TITLE: Effect of lactic acid bacteria on mercury toxicokinetics

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Abstract

The capacity of two LAB strains to inhibit inorganic [Hg(II)] and organic (methyl-Hg; MeHg) mercury translocation through monolayers of co-cultures of NCM460 and HT29-MTX colonic cells was evaluated. *Lactobacillus casei* BL23 and *Lactobacillus acidophilus* ATCC4356 reduced the permeability of Hg(II) and MeHg from aqueous solutions through NCM460/HT29-MTX monolayers (20 to 94% reduction). However, assays using the bioaccessible (soluble) Hg fraction obtained by *in vitro* gastrointestinal digestion of Hg-contaminated swordfish only showed a reduction (42%) with the BL23 strain. *In vivo* experiments carried out in mice receiving an acute dose of Hg(II) or MeHg (0.5 mg/kg body weight/day) with or without lactobacilli resulted in significant decreases of the bioavailability of MeHg with both strains and increased excretion of Hg in feces after treatment with the lactobacilli. However, Hg(II) bioavailability or excretion was not affected. Hg accumulation in liver and kidney remained similar in LAB-treated or non-treated animals. This is the first study of the impact of LAB on Hg(II) and MeHg toxicokinetics and shows that some LAB strains have potential to diminish MeHg bioavailability. Furthermore, it has established the basis for new studies

on the protective effect of LAB under conditions resembling subchronic and chronic Hg exposures.

Keywords: Mercury, *Lactobacillus*, bioavailability, NCM460, HT29-MTX, mouse.

Highlights

Lactobacillus strains decreased Hg permeability in NCM460/HT29-MTX cell monolayers.

Lactobacillus casei BL23 decreased methylmercury bioavailability in acutely exposed mice.

Mercury accumulation in liver and kidney was not affected by *Lactobacillus* supplementation.

ABBREVIATIONS

AUC: area under the curve

CFU: colony forming units

DMEM: Dulbecco's modified Eagle's medium

EFSA: European Food Safety Authority

FBS: fetal bovine serum

HBSS: Hank's balanced salt solution

HEPES: N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid

LAB: lactic acid bacteria

LY: Lucifer yellow

MeHg: methylmercury

PBS: phosphate-buffered saline

TEER: transepithelial electric resistance

1. Introduction

Mercury (Hg) is a toxic trace element whose presence in nature is mainly due to anthropogenic causes. A large part of the world population is exposed to Hg through food, where it is mostly found as divalent inorganic Hg [Hg(II)] or methylmercury (MeHg). The toxic effects of Hg are well known; the critical target for the toxicity of inorganic Hg is the kidney, and it also produces toxic effects on the liver, and nervous, immune and reproductive systems (EFSA, 2012). MeHg causes adult and developmental neurotoxicity (EFSA, 2012) and is also classified by the International Agency for Research on Cancer as possibly carcinogenic for humans (IARC, 1993).

Environmental Hg exposure, mainly due to mining activities, affects populations in Africa, Asia and Latin America. For example, Hg contents in biomarkers exceeds the reference values in more than 70% of individuals in populations of the Colombian, Brazilian and Peruvian Amazonia (Ashe, 2012; Malm, 1998; Olivero-Verbel et al., 2016). In countries where environmental Hg contamination is negligible, dietary Hg exposure can also exceed the recommended limits. A study conducted in Spain revealed a large proportion of newborns with cord blood Hg concentrations above the limit proposed by the U.S. Environmental Protection Agency (5.8 µg/L for MeHg), due mainly to oily fish consumption by their mothers during pregnancy (Ramon et al., 2011). The last report of the European Food Safety Authority (EFSA) shows that the mean dietary exposure exceeds the tolerable weekly intake (TWI) for MeHg in toddlers and other children in some surveys and the 95th percentile dietary exposure is close to or above the TWI for all age groups (EFSA, 2012).

International organizations such as EFSA or the World Health Organization recommend reducing exposure to this element, especially for the most susceptible population groups (pregnant women and children). Health and food safety agencies from many countries

have recommended limiting consumption of certain fish products, especially for these groups. However, due to the high nutritional value of some fish products, avoiding their consumption is not an adequate measure. Among many existing possibilities for reducing exposure, the reduction of the entry of the toxic element into the systemic circulation after ingestion (bioavailability) has been considered.

Certain strains of lactic acid bacteria (LAB), normal inhabitants of the gastrointestinal tract and commonly present in many fermented food products, possess an elevated ability to remove metals from aqueous solutions (Chiocchetti et al., 2018). Studies about the impact of LAB in cadmium (Cd) or lead (Pb) accumulation have been carried out in animal models with good results (Li et al., 2017; Yi et al., 2017; Zhai et al., 2013; Zhai et al., 2015).

Despite the evidence supporting the beneficial effects of LAB on the relief of the toxicity caused by some heavy metals, research about Hg, and most specifically MeHg, is scarce. A human intervention study in Tanzania showed that intake of milk fermented with *Lactobacillus rhamnosus* GR-1 by pregnant women had a protective effect against increases in Hg and arsenic in blood (Bisanz et al., 2014), although the exposure levels of the studied cohort were not relevant. Alcántara et al. (Alcántara et al., 2017) evidenced the biosorption of Hg(II) and MeHg on the surface of lactobacilli and it has been shown that lactobacilli-Hg complexation also occurs during a simulated gastrointestinal digestion (Jadán-Piedra et al., 2017), suggesting that these bacteria could be used as a strategy for diminishing Hg intestinal absorption. Oral gavage administration of *Lactobacillus plantarum* cells or *Bacillus coagulans* spores to rats reduced the burden caused by Hg(II) exposure (Majlesi et al., 2017). A recent study in mice in which a *Lactobacillus reuteri* strain was orally administered has also shown that this bacterium resulted in a diminished Hg(II) toxicity, protecting the animals from the

Hg(II)-triggered oxidative stress and inflammation by acting on the MAPK and NF- κ B pathways (Jiang et al., 2018).

On the basis of previous studies (Jadán-Piedra et al., 2017), we have selected two *Lactobacillus* strains that displayed a good retention of Hg(II) and MeHg on their surfaces. The goals of the present work are, first, to verify whether complexation of Hg(II) and MeHg by these LAB has an effect on Hg permeability in a model of human intestinal epithelium, and second, to test their efficacy in reducing the arrival of both mercurial forms to the systemic circulation after oral intake in a mice model.

2. Materials and Methods

2.1. Mercury species

The solutions of Hg(II) and MeHg were prepared by diluting commercial standards of Hg(NO₃)₂ (1 g/L, Merck) and CH₃HgCl (1 g/L, Alfa Aesar), respectively.

2.2. Swordfish bioaccessible fraction

Cooked swordfish was submitted to an *in vitro* gastrointestinal digestion following the procedure described by Jadán-Piedra et al. (Jadán-Piedra et al., 2016) The resulting bioaccessible fraction was heated before the transport assay for 10 min at 90 °C in order to inhibit digestive enzymes. To facilitate cell viability, glucose (final concentration 1 g/L, Sigma) was added and osmolarity was adjusted with NaCl (3 mM, Panreac) to 300 $\pm$ 30 mOsm/kg, using a freezing point osmometer (Automatic Micro-Osmometer Type 15 Löser, Löser Messtechnik).

2.3. Strains and growth conditions.

The LAB strains used were *Lactobacillus acidophilus* BL17 (American Type Strain Culture Collection - ATCC4356) and *Lactobacillus casei* BL23 (Mazé et al., 2010). These strains were selected on the basis of their capacity to bind Hg species in *in vitro* tests under emulated gastrointestinal conditions (Jadán-Piedra et al., 2017). Lactobacilli were routinely grown in de Man-Rogosa-Sharpe (MRS) broth (Difco) under static conditions at 30 °C (BL17) or 37 °C (BL23). Bacterial cells were harvested by centrifugation (5500×g, 10 min) and washed with phosphate-buffered saline (PBS). The bacterial pellets were resuspended in PBS and adjusted to different OD (optical density at 595 nm) for subsequent assays.

2.4. *In vitro* studies on mercury intestinal permeability

2.4.1. Cell culture

The NCM460 cell line was obtained from Incell Corporation and it was used at passages 15 to 30. The HT29-MTX cell line was kindly provided by Dr. Técla Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S 938, Paris, France) and used between passages 16 and 24. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 0.6 g/L glutamine. For NCM460 cells, DMEM was supplemented with 10% (v/v) fetal bovine serum (FBS), 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (NCM-DMEMc). HT29-MTX medium was supplemented with 10% (v/v) FBS, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (HT-DMEMc).

The cells were incubated at 37 °C in an atmosphere with 95% relative humidity and a CO₂ flow of 5%. The medium was changed every 3 days. When the cell monolayer

reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (ethylene diamine tetraacetic acid, 0.22 g/L) and reseeded at a density of $5-6 \times 10^4$ cells/cm². All the reagents used were obtained from Hyclone.

2.4.2. Cellular permeability assays

The assays were performed in 12-well plates with polyester membrane inserts (12 mm diameter, 0.4 μ m pore-size; Transwell[®], Corning). NCM460/HT29-MTX co-cultures were prepared at an 80/20 proportion. This ratio was chosen on the basis of the habitual percentages of both cellular types in the intestine (Hilgendorf et al., 2000). The cells were seeded at an initial density of 7.5×10^4 cell/cm² in 0.5 mL of HT-DMEMc added to the apical (top) compartment. HT-DMEMc medium (1.5 mL) was added to the basolateral compartment (bottom). The cells were incubated at 37 °C under an atmosphere of 95% relative humidity and 5% CO₂ for ten days with medium changes every three days. During this time the development and confluence of the cellular monolayer was checked by measuring the transepithelial electric resistance (TEER) with a Millicell[®]-ERS voltmeter (Millipore Corporation). The reference values for considering an adequately formed monolayer for the assays were 170-190 Ohms cm².

For permeability assays, the cellular medium was withdrawn. Then, 0.5 mL of Hg(II) or MeHg standards (0.5 μ g/mL) prepared in Hank's balanced salt solution with NaHCO₃ (HBSS, Lonza) containing 10 mM HEPES or 0.5 mL of the previously obtained bioaccessible fraction of swordfish (Section 2.2), were added to the apical compartment with or without the bacterial strains (final ODs ranging from 0.3 to 1.2). Lucifer yellow (LY; Sigma) was also added to the apical side at 100 μ M, a concentration that does not interfere with Hg transport (Vázquez et al., 2015; Vázquez et al., 2014), as a marker of

monolayer integrity. HBSS medium with 10 mM HEPES (1.5 mL) was added to the basolateral compartment.

At different times (30, 60, 90 and 120 min for Hg standards; 30, 45, 60 and 75 min for bioaccessible fractions), 0.7 mL-aliquots were collected from the basolateral compartment and replaced by an equal volume of fresh medium (HBSS with 10 mM HEPES). Mercury was quantified in the aliquots by microwave oven-assisted digestion and cold vapor atomic fluorescence spectrometry following the procedure described by Jadán-Piedra et al. (Jadán-Piedra et al., 2016). The methodology exhibited a linear range of 0.01 ng Hg/mL to 2 ng Hg/mL. The instrumental detection (LOD) and quantification (LOQ) limits were 0.003 ng Hg/mL and 0.01 ng Hg/mL respectively. Considering the sample mass (0.03-0.5 g) and the volume (10 mL) employed for tissue analysis, LOQ values ranged between 0.2 and 3 ng Hg/g. The mean recovery calculated at two levels of fortification of tissue homogenates was in the range of 92-110%. The method allowed accurate analysis of the fish protein muscle certified reference material for trace metals DORM-3 (National Research Council of Canada, NRCC) (certified Hg concentration: 0.382 ± 0.06 mg/kg).

Apparent permeability coefficients were calculated as $P_{app} = (dC/dt) \times (V_r/AC_o)$, where dC/dt is the flux ($\mu\text{g/s}$) determined by the linear slope of the equation describing the variation of Hg concentrations, corrected by the dilution, over time; V_r is the volume of the basolateral compartment (1.5 mL); A is the cell monolayer surface (1.12 cm^2) and C_o is the initial concentration of Hg added to the apical compartment ($0.5 \mu\text{g/mL}$ for Hg(II) and MeHg standards).

LY in aliquots collected at different times from the basolateral compartments was measured using a microplate reader (POLARstar OPTIMA, BMG-Labtech) at 485 nm excitation wavelength and emission at 520 nm. P_{app} for LY was calculated as described

for Hg. The assays were considered valid when P_{app} for LY was lower than 1×10^{-5} cm/s.

2.5. Mercury toxicokinetics in mice

2.5.1. Animals

Forty-two 4-weeks BALB/c female mice were obtained from Charles River. The animals had a weight ranging from 20 to 25 g and during the study they were kept in controlled environmental conditions (cycles of 12 h of light and dark, room temperature of 22 °C and humidity of 75%) at the facilities of the Animal Production Section from the University of Valencia. The mice were fed *ad libitum* with standard rodent maintenance feed. The protocols applied to the animals were designed in conformity with the Spanish regulations for the use of experimental animals (BOE, 2013) and were approved by the Ethical Committee for Use of Laboratory Animals of the University of Valencia and the Agriculture, Fisheries, and Food Council of the Generalitat Valenciana (Spain).

2.5.2. Mice experiments

Six groups of seven animals were housed separately. Four groups received a daily bacterial dose (5×10^8 CFU in 50 μ L PBS) by oral gavage for a total of eight days. Two groups were dosed with *L. casei* BL23 and two groups received *L. acidophilus* BL17. The remaining two groups (controls) were given 50 μ L of PBS by gavage. After the eight days (preconditioning), the mice started to receive daily a single dose (0.5 mg/kg body weight) of Hg(II) (three groups; BL23, BL17 and control) or MeHg (three groups; BL23, BL17 and control) with or without the bacteria for three days. The Hg concentrations for dosing were chosen taking into account toxicological studies in mice,

where a NOAEL (non-observed adverse effects level) of 0.93 mg/kg body weight/day is established for renal effects in animals exposed to Hg(II) by gavage for 14 days (NTP, 1993). For MeHg, mice studies show LOAELs (lowest observed adverse effect level) for neurotoxic effects at doses of 4 mg/kg body weight/day for 7 days and 0.6 mg/kg body weight/day for two-years exposure (NRC, 2000).

The relative bioavailability was determined after the first administration of the treatment bacteria/Hg. Blood samples (~100 µL) were collected by puncturing the submandibular venous sinus at different times (0, 60, 300 and 1440 min after dosing). Blood samples were recovered in heparin tubes (Microvette, Sarstedt) and centrifuged (1500×g for 5 min at 4 °C) for plasma isolation. Relative bioavailability (F%) was calculated as $F\% = (AUC_a / AUC_r) \times 100$, where AUC corresponds to the area under the curve representing Hg plasma content (µg/mL) versus time (min). AUC_a are the values from animals administered with Hg and LAB, whereas AUC_r are the values from control animal receiving only Hg(II) or MeHg. Calculations were carried out with SigmaPlot v13.5.

Additionally, fresh fecal pellets were recovered from individual mice 24 h after each Hg dosing in order to determine Hg fecal excretion. At the end of the experiment, animals were anesthetized by inhalation of isoflurane and euthanized by cervical dislocation. Liver and kidneys were extracted and thoroughly washed with PBS for determining Hg tissular accumulation. Mercury quantification in samples (plasma, feces and organs) was performed using the protocol describe previously (section 2.4.2).

2.5. Statistics

The statistical analyses of results were performed by Student t-test or one-way ANOVA with multiple *post hoc* comparisons by Tukey HSD test (SigmaPlot v13.5). Differences

were considered significant at $p < 0.05$. The calculation of the sample sizes for *in vivo* assays were carried out with GPower 3.1, with $\alpha = 0.05$ and a statistical power ($1-\beta$) of 0.8.

3. RESULTS

3.1. *In vitro* evaluation of the effect of lactic acid bacteria on Hg permeability

To assess the effect of the selected LAB strains on the intestinal permeability of Hg species we employed an *in vitro* model consisting in a co-culture of two intestinal epithelial cells: NCM460, an epithelial colonic cell line and the mucus producing cell line HT29-MTX. The treatments assayed in the present study had no effects on the integrity of the cell monolayers, whose permeability coefficients of LY were lower than 1×10^{-5} cm/s. However, it was evidenced that addition of LAB to the media resulted in a significant reduction of LY permeability (15-25 % reduction; data not shown), indicating that these microorganisms could decrease the paracellular transport.

Table 1 shows the P_{app} for Hg(II) and MeHg in HBSS medium through co-cultures of NCM460/HT29-MTX cells (80/20 proportion) in the absence or presence of the two lactobacilli strains assayed. *L. casei* BL23 and *L. acidophilus* BL17 strains significantly reduced Hg(II) permeability (74-94%) when applied at an OD of 0.3. However, this cell density did not result in reduced permeability for MeHg. Increasing the amount of bacteria to an OD of 0.6 did not modify permeability for MeHg and significant reductions (20-31 %) were only achieved at an OD of 1.2 (Table 1).

We also determined the P_{app} of Hg present in the bioaccessible fraction of naturally-contaminated swordfish obtained after cooking and performing a simulated gastrointestinal digestion (Table 2). Previous Hg speciation studies evidenced that most of the metal present in swordfish corresponds to MeHg (Torres-Escribano et al., 2010).

The cooked sample of swordfish presented a concentration of 3.6 ± 0.3 mg Hg/kg. After application of the gastrointestinal digestion, the concentration of Hg in the bioaccessible fraction was 1.6 ± 0.2 mg/kg. The presence of the BL23 strain at an OD of 1.2 reduced by 43% the P_{app} of Hg, whereas no reduction was observed with BL17 strain.

3.2. Effect of lactic acid bacteria on Hg toxicokinetics *in vivo*

3.2.1. Effect of lactic acid bacteria on Hg bioavailability

The areas under the curve (AUC) for Hg of animals orally given Hg(II) or MeHg in the absence or presence of lactobacilli are depicted in Fig. 1. Administration of both lactobacilli to animals by gavage (5×10^8 CFU) did not affect the plasmatic contents of Hg(II), but it resulted in a significant reduction of the MeHg concentration. Relative bioavailabilities calculated by comparing the AUCs of the co-exposures (metal + LAB) with those of the controls only treated with Hg are shown in Table 3. Under the assayed conditions, bacteria reduced MeHg bioavailability (median: 39 %); however, Hg(II) bioavailability was not affected.

3.2.2. Effect of lactic acid bacteria on Hg fecal excretion

Figures 2A and 2B show the Hg fecal excretion of mice exposed to Hg(II) and MeHg, respectively. The data evidenced that the treatment with LAB and later co-exposure with Hg(II) did not influence Hg excretion, with the exception of BL17 strain during the first day of exposure (increase in excretion of 16-270%). This increase, however, was not reflected in Hg plasma concentrations, where no differences in LAB-treated and untreated animals was seen for Hg(II) (Table 3).

For MeHg a different situation was found, as the animals treated with both lactobacilli strains showed a significant increase in fecal elimination of Hg at the third day. The

BL17 strain led to an increased Hg excretion compared to control animals that ranged between 38 and 106 % (median 72%). Similarly, an increase in Hg excretion through feces was observed for BL23 strain: 13-124% increase; median 68%.

3.2.3. Effect of lactic acid bacteria on Hg tissular accumulation

Accumulation of Hg in liver and kidney of mice after three days of exposure is shown in Figures 3A and 3B, respectively. Considering the differences detected in plasma levels and fecal excretions between both Hg forms, it was expected that tissular accumulation was much higher for MeHg compared to Hg(II). The higher absorption of MeHg over Hg(II) has been previously observed (Sasser et al., 1978) due to its lipophilic character (Halbach, 1990). This was true for liver (medians: MeHg 2534 ng/g; Hg(II) 250 ng/g) but not for kidney, where the differences in accumulation were not statistically significant between groups (medians: MeHg 3895 ng/g; Hg(II) 2364 ng/g). Furthermore, no differences were observed in accumulation between animals treated or untreated with both LAB strains.

4. DISCUSSION

Results reported here constitute the first effort in analyzing the capacity of LAB to reduce Hg transport across an intestinal epithelium exposed to a real contaminated food. The use of LAB strains for reducing metal exposure presents several advantages. First they are GRAS microorganisms that are normal inhabitants of the gastrointestinal tract; and second, they may display a protective effect that counteracts the toxicity (reduction in pro-oxidant response linked to metal exposure) associated to some metals, as evidenced in animal trials (Jiang et al., 2018; Majlesi et al., 2017; Tian et al., 2012; Zhai et al., 2015). In this study we have shown that certain LAB strains that were previously

characterized by their capacity to bind Hg(II) and MeHg during a simulated gastrointestinal digestion (Jadán-Piedra et al., 2017), reduce the *in vitro* Hg translocation across intestinal cells (Table 1). A number of mechanisms may account for the observed reduction in the translocation of Hg but the mechanisms linked to this reduction are probably different for aqueous solution and swordfish-derived Hg. Metal adsorption onto the bacterial surface, which renders the metal non available for transport through the cell monolayers, possibly explains this effect in aqueous solutions (Alcántara et al., 2017). A very recent study carried out with *Lactobacillus rhamnosus* GR-1 also showed that this strain was able to reduce translocation of Cd(II) and Pb(II) through a Caco-2 monolayer probably by sequestering them from solution (Daisley et al., 2018).

Jadán-Piedra et al. (Jadán-Piedra et al., 2017), however, showed that the capacity for capturing swordfish-derived Hg by *L. casei* BL23 strain was dramatically lowered compared to Hg present in aqueous solutions, suggesting that Hg sequestration by lactobacilli was impaired under these conditions. The observed lower transport through the cell monolayers is possibly related to a modulation of the epithelial permeability. In the present study, we showed that the bacterial presence reduced paracellular transport of the LY marker through the cell monolayer, indicating a bacterial regulation of the paracellular permeability. Previous studies have shown that the permeability of the epithelium is largely governed by the proteins that make up the intercellular junctions (Anderson and Van Itallie, 2009). Some LAB are capable of increasing the expression of these proteins, augmenting the barrier function and generating a less-permeable epithelium (Ulluwishewa et al., 2011). In fact, Jiang et al. (Jiang et al., 2018) evidenced that *Lactobacillus brevis* avoided the drop in expression of tight junction proteins that was shown after Hg(II) exposure in mice. Zhai et al. also observed different modes of

protection by lactobacilli against Cd(II) toxic effects that go beyond the mere metal sequestration, and are the result of a reinforcement of the intestinal barrier (Zhai et al., 2016). The likely reduction in the paracellular translocation generated by the lactobacilli should not affect the transport of Hg salts in aqueous solution, whose paracellular transport is negligible (Vázquez et al., 2015; Vázquez et al., 2014). However, it can affect permeability of other Hg forms present in the bioaccessible fractions of swordfish, where the metal could be forming complexes with thiolated compounds (George et al., 2011; Harris et al., 2003).

Considering the *in vitro* permeability data obtained in this study, it was expected that animals treated with both LAB strains would present reductions in Hg(II) and MeHg bioavailability accompanied by an increased fecal excretion and lower concentrations at target organs (liver and kidney). The employed LAB strains diminished MeHg bioavailability and augmented its fecal excretion, but this had no effect in the Hg contents of the analyzed organs. Previous studies have proven the efficacy of *Lactobacillus plantarum* (Zhai et al., 2013) or *Leuconostoc mesenteroides* (Yi et al., 2017) strains reducing hepatic and renal accumulation of Cd and Pb. Regarding Hg, only two *in vivo* experiments involving Hg(II) and bacteria able to capture this metal have been previously reported, but information about MeHg is not available. Dosing of a strain of *L. plantarum* or *Bacillus coagulans* spores (10^9 CFU/animal) reduced Hg(II) tissue accumulation after 48 days of treatment in rats (Majlesi et al., 2017). In the recent study of Jiang et al. (Jiang et al., 2018), administration of a *L. brevis* strain (10^9 CFU/animal) resulted in increased Hg fecal excretion after a single Hg(II) dose of 10 mg/Kg body weight, and the accumulation of Hg in the muscles of mice was reduced. However, at the bacterial concentrations employed in our study (5×10^8 CFU/animal), no significant effects were observed in Hg(II) toxicokinetics. This was true even if

reduction in P_{app} for Hg(II) by the bacteria in the *in vitro* experiments was more efficient compared to MeHg.

The interactions that can take place between Hg and dosed lactobacilli at the lumen, the enterohepatic circulation and the presence of the intestinal microbiota, among other factors, could affect the capacity of the strains to bind the mercurial species or favor the release of the already bound Hg, explaining the divergence between *in vitro* and *in vivo* experiments, especially for Hg(II). A recent study with *in vitro* cultures of human microbiota from two subjects has shown that MeHg demethylation occurred in a individual dependent manner and was influenced by nutrient availability (Guo et al., 2018). A similar situation has been previously observed for other metallic cations. Thus, Zhai et al. (Zhai et al., 2015) showed that certain LAB strains that were effective in reducing Cd(II) solubility *in vitro*, did not display an elevated capacity to modify the metal toxicokinetics in laboratory animals. Li et al. (Li et al., 2017) also evidenced that *Lactobacillus bulgaricus* KLDS1.0207, a strain able to capture 80% of Pb(II) present in aqueous solutions, did not modify substantially Pb hepatic and renal concentrations after two weeks of exposure to the bacteria followed by an acute Pb(II) exposition in mice. The observed differences in the protective effects of LAB on metal (and specifically Hg) accumulation reported to date (Li et al., 2017; Majlesi et al., 2017) suggest that strain-dependent modes of action, rodent strain, supplied diet, metal doses and duration of the treatments (acute, subchronic or chronic exposures) may significantly affect the efficacy of LAB as protective agents against metal toxicity and should be investigated.

The results obtained in this study evidence that strains of *L. casei* and *L. plantarum* could represent promising strategies for reducing MeHg bioavailability. It is worth mentioning that the major source for Hg exposure in humans is MeHg derived from

seafood. The assays with cell cultures show that *L. casei* BL23 reduced Hg permeability from swordfish, but *in vitro* studies do not necessarily correlate with *in vivo* results. Therefore, it is necessary to test whether MeHg toxicokinetics is modified when MeHg is conveyed through food. Furthermore, more assays are necessary in order to confirm that LAB-modulated MeHg bioavailability has an impact on Hg organ accumulation, especially under scenarios considering longer exposure times, lower doses and Hg vehicles approaching the actual conditions of exposure in humans.

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Table 1. Effect of various lactic acid bacteria on the *in vitro* permeability of Hg(II) and MeHg. Apparent permeability coefficients (P_{app}) of Hg in NCM460/HT29-MTX co-cultures exposed to Hg(II) or MeHg (0.5 mg/L) and to the bacterial strains (37 °C, 2 h). BL17 is *L. acidophilus* ATCC4356 and BL23 is *L. casei* BL23.

Species	Treatment	P_{app} (cm/s) $\times 10^{-6}$ ^a	Reduction in P_{app}
Hg(II)	w/o ^b	7.05 $\pm$ 0.83	NA ^c
	BL17 (OD 0.3)	1.75 $\pm$ 0.23	75.2 $\pm$ 3.2* ^d
	BL23 (OD 0.3)	0.57 $\pm$ 0.11	91.9 $\pm$ 1.6*
MeHg	w/o	5.88 $\pm$ 0.65	NA
	BL17 (OD 0.3)	5.68 $\pm$ 0.24	— ^e
	BL23 (OD 0.3)	5.39 $\pm$ 0.49	—
MeHg	w/o	7.23 $\pm$ 1.45	NA
	BL17 (OD 0.6)	5.85 $\pm$ 0.63	19.1 $\pm$ 8.7
	BL23 (OD 0.6)	7.80 $\pm$ 1.19	—
MeHg	w/o	5.99 $\pm$ 0.43	NA
	BL17 (OD 1.2)	4.16 $\pm$ 0.27	30.6 $\pm$ 4.5*
	BL23 (OD 1.2)	4.79 $\pm$ 0.25	20.1 $\pm$ 4.2*

^a Means $\pm$ standard deviations (n=4).

^b w/o; no bacteria added.

^c Not applicable.

^d Asterisks indicate statistically significant differences for P_{app} values in comparison to control experiments performed without bacteria ($p < 0.05$).

^e Reduction in P_{app} below 10%.

Table 2. Mercury permeability from the bioaccessible fraction of swordfish.

Apparent permeability coefficients (P_{app}) of Hg in NCM460/HT29-MTX co-cultures exposed or not to lactobacilli and to Hg present in the bioaccessible fraction of cooked swordfish (37 °C, 2 h). BL17 is *L. acidophilus* ATCC4356 and BL23 is *L. casei* BL23.

Treatment	P_{app} (cm/s) $\times 10^{-6}$ ^a	Reduction of P_{app} (%)
w/o ^b	1.59 $\pm$ 0.29	
BL23 (OD 1.2)	0.91 $\pm$ 0.14	42.3 $\pm$ 8.9* ^c
BL17 (OD 1.2)	1.45 $\pm$ 0.01	8.5 $\pm$ 5.2

^a Means $\pm$ standard deviations (n=4).

^b w/o; no bacteria added.

^c Asterisk indicates statistically significant differences for P_{app} values in comparison to control experiments performed without bacteria ($p < 0.05$).

Table 3. Relative bioavailability of Hg(II) and MeHg in exposed animals.

Strain	F(%) ^a Hg(II)	F(%) MeHg
BL23	89 ± 11	61 ± 14 ^{*b}
BL17	108 ± 11	58 ± 17 [*]

^a Values (F%) are expressed as percentages with respect to animals treated with the mercurial forms only (mean ± SD, n=7).

^b Asterisks indicate significant reductions compared to animals where no bacteria were administered (p < 0.05).

Figure legends

Figure 1. Mercury plasmatic levels in mice. AUC values for plasmatic Hg in mice treated with Hg(II) or MeHg with or without LAB. The data were obtained after a single Hg(II) or MeHg dose (0.5 mg/kg body weight) by oral gavage in the presence or absence of lactobacilli at the first day of Hg exposure. The values are expressed as $\mu\text{g Hg/mL/min}$ (n=7). Control indicates animals exposed to Hg without supplementation with lactobacilli. Asterisks show significant reductions compared to control animals ($p < 0.05$). BL17 is *L. acidophilus* ATCC4356 and BL23 is *L. casei* BL23.

Figure 2. Mercury fecal excretion in mice. Concentrations of Hg in feces from animals treated with Hg(II) (0.5 mg/kg body weight) (A) or MeHg (0.5 mg/kg body weight) (B) in the presence or absence of the two assayed lactobacilli. Values are expressed as ng Hg/g of feces (n=7). Asterisks show significant increases compared to animals not receiving bacteria ($p < 0.05$). BL17 is *L. acidophilus* ATCC4356 and BL23 is *L. casei* BL23.

Figure 3. Tissular accumulation of mercury in mice. Mercury concentrations in liver (A) and kidney (B) of mice orally given Hg(II) (0.5 mg/kg body weight) or MeHg (0.5 mg/kg body weight) with or without the assayed bacteria. Values are expressed as ng Hg/g tissue (n=7). w/o; no bacteria were added. BL17 is *L. acidophilus* ATCC4356 and BL23 is *L. casei* BL23.

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