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

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

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12

13 **Abstract**

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

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

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

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

23

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

25

26

27

28 Background

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

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

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

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

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

60 development (reviewed in Hill *et al.*, 2014). Different policies are used in the Member states
61 which result in a lack of clear recommendations for the appropriate and accurate
62 communication of probiotic statements to the different stakeholders including researchers,
63 industries, legislators, consumers and health-care professionals, who are responsible for the
64 different steps of bringing probiotic to the consumer (Van Buul and Brouns, 2015).
65 At the same time as probiotics proliferate in the market, policy makers and regulators are
66 simultaneously, and usually on an *ad hoc* basis, trying to critically develop the most
67 appropriate regulatory structure for probiotics, which needs on the one hand to be rigorous in
68 defining the level of accuracy required in claim dossiers, but on the other hand needs to be
69 flexible enough to stimulate research and innovation, and thus encourage the release of new
70 health-promoting products (Hoffman *et al.*, 2014). The second part of this paradigm is
71 arguably not working.

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

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

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

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

99 In 2015, the genome sequences of almost all *Lactobacillus* type strains and some historically
100 associated genera were determined (Sun *et al.*, 2015; Zheng *et al.*, 2015), thus providing a
101 definitive genomic resource for mining all relevant phylogenetic and functional information.
102 This data repository should also prove useful for understanding the species-restricted
103 distribution of probiotic traits, thus supporting probiotic claim substantiation.
104 Despite the unprecedented availability of genome sequences and increasing functional
105 information about lactobacilli, the development of functional products containing these
106 bacteria is challenged by the laborious nature of currently prescribed taxonomic
107 characterization, the shortcomings regarding the validation of their beneficial mechanisms,
108 and the drawbacks attached to determining their safety for consumption, issues that we will
109 now expand upon.

110

111 **Taxonomic characterization of *Lactobacillus* probiotics**

112 Isolation and the full characterization of a candidate probiotic is the first essential
113 requirement for a novel food marketing authorization and a health claim submission (EFSA,
114 2017; EFSA 2016b). The taxonomic determination of the genus, the species and the strain
115 contained in a probiotic product provides useful preliminary information regarding the main
116 physiological and metabolic properties of the organism, and allows its discrimination from
117 other closely related but potentially non-beneficial strains (ILSI 2013).

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

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

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

131 The widespread use and characterization of lactobacilli are both hindered by the complex
132 taxonomic structure of the genus, reflected in a poor correlation between the phylogenetic
133 relationship and the physiological properties of *Lactobacillus* species (Zheng *et al.*, 2015).
134 Moreover, the ongoing description of novel species, whose number increased from 152
135 (Salvetti *et al.*, 2012) to more than 190 in the last 3 years

136 (<http://www.bacterio.net/lactobacillus.html>), has resulted in significant taxonomy changes
137 within the genus, causing confusion and leading to the mis-identification of lactobacilli.

138 Although 16S rRNA gene sequence analysis is the standard method for *Lactobacillus* species
139 identification thanks in part to the availability of up-to-date and internationally recognised
140 databases (ie. EzTaxon, <http://www.ezbiocloud.net/eztaxon>), there are still shortcomings to
141 this approach, such as the low taxonomic resolution afforded by 16S rRNA gene comparison
142 especially when trying to separate closely related species (i.e *Lb. plantarum/Lb.*

143 *paraplantarum/Lb. pentosus* or *Lb casei/Lb. paracasei/Lb. rhamnosus*). To overcome this,
144 housekeeping genes as *pheS*, *rpoA* (Naser *et al.*, 2007) and *recA* (Torriani *et al.*, 2001) have
145 been used as alternative phylogenetic markers which provide a higher discrimination between
146 lactobacilli. Although the application of these molecular markers offers useful potential in the
147 probiotic field, data interpretations by taxonomic experts remains crucial to ensure reliability
148 of the identification results (Sanders *et al.*, 2010).

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

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

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

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

187 **Validation of the probiotic potential of *Lactobacillus* spp.**

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

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

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

198 Validation of genome-based analysis with experimental approaches is necessary to link
199 annotated gene sequences to their predicted traits, and this also represents a prerequisite for
200 the construction of databases of probiotic markers with translational predictive value.

201 Although the molecular analyses of probiotic properties do not entirely substitute for
202 experimental tests, *in silico* approaches constitute an important step in the development of
203 more efficient and precise screens for probiotic strains.

204 EFSA approves health claims if the substantiation is based on generally accepted scientific
205 evidence, using an assessment process of the highest possible standard (EC No. 1924/2006).

206 The approach adopted shall consist primarily of human studies and according to a hierarchy
207 of study designs which supports the relative strength of evidence (EC. No. 353/2008).

208 Although a workflow of the key steps in the process of authorisation of health claims made
209 on foods is outlined by EFSA (EFSA, 2017; EFSA, 2016b), no official procedures or
210 workflows for selecting probiotics are available (i.e. validated biomarkers for *in vitro*
211 screening) and this makes it difficult to determine the real probiotic potential of
212 microorganisms and the physiological effect they exert.

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

217 In addition, successful probiotic claim substantiation is also impeded by EU laws which do
218 not recognise the possibility that food can prevent, treat or cure a disease, leaving scientists,
219 marketers, food producers and also legislators in an ambiguous impasse (Katan *et al.*, 2012).

220 In a recent attempt to solve these issues, EFSA released updated general scientific guidance
221 for stakeholders on health claim applications in which the Panel on Dietetic Products,
222 Nutrition and Allergies (NDA) outlines the principles to be applied for the scientific
223 evaluation of health claim applications and the issues to be considered by applicants for the
224 compilation of applications (EFSA, 2017; EFSA, 2016b).

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

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

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

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

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

251 A detailed analysis of the nutrition and health claims that feature *Lactobacillus* strains
252 revealed that the main reasons of rejection were i) insufficient characterization of the food
253 and poor scientific assessment of the claimed effect (i.e. *Lb. plantarum* 299; EFSA, 2010), ii)
254 the absence of a beneficial physiological effect based on the scientific evidence assessed (*Lb.*
255 *acidophilus* NCFM ATCC SD5221; EFSA, 2011) iii) the non-recognition of the property of
256 preventing, treating or curing a human disease to food (i.e. *Lb. paracasei* LPC 01; EFSA,
257 2012a).

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

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

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

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

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

279

280 **Safety assessment of *Lactobacillus* species**

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

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

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

295 pathogenicity/toxicogenicity, and their ability to produce biogenic amines (Sanders *et al.*,
296 2010).

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

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

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

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

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

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

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

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

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

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

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

372

373 **Conclusions**

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

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

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

394

395 **Conflict of interest**

396 The authors declare no conflict of interest.

397

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403

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440 2859, 3521, 3774, 3896), “contribution to body defences against external agents” (ID 3635),
441 stimulation of immunological responses (ID 1479, 2064, 2075, 3139), reduction of
442 inflammation (ID 546, 547, 641, 2505, 2862), increase in renal water elimination (ID 2505),
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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
<i>Lb. acidophilus</i>	<i>Lb. acidophilus</i>	<i>Lb. acetotolerans</i>	<i>Lb. acidophilus</i>
<i>Lb. amyolyticus</i>	<i>Lb. bulgaricus</i>	<i>Lb. acidifarinae</i>	<i>Lb. brevis</i>
<i>Lb. amylovorus</i>	<i>Lb. casei</i>	<i>Lb. acidipiscis</i>	<i>Lb. buchneri</i>
<i>Lb. alimentarius</i>	' <i>Lb. casei</i> subsp. <i>rhamnosus</i> '	<i>Lb. acidophilus</i>	<i>Lb. casei</i>
' <i>Lb. aviaries</i> '	<i>Lb. fermentum</i>	<i>Lb. alimentarius</i>	<i>Lb. crispatus</i>
<i>Lb. brevis</i>	<i>Lb. subsp. lactis</i>	<i>Lb. amyolyticus</i>	<i>Lb. coryniformis</i>
<i>Lb. buchneri</i>	<i>Lb. lactis</i>	<i>Lb. amylovorus</i>	<i>Lb. delbrueckii</i>
<i>Lb. casei</i>	<i>Lb. paracasei</i> subsp. <i>paracasei</i>	<i>Lb. brevis</i>	<i>Lb. fermentum</i>
' <i>Lb. cellobiosus</i> '	<i>Lb. plantarum</i>	<i>Lb. buchneri</i>	<i>Lb. gasseri</i>
<i>Lb. coryniformis</i>	<i>Lb. reuteri</i>	<i>Lb. cacaonum</i>	<i>Lb. helveticus</i>
<i>Lb. crispatus</i>	<i>Lb. rhamnosus</i>	' <i>Lb. casei</i> subsp. <i>casei</i> '	<i>Lb. iners</i>
<i>Lb. curvatus</i>	<i>Lb. sakei</i>	<i>Lb. collinoides</i>	<i>Lb. johnsonii</i>
<i>Lb. delbrueckii</i>		<i>Lb. composti</i>	<i>Lb. kefiranoformis</i>
<i>Lb. diolivorans</i>		<i>Lb. coryniformis</i> subsp. <i>coryniformis</i>	<i>Lb. kitasatonis</i>
<i>Lb. farciminis</i>		<i>Lb. crispatus</i>	<i>Lb. mucosae</i>
<i>Lb. fermentum</i>		<i>Lb. crustorum</i>	<i>Lb. pentosus</i>
<i>Lb. gallinarum</i>		<i>Lb. curvatus</i> subsp. <i>curvatus</i>	<i>Lb. paracasei</i>
<i>Lb. gasseri</i>		<i>Lb. delbrueckii</i> subsp. <i>bulgaricus</i>	<i>Lb. plantarum</i>
<i>Lb. helveticus</i>		<i>Lb. delbrueckii</i> subsp. <i>delbrueckii</i>	<i>Lb. rhamnosus</i>
<i>Lb. hilgardii</i>		<i>Lb. delbrueckii</i> subsp. <i>lactis</i>	<i>Lb. reuteri</i>
<i>Lb. johnsonii</i>		<i>Lb. dextrinicus</i>	<i>Lb. sakei</i>
<i>Lb. kefiranoformis</i>		<i>Lb. diolivorans</i>	<i>Lb. salivarius</i>
<i>Lb. kefiri</i>		<i>Lb. fabifermentans</i>	
<i>Lb. mucosae</i>		<i>Lb. farciminis</i>	
<i>Lb. panis</i>		<i>Lb. fermentum</i>	
<i>Lb. collinoides</i>		<i>Lb. fructivorans</i>	
<i>Lb. paracasei</i>		<i>Lb. frumenti</i>	
<i>Lb. paraplantarum</i>		<i>Lb. gasseri</i>	
<i>Lb. pentosus</i>		<i>Lb. ghanensis</i>	
<i>Lb. plantarum</i>		<i>Lb. hammesii</i>	
<i>Lb. pontis</i>		<i>Lb. harbinensis</i>	
<i>Lb. reuteri</i>		<i>Lb. helveticus</i>	
<i>Lb. rhamnosus</i>		<i>Lb. hilgardii</i>	
<i>Lb. sakei</i>		<i>Lb. homohiochii</i>	
<i>Lb. salivarius</i>		<i>Lb. hordei</i>	
<i>Lb. sanfranciscensis</i>		<i>Lb. jensenii</i>	
		<i>Lb. johnsonii</i>	
		<i>Lb. kefiri</i>	
		<i>Lb. kefiranoformis</i> subsp. <i>kefiranoformis</i>	
		<i>Lb. kefiranoformis</i> subsp. <i>kefirgranum</i>	
		<i>Lb. kimchii</i>	
		<i>Lb. kisonensis</i>	
		<i>Lb. mali</i>	
		<i>Lb. manihotivorans</i>	
		<i>Lb. mindensis</i>	
		<i>Lb. mucosae</i>	
		<i>Lb. nagelii</i>	
		<i>Lb. namurensis</i>	
		<i>Lb. nantensis</i>	
		<i>Lb. nodensis</i>	
		<i>Lb. oeni</i>	
		<i>Lb. otakiensis</i>	
		<i>Lb. panis</i>	

		<i>Lb. parabrevis</i> <i>Lb. parabuchneri</i> <i>Lb. paracasei</i> subsp. <i>paracasei</i> <i>Lb. parakefiri</i> <i>Lb. paralimentarius</i> <i>Lb. paraplantarum</i> <i>Lb. pentosus</i> <i>Lb. perolens</i> <i>Lb. plantarum</i> subsp. <i>plantarum</i> <i>Lb. pobuzihi</i> <i>Lb. pontis</i> <i>Lb. rapi</i> <i>Lb. reuteri</i> <i>Lb. rhamnosus</i> <i>Lb. rossiae</i> <i>Lb. sakei</i> subsp. <i>carneus</i> <i>Lb. sakei</i> subsp. <i>sakei</i> <i>Lb. salivarius</i> subsp. <i>salivarius</i> <i>Lb. sanfranciscensis</i> <i>Lb. satsumensis</i> <i>Lb. secaliphilus</i> <i>Lb. senmaizukei</i> <i>Lb. siliginis</i> <i>Lb. similis</i> <i>Lb. spicheri</i> <i>Lb. suebicus</i> <i>Lb. sunkii</i> <i>Lb. tuccei</i> <i>Lb. vaccinostercus</i> <i>Lb. versmoldensis</i> <i>Lb. yamanashiensis</i>	
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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&type=basic&search=Lactobacillus

607 ³: EFFCA Inventory of microorganisms with beneficial use (International Journal of Food Microbiology 2012, 154, pp.87-97), <http://www.fffca.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.

611
612

613 **Figure caption**

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

616

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

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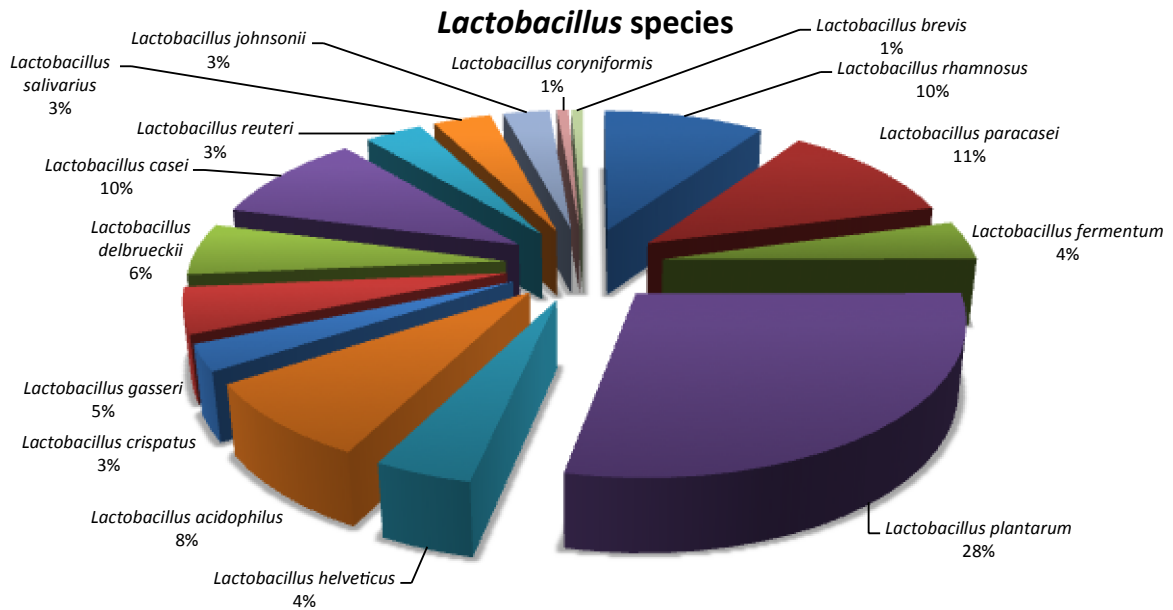
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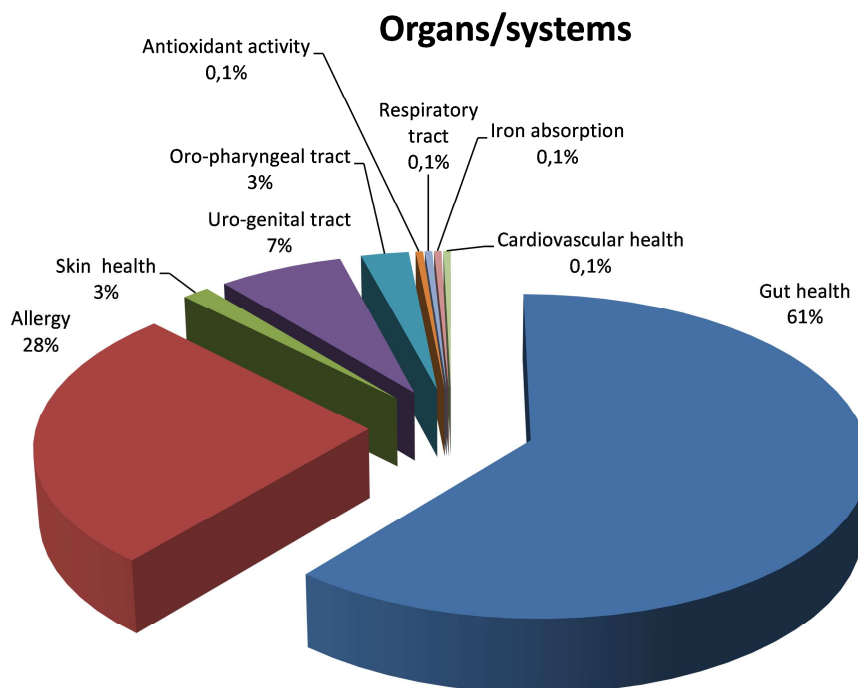
11 Corresponding author: Paul W. O'Toole

12

13

14 **Figure 1**15 **A**

16

17 **B**

18

19

20 Colour in print is not required.

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

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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.