



## Article

# Development of a Taxon-Specific Real-Time PCR Method Targeting the *Bacillus subtilis* Group to Strengthen the Control of Genetically Modified Bacteria in Fermentation Products

Marie-Alice Fraiture , Andrea Gobbo, Nina Papazova and Nancy H. C. Roosens \*

Sciensano, Biological Health Risk, Transversal Activities in Applied Genomics (TAG), Rue Juliette Wytsman 14, 1050 Brussels, Belgium; Marie-Alice.Fraiture@sciensano.be (M.-A.F.); andrea.gobbo@telenet.be (A.G.); Nina.Papazova@sciensano.be (N.P.)

\* Correspondence: Nancy.Roosens@sciensano.be; Tel.: +32-(0)-2642-5258

**Abstract:** Most of the bacteria that are used to produce fermentation products, such as enzymes, additives and flavorings, belong to the *Bacillus subtilis* group. Recently, unexpected contaminations with unauthorized genetically modified (GM) bacteria (viable cells and associated DNA) that were carrying antimicrobial resistance (AMR) genes was noticed in several microbial fermentation products that have been commercialized on the food and feed market. These contaminations consisted of GM *Bacillus* species belonging to the *B. subtilis* group. In order to screen for the potential presence of such contaminations, in this study we have developed a new real-time PCR method targeting the *B. subtilis* group, including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and *B. velezensis*. The method's performance was successfully assessed as specific and sensitive, complying with the *Minimum Performance Requirements for Analytical Methods of GMO Testing* that is used as a standard by the GMO enforcement laboratories. The method's applicability was also tested on 25 commercial microbial fermentation products. In addition, this method was developed to be compatible with the PCR-based strategy that was recently developed for the detection of unauthorized GM bacteria. This taxon-specific method allows the strengthening of the set of screening markers that are targeting key sequences that are frequently found in GM bacteria (AMR genes and shuttle vector), reinforcing control over the food and feed chain in order to guarantee its safety and traceability.

**Keywords:** unauthorized genetically modified microorganisms; real-time PCR detection; enzymes; additives and flavorings; food and feed safety concerns; *Bacillus subtilis* group



**Citation:** Fraiture, M.-A.; Gobbo, A.; Papazova, N.; Roosens, N.H.C. Development of a Taxon-Specific Real-Time PCR Method Targeting the *Bacillus subtilis* Group to Strengthen the Control of Genetically Modified Bacteria in Fermentation Products. *Fermentation* **2022**, *8*, 78. <https://doi.org/10.3390/fermentation8020078>

Academic Editor: Kurt A. Rosentrater

Received: 13 January 2022

Accepted: 10 February 2022

Published: 12 February 2022

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## 1. Introduction

Genetically modified microorganisms (GMM) are commonly used by the food and feed industries in order to produce microbial fermentation products, including enzymes, additives and flavorings. Since 2014, several unexpected contaminations with GMM, both viable strains and their associated DNA, in microbial fermentation products that were commercialized on the European (EU) food and feed chain were observed, leading to numerous RASFF (Rapid Alert System for Food and Feed) notifications according to the EC/2003/1830 regulation [1–14]. In addition, such GMM are frequently carrying antimicrobial resistance (AMR) genes as a selection marker [5–12,15]. Consequently, health concerns that are related to the presence of AMR genes in the food and feed chain were raised regarding the horizontal transfers of AMR genes to pathogens and gut microbiota, resulting in treatment failure [16–20]. Therefore, to ensure the safety and traceability of the food and feed chain as well as the freedom of the consumer's choice, the control of GMM contaminations in microbial fermentation products was demanded by the competent authorities [4–12].

The current strategy that has been implemented by enforcement laboratories in order to control genetically modified organisms (GMO) in the food and feed chain is mainly tar-

getting genetically modified (GM) plants. This strategy is composed of two main successive steps using real-time PCR methods. More precisely, a first-line screening analysis step, one that is targeting the taxon-specific markers and genetic elements that are commonly found in GM plants, is applied in order to screen for the potential presence of GM plants. A second-line analysis step is then applied in order to identify the specific GM plant events in targeting their unique junctions between the host genome and the transgenic cassette. The second-line methods are provided by the GM plant developer companies [21–23]. However, in order to control GMM contaminations, all of the sequences that are related to GMM are confidential and, in contrast to the GM plants that are intended for human and animal consumption, no methods that are specific to GMM strains are provided by manufacturers to the enforcement laboratories, therefore hampering the the establishment of a similar workflow for GMM testing [5–12,15].

In order to overcome these issues, publicly available data from patents and scientific literature were recently used in order to develop a set of first-line screening real-time PCR methods targeting the genetic elements that are frequently found in the GM bacteria that are used to produce microbial fermentation products [5–7,9]. More precisely, these methods target three AMR genes, being *cat* (GenBank: NC\_002013.1), *aadD* (GenBank: M19465.1) and *tet-L* (GenBank: D00946.1) conferring a resistance to chloramphenicol, kanamycin and tetracycline, respectively, as well as a shuttle vector pUB110 carrying *aadD* (GenBank: M19465.1) [5–7,9]. If at least one of these screening markers is detected, a potential GMM contaminations can be suspected. A second-line analysis step was also developed in order to prove the presence of specific GM bacterial strains using real-time PCR methods targeting their unnatural associations of sequences. These sequences, which are publicly unavailable, were previously characterized by sequencing the DNA that was extracted either from GM bacterial isolates or from commercial microbial fermentation products with GMM contaminations [3,8–11,13]. Currently, these second-line methods allow the identification of the GM *Bacillus velezensis* strain that produces protease, the GM *B. amyloliquefaciens* strain that produces alpha-amylase, the GM *B. subtilis* strain that produces vitamin B2 and the GM *B. amyloliquefaciens* strain that produces protease. All of these first- and second-line real-time PCR methods were developed in order to comply with the *Minimum Performance Requirements for Analytical Methods of GMO Testing* of the European Network of GMO Laboratories, being used as a standard in GMO enforcement laboratories [5–7,9,24].

However, in contrast to the GM plant detection strategy, no taxon-specific method for GMM detection is currently available in the first-line screening analysis step. PCR-based methods, including real-time PCR and conventional PCR followed by sequencing, that are specific to key markers such as the bacterial genus (e.g., 16S-rRNA gene) [25,26] and bacterial species of interest (e.g., *B. subtilis*, *B. licheniformis*) [12,27–30] do exist. Nonetheless, the real-time PCR technology is usually preferred as it has been widely mastered by enforcement laboratories which are controlling GMO. Given that the enforcement laboratories carry out their analyses commonly under accreditation, the presently used real-time PCR methods should also comply with the *Minimum Performance Requirements for Analytical Methods of GMO Testing* of the European Network of GMO Laboratories. Moreover, in order to efficiently strengthen the GMM detection strategy, the taxon-specific method needs to be compatible with the set of methods that are used in the first-line screening analysis step (e.g., real-time PCR technology, TaqMan chemistry and PCR conditions) [5–7,9,24]. To this end, a taxon-specific real-time PCR method was developed in this study in order to target *Bacillus* species belonging to the *B. subtilis* group. More precisely, this *B. subtilis* group is composed in total of 10 closely related *Bacillus* species, including *B. amyloliquefaciens*, *B. atrophaeus*, *B. licheniformis*, *B. mojavensis*, *B. pumilus*, *B. sonorensis*, *B. subtilis*, *B. tequilensis*, *B. vallismortis* and *B. velezensis*. This screening method, named BSG, covers the majority of the GM bacterial species that have been reported as being used by the food and feed industry in order to produce microbial fermentation products, namely *B. amyloliquefaciens*, *B. licheniformis*, *B. subtilis* and *B. velezensis* [3,6,9–11,13,28,31–33]. The performance of the BSG method was assessed at the specificity and sensitivity levels, according the *Minimum*

Performance Requirements for Analytical Methods of GMO Testing of the European Network of GMO Laboratories [24]. The applicability of the BSG method was also tested using several commercial microbial fermentation products. In addition, this taxon-specific method was designed to be compatible with the first-line screening analysis step of the GMM detection strategy. Therefore, by completing the set of real-time PCR screening methods, the BSG method can strengthen the control over GM bacterial contaminations in microbial fermentation products that are commercialized in the food and feed chain.

## 2. Materials and Methods

### 2.1. Materials

For the specificity and sensitivity assessments of the BSG method, DNA from the control plasmid (GeneCust, Boynes, France), artificially synthesized to carry one copy of the targeted sequence from *B. subtilis*, was used. DNA from *Homo sapiens* (G3041 Promega, Madison, WI, USA), *Zea mays* (ERM-BF413ak), wild-type microbial species and GM bacterial strains (*B. subtilis* RASFF2014.1249 and *B. velezensis* RASFF2019.333) were obtained as described in Tables 1–3 [5–11]. For the applicability assessment of the BSG method, DNA from several commercial microbial fermentation products (samples n° 1–25) was obtained as described in Table 4. The presence of GMM contaminations was previously investigated in these samples [5–11]. The DNA’s concentration was measured by spectrophotometry using NanoDrop® 2000 (Thermo Fisher, Waltham, MA, USA) and the DNA’s purity was evaluated using the A260/A280 and A260/A230 ratios. The DNA concentration from the control plasmid was also measured by fluorometry using Qubit 3.0 Fluorometer (Thermo Fisher, Waltham, MA, USA). The DNA were also previously assessed as amplifiable [3,4,8–11].

**Table 1.** Oligonucleotides of the real-time PCR BSG method targeting bacterial species belonging to *B. subtilis* group that are used to produce microbial fermentation products. On the targeted sequences, the location of the used oligonucleotides is indicated in bold.

Targeted Sequences			
B_pumilus_CP054310.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_licheniformis_CP045814.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_amyloliquefaciens_CP054479.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_velezensis_CP055160.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_subtilis_CP054177.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_pumilus_CP054310.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_licheniformis_CP045814.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_amyloliquefaciens_CP054479.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_velezensis_CP055160.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_subtilis_CP054177.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_pumilus_CP054310.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_licheniformis_CP045814.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_amyloliquefaciens_CP054479.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_velezensis_CP055160.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_subtilis_CP054177.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_pumilus_CP054310.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_licheniformis_CP045814.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_amyloliquefaciens_CP054479.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_velezensis_CP055160.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
B_subtilis_CP054177.1	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA	CGTGCGCCCTTTCTA
Names	Oligonucleotides Sequences	Annealing temperature	Expected amplicon sizes
BSG-F	CGTGCGCCCTTTCTAAC	64 °C	137–165 bp
BSG-P	FAM-CAAAGAACACGTTGGTGGAGCCTAGC-TAMRA		
BSG-R	TTGACGCAGGAGGT		

### 2.2. Development and Validation of the BSG Method

Based on previous studies [28,30], the 16S-23S region was selected in order to develop a taxon-specific real-time-PCR method allowing the targeting of the *B. subtilis* group, including *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilus* and *B. velezensis* (Table 1) [28,30,32,33].

**Table 2.** Specificity assessment of the developed real-time PCR BSG method. The presence and absence of amplification are symbolized by “+” and “–”, respectively. For each result, the experiment was carried out in triplicate on 10 ng of each sample. The means of the observed  $C_q$  are indicated in brackets.

Kingdom	Genus	Species	Strain Number	BSG Method	
Fungi	<i>Aspergillus</i>	<i>acidus</i>	IHEM 26285	-	
	<i>Aspergillus</i>	<i>aculeatus</i>	IHEM 05796	-	
	<i>Aspergillus</i>	<i>fijiensis</i>	IHEM 22812	-	
	<i>Aspergillus</i>	<i>melleus</i>	IHEM 25956	-	
	<i>Aspergillus</i>	<i>niger</i>	IHEM 25485	-	
	<i>Aspergillus</i>	<i>oryzae</i>	IHEM 25836	-	
	<i>Candida</i>	<i>cylindracea</i>	MUCL 041387	-	
	<i>Candida</i>	<i>rugosa</i>	IHEM 01894	-	
	<i>Chaetomium</i>	<i>gracile</i>	MUCL 053569	-	
	<i>Cryphonectria</i>	<i>parasitica</i>	MUCL 007956	-	
	<i>Disporotrichum</i>	<i>dimorphosporum</i>	MUCL 019341	-	
	<i>Fusarium</i>	<i>venenatum</i>	MUCL 055417	-	
	<i>Hansenula</i>	<i>polymorpha</i>	MUCL 027761	-	
	<i>Humicola</i>	<i>insolens</i>	MUCL 015010	-	
	<i>Kluyveromyces</i>	<i>lactis</i>	IHEM 02051	-	
	<i>Leptographium</i>	<i>procerum</i>	MUCL 008094	-	
	<i>Mucor</i>	<i>javanicus</i>	IHEM 05212	-	
	<i>Penicillium</i>	<i>camemberti</i>	IHEM 06648	-	
	<i>Penicillium</i>	<i>chrysogenum</i>	IHEM 03414	-	
	<i>Penicillium</i>	<i>citrinum</i>	IHEM 26159	-	
	<i>Penicillium</i>	<i>decumbens</i>	IHEM 05935	-	
	<i>Penicillium</i>	<i>funiculosum</i>	MUCL 014091	-	
	<i>Penicillium</i>	<i>multicolour</i>	CBS 501.73	-	
	<i>Penicillium</i>	<i>roqueforti</i>	IHEM 20176	-	
	<i>Pichia</i>	<i>pastoris</i>	MUCL 027793	-	
	<i>Rhizomucor</i>	<i>miehei</i>	IHEM 26897	-	
	<i>Rhizopus</i>	<i>niveus</i>	ATCC 200757	-	
	<i>Rhizopus</i>	<i>oryzae</i>	IHEM 26078	-	
	<i>Saccharomyces</i>	<i>cerevisiae</i>	IHEM 25104	-	
	<i>Sporobolomyces</i>	<i>singularis</i>	MUCL 027849	-	
	<i>Talaromyces</i>	<i>cellulolyticus/pinophilus</i>	IHEM 16004	-	
	<i>Talaromyces</i>	<i>emersonii</i>	DSM 2432	-	
	<i>Trametes</i>	<i>hirsuta</i>	MUCL 030869	-	
	<i>Trichoderma</i>	<i>citrinoviride</i>	IHEM 25858	-	
	<i>Trichoderma</i>	<i>longibrachiatum</i>	IHEM 00935	-	
	<i>Trichoderma</i>	<i>reesei</i>	IHEM 05651	-	
	<i>Trichoderma</i>	<i>viride</i>	IHEM 04146	-	
	Bacteria	<i>Arthrobacter</i>	<i>ramosus</i>	LMG 17309	-
		<i>Bacillus</i>	<i>amyloliquefaciens</i>	LMG 98140	+ ( $C_q$ : 16.6)
		<i>Bacillus</i>	<i>amyloliquefaciens</i>	LMG12325	+ ( $C_q$ : 18.6)
<i>Bacillus</i>		<i>amyloliquefaciens</i>	LMG12329	+ ( $C_q$ : 16.7)	
<i>Bacillus</i>		<i>amyloliquefaciens</i>	LMG12331	+ ( $C_q$ : 19.9)	
<i>Bacillus</i>		<i>amyloliquefaciens</i>	LMG12326	+ ( $C_q$ : 19.8)	
<i>Bacillus</i>		<i>amyloliquefaciens</i>	LMG12327	+ ( $C_q$ : 19.1)	
<i>Bacillus</i>		<i>brevis</i>	LMG 7123	-	
<i>Bacillus</i>		<i>cereus</i>	ATCC 14579	-	
<i>Bacillus</i>		<i>circulans</i>	LMG 6926T	-	
<i>Bacillus</i>		<i>coagulans</i>	LMG 6326	-	
<i>Bacillus</i>		<i>firmus</i>	LMG 7125	-	
<i>Bacillus</i>		<i>flexus</i>	LMG 11155	-	
<i>Bacillus</i>		<i>lentus</i>	TIAC 101	-	

Table 2. Cont.

Kingdom	Genus	Species	Strain Number	BSG Method
	<i>Bacillus</i>	<i>licheniformis</i>	LMG 6933T	+ (C <sub>q</sub> : 17.9)
	<i>Bacillus</i>	<i>licheniformis</i>	LMG6934	+ (C <sub>q</sub> : 19.0)
	<i>Bacillus</i>	<i>licheniformis</i>	LMG7558	+ (C <sub>q</sub> : 18.2)
	<i>Bacillus</i>	<i>licheniformis</i>	LMG7634	+ (C <sub>q</sub> : 17.9)
	<i>Bacillus</i>	<i>licheniformis</i>	LMG7631	+ (C <sub>q</sub> : 18.3)
	<i>Bacillus</i>	<i>megaterium</i>	LMG 7127	-
	<i>Bacillus</i>	<i>pumilus</i>	DSMZ 1794	+ (C <sub>q</sub> : 16.5)
	<i>Bacillus</i>	<i>smithii</i>	LMG 6327	-
	<i>Bacillus</i>	<i>subtilis</i>	LMG 7135 T	+ (C <sub>q</sub> : 19.0)
	<i>Bacillus</i>	<i>subtilis</i>	W04-510	+ (C <sub>q</sub> : 22.4)
	<i>Bacillus</i>	<i>subtilis</i>	E07-505	+ (C <sub>q</sub> : 20.6)
	<i>Bacillus</i>	<i>subtilis</i>	S10005	+ (C <sub>q</sub> : 21.0)
	<i>Bacillus</i>	<i>subtilis</i>	SUB033	+ (C <sub>q</sub> : 21.3)
	<i>Bacillus</i>	<i>subtilis</i>	BNB54	+ (C <sub>q</sub> : 29.2)
	<i>Bacillus</i>	<i>subtilis</i>	GMM RASFF2014.1249	+ (C <sub>q</sub> : 19.7)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 12384	+ (C <sub>q</sub> : 20.0)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 17599	+ (C <sub>q</sub> : 16.4)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 22478	+ (C <sub>q</sub> : 16.1)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 23203	+ (C <sub>q</sub> : 16.4)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 26770	+ (C <sub>q</sub> : 16.3)
	<i>Bacillus</i>	<i>velezensis</i>	LMG 27586	+ (C <sub>q</sub> : 16.4)
	<i>Bacillus</i>	<i>velezensis</i>	GMM RASFF2019.3332	+ (C <sub>q</sub> : 17.8)
	<i>Cellulosimicrobium</i>	<i>cellulans</i>	LMG 16121	-
	<i>Corynebacterium</i>	<i>glutamicum</i>	LMG 3652	-
	<i>Enterococcus</i>	<i>faecium</i>	LMG 9430	-
	<i>Escherichia</i>	<i>coli</i>	LMG2092T	-
	<i>Geobacillus</i>	<i>caldoproteolyticus</i>	DSM 15730	-
	<i>Geobacillus</i>	<i>pallidus</i>	LMG 11159T	-
	<i>Geobacillus</i>	<i>stearothermophilus</i>	LMG 6939T	-
	<i>Klebsiella</i>	<i>pneumonia</i>	LMG 3113T	-
	<i>Lactobacillus</i>	<i>casei</i>	LMG 6904	-
	<i>Lactobacillus</i>	<i>fermentum</i>	LMG 6902	-
	<i>Lactobacillus</i>	<i>plantarum</i>	LMG 9208	-
	<i>Lactobacillus</i>	<i>rhamnosus</i>	LMG 18030	-
	<i>Lactococcus</i>	<i>lactis</i>	LMG 6890T	-
	<i>Leuconostoc</i>	<i>citreum</i>	LMG 9824	-
	<i>Microbacterium</i>	<i>imperiale</i>	LMG 20190	-
	<i>Paenibacillus</i>	<i>alginolyticus</i>	LMG 18723	-
	<i>Paenibacillus</i>	<i>macerans</i>	LMG 6324	-
	<i>Protaminobacter</i>	<i>rubrum</i>	CBS 574.77	-
	<i>Pseudomonas</i>	<i>amyloclavata</i>	ATCC-21262	-
	<i>Pseudomonas</i>	<i>fluorescens</i>	LMG1794T	-
	<i>Pullulanibacillus</i>	<i>naganoensis</i>	LMG 12887	-
	<i>Streptomyces</i>	<i>aureofaciens</i>	LMG 5968	-
	<i>Streptomyces</i>	<i>mobarraensis</i>	DSM 40847	-
	<i>Streptomyces</i>	<i>murinus</i>	LMG 10475	-
	<i>Streptomyces</i>	<i>netropsis</i>	LMG 5977	-
	<i>Streptomyces</i>	<i>rubiginosus</i>	LMG20268	-
	<i>Streptomyces</i>	<i>violaceoruber</i>	LMG 7183	-
	<i>Streptoverticillium</i>	<i>mobarraense</i>	CBS 199.75	-
<b>Plantae</b>	<i>Zea</i>	<i>mays</i>	/	-
<b>Animalia</b>	<i>Homo</i>	<i>sapiens</i>	/	-

**Table 3.** Sensitivity assessment of the developed real-time PCR BSG method using a control plasmid carrying one copy of the targeted sequence from *B. subtilis*. The presence and absence of amplification are symbolized by “+” and “–”, respectively. For each target copy number, 12 replicates were tested. The number of positive replicates out of the 12 replicates tested is indicated and the means of the observed C<sub>q</sub> are indicated in brackets.

	Estimated Target Copy Number						
	100	20	10	5	1	0.1	0
<b>BSG method</b>	+	+	+	+	+	–	–
	(12/12)	(12/12)	(8/12)	(6/12)	(1/12)	(0/12)	(0/12)
	(C <sub>q</sub> : 38.2)	(C <sub>q</sub> : 41.1)	(C <sub>q</sub> : 42.0)	(C <sub>q</sub> : 42.6)	(C <sub>q</sub> : 43.5)		

**Table 4.** Applicability assessment of the developed real-time PCR BSG method using commercialized microbial fermentation products. The BSG method was applied on 25 samples. For each sample, labelled information related to enzyme/additive microbial production sources, forms and application fields is indicated. Each sample was tested at a concentration of 10 ng in duplicate. The presence or absence of PCR amplification is symbolized by “+” or “–”, respectively. The means of the observed C<sub>q</sub> are indicated in brackets. Available information related to previously reported contaminations (GMM, Bacterial DNA) is also indicated for each sample. \* Successful isolation of the GMM strain previously reported.

Samples	Sources	Forms	Application	BSG Method	Available Information	References	
1	Feed additive vitamin B2	Unknown	Solid	Livestock farming	+	GM <i>B. subtilis</i> producing vitamin B2 *	RASFF2014.1249, [3,14]
2	Feed additive vitamin B2	Unknown	Solid	Livestock farming	+	GM <i>B. subtilis</i> producing vitamin B2	RASFF2018.2755, [9,12,14]
3	Food enzyme neutral protease	<i>Bacillus</i> sp.	Solid	Baking, distillery, brewing	+	GM <i>B. velezensis</i> producing protease *	RASFF2019.3332, [4,8,14]
4	Food enzyme alpha-amylase	<i>Bacillus</i> sp.	Solid	Distillery, brewing	+	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	RASFF2019.3332, [4,10,14]
5	Food enzyme alpha-amylase	<i>Bacillus</i> sp.	Liquid	Distillery, brewing	+	GM <i>B. velezensis</i> producing protease	RASFF2020.2576, [4,10,14]
6	Food enzyme alpha-amylase, protease, cellulose, xylanase, beta-glucanase	<i>Aspergillus</i> sp., <i>Bacillus</i> sp., <i>Trichoderma</i> sp.	Solid	Distillery, brewing	+	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	RASFF2019.3332, [4,10,14]
7	Food enzyme alpha-amylase	<i>Aspergillus</i> sp.	Solid	Distillery, brewing, baking	+	GM <i>B. velezensis</i> producing protease	RASFF2019.3332, [4,10,14]
8	Food enzyme alpha-amylase	Unknown	Liquid	Unknown	+	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	RASFF2019.3332, [4,10,14]
9	Food enzyme alpha-amylase	Unknown	Liquid	Distillery	+	GM <i>B. velezensis</i> producing protease	RASFF2020.2870, [4,10,14]
						GM <i>B. amyloliquefaciens</i> producing alpha-amylase	

**Table 4.** Cont.

Samples	Sources	Forms	Application	BSG Method	Available Information	References	
10	Food enzyme alpha-amylase	Bacteria	Liquid	Distillery, brewing	+ (C <sub>q</sub> : 19.8)	GM <i>B. velezensis</i> producing protease GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease	RASFF2020.2846, [14]
11	Food enzyme alpha-amylase	Bacteria	Solid	Distillery, brewing	+ (C <sub>q</sub> : 22.6)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease	RASFF2020.2579, [14]
12	Food enzyme alpha-amylase	Unknown	Solid	Distillery	+ (C <sub>q</sub> : 19.4)	GM <i>B. amyloliquefaciens</i> producing protease GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease*	RASFF2020.2577, [14]
13	Food enzyme alpha-amylase	Unknown	Solid	Distillery	+ (C <sub>q</sub> : 19.5)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease*	RASFF2020.2582, [14]
14	Food enzyme alpha-amylase	Unknown	Solid	Distillery, brewing	+ (C <sub>q</sub> : 31.2)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease*	RASFF2020.2582, [14]
15	Food enzyme alpha-amylase	Unknown	Solid	Distillery, brewing	+ (C <sub>q</sub> : 31.4)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease	RASFF2020.2570, [14]
16	Food enzyme alpha-amylase	Unknown	Solid	Distillery, brewing	+ (C <sub>q</sub> : 36.9)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase GM <i>B. velezensis</i> producing protease	RASFF2020.2572, [14]
17	Food enzyme alpha-amylase	<i>Bacillus</i> sp.	Liquid	Distillery, brewing	+ (C <sub>q</sub> : 25.1)	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	[4]
18	Food enzyme protease	<i>Bacillus</i> sp.	Solid	Baking	+ (C <sub>q</sub> : 43.8)	GM <i>B. amyloliquefaciens</i> producing protease	RASFF2021.1641, [11,14]
19	Feed additive vitamin B2	Unknown	Solid	Livestock farming	+ (C <sub>q</sub> : 38.0)	No known GMM Bacterial DNA	[4,12]
20	Food enzyme transglutaminase	Unknown	Solid	Protein processing	—	No known GMM Bacterial DNA	[4]
21	Food enzyme transglutaminase	Unknown	Solid	Protein processing	—	No known GMM Bacterial DNA	[4]
22	Food enzyme unknown	Unknown	Solid	Baking	—	No known GMM Bacterial DNA	[4]
23	Food enzyme beta-glucosidase	<i>Aspergillus niger</i>	Solid	Distillery, fruit processing	—	No known GMM No bacterial DNA	[4]
24	Food enzyme alpha-amylase	Unknown	Liquid	Distillery, brewing	—	No known GMM No bacterial DNA	[4]
25	Food enzyme rennet	<i>Rhizomucor miehei</i>	Solid	Diary processing	—	No known GMM No bacterial DNA	[4]

The targeted 16S-23S region from all of these species was aligned using the Clustal Omega software with the default parameters as well as a set of primers and a probe that was designed using the software Primer3. This taxon-specific real-time-PCR method, named BSG, was developed in order to be integrated into the first-line screening step of the current GMM detection strategy. Therefore, each real-time PCR assay was applied on a 25 µL reaction volume containing 1X TaqMan® PCR Mastermix (Diagenode, Liège, Belgium), 400 nM of each primer (Eurogentec, Liège, Belgium), 200 nM of the probe and 5 µL of DNA. The real-time PCR program consisted of a single cycle of DNA polymerase activation

for 10 min at 95 °C followed by 45 amplification cycles of 15 sec at 95 °C (the denaturing step) and 1 min at 64 °C (the annealing–extension step). All of the runs were performed on a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A no template control (NTC) was included in each assay.

### 2.2.1. Specificity Assessment

First, an *in silico* analysis of the developed BSG method was performed using SCREENED v1.0 [34,35]. The parameter settings were maximum 10% of mismatches in the annealing site, minimum 90% of alignment length in the annealing site and no mismatch in the last five nucleotides at the 3' end for the primers. This tool uses a two-step BLAST approach. First, the sequence(s) that were targeted by the developed BSG method were extracted from a dataset belonging to the NCBI Nucleotide RefSeq database (accessed on March 2021; filter: genome AND refseq AND complete). Subsequently, the hybridization properties of the targeted regions and the developed set of primers and probe were investigated. As the input for the BSG method, the sequences from the designed set of primers and probe and the expected amplicons that were generated from the targeted *Bacillus* species were used (Table 1 and Supplementary Tables S1 and S2).

Second, the developed BSG method was tested in triplicate on 10 ng of DNA from positive and negative controls (Table 2). For the positive controls, 24 wild-type strains belonging to *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. velezensis* as well as 2 GM strains corresponding to *B. subtilis* (RASFF2014.1249) and *B. velezensis* (RASFF2019.3332) were used. For the negative controls, 75 wild-type microbial strains belonging to fungal and bacterial species that are frequently used by the food and feed industry in order to produce microbial fermentation products were used. In addition, DNA from an animal (*Homo sapiens*) and a plant (*Zea mays*) was included.

### 2.2.2. Sensitivity Assessment

Serial dilutions of the DNA from the control plasmid carrying one copy of the targeted sequence from *B. subtilis*, going from 100 to 0.1 estimated target copy number, were prepared and each dilution point was tested in 12 replicates (Table 3).

The calculation of the estimated target copy number was based on the control plasmid size (2957 bp), as previously described [5–11]. The limit of detection LOD<sub>95%</sub>, defined as the number of copies of the target that were required to ensure a 95% probability of detection (POD), was determined by using the QuoData web application (Table S3). The plausibility check of the POD curve indicated no irregularities and that the POD curve was associated to a LOD<sub>95%</sub> below 25 estimated target copy numbers [24,36,37].

### 2.2.3. Applicability Assessment

Several commercial microbial fermentation products, including enzymes and additives, were used in order to evaluate the applicability of the developed BSG method (Table 4).

From each sample (n°1–25), 10 ng of the DNA was tested in duplicate (Table 4). These products (samples n°1–25) from different brands were labelled as containing vitamin B2, protease, alpha-amylase, cellulase, xylanase, beta-glucanase, transglutaminase, beta-glucosidase or rennet. These products, in liquid or solid forms, are intended for use in various sectors such as livestock farming, brewing, distillery, baking, dairy processing, protein processing or fruit processing. The selection of these samples was performed based on the available information that was related to previously reported contaminations, allowing the present study to cover three possible scenarios (Table 4). First, as was observed with the samples n°1–18, the sample is contaminated by a known GMM, including the vitamin B2-producing GM *B. subtilis* strain, protease-producing GM *B. velezensis* strain, alpha-amylase-producing GM *B. amyloliquefaciens* strain and protease-producing GM *B. amyloliquefaciens* strain. Second, the sample is contaminated with bacterial DNA that is not related to the currently known GM bacterial strains. Based on previously obtained results,

the samples n°19–22 were selected for this scenario as presenting a positive signal for the 16S-rRNA marker and a negative signal for the second-line methods targeting the currently known GM strains. Third, the sample is not contaminated with bacterial DNA, as was observed for the samples n°23–25 for which no positive signal for the 16S-rRNA marker was previously observed.

2.3. Illustration of the Reinforcement of the First-Line Screening Analysis Step Using the BSG Method

Few commercial microbial fermentation products (samples n°2–4, 6, 19–20, 22, 25) that are described in Table 4 were used in order to illustrate the reinforcement of the GMM detection strategy by completing the set of first-line screening methods with the BSG method (Table 5).

**Table 5. Illustration of the reinforcement of the first-line screening analysis step using the BSG method.** The full set of first-line screening methods (BSG, cat, tet-I, aadD and pUB110 methods) was applied on commercialized microbial fermentation products described in Table 4. Each sample was tested at a concentration of 10 ng in duplicate. The presence or absence of PCR amplification is symbolized by “+” or “–”, respectively. The results for the BSG method were generated in this study while the results for the other methods were previously generated.

Samples	First-Line Screening Methods					Reported GMM Contaminations	References
	BSG	cat	tet-I	aadD	pUB110		
2	+	+	–	+	–	GM <i>B. subtilis</i> producing vitamin B2	RASFF2018.2755, [9,12,14]
3	+	–	–	+	+	GM <i>B. velezensis</i> producing protease	RASFF2019.3332, [4,8,14]
4	+	–	–	+	+	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	RASFF2019.3332, [4,10,14]
6	+	–	+	+	+	GM <i>B. velezensis</i> producing protease	RASFF2019.3332, [4,10,14]
19	+	–	–	–	–	GM <i>B. amyloliquefaciens</i> producing alpha-amylase	[4,12]
20	–	–	–	+	–	/	[4]
22	–	–	–	–	–	/	[4]
25	–	–	–	–	–	/	[4]

The results for the BSG method that were generated in this study are as described in the Section 2.2, while the results for the cat, tet-I, aadD and pUB110 methods were previously generated [4,8–10,12].

3. Results and Discussion

3.1. Development of the BSG Method

Among the 37 GM bacterial strains that have been reported as being used by the food and feed industry to produce enzymes, additives and flavoring, 75.6% of them belong to the *B. licheniformis* and *B. subtilis* species [6,31]. These two bacterial species belong to the *B. subtilis* group, which is composed in total of 10 closely related *Bacillus* species, including *B. amyloliquefaciens*, *B. pumilus* and *B. velezensis* (which are also used to produce microbial fermentation products), as well as *B. atrophaeus*, *B. mojavensis*, *B. sonorensis*, *B. tequilensis* and *B. vallismortis*. These *Bacillus* species present, at minimum, 99% similarity at the 16S rRNA sequence level as well as very few or no phenotypic and biochemical distinctions [28,32,33]. To date, all of the GMM contaminations that have been observed on the food and feed market have concerned bacterial species belonging to the *B. subtilis* group, being a vitamin B2-producing GM *B. subtilis* strain, a protease-producing GM *B. velezensis* strain, an alpha-amylase-producing GM *B. amyloliquefaciens* strain and a protease-producing GM

*B. amyloliquefaciens* strain [2,3,8–11,13,38]. The presence of such bacterial species represents, thus, a strong indicator of the potential GMM contaminations in a given sample. Therefore, a real-time PCR method targeting bacterial species from the *B. subtilis* group was developed and designed in this study to be compatible with the first-line screening condition of the GMM detection strategy, allowing the strengthening of the latter.

To this end, this taxon-specific real-time PCR method, named BSG, was designed in this study on a part of the 16S-23S region (Tables 1 and S1). This sequence of interest was previously identified [28,30] as being specific to the *B. subtilis* group, including the bacterial species that are commonly used to produce microbial fermentation products (*B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens*, *B. pumilus* and *B. velezensis*) [4–7,25]. The 16S-23S region belongs to the bacterial ribosomal RNA operon, playing a key role in protein synthesis. The number of copies per genome of this ribosomal RNA operon varies between and within bacterial species, ranging from 6 to 15 for *Bacillus* sp. The developed BSG method, generating PCR amplicons with a size range from 137 to 165 bp, is the first real-time PCR method that was designed to specifically target the *B. subtilis* group in order to control the GMM contaminations in microbial fermentation products [28,30,32,33,39,40] (Tables 1 and S1).

### 3.2. Specificity Assessment of the BSG Method

The specificity of the developed BSG method was first confirmed in silico using SCREENED on a dataset that was composed of animal (75548 items), archaea (54464 items), bacteria (11507253 items), fungi (86250 items), plant (46015 items), protist (89957 items) and virus (10108 items) sequences that were extracted from the NCBI RefSeq Nucleotide database, allowing the present study to theoretically determine the generation of a PCR amplification (Tables S1 and S2) [34,35]. On this basis, a PCR amplification was only predicted for 167 bacterial sequences from the *Bacillus* genus. Among these 167 sequences, 15 belonged to unclassified *Bacillus* species and 152 belonged to the *B. subtilis* group, including *B. amyloliquefaciens* (14 sequences), *B. licheniformis* (11 sequences), *B. mojavensis* group (4 sequences), *B. pumilus* group (29 sequences) *B. subtilis* (42 sequences), *B. vallismortis* (1 sequence) and *B. velezensis* (51 sequences). No PCR amplification was predicted for non-targeted sequences, including *Bacillus* species not belonging to the *B. subtilis* group (for example, species of the *B. brevis*, *B. cereus*, *B. circulans* and *B. megaterium* groups) (Tables S1 and S2).

Second, the specificity of the BSG method was experimentally assessed. As positive controls, 26 bacterial strains from 5 *Bacillus* species belonging to the *B. subtilis* group (*B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. subtilis* and *B. velezensis*) were used. Among these 26 bacterial strains, 2 GMM strains were included, namely a vitamin B2-producing GM *B. subtilis* strain (RASFF2014.1249) and a protease-producing GM *B. velezensis* strain (RASFF2019.3332). As negative controls, 29 bacterial species from 17 non-*Bacillus* genera (*Arthrobacter*, *Cellulosimicrobium*, *Corynebacterium*, *Enterococcus*, *Escherichia*, *Geobacillus*, *Klebsiella*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Microbacterium*, *Paenibacillus*, *Protaminobacter*, *Pseudomonas*, *Pullulanibacillus*, *Streptomyces* and *Streptoverticillium*), and 9 *Bacillus* species not belonging to the *B. subtilis* group (*B. brevis*, *B. cereus*, *B. circulans*, *B. coagulans*, *B. firmus*, *B. flexus*, *B. lentus*, *B. megaterium* and *B. smithii*) were tested. In addition to the bacterial strains, 37 fungal species as well as plant and animal samples were used as negative controls (Table 2). As expected, no amplification was observed for all of the negative controls while all of the positive controls presented a positive signal. Although all of the positive controls were tested at the same DNA concentration, a variation in their  $C_q$  values was observed. This could be explained by the natural copy number variation of the 16S–23S region that was targeted by the developed BSG method [28,32,33].

Based on these results, the developed BSG method was assessed as specific because, firstly, a positive signal was only observed in the samples that were presenting the targeted sequences and, secondly, no false positive and negative signals were observed. This method currently represents the first taxon-specific real-time PCR method targeting GMM

contaminations in microbial fermentation products for which a large spectrum of targeted and non-targeted sequences that are related to microorganisms that are used to produce fermentation products were investigated.

### 3.3. Sensitivity Assessment of the BSG Method

In order to assess the sensitivity of the developed BSG method, DNA from a control plasmid that was artificially synthesized to carry one copy of the targeted sequence from *B. subtilis* was used to prepare the serial dilution points, from 100 to 0.1 estimated target copy number (Tables 3 and S3).

A positive signal was observed as low as 20 estimated target copies for all 12 of the replicates. Moreover, a positive signal was detected as low as 1 estimated target copy. Based on these results, the LOD<sub>95%</sub> was subsequently determined at 22 estimated target copies, indicating that the developed BSG method is sensitive. This taxon-specific method represents the first real-time PCR method that is designed to specifically target the *B. subtilis* group with performance complying with the *Minimum Performance Requirements for Analytical Methods of GMO Testing* of the European Network of GMO Laboratories, being used as a standard in GMO enforcement laboratories, allowing to support enforcement laboratories for the control of GMM contaminations in microbial fermentation products [24,28,30,39,40].

### 3.4. Applicability Assessment of the BSG Method

The applicability of the developed BSG method was tested using several commercial microbial fermentation products that are intended for use in various sectors, such as livestock farming, baking, distillery, brewing, dairy processing, protein processing or fruit processing (Table 4).

Among the 25 investigated samples, 18 of them were previously notified for a contamination with the vitamin B2-producing GM *B. subtilis* strain (samples n°1–2), the protease-producing GM *B. velezensis* strain (samples n°3–16), the alpha-amylase-producing GM *B. amyloliquefaciens* strain (samples n°3–17) or the protease-producing GM *B. amyloliquefaciens* strain (sample n°18). As expected, all of these samples presented a positive signal for the BSG method, highlighting the possibility of using this taxon-specific method as an indicator of potential GMM contaminations (Table 4).

Regarding the 7 remaining samples (n°19–25), no GMM contamination was previously reported based on the negative signals for the second-line methods that were targeting the currently known GM strains (Table 4). Among these 7 samples, a contamination with bacterial DNA was previously observed for the samples n°19–22 (a positive signal for the 16S-rRNA marker) and discarded for the samples n°23–25 (a negative signal for the 16S-rRNA marker) (Table 4). Using the BSG method, a positive signal was detected for the sample n°19 while the samples n°20–25 presented a negative signal (Table 4). On this basis, the previously reported bacterial DNA contamination was associated to bacterial species that are related to the *B. subtilis* group, as in the case of the sample n°19, or not, as in the case of the samples n°20–22. In addition, given that no bacterial DNA contamination was previously observed in the samples n°23–25, the negative signal for the BSG method was expected. According to all of these results, the applicability of the developed BSG method was confirmed.

### 3.5. Illustration of the Reinforcement of the First-Line Screening Analysis Step Using the BSG Method

The BSG method was developed in order to strengthen the GMM detection strategy by completing the set of first-line screening methods targeting the key sequences that are frequently found in GM bacteria. To illustrate this reinforcement, the presence or absence of PCR amplification that was observed for the full set of first-line screening real-time PCR methods (BSG, cat, tet-L, aadD, pUB110) were indicated for few commercial microbial fermentation products (samples n°2–4, 6, 19–20, 22, 25) (Table 5).

For the samples n°2–4 and 6, positive signals for the cat, tet-L, aadD and/or pUB110 methods as well as for the BSG method were observed, indicating potential contaminations with GM bacteria belonging to *B. subtilis* group. This hypothesis was confirmed by the previous detection of the vitamin B2-producing GM *B. subtilis* strain (sample n°2), the protease-producing GM *B. velezensis* strain (samples n°3–4, 6) and the alpha-amylase-producing GM *B. amyloliquefaciens* (samples n°3–4, 6) (Table 5). For the sample n°19, a negative signal was obtained for all of the methods that were targeting key genetic elements that are found in GM bacteria while the BSG method presented a positive signal, suggesting a contamination with bacteria from the *B. subtilis* group (Table 5). Regarding the sample n°20, the positive signal for the aadD method associated to the negative signal for the BSG method suggests a potential contamination that is not related to bacteria belonging to the *B. subtilis* group, including bacteria naturally carrying the detected AMR gene (e.g., *Staphylococcus* sp.) or a new unknown GMM (Table 5). Finally, for the samples n°22 and 25, only negative signals were observed for all of the screening markers, discarding contamination with bacteria from *B. subtilis* group as well as bacteria naturally carrying the targeted AMR genes and shuttle vectors (e.g., *Enterococcus* sp., *Staphylococcus* sp. or *Streptococcus* sp.) (Table 5) [5–9]. Based on all of these results, the added value of the developed BSG method to strengthen the first-line screening analysis step was thus highlighted, allowing the indication of potential contaminations with bacteria from the *B. subtilis* group, GM or not.

The BSG method was developed in order to be used as an indicator of the potential presence of GMM contaminations. Similar to its application to GM plants, this taxon-specific BSG method can also be applied to specifically target GM *Bacillus* strains, supporting the identification of such GMM. However, as the targeted 16S-23S region is naturally present in several copies per bacterial genome, ranging from 6 to 15 copies for *Bacillus* species, another target will need to be selected in case a taxon-specific method is required in the future for the quantification of GMM [24,28,30,39,40]. In addition, given the high copy number of the targeted 16S-23S region that is naturally present in bacteria, potential issues for the interpretation of the results may be encountered in the case of GMM contaminations at trace level (e.g., a late positive signal for the BSG method and a negative signal for the method specifically targeting a GM *Bacillus* strain). It should also be mentioned that the sequences that are targeted by the other screening methods (cat, tet-L, aadD and pUB110) may also be present in a GMM at a high copy number. Indeed, these sequences were previously found as being carried by plasmids or introduced multiple times in the host genome [2–13,38]. Consequently, in case of late signals from screening methods, the need for further investigations will depend on the nature of the contaminated products as well as the final decision of the competent authorities.

#### 4. Conclusions

In order to control GMO, including GMM, in the food and feed chain, an efficient screening tool covering a large spectrum of key targets is essential to allow the identification of samples with potential GMO contaminations. To this end, a combination of taxon-specific and genetic element-specific methods are needed.

Regarding the control of GM bacteria, only screening methods that were targeting key genetic elements that are commonly found in GMM were only available and no taxon-specific method, designed to be compatible with the technical requirements of the GMM detection strategy as well as harboring performance in line with the *Minimum Performance Requirements for Analytical Methods of GMO Testing* of the European Network of GMO Laboratories used as a standard in GMO enforcement laboratories, was available to target GMM contaminations.

In order to meet this need, the BSG method was therefore developed and validated in this sense in the present study. This taxon-specific real-time PCR method was designed to target bacterial species belonging to the *B. subtilis* group, covering most of the GM bacterial species that are used by the food industry to produce microbial fermentation

products [3,6,8–11,13,24,31]. The performance of the BSG method, successfully assessed as specific and sensitive, complies with the requirements of the GMO enforcement laboratories. In addition, the BSG method also represents an interesting tool for screening viable *B. subtilis* group strains isolable from the food–feed matrix.

By completing the set of screening methods of the first-line screening analysis step, the BSG method strengthens the GMM detection strategy that is used to control the GMM contaminations in the food and feed chain. Indeed, the sequences that are targeted by the cat, tet-L, aadD and pUB110 methods are usually not naturally carried by the bacterial species from the *B. subtilis* group [5–9]. Therefore, when combined with a positive signal for at least one of the cat, tet-L, aadD and pUB110 methods, the detection of a positive signal for the BSG method is considered as a strong indicator of potential GM bacterial contaminations, allowing the identification of suspicious samples for further analysis.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/fermentation8020078/s1>, Table S1: In silico analysis for the specificity of the BSG method using SCREENED, Table S2: Misclassification of NZ\_CLNG01000048.1, Table S3: Calculation of LOD<sub>95%</sub> based on the POD curve for the developed real-time PCR BSG method.

**Author Contributions:** Conceptualization, M.-A.F. and N.H.C.R.; methodology, M.-A.F. and N.H.C.R.; formal analysis, M.-A.F., A.G., N.P. and N.H.C.R.; writing—original draft preparation, M.-A.F.; writing—review and editing, M.-A.F., A.G., N.P. and N.H.C.R. All authors have read and agreed to the published version of the manuscript.

**Funding:** The research that yielded these results was both funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract [RT 17/5 SPECENZYM] and the Transversal activities in Applied Genomics (TAG) Service from Sciensano.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** Not applicable.

**Acknowledgments:** The research that yielded these results was both funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract [RT 17/5 SPECENZYM] and the Transversal activities in Applied Genomics (TAG) Service from Sciensano. The Sanger sequencing was performed by TAG. The authors would like also to thank Kevin Vanneste for his support in the bioinformatics analysis.

**Conflicts of Interest:** The authors declare no conflict of interest.

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