In vitro and in vivo evaluation of Weissella cibaria and Lactobacillus plantarum for their protective effect against cadmium and lead toxicities

O. Ojekunle, K. Banwo and A.I. Sanni

Department of Microbiology, University of Ibadan, Ibadan, Nigeria

Significance and Impact of the Study: This present study highlights the presence of lactic acid bacteria (LAB) from traditional fermented foods that were cadmium and lead resistant and possessed probiotic potentials. Weissella cibaria WD2 and Lactobacillus plantarum CaD1 selected for the in vivo studies ameliorated the build-up of cadmium and lead in the organs of the animals. This indicated that good cadmium and lead binding and probiotic lactic acid bacteria can be used to prevent exposure to these heavy metals.

Abstract

Thirty-two lactic acid bacteria (LAB) isolates were obtained from fermenting cassava mash and wara (African soft cheese) and screened for their resistance to cadmium and lead toxicities at 550–1050 mg l⁻¹ and probiotic potentials. Four LAB strains that tolerated the heavy metals at 1050 mg l⁻¹ were selected for antioxidative capacities, tolerance to acid, bile salts and simulated gastric and intestinal tract and safety status. The results revealed that Weissella cibaria WD2 and Lactobacillus plantarum CaD1 exhibited comparatively higher antioxidative capacities, survived in simulated gastric and intestinal transit, tolerated acid and bile salt and possessed safety status. The two strains were employed for the in vivo studies, which was monitored in male albino Wistar rats using skim milk as a carrier for the cultures over a period of 28 days. The rats given the cultures of W. cibaria WD2 and L. plantarum CaD1 in addition with the administration of heavy metals had improved renal and hepatic impairment, while damage was observed in rats fed with cadmium and lead only. Weissella cibaria WD2 and L. plantarum CaD1 demonstrated probiotic potentials and safety status. These strains can be used to effectively amend hepatic and renal histopathological alterations in rats caused by ingestion of cadmium and lead.

Introduction

Cadmium (Cd) and Lead (Pb) are toxic heavy metals that persist in the environment, in soils and sediments for many years because of its nonbiodegradable properties, which causes diverse serious health challenges in both humans and animals. The risk of human contact to Cd and Pb has increased with the cumulative contamination of the food chain by these heavy metals and the inadequate safety from contaminated food (Nordberg et al. 2011; El-shanshoury et al. 2012; Zhai et al. 2015; Kumar et al. 2017). Cd and Pb exposure affects delicate part of human body, which causes various toxic disorders including skeletal and cardiovascular dysfunctions (Bhakta et al. 2012; Zhai et al. 2015), hepatic and renal damage (El-shanshoury et al. 2012) and reproductive illnesses (Zhai et al. 2015). In addition to its extraordinary cumulative properties, Cd and Pb can disrupt a number of biological systems, usually at doses that are much lower than most toxic metals (Zhai et al. 2015; Kumar et al. 2017). Humans are subject to exposure of cadmium and lead pollution through contamination of air that we breathe, food from tainted soil and water from pipelines,
manufactured goods and occupational hazards. Comparatively, large quantities are found in commercial fertilizer, thus the rise in the top soil and plant uptake of the heavy metals contents, which leads to increase in intake of dietary cadmium and lead (Bhakta et al. 2012). Cadmium and lead stimulates free radical production, resulting in oxidative deterioration of lipids, proteins, DNA, and initiating various pathological conditions in humans and animals by bioaccumulation in the tissues mainly the liver and kidney (Bhakta et al. 2012; El-shanshoury et al. 2012; Zhai et al. 2013).

In order to avoid the penetration of cadmium and lead in the food chain, biological materials such as bacteria, yeasts and fungi have been employed in their removal and recovery due to good performance and availability (El-shanshoury et al. 2012). Previous studies have shown that lactic acid bacteria which include Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Bifidobacterium breve and Bifidobacterium lactis can bind and remove heavy metals in vitro from water and the environment (Bhakta et al. 2012; Zhai et al. 2015). Lactic acid bacteria with probiotic potentials have been reported to have antioxidative properties, which are effective against heavy metals, induced oxidative stress in mice, fishes and the environment (Bhakta et al. 2012; Zhai et al. 2013, 2015). Numerous investigations indicate that probiotic bacteria can play an important role in the body’s natural processes of detoxification and elimination of cadmium and lead due to their antioxidative characteristics (Bhakta et al. 2012; Zhai et al. 2013, 2015). Due to these important characteristics, lactic acid bacteria-fermented foods can serve as a protective dietary control to heavy metal contamination (Zhai et al. 2013, 2015; Kumar et al. 2017). The aim of this study was to screen probiotic lactic acid bacteria from two Nigerian traditional foods (fermented cassava mash to make gari and wara (African soft cheese) for their in vitro and in vivo protective capacities against acute cadmium and lead toxicities.

Results and discussion

Identification of cadmium- and lead-resistant LAB

Thirty-two LAB isolates tolerated cadmium and lead at 550 mg l\(^{-1}\) while only four tolerated at 1050 mg l\(^{-1}\). The four isolates were tentatively characterized as Lactobacillus sp. CaD1, Lactobacillus sp. CaD2, Enterococcus sp. WD1 and Leuconostoc sp. WD2 (Table 1). This is in agreement with the report of Zhai et al. (2015) who observed that L. plantarum and L. rhamnosus possessed high resistance against cadmium at a minimum inhibitory concentration (MIC) over 1000 mg l\(^{-1}\). The tolerance of lactic acid bacteria strains to cadmium and lead could be because of some mechanisms such as complex formation, ion exchange, adsorption, chelation and microprecipitation (Bhakta et al. 2012; Zhai et al. 2015). These mechanisms are proposed to be involved in heavy metal biosorption. Lactic acid bacteria had been reported to tolerate cadmium and lead at about 50 and 100 mg l\(^{-1}\), where L. plantarum CCFM8610 had a binding capacity of above 31.34% wet biomass against cadmium (Zhai et al. 2015; Kumar et al. 2017). The results from this study show that binding conditions of our strains to cadmium and lead toxicities is due to the freshly cultured nature of the strains.

<table>
<thead>
<tr>
<th>Food samples</th>
<th>Cadmium-tolerant isolates (mg l(^{-1}))</th>
<th>Lead-tolerant isolates (mg l(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>550 1000 1050</td>
<td>550 1000 1050</td>
</tr>
<tr>
<td>Fermenting cassava</td>
<td>22 7 2</td>
<td>20 7 2</td>
</tr>
<tr>
<td>Wara</td>
<td>10 5 2</td>
<td>12 5 2</td>
</tr>
<tr>
<td>Total</td>
<td>32 12 4</td>
<td>32 12 4</td>
</tr>
</tbody>
</table>

Tolerance to bile salts and simulated GI tract conditions

The tolerance to bile salts and simulated gastric and intestinal juices of the four strains is shown in Table 2. Lactobacillus sp. CaD1 exhibited the highest tolerance of 98.9 and 82.1%, while Enterococcus sp. WD1 the least of 56.6 and 33.7% to 0.3 and 1.0% bile salts concentrations respectively. The bile salt tolerance test showed that the LAB strains in this study possess good resistance to bile. Resistance to bile is an important criterion for probiotic organism, which ensures colonization and metabolic activity of the strain in the small intestine (Banwo et al. 2012; Bhakta et al. 2012).

The highest survival rate of 98.71% was observed in Lactobacillus sp. CaD1 for simulated gastric juice, while Lactobacillus sp. CaD2 the least of 79.24% after incubation in the simulated intestinal juice. The strains evaluated in this study exhibited potentials to survive passage through the gastrointestinal tract after exposure to simulated conditions of gastric and intestinal passage. As the stress condition of the stomach and small intestine transit might interact and thereby affect the viability of the strains in a synergistic fashion, successful combination of the tolerance to bile salts, gastric and intestinal fluids in determinations of simulated gastrointestinal transit is very important (Banwo et al. 2012; Zhai et al. 2015).

Antioxidative activity and safety assessment of the LAB strains

The antioxidative, haemolytic and gelatinase activities of the lactic acid bacteria strains are displayed in Table 3.
Table 2 Tolerance of the lactic acid bacteria strains to bile salts, simulated gastric and intestinal juices

<table>
<thead>
<tr>
<th>Strains</th>
<th>Bile salts tolerance</th>
<th>Survival rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3%</td>
<td>0.5%</td>
</tr>
<tr>
<td>Lactobacillus sp. CaD1</td>
<td>98.9 ± 0.1</td>
<td>83.6 ± 0.2</td>
</tr>
<tr>
<td>Lactobacillus sp. CaD2</td>
<td>86.1 ± 0.2</td>
<td>84.7 ± 0.3</td>
</tr>
<tr>
<td>Enterococcus sp. WD1</td>
<td>56.6 ± 0.3</td>
<td>34.3 ± 0.1</td>
</tr>
<tr>
<td>Leuconostoc sp. WD2</td>
<td>79.4 ± 0.3</td>
<td>68.6 ± 0.3</td>
</tr>
</tbody>
</table>

CaD: isolates from cassava mash; WD: isolates from Wara; SGJ: simulated gastric juice after 3 h of incubation; SIJ: simulated intestinal juice after 8 h of incubation.

Values represent mean ± SD of three independent experiments.

Table 3 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging and reducing abilities of the intact cells and gelatinase and haemolysis activities of lactic acid bacteria strains

<table>
<thead>
<tr>
<th>Strains</th>
<th>DPPH scavenging ability (%)</th>
<th>Reducing ability (equivalent cysteine μmol l⁻¹)</th>
<th>Gelatinase</th>
<th>Haemolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus sp. CaD1</td>
<td>72.48 ± 0.34</td>
<td>73.72 ± 0.22</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>Lactobacillus sp. CaD2</td>
<td>68.54 ± 0.30</td>
<td>51.03 ± 0.41</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>Enterococcus sp. WD1</td>
<td>41.08 ± 0.25</td>
<td>67.89 ± 0.26</td>
<td>–</td>
<td>Y</td>
</tr>
<tr>
<td>Leuconostoc sp. WD2</td>
<td>64.32 ± 0.43</td>
<td>67.06 ± 0.25</td>
<td>–</td>
<td>Y</td>
</tr>
</tbody>
</table>

Y: Gamma haemolysis; –: negative; CaD: isolates from cassava mash; WD: isolates from Wara.

Values represent mean ± SD of three independent experiments.

Lactobacillus sp. CaD1 had the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging ability of 72.48% while Enterococcus sp. WD1 the least of 41.08%. Lactobacillus sp. CaD1 had the highest reducing ability of 73.72% while Lactobacillus sp. CaD2 the least of 51.03. The intact cells were used in this study, because it can defend the intestinal epithelial cells against the impairment caused by heavy metal-induced oxidative stress. The intracellular cell-free extract can be absorbed from the small intestine into the blood which provide protection against oxidative stress in the liver and kidney (Jain et al. 2009; Zhao and Shah 2014; Zhai et al. 2015). The strains were nonhaemolytic and exhibited negative activity to gelatinase test. The safety assessment of strains to be used for in vivo studies is vital so that such strains will not become virulent when administered. Haemolysin plays an important role in virulence of some lactic acid bacteria which may increase the emergence of opportunistic infections in humans and animals (Yoon et al. 2008). Gel genes may be silent and phenotypically undetectable but they are present frequently in clinical isolates than in food-based noninfectious isolates (Yoon et al. 2008; Banwo et al. 2012).

Selection of the LAB strains used for animal studies

Lactobacillus sp. CaD1 and Leuconostoc sp. WD2 strains that gave the best results based on the screening were selected and sequenced. The strains had 97–99% homology with L. plantarum and Weissella cibaria respectively. The name W. cibaria WD2 and L. plantarum CaD1 with accession numbers KX780366 and KX780367 respectively. The living and dead cells of L. plantarum CCFM8610 were used for in vivo studies and it gave excellent protective effects in mice more than L. rhamnosus GG, which was the reference strain (Zhai et al. 2013). To the best of our knowledge, there is paucity of reports on the cadmium or lead toxicity binding capacity of Weissella species. Although, there are reports on the probiotic potentials of these species isolated from food matrices, humans and animal faeces (Ayeni et al. 2011; Yang et al. 2014; Zhang et al. 2014). However, only few studies investigated the potential of Weissella in vivo for biotechnological and probiotic purposes, a thorough strain-specific safety assessment must be administered (Fusco et al. 2015).

Animal experiments

Histopathological studies of the liver and kidney of the experimental animals

Some representative photomicrographs of the untreated and treated liver and kidney of the animals are shown in Fig. 1a,b. Liver cirrhosis was observed in the group of rats fed with cadmium in water (white arrow Fig. 1a D). The hepatocyte show normal morphology in the liver tissue exposed to cadmium and W. cibaria WD2. The liver tissue exposed to cadmium and W. cibaria WD2 showed that the sinusoids appear dilated without infiltration of...
inflammatory cells (blue arrow Fig. 1a B). The renal tubule appear thickened with increased connective tissues, the interstitial spaces appear normal in the kidney tissue of the group of rats fed with lead and treated with \textit{L. plantarum} CaD1 (white arrow Fig. 1b C), while the renal tubules appear collapsed and some diminished luminal spaces, was observed in the kidney tissue exposed to lead and no lactic acid bacteria (blue arrow Fig. 1b D). This indicated that the tubules have mild tubular necrosis (deep blue arrow Fig. 1b D).

Oxidative stress is an important process of heavy metal toxicity, which is characterized by increase in lipid peroxidation and a decrease in the antioxidant enzyme activity. This can cause damage to the cell membrane, thus leading to the necrosis of hepatocytes (Zhai et al. 2013). This may be the reason for the hepatic damage and cirrhosis observed in the histopathology of the liver and kidney of the rats that were exposed to acute lead and cadmium without treatment with lactic acid bacteria. This is in agreement with Zhai et al. (2013) who observed same in their histopathological studies. The living cells of \textit{W. cibaria} WD2 and \textit{L. plantarum} CaD1 significantly alleviated the hepatic injury and the tissue damage. Therefore, this indicates that living cells of \textit{W. cibaria} WD2 and \textit{L. plantarum} CaD1 have protective characteristics \textit{in vitro} and \textit{in vivo} against acute cadmium and lead toxicities.

In conclusion, four lactic acid bacteria strains had good cadmium- and lead-binding capacities above 1000 mg l\(^{-1}\), while two strains (\textit{L. plantarum} CaD1 and \textit{W. cibaria} WD2) demonstrated the desirable features. The two strains possessed the safety status, which qualified them to be employed for the \textit{in vivo} studies. The study revealed that the addition of the metals with the lactic acid bacteria in the skim milk medium fed to the animals increased the binding capacities of the heavy metals after administration. This study showed that \textit{L. plantarum} CaD1 and \textit{W. cibaria} WD2 could be used as potential probiotics against acute cadmium and lead toxicities without any side effects.

**Materials and methods**

**Estimation of bacterial tolerance to cadmium and lead**

The method of Zhai et al. (2015) was used for cadmium while Garhwal et al. (2014) was used for lead with slight modifications. The LAB isolated from fermenting cassava mash and \textit{wara} were exposed to Cadmium chloride and Lead nitrate (Sigma-Aldrich, Saint Louis, MO) solution to estimate their tolerance. The tolerance of each isolate was determined by the minimum inhibitory concentration (MIC). Cadmium chloride and Lead nitrate solution were filter sterilized and added to MRS agar medium to final concentrations ranging from 50 to 1050 mg l\(^{-1}\). Duplicate plates were prepared for each concentration and plates without Cd and Pb were used as control. The growth of the test isolates was recorded after 48 h of incubation at 37°C.

**Determination of probiotic potentials and safety of LAB strains**

**Resistance to bile salts**

The ability of the test isolates to grow in the presence of bile was determined according to the method of Vinderola and Reinheimer (2003) as modified by Banwo...
et al. (2012). Each isolate was inoculated (2% v/v) into MRS broth with 0-3, 0-5 and 1% (w/v) of bile salts (Sigma-Aldrich). This was expressed as the percentage of growth at OD560 nm in the presence of bile salts compared with the control (without bile salts).

**Determination of tolerance of LAB strains to simulated GI tract conditions**

A modification of the method of Zhai et al. (2015) was used. Simulated gastric juice was prepared by dissolving pepsin (1 : 10 000, Sigma-Aldrich, Saint Louis, MO, USA) in sterile saline (0-5% w/v) and adjusted to pH 2-0 with concentrated HCl to a final concentration of 3 mg ml−1 and the isolates were incubated in it for 3 h. The simulated small intestinal juice was prepared by adding trypsin (1 : 250, Sigma) to sterile saline (0-5% w/v, adjusted to pH 8-0 with 0-1 mol l−1 NaOH) at a final concentration of 1 g l−1 with 0-3% bile salts (Sigma).

The gastric and intestinal juices were sterilized by filtration using a 0-22-µm membrane (Millipore, Bedford, MA). The isolates were harvested by centrifugation at 8000g for 15 min and inoculated at a concentration of 1 × 10⁹ CFU per ml. The survival rate was calculated as follows:

\[
\text{Survival rate(%) = \frac{\log \text{CFU } N_i}{\log \text{CFU } N_0} \times 100,}
\]

where: \(N_i\) = Total viable count of each isolate after treatment with simulated GI juices; \(N_0\) = Total viable count of each isolate before treatment with simulated GI juices.

**Determination of antioxidative activities of the LAB strains**

**Preparation of the intact cells of the LAB strains**

The overnight cultured LAB was centrifuged at 8000g for 20 min to harvest the intact cells. The cells were washed with phosphate buffer solution (PBS, pH 7-2) and suspended in the same buffer at a concentration of 10⁹ CFU per ml (Zhai et al. 2015).

**DPPH scavenging activity**

The method described by Zhai et al. (2015) was used. A mixture of 1 ml of the intact cells of the LAB strains and 1 ml of freshly prepared DPPH solution (0-2 mol l−1 in methanol; Sigma) were incubated for 30 min in the absence of light. The mixture of the DPPH and PBS (pH 7-2) was used as blank sample. After centrifugation at 7000g for 10 min, the scavenged DPPH was analysed by measuring the decrease in absorbance at 517 nm. The scavenging ability was calculated in percentage as follows:

\[
\text{Scavenging activity(%) = \left[1 - \frac{A_{517}(\text{sample})}{A_{517}(\text{blank})}\right] \times 100%}
\]

**Reducing activity assay**

The reducing abilities of the LAB strains were determined according to the method of Zhai et al. (2015). A 0-5 ml volume of the intact cells of LAB strains was mixed with 0-5 ml of 1% potassium ferricyanide and 0-5 ml of PBS (pH 6-6). The mixture was incubated at 50°C for 20 min and rapidly cooled, after which 0-5 ml of 10% trichloroacetic acid (TCA) was added. After centrifugation at 2000g for 5 min, 1 ml of the upper phase was mixed with 1 ml of 0-1% ferric chloride. After 10 min of incubation, the absorbance of the mixture was measured at 700 nm. Cysteine was used as the standard for expression of the reducing activity.

**Genotypic characterization of the LAB strains**

The genomic DNA of the LAB strains were extracted using the modified lysozyme-heat lysis method as previously described by Kostinek et al. (2005). The partial 16S rRNA nucleotide sequence was amplified by PCR using universal primers designed for lactic acid bacteria, forward was (5'- GAG TTT GAT CCT GGC TCA G -3') and the reverse was (5' - AGA AAG GAG GTG ATC CAG CC -3') (Kostinek et al. 2005; Banwo et al. 2012). The PCR products were cleaned using Qiagen spin columns (Qiagen, Shanghai, China) according to the manufacturer's instructions and subsequently sequenced in a commercial laboratory (Beijing, China).

**Haemolysis and production of gelatinase**

The strains were cultured in MRS broth at 37°C for 12–18 h and then transferred onto blood agar (Difco Laboratories, Detroit, MI, USA) plates supplemented with 5% defibrinated whole sheep blood as described by Yoon et al. (2008). After 24–48 h, the haemolytic reaction was recorded.

To determine the presence of gelatinase activity, the plate assay method on Todd–Hewitt agar containing 30 g l−1 of gelatin was carried out as described by (Ben-Omar et al. 2004; Banwo et al. 2012).

**In vivo protective potentials of LAB strains against Cadmium and Lead**

**Experimental animals and design**

In vivo protective characteristics of the LAB strains was carried out using the method of Zhai et al. (2013). A total of 40 male Wistar rats weighing 48–58 g and aged 4–5 weeks old were obtained from Central Animal House, University of Ibadan. They were adapted to laboratory handling for a period of 2 weeks. The rats were fed with standard commercial rat feed and drinking water was
given ad libitum. The rats were randomly divided into five treatment groups of four rats in each group making a total of 20 for lead and cadmium respectively (Table 4). The control animals received drinking water and skim milk while the infected group received lead and cadmium (4-24 µg ml⁻¹) dissolved in water only. The infected and treated group received cadmium and lead with the living cells of L. plantarum CaD1 and W. cibaria WD2 in skim milk. The rats were fasted for 12 h before exposure to the heavy metals. The in vivo study was carried out for a period of 28 days. All animal procedures were performed in accordance with the Ethics Committee of the Animal Care and Use for Research, Faculty of Veterinary Medicine, University of Ibadan in accordance with the recommendations of the University for the Proper Care and Use of laboratory animals for research (UI-ACUREC/App/2015/062). The animals were housed in plastic cages at the Central Animal House, University of Ibadan (UI) Vivarium. The cages were laid with wood shavings, which served as bedding for the animals, which were constantly replaced at a 2-day interval, thereby keeping the cages clean, and dry (Zhai et al. 2013).

**Histopathological studies**

The animals were sacrificed by cervical dislocation after light ether anaesthesia. On the 28th day, livers and kidneys were excised, and cleaned in normal saline. The tissues were fixed in 10% formalin saline for 48 h. Tissues were embedded in paraffin and serial sections of 5-µm thickness were made using rotary microtome. The sections were stained with haematoxylin-eosin (H and E) for light microscopy examination. The results of the histopathological studies were analysed with a semi statistical evaluation (Babalola et al. 2010; Zhai et al. 2013).

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**Conflict of Interest**

The authors declare no conflict of interest.

**References**


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**Table 4** Animal groups and treatments for cadmium and lead toxicities

<table>
<thead>
<tr>
<th>Groups</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (Control)</td>
<td>Drinking water</td>
</tr>
<tr>
<td>B</td>
<td>Weissella cibaria WD2 + skim milk + 4-24 µg ml⁻¹ Cadmium</td>
</tr>
<tr>
<td>C</td>
<td>Lactobacillus plantarum CaD1 + skim milk + 4-24 µg ml⁻¹ Lead</td>
</tr>
<tr>
<td>D</td>
<td>Cadmium/Lead (4-24 µg ml⁻¹ of each) + drinking water</td>
</tr>
<tr>
<td>E</td>
<td>Weissella cibaria WD2/L. plantarum CaD1 + skim milk</td>
</tr>
</tbody>
</table>

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