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COMPREHENSIVE REVIEWS IN FOOD SCIENCE AND FOOD SAFETY

Propionic acid bacteria in the food industry: An update on essential traits and detection methods

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Abstract

Propionic acid bacteria (PAB) is an umbrella term for a group of bacteria with the ability to produce propionic acid. In the past, due to this common feature and other phenotypic similarities, genetically heterogeneous bacteria were considered as a single genus, *Propionibacterium*. Members of this genus ranged from "dairy propionibacteria," which are widely known for their role in eye and flavor formation in cheese production, to "cutaneous propionibacteria," which are primarily associated with human skin. In 2016, the introduction of two new genera based on genotypic data facilitated a clear separation of cutaneous (*Cutibacterium* spp.) from dairy PAB (*Propionibacterium* spp., *Acidipropionibacterium* spp.). In light of these taxonomic changes, but with particular emphasis on dairy PAB, this review describes the current state of knowledge about metabolic pathways and other characteristics such as antibiotic resistance and virulence factors. In addition, the relevance of dairy PAB for the food industry and cheese production in particular is highlighted. Furthermore, methods for cultivation, detection, and enumeration are reviewed, incorporating the current taxonomy as well as the potential for routine applications.

KEYWORDS

Acidipropionibacterium, dairy, identification, metabolism, *Propionibacterium*

1 | INTRODUCTION

The first important appearance of propionic acid bacteria (PAB) can be dated to 1878, when Fitz conducted his work on the biochemistry of PAB (Fitz, 1884; Stackebrandt et al., 2006). After this study, this bacterial group was overlooked until approximately 25 years later, when von Freudenreich and Orla-Jensen investigated propionic acid fermentation. These authors were also the first to isolate pure cultures from Emmental cheese (Hettinga & Reinbold, 1972b). At this point, most of the research was concerned with the effects of dairy PAB in cheese production and the issue of taxonomy (Hettinga & Reinbold, 1972b). Virtanen turned his attention more toward understanding the chemical mechanisms of propionic acid fermentation, and in 1928, Van Niel recognized eight species after reviewing earlier works and elaborating his own investigations (Hettinga & Reinbold, 1972b; Stackebrandt et al., 2006). However, the greatest contribution in the investigation of the fermentative abilities of PAB was made by Wood and Werkman, and even today, the metabolic pathway of the reduction of pyruvate to propionate is referred to as the Wood–Werkman cycle (Hettinga & Reinbold, 1972b; Thierry et al., 2011). Since then, many new insights concerning PAB, but also

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TABLE 1 Reorganization of the former genus *Propionibacterium* (Parte et al. 2020)

Propionibacteriaceae		
***Propionibacterium* spp.**	***Acidipropionibacterium* spp.**	***Cutibacterium* spp.**
P. acidifaciens	*A. acidipropionici*[a]	*C. acnes*
P. australiense	*A. damnosum*	*C. avidum*
P. cyclohexanicum[b]	*A. jensenii*[a]	*C. granulosum*
P. freudenreichii[a]	*A. microaerophilum*[b]	*C. modestum*
P. ruminifibrarum	*A. olivae*	*C. namnetense*
	A. thoenii[a]	
	A. virtanenii	

[a]Species considered as "dairy propionic acid bacteria" (dairy PAB).
[b]Sometimes included in the dairy PAB group, even though rarely encountered in the dairy environment.

regarding single species, have come to light, and the genera as well as the species have been restructured and reclassified on a number of occasions (Stackebrandt et al., 2006). The latest reclassification, on which today's taxonomy is built, occurred in 2016 (Scholz & Kilian, 2016).

2 | TAXONOMY

PAB belong to the phylum of Actinobacteria with a high GC content of approximately 57–70% of their genome. The family of *Propionibacteriaceae* contains 25 genera (Parte et al., 2020). Among these, three are recognized as PAB: *Acidipropionibacterium* spp., *Cutibacterium* spp., and *Propionibacterium* spp. (Deptula et al., 2017b; Patrick & McDowell, 2012; Turgay et al., 2020). Scholz and Kilian also proposed a fourth genus, *Pseudopropionibacterium*, with two assigned representatives, but despite the article's publication, the name is considered illegitimate, and the two species are currently assigned to the genus *Arachnia*, namely, *Arachnia propionica* and *Arachnia rubra* (Parte et al., 2020; Scholz & Kilian, 2016). Prior to the currently valid classification of PAB into three genera in 2016, all PAB were assigned to the genus *Propionibacterium* (Scholz & Kilian, 2016). As the first propionibacteria were isolated from cheese and the dairy and cattle environment, the designation "dairy propionic acid bacteria" evolved. Today, some members of the genera *Propionibacterium* and *Acidipropionibacterium* are referred to as "classical" or "dairy" PAB, even though not all members are found in the dairy environment. Species belonging to dairy PAB are identified as such in Table 1, and the relevance of dairy PAB for the food industry is described in more detail in Section 4. The genus *Cutibacterium* was long regarded as *Corynebacterium*, until in 1946 it was proven that propionic acid is a major end product of its metabolism, which led to its allocation as *Propionibacterium*. Subsequently, members of this genus were often referred to as "cutaneous propionibacteria" due to their association with human skin (Patrick & McDowell, 2012).

One aspect that accentuates the relevance of the differentiation between propionibacteria and acidipropionibacteria is the cell wall composition. The genera *Propionibacterium* and *Acidipropionibacterium* here differ, as members of propionibacteria containing meso-2,6-diaminopimelic acid and acidipropionibacteria are characterized by LL-2,6-diaminopimelic acid, with the exception of *Propionibacterium cyclohexanicum*, which contains neither (Patrick & McDowell, 2012; Turgay et al., 2020). The differentiation of cutibacteria from the other two genera by peptidoglycan comparison is hardly possible. In fact, the presence of LL-2,6-diaminopimelic acid and C_{15} iso- and anteiso acids as principal fatty acids of cell lipids and the production of propionic acid are reasons for the assignment of the former corynebacteria to propionibacteria (Patrick & McDowell, 2012). Nevertheless, the genus *Cutibacterium* is clearly separable from the genera *Propionibacterium* and *Acidipropionibacterium*, as it has a 5–10% lower genomic DNA GC content (Scholz & Kilian, 2016).

An overview of all species currently assigned to the three different genera can be found in Table 1 (status as of July 2021). At the species level, the most prominent representatives as well as type species of the three PAB genera are *Acidipropionibacterium acidipropionici, Cutibacterium acnes*, and *Propionibacterium freudenreichii* (Scholz & Kilian, 2016). *A. acidipropionici* is known for its beneficial effect on bovine rumen and potential in propionic acid production (Patrick & McDowell, 2012; Scholz & Kilian, 2016). *C. acnes* is frequently isolated from human skin and has been tied to the skin disorder acne vulgaris, as it colonizes hair follicles in affected patients. Colonization is one of the four processes during the formation of acne lesions, but the exact course of events during the formation of acne vulgaris as well as the effect of other factors influencing the disease still remain unclear (Williams et al., 2012). *P. freudenreichii* is foremost known for its role in Emmental and Swiss-type cheese production. In addition, the organism has been associated with the industrial production of vitamin B12 and strain-dependent probiotic

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and anti-inflammatory properties due to the production of several beneficial metabolites as well as bifidogenic factors (Altieri, 2016; Rabah et al., 2017; Rabah et al., 2018, Thierry et al., 2015; Turgay et al., 2020). *P. freudenreichii* was formerly divided into the subspecies *freudenreichii* and *shermanii* based on its ability of lactose fermentation and nitrate reductase activity (Dalmasso et al., 2011). However, because the genes coding for lactose fermentation are surrounded by integrases and transposases, indicating acquisition by horizontal gene transfer, and the genes coding for nitrate degradation are disrupted by a frameshift in *P. freudenreichii* strains that are unable to degrade nitrate, the division is no longer valid (Dalmasso et al., 2011; Deptula et al., 2017b). Additionally, when tested for the trait, authors have reported an inhibitory effect of potassium nitrate even at low dosage on nitrate reductase-positive organisms (Freitas et al., 2015b).

3 | METABOLISM

PAB are Gram-positive, nonmotile, nonsporulating bacteria. They appear as short rods, albeit their morphology can differ, depending on the conditions and their phase of growth. Hence, they are also referred to as pleomorphic (Fröhlich-Wyder et al., 2017; Turgay et al., 2020). They can also appear coccoid, bifid, branched, or filamentous and may occur single, paired, or in short chains with V or Y configurations; however, the formation of clumps that have been characterized as resembling "Chinese characters" is also possible (Patrick & McDowell, 2012; Turgay et al., 2020). Cutibacteria appear to be longer and thinner than the dairy species, especially during the early stages of growth (Patrick & McDowell, 2012). PAB are anaerobic to aerotolerant, and optimal growth occurs at a pH of 6–7, with a minimum pH of 4.6 and maximum of 8.5 (Fröhlich-Wyder et al., 2017). Dairy PAB show a sensitivity to salt, but to which extent PAB growth is impaired is strain dependent and also influenced by pH (Fröhlich-Wyder et al., 2017). In a whey-based culture, for instance, growth and propionic acid production were completely inhibited by the addition of 6% NaCl, corresponding to an a_w of 0.955 (Bisig et al., 2019). Moreover, in Swiss-type cheeses the volatile fatty acid content as well as CO_2 production by dairy PAB are also influenced by the salt concentration (Fröhlich-Wyder et al., 2017). Growth of PAB has been reported at temperatures lower than 14°C, with a strain-dependent optimum at 25–35°C (Jakob et al., 2016; Turgay et al., 2011). They may survive exposure to higher temperatures, but the ability and temperature endurance are highly strain dependent and are further discussed in Section 4 (Fröhlich-Wyder et al., 2017). Dairy PAB grow rather slowly, with generation times of approximately 5 h under optimal conditions (Falentin et al., 2010a). Interestingly, Patrick & McDowell (2012) reported faster growth of the cutaneous group than among dairy PAB, with an average incubation time of 7 and 14 days, respectively, but incubation times longer than 14 days for dairy PAB is rarely reported (Tharmaraj & Shah, 2003). Studies suggest, nevertheless, that contamination with *C. acnes* is often underdiagnosed, and prolonged incubation times for cutibacteria would improve their detection (Dagnelie et al., 2018; Foster et al., 2020). On solid media, PAB form lenticular colonies with a diameter of 1–4 mm (Turgay et al., 2020). The color of the colonies ranges from cream to orange or red brown (Patrick & McDowell, 2012; Turgay et al., 2020). The manifestation of color depends on the one hand on the studied species (see Table 2) and on the other hand the growth conditions, though this may differ between aerobic and anaerobic incubation (Turgay et al., 2020).

In terms of sugar utilization, the abilities are highly strain dependent, but all PAB can ferment a variety of substances, including carbohydrates, polyols, and organic acids (Turgay et al., 2020). Most PAB are able to utilize sugars such as lactose, galactose, or D-glucose but also alcohols such as glycerol (Freitas et al., 2013; Patrick & McDowell, 2012). In terms of nitrogen requirements, *P. freudenreichii* is prototrophic for all amino acids and nucleotides, whereas concerning vitamins, some PAB strains require only pantothenate and biotin, while others additionally need thiamine and p-aminobenzoic acid (Falentin et al., 2010a). An overview of exemplary factors influencing dairy PAB growth in contrast to *C. acnes* is given in Table 2. Bergey's Manual of Systematic Bacteriology states that more than 90% of *P. freudenreichii* strains are capable of arabinose, erythritol, esculin, fructose, galactose, glucose, glycerol, and mannose degradation (Patrick & McDowell, 2012). Furthermore, Patrick & McDowell (2012) reported that between 40% and 90% of *P. freudenreichii* were able to degrade adonitol, inositol, and ribose, while 10–40% could degrade lactose and melibiose. Loux et al. (2015) disclosed that the numbers of sugars utilized by the different strains of *P. freudenreichii* in their study ranged from 10 to 15. Additionally, each strain displayed a unique fermentation profile. The percentages given by Bergey's Manual for the utilization of sugars by the different strains could not be confirmed by Loux et al. (2015), as, for example, all tested strains were capable of fermenting ribose. A similar picture can be observed for lactose catabolism (80% of the tested strains of Loux et al. vs. 10–40% in Patrick & McDowell). However, most of the strains in Loux et al. (2015) originated from cheese and the cheese environment and therefore may have been subjected to domestication. The question arises, whether this change can be seen as a development over time or instead due to a different strain selection, noting that in publications from the late 1990s,

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TABLE 2 Characteristics of dairy PAB and *C. acnes* according to Patrick & McDowell (2012)

	Species				
Characteristic	***P. freudenreichii***	***A.acidipropionici***	***A.jensenii***	***A.thoenii***	***C.acnes***
Optimum growth temperature [°C]	30–32	30–32	30–32	30–32	36–37
Color[b]	Cream	Cream to orange	Cream to red brown	Orange to red brown	Grayish, semi-opaque[c]
Growth requirement	Biotin (sd) Pantothenate Thiamine (sd)	Biotin Pantothenate	Biotin Pantothenate p-aminobenzoic acid (sd)	Biotin Pantothenate Thiamine (sd)	Pantothenate
Growth stimulation	Thiamine (sd)	Thiamine	Thiamine p-aminobenzoic acid (sd)	Thiamine (sd)	Biotin Nicotinamide Thiamine
Acid production					
–Inositol	i+	+	i+	i+	i–
–Lactose	i–	+	i+	i–	–
–Maltose	–	+	i+	i+	–
–Sucrose	–	+	+	i+	–
Esculin hydrolysis	+	+	+	+	–
Indole production	i	–	–	–	i+
Nitrate reduction	sd	+	–	–	i+
Growth in 20% bile	+	+	+	–	+
Gelatin hydrolysis	–	–	–	–	+
ß-hemolysis	–	–[a]	sd	sd	i+
Pigmentation[d]	–	–	sd	sd	–
CAMP reaction	–	–	–	–	sd

Note: +, positive in >90% of isolates; –, negative >90% of isolates; i, 11–89% of isolates are positive; i+, 40–90% of isolates are positive; i–, 10–40% of isolates are positive; sd, strain dependent,

[a]Slight ß-hemolysis under confluent area of growth possible.

[b]According to Turgay et al. (2020), depending on aerobic/anaerobic conditions.

[c]May turn orange to pink if kept for 3 weeks.

[d]Corresponding to granadaene production according to Deptula et al. (2019).

P. freudenreichii was still deemed unfit for lactose fermentation (Piveteau, 1999).

An overview of metabolic pathways and catalyzing enzymes of *P. freudenreichii* is provided in Figure 1. *P. freudenreichii* was chosen for illustration as the representative PAB because it is the most prominent example of dairy PAB in cheese production and its environment.

Particularly characteristic for all PAB is the production of propionate, acetate, and carbon dioxide via the Wood–Werkman cycle, but ratios of propionate and acetate may vary widely (Deptula et al., 2017b; Stackebrandt et al., 2006; Thierry et al., 2011). In dairy PAB, carbohydrates are either oxidized to produce pyruvate via glycolysis or the pentose phosphate pathway or utilized for trehalose production (Turgay et al., 2020). Disaccharide trehalose is composed of two linked glucose molecules and formed as a response to stresses as a protective function (Turgay et al., 2020). Pyruvate can be converted into lactate or alanine (Turgay et al., 2020). Two other pathways for the further metabolism of pyruvate are possible: one pathway involves the conversion of pyruvic acid to acetic acid via the pyruvate dehydrogenase activity (Figure 1 (d)), leading to the production of acetate and CO_2 and generating NADH (Turgay et al., 2020). The other pathway reduces pyruvate to propionate via the Wood–Werkman cycle (Thierry et al., 2011; Turgay et al., 2020). As the Wood–Werkman cycle continues, the NADH produced during glycolysis is utilized, and extra adenosine triphosphate (ATP) is formed (Thierry et al., 2011). The extent of pyruvic-acid transformation into propionate or into acetate and CO_2 can be seen as a function of the amount of co-enzymes reduced during substrate oxidation to pyruvic acid to maintain the redox balance, but proportions between the two pyruvate pathways also differ depending on strain properties, substrate, and environmental conditions (Falentin et al., 2010a; Thierry et al., 2011; Turgay et al., 2020). One of the key reactions is the transfer of a carboxyl group from methylmalonyl–CoA to pyruvic acid so propionyl–CoA

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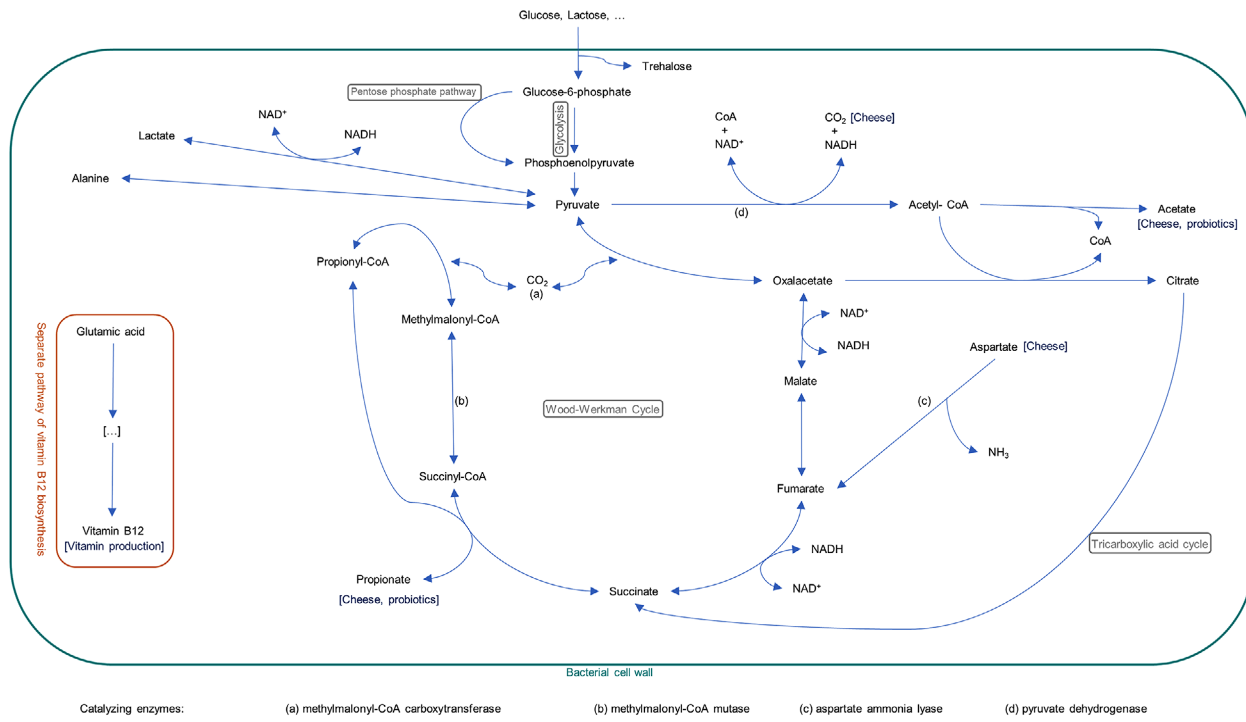

FIGURE 1 Metabolism of *P. freudenreichii* (based on Falentin et al., 2010a; Piveteau, 1999; Thierry et al., 2011; Turgay et al., 2020)

and oxaloacetic acid can be formed without the intervention of free CO_2 (Falentin et al., 2010a; Thierry et al., 2011). The enzyme responsible for the transfer, biotin-dependent methylmalonyl–CoA carboxytransferase (a; E.C. 2.1.3.1), has thus far only been found in propionibacteria. It consists of three polypeptide subunits, one of which has been used as a molecular marker for investigations of the probiotic abilities of *P. freudenreichii* in the human digestive tract (Turgay et al., 2020). The conversion from succinyl–CoA to methylmalonyl–CoA is catalyzed by methylmalonyl–CoA mutase (b; E.C. 5.4.99.2), which is vitamin B12 dependent (Turgay et al., 2020).

The utilization of aspartate in *P. freudenreichii* is also possible, but the extent of aspartase activity is strain dependent (Turgay et al., 2020). Aspartate is deaminated to fumarate and consequently enters the Wood–Werkman cycle (Falentin et al., 2010a). Utilization of aspartate leads to regeneration of oxidized co-enzymes and ATP (Falentin et al., 2010a). In *P. freudenreichii*, two enzymes have been tied to aspartate catabolism: aspartate oxidase (E.C. 1.4.3.16), converting aspartic and fumaric acids into succinic and imminosuccinic acids, and aspartate–ammonia lyase (c, E.C. 4.3.1.1), catalyzing deamination of aspartic acid and subsequent formation of fumaric acid and ammonia (Turgay et al., 2020). Various strains possess two neighboring genes encoding aspartate–ammonia lyase, which is important to note in the context of cheese-making (Turgay et al., 2020). Such strains produce more succinic acid than ones with one coding gene, and thus CO_2 formation is enhanced as well (Thierry et al., 2011). In the absence of other substrates, aspartic and propionic acids can be metabolized too; in this case the Wood–Werkman cycle is reversed, and propionic acid is converted into succinic acid (Turgay et al., 2020).

The biosynthesis of vitamin B12 by *P. freudenreichii* is a process that, when in culture, occurs at the later stages of growth and is therefore presented separately in Figure 1 (Deptula et al., 2017b). The starting point for vitamin B12 production is glutamic acid, which is converted into porphobilinogen by multiple reactions and afterward polymerized to preuroporphyrinogen. Further reactions are required until the active form of vitamin B12 is finally formed (Turgay et al., 2020). Interestingly, Deptula et al. (2017b) observed cobalamin as well as pseudo-cobalamin at the beginning of incubation in their study, but the level of pseudo-cobalamin was below the limit of detection at the end of incubation, indicating a conversion process to the active form. It is worth noting that the formation of the active form requires aerobic and anaerobic conditions (Turgay et al., 2020). The lower ligand of the active form cannot be produced under strictly anaerobic conditions, explaining the observations made by Deptula et al. (2017b). Additionally, blue light is necessary (Turgay et al., 2020).

Even though phylogenetic analysis of the 16S rRNA gene sequences revealed a distinct and coherent clade of *Acidipropionibacterium*, *Cutibacterium*, and *Propionibacterium* within the family *Propionibacteriaceae*, the dairy

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and cutaneous species form two separate clusters (Scholz & Kilian, 2016; Stackebrandt et al., 2006). Considering the different habitats of cutibacteria and dairy PAB, it is logical that some genes are lost during adaptation to the human host. Cutibacteria possess a smaller genome than the dairy group. Only *P. freudenreichii* is an exception in this regard, as it possesses an even smaller genome than cutibacteria (Scholz & Kilian, 2016). Scholz and Kilian (2016) found that *C. acnes* no longer possesses levanase activity, an enzyme-catalyzing degradation of fructose polymers found in grasses, nor the genes necessary for transporting branched-chain amino acids and glutamate decarboxylase. However, due to adaptation to the new host, not only were genes lost, but new genes were also acquired. The authors reported a total of 108 genes have been uniquely found in cutibacteria. In the investigated strains, new genes for iron absorption were found as well as differences in genes related to sugar uptake and lipases, namely, triacylglycerol lipase and pyrophosphokinase, which enable survival in the hostile sebaceous follicles (Scholz & Kilian, 2016).

In conclusion, especially dairy PAB possess a unique metabolism that allows them to use various energy sources and produce several compounds interesting for industrial applications. The ability of *A. acidipropionici* to produce large quantities of propionic acid, for instance, shows potential for industrial-scale production of this organic acid (Assis et al., 2020; Deptula et al., 2018). When used as a probiotic, *P. freudenreichii* has been reported to withstand digestive stresses and inhere to the intestinal epithelial cells, where the organism produces a number of metabolites, for example, short-chain fatty acids and surface proteins that positively influence human and animal gastrointestinal health (Nair et al., 2019; Rabah et al., 2017; Rabah et al., 2018). Additionally, fortification of foods with *P. freudenreichii* has been explored to increase the nutritional value leading to enhanced vitamin B12 uptake by the consumer. *P. freudenreichii* is one of the most frequently used organisms for industrial vitamin B12 production, and B12 formation is a very complex process influenced by many factors (Assis et al., 2020). Additional studies to further improve the industrial-scale utilization of *P. freudenreichii* are encouraged.

4 | DAIRY PAB IN CHEESE PRODUCTION

Dairy PAB include the species *P. freudenreichii*, *Acidipropionibacterium thoenii*, *A. jensenii*, and *A. acidipropionici*, among which *P. freudenreichii* is encountered most frequently in cheese production (see Table 1) (Blasco et al., 2015; Todesco et al., 2000). Only rarely are *A. microaerophilum* and *P. cyclohexanicum* considered dairy PAB, as most authors do not include them because they have not or have only extremely seldomly been encountered in dairy samples and were first isolated from olive-mill waste water and spoiled orange juice (Beresford et al., 2001; Jakob et al., 2016; Koussémon et al., 2001; Turgay et al., 2020; Walker & Phillips, 2007). With the recent division into three genera, referring to propionibacteria in the context of dairy practices can be ambiguous because *P. freudenreichii* is the only species of the genus *Propionibacterium* possessing dairy relevance, whereas three species of the genus *Acidipropionibacterium*, namely, *A. acidipropionici*, *A. jensenii*, and *A. thoenii*, are relevant for the dairy industry as well. This fact must be kept in mind, especially regarding older literature that refers to the above-named organisms only as (dairy) propionibacteria. The dairy-relevant propioni- and acidipropionibacteria have been isolated from milk, cheese, and fermented products, in addition to the cattle environment, where they are present in the rumen and intestines of ruminants and in silage (Falentin et al., 2010a; Fröhlich-Wyder et al., 2017). Furthermore, biofilms and deposits of heat-resistant bacteria, including dairy PAB, can on occasion be found in insufficiently cleaned milking equipment (Turgay et al., 2018; Turgay et al., 2016).

Acidipropionibacteria as well as propionibacteria can be used as protective cultures in food. *A. jensenii* and *A. thoenii*, for instance, produce various bioprotective substances such as bacteriocins and antifungal compounds that may harbor potential for industrial applications (Altieri, 2016; Thierry et al., 2015; Turgay et al., 2020). *P. freudenreichii* and *A. acidipropionici* have long been documented in food and cheese production, and both possess the qualified presumption of safety (QPS) status. *P. freudenreichii* also has generally recognized as safe (GRAS) status (Deptula et al., 2018; Rabah et al., 2017). Hence, *P. freudenreichii* can be used as the starter culture in a wide a range of different cheeses in which propionic acid fermentation is desired, such as Emmental and Swiss-type cheeses (Fröhlich-Wyder et al., 2017). In stark contrast to the positive effects of PAB starter cultures are the deleterious effects on cheese quality when the presence of PAB is undesirable. In cheese production, dairy PAB may also cause a range of quality defects, such as late fermentation, spotting in the cheese matrix, or slits and cracks in the cheese body (Fessler et al., 1999a; Jimeno et al., 1995; Thierry et al., 2011). Establishing a clear dividing line between PAB traits causing positive effects and those causing quality defects is often difficult and dependent on the type of cheese.

Emmental cheeses with a protected designation of origin (PDO) or protected geographical indication (PGI) are made from high-quality raw milk. In the production process, these cheeses undergo propionic acid fermentation during the aging period, a duration of 5 months to 1 year

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or more (Fröhlich-Wyder et al., 2017). During this long ripening time, a characteristic taste and eyes in the cheese body develop. In contrast, Emmental cheeses produced according to the Codex Standard CXS 269–1967, sometimes also referred to as "generic" Emmental, are frequently made from pasteurized milk and have shorter ripening times (Codex Alimentarius Commission, 2019; Fröhlich-Wyder et al., 2017). Moreover, a broad range of semi-hard cheeses can be made using mesophilic, and defined PAB as starter cultures. Their production technology combines those of Gouda and Emmental cheese, and thus they are also referred to as "Goutaler" (Fröhlich-Wyder et al., 2017). Some cheeses such as Comté (PDO) or Fontina (PDO) are made from raw milk but do not necessarily have to undergo propionic acid fermentation. However, if dairy PAB are present in raw milk, propionic acid fermentation is tolerated (Fröhlich-Wyder et al., 2017). Due to their positive properties and the resulting sweet and nutty aroma, dairy PAB may also be incorporated in cheeses that are normally produced without dairy PAB, such as Feta or Raclette cheese (Angelopoulou et al., 2017; Fröhlich-Wyder et al., 2017; Thierry et al., 2005).

Because cheese types also feature different characteristics and sensory profiles, the choice of adequate PAB starter cultures is crucial, with one important criterion being aspartase activity. High aspartase activity can lead to enhanced fermentation and production of CO_2 (Blasco et al., 2011; Fröhlich-Wyder et al., 2017). CO_2 migrates through the curd and forms the characteristic cheese eyes. However, if the rate of fermentation is too high, the cheese body is unable to withstand the gas pressure, leading to slits and cracks in the curd. Furthermore, high aspartase metabolism leads to higher amounts of succinic acid and ammonia, which both strongly influence cheese flavor (Fröhlich-Wyder et al., 2017). Thus, high aspartase activity is undesirable if cheeses are ripened for 12 months or longer, as with Emmental PDO cheese, as they will be more prone to late fermentation (Fröhlich-Wyder et al., 2017). On the other hand, using cultures with a high aspartase activity can reduce the ripening time during warm room storage by up to 10 days and contribute to flavor development in cheeses with short ripening times (Fröhlich-Wyder et al., 2017). An example in which such a scheme is used to more quickly market the end product is "generic" Emmental, or Swiss-type cheeses made from pasteurized milk (Fröhlich-Wyder et al., 2017).

Even though dairy PAB starters are widely used in cheese, another notable feature is that PAB, which naturally occur in the dairy environment, have maintained their diversity. Indeed, wild-type PAB differ significantly from available starter cultures (Fröhlich-Wyder et al., 2017). In cheese production, differentiating between commercial starter cultures and potentially detrimental wild-type strains is important. Generally, dairy PAB are easily inactivated by higher temperatures. Consequently they do not withstand heating regimes during pasteurization, but *P. freudenreichii* may endure temperatures of up to 55°C for approximately 30 min, as applied during the cooking or scalding of Emmental cheeses (Fröhlich-Wyder et al., 2017; Turgay et al., 2020). Acidipropionibacteria are more heat sensitive but, depending on the cooking or scalding scheme of the manufactured cheese, may survive the process as well (Fessler et al., 1999a). Cheese milk therefore risks contamination by wild-type dairy PAB even after heating procedures (Blasco et al., 2011). Cheese quality defects like late fermentation are rather pressing for raw-milk Emmental and Swiss-type cheeses, and late fermentation by dairy PAB is enhanced by long ripening times (Bachmann et al., 2011; Fröhlich-Wyder et al., 2017; Turgay et al., 2011). If propionic acid fermentation must be avoided, Bachmann et al. (2011) suggest that PAB counts should be lower than 30 CFU/mL in raw cheese milk, whereas initial counts lower than 10 CFU/mL are occasionally recommended (Rossi et al., 1999). Of course, the required thresholds vary among different cheese types.

In addition to blowing defects, dairy PAB can also cause reddish to brown spots in cheese (Fröhlich-Wyder et al., 2017). This phenomenon is mostly encountered during winter (Baer & Ryba, 1999). Spotting has been associated with insufficient addition of PAB starter cultures to raw milk and consequent emergence of wild-type PAB contaminants, whose large colonies become visible as undesired spots. *A. jensenii* and *A. thoenii*, for instance, are pigmented and have been isolated from brown to red spots (Fessler et al., 1999a). This quality defect can be avoided effectively in cheeses with desired propionic acid fermentation by the addition of sufficient PAB starter cultures (Fessler et al., 1999a; Rossi et al., 1999).

As stated, PAB growth exhibits a great influence on cheese quality. The growth of PAB, moreover, is affected by a number of different factors, such as the NaCl content of the cheese, the presence of certain metabolites or substances, and synergistic and antagonistic effects caused by the presence of certain members of the indigenous milk microbiota, for example, lactic acid bacteria (LAB) (Jimeno et al., 1995; O'Sullivan & Cotter, 2017; Piveteau et al., 2000; Thierry et al., 2015). Lactic acid production has an inverse relationship with PAB growth: the slower the acid production, the faster PAB growth happens, and the faster the acid production the slower PAB growth occurs (Fröhlich-Wyder et al., 2017). Facultatively heterofermentative nonstarter lactobacilli (FHL) also greatly influence PAB growth in cheese, as they ferment hexoses in the medium almost exclusively to lactic acid (Fröhlich-Wyder et al., 2017). FHL are found as a component of the indigenous microbiota in raw milk, but they may also be deliberately added in the

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production of Swiss-type cheeses and can be used to reduce PAB fermentation (Fröhlich-Wyder et al., 2017; Quigley et al., 2011). The control of propionic acid fermentation by FHL addition is more effective if *P. freudenreichii* starters have a low aspartase activity because dairy PAB with high activity are less inhibited by FHL (Turgay et al., 2011). Dairy PAB with high aspartase activity may be introduced into the cheese in the form of wild-type PAB and can cause the above-stated quality defects, particularly in cheeses with long ripening times (Fröhlich-Wyder et al., 2017).

Further antagonistic effects against PAB growth have been observed for *Lacticaseibacillus casei*, *Lb. rhamnosus* (old genus name *Lactobacillus*), and *Lactiplantibacillus* (old genus name *Lactobacillus*) *plantarum* for reasons that are not well understood. Growth inhibition seems to be caused by competition for limiting substances without facilitation by an inhibiting substance (Beresford et al., 2001). In a study of 20 lactobacilli strains, 9 had antagonistic effects on PAB (Beresford et al. 2001). Fröhlich-Wyder et al. (2017) elucidated some antagonistic mechanisms for *Lb. rhamnosus*, hypothesizing that diacetyl may be a causative agent due to its lethal effect on PAB. Furthermore, acetate and formate also inhibit PAB growth. In contrast, *Lactobacillus helveticus*, which is often used as a starter culture in combination with PAB, promotes PAB growth (Fröhlich-Wyder et al., 2017). This LAB species seems to remove an inhibitory substance, which remains unidentified. Indeed, *L. helveticus* is often replaced by other LAB to avoid extensive PAB growth in Emmental PDO cheese (Fröhlich-Wyder et al., 2017).

Interestingly, PAB growth does not occur in the presence of whey unless it is either substituted with lactate and casein hydrolysate or pretreated with LAB, although these options are only effective when the initial cell density of PAB is >10^5–10^6 CFU/mL (Beresford et al., 2001; Cousin et al., 2012; Piveteau et al., 2000). LAB transform lactose to lactate, which is the preferred carbon source for dairy PAB. After LAB lysis, peptidases are released, which cause the liberation of peptides and free amino acids and thus the growth of PAB (Deptula et al., 2017a; Fröhlich-Wyder et al., 2017; Turgay et al., 2020). Whey, however, also seems to contain a heat-stable inhibitor for PAB growth (Beresford et al., 2001; Deptula et al., 2017a). Piveteau et al. (2000) suggested that the presence of immunoglobulins, transferrin, lactoferrin, lactoferrin-derived, or casein-derived proteins might cause inhibition of PAB. Controversially, Deptula et al. (2019) observed that pigmented strains of *A. thoenii* and *A. jensenii* are somehow protected against growth inhibition caused by whey filtrates.

In conclusion, despite being beneficial for certain cheeses, dairy PAB can cause a range of defects in cheese and consequently lead to decreases in quality and significant monetary losses for producers (Blasco et al., 2011; Daly et al., 2010). Thus, the effective detection of dairy PAB in raw milk to ensure the production of high-quality, long-ripened cheeses is of utmost importance for the dairy industry.

5 | BIOFILM FORMATION, VIRULENCE FACTORS, ANTIBIOTIC RESISTANCE, AND BACTERIOPHAGE SENSITIVITY

As previously discussed, dairy PAB are tightly connected to the food production environment, especially for those possessing GRAS and QPS status. In addition, some strains of *P. freudenreichii* and *A. acidipropionici* are also considered as probiotics (Rabah et al., 2017). The absence of antimicrobial resistance in dairy PAB is highly desirable to limit the risk of spreading antimicrobial resistance to the intestinal microbiota (Altieri, 2016). Furthermore, due to their application in food production, it is advisable that PAB do not possess any virulence factors (Deptula et al., 2019). Antibiotic resistance, as well as the biofilm-forming ability of dairy PAB and the occurrence of bacteriophages among PAB, will be discussed in the following section.

Dairy PAB possess the ability of biofilm formation and can be found in insufficiently cleaned milking equipment, heat-resistant biofilms, and deposits in the milking system. To investigate the effects on biofilm formation, Bevilacqua et al. (2019) treated *P. freudenreichii* and *A. jensenii* strains with low-power ultrasound. Interestingly, this led to increased bacterial metabolism and ability to adhere to inert surfaces and thereby better biofilm-forming ability. Better nutrient transportation in deeper layers of the biofilm and a higher degree of sessile cells when compared to an untreated population may have been the reason (Bevilacqua et al., 2019). The ability of *P. freudenreichii* to form biofilms was recently shown to be facilitated by the production of exopolysaccharides (EPS), which can be enhanced if the organism is exposed to unfavorable growth conditions. However, the formation of EPS in propionibacteria and acidipropionibacteria is strain dependent (Cavero-Olguin et al., 2019). For *A. acidipropionici*, biofilm formation reports are scarce, and biofilms have been characterized as weak (Dishisha et al., 2012). Immobilization of an organism is nevertheless a frequent approach to create high-density batch fermentations with improved fermentation kinetics. Cavero-Olguin et al. (2019) investigated biofilm formation of *A. acidipropionici* in repeated batch fermentations for propionic acid production. In batch culture, a modification of the immobilization matrix was necessary in order to obtain immobilization of *A. acidipropionici*, but biofilm formation could be observed (Cavero-Olguin et al., 2019). Utilization of the *A. acidipropionici* biofilm for repeated fermentations

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was possible, but Cavero-Olguin et al. (2019) reported concerns regarding the stability of the system. Due to the limited amount of data on biofilm formation of acidipropionibacteria and propionibacteria, further studies on the topic are encouraged.

Dairy propionibacteria are known to have natural resistances to a few antibiotics, one of them being nalidixic acid, which does not seem to be encoded by a mobile genetic element or plasmid (Altieri, 2016). Altieri (2016) based this conclusion upon a study by Darilmaz and Beyatli (2012), who tested 29 different dairy PAB cheese isolates and 5 reference strains: *P. freudenreichii* (former subsp. *shermanii*) DSM 20270; *P. freudenreichii* (former subsp. *freudenreichii*) DSM 20271; *A. jensenii* DSM 20235; *A. acidipropionici* DSM 20272; and *A. thoenii* DSM 20276. Stackebrandt et al. (2006) described no unusual or very consistent resistance patterns but stated that all strains are highly resistant to sulfonamides and more resistant to semisynthetic penicillin than to penicillin G. In disk sensitivity tests, growth was reported in the presence of up to 1000 μg/mL sulfadiazine (Stackebrandt et al., 2006). While the data in Stackebrandt et al. are based on a study by Reddy et al. (1973) and due to the study's age the strain designations are outdated, all test strains belong to today's four dairy PAB species. Moreover, dairy PAB harbor resistance against vancomycin and ciprofloxacin as well as sensitivity to ampicillin and chloramphenicol (Campaniello et al., 2015). In addition to a moderate susceptibility to gentamycin and streptomycin, Campaniello et al. (2015) reported susceptibility to erythromycin, trimethoprim, and tetracycline, the last being reversible. They performed tests with *P. freudenreichii* DSM 20270, *A. jensenii* DSM 20279, *A. acidipropionici* DSM 20272, and *A. thoenii* DSM 20276. In a different study, Bevilacqua et al. (2019) reported susceptibility to clarithromycin for strains *P. freudenreichii* DSM 20271 and *A. jensenii* DSM 20535. In contrast to Campaniello et al. (2015), Bevilacqua et al. (2019) reported complete inhibition of *P. freudenreichii* and *A. jensenii* growth by ciprofloxacin and vancomycin, for which Campaniello et al. (2015) reported resistance, and observed also a resistance of *A. jensenii* toward trimethoprim, whereas growth of *P. freudenreichii* was inhibited. Tharmaraj and Shah (2003), who studied *P. freudenreichii* (former) ssp. *globosum* (type standard 10360 DSM Gist brocade Australia Pty. Ltd.) as well as *P. freudenreichii* (former) ssp. *shermanii* (PS1 obtained from Chr. Hansen Pty. Ltd.) observed less antibiotic resistance than that reported by Campaniello et al. (2015). In the patent specification for Pal Propiobac™, a selective medium for PAB cultivation from food matrices, it is stated that dairy PAB feature resistance against fosfomycin, aminosides, polypeptides, imidazoles, and first- and second-generation quinolones (Madec et al., 1994). In summary, an absolute statement on antibiotic resistance of dairy PAB is rather complex and furthermore complicated by the fact that most other data on antibiotic resistance is either based on studies with cutaneous species or those that are no longer aligned with the discussed genera due to reorganization (Thierry et al., 2011). As an example, the resistance to aminoglycosides, peptide antibiotics, and nitroimidazole compounds reported in the third edition of "The Prokaryotes" for propionibacteria are based on data concerning *P. propionicum* (Stackebrandt et al., 2006), although the organism has since been reclassified as *A. propionica*, making the validity of these data questionable (Parte et al., 2020).

Because *P. freudenreichii* is also used in starter cultures, the appearance of bacteriophages could potentially be disastrous. However, studies have shown that *P. freudenreichii* phages are rather common and appear to be a chronic rather than an episodic phenomenon (Gautier et al., 1995). Gautier et al. (1995) reported contamination in 50% of their sampled cheeses and stated that, of 44 strains used, only eight were effective for phage detection and of these, four were sensitive to more than one bacteriophage. The analysis of *P. freudenreichii* (formerly subsp. *shermanii*) strain JS demonstrated the possession of a complete clustered regularly interspaced short palindromic repeats and CRISPR-associated proteins (CRISPR Cas) system for defense against phages as well as invading nucleic acids, underlining the findings of Gautier et al. (1995) (Falentin et al., 2010a; Ojala et al., 2017). CRISPR Cas systems, which can be found in bacteria and archaea, are adaptive immune defense systems in which each system is built of two components (Jackson et al., 2017). One contains memory storage (CRISPR array), and the other (Cas genes) encodes the machinery driving immunity (Jackson et al., 2017). A great benefit of the system is the possibility of CRISPR adaptation, which is the process of updating the CRISPR array in response to infection, by the incorporation of short DNA fragments of the invader to form spacers, thus protecting against future encounters (Jackson et al., 2017). Turgay et al. (2020) reported the investigation of *P. freudenreichii* spacers, which confirmed immunity against the seven already sequenced phages B22, Anatole, E1, Doucette, E6, G4, and B3.

Neither *A. thoenii* nor *A. jensenii* possess GRAS or QPS status, yet both organisms have been detected in foods, as these two acidipropionibacteria can be found in silage and the dairy environment. They are often found in milk, fermented milk products, and cheese, as for instance *A. jensenii* was the species most isolated from Leerdammer samples by Britz and Riedel (Britz & Riedel, 1994; Fessler et al., 1999b; Zarate, 2012). In addition, probiotic *A. jensenii* 702 has been incorporated in yogurt and ice cream made from goat milk (Huang et al., 2003; Ranadheera et al., 2012, 2013). Due to their occurrence in food and potential use

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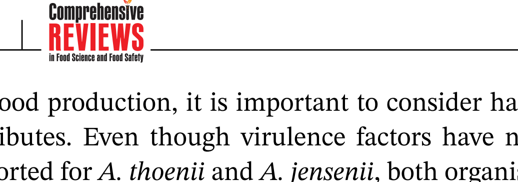

in food production, it is important to consider hazardous attributes. Even though virulence factors have not been reported for *A. thoenii* and *A. jensenii*, both organisms can have *β*-hemolytic activity, which is a virulence property highly undesirable for any organism tied to food production (Altieri, 2016; Deptula et al., 2019). The *β*-hemolytic activity of both organisms is only given if they show pigmentation, which is often observed in cheese with brown or reddish spots. In these cases, the level of hemolytic activity is correlated with the amount of pigment produced (Vanberg et al., 2007). The produced red polyene was identified as granadaene, although the metabolic pathways for its production are yet unknown (Turgay et al., 2020). Interestingly, in *C. acnes* subsp. *acnes*, ß-hemolysis was added to the subspecies description. Further virulence-associated factors of *C. acnes* are discussed in more detail in Mayslich et al. (2021). While pigmentation was not observed in *C. acnes*, in the species *A. rubra*, sometimes also falsely referred to as *Pseudopropionibacterium rubrum*, pigmentation was observed when isolated from the human mouth in 2018, though hemolysis was not (Deptula et al., 2019). Therefore, some controversy remains concerning the interaction of pigmentation and hemolysis in these genera. The same pigment can also be found in *Streptococcus agalactiae* and has been linked with group B streptococcal (GBS) infections associated with preterm birth, fetal injury, and neonatal mortality, and it has recently been demonstrated that GBS pigment and hemolysin are one and the same (Armistead et al., 2019; Rosa-Fraile et al., 2014; Whidbey et al., 2013). It is encoded in the *cyl* operon, coding for 12 genes in GBS (Whidbey et al., 2013). With the exception of one, named *cylK*, homologues of all *cyl* genes are present in *A. jensenii*, only with a different gene organization (Rosa-Fraile et al., 2014). Thus, the exclusion of pigmented strains from food production may be desirable.

6 | CULTIVATION OF PAB

Traditional plating methods for the identification of dairy PAB have been reported frequently, as have the troubles of culturing PAB. This is partially based on sample composition: dairy samples possess a complex and diverse microbiota, which complicates targeted cultivation of only a single species or genus (O'Sullivan & Cotter, 2017; Sohier et al., 2014).

Another challenge when culture methods are used is that PAB tend to form clumps or aggregates in the diluents, which leads to an underestimation of the true count. Furthermore, Kerjean et al. (2000) stated that growth is strongly dependent on the inoculation amount and medium. For instance, if growth in skimmed milk is desired, it needs to be inoculated with at least 10^7 CFU/mL, whereas in a rich complex medium, levels of 10^5 CFU/mL are sufficient for cultures to develop (Kerjean et al., 2000). The selective agents, if used, are reported to sometimes inhibit growth of the desired bacteria as well (Babot et al., 2011). With regard to milk samples, it has been reported that when tested undiluted, components of milk can affect the selective agent of a medium, resulting in a lack of typical colonies (Thierry & Madec, 1995). Even if a selective agent is applied in the medium, growth of other undesired microorganisms cannot always be excluded completely and is highly dependent on the sample (Thierry & Madec, 1995).

Various media have been used for cultivating PAB. An overview of published growth media is given in Table 3. Some media have been reported specifically for the detection of dairy PAB from food, some for the enumeration of cutibacteria from clinical samples, and others universally for PAB growth. Of these general-purpose media, basal agar with galactose and Man, Rogosa, and Sharpe (MRS) agar yielded the best countable results for *P. freudenreichii* (Tharmaraj & Shah, 2003). Patrick & McDowell (2012) also reported glucose broth and trypticase-yeast extract-glucose medium with 0.05% Tween 80 for PAB. For maintenance of PAB, Patrick & McDowell reported media such as blood agar or brain heart infusion medium, which are also used for the detection and diagnosis of *C. acnes* in clinical samples, which is discussed in more detail in Foster et al. (2020).

For the enumeration of dairy PAB in food samples, PAB are often cultivated on yeast extract lactate (YEL) medium, which was first described by Malik et al. (1968). Additionally to YEL, growth in MRS medium supplied with 0.5 g/L L-cysteine-hydrochloride is possible under anaerobic conditions, though neither YEL nor MRS media are selective (Dasen et al., 1998). A method reported to overcome the problem of lacking selectivity for dairy PAB enumeration is the subtraction method. Samples are cultivated on YEL medium and incubated under anaerobic conditions at 30°C, and colony counts are determined after 3 and 7 days, respectively. The counts of LAB on day 3 are afterward subtracted from the total counts on day 7 to estimate the "true" count of propionibacteria in the sample (Tharmaraj & Shah, 2003).

Another challenge described by Beloti et al. (1999) is the difficulty when milk samples are tested using plate count agar (PCA) due to the lack of contrast between medium and colony. Because milk testing can easily be inaccurate due to the opacity of the plates, especially at lower dilutions, 2, 3, 5-triphenyltetrazolium chloride (TTC) is recommended as an addition to improve results and simplify colony counting. The best results for PCA were achieved at a concentration of 0.015%-added TTC, at which almost no

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TABLE 3 Culture media for propionic acid bacteria (PAB) enumeration

Application field	Media (components)	State	Species used	Reference
General purpose	Basal agar + galactose	s	*P. freudenreichii*	Tharmaraj and Shaw (2003)
	MRS[a]	l, s	*P. freudenreichii*	Tharmaraj and Shaw (2003)
	Glucose broth	l	PAB	Patrick & McDowell (2012)
	Trypticase-yeast extract	s	PAB	Patrick & McDowell (2012)
	Glucose + 0.05% Tween 80			
Maintenance and characterization	Blood agar[b]	s	PAB	Patrick & McDowell (2012)
	Brain heart infusion-yeast	l	PAB	Freitas et al. (2015a),
	Extract + 30% glycerol		PAB	Patrick & McDowell (2012
	MRS + L-cysteine	l, s		Dasen et al. (1998)
	Hydrochloride (0.05%)			Patrick & McDowell (2012)
Food	MF95C[c]	l	Dairy PAB[d]	Fessler et al. (1998)
	Lithium glycerol	l, s	Dairy PAB[d]	Freitas et al. (2013),
	Plate count agar + TTC[e]	s	Dairy PAB[d]	Thierry and Madec (1995)
	Yeast extract lactate	l, s	Dairy PAB[d]	Beloti et al. (1999)
				Freitas et al. (2013)
				Malik et al. (1968)
				Patrick & McDowell (2012)
				Stackebrandt et al. (2006)
Clinical	Proteose peptone yeast	l	*C. acnes*	Patrick & McDowell (2012)
	Tryptone-yeast extract	s	*Cutibacterium* spp.	Patrick & McDowell (2012)
	Glucose + furazolidone			
Cell stock	Vitamin B12 free medium	l	*P. freudenreichii*	Assis et al. (2020)
Vitamin B12 production	Liquid acid protein residue of soybean	l	*P. freudenreichii*	Assis et al. (2020)
	Whey-based medium + yeast extract	l	*P. freudenreichii*	Deptula et al. (2017)
Stress tolerance mechanism testing	Chemically defined medium	l	*P. freudenreichii*	Gagnaire et al (2015)
	Emmental juice-like medium	l	*P. freudenreichii*	Gagnaire et al. (2015)
PAB interactions with LAB[f]	Acidified reconstituted skim milk	l	Dairy PAB[d]	Piveteau et al. (1999)

s, solid; l, liquid.
[a]Man, Rogosa, and Sharpe.
[b]Various blood types reported depending on organism (sheep, horse, bovine, rabbit, pig, human).
[c]Medium mimicking the watery phase of cheese.
[d]Dairy PAB include the species *P. freudenreichii*, *A. acidipropionici*, *A. jensenii*, and *A. thoenii*.
[e]2, 3, 5-triphenyltetrazolium chloride.
[f]Lactic acid bacteria.

inhibition of growth was observed when compared to PCA with no dye (Beloti et al., 1999).

To improve selectivity, Thierry and Madec proposed a medium containing lithium glycerol (LG) and antibiotics (Thierry & Madec, 1995). This medium has been patented and is manufactured by STANDA Industries under the name Pal Propiobac™ (Madec et al., 1994). It has been characterized as adequate for the enumeration of PAB from mixed cultures or food samples (Freitas et al., 2013). The dairy PAB flora on LG agar represents on average about 65%, in contrast to only 4% on YEL agar. Thierry and Madec (1995) reported the medium to be selective based on the definition by Reuter (1985). During milk testing, however, growth of a number of salt-resistant cocci, presumably enterococci, which are known to be resistant to a number of selective agents, was observed (Thierry & Madec, 1995). Authors have also reported that sometimes even PAB are inhibited when solely LG broth is used. This may be overcome by adding Petrifilm™ AC plates to the LG broth (Freitas et al., 2013). Thus, more visible colonies are formed, potentially due to the added nutrient source and TTC (Freitas et al., 2013).

Pioneer Hi-Bred applied for a US patent in 1991 for a selective medium for the isolation of PAB from agricultural samples, in particular silage. The medium contains lactate as the primary energy source, adding a combination of antibiotics, namely, netilmicin and heavy metal salts. Alternatively, instead of adding a combination of these components, single use of one constituent is possible as well (Tomes et al., 1991). Regarding heavy metal salts,

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water-soluble cadmium or arsenic salts were proposed because PAB were found to be resistant, even though different results have been reported in the literature (Tomes et al., 1991). With the application of either a single component or all components together, the authors reported the medium to be selective for *Propionibacterium*. In the current perspective, however, the reported selectivity for propionibacteria should be questioned, as the former taxonomy of propionibacteria at the time of the patent application would today include members of three different genera. Furthermore, the medium was criticized for the obvious disadvantage of the resulting heavy metal waste, as well as for health hazards to the staff (Madec et al., 1994).

Some media for PAB cultivation have been developed and applied for very specific purposes. Mimicking the conditions found in cheese-making, these media include, for instance, the MF94C medium used by Fessler et al. (1999a), the Emmental cheese juice-like medium (EJM) of Gagnaire et al. (2015), and acidified reconstituted skim milk by Piveteau et al. (2000). Piveteau et al. (2000) compared growth of dairy PAB in complex media to growth in milk and whey in order to demonstrate the presence of an inhibiting factor present in whey. They found that growth of dairy PAB was not comparable among the different media and highly dependent on the inoculation level. By inoculating reconstituted skim milk with LAB starter cultures prior to dairy PAB inoculation, the inhibition could be overcome, and dairy PAB grew even from low inoculation levels. In contrast, CDM and EJM were utilized by Gagnaire et al. (2015) to investigate the stress tolerance of *P. freudenreichii*. During cheese-making, the organism is exposed to unfavorable conditions. Gagnaire et al. (2015) examined whether exposure to these conditions influences the organism's ability to better withstand stressful conditions such as digestive stresses in order to assess the possibility of cheeses as a delivery vehicle for probiotics.

For the utilization of *P. freudenreichii* for preservation of cell stock and vitamin production, different media have been reported. Even though growth in whey-based media is rather poor, Deptula et al. used a modified whey-based medium for cultivation because whey-based media are often used for industrial vitamin B12 production (Deptula et al., 2017b). Assis et al. (2020) suggested a medium for vitamin production utilizing the liquid acid protein residue of soybeans, an agroindustry residue representing a cheap and animal-derivate-free alternative to a whey-based medium.

Although cultivation of PAB is rather tedious, one advantage is that *P. freudenreichii* is known to remain culturable when inoculated into a fresh medium, even when stored in a spent medium at room temperature for more than 6 months in a laboratory (Aburjaile et al., 2016). This is in line with the finding of Greenblatt et al., who demonstrated the ability of the phylum Actinobacteria to withstand extreme and long-term nutrient shortages (Greenblatt et al., 2004). Nevertheless, data suggest strain dependency, as the extent and ability of long-term survival seemed to differ among *P. freudenreichii* strains (Aburjaile et al., 2016). According to Aburjaile et al. (2016), this is potentially due to the bacteria entering a viable but not culturable (VBNC) state. Generally, for the analysis of samples, the VBNC state may cause misrepresentation if samples are solely assessed by cultivation (Sánchez et al., 2006). Various levels of viability and metabolic activity, which might escape detection by cultivation, can be detected using culture-independent methods, which will be described in more detail in Sections 8 and 9 (Aburjaile et al., 2016).

7 | IDENTIFICATION OF PAB

To ensure accuracy and reliability of cultivation methods, additional identification to verify the generated results is recommended (Sohier et al., 2014).

7.1 | Identification based on fermentation profiles

One option for further identification of PAB is assessment of biochemical characteristics. Several authors have published identification keys based on fermentation profiles, and an extensive summary of biochemical characteristics for the identification of *Acidipropionibacterium*, *Cutibacterium*, and *Propionibacterium* can be found in Bergey's Manual of Systematic Bacteriology (Hettinga & Reinbold, 1972a; Patrick & McDowell, 2012; Stackebrandt et al., 2006). Nevertheless, identification of species based on appearance and fermentation profiles used to be difficult due to their inhomogeneous appearance and differing results of operators in fermentation profiles (Deptula et al., 2018; Stackebrandt et al., 2006). To this end, commercial identification kits may help. Stackebrandt et al. (2006) reported the potential application of API test strips (bioMérieux SA), considering the API Rapid 32A test as well as the API Coryne system, but for the latter, identification problems were reported. Deptula et al. (2018) used the API CHL 50 test strip to assess differences in fermentation profiles of acidipropionibacteria, though without testing for sample identification. Finally, Zhang et al. (2017) used the API 20A test system for the identification of *C. acnes*, although all identified organisms in their study belonged the genus *Cutibacterium*. The usefulness of API test strips particularly for the identification of cutibacteria is attested in the product brochure of the manufacturer,

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bioMérieux SA, but their suitability for the identification of dairy PAB is not well documented and questionable.

As the previous section has elucidated, cultivation of PAB is tedious, and identification by a selective medium is impossible. Even though identification by assessing fermentative abilities is possible, it is a time-intensive and frequently unreliable procedure. Therefore, identification by alternative methods such as mass spectrometry, fluorescence in situ hybridization (FISH), flow cytometry, or PCR-based techniques may be preferable.

7.2 | Identification using mass spectrometry

The first record of a successful analysis of whole microbial cells using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) can be dated to 1996, and since then it has steadily gained popularity due to its good reproducibility (Vorob'eva et al., 2011). The technique yields promising results and can be used for the identification of PAB subsequent to cultivation. Two great advantages are quick analysis and uncomplicated sample preparation (Vorob'eva et al., 2011). Nonetheless, most publications using MALDI-TOF MS describe its application in a medical context for the detection of *C. acnes*. A revision of the Bruker MALDI Biotyper® Reference Library, Version 9, confirms *A. propionica* is still listed as *P. propionicum*. Furthermore, the obsolete classification into subspecies of *P. freudenreichii* is also still in practice. In terms of the division into the three genera *Propionibacterium*, *Acidipropionibacterium*, and *Cutibacterium*, the species lists and main spectra (MSP) are updated. In total, 47 entries are listed. For the dairy-relevant propionibacteria, only three MSP are included, while for acidipropionibacteria, nine MSP are recorded. In contrast, 21 entries are listed for *C. acnes*, again emphasizing the importance of MALDI-TOF MS as a detection method for medical purposes. The BIOTECON Diagnostics D-Mass-02 MALDI-TOF MS Database, Version 1 from March 2020, an additional reference library for the dairy sector, contains three entries for *Propionibacteriaceae*, but none belonging to dairy PAB. One MSP each for *P. acidifaciens* and *P. australiense* are included, as well as one MSP for *A. propionica*, which is falsely accounted as *P. propionicum*. The libraries were accessed using the MTB Compass Explorer, Version 4.1.100, in August 2020. In brief, MALDI-TOF MS is a great tool for the identification of acidipropionibacteria and propionibacteria due to its simple handling and rapid analysis, but an extension of the reference libraries with more spectra belonging to the dairy PAB group would be desirable.

8 | FLUORESCENCE IN SITU HYBRIDIZATION AND FLOW CYTOMETRY

Considering the troubles of cultivating dairy PAB from food matrices, direct detection without prior cultivation may be advantageous. FISH and flow cytometry are two methods that allow such direct detection and quantification.

FISH has been applied for approximately 50 years and was rather popular in the 1990s, but a few more recent studies have employed the method to enumerate dairy microbes (Sohier et al., 2014). It is based on the hybridization of fluorescent-labeled target-specific probes, that bind specifically to the selected DNA or rRNA sequence (Salimi et al., 2020; Wei et al., 2019). After hybridization, the fluorescent probe can be detected by microscopy (Salimi et al., 2020). To the best of our knowledge, sensitivity data for PAB have not been published. Salimi et al. (2020) reported the detection of *Escherichia coli* with a detection limit as low as 1 CFU/ 25 g or mL of food upon utilization of peptide nucleic acid FISH (PNA FISH), and a comparably low detection limit has been reported in a few other publications as well, albeit only in combination with extended periods of previous cultural enrichment (Rohde et al., 2015). Although great amounts of fat or protein in the food matrix might disturb hybridization or cause autofluorescence, pretreatment of the sample can improve results (Rohde et al., 2015). For instance, Rohde et al. (2015) state for milk and dairy samples such a pretreatment can include homogenization, single, or multiple centrifugation steps and the use of a sodium citrate buffer in order to obtain a clean cell pellet without inhibiting substances from the initial sample matrix.

FISH was characterized to show good correlations among bacterial counts in cheese, thereby allowing highly specific bacterial detection and providing information on spatial detection of microorganisms (Sohier et al., 2014). Babot et al. (2011) successfully employed FISH for the identification of PAB from cheese samples. Prior to its application in cheese, the protocol for PAB detection was optimized with cultured PAB, suggesting measurements after approximately 30 h of incubation due to the slow growth of PAB, in addition to a lysozyme treatment for improved cell fluorescence. Comparison of FISH measurements with traditional plate counts revealed no significant differences in numbers (Babot et al., 2011). Consequently, the method is suitable for rapid and accurate enumeration of PAB from cheese samples, and a major advantage of its use is the potential detection of morphological changes of the cell, which would remain unnoticed by PCR-based methods (Babot et al., 2011). Nevertheless, Babot et al.

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(2011) also reported high variability with their measurements. They reasoned their success was dependent on the number of 16S rRNA molecules present, which varied depending on, for example, the growth phase and growth rates. Fluorescent labeling with microscopic technologies might provide good results but is no solution for the dairy industry because its application in routine analysis is difficult (Sohier et al., 2014). A major drawback is the rather difficult setup in combination with artefacts and interference with the food matrix (Sohier et al., 2014). At times the detection of low numbers of bacterial cells has been reported difficult, creating a possible challenge in the detection of PAB contamination in raw milk (Liehr, 2009).

Instead of subsequent detection of microorganisms by microscopy, the use of flow cytometry after fluorescent labeling has been reported as well (Rohde et al., 2015). The so-called flow FISH has several advantages in its rapidity and high-throughput potential over the classical procedure (Rohde et al., 2015). Furthermore, it is a prominent tool to illustrate the microbial composition of food products and is especially well suited for liquid samples (Rohde et al., 2015). It has been applied for the detection of *B. cereus* spores in spiked ultra-heat-treated milk samples, but in order to generate results, pretreatment of the milk sample was necessary to remove the milk fat, making pretreatment before application with raw milk, due to its high fat content, a prerequisite (Laflamme et al., 2009). Laflamme et al. (2009) reported the specific detection of 10^3 CFU/mL *B. cereus* spores, yet dairy PAB in raw milk would need to be detected at much lower levels. Hence, the applicability of the method for the targeted detection of dairy PAB is questionable.

Also, Sohier et al. (2014) suggested flow cytometry as an alternative technique that could be implemented in the dairy industry. Currently, flow cytometry is used to assess the quality of fermented products, and methods have been developed by the International Organization for Standardization in collaboration with the International Dairy Federation for the quantification of LAB in starter cultures, probiotics, and fermented products (Michelutti et al., 2020; Sohier et al., 2014). However, the scope of ISO 19344:2015 states that the minimal bacterial concentration for the application of the proposed method should be 10^6 cells/g or mL of sample (ISO, 2015). Such a high minimal bacterial concentration can hardly be surprising, considering that the standard targets were fermented products. Regarding milk samples, Sohier et al. (2014) reported a detection limit of 10^3–10^4 bacterial cells/mL. Although this limit is much lower than the minimum concentration stated in ISO 19344:2015, it remains too high to assess the quality of raw milk intended for hard cheese production because concentrations as low as 10 dairy PAB cells/mL may cause cheese spoilage. On the positive side, a large choice of dyes is available, allowing for the targeting of different cell characteristics and answering a wide set of questions. Automation is also possible, and analysis time is fast. In conclusion, FISH, flow cytometry, and combinations of these methods are promising tools for the direct investigation of food samples, but at this time they are not suitable for the detection of propionibacteria and acidipropionibacteria in raw cheese milk at low levels.

9 | PCR METHODS FOR THE DETECTION AND ANALYSIS OF PAB

Various PCR-based methods have been reported for the detection and characterization of dairy PAB. Similar to cultivation, molecular methods have been described as work intensive and tedious, although for different reasons. Challenges comprise labor-intensive protocols, several delicate steps such as nucleic acid extraction, amplification, and the generation of significant data (Chiron et al., 2018).

DNA/RNA extraction, for instance, may be demanding, as intact cells and isolated cell walls of PAB, with the only exception being *P. freudenreichii*, have been reported as lysozyme resistant unless acetylated (Stackebrandt et al., 2006). Furthermore, if screening for PAB is performed from a food matrix, cell components such as fats or carbohydrates can impair a high qualitative extraction (Quigley et al., 2011).

Considering the more recent literature, a variety of primers have been used in different contexts; some were applied universally, others only for dairy PAB, and few were considered genus specific or species specific at the time of writing of the cited papers (see Table 4). In view of the reorganization of the three PAB genera, this needs to be examined critically, as some primers may be too specific or not enough.

9.1 | Species identification

Species identification of PAB using biochemical methods has been regarded as challenging, as these methods have at times yielded reliable results or failed completely to this end (Fessler et al., 1999b). Fortunately, due to the abundance of PCR-based methods, which have been developed in the meantime, some of the difficulties have been overcome, especially the troubles regarding the distinction of *A. jensenii* and A. *thoenii*, which has been characterized as problematic with cultural detection methods (Fessler et al., 1999a; Turgay et al., 2016).

A range of possible primer systems that target dairy PAB is shown in Table 4. The discussed primers are

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TABLE 4 Primer for genus- and species-specific identification of dairy PAB

Method	Target gene	Primer pair	AS* [bp]	Target	Reference
Sequencing	16S rRNA	bak11w, bak4	1500	PAB	Dasen et al. (1998)
	16S rRNA	bak4, gd1	900	*Propionibacterium*[a] spp.	Freitas et al. (2013)
	cylG	fw 5', rev 5'	518	Hemolytic dairy PAB[b]	Deptula et al. (2019)
Species specific PCR	16S-23S ISR[1] 16S rRNA	PfrI, PfrII PffI, PfrII PacI, PacII PjeI, PjeII PthI, PthII PB1-PB2 PF, PB2 PA, PB2 PJ, PB2 PT3, PB2	346 241 304 331 267 610 867 868 864 865	*P. freudenreichii* *P. freudenreichii* subsp. *freudenreichii*[a] *A. acidipropionici* *A. jensenii* *A. thoenii* dairy PAB[b], *C. acnes*[a] *P. freudenreichii* *A. acidipropionici* *A. jensenii* *A. thoenii*	Tilsala-Timisjärvi & Alatossava (2001) Rossi et al. (1999)
	groL2[2]	Pfrsh_grol2_fw, Pfrsh_grol2_rev	380	*P. freudenreichii* *A. acidipropionici* *A. jensenii* *A. thoenii*	Turgay et al. (2016)
	pduP[3]	Pac_pduP_fw, Pac_pduP_rev	420		
	Ppk[4]	Pj_ppk_fw, Pj_ppk_rev	409		
	aroE[5]	Pth_aroE_fw, Pth_aroE_rev	500		

*AS, amplicon size.
[1]Intergenic spacer region,
[2]heat-shock protein,
[3]CoA-dependent propionaldehyde dehydrogenase,
[4]polyphosphate kinase,
[5]shikimate 5-dehydrogenase.
[a]Reported genus specific before the re-structuring of the family.
[b]Dairy PAB include the species *P. freudenreichii*, *A. acidipropionici*, *A. jensenii*, and *A. thoenii*.

further divided into categories for use in sequencing-based approaches or genus-specific and species-specific PCR detection.

Dasen et al. (1998) developed a multiplex PCR approach for the identification of PAB by subsequent sequencing utilizing the forward primers bak11w and gd1 and the reverse primer bak4. Upon sequencing of the PCR product, the authors reported almost complete coverage of 16S rDNA without amplification of DNA from other organisms. Detection of propionibacteria was possible due to the amplification of a specific 900 bp fragment amplified with the primers gd1 and bak4, which were also used by Freitas et al. (2013). However, as these primers could not present an amplification product with template DNA from other organisms, the primer bak11w was included as a control in the multiplex approach. Dasen et al. (1998) consequently ensured that the absence of amplification product was not caused by limited DNA extraction or failed amplification. They reported a detection limit for the applied method at a template DNA concentration of 35 pg.

Deptula et al. (2019) reported on the potential negative effect of pigmented hemolytic cultures in food with the development of a PCR approach using primers to target the *cylG* gene in pigmented strains. They obtained a product of 518 bp and confirmed the identity of the amplified strains by sequencing. This gene showed a high degree of conservation, and the *cyl* cluster was altogether absent in strains that were not pigmented and thus not hemolytic. Targeting this region was therefore appealing because it guarantees the exclusion of hemolytic strains in food production, thus assuring product safety.

Tilsala-Timisjärvi and Alatossava (2001) characterized the 16S-23S and 23S-5S intergenic spacer region of PAB and were able to use the obtained data for primer design. Based on sequencing data, they developed five primers for species-specific PCR detection. As the study was published in 2001, an outdated differentiation into the subspecies of *P. freudenreichii* subsp. *freudenreichii* and *P. freudenreichii* subsp. *shermanii* was also performed. The primer set PffI, PfrII, which specifically targets the single nucleotide difference in the 16S-23S spacer region of *P. freudenreichii* subsp. *freudenreichii*, cannot be used for species-specific detection, as it may not detect *P. freudenreichii* strains incapable of nitrate degradation. Since it

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may be helpful for screening nitrate reductase activity in *P. freudenreichii*, the primer set PfrI, PfrII is nevertheless still applicable, as it was not designed for differentiation of subspecies but only detection of the species *P. freudenreichii*.

Rossi et al. (1999) proposed the primer pair PB1, PB2, which enabled genus-specific detection of propionibacteria by the amplification of a 610 bp fragment at the time of publication. Today, due to taxonomic reclassification, this primer pair cannot be deemed genus specific. Moreover, it would also amplify the DNA of species that do not fall within the dairy PAB group, rendering its application for dairy PAB detection ineffective. However, Rossi et al. (1999) also designed another forward primer for species-specific detection of each dairy PAB, which they combined with the reverse PB2 primer, yielding products approximately 865 bp in size. Rossi et al. (1999) also designed primers targeting the 16S rDNA region. These primers vary considerably in amplicon length, as the product is approximately 500 bp longer than those amplified using the primers of Tilsala-Timisjärvi and Alatossava (2001). Furthermore, all species-specific primer pairs published by Rossi et al. (1999) yield products of highly similar size, which may be problematic if, for instance, multiplexing and differentiation based on amplicon size is intended. The limit of detection of these primer sets, when applied for the analysis of milk and cheese samples, proved consistent among all sets and was reported to detect multiples of 10 cells/g or mL of sample. A lower sensitivity, however, was observed when forage and soil samples were analyzed (Rossi et al., 1999).

Turgay et al. (2016) published primers based on sequencing data of the strains *P. freudenreichii* FAM 14176, *A. acidipropionici* FAM 19036, *A. jensenii* FAM 19038, and *A. thoenii* FAM 22284. In contrast to the primers discussed above, the primer systems published by Turgay et al. (2016) do not target 16S rDNA but amplify fragments of the *groL2* gene encoding a heat-shock protein of *P. freudenreichii* and, the *pduP* gene encoding the CoA-dependent propionaldehyde dehydrogenase for the identification of *A. acidipropionici*. The *ppk* gene encoding polyphosphate kinase was chosen for the identification of *A. jensenii*, whereas the primer system for *A. thoenii* targeted a fragment of the *aroE* gene encoding shikimate 5-dehydrogenase. The amplicon length ranged from 380 to 500 bp, and all values can be found in Table 4. The PCR products, which had been obtained using conventional PCR, were subsequently used for the construction of qPCR standards. These standards were used for the development and validation of a qPCR assay, which will be described in more detail in the following section.

9.2 | Quantitative PCR

Over the last decades, culture-independent molecular methods have received increasing attention and thus were subjected to constant improvement (Postollec et al., 2011). Application of qPCR in food microbiology was first reported in 1999 for the analysis of a fermented food matrix, and today it is widely used to detect, identify, and quantify bacterial populations (Postollec et al., 2011). In comparison to culture-based methods, it is much faster, allows detection of dead or VBNC cells, and enables quantification based on the measurement of gene numbers. All in all, it is more specific and may be more sensitive than culture-based approaches and nucleic acid isolation, and further preparation for qPCR can be automated. It may be suitable for routine analysis in the future, although today there is still a need for progress, considering the specific needs of the industry, in robustness, accuracy, and validation processes as well as knowledge on the limits of the methods (Postollec et al., 2011; Sohier et al., 2014). Another drawback reported by Sohier et al. (2014) is its sensitivity to small variations in sample preparation, amplification, and data expression as well as cautious handling, which is required to prevent such variations.

The quantification of several targets in one run using a multiplex assay is possible and can ease especially the detection of dairy PAB in a milk sample. The selection of adequate target genes and development of specific primers are essential for qPCR because effective detection of several different targets in a single run is possible albeit challenging. Furthermore, Quigley et al. (2011) stated that even though qPCR offers many benefits such as enhanced precision and specificity in cases of low cell numbers, reproducibility can be poor.

Various approaches designed for the detection and quantification of dairy PAB are summarized in Table 5. Falentin et al. (2010b) investigated the specific metabolic activity of starter cultures and analyzed stress levels of *P. freudenreichii* in Emmental cheese during manufacture and ripening. For this purpose, they used qPCR as well as reverse transcriptase (RT) qPCR and compared the results to traditional plate counts. They stated that the detection of bacterial counts below 10^3 cells/g of food worked better using plate counts, as these remained more accurate. Furthermore, they concluded that lower numbers than 10^3 cells/g of food might be detectable by their qPCR assay, but the cycle threshold may be out of the linearity range and thus yield unreliable results.

Turgay et al. (2016) developed four primer systems to be applied for the screening of vat-milk samples for dairy PAB at the species level. The authors reported an interesting improvement in the recovery of target cells from milk

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TABLE 5 Primer applied for quantitative PCR detection of dairy PAB

Target gene	Primer Pair	AS*[bp]	Target	Reference
16S rRNA	16SPfs	144	*P. freudenreichii*	Falentin et al. (2010b)
tuf[1]	TufPfs1	102		
GroL1[2]	Grol1Pfs	164		
GroL2[2]	Grol2Pfs	104		
groL2[2]	2Pfr_groL2-F 2Pfr_groL2-R S-Pac_groL2-FAM[b]	103	*P. freudenreichii*	Turgay et al. (2016)
pduP[3]	Pac_pduP-F2 Pac_pduP-R2 S-Pac_pduP2-FAM[b]	111	*A. acidipropionici*	
Ppk[4]	Pj_ppk-F Pj_ppk-R S-Pj_ppk-FAM[b]	89	*A. jensenii*	
aroE[5]	Pth_aroE-F3 Pth_aroE-R3 S-Pth_aroE-FAM[b]	128	*A. thoenii*	
ADP-ACS[6]	3dPAB-(ADP-ACS)-F 3dPAB-(ADP-ACS)-R 3dPAB-(ADP-ACS)-FAM[b]	128	dairy *Acidipropionibacterium* spp.	Turgay et al. (2018)

*AS, amplicon size.
[1]Peptide chain elongation factor Tu,
[2]heat-shock protein,
[3]CoA-dependent propionaldehyde dehydrogenase,
[4]polyphosphate kinase,
[5]shikimate 5-dehydrogenase,
[6]ADP-forming acetyl-CoA synthetase gene.
[b]Sequence specific fluorescence probe.

samples upon use of a second microorganism, for example, *Lb. casei*, which had been added to the sample prior to DNA extraction. They stated that high numbers of bacterial cells could competitively prevent adhesion of the target cells to milk-fat globules and thus increase pellet formation prior to DNA extraction. In total, Turgay et al. (2016) screened 51 samples, of which 40 were positively tested with plate count methods and 41 using qPCR, but only 12 samples allowed quantification. Cross-reaction of *A. thoenii* and *A. jensenii* with the primer system for *P. freudenreichii* was reported, whereas *A. acidipropionici* and *A. jensenii* cross-reacted with the primer system for *A. thoenii* (Turgay et al., 2016). Even though these cross-reactions were observable, they were easily distinguishable from the targeted amplification, as the cycle threshold at which they occurred varied greatly compared to the target species. For spiked milk samples, Turgay et al. (2016) reported a limit of quantification in the range of 10^1–10^2 CFU/mL and a detection limit lower than 10 CFU/mL (Turgay et al., 2016).

In a second study by Turgay et al. (2018), a primer system called 3dPAB-(ADP-ACS) was developed for the simultaneous detection of three thermo-sensitive dairy acidipropionibacteria (*A. acidipropionici, A. thoenii, A. jensenii*). It was used to screen vat-milk samples for the presence of dairy PAB. One benefit of the new primer system is the reduction of required test assays from four to two when screening for dairy PAB. Acidipropionibacteria were targeted with the new primer system, and *P. freudenreichii* with the primers used in Turgay et al. (2016). Consequently, the workload for the operator was significantly reduced. The target gene sequence encoding for the 3dPAB-(ADP-ACS) primer system is present in all dairy *Acidipropionibacterium* species, but cross-reactions were observed with non-dairy *Acidipropionibacterium damnosum* and *A. microaerophilum* (Turgay et al., 2018). However, as these two species were thus far not reported as milk contaminants, it is highly probable that the tested vat milk had been contaminated with one of the three dairy PAB. *P. freudenreichii* contamination was observed twice as frequently as the other three dairy acidipropionibacteria, and even in cheeses made from seemingly uncontaminated milk, signs of PAB fermentation were observed, or the cheese was downgraded in quality. It is important to point out that PAB contamination below the limit of detection of the applied qPCR is still sufficient to cause late fermentation in cheese. In this regard, the method failed to predict propionic acid fermentation in raw-milk cheeses. Based on these results, the authors concluded that the method may not be suitable

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TABLE 6 Primers reported dairy PAB typing

Target gene	Primer	Target	Reference
16S-ARDRA			
16S rRNA	pA, pH	*Propionibacterium* spp., *Acidipropionibacterium* spp.	Blasco et al. (2015)
RFLP			
16S rRNA	16sP1, 16sP4	Dairy PAB[3]	Riedel et al. (1998)
RAPD-PCR			
n.s.[a]	SK2	Dairy PAB[b]	Fessler et al. (1999a)
n.s.[a]	DF4	*P. freudenreichii*	Fessler et al. (1999b)
n.s.[a]	M13	Dairy PAB[b]	Freitas et al. (2015a)
n.s.[a]	OPL-05	Dairy PAB[b]	Rossi et al. (1998)
MLST			
adk[1]	adk-F3, adk-R3	*P. freudenreichii*	Dalmasso et al. (2011)
fumC[2]	fumC2-F, fumC2-R		
gtf[3]	Gtf-F2, Gtf-R1		
pf1637[4]	pf1637-F3, pf1637-R3		
pf169[4]	pf169-F, pf169-R		
recA[5]	recA-F, recA-R		
rpoB[6]	rpoB2-F, rpoB2-R		

[1]Adenylate kinase,
[2]fumarate hydratase,
[3]cell-wall polysaccharide synthase,
[4]carboxylic ester hydrolases,
[5]DNA recombinase A,
[6]RNA-polymerase ß-subunit.
[a]Nonspecific, as the primer binds randomly to the sample DNA (Geary & Forsyth, 1996).
[b]Dairy PAB include the species *P. freudenreichii*, *A. acidipropionici*, *A. jensenii*, and *A. thoenii*.

for screening vat milk. However, it may be useful to assess each supplier's milk individually, and the "dilution" of contaminants by pooling the milk of several suppliers could be avoided. At higher contamination levels the assay offers great potential for the screening of raw cheese milk.

9.3 | Fingerprinting techniques

Even though data on the genomic biodiversity of dairy PAB is scarce, a range of fingerprinting techniques have been applied to characterize dairy PAB on the subspecies or strain level, although most studies focus on *P. freudenreichii* (Thierry et al., 2011).

An overview of primers that have been used for fingerprinting dairy PAB is provided in Table 6. Blasco et al. (2015) used primers pA and pH for 16S-amplified ribosomal DNA restriction analysis (16S-ARDRA) for the differentiation of over 100 different *Propionibacterium* and *Acidipropionibacterium* strains. Restriction of the amplified fragment was carried out afterward using the enzymes *HaeIII* and *AluI*. *HaeIII* did not allow discrimination of *A. acidipropionici* and *A. microaerophilium*, but *AluI* allowed species-specific identification. In addition, the strains were characterized by restriction fragment-length polymorphism–pulsed-field gel electrophoresis (RFLP–PFGE) using *XbaI*, *SpeI*, and *SspI* (Blasco et al., 2015). For RFLP–PFGE, Blasco et al. (2015) reported a reproducibility of 100% for all enzymes used and an overall high discriminatory power of the method that allowed grouping of the different strains into three clusters at a similarity level of 64.5%.

Riedel et al. (1998) employed 16S rDNA RFLP for the successful analysis of 135 dairy-PAB isolates from different origins. They used the primers 16sPI-16sP4, amplifying an approximately 1110 bp fragment, and 16sP3-16sP4, amplifying an approximately 250 bp fragment. The fragments amplified with the primers 16sPI-16sP4 were treated with *HaeIII* and *AluI*, whereas the amplicons generated with 16sP3-16sP4 were treated with *HpaII*. However, *HpaII* did not lead to satisfying results, as restriction endonuclease profiles of *P. freudenreichii*, *A. acidipropionici*, and *A. thoenii* strains were identical and thus distinction was not possible. This study, published in 1998, includes many species designations that are no longer used, but all species belong to the dairy PAB group. In addition *P. freudenreichii* was separated into subspecies, which Riedel et al. criticized openly, as they could not justify the subdivision due to the high degree of DNA similarity and low degree of polymorphism in the 16S rDNA of the subspecies.

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Random amplified polymorphic DNA (RAPD) fingerprinting is a simple and widely used technique to differentiate bacterial strains (Fessler et al., 1999b; Thierry et al., 2011). The use of RAPD has also been successfully applied to study the biodiversity of propionibacteria found in the dairy industry, with the primer M13 used for analysis (Freitas et al., 2015a). Freitas et al. (2015a) applied RAPD in combination with PFGE and were able to distinguish between the analyzed strains. However, PFGE provided better resolution among the strains of the same species than RAPD. The reproducibility of RAPD has sometimes been reported as poor, but utilization of a second primer for multiplex RAPD can improve reproducibility compared to conventional RAPD (Yang et al., 2017).

Fessler et al. (1999a) used the RAPD primers SK2 and DF4 for the analysis of 70 isolates of dairy PAB isolated from brown spots of Swiss hard and semi-hard cheeses. They were able to identify 37 different profiles among their 70 isolates. However, they reported trouble regarding the distinction of different *A. acidipropionici* strains. They interestingly used the method in a second publication with the same primers, in which they encountered four strains from the same milk that exhibited the same RAPD profile but not the same plasmid profile (Fessler et al., 1999b). Fessler et al. (1999b) assumed that the differences in plasmid profile either indicate a horizontal gene transfer or spontaneous loss of plasmids among some strains. Also, Rossi et al. (1998) used RAPD fingerprinting for their analysis of different dairy-PAB strains originating from milk and Italian cheeses. The primer OPL-05 provided bands for easy visual comparison and enabled only identification at species level. They also tested primers OPL-01 and OPL-02, which allowed better differentiation than primer OPL-05, yet a disadvantage of both RAPD-PCR and PFGE is that they reveal hardly any information about nucleotide diversity and mode of evolution (Dalmasso et al., 2011; Rossi et al., 1998).

Another typing tool for the study of bacterial strains is multilocus sequence typing (MLST). This method allows quick characterization of a large number of samples but also exhibits a high degree of resolution (Dekio et al., 2012). Furthermore, it permits a phylogenetic analysis of the studied population, as it is based on DNA sequence analysis (Dalmasso et al., 2011). In 2011, Dalmasso et al. published the first MLST scheme for *P. freudenreichii*, which was developed based on the investigation of 113 strains. The applied primers are listed in Table 4 and were also used in the study of Freitas et al. (2015a), who characterized propionibacteria from Brazilian dairy farms. However, Freitas et al. (2015a) observed better discrimination levels by PFGE in comparison to MLST. They assumed that its ability to detect only a few mutations may limit the effectiveness of MLST, whereas PFGE is capable of detecting significant genome rearrangements.

10 | GENOME DATA AVAILABILITY AND 16S rDNA REFERENCE DATABASES

With the emergence of fast sequencing techniques and increase of bioinformatics tools, the availability of genome data and 16S rDNA reference databases have become crucial. In this study, genome data availability has been assessed by consulting the National Center for Biotechnology Information genome database in February 2021. The importance of certain PAB species for the dairy industry, as well as applications described above such as vitamin production or usage as a probiotic, is reflected in the different numbers of genome assemblies available today. Most entries are available on the frequently used starter culture *P. freudenreichii* (49 entries), even though *A. acidipropionici* (eight entries) and *A. jensenii* (five entries) are receiving increased attention. For *A. thoenii*, only one entry can be found in the database. However, compared to the 285 genome assemblies of *C. acnes* with potential clinical relevance, data on dairy PAB still appear scarce.

The first genome sequence of a dairy PAB was announced in 2010, shedding light on the genome of *P. freudenreichii* and presenting 2 rRNA operons and 45 tRNAs (Deptula et al., 2017b; Falentin et al., 2010a). The results are consistent with the general characterization of dairy PAB to be slow growing, robust, and capable of growth even in low-nutrient environments, which is characteristic for organisms with few ribosomal operons (Falentin et al., 2010a). The characterization of strain *P. freudenreichii* (formerly ssp. *shermanii*) JS revealed a single circular chromosome of 2,675,045 bp and an overall GC content of 67.23% (Ojala et al., 2017). No plasmids were detected (Ojala et al., 2017). *A. acidipropionici*, however, exhibited a circular chromosome of 3,656,170 bp with a GC content of 68.8% and an additional low copy plasmid of 6868 bp with a GC content of 65.4% (Parizzi et al., 2012). Because *P. freudenreichii* has been used in the cheese-making process for a long time, it can be assumed that the reduced number of genes is a consequence of ongoing specialization of the organism and its continued use in the dairy industry (Parizzi et al., 2012). In the genome of *A. acidipropionici*, 32 pseudogenes could be detected, which supports as well the assumption of Parizzi et al. (2012) of specialization of this organism.

As next-generation sequencing technologies have been promoted due to advances in technology, the investigation of microbial communities has thrived as well. For microbiome studies based on metabarcoding methods targeting 16S rDNA, reliable species assignment can be troublesome

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because short-fragment strategies only including single or pairs of hypervariable regions often fail (Meola et al., 2019). Selection of the correct regions is therefore essential to maximizing resolution and conclusively identifying a dairy microbiome (Meola et al., 2019). Furthermore, adequate reference databases are of utmost importance. For this reason, Meola et al. (2019) created a new comprehensive reference database for 16S rDNA classification of dairy products that also includes data on dairy propionibacteria. The improvement of reference databases for specific environments such as dairy samples represents a key step toward further method development in this field.

Considering the vast advances in technology, it can be expected that in the course of the next years not only the amount of available genome data but also the databases will expand, leading to improvement in methods as well as development of new methods that should simplify dairy PAB detection, quantification, and identification in the food environment.

11 | CONCLUSION

The studies reviewed herein underline the complexity and recent changes within the family of *Propionibacteriaceae*, not only considering the consequences of taxonomic restructuring but also the versatile metabolism and characteristics of PAB as well as available methods for cultivation, enumeration, and identification. With regard to the food industry's needs, detection of low levels of PAB remains challenging. However, unnoticed PAB contamination of raw milk may lead to cheese spoilage and monetary losses for cheese producers. Due to the importance of PAB for the food industry, in-depth knowledge concerning these organisms and their prevalence in food matrices is important and may contribute to more efficient and sustainable food production. Thus, further studies analyzing PAB are encouraged.

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AUTHOR CONTRIBUTIONS

Johanna Burtscher, Konrad J. Domig, and Carola Bücher conceptualized the idea of the manuscript. Carola Bücher collected relevant literature and drafted the manuscript. Carola Bücher, Johanna Burtscher, and Konrad J. Domig reviewed and edited the manuscript. All authors read and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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