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Genomic approach to studying nutritional requirements of *Clostridium tyrobutyricum* and other Clostridia causing late blowing defects

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ABSTRACT

Clostridium tyrobutyricum is the main microorganism responsible for the late blowing defect in hard and semi-hard cheeses, causing considerable economic losses to the cheese industry. Deeper knowledge of the metabolic requirements of this microorganism can lead to the development of more effective control approaches. In this work, the amino acids and B vitamins essential for sustaining the growth of *C. tyrobutyricum* were investigated using a genomic approach. As the first step, the genomes of four *C. tyrobutyricum* strains were analyzed for the presence of genes putatively involved in the biosynthesis of amino acids and B vitamins. Metabolic pathways could be reconstructed for all amino acids and B vitamins with the exception of biotin (vitamin B7) and folate (vitamin B9). The biotin pathway was missing the enzyme amino-7-oxononanoate synthase that catalyzes the condensation of pimeloyl-ACP and L-alanine to 8-amino-7-oxononanoate. In the folate pathway, the missing genes were those coding for *para*-aminobenzoate synthase and aminodeoxychorismate lyase enzymes. These enzymes are responsible for the conversion of chorismate into *para*-aminobenzoate (PABA). Two *C. tyrobutyircum* strains whose genome was analyzed *in silico* as well as other 10 strains isolated from cheese were tested in liquid media to confirm these observations. 11 strains showed growth in a defined liquid medium containing biotin and PABA after 6–8 days of incubation. No strain showed growth when only one or none of these compounds were added, confirming the observations obtained *in silico*. Furthermore, the genome analysis was extended to genomes of single strains of other *Clostridium* species potentially causing late blowing, namely *Clostridium beijerinckii*, *Clostridium sporogenes* and *Clostridium butyricum*. Only the biotin biosynthesis pathway was incomplete for *C. butyricum* and *C. beijerincki*. In contrast, *C. sporogenes* showed missing enzymes in biosynthesis pathways of several amino acids as well as biotin, folate, and cobalamin (vitamin B12). These observations agree with the results of growth experiments of these species in liquid media reported in the literature. The results of this study suggest that biotin and folate are potential targets for reducing late blowing in cheese and highlight the usefulness of genomic analysis for identifying essential nutrients in bacteria.

1. Introduction

Late blowing refers to one of the most important fermentation defects observed in hard and semi-hard cheeses. It is characterized by the formation of butyric acid, carbon dioxide, and hydrogen during the maturation of cheese, leading to loss of its commercial value (reviewed by Bachmann, 1999; Doyle et al., 2015). The main cause of late blowing is *Clostridium tyrobutyricum*, a spore-forming, obligate anaerobic bacterium. *Clostridium* spores, present naturally in soil and at high concentrations in silage, survive the passage through the intestines of cows and accumulate in feces. The contamination of milk with these spores occurs primarily during milking. Spores survive pasteurization and *Clostridium tyrobutyricum* then outgrows in cheese during maturation. As few as 50 spores per liter of milk can induce the development of late blowing (Klijn et al., 1995; Bachmann, 1999; Doyle et al., 2015). Other Clostridia that can cause late blowing, although in milder form, are *Clostridium beijerinckii*, and *Clostridium sporogenes*. *Clostridium*

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butyricum has been detected from cheeses with late blowing symptoms, however its role in cheese spoilage is still unclear (Cocolin et al., 2004; Gómez-Torres et al., 2015). In addition to good farm-management practices aiming to avoid the contamination of milk with *Clostridium* spores, several strategies have been developed to minimize late blowing (Doyle et al., 2015). Elimination of spores from milk can be achieved by bactofugation or microfiltration. However, these physical treatments require expensive equipment and change the composition of milk (Ávila et al., 2014). Alternatively, chemical methods include the use of nitrates to inhibit spore germination or lysozyme to hydrolyze linkages in the cell wall (Wasserfall and Teuber, 1979; Ávila et al., 2014). Issues concerning these practices include the risk of formation of carcinogenic compounds and allergic reactions (Fremont et al., 1997; EFSA, 2010). Moreover, bacteriocins such as nisin are not often used in Switzerland owing to lack of acceptance from manufacturers and consumers (Broughton et al., 1996; Switzerland Cheese Marketing AG, 2015). Therefore, the development of new control strategies with minimal impact on cheese quality and safety is desirable. A conceivable approach is the limitation of essential nutrients important for the growth of Clostridia. This can be achieved, for example, by using suitable starter and non-starter lactic acid bacteria that consume these nutrients directly in cheese. *Clostridium tyrobutyricum* grows in cheese using lactate and acetate as carbon sources (Ivy and Wiedman, 2014). However, the high concentrations of these compounds in cheese prevent their use as potential targets. Hence, attention should be focused on other nutrients such as amino acids and B vitamins. Unfortunately, the information available on amino acids and B vitamins essential to sustain the growth of *C. tyrobutyricum* and other late blowing–causing Clostridia is scarce. The only work reporting the growth of *C. tyrobutyricum* in a defined medium is that of Tidswell et al. (1991). In this study, a single strain involved in the industrial reduction of ketones was grown in a medium containing biotin and para-aminobenzoate (PABA). This is in agreement with the general observation that saccharolytic *Clostridium* species require biotin for growth and a few species of this group require PABA as well (Ljungdahl et al., 1989). However, the nutritional requirements of *C. tyrobutyricum* need to be confirmed conclusively using several strains, including those isolated from dairy products. Regarding the other late blowing–causing species, *C. butyricum* has been shown to require only biotin for growth (Cummins and Johnson, 1971; Himmi et al., 1999). In contrast, studies conducted using several strains of *C. sporogenes* indicated that this species requires biotin and PABA, as well as several amino acids to grow (Kindler et al., 1956; Lovitt et al., 1987). Attempts to cultivate *C. beijerinckii* in defined media supplemented with several amino acids and B vitamins have failed (Cummins and Johnson, 1971; Vos et al., 2011).

The determination of essential nutrients to sustain the growth of a microorganism can be a tedious process involving the preparation of growth media containing different combination of compounds. In the last decade, sequencing technologies with high throughput and low cost have been developed. These, together with automatic annotation tools, have made it possible to quickly sequence and analyze entire genomes of microorganisms (Heard et al., 2010; Edwards and Holt, 2013). Among the many new opportunities, the identification of essential nutrients can be accelerated by investigating the presence of the genes involved in various biosynthetic pathways. Examples in the literature where genome analysis led to the development of growth media include those involving *Lactobacillus plantarum* or *Campylobacter jejuni* (Teusink et al., 2005; Alazzam et al., 2011). Moreover, this *in silico* approach not only provides an accurate overview of the metabolic requirements of a particular organism but also highlights the genetic causes behind them.

This study aimed to determine the amino acids and B vitamins essential for the growth of *C. tyrobutyricum* through a comprehensive genomic analysis followed by experiments in defined liquid media. The *in silico* analysis was also extended to other late blowing–causing *Clostridium* species, namely *C. butyricum*, *C. beijerinckii*, and *C. sporogenes*.

2. Materials and methods

2.1. Reconstruction of vitamin and amino acid biosynthesis pathways through genome analysis

2.1.1. Bacterial genomes

Reconstruction of vitamin and amino acid biosynthesis pathways was performed for the genomes of four *Clostridium tyrobutyricum* strains and was extended subsequently to the genomes of single strains of *Clostridium butyricum*, *Clostridium beijerinckii*, and *Clostridium sporogenes* retrieved from the GenBank database. Basic information about these genomes is listed in Table 1.

2.1.2. Genome analysis

Clostridium genomes were analyzed for the presence of putative genes coding for the enzymes involved in amino acid and B vitamin biosynthesis using the following strategy. As the first step, all necessary information about amino acid and B vitamin biosynthesis pathways was collected from KEGG maps (Kanehisa and Goto, 2000; Kanehisa et al., 2014) and the literature listed in Table S1.1 and S1.2 in Supplementary File 1. A theoretical metabolic pathway was proposed for each vitamin and amino acid, which served as template in the subsequent analysis. These biosynthesis pathways are shown in Supplementary Files 2 and 3 for amino acids and B vitamins, respectively. The seven *Clostridium* spp. genomes were then submitted to RAST (Rapid Annotations using Subsystems Technology) for gene annotation (Aziz et al., 2008). The annotated genomes were searched for genes coding for the enzymes involved in the amino acid and B vitamin biosynthesis pathways using the "KEGG metabolic analysis" tool in SEED Viewer (Overbeek et al., 2005). In the case of more than one candidate for a particular reaction, a single gene was selected. In the case that a gene could not be identified using the SEED Viewer, its presence was investigated further using the Local BLAST (Basic Local Alignment Search Tool) function in CLC Workbench version 6.0.2 (CLC Bio). For this purpose, translated amino acid sequences of homologous genes belonging to *Escherichia coli* and other *Clostridium* species were retrieved from GenBank and used as templates. The GenBank accession numbers of these protein sequences are listed in Tables S4.1 and S4.2 in Supplementary File 4. Finally, the translated protein sequence of each identified gene was checked for completeness using the protein BLAST function of National Center for Biotechnology Information (NCBI) (McGinnis and Madden, 2004).

Nucleotides and translated amino acid sequences of all putative genes identified in *C. tyrobutyricum* FAM22552 and FAM22553, *C. beijerinckii* G117, *C. butyricum* DSM10702, and *C. sprogogenes* PA3679 are listed in Supplementary Files 2 and 3 for amino acids and B vitamins, respectively. To confirm the results obtained with RAST, a second annotation of the genomes was performed using Prokka (ver. 1.11; Seeman, 2014). A fasta file containing all the sequences of translated genes annotated with Prokka for the genome of *C. tyrobutyricum* FAM22552 was then blasted using CLC Workbench against the amino acids and B vitamins biosynthesis genes identified with the strategy described above for this genome. The presence of orthologous genes in the other *Clostridium* genomes was then assessed by constructing orthologous gene clusters using OrthoMCL (version 2.0.9; Li et al., 2003). OrthoMCL was also used to

Table 1
Clostridium genomes used in this study.

Species	Strain	Origin	GenBank Accession number	Assembly method	Size (mbp)	Contigs	Coverage	Annotated genes (RAST/Prokka)	Reference
C. tyrobutyricum	FAM22552	Semi-hard red-smear cheese	JTER00000000	SPAdes/SSPACE	3.05	58	687	3030/3087[a,b]	Storari et al. (2015)
	FAM22553	Semi-hard red-smear cheese	JTES00000000	SPAdes/SSPACE	3.08	62	606×	3089/3017[a,b]	Storari et al. (2015)
	UC7086	Grana Padano cheese	ANOE00000000.1	Velvet	3.06	110	10×	3035/3081[a,b]	Bassi et al. (2013)
	ATCC25755	Type strain	APMH01000000	Velvet	3.01	76	100×	3061/2924[a,b]	Jiang et al. (2013)
C. beijerinckii	G117	Soil	AKWA00000000.1	SOAPdenovo	5.81	89	90×	5195/5103	Wu et al. (2012)
C. butyricum	DSM10702	Pig intestine	AQQF00000000.1	Velvet	4.60	207	240×	4180/4229	Xin et al. (2013)
C. sporogenes	PA3679	Spoiled canned corn	AGAH00000000.1	Velvet	4.18	107	200×	4008/3925	Bradbury et al. (2012)

[a] Core genome size based on Prokka annotation of *C. tyrobutyricum* (4 strains) = 2409 CDSs.
[b] Pangenome size based on Prokka annotation *of C. tyrobutyricum* (4 strains) = 3661 CDSs.

determine the size of core- and pangenome of the four *C. tyrobutyricum* strains (see Table 1).

2.2. Growth of Clostridium tyrobutyricum in defined media

2.2.1. Bacterial strains and inoculum preparation

All *C. tyrobutyricum* strains used in the growth experiments in this study were isolated from cheese. These are listed in Table 2 (named with the acronym FAM followed by a number) and are deposited to the Culture Collection of Agroscope (Liebefeld, Bern, Switzerland). Two different procedures were followed to prepare the inoculum depending on the strain and the experiment. *C. tyrobutyricum* FAM22552 and FAM22553 were first grown in Reinforced Clostridial Medium (RCM) composed of 10.0 g L^{-1} meat extract (Lab-Lemco, Oxoid, LP0029), 10.0 g L^{-1} casein peptone (Merck, 1.07213), 3.0 g L^{-1} yeast extract (BBL, 211929), 5.0 g L^{-1} D(+)-glucose monohydrate (Merck 1.08342), 1.0 g L^{-1} starch, 5.0 g L^{-1} sodium chloride (Merck, 1.06404), 3.0 g L^{-1} sodium acetate (Merck, 1.06267), 0.5 g L^{-1} L-cysteinehydrochloride monohydrate (Fluka, 30130), 3.0 g L^{-1} calcium chloride dihydrate (Merck, 2382) at 37 °C anaerobically in airtight boxes with GENbox anaer generators (Biomérieux) for 7 days. Cells were collected by centrifugation (30 min, 4630*g*, 8 °C), suspended in sterile tap water, centrifuged again, and finally suspended in 30 mL of a sterile tap water:biomilk solution (2:1). The biomilk solution contained per liter tap water 105.2 g biomilk (Emmi AG). Cell counts on RCM agar (37 °C, 7 days, anaerobic) were about 7×10^5 CFU mL^{-1} and 2×10^7 CFU mL^{-1} for *C. tyrobutyricum* FAM22552 and FAM22553, respectively. The two bacterial stock suspensions were aliquoted and stored at −20 °C. Just before inoculation of the growth media, 50 μL of stock bacterial suspension were added to 950 μL of H_2O in a sterile Eppendorf tube. The tube was then centrifuged at 6000 g for 5 min, and the resulting pellet was resuspended in 1 mL H_2O. This washing step was repeated twice more to avoid potential contamination of the defined growth media with the nutrients present in the original stock bacterial suspensions.

A second procedure was used to prepare the inoculum of the other 10 strains. These were grown on RCM agar plates for at least 3 days. Subsequently, 5 colonies of each plate were transferred to 1 mL H_2O and washed according to procedure described above.

Table 2
Clostridium tyrobutyricum strains used in in vitro growth experiment in this study.

Species	Strain	Origin
C. tyrobutyricum	FAM22552	Semi-hard red-smear cheese
	FAM22553	Semi-hard red-smear cheese
	FAM1353	Hard cheese
	FAM1519	Processed cheese
	FAM1526	Hard smear cheese
	FAM1600	Hard smear cheese
	FAM1617	Processed cheese
	FAM22547	Semi-hard smear cheese
	FAM22549	Hard cheese
	FAM22550	Hard cheese
	FAM22554	Semi-hard red-smear cheese
	FAM22555	Semi-hard red-smear cheese

2.2.2. Defined growth media

The basic medium (BM) used in this study was a modification of the medium developed by Bhat and Barker (1947), and it contained per liter of distilled water 4.4 g D(+)-glucose monohydrate, 0.72 g sodium acetate, 0.5 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, 0.5 g K_2HPO_4, and 0.5 g L-cysteine hydrochloride. The BM was then modified by adding 0.8 mg para-aminobenzoic acid (BM + PABA), 0.04 mg biotin (BM + biotin) or 0.8 mg PABA and 0.04 mg biotin (BM + PABA + biotin) per liter of basic medium. The pH of the defined media was around 5.4. A fifth medium, BM + yeast, serving as positive control contained 1 g yeast extract per liter in addition to PABA and biotin. Information about the stock solutions used to prepare these media, as well as sterilization procedures and storing conditions applied to them, are specified in Table S5.1, Supplementary File 5.

2.2.3. Growth of Clostridium tyrobutyricum in defined growth media

The growth of the *C. tyrobutyricum* FAM22552 and FAM22553 strains (Table 5) was tested in triplicate in 20 mL of each growth medium (BM, BM + PABA, BM + biotin, BM + PABA + biotin, and BM + yeast) in sterile 50 mL glass bottles. Growth media were inoculated with 20 μL of inoculum suspensions. The bottles were incubated anaerobically at 37 °C for 14 days (open caps) and visually screened daily for turbidity. After incubation, optical density (OD) values were determined and butyric acid concentration was measured in one replicate for each tested condition.

In a second experiment, the growth of 10 other strains of *C. tyrobutyricum* (Table 2) was tested. To this end, 20 μL of inoculum suspensions of these strains were added to 20 mL of growth medium (BM, BM + PABA, BM + biotin, BM + PABA + Biotin and BM + yeast). OD values were determined after 14 days of anaerobic incubation at 37 °C.

2.2.4. Growth over time

The growth of *C. tyrobutyricum* FAM22553 was monitored over time. For this purpose, 50 mL of the defined growth medium containing PABA and biotin (BM + PABA + biotin) and the growth medium containing yeast extract (BM + yeast) were prepared in triplicate for 5 different incubation periods and inoculated with

50 μL of inoculum solution. Growth was assessed after 0, 2, 6, 9, and 14 days of incubation (anaerobic, 37 °C) by measuring OD and butyric acid concentration.

2.2.5. Butyric acid quantification

To quantify the butyric acid production of *C. tyrobutyricum*, about 10 mL of growth medium was centrifuged (4630g, 15 min). The supernatant was filtered with a 0.45 μm filter (DIA-Nielsen 5120389D) and stored at −20 °C until analysis. One gram of the sample was then weighed into a headspace vial, together with 200 μL of 10% HCl and 200 μL of ethanol. The vial was sealed and incubated at 95 °C for 3 h. Thereafter, 1 mL of the headspace was analyzed with a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Basel, Switzerland) equipped with a Hewlett Packard HP-5 cross-linked (5%-phenyl)-methylpolysiloxane fused silica capillary column (50 m × 0.32 mm × 0.52 mm) and a flame ionization detector (FID). The transfer to the capillary column was performed in split mode (1:8) using helium as the carrier gas. The GC temperature program was 50 °C (7 min) at 5 °C min^{-1}–60 °C at 32 °C min^{-1}–268 °C (4.5 min). The temperatures of the injection port and detector were 150 °C and 320 °C, respectively. The butyric acid was identified on the basis of retention time using external standards. The amount of the butyric acid was calculated from an external calibration curve corrected with an internal standard (3,3-Dimethylbutyric acid). The limit of detection was 0.005 mmol kg^{-1} based on a signal to noise (S/N) ratio of 3.

2.2.6. Optical density (OD) measurement

To measure OD, 500 μL of growth medium was removed from each bottle immediately after inoculation and after the incubation period. Of this, 200 μL was transferred in duplicate to a 96-well plate (CytoOne). OD of the transferred medium was determined using an ELx808™ Absorbance Microplate Reader (BioTek) with the following settings: temperature 25 °C, shake with medium intensity for 30 s, and read absorbance at wavelength of 600 nm. Mean OD values were determined from the two technical replicates. Final ODs were calculated by subtracting the ODs determined at the beginning from those determined at the end of the incubation period. OD values < |0.01| were defined as 0.

3. Results and discussion

3.1. Reconstruction of B vitamin and amino acid biosynthesis pathways

3.1.1. Amino acids and B vitamins biosynthesis in Clostridium tyrobutyricum

The genomes of four *Clostridium tyrobutyricum* strains were analyzed for the presence of putative genes coding for enzymes involved in the biosynthesis of amino acids and B vitamins. For this purpose, the theoretical biosynthetic pathway of each amino acid and B vitamin was retrieved from the literature (see Tables S1.1 and S1.2 in Supplementary Files 1 for information about the literature used). Biosynthesis pathways, as well as the DNA and the translated amino acid sequences of their corresponding putative genes found in the genomes of *C. tyrobutyricum* FAM22552 and FAM22553, are reported in Supplementary Files 2 and 3 for amino acids and B vitamins, respectively. Literature taken into account (Tables S1.1 and S1.2) indicated that for most amino acids and B vitamins only one conserved biosynthesis pathway has been described for bacteria. However, for some exceptions such as L-alanine, L-aspartate, L-lysine, and cobalamin (vitamin B12), different pathways could be found in the literature. Moreover, in addition to their *de novo* synthesis, several amino acids can also be converted into each other, for example, L-aspartate to L-asparagine and L-phenylalanine to L-tyrosine. This information was considered in the analysis. The genomes were annotated with RAST, and the biosynthesis pathways analyzed with SEED Viewer. This led to the identification of most of the involved putative genes. Enzymes catalyzing reactions with incomplete or recently assigned EC numbers were the main causes underlying the failure of gene identification. Moreover, some enzymes have not yet been identified or have not been updated in RAST. These issues were also reported in previous *in silico* reconstruction studies (Teusink et al., 2005; Guimaraes et al., 2008). The presence of the genes that could not be identified was investigated further by blasting homologs belonging to *E. coli* and *Clostridium* spp. (see Tables S4.1 and S4.2 in Supplementary File 4). To confirm the reliability of RAST annotation, a second annotation was performed with Prokka. Amino acids and B vitamins biosynthesis genes identified with RAST annotation for *C. tyrobutyricum* FAM22552 were then searched using BLAST in the translated genes annotated with Prokka for *C. tyrobutyricum* FAM22552. All genes annotated with RAST were found to be annotated also with Prokka. A minority of them showed a difference in nucleotide length (9–30 nucleotides) and a discordant assigned function. A BLAST search in NCBI database clarified the putative function of these genes. To rapidly check if the two annotations were comparable also for the other genomes, orthologs of biosynthesis genes identified in *C. tyrobutyricum* FAM2252 were searched in the Prokka annotations of the other Clostridium draft genomes by gene clustering using OrthoMCL. Doing this it was possible to identify almost all orthologs in each *Clostridium* genome. A manual search using BLAST showed that the few missing genes were nevertheless present in the Prokka annotation.

Using this strategy, the biosynthesis pathways of 19 amino acids could be reconstructed completely for the four *C. tyrobutyricum* genomes (see Table 3). The only exception was the gene encoding glutamine synthase, which catalyzes the conversion of L-glutamate to L-glutamine (EC 6.3.1.2) in the glutamine biosynthesis pathway. In fact, only partial sequences that were approximately 40 amino acids shorter than their homologs (about 630 amino acids long) in the NCBI database could be identified for *C. tyrobutyricum* FAM22552, FAM22553 and UC7086. Interestingly, this gene was identified at the very end of a scaffold in each of these *C. tyrobutyricum* draft genomes. Common assembly errors can lead to partially or entirely missing genes or fragmentation of genes into pieces lying on different scaffolds (Denton et al., 2014). Since *C. tyrobutyricum* ATCC showed a complete gene, it was assumed that a functional glutamine synthase gene is normally present in *C. tyrobutyricum*. An additional observation was made in the common biosynthesis pathway of aromatic amino acids (L-phenylalanine, L-tryptophan, and L-tyrosine). Two putative genes coding for enzymes responsible for the conversion of 3-dehydroquinate to 3-dehydroshikimate (3-dehydroquinate dehydratase I; EC 4.2.1.10) and shikimate to shikimate 3-phosphate (shikimate kinase; EC 2.7.1.71) have been found to be fused together in each of the studied draft genomes. Bifunctional enzymes catalyzing consecutive and non-consecutive reactions have been discovered in various metabolism pathways, particularly in those of amino acids (James and Viola, 2002). Taken together, these observations indicate that *C. tyrobutyricum* does not require any amino acids for growth.

In terms of the biosynthesis of B vitamins, the analysis showed that all strains have all putative genes coding for the enzymes involved in the formation of thiamine (B1), nicotinate (B3) and nicotinamide, pantothenate (B5), and pyridoxyl 5-phosphate (B6; see Table 4). For vitamin B6, it is important to note that only pyridoxal 5′phosphate synthase, which catalyzes the *de novo* synthesis of pyridoxal 5′pohsophate from glutamine, ribulose 5-phosphate, and glyceraldehyde 3-phosphate, was identified (Supplementary File 3, Tables S3.1.5 and S3.2.5). In fact, vitamin B6 is an umbrella

Table 3
Amino acid biosynthesis pathways complete ( ) and incomplete (✗) in *C. tyrobutyricum* FAM22552, FAM22553, UC7086 and ATCC25755; *C. beijerinckii* G117; *C. butyricum* DSM10702; and *C. sporogenes* PA3679.

Amino acid	*C. tyrobutyricum*				*C. beijernickii*	*C. butyricum*	*C. sporogenes*
	FAM22552	FAM22553	UC7086	ATCC25755	G117	DSM10702	PA3679
Alanine	+	+	+	+	+	+	+
Arginine	+	+	+	+	+	+	−
Asparagine	+	+	+	+	+	+	+
Aspartate	+	+	+	+	+	+	+
Cysteine	+	+	+	+	+	+	+
Glutamate	+	+	+	+	+	+	+
Glutamine	+[a]	+[a]	+[a]	+	+	+	+
Glycine	+	+	+	+	+	+	+[c]
Histidine	+	+	+	+	+	+	+
Isoleucine	+	+	+	+	+	+	−
Leucine	+	+	+	+	+	+	−
Lysine	+	+	+	+	+	+	+
Methionine	+	+	+	+	+	+	+
Phenylalanine	+[b]	+[b]	+[b]	+[b]	+	+	−
Proline	+	+	+	+	+	+	−
Serine	+	+	+	+	+	+	−
Threonine	+	+	+	+	+	+	+
Tryptophan	+[b]	+[b]	+[b]	+[b]	+	+	−
Tyrosine	+[b]	+[b]	+[b]	+[b]	+	+	−
Valine	+	+	+	+	+	+	−

[a] A portion of the gene catalyzing the reaction of L-glutamate to L-glutamine (EC 6.3.1.2) was missing. This was assumed to be due to the fact that this gene is located at the very end of a scaffold in each *C. tyrobutyricum* genome.
[b] The genes of the enzymes 3-dehydroquinate dehydratase I and shikimate kinase are fused together. They catalyze the conversion of 3-dehydroquinate to 3-dehydroshikimate (EC 4.2.1.10) and of shikimate to shikimate 3-phosphate (EC 2.7.1.71), respectively.
[c] Glycine can be synthesized only if serine is provided.

term that includes the compounds pyridoxine, pyridoxal, and pyridoxamine in addition to their 5′-phosphoesters. Pyridoxal 5′-phosphate is the biologically active form, acting as a coenzyme in many important metabolic pathways. The different vitamers in the so-called salvage pathway can be interconverted (reviewed by Mooney et al., 2009). The enzymes involved in this salvage pathway could not be identified in any *C. tyrobutyricum* genome. Consequently, *C. tyrobutyricum* could not use any of the B6 vitamers potentially present in the growth medium, with the exception of pyridoxal 5′-phosphate. In thiamine (B1) biosynthesis, the phosphatase responsible for the conversion of thiamine monophosphate to thiamine has not been fully characterized (Hasnain et al. 2016). Hence, its presence could not be verified. A similar case was found in the riboflavin (B2) biosynthesis pathway, where the enzyme performing the conversion of 5-amino-6-(5′-phosphoribitylamino)uracil to 5-amino-6-ribitylaminouracil (EC 3.1.3.−) has not yet been identified (Haase et al., 2013). Hence, its presence could not be verified. The same was true for the enzyme catalyzing the conversion of cob(II)yrinic acid a,c-diamide to cob(I)yrinate a,c-diamide (EC 1.16.8.1) in the biosynthesis of cobalamin (B12) through the anaerobic pathway (Lawrence et al., 2008, 2014). Given that all other enzymes of these two pathways were present in the four genomes, it was assumed that *C. tyrobutyricum* is capable of *de novo* synthesis. Concerning biotin (B7) biosynthesis, the enzyme 8-

Table 4
B vitamin biosynthesis pathways complete ( ) and incomplete (✗) in *C. tyrobutyricum* FAM22552, FAM22553, UC7086 and ATCC25755; *C. beijerinckii* G117; *C. butyricum* DSM10702; and *C. sporogenes* PA3679.

B vitamin	*C. tyrobutyricum*				*C. beijernickii*	*C. butyricum*	*C. sporogenes*
	FAM22552	FAM22553	UC7086	ATCC25755	G117	DSM10702	PA3679
Thiamine (B1)	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]	+[a]
Riboflavin (B2)	+[b]	+[b]	+[b]	+[b]	+[b]	+[b]	+[b]
Nicotinate (B3) and Nicotinamide	+	+	+	+	+	+	+
Pantothenate (B5)	+	+	+	+	+	+	+
Pyridoxal 5-phosphat (B6)	+	+	+	+	+	+	+
Biotin (B7)	−	−	−	−	−	−	−
Folate (B9)	−[c,d]	−[c,d]	−[c,d]	−[c,d]	+[d]	+[d]	−[c,d]
Cobalamin (B12)	+[e]	+[e]	+[e]	+[e]	+[e]	+[e]	−[e,f]

[a)] Thiazole tautomerase (EC 5.3.99.10) has been only recently characterized (Hazra et al. 2011). The penultimate step of thiamin biosynthesis (dephosphorylation of thiamine monophosphate; 3.1.3.-) involves a not fully characterized thiamine monophosphatase (Hasnain et al. 2016).
[b)] The specific enzyme(s) catalyzing the reaction of 5-amino-6-(5′-phospho-ribitylamino)uracil to 5-amino-6-ribitylaminouracil (EC. 3.1.3.-) have not yet been identified (Haase et al., 2013).
[c)] The enzymes involved in the biosynthesis of PABA from chorismate are missing. Dihydroneopterin triphosphate pyrophosphatase (3.6.1.-) has been characterized only in Gram negative bacteria (Gabelli et al., 2007).
[d)] The conversion of 7,9-dihydroneopterin triphosphate to 7,8 dihydroneopterin is poorly understood (reviewed by Bourne, 2014).
[e)] The enzyme catalyzing the reaction of cob(II)yrinic acid a,c-diamide to cob(I)yrinate a,c-diamide (EC 1.16.8.1) has not yet been identified in organisms operating the anaerobic pathway (Lawrence et al., 2008, 2014).
[f] The enzyme involved in the reaction of dimethylbenzimidazole to α-ribazole 5′-phosphate (EC 2.4.2.21) is missing.

amino-7-oxononanoate synthase could not be identified in any *C. tyrobutyricum* genome (Fig. 1 and Table 3). This enzyme catalyzes the condensation of pimeloyl-ACP and L-alanine to 8-amino-7-oxononanoate (EC 2.3.1.47). This suggests that *C. tyrobutyricum* cannot synthesize biotin. Moreover, the folate (B9) biosynthesis pathway was missing the two enzymes responsible for the conversion of chorismate to PABA (Table 4 and Fig. 2). These are PABA synthase and aminodeoxychorismate lyase, which catalyze the formation of 4-amino-4-deoxychorsimate from chorismate (EC 2.6.1.85) and PABA from 4-amino-4-deoxychorsimate (EC 4.1.3.38), respectively. Folate molecules are formed by the condensation reaction of 2-amino-4-hydroxy-6-hydromethyl-7,8-dihydropteridine-P2 (DHPPP) and PABA (reviewed by Rossi et al., 2011). These two precursors are synthesized separately, DHPPP from guanosine triphosphate (GTP) and PABA from chorismate, which is also an intermediate in the synthesis of aromatic amino acids (Rossi et al., 2011). It is important to note that in the biosynthesis of DHPPP the conversion of 7,9-dihydroneopterin triphosphate to 7,8 dihydroneopterin is poorly understood (reviewed by Bourne, 2014). It has been shown that in *Lactococcus lactis* and *E. coli* a Nudix hydrolase is involved in the conversion of 7,8-dihydroneopterin triphosphate to 7,8 dihydroneopterin monophosphate. A nonspecific hydrolase is supposed to convert this in 7,8-dihydroneopterin (Klaus et al., 2005; Gabelli et al., 2007). Since Nudix hydrolases show high heterogeneity it is difficult to identify putative orthologs by sequence homology (de Crécy-Lagard et al., 2007). In conclusion, the results of the *in silico* analysis suggest that *C. tyrobutyricum* growth requires only biotin and PABA or folate.

3.1.2. Amino acids and B vitamins biosynthesis in Clostridium butyricum, C. beijerinckii, and C. sporogenes

The *in silico* analysis was extended to the genomes of *C. beijerinckii* G117, *C. butyricum* DSM10702, and *C. sporogenes* PA3679. Unfortunately, at the moment, no genome of strains isolated from cheese is available for these species. Therefore, the observations made here should be confirmed by analyzing the genomes of strains isolated from cheese when these become available. The identified nucleotide and amino acid sequences of putative genes involved in the biosynthesis of amino acids and B vitamins in these strains are listed in Supplementary Files 3 and 4. Similar to *C. tyrobutyricum*, the genomes of *C. beijerinckii* G117 and *C. butyricum* DSM10702 were found to contain all the genes encoding the enzymes responsible for the biosynthesis of all 20 amino acids (Table 3). In contrast, various pathways could not be completed for *C. sporogenes* PA3679. More precisely, it was not possible to identify several genes coding for enzymes engaged in the biosynthesis of arginine, isoleucine, leucine, phenylalanine, proline, serine, tryptophan, tyrosine, and valine (Table 3). Concerning the biosynthesis of B vitamins, the findings for each strain were similar to the ones obtained for *C. tyrobutyricum* (Table 3). In fact, all three strains possessed the putative genes coding for the enzymes involved in the biosynthesis of thiamine (B1), nicotinate (B3) and nicotinamide, pantothenate (B5), and pyridoxal 5-phosphate (B6). The riboflavin (B2) biosynthesis pathway, excluding the yet-to-be-characterized enzyme responsible for the formation of 5-amino-6-ribitylaminouracil, could also be reconstructed for each strain. The same was true for cobalamin (B12) biosynthesis in *C. butyricum* and *C. beijerinckii*. However, nicotinate mononucleotide-dimethylbenzimidazole phosphoribosyltransferase, which catalyzes the conversion of dimethylbenzimidazole to the cobalamin precursor α-ribazole 5′-phosphate (EC 2.4.2.21), could not be identified in *C. sporogenes* PA3679 (Fig. 3). The three strains showed an incomplete pathway for biotin biosynthesis (Fig. 1). The enzyme 8-amino-7-oxononanoate synthase, the same as the one missing in *C. tyrobutyricum*, could not be identified in any of the analyzed genomes. In addition to this enzyme, the *C. beijerinckii* G117 and *C. sporogenes* strains were also missing dethiobiotin synthase and adenosylmethionine-8-amino-7-oxononanoate aminotransferase, respectively. Dethiotin synthase catalyzes the reaction converting 7,8-diaminononanoate to dethiobiotin (EC 6.3.3.3), whereas adenosylmethionine-8-amino-7-oxononanoate aminotransferase mediates the formation of 7,8-diaminononanoate from 8-amino-7-oxononanoate (EC 2.6.1.62). Concerning the biosynthesis of folate, the pathways of *C. beijerinckii* G117 and *C. butyricum* DSM10702 were complete, whereas *C. sporogenes* PA3679 was lacking the enzymes engaged in the synthesis of PABA from chorismate (Fig. 2).

According to this analysis, *C. butyricum* DSM10702 can synthesize all amino acids and B vitamins except biotin. This is in agreement with the results of previous work, where *C. butyricum* strains were grown in liquid media containing only this vitamin, thus confirming its essential role for this species (Cummins and Johnson, 1971; Himmi et al., 1999). Moreover, biotin was the only essential nutrient identified for *C. beijerinckii* G117. However, previous studies did not succeed in growing strains of this species in a minimal medium supplemented with amino acids and B vitamins,

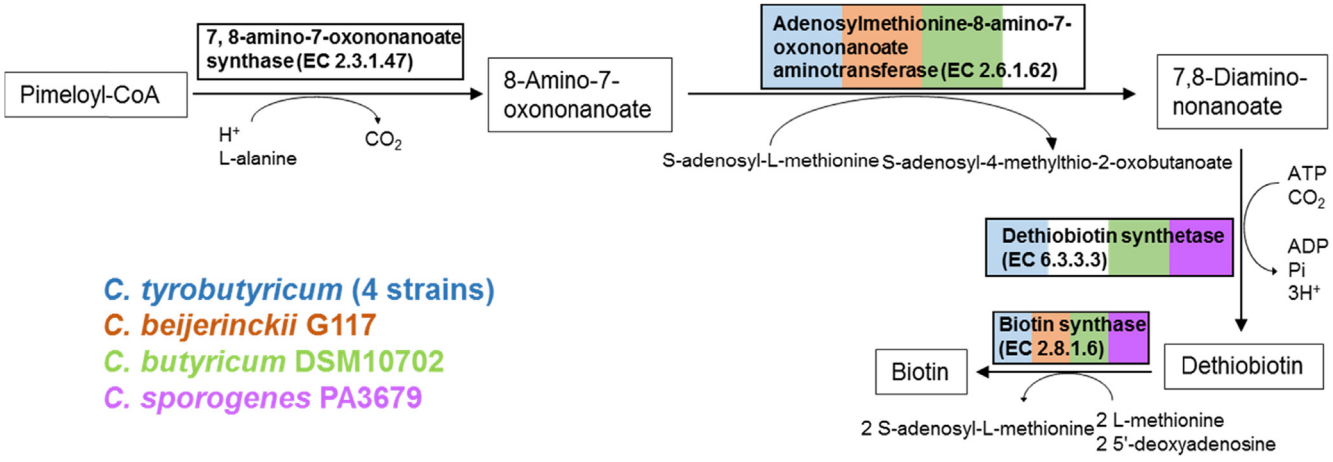

Fig. 1. Biotin biosynthesis pathway in *C. tyrobutyricum* FAM22552, FAM22553, UC7086, and ATCC 25755, *C. beijerinckii* G117, *C. butyricum* DSM10702 and *C. sporogenes* PA3679. 8-amino-7-oxononanoate synthase ((EC 2.3.1.47) was absent in analyzed draft genomes. Adenosylmethionine-8-amino-7-oxononanoate aminotransferase (EC 2.6.1.62) and dethiobiotin synthetase (EC 6.3.3.3) were found to be absent in the genomes of *C. sporogenes* PA3679 and *C. beijerinckii* G117, respectively.

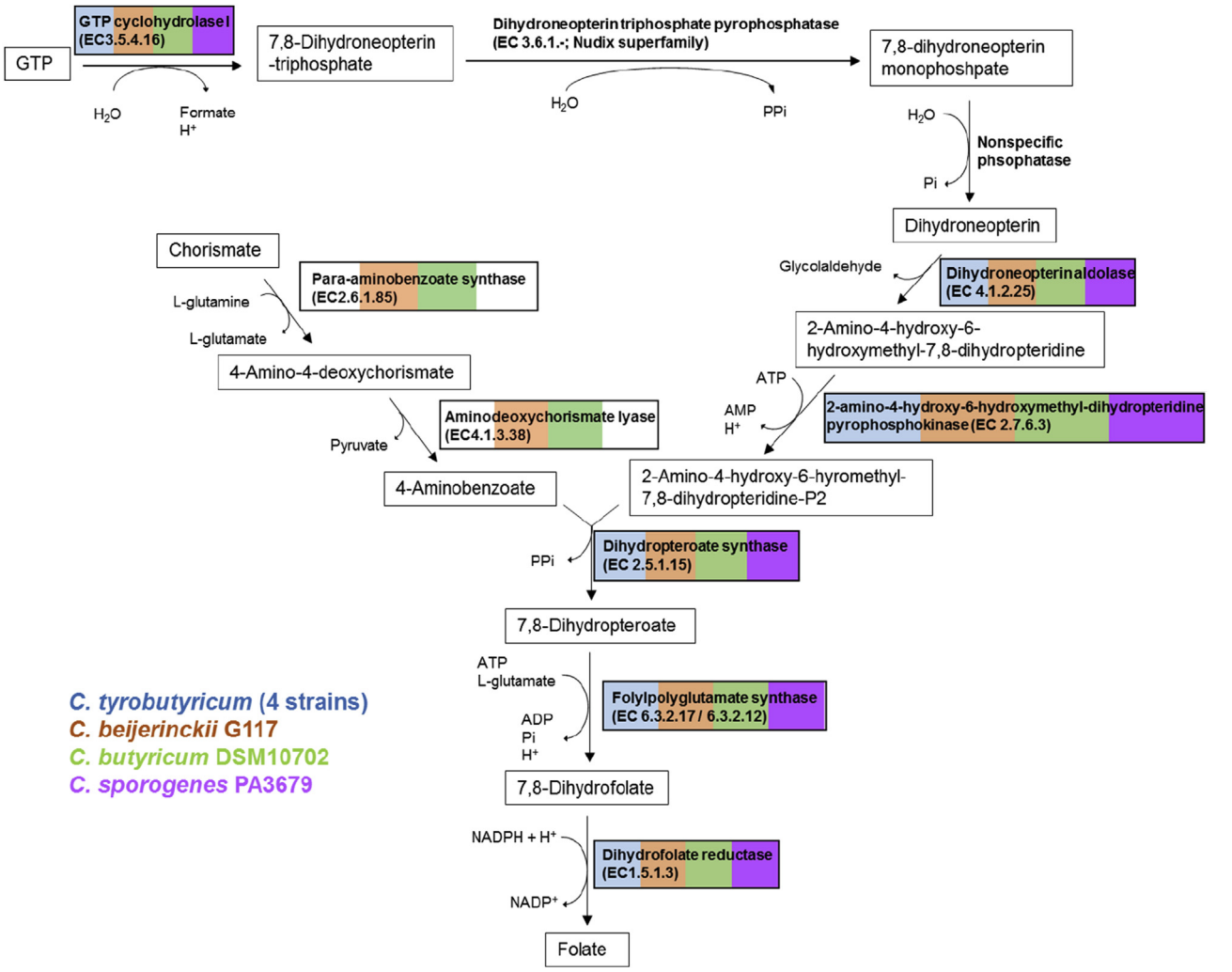

Fig. 2. Folate biosynthesis pathway in *C. tyrobutyricum* FAM22552, FAM22553, UC7086, and ATCC 25755, *C. beijerinckii* G117, *C. butyricum* DSM10702 and *C. sporogenes* PA3679. Genes coding for enzymes responsible for the conversion of chorismate into 4-aminobenzoate (para-aminobenzoate synthase, EC 2.6.1.85; aminodeoxychorismate lyase, EC 4.1.3.38) were absent in all *C. tyrobutyricum* genomes as well as in that of *C. sporogenes* PA3679. The conversion of 7,9-dihydroneopterin triphosphate to 7,8 dihydroneopterin is poorly understood.

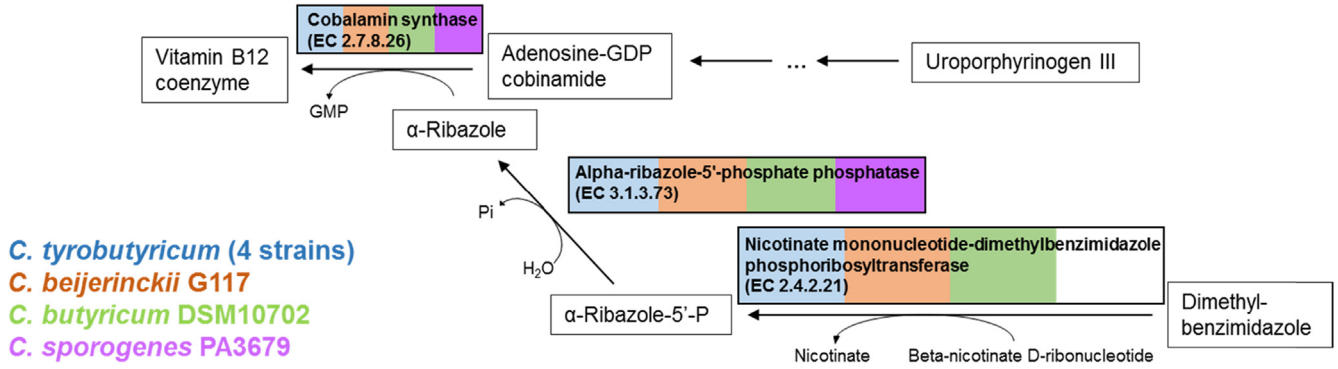

Fig. 3. Cobalamin (vitamin B12) biosynthesis in *C. tyrobutyricum* FAM22552, FAM22553, UC7086, and ATCC 25755, *C. beijerinckii* G117, *C. butyricum* DSM10702 and *C. sporogenes* PA3679 (shortened). The gene coding for nicotinate mononucleotide-dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21) was absent in *C. sporogenes* draft genome.

but growth occurred only when yeast extract was provided (Cummins and Johnson, 1971; Vos et al., 2011). These observations suggest that *C. beijerinckii* might require only biotin in terms of B vitamins and no amino acids, but it needs other essential growth factors such as carbon sources or minerals to grow in defined media. The *C. sporogenes* PA3679 genome, in contrast, presented several incomplete pathways. The requirement of several amino acids to grow *C. sporogenes* in defined media has been reported in

many studies (Kindler et al., 1956; Belokopytov et al., 1982; Lovitt et al., 1987). However, each of these works found a different set of essential amino acids. These apparently contradictory results could be explained by the genetic diversity of the *C. sporogenes* strains used and by the fact that amino acids such as proline and serine can be synthesized from arginine and glycine if they are present in the growth medium (Muth and Costilow, 1974; Cunin et al., 1986; Eggeling, 2007). In terms of the B vitamin pathways, the essential role of biotin and PABA in the growth of *C. sporogenes* was described previously (Kindler et al., 1956). The absence of nicotinate mononucleotide-dimethylbenzimidazole phosphoribosyltransferase in cobalamin biosynthesis was also reported in *Listeria innocua* and other gram-positive bacteria, where an alternative pathway for α-ribazole salvaging was described (Gray and Escalante-Semerena, 2010). This could be the case for *C. sporogenes* as well.

3.2. Growth of *Clostridium tyrobutyricum* in defined media

Clostridium tyrobutyricum FAM22552 and FAM22553 were grown in defined liquid media to confirm the roles of biotin and PABA as essential nutrients (Table 5). Turbidity was observed in the growth medium containing yeast extract (BM + yeast) after two days and in the medium containing PABA and biotin (BM + PABA + biotin) after 6–8 days of incubation. No growth was observed after 14 days of incubation in the media containing no vitamins (BM), biotin (BM + biotin), or PABA (BM + PABA). Optical density (OD) values after 14 days were higher in the BM + yeast medium (0.487 and 0.587 for FAM22552 and FAM22553, respectively) than in the BM + PABA + biotin medium (0.302 and 0.244 for FAM22552 and FAM22553, respectively). These findings were also confirmed by the quantification of butyric acid produced during growth, which showed concentrations of around 10 mmol kg^{-1} for BM + yeast and 6 mmol kg^{-1} for BM + PABA + biotin (Table 6). This analysis was then extended to ten additional *Clostridium tyrobutyricum* strains, and the results obtained were comparable to those obtained for FAM22552 and FAM22553 (Table 5). For nine out of ten strains, turbidity was visible after two days in BM + yeast and after 6–8 days in BM + PABA + biotin. The only exception was FAM1353, which could not grow in BM + PABA + biotin. This can possibly be attributed to mutations leading to the loss of function of the enzymes involved in biosynthesis pathways of vital importance.

To further characterize the growth of *C. tyrobutyricum*, the OD and butyric acid concentration were quantified at five time points during the incubation of FAM22553 in BM + PABA + biotin and in BM + yeast (Fig. 1). During the first two days of incubation in BM + yeast, the OD increased from 0 to 0.728 ± 0.004. A decrease was then observed at day 6 (0.631 ± 0.005). Thereafter, the OD remained relatively constant until the end of incubation at day 14. Concerning the BM + PABA + biotin medium, growth was first visible after 6 days of incubation (Fig. 4). Subsequently, OD values increased and peaked on day 9 (0.300 ± 0.037). The progression of growth correlated well with the measured concentration of butyric acid in both media (Fig. 5). Monitoring of the growth of FAM22553 over time indicates that in the defined medium, *C. tyrobutyricum* remains latent for 6 days before starting to grow. Faster proliferation in complex media containing yeast extract was also observed in different studies investigating the germination of *Bacillus* and *Clostridium* species (Demain, 1958). These studies also showed that the amino acids provided by the growth media influence the duration of germination (Demain, 1958; Ramirez and Abel-Santos, 2010). Hence, it is assumed that the faster proliferation in the BM + yeast medium is due to a stronger promotion of germination owing to the presence of a greater number of growth factors.

In vitro results presented here confirm the role of biotin and PABA/folate as essential nutrients to sustain the growth of *C. tyrobutyricum*. The possible exploitation of these vitamins to control late blowing depends on their concentration in cheese and the potential to lower them using starter and non-starter lactic acid bacteria. Biotin content in cheese was shown to range between 7 and 59 μg kg^{-1}, which is comparable to the concentration used in this study (40 μg L^{-1}; Hoppner and Lampi, 1992). Unfortunately, to our knowledge there is no report on the concentration of PABA in cheeses, whereas folate occurrence varies between 40 and 430 μg kg^{-1} (Wigertz et al., 1997). Assuming that all PABA consumed in the growth medium is converted to folate and the concentration of PABA in cheese is negligible, the concentration of PABA used in the present work (800 μg L^{-1}) would be comparable to that of folate in cheeses. Preliminary experiments with three months old semi-hard red-smear cheeses in our laboratory showed a concentration of about 10 μg kg^{-1} for biotin and between 100 and 200 μg kg^{-1} for folate (Storari and Arias, unpublished). These concentrations agree with those reported in the literature. Work is currently underway to assess the levels below which the concentrations of these compounds pose a problem to the growth of *Clostridium* spp. in defined media resembling cheese and in cheese itself. In parallel, strains of LABs described in the literature as folate and biotin consumers or showing no biosynthesis genes in their genomes are tested in milk and in cheese to select the best candidates to lower the concentration of free vitamins in cheeses (Rossi et al., 2011).

Table 5
Growth of *C. tyrobutyricum* strains measured by optical density (OD600) in defined growth media after 14 days of incubation.

Strain	Optical density (OD_{600})				
	BM + yeast	BM + PABA + biotin	BM + PABA	BM + biotin	BM
FAM 22552[a]	0.487 ± 0.040	0.302 ± 0.012	0	0	0
FAM 22553[a]	0.587 ± 0.015	0.244 ± 0.024	0	0	0
FAM 1353	0.519	0	0	0	0
FAM 1519	0.333	0.194	0	0	0
FAM 1526	0.478	0.196	0	0	0
FAM 1600	0.577	0.230	0	0	0
FAM 1617	0.565	0.257	0	0	0
FAM 22547	0.411	0.202	0	0	0
FAM 22549	0.597	0.244	0	0	0
FAM 22550	0.500	0.212	0	0	0
FAM 22554	0.425	0.261	0	0	0
FAM 22555	0.599	0.357	0	0	0

$0 \triangleq < |0.01|$
[a] Strains grown in triplicate.

Table 6
Butyric acid production of *Clostridium tyrobutyricum* FAM22552 and FAM22553 in defined growth media after 14 days of incubation.

Strain	Butyric acid [mmol kg^{-1}]				
	BM + yeast	BM + PABA + biotin	BM + PABA	BM + biotin	BM
FAM 22552	9.542	6.441	n.d.	n.d.	n.d.
FAM 22553	8.415	6.193	n.d.	n.d.	n.d.

n.d. = not detected (concentration below 0.005 mmol kg^{-1}).

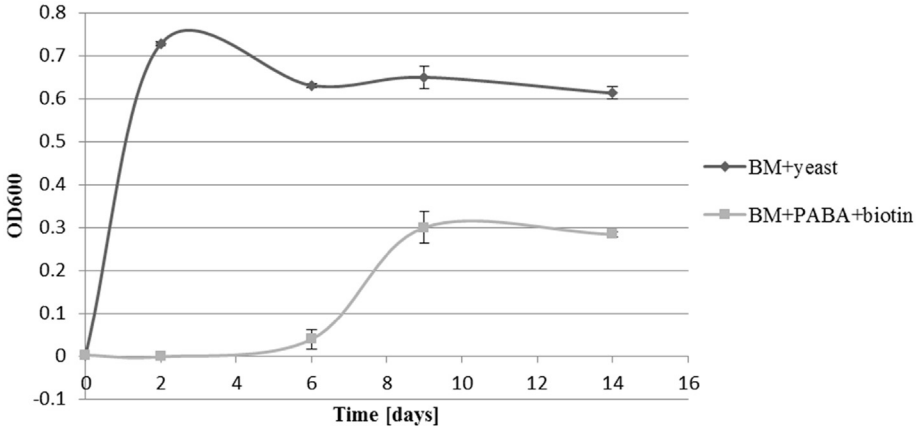

Fig. 4. Optical Density (OD_{600}) values measured during growth of *C. tyrobutyricum* FAM22553 in defined growth medium (BM + PABA + biotin) and medium with yeast extract (BM + yeast).

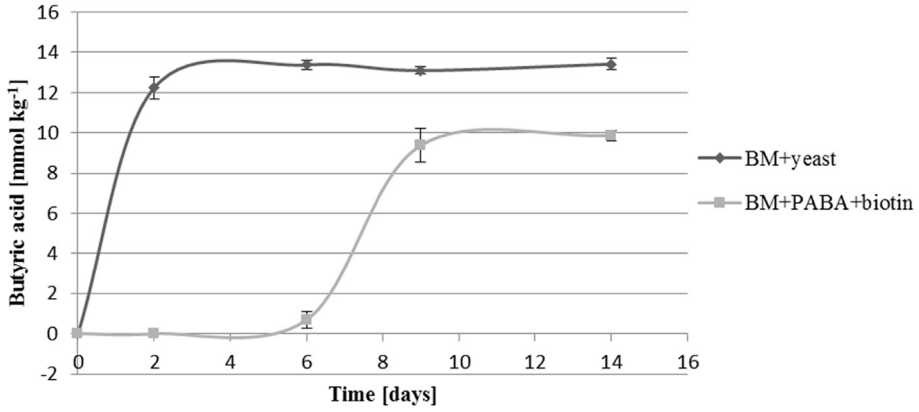

Fig. 5. Butyric acid production during growth *of C. tyrobutyricum* FAM22553 in defined growth media.

4. Conclusion

Late blowing is associated with severe economic losses for the cheese industry each year (Gomez-Torres et al., 2014). Strategies used to prevent this defect include physical treatments such as bactofugation, microfiltration and the use of additives such as nitrate or lysozyme. However, these treatments entail negative effects for manufacturers and consumers, including costly equipment and potential health risks of additives (Fremont et al., 1997; EFSA, 2010; Ávila et al., 2014). More effective control strategies could be developed by exploiting specific weak points of late blowing–causing microorganisms. In this sense, the first candidates as targets may be amino acid and B vitamin requirements. In the present study, biotin and PABA, a precursor of folate, were identified as the only growth requirements of *Clostridium tyrobutyricum*. This matches with the nutrients that Tidswell et al. (1991) added to their minimal medium. Moreover, biotin is supposed to be essential also for the growth of *C. butyricum*, *C. beijerinckii*, and *C. sporogenes*. Biotin plays a crucial role in various physiological processes. It is a prosthetic group serving in general as a carbon dioxide carrier for carboxylases, transcarboxylases, and decarboxylases (Moss and Lane, 1971). These are involved in various metabolic processes, including fatty acid biosynthesis and gluconeogenesis (Wood and Barden, 1977). Folate is converted to its biologically active form tetrahydrofolate (H_4folate) by the enzyme dihydrofolate reductase (Maden, 2000). H_4folate is a carrier of C_1 fragments, which are needed for various essential biosynthesis processes, including purine and thymidylate synthesis (Vorholt, 2002). Since both biotin and folate play crucial roles in various metabolic processes, they are promising targets to prevent late blowing in cheese.

To conclude, it is important to highlight the usefulness of genome analysis to quickly obtain a realistic view of the nutritional requirements of bacterial strains and species. In fact, we found a strong correlation between the results of the *in silico* analysis and

the growth of *Clostridium* strains in defined media used here and in other studies. This approach has the potential to become a standard in the process of screening for strain selection in food microbiology.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fm.2016.05.013.

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