Food Chemistry 305 (2020) 125431

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Strategy for the identification of micro-organisms producing food and feed products: Bacteria producing food enzymes as study case

Marie Deckers[a], Kevin Vanneste[a], Raf Winand[a], Sigrid C.J. De Keersmaecker[a], Sarah Denayer[b], Marc Heyndrickx[c,d], Dieter Deforce[e], Marie-Alice Fraiture[a,1], Nancy H.C. Roosens[a,*,1]

[a] Transversal activities in Applied Genomics (TAG), Sciensano, Brussels, Belgium
[b] Foodborne pathogens, Sciensano, Brussels, Belgium
[c] Technology and Food Sciences Unit, ILVO, Melle, Belgium
[d] Department of Pathology, Bacteriology and Avian Diseases, Ghent University, Merelbeke, Belgium
[e] Laboratory of Pharmaceutical Biotechnology, Ghent University, Ghent, Belgium

ARTICLE INFO

Keywords:
Screening
Identification
PCR technology
16S-rRNA gene sequencing
Food enzymes
Producing organisms
Bacteria

ABSTRACT

Recent European regulations require safety assessments of food enzymes (FE) before their commercialization. FE are mainly produced by micro-organisms, whose viable strains nor associated DNA can be present in the final products. Currently, no strategy targeting such impurities exists in enforcement laboratories. Therefore, a generic strategy of first line screening was developed to detect and identify, through PCR amplification and sequencing of the 16S-rRNA gene, the potential presence of FE producing bacteria in FE preparations. First, the specificity was verified using all microbial species reported to produce FE. Second, an in-house database, with 16S reference sequences from bacteria producing FE, was constructed for their fast identification through blast analysis. Third, the sensitivity was assessed on a spiked FE preparation. Finally, the applicability was verified using commercial FE preparations. Using straightforward PCR amplifications, Sanger sequencing and blast analysis, the proposed strategy was demonstrated to be convenient for implementation in enforcement laboratories.

1. Introduction

In the food and feed industry, microbial strains are increasingly being used to produce additives, flavourings and enzymes. Among these microbial strains, most of them are often genetically modified (Raveendran et al., 2018; Singh, Kumar, Mittal, & Kumar, 2016). In the particular case of food enzymes (FE), products are placed on the market under the form of FE preparations, containing a blend of a single or multiple FE's combined with additional substances (i.e., additives, diluents, preservatives and stabilizers) for their stabilization and conservation (European Parliament and Council of the European Union, 2008; Pariza & Johnson, 2001; West-Barnette & Srinivasan, 2013). Since 1971, FE are evaluated worldwide on a voluntary basis by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) (JEFCA, 2006; Spök, 2006). FE are mostly regulated as food additives or as processing aids, e.g. in the United States of America, Australia and Canada (Magnuson et al., 2013). Before 2008, regulations on FE preparations existed only at the national level in a few member states within the European Union (EU), namely in France (Cerutti, Boudot, Bournigal, & Rousseau, 2006) and in Denmark (Regulation (EU) 2015/2283). In light of the EU decision to harmonize regulations related to the commercialization of FE preparations, the EC/1331/2008, EC/1332/2008 and EC/1333/2008 regulations were delivered in 2008 (European Parliament and Council, 2008; European Parliament and Council of the European Union, 2008; European Parliament and the Council of the European Union, 2008). The first regulation, EC 1331/2088, establishing a common authorization procedure for food additives, FE and food flavourings. The second regulation, EC 1332/2008, harmonizes the rules on enzymes used in food in the EU and requires the submission of applications for authorization. The last regulation, EC 1333/2008, harmonizes the use of food additives. Additionally, the FE's Invertase and Lysozyme, also used as food additive, were already

* Corresponding author.
E-mail addresses: marie.deckers@sciensano.be (M. Deckers), kevin.vanneste@sciensano.be (K. Vanneste), raf.winand@sciensano.be (R. Winand), sigrid.dekeersemaecker@sciensano.be (S.C.J.D. Keersmaecker), sarah.denayer@sciensano.be (S. Denayer), marc.heyndrickx@ilvo.vlaanderen.be (M. Heyndrickx), dieter.deforce@ugent.be (D. Deforce), marie-alice.fraiture@sciensano.be (M.-A. Fraiture), nancy.roosens@sciensano.be (N.H.C. Roosens).
[1] Equal contribution

https://doi.org/10.1016/j.foodchem.2019.125431
Received 26 February 2019; Received in revised form 30 July 2019; Accepted 27 August 2019
Available online 04 September 2019

submitted to the commission regulation (EU) No 231/2012 (*COMMISSION REGULATION (EU) No 231/2012*, 2012). Following the regulation EC/1332/2008, 304 FE dossiers have been submitted between 2011 and 2015 to the EU Commission for their safety evaluation by the European Food Safety Authority (EFSA) (Anadón et al., 2009; European Commission, 2016). Although a list of authorized FE for the EU market will be elaborated, only national regulations are followed in the meantime.

Currently, the quality control of commercialized FE preparations is, to our knowledge, under the responsibility of the FE manufacturers themselves in the EU as well as in the rest of the world (Spök, 2006; West-Barnette & Srinivasan, 2013). It is therefore assumed that the company that releases the final product on the market, has verified the criteria requirements regarding its purity. However, accidental contaminations still occur. In particular in the EU food and feed markets, the presence of such contaminants has already been reported in commercialized fermentation products. For instance, in 2013, a high level of chloramphenicol, an antibiotic for which a zero tolerance is applied in the EU, was first detected in xylanase and afterwards also in other FE preparations (RASFF 2013.1017) (RASFF portal; Standing Committee on the Food Chain and Animal Health, 2005). Similarly, in 2014, a microbiological contamination was demonstrated by the presence of a genetically modified *Bacillus subtilis* strain, used to produce vitamin B2, in a vitamin B2 feed additive powder imported from China (Barbaupiednoir, De Keersmaecker, Delvoye, et al., 2015; Paracchini et al., 2017) (RASFF 2014.1219, RASFF 2018.2755) (RASFF portal). These findings support the potential presence of contaminants in microbial fermented food and feed products, such as FE, despite the purity verifications by the producing companies. They also emphasize the need for appropriate detection methods targeting impurities in FE preparations at the enforcement laboratories level. However, to the best of our knowledge, no strategy for the detection of such potential contaminants in FE preparations currently exists in the enforcement laboratories worldwide, including in the EU.

Among the potential impurities, the presence of producing organisms can be considered as crucial in enzymes, flavourings and additives. For feed additives, the guidance following regulation (EC) No 1831/2003 clearly states that the production strain, including both viable strains and/or their corresponding DNA, must be absent in the final feed additive product (Rychen et al., 2018). For food enzymes, the absence of the production strain or its DNA must be proven (Commission of the European Communities, 1991). Clarifications regarding the characterization of FE producing microorganisms have been provided in an EFSA statement (Pariza & Johnson, 2001). Regarding public health concerns, the presence of producer micro-organisms is particularly critic if it concerns a genetically modified microbial strain. This because they are frequently harboring antimicrobial resistance genes as selection markers, that could be acquired via horizontal transfer by pathogens and gut microbiota (Rozwandowicz et al., 2018; Xiong et al., 2018). Therefore, the identification of the potential presence of FE producing micro-organisms or their DNA represents an important first line screening strategy that could be used by enforcement laboratories in order to know if further analyses would be needed to confirm the suspected accidental contamination.

In this context, we developed a first line generic screening strategy allowing to both detect and identify FE producing bacteria. This strategy is based on an available PCR method specific to the 16S-rRNA gene region, described in several studies as a molecular marker allowing bacterial phylogenetic classifications, and the identification of bacteria by sequencing (Dorn-In, Bassitta, Schwaiger, Bauer, & Hölzel, 2015; Srinivasan et al., 2015). Firstly, the potential presence of FE producing bacterial strains is screened by a PCR amplification targeting the V3–V4 regions of the 16S-rRNA gene (Dorn-In et al., 2015). Secondly, the generated amplicons are sequenced by Sanger sequencing. The generated sequences are then identified down to the genus and/or species level using an in-house 16S-rRNA gene database, developed and curated in this study. The performance of the proposed generic strategy of first line screening was assessed. To this end, the specificity was tested on all FE producing microbial strains mentioned in the list of 304 FE dossiers submitted to EFSA. Moreover, the robustness of the in-house database, containing all available 16S-rRNA gene region sequences from FE producing micro-organisms extracted from NCBI, was assessed through phylogenetic analyses. Using a FE preparation that was artificially contaminated by a FE producing bacteria, the sensitivity was evaluated. In addition, to illustrate the applicability, commercial FE preparations were analysed using our proposed generic strategy.

2. Materials and methods

2.1. Microbial strains

All wild type (WT) species corresponding to the bacteria, fungi and yeast strains mentioned as FE producing micro-organisms in the list of 304 FE dossiers submitted to EFSA (European Commission, 2016) were collected. *Chryseobacterium proteolyticum* was not collected as it has not been published under the rules of the International Code of Nomenclature of Bacteria, which means this species has not been deposited in a recognized culture collection. Information regarding the strain number, batch number, species origin and culture conditions are provided in Table 1. All collected strains were cultured according to the culture collection recommendations on specific growth media and at optimal temperatures.

2.2. DNA extraction and concentration

DNA was extracted from the cultured pure strains listed in Table 1, using the *Quick*-DNA™ Fungal/Bacterial Miniprep Kit (ZYMO research), according to the manufacturer's instructions. The DNA extraction of the FE matrices used for the applicability evaluation (see Section 2.5) was performed using the NucleoSpin® Food kit (Macherrey-Nagel) according to the manufacturer's instructions. DNA extraction of *Glycine max* and *Oryza sativa* was carried out as previously described (Fraiture et al., 2014). Using the Qubit 4.0 Fluorometer, the DNA concentration was measured with the dsDNA Broad range (BR) Assay Kit (Life Technologies) according to manufacturer's instructions.

2.3. PCR amplification of 16S-rRNA gene region and Sanger sequencing

For each PCR reaction, a standard 25 µl reaction volume was applied containing 1× mastermix, 250 nM of each primer (335-F: 5′-TAATACGACTCACTATAGGCADACTCCTACGGGAGGC-3; 796-R: 5′-TATTTAGGTGACACTATA ATCCTGTTTGMTMCCCVCRC-3′, with T7 and SP6 primer (underlined) recognition sites respectively added to the 5′ end for down-stream sequencing purposes) (Dorn-In et al., 2015) and 5 µl of DNA (2 ng/µl), according to the manufacturer's instructions from the KAPA Taq EXtra HotStart ReadyMix PCR kit (KAPA Biosystems). The PCR program consisted of an initial denaturation of 3 min at 95 °C followed by 35 amplification cycles of 30 s at 95 °C, 30 s at 50 °C and 1 min at 72 °C, and a final extension of 1 min at 72 °C. The run was performed on a Swift Aeris Thermal Cycler (ESCO). For each assay, a non-template control (NTC) and negative controls, consisting out of human (TaqMan™ Control Genomic DNA, ThermoFisher), plant (*Glycine max* and *Oryza sativa*) and the fungal and yeast species mentioned in Table 1, were included. The final PCR products were analysed by electrophoresis using the D1000 screentapes and reagents on a Tapestation 4200 device (Agilent, Belgium), according to manufacturer's instructions, in order to visualize the profiles of the generated amplicons. For each sample, the generated amplicon was then purified using the ExoSAP-IT® PCR Product Clean-up (Thermo Fisher). In case of multiple amplicons, the final PCR product was separated by electrophoresis on a 1% agarose gel (INVITROGEN, CA, USA) (100 V, 400 mA, 40 min). Amplicons of interest were excised from the gel and purified using the

Table 1
Overview of all FE producing micro-organisms mentioned in the list of 304 FE dossiers submitted to EFSA that were collected in this study. For each organism, representative strains were selected and the strain number, batch number, name of the original collection (origin) and culturing conditions used are indicated. Presence of the organism in the QPS (qualified presumption of safety) list of EFSA is also indicated.

Kingdom	Genus	Species	Origin			Culturing conditions		QPS
			Strain number	Collection	Batch number	Medium	Temperature	
Bacteria	*Arthrobacter*	*ramosus*	LMG 17309	BCCM	17309 LYO 04/13	NA	28 °C	No
	Bacillus	*licheniformis*	MB 392	ILVO		NA	30–37 °C	Yes
	Bacillus	*subtillis*	MB 4578	ILVO		NA	30–37 °C	Yes
	Bacillus	*circulans*	MB 367	ILVO		NA	30–37 °C	No
	Bacillus	*pumilus*	QA 55	ILVO		NA	30–37 °C	Yes
	Bacillus	*amyloliquefaciens*	LMG 9814	BCCM	09814 LYO 06/09	NA	30 °C	Yes
	Bacillus	*flexus*	LMG 11155	BCCM	11155 LYO 09/00	NA	30 °C	Yes
	Cellulosimicrobium	*cellulans*	LMG 16121	BCCM	16121 LYO 06/95	NA	28 °C	No
	Chryseobacterium	*proteolyticum*[a]	/	/	/	/	/	No
	Corynebacterium	*glutamicum*	LMG 3652	BCCM	03652 LYO 05/11	NA	30 °C	No
	Escherichia	*coli*	MB 1068	ILVO		NA	30–37 °C	No
	Geobacillus	*stearothermophilus*	MB 394	ILVO		NA	55 °C	No
	Geobacillus	*pallidus*	MB 401	ILVO		NA	55 °C	No
	Geobacillus	*caldoproteolyticus*	DSM 15730	DSMZ	DSM 15730-0703-001	Caso agar	55 °C	No
	Klebsiella	*pneumoniae*	MB 4414	ILVO		NA	30–37 °C	No
	Lactobacillus	*fermentum*	LMG 6902	BCCM	06902 LYO 03/14	MRS	37 °C	Yes
	Lactococcus	*lactis*	MB 96	ILVO		MRS	30 °C	Yes
	Leuconostoc	*citreum*	LMG 9824	BCCM	09824 LYO 03/05	MRS	30 °C	Yes
	Microbacterium	*imperiale*	LMG 20190	BCCM	20190 LYO 12/11	14	28 °C	Yes
	Paenibacillus	*macerans*	LMG 6324	BCCM	063240 LYO 08/00	14	28 °C	No
	Paenibacillus	*alginolyticus*[a]	/	/	/	/	/	No
	Protaminobacter	*rubrum*	CBS 574.77	CBS		19 (pepton agar)	30 °C	No
	Pseudomonas	*fluorescens*	MB 4440	ILVO		NA	28–30 °C	No
	Pseudomonas	*amyloderamosa*[b]	ATCC-21262	ATCC	39531	NA	30 °C	No
	Pullulanibacillus	*naganoensis*	LMG 12887	BCCM	12887 LYO 06/08	232	30 °C	No
	Streptomyces	*violaceoruber*	LMG 7183	BCCM	07183 LYO 03/86	78	28 °C	No
	Streptomyces	*murinus*	LMG 10475	BCCM	10475 LYO 06/03	78	28 °C	No
	Streptomyces	*netropsis*	LMG 5977	BCCM	05977 LYO 03/89	78	28 °C	No
	Streptomyces	*mobaraensis*	DSM 40847	DSMZ	DSM 40847-0616-001	Yeast malt extract agar	24 °C	No
	Streptomyces	*rubiginosus*	LMG20268	BCCM	20268 LYO 06/01	78	28 °C	No
Fungi	*Aspergillus*	*oryzae*	IHEM 25836	BCCM	IHEM LY2012-1051	Medium S10	25 °C	No
	Aspergillus	*niger*	IHEM 05296	BCCM	IHEM LY2016-0075	Medium S10	25 °C	No
	Aspergillus	*niger* agg.[a]	/	/	/	/	/	No
	Aspergillus	*niger macrosporus*[a]	/	/	/	/	/	No
	Aspergillus	*niger awamori*	IHEM 25485	BCCM	IHEM LY2012-0133	Medium S10	25 °C	No
	Aspergillus	*fijiensis*	IHEM 22812	BCCM	IHEM LY2012-0198	Medium S10	25 °C	No
	Aspergillus	*acidus*	IHEM 26285	BCCM	IHEM LY2014-0334	Medium S10	25 °C	No
	Aspergillus	*aculeatus*	IHEM 05796	BCCM	IHEM LY2016-0579	Medium S10	25 °C	No
	Aspergillus	*melleus*	IHEM 25956	BCCM	IHEM LY2013-0309	Medium S10	25 °C	No
	Chaetomium	*gracile*	MUCL 053569	BCCM	FRT-2011-0283	PDA	25 °C	No
	Chaetomium	*erraticum*[a]	/	/	/	/	/	No
	Cryphonectria	*parasitica*	MUCL 007956	BCCM	OIL-2017-0171	MA1	20 °C	No
	Sporobolomyces	*singularis*	MUCL 027849	BCCM	FRT-1996-1295	MYA2	24 °C	No
	Disporotrichum	*dimorphosporum*	MUCL 019341	BCCM	FRT-1999-0506	MYA2	23 °C	No
	Boletus	*edulis*	MUCL 043104	BCCM	FRT-2001-0009	MA2	25 °C	No
	Fusarium	*venenatum*	MUCL 055417	BCCM	FRT-2014-0324	PDA	25 °C	No
	Hansenula	*polymorpha*	MUCL 027761	BCCM	FRT-2008-0858	MYA2	25 °C	No
	Humicola	*insolens*	MUCL 015010	BCCM	FRT-2000-1490	PDA	37 °C	No
	Kluyveromyces	*lactis*	IHEM 02051	BCCM	IHEM LY2007-0748	Medium S	25 °C	Yes
	Leptographium	*procerum*	MUCL 008094	BCCM	FRT-1999-2437	DYAA	20 °C	No
	Mucor	*javanicus*	IHEM 05212	BCCM	IHEM LY2012-1038	Medium S10	25 °C	No
	Penicillium	*roqueforti*	IHEM 20176	BCCM	IHEM LY2003-0624	Medium S10	25 °C	No
	Penicillium	*camemberti*	IHEM 06648	BCCM	IHEM LY2016-0256	Medium S10	25 °C	No
	Penicillium	*multicolor*	CBS 501.73	CBS		MEA	24 °c	No
	Penicillium	*citrinium*	IHEM 26159	BCCM	IHEM LY2014-0060	Medium S10	25 °C	No
	Penicillium	*decumbens*	IHEM 05935	BCCM	IHEM LY2002-0039	Medium S10	25 °C	No
	Penicillium	*chrysogenum*	IHEM 03414	BCCM	IHEM LY2002-0279	Medium S10	25 °C	No
	Penicillium	*funiculosum*	MUCL 014091	BCCM	FRT-2000-2027	MYA2	25 °C	No
	Rhizomucor	*miehei*	IHEM 26897	BCCM	IHEM LY2016-0179	Medium S10	37 °C	No
	Rhizopus	*oryzae*	IHEM 26078	BCCM	IHEM LY2013-0805	Medium S10	25 °C	No
	Rhizopus	*niveus*	ATCC 200757	ATCC	2547375	PDA	25 °C	No
	Talaromyces	*pinophilus*	IHEM 16004	BCCM	IHEM LY200-2056	Medium S10	25 °C	No
	Talaromyces	*emersonii*	DSM 2432	DSMZ	DSM 2432-0807-001	OAT FLAKE MEDIUM	40 °C	No
	Trametes	*hirsute*	MUCL 030869	BCCM	FRT-1996-0924	MA1	20 °C	No
	Trichoderma	*reesei*	IHEM 05651	BCCM	IHEM LY2006-0407	Medium S10	25 °C	No
	Trichoderma	*citrinoviride*	IHEM 25858	BCCM	IHEM LY2013-0070	Medium S10	25 °C	No
	Trichoderma	*viride*	IHEM 04146	BCCM	IHEM LY2002-0631	Medium S10	25 °C	No

(*continued on next page*)

Table 1 (*continued*)

Kingdom	Genus	Species	Origin			Culturing conditions		QPS
			Strain number	Collection	Batch number	Medium	Temperature	
Yeasts	*Candida*	*cylindracea*	MUCL 041387	BCCM	FRT-1998-2273	DYPA	25 °C	Yes
	Candida	*rugose*	IHEM 01894	BCCM	IHEM LY2002-0071	Medium S	25 °C	No
	Pichia	*pastori*	MUCL 027793	BCCM	FRT-2015-0116	MYA2	24 °C	No
	Saccharomyces	*cerevisiae*	IHEM 25104	BCCM	IHEM LY2011-0436	Medium S	25 °C	Yes

Collections: BCCM = Belgian Coordinated Collections of Micro-organisms; ILVO = Research Institute for Agriculture, Fisheries and Food; DSMZ = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.
Medium: BHI = brain heart infusion; DYAA = dextrose 1% yeast extract asparagine agar; DYPA = dextrose yeast extract peptone water; MA = malt agar; MEA = malt extract agar; MRS = De Man, Rogosa and Sharpe; MYA2 = malt 2% yeast extract agar; NA = nutrient agar; PDA = potato dextrose agar; S10 = Sabouraud diluted; 14 = Tryptone soy agar; 78 = GYM STREPTOMYCES MEDIUM; 232 = Trypticase Soy Broth supplemented with 1% soluble starch, pH 5.5.
[a] Species that were unavailable in the consulted collections.
[b] Bacterial species for which no sequence information was available in the 16S RefSeq Nucleotide sequence records (NCBI).

Wizard® SV Gel and PCR Clean-Up System (Promega, WI, USA). The purified PCR products/excised amplicons were subsequently sequenced on a Genetic Sequencer 3130XL using the Big Dye Terminator Kit v3.1 (Applied Biosystems).

2.4. Collection of 16S-rRNA gene region sequences to build the in-house curated database of FE producing bacteria

For the 30 bacterial species indicated as FE producer in the EFSA (European Commission, 2016) list (see Table 1), all available sequences from the NCBI 16S RefSeq Nucleotide sequence records database (accessed February 2018) were collected (O'Leary et al., 2016), resulting in a total set of 107 reference sequences for 29 species (Table 1). No sequence record was available for *Pseudomonas amyloderamosa*, for which no representative entry is therefore present in our database. The amplicons of 16S-rRNA gene regions targeted by our PCR, using the primers mentioned in Section 2.3, were afterwards manually extracted from these sequence records in order to construct a 16S-rRNA gene region in-house curated database (see Supplementary file I). Own generated 16S-rRNA sequences during this study were afterwards added to the in-house database.

2.5. Species identification

The megablast program (Camacho et al., 2009) from the BLAST suite (v2.8.0) was used to perform species identification employing the generated amplicon sequences (see Section 2.3) as query, and the entire in-house 16S-rRNA gene region database (see Section 2.4) as subject (using default settings). The first BLAST hit (sorted based on e-value) was considered to represent the FE producing bacteria. A series of additional phylogenetic analyses were performed to investigate the value of using the 16S-rRNA gene as a marker for identification of the FE producing bacteria. Firstly, for all generated 16S-rRNA gene region amplicon sequences (see Section 2.3), the forward and reverse sequences were assembled into a consensus sequence. All resulting sequences were then aligned by Muscle using the MEGA software (version 7.0.18) using the following settings: max Iterations: 100 (all other settings were left at their default values). Next, model selection (MS), and tree building (TB) using the best model identified by MS, were performed with MEGA using the following settings: Gaps/missing data treatment: Partial deletion (MS + TB), Site coverage cut-off: 50% (MS + TB), Branch swap filter: very weak (MS), Number of bootstrap replications: 100 (TB), ML Heuristic Method: Subtree-Pruning-Regrafting - Fast (SPR Level 3) (TB). All other settings were left at their default values. Secondly, the same analysis was performed for the entire in-house 16S-rRNA gene region database supplemented with all generated 16S-rRNA gene region amplicon sequences (see Section 2.3). For both analyses, the resulting bootstrap consensus tree was visualized using FigTree (version 1.4.3, available at https://github.com/rambaut/figtree/releases/tag/v1.4.3) employing a midpoint rooting. To reduce the size of the trees and facilitate their subsequent interpretation, all terminal branches that represent either the same genus or species were collapsed, resulting in two interpretations of each tree (Supplementary files IV and V).

2.6. Sensitivity analysis

The sensitivity of the proposed strategy was tested using the commercialized liquid FE preparation α-amylase (Termamyl®, Novozymes Corp.) produced by *Bacillus licheniformis*. This FE preparation was artificially contaminated with different concentrations of the wild-type *Bacillus licheniformis*. More precisely, a single colony of *B. licheniformis* (MB 392) was cultured overnight (16 h) at optimal growth conditions (Table 1, Fig. 2). This fresh culture (10 µl) was diluted in 10 ml fresh BHI medium and grown to an OD_{600} of 0.7 to constitute the mother dilution (D-0), that was used to perform a 10-fold serial dilution until D-8. 100 µl of each dilution (D-0 to D-8) was plated on NA medium for the enumeration of colonies (grown overnight at 37 °C) (Fig. 2A). 200 µl of each dilution (D-0 to D-8) was mixed with 200 µl of the commercialized FE, α-amylase, and 200 µl of each mixture was also plated on NA medium for enumeration (grown overnight at 37 °C) (Fig. 2). The plates of the D-0 to D-8 dilutions, from both the pure culture and the artificially spiked FE preparation samples, gave similar number of colonies, demonstrating that the used FE preparation seems to have no impact on the growth of *Bacillus licheniformis*.

From both the non-spiked and the spiked D-0 to D-6 solutions, 200 µl was used for DNA extraction using the NucleoSpin® Food kit (Macherrey-Nagel), according to the manufacturer's instructions with the addition of an initial beating step of $2 * 2$ min at 5000 rpm (MiniLys, Bertin Instruments) (see Section 2.2) in order to analyse the sensitivity of the proposed generic strategy of first line screening. The estimated colony numbers used for each PCR amplification are indicated in Fig. 2C. As a control, the proposed generic strategy of first line screening was also applied on an isolated colony, obtained from the previous plating of the D-0 to D-8 dilutions (see Fig. 2A).

2.7. Applicability assessment

The applicability of the proposed strategy was verified using six commercially available FE preparations: (1) papain (Vitalingo), from Papaya, under a solid form; (2) lactase (Lactose-OK) produced by *Aspergillus oryzae*, under a solid form; (3) microbial rennet (Lactoferm-Brouwland), produced by *Rhizomucor miehei*, under a solid form; (4) flour treatment agent (Molen 'de père'), from an unknown origin, under a solid form; (5) α-amylase (Dextzyme HT, The Alchemist's Pantry), produced by *Bacillus licheniformis*, under a solid form; (6) neutral protease (Pureferm, The Alchemist's Pantry), produced by *Bacillus subtilis*, under a solid form (Fig. 3A). Of all these FE preparations DNA was

extracted using the NucleoSpin® Food kit (Macherrey-Nagel) according to the manufacturer's instructions with the addition of an initial beating step of 2 ∗ 2 min at 5000 rpm (MiniLys, Bertin Instruments) in order to verify the applicability of the proposed generic strategy of first line screening.

Additionally, for the α-amylase product (Dextzyme HT, The Alchemist's Pantry) and the neutral protease product (Pureferm, The Alchemist's Pantry) a liquid culture, composed of 1 g of FE powder and 5 ml of BHI, was grown overnight at 37 °C. This mixture was plated on NA medium (grown overnight at 37 °C) and isolated colonies were submitted to the proposed generic strategy of first line screening.

3. Results and discussion

3.1. Overview of FE producing micro-organisms

Within the list of 304 FE dossiers submitted to EFSA (European Commission, 2016) 53%, 2% and 32% are produced by fungi, yeasts and bacteria, respectively (Table 1). The remaining 13% are extracted from animals or plants. In total, 71 different species of micro-organisms are mentioned for the production of FE. Within the FE produced by bacteria, the majority is produced by *B. subtilis* (23.8%) and *B. licheniformis* (21.6%). Besides WT strains, genetically modified strains are often used for the production of FE, representing 50.5% of all the mentioned bacterial FE production strains.

Of the 71 different species, only 14 (19.7%) have been added by EFSA to the qualified presumption of safety (QPS) list of biological agents (Ricci et al., 2017). All other species could not be granted the QPS status because they produce mycotoxins, are linked to human disease, lack a sufficient body of knowledge on a history of safe use, or other reasons.

3.2. Specificity of the 16S strategy to detect and identify FE producing micro-organisms

The use of conventional PCR methods, specifically targeting the V3–V4 16S-rRNA gene regions, followed by sequencing is a well-known approach for the detection and identification of bacterial species (Dorn-In et al., 2015; Lebonah et al., 2014). However, in this study such an approach is tested for the first time to systematically detect and identify FE producing bacterial strains. In order to test the specificity of the proposed generic strategy of first line screening, all microbial strains used to produce FE, as mentioned in in the list of 304 FE dossiers submitted to EFSA, were collected and analysed, including 28 bacterial species, 34 fungal species and 4 yeast species. As expected, the DNA from all tested bacterial strains was positively amplified by the conventional PCR method. Whereas, no PCR amplification was obtained for all tested fungal and yeast strains, nor for the plant samples (*Glycine max* and *Oryza sativa*) and the human sample that were integrated in the analysis to complete the specificity study (Table 2A, Supplementary files II and III). More precisely, a single amplicon of the expected size (390–420 bp) was observed for the DNA of each tested bacterial strain, except for *Paenibacillus macerans* (Supplementary file II) for which 5 amplicons were observed, including four with a weak signal intensity, that were discarded for further analysis, and one at the expected size (416 bp) with a strong signal intensity that was selected for subsequent analysis (Supplementary file II). These results confirm the ability of the conventional PCR to specifically amplify the bacterial 16S-rRNA gene region (i.e. a signal is obtained for all bacterial strains but not for other kingdoms) (Dorn-In et al., 2015).

All obtained amplicons from the conventional PCR amplification were sequenced using the Sanger technology, after which the identity of each generated sequence was obtained by blasting against an in-house 16S-rRNA gene region database consisting of all available 16S-rRNA gene region sequences belonging to the FE producing bacterial strains from the NCBI 16S RefSeq Nucleotide sequence records database. To

Table 2
Assessment of the proposed new generic strategy to detect contaminations of FE producing bacteria in FE preparations.

(A) Summarized results from the 16S-rRNA gene PCR-based sequencing, applied on the FE producing micro-organisms mentioned in Table 1. Plants and human samples were used as negative controls.

(B) Overview of all collected bacterial strains with their generated 16S-rRNA gene amplicon using the new generic strategy. For each strain, the generated amplicon sequence was analysed by blasting against an in-house curated 16S-rRNA gene database containing 16S-rRNA gene sequence information for all FE producing bacterial strains, for which the correct species was always assigned as first hit. For each bacterial strain, the range in percent identity of hits to the correct species is indicated in the column “Percent identity range for the expected correct species”. The first hit and its identity to an incorrect species, is also indicated.

A

	16S-rRNA gene region amplification
Bacteria (28)	28/28
Fungi/yeast (34)	0/34
Yeast (4)	0/4
Plants (2)	0/2
Human (1)	0/1

FE producing bacteria	Generated 16S rRNA gene region amplicon with the developed PCR method from this study	Size (bp)	BLAST against curated in-house 16S rRNA gene region database consisting out of FE producing strains	
			Percent identity range for the expected correct species[b]	First hit, and its percent identity, to an incorrect species
Arthrobacter ramosus	AGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAG GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGG TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG CGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTC TGCTGTGAAAGACCGGGGCTCAACTCCGGTTCTGCAGTGGGTACGGGCAGACTAGAG TGATGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGA GGAACACCGATGGCGAAGGCAGGTCTCTGGGCATTAACTGACGCTGAGGAGCGAAA	396	100%[1]	*Microbacterium imperiale*, 95%
Bacillus licheniformis	AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGA ATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA CTGACGCTGAGGCGCGAAA	417	100%[3]–99%[1]	*Bacillus subtilis*, 99%
Bacillus subtillis	AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGA ATAGGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA CTGACGCTGAGGAGCGAAA	417	100%[7]–99%[1]	*Bacillus amyloliquefaciens*, 99%
Bacillus circulans				

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTA
ACTGCTTGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGCGGTCCTTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT
GGAAACTGGGGGACTTGAGTGCAGAAGAGAAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTTTGGTCTGTAAC
TGACGCTGAGGCGCGAAA

416

100%[3]–99%[2]

Bacillus flexus, 97%

Bacillus pumilus

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTA
ACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTC
GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATT
GGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAC
TGACGCTGAGGAGCGAAA

416

100%[5]

Bacillus amyloliquefaciens, 97%

Bacillus amyloliquefaciens

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAA
ATAGGGCGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCT
CGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAA
CTGACGCTGAGGAGCGAAA

417

100%[5]

Bacillus subtilis, 99%

Bacillus flexus

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTACAAGAGTA
ACTGCTTGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCATT
GGAAACTGGGGAACTTGAGTGCAGAAGAAAAGCGGAATTCCACGTGTAGCGGTG
AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTTTTTGGTCTGTAAC
TGACGCTGAGGCGCGAAA

416

100%[2]–98%[1]

Bacillus circulans, 97%

Cellulosimicrobium cellulans

AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGCAAGTGACGG
TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTC
TGGTGTGAAAACTCGAGGCTCAACCTCGAGCTTGCATCGGGTACGGGCAGACTAGAG
TGCGGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGA
GGAACACCGATGGCGAAGGCAGGTCTCTGGGCCGCAACTGACGCTGAGGAGCGAAA

396

100%[1]–99%[2]

Microbacterium imperiale, 96%

Chryseobacterium proteolyticum[a]

NA

/

/

/

Corynebacterium glutamicum

AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGGG
GGATGACGGCCTTCGGGTTGTAAACTCCTTTCGCTAGGGACGAAGCCTTATGGTGAC
GGTACCTGGAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAG
GGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGTGGTTTGTCGCG
TCGTCTGTGAAATCCCGGGGCTTAACTTCGGGCGTGCAGGCGATACGGGCATAACTT
GAGTGCTGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAG
GAGGAACACCAATGGCGAAGGCAGGTCTCTGGGCAGTAACTGACGCTGAGGAGCG
AAA

399

99%[2]

Arthrobacter ramosus, 91%

Escherichia coli

AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTAT
GAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAAAGTTA
ATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCA
TCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAG
ACTGACGCTCAGGTGCGAAA

416

100%[1]–99%[2]

Klebsiella pneumoniae, 95%

Geobacillus stearothermophilus

AGCAGTAGGGAATCTTCCGCAATGGGCGAAAGCCTGACGGAGCGACGCCGCGTGAG
CGAAGAAGGCCTTCGGGTCGTAAAGCTCTGTTGTGAGGGACGAAGGAGCGCCGTTCG
AAGAGGGCGGCGCGGTGACGGTACCTCACGAGAAAGCCCCGGCTAACTACGTGCCA
GCAGCCGCGGTAATACGTAGGGGGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGC
GCGCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGT
CATTGGAAACTGGGGGACTTGAGGGCAGGAGAGGAGAGCGGAATTCCACGTGTAGC
GGTGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCT
GCACCTGACGCTGAGGCGCGAAA

417

100%[5]

Geobacillus caldiproteolyticus, 94%

Geobacillus pallidus

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG
CGAAGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGGGAAGAACAAGTGCCGTTCG
AACAGGGCGGTACCTTGACGGTACCTGACGAGGAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTTCCTTAAGTCTGATGTGAAATCTCGCGGCTCAACCGCGAGCGGCCA
TTGGAAACTGGGGAACTTGAGTGCAGGAGAGGGGAGCGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGCCTGTA
ACTGACGCTGAGGCGCGAAA

417

99%[1]

Geobacillus caldiproteolyticus, 95%

Geobacillus caldiproteolyticus

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAG
CGAAGAAGGTCTTCGGATTGTAAAGCTCTGTTGTTAGGGAAGAAGAAGGTGCCGTTCG
AACAGGGCGGTACCGTGACGGTACCTAACGAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGCGGTCTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCA
TTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAGCGGAATTCCACGTGTAGCGG
TGAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTA
ACTGACGCTGAGGCGCGAAA

417

100%[2]

Bacillus licheniformis, 96%

Klebsiella pneumoniae

AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTGGGGAGGAAGGCGTTAAGGTTA
ATAACCTTGGCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCA
TTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG
ACTGACGCTCAGGTGCGAAA

416

99%[3]–98%[8]

Protaminobacter rubrum, 95%

Lactobacillus fermentum

AGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACCGCGTGAGT
GAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGTA
ACTGTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGT
GCAGGCGGTTTTCTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATC
GGAAACTGGATAACTTGAGTGCAGAAGAGGGTAGTGGAACTCCATGTGTAGCGGTG
GAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTACCTGGTCTGCAAC
TGACGCTGAGACTCGAAA

416

100%[2]–98%[1]

Bacillus pumilus, 88%

Lactococcus

lactis

AGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAGT
GAAGAAGGTTTTCGGATCGTAAAACTCTGTTGGTAGAGAAGAACGTTGGTGAGAGTG
GAAAGCTCATCAAGTGACGGTAACTACCCAGAAAGGGACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTCCCGAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAG
CGCAGGTGGTTTATTAAGTCTGGTGTAAAAGGCAGTGGCTCAACCATTGTATGCATT
GGAAACTGGTAGACTTGAGTGCAGGAGAGGAGAGTGGAATTCCATGTGTAGCGGTG
AAATGCGTAGATATATGGAGGAACACCGGTGGCGAAAGCGGCTCTCTGGCCTGTAAC
TGACACTGAGGCTCGAAA

416

100%[7]

Pullulanibacillus naganoensis, 87%

Leuconostoc

citreum

TGCAGTAGGGAATNTTCCACAATGGGCGCAAGCCTGATGGAGCAACGCCGCGTGTGT
GATGAAGGCTTTCGGGTCGTAAAGCACTGTTGTATGGGAAGAAATGCTAAAATAGGG
AATGATTTTAGTTTGACGGTACCATACCAGAAAGGGACGGCTAAATACGTGCCAGCA
GCCGCGGTAATACGTATGTCCCGAGCGTTATCCGGATTTATTGGGCGTAAAGCGAGC
GCAGACGGTTGATTAAGTCTGATGTGAAAGCCCGGAGCTCAACTCCGGAATGGCATT
GGAAACTGGTTAACTTGAGTGTTGTAGAGGTAAGTGGAACTCCATGTGTAGCGGTGG
AATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTACTGGACAACAACT
GACGTTGAGGCTCGAAA

416

99%[1]

Lactobacillus fermentum, 84%

Microbacterium

imperiale

AGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTGATGCAGCAACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTTAGCAGGGAAGAAGCGAGAGTGACGG
TACCTGCAGAAAAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTATCCGGAATTATTGGGCGTAAAGAGCCGTAGGCGGTTTGTCGCGTCT
GCTGTGAAATCCCGAGGCTCAACCTCGGGCCTGCAGTGGGTACGGGCAGACTAGAGT
GCGGTAGGGGAGATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAG
GAACACCGATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAA

395

99%[1]–98%[1]

Cellulosimicrobium cellulans, 95%

Paenibacillus

macerans

AGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGTTGCCAGGGAAGAACGTCTTNTGGAGTA
ACTGCCANGAGAGTGACGGTACCTGAGAAGAAAGCCCCGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCGC
GCAGGCGGCTGTTTAAGTCTGGTGTATAATCCTGGGGCTCAACTCCGGGTCGCACTG
GAAACTGGACGGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGA
AATGCGTAGAGATGTGGAGGAACACCAGTGGCGAGAGGCGACTCTCTGGGCTGTAA
CTGACGCTGAGGCGCGAAA

416

99%[2]–93%[1]–92%[1]

Paenibacillus alginolyticus, 89%

Paenibacillus

alginolyticus[a]

NA

/

/

/

Protaminobacter

rubrum

AGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGT
GAAGAAGGCCTTAGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGGTAGTGTGTTA
ATAGCACATTGCATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGC
AGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACGTGGGAACTGCA
TTTGAAACTGGCAAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGT
GAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAG
ACTGACGCTCAGGTGCGAAA

416

99%[3]

Klebsiella pneumoniae, 95%

Pseudomonas

fluorescens

AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT
GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCATTAACCTAA
TACGTTAGTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCA
GCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGC
GTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATT
CAAAACTGACTGACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGA
AATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTAATACT
GACACTGAGGTGCGAAA

416

100%[4]–99%[1]

Klebsiella pneumoniae, 87%

Pseudomonas

amyloderamosa

AGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCAATGCCGCGTGTGT
GAAGAAGGCCTTCGGGTTGTAAAGCACTTTTATCAGGAGCGAAATACTACCGGCTAA
TATCCGGTGGGGCTGACGGTACCTGAGGAATAAGCACCGGCTAACTTCGTGCCAGCA
GCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGC
GTAGGCGGTTATTTAAGTCTGTTGTGAAATCCCCGGGCTCAACCTGGGAATGGCAAT
GGATACTGGATAGCTAGAGTGTGATAGAGGATGGTGGAATTCCCGGTGTAGCGGTG
AAATGCGTAGAGATCGGGAGGAACATCAGTGGCGAAGGCGGCCATCTGGATCAACA
CTGACGCTGAGGCACGAAA

416

/

No *P. amyloderamosa* sequence available in the in-house database.

Klebsiella pneumoniae, 84%

Pullulanibacillus

naganoensis

AGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGCAACGCCGCGTGAG
CGATGAAGGTCTTCGGATCGTAAAGCTCTGTTGTCAGAGAAGAACACGTGATAGAGG
AAATGCTATCACCTTGACGGTATCTGACCAGAAAGCCCCGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGGGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGCGCG
CGCAGGCGGCTTCTTAAGTCTGATGTGAAAGCCCACGGCTCAACCGTGGAGGGTCAT
TGGAAACTGGGGAGCTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGT
GAAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAAGCGGCTCTCTGGTCTGTAA
CTGACGCTGAGGCGCGAAA

416

100%[1]

Bacillus pumilus, 94%

Streptomyces

violaceoruber

AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGG
TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTC
GGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAG
TTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA

396

100%[4]

Streptomyces mobaraensis, 99%

Streptomyces

murinus

AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG
GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGG
TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTC
GATTGTGAAAGCTCGGGGCTTAACCCCGAGTCTGCAGTCGATACGGGCTAGCTAGAG
TGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGA
GGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAA

396

100%[3]

Streptomyces violaceoruber, 98%

Streptomyces

netropsis

AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG

	GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGG TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTTGCGTC GGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAG TGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGA GGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAA
396	
100%[3]	
Streptomyces mobaraensis, 98%	
Streptomyces mobaraensis	
	AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGG TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTC GGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCAGGCTAGAG TTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
396	
100%[2]	
Streptomyces rubigiosus, 99%	
Streptomyces rubiginosus	
	AGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAG GGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGG TACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGG CGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTC GGTTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAG TTCGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAA
396	
99%[2]	
Streptomyces violaceoruber, 99%	

[a] Was not available in the consulted public strain collections.

[b] The superscript represents the number of sequences that had that specific percentage as BLAST result available in the in-house database. The expected strain is the FE producing micro-organism that is analysed and is indicated in the first column “FE producing bacteria”.

verify the absence of ambiguity in the blast results, the first hit to an incorrect species was also investigated. These results are presented in Table 2B. For all bacterial species present in the FE list (Table 1), blasting the generated amplicon sequence against the in-house database always assigned the correct species as first hit with a percent sequence identity ranging between 92% and 100% and a query coverage of 100%. Moreover, the percent identity of the first hit corresponding to the correct species was always higher than the percent identity of their first incorrect species hit, with the exception of *Streptomyces rubiginosus*, *B. subtilis* and *B. licheniformis* for which there was no difference between the percent identity of the correct species and first incorrect species hit, although the latter one always belonged to the same genus. If an identification down to the species level is required for these more problematic species, species-specific PCR methods could be performed (De Clerck, Van Mol, Jannes, Rossau, & De Vos, 2004). This suggests that the proposed strategy based on conventional PCR amplification and sequencing of the 16S-rRNA gene region, allows to identify FE bacterial strains at least down to the genus level, based on their first hit in a 16S-rRNA gene region database, for all 29 tested bacterial strains known to produce FE, and for the majority of cases even down to the species level. These results were also confirmed through additional phylogenetic analyses of the obtained 16S-rRNA gene region sequences (see Section 3.3).

3.3. Assessment of the in-house 16S-rRNA gene region database

The proposed generic strategy based on 16S-rRNA gene region amplification and Sanger sequencing followed by blasting against a 16S-rRNA gene region database, containing records for all FE producing bacterial strains, always allowed for the correct identification of the FE bacterial strain by assignment to the correct species as first hit. Although the same approach has also been used by others (Fontana, Favaro, Pelliccioni, Pistoia, & Favalli, 2005), some caution is needed for the interpretation of the results. In particular, the 16S-rRNA gene region has low phylogenetic power at the species level and poor discriminatory power for some genera (Mignard & Flandrois, 2006), which is especially problematic for the genus *Bacillus*. Within the *Bacillus* genus some species display very high sequence similarity in their 16S-rRNA gene region so that their small 16S-rRNA gene differences cannot justify choosing the first hit as definitive identification (Janda & Abbott, 2007). Although, the extent of this problematic is considerably reduced in our case because the identification is limited to 27 of the 30 FE producing bacterial strains present in the list of 304 dossiers submitted to EFSA (no *Chryseobacterium proteolyticum* and *Paenibacillus alginolyticus* strains were available from public collections and no *Pseudomonas amyloderamosa* sequence information is available in our in-house constructed database). We performed an additional phylogenetic investigation to characterize the extent of any such bias that may be present in our set-up and which is not directly apparent based on results presented in Table 2. More specifically, we constructed two bootstrap consensus phylogenetic trees based on all 16S-rRNA gene region sequences present in our in-house curated 16S-rRNA gene region database, and the former supplemented with all generated bacterial amplicon 16S-rRNA gene region sequences listed in Table 2. The resulting trees are provided in Supplementary files IV and V, and are presented as a sunburst chart to simplify their interpretation in Fig. 1. The inner and outer rings represent all bacterial genera and species, respectively, and are listed in black in case all their corresponding 16S-rRNA gene region sequences were unambiguously grouped together in one cluster in the constructed phylogenetic trees or alternatively in grey if this was not the case. Fig. 1 illustrates that all 16S-rRNA gene region sequences always properly clustered down to the correct genus level, and in the majority of cases (82.2%) also down to the correct species level for the selected FE bacterial strains with the remaining 17.8% consisting out of *B. subtilis*, *B. licheniformis*, *Streptomyces rubiginosus*, *S. violaceoruber* and *S. mobaraensis*. Although these results are derived from a limited set of 16S-rRNA gene region sequences obtained specifically from FE producing bacterial strains, they are in line with general estimates for identification of bacteria based on 16S-rRNA gene sequencing. Generally, identification is possible to the genus level (> 90%) and to a lesser extent to the species level (65%–83%) (Janda & Abbott, 2007), confirming that all FE producing bacteria can be identified down to the genus level with this strategy and the majority also down to the species level. For the exceptions listed above, although the first hit always correctly assigned the correct species, a more robust identification could for instance be obtained using an additional specific (q)PCR method. For the future development of these (q)PCR methods we will focus in particular on *B. subtilis* and *B. licheniformis* because combined they account 45.4% of FE produced by bacteria.

3.4. Sensitivity of the strategy on a spiked industrial enzyme sample

The sensitivity of the proposed generic strategy of first line screening was investigated using an artificially spiked liquid α-amylase FE preparation, produced by *B. licheniformis*, one of the most frequent FE producing organism used in the FE industry, with various concentrations (from $9.2 * 10^5$ to $9.2 * 10^{-3}$ CFU/DNA extraction) of the FE producing bacteria to mimic an accidental contamination (Table 1, Fig. 2). As a control, an isolated colony from the spiked FE preparation was also submitted to the proposed generic strategy of first line screening.

First, using the conventional PCR targeting the V3–V4 16S-rRNA gene regions, no PCR amplicon was observed for the non-spiked FE preparation while an amplicon of the expected size (417 bp) was observed for the spiked FE preparation up to the dilution D-2, corresponding to $4.6 * 10^2$ CFU (Fig. 2B, Table 2B). The subsequent sequencing analysis of the generated amplicons confirmed the

Fig. 1. 16S-rRNA gene region amplification and Sanger sequencing allows identification of FE bacterial strains down to the genus level, and for the majority of the cases to the species level, for FE producing bacterial strains. The inner and outer rings represent all bacterial genera and species, respectively. Genera and species names are indicated in black if all their 16S rRNA gene region sequences unambiguously grouped together in one cluster based on phylogenetic analysis, and in grey otherwise. *Pseudomonas amyloderamosa* is indicated with a white background because no reference 16S rRNA gene region sequences were available for this species and therefore did not allow identification. Chryseobacterium proteolyticum and *Paenibacillus alginolyticus* are indicated with a white background because no bacterial culture was available in the reference collections.

identification of *B. licheniformis* (100% sequence identity) (Fig. 2C). However, it should be noticed that, although this identification analysis led to the correct species, the discriminatory power of the proposed strategy only allows a confident identification down to the genus level (see Fig. 1).

3.5. Applicability assessment of the strategy on commercial FE preparations

The applicability of the proposed generic strategy was analysed on six commercial FE preparations (Fig. 3A). These commercial FE preparations are produced using bacterial strains (α-amylase from *Bacillus licheniformis* and neutral protease from *Bacillus subtilis*), fungal strains (lactase from *Aspergillus oryzae* and microbial rennet from *Rhizomucor miehei*) or plants (papain from Papaya). In addition, for one selected FE preparation (Flour treatment agent) no information regarding the producing organism(s) is available. The use of the conventional PCR analysis, targeting the V3–V4 16S-rRNA regions, on the extracted DNA from the six commercial matrices showed the presence of a PCR amplicon of 417 bp for the two FE preparations produced by *B. licheniformis* and *B. subtilis* while no PCR amplification was observed for the four other commercial matrices (Table 1, Fig. 3B). These PCR amplifications demonstrate the presence of DNA from bacteria inside these 2 tested FE preparations. Following to the sequencing of the observed amplicon for each of these 2 FE preparations, the blast analysis of the generated sequences against our in-house curated 16S-rRNA gene region database allowed to identify *B. licheniformis* (100% sequence identity) in the α-amylase FE preparation and *B. amyloliquefaciens* or *B. subtilis* (respectively 100% and 99.28% identity) in the neutral protease FE preparation. The identified bacterial species corresponded to the mentioned FE producing bacterial species (Fig. 3A and D). The difficulty of identification down to the species level for *B. subtilis* was expected, as previously mentioned in Sections 3.2 and 3.3. These results therefore illustrate the applicability of the proposed generic strategy of first line screening on FE preparations.

In addition, for each of the two FE preparations in which bacterial DNA was detected, the viability status of the identified bacteria was determined using a classical microbiology analysis. For each of these FE preparations, living bacterial colonies were observed (Fig. 3C) and submitted to the proposed generic strategy of first line screening (Fig. 3D).

The obtained sequencing results of the colony amplicons were similar to the above mentioned results from the direct DNA extraction of the FE preparations. These results allow confirming the presence of viable strains belonging to the *Bacillus* strains in the tested matrices. The high level of bacterial contamination combined with the identified bacterial genus strongly suggest that the FE producing bacterial strains are accidentally present in these FE preparations. However, environmental microbial contaminations, which could potentially occur during the fermentation and packaging processes, cannot be excluded. To confirm the origin of the contamination, further analysis is thus needed in a second step.

4. Conclusion

In order to allow the detection and identification of accidental contaminations of the FE producing bacteria in FE preparations, a new generic strategy of first line screening is proposed using a conventional PCR approach to amplify 16S-rRNA regions, followed by sequencing for characterization. The strategy specificity was successfully tested on all FE producing microbial species mentioned in the 304 FE dossiers submitted to EFSA. Results from the blast approach of the 16S-rRNA gene

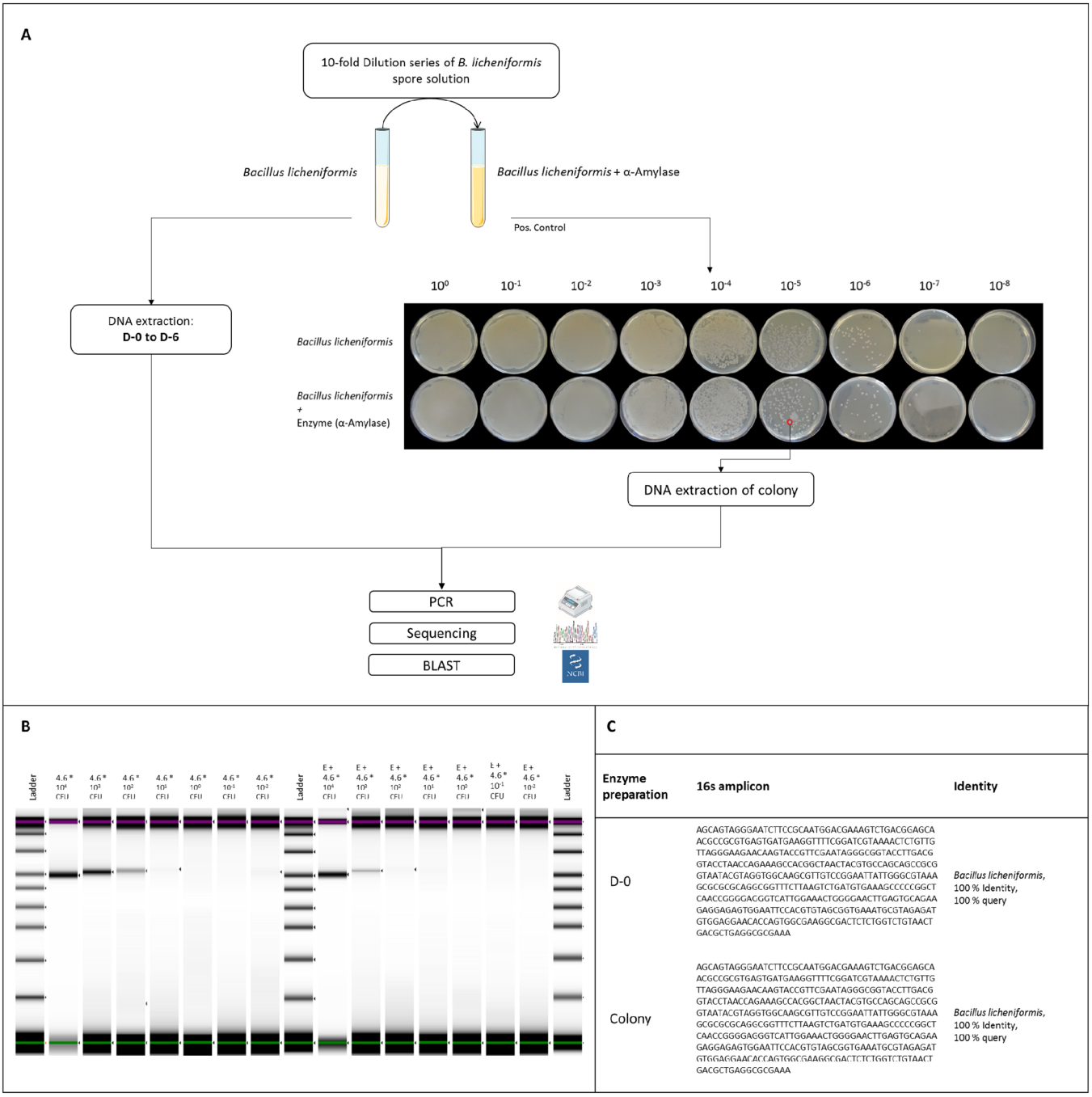

Fig. 2. Overview of the sensitivity assessment of the proposed generic strategy of first line screening.
An α-amylase sample was artificially contaminated with various concentrations of *Bacillus licheniformis* (indicated by the manufacturer as being the producing organism of this enzyme preparation).
A: A 10-fold dilution series was performed of a *B. licheniformis* liquid culture, which was spiked in the liquid α-amylase sample. Both the liquid cultures and the spiked solutions were analysed using the proposed generic strategy of first line screening. As a control, all dilutions were also plated on NA and the obtained colonies were analysed using the proposed generic strategy of first line screening.
B: Visualization of the PCR results, representing both the amplifications of the *B. licheniformis* dilution series and the amplifications of the dilution series artificially spiked in the α-amylase preparation.
C: The sequences generated using the proposed generic strategy of first line screening applied on the DNA extracted from the *B. licheniformis* D-0 liquid culture and from the isolated colony. Both sequences could be identified as *B. licheniformis*.

region sequences against the constructed in-house 16S-rRNA gene region database, containing 16S-rRNA reference sequences of FE producing bacterial species, show the ability to identify all tested FE producing bacteria at least down to the genus level. This was also confirmed by additional phylogenetic analyses. Regarding the sensitivity, the conventional PCR method amplifying the 16-rRNA region was able to reach an estimated concentration of 460 CFU/reaction. This suggests that the application of this strategy would be appropriate to detect contaminations at high levels and to identify the genus/species of isolated bacteria. In order to detect contaminations at lower levels, other approaches would need to be applied, such as specific qPCR's targeting genera or species of the FE producing bacterial strains. The applicability

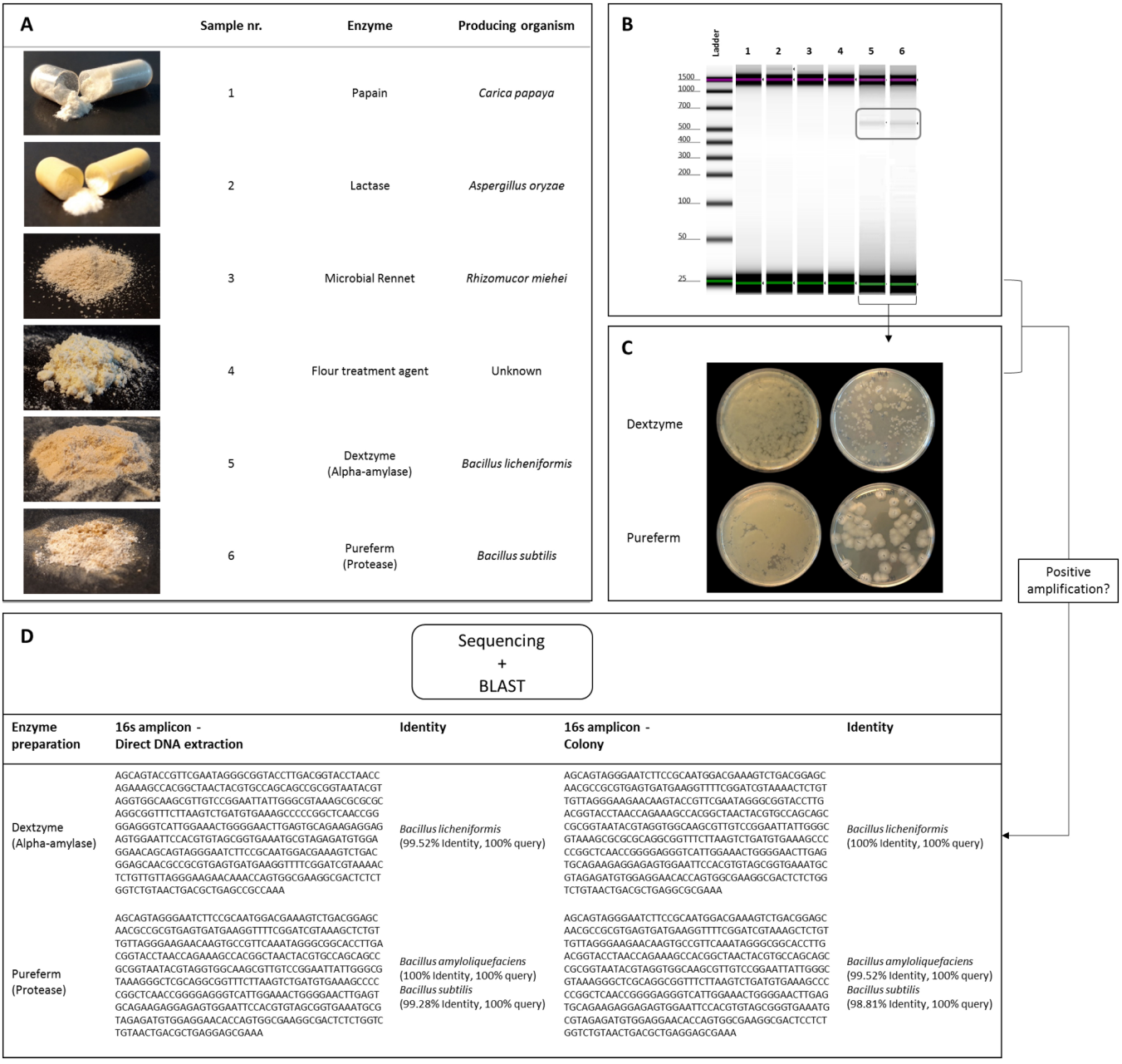

Fig. 3. Overview of the applicability assessment of the proposed generic strategy of first line screening.
A: All tested FE preparations with their corresponding producer organism.
B: Visualization of the generated PCR products from the tested FE preparations.
C: Bacteria viability assessment of the two FE preparations showing a positive PCR amplification (B).
D: Analysis of the generated sequences, of the PCR products from the two FE preparations showing a positive PCR amplification (B) and of the viable colonies observed in (C).

of the proposed generic strategy was also successfully tested using commercially available FE preparations. These results have allowed to highlight the contamination of *Bacillus* species in two FE preparations. Given that these bacterial species were mentioned as FE producer for these two FE preparations, these results strongly suggest an accidental contamination of the FE producing bacterial strains, even though an environmental microbial contamination cannot be excluded. However, after this first line screening, additional analyses using or PCR markers, specific to certain bacterial species and genetically modified organisms, or whole genome sequencing approaches (Barbau-piednoir, De Keersmaecker, Delvoye, et al., 2015; Barbau-piednoir, De Keersmaecker, Wuyts, et al., 2015; Paracchini et al., 2017) would need to be performed for a deeper characterization and to confirm the presence of the FE producer micro-organisms, or its corresponding DNA, in the tested sample. In any case, the results obtained in this study clearly demonstrate the added value of the proposed generic strategy of first line screening to detect the accidental contamination of bacteria, genetically modified or not, in FE preparations.

Our proposed new generic strategy, adequate as a first screening to verify the potential presence of FE producing bacteria, could also be applied on related microbial fermentation products such as food and feed additives and flavouring products. However, for such products, it would be needed to extend the spectra of species specifically used for their production.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The research that yielded these results, was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract [RT 17/5 SPECENZYM] and by the NRL-GMO (Sciensano, Belgium).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125431.

References

Anadón, A., Bell, D., Binderup, M., Bursch, W., Castle, L., Engel, K., … Wölfle, D. (2009). Guidance of the Scientific Panel of Food Contact Materials, Enzymes, Flavourings and Processing Aids (CEF) on the submission of a dossier on food enzymes for safety evaluation by the Scientific Panel of Food Contact Material, Enzymes, Flavourings an, 1305(2009), 1–26.

Barbau-piednoir, E., De Keersmaecker, S. C. J., Delvoye, M., Gau, C., Philipp, P., & Roosens, N. H. (2015). Use of next generation sequencing data to develop a qPCR method for specific detection of EU-unauthorized genetically modified Bacillus subtilis overproducing riboflavin. *BMC Biotechnology, 15*(1), 1–10. https://doi.org/10.1186/s12896-015-0216-y.

Barbau-piednoir, E., De Keersmaecker, S. C. J., Wuyts, V., Gau, C., Pirovano, W., Costessi, A., … Roosens, N. H. (2015). Genome sequence of EU-unauthorized genetically modified Bacillus subtilis strain 2014-3557 overproducing riboflavin, isolated from a vitamin B2 80% feed additive. Genome Announcements, 3(2). doi:https://doi.org/10.1128/genomeA.00214-15.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., & Madden, T. L. (2009). BLAST+: Architecture and applications. *BMC Bioinformatics, 10*, 421. https://doi.org/10.1186/1471-2105-10-421.

Cerutti, G., Boudot, J., Bournigal, J.-M., & Rousseau, L. (2006). Arrêté du 19 octobre 2006 relatif à l'emploi d'auxiliaires technologiques dans la fabrication de certaines denrées alimentaires. Retrieved from https://www.legifrance.gouv.fr/affichTexte.do?cidTexte=LEGITEXT000020667468#LEGISCTA000020667473.

Commission of the European Communities. (1991). Guidelines for the presentation of data on food enzymes.

COMMISSION REGULATION (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council. (2012). Official Journal of the European Union (Vol. 22).

De Clerck, E., Van Mol, K., Jannes, G., Rossau, R., & De Vos, P. (2004). Design of a 5′ exonuclease-based real-time PCR assay for simultaneous detection of Bacillus licheniformis, members of the "B. cereus group" and B. fumarioli in gelatine. *Letters in Applied Microbiology, 39*(1), 109–115. https://doi.org/10.1111/j.1472-765X.2004.01550.x.

Dorn-In, S., Bassitta, R., Schwaiger, K., Bauer, J., & Hölzel, C. S. (2015). Specific amplification of bacterial DNA by optimized so-called universal bacterial primers in samples rich of plant DNA. *Journal of Microbiological Methods, 113*, 50–56. https://doi.org/10.1016/j.mimet.2015.04.001.

European Commission. (2016). Food enzyme applications submitted to the Commission within the legal deadline (from 11 September 2011 to 11 March 2015). Retrieved from http://oxfordhandbooks.com/view/10.1093/oxfordhb/9780199546282.001.0001/oxfordhb-9780199546282-e-24.

European Parliament and Council (2008). Regulation (EC) No 1331/2008 of the European Parliament and of the Council of 16 December 2008 establishing a common authorisation procedure for food additives, food enzymes and food flavourings. *Official Journal of the European Union, 354*, 16–33. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:354:0001:0006:EN:PDF.

European Parliament and Council of the European Union. (2008). Regulation (EC) No 1332/2008 of the European Parliament and of the Council of 16 December 2008 on food enzymes and amending Council Directive 83/417/EEC, Council Regulation (EC) No 1493/1999, Directive 2000/13/EC, Council Directive 2001/112/EC and(..). Official Journal of the European Union L 354, 51(1332), 7–15.

European Parliament and the Concil of the European Union. (2008). Regulation (EC) No 1333/2008 of the European Parliament ans of the Council of 16 December 2998 on food additives. Official Journal of the European Union, (L 354), 16–33.

Fontana, C., Favaro, M., Pelliccioni, M., Pistoia, E. S., & Favalli, C. (2005). Use of the MicroSeq 500 16S rRNA gene-based sequencing for identification of bacterial isolates that commercial automated systems failed to identify correctly. *Journal of Clinical Microbiology, 43*(2), 615–619. https://doi.org/10.1128/JCM.43.2.615.

Fraiture, M. A., Herman, P., Taverniers, I., De Loose, M., Deforce, D., & Roosens, N. H. (2014). An innovative and integrated approach based on DNA walking to identify unauthorized GMOs. *Food Chemistry, 147*, 60–69.

Janda, J. M., & Abbott, S. L. (2007). Minireview. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: Pluses, perils, and pitfalls. Journal of Clinical Microbiology, 45(9), 2761–2764. doi:https://doi.org/10.1128/JCM.01228-07.

JEFCA (2006). *Compendium of food additive specifications*.

Lebonah, D. E., Dileep, A., Chandrasekhar, K., Sreevani, S., Sreedevi, B., & Kumari, J. P. (2014). DNA barcoding on bacteria: A review. *Advances in Biology, 2014*, 9.

Magnuson, B., Munro, I., Abbot, P., Baldwin, N., Lopez-Garcia, R., Ly, K., ... Socolovsky, S. (2013). Review of the regulation and safety assessment of food substances in various countries and jurisdictions. *Food Additives and Contaminants - Part A Chemistry, Analysis, Control, Exposure and Risk Assessment, 30*(7), 1147–1220. Retrieved from http://ovidsp.ovid.com/ovidweb.cgi?T=JS&PAGE=reference&D=emed11&NEWS=N&AN=2013434205.

Mignard, S., & Flandrois, J. P. (2006). 16S rRNA sequencing in routine bacterial identification: A 30-month experiment. *Journal of Microbiological Methods, 67*, 574–581. https://doi.org/10.1016/j.mimet.2006.05.009.

O'Leary, N. A., Wright, M. W., Brister, J. R., Ciufo, S., Haddad, D., McVeigh, R., ... Pruitt, K. D. (2016). Reference sequence (RefSeq) database at NCBI: Current status, taxonomic expansion, and functional annotation. *Nucleic Acids Research, 44*(D1), D733–D745. https://doi.org/10.1093/nar/gkv1189.

Paracchini, V., Petrillo, M., Reiting, R., Angers-Loustau, A., Wahler, D., Stolz, A., ... Grohmann, L. (2017). Molecular characterization of an unauthorized genetically modified Bacillus subtilis production strain identified in a vitamin B2feed additive. *Food Chemistry, 230*, 681–689. https://doi.org/10.1016/j.foodchem.2017.03.042.

Pariza, M. W., & Johnson, E. A. (2001). Evaluating the safety of microbial enzyme preparations used in food processing: Update for a new century. *Regulatory Toxicology and Pharmacology, 33*(2), 173–186. https://doi.org/10.1006/rtph.2001.1466.

RASFF portal https://webgate.ec.europa.eu/rasff-window/portal/?event=SearchForm&cleanSearch=1.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., … Pandey, A. (2018). Applications of microbial enzymes in food industry. Food Technology and Biotechnology, 56(1), 16–30. doi:10.17113/ftb.56.01.18.5491.

Regulation (EU) 2015/2283 of the European Parliament and of the Council of 25 November 2015 on novel foods, amending Regulation (EU) No 1169/2011 of the European Parliament and of the Council and repealing Regulation (EC) No 258/97 of the European Parliament and of the Council and Commission Regulation (EC) No 1852/2001 (text with EEA relevance).

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Girones, R., … Salvador, P. (2017). Scientific opinion on the update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA * (Vol. 15). doi:https://doi.org/10.2903/j.efsa.2017.4664.

Rozwandowicz, M., Brouwer, M. S. M., Fischer, J., Wagenaar, J. A., Gonzalez-Zorn, B., Guerra, B., ... Hordijk, J. (2018). Plasmids carrying antimicrobial resistance genes in Enterobacteriaceae. *Journal of Antimicrobial Chemotherapy, 73*(5), 1121–1137. https://doi.org/10.1093/jac/dkx488.

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. D. L., Bories, G., ... Galobart, J. (2018). *Guidance on the characterisation of microorganisms used as feed additives or as production organisms, 16(February), 1–24.* https://doi.org/10.2903/j.efsa.2018.5206.

Singh, R., Kumar, M., Mittal, A., & Kumar, P. (2016). Microbial enzymes: Industrial progress in 21st century. 3. *Biotech, 6*(2), 1–15. https://doi.org/10.1007/s13205-016-0485-8.

Spök, A. (2006). Safety regulations of food enzymes. *Food Technology and Biotechnology, 44*.

Srinivasan, R., Karaoz, U., Volegova, M., MacKichan, J., Kato-Maeda, M., Miller, S., ... Lynch, S. V. (2015). Use of 16S rRNA gene for identification of a broad range of clinically relevant bacterial pathogens. *PLoS One, 10*(2), 1–22. https://doi.org/10.1371/journal.pone.0117617.

Standing Committee on the Food Chain and Animal Health. (2005). Section on genetically modified food and feed. https://ec.europa.eu/food/sites/food/files/plant/docs/sc_modif-genet_summary02_en.pdf.

West-Barnette, S., & Srinivasan, J. R. (2013). Manufacturing process and composition. In P. M. Visakh, S. Thomas, L. B. Iturriaga, & D. R. Pablo (Eds.), Advances in food science and technology (pp. 219–222).

Xiong, W., Wang, Y., Sun, Y., Ma, L., Zeng, Q., Jiang, X., ... Zhang, T. (2018). Antibiotic-mediated changes in the fecal microbiome of broiler chickens define the incidence of antibiotic resistance genes. *Microbiome, 6*(1), 34. https://doi.org/10.1186/s40168-018-0419-2.