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The Citrate Metabolism in Homo- and Heterofermentative LAB: A Selective Means of Becoming Dominant over Other Microorganisms in Complex Ecosystems*

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Abstract

The citrate metabolism has been extensively studied in lactic acid bacteria (LAB) for its aroma compound production. Among the 4-carbon (C_4) by-products obtained from citrate fermentation, diacetyl is one of the better known products for its contribution to the buttery aroma of dairy products. A lot of documents deal with ways to improve diacetyl concentration in food matrices. Apart from these organoleptic advantages, in a microbial ecosystem, the citrate metabolism gives selective advantages to citrate positive microorganisms. Citrate metabolism allows the LAB to use another carbon source for their growth, withstand acidic conditions and generate a "proton motive force" (PMF). Moreover, the citrate/glucid co-metabolism leads to the fast release of organic compounds known for having bacteriostatic effects. Under specific conditions, the C_4 pathway liberates diacetyl which is bacteriostatic. In this review we first describe the citrate metabolism and the enzymes involved in the two homo- and heterofermentative LAB *Lc diacetylactis* and *Leuconostoc* spp. Moreover, the way to shift the metabolic pathway toward the production of aromatic compounds is discussed for both of these fermentative types of bacteria. Finally, the selective advantages of citrate metabolism for LAB in complex microbial ecosystems are delineated.

Keywords

Citrate Metabolism, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, *Leuconostoc* spp., Complex Microbial Ecosystems, Homo- and Heterofermentative Lactic Acid Bacteria

*The homo- and heterofermentative lactic acid bacteria (LAB) are illustrated by *Leuconostoc* and *Lactococcus* species.
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1. Introduction

The citrate molecule is abundant in nature. It is an important source of energy for many bacteria in food matrices such as fruits, vegetables or cheeses. For instance, the citrate concentration in raw cow milks ranges from 1.25 to 2.00 g/L. In the food industry, citrate salts are added as buffer substances or sometimes as fungistatic agents. This molecule is also a key compound for different metabolic routes, such as the Krebs Cycle. Most bacteria possess transport systems in the cytoplasmic membrane that mediate the uptake of citrate, e.g. *Klebsiella pneumoniae*, *Salmonella typhymurium*, *Escherichia coli*, *Bacillus subtilis* and some lactic acid bacteria (LAB) [1]. Internalized citrate can be utilized as a carbon and energy source under aerobic as well as under anaerobic conditions. If the aerobic metabolism implies a functional tricarboxylic acid cycle, under anaerobic conditions the fermentative pathways are different. For instance, three pathways have been described in *Enterobacteriaceae* [2]. The citrate positive LAB ferment the citrate under strict anaerobic condition [3]. After the depletion of lactose at the end of the cheesemaking process, citrate is a possible carbon source used by the mesophilic lactobacilli to support their growth during cheese ripening [4]. Under acidic conditions and in the presence of citrate, citrate metabolism liberates 4-carbon (C_4) compounds: diacetyl, acetoin, and 2,3-butanediol. Among them, diacetyl and acetoin are known to exhibit nutty and buttery aromatic notes. *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, few *Leuconostoc*, few *Enterococcus*, *Lactobacillus plantarum* and *Oenococcus oeni* are known as diacetyl and acetoin producers [5]-[9]. The improvement of diacetyl yields in food technology has been extensively studied in the two QPS—Qualified Presumption of Safety—LAB, *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* (hereafter designated as LD) and some citrate positive *Leuconostoc* spp. (cpLN hereafter). Citrate metabolism is regulated by different environmental factors whatever the type of metabolic route involved, homo or heterofermentative. Besides, the metabolic engineering involved in rerouting the carbon metabolism has been discussed in numerous studies (for reviews, see [7] [10] [11], for original articles, see [12]-[15]). Citrate metabolism can also be seen as a selective advantage for LAB in a complex microbial ecosystem. Indeed, citrate positive LAB show a higher resistance to lactate toxicity [16]. The citrate/glucid co-metabolism, which increases the maximum specific growth rate and the growth yield under acidic conditions, leads to a rapid ground invasion and the associated barrier effect [17] [18]. This additional catabolism generates a “proton motive force” (PMF). As well as energy-rich phosphate-bond intermediates such as ATP (adenosine triphosphate), the PMF is a metabolic energy source required for all the energy-requiring processes in the living cells. As sugar fermentation provides low ATP yields, the electrochemical energy generated via a proton gradient appears essential to support the metabolic activity of fermentative microorganisms. Besides, in their natural environment, LAB are more likely to be subjected to sugar starvation. The PMF generated by citrate metabolism enables the LAB to withstand energy depletion for short periods of time. The viability of the culture depends on the physiological state of the cell before the onset of the starvation period. When nutritional conditions are restored, glucidic fermentation restarts with the concomitant production of ATP and the recovery of LAB growth [19]. The citrate/glucid co-metabolism leads to the fast release of acetic acid in great amounts. Weak acids are known to have bacteriostatic effects at pH values below their pKa. Under specific conditions, this pathway liberates significant amounts of diacetyl also known as an antimicrobial agent.

In this review, we first describe the diverse pathways and key-regulators involved in the production of aromatic compounds for homo (such as LD) and heterofermentative (such as LN) bacteria. In particular, we emphasize the different factors that can inhibit or activate each metabolic step. Then we delineate the selective advantages of this metabolism in complex microbial ecosystems.

2. Part 1: Citrate Metabolic Pathway

2.1. Initial Breakdown of Citrate to Obtain Pyruvate

The first steps of the citrate metabolism include the internalization of the molecule followed by its breakdown to obtain pyruvate. This is observed in both homo- (**Figure 1**) and heterofermentative (**Figure 2**) LAB. This catabolism is unstable for LD and cpLN because one or the two gene clusters involved in the internalization and the first steps of the citrate breakdown are plasmid-encoded. Three steps lead to pyruvate synthesis. Firstly, citrate is taken up by the citrate permease (CitP), a member of the 2-hydroxycarboxylate family transporters. Then citrate breakdown inside the cell involves its conversion into acetate and oxaloacetate (OxA) by the action of the citrate lyase (CL). Finally, OxA is decarboxylated by the oxaloacetate decarboxylase (OAD) into pyruvate and carbon dioxide.

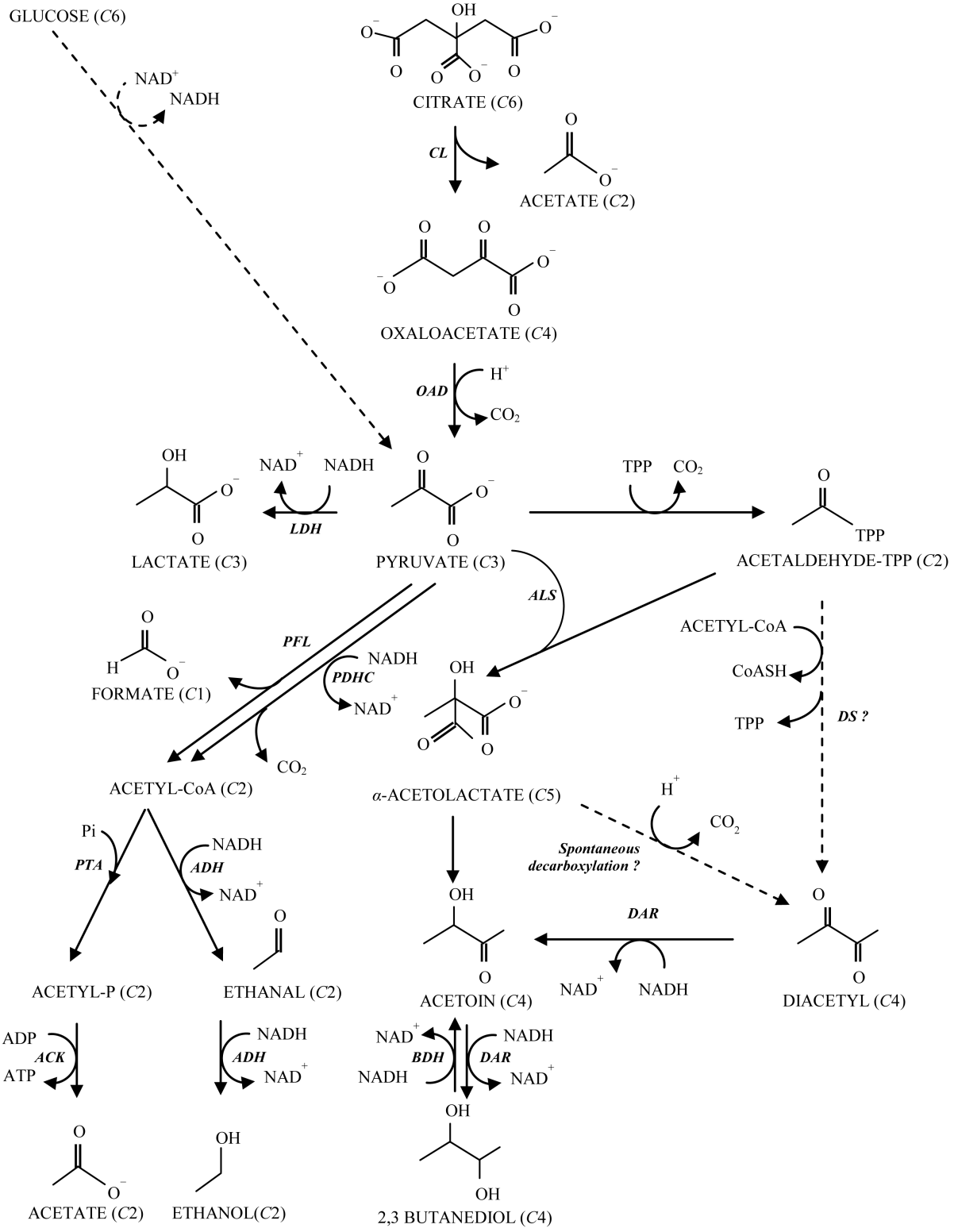

Figure 1. Glucidic and citrate fermentation by the homofermentative citrate positive LAB such as LD. CL: citrate lyase, OAD: oxaloacetate decarboxylase, LDH: lactate dehydrogenase, ALS: α-acetolactate synthase, ALD: α-acetolactate decarboxylase, DS: diacetyl synthase, DAR: diacetyl/acetoïn reductase, BDH: 2,3-butanediol dehydrogenase, PFL: pyruvate formate lyase, PDHC: pyruvate dehydrogenase complex, PTA: phosphotransacetylase, ACK: acetate kinase, ADH: aldehyde/alcohol dehydrogenases, TPP: thiamine pyrophosphate CoA: coenzyme A.

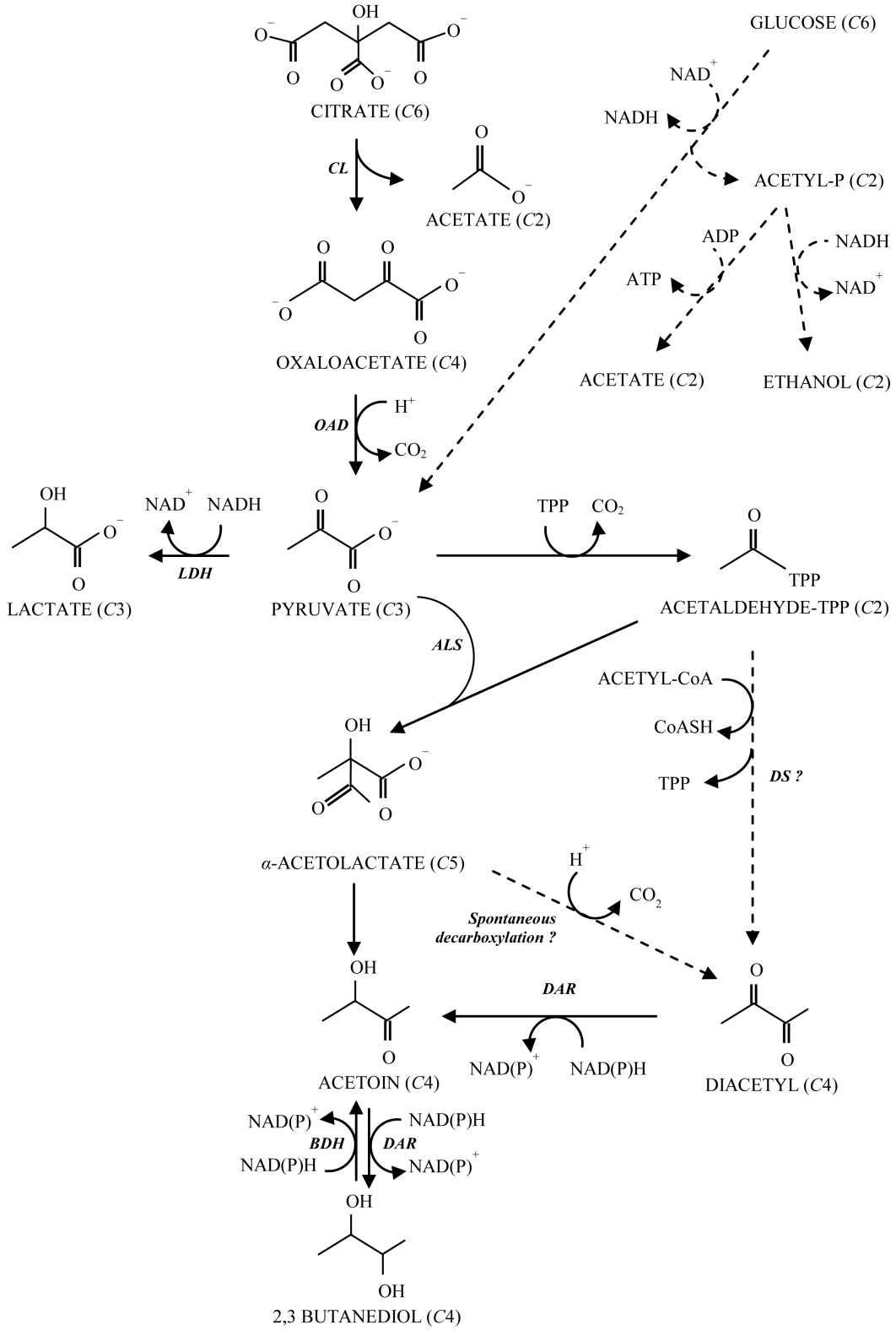

Figure 2. Glucidic and citrate fermentation by the heterofermentative citrate positive inLAB such as cpLN. CL: citrate lyase, OAD: oxaloacetate decarboxylase, LDH: lactate dehydrogenase, ALS: α-acetolactate synthase, ALD: α-acetolactate decarboxylase, DS: diacetyl synthase, DAR: diacetyl/acetoïn reductase, BDH: 2,3-butanediol dehydrogenase, TPP: thiamine pyrophosphate, CoA: coenzyme A.

All the genes involved in the uptake and the citrate conversion into pyruvate are plasmidic for cpLN. Studies on *Ln paramesenteroides* revealed a low level of constitutive transcription of the gene cluster *citI-citMCDEFGRP* [20]. The transcription of these genes is enhanced by the presence of citrate in the culture medium irrespective of the medium pH [21] (**Table 1** [1] [16] [20]-[26] and **Table 2** [27] [28]).

For LD, CitP is encoded by the plasmidic *citQRP* operon, whereas the CL and the oxaloacetate decarboxylase are encoded by the chromosomal *citM-citI-citCDEFXG* cluster. The genes involved in the citrate transport throughout the cytoplasmic membrane and its consecutive conversion into pyruvate are constitutively expressed [16]. The transcription of the two clusters is activated by acidic conditions [23]. For more details on the regulation of the citrate transport and its fermentation into C_4 compounds formation in LAB see the reference [1].

2.2. Pyruvate Metabolism in Homofermentative LAB

Pyruvate can then follow three different routes. Pyruvate is a compound common to the glucidic and the citrate pathways. In the presence of sugars, nicotinamide (NAD) cofactors reduced during the Embden-Meyerhof-Parnas pathway (EMP) are reoxidized by lactate dehydrogenase (LDH) to form lactate. The transcription of the gene encoding for LDH is activated by fructose 1,6-diphosphate. The latter is also an allosteric activator of the LDH. Thus, pyruvate is rapidly converted into lactate and NADH is regenerated [7]. For example, the glucose/citrate co-metabolism in LD leads to the lactate conversion of 80% of the citrate metabolized [29] (**Table 3** [3] [7] [10] [24] [30]-[34]).

Where sugar is limited, LD switches from an homolactic to a mixed acid fermentation. Under strict anaerobic conditions, the pyruvate formate lyase (PFL) converts pyruvate into formate and acetyl-coA. The PFL is inhibited by a pH inferior to 6 and by two metabolic intermediates of the EMP pathway: glyceraldehyde-3P and dihydroxy-acetone-P [3] [32]. Under strong aerobic conditions, pyruvate is decarboxylated by the pyruvate dehydrogenase complex (PDHC) to give acetyl-CoA, CO_2 and an additional reduced nicotinamide cofactor. One subunit of the enzymatic complex is inactivated by NADH; therefore, the PDHC of *Lc lactis* has a very low activity under anaerobic conditions [3] [35]. The PDHC requires the presence of lipoic acid as a co-factor to be active [36]. Regardless of the enzymes involved in the formation of acetyl-CoA, the activities of phosphotransacetylase (PTA) and acetate kinase (ACK) lead to the release of acetate. This pathway allows one ATP molecule to be produced per acetyl-CoA. Otherwise, aldehyde/alcohol dehydrogenases (ADH) generate acetaldehyde or ethanol, respectively. This pathway allows one NADH molecule to be reoxidized (**Table 4** [3] [30] [32] [34]-[40]).

Supposing citrate to be the sole carbon source, reference [41] recently highlighted another pathway leading to acetate formation without the help of any acetate kinase or ATP formation. This is likely to involve pyruvate oxidase, but this pathway has to be explored further.

2.3. Pyruvate Metabolism of Heterofermentative LAB

Concerning heterofermentative LAB, the pentose phosphate pathway leads to four different end-products: CO_2, lactate, acetate or ethanol according to the NADH:NAD ratio. In contrast with homofermentative LAB, the mixed acid pathway does not exist for heterofermentative LAB. Acetate and ethanol are produced from the pentose phosphate pathway. Pyruvate arising from citrate leads to the formation of lactate by LDH, with the use of NADH. A low level of NADH leads to acetate and ATP formation but with less ethanol produced from the glucidic source; the citrate acts then as an electron acceptor [8]. Under specific conditions (see below), C_4 compounds are produced.

2.4. C_4 Pathway in Homo- and Heterofermentative LAB and the Key Regulation of the Transcription

Under aerobic and acidic conditions, the citrate/sugar co-metabolism leads to the production of pyruvate in excess; this is explained by the lower activity or the inactivation of the enzymes involved in pyruvate conversion. The increase in pyruvate concentration leads to a growth in pyruvate flux towards the C_4 pathway. In the presence of thiamin pyrophosphate (TPP), the pyruvate is decarboxylated to form CO_2 and acetaldehyde thiamine pyrophosphate (active acetaldehyde). The α-acetolactate synthase (ALS) condenses the latter with a second molecule of pyruvate, yielding one mole of α-acetolactate (AL). For LD and cpLN, ALS is constitutively expressed

Table 1. Genes localization and transcription type of gene clusters implied in the first steps of citrate metabolism in LD and cpLN.

Transporter or enzymes	Microorganisms	
	LD[a]	cpLN
CitP	*citQRP* operon harboured by a 8 kb plasmid, Constitutively expressed and enhanced under acidic conditions (pH 4.5) [16] [22] [23].	*citI-citMCDEFGRP* cluster harboured by a 22 kb plasmid, Low constitutive transcription, enhanced by citrate addition [20] [21].
CL and OAD	Chromosomal *citM-citI-citCDEFXG* cluster, Constitutively expressed, and enhanced under acidic conditions (pH 4.5 to 5) [1] [16] [24] [25].	

[a]A few atypical citrate-positive *Lactococcus strains* harboured a plasmid with *citI-citMCDEFGRP* cluster [26].

Table 2. Activators and characteristics of CitP in LD and cpLN.

CitP	Microorganisms	
	LD	cpLN
Activating factors/ optimal activity parameters	Activators: Cu^{2+} and Co^{2+} [27].	Optimum pH for *Ln lactis:* 6 [28].

Table 3. Genes localization, transcription type, activators, inhibitors and characteristics of LDH implied in glucidic and citrate metabolism in LD and cpLN.

LDH	Microorganisms	
	LD	cpLN
Genes localization	Chromosomic genes [30].	Chromosomic genes [31].
Activating factors/ optimal activity parameters	Activators: fructose-1,6-diphosphate (FDP) (transcriptional and allosteric activation) and NADH [7], Carbon source: glucose, lactose, Sugar in excess [32].	No activators found—no regulation found [3] [33], Optimal pH for *Ln lactis*: 7.5 [24].
Inhibitory factors	Oxygen which is associated to a lower FDP concentration [32], phosphoenolpyruvate (PEP) and inorganic phosphates [10], Sugar-limited conditions, high aeration, Low NADH/NAD ratio (inferior to 0.03) and inorganic phosphate [34].	pH inferior to 5 [33].
Enzymes characteristics[a]	Specific activity of 0.0043 U/mg, Michaelis constant (Km) for pyruvate: 3 mM at pH 7.2 [34].	For *Ln lactis*, the kinetic parameters decrease as the pH decreases: the Km for NADH, pyruvate, and the Vmax decrease 30-, 20- and 8-fold respectively as the pH decreases from 8 to 5 [33], For *Ln lactis*: Km for NADH are 0.307 and 0.023 mM at pH 7 and 5 respectively, Km for pyruvate are 1.23 and 0.062 mM at pH 7 and 5 respectively [33], Km for pyruvate: 1.05, 0.25 and 0.13 mM at pH values of 7.5, 6.5 and 5.0 respectively [24], The optimum pH decreases as the level of pyruvate decreases [33]. For *Ln mesenteroides* subsp. *cremoris*: Km for pyruvate: 0.3 mM, Km for NADH: 0.03 mM [31].

[a]Some of these characteristics are given for specific environmental factors and for specific analytical methods, for more details, refer to the relevant article.

Table 4. Genes localization, transcription type, activators, inhibitors and characteristics of PFL and PDHC implied in glucidic and citrate metabolism in LD.

In LD	Enzymes	
	PFL	PDHC
Genes localization	Chromosomic genes [30].	Chromosomic genes [30].
Activating factors/ optimal activity parameters	Anaerobic conditions, Optimal pH: superior to 7 [32], Carbon source: galactose [37].	Oxygen [3], Lactose limitation [40], pH optimum: 5.5 - 6.5 [32].
Inhibitory factors	Oxygen [3] [32] [39], pH inferior to 6 [3], Triose phosphates as glyceraldehyde-3-phosphate and dihydroxyacetone phosphate [34] [38] [39].	NADH, Anaerobic condition [35].
Enzymes characteristics[a]	Km for pyruvate: 1 mM at pH 7.2 [34].	Enzyme requires to be functional: lipoic acid, divalent cations, thiamine pyrophosphate (TPP) and CoA [35] [36], Specific activity: 6.5 U/mg, Km for pyruvate: 1 mM, Km for TPP: 3.2 µM [35].

[a]Some of these characteristics are given for specific environmental factors and for specific analytical methods, for more details, refer to the relevant article.

at low concentration and partially induced in an acidic environment—around pH 5—and in the presence of citrate [24] [35]. The optimal pH of ALS is around 5.5 for LD and cpLN [25] [28] (**Table 5** [1] [3] [8] [24] [25] [28] [30] [35] [42]-[46]).

The ALS of cpLN has a lower Michaelis constant for pyruvate (Km: 10 mM) than that of LD (Km: 50 mM) [35] [42]. A high Km value for pyruvate favors the elimination of this toxic metabolite without there being any competition between the ALS and the enzymes involved in the pyruvate catabolism. And, the ALS of LD shows a higher activity than that of cpLN with 80 mM of pyruvate [45]. There are two groups of ALS which present a high amino acid homology. One is involved in the biosynthesis of the branched-chain amino acids (BCAA) leucine, valine and isoleucine (and frequently named acetohydroxyl acid synthase, AHAS), and the other in the catabolic pathway of pyruvate [1]. The anabolic AHAS has an optimum pH of 8.0, requires flavin adenine dinucleotide (FAD) and $MgCl_2$ and is subjected to a negative feedback control by BCAA. The catabolic ALS does not need these co-factors and optimally works at pH 6.0 [42] [46]. These two enzymes lead to α-acetolactate (AL) formation. As the AHAS is inhibited by a lot of herbicides (for a review, see [47]), we can suppose—even if no information has been published to confirm it—that the ALS activity is also depleted by the same types of inhibitors. The anabolic AHAS has been detected in *Lc lactis* but not in *Leuconostoc* spp.

AL is then decarboxylated into acetoin by the α-acetolactate decarboxylase (ALD). In the specific case of LD, this conversion is modulated by different factors, among them the BCAA metabolism [42]. Compared with wild strains, dairy *Lactococcus* strains have lost the ability to synthesize these amino acids. From an evolutionary point of view, this loss could be explained by the transfer of the microorganisms from their poor primary ecological niche—vegetable leaves—to a richer one, the milk; these functions would have then become superfluous [48] [49]. As indicated above, AL is decarboxylated into acetoin by the ALD. Consequently, in the "prototrophic" wild LD evolving in an oligotrophic environment, the ALD plays a dual role in the cell: either the AL flux is orientated towards the BCAA pathway, or it is transformed into C_4 compounds. Consequently, the expression of the ALD is strictly regulated at both transcriptional and post-transcriptional levels [1]. In addition, ALD is allosterically activated by leucine. In spite of the incapacity of the dairy *Lactococcus* strains to synthesize BCAA, the ALD activation by these amino acids is conserved [45]. The gene encoding the ALD of LD seems to be constitutively expressed, in accordance with the basal transcription level of the ALS [50] (**Table 6** [1] [8] [24] [25] [30] [45] [50]).

In the presence of oxygen, AL is also spontaneously decarboxylated into diacetyl. However, other enzymatic pathways involving cofactors or ALD might lead to diacetyl formation [51]-[53]. At the present time, this remains a supposition.

The diacetyl/acetoin reductase (DAR) catalyzes the acetoin reduction into 2,3-butanediol and the diacetyl reduction into acetoin. This latter reaction is irreversible whereas 2,3-butanediol can be reoxidized into acetoin by

Table 5. Genes localization, transcription type, activators, inhibitors and characteristics of ALS in LD and cpLN.

ALS	Microorganisms	
	LD	cpLN
Genes localizationand transcription type	Chromosomic genes, constitutively expressed, enhanced under acidic conditions (pH 5) and partially induced by the presence of citrate [1] [24] [25] [30].	Chromosomic genes, low constitutive transcription, partially induced by citrate addition, enhanced under aerobic conditions and sugar-limited source [8] [28] [42].
Activating factors/optimal activity parameters	Oxygen [43] [44], Optimum pH: 5.5 - 6 and 60% of the maximum activity at pH 6.5 [35] [45].	Optimum pH for *Ln lactis*: between 5 and 6 [28] [42] [45], Optimum pH for *Ln mesenteroides* subsp. *mesenteroides* and *Ln mesenteroides* subsp. *cremoris*: 5.1 - 5.3 and 20% of the maximal activity at pH 6.5 [42] [46].
Inhibitory factors	-	ALS of *Ln lactis* and *Ln mesenteroirdes* subsp. *cremoris* is inhibited by several intermediates of glucose metabolism [42] [46].
Enzymes characteristics[a]	Enzyme requires to be functional: divalent cations and TPP [3], Specific activity: 103 U/mg, Km for pyruvate: 50 mM, Km for TPP: 3.2 µM [35], Specific activity: ranged from 0.1 to 0.38 U/mg for 4 strains of LD with 80 mM of pyruvate, Km for pyruvate: 30 mM [45].	Enzyme requires to be functional: divalent cations and TPP [3], Km for pyruvate for *Ln mesenteroirdes* subsp. *cremoris*: 10 mM [42], For *Ln lactis*, *Ln mesenteroides* subsp. *mesenteroides* and *Ln mesenteroides* subsp. *cremoris*: Specific activity: ranged from 0.018 and 0.219 U/mg with 80 mM of pyruvate, Km for pyruvate: 10 mM [45].

[a]Some of these characteristics are given for specific environmental factors and for specific analytical methods, for more details, refer to the relevant article.

Table 6. Genes localization, transcription type, activators, inhibitors and characteristics of ALD in LD and cpLN.

ALD	Microorganisms	
	LD	cpLN
Genes localizationand transcription type	Chromosomic genes, constitutively expressed, and enhanced under acidic conditions (pH 5) [1] [25] [30] [50].	Chromosomic genes [8].
Activating factors/optimal activity parameters	pH optimum: 5.5 and 60% of maximum of activity at pH 6.5 [45], Activation by leucine, isoleucine, valine and notably allosteric leucine activation [45] [50].	Optimum pH for *Ln lactis*: 5.4 - 6 [24] [45], Optimum pH for *Ln mesenteroides* subsp. *mesenteroides* and *Ln mesenteroides* subsp. *cremoris*: 5.1 and 20% of the maximal activity at pH 6.5 [45].
Inhibitory factors	-	-
Enzymes characteristics[a]	Specific activity: ranged from 0.058 to 0.2 U/mg for 4 strains of LD with 48.4 mM of AL, Km for AL: 60 mM [45].	For *Ln lactis*, *Ln mesenteroides* subsp. *mesenteroides* and *Ln mesenteroides* subsp. *cremoris*: Specific activity ranged from 0.109 to 0.95 U/mg with 3.6 mM of AL, Km for AL: 0.03 mM [45].

[a]Some of these characteristics are given for specific environmental factors and for specific analytical methods, for more details, refer to the relevant article.

butanediol dehydrogenase (BDH). The LD strains possess one or two enzymes displaying both DAR and BDH activities in the presence of the co-enzyme NAD [54]. In *Ln pseudomesenteroides*, the same enzyme is involved in the DAR and BDH activities. The diacetyl reductase and the BDH require NADH and NAD^+ as cofactors, respectively, whereas the acetoin reductase activity can either rely on NADH or NADPH. However, the diacetyl and acetoin reductase activities of *Leuconostoc lactis* occur only with NADPH as a co-factor [28]. Whereas the reductive activity is optimal under slightly acidic conditions—between pH 5.2 to 6.6—the oxidative activity is optimal in an alkalinized environment, that is between pH 7.5 to 10 [54]. Under acidic conditions, acetoin and diacetyl compete for the enzyme, though the DAR has a lower Km for acetoin (Km: 0.2 and 0.3 mM for LD and cpLN, respectively) than for diacetyl (Km: 9.0 and 5.1 mM for LD and cpLN, respectively). These Km values might indicate that the DAR activity would become vigorous as soon as the diacetyl concentration reaches a

toxic level for the cells [55]. Acetoin exerts an inhibitory effect on the acetoin reductase activity if its concentration exceeds 1 mM [3]. When the pH, the growth temperature and the storage temperature diminish to 4.5, 18˚C and between 2˚C and 5˚C, respectively, the reductase activity of the DAR is strongly inhibited in cpLN and LD cells; the diacetyl pool is favored [24] [56]-[59]. The gene encoding for the DAR/BDH is carried on the chromosome in *Lc lactis* whereas in *Ln pseudomesenteroides*, this enzyme seems to be plasmid-encoded [60] (**Table 7** [1] [24] [25] [28] [30] [54] [55] [60]).

3. Part 2 Selective Advantages of the Citrate Metabolism in a Complex Bacterial Ecosystem

3.1. Energetic States of LAB from a Bioenergetic Perspective

As any leaving systems, the bacterial metabolism needs energy to function. Energy can be used for chemical reactions, to allow the cell to move or to translocate molecules inside or outside the membrane. Energy can come from two sources, the degradation of organic molecules, for instance via fermentation, and the electrochemical energy created by an ion gradient (proton in LAB) known as PMF. Energy is "stored" in the energy-rich phosphate-bond intermediates such as ATP or used directly. In LAB, fermentation is much less efficient in yielding ATP than sugar oxidation in aerobic microorganisms—2 moles of ATP/mole of glucose for homolactic fermentation versus 38 moles of ATP/mole of glucose. The metabolic energy obtained from fermentation is usually insufficient for the energy-requiring processes to operate in the cells [19]. Energy originating from ion gradients is thus essential. The PMF is an electrochemical gradient across the membrane involving two components, a membrane potential (inside negative) and a pH gradient (inside alkaline for LAB). Two systems can generate a PMF: the "primary" and the "secondary" transport systems. In the primary system, the translocation of ions through the cytoplasmic membrane is coupled with the energy release as a consequence of a chemical or redox reaction; to give an example, we can cite the respiratory chain and the membrane bond F0-F1-ATPase. But generally, fermentative bacteria do not possess the proton pumping electron transfer system, except for some LAB genetically equipped for respiratory metabolism, for example, *Lc lactis*, *Lc garviae*, *Ln mesenteroides*, *Weissella paramesenteroides*, *Enterocococcus faecalis*, (for a review see [61]. In fermentative bacteria, the PMF is usually generated via the "secondary" transport system. This system converts the (electro)-chemical energy of one solute into the (electro)-chemical energy of another solute (chemo-chemical coupling). The secondary transport system refers to uniporters catalyzing the translocation of one solute across the cytoplasmic membrane, symporters catalyzing the translocation of two or more solutes in the same direction and antiporters catalyzing the exchange of two molecules in the opposite direction.

Table 7. Genes localization, transcription type, activators, inhibitors and characteristics of DAR/BDH in LD and cpLN.

DAR/BDH	Microorganisms	
	LD	cpLN
Genes localizationand transcription type	Chromosomic genes, enhanced under acidic conditions (pH 5) and partially repressed by the presence of citrate [1] [24] [25] [30].	Plasmidic genes for *Ln pseudomesenteroides* [60], *Ln lactis*: low constitutive transcription partially repressed by citrate addition [28].
Activating factors/ optimal activity parameters	Optimum pH for the reduction of diacetyl: between 5.8 and 6.1, Optimum pH for the oxidation of 2,3-butanediol: between 8.5 and 10 [54].	Optimal pH for *Ln lactis*: 5.7 [28], Optimal pH for *Ln pseudomesenteroides*: 5.5 for the reductive activity and 7.5 for the oxidative activity, Optimal temperature for *Ln pseudomesenteroides*: 40˚C, and active from 20˚C to 55˚C [55].
Inhibitory factors	pH of 4.5 [54].	-
Enzymes characteristics[a]	The diacetyl reductase activity is 7- and 4.7-fold higher (for the two DAR) than the acetoin reductase activity with 10 mM of substrate. When the two substrates are present, acetoin is the preferred substrate for the two DAR [54]. Km for diacetyl: 9 mM, Km for acetoin: 0,2 mM, Acetoin acts as inhibitor at concentrations above 1 mM [3], Coenzyme: specific for NADH [28].	Km for diacetyl, acetoin and meso-2,3-butanediol for *Ln pseudomesenteroides*: 5.1, 0.34 and 1.67 mM at pH 5.5, 5.5 and 7.5; respectively [55]. Coenzyme: *Ln lactis*: specific for NADPH [28], *Ln pseudomesenteroides*: diacetyl reductase requires only NADH as coenzyme for the diacetyl reduction and either NADH or NADPH for the acetoin reduction. The enzyme catalyzes the oxidation of meso-2,3-butanediol only with the NAD^+ [55].

[a]Some of these characteristics are given for specific environmental factors and for specific analytical methods, for more details, refer to the relevant article.

In LAB, a PMF can be generated by the fermentation of citrate for instance. The CitP is a secondary active transport system which allows the translocation of substrates with $R_1R_2C(OH)COOH$ motif—citrate, malate, lactate, OxA—inside the cell [41] [62]. In the presence of lactate, which can originate from the citrate/sugar co-metabolism, the citrate is internalized inside the cell concomitantly with the externalization of lactate. This $Hcitrate^{2-}/lactate^{-}$ antiport generates an electrical potential across the membrane ($\Delta\Psi$) and the internalization of one electron per pair of molecules translocated. In the absence of lactate, the symport $Hcitrate^{2-}/H^{+}$ occurs with the same consequence on the membrane potential, namely the translocation of one negative charge into the cell. Moreover, the subsequent steps of the citrate catabolism lead to proton consumption as well. This depletion participates in the generation of the PMF which then results both from an electrical and a chemical potential of electrons (ΔpH) across the membrane [62]. Thus, the citrate metabolism can be considered as a PMF-generating system resulting from the action of the CitP secondary active transporter and the proton consumption in the cytoplasm. Overall, this leads to the alkalization of the inner part of the cell. The term "secondary PMF-generating pathway" is frequently used to refer to the citrate metabolism [16].

Besides its energy supplier function, the PMF system allows the LAB to better withstand sugar starvation. Indeed, LAB can encounter drastic fluctuations in the composition of their environment. As a consequence of carbon source depletion, the ATP rate generated by glycolysis can decrease rapidly. The PMF system, active for several hours (as long as 24 h in certain cases), enables the bacteria to survive by maintaining the energy pool inside the cell [19] [63]. Therefore, the citrate metabolism provides a growth advantage to citrate positive LAB.

3.2. Acidic Stress Resistance

During the fermentation step, organic acids are produced—mainly lactic and acetic acids—lowering the pH of the medium. The proportion of acid-protonated forms [AH] outside the cell increases as the pH decreases, whereas the dissociated form of acids $[A^{-}]$ diminishes. This evolution depends on the pK_A of each acid. Un-dissociated [AH] forms passively cross the cytoplasmic membrane, *i.e.* without any energy requirement. Inside the cells, the pH is generally close to neutrality (between 6 and 7). Consequently, [AH] dissociate to form $[A^{-}]$ with the concomitant release of a proton $[H^{+}]$ and an electron which contribute both to acidify the cytoplasm; this can compromise the pH homeostasis. The cell has to prevent $[A^{-}]$ accumulation by rejecting it outside and to stabilize its internal pH. This requires energy. Weak acids and low external pH have synergic deleterious effects on cell viability. As the acidification progresses, the preservation of an active metabolism becomes more and more difficult. The increasing need for energy is supplied by the carbon metabolism, which generates growing quantities of organic acids. In the absence of pH buffering or medium regeneration, this deadly circle leads to the progressive poisoning of the cell. The population growth finally stops or the population may even begin to decrease.

In LD, the citrate metabolism pathway is constitutively expressed and enhanced under acidic conditions, *i.e.* during the late exponential growth phase. Moreover, acidic conditions—pH 5 or below—favor the activity of several enzymes, ALS, ALD and DAR for example, and the transport activity of the CitP system (optimal pH between 4.5 and 5.5) [1] [22]. The citrate metabolism is all the more protective as the lactate toxicity is the most deleterious; cells are thus more resistant at low pH [16].

In the subsequent steps of the citrate fermentation pathway, the decarboxylations of OxA and AL are both proton-consumers. Each citrate molecule catabolized leads then to the "consumption" of two scalar protons; this contributes to the cytoplasm alkalization. Hence, the citrate metabolism plays an important role in the regulation of the intracellular pH homeostasis necessary for the maintenance of metabolic activities and growth. This effect is extended by the related release of neutral compounds, ethanol, CO_2, acetoin, diacetyl and 2,3-butanediol. A moderate re-alkalization of the culture medium is then observed [18] [64] [65]. This re-alkalization seems to improve the efficiency of the use of citrate and the sugar metabolism [22].

These additional benefits of citrate metabolism could explain the growth stimulation of LD observed when citrate is added to the medium (even if this activation remains marginal compared to that of cpLN). Indeed, the specific growth rate increases from 10% to 15% for LD after citrate addition, and from 50% to 100% for cpLN [64] [66]-[68]. As for the heterofermentative bacteria, the citrate/glucid co-metabolism also improves the acid resistance at low pH and participates in the energetic metabolism of the cell; but the key regulators of the citrate metabolism are different (see below).

3.3. Effects on Growth

In cpLN, the transcription gene coding for the CitP and the two enzymes involved in the first steps of citrate de-

gradation into pyruvate (CL, OAD) are induced by the presence of citrate regardless of the external pH. In the absence of substrate, CitP, CL and OAD are only present at low basal levels [20] [21]. This is contrary to LD, where the transporter and the enzymes CL and OAD are constitutively expressed and the transcription of the gene cluster is greatly improved under acidic pH [16]. Thus in cpLN, the citrate is co-metabolized with a glucidic source during the exponential growth phase. The heterolactic fermentation of sugars leads to the formation of CO_2, lactate and acetate or ethanol depending on the NADH:NAD^+ ratio. When glucose is the unique source of carbon, a high NADH:NAD^+ ratio favors the formation of ethanol concomitantly with the regeneration of NAD^+. In the case of a citrate/glucid co-metabolism, the initial steps lead to pyruvate formation without any NADH. Moreover, the LDH of cpLN under neutral or slightly acidic conditions (above pH 5) is not subject to any regulation; all the pyruvate is therefore converted into lactate with the concomitant re-oxidation of NADH. Consequently, there is a lack of NADH (or any other reduced molecule) to reduce the acetyl-P formed in the pentose phosphate pathway. Instead of ethanol, acetate is generated. This pathway is energetic. As a result, the maximum specific growth rate greatly increases. The growth yield, *i.e*. biomass produced per mole of metabolized sugar, is also improved, even if it depends on the strains and the physico-chemical conditions [17] [66]. The same observations have been made for LD under acidic conditions with the citrate/sugar co-metabolism [18].

3.4. Biopreservation

During the citrate/glucose co-metabolism of cpLN, the lactate production is 1.63 fold higher when citrate concentration varies from 0 to 25 mM, although the level of the LDH is divided by about 3 [69]. The citrate/glucose co-metabolism also leads to a better utilization of glucose by cpLN and LD and so to greater lactate excretion [22]. Lactate is usually considered as a food preservative for its antimicrobial activities. Several mechanisms of inhibition have been proposed. Lactate, like many organic acids, has the ability to cross the cell membrane passively in its un-dissociated form; thereafter, it dissociates inside the cell into two parts, a salt and a proton. The proton lowers the inner pH. This mechanism has been described above in this article. Even if the pK_A value of lactate is lower compared with that of acetate and propionate—3.86, 4.73, and 4.87, respectively—lactate is a more efficient inhibitor compared with other organic acids at identical concentrations [70]. A higher lactate concentration is then supposed to deplete the other microflora and among them, the undesired microbes, pathogens and spoiling cells.

At high concentrations, lactate is also known to lower the water activity by sequestering a part of the water available in the medium. Other specific inhibitory effects have been suggested. Organic acids are able to chelate cations, which render them inaccessible to support the growth of some microorganisms; this is the case for *Listeria monocytogenes*. Although this inhibition mechanism is globally understood, further studies have to be made for its complete deciphering and the implications on the bacteriostatic effects [71].

Moreover, citrate metabolism systematically leads to acetate formation, in particular as a result of the action of the CL. This release is observed whatever the metabolism, homo- or heterofermentative, of LAB. In a complex microbial ecosystem, the other microorganisms are thus subjected to the synergestic inhibitory effects of these organic acids produced in a more and more acidic environment [72].

At low pH—5 and below—the apparent Km value of the cpLN LDH tends to decrease. More pyruvate is available for the C_4 compounds formation pathway as a consequence of the saturation of the LDH [24]. In the case of LD, anaerobic and acidic conditions inhibit the mixed acid fermentation pathway and the pyruvate coming from the citrate/glucid co-metabolism is produced in excess. The toxic pyruvate is converted by the ALS into AL. Under aerobic conditions, this compound is decarboxylated into diacetyl. Diacetyl has a broad antimicrobial spectrum. For instance, the Gram negative bacteria and yeasts are inhibited at 200 ppm and the Gram positive non-lactic acid bacteria at 300 ppm of diacetyl [73]. According to reference [74], most of the LAB is not affected by concentrations ranging between 100 to 350 ppm. But, our results obtained with two strains of *Lc lactis* subsp *lactis* and one *Lc lactis* subsp *cremoris* suggested a significant effect at a 100 ppm concentration. However, the diacetyl concentration detected in fermented food matrices is not so high, around 2 ppm in wine and 5 ppm in dairy products [5] [75]. These rates have no visible effect on the *Lactococcus* growth compared with the control samples [76]. Diacetyl could, however, act synergistically with other factors and is, besides, an unstable compound [77]. Being volatile and influenced by the medium composition, diacetyl concentration can vary a lot and could be under-estimated. The accuracy of the published results is also debatable if we consider the different methods of diacetyl extraction and quantification used [78] [79]. For example, according to refer-

ence [80], the diacetyl concentration in fermented raw milk ranges between 48 to 133 ppm. This concentration could then have an inhibitory effect on LAB and other microflora.

3.5. Observation of the Selective Advantage of Citrate Metabolism in a Complex Bacterial Ecosystem

As a consequence of those four points, we can imagine that in complex ecosystems, citrate positive lactic acid bacteria are favored. In the same way, reference [81] highlights an antagonistic effect of citrate positive facultative heterofermentative lactobacilli towards citrate negative propionic acid bacteria in hard Swiss-type cheese. This could explain the observations we made when we cultured together a mix of four lactococci in milk. One strain was a LD. After clotting, the curd was removed and the whey used to re-seed the following day's milk. This is called "backslopping". This procedure was followed during 10 consecutive days. From day to day, we observed the progressive dominance of the LD strain. After 8 days, only LD was detected on Petri dishes [76]. We also observed the composition of the dominant LAB populations in a natural whey starter used to make Rocamadour cheeses during four months (Demarigny, personal communication). The same result was found. Progressively, the LD population overwhelmed the other lactococci. And consequently, the organoleptic characteristics of the cheeses were altered.

As noticed above for LD, we can argue that this metabolism allows the cpLN to take advantage over the other microflora. Recently, we indicated that many naturally fermented tropical vegetable products were characterized by the systematic presence of lactobacilli together with leuconostocs [82]. Among the different hypotheses put forward, we proposed that the energetic metabolism of cpLN gave them a selective advantage to settle even under harsh conditions—low pH, the presence of many other microorganisms.

4. Conclusion

The LAB citrate metabolism has been extensively studied, especially during the nineties. Although many articles have been published on this subject, to our knowledge, they generally tended to have biochemical objectives, the final goal being the improvement of the buttery and creamy aroma in dairy products. As we have tried to show in this review, the role of the citrate metabolism largely exceeds these considerations. Citrate positive bacteria seem to be favored, in particular when the environment becomes harsh. This is the case in fermented food products. The acidification decreases the pH at sub-lethal levels. The energetic benefit of this metabolism allows bacteria to better withstand and, even, to dominate. Moreover, the citrate metabolism contributes to the production of toxic compounds, organic acids and diacetyl, active against many undesired (and unfortunately desired) bacteria. This could explain the capacity of LD and cpLN to colonize natural starters and, in the case of cpLN, to be systematically present in vegetable food products. As such, it would seem interesting to select these microorganisms as starters, and not only for aroma production. Inevitably, some questions arise from this review. In particular, if we consider the advantages of the citrate metabolism, we can wonder why citrate positive bacteria are not the sole populations enumerated in complex natural starters. This question can be enlarged to all the citrate positive bacteria—including a lot of pathogenic Gram negative bacteria. We tried to focus on the different factors that influence the citrate metabolism. Is it then possible to use this knowledge to pilot the food making of traditional foods, to prevent/to control growth of undesirable (food spoiling and/or foodborne pathogenic) microorganisms or to improve their "typicality"? On the fringe of these questions, many technical problems have to be solved. Among them, the instrumental means to detect diacetyl have to be improved. Within the perspective of protecting people against infections, the ecological implications of citrate metabolism might be crucial in the future, and not only in Western countries. In many regions worldwide, the consumption of contaminated food products is a major sanitary concern. The addition of citrate positive bacteria in fermented food products could be then a means, among others, to better control the occurrence of infections or intoxications due to foodborne pathogenic microorganisms.

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