***Lactobacillus reuteri*: direct passage from ingested yogurts to urine microbiota**

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Short title: Bacterial passage from yogurt to urine

22 **Abstract**

23 **Background:** Serendipitously, it was observed that fecal transplantation made for
 24 *Clostridium difficile* may cure chronic urinary tract infections. This led us to evaluate the
 25 passage in the urine of probiotics contained in yoghurt, which have been claimed to prevent
 26 urinary infections.

27 **Results**

28 A commercial yogurt that contained 3 probiotics (*Bifidobacterium animalis*, *Lactobacillus*
 29 *delbrueckii* and *Lactobacillus reuteri*) was consumed by 28 healthy subjects. We performed
 30 by culturomics, urine analysis before and after feeding these yogurts. Genome sequencing of
 31 the bacterial strains absent before yogurt consumption and present after consumption was
 32 performed. Testing more than 40,000 colonies by MALDI-TOF, we observed in two men and
 33 one woman (11% of subjects included), the urine colonization of the same *Lactobacillus*
 34 *reuteri* present in yogurts, with the same genome with 50 genes never identified in other
 35 lactobacilli.

36 **Conclusion:** This confirms that, as ingested, *Lactobacillus salivarius* passes into the milk of
 37 lactating women, some bacteria, particularly *Lactobacillus* can colonize body fluids
 38 previously considered sterile after ingestion via the digestive route. Although the
 39 consequences of this passage remain unknown, we prove for the first time that there is a
 40 digestive passage in the urine after consumption of probiotics, including fermented products
 41 sold commercially.

42 **Keywords:** *Lactobacillus reuteri*; urine microbiota; culturomics; probiotics

43 Background

44 The role of probiotics in the control of urinary tract infections remains controversial
 45 [1]. In addition, the mechanism of its putative activity has not yet been clarified. Nevertheless,
 46 it was demonstrated that after oral ingestion, *Lactobacillus salivarius* and *L. fermentum* could
 47 be identified in human milk [2]; patients treated with these probiotics improved more and had
 48 fewer recurrence of mastitis compared to patients treated with antibiotics [2]. In addition,
 49 more recently, *L. salivarius* oral administration in pregnant women has also demonstrated its
 50 efficiency to prevent infectious mastitis [3]. Therefore, it exists a passage from digestive tract
 51 to the biologic fluids. At the same time, a paradigm shift has recently been observed,
 52 concomitantly through metagenomics and culture techniques, demonstrating that urine is not
 53 sterile [4, 5]. A recent study hypothesizes interconnection from vaginal microbiota and urine
 54 bladder microbiota in women by culture. However, it is neglecting oxygen intolerant bacteria
 55 which were revealed by metagenomic studies [6] and do not explain male urine microbiota.
 56 Here, we have tested the passage of *Lactobacillus* spp. from dairy products after ingestion by
 57 healthy individuals. The genome sequences of the cultured strains were analyzed in order to
 58 confirm the strain identity.

59 Results

60 In a preliminary work involving 4 different yogurts, we tested after ingestion in urine
 61 samples of 8 individuals, 6,144 different colonies identifying 79 different bacterial species
 62 (Supplementary table 1). In a 36-year-old man (individual 1), *Lactobacillus reuteri* recovers
 63 in the urine sample after 24 hours of Bifidus yogurt consumption (*L. reuteri*, CSUR P4904).
 64 Indeed, we selected this yogurt for the second step of our study involving 20 volunteers.
 65 Culturomics analysis of this yogurt (Bifidus, Carrefour), identified three different species of
 66 probiotics: *Bifidobacterium animalis* (7.10^8 CFU/mL), *Lactobacillus delbrueckii* (4.10^7
 67 CFU/mL) and *Lactobacillus reuteri* (2.10^5 CFU/mL) (CSUR P4870). We tested the urine of

volunteers after 7 days of daily yogurt consumption. We recovered from urine, 34,650 colonies by MALDI-TOF (Table 1). Overall, we identified 151 bacterial species. In 2 individuals, *Lactobacillus reuteri* was absent of the urine sample before yogurts consumption whereas it was present after a week consumption. The 2 individuals were a 30-year-old women (individual 14, *L. reuteri*, CSUR P5461) and a 35-year-old man (individual 25, *L. reuteri*, CSURP5460) (Table 1). Dendrogram performed using the 4 *L.reuteri* strains of this study detected by MALDI-TOF (CSUR4870= yogurt, CSUR 4904= individual 1, CSUR5461= individual 14, CSUR5460=individual 25), as well as 7 other *L.reuteri* strains for which spectra were available in our database revealed 4 different clusters. Three of the strains were included on a same cluster, while the last strain was classified in a close cluster (**Figure 1**).

The genomes of the four isolates of *L. reuteri* strains were sequenced and assembled (supplementary table 1). The genome sizes of the four strains were 2,039,275 (+/- 40) bp with a GC content of ~39%. The number of total encoding genes was similar between four strains with an average of 1,631 (+/- 28). In order to study the evolutionary relationships among the different strains of *L. reuteri*, a phylogenetic reconstruction based on the core-genomes alignment of all these strains was carried out (**Figure 2**). The 4 strains of *L reuteri* (CSUR P4870, CSUR P4904, CSUR P5460, CSUR P5461) were distantly separated from all other strains with strongly supported bootstrap values. These 4 strains were grouped along the same branch in a single clade confirming the common origin for these strains. Single nucleotide polymorphisms analyses were performed revealing a close proximity between the strains CSUR P4870, CSUR P4904, CSUR P5460, and CSUR P5461 with only 181 to 432 SNIPS differencing them, as compared to all other strains (**Table 2**). Accordingly, these results confirmed the common origin for these four strains.

Orthologous genes were identified by a bidirectional blast approach of all CDS of the 15 genomes of *L. reuteri* using ProteinOrtho (**Figure 3**). This approach detected 1,283 orthologs shared by all 15 genomes, i.e. in average 67% of the CDS of each strain are part of the core proteome. A specific group of 50 encoding-genes was only identified in the *L. reuteri* (CSUR P4870, CSUR P4904, CSUR P5460, CSUR P5461), mostly composed of hypothetical proteins (41 proteins) (**Supplementary Table 1**). Among proteins with assigned functions, we identified proteins involved in metabolism of nitrogen (carbon-nitrogen bonds and other peptide bonds), in transfer of nitrogenous group such as transaminase and in carbohydrate active -transport system. Interestingly, a candidate protein was annotated as an oxalyl-CoA decarboxylase. Finally, all genomes contain two clusters of polyketide synthases and one cluster of nonribosomal peptide synthetases (**Table 3**). The genes order was relatively conserved among genomes and the clusters were found in syntenic regions, with the exception of the genome of *L. reuteri* TD1 that contains one non-modular cluster of polyketide synthases. The lengths of the clusters were very similar among genomes with a mean of 4,148 and 12,566 bp for the polyketide synthases clusters and 3,851 bp for the cluster of nonribosomal peptide synthetases (**Table 3**).

Discussion

Here, we demonstrated the proof of concept that the consumption of dairy products can lead to the presence of fermenting bacteria in the urine human fluids. We are strongly confident in our data as we included negative and positive controls for each culturomics and genomic step [7]. In addition, we observed the same results in 3 different individuals among the 28 tested (10.7%), including 2 collected in men. This seems to rule out the usual pattern of vaginal contamination of urine with *Lactobacillus*. To the best of our knowledge, none study have previously demonstrated that oral administration of probiotic could be followed by a urinary passage from the gut. Coupled with genome analysis allowing to hypothesize that

specific bacterial characteristics facilitated modification of urine microbiota, this opens several reflections on the possible colonization of the internal mucosa by transient bacteria through the digestive tract.

The precise role, the duration of excretion of these *Lactobacillus* in urine after a single dose, and the significance of this passage are not currently fully understandable. The decrease of the diversity of the human urinary microbiota is a risk factor of urinary tract infection. The role of the administration of probiotic in protecting against urinary tract infection in both men and women remains controversial [1, 13]. Nevertheless, intra urethral *Lactobacillus casei* administration improves urinary tract infection caused by *E. coli* in mice [14]. If many questions remain unanswered, our results should help to end the debate over the meaning of *Lactobacillus* in the urine, which has long been thought to be exclusively female and of vaginal origin [6]. The origin from the digestive tract by oral ingestion of yogurts in the urine supposes a direct passage from the digestive tract to the bladder through the blood or lymphatic system and opens a new field of reflection.

Interestingly, oxalyl-CoA decarboxylase is known to be a major enzyme involved in the bacterial detoxification of oxalate within the human intestinal tract [15]. Oxalate is a toxic compound widely distributed in food [16]. The possible role of oxalate-degrading anaerobic bacteria in oxalate-related diseases has already been studied, and a direct correlation between oral administration of probiotic bacteria and the reduction of urinary oxalate excretion has been evidenced in rats and humans [17]. This suggests that administration of probiotics could have a role in the prevention of oxalate urinary lithiasis. *Lactobacillus* spp. have been reported for a long time as having an antimicrobial activity and are acidifying culture media. Both of these actions may be of interest in preventing bladder infections.

Conclusion

Finally, it was recently demonstrated that fecal microbiota transplantation led not only to the resolution of recurrent *Clostridium difficile* infections, but also to a significantly decrease of recurrent urinary tract infections frequency, and improved antibiotic susceptibility profile of causative microorganisms lacked explanation [18]. The possible passage of microorganisms from digestive tract could lead to a paradigm shift in the understanding of urinary tract infections and beyond that on the relationships between urinary microbiota and human health [1, 19].

Material and Methods

Patients and samples

Healthy individuals, aged from 24 to 44 years old, were recruited. Inclusion criteria were absence of urinary tract infection symptoms, absence of treatment with antibiotics in the last month, absence of yogurt consumption in the last 2 days. The first step of the study included 8 individuals (4 male and 4 female). Microbiological analysis of the urine was performed before and after the consumption of 2 yogurts (125g) a day. Two individuals have consumed 4 different yogurts (Bifidus, Carrefour, France; Nature sucré, Carrefour, France, Activia brassé Nature, Danone; Danacol, Danone, France). We selected these yogurts among their large consumption in France and we directly bought in supermarket these yogurts with a date of expiry compatible with their consumption.

The second step of the study included 20 volunteers (10 male and 10 female). Microbiological analysis of the urine was performed before the consumption of one of the selected yogurt (Bifidus, Carrefour, France) and after the consumption of 2 yogurts (125g) by 24 hours during 1 week. Urines were collected after local disinfection. This study was approved by the Ethics Committee of the IHU Méditerranée Infection under Number 2016-011.

Culturomics analysis:

Urine samples, as well as yogurt samples, were directly inoculated in Man Rogosa Sharpe (MRS) agar (Biomérieux, Marcy l'Etoile, France) and in 5% sheep blood agar (Biomérieux, Marcy l'Etoile, France) in both aerobic and anaerobic atmosphere using GENbag anaer system at 37°C. Culture were preserved during 5 days and all the colonies were tested by MALDI-TOF mass spectrometry (Bruker Daltonik, Leipzig, Germany) [7].

Identification methods

The growing colonies were first identified using our systematic matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF-MS) screening on a MicroFlex LT system spectrometer (Bruker Daltonics) [8]. Each deposit was covered with 2 mL of a matrix solution (saturated α -cyano acid-4-hydroxycinnamic in 50% acetonitrile and 2.5% trifluoroacetic acid). For each spectrum, a maximum of 100 peaks was used, and these peaks were compared to those of previous samples in the computer database of the Bruker Base and in our homemade database, including previously identified bacterial spectra. Protein profiles are regularly updated based on the results of clinical diagnoses and on new species, providing new spectra, our database containing 7,462 bacterial spectra at the time of our study.

If, after three attempts, the bacterial colony could not be accurately identified by MALDI-TOF or if the bacterium had not previously been isolated from the human gut, 16S rRNA gene-based identification was performed by amplifying and sequencing the bacterial 16S rRNA gene extracted from the pure culture using the previously described DNA extraction method.

Strain deposition, 16S rRNA and genome sequencing accession numbers

All the strains of *Lactobacillus reuteri* isolated in this study were sequenced and deposited in the Collection de Souches de l'Unité des Rickettsies (CSUR, WDCM 875) and are readily available at (<http://www.mediterranee>

infection.com/article.php?leref=14&titre=collection-de-souches&PHPSESSID=cncregk417f197gheb8k7u7t07) (Supplementary Table 2). The 16S rRNA accession numbers and the draft genomes are deposited with an available GenBank accession number (Supplementary Table 2).

Genome sequencing

Genomic DNAs of *Lactobacillus reuteri* CSURP4870, *Lactobacillus reuteri* CSURP4904, *Lactobacillus reuteri* CSURP5460 and *Lactobacillus reuteri* CSURP5461 were sequenced on the MiSeq Technology (Illumina, Inc, San Diego CA 92121, USA) with paired end and barcode strategy in order to be mixed with others projects constructed according to the Nextera XT library kit (Illumina). The gDNAs were quantified by a Qubit assay with the high sensitivity kit (Life technologies, Carlsbad, CA, USA) to 12.6 ng/μl, 2.4 ng/μl, 33.9 ng/μl and 23.7 ng/μg respectively, and dilutions were performed to require an input of 1 ng of DNA. The « tagmentation » step fragmented the genomic DNA. Then, limited cycle PCR amplification (12 cycles) completed the tag adapters and introduced dual-index barcodes. After purification on AMPure beads (Lifetechnologies, Carlsbad, CA, USA), the libraries were then normalized on specific beads according to the Nextera XT protocol (Illumina). Normalized libraries are pooled for sequencing on the MiSeq. Automated cluster generation and paired-end sequencing with dual index reads was performed in a single 39-hour run in a 2x251bp. Within this pooled run, the numbers of paired-end reads were 577,861 for *Lactobacillus reuteri* CSURP4870, 723,210 for *Lactobacillus reuteri* CSURP4904, 1,732,818 for *Lactobacillus reuteri* CSURP5460 and 1,514,107 for *Lactobacillus reuteri* CSURP5461. The paired end reads were trimmed and filtered according to the read qualities.

Bioinformatic and evolutionary analyses

All complete genomes from *Lactobacillus reuteri* were downloaded from NCBI : *Lactobacillus reuteri* DSM 20016, GCA_000016825.1; *Lactobacillus reuteri* JCM 1112,

GCA_000010005.1; *Lactobacillus reuteri* SD2112, GCA_000159455.2; *Lactobacillus reuteri* ATCC 53608, GCA_000236455.2; *Lactobacillus reuteri* I5007, GCA_000410995.1, *Lactobacillus reuteri* TD1, GCA_000439275.1, *Lactobacillus reuteri* IRT, GCA_001046835.1, *Lactobacillus reuteri* ZLR003, GCA_001618905.1, *Lactobacillus reuteri* I49, GCA_001688685.2. After sequencing, reads from strains *Lactobacillus reuteri* (CSUR4870, CSUR 4904, CSUR5460, and CSUR5461) were mapped on the reference *L. reuteri* JCM 1112 using CLC Genomics workbench 7 with the following thresholds: 0.7 for coverage and 0.8 for similarity. Consensus sequences were extracted from the mapped reads (>3 reads threshold). All genomes were annotated using Prokka using default parameters [9]. For orthologous detection, we applied Proteinortho with default values [10]. All orthologous genes were aligned using Muscle, then concatenated [11]. Phylogenetic reconstruction was performed using RaxML (Randomized Axelerated Maximum Likelihood) with the GTRCAT model and bootstrap value of 100 [12]. Single nucleotide polymorphisms and insertion deletion events were calculated based on aligned orthologous sequences.

Comparative Genomic and Pan-genome Analysis

Gene prediction and annotation of all genomes were done with Prokka. To estimate the size of the pan-genome among *Lactobacillus reuteri*, their predicted proteins were clustered using the Proteinortho tool [10] using the reciprocal best hits strategy with 1×10^{-3} , 30% and 70% as thresholds for the BLASTp e-value, and identity and coverage of amino acid sequences, respectively.

Figure 1: Dendrogram including the 4 *L. reuteri* strains and 7 strains included in our

MALDI-TOF database

Figure 2. Phylogenetic tree based on core-genome alignment of different *Lactobacillus*

reuteri strains.

Figure 3: Pan-genome of *L. reuteri* species. Core genome is depicted in dark, accessory genes in light grey. Studied strains of this work are in bold.

Table 2. Table of SNPs and indels events between *L. reuteri* stains.

Table 3: List and number of bacteriocins, polyketide synthases and nonribosomal peptide synthetases detected in the sequenced genomes.

Supplementary table 1. Nucleotide content and gene count levels of the *L. reuteri* genomes (strains CSUR P4870, P4904, P5460 and P5461)

Supplementary Table 2: accession numbers of the strains CSUR P4870, P4904, P5460 and P5461

250 **DECLARATIONS**

251 **Ethical approval and consent to participate:** The healthy individuals have consent to
 252 participate. This study was approved by the Ethics Committee of the IHU Méditerranée
 253 Infection under Number 2016-011.

254 **Consent for publication:** not applicable

255 **Availability of data and material:** Strain deposition numbers, 16S rRNA and genome
 256 sequencing accession numbers are available on supplementary Table 2.

257 **Competing interests:** The authors declare that they have no competing interests

258 **Funding**

259 This work has received financial support from the French Government through the Agence
 260 Nationale pour la Recherche, including the “Programme d’Investissement d’Avenir” under
 261 the reference Méditerranée Infection 10-IAHU-03.

262 **Author’s contribution:** JCL and DR designed the study; JCL and FM performed the
 263 experiments; JCL, FM, VM, HC, JD, AL and DR contributed new reagents/analytic tools;
 264 JCL, FM, VM, HC, JD, AL and DR analyzed the data; JCL, FM, AL and DR wrote the paper.

265 **Competing financial interest:** The authors declare no competing financial interests

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Figure 1: Dendrogram including the 4 *L. reuteri* strains and 7 strains included in our MALDI-TOF database

Figure 2. Phylogenetic tree based on core-genome alignment of different *Lactobacillus reuteri* strains.

333

334 **Table 1**

Number of healthy individual	Age	Sex
24 hours yogurt consumption		
1	36	M *
2	25	F
3	30	F
4	26	M
5	28	M
6	32	F
7	35	F
8	22	M
7 days yogurt consumption		
9	25	F
10	28	F
11	29	F
12	27	F
13	25	F
14	30	F *
15	29	F
16	28	F
17	28	F
18	39	F
19	34	M
20	32	M
21	30	M
22	33	M
23	30	M
24	30	M
25	35	M *
26	28	M
27	28	M
28	29	M

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337 **Table 2. Table of SNPs and indels events between *L. reuteri* stains.**

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	<i>L. reuteri</i> IRT	<i>L. reuteri</i> ATCC-1	<i>L. reuteri</i> DSM20016	<i>L. reuteri</i> JCM1112	<i>L. reuteri</i> ATCC53608	<i>L. reuteri</i> ZLR003	<i>L. reuteri</i> I5007	<i>L. reuteri</i> CSUR P4870	<i>L. reuteri</i> CSUR P4904	<i>L. reuteri</i> CSUR P5460	<i>L. reuteri</i> CSUR P5461	<i>L. reuteri</i> SD2112	<i>L. reuteri</i> I49	<i>L. reuteri</i> L6798	<i>L. reuteri</i> TD1
<i>L. reuteri</i> IRT															
<i>L. reuteri</i> ATCC-1	834														
<i>L. reuteri</i> DSM20016	475	766													
<i>L. reuteri</i> JCM1112	619	641	267												
<i>L. reuteri</i> ATCC53608	60524	59331	60570	60783											
<i>L. reuteri</i> ZLR003	64523	64135	63991	65175	12388										
<i>L. reuteri</i> I5007	53033	54098	54838	55358	16290	15984									
<i>L. reuteri</i> G1570	48731	48433	47822	48957	55694	55414	55561								
<i>L. reuteri</i> G1583	48825	48614	47915	49044	55816	55529	55665	181							
<i>L. reuteri</i> G1614	48648	48297	47703	48872	55950	55332	55548	432	400						
<i>L. reuteri</i> G1615	48727	48409	47782	48952	56020	55406	55610	335	312	206					
<i>L. reuteri</i> SD2112	66832	66796	67330	67781	65786	69296	68972	16364	16460	16313	16361				
<i>L. reuteri</i> I49	50930	50778	50102	51303	61236	64152	58355	45764	45985	45815	45845	58180			
<i>L. reuteri</i> L6798	51812	51125	51212	52295	71651	64353	63457	45053	45270	45154	45170	57195	46452		
<i>L. reuteri</i> TD1	52276	51259	51655	52743	62529	72571	62734	46208	46423	46367	46377	59498	50280	16913	

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Table 3: List and number of bacteriocins, polyketide synthases and nonribosomal peptide synthetases detected in the sequenced genomes:

	PKS	NRPS	Bacteriocin	name	name	name
SD2112	2	1	3	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	Nukacin (Staphylococcus warneri)
TD1	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
JCM1112	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
DSM20016	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
G1570	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
G1583	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
G1614	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	
G1615	2	1	2	Plantaricin (Lactobacillus plantarum)	circularin_A/uberolysin_family_circular_bacteriocin (Enterococcus faecalis)	

346 **Supplementary table 1. Nucleotide content and gene count levels of the *L. reuteri* genomes (strains CSUR P4870, P4904, P5460 and**
 347 **P5461)**

	L. reuteri CSUR P4870	L reuteri CSUR P4904	L reuteri CSUR P5460	L. reuteri CSUR P5461
Size (bp)	2,039,305	2,039,310	2,039,225	2,039,262
% G-C content	39	39	39,2	39,2
Number total of genes	1,679	1,676	1,740	1,740
Number total of protein encoding genes	1,607	1,607	1,656	1,656
Number total of tmRNA Genes	1	1	1	1
Number total of tRNA Genes	65	65	65	65
Number total of RNA (5S, 16S, 23S) Genes	6	6	18	18

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352 **Supplementary Table 2**

Strain name	16S number	Genome accession number
P4870	LR594461	CABFIV010000001-CABFIV01000028
P4904	LR594463	CABFIX010000001-CABFIX01000028
P4904	LR594460	CABFIU010000001-CABFIU01000031
P4904	LR594462	CABFIW010000001-CABFIW01000031

353