^{--}, SO_4^{--} and Cl^{-} ions.

Commercial media also present problems. The same media produced by different manufacturers may differ greatly, and may not contain the same amount of cations. Bovallius and Zacharias (1971) have pointed this out, especially as regards Mg^{++} content in different commercial media.

Although the requirements of several lactic acid bacteria for organic nutrients are well known (Cogan, 1980; Desmazeaud, 1983), knowledge of mineral interactions within the same group is limited. It has been reported that mineral requirements for lactic acid bacteria

growth are complex. Mabbitt and Zielinska (1956) reported a change in the mineral composition of Rogosa medium (Rogosa *et al.*, 1951) which improved the growth of *L. lactis, L. bulgaricus* and *L. helveticus.*

In their review, Reiter and Moller-Madsen (1963) noted only a few studies on the metal requirements of starters. The problem is still unresolved. Dilanyan *et al.* (1971) showed that addition of trace elements (Cu, Mn, B, Sn, Ni, Co, Zn, Fe, I) to milk in "optimal doses" stimulated lactic acid bacteria activity in pickled cheeses. In the formulation of medium of a suitable composition to support

Table I. Implication of some metal ions in the reactivation of EDTA-inhibited proteolytic enzymes from lactic acid bacteria.

Enzyme	*Strain*	*Inhibition* *	*Metal ion reactivation*	*References*
Aminopeptidase	*S. cremoris*	PCMB; EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Tripeptidase	and	EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Dipeptidase	*S. lactis*	EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Dipeptidase		EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Dipeptidase	*S. cremoris*	EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Dipeptidase	*S. lactis*	PCMB; EDTA	Co^{++}; Mn^{++}; Zn^{++}	
Dipeptidase	*S. cremoris*	ME; EDTA	Mg^{++}	Law (1979)
Dipeptidase		ME; EDTA	Mg^{++}	
Tripeptidase	*S. lactis*	ME; EDTA	Mg^{++}	
Dipeptidase	*S. lactis*	PCMB; ME; EDTA	Mg^{++}	
Tripeptidase		PCMB; ME; EDTA	Mg^{++}	
Aminopeptidase	*L. lactis*	EDTA; Fe^{+++}; Cu^{++}; Hg^{++}	Co^{++}; Zn^{++}	Eggimann & Bachmann (1980)
Aminopeptidase	*S. diacetylactis*	EDTA	Co^{++}; Mn^{++}; Mg^{++}	Zevaco & Desmazeaud (1980)
Dipeptidase		EDTA	Co^{++}; Zn^{++}	Desmazeaud & Zevaco (1977)
Metalloprotease	*S. cremoris*	EDTA	Ca^{++}	Ohmiya & Sato (1975)
Aminopeptidase	*S. thermophilus*	EDTA	Co^{++}; Mn^{++}	Rabier & Desmazeaud (1973)
Carboxypeptidase Aminopeptidase Dipeptidase	*L. plantarum*	EDTA	Co^{++}; Mn^{++}	El Soda *et al.* (1978)
Proteinase	*L. bulgaricus*	EDTA		Argyle *et al.* (1976)

* PCMB, p-chloromercuribenzoic acid; ME, 2-mercaptoethanol; PMSF, phenylmethylsulphonylfluoride; EDTA, ethylenediaminetetraacetic acid.

Lactobacillus growth, Ledesma *et al.* (1977) indicated that no growth was obtained in the absence of Mn^{++} and/or Fe^{++}. When studying *S. lactis* nutrition, Niven (1944) included NaCl, $MgSO_4$, $FeSO_4$ and $MnCl_2$ in the medium, but said nothing upon the effect of these salts on growth or lactic acid production.

The purpose of this review is to establish the present state of knowledge of relationships between minerals (magnesium, calcium, iron, manganese, potassium, sodium, caesium, cobalt, copper, cadmium, zinc, thorium, zirconium, uranium, thallium) and lactic acid bacteria, with emphasis on dairy starters.

Magnesium

Nutrition

Magnesium is the major divalent cation in all living cells. In bacterial cells, the intracellular Mg^{++} content is equivalent to 20—40 mM Mg^{++} (Silver and Clark, 1971), compared to 100—500 mM for K^+. Most of the Mg^{++} (> 95 %) is probably bound to intracellular polynucleotides. Magnesium transport across bacterial membranes has been reviewed by Jasper and Silver (1977).

In milk, the Mg^{++} concentration fluctuates between 4.2 and 6.25 mM, depending on the geographic region (Veisseyre, 1975) (Table II). Supplementation of milk with 1—2.1 mM Mg^{++} permitted both a stimulation of growth of *S. thermophilus* and *S. lactis* and a better survival rate of the lactic streptococci (Amouzou *et al.*, 1985). The authors postulated that this effect resulted from an increase in the free ionic form of Mg^{++} in the medium. Sabine and Vaselekos (1967) have shown that Mg^{++} and Mn^{++} are the only cations required by *L. acidophilus,* while Rogosa and Mitchell (1950) claimed that Mg^{++} was indispensable for growth of *L. helveticus.* Magnesium also appeared to be essential for the growth of *L. lactis* and *L. delbrueckii* (0.1—0.2 mg $MgSO_4$ ml^{-1}). A Mg^{++} deficiency was found to cause filamentous forms, delayed cell division and growth and a tendency for the bacteria to stain Gram-negative (Nowakowska-Waszczuck, 1965a,b).

The production yield of lactic acid from molasses, yeast extract and $(NH_4)_2HPO_4$ by *L. delbrueckii* was increased up to 70%

Table II. Average content of macronutrient minerals (Ca, P, Mg, K, Na) and trace elements in cow milk (from Gueguen, 1979).

Ca	1.20	g•kg^{-1}
P	0.90	
Mg	0.12	
K	1.50	
Na	0.45	
Zn	2 000 — 5 000	µg•kg^{-1}
Fe	200 — 800	
Cu	50 — 250	
I	15 — 150 *	
Mo	20 — 100 *	
Mn	30 — 60	
Se	10 — 50	
Ni	20 — 30	
As	10 — 30	
F	10 — 30	
Cr	10 — 20	
Co	0.5 — 1.0	
Si	1 500 — 7 000	
Rb	1 000 — 3 000	
Al	500 — 1 000	
Br	100 — 500 *	
Ba, Sr	150 — 250	
V, B	100 — 200	
Sn	80 — 150	
Ti, W	50 — 100	
Cd, Ag	20 — 50	
Li, Sb	20 — 40	
Pb	10 — 40	
Hg	10 — 30	

* These values vary according to diet.

when $MgSO_4$ was added (Aksu and Kutsal, 1986).

Enzymatic implications

The essential requirement of Mg^{++} for growth is generally explained by the fact that it acts as an activator of different metabolic reactions : cell division, stabilization of nucleic acids (DNA and RNA) (Webb, 1948, 1949, 1951a,b, 1953), peptide hydrolysis (Johnson and Berger, 1942), and synthesis of the «Gram-complex» (Henry and Stacey, 1946). A deficiency in magnesium (0.041—0.2 mM) resulted in slower cell division and formation of filamentous cells (Webb, 1951b). Shankar and Bard (1955) noted that Mg^{++} was essential for the phosphokinases involved in glycolysis, while Harvey and Collins (1963) reported strong evidence that the real substrate for citritase in *S. diacetylactis* is an Mg—citrate complex, rather than citrate.

It is well known that many enzymes which require Mg^{++} for activation may also be activated by Mn^{++} (Nilsson *et al.*, 1942), but some Mg^{++} activated enzyme reactions associated with growth and metabolism cannot be replaced by Mn^{++}. Mg^{++} is directly involved in the formation of the «Gram-complex».

Thomas and Batt (1968) observed that Mg^{++} increased the survival rates of *S. lactis* cells, resuspended in phosphate buffer. At high bacterial concentrations, the survival rate increased because Mg^{++} was excreted in the medium by the bacteria. In 1969, the same authors observed that bacteria suspended in buffer without Mg^{++} had a reduced RNA content (20.8 to 5.5% in 28 h). In the presence of 1 mM $MgSO_4$, this decrease was lower (20.7 to 10.4%). Although considerable RNA may be degraded without affecting viability, it seems likely that a degree of ribosome stability is important for *S. lactis* survival.

Manganese

Transport

The specificity and kinetics of Mn^{++} uptake by *L. plantarum* have been studied : a K_m of 0.2 µM (comparable to other organisms) and a V_M of 23.8 nmol mg^{-1} protein min^{-1} have been found.

This V_M was 10^4 times greater than the value found in *E. coli*. It is not at present known if this is due to a higher number of carriers or more rapid Mn^{++} transport (Archibald and Duong, 1984). Eighty percent of the cellular Mn^{++} is nondialyzable. The large amount of nondialyzable polyphosphate, «metachromatic» or «volutin» granules, combined with its intensely polyanionic nature makes it a good candidate as an Mn^{++} sequestering or storage molecule (Archibald, 1986).

Nutrition

Vegetable extracts have been routinely used for enhancement of growth of lactic acid bacteria for many years. The beneficial growth of tomato juice was reported by Mickle (1924), Mickle and Breed (1925) and Briggs (1953).

The acid and heat resistant growth factor present in tomato juice (Kuicken *et al.*, 1943; Metcalf *et al.*, 1946) was also reported in asparagus juice (Snell and Lewis, 1953), and was identified as Mn^{++} by Stamer *et al.* (1964). Of the 71 strains of lactic acid bacteria tested, 63 showed a

definite requirement for Mn^{++} or tomato juice, which contains 0.011% Mn. This work was a confirmation of the studies of Zlataroff and Kaltschewa (1936), Snell *et al.* (1937), Moeller (1939), Woolley (1941) and Orla-Jensen (1943). When assaying pantothenic acid, Woolley (1941) obtained quicker growth and acid production by *L. casei* in the presence of Mn^{++}.

Bentley *et al.* (1947) have described a method for determining manganese using *L. arabinosus*. The response was quantitative from 1.8 to 9.1 10^{-4} mM Mn^{++}. Mn^{++} is essential for *Leuconostoc mesenteroides,* all lactobacilli and probably for *S. faecalis*. McLeod (1951) demonstrated that *S. faecalis* required Mg^{++} and Mn^{++} for growth. The amount of Mn^{++} required is, however, always decreased when Mg^{++} or Ca^{++} is added (McLeod and Snell, 1947). Trace amounts of Mn^{++} prevented cellular autolysis of *S. faecalis* (McLeod, 1951). Mn^{++} cannot be replaced by Mg^{++}, Ca^{++} or Sr^{++} for growth of *L. arabinosus* and *L. pentosus,* but these ions can counteract Zn^{++} inhibition (McLeod and Snell, 1950). Heterofermentative lactobacilli required Mn^{++} for optimum growth (Evans and Niven, 1951).

Among the mineral elements tested on *Leuconostoc dextranicum,* Whiteside-Carlson and Rosano (1951) showed that only Mn^{++} and PO_4^{--} ions were required for growth and dextran synthesis. De Man (1961) observed that small amounts of Mn^{++} added to milk stimulated growth of *Leuconostoc cremoris* and other betacocci, but not lactic streptococci. Galesloot and Hassing suggested in 1962 that the seasonal effect on aroma production could be explained by fluctuations in Mn^{++} levels. De Man and Galesloot (1962) showed that addition of Mn^{++} to milk eliminated the seasonal effect on aroma production with betacocci. Later, Menger *et al.* (1967) confirmed these studies : «The seasonal variation of the Mn^{++} content of the milk powders corresponds with the seasonal variation of the activity of *L. cremoris*». Grigorov and Chebotarev (1979) showed that addition of Mn and Mg ions alone or together increased by a factor of 4 the colony count and lactate production by *Leuconostoc cremoris.* No effect was observed on *S. cremoris.*

Manganese, magnesium and iron are generally believed to be required for *L. acidophilus.* Sabine and Vaselekos (1967) have shown that only Mn^{++} and Mg^{++} are essential for *L. acidophilus.*

Efthymiou and Joseph (1972) showed that 3 strains of pediococci required Mn^{++} for maximal growth in Rogosa basal medium, whereas 22 strains of enterococci were unaffected by this supplementation.

Nowakowska-Waszczuk (1965c) observed that the growth of *L. delbrueckii* was inhibited even at low Mn^{++} concentration. With *L. lactis,* Mn^{++} could partially substitute for Mg^{++}.

Biological functions of manganese

Reported enzymatic functions of manganese are numerous. This metal is involved in some LDHs, RNA polymerase, malolactic enzyme, xylose isomerase, manganicatalase, manganisuperoxide dismutase and NADH oxidase activities (Archibald, 1986).

De Vries *et al.* (1970) showed that the nicotinamide adenine dinucleotide (NAD) L-lactate dehydrogenase of *L. casei* required fructose-1,6-bisphosphate (FBP) and Mn^{++} ions for activity. Manganous ions increased the pH range of activity of this enzyme by increasing its affinity for FDP. Other divalent metals, except Mg^{++}, will effectively substitute for Mn^{++} (Holland and Pritchard, 1975).

Recently, studying lactic acid racemases (such an enzyme is unusual in lactic acid bacteria; Desmazeaud, 1983) and ribose fermentation induction in *Lactobacillus curvatus,* Stetter and coworkers found that both processes are dependent on Mn^{++} content. Later, they concluded that Mn^{++} was involved in the transcription process, possibly *via* a manganese dependent RNA polymerase (Stetter and Kandler, 1973; Stetter and Zillig, 1974).

Yamanaka and Higashihara (1962) have shown that 5.10^{-3} M Mn^{++} greatly promoted D-xylose and L-arabinose isomerase in lactic acid bacteria.

Many aerotolerant lactic acid bacteria have neither catalase (EC 1.11.1.6) (which catalyzes the reaction : $2H_2O_2 \longrightarrow 2H_2O + O_2$) nor superoxide dismutase (SOD) (EC1.15.1.1.) (SOD catalyzes the dismutation of O_2^- : $2O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$). These enzymes are believed to be necessary for aerobic growth. O_2^- is a detrimental but unavoidable by-product of aerobic metabolism, and therefore it was felt that all cells exposed to oxygen should contain an SOD. Most of the aerobes were found to contain both SOD and catalase; the aerotolerant anaerobes contained SOD but no catalase; and the strict anaerobes contained neither SOD nor catalase (McCord *et al.,* 1972).

A considerable amount of work, mainly done by Archibald and Fridovich, has correlated oxygen sensitivity with the high levels of Mn^{++} and very low level of Fe in these bacteria. *L. plantarum,* which respires at substantial rates, can accumulate more than 7 mM H_2O_2 in the culture medium, yet possesses no SOD or catalase activity (Archibald and Fridovich, 1981a). Archibald and Fridovich's studies (1981b) support the conclusion that a high cellular level of Mn^{++} (20—25 mM) provides some lactobacilli with an important defense mechanism against endogenous O_2. Moreover, Gotz *et al.* (1980a,b) found that O_2^- elimination was not an enzymatic reaction and suggested that Mn^{++}-phosphate(s) might be responsible for the observed elimination of O_2^-. Those lactic acid bacteria which contained high intracellular levels of Mn^{++} were devoid of true SOD. Curiously, the bacteria containing high Mn^{++} levels (pediococci, leuconostocs and lactobacilli) live on plants, which contain chloroplasts with high Mn^{++} concentrations, while the lactic acid bacteria associated with milk do not accumulate high levels of Mn^{++}, possibly because the content of Mn in milk is low (Table II). Martin *et al.* (1984) showed that Mn^{++} and Fe^{++} or Fe^{+++} were necessary for aerobic but not anaerobic growth of *S. mutans.*

Despite the biotoxicity of H_2O_2, several streptococci and lactobacilli have been shown to accumulate millimolar levels of H_2O_2 and yet remain viable (Whittenbury, 1964). How are such levels of H_2O_2 tolerated ? With the exception of some catalase positive strains (*L. plantarum* : Wurtz, 1953; *L. delbrueckii* : Vankova, 1957; *lactobacilli* : Dacre and Sharpe, 1956), aerotolerant lactic acid bacteria strains ordinarily lack catalase. But Johnston and Delwiche (1965), and Ingram (1975) have shown that certain strains of lactobacilli and pediococci incorporate hematin into catalase during growth, with the concomitant formation of cyanide- and azide-sensitive catalase. The production of the enzyme is stimulated by the presence of Fe and Mn ions in the growth medium.

A manganicatalase has been found in various species of *Pediococcus, Lactobacillus, Leuconostoc* and *Streptococcus.* The scavaging of H_2O_2 is a dismutation :

$$E\text{—}Mn\ (III) + H_2O_2 + 2H^+ \longrightarrow E\text{—}Mn\ (V) + 2H_2O$$
$$E\text{—}MN\ (V) + H_2O_2 \longrightarrow E\text{—}Mn\ (III) + 2H^+ + O_2.$$

The occurrence of this enzyme did not correlate with the considerable amounts of intracellular Mn (Archibald, 1986).

Some superoxide dismutases required Mn, Fe, or Co and Zn (Kirby *et al.*, 1980). They have been distinguished by selective inhibition or inactivation : CN^- inhibits the CuZn SOD but not Mn SOD or Fe SOD; H_2O_2 inactivates Cu, Zn and Fe enzymes but not the Mn enzyme; N^- inhibits these enzymes in the following order : Fe SOD > Mn SOD > Cu Zn SOD (Kirby *et al.*, 1980).

These authors have identified an Mn SOD in *S. lactis* ATCC 19435.

The toxic hydroxyl radical OH is produced by a Fenton type reaction (Walling, 1975; Cohen and Sinet, 1980) :

$$H_2O_2 + Fe^{++}\ (\text{or } Cu^+) \longrightarrow OH^- + OH + Fe^{+++}\ (\text{or } Cu^{++}).$$

The presence of low molecular weight forms of Fe or Cu is believed to exacerbate oxygen damage to cells. But it was found that *L. plantarum* contained < 2 Fe atoms per cell (in contrast with the 10^6 atoms of Fe in an *E. coli* cell) (Archibald, 1983). The ability of this organism to tolerate a high level of H_2O_2 may be due to much lower toxicity of this compound in a cell in which Fenton type reactions are much less likely to occur.

The responses of lactic acid bacteria to oxygen have been reviewed recently by Condon (1987).

Iron

Transport

From a transport point of view, Fe is probably the best studied element (for a detailed review, see Neilands, 1974). Fe transport is mediated by a number of high affinity molecules (catechols and hydroxamic acids) which chelate this ion. The complex, called a siderochrome or siderophore, is subsequently transported to the cell. The Czaky test (1948), which detects bound hydroxylamines, is negative for the lactic acid bacteria, suggesting the absence of hydroxamate type siderochromes (Burnham and Neilands, 1961).

Nutrition

Effect on growth

McLeod and Snell (1947) found no enhanced growth of several lactobacilli on supplementation with iron in an iron-deficient medium. However, iron was found to be an essential element for one *S. cremoris* strain (requiring 1.8×10^{-7} mM) and one *S. lactis* strain (3.6×10^{-8} mM). Vanadium in higher concentrations could replace iron (Reiter and Moller-Madsen, 1963).

Certain milk proteins, *e.g.* lactoferrin, have a bacteriostatic effect on growth ions. This effect has been shown by Ashton and Busta (1968) and Oram and Reiter (1968) for *Bacillus stearothermophilus* and *B. subtilis.* The protein complexes the ions and decreases the already very low Fe^{++} content of the medium. Addition of external Fe^{++} saturates the chelating sites of this protein and allows the medium to support growth of the iron-requiring bacteria.

Several studies have shown that addition of trace metals to milk increases cheese ripening (Hofi *et al.*, 1970; Saakjan, 1982).

Effect on acid production

The intensity of acid production by *S. thermophilus, L. casei* and *L. helveticus* is

unaffected by addition of Fe to milk. However, *S. lactis* and *S. cremoris* produced somewhat less acid in iron-deficient milk compared to milk containing iron. Sabine and Vaselekos (1967) found that the presence of ethylene diamine tetraacetic acid (EDTA), Fe^{++} and Mg^{++}, separately or in combination did not affect *L. acidophilus* growth. Fe^{++} or Fe^{+++} had no effect on streptococci acid production.

Production of lactic acid from molasses by *L. bulgaricus, L. delbrueckii* and a mixed population of the two was studied by Tiwari and Pandey (1980). The Fe—EDTA complex had a slightly positive effect on *L. bulgaricus* growth, but other combinations tested (EDTA alone or with Mn, Ca and Zn) were ineffective.

Addition of ferric chloride (9×10^{-5} mM) and cobalt chloride to paneer whey increased *L. bulgaricus* acid production, while 10 ppm of the same salts inhibited fermentation (Tewari *et al.*, 1985).

Moreover, Pulay *et al.* (1959) showed than *S. cremoris* required no iron for aroma production.

The prime function of iron in aerobic metabolism is the reduction of oxygen by means of the cytochrome chain and concomitant generation of chemical energy. But anaerobic species may also contain high quantities of iron (Neilands, 1974). Cells which contain little or no iron compounds would be useful in the study of iron flux in microbial metabolism. All lactic acid bacteria lack cytochromes and derive their energy from fermentation. Thus they should be useful in this regard.

Enzymatic functions

In the course of investigations on the role of Mn^{++} in *L. plantarum,* Archibald (1983) did not detect any intracellular iron or Fe requirements. A decrease of the Fe content in the medium from 23 µM to < 0.2 µM had no effect on the growth of this organism (Archibald and Fridovich, 1981a). Atomic absorption analysis revealed that *L. plantarum* cells contained < 0.1 µM intracellular Fe. *E. coli* B cells contain about 900 µM Fe; these values are equivalent to 2 atoms of Fe/cell and 10^6 atoms, respectively, in Fe-limited *E. coli* cell ! Stephenson (1949) reported the cellular Fe content to be between 0.0036 and 0.0175% (dry weight). Bowen (1966) confirmed these values : 0.25 mg/g (dry weight). Why does *L. plantarum* avoid accumulation of Fe ? Archibald (1983) suggested the following hypothesis : trace levels of Fe catalyse a variety of oxygen free radical reactions, especially of the OH generating Fenton type, thus greatly exacerbating the toxicity of H_2O_2 and O_2^- (Fenton, 1894). *L. plantarum* remains viable in high concentrations of H_2O_2 (7 mM). This fact and the acid tolerance may provide an advantage in its natural habitat.

Implications in enzymatic activities

Detailed information of Fe-requiring enzymes are very rare; Reiter and Moller-Madsen (1963) reported an Fe-activated aldolase in *S. lactis,* while Yamanaka and Higashihara (1962) showed that ferrous ions were needed for D-xylose isomerase production.

Ribonucleotide reductase of *L. leichmanii,* which catalyzes reduction of all 4 ribonucleoside phosphates, has been studied by Panagou *et al.* (1972). This enzyme lacks the iron-containing B_2 component and has an absolute requirement for the coenzyme B_{12}; although it is a single polypeptide (M.W. 76,000), it is subject to allosteric regulation.

Calcium

Transport

Exclusion of calcium from the cytoplasm appears to be a universal attribute of bacteria, except under special circumstances such as sporulation; however, the exact role of calcium in bacterial physiology remains unknown (Harold, 1977).

Specific exclusion mechanisms operate to maintain intracellular Ca^{++} at a concentration that is significantly lower than that in the extracellular medium. In the majority of cases, calcium flux is mediated by a secondary transport system (Ca^{++} : H^+ or Ca^{++} : Na^+ antiporters) which are driven by cation (H^+ or Na^+) motive gradients (Rosen and Kashket, 1978). Driessen *et al.* (1985) and Drissen and Konings (1986) reported that the pH gradient was dependent on calcium accumulation, presumably *via* a calcium or calcium phosphate : proton antiporter, in rightside-out vesicles of *S. cremoris* fused with proteoliposomes containing bacteriorhodopsin. However, data reported by Ambudkar *et al.* (1986) indicate that in *S. lactis,* as well as *S. faecalis* and *S. sanguinis,* calcium flux is catalyzed by an ATP-linked primary pump. It appears that this sytem is a member of the E_1E_2 family of cation translocating ATPases.

Demott and Cragle (1960) observed that the strontium 89 : calcium 45 ratio is higher in whey than in cheese. This preferential accumulation of Ca in the curd was due to the intracellular quantities of Ca^{++} accumulated by *S. lactis* (Demott and Holt, 1962). In 1968, Hurst and Lazarus found that high intracellular concentrations of Ca^{++} occur during the logarithmic growth phase of *S. lactis* (7.5 10^{-3} mM) which returned to about 10^3 mM during stationary phase. Moreover, a complex inverse relationship was found between antibiotic activity (nisin) and calcium content. The formation of hypothetical complex between nisin and calcium was suggested, but no definite proof was given.

Nutrition

McLeod and Snell (1947) showed that Ca^{++} ions were not essential for growth of several lactic acid bacteria. They did not study *L. delbrueckii,* in which serine utilization is stimulated by Ca (Meinke and Holland, 1950). Moreover, during studies with *L. casei, L. arabinosus, Leuconostoc mesenteroides* and *S. faecalis,* only *L. casei* growth was enhanced by calcium addition. Calcium stimulated early growth of *L. casei* in an amino acid medium (Eades and Womack, 1953) and in media containing limiting amounts of serine (Camien and Dunn, 1952). It is interesting to note that in the presence of Ca^{++}, short chains were formed, while in the absence of this ion the cells were in longer chains. Wright and Klaenhammer (1981) showed that Ca supplementation of MRS resulted in a morphological transition of *L. acidophilus* from filamentous to bacilloid rods, which were more resistant to freezing.

McDonald (1957) suggested that Ca^{++} and Mg^{++} ions stimulated *S. lactis* and *S. cremoris* growth. The nutritional requirements of 124 strains of lactic acid bacteria were investigated by Nurumikko and Karha (1961), who found that 30 strains of *S. thermophilus* required Ca^{++} for growth, which could not be replaced by other common metal ions.

Nurumikko and Karha (1962) determined the effect of various media

components on the growth of *S. thermophilus* in the presence and absence of calcium. The slightly stimulatory effect of the emulsion of oleic acid and Tween 80 was more marked in the presence of calcium. The requirements of different amino acids did not depend on the calcium content of the medium.

Ca^{++} effects on phages

Since the original studies of Stassano and de Beaufort (1925), much work has been done on the possibility of limiting phage by multiplication lowering the calcium content of the media. Several early workers (Reiter, 1949; Shew, 1949; Collins *et al.*, 1950; and Potter and Nelson, 1952a) showed that Ca^{++} ions were necessary for phage proliferation in lactic streptococci. Cherry and Watson (1949) showed that calcium chloride promoted lysis of *S. lactis* cells. Potter and Nelson (1952b) also observed that phage adsorption was not the limiting step in absence of calcium. These results were contradicted by other data (Puck *et al.*, 1951). To reduce the available Ca^{++} several authors suggested that milk be treated with phosphates (Reiter, 1956a; Babel, 1958; Hargrove, 1959; Hargrove *et al.*, 1961; Kadis and Babel, 1962; Bester and Lombard, 1962; Henning *et al.*, 1965; Robertson, 1966; Zottola and Marth, 1966) or citrate (Stassano and de Beaufort, 1925; Doull and Meanwell, 1953), or polyelectrolytes (polysaccharides such as pectin or dextranglycollate) (Reiter, 1956b). These compounds sequester the available calcium due to their polymeric electrolyte nature. However, Sozzi (1972) showed that lysis may occur even at low Ca concentrations. Moreover, media treated with phosphates did not allow optimal growth of all starters (Czulak and Keogh, 1957; Tybek, 1959) especially thermophilic strains (*L. helveticus, S. thermophilus*) (Sozzi, 1972).

Lagrange and Reinbold (1968) reported that the cost of culture medium was the major expense in starter preparation and emphasized the need for more economical culture media. Richardson and coworkers at Utah State University tried to overcome this problem by growing cultures under pH control in cheese whey fortified with reduced quantities of inorganic phosphate and yeast extract (Ausavanodom *et al.*, 1977; Richardson *et al.*, 1977). The cultures propagated in the acid whey medium have been used successfully to manufacture Cottage cheese (Chen and Richardson, 1977), Cheddar cheese and Italian and Swiss type cheese cultures (Reddy and Richardson, 1977). A new formulation of this phospate—whey medium was developed in 1982 to increase cell population and culture activity (Wright and Richardson, 1982).

Calcium and the cell wall

The role of calcium in the cell wall is not clear. Mills and Thomas (1978) have shown that the liberation of proteinase from cell walls of *S. lactis* and *S. cremoris* stopped when $CaCl_2$ was added to the buffer, or when the temperature was raised or when the pH reached 5.5. Thomas *et al.* (1974) thought that calcium linked the cell wall and the enzyme, while Exterkate (1979) felt that calcium stabilized proteinase activity. Monnet (1986) thought that calcium acted by expanding and contracting the cell wall. Marquis (1968) found that *B. megaterium* walls behaved as flexible amphoteric polyelectrolytes and that their compactness in aqueous suspensions was

affected by changes in environmental ionic strength and pH. $CaCl_2$ added to water suspensions of isolated cell walls caused a contraction of the structures (NaCl, NaBr, Na_2SO_4, KCl, LiCl and $MgCl_2$ were also effective in this regard).

Ca^{++}/Mg^{++} stimulated membrane bound ATPase complex

Metabolic energy in lactic streptococci is generated by substrate level phosphorylation and by flux of end products (lactate) in symport with protons (Fig. 1) (Otto *et al.,* 1980). The aim of this paragraph is not to describe the 2 groups of metabolic energy-generating mechanisms (for a review, see Konings and Otto, 1983), but to present one of the 2 most important proton motive force-generating mechanisms, which is the Ca^{++}/Mg^{++} stimulated membrane bound ATPase complex (the other is the cytochrome-linked electron transfer system) (see Strittmatter, 1959; Sijpesteijn, 1970). The driving force for proton extrusion in the ATPase complex is the free energy for hydrolysis of ATP ($\Delta G'_{ATP}$). This ATPase can also catalyse the reverse reaction and synthesize ATP. In order to avoid the hydrolysis of a considerable fraction of the ATP formed by substrate level phosphorylation, streptococci utilize an electrogenic excretion of lactate with 2 protons for disposal of metabolic end-product, but also to form an energy supply. The ATPase complex can synthesize ATP. Evidence in favour of this idea has been obtained in glycolysing cells of *S. lactis* by Harold (1977). Michels and coworkers (1979) have calculated the generation of the electrochemical proton gradient during lactate efflux for a model cell (spherical with a diameter of 1 μm and performing a homolactic fermentation of glucose).

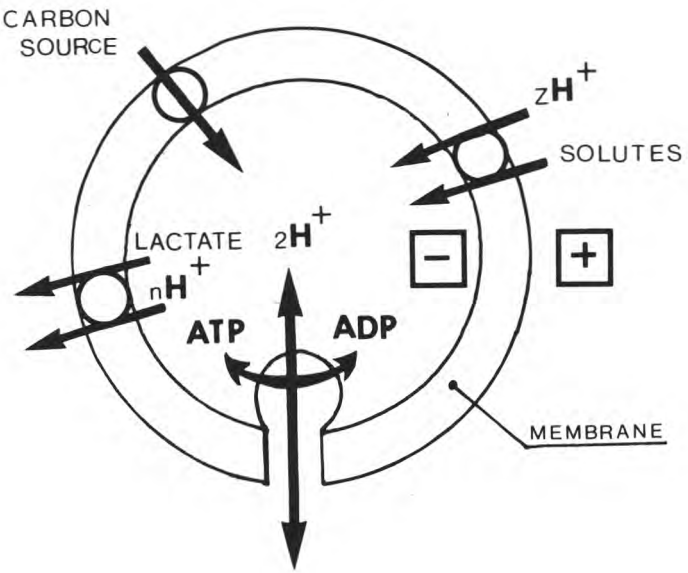

Fig. 1. Oversimplified metabolic energy transformation processes in streptococci (adapted from Konings and Otto, 1983).

Potassium

Transport

Fermenting conditions are required for K^+ translocation across the streptococcal membrane (Harold and Altendorf, 1974). Potassium is a cofactor of many enzymes, and a high cytoplasmic K^+ level is required for protein synthesis. K^+ transport appears to play an important role in the control of the cytoplasmic pH (Harold, 1977).

More recently, Kashket and Barker (1977), have shown that energized *S. lactis* cells accumulate K^+ ions, presumably in exchange for H^+. Addition of KCl to cells fermenting glucose or arginine at pH 5 showed little change in the electrochemical gradient of hydrogen ions ($\Delta p = \Delta\psi - \Delta pH$) (which is the mode of energy conservation in bacteria; for more information about energy conservation in bacteria, see Harold, 1972), but lowered the $\Delta\psi$ (membrane potential) while increasing the transmembrane pH gradient (ΔpH). These parameters bear the relationship $\Delta pH = \Delta\psi - 2{,}3\ RT/F\ (pH_{out} - pH_{in})$.

Data presented by Kashket *et al.* (1980) suggest that the K^+ concentration and the pH of the medium are the 2 main factors determining the relative contribution of ΔpH and $\Delta\psi$ to the proton motive force in both growing and resting cells of *S. lactis* ATCC 7962. With no pH gradient nor electrical potential across the plasma membrane, Harold and Van Brunt (1977) showed that *S. faecalis* grows normally if a high concentration of external K^+ is provided. The uptake of thallous ions by *S. lactis* cells required a fermentable energy substrate plus Na^+ ions and was inhibited by K^+ ions (Kashket, 1979).

Nutrition

K^+ is a major nutrient for microorganisms and is often present in amounts equal to or even greater than phosphorus (Tempest and Wouters, 1981). Levels of > 50 mM are common. K^+ is contained within the cells in an unmodified and largely unbound state, which is unique among the major nutrients. The K^+ is generally greater in Gram-positive than in Gram-negative bacteria. Potassium is required for growth of *S. faecalis* and *L. helveticus* (Barton-Wright, 1945) and *L. casei* (Rogosa, 1944), who suggested that *L. casei* could be used to assay potassium, since there was a linear response of growth to the potassium concentration in the medium. Lester (1958) showed that in complex medium, K^+ on *S. faecalis* growth was 3 times more effective than Rb^+ and 17 times greater than Cs^+.

McLeod and Snell (1947) have shown that potassium, manganese and phosphate are essential for growth of *Leuconostoc mesenteroides, S. faecalis* and a number of *Lactobacilli* in a synthetic medium commonly used for vitamin assays.

In 1948, the same authors found that a competitive relationship exists between K^+, NH_4^+ and Na^+ for all of the lactic acid bacteria that were tested (*L. arabinosus; L. casei; Leuconostoc mesenteroides* and *S. faecalis*). Rubidium replaced K^+ except with *Leuconostoc mesenteroides* which is inhibited; however, the inhibition was competitively alleviated by more potassium.

Sodium

Transport

In their normal physiological condition, Na^+ ions are rejected by bacterial cells

(Silver, 1978), which maintain a constant int/ext ratio of 1/3—1/50, depending on the osmotic pressure of the medium. Transport could be stimulated or inhibited by Na^+ ions, depending on the bacteria (Harold, 1977).

Barker and Kashket (1977) showed that addition of NaCl had a minor effect on $\Delta\psi$ and no effect on ΔpH of *S. lactis*. At pH 6.0, NaCl lowered the $\Delta\psi$ from 90 to 80 mV and dissipated $\Delta\psi$ of 15—20 mV at pH 7.5 in resting *S. lactis* cells. This relative lack of effect of NaCl on ΔpH may be due to a flux in H^+, in exchange for Na^+, that is low enough for the ATPase to compensate by increasing the H^+ efflux (Kashket *et al.*, 1980).

Nutrition

The results depend on the species and especially on the medium. Numerous experiments have been reported on the effect of NaCl on lactic acid bacteria. Generally, these dealt with its influence on growth or acid production (Olson and Qutub, 1970). Orla-Jensen (1919) found that most lactic acid bacteria were not inhibited by 2.5% salt, but considerable inhibition at 5.5% and complete inhibition at 10.0% were apparent. Sherman (1937) was unable to use a criterion for identification of *S. lactis* and *S. cremoris*.

McDowall and Whelan (1934) showed that addition of 1—2% salt stimulated acid production by a mixed culture of lactic streptococci in raw milk. Concentrations > 3% gave definite inhibitions. Solol'Skaya (1955) observed stimulation of *S. lactis* by 0.5% salt and inhibition at 2%, but Walter *et al.* (1958) observed little inhibition at the latter concentration. In contrast, *S. cremoris* showed inhibition at 1.4% and complete inhibition at 2% salt. Irvine and Price (1961) observed a more rapid acid production in the curd of Dariworld cheese at 2% salt, while Elliker *et al.* (1956) reported increased numbers and sizes of colonies of lactic streptococci when sodium chloride and sodium acetate were added to the medium.

Stepwise increases in NaCl concentration up to 6.5% had a less inhibitory effect on acid production by *S. lactis* and *S. paracitrovorus* than one single addition (Rasic, 1965).

El Gendy *et al.* (1983) isolated 145 lactic acid bacteria from 12% salted raw milk samples (126 lactobacilli), from high salted Domiati cheeses, and Karlikanova and Ramazanov (1979) selected a salt-resistant starter (*S. faecalis, L. plantarum, S. lactis, S. cremoris, S. lactis* ssp. *diacetylactis* and *Leuconostoc dextranicum*) for Osetonskii cheese.

Other trace metals

Mercury

Transformation of Hg is the best documented metal transformation in bacteria (Summers and Silver, 1978). No information is available concerning this aspect in lactic acid bacteria. However, $HgCl_2$ has been employed as a preservative for raw milk.

Cadmium

Cadmium delays the initiation of bacterial growth and inhibits growth. Like Hg, Cd is transported by Mn^{++} carriers (Summer and Silver, 1978). Industrial activities such as smelting and refining of zinc and lead ores and disposal of waste plastics are the most significant sources of environmental contamination by Cd

(Webb, 1975). In Finland, Koiranen *et al.* (1978) found between 9.6 x 10^{-6} mM and 6.3 x 10^{-5} mM of Cd in milk, and Murthy and Rhea (1968) found 1.5—2.6 x 10^{-4} mM in the United States. Using skim milk containing 6 x 10^{-5} mM Cd as substrate, Korkeala *et al.* (1984) showed that growth and acid production of *L. lactis, L. helveticus,* and *S. thermophilus* were partly inhibited by Cd.

Svec (1948) showed that addition of cadmium chloride (1.75 x 10^{-3}—8.7 x 10^{-2} mM) to a medium containing 3 x 10^{-4} mM of Cd caused complete inhibition of growth of *L. casei, L. delbrueckii* and *L. arabinosus.* Yamanaka and Higashihara (1962) have also noted Cd inhibition of lactic acid bacteria growth at 10^{-3} M.

These data suggest that the Cd content of milk may be high enough to interfere with dairy processes and cause economic loss.

Caesium

Caesium ion could not replace K^+ in *L. casei, S. faecalis, L. mesenteroides* or *L. arabinosus* (McLeod and Snell, 1947). Cs^+ was inhibitory at concentrations of 2.25 mM for *L. arabinosus.*

Copper

There is no evidence for a specific transport system for copper (Silver, 1978). There are a few bacterial copper proteins that appear to serve an electron transport role, but no studies on lactic acid bacteria are known.

Lactic acid bacteria are inhibited in whey based medium containing 3 x 10^{-2}—8 x 10^{-2} mM copper, while in milk more than 8 x 10^{-2} mM was required for inhibition (Zollikofer and Hoffmann, 1962). Pamir (1964) showed that 0.3 mM copper had a toxic effect on growth of *L. casei* and *L. bulgaricus* and Kiermeier *et al.* (1961) observed an inhibitory effect of 8 x 10^{-2} mM copper on *S. thermophilus.* Additions of 1.6 x 10^{-4}—1.6 x 10^{-3} mM Cu had no effect on acidity production for *L. casei, L. arabinosus* and *L. delbrueckii* (Svec, 1948). Growth and acid production were affected by higher levels of copper. Data presented by Efstathiou and McKay (1977) showed that lactose metabolizing strains of *S. lactis* were less resistant to arsenate (7.5—60.2-fold), arsenite (2.25—3.0-fold) and chromate (6.6—9.4-fold), but more sensitive to copper (10—13.3-fold) than non-lactose-metabolizing derivatives.

Addition of Cu^{++} at 39 x 10^{-2} mM had no significant effect on growth and acid production of *S. lactis, S. cremoris, S. lactis* subsp. *diacetylactis and Leuconostoc* spp. But 2 mM caused marked inhibition of acid formation (Gudkov *et al.*, 1979).

The genetic information for this resistance is probably borne on the lactose plasmid. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions is common in bacteria (Foster, 1983). The copper sensitivity marker may be interesting to select copper-resistant variants. This fact may be of commercial consequence, since Maurer *et al.* (1975) pointed out that use of copper vats improves the flavour of Swiss cheese. Therefore, copper-resistant starters must be used, because these authors showed that commercial strains of *L. bulgaricus* and *S. thermophilus* exhibited loss of viability and lowered acid production in the presence of copper. This work confirmed the experiments of Mueller *et al.* (1952), which indicated that 0.3 mM copper exerted an unfavourable influence on the course of Swiss cheese ripening by its pronounced effect on *S. thermophilus,*

L. casei, L. bulgaricus and *Propionibacterium shermanii*. Cu concentrations < 16 x 10^{-3} mM had favourable effects on starter growth in the manufacture of low temperature scalded cheese varieties, while concentrations > 3 x 10^{-3} mM were inhibitory (Kolodkin, 1978).

Kanedo *et al.* (1987) observed increased diacetyl formation in *S. lactis* subsp. *diacetylactis* on adding Cu^{++}, Fe^{++}, Fe^{+++}, Co^{++} and Mo^{6+} to the medium. This strain produced 5.4 times more diacetyl in the presence of 1 mM Cu^{++} while suppressing citrate utilization.

Cobalt

Shorb (1947, 1948) reported growth stimulation of *L. lactis* by crystalline vitamin B_{12} and liver extracts. Cobalt is the center of the vitamin B_{12} complex, which is essential for growth of a number of bacteria including *L. casei* (Hoff-Jorgensen, 1949) and *L. bifidus* (Skeggs *et al.*, 1949). Only some strains of *L. bifidus* are affected by Co (Tomarelli *et al.*, 1949; Kitay *et al.*, 1949). Cobalt chloride (1.7 x 10^{-4}—1.7 mM) had no effect on *L. casei, L. arabinosus* and *L. delbrueckii* (Svec, 1948). For certain lactobacilli, vitamin B_{12} can be replaced by one or more purine or pyrimidine nucleosides occurring in desoxyribose nucleic acid (Hoff-Jorgensen, 1949). Hutner *et al.* (1950) supposed that B_{12} is related to the enzymatic synthesis of these nucleosides and not *vice versa*. Co-ions were effective for *L. arabinose* isomerase production in *L. brevis* and *L. gayonii* (Yamanaka and Higashihara, 1962).

Molybdenum

Molybdenum produced no effect on acid production or growth of 3 lactobacilli tested by Svec (1948).

Zinc

Zinc transport, accumulation, and function in microbial cells have been comprehensively reviewed by Failla (1977). Eukaryotes require 10 times more zinc for growth than prokaryotes (Silver, 1978). Zinc was added by Svec (1948) in the Landy and Dicken medium for vitamin assay to study its effect on acid production by 3 lactobacilli (*L. casei, L. arabinosus* and *L. delbrueckii*). Slight stimulation resulted from the addition of 1.6 x 10^{-2} mM to the medium (which already contains 1.6 x 10^{-2} mM), while an addition of 0.3 mM produced a precipitate. McLeod and Snell (1950) observed that Zn^{++} was toxic for *L. arabinosus*. This toxicity could be overcome by increasing the Mn^{++} content of the medium. They suggested that Zn^{++} ions interfered with the formation of one or more metabolically essential metalloproteins. Zn^{++} toxicity for *Leuconostoc mesenteroides* was not reversed by addition of Mg^{++}, Mn^{++}, Ca^{++} and Sr^{++} (McLeod and Snell, 1950). Zn^{++} is toxic at concentrations of 0.3 mM for *L. casei*. The toxicity could be prevented by EDTA in *L. casei* and *L. bulgaricus* (Pamir, 1964).

Thorium, uranium, thallium and zirconium

Tiwari and Pandey (1980) have studied the influence of thorium (Th), uranium (U), zirconium (Zr), and thallium (Tl) on lactic acid production by *L. bulgaricus*. Th and Zr at 2 x 10^{-2} mM and 4 x 10^{-2} mM, respectively, had a slightly stimulatory effect on acid production. Under the same conditions U (10^{-2} mM) and Tl (10^{-2} mM) reduced this production, like Th at 4.0 x 10^{-2} mM.

Conclusions

It is clear that a number of studies are contradictory, which is not surprising in view of the difficulties encountered in the areas in question.

Table III. Cation radioisotopes utilized in biochemical experimentation (adapted from Silver and Bhattacharyya, 1974).

Isotope	*Half-life*	*Principal radiation emitted (meV)*		
		β⁻	*γ*	*X-ray*
^{22}Na	2.6 yr		1.3 0.5	
^{24}Na	15 h	1.391	1.368	
^{42}K	12.4 h	3.53 2.03	1.52	
^{86}Rb	18.8 days	1.77	1.08	
^{131}Cs	9.7 days	0.023—0.034		0.029—0.034
^{137}Cs	30 yr	0.5 1.2	0.66	
^{28}Mg	21.3 h	0.45	1.78 1.35 0.95	
^{45}Ca	165 days	0.25		
^{47}Ca	4.5 days	0.690	0.489	
^{51}Cr	27.7 days		0.320	
^{85}Sr	64.7 days		0.514	0.013—0.015
^{90}Sr	28 yr	0.54		
^{54}Mn	314 days		0.84	
^{55}Fe	2.7 yr			0.0059
^{59}Fe	44.5 days	0.131 0.273 0.466	0.192 1.099 1.291	
^{57}Co	271 days		0.14 0.122 0.136	
^{60}Co	5.3 yr	0.31	1.2 1.3	
^{63}Ni	92 yr	0.067		
^{64}Cu	12.8 h	0.57	0.51	
^{65}Zn	245 days		1.11	
^{133}Ba	10.7 yr	0.024 0.045 0.075—0.08	0.081 0.276 0.303 0.356 0.38	0.031 0.036
^{197}Hg	64.1 h	0.063—0.06 0.074	0.077	0.067—0.081
^{203}Hg	46.8 h	0.194 0.212	0.279	0.082—0.085

Moreover, very few studies have considered the distribution of trace elements in the medium between the protein and non-protein fractions. This is an important parameter. Brulé and Fauquant (1982) have shown that ≈ 95% of the Mn and Zn is bound to the casein in milk and 50—75% of the Cu and Fe and 18—33% of iron were present in whey proteins fraction. The availability of these ions to lactic acid bacteria is questionable.

Traditional chemical methods (gravimetry, titrimetry, fluorimetry, absorptiometry) are employed less and less in the study of the metal requirements of bacteria. Advances in physics, chemistry and technology have introduced more efficient methods for studying the role of metals in the metabolism of microorganisms. Radioisotopes are currently available for most cations of biological interest (Table III). Silver and Bhattacharyya (1974) have described procedures for studying cation transport systems with radioisotopes.

Atomic absorption spectroscopy, flame emission spectroscopy, emission spectroscopy, polarography, X-ray spectroscopy, mass spectroscopy and analysis by ion activation appear to be promising methods (Laurent, 1981).

No doubt the exact determination of the complex relationship between lactic acid bacteria and metals will lead to a better understanding of their physiology, and to a better formulation of media for their growth and activity in the dairy product area. New techniques and increased knowledge regarding other bacteria will be two strong tools for researchers working on metal requirements of lactic acid bacteria.

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