Taxonomy, physiology and growth of *Lactococcus lactis*: a review

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Summary

Lactococcus lactis *species is one of the most important groups of lactic acid bacteria that are used in the dairy industry. The major functions of this species in dairy fermentation are the production of lactic acid from lactose, hydrolysis of casein and citric acid fermentation. Thus their metabolic end products and enzymes directly or indirectly have significant influence in determining the texture and flavour of the final products. In recent years, genetics and physiological properties of lactococci have considerable changed. Therefore, both for basic research and for application purposes in this paper the general view of the new taxonomic classification of* ***Lactococcus lactis****, the role of their plasmids and the physiology and nutritional requirements during growth are discussed.*

Key words: Lc. lactis, taxonomy, physiology and growth

Introduction

Strains belonging to the species *Lactococcus lactis*, are the most important organisms in the manufacture of fermented dairy products such as sour milk, cream, butter, fresh cheeses and many varieties of semi-hard cheeses. Research on the genetic and physiological properties of these bacteria has expanded rapidly in the last decade. Therefore, for the better understanding of *Lactococcus lactis* species this paper discusses some of theirs most important characteristics.

Lactococcus lactis

The majority of the microorganisms from the Lancefield group of N streptococci have been transferred to the *Lactococcus* genus. The mobile

group of N streptococci has been integrated into the *Vagococcus* genus. The genus includes five species: *Lc. garvieae, Lc. piscium, Lc. plantarum, Lc. raffinolactis* and *Lc. lactis*. But, among species of this genus only *Lc. lactis* is used in dairy technology.

This species have two subspecies and a biovar: *Lc. lactis* subsp. *lactis; Lc. lactis* subsp. *cremoris; Lc. lactis* subsp. *lactis* biovar *diacetylactis* (Schleifer et al., 1985; Stiles and Holzapfel, 1997.). The literature, according to Salama et al., (1995.), on the basis of evidence gathered, identifies green plants as the natural habitat for lactococci, particularly for *Lc. lactis* subsp. *lactis,* and *Lc. lactis* subsp. *lactis* biovar *diacetylactis*. The natural source of the *Lc. lactis* subsp. *cremoris,* has still not been confirmed and is the subject of numerous controversies.

Due to the phosphoenolpyruvate-phosphotransferase system (PEP-PTS), which ensure their efficient uptake and fermentation of lactose, some of these organisms have been adapted well to growth in milk and today the most recognised habitat for lactococci are dairy products (Axelsson, 1998.).

Lactococci are homofermentative microaerophilic Gram-positive bacteria which grow at a temperature of 10 ^{o}C, but not at 45 ^{o}C, and produce L(+) lactic acid from glucose. They are characterised by ovoid cells which appear individually, in pairs, or in chains. It often happens that cells of lactococci themselves extend into a chain, which makes them difficult to differentiate from lactobacilli. The group consisting of *Streptococcus, Enterococcus* and *Leuconostoc* also forms cocci that occur as chains or pairs, so it is difficult to distinguish these genera from *Lactococcus* genera on a morphological basis. (Wijtzes et al., 1997.). Among the lactococci, *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* differs from *Lc.lactis* subsp. *lactis* and *cremoris* in theirs ability to utilise citrate with production of diacetyl. These strains possess citrate permease that enables them, without modification, to transport citrate into a cell (Kempler and McKay, 1981). However, as citrate utilisation is plasmid mediated it is an unstable characteristic of these bacteria, which resulting in them being classified as a variety of *Lc. lactis* subsp. *lactis* **(**Stiles and Holzapfel, 1997.). *Lactococcus lactis* do not posses flagella and do not create endospores, while some of its strains are capable of excreting extracellular polysaccharide substances (Marshall and Tamime, 1997.).

Lc.lactis is also characterised by numerous phenotype variations, and it is sometimes difficult to recognize the differences among them. Thus, according

to Bergeys' Manual (1994.), *Lc. lactis* subsp. *lactis* biovar *diacetylactis* produces ammonia from arginine. Collins (1977.), on the other hand, lists strains that do not possess that property, but are the *diacetylactis* strains nevertheless. *Lc. lactis* subsp. *lactis* is usually differentiated from the subspecies *cremoris* on the basis of the maximum growth temperature and inability to produce ammonium from arginine (Petterson, 1988.). Davey and Heap (1993.), however, established the existence of *Lc. lactis* subsp. *cremoris* strains that manifest the arginine metabolism. Although research on genetic of lactic acid bacteria including genus *Lactococcus* began in the early 1970s there are still discrepancy in their fully characterization, especially between phenotypic and genotypic characteristics (Godon, et al. 1992; Salama et al. 1995).

Plasmids

One of the characteristics of the *Lc. lactis* strains is that theirs most industrially important traits are plasmid encoded. This means that plasmids carry genes for properties such as lactose catabolism and proteinase production as well as bacteriophage resistance (Gasson, 1993). The other important metabolic plasmid and theirs functions in lactococcal strains have also been described. Thus, we today know the plasmid genes controlling the metabolism of sucrose, galactose, mannose, xylose, glucose, citrate utilization, phage resistance and DNA restriction and modification, cell aggregation, production of bacteriocines, mucoidness and resistance to inorganic ions (McKay et al., 1976; McKay and Baldwin 1978; Larsen and McKay, 1978; Kempler and McKay, 1979a; Kempler and McKay, 1979b; Walsh and McKay, 1981; Crow et al., 1983; Davey, 1984; Thompson and Collins, 1989; Smith et al., 1992; von Wright and Sibakov, 1998; Miklič-Anderlič et. al. 2000.). In addition, lactococci usually contain many apparently cryptic plasmides (Thompson and Collins, 1989.) ranging in size from 1 to > 100 kilobase pairs (kbp). Bearing in mind that a plasmid DNA is replicated independently of the chromosome (Cords et al., 1974.), any mutation or rearrangements could cause the loss of plasmid in daughter cells. As they are not necessary for cell survival, the bacterial cell may have lost one or more plasmids spontaneously, by protoplast regeneration, transduction, conjugation and transformation, as a consequence of the function encoded by this plasmid (Kempler and McKay, 1981; Davies and Gasson, 1981; Chopin, 1993.). It was also found that

frequently repeated cultivations in milk or changed culture conditions lead to the loss of plasmid (McKay et al., 1976; Gasson and Davies, 1984; Foucaud et. al., 1990; Davidson and Hillier, 1995.).

The plasmids size for the technologically useful traits varies from 17 to more than 50 kbp for lactose fermentation and proteinase activity and is relatively large (Otto et al., 1982; Thompson and Collins, 1989; Foucaud et al., 1990.). The average size of very important citrat plasmid which encoded production of diacetyl is 8,7 kbp (Thompson and Collins, 1989; Bandell et al., 1998.). Plasmids-encoded bacteriocines are also large and their size is from 81-133 kbp (von Wright and Sibakov, 1998). Strains of Lc. *lactis* usually possess from 2 to 11 DNA plasmids, while the most common are between 4 and 7 (McKay, 1983.).

Physiology and Growth of Lactococci

Protein metabolism

Like all other lactic acid bacteria, lactococci are highly fastidious with regard to the medium in which they grow. Their growth requires proteins, peptides, specific amino acids, derivates of nucleic acids and vitamins, all of which serve as building units in the synthesis of their own cell compounds. In milk, the concentration of isoleucine, leucine, valine, histidine and methionine, which are essential to the majority of lactococci, is less than 1 mg/L. This content of free amino acids, that is initially present in milk, provides sufficient nitrogen for only 2% of the final cell density (Juillard et al., 1996.). Thus, for their optimal growth in milk, lactococci depends on their own proteolytic system to obtain the amino acids needed for growth to high cell densities. Casein, which composes 80% of all proteins present in milk, becomes the primary nitrogen source after nonprotein nitrogen is depleted (Steele, 1998.). The enzymes that form proteolytic system of lactococci and that are active in hydrolysis of casein are a cell wall-associated proteinase, an extracellular peptidase (s), amino acid transport system, peptide transport system and intracellular peptidases **(**Smid et al., 1991; Tjwan Tan et al., 1993; Juillard et al. 1998). But the key enzyme in proteolysis is a cell-wall associated proteinase (PI- or PIII- type proteinase [PrtP]) which cleavage more than 40% of the peptide bonds into more than 100 different oligopeptides (Juillard et al. 1995.). Then, for the uptake of nitrogenous compounds by

the cell, lactococci utilise three distinct transport systems di- and tripeptide and an oligopeptide transport system. The intracellular located peptidases then hydrolyse peptides into amino acids required for growth (Poolman et al., 1995; Meijer and Hugenholtz, 1997; Wang et al., 1998.). It is worth emphasising that both proteinase and the oligopeptide transport systems play a crucial role in amino acids, peptides and casein utilisation by lactococci when they grow in milk.

However, it has been established that the enzymatic system present within the *Lc. lactis* strains varies both biochemical and genetically (Exterkate et al., 1991; 1993; Bruinenberg and Limsowtin, 1995.)**.** Thus, the activity level of cell proteinase and lysilaminopeptidase in the *Lc. lactis* subsp. *cremoris* strains, with a faster milk coagulation effect, has been found to be twice as high as *Lc.lactis* subsp. *lactis* with a faster milk curdling ratio. Although both strains, those with a faster coagulation effect and those with a slower coagulation effect, have significant level of caseolytic activity (Crow et al., 1994.). Electrophoretic study of proteolytic enzymes has established that significant differences also exist between the proteolytic, lactococci and lactobacilli systems (Sasaki et al., 1995.).

Recently, it was estimated that wild strains of lactococci required only between 1 and 4 amino acids for growth. So, in comparison with strains used in dairy industry these strains probably possess more active amino acid convertase (Ayad et al., 1999.). Since, several aminotransferases have been detected this may be suggested that lactococi have also enzymatic potential required for degradations of aromatic and branched-chain amino acids into volatile aroma compounds (Roudot-Algaron and Yvon, 1998.).

Lactose metabolism

The lactose metabolism of the *Lactococcus lactis* species differs from the lactose metabolism of other lactic acid bacteria. The difference is in the simultaneous catabolism of glucose and galactose. The gene responsible for lactose breakdown is carried by plasmid (Lac plasmid), and encodes the enzymes that transport lactose to a cell (McKay et al., 1976; McKay and Baldwin, 1978; Crow et al., 1983.).

Lactose is phosphorylated by phosphoenolpyruvate (PEP) during translocation by PEP-dependant phosphotransferase system (PEP: PTS). The intracellular lactose phosphate is subsequently hydrolysed to glucose and

galactose by different enzyme ß-D-phosphogalactosidase. The galactose is then catabolized via the Tagatose pathway at the same time as the glucose is catabolised via Embden-Mayerhof-Parnas pathway (Marshall and Tamime, 1997.). In both pathways, an aldolase cleaves a diphosphate intermediate compound to produce the triose phosphate-dihydroxyacetone phosphate and glyceraldehyde phosphate. These triose pathways are converted to pyruvate and then to lactate by enzyme L-lactate dehydrogenase (Steele, 1998.). The function of that pathways for the lactococci is to generate energy and lactic acid is by product. On the other hand thus organism eliminate pyruvate that is toxic for a cell, particularly when the medium pH is low (Tsau et al., 1992.). The utilisation of pyruvate in a cell is fourfold: reductive (lactate dehydrogenase), oxidative (by means of the pyruvate dehydrogenase complex), non-oxidative transfer (pyruvate formatelyase) and through α-acetolactate synthesis (Verhue and Tjan, 1991; Starrenburg and Hugenholtz, 1991; Hugenholtz and Starrenburg, 1992.).

Citrate metabolism

Among lactococci only *Lc. lactis* subsp. *lactis* biovar *diacetylactis* has the ability to metabolise the citrate present in milk. The metabolic end products of citrate metabolism are diacetyl, acetoin, 2,3 butanediol, acetic acid and carbon dioxide (Harvey and Collins, 1961; Cogan, 1995; Steele, 1998.) which contribute to the flavour development in fermented dairy products. Citrate is transported without modification into the cell. In *Lc. lactis* subsp. *lactis* biovar *diacetylactis* this reaction is catalysed by the citrat permease (CitP), which is encoded by the plasmid citP gene that is induced into the citQRP operon. The induction is not, however, conditioned by the presence of citrate in the medium, as was previously believed, but by the lactic acid produced through co-metabolism of glucose and citrate when the pH value of the medium is low (Garcia-Quintans et al., 1998; Bandell et al., 1998.).

The presence of CitP is essential for citrate utilization, since in its absence no citrate metabolism is observed although all enzimes involved in conversion of citrate are present inside the cells (Lopez et al., 1998.). During citrate metabolism three decarboxylation reactions occur: oxalacetate to pyruvate, pyruvate to acetaldehyde-thiaminopyrophosphate (TPP) and α-acetolactate to acetoin. Quantitatively, acetoin is the most important product of citrate metabolism and occurs as a mechanism of preventing accumulation of pyruvate Diacetyl and CO_2 are produced in small quantities, but their

commercial value is significant since they are responsible for both the texture and flavour of fermented products (Cogan, 1995.). Diacetyl is produced by chemical decomposition of α-acetolacete (nonenzymatic) and this reaction occurs at the intracellular level. This reaction is favoured by aeration and low pH (Cogan, 1995; Axelsson, 1998; Rondags et al., 1998.).

The ability to metabolised citrate also has the strains of *Leuconostoc* spp. However, significant differences in enzyme activities exist in the production of acetoin and diacetyl, and the use of citrate, between them. *Leuconostoc* spp. produce little or no acetoin in the presence of citrate while the *Lc.* and. *lactis* subsp. *lactis* biovar *diacetylactis* produce large quantity of acetoin when citrate is present and a small quantity of acetoin when there is no citrate (Cogan, 1975; Drinan et al., 1976; Hugenholtz and Starrenburg, 1992.). *Leuconostoc* spp. from *Lc. lactis* subsp. *lactis* biovar *diacetylactis* is also distinguished by the inducible nature of citrate lyase and α-acetolacetate synthase. While both these enzymes are constitutively present in citrate metabolism of *Lc. lactis* subsp. *lactis* biovar *diacetylactis* in *Leuconostoc* spp enzymes are induced in the presence of citrate (Cogan, 1981; Hugenholtz and Starrenburg, 1992.). This explains the differences in the mechanism and regulation of citrate utilization and diacetyl production in these two species. However, the process of diacetyl formation by both species is still poorly understood and therefore difficult to control during the manufacture of dairy products. Recently much work has been done on citrate metabolism by lactococci and leuconostocs, but many questions remains to be answered that will enable better understanding of many interesting genetic phenomena which are specific for those bacteria. (Starrenberg and Hugenholtz, 1991; Hugenholtz and Starrenberg, 1992; Hugenholtz et al., 1993; Bassit et al., 1995.).

Bacteriocin production

Certain strains of the species *Lc. lactis* produce a multitude of different antagonistic compounds, including antimicrobic proteins or bacteriocins. These compounds occur as a final products of the metabolism process. The ability of some strains to produce bacteriocins is significant from technological scientific aspects (Klaenhammer, 1993; Rogelj and Bogovič- Matijašič, 1994; De Vuyst and Vandamme, 1994.).

Bacteriocins are proteins or protein complexes containing bacteriocidal properties. Lactococcal bacteriocins are small thermostable proteins that destroy closely related bacteria. The exception is the nisine molecule which activity is directed towards the wide range of gram-positive bacteria, including *Listeria monocytogenes* (Harris et al., 1991.). Nisine, which is produced by some strains of *Lc. lactis* subsp. l*actis*, is the best-known bacteriocin and it is now well established that nisin production and immunity are coded by a transmissible chromosomal gene block (von Wright and Sibakov, 1998.). It consists of 34 amino acids, has a molecular mass of 3354 (De Vuyst, 1995; Kuipers et al., 1995.), belongs to the group of lantibiotics, and has a practical use in food preservation. It is successfully used in the production of cheeses, melted cheeses, milk desserts, fermented drinks and canned vegetables (Fowler and Gasson, 1991; Rodrigez et al., 1995.). In 1988 the US Food and Drug Administration (FDA) accepted it as a preservative for prevention of delayed clostridial bloating in cheeses.

In addition to *Lc. lactis* subsp. *lactis*, the certain strains of *Lc. lactis* subsp. *cremoris* and *Lc. lactis* subsp. *lactis* biovar *diacetylactis* possess an ability to produce different bacteriocins with a range of inhibition. However, their possible value in the growth control of microorganisms causing decay, and of pathogenic microorganisms, remains to be investigated (Cogan et al. 1997.).

Conclusion

The metabolic properties of the strains within the *Lc. lactis* species have a direct or indirect influence on the organoleptic, nutritional and hygienic quality of fermented dairy products, which makes knowledge of their characteristics extremely important from an economic aspect. However, this is no easy task because, as this paper has shown, the bacteria in question possess quite unique morphological and phylogenetic properties. The three genes responsible for lactic acid synthesis and organised into a single transcription unit or operon, which have been established in the chromosome, have not been found in any other bacterium (Griffin and Gasson, 1993; Davidson and Hillier, 1995.). A number of genes important for their industrial use are carried on plasmids. While those genes have been characterised the study of chromosomal genes, which provide more than 95% of genetic information of the cell for lactococci is still limited (Chopin, 1993.). Considering that lactococci are more than just lactic acid producer, those bacteria will excite the

interest of both scientists and dairy experts, as well as presenting a challenge in numerous genetic, physiological and technological research projects.

TAKSONOMIJA, FIZIOLOGIJA I RAST SOJEVA ***LACTOCOCCUS LACTIS***

Sažetak

Sojevi ***Lactococcus*** *lactis vrste pripadaju najznačajnijoj grupi organizama koji se koristi u mljekarskoj industriji. Važnost tih bakterija je u njihovoj sposobnosti hidrolize laktoze, kazeina i citrata iz mlijeka. Razgradni produkti i oslobođeni bakterijski enzimi direktno ili indirektno utječu na teksturu i okus finalnog proizvoda. Saznanja o genetičkim i fiziološkim svojstvima sojeva* ***Lactococcus lactis*** *vrste zadnjih godina znatno su se izmijenila. Na osnovi tih saznanja, koja nisu samo značajna za znanstvena istraživanja već imaju i praktičnu primjenu, u radu je prikazana nova taksonomska klasifikacija sojeva* ***Lactococcus lactis*** *vrste. Objašnjena je uloga plazmida za najvažnija svojstva u mliječnim fermentacijama, te fiziološki i nutritivni zahtjevi u vrijeme rasta sojeva* ***Lactococcus lactis*** *vrste u mlijeku.*

Ključne riječi: ***Lc.lactis****, taksonomija, fiziologija i rast.*

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