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Phylotype-level profiling of lactobacilli in highly complex environments by means of an ITS-based metagenomic approach.

Key words: *Lactobacillus*, microbiota, ITS, Next Generation Sequencing, Illumina.

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Abstract

The genus *Lactobacillus* is a widespread taxon, members of which are highly relevant to functional and fermented foods, while they are also commonly present in host-associated gut and vaginal microbiota. Substantial efforts have been undertaken to disclose the genetic repertoire of all members of the genus *Lactobacillus*, yet their species-level profiling in complex matrices is still undeveloped due to the poor phylotype resolution of profiling approaches based on the 16S rRNA gene. To overcome this limitation, an ITS-based profiling method was developed to accurately profile lactobacilli at species-level. This approach encompasses a genus-specific primer pair combined with a database of ITS sequences retrieved from all available *Lactobacillus* genomes and a script for the Qiime software suite that performs all required steps to reconstruct a species-level profile. This methodology was applied to several environments, i.e., human gut and vagina, cecum of free range chickens, as well as whey and fresh cheese. Interestingly, data collected confirmed a relevant role of lactobacilli present in functional and fermented foods in defining the population harbored by the human gut, while, unsurprisingly perhaps, the cecum of free range chickens was observed to be dominated by lactobacilli characterized in birds living in natural environments. Moreover, vaginal swabs confirmed the existence of previously-hypothesized community state types, while analysis of whey and fresh cheese revealed a dominant presence of single *Lactobacillus* species used as starters for cheese production. Furthermore, application of this ITS profiling method to a mock *Lactobacillus* community allowed a minimal resolution level of <0.006 ng/µl.

Importance

The genus *Lactobacillus* is a large and ubiquitous taxon of high scientific and commercial relevance. Despite the fact that the genetic repertoire of lactobacilli species has been extensively characterized, the ecology of this genus has been explored by metataxonomic techniques that are accurate down to the genus or phylogenetic group level only. Thus, the distribution of lactobacilli in environmental or processed food samples is relatively unexplored. The profiling protocol described here relies on the use of the Internally Transcribed Spacer to perform an accurate classification in a target population of lactobacilli with <0.006 ng/µl sensitivity. This approach was used to analyze five sample types collected from both human and animal host-associated microbiota as well as from the cheese production chain. Availability of a tool for species-level profiling of lactobacilli may be highly useful for both academic research and a wide range of industrial applications.

Introduction

The genus *Lactobacillus* is a widespread and diverse taxon encompassing more than 170 species and 17 subspecies, which are classified as Gram-positive, non-spore-forming and catalase-negative facultative anaerobes (1, 2). Moreover, based on their metabolic capability to produce lactic acid as the main metabolic end product of carbohydrate fermentation, lactobacilli are classified as members of the Lactic Acid Bacteria (LAB). Notably, 16S rRNA gene-based phylogenetic analyses revealed the existence of 22 distinct phylogenetic groups of *Lactobacillus* species (24 including pediococci) (2-4).

Regarding their ecological distribution, lactobacilli are found in a wide range of environments, including plants, water, soil, silage and different body sites of humans and other animals as members of host-associated microbiomes, such as those colonizing the oral cavity, the vagina and the gastrointestinal tract (GIT) (4, 5). Moreover, 37 species of this genus have been granted the Qualified Presumption of Safety (QPS) status by the European Food Safety Authority (EFSA) (6). Thus, they are extensively used in the food industry, in particular in fermented foods due to their high performance in lactic acid fermentation coupled with high tolerance for low pH, preservative and organoleptic properties, and production of exopolysaccharides that contribute to the texture of foods (2, 7). In this context, members of the genus *Lactobacillus* have in recent years gained significant scientific and commercial interest as health-promoting microorganisms, as evidenced by the fact that 22 species encompass strains patented as probiotics in Europe (8).

The high commercial and scientific relevance of lactobacilli coupled to the recent introduction of next-generation sequencing technologies has recently led to genome decoding of all (then) known *Lactobacillus* species (3, 7). The retrieved genomic data has been exploited for comparative genomic analyses, and has allowed identification of many shared or distinct genetic features of this genus. Furthermore, this genomic information has permitted the reconstruction of their metabolic potential, has shed light on host-microbe interactions, such as adhesion to the mucus

layer and modulation of the immune system of the host, and has revealed particular microbe-microbe interactions with other commensals or (opportunistic) pathogens (1, 7, 8).

Despite the large body of data concerning the physiology and genetics of lactobacilli, knowledge about the ecology and distribution in environmental or host-associated niches of individual species relies mainly on culture-dependent studies. This is partly due to the resolution limit of currently used metagenomic approaches. Although microbial profiling based on partial 16S rRNA gene is able to discriminate between phylogenetic groups of lactobacilli due to the high phylogenetic diversity of this genus, it cannot provide an accurate species-level resolution. Moreover, the majority of the currebt exiting studies of lactobacilli populations based on 16S rRNA gene profiling do not even perform phylogenetic group-level analyses. To offer a more refined taxonomic view of lactobacilli in a given environment or sample, we developed a profiling approach based on amplification of the internally transcribed spacer (ITS) sequence. Notably, due to their high variability, ITS sequences have previously been exploited in a wide range of studies encompassing the identification of unique species-specific restriction patterns of lactobacilli, as well as the identification and characterization of *Leuconostoc* strains and for the genotyping of *Streptococcus pneumoniae* strains (9-11). The developed methodology in the current work is able to determine the composition of lactobacilli-containing communities down to the species level. The method was validated through the analysis of a sample artificially constituted by DNA of 14 lactobacilli taxa at known concentration. Furthermore, we applied this methodology for the precise investigation of bacterial communities harbored by human-, animal- and food-associated matrices that were previously explored down to the genus-level only.

Results and discussion

Analysis of ITS variability within the *Lactobacillus* genus.

Genomes of 1523 strains assigned to the genus *Lactobacillus* and corresponding to 176 species were retrieved from the NCBI genome database, and then processed using the MEGAnnotator software (12) for prediction of rRNA genes in order to ensure the same high-quality standard for all sequences of ribosomal loci included in this study (Table S1). Notably, the genomic sequences of 892 *Lactobacillus* strains, representing the 58.6 % of the total strain pool analyzed, did not harbor complete rRNA loci, i.e. encompassing complete 5S, 16S and 23S rRNA genes. In contrast, at least one complete ribosomal rRNA genes locus was identified for 631 of the 1523 analyzed strains, corresponding to 70 species and a custom script was then used to extract a total of 1788 Internally Transcribed Spacer (ITS) sequences. Assembly of draft genomes generally generates the collapse of reads that correspond to rRNA genes into a single rRNA locus. However, availability of multiple draft sequences of a given *Lactobacillus* taxon, complemented with analysis of 217 complete genomes of *Lactobacillus* species, allowed us to retrieve an average of 25.5 ITS sequences per species. Interestingly, 137 of the 1788 retrieved ITS sequences include stretches of >3 undefined (N) nucleotides, thus highlighting a high rate of assembly-related issues and/or low-quality regions in genomes deposited at the NCBI genome database. Comparative analysis of the 1651 complete ITS sequences without multiple contiguous nucleotide ambiguities revealed that 92.5 % of the ITS sequences range between 200 and 500 bp.

As previously observed for bifidobacteria (13, 14), alignment of ITS sequences from *Lactobacillus* genomes shows a high level of diversity, probably due to a high mutation frequency, and corresponding to a high evolutionary rate, as reflected by multiple substitutions at a given nucleotide position and indicative of mutational saturation of such ITS sequences. While this particularly high mutation frequency prevents phylogeny inference (15), it is suitable for metagenomic amplicon-based profiling below the genus level, as previously validated for members of the genus *Bifidobacterium* (13).

Design of a PCR primer pair for ITS-profiling of the *Lactobacillus* genus.

Many profiling approaches have been developed to accurately reconstruct the taxonomic composition of complex bacterial communities. These include methods based on low-coverage sequencing of full-length 16S rRNA genes and the use of technologies providing long reads, i.e. Sanger and PacBio. Nevertheless, despite the fact that full-length sequencing of the 16S rRNA gene allows high accuracy in taxonomic assignment, the low sequencing coverage permits the detection only of dominant taxa and prevents profiling of bacteria present at low relative abundance in a given population (10). Furthermore, the use of alternative marker genes has also been proposed, though their use remains limited due to difficulties in the definition of universal primers as well as in the lack of a complete reference database. The advent of next-generation sequencing, characterized by high coverage and short reads, facilitated the amplification and sequencing of partial 16S rRNA genes, i.e. 16S rRNA gene profiling. This metagenomic method has in recent years been used as the gold standard for taxonomic characterization of environmental and host-associated microbiomes. While this methodology covers all bacterial biodiversity, it is generally only accurate for the reconstruction of taxonomic profiles down to genus level (16) or down to phylogenetic groups in case of genera with a high level of phylogenetic diversity, e.g. the genus *Lactobacillus* (3, 4) since it relies on sequencing of a small region of the whole 16S rRNA gene through next-generation sequencing. To overcome this limitation and to obtain species-level resolution, the use of the ITS sequence as an alternative molecular marker has been proposed (13). In order to develop a universal primer pair suitable for profiling of all members of the *Lactobacillus* genus, we aligned the 16S and 23S rRNA genes flanking the 1651 complete ITS sequences without stretches of undefined nucleotides that were retrieved from lactobacilli genomes deposited at the NCBI database. Manual inspection of the alignments allowed the identification of ‘universal’ primers located at the 5’-end of the 16S rRNA gene and at the 3’-end of the 23S rRNA gene, i.e., Probio-lac_Uni (CGTAACAAGGTAGCCGTAGG) and Probio-lac_Rev

(GTYVCGTCCTTCWTCGSC), respectively (Figure 1). Sequence conservation amongst the aligned 16S and 23S rRNA genes is reported in Figure 1 through a WebLogo representation. These primers generate an amplicon of an average length of 380 bp covering the complete ITS region and suitable for 2 X 250 bp paired-end Illumina sequencing followed by single-end bioinformatic analysis of both paired reads (see below). Analysis of single-end reads provided reliable assignment to species level even in cases where a tRNA gene was located within the ITS region (see below). Notably, the final sequence of the primers was defined after multiple iterative alignments to the Silva SSU and LSU databases (17) using the Silva TestProbe v. 3.0 tool (https://www.arb-silva.de/search/testprobe/). The latter approach led to introduction of specific IUPAC bases in order to maximize alignment of the primers to all currently available 16S and 23S rRNA gene sequences of lactobacilli corresponding to all known species of this genus, while minimizing alignment to non-lactobacilli ribosomal RNA genes. The usefulness of the Probio-lac_Uni/Probio-lac_Rev primer pair was *in vitro* validated through successful amplicon generation in the case of 31 lactobacilli species belonging to the 23 phylogenetic groups identified previously in the genus *Lactobacillus* (3, 4) (Figure S1). In contrast, no amplification was observed when the Probio-lac_Uni/Probio-lac_Rev primer pair was used to amplify DNA extracted from nine non-*Lactobacillus* taxa (Figure S1). Interestingly, for all tested lactobacilli we observed two PCR fragments, each with a molecular size ranging from 300 to 350 bp, and 500 to 550 bp, corresponding to the ITS region with and without a tRNA gene (see below for details), respectively (Figure S1) (Figure 1). Such ITS patterns confirmed those displayed in previous studies targeting the amplification of the ITS region of lactobacilli (18). Notably, for few taxa we observed a faint amplification fragment of 500 to 550 bp, which might suggest a lower copy number of ITS regions encompassing tRNA genes in the same genome.

The Probio-lac_Uni/Probio-lac_Rev primer pair was employed for *in silico* PCR amplification of the 631 genomes of the genus *Lactobacillus* encoding at least one rRNA genes locus. This approach facilitated the development of a database encompassing 1651 complete ITS sequences without

multiple ambiguous nucleotides, and flanked by partial 16S and 23S rRNA sequences, together constituting the *Lactobacillus* ITS Amplicon database (LITSA database).

Cross-alignment of all retrieved LITSA sequences using MatGAT software (19) was performed in order to evaluate the level of identity between predicted amplicons (Table S2) and to evaluate possible limits imposed by actual lactobacilli taxonomy to the proposed ITS profiling methodology. Notably, this analysis highlighted cases in which comparison of multiple LITSA sequences from the same strain showed low identity. In-depth investigation revealed that 46 of the 62 lactobacilli species included in the LITSA database contain at least one ITS sequence that harbors two tRNA genes (for Alanine and Isoleucine) (Figure 1). Notably, despite the fact that this prediction is limited due to the small number of complete genomes available, the presence of tRNA genes in one or multiple rRNA loci appears to be a common feature of genomes from members of the *Lactobacillus* genus.

Furthermore, cross-alignment analysis also revealed that the majority of the 62 *Lactobacillus* species, for which a complete LITSA sequence was available, can be discriminated (Table S2), with the exception of putatively misclassified strains and/or species (see below). In this context, despite the fact that lactobacilli are known to possess a very high level of phylogenetic diversity (3, 4), strains corresponding to 18 species showed an average LITSA sequence identity of >97 % with at least one other *Lactobacillus* species, thus showing a very close phylogenetic relationship between such taxa (Table 1). Amongst lactobacilli, *Lactobacillus casei* and *Lactobacillus paracasei* strains possess an average LITSA sequence identity of 99 %, while the amplicon sequences of *Lactobacillus pentosus*, *Lactobacillus plantarum* and *Lactobacillus paraplantarum* strains show up to 100 % identity (Table S2). An in-depth analysis of each strain revealed that 23 of the 25 strains classified as *L. casei* share an average LITSA sequence identity ≤96.1 % with the type strain *L. casei* ATCC 393, while the average identity with the type strain *L. paracasei* ATCC 394 is ≥99.7 % (Table S2). In contrast, the putative lactobacilli species *Lactobacillus* sp. FMNP02 shares 99.7 %

identity with *L. casei* ATCC 393 (Table S2), thus representing a possible misclassification of the latter strain.

In our attempts to obtain insights into the phylogeny of *L. pentosus*, *L. plantarum* and *L. paraplantarum*, we observed an average LITSA sequence identity of 98.9 % between *L. pentosus* and *L. plantarum* strains (Table S2). Moreover, the two strains of *L. paraplantarum*, for which we were able to predict an rRNA gene locus, show an average LITSA identity of 99.5 % with *L. plantarum* strains (Table S2), thus indicating that such taxa may belong to the same species and therefore cannot be discriminated using metataxonomic techniques. Nevertheless, evaluation of the average nucleotide identity is needed to confirm this hypothesis. Furthermore, we could not retrieve an *in silico* Probio-lac_Uni/Probio-lac_Rev-corresponding amplicon for the type strains of *L. pentosus* and *L. paraplantarum* due to absence of a complete ITS region in the deposited genomes, and we were therefore unable to evaluate their amplicon identity with the LITSA sequences of *L. plantarum* strains.

Notably, these observations suggest that major issues in the classification of the genus *Lactobacillus* still exist, resulting in the unfeasibility of distinguish a number of species through ITS profiling. Thus, as has been proposed previously, it is desirable that a re-evaluation of the taxonomy of lactobacilli is undertaken based on a phylogenomic approach (20, 21), as was also corroborated by recent studies (3, 4, 7).

Development of a bioinformatic tool for ITS-profiling of the *Lactobacillus* genus.

The length of the amplicon produced by the Probio-lac_Uni/Probio-lac_Rev primer pair may exceed 600 bp, particularly when tRNA-encoding sequences are present in the ITS sequence. Thus, sequencing produced non-overlapping paired-end reads even with the maximum length obtainable using Next-Generation Illumina sequencing, i.e. 2 X 250 bp paired-end reads, using the MiSeq Reagents Kit v3 600 cycles chemistry. Nevertheless, each forward and reverse read covers 42 and 60 nucleotides corresponding to the 16S rRNA gene 3'-end and the 23S rRNA gene 5'-end,

229 respectively, which are followed by 190-208 bp of hyper-variable ITS sequence suitable for
230 profiling at species-level (Figure 1). Thus, we developed a package for QIIME software suite v1.9.1
231 (22) that encompasses the LITSA database and a bash script for analysis of both forward and
232 reverse reads of the *Lactobacillus* ITS profiling data (probiogenomics.unipr.it/pbi). Notably, the
233 LITSA database will be updated regularly to include additional ITS sequences as new lactobacilli
234 genome sequences become available, thus increasing the number of lactobacilli species that can be
235 profiled. The script performs quality-filtering, *de novo* OTU clustering at 100 % identity and
236 taxonomic classification of OTU reference sequences through RDP classifier with a confidence
237 level of 0.80. Notably, these cut-off values permit discrimination of closely related taxa. Due to the
238 average size of the amplicon, the paired-end reads are not joined prior classification. Instead, the
239 script analyzes both the forward and the reverse reads altogether and provides an average profile.
240 Notably, the different number of rRNA loci predicted in the genomes of *Lactobacillus* species may
241 generate biases in the retrieved profiles. Thus, we evaluated the average number of ITS regions
242 present in the 217 available complete *Lactobacillus* genomes. This analysis provided data for
243 normalization of 45 of the 62 species of lactobacilli for which a LITSA sequence could be retrieved.
244 Moreover, the average number of rRNA genes loci of the remaining 17 species with only draft
245 genomes was set at 5.6, i.e., the average obtained for all the species with at least a complete
246 genome. Notably, the *Lactobacillus* ITS profiling analysis script includes a normalization step
247 based on the number of rRNA genes loci predicted for all the 62 *Lactobacillus* species for which a
248 LITSA sequence could be retrieved. The output produced by the script is summarized in the
249 "output" folder, which contains the predicted taxonomic profile based on the LITSA database (both
250 non-normalized and normalized for the number of rRNA loci) and the OTU table in tabular text
251 format that reports the reference sequence and associated taxonomy. All *Lactobacillus* ITS profiles
252 reported in this manuscript correspond to the average between forward and reverse read profiles
253 after normalization for the number of predicted rRNA genes loci.
254

Assessing detection sensitivity and accuracy using the *Lactobacillus* ITS profiling protocol

In order to provide an evaluation of the sensitivity and accuracy of the Probio-lac_Uni/Probio-lac_Rev primer pair, 14 *Lactobacillus* type strains were employed to artificially compose a mock community (Table S3). The DNA extracted from each taxon grown in pure culture was added to the mix at known amount, ranging from 0.006 ng to 50 ng of DNA, corresponding to 0.006 % to 50 % of the total DNA pool (Figure 2). Sequencing of the mock sample was performed using an Illumina MiSeq with 2X250 bp chemistry, producing 45,146 quality-filtered paired-end reads. Interestingly, *Lactobacillus* ITS profiling of this dataset successfully profiled all *Lactobacillus* species included in this sample, except *Lactobacillus vaginalis* and *Lactobacillus pontis*, for which we could not retrieve a LITSA sequence from analysis of available genome sequences (Figure 2). Thus, even though the Probio-lac_Uni/Probio-lac_Rev primer pair produces an amplicon for these species, the latter cannot be taxonomically classified due to absence of *L. vaginalis* and *L. pontis* in the present version of the LITSA database. This is a temporary limitation and the LITSA database will be updated regularly (probiogenomics.unipr.it/pbi) to include LITSA sequences of newly sequenced genomes in order to cover all the lactobacilli species that currently cannot be profiled. Moreover, comparison of the retrieved profile with the expected composition revealed a strong correlation for each taxon with few discrepancies (Figure 2). The causes of such differences between expected and observed relative abundance may be imputed to the lack of sufficient information in the LITSA database, at this time, regarding the average number of rRNA loci per genome used for normalization of the ITS profiling data.

Furthermore, since PCR amplicon size has been identified as a source of bias in ITS-based profiling studies of fungi (23), we evaluated the presence of possible biases introduced by amplification of lactobacilli ITS sequences of different length due to the presence or absence of tRNA genes (see above). The 14 *Lactobacillus* species that constitute the mock community (Table S3) were subjected to manual characterization of corresponding rRNA loci. Notably, the ten species for which a complete genome was available, confirmed what had been observed for the *in vitro* PCR,

281 i.e. presence of longer ITS sequences that encompass two tRNA genes (Figure 1; Figure S1).
282 Interestingly, the different intensities observed in the PCR fragments, i.e. 300-350 bp and 500-550
283 bp (Figure S1), did not influence
284 expected relative abundance of the mock community (Figure 2). Notably, detection of *Lactobacillus*
285 *rhamnosus* whose concentration in the mock community is 0.006 ng/µl indicates that the limit of
286 detection of the lactobacilli ITS profiling is <0.006 ng/µl, corresponding to $1.85*10^3$ cells/ µl.
287
288 **Validation of the *Lactobacillus* ITS profiling protocol through analysis of samples from**
289 **multiple environments.**
290 *Lactobacillus* is a highly diverse microbial genus, members of which are found in a wide range of
291 environments (5). To perform a comprehensive testing of the performances of the *Lactobacillus* ITS
292 profiling protocol, we analyzed a total of 25 samples encompassing five human faecal samples, five
293 human vaginal swab samples , five free range chicken cecal samples, five whey samples and five
294 parmesan cheese samples (Table S4). Sequencing was performed with an Illumina MiSeq
295 instrument using 2x250 bp chemistry, producing an average of 15,529 forward and 15,293 reverse
296 quality-filtered reads per sample (Table S4).
297 Interestingly, analysis of the human faecal samples revealed the presence of human gut colonizers,
298 such as *Lactobacillus rhamnosus*, along with a range of lactobacilli used in functional or fermented
299 foods that are typically part of the human diet, such as *L. plantarum*, *Lactobacillus helveticus*,
300 *Lactobacillus delbrueckii* and *Lactobacillus sakei* (Figure 3).
301 Moreover, the obtained profiles of the five human vaginal swab samples confirmed the proposed
302 existence of community state types (CSTs) of the vaginal microbiota dominated by specific
303 *Lactobacillus* taxa (24, 25). In fact, HV1 is dominated by *Lactobacillus gasseri*, while
304 *Lactobacillus iners* and *Lactobacillus crispatus* are the most abundant lactobacilli taxa in HV2/HV5
305 and HV3/HV4, respectively (Figure 3). Furthermore, in all five reconstructed human vaginal
306 profiles, *L. helveticus* is the second most abundant *Lactobacillus* species, as observed in the

307 aforementioned CSTs (24, 25) (Figure 3). Thus, based on the classification proposed by DiGiulio et
308 al. (24), HV1 can be classified as a CST 2, while HV2/HV5 falls within the CST 3, whereas
309 HV3/HV4 can be attributed to CST 1.
310 To demonstrate the relevance of an efficient methodology for precise cataloguing of the
311 *Lactobacillus* species for which a complete LITSA sequence is available in different environments,
312 we analyzed five free range chickens cecal samples. The retrieved profiles revealed a high relative
313 abundance (ranging from a total of 53.1 % to 96.8 %) of *Lactobacillus* species previously
314 characterized in poultry or other birds, such as *Lactobacillus salivarius, Lactobacillus reuteri,*
315 *Lactobacillus ingluviei, Lactobacillus amylovorus, Lactobacillus agilis, Lactobacillus aviarius* and
316 *Lactobacillus johnsonii* (26-33) (Figure 3). Notably, samples FRC1, FRC2 and FRC3 showed a
317 similar profile with high abundance of *L. salivarius, L. ingluviei* and *L. amylovorus*, reflecting the
318 fact that they were kept in the same hen house (Figure 3). Accordingly, samples FRC4 and FRC5,
319 collected in two additional hen houses, showed different profiles characterized by high abundance
320 of *L. aviarius* and *L. johnsonii*, respectively (Figure 3).
321 For milk and milk-related products, profiling of five whey and five fresh parmesan cheeses (at 1
322 day of ripening) samples revealed, as expected, similar profiles dominated by *L. helveticus* and *L.*
323 *delbrueckii* (Figure 3), which represent two lactobacilli species typically used as starter cultures for
324 the production of cheese (34). These data indicate that the *Lactobacillus* ITS profiling approach also
325 represents a valuable tool for monitoring the population of lactobacilli across the cheese production
326 chain.
327 Results obtained from ITS-profiling were also compared to profiles reconstructed through analysis
328 of OTUs generated at 99 % identity from 16S rRNA profiling data (Figure 3) (Table S5). Notably,
329 only OTUs classified as lactobacilli have been included in the representation, thus the relative
330 abundance of unclassified lactobacilli reported in the bar plot do not include additional OTUs that
331 could not be attributed to this genus. Moreover, lactobacilli species whose relative abundance is
332 below 5 % in each sample were collapsed under “Others <5 %” in the bar plot representation.

333 Interestingly, the ITS profiling approach provided a more accurate species-level reconstruction of
334 the lactobacilli populations when used to analyze human faecal and vaginal samples as well as free
335 range chicken faecal samples. Moreover, it confirmed and partially improved the simple lactobacilli
336 community of whey and fresh Parmesan cheese samples observed through 16S rRNA gene
337 profiling. In fact, differences in the profiles obtained through 16S rRNA gene and ITS profiling can
338 be observed in all cases (Figure 3) (Table S5). Such differences are caused by the limited number of
339 *Lactobacillus* species that could be discriminated based on partial16S rRNA gene sequence respect
340 to ITS sequence (Figure 3) (Table S5).
341 Altogether, these results confirm the performance of the *Lactobacillus* ITS profiling protocol
342 observed from analysis of the artificial sample and validate their use, complemental to 16S rRNA
343 gene profiling, for analysis of a wide range of complex environmental and host-associated matrices.
344

345 **Conclusions**

346 We developed a newly designed method for characterization of the *Lactobacillus* population in
347 complex environments based on the use of the internally transcribed spacer (ITS), which represents
348 a hypervariable region located between the 16S and the 23S rRNA genes that allows high-accuracy
349 species-level profiling. The accuracy and sensitivity of this method allowed profiling of complex
350 communities of lactobacilli with a successful identification of taxa with abundance of $1.85*10^3$
351 cells/ µl, which is even lower to what was previously identified for a similar approach developed for
352 the profiling of bifidobacterial communities (13). Notably, despite the fact that the current LITSA
353 database allows the precise profiling of just 62 species, the ITS-profiling approach represents a new
354 metagenomic tool for species-level profiling of complex lactobacilli communities that complements
355 phylogenetic group assignments that can be obtained from 16S rRNA gene profiling data.
356 Moreover, the database will be regularly updated to represent additional lactobacilli species as
357 genomes encompassing complete LITSA sequences are becoming available. When the ITS
358 lactobacilli profiling method was applied to different biological samples, encompassing the stool of

359 human as well as birds, vaginal swabs and cheese, it allowed the reconstruction of the cataloguing
360 of lactobacilli communities residing in these environments. Altogether, these results highlight that
361 ITS-mediated profiling of populations of lactobacilli could be useful not only for academic
362 purposes, but also for industrial applications such as tracing the microbial composition of probiotic
363 products based on lactobacilli as well as of starter cultures in food manufacture.
364
365 **Material and methods**
366 **Sample collection**
367 In the frame work of a more extensive bacterial cataloguing project, this study enrolled stool,
368 vaginal swab, fresh parmesan cheese (one day of ripening), whey and cecal (from free range
369 chickens) samples.
370 Five fresh stool samples obtained from human healthy volunteers and five cecal samples retrieved
371 from free range chickens were immediately frozen upon collection at -80°C until processing for
372 DNA extraction. The DNA extraction was performed using the QIAamp DNA Stool Mini Kit
373 following the manufacturer's instructions (Qiagen, Manchester, UK). Additionally, five vaginal
374 swab samples were collected in sterile tubes containing 1 ml of DNA-RNA shield from ZYMO
375 Research until bacterial DNA extraction using ZymoBIOMICS^{TM} DNA Miniprep Kit (ZYMO
376 Research). Furthermore, 10 ml samples of whey and 2-4 gr of fresh parmesan cheese were collected
377 in sterile tubes and the DNA was extracted using the DNeasy Mastitis Mini Kit (Qiagen Ltd,
378 Strasse, Germany) following the manufacturer's instructions (Qiagen Ltd). Notably, whey samples
379 and cheese samples at one day of ripening were collected from the same Parmesan cheese producer
380 in Parma, Italy.
381 **Ethical statement.** This study was carried out in accordance with the recommendations of the
382 ethical committee of the University of Parma and was approved by the "Comitato di Etica
383 Università degli Studi di Parma", Italy. All animal procedures were performed according to national
384 guidelines (Decreto legislativo 26/2014).

385 **Bacterial growth conditions and DNA extraction.** Type strains of several lactobacilli taxa (Table
386 S3) were growth in Man-Rogosa-Sharpe (MRS) medium (Scharlau Chemie) supplemented with
387 0.05 % (w/v) L-cysteine hydrochloride and incubated in an anaerobic atmosphere (2.99 % H2,
388 17.01 % CO2 and 80 % N2) in a chamber (Concept 400; Ruskin) at 37°C for 24 h. In addition, nine
389 non-lactobacilli microorganisms were used in this study. These included *Bifidobacterium bifidum*
390 LMG11041, which was cultivated in MRS broth as *Lactobacillus* strains; *Collinsella intestinalis*
391 DSM 13280, *Escherichia coli* LMG 2092, and *Klebsiella pneumoniae* CECT 143, which were
392 grown in de MRS broth (Difco, Detroit, MI) supplemented with 0.05% (w/v) l-cysteine (MRSC;
393 Sigma, St. Louis, MO). *Prevotella copri* DSM 18205 and *Blautia coccoides* DSM 935 were
394 cultivated in a combination of Reinforced Clostridial Broth (Merck, Darmstadt, Germany) and
395 Brain-Heart Infusion (Difco), supplemented with 5% (v/v) heat-inactivated fetal bovine serum
396 (LabClinics, Barcelona, Spain) respectively. For *Bacteroides thetaiotaomicron* DSMZ 2079, the
397 latter medium was supplemented with 0.005 % hemin (Sigma) and 0.005 % Vitamin K1 (Sigma).
398 *Faecalibacterium prausnitzii* DSM 17677 was grown in Wilkins-Chalgren Anaerobe broth (Merck),
399 following the recommendations included in the DSMZ medium 339. Finally, an active culture of
400 *Methanobrevibacter smithii* DSM 861 grown in *Methanobacterium* medium (DSMZ 119) was
401 directly supplied by DSMZ.
402 Bacterial DNA was extracted using GenEluteTM Bacterial Genomic DNA kits (SIGMA-
403 ALDRICH) following the manufacturer's instructions. Taxonomic identity of the microorganisms
404 was validated by sequencing the V3 variable region of the 16S rRNA gene using primer pair
405 Probio_Uni and/Probio_Rev (14).
406 ***Lactobacillus* mock community**
407 The cultures of fourteen different *Lactobacillus* strains were grown separately on Man-Rogosa-
408 Sharpe (MRS) medium (Scharlau Chemie) supplemented with 0.05 % (w/v) L-cysteine
409 hydrochloride and incubated in an anaerobic atmosphere (2.99 % H2, 17.01 % CO2 and 80 % N2)
410 in a chamber (Concept 400; Ruskin) at 37°C until they reached late log phase. The bacteria were

411 enumerated by counting colonies on solid medium and the optical density at 600 nm was
412 determined. The final bacterial cell concentration was approximately 10^7 cfu/ml. Chromosomal
413 DNA of each strains was extracted as previously described and subsequently mixed.
414 Specifically, the mock community consists of a pool of known concentration of fourteen different
415 *Lactobacillus* strains to obtain the final quantity of DNA indicated in Table S3. Furthermore, the
416 mix was prepared by combining equal volumes (20 µL) of DNA.
417 The DNA from the mix was diluted to produce a final DNA concentration of 2 ng/µL, and 4 µL of
418 these dilutions were used in each PCR reaction. For the PCR reaction, the primer pair Probio-
419 lac_Uni/Probio-lac_Rev was used and the generated amplicons were sequenced using Illumina
420 MiSeq as described below.
421 ***Lactobacillus* ITS-specific primer design and gene amplification**
422 The bioinformatics platforms MEGAnnotator (10) and METAnnotatorX (unpublished data) were
423 used to perform 16S and 23S rRNA genes prediction in all 1523 sequenced lactobacilli genomes
424 deposited at the NCBI Genomes database. Primers Probio-lac_Uni
425 (CGTAACAAGGTAGCCGTAGG)/Probio-lac_Rev (GTYVCGTCCTTCWTCGSC) were
426 manually designed based on the alignment of all 16S and 23S rRNA sequences to generate an
427 amplicon encompassing the 3'-end of the 16S rRNA gene, the ITS region and the 5'-end of the 23S
428 rRNA gene. Specificity test was performed using the Silva TestProbe v. 3.0 tool (https://www.arb-
429 silva.de/search/testprobe/) that allows alignment of primers sequences to the Silva SSU and LSU
430 databases (15). A custom bioinformatics script was then used to create a database of all the Probio-
431 lac_Uni/Probio-lac_Rev-generated lactobacilli ITS amplicon sequences (LITSA database),
432 encompassing the Internally Transcribed Spacer (ITS) region and partial 16S and 23S rRNA genes.
433 The PCR conditions used for *Lactobacillus* ITS profiling using the Probio-lac_Uni/Probio-lac_Rev
434 primer pair were 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 58 °C, and 40 s at 72 °C,
435 followed by 10 min at 72 °C. Amplification was carried out using a Verity Thermocycler (Applied
436 Biosystems). The integrity of the PCR amplicons was analyzed by gel electrophoresis. An

437 additional specificity test was performed by PCR using the DNA extracted from all known
438 *Lactobacillus* species as well as *B. bifidum* ATCC11041, *C. intestinalis* DSM 13280, *E. coli* LMG
439 2092, *K. pneumoniae* CECT 143, *P. copri* DSM 18205, *Bl. coccoides DSM 935*, *Bc.*
440 *thetaiotaomicron* DSMZ 2079, *F. prausnitzii* DSM 17677 and *M. smithii* DSM 861.
441 WebLogo representation of primer sequence conservation among the retrieved 16S and 23S rRNA
442 genes flanking complete ITS sequences was obtained through the WebLogo website
443 (http://weblogo.berkeley.edu/) (35).

444 **Illumina MiSeq sequencing of ITS gene-based amplicons**

445 Illumina adapter overhang nucleotide sequence was added to the PCR amplicons obtained following
446 amplification of the ITS region, as previously described (13). The library of ITS amplicons was
447 prepared following the 16S Metagenomic Sequencing Library Preparation Protocol (Part No.
448 15044223 Rev. B-Illumina). Sequencing was performed using an Illumina MiSeq sequencer with
449 MiSeq Reagent Kit v3 chemicals.

450 **ITS-based microbiota analysis**

451 Fastq files obtained from metagenomic sequencing of each sample were analyzed using a custom
452 script for QIIME software suite (22) and the LITSA database available at
453 (http://probiogenomics.unipr.it/pbi/index.html).
454 Input data were processed in the following steps: filtering of the reads based on length > 100 nt
455 (primers included) to avoid primer dimers, overall quality > 25 and the presence of forward and
456 reverse primers in the forward and reverse reads, respectively, creation of OTUs constituted by
457 identical sequences using prefix_suffix method and removal of OTUs represented by < 10
458 sequences. Taxonomy assignment was performed using RDP method (RDP classifier with a
459 confidence level of 0.80) and the LITSA database constituted by ITS sequences retrieved from the
460 1523 *Lactobacillus* genomes available at the NCBI Genome database. This script is easily
461 modifiable to obtain a profiling based on a different sequence, though will depend on the
462 availability of a corresponding database.

463 **Evaluation of the sensitivity of the Probio-lac_Uni/Probio-lac_Rev primer pair**

464 The artificial sample used for the evaluation of the detection sensitivity and accuracy of the Probio-
465 lac_Uni/Probio-lac_Rev primer set was generated using known DNA amounts, ranging from 50 to
466 0.006 ng, of 14 different *Lactobacillus* taxa (Table S3).

467 **Microbiota identification by 16S rRNA gene- amplification, -sequencing and data analysis**.

468 Partial 16S rRNA gene sequences were amplified from extracted DNA using primer pair
469 Probio_Uni / Probio_Rev, which target the V3 region of the 16S rRNA gene sequence (16). 16S
470 rRNA gene amplification and amplicon checks were carried out as previously described (16). 16S
471 rRNA gene sequencing was performed using a MiSeq (Illumina) at the DNA sequencing facility of
472 GenProbio srl (www.genprobio.com) according to a previously reported protocol (16). Following
473 sequencing, the .fastq files were processed using a custom script based on the QIIME software suite
474 (22). Paired-end read pairs were assembled to reconstruct complete Probio_Uni / Probio_Rev
475 amplicons. Quality control retained sequences with a length between 140 and 400 bp and mean
476 sequence quality score >20 while sequences with homopolymers >7 bp and mismatched primers
477 were omitted. 16S rRNA gene Operational Taxonomic Units (OTUs) were defined at ≥ 99 %
478 sequence homology using uclust (36) and OTUs with less than 10 sequences were filtered. All reads
479 were classified to the lowest possible taxonomic rank using QIIME (37) and a reference dataset
480 from the SILVA database (Quast et al., 2013).

481

482 **Nucleotide sequence accession numbers**

483 The raw ITS and 16S rRNA gene sequences reported in this article have been deposited in the
484 NCBI Short Read Archive (SRA) under the accession number PRJNA434072.

485

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609

610

611 **Table 1:** List of *Lactobacillus* species with LITSA sequence identity ≥97 % with another
612 *Lactobacillus* species. The percentage reported corresponds to the highest identity observed among
613 all LITSA sequences identified in strains of the two species compared.

Species	LITSA % identity with the closest species	Closest species
Lactobacillus acidophilus	98	*Lactobacillus amylovorus*
	97	*Lactobacillus crispatus*
Lactobacillus amylovorus	98	*Lactobacillus acidophilus*
	97	*Lactobacillus crispatus*
Lactobacillus buchneri	99	*Lactobacillus parabuchneri*
Lactobacillus casei	99	*Lactobacillus paracasei*
	100	*Lactobacillus rhamnosus*
Lactobacillus crispatus	97	*Lactobacillus acidophilus*
	97	*Lactobacillus amylovorus*
Lactobacillus curvatus	98	*Lactobacillus sakei*
Lactobacillus gallinarum	99	*Lactobacillus helveticus*
Lactobacillus gasseri	99	*Lactobacillus johnsonii*
Lactobacillus helveticus	99	*Lactobacillus gallinarum*
Lactobacillus johnsonii	99	*Lactobacillus gasseri*
Lactobacillus parabuchneri	99	*Lactobacillus buchneri*
Lactobacillus paracasei	99	*Lactobacillus casei*
	100	*Lactobacillus rhamnosus*
Lactobacillus paraplantarum	100	*Lactobacillus pentosus*
	100	*Lactobacillus plantarum*
Lactobacillus pentosus	100	*Lactobacillus paraplantarum*
	99	*Lactobacillus plantarum*
Lactobacillus plantarum	100	*Lactobacillus paraplantarum*
	99	*Lactobacillus pentosus*
Lactobacillus rhamnosus	100	*Lactobacillus paracasei*
	100	*Lactobacillus casei*
Lactobacillus sakei	98	*Lactobacillus curvatus*

614

615 **Figure legends**

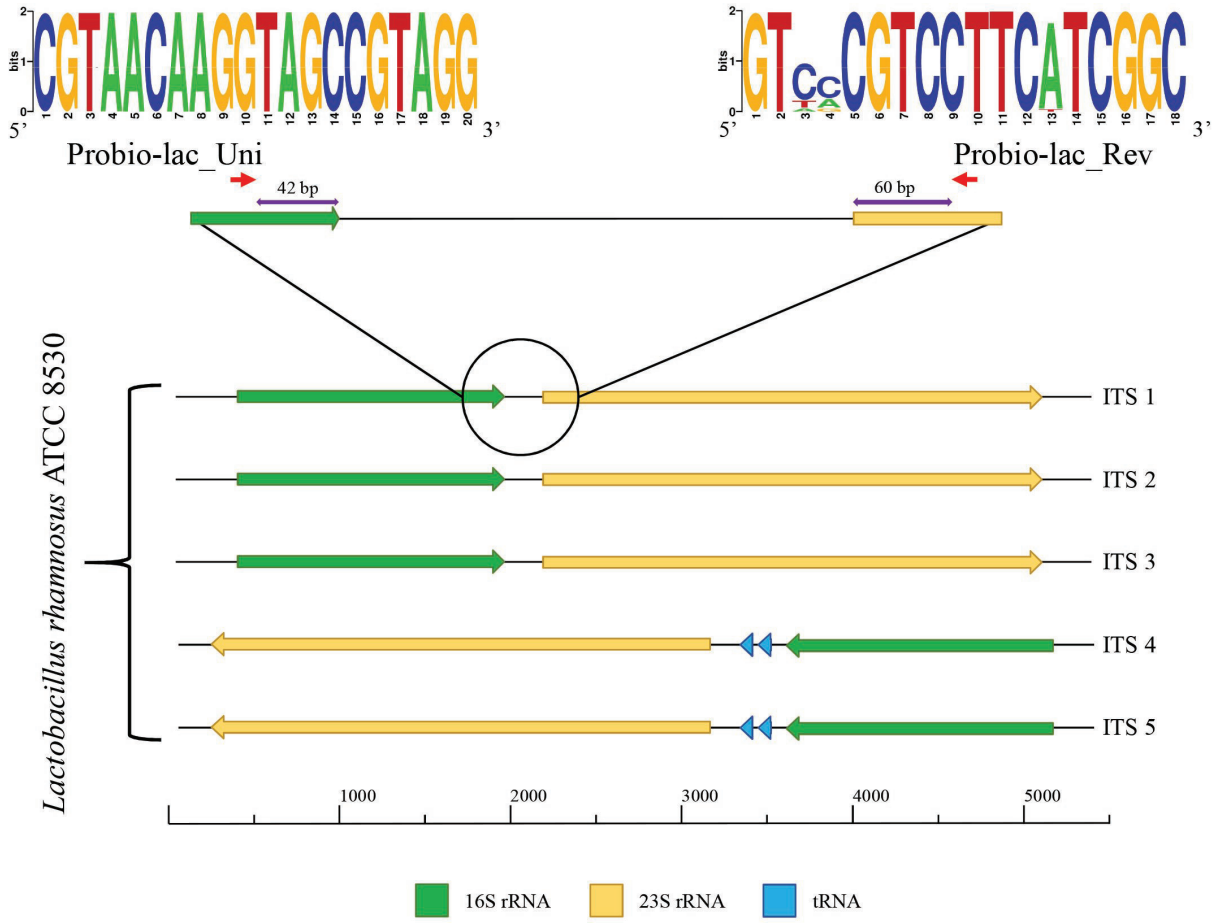
616 **Figure 1:** Genetic map of the Internally Transcribed Sequence (ITS) region of *Lactobacillus* with
617 and without tRNA genes. Panel a depicts the genetic organization of the five complete ITS regions
618 predicted in the complete genome of *Lactobacillus rhamnosus* ATCC 8530, used here as a test case.
619 Primer sequence conservation is shown through a WebLogo representation where the overall height
620 of the stacks indicates the sequence conservation at that position, while the height of symbols within
621 the stacks indicates the relative frequency of nucleic acids at that position. Panel b illustrates the
622 details of ITS regions identified in the complete genomes of species included in the mock sample
623 for which a complete genome was available. ITS sequences without tRNA genes are highlighted in
624 green, while ITS regions encoding tRNA genes are marked in blue. Black and red text indicates
625 forward and reverse strand orientation, respectively.

626

627 **Figure 2:** Evaluation of the sensitivity and accuracy of the *Lactobacillus* ITS profiling protocol.
628 The graph shows the expected and observed relative abundance of 14 *Lactobacillus* taxa
629 constituting an artificial sample. An exponential trendline is reported for the expected and observed
630 data.

631

632 **Figure 3:** ITS and 16S rRNA gene profiling of *Lactobacillus* species in five ecological niches. The
633 profile of the *Lactobacillus* population obtained for: a) five human faecal samples (HG); b) five
634 human vaginal swab samples (HV); c) five free range chicken faecal samples (FRC); d) five whey
635 samples (WH), and e) five parmesan cheese samples (PC) is depicted in the corresponding bar
636 plots. Only species with relative abundance >5% in at least a sample are reported. Species below
637 5% are collapsed in "Others <5 %.



B)

	ITS 1	ITS 2	ITS 3	ITS 4	ITS 5	ITS 6	ITS 7	ITS 8	ITS 9
Lactobacillus curvatus FBA20	211 bp	426 bp	211 bp	211 bp	211 bp	426 bp	-	-	-
Lactobacillus acidophilus NCFM	130 bp	377 bp	130 bp	132 bp	-	-	-	-	-
Lactobacillus brevis NPS QW 145	205 bp	205 bp	205 bp	205 bp	885 bp	-	-	-	-
Lactobacillus delbrueckii KCTC 13731	208 bp	447 bp	208 bp	447 bp	208 bp	208 bp	447 bp	443 bp	208 bp
Lactobacillus amylovorus DSM20531	192 bp	192 bp	438 bp	438 bp	192 bp	-	-	-	-
Lactobacillus fermentum FTDC8312	192 bp	193 bp	338 bp	193 bp	338 bp	-	-	-	-
Lactobacilllus helveticus H10	192 bp	442 bp	192 bp	460 bp	-	-	-	-	-
Lactobacillus plantarum ZS2058	425 bp	196 bp	196 bp	196 bp	425 bp	-	-	-	-
Lactobacillus rhamnosus ATCC 8530	211 bp	211 bp	211 bp	424 bp	424 bp	-	-	-	-
Lactobacillus ruminis ATCC 27782	427 bp	205 bp	305 bp	405 bp	205 bp	205 bp	-	-	-

Figure 1

Figure 2

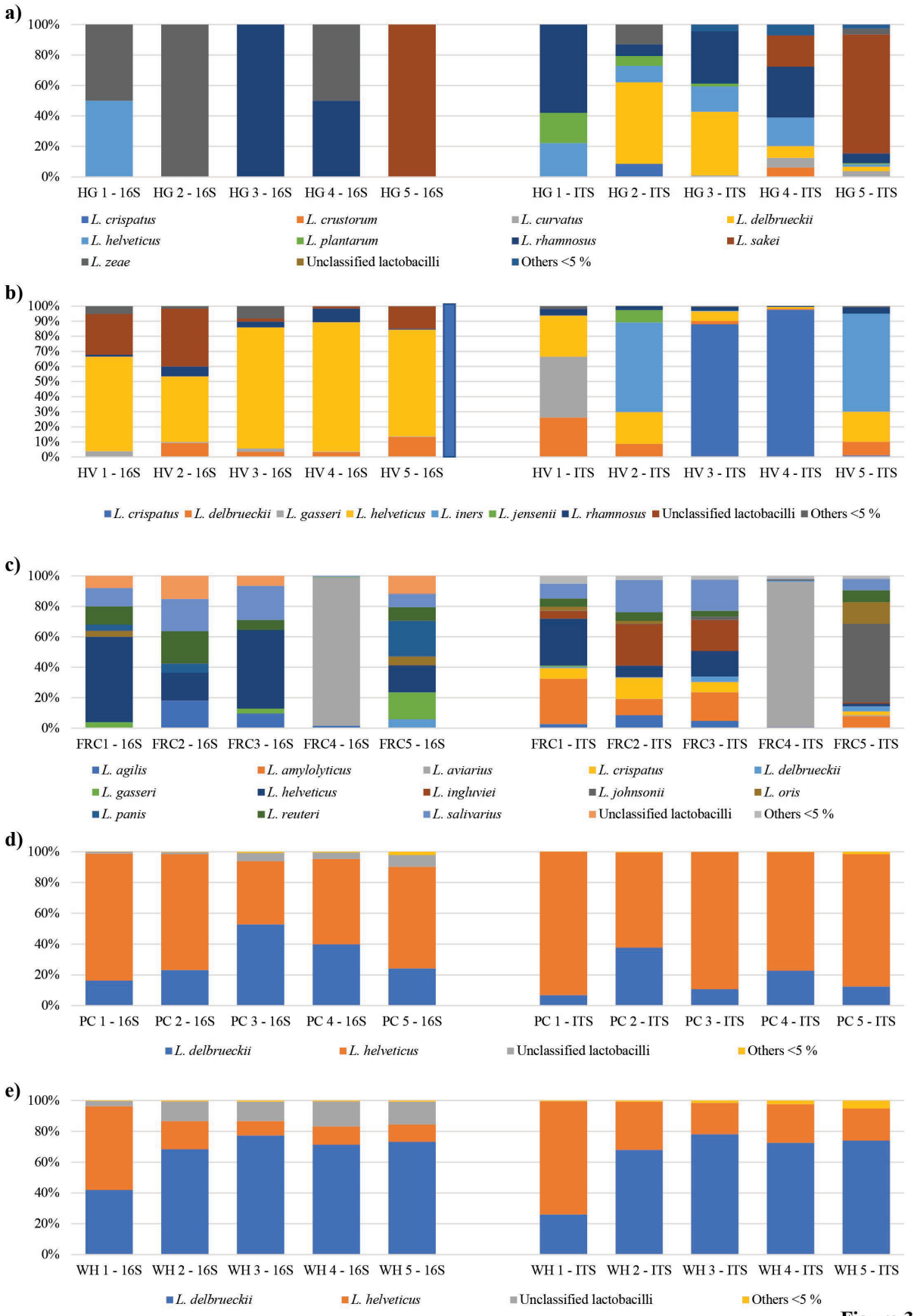


Figure 3