Assessment of Pb(II), Cd(II) and Al(III) Removal Capacity of Bacteria From Food and Gut Ecological Niches: Insights Into Biodiversity to Limit Intestinal Biodisponibility of Toxic Metals

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Article

Assessment of Pb(II), Cd(II) and Al(III) removal capacity of bacteria from food and gut ecological niches: insights into biodiversity to limit intestinal biodisponibility of toxic metals

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Abstract: Hazardous toxic metals, such as lead and cadmium, and to a lesser extent aluminum, are extensively recognized as detrimental for health following ingestion within food and water, or following inhalation. Gut and food-derived microbes, by interacting with heavy metals, may actively or passively modulate their bioavailability inside the gut, either by adsorption or by sequestration. Such a bioremediation within the gut implies the selection of safe microbes, based on their specific capacities to immobilize metals. We investigated the metal removal ability of 225 bacteria toward the potential harmful trace elements lead, cadmium and aluminum *in vitro*, using Inductively Coupled Plasma Mass Spectrometry analysis. Interspecies and intraspecies comparisons were addressed and discussed among bacteria from the phylum Firmicutes, which are mostly lactic acid bacteria, including *Lactobacillus spp*, with some *Lactococcus*, *Pediococcus* and *Carnobacterium* representatives, Actinobacteria as well as Proteobacteria. The effect on mixture of lead and cadmium was also investigated. Although the purpose of such a screening is so far not to elucidate each of the various strain specific- and metal dependent- mechanisms of heavy metal removal, we identified potential bacteria which are able to alleviate Pb(II) and Cd(II) concerns in order to propose performing candidate probiotics for metal xenobiotic bioremediation.

Keywords: Bioremediation; gut microbiota; lactic acid bacteria, Enterobacterales; lead; cadmium; aluminum; Probiotics; ICP-MS;

1. Introduction

Hazardous toxic metals, such as lead and cadmium, and to a lesser extent aluminum, are non-essential and non-biodegradable elements, which are extensively recognized as detrimental for health. They are a cause for several toxicological concerns, either (i) as poisoning after acute environmental exposure, (ii) or following long-term contamination at low doses throughout the food chain or (iii) by inhalation. Considering the human (and animal)

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exposome, heavy metals and other related detrimental elements may enter the body by the oral route after inhalation or ingestion of contaminated drinking water, beverages and food. These metals first interact with the digestive tract and its ecosystem and may further accumulate within target tissues ensuing blood distribution [1,2].

Lead (Pb) is a widespread heavy metal ion considered as 'probably' carcinogenic according to the American Environmental protection agency (EPA) and the international agency for research on cancer (IARC), as group 2A substances. Chronic Pb exposure leads to anemia, increase of blood pressure, persistent vomiting and neuropsychiatric disorders, including encephalopathy, delirium, convulsions and even coma in severe cases [3,4]. Of note, children are highly susceptible to Pb exposure, which may cause mental retardation [5] and recent studies suggest that childhood Pb exposure is a risk-factor of developing neurodegenerative diseases in adulthood [6]. Pb accumulates mostly in liver, kidneys and bones. Safe drinking water should contain less than 10 $\mu g.L^{-1}$ Pb, a threshold that is very often exceeded [7].

Cadmium (Cd) is classified as a group I carcinogenic compound by IARC [8]. Cd is nephrotoxic and may induce various health concerns, comprising kidney tubular damage, as well as skeletal damages (osteoporosis), brain and testis impairments [9–11]. Other Cd-related concerns such as metabolic diseases and contribution to respiratory infection susceptibility have also recently been suggested [12,13]. A Cd intake level of 23.2 µg/day, which is less than half the safe intake stated by the current guidelines, may increase the risk of chronic kidney disease, mortality from heart disease, cancer of any site and Alzheimer's disease [14]. Moreover, epidemiological studies examining the adverse effects of co-exposure to Cd and Pb have shown that Pb may enhance the nephrotoxicity of Cd and *vice versa*. In addition, Cd is also involved in the modulation of inflammatory responses [15], including in the gastrointestinal tract [16]. Perinatal and early life exposure to Cd is involved in poor birth outcomes and has adverse effects on neurodevelopment and metabolism functions of the child [17].

Aluminum (Al) has no known physiological function and accumulates in the liver, kidneys, bones, testis, as well as in the brain and nervous system where it exhibits toxicity in humans and animals [18,19]. Al has also been suggested to be involved in neurological disorders such as Alzheimer's disease, autism spectrum disorders and multiple sclerosis [20]. Considering the digestive tract, Al induces epithelial barrier dysfunction, abdominal pain and inflammation [21,22] and is highly suspected to be involved in inflammatory bowel diseases [23–25]. Al has adverse effects on reproduction [26], and *in utero* exposure has negative impacts even at low concentrations [27].

Collectively, ingested metal xenobiotics may also contribute to dysbiosis by targeting the host's gut microbiota and the corresponding key functions on intestinal homeostasis [16,28]. Hence, metal contaminants may indirectly alter the host's health following subtle microbial changes within the gut [29,30], affecting intestinal integrity and possibly contributing to a broad range of metabolic and/or chronic immune diseases and other neurological disorders.

In turn, gut microbes also interact with metals inside the gut, either by biotransformation, or by sequestration. It is clear that microorganisms can actively, or passively, control bioaccessibility and further bioavailability of heavy metals [31]. We and others have previously demonstrated the overall role of the gut microbiota as a barrier towards heavy metal dissemination, using germ-free mice [2,32] or broad spectrum antibiotics [33]. However, not all bacteria may have the same capacities to limit toxic metal bioavailability. Whereas the use of environmental bacteria as biosorbents for heavy metals has been widely employed to remove metals from contaminated soils and wastewaters [34], introducing bioremediation within the gut requires the selection of safe microbes based on their specific capacities to immobilize metals [35]. Food grade bacteria as well as gut-isolated microorganisms are thus the best candidates to be screened in order to alleviate metal toxicity, regarding their ecological niches [36]. In this context, lactic acid bacteria (LAB) have demonstrated obvious performances, showing efficient binding and/or internalization of metals *in vitro* [37–41], especially cadmium and lead.

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Although such LAB-mediated metal removal capacity was partly shown to be strain-dependent, scarce studies have explored species and strains diversity. Only few restricted types of LAB are generally analyzed in distinct and designed heterogenous studies among lactobacilli, enterococci and *Weissella* spp [41,42] together with dairy propionibacteria and bifidobacteria [38,43]. In addition, such properties were rarely sought in non-lactic acid bacteria, with a limited number of evaluations in few proteobacteria species (*E. coli*) and gut-isolated anaerobic bacteria (*Akkermansia muciniphila*, *Faecalibacterium prausnitzii* and *Oscillibacter ruminantium* single strain isolates) [33,44]. Other genera showing noticeable detoxification potentials *in vitro,* such as *Pseudomonas*, *Stenotrophomonas* or *Bacillus,* are not appropriate for intestinal compartment targeting. Only LAB such as *L. plantarum*, *L. casei*, *L. rhamnosus* and *L. delbrueckii* strains have been so far selected *in vitro* and confirmed to have detoxification abilities *in vivo*. Various selected food microbes can thus prevent the absorption of heavy metals in the gut (and dissemination in various tissues) and remove them upon defecation. Promising proofs of concept of efficacy were demonstrated in preclinical models of acute and chronic heavy metal toxicity in mice for lead [33,45,46], cadmium [47,48] and aluminum [49,50].

Cell surface associated compounds of probiotic lactobacilli sustain the strain-specificity dogma of strain's functionality [51]. The mechanism responsible for binding of metals to bacterial cell wall is highly suggested to depend on the huge variety of surface molecules of individual bacterial species and strain [52], including teichoic and lipoteichoic acids and peptidoglycan. In line, considering other binding site alike S-layer proteins, cell surface proteins and polysaccharides, we can question the distinct biosorbent properties of other Gram positive and Gram negative bacteria.

Here, we thus addressed the variability, among species and strains, by analyzing the ability of bacteria to remove the potential harmful trace elements lead, cadmium and aluminum *in vitro*. The purpose here is not to elucidate the mechanism of biosorption or bioaccumulation by plotting adsorption isotherms, but rather to compare the intrinsic aptitudes to cope with heavy metals among bacteria from the phylum Firmicutes, Actinobacteria and Proteobacteria. It comprises many LAB (99), several bifidobacteria (11), dairy propionibacteria (21), and cutibacteria (4), together with other gut-friendly bacteria such as Enterobacterales (90). This study thus aims to explore the strain diversity and the metal dependency of the overall toxic metal removal capacity of various food and gut bacteria. It also serves to identify the best candidates for preclinical assays and further veterinary and clinical applications.

2. Materials and Methods

2.1. Chemicals, reagents and instruments

Chemicals and reagents were purchased from Sigma–Aldrich Chemical (Saint-Quentin-Fallavier, France), unless otherwise stated. Ultrapure water corresponds to PURELAB Option-Q; Veolia Water (Antony, France). Ultraflex III MALDI-TOF/TOF instrument and Flex Analysis software were from Bruker Daltonik GmbH (Bremen, Germany). Determinations of metal concentrations in diluted samples were performed using Inductively Coupled Plasma - Mass Spectrometry (ICP-MS) THERMO ICAP™ Qc (Thermo Scientific, Courtaboeuf Cedex, France).

2.2. *Bacterial strains collections and culture conditions*

A set of 225 bacterial strains of distinct origins was used in this study. Lactic acid bacteria (LAB) mostly came from the well characterized DSM and ATCC collections previously used for comparative genomics of lactobacilli and associated genera [53].

Propionibacteria sampling consists of 21 *P. freudenreichii* strains from the Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire collection (CIRM-BIA; STLO, INRAE, Rennes, France), previously characterized throughout comparative genomics for immunomodulatory potentials [54].

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Most *Escherichia coli* strains belong to the ECOR standard reference strains of *E. coli* collection [55]. The later includes isolates from a variety of hosts and geographic regions, covering A, B1, B2, D, and E phylogroups, and were kindly provided by Dr Laurent Debarbieux (Institut Pasteur Paris). Other *E. coli* type strains or characterized as adherent- and invasive pathovars (AIEC) were described previously [56]. Strains of *Serratia marcescens* (Db10, JUb9, SM25, SM38 and SM45) were kindly provided by Dr Elizabeth Pradel [57]. Some cheese-derived *Hafnia alvei* strains (Gb01, E215, 920 and Grignon) were described elsewhere [58]. Finally, few bacterial strains (9 *Bifidobacterium species*, 4 *Cutibacterium acnes*, 2 *Enterobacter,* 2 *Hafnia alvei* and 5 *Klebsiella*) were sourced from historical clinical gut or fecal samples of human origin, food or as re-isolates from commercial probiotics products (Bb12 and Morinaga) belonging to the Faculty of Pharmacy of Lille (FPL) collection, University of Lille. Identification of those strains were determined by selective media and the species level was confirmed using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) and are referenced by internal FPL numbers.

Strains of *Lactobacillus* and associated genera (*Fructobacillus, Leuconostoc, Lactococus, Pediococcus,* and *Weissella*) were cultivated statically in MRS (de Mann, Rogosa and Sharpe medium), *Carnobacterium* and *Staphylococcus* in BHI (Brain heart Infusion) at 30 or 37 °C, according to their optimal growth. Bifidobacteria were grown anaerobically using anaerobic generator packs (GENbaganaer, Biomérieux, France) in MRS supplemented with 0.1% (w/v) L-cysteine hydrochloride. *P. freudenreichii* strains were grown at 30 °C under microaerophilic conditions, without agitation, in YEL (Yeast Extract Lactate medium) [59]. Strains of Enterobacterales (*Enterobacter*, *Escherichia*, *Hafnia, Klebsiella* and *Serratia*) were grown in LB (Luria Bertani medium) at 37 °C without shaking. Bacterial cultures duration ranged from overnight to 72h depending on the bacterial strain in order to reach the stationary phase.

2.3. Metal-removal capacity assays

(See also Preamble)

Eight mL of stationary phase bacterial cultures were standardized at optic density (OD) 600 nm of 2.5 and washed twice in Ringer's solutions. Pellets were further suspended with 8 mL of the corresponding ion's metal solutions (Ringer, pH 7.0) at 25 ppm ($PbCl_2$, $AlCl_2$) or 1 ppm ($CdCl_2$) and gently mixed using a rotary agitator (12 rpm), at room temperature for 1h. Samples were then centrifuged and washed twice before metal quantification by inductively coupled plasma mass spectrometry (ICP-MS). The pellets were suspended in 500 µL of 70% nitric acid, and heated at 98°C for 15 minutes. The samples were finally diluted in mQ water and further assessed by ICP-MS method. For each strain, a percentage of chelation/removal capacity is defined as the *ratio* of residual metal mass quantitated in the pellet toward the initial amount in the incubation medium. All assays were performed in triplicate, corresponding to three distinct bacterial cultures.

2.4. Statistical analyses

GraphPad Prism was employed for graph preparation and statistical evaluation. All analyses were performed by comparing experimental groups with their respective controls in the nonparametric, one-way analysis of variance (the Mann-Whitney U test) or a two-tailed Student t test, as appropriate (GraphPad Prism, version 6.0, GraphPad Software Inc, San Diego, CA, USA). Quantitative variables were quoted as the mean +/- standard error (SD). Data with p values ≤ 0.05 were considered to be significant.

3. Results

3.1. Preamble

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We first ensured that our methods were reliable and appropriate enough to screen distinct bacterial strains for their ability to remove selected metals. It has been previously shown that many factors may influence the levels of metal binding by bacteria, such as contact time, temperature, pH, and salt concentration of metal solutions, as well as washing buffers and inoculum size [37,60,61]. Thus, several key parameters were defined to mimic the gut environment unless fixed for convenience, *e.g*. temperature. The binding evaluation was thus done in physiological saline buffer (Ringer's solution), at a neutral pH (7.1) and at room temperature (22 +/-2 °C), in time-separated and bacterial culture triplicates to test reproducibility. Lead and aluminum concentration at 25 ppm were retained as a realistic dose to evaluate metal sequestration, whereas a lower dose of 1 ppm of cadmium was necessary to allow discriminant selection of strains. Indeed, cadmium at 25 ppm was not appropriate to identify clear differences among bacteria and most strains had very low cadmium binding capacity in a first pre-screening step (data not shown), below 5% of removal capacity. Consequently, only the most promising strains (over 5%) were further assessed at 1 ppm. Of note, these doses for Pb, Cd and Al correspond to similar orders of magnitude reported previously [41,44,50,52]. We thus ranked strains according to removal capacity as weak, low, moderate and high, respectively for 0-25%, 26-50%, 51-75% and 76-100% for Pb, whereas narrower intervals of 0-10%, 11-20%, 21-30% and over 30% correspond to Cd and Al.

The screening of bacterial strains is based on the residual metal fraction robustly associated to the pellets. However, we also confirmed the stability and irreversibility of binding, in order to discriminate strains from weak to strong metal binding capacity for both lead and cadmium. In this aim, we performed two serial cycles of washes (consisting in two cycles of pellet resuspension in metal-free solutions followed by centrifugation). Indeed, the residual quantity of metal in the second and last wash samples were very low or undetectable and considered as negligible. This is illustrated by selected examples of strains with distinct binding efficiency on **supplementary figure 1A and 1B**. However, binding of aluminum to bacteria was more labile and appeared partly reversible after rinsing, although the test using 2 wash steps is quite reproducible (**supplementary figure 1C).** Consequently, aluminum binding is somewhat overestimated in our assay but allows to discriminate strains and may reflect the intrinsic capacity of bacteria to interact with the metal in physiolocal conditions, *i.e.* the gastrointestinal tract.

3.2. Lactic acid bacteria (LAB) exhibit variable lead removal capacities

Among the 99 individual lactic acid bacteria strains tested for their capacity to remove Pb(II) salts at 25 ppm, most (>2/3) were able to immobilize an average of 50% to up to 90% of this metal in solution (**Figure 1A**) while others part showed moderate or quite weak biosorption potentials. When considering only the genus *Lactobacillus*, covering 65 distinct species and 76 strains, the removal capacity of lead ranged from 6% +/- 2.5 to 92% +/- 8.5. No particular consistency could be identified at the species level and some strains belonging to the same species could show extremely different properties such as *L. acidophilus*, *L. casei*, *L. paracasei L. rhamnosus* (**Figure 2A**). The two strains of *L. fermentum* were particularly efficient in lead removal, whereas 2 *L. plantarum* strains were unexpectedly poor lead biosorbers (**Figure 2B**).

The lead removal capacity is also variable among strains from other genus comprised within the LAB. *Carnobacterium spp* and *Pediococcus spp,* together with *Leuconostoc* and *Fructobacillus* members as well as the *Weissella spp,* demonstrated substantial lead removal properties (**Figure 2C**). Such potential is not related to the intrinsic shape of bacteria (*i.e.* rods or cocci) because the 3 enterococci and 4 pediococci tested were quite good metal biosorbers (> 50%) while *Lactococcus lactis* and 4 distinct strains of *Staphylococcus aureus* were not (mostly < 30%) (**Figure 2D**).

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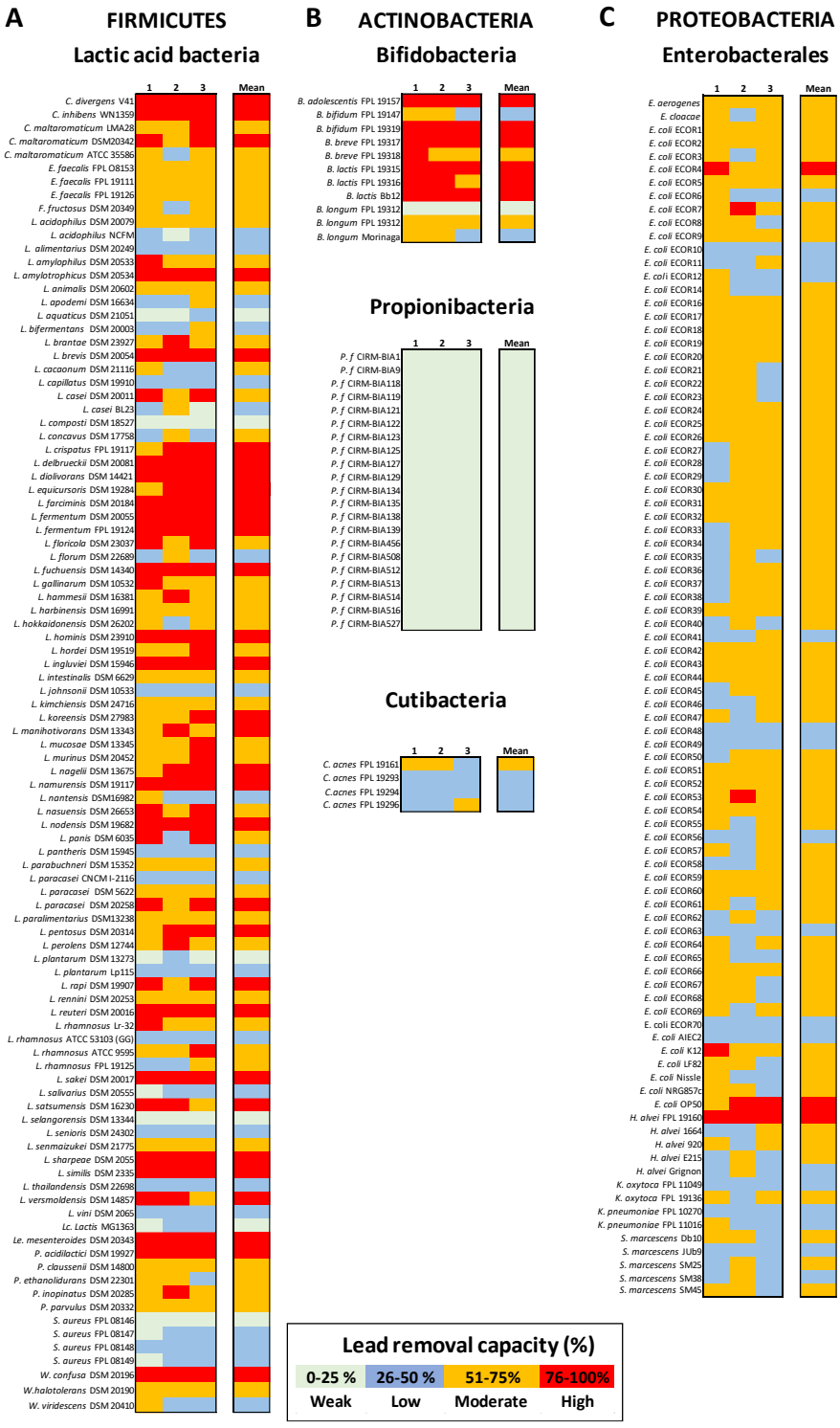

Figure 1. Pb(II) removal capacity of bacteria from distinct taxonomic phyla. Panel (**A**): Lactic acid bacteria. Panel (**B**) Actinobacteria, comprising bifidobacteria, propionibacteria and cutibactera. Panel (**C**): Proteobacteria, as Enterobacterales. Metal removal capacities were expressed as % of initial metal quantity in solution as mean +/- SD of three determinations. They are represented by colored heat maps as weak (0 to 25%), low (26 to 50), moderate (51 to 75%) and high (76 to 100%) performing bacterial strains, respectively in pale green, blue, orange and red, to remove lead.

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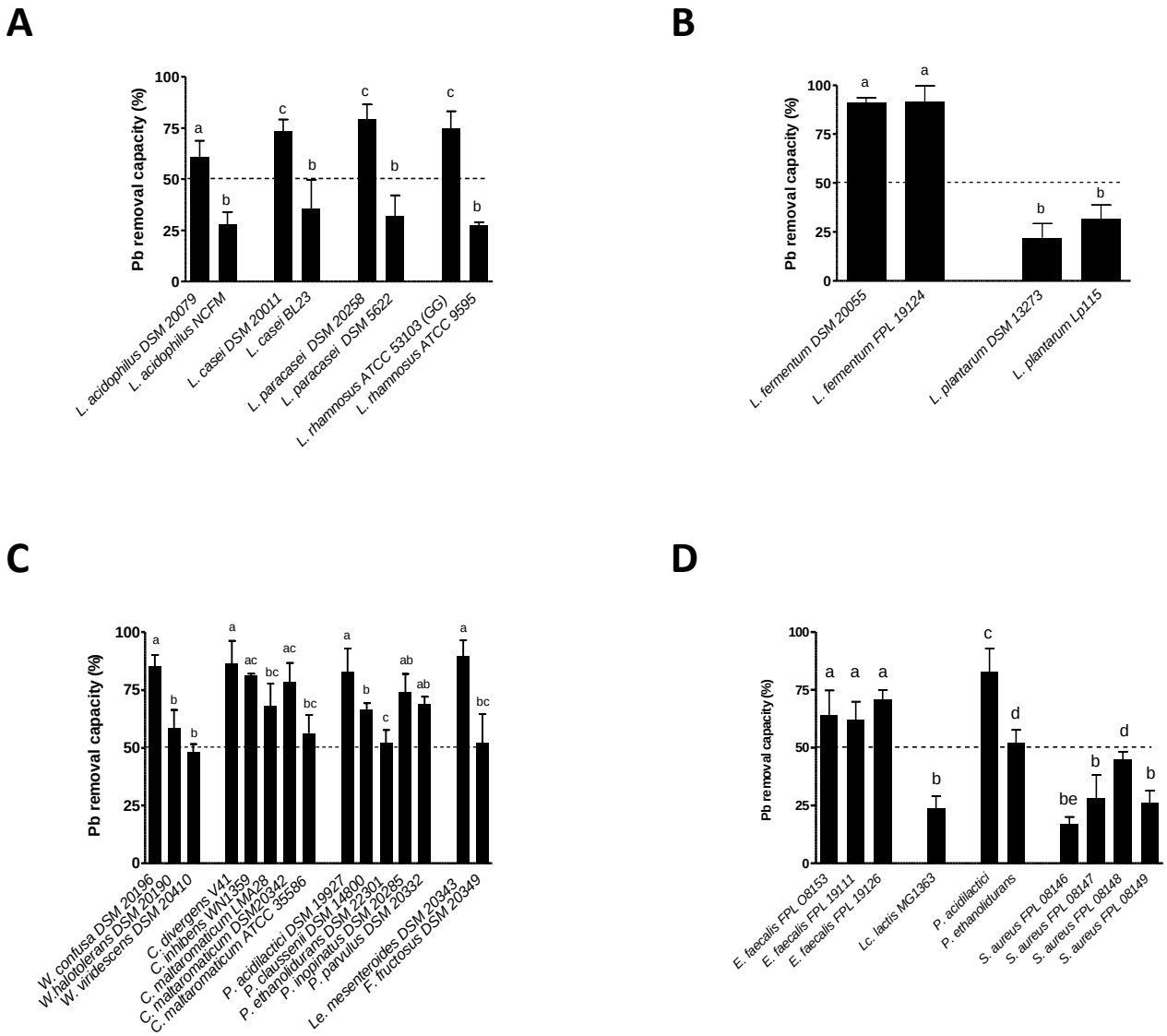

Figure 2. Pb(II) removal capacity of distinct strains of lactic acid bacteria. Panel (**A**): Selected examples of 5 couples of strains from the species with distinct performances. Panel (**B**): Selected examples of 2 couples of strains from the species with similar performances. Panel (**C**): Selected examples of 4 groups of strains from the same genus with distinct performances. Panel (**D**): Selected examples of strains from 4 groups of cocci with distinct performances. Values are mean +/- SD of three determinations. Different letters indicate the significant differences (P< 0.05) among the strains.

3.3. Actinobacteria cover distinct lead biosorption potentials

Within the phylum of Actinobacteria, bifidobacteria exhibit very poor or extremely high lead removal capacities, depending on the species and related strains (**Figure 1B**), some strains reaching the value of nearly 90% whereas other could only bind 6.6% of the solubilized lead. This is not related to the species, as distinct *B. longum* strains may exhibit up to 10-fold higher binding capacities than others, *i.e.* 6.75% +/- 0.9 *versus* 65.4% +/- 7.2 ($p < 0.001$). Surprisingly, none of the *Propionibacterium freudenreichii* strains from dairy origins was able to alleviate the concentration of lead salts, characterized by an average of 10.25% +/- 4.4 of binding. In contrast, 4 *Cutibacterium acnes* strains (previously referenced as *Propionibacterium acnes*), showed a binding of 41%, 44%, 49%, and 56.8%, respectively.

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3.4. Enterobacterales are moderate performers for lead chelation

We evaluated 90 strains belonging to the class of Gamma-Proteobacteria and to the order of Enterobacterales, comprising 68 *E. coli* strains from the ECOR library, extended to 5 other *E. coli* strains showing either probiotic properties (*E. coli* Nissle 1917), pathobiont traits such adherent and invasive capacities *e.g.* LF82 and NRG857C or no particular criterion from a physiological point of view (*E. coli* K12). In addition, other genera were considered, with 5 dairy isolates of *Hafnia alvei*, 2 *Klebsiella* spp, 2 *Enterobacter* spp and 5 *Serratia marcescens* strains from clinical collections, as commensal prototypes. The lead removal capacity of all the Enterobacterales strains tested was quite uniform (54.14% +/- 6.7), demonstrating moderate and consistent values for enteric Gram-negative bacilli. Indeed, nearly 90% of the strains showed lead removal capacities between 45 and 65%. Only 2 *E. coli* strains and a single *Hafnia alvei* representative were able to immobilize lead up to 75% from a 25 ppm lead solution.

3.5. Bacteria-mediated cadmium removal capacity is phylum, genus and strain specific

As explained in the preamble, the dose of 25 ppm is not appropriate to discriminate bacterial strains with respect to binding of cadmium Cd(II). Nor is it relevant to mimic realistic contamination events. Here, we thus addressed the ability of several bacteria to lower cadmium salts from 1 ppm solution. Among the 95 LAB strains tested, most of them (around 90%) exhibited weak or low cadmium removal properties, below the value of 20% of binding (**Figure 3A**). Interestingly, few strains comprising 3 *Pediococcus* spp, a *Carnobacterium divergens* and to a lesser extent a single *L. rhamnosus* and a *Leuconostoc mesenteroides* have cadmium binding capacities over 25% and up to 50% +/- 15.7 for a *P. acidilactici* isolate.

Considering the phylum of Actinobacteria, bifidobacteria are characterized by variable binding of cadmium, depending more on the strain than on the species. Indeed, *B. breve* strains removal capacity ranged from 6.2% +/- 0.6 to 40.7% +/- 6.7 ($p<0.01$) and *B. longum* strains from 3.6% +/- 1.7 to 18.9% +/- 5.8 ($p<0.01$) Most of the *Propionibacterium freudenreichii* strains were consistently weak cadmium biosorbers, whereas *C. acnes* strains were distinctly either low or moderate in their overall capacity to remove cadmium (**Figure 3B**). Lastly, near all members of the 60 Enterobacterales tested were weak or low cadmium chelators, except for 4 *E. coli* strains *i.e.* ECOR 64, ECOR 66, *E. coli* LF82 and *E. coli* Nissle having moderate cadmium removal potential (respectively 24.2% +/- 3.6, 21.2% +/- 5.6, 20.5% +/- 2.2 and 25.2% +/- 3.5) (**Figure 3C**).

3.6. Bacteria-mediated aluminum removal capacity is also genus and strain dependent

As a matter of fact, Al(III) from aluminum chloride solution can also distinctly bind to bacteria, depending on their origin and phylogenic diversity. Aluminum removal capacity by LAB is discrepant from various species, ranging from 5 to 28% of 25 ppm solutions with an average of 14.8% +/- 4.7 (**Figure 4A**). Similarly, the ability of bifidobacteria and propionibacteria to bind aluminum is quite weak and rarely exceed 10% (8.9% +/- 2.8 and 9.4% +/- 2.6, respectively). Strains of *Cutibacterium acnes* were more efficient and showed moderate binding levels (means of 24.3% +/- 4.7) (**Figure 4B**). In contrast, representatives of Enterobacterales exhibited usually higher values for this phenotype, ranging from 12% up to 30% and a median value of 20.4% +/- 4.7 (**Figure 4C**). Few *E. coli* strains were particularly favorable, *i.e.* ECOR37, ECOR40, ECOR50 and ECOR64, ranging from 25 to 30 %, whereas the overall *Hafnia* and *Serratia* strains seem to be less promising, below 15%.

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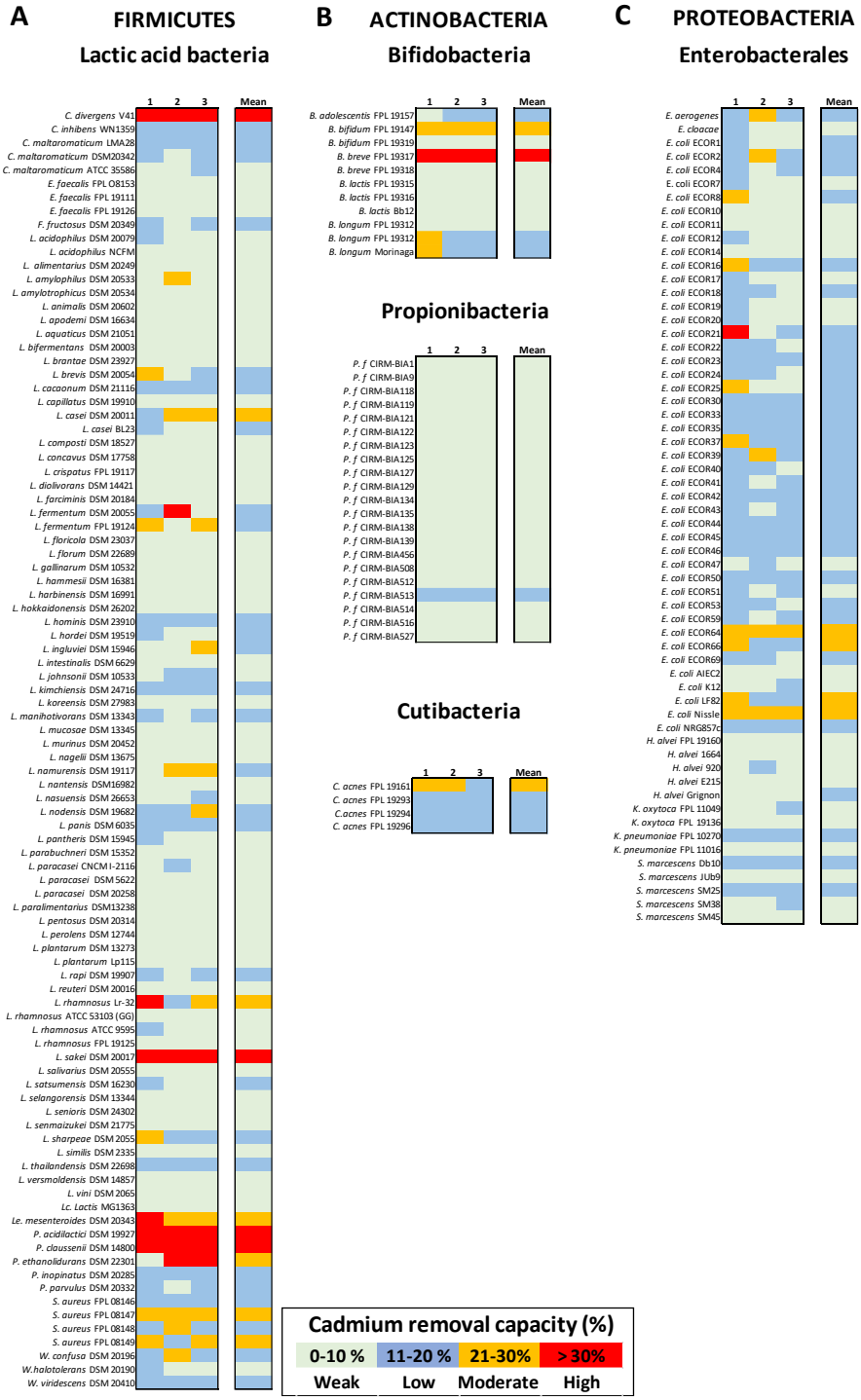

Figure 3. Cd(II) removal capacity of bacteria from distinct taxonomic phyla. Panel (**A**): Lactic acid bacteria. Panel (**B**) Actinobacteria, comprising bifidobacteria, propionibacteria and cutibactera. Panel (**C**): Proteobacteria, as Enterobacterales. Metal removal capacities were expressed as % of initial metal quantity in solution as mean +/- SD of three determinations. They are represented by colored heat maps as weak (0 to 10%), low (11 to 20), moderate (21 to 30%) and high (over 100%) performing bacterial strains, respectively in pale green, blue, orange and red, to remove cadmium.

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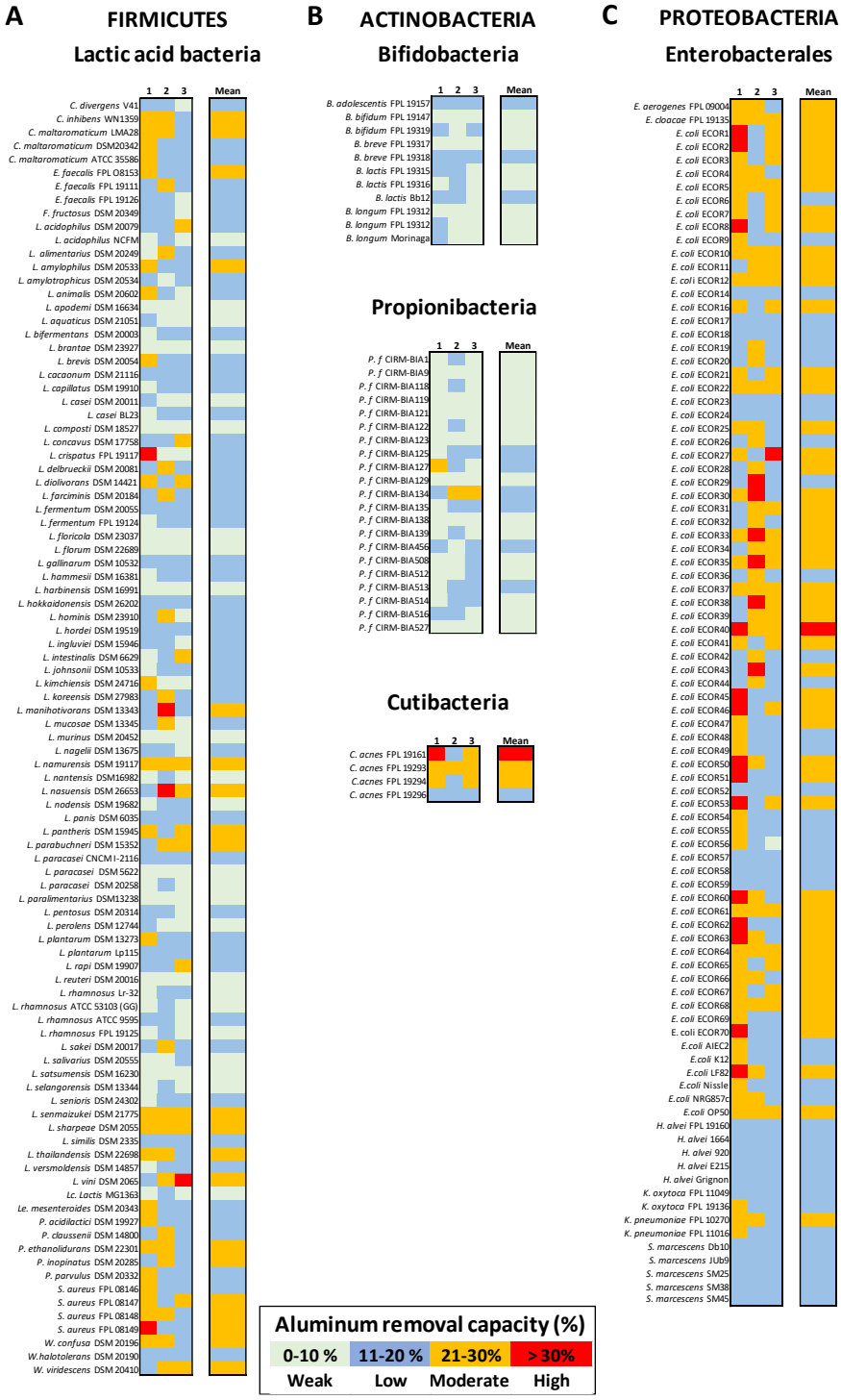

Figure 4. Al(III) removal capacity of bacteria from distinct taxonomic phyla. Panel (**A**): Lactic acid bacteria. Panel (**B**) Actinobacteria, comprising bifidobacteria, propionibacteria and cutibactera. Panel (**C**): Proteobacteria, as Enterobacterales. Metal removal capacities were expressed as % of initial metal quantity in solution as mean +/- SD of three determinations. They are represented by colored heat maps as weak (0 to 10%), low (11 to 20), moderate (21 to 30%) and high (over 100%) performing bacterial strains, respectively in pale green, blue, orange and red, to remove aluminum.

3.7. The ability of bacteria to remove lead and cadmium is poorly affected in mixture conditions

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Because co-exposure to lead and cadmium may commonly happen due to their co-occurrence in food, water and environment, we also addressed the respective binding of both elements when these metals were mixed together. We thus compared the binding capacity for lead at 25 ppm, cadmium at 1 ppm and for the corresponding mixture (*i.e.* Pb 25 ppm and Cd 1 ppm) of a set of arbitrary selected 16 representative Gram positive (**Figure 5A**) and 16 Gram negative bacteria (**Figure 5B**). Interestingly, the intrinsic removal capacity of bacteria was fairly not influenced by the presence of the other metal, except for few strains (out of 32) showing rare but significant lowering of 20 to 40% of the initial baseline values ($p < 0.05$), irrespective of the metal considered.

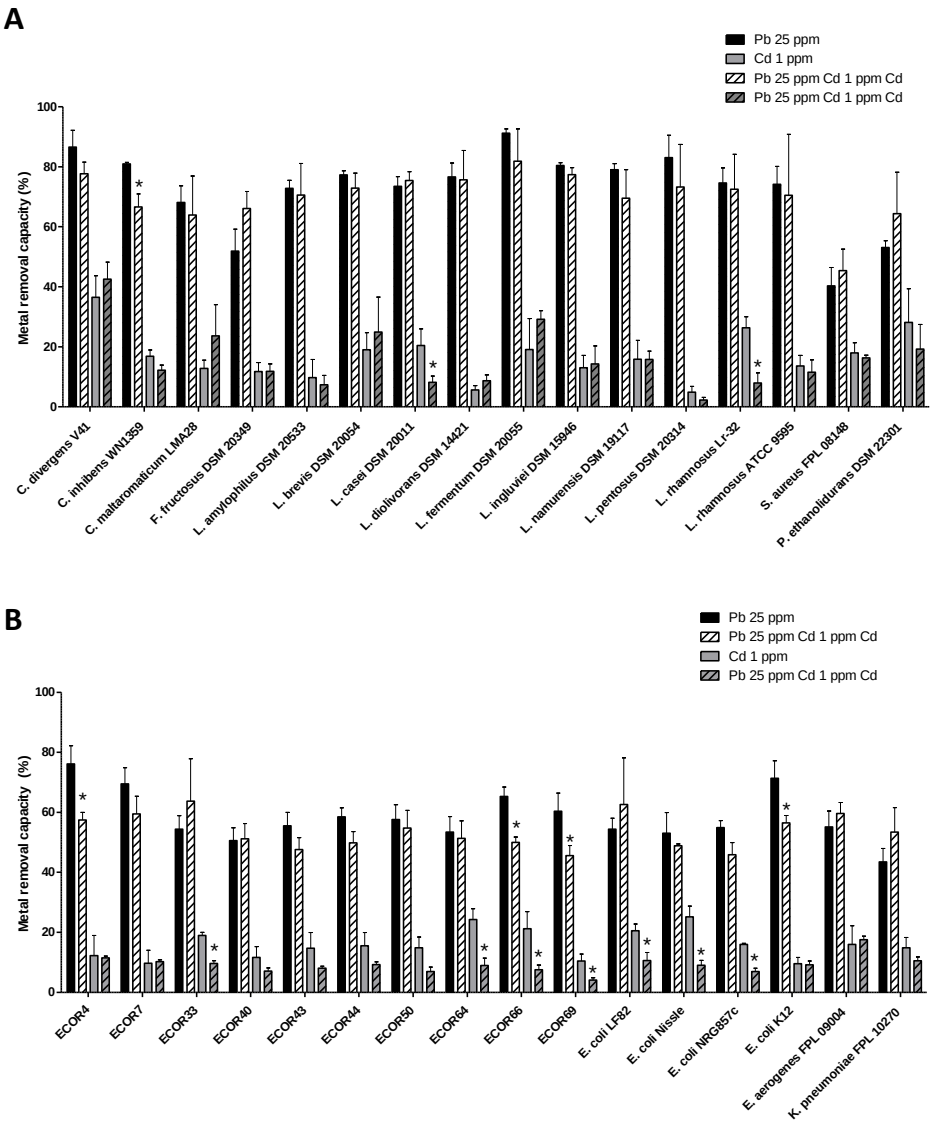

Figure 5. Ability of bacteria to remove lead and cadmium in pure or mixed solutions. Metal removal capacity was determined for lead at 25 ppm (black), for cadmium 1 ppm (grey), alone or as mixtures of both (25 ppm lead and 1 ppm Cd) for lead (hatched black) and cadmium (hatched grey). Values are expressed as % of initial metal quantity in solution as mean +/- SD of three determinations. * indicates the significant differences of mixture ($p < 0.05$) compared with corresponding metal alone.

4. Discussion

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Here, we addressed the metal removal capacity in more than 200 bacterial strains, mostly lactic acid bacteria and associated genera, as well as representatives of gut enterobacteria inhabitants. We considered lead, cadmium, aluminum, and to a lesser extent, a mixture of lead and cadmium. Of note, all tested bacteria were able to survive at the corresponding doses of metals used, *i.e.* 25 ppm for lead and aluminum and 1 ppm for cadmium (data not shown) and as previously described elsewhere for several LAB strains [41]. Considering cadmium, the removal capacity of distinct strains was not correlated to the minimal inhibitory concentration (MIC) established at higher doses (data not shown). The later suggests that exploring metal tolerance is not appropriate for screening purposes, as also demonstrated for lead with various *L. plantarum* strains [62]. The viability of bacteria is so far not necessary to allow significant metal biosorption. Indeed, binding isotherms in the Langmuir model showed that the maximum binding capacity (Q_{max}) could either be significantly higher or lower in boiled or live forms for two probiotic strains (*Lactobacillus rhamnosus* and *Propionibacterium freudenreichii*) [37]. Others have demonstrated that dead and live bacteria had similar lead and cadmium binding capacity (33,37,40,43). However, some slightly higher removal efficiency of lead by living forms could be observed, owing to the occurrence of cell-specific intracellular metal accumulation [63].

Other strategies based on lactobacilli surface characteristics, such as hydrophobicity and electrostatic properties, failed to identify relevant selection criteria for lead and cadmium removal [64]. Yet, no rationale is established to select strains with high detoxification potential. In our study, we thus empirically characterized the removal metal capacity of bacterial living biomasses without *a priori*, considering the cross-species and strain diversity. We used gut friendly (nonpathogenic) bacteria, either originated from intestinal ecological niches or derived from food, mostly regarded as Generally Regarded as Safe (GRAS), because safety is essential for further *in vivo* applications. In contrast with many studies exploring the efficiency of metal removal within metal solutions in deionized water, we therefore used Ringer's solution at neutral pH, both for the binding assays and the washing of the pellets in order to achieve isotonicity close to biological conditions. Incubation time by bacteria and the distinct metals was set up at one hour to partly mimic the food transit time and the possible contact time within the gut.

Although LAB-mediated metal removal capacity has already been shown to be strain dependent, very few studies have explored the cross-species and strain diversity. Only few restricted types of LAB are generally analyzed and often in heterogeneous design studies among lactobacilli, enterococci and *Weissella* spp [41,42]. In line, data considering dairy propionibacteria and bifidobacteria are scarce and included a limited number of species and strains [38,43]. Studies that comprise proteobacteria are also poorly documented. We have here extended and confirmed the huge functional diversity throughout bacterium specimen and the distinct metals for Gram-positive and Gram-negative bacteria. Mechanisms of metal removal have been described elsewhere [52,65]. They include ion exchange, chelation, adsorption by physical forces and intracellular sequestration and are known to be strain-dependent. The role of hydroxyl (from the peptidoglycan), carboxyl and phosphate groups (from surface proteins) influenced by pH and specificity and abundance, is assumed to be a key determinant, together with the contribution of capsular polysaccharide for metal binding sites. Thus, the overall removal capacity of a single cell is complex and multifactorial. Anyway, although many variables such as culture conditions, culture medium types and growth phase are involved, independently of the core and individual specific bacterial genes, the comparative genomic among lactic acid bacteriacan additionally be used help to identify specific genes amplifying or lowering factors for metal removal.

We found that LAB and bifidobacteria have generally moderate to high lead removal capacities, whereas dairy propionibacteria consistently have weak performances. Gram negative bacteria have almost low to moderate aptitudes to immobilize lead. We could identify good candidates toward lead among lactobacilli, and bifidobacteria, as previously described for *L. sakei*, *L. delbruckii*, *L. fermentum* and *B. bifidum* strains [45]. In line with our results, *Weissella* and *Pediococcus* spp were also described with high levels of lead removal [41]. To our knowledge, we first describe the promising potential of *Carnobacterium* spp. Out of 220 strains, only five were identified with high capacity to interact

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with cadmium, comprising a *B. breve*, a *L. sakei*, a *Carnobacterium divergens* and two *Pediococcus* strains, suggesting that some LAB not generally considered as probiotics may have interesting properties for cadmium bioremediation purposes. In contrast with previous reports [45,48], the few strains of *L. rhamnosus* and *L. plantarum* from our set of bacteria exhibited poor lead and cadmium lowering properties. Again, metal removal capacities are highly strain-dependent and yet cannot be generalized at the species taxonomic level. Interestingly, among the 20 strains of *Propionibacterium*, all are weak chelators for both lead and cadmium, although they demonstrated very strain-specific surface proteins and exopolysaccharide production abilities, related to various immunological functional properties [66].

Existing data on aluminum removal capacity by bacteria are quite rare and have only reported potentials for *L. plantarum* and *L. reuteri* strains [50], exceeding the % of removal we could reach in our study (25%) in a similar experimental design. Noticeably, *Enterobacteria* demonstrated more consistent and higher capacities for aluminum removal than LAB and *Actinobacteria*.

Another interesting and promising result from our study is the overall maintenance of the removal capacity of a selected strain when both cadmium and lead are applied together. This encourages us to select strains with shared high lead and cadmium removal capacities. In line, synergistic issues to question the impact of mixture of strains on a single (or a mixture of) metal(s) have also to be explored [67].

The proof of concept that some bacterial strains which possess high *in vitro* metal removal capacities can also have these potentials *in vivo* in models of acute and chronic poisoning in mice [33,45–48,50], rats [67] and humans [68] has been clearly demonstrated. The first screening step we described here needs to be further assessed *in vivo* considering the physiology of the gastrointestinal tract in the presence of other essential metals, trace elements and organic molecules. Experimental protocols in preclinical models must include negative controls by comparing strains with poor and high removal capacities, in order to ensure the intrinsic contribution of the selected bacteria as a detoxification tool. In order to develop probiotics for toxic metal removal, appropriate strains and even cocktails of several bacteria should clearly be evaluated. On the one hand, the interaction with other bacteria inhabiting the intestine has also to be considered, as the baseline role of resident commensal microbiota is a key factor [33]. On the other hand, distinct strategies used to modify the gut microbiota, including the prebiotics, may also interfere with bacteria and heavy metal equilibrium [69]. Because heavy metals influence the structure, the diversity, and the functionality of the gut microbiota [29] (including heavy metal sequestration), the bidirectional relationship of dysbiosis and heavy metals in various pathologies, and the interconnected use of probiotics with multipurpose functions, is highly complex [28] and will need to be integrated for a personalized medicine perspective [70].

5. Conclusion

Collectively, our results revealed the huge bacterial diversity in terms of ability to remove metal such as lead, cadmium, aluminum, or a mixture of lead and cadmium, *in vitro*. By exploring the cross-species and strain diversity of lactic acid bacteria, bifidobacteria, propionibacteria and enterobacteria, we underlined the strain- and metal-dependency of bacterial metal removal. These results open new perspectives: (i) to define probiotic candidates, with a wide and long history of safe use, to be considered as a dietary therapeutic strategy against heavy metal contamination.This may be valuable both to counteract low dose accumulation and chronic toxicity and/or to alleviate heavy metal poisoning, concomitantly with conventional chelation, antioxidant and anti-inflammatory therapies in human and veterinary developments, and (ii) to understand the role of some resident gut microbes in the toxic metal balance within the gut microbiota.

Supplementary Materials: The following are available online at httpps://www. xxx, Figure S1: Selected examples of bacterial strains with distinct metal removal efficiency demonstrating the accuracy of the assays for lead (A), cadmium (B) and aluminum (C). Metals were quantified in the binding supernatant, in the two washing buffers, and in the final bacterial pellet.

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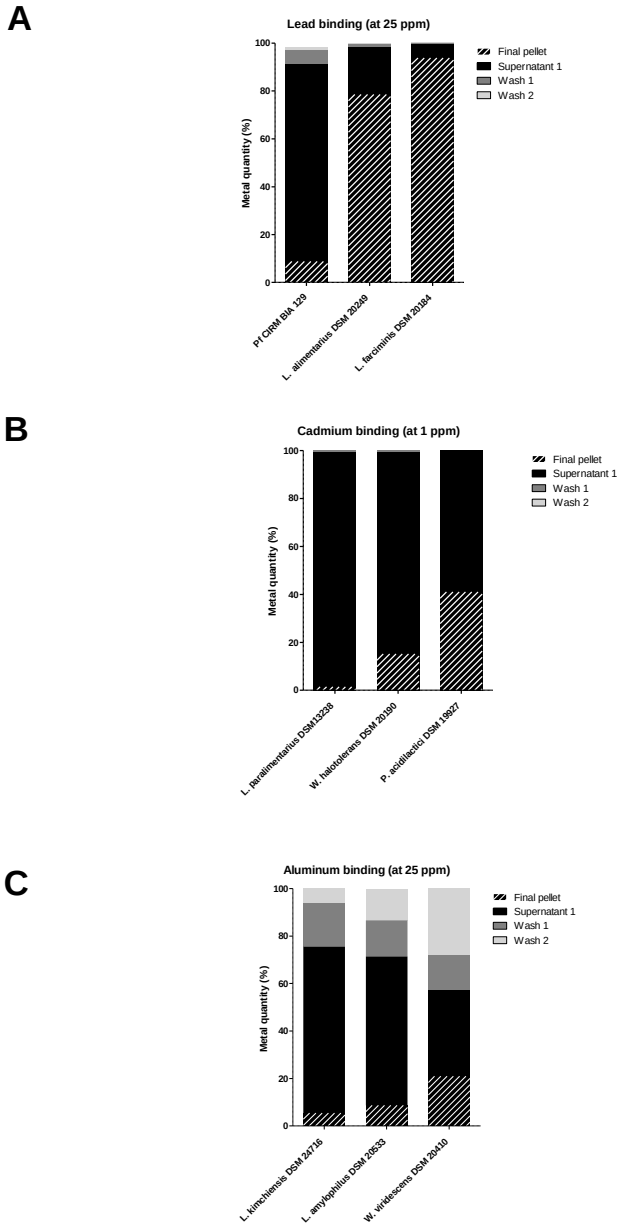

Figure S1: Selected examples of bacterial strains with distinct metal removal efficiency demonstrating the accuracy of the assays for lead (A), cadmium (B) and aluminum (C). Metals were quantified in the binding supernatant, in the two washing buffers, and in the final bacterial pellet.