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FROM CASEIN TO CHEESE: THE ROLE OF LACTOCOCCUS LACTIS

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1. SUMMARY

Lactococci have limited biosynthetic abilities. In addition to the energy sources many solutes have to be taken up. Essential and non-essential amino acids are accumulated via specific transport systems. The concentration of these solutes in milk is insufficient to support optimal growth. Cells secrete proteinases and peptidases to hydrolyse casein. During milk fermentation, the growth requirements of lactococci are satisfied by the peptides and amino acids released upon casein degradation. When present in limiting amounts. amino acids are acquired by uptake and subsequent hydrolysis of peptides. The effects of an imbalance of the supply of amino acids on the growth and metsbolic activities of these bacteria are discussed.

2. INTRODUCTION

The Dutch cheese industry uses starter cultures composed of lactic acid bacteria from different genera. Lactococcus lactis species are quantitatively the most important bacteria in these cultures and their metabolic activities are to a large extent responsible for the organoleptic properties of the cheese. L. lactis is the most extensively studied lactic acid bacterium. Many of its physiological and genetic properties are well analyzed and these studies have been reviewed recently (Laan et al., 1989; Konings et al., 1989; Kok, 1990; Driessen, 1989; Driessen and Konings, 1990). Lactococci are gram-positive bacteria which are very fastidious and require exogenous sources of vitamins, nucleotides and amino acids for growth. The limited biosynthetic capacity means that growth of these bacteria strongly depends on the composition of the medium. This lecture will address the following questions: "How does L. lactis obtain the amino acids during growth in milk and how is this amino acid supply affected by the medium conditions?" Since uptake of essential nutrients such as amino acids from the medium into the

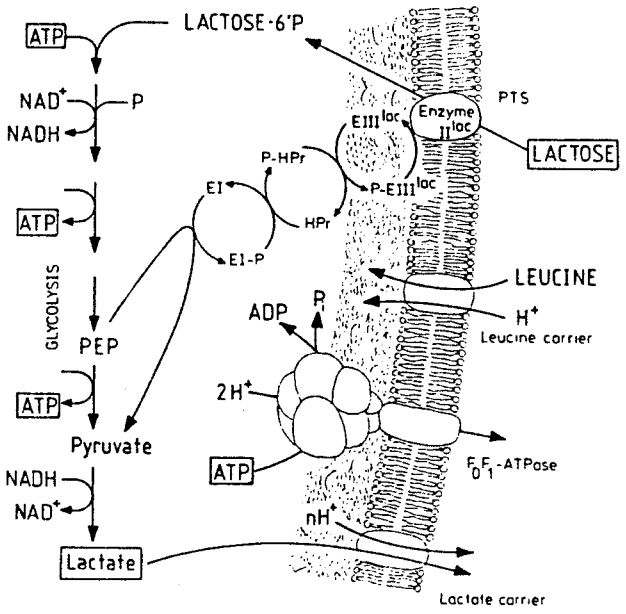

FIGURE 1:
Scheme of lactose fermentation to lactate and the production of metabolic energy in Lactococcus lactis.

cells requires metabolic energy, the mechanisms by which metabolic energy can be obtained will first be described.

3. LACTOSE FERMENTATION

The most important energy source during growth in milk is lactose. Lactose is taken up by a phosphoenolpyruvate-dependent group translocation system (PTS) (Fig 1).
While translocated lactose is phosphorylated to lactose-6-phosphate and within the cytoplasm split into galactose-6-phosphate and glucose. Both metabolites are further degraded: galactose -6-phosphate via the tagatose-6-phosphate pathway and glucose via the glycolytic pathway. In these processes lactate is formed as the main metabolic endproduct. Metabolic energy is obtained in the form of ATP generated by substrate level phosphorylation (Fig 1). ATP will be used as a source of energy for biosynthetic and other metabolic energy requiring processes. It can also supply the energy for the extrusion of protons across the cytoplasmic membrane via the membrane bound ATPase. In this way an electrochemical gradient of protons across the cytoplasmic membrane can be generated. This gradient is composed of two components: an electrical potential across the membrane, inside negative versus outside, formed by the translocation of positive charges (with the protons), and a pH-gradient, inside alkaline versus outside, formed by the removal of protons from the cytoplasm and the accumulation of the protons in the external medium. Both gradients exert a force on the protons from the outside to the inside. This force is termed the proton-motive force and can be used to drive energy-requiring processes at the cytoplasmic membrane, such as the uptake of nutrients. The endproduct of lactose fermentation,

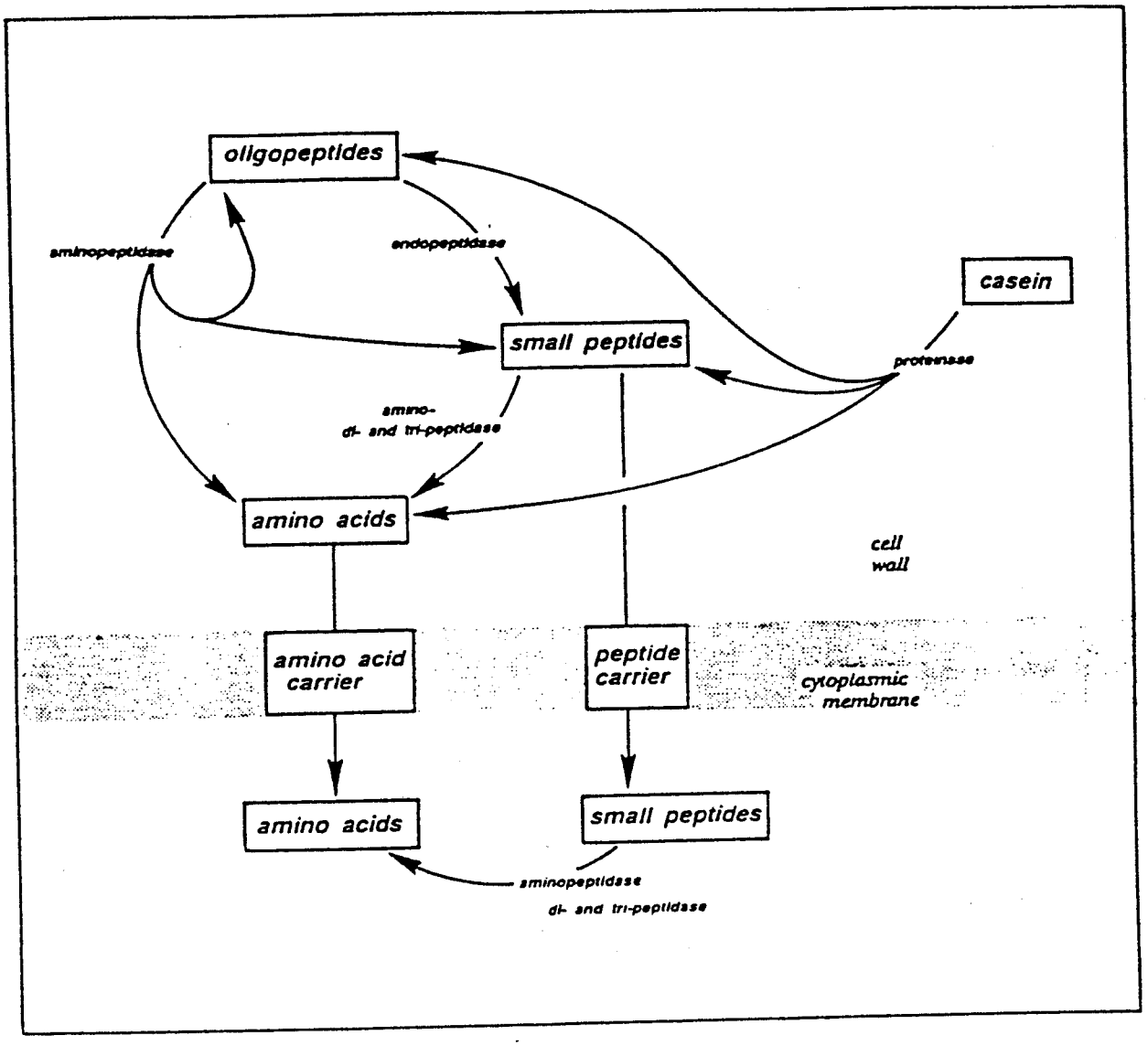

FIGURE 2:
Scheme of the breakdown and utilization of casein by Lactococcus lactis (from Laan et al., 1989).

lactate, has to be excreted into the external medium. This excretion is mediated by a membrane-bound protein and is coupled to the movement of protons from the cytoplasm to the external medium (Fig 1). This coupled movement of lactate and protons also can result in the generation of a proton motive force (Konings, 1985). In this way the energy present in the lactate gradient high concentration inside versus low concentration outside) can be converted into useful metabolic energy (see for review Konings et al., 1989).

Lactose fermentation thus leads to the generation of two important metabolic energy sources: ATP and an electrochemical gradient of protons (proton motive force). Both these energy sources are needed for the amino acid supply of L. lactis.

4. PROTEOLYSIS OF CASEIN

The concentrations of the amino acids in milk is not sufficient for optimal growth of L. lactis. High growth rates can only be obtained if the organisms degrade casein to amino acids and peptides which can be taken up by the organism. For this degradation L. lactis produces several proteolytic enzymes which are exposed to or secreted in the external medium (Fig 2).
The initial step of casein hydrolysis is performed by a proteinase which is attached to the membrane or released in the medium. Proteolysis of casein by the proteinase results in the release of peptides which are too large to be taken up by the organism. Further degradation of these oligopeptides occurs by various peptidases with different specificities and activities (Laan et al., 1989). The combined action of these peptidases leads to the formation of amino acids and peptides which are sufficiently small for translocation across the membrane. Peptides which are translocated across the membrane have to be hydrolysed internally to serve as source of amino acids for the organism. For this hydrolysis several other peptidases are present in the cytoplasm. Currently, the peptidases of L. lactis are studied in great detail. These studies will provide information about their localization and their role in the hydrolysis of casein (see also Fox this issue). The combined action of proteinases, external peptidases and internal peptidases determines which amino acids from casein become available for growth of L. lactis. To a large extent the growth properties of this organism in the initial stage of cheese production depends on these activities. It is important to realize that the amino acid composition of the peptides and amino acids which become available for L. lactis is not optimal for growth. Quantitatively, the availability of amino acids does not match the amino acid composition of the proteins of L. lactis. This means that some amino acids have to be metabolized to form other amino acids or serve other metabolic requirements. Also some amino acids will possibly be converted to flavor compounds. For instance biosynthesis of biogenic amines could be related to an excess intracellular supply of certain amino acids. In this way the unbalanced supply of amino acids via the proteolysis of casein can contribute significantly to flavor development during cheese formation. These processes could have a similar impact on flavor production of cheese as the production of flavor peptides during casein hydrolysis.

4.1 PEPTIDE AND AMINO ACID UPTAKE SYSTEMS

Translocation of hydrophilic compounds such as most of the amino acids and peptides that cross the cytoplasmic membrane of L. lactis, requires the involvement of specific transport proteins in the membrane. The mechanisms of transport of different solutes across the cytoplasmic membranes of L. lactis has been studied extensively and recently reviewed (Konings et al., 1989; Driessen, 1989). The different mechanisms operating in L. lactis are schematically shown in Fig 3.

The transport systems which utilize ATP or some other form of phosphate-bond energy for the translocation of a solute are termed primary transport systems. The membrane bound ATPase which translocates protons at the expense of ATP and generates a proton motive force belongs to this category. Also the ATP- or phosphate-bond driven transport systems for amino acids (such as

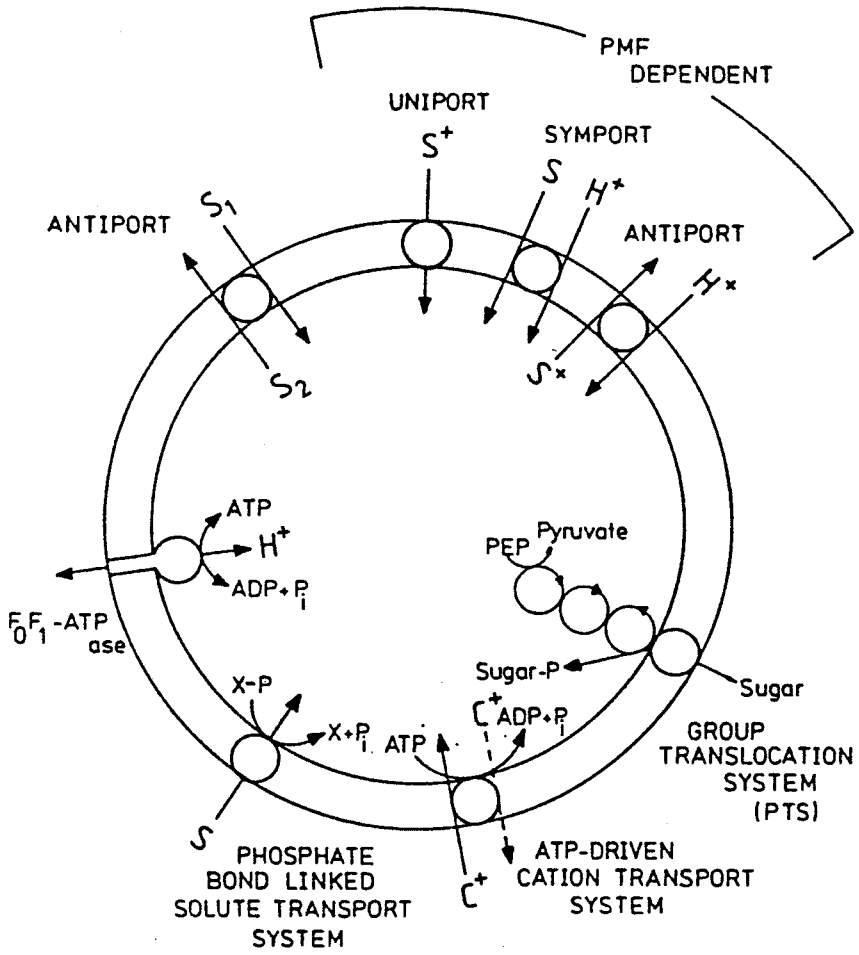

FIGURE 3:
Schematic presentation of carrier-mediated solute transport in Lactococcus lactis. The solid and dashed arrows of ATP-driven cations (C) transport systems indicate mechanisms for uptake or extrusion of cations, respectively. Solutes and protons are indicated by S and H+, respectively (from Konings et al., 1989).

glutamate, see below) and inorganic cations are primary transport systems. A completely different class of transport systems are the group translocation systems (PTS) which utilize phosphoenolpyruvate as energy source. A PTS involved in the translocation of lactose has already been mentioned above. These transport systems require a membrane bound protein and several cytoplasmic proteins. PTS are only involved in the uptake of sugars in L. lactis. A third group of transport systems is driven by the proton motive force. Specific membrane proteins mediate the translocation of solutes across the membrane. These systems belong to the category of the secondary transport systems. Three different mechanisms are found: "the uniport systems" which translocate only one solute; "the symport systems" which couple the movement of a solute to the movement of proton(s) in the same direction and the "anti-port systems" which couple the outward movement of a solute to the inward movement of proton(s) or

another solute. Lactate efflux described above is mediated by a proton symport system. These transport processes are driven by the proton motive force or one of its components. The electrical potential across the membrane pulls positive charges from the outside to the inside (or negative charge in the opposite direction) and functions as a driving force for solute transport whenever charge translocation occurs. The pH-gradient pulls protons from the outside to the inside and functions as a driving force for solute transport whenever proton translocation occurs. When both protons and charge are translocated in a transport process both the electrical potential and the pH-gradient (and thus the total proton motive force) function as driving forces for transport. The secondary transport systems also comprise the antiport systems (Fig 3) in which the movement of two solutes in opposite directions is coupled by a specific transport protein. When no charge and protons is translocated in this antiport only the solute gradients and not the electrical potential nor the pH-gradient function as driving forces for transport. In the following sections exsmples of amino acid or peptide uptake systems will be described. The relation of these transport activities to the growth of L. lactis will be indicated.

4.2 PROTON MOTIVE FORCE DRIVEN AMINO ACID TRANSPORT

The amino acids, L-leucine, L-isoleucine and L-valine; L-alanine and glycine; L-serine and L-threonine; and L-lysine, are transported by separate secondary transport systems (Table 1).

In the presence of lactose as a source for the generation of a proton motive force these amino acids are take up at high rates. In a mixture of amino acids such as that found during growth in milk, only the amino acids which are transported by the same transport protein such as leucine, isoleucine and valine) will compete for uptake. The specific growth rate (μ) of L. lactis subsp. cremoris strains in milk is 10 - 40 % lower than in other nutritious media. The supply of some amino acids during growth in milk can indeed be limiting. This is evident from the observation by Hugenholtz et al. (1987) in that the supply of the amino acid leucine was found to espeically limit the growth rate.

4.3 ATP-DRIVEN AMINO ACID TRANSPORT SYSTEMS

The amino acids, L-asparagine; and L-glutamate and L-glutamine, are transported by ATP-(or phosphate-bond-)driven transport systems (Table 2). ATP-driven transport systems catalyze unidirectional vectorial reactions which lead to the accumulation of the solute internally. The activity of these systems is strongly regulated by the internal pH. The transport system for L-glutamate has been studied in great detail Poolman and Konings, 1988). This system recognizes only the uncharged form of glutamate, i.e. the undissociated glutamic acid. Glutamate is an essential amino acid for growth of L. lactis. The concentration of undissociated glutamic acid decreases with increasing pH of the medium, and growth of L. lactis on a chemically defined medium with supplemented glutamate decreases above pH 6.5 and stops completely above pH 7.5 (Fig 4).

The other substrate for this transport system is glutamine. This solute remains uncharged above pH 6.5 and, in contrast to glutamate transport, uptake of glutamine continues at high pH values. As a result glutamine can function as a source of glutamate at high pH values and sustain growth under these conditions. The limitation of the supply of glutamate at high pH is a reason why L. lactis fails to grow in milk at high pH-values.

TABLE: 1

Specificity and Micheelis Constants K) for Amino Acid Transport in Membrane Vesicles and Intact Cells of Amino L. lactis

H^+ amino acid symport	K_t (μM)	Phosphate-bond driven	K_t (μM)	Antiport K_t (μM)
L-leucine	6.5	L-glutamic acid	1.8	L-arginine 55
L-isoleucine	8	L-glutamine	2.5	L-ornithine 40
L-valine	12	L-asparagine	3.0	
L-alanine	52	L-aspartate	250	
glycine	330			
L-serine	42			
L-threonine	285			
L-lysine	16			

(From Driessen, 1989)

4.4 AMINO ACID ANTIPORT SYSTEMS

L. lactis subsp. lactis has the capacity to degrade arginine via the arginine-deiminase pathway (Fig 5). In this pathway arginine is converted to ornithine and carbamoyl-phosphate. Carbamoyl-phosphate is hydrolysed to NH_3 and CO_2, by a reaction that is coupled to the formation of ATP. Arginine metabolism supplies the organism with an additional source of ATP. The production and excretion of ammonia results in an increase of the medium pH that counteracts the acidification caused by lactate excretion. As a result growth of L. lactis on arginine- and lactose-containing media can continue for a longer period and can lead to a more extensive fermentation of lactose. Since the metabolism of arginine leads to the formation of only one ATP per arginine molecule converted, the process will be energetically favorable if no metabolic energy is required for arginine uptake or ornithine excretion. As is shown in Fig 5, arginine transport occurs in antiport with ornithine. This process is driven by the arginine gradient which is directed from the outside to the inside since the arginine concentration internally is low due to its metabolism, and by the ornithine gradient which is high inside due to the conversion of arginine and low outside. Both the arginine and the ornithine gradient work together to drive the antiport process, thus metabolic energy is not required. Metabolism of arginine provides L. lactis with an energetically attractive process for the generation of ATP.

4.5 PEPTIDE TRANSPORT

The mechanisms involved in the uptake of peptides have been studied recently (Smid et al., 1989a;b). The most important peptide uptake system in L. lactis was found to be a secondary di-/tripeptide

TABLE 2

Specific Growth Rates of Lactococcus lactis in CDM[a] Supplemented with an Amino Acid Mixture

[a] Proline or proline-containing peptides were added at the concentrations indicated.

[b] All measurements were performed in duplicate. (Smid and Konings, 1990).

	Maximum specific growth rate (h^{-1}) of strain	
Proline source (mM)	Wg2	ML_3
No proline	ND[b]	0.40
Proline (0.5)	0.01	0.42
Proline (1.0)	0.35	0.81
Leucyl-proline (0.05)	0.68	0.91
Leucyl-proline (1.0)	0.64	0.90
Prolyl-methionine (0.05)	0.59	0.87
Methionyl-proline (0.05)	0.56	0.81
Prolyl-histidyl-valine (0.05)	0.11	0.51
Prolyl-valyl-asparagine (0.05)	0.01	0.39
Prolyl-prolyl-glycine (0.05)	<0.01	0.39
Valyl-prolyl-leucine (0.05)	0.04	0.73
Histidyl-prolyl-valine (0.05)	0.04	0.47
Glycyl-prolyl-alanine (0.05)	0.01	0.44
Isoleucyl-prolyl-isoleucine (0.05)	0.01	0.79

uptake system. This system has a very broad substrate specificity and translocates a wide variety of dipeptides and tripeptides in symport with protons (Fig 6).

In addition a transport system is present that transports tetra- and pentapeptides (Smid, E.J., unpublished results). The activity of this system is much lower than that of the di-tripeptide transport system. The energetic properties of the oligopeptide transport system have not yet been analyzed. As a result of the broad substrate specificity of the di-/tripeptide transport system, many different peptides derived from casein by the proteolytic system will compete for uptake. The addition of other dipeptides to such a growth medium can disturb the balance in the supply of amino acids. These peptides inhibit uptake of nutritionally important peptides and can lead to inhibition of growth. An example of such an inhibitory effect on growth by the addition of peptides is shown in Table 2. The balanced supply of amino acids via amino acid and peptide uptake systems is thus essential for optimal growth of L. lactis

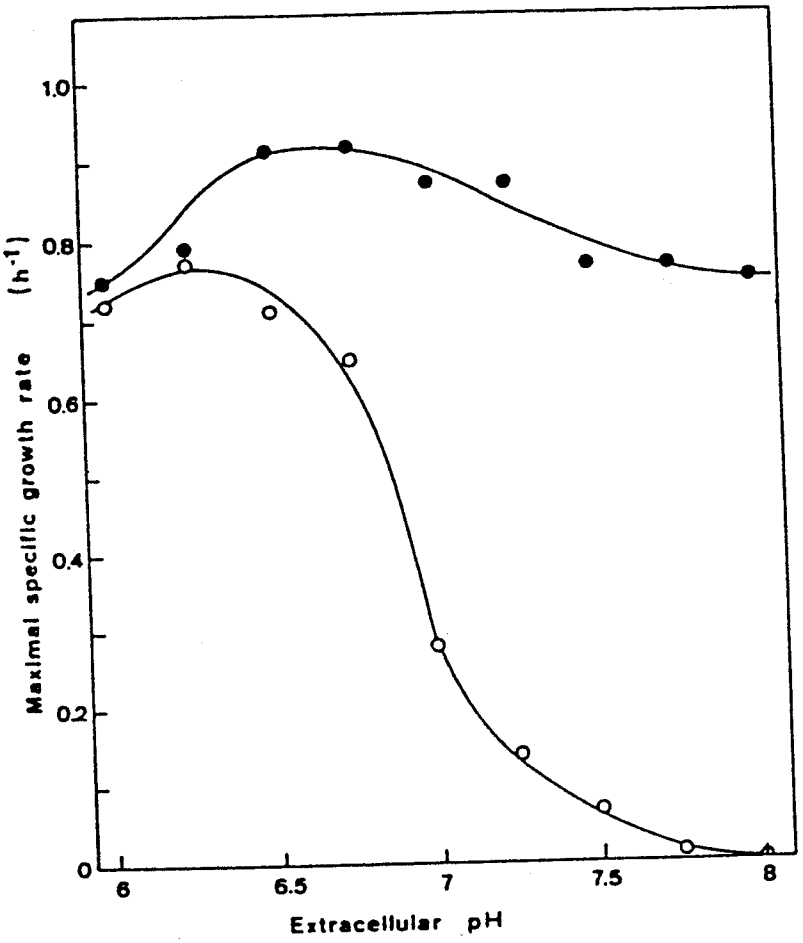

FIGURE 4:
pH dependence of growth of Lactococcus lactis on chemically defined medium at 30°C with 2.6 mM of L-glutamate (o) or L-glutamine (o) as sources of L- glutamate for biosynthesis (from Poolman and Konings, 1988).

on casein-containing media. The importance of the peptide uptake system for achieving such a balanced supply of amino acids is further demonstrated by the growth properties of a di-/tripeptide uptake-deficient mutant of L. lactis. This mutant was selected by growth of mutagenized cells on media containing the toxic dipeptide, ala-ß-chloroalanine. Hydrolysis of this dipeptide after uptake leads to the intracellular release of chloroalanine that cannot be used for biosynthesis. A di-/tripeptide uptake deficient mutant is resistant to this toxic dipeptide. Growth of this mutant on a chemically defined medium with casein as sole source of amino acids was found to be completely impaired while growth on the same medium with amino acids was essentially the same as the wild type strain (Fig 7). These observations demonstrate that L. lactis can obtain certain essential amino acids from casein only via di-/tripeptide transport and not via uptake of the amino acids itself.

The important role of the di-/tripeptide transport system in the balanced supply of amino acids is further demonstrated for the uptake of proline. L. lactis does not appear to possess a secondary

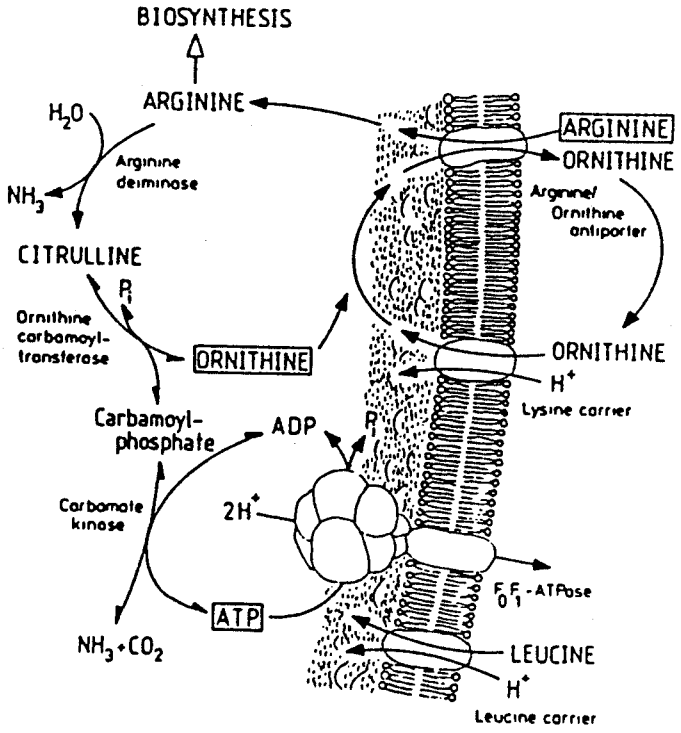

FIGURE 5:
Scheme of the arginine deiminase pathway in Lactococcus lactis (from Driessen and Konings, 1990).

transport system for proline. Uptake of proline is independent of metabolic energy and the rate of uptake increases linearly with the external concentration (Fig 8).

This indicates that transport of proline across the cytoplasmic membrane occurs passively without the involvement of specific membrane proteins. As calculated from the amino acid composition of L. lactis cells, a specific growth rate of 1 requires a rate of proline uptake that can only be achieved when the external proline concentration is equal to 5 mM. Fig 9 shows that the growth rate of L. lactis on a chemically defined medium with all amino acids except proline in excess depends on the proline concentration. This is observed for a proline-auxotrophic strain (L. lactis subsp. cremoris Wg2) as well as for a strain which can synthesize proline (L. lactis subsp. lactis ML_3). Unless the proline requirement can be met by other means, the rate of proline uptake via diffusion severely limits the growth rate of L. lactis at low proline concentrations. Such a possibility is provided by the di-/tripeptide transport system that exhibits a very high affinity for proline-containing dipeptides (Fig 10). Uptake of proline-containing dipeptides can supply the organism with sufficient amounts of proline to allow maximal growth (Table 3). These observations show that proline-containing dipeptides are superior to proline in stimulating growth of L. lactis. It emphases the important role of di-/tripeptide transport systems in the overall pathway of casein utilization.

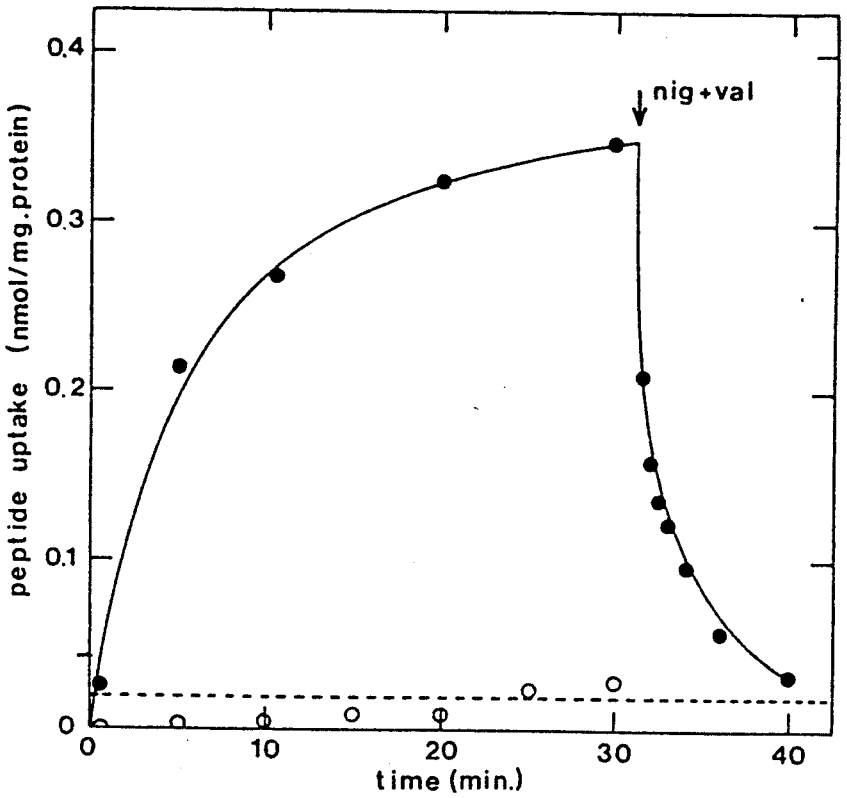

FIGURE 6:

Uptake of ala-glutamate by membrane vesicles of Lactococcus lactis ML_3 in the presence of an electrochemical proton gradient (o) or in its absence (o). At the arrow the ionophores valinomycin (100 nM) and nigericin (10 nM) were added (from Smid et al., 1989a).

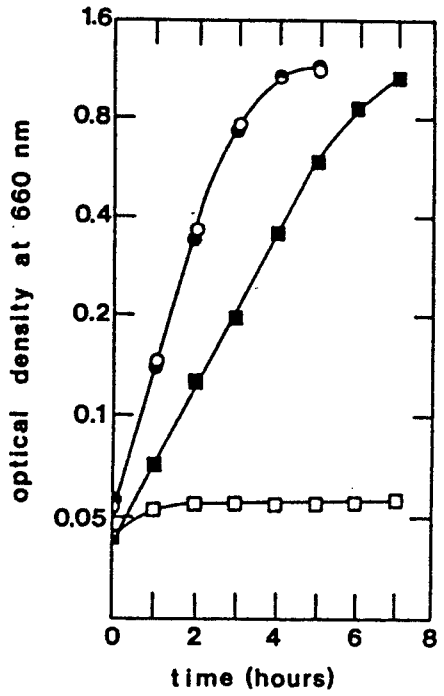

FIGURE 7:

Growth of Lactococcus lactis ML_3 wild type (o and ■) and the di-/tripeptide transport deficient mutant (o and) in chemically defined medium with 0.5% (wt/vol) casein (and ■) or 0.5 % casein plus a complete amino acid mixture (o and o) (from Smid et al., 1989b).

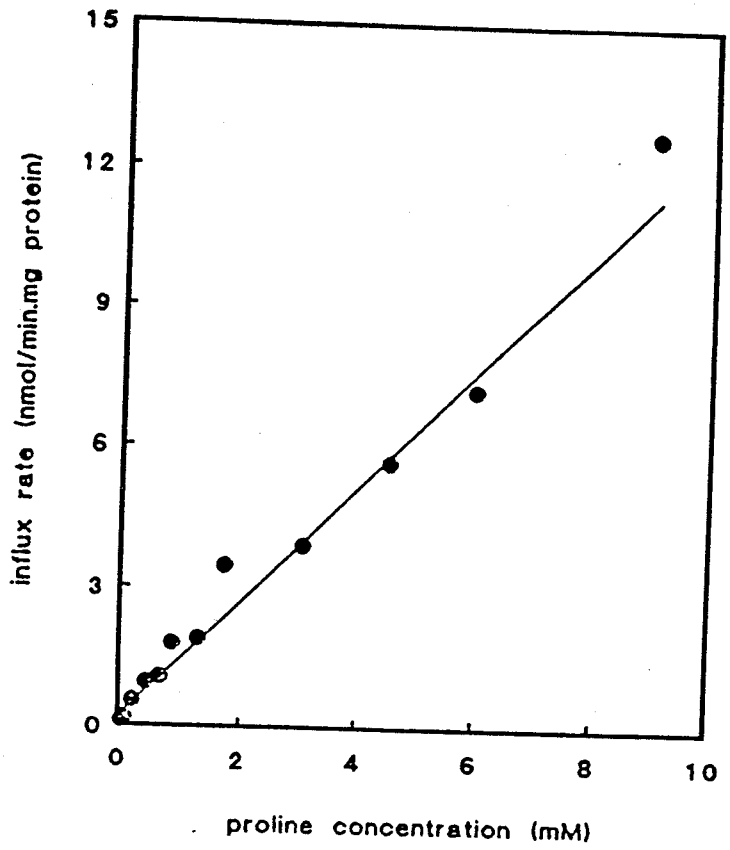

FIGURE 8:

Kinetics of proline influx in intact cells of Lactococcus lactis subsp. lactis ML_3 (pH 6.5) (from Smid en Konings, 1990).

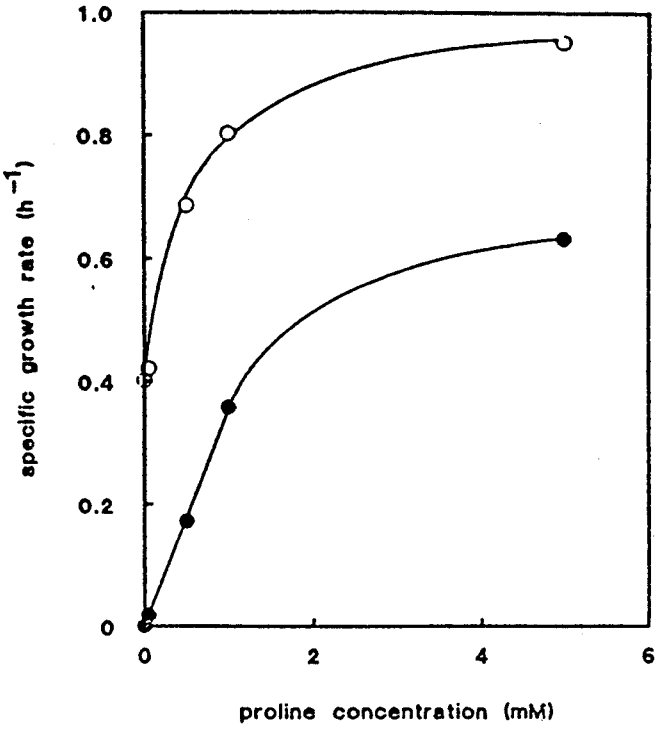

FIGURE 9:

Relation between the proline concentration and specific growth rate of Lactococcus lactis subsp. cremoris Wg2 (o) and L. lactis subsp. lactis ML_3 (o). Cells were grown in a chemically defined medium supplemented with a complete amino acid mixture except for proline (from Smid and Konings, 1990).

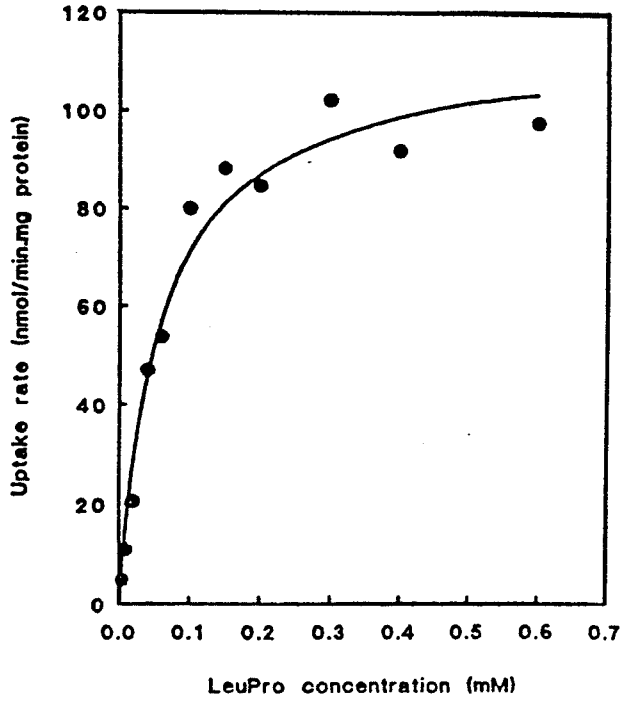

FIGURE 10

Kinetics of leucyl-proline uptake in glycolyzing cells of Lactococcus lactis ML_3,. A K_t of 50 M for leucyl-proline uptake was calculated (from Smid and Konings, 1990).

TABLE 3

Specific Growth Rates of Lactococcus lactis subsp. lactis ML_3 Grown in CDM[a] Supplemented with Various Dipeptides

Substrate added (nM)	Maximum specific growth rate (h^{-1})
None	-0.38
Leucyl-proline (0.05)	0.21
Leucyl-proline (1)	0.17
Prolyl-methionine (0.05)	0.23
Prolyl-methionine (l)	0.14
Leucyl-leucine (0.05)	0.18
Leucyl-leucine (1)	0.18

[a] CDM was supplemented with 0.25 (wt/vol) casein as the organic nitrogen source. Substrates were added at concentrations indicated. All measurements were performed in duplicate.

(From Smid and Konings, 1990)

5. CONCLUSIONS

The process of casein utilization by L. lactis during growh in milk involves the activities of proteinases, external peptidases, amino acid and peptide transport systems and internal peptides. The activities of all these systems determine which amino acids enter the enzymatic machinery in the cytoplasm. For optimal growth this supply of amino acids has to be balanced. Manipulation of the proteinase or peptidase activities can disturb this supply. This may lead to a decreased uptake of amino acids and consequently to decreased growth and metabolic activities of L. lactis.

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