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ACCEPTED MANUSCRIPT

When regulation challenges innovation: the case of the genus *Lactobacillus*

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Abstract

The majority of probiotic bacteria belong to the genus *Lactobacillus* which includes a large number of safe species integral to fermented food production.

In the European Union the conversion of ensuing data into successful claims that are compliant with regulatory requirements has proved difficult. Furthermore, the study of lactobacilli has been challenging because of their phenotypic and genomic diversity.

Here issues pertaining to the marketing authorization of novel foods and probiotics are outlined, taking *Lactobacillus* genus as reference.

We highlight the drawbacks regarding the taxonomic characterization and the safety assessment of these bacteria and the validation of their beneficial mechanisms.

Keywords: probiotics, *Lactobacillus*, legislation, safety, characterization, substantiation

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Background

In recent decades the Western diet has dramatically changed, being now characterized by high amounts of processed foods, refined sugars, refined fats and oils. This dietary shift has contributed to the increased incidence of chronic diseases such as type II diabetes, coronary heart disease and some cancers (Tilman and Clarke, 2014). To tackle the scale of this social problem, the European Union has been promoting actions that aim to meet the consumers' need for safe, healthy, high quality and affordable food, and developing new dietary solutions and innovations focused on preventing chronic diseases and disorders (https://ec.europa.eu/programmes/horizon2020/en/h2020-section/societal-challenges).

Although a number of novel functional foods have recently been introduced in the market, probiotics still remain the most popular. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Hill *et al*., 2014; FAO/WHO, 2001). Many organisms now considered probiotic have traditionally been used as starter cultures in the manufacture of fermented foods. Probiotics available today comprise a much broader range of products including pharmaceuticals, a large variety of foods including juices, nutrition bars, infant formulas, relishes and condiments, sweeteners, waters, pizza crust, and other products such as gum, lozenges, dietary supplements, toothpaste, and cosmetics (Hoffman *et al*., 2014).

The health and wellness claims associated with probiotics have led consumer demand for these products to grow at a fast pace: the market for probiotic ingredients is projected to reach USD 46.55 billion by 2020, with Europe and the Asian-Pacific region estimated to be the largest and the fastest-growing markets, respectively (http://www.marketsandmarkets.com/PressReleases/probiotics.asp).

The lack of a well-established regulatory status of probiotic products at international level has led some manufacturers to market probiotic products in Europe without any pre-market approval (Caselli *et al*., 2013). This has led to the misuse of the term "probiotic", which have been used for some foods in Europe even in the absence of an approved health claim (Sanders, 2015; Katan *et al*., 2012).

Despite the fact that the European food industry has guidelines governing how to produce and market probiotic products, and the EU recognises probiotic bacteria as having the status of nutrients (EU regulation 1924/2006), substantial confusion reigns due to the application to probiotic foods of regulatory schemes initially designed to regulate pharmaceutical

development (reviewed in Hill *et al*., 2014). Different policies are used in the Member states which result in a lack of clear recommendations for the appropriate and accurate communication of probiotic statements to the different stakeholders including researchers, industries, legislators, consumers and health-care professionals, who are responsible for the different steps of bringing probiotic to the consumer (Van Buul and Brouns, 2015).

At the same time as probiotics proliferate in the market, policy makers and regulators are simultaneously, and usually on an *ad hoc* basis, trying to critically develop the most appropriate regulatory structure for probiotics, which needs on the one hand to be rigorous in defining the level of accuracy required in claim dossiers, but on the other hand needs to be flexible enough to stimulate research and innovation, and thus encourage the release of new health-promoting products (Hoffman *et al*., 2014). The second part of this paradigm is arguably not working.

The approval of health claims for probiotic-containing foods by the European Food Safety Authority (EFSA), which was appointed by the EU to provide scientific opinion on candidate claims and to protect the consumer from misleading information, has become very challenging due to the requirements for validating probiotic mechanisms in the target consumer, for proper strain characterization, and for conformity to required product characteristics (EFSA, 2016b; Miquel *et al*., 2015). Although a large volume of data about the beneficial effects of some probiotics has been obtained, precise mechanisms of probiotic action remain largely elusive except for a few examples, and thus the conversion into actual claims and compliance with the regulatory requirements in particular regions have proved difficult.

Probiotic properties of *Lactobacillus* species include competitive exclusion of medically significant pathogens (Kanmani *et al*., 2013); immune system modulation (Klaenhammer *et al*., 2012), and the reduction of antibiotic therapy side effects (Lönnermark *et al*., 2010). From a regulatory point of view, the *Lactobacillus* genus includes 36 species that have been assigned Qualified Presumption of Safety (QPS) status by EFSA (EFSA, 2016a) and 12 species are Generally Recognised as Safe (GRAS) by the U.S. Food and Drug Administration (FDA) (http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices). This means that they are suitable to be used as food/feed additives and they do not need *a priori* risk assessment.

Furthermore, lactobacilli constitute 43% (84 species) of the total number of microorganisms with certified beneficial use (195 species representing 28 genera of phyla Actinobacteria,

Firmicutes and Proteobacteria), (Bourdichon *et al.*, 2012), with 22 of them represented by strains that are patented in Europe due to their potential probiotic properties (Table 1). Despite their particular relevance, exploiting lactobacilli has always been very challenging due to their unusual phenotypic and genotypic diversity, unclear species identity and uncertain degree of relatedness between them and other commercially important lactic acid bacteria (Sun *et al*., 2015).

In 2015, the genome sequences of almost all *Lactobacillus* type strains and some historically associated genera were determined (Sun *et al*., 2015; Zheng *et al*., 2015), thus providing a definitive genomic resource for mining all relevant phylogenetic and functional information. This data repository should also prove useful for understanding the species-restricted distribution of probiotic traits, thus supporting probiotic claim substantiation.

Despite the unprecedented availability of genome sequences and increasing functional information about lactobacilli, the development of functional products containing these bacteria is challenged by the laborious nature of currently prescribed taxonomic characterization, the shortcomings regarding the validation of their beneficial mechanisms, and the drawbacks attached to determining their safety for consumption, issues that we will now expand upon.

Taxonomic characterization of *Lactobacillus* probiotics

Isolation and the full characterization of a candidate probiotic is the first essential requirement for a novel food marketing authorization and a health claim submission (EFSA, 2017; EFSA 2016b). The taxonomic determination of the genus, the species and the strain contained in a probiotic product provides useful preliminary information regarding the main physiological and metabolic properties of the organism, and allows its discrimination from other closely related but potentially non-beneficial strains (ILSI 2013).

The ideal characterization of microorganisms should include both genotypic and phenotypic tests; the combination of these data strands allows identity of the microorganism at both the species and strain level (EFSA, 2015).

Taking account of the current state-of-the-art techniques for identification and molecular characterisation of microorganisms, EFSA recommends sequence analysis of at least two robust taxonomic markers (i.e. 16S rRNA gene sequence) or fully assembled and validated whole-genome sequence analysis for species identification. Genome sequencing is also suggested for strain typing, but this can also be achieved by other internationally accepted genetic typing molecular methods like whole genome mapping (WGM) or optical mapping

analysis. The bacterium is considered to be sufficiently characterised only when these two criteria are fulfilled. In addition, the EFSA advocates that the strain is deposited in at least one recognised international culture collection and encourages naming of strains according to the International Code of Nomenclature (EFSA, 2016b).

The widespread use and characterization of lactobacilli are both hindered by the complex taxonomic structure of the genus, reflected in a poor correlation between the phylogenetic relationship and the physiological properties of *Lactobacillus* species (Zheng *et al*., 2015). Moreover, the ongoing description of novel species, whose number increased from 152 (Salvetti *et al*., 2012) to more than 190 in the last 3 years (http://www.bacterio.net/lactobacillus.html), has resulted in significant taxonomy changes within the genus, causing confusion and leading to the mis-identification of lactobacilli. Although 16S rRNA gene sequence analysis is the standard method for *Lactobacillus* species identification thanks in part to the availability of up-to-date and internationally recognised databases (ie. EzTaxon, http://www.ezbiocloud.net/eztaxon), there are still shortcomings to this approach, such as the low taxonomic resolution afforded by 16S rRNA gene comparison especially when trying to separate closely related species (i.e *Lb. plantarum/Lb. paraplantarum/Lb. pentosus* or *Lb casei*/*Lb. paracasei*/*Lb. rhamnosus*). To overcome this, housekeeping genes as *pheS*, *rpoA* (Naser *et al*., 2007) and *recA* (Torriani *et al*., 2001) have been used as alternative phylogenetic markers which provide a higher discrimination between lactobacilli. Although the application of these molecular markers offers useful potential in the probiotic field, data interpretations by taxonomic experts remains crucial to ensure reliability of the identification results (Sanders *et al*., 2010).

When the genomes of the type strains of around 175 *Lactobacillus* species were recently sequenced (Sun *et al*., 2015; Zheng *et al*., 2015), the ensuing analysis of the Average Nucleotide Identity (ANI) and the phylogenomics based on the core genes showed that the genus *Lactobacillus* is paraphyletic, intermixed with other five genera of order Lactobacillales (*Pediococcus, Weissella, Leuconostoc, Oenococcus* and *Fructobacillus*) and displaying a level of genomic diversity that is larger than that which is typical for a taxonomic family (Sun *et al*., 2015). Thus the (currently defined) genus *Lactobacillus* presents problems for strain and species distinction at short phylogenetic range, and problems for clade distinction at long phylogenetic range. None of this has aided providing industries, regulators or consumers with confident identification of commercial strains, as evidenced by some notable re-naming of high-profile strains such as La1 (Ashraf and Shah, 2014).

The vast genomic diversity of the genus *Lactobacillus* and its polyphyletic structure strongly suggests to us the necessity for the formal revaluation of its taxonomic scheme and its feasibility to be split in more homogeneous genera (Sun *et al*., 2015; Salvetti *et al*., 2012). The creation of more uniform taxonomic nuclei within the *Lactobacillus* genus is also expected to help prevent mis-identification issues which are still the major cause of mislabelling of probiotic food products reported worldwide (Hill *et al*., 2016; Van Loveren *et al*., 2012). This is not only essential to protect consumers from incorrect information, as food marketers sometimes give their probiotic strains trade names such as '*Lactobacillus immunitas'* or '*Lactobacillus defensis'* (Katan *et al*., 2012), but also for correct scientific communication and knowledge exchange between regulatory agencies and health-care providers.

Considering the data summarized in Table 1, it is noteworthy that incorrect names are enshrined both in the QPS list of EFSA and in the GRAS notices of the FDA such as *Lb. cellobiosus* (which should be replaced by *Lb*. *fermentum*) or *Lb*. *casei* subsp. *rhamnosus* (which is now *Lb*. *rhamnosus*)Furthermore, incorrect and trade/proprietary names are also found in the page dedicated to "*Lactobacillus*" in the MedlinePlus website, the National Institutes of Health's website for patients and health-care providers: (https://www.nlm.nih.gov/medlineplus/druginfo/natural/790.html).

Probiotic stakeholders are encouraged to refer to international organisations such as the Subcommittee on the Taxonomy of *Bifidobacterium*, *Lactobacillus* and related organisms (http://icsp.org/subcommittee/bifidobacterium_lactobacillus/) which provides the probiotic community with updated classification tools for research and application of *Lactobacillus* probiotics (Mattarelli *et al*., 2014), as well as the International Life Science Institute, the International Scientific Association for Probiotics and Prebiotics, the International Dairy Federation, the European Food and Feed Culture Association, whose panels of experts can advise which technique is necessary and sufficient so that probiotic strains are correctly labelled and ensure clear communication between stakeholders involved.

Validation of the probiotic potential of *Lactobacillus* spp.

Approval of probiotic claims requires a full analysis of the mechanism(s) of action which is usually accomplished through a combination of *in vitro* and *in vivo* screening assays and "omics" technologies (Papadimitriou *et al*., 2015).)

Powerful genetic and omics analyses have allowed the investigation of the molecular mechanisms that underpin probiotic properties and unveiled a plethora of genes as potential markers for the identification of probiotic strains, including genes/proteins involved in stress

response (acid and bile), adhesion, metabolism of human milk oligosaccharides and mucus, modulation of the immune system, production of antimicrobial compounds, quorum sensing, production of nutrients and other beneficial processes such as the metabolism of prebiotics (Ventura *et al*,. 2009; Lebeer *et al*., 2008).

Validation of genome-based analysis with experimental approaches is necessary to link annotated gene sequences to their predicted traits, and this also represents a prerequisite for the construction of databases of probiotic markers with translational predictive value. Although the molecular analyses of probiotic properties do not entirely substitute for experimental tests, *in silico* approaches constitute an important step in the development of more efficient and precise screens for probiotic strains.

EFSA approves health claims if the substantiation is based on generally accepted scientific evidence, using an assessment process of the highest possible standard (EC No. 1924/2006). The approach adopted shall consist primarily of human studies and according to a hierarchy of study designs which supports the relative strength of evidence (EC. No. 353/2008). Although a workflow of the key steps in the process of authorisation of health claims made on foods is outlined by EFSA (EFSA, 2017; EFSA, 2016b), no official procedures or workflows for selecting probiotics are available (i.e. validated biomarkers for *in vitro* screening) and this makes it difficult to determine the real probiotic potential of microorganisms and the physiological effect they exert.

The lack of sufficient efficacy data has undermined the acceptance of health claim dossiers: in the foodprobiotic area, none of over 300 nutrition and health claims submitted to EFSA since 2009 was considered sufficiently substantiated (http://ec.europa.eu/nuhclaims/)(Glanville *et al*., 2015).

In addition, successful probiotic claim substantiation is also impeded by EU laws which do not recognise the possibility that food can prevent, treat or cure a disease, leaving scientists, marketers, food producers and also legislators in an ambiguous impasse (Katan *et al*., 2012). In a recent attempt to solve these issues, EFSA released updated general scientific guidance for stakeholders on health claim applications in which the Panel on Dietetic Products, Nutrition and Allergies (NDA) outlines the principles to be applied for the scientific evaluation of health claim applications and the issues to be considered by applicants for the compilation of applications (EFSA, 2017; EFSA, 2016b).

Furthermore, the EFSA also updated the guidance document on the scientific requirements for the substantiation of health claims related to gut and immune function (EFSA, 2016c; EFSA, 2015) where it provides clearer definitions of the supporting evidence required for

health claims applied to food products, the reproducibility and consistency of the effect of the constituent for which a health claim is proposed, the definition of physiological effect in the context of food and the use of authorised health claims by stakeholders. Focusing on the characterisation of the claimed effect of the constituent (including probiotic microorganisms); the EFSA opinion specifically highlights the fact that data on genetic regions derived through whole genome sequencing, in combination with other experiments performed *in vivo*, is a solid approach to characterise the mechanisms at the basis of a specific function or health benefit (EFSA, 2015).

Lactobacilli occupy a particular position in this context: of the submitted nutritional and health claim applications mentioned above, 264 submissions (all of them rejected by EFSA) include strains belonging to 15 *Lactobacillus* species, either developed as sole active ingredients or in combination with other microorganisms, which in turn refer to the functioning of nine specific organs and systems, in particular the gut (61% of the claims with *Lactobacillus* strains) (Figure 1).

The most numerous species among these applications are *Lb. plantarum* (28%), *Lb. paracasei* (11%), *Lb. rhamnosus* (10%) and *Lb. casei* (10%): a review of the literature in PubMed showed that strains belonging to these species for which a claim has been submitted are cited in more than 700 papers, with *L. rhamnosus* GG and *L. casei* Shirota covering more than 200 papers each.

Although the genus *Lactobacillus* is one of the most investigated genera in food microbiology and human nutrition, surprisingly only 7-8% of the *Lactobacillus* species (15 species out of more than 190) have been formally explored as probiotics by way of a health claim submitted to the regulatory agencies.

A detailed analysis of the nutrition and health claims that feature *Lactobacillus* strains revealed that the main reasons of rejection were i) insufficient characterization of the food and poor scientific assessment of the claimed effect (i.e. *Lb. plantarum* 299; EFSA, 2010), ii) the absence of a beneficial physiological effect based on the scientific evidence assessed (*Lb. acidophilus* NCFM ATCC SD5221; EFSA, 2011) iii) the non-recognition of the property of preventing, treating or curing a human disease to food (i.e. *Lb. paracasei* LPC 01; EFSA, 2012a).

Since the majority of the nutrition and health claims that involve lactobacilli target the functioning of the gastrointestinal tract and the improvement of gut health, the application of the novel directives provided in the recent guidances by EFSA is expected to support the

successful resubmission of these claims and may allow the marketing authorization of new *Lactobacillus* products.

In this framework, the genome of *Lactobacillus* type strains (Sun *et al*., 2015) and probiotic strains (e.g. *Lb. rhamnosus* GG, Kankainen *et al*., 2009) constitute a solid basis for claim substantiation in combination with *in vivo* approaches (as suggested by EFSA), but they also expand the pool of *Lactobacillus* species to be investigated as probiotics (i.e. other *Lactobacillus* species isolated from humans such as *Lb. gastricus*, *Lb. antri* or *Lb. kalixensis* (Roos *et al*., 2005) and contribute to the creation of a custom database of *Lactobacillus* probiosis marker genes.

Finally, defining the mechanisms of probiotic action through genome-based analysis may also be useful for the optimization of some critical parameters during the industrial process: the production of bioactive metabolites, in fact, can be predicted from the genome sequence, facilitating construction of metabolic models that incorporate the biochemical reactions of an organism together with information on biomass assembly reaction and exchange fluxes with the external enviroment (Fondi *et al*., 2015).

The development of such a strategy allows predictive modelling of optimal industrial conditions to be used, facilitating the selection and optimization of probiotics and/or beneficial compounds production (Saulnier *et al*., 2011).

Safety assessment of *Lactobacillus* species

The safety of probiotics is linked to their intended use, the potential vulnerability of the consumer or patient, the dose and duration of consumption and both the manner and frequency of administration.

In the EU, *a priori* safety is generally accepted for microorganisms that have been awarded QPS status. Microorganisms that have not been used in food in Europe prior to 1997 must to be assessed for their safe use before being authorized for sale on the European market, as stated by the UE 97/618/EC recommendation and regulation N. 258/97.

In 2010, Sanders and colleagues described the factors that should be addressed to assess the safety of probiotics, in particular i) the immunological effects in certain vulnerable populations including the immunocompromised, the critically ill, patients with inflammatory bowel disease and full-term or premature infants with undeveloped immune functions; and ii) the microbiological and metabolic issues, including the correct identification and labelling of probiotic bacteria, the evidence for their long-term colonization of the host, the assessment of antibiotic resistance and its transferability, their genetic stability and viability, their

pathogenicity/toxicogenicity, and their ability to produce biogenic amines (Sanders *et al*., 2010).

More recently, Miquel and colleagues (2015) reported an updated list of criteria considered as essential for the safety of probiotic products (required for both novel food and health claim regulation) including the survival in GI tract conditions, preservation of the homeostasis of the gut barrier components, adhesion and translocation risk, and metabolic and other remote effects (such as genotoxicity and platelet aggregation) (Miquel *et al*., 2015).

It is evident that the lack of the mechanistic understanding of probiotic activity together with incorrect species identification and mislabelling of probiotics (discussed above) is a major drawback for the prediction of safety of a probiotic intervention and for the creation of an exhaustive list of criteria to be assessed (Sanders *et al*., 2010).

Due to these shortcomings, the biological relevance of the requirements listed above is still the subject of debate and no formal guidance exists for the safety assessment of probiotic bacteria (Miquel *et al*., 2015).

As already mentioned, the majority of *Lactobacillus* species have a long history of apparently safe use in industrial and agricultural applications; moreover, they are among the dominant populations in microbial communities of traditional fermented foods and are part of natural starter cultures. Despite being occasionally involved in human diseases (like bacteremia and/or systemic septicaemia in already immunocompromised patients), the daily consumption of large quantities of lactobacilli in a variety of fermented foods by people of all ages and health statuses apparently does not have ill effects, and they have generally been considered to be non-pathogenic (EFSA, 2007).

However, several intrinsic properties of lactobacilli related to their metabolic activities may be implicated in human health risk, such as the production of biogenic amines (histamine, tyramine, and others), bile salt deconjugase activity, enzymatic activities which may have undesirable toxicological effects (like azoreductases and nitroreductases), the degradation of hyaluronic acid, the platelet aggregation activity (Collins *et al*., 2012), or the colonization and the production of toxic metabolites (Bernardeau *et al*., 2008). In addition, a considerable number of antibiotic resistant lactobacilli has been reported, which could theoretically act as donors or reservoirs for antibiotic resistance genes, thus with the potential risk of transferring the genes to pathogenic bacteria in the food matrices as well as in the gastrointestinal tract.

The lack of official guidance for the safety assessment for *Lactobacillus* species with intended use as food or feed additives has led to the release of papers that address this issue inconsistently: in fact a search in PubMed shows publications that report different

combinations of assays (from genome-based techniques and phenotypic assays, to the use of mouse models and human clinical trials) providing only partial safety estimations which are also difficult to compare.

Because apparently no particular safety concerns exist for lactobacilli for use in the general population in foods at typical consumption levels, EFSA recommends that *Lactobacillus* strains be assessed for their susceptibility to antibiotics: in the guidance released in 2012, EFSA reports the Minimum Inhibitory Concentrations (MIC) cut-off values for nine antibiotics (ampicillin, vancomycin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline and chloramphenicol) to define if the lactobacilli being used are susceptible or resistant to antimicrobials and thus their suitability for use as feed/food additives. In addition, EFSA proposes a scheme for antimicrobial resistance assessment including the analysis of the distribution of known antimicrobial resistance genes, based upon the Antibiotic Resistance database (ARDB, http://ardb.cbcb.umd.edu/) (EFSA, 2012b). Despite the presence of this specific guidance, some drawbacks still exist: the cut-off values are reported for only some groups of lactobacilli and not at species-by-species level (Goldstein *et al*., 2015), while the ARDB is an obsolete tool as it was most recently updated in 2009.

Thanks to the recent explosion in the genome sequencing of microorganisms, other databases have been developed like the Comprehensive Antibiotic Resistance database (CARD, arpcard.mcmaster.ca/) and the Virulence Factor database (VFDB, http://www.mgc.ac.cn/VFs/), which, on one hand, allow the fast detection of putative antibiotic resistance genes or virulence factors, but, on the other hand, a big effort is required to assess if the “hits” or determinants identified in a given genome sequence represent a real safety concern. In fact, the trait of adhesion to the host is a virulence factor in pathogenic bacteria, but it may represent a marker of probiosis in health-promoting microorganisms. Furthermore, many traits considered as virulence determinants in the VFDB are mis-annotated (e.g. proteins with membrane-spanning alpha helices may be mis-identified as toxins, and ATP-binding cassette proteins may be flagged simply because this class of proteins is associated with some virulence loci in pathogens).

To tackle this particular issue in future, the availability of the genome sequences of all *Lactobacillus* type strains will be an invaluable resource for the forensic detection of *bona fide* antibiotic resistance determinants, virulence factors or other genes responsible for undesirable metabolite production in lactobacilli. The parallel execution of phenotypic assays on all lactobacilli such the determination of the antibiotic MIC will allow robust genotype-

phenotype matching for the first time across the whole genus. Similarly, assays for traits such as the decarboxylase activity linked with biogenic amines production compared to genomic searches for the relevant determinants can provide a more robust body of knowledge upon which more specific databases for the analysis of the safety of lactobacilli can be developed. In addition to supporting researchers and scientists in achieving much more consistent data on *Lactobacillus* safety, these tools can also help regulatory agencies to define more precise recommendations (for instance, revised MIC cut-off values for all *Lactobacillus* species, if appropriate), which would be useful for the safe marketing authorization of new products containing lactobacilli.

Conclusions

In this perspective we highlight drawbacks in the scientific approach and the regulatory procedure to obtain market authorization for probiotics, taking the genus *Lactobacillus* as a reference. We believe that the unprecedented availability of the genomic, phenotypic and functional data of *Lactobacillus* strains (including type strains, non-probiotic strains, probiotic strains, and widely used starter cultures) represents the ideal resource for the development of new and more focused scientific protocols and regulatory procedure to assess the safety and the beneficial effects of *Lactobacillus* probiotics and for successful health claim substantiation. This compliance could then be further used as a *rationale* for probiotic microorganisms belonging to other genera as *Bifidobacterium* or *Bacillus*.

Such a straightforward regulatory system would stimulate more systematic research and innovation in probiotics, ensure effective communication of probiotic knowledge to consumers and health-care providers, and strengthen their confidence in probiotic and health claims through coherent recommendations and product labels, and finally improve the industry with high-quality and profitable products (Sanders *et al*., 2015).

A well-established framework for regulation and authorization of existing probiotics whereby the stakeholders agree almost unanimously is also necessary to face the next challenge for the market authorization of the next-generation probiotics belonging to ‘unconventional’ species isolated from the human gut, such as *Faecalibacterium prausnitzii*, *Akkermansia muciniphila*, and *Eubacterium hallii*, identified through our growing understanding of the composition of the gut microbiota and its role in health and disease.

Conflict of interest

The authors declare no conflict of interest.

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References

Ashraf, R., & Shah, N. P. (2014). Immune system stimulation by probiotic microorganisms. *Critical Reviews in Food Science and Nutrition*, *54*, 938-956.

Bernardeau, M., Vernoux, J. P., Henri-Dubernet, S., & Guéguen, M. (2008). Safety assessment of dairy microorganisms: the *Lactobacillus* genus. *International Journal of Food Microbiology*, *126*, 278-285.

Bourdichon, F., Casaregola, S., Farrokh, C., Frisvad, J. C., Gerds, M. L., Hammes, W. P., Harnett, J., Huys, G., Laulund, S., Ouwehand, A., Powell, I. B., Prajapati, J. B., Seto, Y., Ter Schure, E., Van Boven, A., Vankerckhoven, V., Zgoda, A., Tuijtelaars, S., & Hansen, E. B. (2012). Food fermentations: Microorganisms with technological beneficial use. *International Journal of Food Microbiology*, *154*, 87-97.

Caselli, M., Cassol, F., Calò, G., Holton, J., Zuliani, G., & Gasbarrini, A. (2013). Actual concepts of "probiotics": is it more functional to science or business? *World Journal of Gastroenterology*, *19*, 1527-1540.

Collins, J., van Pijkeren, J. P., Svensson, L., Claesson, M. J., Sturme, M., Li, Y., Cooney, J. C., van Sinderen, D., Walker, A. W., Parkhill, J., Shannon, O., & O'Toole, P. W. (2012). Fibrinogen-binding and platelet-aggregation activities of a *Lactobacillus salivarius* septicaemia isolate are mediated by a novel fibrinogen-binding protein. *Molecular Microbiology*, *85*, 862-877.

EFSA - European Food Safety Authority. (2007). Introduction of a Qualified Presumption of Safety (QPS) approach for assessment of selected microorganisms referred to EFSA. *EFSA Journal*, *587*, 1-16.

EFSA - European Food Safety Authority. (2010). Scientific Opinion on the substantiation of health claims related to *Lactobacillus plantarum* 299 (DSM 6595, 67B) (ID 1078) and decreasing potentially pathogenic intestinal microorganisms pursuant to Article 13(1) of Regulation (EC) No 1924/20061. *EFSA Journal*, *8*, 1-14.

EFSA - European Food Safety Authority. (2011). Scientific Opinion on the substantiation of health claims related to various foods/food constituents and "immune function/immune system" (ID 573, 586, 1374, 1566, 1628, 1778, 1793, 1817, 1829, 1939, 2155, 2485, 2486, 2859, 3521, 3774, 3896), "contribution to body defences against external agents" (ID 3635), stimulation of immunological responses (ID 1479, 2064, 2075, 3139), reduction of inflammation (ID 546, 547, 641, 2505, 2862), increase in renal water elimination (ID 2505), treatment of diseases (ID 500), and increasing numbers of gastro-intestinal microorganisms (ID 762, 764, 884) pursuant to Article 13(1) of Regulation (EC) No 1924/20061. *EFSA Journal*, *8*, 1-29.

EFSA - European Food Safety Authority. (2012a). Scientific Opinion on the substantiation of health claims related to *Lactobacillus paracasei* LPC 01 (CNCM I-1390) and treatment of disease (ID 3055, further assessment) pursuant to Article 13(1) of Regulation (EC) No 1924/20061. *EFSA Journal*, *10*, 1-14.

EFSA - European Food Safety Authority. (2012b). Guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance. *EFSA Journal*, *10*, 1-10.

EFSA - European Food Safety Authority. (2015). Outcome of a public consultation on the discussion paper for the revision of the guidance on the scientific requirements for health claims related to gut and immune function. Online: http://www.efsa.europa.eu/en/supporting/pub/758e.

EFSA - European Food Safety Authority. (2016a). Update of the list of QPS-recommended biological agents intentionally added to food or feed as notified to EFSA 4: suitability of taxonomic units notified to EFSA until March 2016. *EFSA Journal*, *14*, 4522.

EFSA - European Food Safety Authority. (2016b). General scientific guidance for stakeholders on health and claim applications. *EFSA Journal, 14*, 4367.

EFSA - European Food Safety Authority. (2016c). Guidance on the scientific requirements for health claims related to the immune system, the gastrointestinal tract and defence against pathogenic microorganisms. *EFSA Journal, 14*, 4369.

EFSA - European Food Safety Authority (2017). Scientific and technical guidance for the preparation and presentation of a health claim application (Revision 2). *EFSA Journal, 15*, 4680.

FAO/WHO - Food and Agricultural Organization of the United Nations and World Health Organization. (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization [online], http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf (2001).

Fondi M., & Liò P. (2015). Genome-scale metabolic network reconstruction. *Methods in molecular biology*, *1231*, 233-256.

Glanville, J., King, S., Guarner, F., Hill, C., & Sanders, M. E. (2015). A review of the systematic review process and its applicability for use in evaluating evidence for health claims on probiotic foods in the European Union. *Nutrition Journal*, *14*, 1-9.

Goldstein, E. J., Tyrrell, K. L., & Citron, D. M. (2015). *Lactobacillus* species: taxonomic complexity and controversial susceptibilities. *Clinical Infectious Diseases*, *60*, 98-107.

Hill, C., Scott, K., Klaenhammer, T. R., Quigley, E., Sanders, M. E. (2016). Probiotic nomenclature matters. *Gut Microbes, 7,* 1-2

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., Morelli, L., Canani, R. B., Flint, H. J., Salminen, S., Calder, P. C., & Sanders, M. E. (2014). The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nature Reviews. Gastroenterology & hepatology*, *11*, 506-514.

Hoffman, D. E., Fraser, C. M., Palumbo, F., Ravel, J., Rowthorn, V., & Schwartz, J. (2014). Probiotic: achieving a better regulatory fit. *Food and Drug Law Journal*, *69*, 237-272.

ILSI – International Life Sciences Institute – Europe. (2013). Probiotics, prebiotics and gut microbiota. Online: http://www.ilsi.org/Europe/Publications/Prebiotics-Probiotics.pdf.

Kankainen, M., Paulin, L., Tynkkynen, S., von Ossowski, I., Reunanen, J., Partanen, P., Satokari, R., Vesterlund, S., Hendrickx, A. P., Lebeer, S., De Keersmaecker, S. C., Vanderleyden, J., Hämäläinen, T., Laukkanen, S., Salovuori, N., Ritari, J., Alatalo, E., Korpela, R., Mattila-Sandholm, T., Lassig, A., Hatakka, K., Kinnunen, K. T., Karjalainen, H., Saxelin, M., Laakso, K., Surakka, A., Palva, A., Salusjärvi, T., Auvinen, P., & de Vos, W. M. (2009). Comparative genomic analysis of *Lactobacillus rhamnosus* GG reveals pili containing a human- mucus binding protein. *Proceedings of the National Academy of Sciences of the United States of America*, *106*, 17193-17198.

Kanmani, P., Satish Kumar, R., Yuvaraj, N., Paari, K. A., Pattukumar, V., & Arul, V. (2013). Probiotics and its functionally valuable products – a review. *Critical Reviews in Food Science and Nutrition*, *53*, 641-658.

Katan, M. B. (2012). Why the European Food Safety Authority was right to reject health claims for probiotics. *Beneficial microbes*, *3*, 85-89.

Klaenhammer, T. R., Kleerebezem, M., Kopp, M. V. & Rescigno, M. (2012). The impact of probiotics and prebiotics on the immune system. *Nature Reviews. Immunology*, *12*, 728-734.

Lebeer, S., Vanderleyden, J., & De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiology and Molecular Biology Reviews*, *72*, 728-764.

Lönnermark, E., Friman, V., Lappas, G., Sandberg, T., Berggren, A., & Adlerberth, I. (2010). Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *Journal of Clinical Gastroenterology*, *44*, 106-112.

Miquel, S., Beaumont, M., Martín, R., Langella, P., Braesco, V., & Thomas, M. (2015). A proposed framework for an appropriate evaluation scheme for microorganisms as novel foods with a health claim in Europe. *Microbial Cell Factories*, *14*, 1-11.

Mattarelli, P., Holzapfel, W., Franz, C. M. A. P., Endo, A., Felis, G. E., Hammes, W., Pot, B., Dicks, L., & Dellaglio, F. (2014). Recommended minimal standard requirements for description of new taxa of the genera Bifidobacterium, *Lactobacillus* and related genera. *International Journal of Systematic and Evolutionary Microbiology*, *64*, 1434-1451.

Naser, S. M., Dawyndt, P., Hoste, B., Gevers, D., Vandemeulebroecke, K., Cleenwerck, I., Vancanneyt, M., & Swings, J. (2007). Identification of lactobacilli by *pheS* and *rpoA* gene sequence analyses. *International Journal of Systematic and Evolutionary Microbiology*, *57*, 2777-2789.

Papadimitriou, K., Zoumpopoulou, G., Foligné, B., Alexandraki, V., Kazou, M., Pot, B., & Tsakalidou, E. (2015). Discovering probiotic microorganisms: *in vitro*, *in vivo*, genetic and omics approaches. *Frontiers in Microbiology*, *6*, 1-28.

Roos, S., Engstrand, L., & Jonsson, H. (2005). *Lactobacillus gastricus* sp. nov., *Lactobacillus antri* sp. nov., *Lactobacillus kalixensis* sp. nov. and *Lactobacillus ultunensis* sp. nov., isolated from human stomach mucosa. *International Journal of Systematic and Evolutionary Microbiology*, *55*, 77-82.

Salvetti, E., Torriani, S., & Felis, G. E. (2012). The genus *Lactobacillus*: a taxonomic update. *Probiotics and Antimicrobial Proteins*, *4*, 217-226.

Sanders, M. E., Akkermans, L. M. A., Haller, D., Hammerman, C., Heimbach, J., Hörmannsperger, G., Huys, G., Levy, D. D., Lutgendorff, F., Mack, D., Phothirath, P., Solano-Aguilar, G., & Vaughan, E. (2010). Safety assessment of probiotics for human use. *Gut Microbes*, *3*, 164-185.

Sanders, M. E. (2015). Probiotics in 2015. Their scope and use. *Journal of Clinical Gastroenterology*, *49*, S2-S6.

Saulnier, D. M., Santos, F., Roos, S., Mistretta, T. A., Spinler, J. K., Molenaar, D., Teusink, B., & Versalovic J. (2011). Exploring metabolic pathway reconstruction and genome-wide expression profiling in *Lactobacillus reuteri* to define functional probiotic features. *PLoS One, 6*, 1-14.

Sun, Z., Harris, H. M., McCann, A., Guo, C., Argimón, S., Zhang, W., Jeffery, I. B., Cooney, J. C., Kagawa, T. F., Liu, W., Song, Y., Salvetti, E., Wrobel, A., Rasinkangas, P., Parkhill, J., Rea, M. C., O'Sullivan, O., Ritari, J., Douillard, F. P., Ross P. R., Yang, R., Briner, A. E., Felis, G. E., de Vos, W. M., Barrangou, R., Klaenhammer, T. R., Caufield, P. W., Cui, Y., Zhang, H., & O'Toole, P. W. (2015). Expanding the biotechnology potential of lactobacilli through comparative genomics of 213 strains and associated genera. *Nature Communications, 6*, 1-13.

Tilman, C., & Clark, T. (2014). Global diets link environmental sustainability and human health. *Nature, 515*, 518-522.

Torriani, S., Felis, G. E., & Dellaglio F. (2001). Differentiation of *Lactobacillus plantarum, L. pentosus*, and *L. paraplantarum* by *recA* gene sequence analysis and multiplex PCR assay with *recA* gene-derived primers. *Applied and Environmental Microbiology, 67*, 3450-3454.

Van Buul, V. J., & Brouns, F. J. (2015). Nutrition and health claims as marketing tools. *Critical reviews in Food Science and Nutrition, 55*, 1552-1560.

Van Loveren, H., Sanz, Y., & Salminen S. (2012). Health claims in Europe: probiotics and prebiotics as case examples. *Annual review of food science and technology, 3*, 247-261.

Ventura, M., O'Flaherty, S., Claesson, M. J., Turroni, F., Klaenhammer, T. R., van Sinderen, D., & O'Toole P. W. (2009). Genome-scale analyses of health-promoting bacteria: probiogenomics. *Nature Reviews. Microbiology, 7*, 61-71.

Zheng, J., Ruan, L., Sun, M., & Gänzle, M. (2015). A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. *Applied and Environmental Microbiology, 81*, 7233-7243.

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601 **Table 1.** *Lactobacillus* species on the QPS list (EFSA), on the GRAS list (FDA), in the EFFCA inventory and
602 for which a patent has been deposited (ESPACENET database).

QPS list (EFSA)[1]	GRAS notice (FDA)[2]	EFFCA Inventory[3]	Patents (ESPACENET)[4]
Lb. acidophilus	*Lb. acidophilus*	*Lb. acetotolerans*	*Lb. acidophilus*
Lb. amylolyticus	*Lb. bulgaricus*	*Lb. acidifarinae*	*Lb. brevis*
Lb. amylovorus	*Lb. casei*	*Lb. acidipiscis*	*Lb. buchneri*
Lb. alimentarius	*'Lb. casei* subsp.	*Lb. acidophilus*	*Lb. casei*
'Lb. aviaries'	*rhamnosus'*	*Lb. alimentarius*	*Lb. crispatus*
Lb. brevis	*Lb. fermentum*	*Lb. amylolyticus*	*Lb. coryniformis*
Lb. buchneri	*Lb.* subsp. *lactis*	*Lb. amylovorus*	*Lb. delbrueckii*
Lb. casei	*Lb. lactis*	*Lb. brevis*	*Lb. fermentum*
'Lb. cellobiosus'	*Lb. paracasei* subsp.	*Lb. buchneri*	*Lb. gasseri*
Lb. coryniformis	*paracasei*	*Lb. cacaonum*	*Lb. helveticus*
Lb. crispatus	*Lb. plantarum*	*'Lb. casei* subsp. *casei'*	*Lb. iners*
Lb. curvatus	*Lb. reuteri*	*Lb. collinoides*	*Lb. johnsonii*
Lb. delbrueckii	*Lb. rhamnosus*	*Lb. composti*	*Lb. kefiranofaciens*
Lb. diolivorans	*Lb. sakei*	*Lb. coryniformis* subsp.	*Lb. kitasatonis*
Lb. farciminis		*coryniformis*	*Lb. mucosae*
Lb. fermentum		*Lb. crispatus*	*Lb. pentosus*
Lb. gallinarum		*Lb. crustorum*	*Lb. paracasei*
Lb. gasseri		*Lb. curvatus* subsp. *curvatus*	*Lb. plantarum*
Lb. helveticus		*Lb. delbrueckii* subsp. *bulgaricus*	*Lb. rhamnosus*
Lb. hilgardii		*Lb. delbrueckii* subsp. *delbrueckii*	*Lb. reuteri*
Lb. johnsonii		*Lb. delbrueckii* subsp. *lactis*	*Lb. sakei*
Lb. kefiranofaciens		*Lb. dextrinicus*	*Lb. salivarius*
Lb. kefiri		*Lb. diolivorans*	
Lb. mucosae		*Lb. fabifermentans*	
Lb. panis		*Lb. farciminis*	
Lb. collinoides		*Lb. fermentum*	
Lb. paracasei		*Lb. fructivorans*	
Lb. paraplantarum		*Lb. frumenti*	
Lb. pentosus		*Lb. gasseri*	
Lb. plantarum		*Lb. ghanensis*	
Lb. pontis		*Lb. hammesii*	
Lb. reuteri		*Lb. harbinensis*	
Lb. rhamnosus		*Lb. helveticus*	
Lb. sakei		*Lb. hilgardii*	
Lb. salivarius		*Lb. homohiochii*	
Lb. sanfranciscensis		*Lb. hordei*	
		Lb. jensenii	
		Lb. johnsonii	
		Lb. kefiri	
		Lb. kefiranofaciens subsp.	
		kefiranofaciens	
		Lb. kefiranofaciens subsp.	
		kefirgranum	
		Lb. kimchii	
		Lb. kisonensis	
		Lb. mali	
		Lb. manihotivorans	
		Lb. mindensis	
		Lb. mucosae	
		Lb. nagelii	
		Lb. namurensis	
		Lb. nantensis	
		Lb. nodensis	
		Lb. oeni	
		Lb. otakiensis	
		Lb. panis	

		Lb. parabrevis *Lb. parabuchneri* *Lb. paracasei* subsp. *paracasei* *Lb. parakefiri* *Lb. paralimentarius* *Lb. paraplantarum* *Lb. pentosus* *Lb. perolens* *Lb. plantarum* subsp. *plantarum* *Lb. pobuzihi* *Lb. pontis* *Lb. rapi* *Lb. reuteri* *Lb. rhamnosus* *Lb. rossiae* *Lb. sakei* subsp. *carnosus* *Lb. sakei* subsp. *sakei* *Lb. salivarius* subsp.*salivarius* *Lb. sanfranciscensis* *Lb. satsumensis* *Lb. secaliphilus* *Lb. senmaizukei* *Lb. siliginis* *Lb. similis* *Lb. spicheri* *Lb. suebicus* *Lb. sunkii* *Lb. tucceti* *Lb. vaccinostercus* *Lb. versmoldensis* *Lb. yamanashiensis*	

603 [1]:EFSA Journal 2016; 14(7): 4522;
604 [2]. updated 20/11/2015;
605 http://www.accessdata.fda.gov/scripts/fdcc/?set=GRASNotices&sort=GRN_No&order=DESC&startrow=1&ty
606 pe=basic&search=Lactobacillus
607 [3]: EFFCA Inventory of microorganisms with beneficial use (International Journal of Food Microbiology 2012,
608 154, pp.87-97), http://www.effca.org/content/inventory-microorganisms
609 [4]: updated 20/11/2015, search performed in Espacenet (http://worldwide.espacenet.com/) using the keywords
610 "*Lactobacillus*" and "probiotic" in "Title" and "Title/Abstract", respectively.
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Figure caption

Figure 1. *Lactobacillus* species involved in health claims applications (A) and the target organs/systems for which *Lactobacillus* species have a beneficial effect (B).

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When regulation challenges innovation: the case of the genus *Lactobacillus*

Elisa Salvetti, Paul W. O'Toole

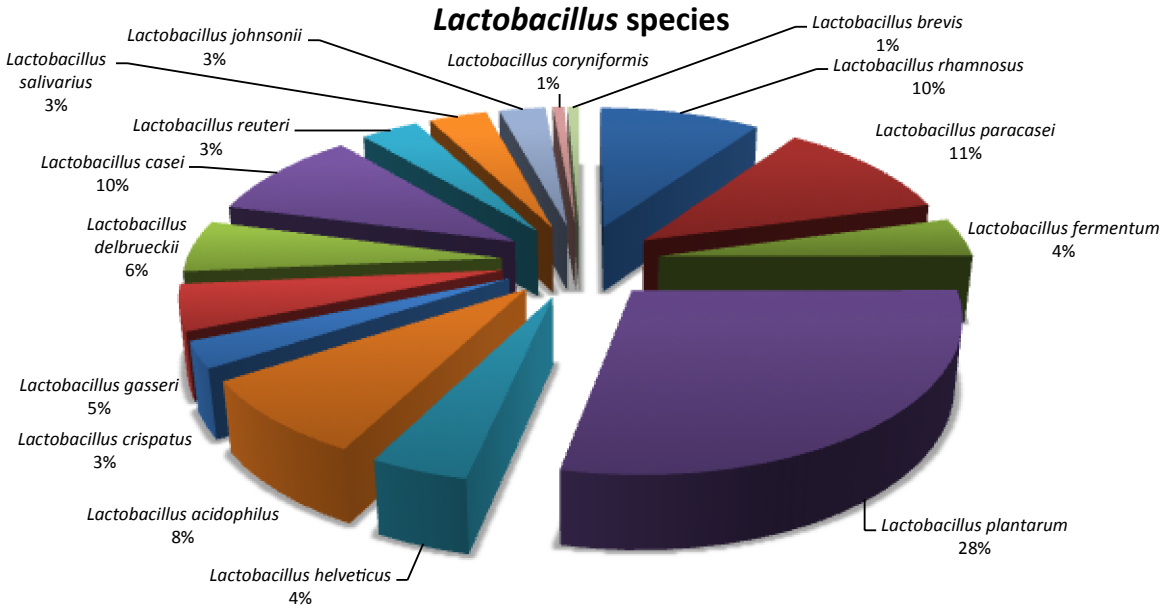
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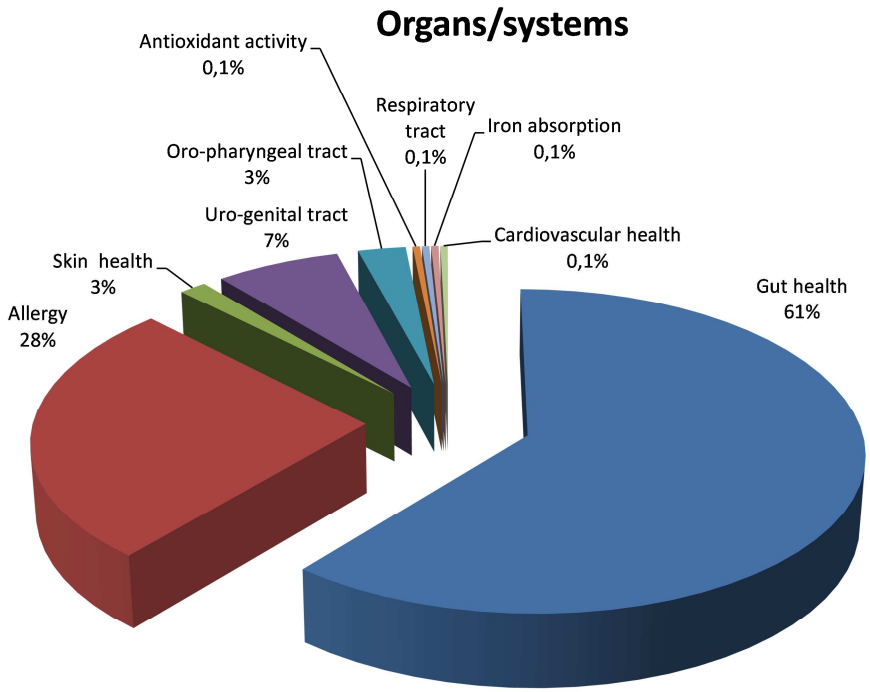
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Figure 1

A



B



Colour in print is not required.

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Highlights

- The approval of health claims for probiotics has become very challenging
- The amount of data for the genus *Lactobacillus* is a resource for regulatory procedures.
- This *Lactobacillus*-centric compliance model can be a *paradigm* for other probiotic bacteria.

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