ROSÂNGELA DE FREITAS

GENETIC AND PHENOTYPIC DIVERSITY OF AUTOCHTHONOUS DAIRY PROPIONIBACTERIA ISOLATED IN BRAZIL

Dissertation thesis presented to the Universidade Federal de Viçosa as part of the requirements of the PostGraduate Progam in Food Science and Technology, to obtain the title of *Doctor Scientiae*.

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SUMMARY

LIST OF ABBREVIATIONS

CC: clonal complexe

CIRM-BIA: Centre International de Ressources Microbiennes – Bactéries d'intérêt alimentaire

F: *P. freudenreichii* subsp. *freudenreichii*

S: *P. freudenreichii* subsp. *shermanii*

GC: gas chromatography

HS: head space

LC: Lithium glycerol

MLEE: Multilocus Enzyme Eletrophoresis

MLST: Multilocus Sequence Typing

MS: mass spectrometry

OD: optimal density

P+: lac+/nit+

P-: lac-/nit-

PCA: Principal Component Analysis

PCR: Polymerase Chain Reaction

PFGE: Pulsed-field Gel Eletrophoresis

RAPD: Randomly Amplified Polymorphic DNA

SDS: Sulfate dodecyl sodium

ST: Sequence Type

UPGMA: Unweighted Pair Group Method with Arithmetic Mean

YEL: Yeast Extract Lactate

TTC : 2,3,5-triphenyltetrazolium chloride

LIST OF FIGURES

Page

LIST OF TABLES

Page

PART 1: LITTERATURE REVIEW

PART 2: THE ALTERNATIVE METHOD TO ENUMERATE DAIRY PROPIONIBACTERIA

PART 3: THE GENETIC CARACTERIZATION OF DAIRY PROPIONIBACTERIA

PART 4 : THE PHENOTYPICAL DIVERSITY OF THE *P. FREUDENREICHII*

RESUMO

FREITAS, Rosângela de, D.Sc., Universidade Federal de Viçosa, abril de 2014. **Diversidade genética e fenotípica de bactérias propiônicas lácteas autóctones isoladas no Brasil**. Orientador: Antônio Fernandes de Carvalho; Coorientadores: Anne Thierry, Luís Augusto Nero e Monique Renon Eller.

As bactérias propiônicas lácteas, principalmente a espécie *P. freudenreichii,* apresentam um papel importante na tecnologia de produção de queijos do tipo suíço, uma vez que os produtos do seu metabolismo contribuem para o desenvolvimento de flavor e olhaduras nos queijos. Os objetivos deste estudo foram I) avaliar um método alternativo de enumeração de bactérias propiônicas, a fim de desenvolver uma metodologia segura e prática para ser empregada por indústrias de lacticínios, e II) caracterizar a diversidade genotípica e fenotípica de cepas *Propionibacterium* lácteas autóctones de fazendas do Campo das Vertentes, Minas Gerais, Brasil. Na primeira etapa deste estudo, cepas de referência de *Propionibacterium* spp., culturas *starter* comercial, e também amostras de queijo tipo Emmental foram submetidas a enumeração de propionibactéria utilizando agar lítio glycerol (LG) e placas Petrifilm™ Aerobic Count (AC) adicionadas de caldo LG. Os resultados obtidos indicam a adequação das placas Petrifilm™ AC adicionadas de caldo LG para enumeração de bactérias propiônicas, como uma ferramenta de monitoramento na indústria láctea. Entretanto, devido ao tempo requerido para o isolamento e purificação das bactérias isoladas a partir do sistema Petrifilm, o agar LG foi selecionado para a segunda parte do trabalho. Em seguida, propionibactérias lácteas foram isoladas de amostras de leite cru, solo, silagem e pastagem provenientes de cinco fazendas. Os isolados foram identificados por Reação em Cadeia de Polimerase (PCR) e a diversidade genotípica foi caracterizada por Randomly Amplified Polymorphic DNA (RAPD) and Pulsed-field Gel Eletrophoresis (PFGE). Cepas identificadas como *P. freudenreichii* foram investigadas com relação à diversidade fenotípica baseado em atividades enzimáticas (fermentação de lactose e redução de nitrato) e pela capacidade por parte das cepas em formar compostos de aroma. Além disso, a relação filogenética de cepas *P. freudenreichii* foi investigado por Multilocus Sequence Typing (MLST), baseado em um esquema MLST existente. Do total de 71

isolados apresentando caracteríticas morfológicas compatíveis com o gênero *Propionibacterium*, 50 foram efetivamente identificados como propionibactéria, no qual 25 *P. freudenreichii*, 22 *P. jensenii* e 3 *P. acidipropionici*. RAPD e PFGE distinguiram 27 e 31 perfis de bandas, respectivamente, demonstrando que o PFGE foi mais efetivo na discriminação das amostras e sugerindo que cada fazenda representa um nicho específico. Dentre as 18 cepas de *P. freudenreichii* provenientes de 3 fazendas das 5 analisadas por MLST, dois Sequence Type (ST) foram identificados, ambos anteriormente descritos no esquema MLST para a espécie. Estes ST não foram relacionados a nenhuma fonte específica e estavam aleatoriamente distribuídos na árvore filogenética. As cepas demonstraram baixo nível de diversidade nucleotídica. Com relação às atividades enzimáticas, dois fenótipos adicionais (“P+” e “P-“) foram identificados, juntamente com os fenótipos clássicos (lac-/nit+ para *P. freudenreichii* subsp. *freudenreichii* e lac+/nit- para *P. freudenreichii* subsp *shermanii*). Não houve correlação entre a capacidade destas cepas em fermentar a lactose e reduzir o nitrato. Adicionalmente, não se observou correlação entre o perfil fenotípico e a capacidade de produção de compostos voláteis. Estes resultados sugerem que o ambiente das fazendas e de produção de leite constitui um reservatório natural de cepas de *Propionibacterium*. As cepas isoladas apresentam alto potencial para futuras aplicações como cultura secundária para aplicação em queijos.

RESUME

FREITAS, Rosângela de, D.Sc., Universidade Federal de Viçosa, Avril, 2014. **Diversité genetique et phénotypique des bactéries propioniques latières autochtones isolées au Brésil**. Encadrant: Antônio Fernandes de Carvalho. Co-encadrants: Anne Thierry, Luís Augusto Nero et Monique Renon Eller.

Les bactéries propioniques laitiéres, particulièrement l'espèce *P. freudenreichii*, présentent un rôle important dans la technologie de production des fromages type suisse, depuis les produits de leur métabolisme contribuent au développement de la flaveur et des ouvertures caractéristiques (les yeux) de ces fromages. Ce travail a donc été effectué dans le but I) évaluer une procédure alternative pour compter (faire le dénombrement des) les propionibactéries, afin de développer une méthodologie fiable et pratique que soit utilisable par les industries laitières, et II) caractériser la diversité génotypique et phénotypique des souches de *Propionibacterium* laitière autochtone de fermes laitières situées dans la région Campo das Vertentes, Minas Gerais, au Brésil. Afin de metrre en place cette démarche, des souches type *Propionibacterium* spp. et des cultures *starter* commercialles, ainsi que des échantillons de fromages type Emmental ont été soumis au dénombrement des propionibactéries en utilisant le milieu solide (agar) lithium glycérol (LG), et les plaques Petrifilm^{TM} aérobic count (AC) ajoutées de bouillon LG. Les résultats obtenus indiquent la pertinence de plaques Petrifilm^{TM} AC ajoutées de bouillon LG pour le dénombrement des bactéries propioniques comme un outil de contrôle de l'industrie laitière. Cependant, en raison du temps nécessaire pour l'isolement et purification de bactérie à partir du système Petrifilm, le LG (milieu solide traditionnel) a été utilisé dans la deuxième étape du travail. De plus, propionibactéries laitiéres ont été isolés du lait cru, du sol, d'ensilage et d'herbe de cinq fermes. Les isolats ont été identifiés par Réaction en chaîne par polymérase (PCR), et leur diversité génotypique a été caractérisée par Randomly Amplified Polymorphic DNA (RAPD) and Pulsed-field Gel Eletrophoresis (PFGE). Les souches identifiées comme *P. freudenreichii* ont été étudiées en rélation à leur diversité phénotypique, ce qui a été basé sur l'activités enzymatique (la fermentation du lactose et la réduction de nitrate), et sur leur capacité à former des composés d'arôme. Puis, nous avons étudié la

relation phylogénétique des souches de *P. freudenreichii* par Multilocus Sequence Typing (MLST), basé sur un schéma existant. Parmi les 71 isolats présentant des caractéristiques morphologiques compatibles avec le genre *Propionibacterium*, 50 ont été effectivement identifiés comme propionibactéries, dont 25 étaient *P. freudenreichii*, 22 *P. jensenii* et 3 *P. acidipropionici*. RAPD et PFGE ont distingés 27 et 31 profils de bandes, respectivement, ce qui démontré que la PFGE a été plus efficace dans la discrimination des échantillons et cella a suggéré aussi que chaque exploitation a été représenté un niche spécifique. Nous avons identifié 2 Sequence Type (ST), parmi les 18 souches de *P. freudenreichii*, en étant tous les deux ST déjà décrites précédemment pour le schéma MLST pour l'espèce. Ces ST ne sont pas liés à une source spécifique et ils ont été répartis au hasard dans l'arbre phylogénétique. Les souches ont montré un faible niveau de diversité nucléotidique entre les souches. Deux phénotypes supplémentaires («P+ » et « P- ») ont été identifiés, ainsi que ceux classiques (lac-/nit+ par *P. freudenreichii* subsp. *freudenreichii* et lac+/ nit- par *P. freudenreichii* subsp. *shermanii*). Il n'y avait pas de corrélation entre la capacité de ces souches à fermenter le lactose et réduire le nitrate. De plus, aucune corrélation n'a été trouvée entre le profil phénotypique et la capacité à produire des composés volatils. Ces résultats suggèrent que l'environnement des exploitations laitières et la production de lait constitue un réservoir naturel de souches de *Propionibacterium*. Les souches isolées présentent un potentiel d'application future en tant que culture secondaire pour la production de fromages.

ABSTRACT

FREITAS, Rosângela de, D.Sc., Universidade Federal de Viçosa, April, 2014. **Genetic and phenotypic diversity of autochtonous dairy propionibacteria isolated in Brazil**. Advisor: Antônio Fernandes de Carvalho. Co-advisors: Anne Thierry, Luís Augusto Nero and Monique Renon Eller.

Dairy propionibacteria, specially the species *P. freudenreichii*, play an important role in the process of production of the Swiss-type cheeses, by the production of metabolites contributing to the development of flavour and eyes in the cheeses. The aims of this work were I) to evaluate an alternative procedure to enumerate propionibacteria to develop a reliable and practical methodology to be employed by dairy industries, and II) to characterize the genotypic and phenotypic diversity of autochtonous dairy *Propionibacterium* strains from dairy farms situated in Campo das Vertentes, Minas Gerais, Brazil. For the first part, *Propionibacterium* spp. type strains, commercial *starter* cultures, and also Emmental-type cheeses samples were subjected to propionibacteria enumeration using Lithium Glycerol (LG) agar, and Petrifilm™ Aerobic Count (AC) plates added to LG broth. The obtained results indicate the adequancy of Petrifilm™ AC plates added to LG broth for the enumeration of propionibacteria as a monitoring tool in the dairy industry. However, due to the time required for the isolation and purification of the bacteria from the Petrifilm system, the LG agar was selected for the second part of this work. Subsequently, dairy propionibacteria provided of raw milk, soil, silage and pasture from five farms were isolated. The isolates were identified by specific Polymerase Chain Reaction (PCR), and their genotypic diversity was characterized by Randomly Amplified Polymorphic DNA (RAPD) and Pulsed-field Gel Eletrophoresis (PFGE). For strains identified as *P. freudenreichii*, we were investigated their phenotypic diversity based both on the enzymatic activities (fermentation of lactose and reduction of nitrate), and their capacity to form aromatic compounds. Moreover, the phylogenetic relation of *P. freudenreichii* strains was investigated by Multilocus Sequence Typing (MLST) following an existing MLST scheme. Of 71 isolates presenting same morphologic characteristics as *Propionibacterium* genus, 50 were effectively identified as propionibacteria, from which 25 were *P. freudenreichii*, 22 were *P. jensenii* and 3 were *P. acidipropionici*. RAPD and PFGE profiles distingued 27 and 31 band profiles

respectively, demonstrated that the PFGE was more effective to discriminated the samples and suggesting that each farm represented a specific niche. On 18 strains of *P. freudenreichii* provided of 3 farms on 5, analyzed by MLST, two Sequence Type (ST) were identified, both already previously described for the MLST scheme for species. These STs were not related to any specific source, and were randomly distributed in the phylogenetic tree. The strains showed low level of nucleotide diversity among strains. For the enzymatic activities two additional phenotypics ("P+" and "P-") were identified, together with the expected ones (lac-/nit+ for *P. freudenreichii* subsp. *freudenreichii* and lac+/nit- *P. freudenreichii* subsp *shermanii*). There was no correlation between the capacity of these strains to ferment lactose and to reduce nitrate. Additionally, no correlation was found between the phenotypic profile and the capacity to produce volatile compounds. These results suggest that the environment of dairy farms and milk production constitute a natural reservoir of *Propionibacterium* strains. The isolated strains present high potential for future applications as a secondary culture for the production of cheeses.

1. GENERAL INTRODUCTION

The food industry has increased their interest in the dairy propionibacteria due to the important role in cheese production, mainly for Emmental cheese. Besides their roles in flavor and eyes development some strains present the capacity to produce folic acid, vitamin B12 and several bioprotective molecules, such as the bacteriocin. Most recently, studies have shown the potential for probiotic activity of some propionibacteria strains. *Propionibacterium freudenreichii,* the main species used as a secondary culture (ripening culture) in the cheese production, is divided into two subspecies based on phenotypic criteria. However, some studies have identified strains presenting different phenotypes then those officially accepted. The lack of reference protocol to the analyses (standardization of phenotypical tests) can be a source of errors in the strain classification, especially the experimental conditions such as the presence of O_2, the temperature and the incubation time, can influence the final result.

Due to the important role in cheese production technology of dairy propionibacteria, the knowledge of the diversity and phylogenetic relation between strains of different ecosystems is important to understant the biologic properties of the strain and to optimize their use in the food industry.The characterization of the diversity at the species can be made by different methods, based on the phenotypic and genotypic characteristics of the strains. The traditional methods, such as the analysis of morphologic and biochemical characteristics (*e.g.* the capacity of fermenting carbohydrates), are used to characterize the phenotypic diversity of microorganisms. However, the methods based on genetic information have been used with success in biodiversity studies. They have the advantage of focusing the genome, not the product of its expression, and are, unlike to phenotyping, independent of the conditions of the culture.

Objective

The main objective of this study is to carry out a genotypic and phenotypic characterization of the autochthonous dairy propionibacteria strains in a region of Brazil with Swiss-type cheese production.

Specific objectives

- To evaluate an alternative procedure to enumerate propionibacteria;
- To collect and isolate dairy propionibacteria strains from different sources (raw milk, soil, silage, and pasture) from farms in the region of Minas Gerais, by using the selective culture medium: Lithium Glycerol Agar (LG);
- To identify by genus-specific PCR, species-specific PCR and by phenotypic characterization the isolated strains;
- To characterize the genotypic diversity of dairy *Propionibacterium* strains byRAPD and PFGE;
- To analyze the phylogenetic relationships in a pool of *P. freudenreichii* strains from two distinct sources (autochthonous strains isolated in Brazil and strains from the CIRM-BIA collection) by MLST;
- To characterize the phenotypic diversity of the *P. freudenreichii* strains 1) considering the enzymatic activities that allow the differentiation of the subspecies *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp *shermanii* and 2) through the capacity of the strains to form aroma compounds by comparing the profiles of the volatile compounds that are formed.

Thus, this work consists of a literature review and of articles describing the study and development of an alternative method of enumeration of propionibacteria (Article 1), the biodiversity of dairy *Propionibacterium* strains from different sources isolated from Brazil (article 2) and the phenotypical diversity of the *P. freudenreichii* strains (Article 3).

2. LITERATURE REVIEW

2.1. Industrial application and diversity of dairy propionibacteria

The dairy (classical) propionibacteria, especially *P. freudenreichii*, are traditionally used as a secondary culture in the production of hard cheeses, such as Emmental, Gruyère, Appenzeller, Raclette, Leerdammer, Maasdammer, Jarlsberg, and Comté (Hojo *et al.*, 2007). These microorganisms are important during cheese ripening, and their function is related to the formation of aroma compounds. The biochemical reactions promoted by these bacteria occur mainly when the cheese is kept in hot chambers (20 ºC to 24 ºC). However, *P. freudenreichii* are still active during the storage of the cheese at cool temperature, as noted by Falentin *et al.* (2010a). Also, Dalmasso *et al.* (2012) identified that the expression of the different genes involved in the formation of aroma compounds remained unaltered at 4 ºC. Under this condition, *P. freudenreichii* CIRM-BIA1 uses alternative metabolic pathways for energy storage as a strategy for adaptation and long-term survival.

The flavor and aroma characteristic to cheeses matured by propionibacteria are due to the production of volatile compounds, mainly from the propionic fermentation. These bacteria are also responsible for the formation of eyes in Swiss-type cheeses; the holes come from the CO_2 originated mainly from the fermentation of lactate and aspartate (Dherbécourt *et al.*, 2010). The production of propionic acid, acetic acid, and CO_2 by the bacteria involves a complex and specific metabolic cycle, where the substrates are metabolized to pyruvate by the glycolytic or the phosphate pentose pathways, generating ATP. Part of the pyruvate produced in these steps is reduced to propionate through the transcarboxylase cycle or the Wood Werkman cycle, and a second part is oxidized to CO_2 and acetate; the

proportions of pyruvate which are directed to each pathway depends on the concentrations of substrates and products, environmental conditions, and on the characteristics of the strains, aiming at maintaining the redox balance (Thierry *et al.*, 2011). The Wood-Werkman cycle was widely investigated for *P. freudenreichii* (Deborde, 1998) and involves succinyl-CoA and methylmalonyl-CoA as intermediaries. The main characteristic of this cycle is the transcarboxylation reaction catalyzed by the enzyme methylmalonyl-CoA carboxytransferase, which transfers the carboxyl group from methylmalonyl-CoA to pyruvate, culminating with the production of oxaloacetate and propionyl-CoA. *P. freudenreichii* also exhibits the capacity of co-metabolizing aspartate and lactate (Crow, 1986). This bacterium, as it utilizes aspartate along with lactate, modulates the conversion of pyruvate by oxidative decarboxylation in order to maintain the oxidation-reduction balance, generating a greater production of acetate and CO_2 from pyruvate when compared to the fermentation of lactate by itself (Falentin *et al.*, 2010b).Additionally, *P. freudenreichii* produces several volatile, branched-chain compounds, especially 2-methylbutanoic and 3-methylbutanoic acids, from the catabolism of isoleucine and leucine, respectively (Thierry & Maillard, 2002). The production of these compounds is closely related to the biosynthesis of fatty acids from the cell wall (Dherbécourt *et al.*, 2008). The synthesis of methylbutanoic acids is constitutive and occurs through the transamination of the branched amino-acid chain, thus forming ketoacids, which are subsequently converted into acids by oxidative decarboxylation (Thierry & Maillard, 2002). *P. freudenreichii* also contributes to the flavor of cheese through the liberation of fatty acids from the degradation of lipids from the product. One main extracellular lipolytic esterase is responsible

for the production of free fatty acids from milk fat triglycerides (Dherbécourt *et al.,* 2010; Abeijon *et al.* 2014). Ten intracellular esterases were identified on the genome *P. freudenreichii* CIRMBIA1, which may be involved with the synthesis of volatile esters associated with the fruity flavor in cheese (Falentin *et al.* 2010b). Despite the fact that different biochemical reactions are related to the characteristics of each cheese, multiple studies have demonstrated that the metabolic pathway used by this bacteria, beyond the concentration of the compounds produced, are strain-dependent (Thierry *et al.*, 2011).

On the other hand, propionibacteria can be the causative agent of cheese defects. For example, the occurrence of cracking (fissures) in the cheese can be caused by the excessive production of CO_2 due to the ability of the strains to metabolize aspartate – what Wyder *et al.* (2001) define as the secondary fermentation – or by the inability of the cheese dough to retain the gas produced by the bacteria, (Daly *et al.*, 2010), which was observed in Swiss (Hettinga & Reinbold, 1975), Gouda (Britz & Jordaan, 1976), mozzarela (Massa *et al.*, 1992), Parmegiano Reggiano, and Grana Padano (Carcano *et al.*, 1995) cheeses. The presence of pigmented strains in foods is responsible for the deterioration characterized by “brown stains”, according to Baer & Ryba (1992).

Dairy propionibacteria are also known as biopreservers, since they exhibit the ability to produce metabolites with antimicrobial activity, such as propionate and acetate (Hugenholtz *et al.*, 2002). The antimicrobial action is based on the capacity of the organic acids to dissociate into the cytoplasm of the microbial cell after its diffusion through the cell wall in a non-dissociated form. This process leads to the acidification of the citoplasm, what causes the inactivates essential enzymes and damages to the DNA (Adams, 2001).

Furthermore, studies have related that strains of *P. freudenreichii*, *P. acidipropionici*, *P. thoenii*, and *P. jensenii* are able to produce antifungal peptides (Jan *et al.*, 2007), and that the inhibitive effect of fungi in foods can be enhanced when the bacteria is used with glycerol, which is commonly used as an additive (Lind *et al.*, 2010).

Some strains of Propionibacteria are used as probiotics due to their capacity of modulating the intestinal microbiota (Collado *et al.*, 2008; Myllyluoma *et al.*, 2008), to their bifidogenic (Furuichi *et al.*, 2006; Bougle *et al.*, 1999) and anti-inflammatory effects (Foligné *et al.*, 2010), to their ability to suppress the activity of procarcinogenic enzymes (Reddy *et al.*, 2000), and to their antimutagenic properties (Vorobjeva *et al.*, 2008; Vorobjeva *et al.*, 2001). Recent studies have shown that the metabolites (short-chain fatty acids) produced by *P. freudenreichii* may induce the apoptosis of cancerous cells (Jan *et al.*, 2002; Lan *et al.*, 2008). Cousin *et al.* (2012) developed a product based on a milk fermented exclusively by *P. freudenreichii* and that could be useful as part of a preventive diet meant to avoid gastric cancer and/or as a food supplement to optimize therapeutic cancer treatments.

The product used as a probiotic vector is important in ensuring the survival of the bacteria to the stress of the digestive tract. Hence, products based on fermented milk are widely used as sources for probiotic bacteria, because they are excellent matrices; beyond other effects, they exhibit a buffering capacity against acid secretions of the superior gastrointestinal tract (Salminem & Playne, 2001). Leverrier *et al.* (2005) evaluated the impact of the incorporation of *P. freudenreichii* in different food matrices on the stress tolerance of these bacteria. The authors concluded that fermented milk

statistically provides greater protection when compared to other foods under the conditions of acid and biliary stress. LeBlanc *et al.* (2006) developed both yogurt and fermented milk with the addition of *P. freudenreichii* B2336 and identified that this type of food can be used in order to reduce the number of patients presenting riboflavin deficiency, a common condition in several countries.

The dairy propionibacteria have low nutritional demand and are capable of surviving and maintaining their viability under different conditions (Thierry *et al.*, 2011). A strain of *P. freudenreichii* present the capacity to synthesize amino-acids and vitamins needed for their survival (Cummins & Johnson, 1992). Another strain of this same species was able to store energy by the polyphenol accumulation (Thierry *et al.*, 2011). In this sense, some bacterial strains have shown the ability to adapt to different stress conditions, as in the production of cheese or as probiotic. Leverrier *et al.* (2004), while evaluating the tolerance of the *P. freudenreichii* strains to acid, thermic and biliary stress, identified that the stress-adaptation proteins were overproduced, while the global protein synthesis was reduced. On the other hand, a study on the cellular process involved in the adaptation to stress by *P. freudenreichii* revealed that the genes that codify response proteins are differently expressed (Falentin *et al.*, 2010b).

Several proteome and transcriptome tools were developed to monitor *in vivo* the genetic expression in different stress conditions such as high and low temperatures, low pH, presence of biliary salts, and conditions found during the production of cheese (Anastasiou *et al.*, 2006; Dalmasso *et al.*, 2012; Falentin *et al.*, 2010; Jan *et al.*, 2001; Leverrier *et al.*, 2003). Dalmasso *et al.* (2012) identified that *P. freudenreichii* CIRM-BIA1 presents a overexpression of the

genes involved in the conversion of lactate, alanine, and serine to pyruvate, as well as in the synthesis of glycogen. Saraoui *et al.* (2013) developed a new strategy to investigate *P. freudenreichii* inside the colon of live animals (pigs) and observed that in this environment the bacterium promotes a metabolic reorientation, with activation of catabolic pathways for amino-acids, gluconate, and other available substrates, as well as the genes that codify for proteins involved in cellular division and replication, confirming the adaptation in a period of 24 hours.

Several dairy propionibacteria strains were isolated from environments such as silage, rumen fluid (Romanov *et al.*, 2004), raw milk, straw, hay, wheat, and cheeses elaborated with the intentional addition of the culture or not (Dalmasso *et al.*, 2011). According to Ogier *et al.*, (2004), several dairy products, including cheeses, represent a complete microbial ecosystem. The diversity of flavors, aromas and textures in cheeses, at times, is related to bacteria present in raw milk and in the farm environment (Martley *et al.*, 1993). Several studies have stated the importance of understanding the population dynamics for the quality of foods (Porcellato *et al.*, 2013; Silva *et al.*, 2012).

Due to the economic relevance related to the use of propionibacteria in the food industry and to the fact that these bacteria may cause defects in the flavor and textures of cheeses, the accurate identification of strains is important in ensuring the quality of the final product.

2.2. The Propionibacterium genus

2.2.1. Taxonomy

The first studies on the biochemistry involved in the production of propionic acid, acetic acid, and carbon dioxide (CO_2) were conducted by Albert Fitz (1879), who first proposed that the fermentation of calcium lactate by bacteria present in cheese with eyes was the origin of these compounds in food. In 1909, a few years after the isolation of the bacterium responsible for the formation of eyes in Emmental cheese (Von Freudenreichii & Orla-Jensen, 1906), Orla-Jensen suggested the name *Propionibacterium* to generically represent all bacteria able to produce propionic acid. The inclusion of these bacteria as a specific genus was confirmed by van Niel (1928). In his studies, these bacteria were, for the first time, organized according to the rules of binary classification, and were grouped in eight species: *P. freudenreichii*, *P. jensenii*, *P. peterssonii*, *P. shermanii*, *P. pentosaceum*, *P. rubrum*, *P. thoenii*, and *P. technicum*. The genus was included in the third edition of "Bergey's Manual of Determinative Bacteriology" (Bergey, 1930), reproducing the scheme defended by this author, after the isolation of several strains of propionibacteria.

Subsequent studies lead to the identification of new species, such as *P. raffinosaceum* (Werkman & Kendall, 1931), *P. zeae*, and *P. arabinosum* (Hictch, 1932), which were added in the fourth and fifth editions of "Bergey's Manual" and kept in the sixth and seventh editions. In the eight edition of "Bergey's Manual of Determinative Bacteriology" (Moore & Holdeman, 1974), the propionibacteria were divided into two major groups, based on a study made by Johnson & Cummins (1972), who classified the strains of propionibacteria in eight genomic groups, according to their DNA-DNA hybridization pattern and to

the peptidoglycan composition of their cell wall. Thus, *P. thoenii*, *P. jensenii*, *P. acidipropionici*, and *P. freudenreichii* (and the three subspecies of this species: *freudenreichii*, *shermanii*, and *globosum*) were recognized as “dairy” (or “classical”) species, while *P. acnes*, *P. avidum*, *P. lymphophylum*, and *P. granulosum* were classified as “cutaneous”. The same classification scheme was maintained in the first edition of Bergey’s Manual of Systematic Bacteriology (Cummins & Johnson, 1986); however, *P. freudenreichii* subsp. *globosum* was excluded. *P. rubrum*, considered a species before, was reclassified as *P. jensenii* based on numerical taxonomy studies (Britz & Riedel, 1991; Britz & Steyn, 1980).

In recent years, new species were accepted as part of the genus. For example, *P. cyclohexanicum*, isolated from spoiled orange juice (Kusano *et al.*, 1997) and *P. microaerophilum*, originated from olive mill wastewater (Koussemon *et al*, 2001), were added to the group of dairy (classical) propionibacteria. In the other hand, *P. acidificiens*, isolated from human carious dentine (Downes *et al.*, 2009), was proposed to be a member of the cutaneous group, once it is closely related to *P. australiense*, originating from granulomatous bovine lesions (Bernard *et al*., 2002). Finally, the strain *P. humerusii*, recently isolated from human humerus, has been proposed as a new species (Butler *et al.*, 2011). Currently, the *Propiniobacterium* genus is composed, between dairy and cutaneous bacteria, of twelve species (Table 1). The specie *P. lymphophilum* was reclassified as *Propionimicrobium lymphophilum* (Stackebrandt *et al.*, 2002) based on the composition – peptidoglycan and fatty acids – of its cell wall, an on its phylogenetic position when is considered the 16S rDNA sequence of this specie.

Table 1. Classification of the species of the genus *Propionibacterium* into two major groups, according to their original habitat.

Dairy (classic)	**Cutaneus**
P. acidipropionici	*P. acidifaciens*
P.cyclohexanicum	*P. acnes*
P. jensenii	*P. australiense*
P. thoenii	*P. avidum*
P. microaerophilum	*P. granulosum*
P.freudenreichii subsp. *freudenreichii*	*P. propionicum*
P.freudenreichii subsp. *shermanii*	

The taxonomic classification of propionibacteria is carried out according to genomic criteria at a species level; however, the classification regarding the subspecies is based on phenotypic characteristics. According to Wayne *et al.* (1987), the subspecies designation can be used for genetically close microorganisms with different phenotypic characteristics. According to Staley & Krieg (1987), the classification of the microorganisms into subspecies is based on subtle phenotypic variations; nonetheless, they must be consistent within the species or within a cluster of strains of a same species. Considering these definitions, *P. freudenreichii* is divided into two subspecies, following two phenotypic criteria: the capacity of fermenting lactose and the activity of the nitrate reductase enzyme. Thus, *P. freudenreichii* subsp freudenreichii produces the nitrate reductase enzyme and does not ferment lactose, while *P.*

freudenreichii subsp *shermanii* presents the opposing characteristics – positive to the use of lactose and negative to nitrate reductase. However, recent studies have identified strains that present different phenotypes: a “positive” phenotype, which is the capacity to ferment lactose and reduce nitrate, and a “negative” phenotype, which corresponds to the non-fermentation of lactose and non-reduction of nitrate (Dalmasso *et al.*, 2011; de Carvalho *et al.*, 1994).

Recent data on the genome of *P. freudenreichii* subsp *shermanii* CIRM-BIA1 can explain this phenotypic variation. Indeed, it was shown that, in this strain, the *lacZ* gene, which codifies for β-galactosidase, is on a transposon or mobile genetic element. So, it's possible that this gene may have been acquired, in this case, by a horizontal transference event, probably via phage infection (Falentin *et al.*, 2010b). On the other hand, a DNA region responsible for the nitrate reduction activity harbours some pseudogenes (Falentin *et al.*, 2010b). These sequences comprise inactive, non functional genes originated from genetic alterations (Kasomatis *et al.*, 2013). Falentin *et al.* (2010b) also identified 22 insertion sequences, which represent 3.47% (in base pairs) of the *P. freudenreichii* subsp *shermanii* CIRM-BIA1 genome. Insertion sequences and mobile genetic elements may promote genome plasticity and induce phenotypic alterations which may contribute to the adaptation of these strains to different environments.

In another study, the phylogenetic analysis of a population of 113 *P. freudenreichii* strains by Multilocus Sequence Typing (MLST) demonstrates that are no phylogenetic relation between subspecies, indicating that the classification on the species level does not reflect the ancestral relations amongst the strains (Dalmasso *et al.*, 2011)

2.2.2. General characteristics

The *Propionibacterium* genus belongs to the Actinobacteria class and it is composed of non-spore forming, non-mobile, anaerobic to slightly aerotolerant, Gram-positive bacteria, with genomes with high G+C contents (between 53 and 68%) (Stackebrandt *et al.*, 1997). In general, bacterial morphology varies according to the strain and to the conditions of the culture; it is recognized as pleomorphic (Cummins & Johnson, 1986). These bacteria are heterofermentative and are able to metabolise a great variety of substrates, such as carbohydrates, alcohols, and organic acids.

The species of *Propionibacterium* are historically divided into two major groups, based on their natural habitat (Johnson & Cummins, 1972). The “dairy or classic” group is mainly composed of bacteria isolated from dairy products and livestock environments, and the “cutaneous” group is formed by bacteria which colonize human and animal skin and intestines, which are recognized as opportunistic pathogens in immunocompromised hosts (Thierry *et al.*, 2011).

P. freudenreichii was the first between dairy propionibacteria to be found in Emmental cheese. Later, *P. acidipropionici*, *P. jensenii*, and *P. thoeni* were isolated not only from milk and dairy products, but also from soil, silage, or dairy production facilities (Cummins & Johnson, 1992). Dairy propionibacteria – *P. freudenreichii* in particular – play an important role in the technology for the Swiss cheese production. Their metabolism relies on the anaerobic conversion of carbohydrates and lactic acids into short-chain fatty acids – especially propionate and acetate – and into CO_2. These products significantly contribute

to the flavor, aroma, and to the development of eyes in cheeses (Britz & Steyn, 1980). Propionibacteria are also capable of producing aromatic (volatile) compounds derived from the catabolism of lipids and amino-acids (Thierry *et al.*, 2005), as free fatty acids and methylbutanoic acids (isovaleric acids), respectively. On the other hand, they have limited participation on the proteolysis (protein degradation) during cheese ripening (Gagnaire *et al.*, 2001).

Additionally, certain species of *Propionibacterium* are able to synthetise a number of bioprotective compounds, such as organic acids and bacteriocins (Ho *et al.*, 2009; Lind *et al.*, 2007), folic acid, proline, and vitamin B12 (Iida *et al.*, 2007), and are known for their probiotic activity (Cousin *et al.*, 2011; Saraoui *et al.*, 2013). Various studies have identified that propionibacteria could survive and maintain their activities while passing through digestive tract (Herve *et al*, 2007; Jan *et al.*, 2001; Leverrier *et al*, 2004), and are capable of adhering to epithelial cells in the intestine (Zarate *et al.*, 2002), synthetizing immunomodulatory enzymes (Foligne *et al.*, 2010), resulting in benefits to the human health, even for the prevention of some types of cancer (Cousin *et al.*, 2012). *In vivo* studies have demonstrated that some strains of dairy propionibacteria may reach high levels of viable cells (approximately 6 $\log_{10}$ UFC.g^{-1}) in the colon, a sufficient number to yield beneficial effects to the physiology of the host (Collins & Gibson, 1999). Jan *et al.* (2002a) observed that *P. freudenreichii* and *P. acidipropionici* may induce the apoptosis of colorectal carcinomas through the secretion of cytotoxic components, such as propionate and acetate.

2.2.3. Selective medium and identification of propionibacteria

Culture media with different degrees of selectivity were proposed for the isolation and identification of dairy propionibacteria. The Yeast Extract Lactate (YEL) medium, a popular and traditional media, is normally employed for enumeration of these bacteria, after incubation at 30 ºC for 5 days, under anaerobioc conditions. However, this protocol does not provide the ideal selectivity level once enterococci and lactobacilli may also form visible colonies in the medium (Thierry *et al.*, 1994). In 1994, Madec *et al.* developed the Lithium Glycerol (LG) as a medium for the isolation and enumeration of *Propionibacterium* ssp. based on their resistance to lithium, and on their capacity of fermenting glycerol under anaerobiosis. LG Agar demonstrated a higher selectivity to dairy propionibacteria when compared to YEL; this selectivity is considered suited for the counting of the group in mixed foods containing different lactic cultures (Thierry *et al.*, 1994; Thierry & Madec, 1995).

The conventional procedures for the enumeration of microorganisms in food, including probiotic starter cultures, are very arduous and time-consuming (Rosetti & Giraffa, 2005) and, may require technologies to ensure the ideal conditions for microbial multiplication (such as anaerobiosis chambers). Thus, alternative methods for the enumeration of microorganisms are mandatory for the food industry, aiming to reduce the time until the final results, as well as to assure their reliability and sensitivity (Bremser *et al*, 2011; Brichta-Harhay *et al*., 2008).

Phenotypic methods could be utilized to characterize the diversity of Propionibacteria in a sample at a species level. Traditional methods, such as

the analysis of morphological and biochemical characteristics (*e.g.* the capacity of fermenting carbohydrates), can be used to analyze the characteristics expressed by microorganisms (Charteris *et al.*, 1997). Nevertheless, due to the limited discriminatory capacity of the methods, added to the strong dependence of the results on environmental conditions, the phenotypic tests have been shown to be insufficient to differentiate bacteria. In order to solve this problem, the methods based on genetic information have been used and have the advantage of focusing the genome, not the product of its expression, and are, contrarily to phenotyping, independent of the conditions of the culture (Germond et al., 2003).

2.3. Genotypic methods for the identification of bacteria

The genotypic (molecular) methods for the identification and classification of bacteria are based on the variations of their genomes sequences, resulting in studies on the evolution and phylogeny of these microorganisms (Juste *et al.*, 2008). These methods are based on the variety of information and diversity of the bacterial chromosomal DNA's, which replace the enzymes as the main markers utilized in the analysis of genetic variation in a population (Hamrick & Godt, 1989). These techniques have revolutionized the studies of microbial ecology, since they are not influenced by the physiological state of the cells, which enables the microbial identification at a strain level (Giraffa & Neviani, 2000).

To be considered as efficient, a molecular typing test has to present a high discriminatory power to allow the differentiation of strains of a same

species. The selection of the most fitting method depends on its cost, the capacity of data processing, and reproducibility (Randazzo *et al*., 2009). However, the use of two or more genotypic methods is, at times, mandatory for the precise differentiation of the microorganisms, as well as for studies on the taxonomy and evolution of a particular species (Perez et al., 2002).

2.3.1. Pulsed-Field Gel Electrophoresis

Pulsed-Field Gel Electrophoresis (PFGE) is a tool considered as “the gold standard” for genotyping, guided by the existence of restriction sites in the bacterial genome, and it is widely used to differentiate same-species strains (Suomalainen *et al*., 2008). The identification and differentiation of the microorganisms is based on the separation of large DNA fragments, which are particular for a similar pattern of restriction regions in the genome, by electrophoresis (under pulsed field). This is possible through the reorientation of the genetic material in a polimeric gel under alternating electric fields (Lai *et al.*, 1989; McCartney, 2002).

The process involves the incorporation of bacterial cells in agarose and fragmentation of the genomic DNA by one or more restriction endonucleases, which recognize specific nucleotide sequences in the bacterial genome. The enzymes have few recognition sites, and this process generates specific fragment profiles for each bacterial genome (Randazzo *et al.*, 2009). The fragments are then separated according to their molecular size, by means of a gradient, alternating-tension system, since the molecules are too large to be split by the conventional electrophoresis process (McCartney, 2002). The

differentiation of the strains occur by obtaining the DNA banding patterns (pulsotype) for each strain or specie, and these patterns are analyzed and compared by softwares available on the market (Olive & Bean, 1999).

The technical resolution of PFGE depends, among other factors, on the composition and concentration of agarose, the tension of the electric current (voltage), the pulse time, and the total time of the electrophoretic run, as well as on the stages before to the electrophoresis (DNA-extraction stages). Thus, it is of utter importance that the integrity of the DNA be preserved, and, in order to achieve this, the extraction of the chromosomal material is conducted after the incorporation of bacterial cells to agarose blocks, which provides the molecules with mechanical protection (Pfaller *et al.*, 1994).

The PFGE technique can be used in epidemiological studies, providing a fast detection of associated outbreaks and sometimes in the differentiation of strains from a same species, as well as in the estimative of bacterial genomes size (Goering, 1993). Gautier *et al.* (1992) conclude that PFGE is a method adequate (suitable) to determine the size of the *Propionibacterium* genome, when its utilized the *Xba*I and the *Ssp*I enzymes in a bacterial population belonging to the four main dairy species: *P. freudenreichii*, *P. jensenii*, *P. thoenii*, and *P. acidipropionici*. Jan *et al.* (2002b), during their study on the survival and beneficial effects of *P. freudenreichii* in the human intestine, showed that the technique demonstrated high efficiency in the differentiation of the strains in distinct stages of the treatment.

Suomalainen *et al.* (2008) used PFGE to detect *P. freudenreichii* subsp *shermanii* JS in human faeces, when administered with other *Propionibacterium* spp. strains, in a study for the probiotic potential evaluation. The authors

identified, by means of the dendrograms generated by this method, that the technique was capable of detect the presence of genetic differences (and not similarities) between the strains and is, therefore, appropriate for this type of study.

2.3.2. Randomly Amplied Polymorphic DNA

The Randomly Amplified Polymorphic DNA (RAPD) technique is a variation of the Polymerase Chain Reaction (PCR) based on the utilization of short, randomly chosen primers, with approximately 10 to 15 nucleotides, for the detection of DNA polymorphisms. The primers used have an arbitrary sequence, which allows for molecular analyses without the need for previous knowledge about the species to be studied (Perry *et al*., 2003).

For the amplification of the genetic material, the primers must anneal to complementary sequences in opposing extremities of a sequence in the bacterial DNA, allowing the exponential amplification of this segment through the action of the enzyme Polymerase (Williams *et al.*, 1990). Finally, the PCR products are visualized as a band in an agarose gel by electrophoresis.

The low cost, simplicity, and rapidity of this technique are the main advantages observed (Bem Amor *et al.*, 2007). However, it may present low reproductivity if there's no appropriate standardization or optimization of the analytic parameters (Randazzo *et al.*, 2009).

Several studies have demonstrated the efficiency of the RAPD method in determining the diversity of microbial populations (Baruzzi *et al.*, 2002; Giraffa & Neviani, 2000, 1999; Jenkins *et al.*, 2002; Klein *et al.*, 1998; Olive & Bean,

1999). According to Fessler *et al.* (1999), the utilization of the RAPD to differentiate strains of dairy propionibacteria that were responsible for the occurrence of browm spots (defects) on cheese and raw milk was considered appropriate. Rossi *et al.* (1998) noted that the method was able to differentiate strains of classic (dairy) propionibacteria from distinct sources (milk, cheese, whey, and feed), with formation of clusters for the species found.

2.3.3. Multilocus Sequence Typing

The Multilocus Sequence Typing (MLST) test was proposed by Maiden *et al.* (1998) as an alternative technique to the Multilocus Enzyme Electrophoresis (MLEE) for the differentiation of virulent strains of *Neisseria meningitis*. The protein analysis, in spite of presenting positive results, has the limitation of verifying the expression of genes that may be affected by environmental conditions. In that sense, the bacterial characterization by MLST is a considerable alternative, since the discrimination of microbial isolates is carried out from the comparison amongst the sequences of gene fragments that constitute bacteria (Jolley *et al.*, 2004).

The MLST technique was developed as a universal approach to epidemiological studies, due to its high reproductivity and to the possibility of data sharing on the Internet (Urwin & Maiden, 2003). Thus, it enables the estimation of the clonal origin, the calculation of the homologous recombination index, and the phylogenetic analysis amongst strains of a same species.

For the development of a MLST scheme, it is necessary to define the internal fragments of the bacterial DNA to be analyzed. These fragments must

be genes which codify for enzymes involved in the main metabolic pathways, named housekeeping genes, which are located in well-conserved regions of the bacterial genome, with an average length of 450 to 500 bp (Maiden *et al.*, 1998). After the amplification and sequencing of these DNA fragments, each allele receives an arbitrary number, and the cluster of alleles of each gene determines an allelic profile, or sequence type (ST), of the isolate (Ndoye *et al.*, 2011). The alleles are considered equally distinct when there are differences of, at least, one nucleotide between the sequences. At present, some scientists have been adding non-conserved genes to the MLST studies (such as virulent genes or antibiotic-resistant genes), aiming to increase the consistency and discriminatory power of this technique (McDowell *et al.*, 2012; Nemoy *et al.*, 2005). Nemoy *et al.* (2005) observed that the addition of two genes which confers antibiotics resistance increased the discriminatory index of the MLST method for beta-galactosidase-producing *E. coli*, from 0.786 to 0.916.

The MLST technique has been used in epidemiologic and evolutionary (phylogenetic) studies a series of pathogenic bacteria, including *Staphylococcus aureus* (Enright *et al.*, 2000), *Campylobacter jejuni* (Dingle *et al.*, 2001), *Enterococcus faecalis* (Nallapareddy *et al.*, 2002), *Escherichia coli* (Nemoy *et al.*, 2005), *Listeria monocytogenes* (Parisi *et al.*, 2010) and, *P. acnes* (McDowell *et al.*, 2011), and non-pathogenic bacteria, such as *Lactobacilus casei* (Diancourt *et al.*, 2007), *Bifidobacterium* (Brisse, 2009), *Streptococcus thermophillus* (Delorme *et al.*, 2010), and *Lactococcus lactis* subsp *lactis* (Passerini *et al.*, 2010).

Considering the dairy propionibacteria, an MLST scheme was developed for *P. freudenreichii*. Dalmasso *et al.* (2011), in a research about the molecular

diversity and the population structure of 113 strains of *P. freudenreichii*, identified 46 STs and determined that the species constitutes a cohesive population with low level of polymorphisms. The authors also concluded that there is no relation between the STs found and the source of the isolates.

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3. THE ALTERNATIVE METHOD TO ENUMERATE PROPIONIBACTERIA

PAPER 1: Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic Count plates added to Lithium Glycerol broth

Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic Count plates added to Lithium Glycerol broth

Resumo

As bactérias propiônicas derivadas de produtos lácteos são importantes culturas *starter* (fermentos) utilizadas na elaboração de queijos tipo suíço e Emmental, e o seu monitoramente é obrigatório para a controle da qualidade adequada. Este estudo objetivou avaliar um método alternativo de enumeração de bactéria propiônica, a fim de desenvolver uma metodologia confiável e prática a ser empregada nas indústrias de laticínios. A atividade inibitória de 2,3,5-cloreto de trifeniltetrazólio (TTC) foi testado em cinco cepas de referência (CIRM 09, 38, 40 e 116); TTC a 0,0025% (p/v) não causou inibição à multiplicação microbiana, com exceção de uma cepa (CIRM 116). Posteriormente, as quatro cepas resistentes ao TTC, três culturas *stater* comerciais (PS-1, PB-I e CHOO) e doze amostras de queijo tipo Emmental foram submetidos a enumeração de propionibacteria utilizando agar lítio glicerol (LG) e placas Petrifilm™ aerobic count (AC) adicionado de caldo LG (incubação sob anaerobiose, a 30 ºC por 7 dias). O Petrifilm™ AC adicionado de caldo LG apresentou contagens mais elevadas que o agar LG ($P<0,05$) para apenas duas cepas de referência (CIRM 39 e 40) e para todos as culturas comerciais. As contagens obtidas em amostras de queijo para ambos os métodos não apresentou diferenças significativas ($P<0,05$). Foram observados índices de correlação significativa entre as contagens registradas em ambos os métodos ($P<0,05$). Estes resultados demonstram a confiabilidade das placas Petrifilm™ AC adicionadas de caldo LG na enumeração seletiva de *Propionibacterium* spp., apesar de algumas limitações observadas em culturas comerciais específicas.

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Selective enumeration of propionibacteria in Emmental-type cheese using Petrifilm™ Aerobic Count plates added to Lithium Glycerol broth

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Propionibacteria derived from dairy products are relevant starter cultures for the production of Swiss and Emmental-type cheeses, and the monitoring of which is mandatory for proper quality control. This study aimed to evaluate an alternative procedure to enumerate propionibacteria, in order to develop a reliable and practical methodology to be employed by dairy industries. 2,3,5-triphenyltetrazolium chloride (TTC) inhibitory activity was tested against five reference strains (CIRM 09, 38, 39, 40 and 116); TTC at 0·0025% (w/v) was not inhibitory, with the exception of one strain (CIRM 116). Subsequently, the four TTC-resistant strains, three commercial starter cultures (PS-1, PB-I, and CHOO) and twelve Emmental-type cheese samples were subjected to propionibacteria enumeration using Lithium Glycerol (LG) agar, and Petrifilm™ Aerobic Count (AC) plates added to LG broth (anaerobic incubation at 30 °C for 7 d). Petrifilm™ AC added to LG broth presented high counts than LG agar ($P<0·05$) for only two reference strains (CIRM 39, and 40) and for all commercial starter cultures. Cheese sample counts obtained by both procedures did not show significant differences ($P<0·05$). Significant correlation indexes were observed between the counts recorded by both methods ($P<0·05$). These results demonstrate the reliability of Petrifilm™ AC plates added to LG broth in enumerating select *Propionibacterium* spp., despite some limitations observed for specific commercial starter cultures.

Keywords: Propionic bacteria, Petrifilm™ AC, cheese, Emmental.

Microorganisms belonging to the genus *Propionibacterium* are Gram-positive, usually pleomorphic, non-spore-forming, non-motile, range from anaerobic to aerotolerant, generally catalase-positive and are able to ferment lactate to short-chain fatty acids, mainly propionate and acetate (Carvalho et al. 1995; Jan et al. 2002; Turgay et al. 2011). *Propionibacterium* spp. have been isolated from the skin or dairy products, and examples of their species are *Prop. acidipropionici, Prop. freudenreichii, Prop. jensenii* and *Prop. thoenii* (Tilsala-Timisjarvi & Alatossava, 2001; Jenkins et al. 2002).

Propionibacteria originating from milk play an important role in the production of Swiss and Emmental-type cheeses: during maturation, lactic acid bacteria produce lactate, which is converted to propionic acid, acetic acid and carbon dioxide, and contribute to the flavour and the emergence of eyes in these cheeses (Piveteau, 1999; Thierry et al. 2004). Additionally, propionibacteria are able to synthesise biopreservative compounds, such as bacteriocins, as well as folic acid, proline and vitamin B12, as shown by several studies that have assessed the probiotic potential of this genus (Mantere-Alhonen, 1995; Meile et al. 2008; Cousin et al. 2011).

Propionibacterium spp. growth in dairy products must be controlled because it is necessary to ensure minimal concentrations in end products to allow for the development of typical characteristics and probiotic activity (Cousin et al. 2011). For monitoring in the dairy products industry, Yeast Extract Lactate (YEL) culture medium is usually employed to enumerate propionibacteria, after incubation at 30 °C for 5 d; however, this protocol does not provide adequate selectivity because enterococci and lactobacilli can also form

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visible colonies (Thierry et al. 1994). Madec et al. (1994) developed a selective medium named Lithium Glycerol (LG) based on the resistance of propionibacteria to lithium and their ability to ferment glycerol under anaerobiosis. LG agar has shown higher selectivity for propionibacteria when compared with YEL; this selectivity is considered adequate for the enumeration of this group in mixed cultures or foods containing different lactic cultures (Thierry et al. 1994; Thierry & Madec, 1995).

The conventional procedures for the enumeration of microbial groups in foods, including starter and probiotic cultures, are very time-consuming and require a variety of apparatus to ensure ideal conditions of microbial growth (as example, anaerobiosis generators and chambers). Thus, reliable alternative methods for enumerating microorganisms are needed for food industries, enhancing laboratory work and shortening the time required to obtain final results (Brichta-Harhay et al. 2008; Bremser et al. 2011). The Petrifilm™ system (3M Microbiology, St. Paul, MN, USA) is an example of such an alternative method that increases the speed of enumeration (McGregor et al. 1995; Bulte et al. 1998; Beloti et al. 2002). As a ready-to-use system, Petrifilm™ represents an improvement in speed and efficiency in conducting analyses, as well as a reduction in the laboratory work required to obtain results (Senyk et al. 1987). Petrifilm™ Aerobic Count (AC) plates have been used in combination with selective culture media for the enumeration of lactic acid bacteria (LAB), with reliable results that justify their use in the monitoring of these microorganisms in fermented foods (Champagne et al. 1994; Pattison et al. 1998; Nero et al. 2006, 2008; Ortolani et al. 2007; Gonçalves et al. 2009; Miranda et al. 2011). However, the performance of this system for the enumeration of propionic acid bacteria has not been evaluated until now.

The aim of this study was to evaluate the performance of Petrifilm™ AC associated with LG broth as an alternative method for enumerating *Propionibacterium* spp. strains and commercial starter cultures, and also propionibacteria from Emmental-type cheeses.

Materials and methods

Microbial cultures and cheese samples

Five reference strains of *Propionibacterium* spp. (*Prop. freudenreichii* subsp. *shermanii* CIRM 01, *Prop. acidipropionici* CIRM 38, *Prop. jensenii* CIRM 39, *Prop. thoenii* CIRM 40, and *Prop. freudenreichii* subsp. *freudenreichii* CIRM 116) were obtained from the Centre International de Ressources Microbiennes – Bactéries d'Intérêt Alimentaire (CIRM-BIA, INRA, Rennes, France) and stored at −20 °C in YEL agar. A loop of each stock culture was added to LG broth and incubated at 30 °C for 24–48 h; the obtained cultures were then streaked on LG agar plates and incubated under the same conditions. Isolated colonies were then transferred to LG broth and incubated at 30 °C to obtain cultures with turbidity similar to McFarland tube 1, which approximately corresponds to 3×10^8 colony forming units per ml (CFU/ml).

Three commercial lyophilised cultures used in Emmental cheese production were included in this study: PS-1 (Chr. Hansen A/S, Horsholm, Denmark), PB-I (Sacco, Cadorago, Italy), and CHOO (Zit™ Eyes, Danisco, Niebüll, Germany). All stock cultures were kept at −20 °C, and upon use, approximately 1 g each culture was transferred to LG broth and incubated at 30 °C for 24 h. Then, colonies isolation and culture preparation steps were performed as described previously for reference strains.

Emmental-type cheeses produced by three different dairy companies (one French and two Brazilian) were purchased from various retail stores and kept under refrigeration until microbiological analysis.

Culture media

LG broth was previously prepared as described by Madec et al. (1994) with the following composition (in one litre): 10 g lithium lactate, 10 g peptone, 10 g yeast extract, 6 g glycerol, 1 g powdered milk, 50 mg bromocresol purple, 328 mg K_2HPO_4 and 56 mg $MnSO_4$ (all chemicals from Sigma Aldrich, St. Louis, MO, USA). LG agar was prepared by adding bacteriological agar at 1·5% (w/v) (Merck KGaA, Darmstadt, Germany) in LG broth. After autoclaving, a cocktail of commercial antibiotics (obtained from the kit Pal Probiobac™, Laboratoires Standa, Caen, France) was added to the LG broth and agar to confer selectivity for propionibacteria.

YEL broth was prepared according Malik et al. (1968) with the following composition (in one litre): 10 g peptone, 20 ml sodium lactate, 10 g yeast extract, 328 mg K_2HPO_4 and 56 mg $MnSO_4$ (all chemicals from Sigma-Aldrich). YEL agar was prepared by adding bacteriological agar at 1·5% (w/v) (Merck) in YEL broth. YEL broth and agar were used as non-selective culture media for propionibacteria.

2,3,5-Triphenyltetrazolium chloride (TTC) reduction by propionibacteria

All reference strains were diluted ten-fold in 0·85% NaCl (w/v) and plated (in duplicate) by pouring using YEL agar added to TTC (Sigma-Aldrich), in order to achieve end concentrations of 0·0025 and 0·0050% (w/v) (Beloti et al. 1999). As controls, the same dilutions were plated on YEL agar without TTC. All plates were incubated at 30 °C for 7 d under anaerobiosis (Anaerocult, Merck); afterwards, the resulting colonies were enumerated and the counts expressed in CFU/ml. During enumeration, the ability of the *Propionibacterium* spp. strains to reduce TTC, thus forming red colonies, was recorded. This experiment was conducted in triplicate.

Propionibacteria enumeration

Immediately after recovering of *Propionibacterium* spp. strains and propionibacteria commercial starter cultures,

Table 1. Mean counts (±SD) of *Propionibacterium* spp. reference cultures plated on Yeast Extract Lactate (YEL) agar with 2,3,5-Triphenyltetrazolium chloride (TTC) at 0·0025 and 0·0050% (w/v)

Propionibacterium spp.	YEL added to TTC		Control†	Statistics‡
Cultures	0·0025%	0·0050%		
CIRM 01	9·70±0·18[a]	9·29±0·91[a]	9·67±0·19[a]	$F_{(2,6)}=0·520$, $P=0·619$
CIRM 38	8·37±0·35[a]	8·31±0·44[a]	8·30±0·34[a]	$F_{(2,6)}=0·025$, $P=0·976$
CIRM 39	9·34±1·01[a]	nr	9·67±0·29[a]	$F_{(1,4)}=0·298$, $P=0·614$
CIRM 40	8·36±1·14[a]	nr	8·60±0·55[a]	$F_{(1,4)}=0·111$, $P=0·756$
CIRM 116	nr	nr	9·77±0·12	—

†Control: *Propionibacterium* spp. cultures were plated in YEAL agar without TTC, to show their real counts without inhibitory agent (TTC); ‡Analysis of Variance (ANOVA), *F*: ANOVA value, *P*: level of significance. Mean values in a same row followed by distinct superscript letters are significantly different ($P<0·05$). nr: not recorded

the obtained cultures were subjected to ten-fold dilution using LG broth. For cheese samples, 25 g of each cheese was obtained with a sterile knife and transferred to individual sterile bags containing 225 ml of a 0·85% NaCl (w/v) solution, homogenised, and subjected to ten-fold dilution using LG broth. Four dilutions of each culture and cheese sample were selected and pour plated on LG agar (duplicate) and in Petrifilm™ AC plates. The plates were incubated at 30 °C for 7 d at anaerobic conditions (Anaerocult, Merck). After incubation, the colonies formed on the plates were counted and the results expressed in CFU/ml (cultures) or CFU/g (cheeses). Reference strains and commercial cultures were tested in triplicate, and twelve cheese samples were evaluated (four from each dairy company).

Molecular identification of Propionibacterium spp.

To verify the adequate selectivity of the tested culture media, a total of 70 colonies recorded from cheese samples plated on LG agar and Petrifilm™ AC added to LG broth were subjected to morphological characterisation by Gram staining and genus-specific PCR (Dasen et al. 1998). The selected colonies were streaked on YEL agar (incubation at anaerobic conditions, Anaerocult, Merck, at 30 °C for 48 h), and isolated colonies were transferred to YEL broth (incubated at 30 °C for 48 h). The purified cultures were subjected to DNA extraction using the Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA). The multiplex PCR reactions contained 12,5 µl of 2 × Go Taq Green Master Mix (Promega), 1 µl of the primer bak4 (AGGAGGTGATCCARCCGCA, reverse, at 100 µM), 1 µl of the primer bak11w (AGTTTGATCMTGGCTCAG, forward, at 100 µM), 0,5 µl of the primer gd1 (TGCTTTCGATACGGG-TTGAC, forward, at 100 µM), 2 µl of extracted DNA (in a minimal concentration of 15 ng/µl), and ultra-pure PCR water (Promega) to a final volume of 50 µl. The PCR conditions were initial denaturation at 95 °C for 3 min; 35 cycles at 95 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s; and a final extension step at 72 °C for 10 min. Amplification products were mixed with 20x GelRed stain (Biotium Inc., Hayward, CA, USA) at a ratio of 5:1, and the products were submitted to electrophoresis in a 1% agarose gel made with 0·5 × TBE. PCR products of 889 bp (primers bak4 and gd1) were recorded as typical for *Propionibacterium* spp., and PCR products of 1508 bp (primers bak11w and bak4) were recorded as positive controls for the reactions.

Statistical analysis of results

The counts of reference strains plated on YEL agar with and without added TTC were compared to verify the inhibition of this substance over the propionibacteria. In addition, the TTC reduction capacity of the cultures was observed.

The counts of reference strains, commercial cultures and cheese samples obtained on LG agar and Petrifilm™ AC added to LG broth were converted to $\log_{10}$, and the mean counts were compared by ANOVA and Tukey tests ($P<0·05$). In addition, the $\log_{10}$ counts were compared by linear regression ($P<0·05$). All analyses were performed using software Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA).

Results and discussion

All tested strains were capable of reducing TTC, forming red colonies in YEL agar, and inhibitory activity of TTC was observed only in specific situations (Table 1): CIRM 39, CIRM 40, and CIRM 116 presented very small colonies when plated on YEL agar added to TTC at 0·0050% (w/v), making proper and reliable enumeration difficult and indicating an inhibitory activity of TTC. However, 0·0025% (w/v) TTC did not inhibit the growth of all reference strains, except CIRM 116 TTC (Table 1). TTC at 0·0050% (w/v) determined an inhibitory activity that resulted in small sized colonies of some *Propionibacterium* spp. strains; however, when the colonies enumeration was possible, the final counts did not differ from the controls in YEL agar without TTC ($P>0·05$, Table 1). This dye is used as a colour indicator in a number of culture media for the enumeration of bacteria (Senyk et al. 1987), as in some Petrifilm™ plate systems (e.g., AC), because it facilitates the visualisation of the resulting colonies by its reduction and the appearance of red pigment (Kenner et al. 1961). Based on these results, CIRM 116 was not considered in the following methods conducted to evaluate the performance of Petrifilm™ AC plates added to LG broth to enumerate propionibacteria.

Table 2. Mean counts (±SD) of Propionibacterium spp. reference strains, commercial cultures, and from Emmenthal cheese type samples subjected to enumeration by Lithium Glycerol (LG) agar and Petrifilm™ AC plates added to LG broth

Group	Sample	*n*	LG agar	Petrifilm™ added to LG broth	Statistics†
Reference strains	CIRM 01	3	$9{\cdot}86 \pm 0{\cdot}10^{a}$	$9{\cdot}82 \pm 0{\cdot}08^{a}$	$F_{(1,4)}=0{\cdot}21$, $P=0{\cdot}668$
	CIRM 38	3	$8{\cdot}02 \pm 0{\cdot}17^{a}$	$8{\cdot}13 \pm 0{\cdot}20^{a}$	$F_{(1,4)}=0{\cdot}61$, $P=0{\cdot}479$
	CIRM 39	3	$6{\cdot}93 \pm 0{\cdot}82^{a}$	$8{\cdot}49 \pm 0{\cdot}30^{b}$	$F_{(1,4)}=9{\cdot}44$, $P=0{\cdot}037$
	CIRM 40	3	$8{\cdot}24 \pm 0{\cdot}30^{a}$	$9{\cdot}11 \pm 0{\cdot}35^{b}$	$F_{(1,4)}=10{\cdot}69$, $P=0{\cdot}031$
	all strains	12	$8{\cdot}26 \pm 1{\cdot}16^{a}$	$8{\cdot}89 \pm 0{\cdot}71^{a}$	$F_{(1,22)}=2{\cdot}57$, $P=0{\cdot}123$
Commercial cultures	PS-1	3	$8{\cdot}24 \pm 0{\cdot}28^{a}$	$9{\cdot}29 \pm 0{\cdot}18^{b}$	$F_{(1,4)}=96{\cdot}15$, $P=0{\cdot}001$
	PB-I	3	$8{\cdot}82 \pm 0{\cdot}06^{a}$	$9{\cdot}54 \pm 0{\cdot}11^{b}$	$F_{(1,4)}=9{\cdot}59$, $P=0{\cdot}036$
	CHOO	3	$5{\cdot}91 \pm 0{\cdot}79^{a}$	$8{\cdot}01 \pm 1{\cdot}08^{b}$	$F_{(1,4)}=30{\cdot}49$, $P=0{\cdot}005$
	All cultures	9	$7{\cdot}56 \pm 1{\cdot}54^{a}$	$8{\cdot}94 \pm 0{\cdot}9^{b}$	$F_{(1,16)}=5{\cdot}44$, $P=0{\cdot}033$
Cheese samples	Company 1	4	$5{\cdot}78 \pm 0{\cdot}41^{a}$	$6{\cdot}73 \pm 0{\cdot}40^{b}$	$F_{(1,6)}=10{\cdot}78$, $P=0{\cdot}017$
	Company 2	4	$7{\cdot}97 \pm 1{\cdot}25^{a}$	$8{\cdot}90 \pm 1{\cdot}11^{a}$	$F_{(1,6)}=1{\cdot}26$, $P=0{\cdot}304$
	Company 3	4	$8{\cdot}41 \pm 0{\cdot}55^{a}$	$8{\cdot}56 \pm 0{\cdot}58^{a}$	$F_{(1,6)}=0{\cdot}14$, $P=0{\cdot}725$
	All companies	12	$7{\cdot}39 \pm 1{\cdot}41^{a}$	$8{\cdot}06 \pm 0{\cdot}21^{a}$	$F_{(1,22)}=1{\cdot}58$, $P=0{\cdot}221$

†Analysis of Variance (ANOVA), *F*: ANOVA value, *P*: level of significance. Mean values in a same row followed by distinct superscript letters are significantly different ($P<0{\cdot}05$)

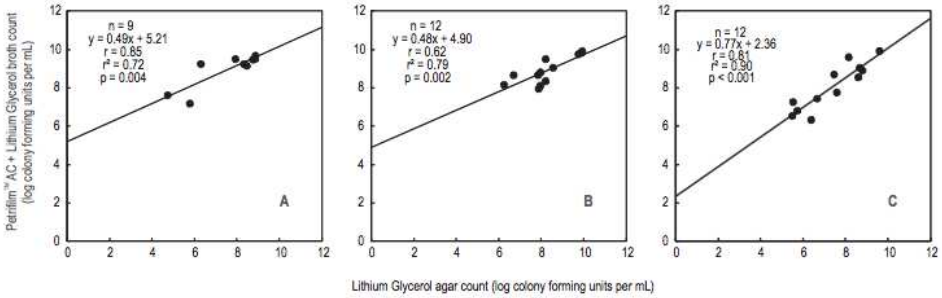

Fig. 1. Linear regression parameters and dispersion of propionibacteria counts (in log of colony forming units per ml) of reference strains (a), and commercial cultures (b), and Emmental-type cheese samples (c) obtained by Lithium Glycerol (LG) agar and Petrifilm™ AC added to LG broth. In each graph: *n*: number of samples; *r*: correlation index; r^2: coefficient of determination; *P*: level of significance.

Figure 1 shows the linear regression parameters and dispersion of propionibacteria counts of reference and commercial cultures and cheese samples obtained by LG agar and Petrifilm™ AC plates associated with LG broth. The correlation coefficients obtained for each data group were significant ($P<0{\cdot}05$), demonstrating the levels of equivalence of both methods. Despite presenting significant correlations (Fig. 1), Petrifilm™ AC added to LG broth trended to present higher colony counts when compared to the conventional plating system, although the differences were not significant difference according to ANOVA ($P>0{\cdot}05$, Table 2). Although the observed differences between the tested methodologies, the results indicate the feasibility of the Petrifilm™ AC for enumerating propionibacteria when compared with the conventional plating system, as observed in previous studies focusing other starter cultures and lactic bacteria, microorganisms that present similar biochemical and technological properties (Champagne et al. 1994; McGregor et al. 1995; Pattison et al. 1998; Nero et al. 2006, 2008; Ortolani et al. 2007; Gonçalves et al. 2009; Miranda et al. 2011).

The morphological analysis of the 70 colonies selected from both LG agar and Petrifilm™ AC plates added to LG broth indicate the adequate selectivity of this culture medium for this genus: all of the colonies presented typical *Propionibacterium* spp. characteristics (Gram positive, pleomorphic). Furthermore, PCR of the 70 isolates resulted in 889 bp products, confirming the identification of *Propionibacterium* spp. (Dasen et al. 1998). Based on the obtained data, the presence of lithium, glycerol, and a cocktail of antimicrobials, associated with incubation under anaerobiosis were sufficient to provide adequate selectivity of the culture medium to enumerate only *Propionibacterium* spp., either by conventional plating procedure or by association with Petrifilm™ AC plates. Although typically employed for propionibacteria enumeration in cheese, LG selective agents can inhibit the growth of some specific strains of this genus (Rossi et al. 2000), which can explain

some of the differences observed in the present study (Table 2). Because commercial cultures usually are composed of several strains of starter cultures (Dworkin et al. 2006), some of them may have been inhibited by the selective agents in LG agar and were not able to form visible colonies (Table 2). In contrast, the Petrifilm™ AC plates added to LG broth may have provided better conditions for strains to form visible colonies, considering the presence of additional nutritive substances of the system, and also the presence of a dye, TTC, that improves the visualisation of colonies.

Propionibacterium spp. were able to reduce TTC properly, and the dye did not show inhibitory activity against the strains, when present at 0·0025% (w/v), except for the CIRM 116 reference strain. When associated with LG broth, the Petrifilm™ AC plates presented adequate selectivity and the proper recovery of *Propionibacterium* spp. reference strains and propionibacteria commercial starter cultures, as well as from the Emmental-type cheese samples. The obtained results indicate the adequacy of Petrifilm™ AC plates added to LG broth for the enumeration of propionibacteria as a monitoring tool in the dairy industry.

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4. THE GENETIC CARACTERIZATION OF DAIRY PROPIONIBACTERIA

PAPER 2: Biodiversity of dairy *Propionibacterium* isolated from dairy farms in Minas Gerais, Brazil

Biodiversity of dairy *Propionibacterium* isolated from dairy farms in Minas Gerais, Brazil

Resumo

As bactérias propiônicas lácteas são utilizadas como culturas secundária (culturas de maturação) na produção de queijos tipo suíço, e algumas estirpes apresentam potencial probiótico. Este estudo investigou a biodiversidade de cepas de *Propionibacterium* lácteas autóctones de fazendas de região produtora de queijo tipo suíço, situadas no Brasil. RAPD e PFGE foram utilizadas como técnicas para a tipagem molecular e MLST foi aplicado em análise filogenética de cepas de *P. freudenreichii*. Os resultados demonstraram considerável diversidade genética na microbiota autóctone, uma vez que três das principais espécies de *Propionibacterium* láctea foram observadas distribuídas aleatoriamente dentre as amostras coletadas em diferentes localidades. Isolados de diferentes fazendas demonstraram perfils genéticos distintos, sugerindo que cada localidade representa um nicho específico. Além disso, os STs identificados em cepas de *P. freudenreichii* por MLST não foram relacionados com nenhuma origem específica e foram distribuídas aleatoriamente na árvore filogenética. As fazendas e o ambiente de produção de leite demonstrou ser um reservatório de cepas de *Propionibacterium*, as quais são importantes para uso futuro como possíveis culturas *starter* ou probióticas, assim como na prevenção de defeitos em queijos.

Biodiversity of dairy *Propionibacterium* isolated from dairy farms in Minas Gerais, Brazil

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Abstract

Dairy propionibacteria are used as ripening cultures for the production of Swiss-type cheeses, and some strains have potential for use as probiotics. This study investigated the biodiversity of autochthonous dairy *Propionibacterium* in farms a Brazilian of region with Swiss-type cheese production. RAPD and PFGE were used for molecular typing and MLST was applied for phylogenetic analysis of strains of *P. freudenreichii*. The results showed considerable genetic diversity of the autochthonous microbiota, since three of the main dairy *Propionibacterium* species were observed to be randomly distributed among the samples collected from different locations. Isolates from different farms showed distinct genetic profiles, suggesting that each location represented a specific niche. Furthermore, the STs identified for the strains of *P. freudenreichii* by MLST were not related to any specific origin and were randomly distributed in the phylogenetic tree. The environment of dairy farms and milk production proved to be a

reservoir for *Propionibacterium* strains, which are important for future use as possible starter cultures or probiotics, as well as in the study of prevention of cheese defects.

1. Introduction

The genus *Propionibacterium* is divided into two groups, namely "cutaneous" *Propionibacterium*, primarily isolated from human skin and commonly associated with infections, and "classical" or "dairy" *Propionibacterium*, isolated from dairy products (McDowell et al., 2012). The latter is particularly relevant to the food industry (Cummins and Johnson, 1986). *P. freudenreichii* was the first propionic acid-producing bacteria isolated from Emmenthal cheese (Von Freudenreich and Orla-Jensen, 1906). Later, *P. acidipropionici*, *P. jensenii*, and *P. thoenii* were classified as dairy species after having been isolated from milk and dairy products, as well as from soil, silage, and dairy plants (Cummins and Johnson, 1992).

The dairy propionibacteria, particularly *P. freudenreichii*, have an important role in the Swiss cheese production process, as their metabolism positively contributes to the flavor and development of the typical holes during ripening (Falentin et al., 2010; Langsrud and Reinbold, 1973; Rossi et al., 1999).

Furthermore, *Propionibacterium* spp. are able to produce folic acid, proline, and vitamin B12 (Iida et al., 2007), and synthesize several different bioprotective compounds such as bacteriocins (Ho et al., 2009; Lind et al., 2007). Some strains produce bifidogenic compounds and present the ability to survive and maintain activity during passage of the digestive tract, so this genus also has relevance for potential use as probiotic cultures (Hervé et al., 2007; Leverrier et al., 2003; Uchida et al., 2011). However, *P. jensenii* and *P. thoenii* are commonly associated with problems in cheese

production, such as formation of red or brown spots, pigmentation, or excessive production of gas (CO_2) (Carcano et al., 1995; de Carvalho et al., 1995).

Propionibacteria have low nutritional requirements and maintain their viability under various environmental conditions (Thierry et al., 2011). At the moment, genetic information related to the ecologic adaption capacity of dairy propionic bacteria is largely unknown and only few studies have reported the biodiversity in different surroundings (niches). Some studies have shown that the genus is homogenous and that *P. freudenreichii* forms an isolated cluster among the dairy species (Britz and Riedel, 1991; Charfreitag and Stackebrandt, 1989). Molecular typing has been used for the identification and differentiation of bacteria, as well as in research on the different aspects related to microbial adaptation in different environments. The characterization of dairy *Propionibacterium* strains by molecular typing allows the traceability of strains with industrially useful properties such as the production of volatile. Some genotypic methods, such as random amplified polymorphic DNA (RAPD) and pulsed field gel electrophoresis (PFGE), have been used to study the biodiversity of microorganisms (Baruzzi et al., 2000; Kaufmann, 1998; Rossetti and Giraffa, 2005). The Multilocus sequence typing (MLST) technique has been used in studies where information about the historic evolution is considered necessary (Maiden et al., 1998). The aim of this study was to describe the biodiversity of autochthonous dairy *Propionibacterium* strains in a Brazilian region with Swiss-type cheese production.

2. Material and Methods

2.1. Reference strains

Five reference strains of *Propionibacterium* spp. (*Propionibacterium* spp. – TL1367; *P. freudenreichii* – TL176; *P. jensenii* – PJ348; *P. thoenii* – PT221; and *P. acidipropionici*

– 690) were obtained from the Centre International de Ressources Microbiennes – Bactéries d'Intérêt Alimentaire. These strains were used as positive controls for the identification of isolates by polymerase chain reaction (PCR).

A total of 12 strains from Centre International de Ressources Microbiennes – bactéries d'interet Alimentaire, INRA, Rennes, France (CIRM-BIA), present in the MLST scheme for *P. freudenreichii* (Dalmasso et al., 2011), were used for the molecular characterization by PFGE and for phylogenetic studies.

2.2. Collection of *Propionibacterium* strains from Brazilian food systems

Samples of raw milk (n=5), soil (n=5), silage (n=4), and pasture (n=4) were aseptically collected in five dairy farms in Campo das Vertentes (Minas Gerais, Brazil) and kept under refrigeration until analysis. The raw milk samples were diluted in 0.85% NaCl (w/v). For solid samples, 25 g of each sample were obtained with a sterile knife and transferred to individual sterile bags containing 225 mL of 0.85% NaCl (w/v), homogenized, and diluted in the same solution. From each sample, the dilutions were pour-plated in duplicate in lithium glycerol (LG) agar, as described by Madec et al. (1994), containing a mixture of commercial antibiotics (Pal-propiobac^{TM}, Laboratoire Standa, Caen, France). The plates were incubated at 30 ºC for 6 days under anaerobic conditions (Anaerocult, Merck KGaA, Darmstadt, Germany). After incubation, colonies that formed in the culture media were randomly selected (10%) and submitted to microscopy studies. The colonies were then streaked (three times) on yeast extract lactate (YEL) plates and incubated at 30 ºC for 5 days under anaerobic conditions. Isolated colonies were then transferred to YEL broth and incubated at 30 °C for 2 days and subjected to molecular analysis.

2.3. Molecular identification of *Propionibacterium* spp.

The purified cultures were subjected to DNA extraction using the DNeasy Kit (QIAGEN, Courtabouef, France). The identification of isolates of *Propionibacterium* spp. was performed by genus-specific and species-specific PCR. The primers and amplification conditions are shown in Table 1.

2.4. RAPD genotyping of *Propionibacterium* strains

In RAPD, the oligonucleotide primer M13 (5'GAGGGTGGCGGTTCT-3') was used. The PCR reactions contained 2.5 μL of Taq buffer (with 2 mM $MgCl_2$), 0.5 μL of Taq DNA polymerase (Qbiogene, Burlingame-California, USA), 0.51 μL of primer (100 μM), 1.0 μL of dNTPs, 1.5 μL of DNA (at a minimal concentration of 25 ng/μL), and PCR water (Sigma, St. Louis, USA) to a final volume of 50 μL. The PCR conditions were: initial denaturation at 94 °C for 2 min; 40 cycles at 94 °C for 1 min, 42 °C for 20 s, and 72 °C for 2 min; and a final extension step at 72 °C for 10 min.

Photographs of GelRed-stained RAPD gels were scanned, and the band profiles were analyzed using BioNumerics, version 4.1 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient. The compiled matrix was used for cluster analysis using the arithmetic average (UPGMA) clustering algorithm.

2.5. Characterization of *Propionibacterium* strains by PFGE

Propionibacterium strains were grown to an optical density (OD_{650}) of 0.3 in LG broth. Cells were harvested from 10 mL of culture, washed with TES buffer, and suspended in TE buffer (pH 8.0) containing 10 mg/mL of lysozyme (Sigma, St. Louis, MO, USA). After incubation at 37 °C for 1 h, the suspension was heated at 50 °C, and 700 mL of

0.5% agarose in 125 mM EDTA (pH 7.0) at the same temperature was added before solidifying the suspension in molds. The agarose blocks were incubated for 15 min at 4 ºC. Proteinase K (20 mg/mL) treatment was performed in proteinase K buffer (10 mM Tris-base, 100 mM EDTA, pH 8.0) containing sulfate dodecyl sodium (SDS) solutions (10%) for 2 h at 55 °C. The agarose blocks were washed in water for 10 min and four times in 20 mM of TE (pH 8.0) for 10 min per wash.

Before restriction enzyme digestion, the agarose blocks were incubated for 1 h at 4 ºC in restriction buffer (4-*Xba*I buffer, Biolabs). Restriction enzyme digestion with *Xba*I was performed at 37 °C for 4 h. Electrophoresis was carried out in 0.5× TBE buffer in a 1% (w/v) agarose gel (PFGE certified agarose, Bio-Rad) with a pulse time of 2 to 20 s, voltage of 6 V/cm, for 20 h at 14 °C, using a CHEF DR II apparatus (Bio-Rad, Hercules, CA, USA). The gel was stained with GelRed and visualized under UV light. Photographs of PFGE gels were scanned, and the band profiles were analyzed using BioNumerics, version 4.1 (Applied Maths, Kortrijk, Belgium). Comparisons between the normalized band profiles were made using the Dice similarity coefficient. The compiled matrix was used for cluster analysis using the unweighted pair group method with the arithmetic average (UPGMA) clustering algorithm.

2.6. Phylogenetic analysis of *P. freudenreichii* by MLST

Considering the genetic profiles that were obtained by RAPD and PFGE, 18 isolates of the *P. freudenreichii* species were selected and subjected to MLST. Twelve CIRM-BIA strains were included in this phase of the study.

After DNA extraction from all cultures, analysis of the DNA sequence corresponding to the intragenic regions of the genome encoding the RNA polymerase β-subunit (rpoB), the adenylate kinase (adk), the carboxylic ester hydrolases (pf169 and pf1637), DNA

recombinase A (recA), the fumarate hydratase (fumC), and the cell-wall polysaccharide synthase (gtf) was performed using the primers and PCR conditions described by Dalmasso et al. (2011).

The PCR products were sequenced by AGOWA genomics (Berlin, Germany). All the sequences were analyzed using MEGA software (version 4) and compared with the sequences available in the database (http://www.pasteur.fr/recherche/genopole/PF8/mlst/). The allelic profiles (STs) identified for the strains isolated in Brazil and the CIRM-BIA strains (identified in the MLST scheme for *P. freudenreichii*, Dalmasso et al., 2011) were grouped into two clonal complexes (CCs) using the software e-BURST.

3. Results

3.1. Isolation and identification of *Propionibacterium* spp.

For this study, five dairy farms were selected based on their importance in cheese production. Among the different isolates from these farms, 71 were chosen based on morphological characteristics compatible with the genus *Propionibacterium.* Isolates from raw milk, soil, silage, and pasture were submitted to genus- and species-specific PCR and 50 isolates were identified as propionibacteria. Among them, 25 were *P. freudenreichii*, 22 *P. jensenii*, and 3 *P. acidipropionici*. These isolates originated from four of the five dairy farms sampled in our study. In farm A, no propionibacteria could be isolated, whereas in farms B and E, strains of all three species were identified. In farm D, only *P. jensenii* was isolated, whereas this species was not isolated from farm C. Twenty-four isolates were found in raw milk, 19 in soil, 1 in silage, and 6 in pasture. In milk and soil, strains of the three species (*P. freudenreichii*, *P. jensenii*, and *P. acidipropionici*) were identified, whereas *P. freudenreichii* and *P. jensenii* were isolated

from pasture and the unique strain that could be isolated from silage was identified as *P. freudenreichii*.

3.2. Characterization of *Propionibacterium* spp. strains by RAPD

The RAPD analysis allowed differentiation of 27 isolates among the 50 analyzed, identifying 8 band profiles (RAPD types) for the 25 *P. freudenreichii* isolates, 16 for the 22 *P. jensenii* isolates, and 3 for the 3 *P. acidipropionici* isolates. With this method, bacteria with the same profile could be identified from different samples and farms. The bacteria formed three distinct clusters, with cluster I formed by two strains of *P. jensenii*, from farms C and E, originating from soil and milk samples, and one strain of *P. acidipropionici* from farm E, originating from milk. The *P. freudenreichii* isolates were grouped in cluster II, which showed 42.5% similarity with cluster I. Cluster III was formed by *P. jensenii*, two strains of *P. freudenreichii* (strains from farms B and C, originating from pasture and milk, respectively), as well as two of the three isolates that were identified as *P. acidipropionici*. This cluster showed 35.5% similarity with the other clusters (Figure 1).

3.3. Characterization of *Propionibacterium* spp. strains by PFGE

Restriction digestion of chromosomic DNA of 50 isolates of *Propionibacterium* spp. with the enzyme *Xba*I yielded 31 different profiles; of these, 12 profiles (restriction patterns) represented *P. freudenreichii*, 16 represented *P. jensenii*, and 3 represented *P. acidipropionici*. Clonal differentiation by PFGE led to the identification of different pulse types (PFGE types) among isolates from the same origin.

Some *P. freudenreichii* strains isolated from different samples presented the same PFGE profile. However, no identical profiles were detected among the different farms

used in this study. The *Propionibacterium* spp. were grouped into five clusters, with more than 42.8% similarity between them (Figure 2). The *P. freudenreichii* strains formed two distinct clusters. Cluster III contained all the isolates from farms B and E, and two isolates from farm C, namely, strains 78 and 91 – isolated from milk and soil, respectively, and one *P. jensenii* strain from farm E (strain 146), with similarity of more than 70% between the strains. Cluster IV contained only *P. freudenreichii* strains from farm C, with at least 73% of similarity between the strains. We identified a cluster (cluster II) containing only *P. jensenii* (about 77% of the isolates) in samples from farms B and D. The last two clusters contained *P. jensenii* as well as *P. acidipropionici* strains.

3.4. Phylogenetic analysis of *P. freudenreichii* strains by MLST

Table 2 shows the allelic profiles and origin data of 18 *P. freudenreichii* strains isolated from three dairy farms, since seven strains among the 25 initially identified ones had shown the same patterns (profiles) in RAPD and PFGE analyses, and were therefore considered identical.

Analysis of the seven housekeeping genes resulted in 12 sequence types (STs), all of which had been previously identified in the MLST scheme for *P. freudenreichii* (Dalmasso et al., 2011). Among the 18 isolates from three dairy farms, two STs were identified, showing allele variation only for the gene *rpoB*. ST 3 could be identified in 13 strains isolated from milk, soil, silage, or pasture. ST 42 was present only in five strains isolated from milk or soil. The number of alleles varied between one (for gene *pfl69*) to four (for genes *fumC* and *pfl637*). STs 1, and 23 were identified in two strains.

The STs identified for the Brazilian isolates and CIRM-BIA strains (identified in the MLST scheme for *P. freudenreichii*) were grouped into two CCs. Each CC was formed

by isolates with either identical allelic profiles (same ST) or STs that differed in one locus at most. CC1, composed by STs 19, 5, 9, 23, 28, 22, 11, 3, 10, and 42 (Figure 3), combineed 33 strains isolated from raw milk, soil, silage, pasture, and different types of cheese, and a strain from an unidentified origin. The STs 38, and 41 were combined into cluster CC2. The ancestral allelic profiles of the two clonal complexes are ST 31 and ST 41, respectively, both present in the MLST scheme for *P. freudenreichii* and identified in strains isolated from Swiss-type cheese from Finland (Dalmasso et al., 2011). ST 44 was identified as a singleton, not being integrated in any other clonal complex.

4. Discussion

Characterization studies of dairy propionibacteria from different origins are important to understand their biodiversity in different habitats, allowing us to relate the isolates taxonomically. Additionally, research in this area facilitates the selection of strains of industrial importance, focussing the interest of the food industry on these bacteria, especially as secondary (ripening) cultures in the production of cheese. At the same time, good knowledge of the strains also helps to prevent the presence of defect-causing strains in certain products.

For the isolation of *Propionibacterium* spp. from different samples of milk, soil, silage, and pasture, LG agar was used as a selective medium. It was developed by Madec et al. (1994) as a means to isolate and enumerate *Propionibacterium* spp., taking advantage of their resistance to lithium and their ability to ferment glycerol under anaerobic conditions. This culture medium has proven to be highly selective in the isolation and enumeration of propionibacteria in previous studies in France (Thierry and Madec, 1995; Thierry et al., 1994) as well as in our study.

Molecular characterization of the isolates, as exemplified in the dendrograms generated with results from RAPD and PFGE, shows that these molecular typing methods are able to identify differences between the studied strains (Figures 1 and 2). Similar results have been found by Suomalainen et al. (2008), using RAPD and PFGE to detect *P. freudenreichii* subsp. *shermanii* JS in human feces after oral administration. Our data show that the PFGE method provides better resolution of the heterogeneity among strains of the same species than the RAPD method, as shown in previous studies (Cai et al., 2007; Jenkins et al., 2002; Meile et al., 2008; Unemo et al., 2007). The difference between the two dendrograms (Figures 1 and 2) can be explained by the sensitivity of the PFGE method in identifying deletions, insertions, and genetic alterations of the cleavage sites of the chromosomic material (Nemoy et al., 2005), whereas RAPD identifies rearrangements in an amplified DNA region (Perry et al., 2003). Jenkins at al. (2002) analyzed the diversity of dairy cultures in Swiss cheese production and showed that the PFGE method discriminated 10 of the 11 *P. freudenreichii* strains analyzed, which showed about 30% similarity between them.

In the studied region, a considerable degree of biodiversity could be detected, as three of the four main species of dairy propionibacteria were isolated. The results showed that the isolates from different geographic regions did not present the same PFGE restriction profiles, and were therefore identified as distinct strains (Figure 2). Although clustered together, many isolates from different farms showed very distinct genetic profiles. The fact that no identical strains (showing the same PFGE band profiles) were found on different farms suggests that each location represents a specific niche. *P. freudenreichii* isolated from milk or cheese from different regions always present different PFGE restriction patterns (Gautier et al., 1996).

The three different species of dairy propionibacteria that we identified were randomly distributed among the samples, showing the adaption capacity of the bacteria to their environment. These findings were confirmed by the fact that different patterns (pulse types) were observed within a unique species identified in the same sample, as well as by the fact that the three different species were isolated from milk and soil, and by the detection of *P. freudenreichii* and *P jensenii* in pasture. On the contrary, Fessler et al. (1999) observed that a contamination was responsible for the presence of four species of dairy propionibacteria in Swiss raw milk, since they concluded that the high degree of diversity of strains identified by RAPD came from pasture and dairy production.

Furthermore, the STs identified in the strains of *P. freudenreichii* by MLST were not related to any specific origin and were randomly distributed in the phylogenetic tree, which corroborate previous studies (Dalmasso et al., 2011). A similar result has been found by McDowell et al. (2011) in the MLST scheme for *P. acnes*, in which ST 6 was identified in 50% of the 123 isolates from different ecological niches such as skin, eyes, and dental infection. In contrast, Cai et al. (2007) were able to identify a relationship between the ST and sample origin in a genotype characterization study of *Lactobacillus casei*. Analysis of six housekeeping genes showed the formation of three clusters closely related to the isolation source, with the formation of a cluster by strains isolated from cheese, one cluster formed by strains coming from silage, and a third cluster containing strains from diverse sources, but with a predominance of isolates from the human gastrointestinal tract and from cheese.

Considering the relationship between PFGE and MLST data of the 18 strains from this study, 11 PFGE profiles could be observed in two distinct clusters. One cluster contained strains presenting the profile ST 42, whereas the other cluster was formed by strains of ST 3, with only 49% similarity between the two clusters. The other clusters

were formed by CIRM-BIA strains from different origins, with each one presenting a specific PFGE and ST profile.

For some isolates displaying the same allelic profile (ST), different restriction patterns could be detected with PFGE. Each of the five strains presenting the ST 42 profile, for example, showed a particular PFGE profile (Table 2). However, all strains displaying the same PFGE patterns also presented the same allelic profile in MLST. We did not find a relationship between the sample origin of the isolates and their PFGE and MLST profiles. Isolates from milk as well as pasture displayed the same PFGE pulse type, and isolates from milk, soil, pasture, and silage presented the same ST. While isolates from different geographical regions did not present the same PFGE profile, some presented the same ST profile.

The MLST method defines genetically consistent groups (clusters), so-called CCs, which derive from a common ancestor (Maiden et al., 1998). With the exception of ST 44, which was identified as a singleton, all of the analyzed strains could be divided into two CCs. Isolates from distinct geographic regions proved to be derived from the same common ancestor as identified in isolates from Swiss-type cheese, reflecting the evolutionary relationship between the analyzed strains. Similar results were obtained in a recent study of the genetic diversity of *P. freudenreichii* using MLST (Dalmasso et al., 2011). The species identified presented a cohesive population with a low level of polymorphism. The fact that the two ancestral STs were identified in strains from Swiss-type cheese, cheese that is ripened by *P. freudenreichii*, can be considered a consequence of the impact that international trade in starter cultures and cheese probably has on the dissemination of strains.

5. Conclusion

The present study shows that the autochthonous microbiota of dairy farms in Minas Gerais, Brazil, presents considerable genetic diversity, since three of the main dairy *Propionibacterium* species were observed to be randomly distributed among the studied samples collected from different locations. The reason for such diversity is likely the adaptation capacity of these bacteria to their surrounding conditions. It is worth stressing that the choice of the typing methods was of extreme importance to the characterization of the diversity of propionibacteria. The environment of dairy farms and milk production proved to be a reservoir for *Propionibacterium* strains that are important for future use as possible starter cultures as well as in the study of prevention of cheese defects.

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Table 1 – Primers used for PCR identification

Primer	Sequence 5' - 3'	*Propionibacterium* species	Reference
Gd1	TGCTTTCGATACGGGTTGAC	*Propionibacterium* sp.	Dasen et al., 1998.
bak4	AGGAGGTGATCCARCCGA		
bak11w	AGTTTGATCTGGCTCAG		
PfrI	AGGAGCCTTTTCGCCATC	*P. freudenreichii* ssp.	Tilsala-Timisjarvi & Alatossava, 2001
PfrII	TAGCTTGTCACACAAAACTC		
PjeI	CTAAGGAGCTGTGACTGTG	*P. jensenii*	Tilsala-Timisjarvi & Alatossava, 2001
PjeII	AGCTTGCAATACACACAAAAC		
PthI	ATGGGCCCTGTGCTCAC	*P. thoenii*	Tilsala-Timisjarvi & Alatossava, 2001
PthII	AGTAGCTTGCAATACACATAC		
PacI	CTGGAAGCTGGCCGTCG	*P. acidipropionici*	Tilsala-Timisjarvi & Alatossava, 2001
PacII	CTTGCAACACAACACATTAC		

Table 2 - Strains, allele types (ATs), sequence types (STs), biotopes, countries of isolation and subspecies.

			AT							
Strain name	**PFGE**	**ST**	***Adk***	***fumC***	***gtf***	***pf1637***	***pf169***	***recA***	***rpoB***	**Origem**
B42	1	3	1	1	2	3	1	1	1	milk/Brazil
B43	1	3	1	1	2	3	1	1	1	milk/Brazil
B44	2	3	1	1	2	3	1	1	1	milk/Brazil
B46	1	3	1	1	2	3	1	1	1	milk/Brazil
B66	1	3	1	1	2	3	1	1	1	pasture/Brazil
B70	3	42	1	1	2	3	1	1	2	milk/Brazil
B75	3	42	1	1	2	3	1	1	2	milk/Brazil
B78	4	3	1	1	2	3	1	1	1	milk/Brazil
B82	5	42	1	1	2	3	1	1	2	soil/Brazil
B86	6	42	1	1	2	3	1	1	2	soil/Brazil
B89	7	42	1	1	2	3	1	1	2	soil/Brazil
B91	4	3	1	1	2	3	1	1	1	silage/Brazil
B141	8	3	1	1	2	3	1	1	1	milk/Brazil
B145	8	3	1	1	2	3	1	1	1	milk/Brazil
B148	9	3	1	1	2	3	1	1	1	milk/Brazil
B158	9	3	1	1	2	3	1	1	1	soil/Brazil
B171	8	3	1	1	2	3	1	1	1	pasture/Brazil
B172	8	3	1	1	2	3	1	1	1	pasture/Brazil
CIRM-BIA121	10	44	2	1	2	6	1	1	1	Swiss cheese/USA
CIRM-BIA122	11	28	1	3	2	3	1	4	1	NA/NA
CIRM-BIA514	12	10	1	1	2	1	1	1	1	hay/France
CIRM-BIA527	13	22	1	4	2	3	1	1	1	Fribourg cheese/Switzerland
CIRM-BIA690	19	19	1	5	2	2	1	2	1	Leerdammer cheese/France
CIRM-BIA9	20	1	1	1	1	1	1	1	1	Cheese/The Netherlands
CIRM-BIA118	21	23	1	1	2	1	1	2	1	Gruyere cheese/France
CIRM-BIA135	22	5	1	1	2	2	1	2	1	Ewe milk/France
CIRM-BIA508	23	23	1	1	2	1	1	2	1	Gruyere cheese/France
CIRM-BIA513	24	9	1	1	1	3	1	2	1	Ras cheese/Egypt
CIRM-BIA516	25	11	1	1	2	3	1	1	1	Yak milk/Nepal
CIRM-BIA1	26	1	1	1	1	1	1	1	1	NA/The Netherlands

NA = Not available. Data of MLST for CIRM-BIA strains from Dalmasso et al., 2011

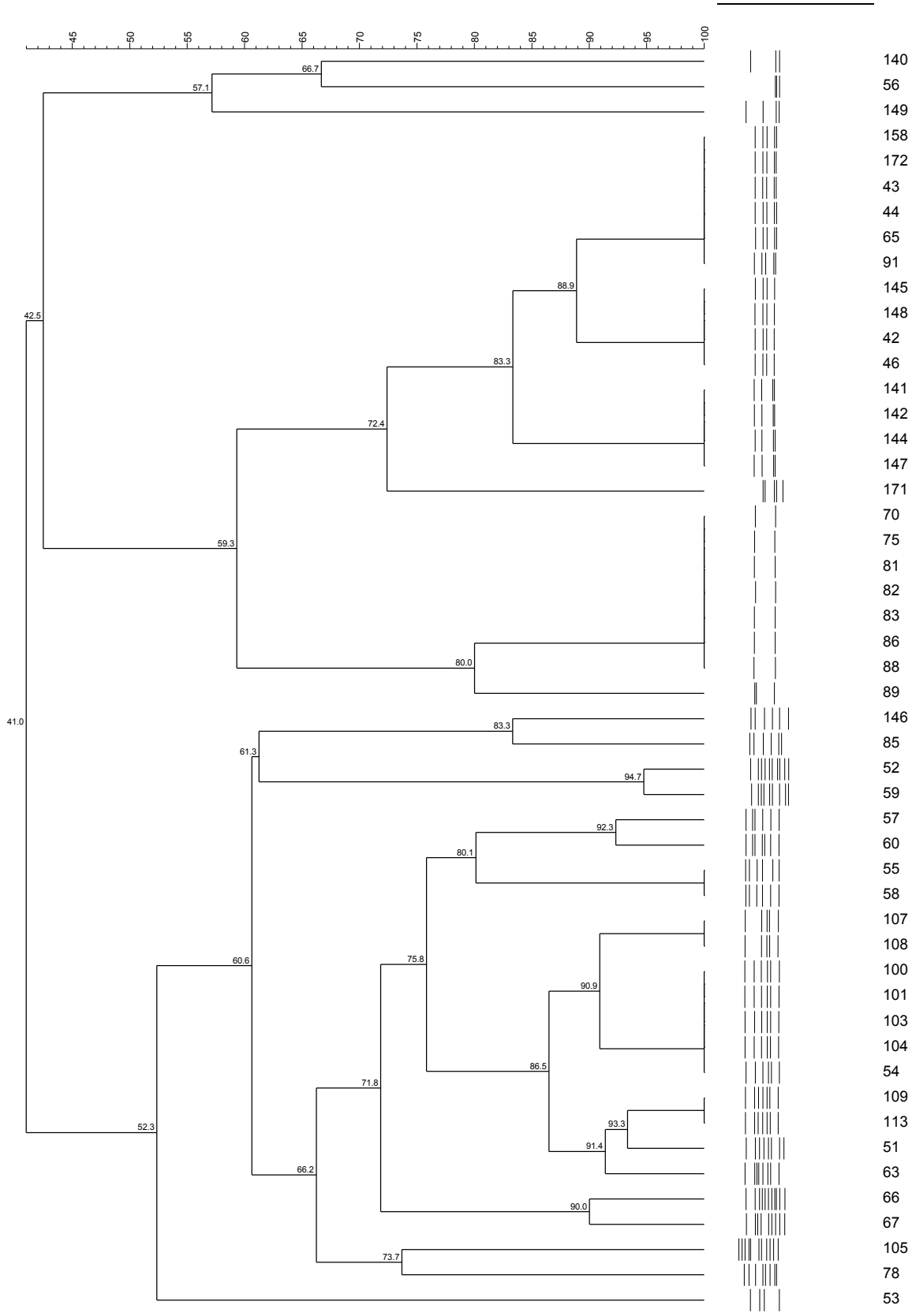

Fig. 1. Dendograms based on UPGMA clustering (Dice coefficient) of RAPD profiles from culture strains of *P. freudenreichii*, *P. jensenii* and *P. acidipropionici*, isolated of dairy farms source: raw mik, soil, silage and pasture.

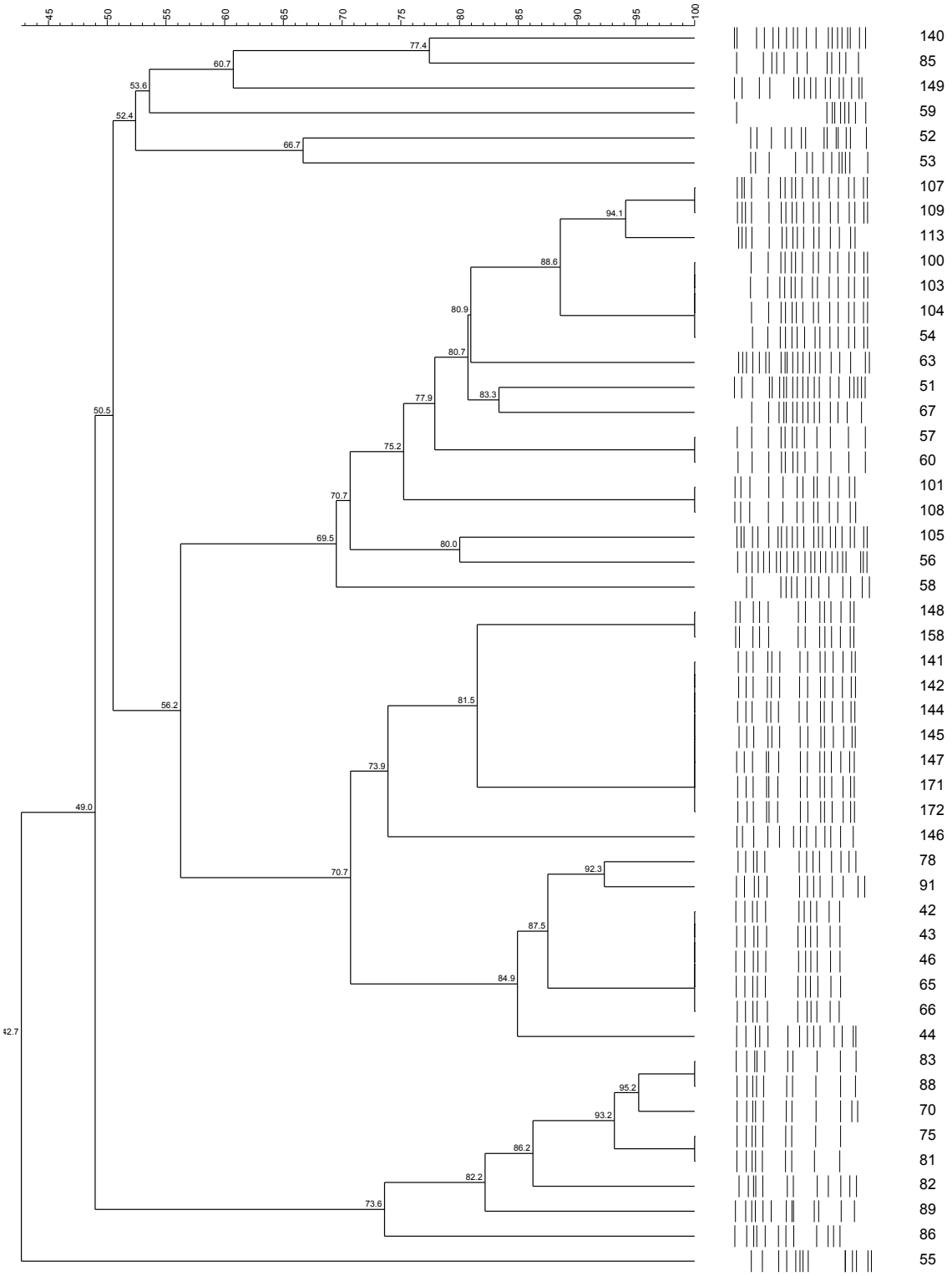

Fig. 2. Dendograms based on UPGMA clustering (Dice coefficient) of PFGE profiles from culture strains of *P. freudenreichii*, *P. jensenii* and *P. acidipropionici*, isolated of dairy farms source: raw mik, soil, silage and pasture.

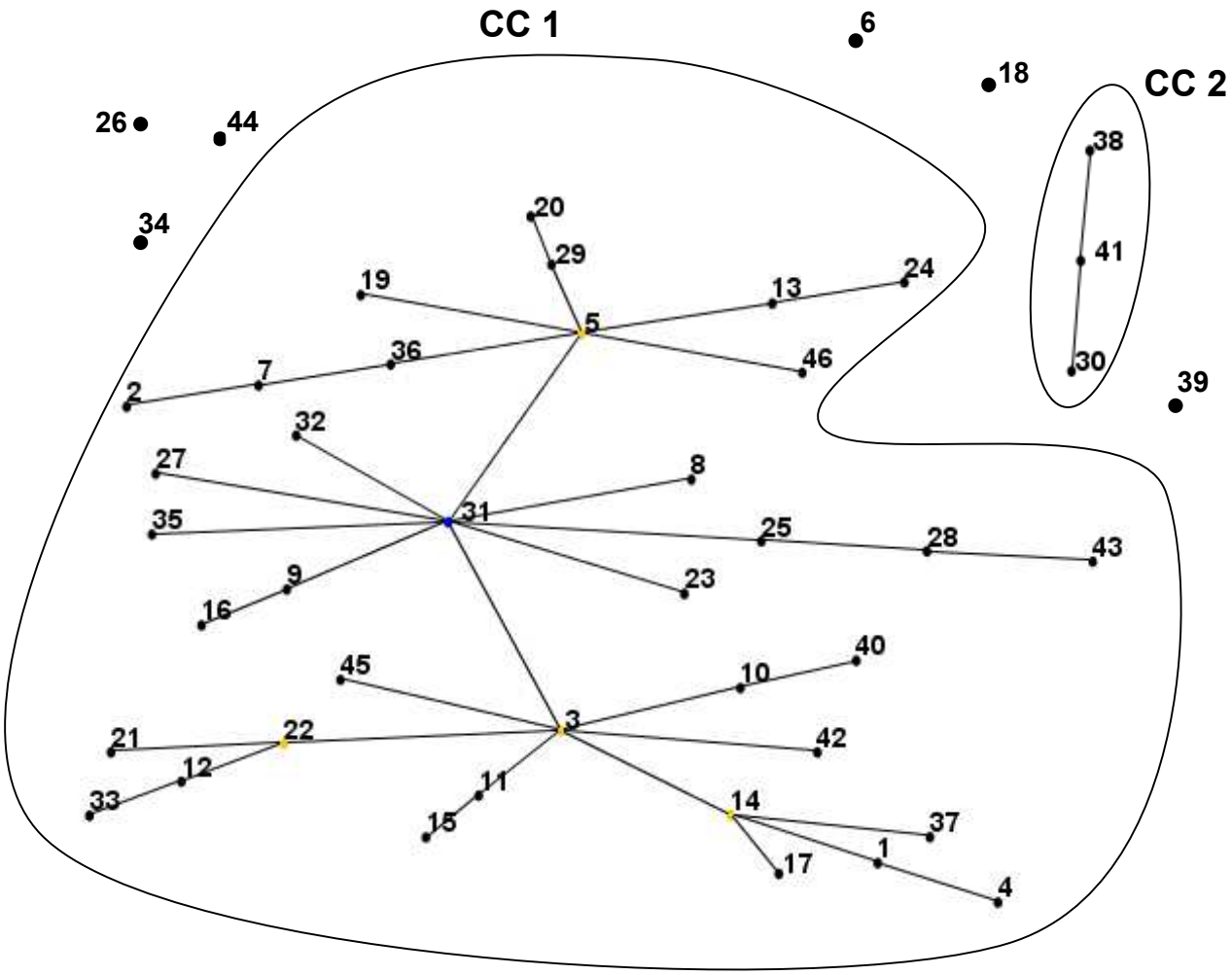

Fig. 3. eBURST analysis of 30 strains of *Propionibacterium freudenreichii* (18 Brazilian strains and 12 CIRM-BIA strains) using an existing MLST scheme of Dalmasso et al, 2011. 2 clonal complex composed of 37 ST for the first and 3 ST for the second complex are present with 6 singletons (ST26, ST18, ST6, ST44, ST39, ST34).
The eigtheen Brazilian strains are distributed between ST3 and ST42. Regarding to the twelve CIRM-BIA strains, they are distributed in 10 ST (ST1, ST5, ST9, ST10, ST11, ST19, ST22, ST23, ST28, ST44).

5. THE PHENOTYPICAL CARACTERIZATION OF *P. FREUDENREICHII*

PAPER 3: New insithts about diversity within *Propionibacterium freudenreichii* argue against its division into subspecies

New insiths about diversity within *Propionibacterium freudenreichii* argue against its division into subspecies

Resumo

Propionibacterium freudenreichii é amplamente utilizada como cultura de maturação (cultura secundária) na produção de queijo tipo suíço. *P. freudenreichii* é atualmente dividida em duas subspécies, *P. freudenreichii* subsp *freudenreichii* e *P. freudenreichii* subsp *shermanii*, de acordo com o fenótipo baseado em dois critérios, fermentação de lactose e redução de nitrato (lac+/nit- para subspécie *shermanii* e lac-/nit+ para subspécie *freudenreichii*). Entretanto, a existência de cepas que não podem ser classificadas segundo este critério (lac+/nit+ ou lac-/nit-) foi também previamente relatado. Além disso, testes fenotípicos podem gerar resultados diferentes dependendo das condições experimentais e nenhum método de referência foi descrito. O objetivo deste trabalho foi i) confirmar a diversidade fenotípica dentro da espécie *P. freudenreichii* com correlação a fermentação de lactose e redução de nitrato, e ii) determinar se a subspécie está relacionada a outras especificidades metabólicas, por exemplo, pelo perfil de compostos de aroma. Inicialmente, nós determinamos as condições relevantes para o teste de habilidade de *P. freudenreichii* quanto a fermentação de lactose e redução de nitrato, utilizando 10 cepas sequenciadas, para as quais eram conhecidos a presença ou ausência do loci lactose e nitrato. Vinte e oito cepas foram caracterizadas. Compostos de aroma foram analisados por headspace com cromatografia gasosa acoplada a espectometria de massa (HS-GC-MS). As condições experimentais utilizadas no teste de fermentação de lactose e redução de nitrato efetivamente influenciaram os resultados, com uma

particular importância para o tempo de incubação, o que pode ocasionar um resultado falso negativo para a redução do nitrato se o tempo for curto (insuficiente) e explicar algumas discrepâncias observadas entre os resultados deste estudo e trabalhos anteriores sobre as mesmas cepas. A aplicação das condições experimentais demonstraram a impossibilidade de classificação para cerca de metade das cepas analisadas (7 lac+/nit+ e 6 lac-/nit- das 28 cepas testadas). A Análise de Componente Principal realizada em 16 compostos aromáticos produzidos pelas bactérias não demonstrou qualquer agrupamento das cepas de acordo com o fenótipo (*freudenreichii*, *shermanii*, lac+/nit+ ou lac-/nit-) foi encontrada difundido no mapa. Portanto, o fenótipo lac/nit de *P. freudenreichii* não está relacionado às suas propriedades importantes para a fabricação de queijos. Considerando em conjunto, os resultados apresentados demonstram que a classificação de *P. freudenreichii* em duas subespécies não parece relevante.

New insiths about diversity within *Propionibacterium freudenreichii* argue against its division into subspecies

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Abstract

Propionibacterium freudenreichii is widely used as a ripening culture in Swiss-type cheese manufacture. *P. freudenreichii* is currently divided into two subspecies, *P. freudenreichii* subsp *freudenreichii* and *P. freudenreichii* subsp *shermanii*, according to their phenotype for two criteria, lactose fermentation and nitrate reduction (lac+/nit- for subsp *shermanii* and lac-/nit+ for subsp *freudenreichii)*. However, the existence of unclassifiable strains (lac+/nit+ or lac-/nit-) has also been previously reported. However, phenotypic tests can generate different results depending on the experimental conditions and no reference method has been described. The aim of this study was i) to confirm the phenotypical diversity within *P. freudenreichii* species for lactose fermentation and nitrate reduction, and ii) to determine whether the subspecies is related to other metabolic specificities for exemple by aroma compound profiling. We first determined the relevant conditions to test the ability of *P. freudenreichii* for lactose

fermentation and nitrate reduction, by using a set of 10 sequenced strains, in which the presence or absence of the lactose and nitrate loci were known. Twenty eight strains were characterized. Aroma were analysed by head space-gas chromatography-mass spectrometry (HS-GC-MS). The experimental conditions used to test lactose fermentation and nitrate reduction effectively influenced the results, with a particular importance in the incubation time, which can lead to false nit- if too short and explain some discrepancies observed between the present results and previous reports for the same strains. The application of retained conditions to all strains demonstrated the existence of nearly half of unclassifiable strains (7 lac+/nit+ and 6 lac-/nit- out of the 28 tested strains). The Principal Component Analysis performed on 16 aroma compounds produced did not show any grouping of the strains according to the phenotype (*freudenreichii*, *shermanii*, lac+/nit+ or lac-/nit-) which appeared widespread on the map. Therefore the lac/nit phenotype of *P. freudenreichii* is not related to their important properties in cheese manufacture. Taken together, the present results demonstrate that the division of *P. freudenreichii* into two subspecies does not appear relevant.

Introduction

Propionibacterium freudenreichii is the main dairy propionibacteria species used as ripening culture in cheese manufacture, in particular Swiss-type cheeses (Cummins et Johnson, 1986). It produces short-chain fatty acids resulting from lactate fermentation, from amino acid catabolism and from milk fat hydrolysis, and CO_2. These products have a significant contribution to the development of the typical flavour and the formation of eyes in Swiss-type cheeses (Thierry et al., 2011). Moreover, *P. freudenreichii* has potentials as bioprotective cultures by producing antimicrobial compounds such as bacteriocins and organic acids (Tharmaraj & Shah, 2009) and antifungal peptides (Jan et al., 2007). It is also able to produce a variety of beneficial compounds for human health, such as vitamin B12 and folic acid (Hugenholtz et al., 2002). Some strains have been proposed as probiotics, due to their ability to modulate intestinal microbiota through their bifidogenic effect and to anti-inflammatory effects and antimutagenic properties, among others (Cousin et al., 2011).

P. freudenreichii is currently divided into two subspecies, on the basis of two phenotypical criteria (Cummins and Johnson, 1986, http://www.bacterio.net). *P. freudenreichii* subsp. *freudenreichii* does not ferment lactose and possess a nitrate reductase activity, whereas it is the opposite for *P. freudenreichii* subsp. *shermanii*. However, former works have occasionally mentioned the occurrence of strains harbouring other phenotypes, detected along with the classical subspecies (de Carvalho et al., 1994; Moore and Holdeman, 1974; Vorobjeva, 1999). More recently, during the characterisation of a collection of 113 strains of *P. freudenreichii*, more than 25% of strains could not assigned a subspecies, and were labelled “pheno+” (lac+/nit+) or “pheno−” (lac-/nit-) (Dalmasso et al., 2011). Regarding the use of *P. freudenreichii* for

cheese ripening, the ability to ferment lactose can be of importance culture in some cheese varieties, therefore resulting in the choice of strains of the subspecies *P. freudenreichii* subsp. *shermanii*. However, some "lac+/nit+" strains could be also used in this case.

Molecular methods have failed up to date to distinguish *P. freudenreichii* subspecies (Dasen et al., 1998; Fessler et al., 1999; Tilsala-Timisjarvi and Alatossava, 2001) and therefore phenotypic methods are still required. Some discrepancies between studies could also have been due to a lack of standardisation of phenotypical tests, since it is known that experimental conditions such as the presence of O_2, temperature and incubation time can influence the results observed (Busse et al., 1996).

The aim of this study was to explore the phenotypical diversity within *P. freudenreichii* species, and its relationship with the current subdivision into two subspecies, *P. freudenreichii* subsp. *freudenreichii* and subsp. *shermanii*. We first determined the more relevant conditions to test *P. freudenreichii* for its activity of lactose fermentation and nitrate reduction, by using a set of sequenced strains for which the expected phenotype could be predicted from the presence or the absence of the loci involved in the fermentation of lactose and the reduction of nitrate. Eighteen strains isolated from various biotopes (soil, silage, pasture, raw milk) in dairy farms in Minas Gerais, Brazil were also included to investigate the intraspecies diversity within a pool of autochthonous strains. For all straisn, we also evaluated their ability to produce aroma compounds to determine whether these important properties were related or not to the subspecies.

Materials and methods

Strains and culture conditions

A total of 28 strains *P. freudenreichii* were used (Table 1). Ten previously sequenced (Falentin et al., submitted) *P. freudenreichii* strains were provided by the International Centre for Microbial Resources collection of bacteria (CIRM-BIA, UMR1253, INRA Rennes, France). Genomic data were analysed with the AGMIAL platform (Bryson et al., 2006).

Eighteen strains isolated from dairy farms situated in Minas Gerais, Brazil were also used (De Freitas, unpublished results). These strains were identified using species-specific PCR (Tilsala-Timisjarvi & Alatossava, 2001) and clones differentiated using pulsed-field gel electrophoresis (De Freitas, umpublished results). Strains were stored in a 30% (v/v) glycerol solution at - 80 °C. Before phenotypical characterization strains were cultured in yeast extract lactate (YEL) medium (Malik et al., 1968) and incubated at 30 °C for 48 h.

For test of the aroma-forming ability of strains, cultures were grown in YEL supplemented by ethanol (2 mM) and butanoic acid (2 mM) to promote the formation of ethyl esters. Cultures were performed in triplicate.

Phenotypic characterisation

Two concentrations of lactose and potassium nitrate were tested for a pool of strains in preliminary tests. Lactose fermentation was tested in a modified API 50CH medium containing: lactose (5 or 20 g / l, Panreac , Lyon, France), tryptone 10 g/l, yeast extract 5 g/l, K_2HPO_4, 0.25 g/l, $MnSO_4$ 0.05 g/l, and bromocresol purple 0.17 g/l). Strains were incubated at 30 °C for 48 h under anaerobiosis (using the Anaerocult A system, Merck, Darmstadt, Germany). The production of acid from lactose was determined from the colour change of bromocresol purple from purple to yellow.

Nitrate reductase activity was detected by means of the Griess reagent (Biomérieux, Marcy l'Etoile, France) after strain incubation on broth containing nitrate (potassium nitrate 0.5 or 1.5 g/l (VWR International, Fontenay-sous-Bois, France), tryptone 10 g/l, yeast extract 5 g/l, glucose 1 g/l) after incubation for 48 h at 30 °C under microaerophilic conditions. The results of tests were read after 2 and 5 days of incubation. All the tests were carried out in triplicate.

Analysis of volatile compounds

Volatiles were extracted, analysed, and identified by head space - gas chromatography - mass spectrometry (HS-GC-MS), using a Turbo Matrix HS-40 trap as a head space (HS) sampler, a Clarus 680 gas chromatograph coupled to Clarus 600T quadrupole mass spectrometer (Perkin Elmer, Courtaboeuf, France), as previously described (Le Boucher et al., 2013). Briefly, samples of 2.5 g culture were used, the volatiles extracted were separated on an Elite 5MS capillary column (60 m x 0.25 mm x 1 µm; Perkin Elmer), with helium as the mobile phase. The initial temperature of the oven was 35 °C, maintained for 5 min. The oven temperature was then increased performed up to 140 °C at a rate of 7 °C.min^{-1} and then up to 280 °C at 13 °C min^{-1}. The mass spectrometer was operated in the scan mode, within a mass range of m/z 25-300, scan time: 0.3sec , interscan delay: 0.03. Ionization was done by electronic impact at 70 eV. The GC–MS data processing was performed as previously described (Le Boucher et al., 2013), using the package XCMS on the R software (Smith, 2006), which converts the raw data to time- and mass-aligned chromatographic peaks areas.

Volatile compounds were identified thanks to the mass spectral data Library NIST and to their retention index. A principal component analysis (PCA) was performed on

preprocessed, $\log_{10}$[x]-transformed and Pareto scaled data, using the package FactomineR of the software R.

Results

Lactose fermentation and nitrate reduction

Preliminary tests were performed on a pool of strains to determine the effect of varying the concentrations of lactose and potassium nitrate on the results of the phenotypic tests classically used to distinguish the two subspecies of *P. freudenreichii*. The results of the tests did not differ at the two lactose concentrations tested. For nitrate reduction, one strain, *P. freudenreichii* B66 was detected positive at 0.5 g/L potassium nitrate, but negative at 1.5 g/L. Therefore the concentrations of 5 g/l lactose and 0.5 g/l nitrate were selected for further tests.

Table 1 summarizes the results of genotype and phenotype of all the strains. The two strains that did not possess the *lacZ* operon showed a negative answer to the test, as expected. The eight strains which possess the *lacZ* operon were effectively capable of fermenting lactose. The change of colour of bromocresol purple from purple to yellow (i.e. positive test) was clear only after 5 days (or 7 days for CIRM-BIA516), of incubation, and not after 2 days. For nitrate reductase, the five strains lacking the complete *nar* operon were effectively found negative regardless of the incubation time. The five strains that possess the *nar* operon showed a positive phenotype, three of them at 2 days of incubation and two of them (CIRM-BIA122 and 513) only after 5 days (Table 1). Out of the 18 Brazilian strains, nine showed the ability to ferment lactose, with 5 strains positive at 2 days and 4 strains only at 5 days. Seven strains were positive for nitrate reductase, with 3 and 4 strains detected positive after 2 and 5 days of incubation, respectively.

Production of aroma compounds

Sixteen main aroma compounds detected in the 28 cultures analysed were selected and subjected to a multivariate analysis (PCA, Fig. 1). A cumulative variation of 69% was explained by the first two principal components. PC1-PC2 plot differentiated the cultures on the basis of their content in many volatiles, including esters and, ketones among others. The four phenopypes *freudenreichii* (F), *shermanii* (S), lac+/nit+ (P+) and lac-/nit- (P-) appeared widespread on the map, showing that the ability of producing aroma compounds is not related to the four sub-groups created from the results of lactose fermentation and nitrate reduction.

Discussion

This study was undertaken to explore the phenotypical diversity within *P. freudenreichii* species, and its relationship with the current subdivision into two subspecies, based on two criteria, lactose fermentation and nitrate reduction.

Phenotypic methods are essential in the taxonomic characterization of microorganisms, but they are time-consuming and the results sometimes ambiguous. To give consistent results phenotypic characterization should be applied by using strictly defined protocols adapted for each species, which are rarely available. In this study, we defined the ad hoc conditions of determination of lactose fermentation and nitrate reduction for *P. freudenreichii* by using 10 sequenced strains, including the two type-strains of the two *P. freudenreichii* subspecies. Our results show that the conditions of test effectively influenced the detection of positive results, as expected. In particular, the incubation time can lead to false nitrate reductase negative results if too short. Difference in the incubation time could be responsible for the discrepancies observed between this study

and previous reports for the same strains. Hence, three strains previously classified as *P. freudenreichii* subsp. *shermanii*, CIRM-BIA122, 513 and 516 (Dalmasso et al., 2011) were identified as nitrate reductase positive after 5 days of incubation, in agreement with the phenotype expected from their genome, resulting in their reclassification as lac+/nit+ (P+, Table 1). In fact, half of the nit+ strains showed a positive response only after 5 days of incubation. Therefore, false nit- or lac- strains can be observed, if the incubation is not enough prolonged, in agreement with the conclusions of other authors (Forbes et al., 2007). Our results also showed that a high concentration of potassium nitrate in the test medium may inhibit the growth of some strains, thus causing false negative results. We used a medium with a reduced concentration of yeast extract, in comparison with the classically used medium, to avoid the risk of false positive reactions of acidification, based on amino acid utilisation when other carbon sources are not available. These results show how a lack of standardisation of the experimental conditions can result in the detection of both false positive and false negative traits.

A collection of Brazilian autochthonous strains were characterised using the conditions defined to test lactose fermentation and nitrate reduction. The phenotype determined for these strains showed that, in addition to the two subspecies classically described, some strains exhibited the phenotype lac+/nit+ or lac-/nit-, confirming previous findings (de Carvalho et al., 1994; Moore and Holdeman, 1974; Vorobjeva, 1999, Dalmasso et al. , 2011). A subspecies called *P. freudenreichii* subsp. *globosum*, previously described as harbouring the phenotype lac+/nit+ (de Carvalho et al., 1994), was suppressed in the most recent editions of the Bergey's Manual of Systematic Bacteriology (Cummins and Johnson, 1986). In the overall, 46% of the strains characterized in the present study could not be classified in one of the two subspecies of *P. freudenreichii*, with 25% of lac+/nit+ and 21% of lac-/nit- strains. In comparison, 27% of the strains characterized

could not be classified into subspecies in the study of Dalmasso et al. (2011). Our results also demonstrate the importance of the combined use of genotypic and phenotypic methods and the integration of reference strains in taxonomic studies. Fernández et al. (2011) found that the combined use of molecular techniques and analysis of phenotypic characteristics confirm that, despite the similarity between the genotypes *lactis* and *cremoris*, there are well defined *Lactococcus lactis* subspecies. Rademaker et al. (2007) evaluated the diversity of pool of 102 *Lactococcus* strains by molecular and phenotypic methods and concluded that the phenotypic differences observed in the current classification is sufficient to distinguish subspecies.

Recent data acquired in the genome of *P. freudenreichii* subsp *shermanii* CIRM-BIA1 offer new insights on the origin of the variation in phenotypes among *P. freudenreichii* species. In the genome, the lactose locus is surrounded by integrases and transposases, suggesting that the *lac* genes may have been acquired through a horizontal transfer, which could explain why this ability is strain dependent (Falentin et al., 2010). In the genome of CIRM-BIA1, the gene corresponding to the beta subunit of nitrate reductase is a pseudogene due to a frameshift, explaining the negative phenotype of this strain for nitrate reduction (Falentin et al., 2010). Pseudogenes are non-functional genes in the genome due to genetic alterations, suggesting a potential mechanism of adaptation (Kasomatis et al., 2013).

A number of genes coding for key enzymes in the metabolism of bacteria have been identified in the recent decades (Gregory et al., 2003, Roderick , 2005). This knowledge could be used to develop specific molecular tests to assess the presence of lactose-fermenting or nitrate-reducing capacities in *P. freudenreichii*.

Regarding the production of aroma compounds by the different strains, the results obtained in the present study showed that these important properties for the cheese

ripening are not related to the phenotypes *freudenreichii*, *shermanii*, lac+/nit+ and lac-/nit-. In other words, the classification of strains into these phenotypes does not bring any information concerning their interest as flavour-producing cultures in cheese.

Conclusion

This study demonstrates that the ability to ferment lactose and reduce nitrate are not correlated in *P. freudenreichii* and confirms the existence of a very large proportion of strains that cannot be unclassied into the currently defined subspspecies. We also showed that the subdivising *P. freudenreichii* strains from their property to ferment lactose or reduce nitrate does not give information about their ability to produce aroma compounds, one of their important property for cheese ripening. Taken together, the present results demonstrates that the division of *P. freudenreichii* into two subspecies does not appear relevant.

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Table 1. Results of phenotypical tests of lactose fermentation and nitrate reductase evaluated after two times of incubation (2 and 5 days), and presence or absence of the operon lactose and nitrate reductase

Strain	Strain origin		*Lactose*		*Nitrate*				
Name	**Biotope**	**Country**	***2 d***	***5 d***	***2 d***	***5 d***	**Phenotype**	**Lac operon**	**Nit operon**
CIRM-BIA121	Swiss cheese	USA	0	0	1.	1	F	0	1
CIRM-BIA122	NA	NA	0	1	0	1	P+	1	1
CIRM-BIA514	Hay	France	0	0	1	Nd	F	0	1
CIRM-BIA9	Emmental cheese	The Netherlands	0	1	0	0	S	1	0
CIRM-BIA118	Gruyère cheese	France	0	1	0	0	S	1	0
CIRM-BIA135	Ewe raw milk	France	0	0	0	0	S	1	0
CIRM-BIA508	Yack cheese	Nepal	0	1	0	0	S	1	0
CIRM-BIA513	Gruyère cheese	France	0	1	0	1	P+	1	1
CIRM-BIA516	Ras cheese	Egypt	0	0	1	nd	P+	1	1
CIRM-BIA1	unk		0	1	0	0	S	1	0
B42	Milk	Brazil	0	1	1.	nd	P+	nd	nd
B43	Milk	Brazil	1	Nd	0	1	P+	nd	nd
B44	Milk	Brazil	0	0	0	1	F	nd	nd
B46	Milk	Brazil	0	0	0	1	F	nd	nd
B66	grass	Brazil	1	Nd	0	1	P+	nd	nd
B70	Milk	Brazil	1	Nd	0	0	S	nd	nd
B75	Milk	Brazil	0	0	0	0	P-	nd	nd
B78	Milk	Brazil	0	1	0	0	S	nd	nd
B82	Soil	Brazil	0	1	1.	nd	P+	nd	nd
B86	Soil	Brazil	1	Nd	0	0	S	nd	nd
B89	Soil	Brazil	0	0	0	0	P-	nd	nd
B91	silage	Brazil	1	Nd	0	0	S	nd	nd
B141	Milk	Brazil	0	1	0	0	S	nd	nd
B145	Milk	Brazil	0	0	0	0	P-	nd	nd
B148	Milk	Brazil	0	0	0	0	P-	nd	nd
B158	Soil	Brazil	0	0	1.	nd	F	nd	nd
B171	grass	Brazil	0	0	0	0	P-	nd	nd
B172	grass	Brazil	0	0	0	0	P-	nd	nd

nd, not determined; unk, unknown

phenotype: *freudenreichii* (F), *shermanii* (S), lac+/nit+ (P+) and lac-/nit- (P-)

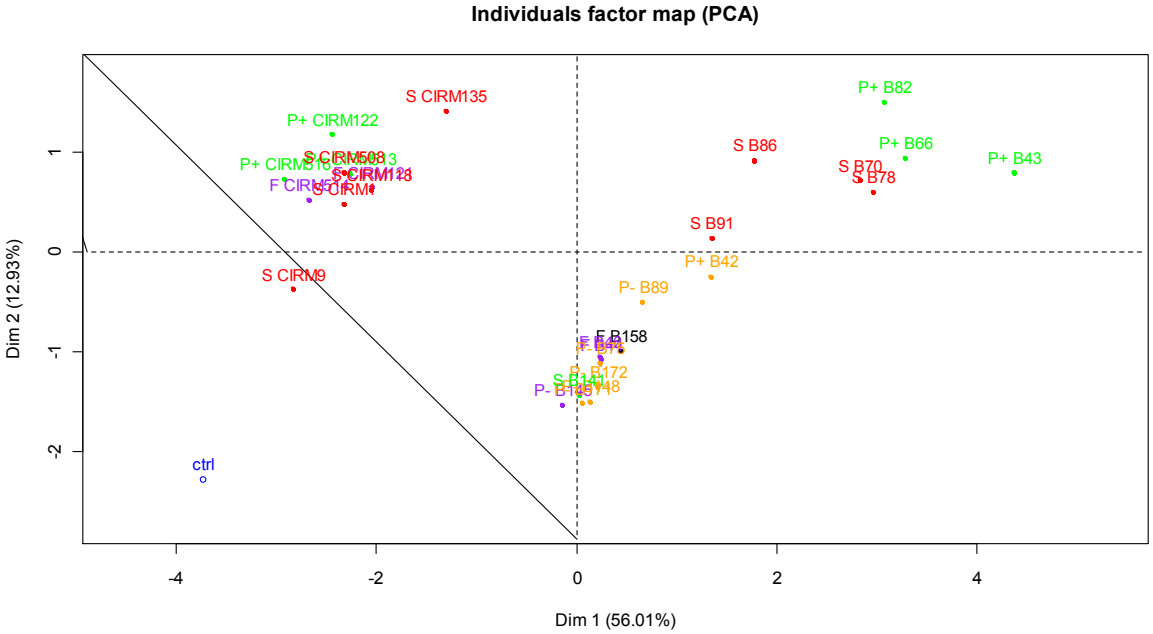

Fig. 1. Results of principal component analysis (PCA) performed on 16 volatile compounds analysed by GC–MS. PCA scores and loadings for the first two principal components. The control medium (ctrl) is projected a supplementary sample. Codes: *freudenreichii* (F), *shermanii* (S), lac+/nit+ (P+) and lac-/nit- (P-)

6. GENERAL CONCLUSION

The methodologies applied in this research allowed the genetic and phenotypic study of the biodiversity of autochtonous propionibacteria from dairy farms located in the Campo das Vertentes region in Minas Gerais, Brazil.

Preliminarily, an alternative method using Petrifilm^{TM} Aerobic Count system associated to the LG selective medium was successfully adapted to replace the traditional methods used for propionibacteria enumeration. This alternative method indicate the adequate selectivity for the enumeration this group in reference and commercial cultures, and in Emmental cheeses. The Petrifilm^{TM} AC plates added to LG broth is faster than traditional method and have the advantages of low cost and simplicity, and it may be used as a monitoring toll in the dairy industry to control the proper quality of products.

Considerable biodiversity was found in the isolates, with *P. freudenreichii, P. jensenii* and *P. acidipropionici* (three of the main species of dairy propionibacteria) identified randomly in the analyzed samples. Remarkably, no identical strains were found in the different farms analyzed, what means that they represent specific niches. The microbial diversity can be explained by the bacteria ability to adapt to the environment.

The results from MLST have shown low level of nucleotidic diversity between the *P. freudenreichii* strains isolated in this work and strains identified in other countries (CIRM-BIA), a possible consequence of strains dissemination through international trade of starter cultures and cheeses.

Results of the phenotypic tests for *P. freudenreichii* classification in subspecies show the need of establishing standard analytical protocols for strain characterization. To provide consistent results phenotypic characterization for *P. freudenreichii* is necessary to check the conditions

(parameters) determined in this work, with greater attention to the concentration of key compounds (lactose and potassium nitrate) and incubation time, and to include in the search the type-strains of the *P. freudenreichii* subspecies.

Additionaly, two phenotypes (P+ and P-) were identified in addition to the classical *P. freudenreichii* subsp *freudenreichii* and *P. freudenreichii* subsp *shermanii.* There was no correlation between the ability of these phenotypes to ferment lactose and reduce nitrate, or between the phenotypic profile and the ability to produce aroma compounds.

Lastly, the results of this research suggest that the farm environment and the raw milk are reservoirs of *Propionibacterium* ssp., and the isolated strains present potential for future applications as secondary cultures for cheese processing.