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Metabolism of lactate and sugars by dairy propionibacteria: A review

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Abstract — Dairy propionibacteria are important organisms for the manufacture of Swiss-type cheese, for the biological production of propionate and vitamin B₁₂ and have probiotic properties. In all these applications, their metabolic activities play a critical role. A complete understanding of propionate fermentation and of the metabolic routes used is therefore necessary. Dairy propionibacteria have a complex metabolism and involves several cycles. Lactate or sugars utilisation yields pyruvate which can be reduced to produce propionate via the transcarboxylase cycles, or oxidised to yield acetate and CO₂. During the coupled oxidation-reduction, ATP is produced by an electron transport system and fumarate acts as the final acceptor. Although propionibacteria are mainly anaerobes, the electron transport system can be used in the presence of oxygen and they possess the citrate cycle, but can not grow under normal atmospheric oxygen pressure. The proportions of propionate acetate and CO₂ produced vary depending on the strain used and this can be explained, to some extent, by their relative ability to utilise pyruvate via reactions of the citrate cycle. The physico-chemical environment during growth affects propionic acid fermentation; it is impaired by the presence of oxygen and nitrate, but fermentation at acidic pHs enhances propionate production. Fermentation in presence of more than one substrate is complex and still poorly understood. When both L- and D-lactate isomers are available, L-lactate is used preferentially. Although sugar fermentation is more efficient, in the presence of lactate and sugars, there is evidence that lactate is used preferentially. Propionic acid fermentation is affected by the utilisation of amino acids, especially aspartate; co-metabolism of lactate and aspartate results in a lower propionate production and a decrease of the ratio propionate:acetate. There is evidence that the utilisation of the products of proteolysis is an important event in the ripening of Swiss-type cheese and could account for the low propionate:acetate ratios observed in Swiss-type cheese. © Inra/Elsevier, Paris.

propionic acid bacteria / lactate / sugar / metabolism

Résumé — **Métabolisme du lactate et des sucres par les bactéries propioniques laitières : une revue.** Les bactéries propioniques laitières sont importantes pour la fabrication de fromages de type emmental, pour la production biologique de propionate et de vitamine B₁₂ ainsi que pour leurs propriétés probiotiques. Dans ces domaines, leurs activités métaboliques ont un rôle crucial. Il est donc

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nécessaire de comprendre de façon approfondie la fermentation propionique et les voies métaboliques empruntées. Les bactéries propioniques possèdent un métabolisme complexe impliquant plusieurs cycles. Le pyruvate issu de l'utilisation du lactate et des sucres peut être réduit, ce qui conduit à la production de propionate via les cycles de la transcarboxylase. Le pyruvate peut aussi être oxydé en acétate et en CO_2 . Au cours de ces réactions d'oxydation-réduction, un transport d'électron, dans lequel l'accepteur final est le fumarate, produit de l'ATP. Bien que les bactéries propioniques soient principalement anaérobies, le transport d'électron est possible en présence d'oxygène et elles possèdent le cycle des acides tricarboxyliques. Elles ne peuvent cependant pas se développer sous les pressions partielles en oxygène atmosphériques habituelles. Les proportions de propionate, d'acétate et de CO_2 produites sont variables en fonction de la souche utilisée ; ceci peut être en partie expliqué par la proportion de pyruvate utilisée via les réactions du cycle des acides tricarboxyliques. L'environnement physico-chimique influence la fermentation propionique. Elle est inhibée par la présence d'oxygène et de nitrate alors que la production de propionate est accrue à des pH acides. La fermentation en présence de plusieurs substrats est complexe et mal comprise. Lorsque les deux isomères du lactate sont présents, le L-lactate est utilisé de façon préférentielle. La fermentation propionique est affectée par l'utilisation d'acides aminés, et plus particulièrement en présence d'acide aspartique. L'utilisation de l'aspartate pendant la fermentation du lactate provoque une diminution de la quantité de propionate produite ainsi que du ratio propionate : acétate. Au cours de l'affinage des fromages de type emmental, l'utilisation des produits de la protéolyse est un événement important qui pourrait être corrélé aux faibles ratios propionate : acétate observés. © Inra/Elsevier, Paris.

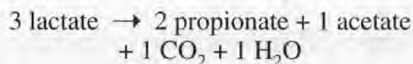
bactérie propionique / lactate / sucre / métabolisme

1. INTRODUCTION

Interest in propionibacteria (PAB) is not recent. They were first isolated from Emmental cheese by Freudenberg and Jensen in 1906 [28] and play a critical role in the ripening and quality of Swiss-type cheese by producing propionate, acetate and CO_2 . The acids are important flavour components and the eyes in the cheese are produced from the CO_2 [30, 31, 33, 40–42, 83, 88, 97]. Their ability to produce large amounts of propionate makes PAB ideal candidates for the biological production of this anti-fungal fatty acid, and several processes have been proposed [9]. Other industrial applications include the production of vitamin B_{12} and their use as possible probiotics [51].

Dairy PAB have the ability to utilise a variety of substrates for energy [30, 31]. Lactate is the main energy source for propionic acid fermentation in cheese, but sugars and other organic compounds such as

glycerol, cellulose and complex by-products of other industries such as gluten, whey, corn steep liquor, distiller's waste, spent potato wash, casein hydrolysates can also be used. The quality of cheese can be related to the extent of propionic acid fermentation both in terms of the actual amounts of propionate, acetate and CO_2 produced as well as the relative proportions of each compound. The molar ratios of propionate, acetate and CO_2 produced vary depending on the substrate used. As early as the 19th century, the stoichiometry of the reaction resulting in the production of propionate from lactate was established by Fitz:



However, in Swiss-type cheese, these theoretical equations are rarely found and the relative concentrations of propionate acetate and CO_2 may be significantly different from the expected molar ratios of 2:1:1.

2. CARBOHYDRATE AND LACTATE DEGRADATION

The early literature concerning PAB has been reviewed by Hettinga and Reinbold [30–32]. All dairy PAB, except *P. freudenreichii* subsp. *freudenreichii* are able to metabolise lactose [30, 31, 37, 89]. This implies that PAB are equipped with a lactose transport system and must be able to hydrolyse lactose as the first step of its utilisation. To my knowledge, there are no reports on transport systems in PAB. β -Galactosidase activity was demonstrated in *P. freudenreichii* subsp. *shermanii* strains by Hartley and Vedamuthu [29]. Depending on the strain tested, toluene-acetone treatment of the cells either increased or decreased the enzymatic activity, while with one strain, almost all the activity was lost in the cell-free extract. There was some evidence that the β -galactosidase system of *P. freudenreichii* subsp. *shermanii* is constitutive rather than inducible as only slight differences in β -gal activity were observed in cultures grown on lactate and different carbohydrates including lactose, glucose and galactose or mixtures of sugar and lactate. The optimal conditions for enzyme activity depended on the strain and treatment used (untreated cells, solvent treated cells or cell-free extract); the optimal temperature and pH ranged from 32 °C to 58 °C and 7.0 to 7.5, respectively.

Hexoses are utilised via the Embden-Meyerhof-Parnas (EMP) pathway. Phosphohexose isomerase, aldolase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and a pyrophosphate phosphofructokinase are present [31, 59, 109, 114]. PAB also contain two glucokinase activities, a polyphosphate dependent and an ATP dependent activity both carried on the same protein [62, 63, 73] and polyphosphate was isolated during growth on glucose and lactate [10]. Another line of evidence for the EMP pathway was furnished by the study of fermentations using radiolabeled glucose [108, 109, 112]. However, the presence of another pathway for the util-

isation of glucose was clearly demonstrated by the distribution of the label in the products depending on the position of the label on the glucose molecule, and the fact that glucose is still utilised in the presence of glycolytic inhibitors. PAB contain the pentose phosphate pathway and all the enzymes of the pathway have been detected [7, 99, 102, 104]. The position of the label in propionate during the fermentation of labeled ribose and gluconate is consistent with the utilisation of pentoses via transketolase-transaldolase conversion to fructose 6-phosphate [108, 109]. However, under anaerobic conditions, most of the glucose is utilised through the EMP pathway alone [108].

During growth on lactate, lactate is initially oxidised to pyruvate via a NAD^+ independent lactate dehydrogenase. The degradation of sugars also leads to the production of pyruvate.

3. PROPIONATE PRODUCTION AND THE TRANSCARBOXYLASE CYCLES

Propionate is the main compound produced by the reduction of pyruvate by PAB. It involves several reactions [31, 98, 108, 109] arranged in cycles (*figure 1*). The first step is the formation of oxaloacetate by transcarboxylation, in which the COOH group of methylmalonyl-CoA reacts with pyruvate to form oxaloacetate and propionyl-CoA. Oxaloacetate is reduced to succinate via malate and fumarate in two NADH requiring reactions. Succinate is then converted to propionate via methylmalonyl-CoA intermediates (succinyl-CoA and propionyl-CoA); the carboxyl group removed from methylmalonyl-CoA is transferred to pyruvate to yield oxaloacetate, thus completing one cycle. Methylmalonyl-CoA is also regenerated from succinyl-CoA during propionate production, thus creating the second of the two transcarboxylase cycles, and can react with a new molecule of pyruvate. Because of the transcarboxylation reaction,

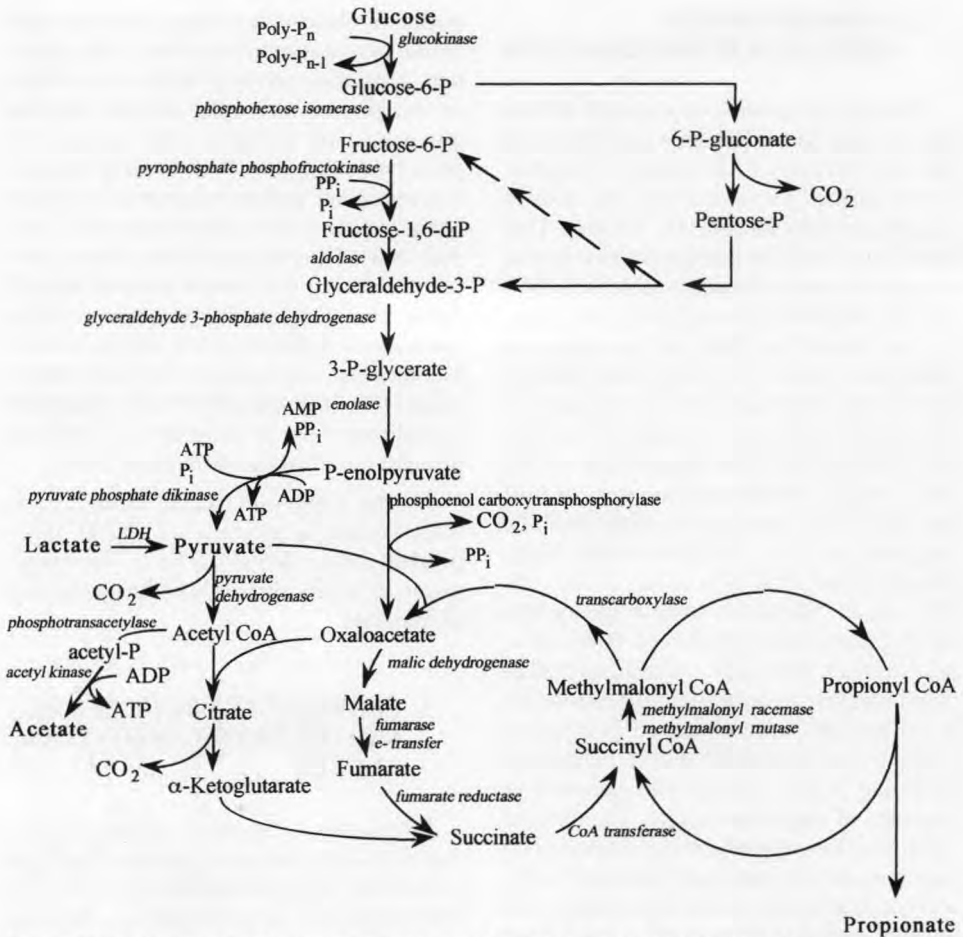


Figure 1. Cycles involved in the propionic acid fermentation (Wood [108]). LDH: lactate dehydrogenase; Poly-P_n: polyphosphate; PP_i: pyrophosphate. For reasons of clarity, only the pyrophosphate dependent conversion of fructose-6-P to fructose-1,6-diP is shown and ATP generation by the electron transfer system is omitted. All the reactions are directed towards propionate production, even though the reactions are reversible.

Figure 1. Cycles de la fermentation propionique (Wood [108]). LDH : lactate déshydrogénase ; Poly-P_n : polyphosphate ; PP_i : pyrophosphate. Pour faciliter la lecture, seule la réaction de conversion du fructose-6-P en fructose-1,6-diP nécessitant des pyrophosphates est montrée. De même, la production d'ATP par le transfert d'électron est omise. Toutes les réactions sont dirigées vers la production de propionate, bien qu'elles soient réversibles.

CO₂ fixation is minimal and is only used to produce catalytic amounts of oxaloacetate when the cycle is broken when for example succinate accumulates as an end product [1]. Under such circumstances, oxaloacetate is generated by condensation of CO₂ with

phosphoenolpyruvate in a reaction catalysed by carboxytransphosphorylase; inorganic phosphate is converted to pyrophosphate and the reaction does not need ATP [60, 90, 91]. PAB can also produce phosphoenolpyruvate from pyruvate; this reac-

tion is catalysed by a dikinase and requires inorganic phosphate and ATP [56, 57, 114]. Because CoA is recycled and free CO₂ fixation is minimal, propionate is produced with a minimum loss of energy.

4. ENZYMES INVOLVED IN PROPIONIC ACID FERMENTATION

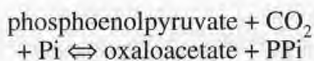
The enzymes involved in propionic acid fermentation (*figure 1*) have been reviewed by Hettinga and Reinbold [31]. Since then, a great deal of attention has been given to the transcarboxylase. This interest is due firstly to the central role of the transcarboxylase reaction in the propionic acid fermentation, and secondly, because it catalyses a carboxylation without the intervention of free CO₂ which is unusual among carboxylases. Transcarboxylase has a complex structure [5, 76, 107, 113, 116]; it comprises three subunits giving it a flexible structure. The central subunit, or 12S subunit, consists of 6 identical polypeptides (Mw 60 000 daltons); it contains 12 CoA ester binding sites. In the complete transcarboxylase complex, this 12S subunit is surrounded by 6 outer 5S subunits. Each 5S subunit comprises 2 identical polypeptides and contains Co²⁺ and Zn²⁺. The central and outer subunits are linked by 12 biotinyl or 1.3S subunits, each containing 2 peptides of 65 and 50 amino acids residues. The complete 26S form of the enzyme dissociates to a 18S form at neutral pH, through the loss of 3 outer subunits. The transcarboxylase reaction is divided in two half-reactions; on the central subunit, the carboxyl group is transferred from methylmalonyl-CoA to the biotinyl subunit by carboxylation of biotin, the second reaction takes place on the outer subunit where the carboxyl group is transferred to pyruvate to form oxaloacetate. Neither ATP nor metal ions are needed for the transcarboxylation reaction. Further characterisation of the structure and functions of the subunits has been facilitated by cloning and expression of the transcarboxylase operon in

E. coli. The monomer of the 12S subunit is a protein of 604 amino acid residues and contains regions with extensive homology with other carboxylases [100]. By deleting amino acids from the carboxyl end of the 12S subunit, Woo et al. [106] demonstrated that this part of the molecule is involved in the stability of the 12S subunit and in the binding with the outer subunits. The 5S subunit monomer contains 519 amino acid residues with considerable homology to yeast pyruvate carboxylase and *Klebsiella pneumoniae* oxaloacetate decarboxylase [76]. The Trp residues of the 5S subunit are involved in binding with the 1.3S subunit and with pyruvate. The structure and function of several regions of the 1.3S subunit have been determined. The amino terminus is involved in the formation of the complex [39]. Biotin is attached by an amide linkage to Lys₈₉ and the penultimate Ile residue is involved in biotinylation [58]. Samols et al. [76] observed a highly conserved tetrapeptide Ala-Met-biotinyl-Met₍₈₇₋₉₀₎ in most biotin carboxylases which is involved in the carboxyl transfer. By using truncated 1.3S subunits, Shenoy et al. [84-86] determined the role of the regions around the biotinyl lysine and that residues 1 to 18 are required for the assembly of the complex and residues 59 to 78 for activity. Finally, there is some evidence for the existence of a fourth protein subunit in the assembly of the complex [87].

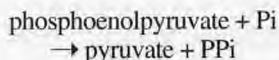
Methylmalonyl-CoA mutase is another interesting PAB enzyme. It requires adenosylcobalamin (coenzyme B₁₂) for activity and catalyses the rearrangement of succinyl-CoA into methylmalonyl-CoA [38]. This enzyme has been the object of several structural studies; it is an $\alpha\beta$ dimer [27, 119] with an apparent Mw of 150 000 daltons. The cloning and sequencing of the structural genes have shown that the two monomers have a close structural homology but are different. The α and β subunits contain 638 residues and 728 residues respectively and have Mw of 69 465 and 80 147 daltons, respectively. The α subunit contains a sequence homology to a portion

of the sequence coding for the methylmalonyl-CoA binding site of transcarboxylase [53, 54]. The enzymatic activity is lost on dissociation of the two subunits. Cobalamin is covalently bound to the enzyme but does not stabilise it [55]. The accepted mode of action of this type of enzyme is the generation of free radicals [105]. The breaking of the Co-C bond of coenzyme B₁₂ induces the change from its Co³⁺ form to its Co²⁺ form and a 5'-deoxyadenosyl free radical is generated. The radical takes a hydrogen atom from succinyl-CoA which consequently is converted into a radical; this new radical undergoes rearrangement of the acyl-CoA group to the position formerly occupied by the hydrogen atom. Finally, a hydrogen atom from the 5'-deoxyadenosyl group is returned to the product-like radical, thus generating methylmalonyl-CoA and a 5'-deoxyadenosyl radical. The rebinding of the Co-C bond regenerates the cofactor in its Co³⁺ form and the enzyme is ready for a new catalytic cycle. The exact mechanism of the reaction is still under investigation [11].

A third peculiarity of PAB is their ability to utilise pyrophosphate instead of ATP for several reactions. Pyrophosphate dependent enzymes have been described in PAB [114]. Phosphoenolpyruvate carboxytransferase catalyses the fixation of CO₂ with phosphoenolpyruvate to yield oxaloacetate [49, 90, 91]:

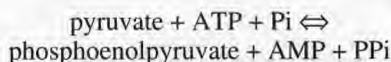


In the absence of CO₂, this enzyme is also involved in the irreversible formation of pyruvate from phosphoenolpyruvate [114]:

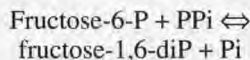


The active enzyme exists in monomeric, dimeric and tetrameric forms and requires Mg²⁺, Mn²⁺ or Co²⁺ for activity. The tetrameric enzyme has been obtained in crystal form, has a molecular weight of 430 kD, a sedimentation coefficient of 15.2S, and is the most active form of the enzyme.

The second pyrophosphate dependent enzyme is pyruvate phosphate dikinase which catalyses the reversible phosphorylation of pyruvate and inorganic phosphate in the presence of ATP [56, 57]:



A third pyrophosphate enzyme is pyrophosphate phosphofructokinase [59] which is involved in the transfer of phosphate from pyrophosphate to fructose-6-P:



ATP-phosphofructokinase activity which is common in other organisms, is low in PAB compared to the pyrophosphate dependent enzyme.

5. ACETATE PRODUCTION AND THE CITRATE CYCLE

The reduction of fumarate to succinate, during propionate production, is coupled to the oxidation of pyruvate to acetate [12, 92]. This oxidation, with acetyl-CoA as an intermediate, results in the production of equimolar concentrations of ATP, acetate and CO₂. Wood [108] observed large variations in the amounts of acetate and CO₂ produced during the fermentation of glucose, and studies involving labelled glucose showed that these differences only appeared at the conversion of acetyl-CoA. It has been proposed that some acetyl-CoA may be utilised through the tricarboxylic acid (TCA) cycle [108]. In this scheme, acetyl-CoA is converted to citrate, which in turn yields succinate via α-ketoglutarate, with the direct consequence that less acetate is produced. Although PAB are essentially anaerobes, Bonartseva et al. [7] detected all the enzymes of the TCA cycle in *P. freudenreichii* subsp. *shermanii* and *P. jensenii*, grown either aerobically or anaerobically.

Growth and metabolism of glucose and lactate by PAB under aerobic conditions has

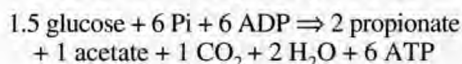
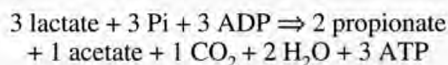
been reported [13, 23, 66, 67, 78]. Oxygen uptake during growth on glycerol, glucose and lactate and oxidation of lactate was observed [93]. However, growth is slower in the presence of oxygen [23]; the formation of propionate, acetate and succinate is inhibited and pyruvate accumulates [81, 101]. Because pyruvate accumulates, energy can not be produced from substrate level phosphorylation, suggesting that it must be produced by oxidative phosphorylation; indeed De Vries et al. [23] observed that, in the presence of O₂ and at limiting lactate concentrations, part of the lactate was completely oxidised to CO₂ via the TCA cycle. The presence of the TCA cycle in PAB could explain differences in the fermentation balances (amounts and relative proportions of propionate, acetate and CO₂ produced) observed between strains as will be illustrated later.

6. GENERATION OF ATP VIA ELECTRON TRANSPORT-COUPLED PHOSPHORYLATION

An electron transport-coupled phosphorylation takes place, in the presence and absence of O₂, during the coupled reduction of fumarate to succinate and oxidation of pyruvate to acetate and CO₂. Components of the respiratory chain have been identified in *P. freudenreichii* subsp. *shermanii* and *P. acidipropionici* and consist of dehydrogenases [24, 79, 93, 94] and menaquinones [80, 95, 96], which act in collecting the reducing equivalents from the substrate and distributing them to the cytochromes [2, 13, 23, 80]. The exact mechanism of electron transport is still not clear and two models, differing in the location and role of cytochrome b in the chain, have been proposed [2, 80]. Schwartz and Sporckenbach [80] suggested that cytochromes b are not involved in the anaerobic transport system; they suggested that cytochromes a, b, d and o were all located in the oxygen linked branch of the chain and

are the remains of a former aerobic metabolism. The second model involves two types of cytochrome b. One is a low potential cytochrome (E'o = -20 mV) that couples NADH oxidation to reduction of fumarate during anaerobic metabolism; the second type has higher potential (E'o = +120 mV and E'o = +90 mV), and along with cytochrome d (E'o = +140 mV), takes part in an aerobic branch of the electron transport. Traces of cytochrome a, c and o were also observed in this study. The variable ability of PAB strains to grow under aerobic conditions could be related to the ratio between the high and low potential cytochromes.

When lactate is used as a substrate, generation of ATP only occurs in the production of acetate and reduction of fumarate to succinate but during sugar metabolism, additional substrate-level phosphorylations occur in the formation of pyruvate. The overall fermentation balances are:



7. INFLUENCE OF THE PHYSICO-CHEMICAL ENVIRONMENT

pH greatly influences the formation of the end products from lactate and carbohydrates. Although optimal growth occurs at pHs between 6 and 7, the production of propionate from lactose, glucose and lactate can be increased by growth at acidic pH [34, 46, 69] whereas acetic acid production is not significantly affected by the pH. The higher production of propionate at acidic pHs can be explained by the formation of non-growth associated propionate, but there is no direct evidence of such a phenomenon in PAB.

Propionic acid can inhibit the growth of propionibacteria under acidic conditions [26], e.g. 100 mmol·L⁻¹ propionate prevented the growth of *P. freudenreichii* subsp.

shermanii during glucose metabolism, at pH 5.2 as observed by Namba et al. [50]. However, this concentration of propionate is very high and would not be reached during normal growth.

Propionate production is affected by the level of oxygen present in the environment. Pritchard et al. [66] reported that *P. freudenreichii* subsp. *shermanii* behaves as a facultative anaerobe under O₂ partial pressures of 0 to 42 mm Hg, has an aerobic metabolism under O₂ partial pressure of 42 to 330 mm Hg but does not grow at 550 mm Hg. Lack of growth at normal O₂ atmospheric pressure is attributed to the inhibition of cytochrome synthesis [23]. Although the viability of the cells is not affected, acid production is inhibited under partially aerobic growth conditions. After restoration of anaerobic conditions, acetate is produced at the same rate as before the aeration phase but propionate production is drastically reduced [35, 36, 81].

Lactate fermentation is also affected by the presence of NO₃⁻; Van Gent-Ruijters et al. [101] reported that, during anaerobic growth on lactate, the production of propionate by *P. pentosaceum* was drastically decreased, production of acetate did not change and pyruvate accumulated. In the presence of NO₃⁻, CO₂ was produced from the TCA cycle and the molar growth yield was increased due to ATP production via the TCA cycle and oxidative phosphorylation coupled to nitrate reduction. NO₂⁻ was produced from NO₃⁻ and its subsequent reduction (after NO₃⁻ was exhausted) gave fermentation patterns similar to those observed in the absence of any inorganic electron acceptor. However, a NO₂⁻ concentration of 9 mmol·L⁻¹ inhibited growth.

8. FERMENTATION OF SUBSTRATE MIXTURES

The utilisation of more than one substrate during propionic acid fermentation is com-

plex and can affect the yields of propionate, acetate and CO₂.

In either complex or defined medium, *P. freudenreichii* subsp. *shermanii* utilises L-lactate, preferentially over D-lactate, in a mixture of both isomers independently of initial pH, initial lactate concentration and initial ratio of the two isomers. When the medium was altered to resemble a ripening Swiss cheese (pH 5.3, 2.1 % NaCl, growth at 22 °C, 145 mmol·L⁻¹ L-lactate and 55 mmol·L⁻¹ D-lactate), the rate of L-lactate utilisation was also higher than the rate of D-lactate utilisation. This preferential utilisation was explained by the fact that L-lactate metabolism results in the production of high intracellular pyruvate concentration which has a stronger inhibitory effect on D-lactate dehydrogenase activity than on L-lactate dehydrogenase activity. With 10 mmol·L⁻¹ lactate as substrate, 20 mmol·L⁻¹ pyruvate did not inhibit L-LDH activity but caused a 75 % decrease in D-LDH activity; 40 mmol·L⁻¹ pyruvate decreased L-LDH and D-LDH activities by 6 and 81 %, respectively [16]. Similar trends are observed with representatives of *P. freudenreichii* subsp. *freudenreichii* and *P. acidipropionici* (Piveteau, unpublished).

As mentioned earlier, higher levels of ATP are produced from similar amounts of sugars than from lactate. As a consequence, higher growth rates and cell yields are obtained during the fermentation of lactose, glucose and galactose than during fermentation of lactate. More propionate and acetate, expressed per mole of pyruvate, are produced during lactate fermentation than during the fermentation of sugars [3, 45–47, 65]. Despite this, PAB utilise lactate faster than sugars when lactate and carbohydrates are both available. In a mixture of lactate and either lactose, glucose or galactose, *P. freudenreichii* subsp. *shermanii* strains use lactate preferentially [45, 65]. Liu and Moon [48] and Crow and Turner [21] found that some carbohydrate was utilised during lactate fermentation by *P. freudenreichii*

subsp. *shermanii*. *P. acidipropionici* also utilised at least some of the lactose, glucose and galactose during lactate metabolism [65]. However, during growth in cheese whey supplemented with fermented milk permeates [52] and in wheys produced by acidification of milk with cultures or addition of lactic acid [64, 65], lactose was not utilised during metabolism of lactate. This preferential utilisation of lactate in media containing both lactate and a sugar is apparently contradictory with the fact that the sugar fermentation is more efficient than the fermentation of lactate. The reason(s) for this is unclear; perhaps the shorter metabolic pathway from lactate than sugars to pyruvate is a factor.

Although lactate is the major substrate metabolised by propionibacteria in Swiss-type cheese, the products of proteolysis are also used. The utilisation of amino acids can affect the yields of propionate, acetate and CO₂. Crow [17] studied the effect of aspartate on lactate fermentation. In either defined or complex medium, more lactate was converted to acetate and CO₂ than to propionate when aspartate was added to the growth medium. Aspartate utilisation resulted in the production of equimolar concentrations of succinate, and NH₃. During co-metabolism of 180 mmol·L⁻¹ lactate and 33 mmol·L⁻¹ aspartate by three *P. freudenreichii* subsp. *shermanii* strains in complex medium, the ratio of propionate to acetate decreased from 2:1 (lactate on its own) to 1.5:1.0. The presence of either casein hydrolysate or aspartate during fermentation of acid whey results in an increase in cell yield, a production of succinate proportional to the amount of amino acids added and a decrease in the molar ratio of propionate:acetate [64, 65]. Moreover, the propionate yield and the ratio of propionate:acetate varies, depending on the source of nitrogen used during glucose metabolism by *P. freudenreichii* subsp. *shermanii* [69] and lactate metabolism by *P. acidipropionici* [46]. Altering the growth

conditions to resemble Swiss cheese increased the rate of aspartate utilisation, suggesting that aspartate metabolism can be important in the ripening cheese [17]. This hypothesis is supported by results of cheese trials using aspartase deficient variants of PAB strains. In cheeses made with a strain of low aspartase activity, the ratio of propionate:acetate was 1.8:1.0 and succinate concentration was the lowest (10.1 mmol·kg⁻¹ cheese) compared to 1.5:1.0 and 20.4 mmol·kg⁻¹ cheese, respectively, in cheeses made with the strain with greater aspartase activity [22]. The amount of succinate detected at the end of warm room ripening depends, to some extent, on the conditions used during cheese making. In commercial cheese and experimental cheese made with *Lb. helveticus*, 18 to 20 mmol·kg⁻¹ of cheese were detected, while 39.4 mmol·kg⁻¹ was found in cheese made with *Lb. bulgaricus* [21]. However, the residual galactose could have been metabolised by the relatively high levels of non starter lactic acid bacteria in the cheeses manufactured with *Lb. bulgaricus* which renders any comparison between the cheeses made with *Lb. helveticus* and with *Lb. bulgaricus* difficult. Sebastiani and Tschager [82] also observed the role of aspartate metabolism in succinate production in Swiss-type cheese. PAB are equipped with a complex intracellular peptidase system [43, 68] and there is strong evidence that PAB can utilise short chain peptides [4, 64, 65]. It is likely that amino acids and peptides, produced by proteolysis during ripening form a pool some of which are subsequently used by PAB; the extent of this utilisation may explain at least some of the variations in the relative concentrations of propionate, acetate, CO₂ and succinate produced.

During co-metabolism of aspartate and lactate, the final ratio of propionate:acetate depends on the initial aspartate:lactate ratio. Crow [17] reported that if 2 moles of aspartate are utilised per mole of lactate, the ratio propionate:acetate is 1:1, while if less than one mole of aspartate is used per mole of

lactate, the ratio will be between 1:1 and 2:1. This worker also observed that in a medium containing aspartate, propionate but not lactate, 3 moles of aspartate are utilised with 1 mole of propionate to yield 3 moles of succinate, 3 moles of NH_3 , 1 mole of acetate and 1 mole of CO_2 . The effect of aspartate metabolism on the amount of propionate produced from lactate was explained by the involvement of the TCA cycle and the intermediates of this cycle (malate and fumarate) were detected during aspartate metabolism [17, 19]. Aspartate is converted to fumarate via aspartase activity [17, 18, 25, 103]. In the scheme as proposed by Crow [17], fumarate has to be reduced to succinate in order to balance the oxidation-reduction status of the cell; the reduction to suc-

cinatate is therefore coupled to the oxidation of pyruvate to acetate (figure 2). Hence, the overall consequence of this increased pool of fumarate is a higher production of acetate and CO_2 and a lower amount of propionate produced.

An alternative mechanism of aspartate metabolism was proposed by Rosner and Schink [75]. In their scheme, propionate acts as a COOH acceptor from oxaloacetate derived from aspartate and is converted to succinate via malate and fumarate. This allows the conversion of oxaloacetate to pyruvate and ultimately to acetate and CO_2 , thus producing reducing equivalents used for the reduction of aspartate to succinate (figure 3).

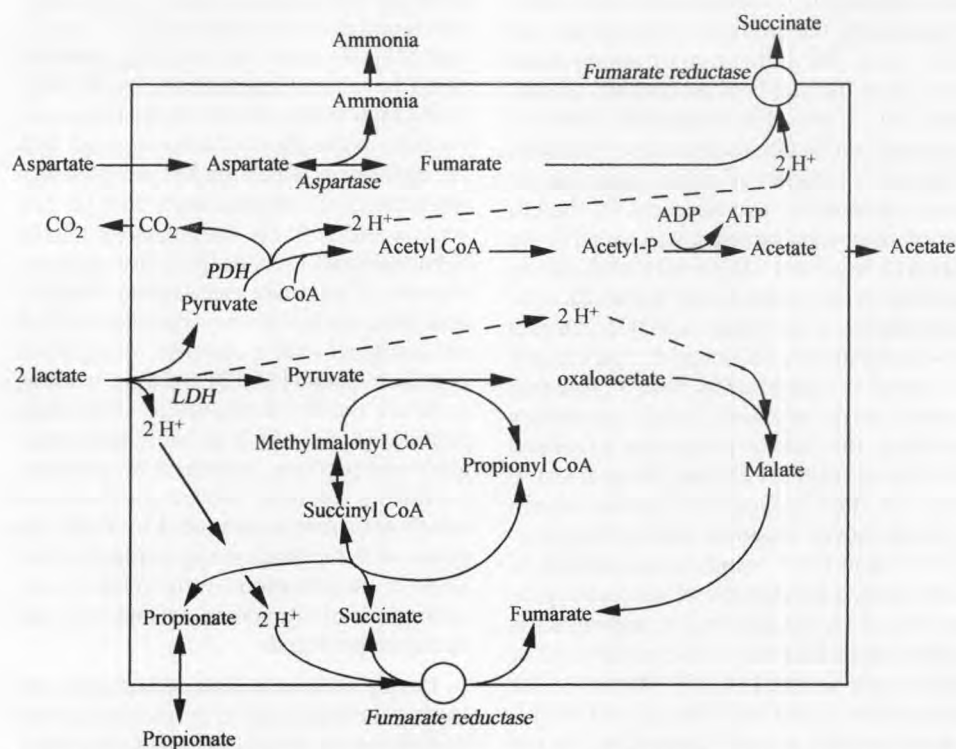


Figure 2. Aspartate and lactate metabolism according to Crow [17]. PDH, pyruvate dehydrogenase. LDH lactate dehydrogenase.

Figure 2. Métabolisme de l'aspartate et du lactate selon Crow [17]. PDH : pyruvate déshydrogénase. LDH : lactate déshydrogénase.

9. VARIABILITY OF THE RATIOS AND YIELDS OF PROPIONATE AND ACETATE

Many studies have dealt with the biological production of propionate from a variety of substrates present in cheap industrial by-products. The amounts of propionate and acetate produced and the ratios propionate:acetate varied according to the conditions and strain used (*table I*); in general, *P. acidipropionici* strains have a better potential for propionate production than other dairy PAB [3, 8, 15, 71, 115]. The strain dependency towards propionate production is confirmed concerning the relative proportions of the fermentation end products obtained from lactate or carbohydrate [3, 20]. Wood [108] reported a range of ratios propionate:acetate and CO_2 :acetate from 2.1 to 14.7 and 1.0 to 6.3, respectively when glucose was the growth substrate; such

great differences between strains can be accounted for by their relative ability to metabolise pyruvate through the TCA cycle. In this event, the acetate yield is mostly affected, and the decrease in acetate produced explains the high ratios found. Depending on the substrate and the medium used, succinic and pyruvic acid are produced at various levels during the fermentation of lactate and carbohydrate. *P. acidipropionici* formed 18 to 38 $\text{mmol}\cdot\text{L}^{-1}$ succinate and 17 $\text{mmol}\cdot\text{L}^{-1}$ to 62 $\text{mmol}\cdot\text{L}^{-1}$ pyruvate during the fermentation of 111 $\text{mmol}\cdot\text{L}^{-1}$ lactose or 222 $\text{mmol}\cdot\text{L}^{-1}$ glucose, but lower amounts (4 $\text{mmol}\cdot\text{L}^{-1}$ succinate, no pyruvate) were detected during the metabolism of 444 $\text{mmol}\cdot\text{L}^{-1}$ lactate [34, 46, 47]. Crow [19, 20] detected 3 to 5 $\text{mmol}\cdot\text{L}^{-1}$ succinate and pyruvate during the fermentation of 155 $\text{mmol}\cdot\text{L}^{-1}$ lactate and 145 $\text{mmol}\cdot\text{L}^{-1}$ lactose. Pyruvate was detected during growth of *P. freudenreichii* subsp. *shermanii* [52] and

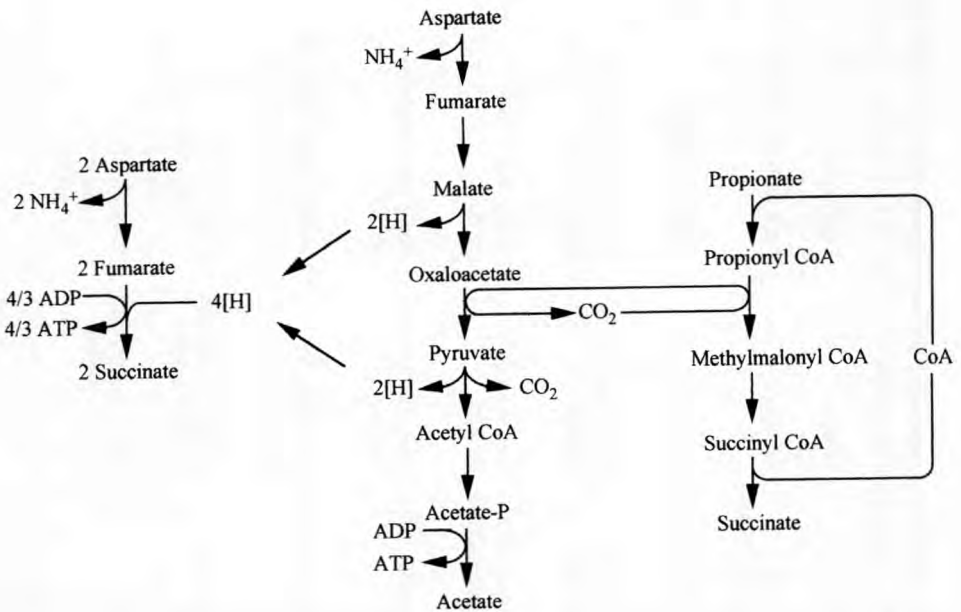


Figure 3. Pathway of aspartate plus propionate fermentation according to Rosner and Schink [75].

Figure 3. Voie de l'utilisation de l'aspartate en présence de propionate selon Rosner et Schink [75].

Table I. Comparison of the relative amounts of propionate and acetate produced by various strains of propionibacteria.**Tableau I.** Comparaison des quantités relatives de propionate et d'acétate produites par diverses souches de bactéries propioniques.

Strain used	Medium	Conditions	Propionate	Acetate	Molar ratio	Recovery (%)	Authors
<i>P. freudenreichii</i> subsp. <i>shermanii</i> PS209	whey lactose	batch fermentation pH 7.0	19 g·L ⁻¹	5 g·L ⁻¹	3.8/1.0	102	Bodie et al. [6]
PS209 + <i>Lb. casei</i> TA101	whey lactose	batch fermentation	30 g·L ⁻¹	10 g·L ⁻¹	3.0/1.0	86	
<i>P. freudenreichii</i> subsp. <i>shermanii</i>	lactose complex medium	steady state continuous pH 4.5 to 7.5			1.6/1.0 to 1.9/1.0	79	Schutz et al. [77]
<i>P. freudenreichii</i> 20271 + <i>Lb. acidophilus</i> 20079	lactose complex medium	steady state continuous pH 5.8	2.4 g·L ⁻¹	1.0 g·L ⁻¹	1.9/1.0		
<i>P. freudenreichii</i> 20271 + <i>Lc. Lactis</i>	lactose complex medium	steady state continuous pH 5.8	1.0 g·L ⁻¹	0.8 g·L ⁻¹	0.9/1.0		
<i>P. freudenreichii</i> subsp. <i>shermanii</i>	<i>Lb. Helveticus</i> fermented whey	agitated immobilised cells	6.6 g·L ⁻¹	4.1 g·L ⁻¹	1.6/1.0	137	Champagne et al. [14]
		static immobilised cells	7.3 g·L ⁻¹	2.6 g·L ⁻¹	2.8/1.0	140	
<i>P. acidipropionici</i> ATCC4875	lactose rich nitrogen complex medium	batch pH 7.12			2.1/1.0	59	Hsu and Yang [34]
		pH 6.15			2.5/1.0	6	
		pH 5.5			3.3/1.0	61	
	lactose low nitrogen complex medium	batch pH 7.03			3.0/1.0	72	
		pH 6.12			2.7/1.0	72	
pH 5.5			3.4/1.0	73			

Table I. (Continued) / Tableau I. (Suite).

<i>P. acidipropionici</i> ATCC4875	complex medium	batch pH 6.6						
	lactate		18 g·L ⁻¹	7 g·L ⁻¹	2.1/1.0	90	Lewis and Yang [46, 47]	
	lactose		15 g·L ⁻¹	5 g·L ⁻¹	2.4/1.0	71		
	glucose		16 g·L ⁻¹	4.5 g·L ⁻¹	2.9/1.0	77		
	lactate	packed bed pH 7.0			1.7/1.0	nc		
	packed bed pH 5.2			1.9/1.0	nc			
<i>P. freudenreichii</i> subsp. <i>shermanii</i> B123	lactate whey based medium	batch	0.1 to 1.5 %	0.1 to 0.6 %	0.8/1.0 to 3.2/1.0	83–85	Marcoux et al. [52]	
		immobilised cells (3rd re-use)	8.3 to 9.4 g·L ⁻¹	3.7 g·L ⁻¹	1.8/1.0 to 2.0/1	122		
<i>P. acidipropionici</i> ATCC4965	lactate whey permeate based medium	sequential fermentation	35g·L ⁻¹	9 g·L ⁻¹	3.1/1.0	80	Colomban et al. [15]	
<i>P. acidipropionici</i> ATCC4875	lactose whey permeate	steady state continuous	24.2 to 27.4 g·L ⁻¹	9.7 to 10 g·L ⁻¹	2.0/1.0 to 2.2/1.0	98–107	Yang et al. [117]	
<i>P. acidipropionici</i> P9	glucose complex medium	batch pH 6.9			2.7/1.0	nc	Ozadali et al. [61]	
	corn steep liquor complex medium	batch pH 6.9			3.0/1.0	nc		

nc: the percentage recovery could not be calculated from the data presented.

nc : le pourcentage de récupération ne pouvait pas être calculé à partir des données obtenues.

P. acidipropionici [117] in whey-based media. Succinate, but not pyruvate was produced during propionate production from whey lactose by immobilised cells of *P. acidipropionici* [118].

The variability of the propionate:acetate ratio by different strains is also found in Swiss-type cheese. A range of ratio from 1.1:1.0 to 2.2:1.0 was reported by Langsrud and Reinbold [42]; Crow and Turner [21] reported ratios of 0.7:1.0 for an experimental Emmental cheese made with *Lb. bulgaricus*, 0.95:1.0 and 1.0:1.0 for two experimental cheeses made with *Lb. helveticus* and 1.5:1.0 for a commercial Emmental cheese. The large variations in the metabolic activity of *Propionibacterium* strains were further confirmed in experimental small scale Swiss-type cheese manufacture where 19 PAB strains could be arranged into four clusters depending on their ability to produce propionate and acetate [72].

10. INFLUENCE OF MINOR FERMENTATION PATHWAYS

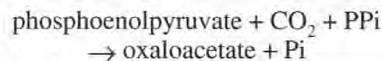
Another factor affecting propionate and acetate production is the formation of new carbohydrates [32]. Trehalose is produced during glucose [109–111] and lactate [74] fermentation. Three polysaccharides two of low Mw (~350–450 and ~200–300) and one of high Mw (> 5 000) containing methylpentose, glucose and galactose were detected during growth of *P. acidipropionici* at high lactose concentration and was related to a low propionate:acetate ratio (< 2:1) [20]. The production of a polysaccharide of Mw < 5 800 was also described during lactose fermentation by *P. acidipropionici* in whey-based medium, and its concentration was proportional to the C:N ratio [70]; it was composed of glucose, galactose, mannose and two methylpentoses (rhamnose and fucose), but a lower proportion of methylpentose was present compared to the polysaccharide studied by Crow [20]. Again the differences are probably due to the dif-

ferences in strain and growth medium used. The effect of the polysaccharide on the propionate and acetate production can be attributed to a direct loss of C for synthesis, but more importantly to the effect of the reduced components, the methylpentoses, which decrease the oxidation-reduction status of the cell; the overall result is an increased production of oxidised products, i.e. acetate, to maintain the oxidation-reduction balance and thus a decrease in propionate production.

The production of diacetyl by dairy PAB was observed during growth in milk by Lee et al. [44]. Only 5 out of 24 strains produced the dicarbonyl, but the pathway is not clear. Citrate is a likely substrate as diacetyl production followed its utilisation. Citrate utilisation and diacetyl production were increased at pH < 6.25. Addition of citrate to the milk increased diacetyl production. Diacetyl was detected during fermentation of pyruvate, citrate and glucose; it is therefore possible that diacetyl production would affect propionate, acetate and CO₂, but little information is available.

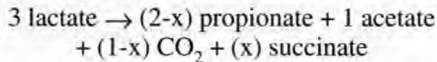
11. FATE OF CO₂ DURING PROPIONIC FERMENTATION

Because of its critical role in eye formation in Swiss-type cheese, it is worth considering the factors which determine the net amount of CO₂ produced during fermentation of lactate and sugars. In the classical pathway of propionic acid fermentation, 1 mole of CO₂ is produced per 3 moles of lactate or 1.5 moles of glucose. However, the net quantity of CO₂ produced can be depleted by fixation; CO₂ can condense with phosphoenol pyruvate (*figure 1*), by the reaction catalysed by the carboxytransphosphorylase:



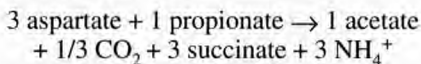
The relevance of CO₂ fixation depends on the growth conditions and is especially

important when the quantity of CO₂ is high [90, 91, 108, 110, 111]. In acidic conditions, CO₂ is less soluble in water and its availability for fixation increases. Such a situation would arise when sugars are used. CO₂ fixation leads to production of equimolar concentrations of succinate and reduces the amount of propionate produced:



On the other hand, involvement of the TCA cycle could lead to extra production of CO₂. Considering only this cycle, 7 moles of lactate would be metabolised into 6 moles of propionate and 3 moles of CO₂, while the hypothetical joint use of the glyoxylate enzymes (from citrate to isocitrate, glyoxylate and malate) with the TCA cycle, and followed by propionate production gives a theoretical 7 moles of lactate being converted to 5 moles of propionate, 2 moles of CO₂ and 1 mole of succinate.

Metabolism of amino acids, and especially aspartate would yield extra CO₂ production during lactate utilisation:



The overall quantity of CO₂ produced can be explained by the relative involvement of each of these processes.

12. CONCLUSION

The metabolism of propionibacteria is complex and involves several cycles not very well understood yet. Although the transcarboxylase cycle is the major pathway leading to the production of propionate, acetate and CO₂, the TCA cycle can be used as another route for the dissimilation of lactate and sugars. The presence of alternative pathways can partly explain the discrepancies from the theoretical molar ratios of end products during lactate and sugar metabolism which are found. Hence, CO₂ can be produced from lactate, according to the Fitz

equation, and from amino acids, but it can also be utilised by fixation with phosphoenolpyruvate, and lead to succinate production. Moreover, the physico-chemical composition of the medium, utilisation of extra substrates such as amino acids or short chain peptides and production of polysaccharides can affect the propionic acid fermentation by reducing the amount of propionate produced compared to acetate. The ability to use those alternative pathways for lactate and sugar dissimilation could explain the great variability observed between different *Propionibacterium* strains.

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