Original article

Removal of Pathogens (*Escherichia coli* O157:H7 and *Salmonella typhi*), and nutrients from wastewater by Lactic Acid bacteria

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Abstract

Outbreaks of various forms of diarrhoea, particularly cholera and typhoid, are a frequent occurrence in low income countries. Poor water sanitation and hygiene practices are frequently implicated for all diarrhoeal out breaks in the low income countries. The present study assessed (i) the probiotic potential of selected strains of lactic acid bacteria in terms of their inhibition activities against *Escherichia coli* O157:H7 and *Salmonella typhi*, and the removal of heavy metals in wastewater. The selected lactic acid bacteria had probiotic properties, namely resistance to low pH (pH2 and 3), tolerance to bile salts and sodium chloride, and significant antimicrobial activities against strains of *E. coli* O157:H7 and *S. typhi* ($p < 0.001$). Strains of lactic acid bacteria were shown to modulate the concentration of aqueous Cu, Mn, NH4 + and Zn with a significant net decrease between Day 7 or 10 ($p \leq 0.05$). The selected lactic acid bacteria were also shown to deploy biofilms on surfaces of sand particles, which are thought to constitute a mechanism by the lactic acid bacteria to hold and kill *E. coli* and *S. typhi* in wastewater. The lactic acid bacteria have exhibited great potential for use in the removal of diarrhoeagenic bacteria and heavy metals from wastewater.

Keywords: *Diarrhoea; Pathogen; Heavy Metal; Inhibition; Lactic acid bacteria (LAB); Removal; Wastewater.*

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Abbreviations

%	percent
~	approximately
/	per
+	plus
<	less than
>	greater than
≤	less than or equal to
μmol/L	micromolars per litre
B. lactis	*Bifidobacterium lactis*
CB	strain of LAB from *P. curatellifolia* fruit cake
Cd	Cadmium
CFU	colony forming units
CFU/ml	colony forming units per units
Cr	Chromium
Cu	Copper
E. coli	Escherichia coli
et al	*et alia* (Latin phrase meaning and others)
Fe	Iron
Fe(III)	Iron(III) oxide
h	hour
H_2O_2	hydrogen peroxide
Hg	Mercury
LAB	lactic acid bacteria
Lb. acidophilus	*Lactobacillus acidophilus*

Lb. casei	*Lactobacillus casei*
Lb. plantarum	*Lactobacillus plantarum*
LIC	low income countries
log	logarithm
mg	milligram
mg/L	milligram per litre
MIC	minimum inhibition concentration
mm	millimetre
P. curatellifolia	*Parinari curatellifolia*
MRS agar	De Man, Rogosa and Sharpe agar
NaCl	sodium chloride
Ni	Nickel
^{o}C	degree of celcius
OD	optical density
pH	potential of hydrogen
Pb	lead
PRO1	probiotic lactibiane WYAGE
PRO2	probiotic FORT
S. typhi	*Salmonella typhi*
SAL	*Salmonella typhi*
T0	time 0
T1	time 1
T2	time 2
T3	time 3
T4	time 4

T5	time 5
TOM	strain of LAB from decomposed tomatoes
v/v	volume for volume
Zn	Zinc
ZOI	zone of inhibition
$(NH_4)_2SO_4$	ammonium sulfate

Introduction

Several countires across the globe, especially low income countries (LIC), frequently face a burden of diarrhoeal outbreaks (Baker, 2016). Zimbabwe, one of the low income countries in the Southern African region, is frequently affected by erratic outbeaks of diarrhoea (Kidia, 2018). Notably, Zimbabwe recorded at least 200 cases of typhoid fever during the first 3 weeks of 2018 alone. Ongoing research continues to identify poor water quality and the release of wastewaters into the country's freshwater channels as the major risk factors for its heavy burden of diarrhoeal diseases. Bioremediation, as compared to conventional treatment methods, is widely seen as a more sustainable and cheaper way of treating municiopal wastewater particularly in resource limited settings (Alufasi *et al.*, 2017). Lactic acid bacteria (LAB) are facultatively anaerobic, and areknown to kill pathogens in the gut of animals as well as harbor other healthful properties. Use of probiotic bacteria, especially LAB is widely reported in aquaculture (Gatesoupe, 1999; Hai, 2015; Melgar Valdes *et al.*, 2013; Verschuere *et al.*, 2000), particularly to control diseases / pathogens of aquatic life (Hai, 2015; Verschuere *et al.*, 2000). Besides the rerported novelty of probiotic microorganisms in aquatic environments, no research has reported the application of probiotic microorganisms in wastewater treatment, or as a means to control diarrhoeal outbreaks. The fact that most LAB are used in aquaculture may imply possible application of these friendly microorganisms in wastewater treatment. Municipal wastewaters are frequently laden with heavy metals (Spiegel *et al.*, 1985), dissolved organic matter (Czerwionka *et al.*, 2012; Maizel and Remucal, 2017), pharmaceutical effluent from hospitals and pharmaceutical industries (Gadipelly *et al.*, 2014; Lindholm-Lehto *et al.*, 2015) as well as solid waste (Lindholm-Lehto *et al.*, 2015). Mining towns such as Bindura, which falls in the heartland of a major mining region in Zimbabwe (housing mines such as Trojan Nickel Mine, Fredda Rebecca Gold Mine (a member Ashanti Gold) as well as several parties of artisanal miners), besides their polluted wastewaters, face an additional challenge of intersecting with / handling mining effluent laden with heavy metals and other toxins (Feng *et al.*, 2000; Muposhi *et al.*, 2015). Bindura Nickel Mine which primarily mines Nickel, is implicated for releasing heavy metals that include zinc, cobalt and cadmium into freshwaters surrounding the town (Muposhi *et al.*, 2015). For a town such as Bindura, which has limited capacity to treat its wastewater of microbial pollutants and without means to remove heavy metals used by its residents, needs a cheap and effient method to remove these health hazards. Heavy metals are metallic elements having a density of over 4 g/cm3 (Rucińska-Sobkowiak, 2016). Further, heavy metals are nondegradable compounds that may exist as either organic or inorganic forms (Jan *et al.*, 2015). Some heavy metals such as zinc (Zn) and (Cu) are essential trace elements, whereas, others such as Lead (Pb) and Cadnium (Cd) are regarded as most toxic heavy metals (Singh *et al.*, 2011). Aquatic and subsurface environments are rich in a number of mineral elements particularly trivalent Fe [Fe(III)] and tri/tetravalent Mn [Mn(III/VI)] that can support a variety of dissimilatory metal-reducing bacteria when oxygen or other terminal electron acceptors are absent (Novotnik *et al.*, 2019). Municipal wastewaters are often laden with heavy metals that include Cd,

chromium (Cr), copper (Cu), mercury (Hg), nickel (Ni), lead (Pb) and zinc (Zn) (Tytla, 2019). Municipal wastewaters in Zimbabwe, especially in mining towns such as Bindura and industrialised cities such as Harare and Bulawayo, pose a major threat to urban populations and those residing in places downstream. Irrigated vegetables in Harare have were shown by Muchuweti *et al.* (2006) to be a primary source of cadnium and lead exposure among the Zimbabwean population. High acquatic concentrations of cadnium and lead have been reported to be released from a number of anthropogenic sources such as fertilisers used in agriculture, dumping of sewage sludge, metal mines and smelters (Wuana and Okieimen, 2011). Oral exposure of Cd is known to be a risk factor for osteoporosis (Engstrom *et al.*, 2012) and prostate cancer (Ju-Kun *et al.*, 2016). On the other hand, Pb disturbs haemoglobin synthesis, renal function and neurological disturbances in children (Levin and Goldberg, 2000). LAB have been reported to remove heavy metals (Monachese *et al.*, 2012) sulphates (Andrea *et al.*, 2017) and nitrates from aqueous solution in vitro. The removal of these nutrients have been observed to be strain dependant. Additionally, heavy metals have been reported to passively bind to the LAB surface by electrostatic and hydrophobic interations (Halttunen *et al.*, 2008). Binding of heavy metals on the LAB could be a promising solution for heavy metal removal from wastewater. Certain strains of LAB are known to bind to zinc, thereby removing it from solution (Mrvčić *et al.*, 2009). Manganese is a redox sensitive element and it undergoes many oxidation–reduction reactions in the natural environment (Novotnik *et al.*, 2019). While Mn is known to be reduced / oxidised (redox) naturally (abiotically) in the environment by iron(III) oxide [Fe(III)], a number of strains of LAB have been shown to modulate the concentration of aqueous manganese in wastewater (Novotnik *et al.*, 2019). Antioxidative properties of LAB may also be another important characteristic for cadnium toxicity protection (Zhai *et al.*, 2015). On this basis, LAB seem to offer great potential as a tool for the removal of selected heavy metals and diarrhoeagenic bacteria from municipal wastewaters. The objective of this study was to determine the potential antidiarrhoeal (against *Escherichia coli* O157:H7 and *S. typhi*), and heavy metal and nutrient removal activities of selected lactic acid bacteria in a synthetic model of wastewater. It was hypothesised that, since LAB survive under anaerobic conditions including aquatic environments, and remove pathogens from-, and impart healthful effects in mammals, they can remove diarrhegenic pathogens, heavy metals and nutrients from wastewater. The study tested the removal of strains of *Escherichia coli* (saprophytic / environmental and O157:H7) by two putative lactic acid bacteria and standard probiotic bacteria from synthetic wastewater. Furthermore, tests for cidal/ static activities of the LAB were done at Bindura University laboratory between February and 2019 using the well diffusion assay. The study aimed to determine the probiotic potential of selected strains of lactic acid bacteria in terms of their inhibition activities against *Escherichia coli* O157:H7 and *S. typhi*, and the removal of heavy metals in wastewater.

Materials and Methods

Isolation and characterisation of putative LAB

Putative LAB were isolated from two selected Zimbabwean fermented food

and beverage, namely, *P. curatellifolia* fruit cake (CB) and decomposing tomatoes (TOM) (Table1) (Chabwinja *et al.*, 2017). Isolation and characteristion of putative LAB was carried out using the protocol of Prabhurajeshwar and Chandrakanth, (2017) with slight modifications. Briefly, 1mL of the sample was mixed aseptically with 9 mL of sterile Ringer's solution and further diluted serially up to 10^{-5}. The diluted sample (0.1mL), was spread over de Man, Rogosa and Sharpe (MRS) agar (Himedia, India) and incubated anaerobically at 37^{o}C for 48h.

After 48h, white colonies that formed were purified by subculturing on MRS agar using the streak plate technique. The purified cultures were kept in MRS agar slants and stored at 4^{o}C with subsequent subculturing/ purification. For long term storage, isolates were stored at -20^{o}C in 30% glycerol (v/v). The isolates were characterised initially based on colony mophology, Gram staining (morphological characterisation) and catalase activity (3% H_2O_2). Finally, putative LAB were confirmed by biochemical profiling and antimicrobial activities.

Table 1: Description of products used as source of putative and commercial LAB

Abbreviation used	Local name of product	English / Latin name	Uses	Origin	Composition / Description
CB	Chambwa (Shona)	*P. curatellifolia* cake	Cake (food)	Mutoko, Zimbabwe	Cake made from extract of *P. curatellifolia* fruits, finger millet powder and water
TOM	Decomposing tomatoes	Tomatoes	Food	Mutoko, Zimbabwe	Rotten tomatoes
PRO1	Commercial LAB	Lactibiane WYAGE	Food supplement/ Tablets	France	*Lb. acidophilus* LA 201, *Lb. plantarum* LA 301 and *Lb. casei* LA 205
PRO2	Commercial LAB	Probiotic FORT	Food supplement/ Tablets	France	*B. lactis*, *Lb. acidophilus*

Gram's staining

A smear of the isolates from glycerol stocks, was prepared on a cleaned grease free slide, and then heat fixed over a burner. The smear was then flooded with crystal violet stain, kept for 1min and the stain was washed with clean tap water. A drop of Gram's iodine was poured on the smear, kept for 1 minute and washed with clean tap water. The smear was flooded with 95% ethanol, kept for 30 seconds and again washed with clean tap water. At last safranin stain was flooded on the smear for 1 minute, and washed with clean tap water. The smear was then air dried and observed under the compound light microsope, first with 10× objective to check staining and under oil immersion 100× objective. Two selected Gram positive putative LAB isolates (CB and TOM) were then used for *in vitro* studies.

Biochemical and cultural characterisation of putative LAB

Catalase activity

Catalase test was perfomed in accordance with the protocol describe by Omemu *et al.* (2018). Briefly, overnight cultures of LAB isolates were grown in MRS broth for 18 h at 37ºC under anerobic conditions. After 18h 3% hydrogen peroxide solution was droped onto 1mL of overnight cultures and isolates which did not give bubbles, were selected since LAB are known to be catalase negative (Jung *et al.*, 2017).

Resistance to low pH

Active fresh cultures of putative LAB were inoculated into MRS broth with pH of 2, 3 and 4. The pH was adjusted with concentrated acetic acid (99%) and 5N NAOH. The inoculated broths were incubated anaerobically at 37oC for 18 to 24 h and growth was monitored by measuring absorbance in a Biobase EL 10B Microplate Reader (Jinan, China), at optical density 620nm (OD_{620}) (Diba *et al.*, 2013) against uninoculated broth. Additionally, viable microorganisms were enumerated at specific time intervals namely 0., 1., 2. and 3 hours with spread plate techniques as previously described by Prabhurajeshwar and Chandrakanth (2017). Serial dilutions were done using Ringer's solution as diluent, and plates were incubated at 37°C under anaerobic conditions for 48 h.

Resistance to bile salts

The ability of the putative LAB strains to tolerate bile salts was determined according to the modified method of Diba *et al.* (2013). The bile tolerance test was examined by inoculating various putative LAB isolates separately into MRS tubes containing 0.3 %, 0.5 % and 1 % bile salts (Oxoid). Inoculated tubes were incubated for 18 to 24 h at 37°C under anaerobic conditionsand bacterial growth was monitored by measuring OD_{620nm}. Bile salt free MRS broth was used as control for this experiment.

Growth at different concentrations of sodium chloride (NaCl)

To deternine NaCl tolerance in the LAB strains, the isolates were grown in MRS brothsuppplimented with different concentrations of NaCl ranging from 1% to 5%. The broth was inoculated with 10µL overnight culture of the isolates and incubated anaerobically at 37°C for 18 to 24h, bacterial growth was monitored by measuring OD_{620nm} and NaCl free MRS broth used as control.

Antibiotic susceptibility test

Antibiotic susceptibility was studied using the disk diffusion method (Georgieva *et al.*, 2015; Silva *et al.*, 2006). Activated cultures of putative and commercial LAB were swabbed

onto MRS agar plates. In this study three selected antibiotics were supplied (streptomycin, kanamycin and penicillin) in the form of dodeca discs (Himedia, India). The zones of inbibition were measured (mm) after incubation at 37°C for 24h.

Gas production from glucose

In order to determine the heterofermentative and homofermentative characteristics of the LAB isolates, carbon dioxide production test was adopted (Nikita and Hemangi, 2012). Citrate lacking MRS broths and inverted Durham tubes were prepared and inoculated with 10% overnight cultures. The test tubes were then incubated at 37 for 72h. Gas occurance in Durham tubes was observed after 72h which is the evidence for carbon dioxide production from glucose.

Antimicrobial studies

In vitro studies

The study tested the cidal / static activities of the LAB / products using the well diffusion assay. Furthermore, the study the removal of strains of *Escherichia coli* O157:H7 and *S. typhi* from synthetic wastewater by putative probiotic LAB and probiotic products.

Bactericidal activities of LAB using the well diffusion assay

The bactericidal assay was tested in accordance with a protocol described by Akbar *et al.* (2019), briefly, strains of *E. coli* O157 and *S. typhi* were inoculated into Nutrient Broth and incubated aerobically at 37^{o}C for 18h. After 18h, OD was adjusted to 0.2 at 620nm (Chabwinja *et al.*, 2017). The cultures were swabbed on EC-O157 Hicrome and Xylose Lysine Deoxycholate (XLD) agar to establish lawns of *E. coli* O157 and *S. typhi* growth respectively. Wells were then drilled into the agars using an 8mm auger, and then 60µL of an overnight culture of putative LAB were introduced into wells in triplicate. Two commercial LAB strains (PRO1 and PRO2) were included and used as reference strains. The plates were dried for 15minutes at room temperature and incubated aerobically at 37^{o}C for 48h. Antimicrobial activities were evaluated interms of zones of clearance and classified as follows: inhibition zones greater than 20, 19 to 17 and less than 16, respectively considered strong, intermidiate and low inhibitions.

Antimicrobial activity of putative LAB on Escherichia coli O157 and S. typhi in synthetic wastewater

Two putative LAB (CB and TOM), two commercial LAB stains (PRO1 and PRO2) were inoculated from glycerol stocks (-20^{o}C), propagated twice in MRS medium and grown in non shaking conditions. Both putative LAB strains (CB and TOM) and the two commercial LAB stains (PRO1 and PRO2), *E. coli* O157 and *S. typhi* were incubated at 37^{o}C for 18h. *S. typhi* and *E. coli* O157 were grown in nutrient broth and incubated as described above. After 18h, OD of all the cultures was adjusted to 0.2 at 620nm (equivalent to a concentration lying between 7 to 8 log CFU/mL) in a Biobase EL 10B Microplate Reader (Jinan, China) (Chabwinja *et al.*, 2017). Wastewater from a stream was collected, dispensed in 250mL conical flasks and supplemented with MRS broth (pH 6.5) in 1:1 ratio to allow LAB to survive and grow, hence referred to as synthetic wastewater. The synthetic wastewater was then sterilised by autoclaving at 121^{o}C for 15min. Antimicrobial activity of putative LAB on *E. coli* O157 and *S. typhi* was evaluated as a function of coincubation time. Putative LAB and *E. coli* O157 were spiked (1mL each in 1:1 ratio) in a 250mL conical flaskwith

100mL sterile synthetic wastewater. Another flask was spiked with *S. typhi* instead of *E. coli* O157. Two commercial LAB cultures (PRO1 and PRO2) were spiked separately in two flasks instead of putative LAB and used as positive controls. As negative controls sterile synthetic wastewater, was left unspiked, spiked separately with *E. coli* O157, *S. typhi*, or putative and commercial LAB cultures. The conical flasks were incubated aerobically at 37ºC with periodic shaking. At the start and at specific intervals (0h, 24h, 48h and 72h), aliquots were removed, serially diluted and plated to determine bacterial colony counts of *E. coli* O157, *S. typhi* and LAB. The results of the viability assay experiments are represented as mean values of the three independent replicates.

Nutrient removal activities of LAB

Removal of Cd, Cu, Mn and *Zn from wastewater*

In-house putative strains of probiotic bacteria kept at Bindura University were added to synthetic wastewater and incubated over a period of 10 days. Sampling for heavy metal detection was done at Day 4, Day 7 and Day 10. Samples were analysed using Varian 1275 Atomic Absorption Spectrophotometer (AAS) (Varian. Associates, Palo Alto, CA) at the Fertiliser, Farm, Feeds and Remedies Institute (Department of Research and Soecialist Services) (Harare).

Nitrate removal from wastewater

To determine ammonium concentration in the wastewater samples the method of Hernandez-Lopez and Vargas-Albores (2003) was adopted with few modifications. 0.01% ammonium nitrate was prepared and serial dilutions were then carried out so as to come up with a standard curve. A concentration of 50µmol/L was then spiked in-situ with th Lactic acid bacteria and OD measured at 620nm using the microplate reader (Chabwinja *et al.*, 2017).

Results and Discussion

Isolation of putative LAB

Five samples from Zimbabwean fermented food and beverages were used as potential sources of putative LAB. Lactic acid bacteria were isolated on MRS agar at 37ºC under anaerobic conditions. The two isolates (TOM and CB) were shown to be Gram positive, catalase negative rods. Two commercial LAB (PRO2 and PRO2) were used as reference isolates for the assays.

Morphological and biochemical characterisation of LAB

All the isolates were subjected to Gram staining and were examined under light microscope. Lactic acid bacteria isolates from decomposed tomatoes (TOM), Chambwa (CB), Lactibiane WYAGE (PRO1) and Probiotic FORT (PRO2) gave blue- purple color with staining, hence they were Gram positive and catalase negative bacteria (Table 2). The most useful test for the determination of strain differences is gas production from glucose. All isolates showed no gas production. The isolates gave different morphological and biochemical characteristics as shown on Table2.

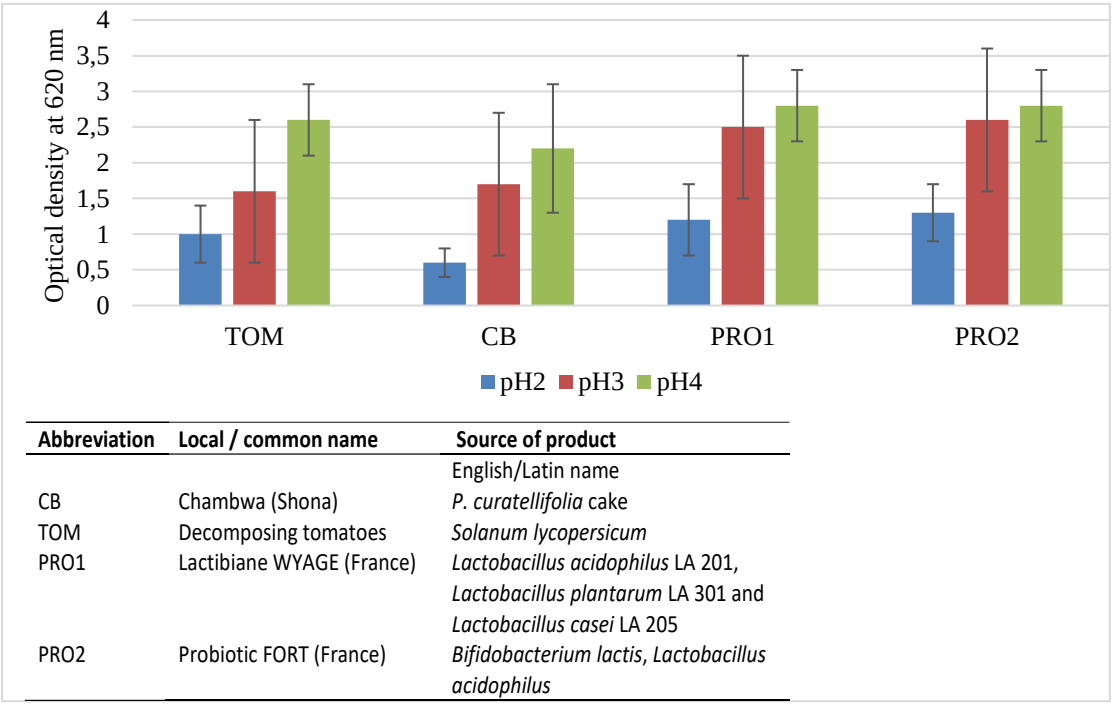
Resistance to low pH

Lactic acid bacteria growth in low pH is important to allow them to tolerate initial stress- and survive in the stomach. Fig. 1 shows the relative survival of the LAB strains (OD_{620mn}), hence tolerance to low (pH2, 3 and 4). Growth was noted for various pH values in the range of 2 to 4. A decrease in LAB growth with increase in the acidity of the medium was also

noted for all the isolates. As shown in the graph, all isolates were stable at pH3 and 4 with an OD of approximately 1.5 and above. However, all isolates were sensitive to low pH (pH 2) as indicated by a low OD which ranges between 0.5 and 1.

Table 2: Morphological and biochemical characteristics of LAB.

Putative LAB	Colony morphological characteristics	Shape	Gram's staining	Catalase test	Gas production fromGlucose
TOM	White, rough, irregular and round	Cocci	Positive (+)	Negative (-)	Negative (-)
CB	Small circular and creamy white	Bacilli	Positive (+)	Negative (-)	Negative (-)
PRO1	White creamy, circular and irregular	Bacilli	Positive (+)	Negative (-)	Negative (-)
PRO2	Small, irregular, smooth and circular	Bacilli	Positive (+)	Negative (-)	Positive (+)



Abbreviation	Local / common name	Source of product
		English/Latin name
CB	Chambwa (Shona)	*P. curatellifolia* cake
TOM	Decomposing tomatoes	*Solanum lycopersicum*
PRO1	Lactibiane WYAGE (France)	*Lactobacillus acidophilus* LA 201, *Lactobacillus plantarum* LA 301 and *Lactobacillus casei* LA 205
PRO2	Probiotic FORT (France)	*Bifidobacterium lactis*, *Lactobacillus acidophilus*

Fig.1: Resistance to low pH of putative and commercial LAB

Tolerance to bile salts and NaCl

The isolated LAB to be called as probiotics should be capable to resist inhibitory constituents of the gastrointestinal track namely bile salt and NaCl. To determine the tolerance of the LAB strains to salts and NaCl, the effect of different concentrations of 0.3 to 1% bile salts) and NaCl in MRS broth were used. The results suggest that all LAB isolates were resistant to bile salt during the incubation period 0f 18 to 24h (Fig. 2). Additionally, LAB isolates were more tolerant to NaCl 1-5% as shown in Fig. 3.

In vitro inhibitory activities of LAB on E. coli and S. typhi

Antimicrobial activities were evaluated in terms diameter of zones of inhibition (ZOI) and classified as follows: i) ZOI > 20mm (highl susceptibility), ii) between 17 and 19mm (intermediate susceptibility) and (iii) less than 16mm (low suceptibility).

As shown in Fig. 4, TOM, CB, PRO1 and PRO2 showed strong inhibition on *E. coli* O157, and intermediate inhibion on *S. typhi* . CB and TOM showed strong inhibition on *E. coli* O157 (zones of clearance >20mm) while the commercial LAB stains (PRO1 and PRO2) showed greater bactericidal activities against both *E. coli* and *S. typhi* (zones of clearance ≥ 24mm). Overall, both CB and TOM shown inhibition capacities that were lower but comparable to that of Streptomycin (~24mm) and Kanamycin (~24mm) against *E. coli* and *S. typhi* . The control strains PRO1 and PRO2 had ZOI that were comparable to that of the two antibiotics (Fig. 4).

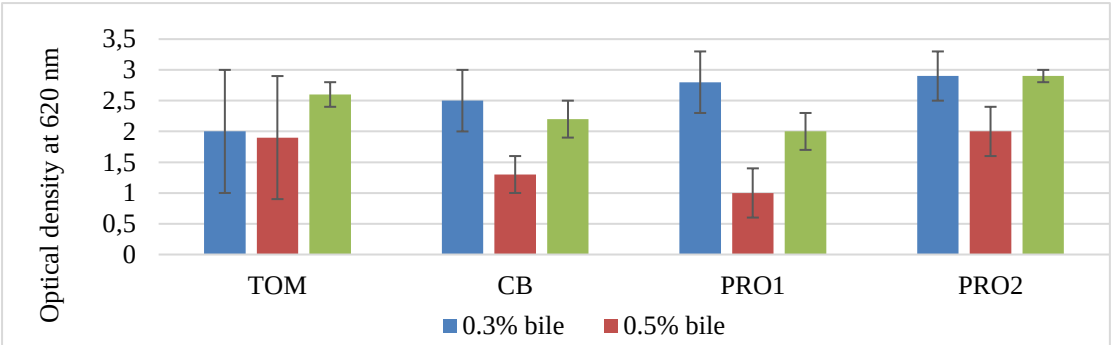


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Fig. 2: Bile salts tolerance of LAB

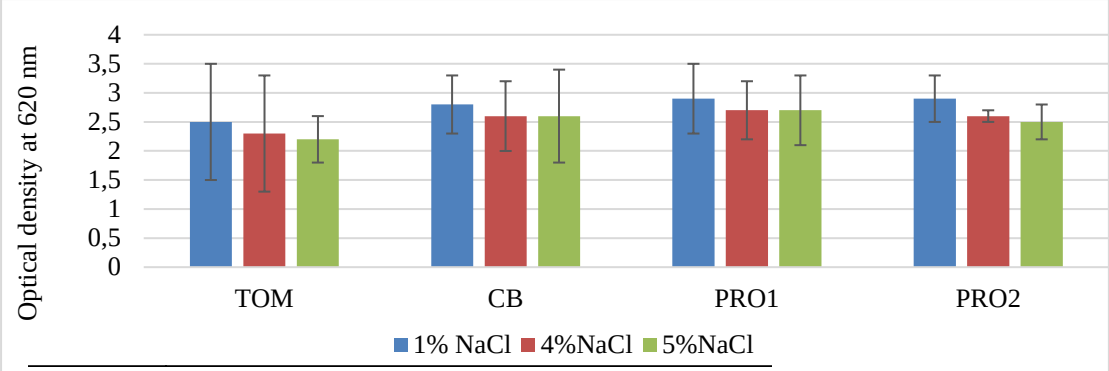

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Fig. 3: Tolerance to NaCl of LAB

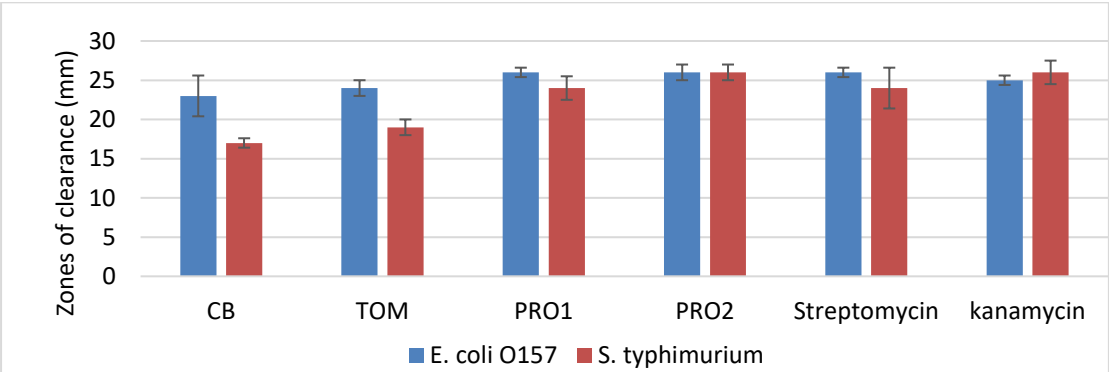

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Fig.4: Bactericidal activities of LAB on *E. coli* O157 and *S. typhi*

Antimicrobial activities of LAB on S. typhi in synthetic wastewater

Table 3 shows the antimicrobial activity of putative LAB on *S. typhi* as a function of contact time. *Salmonella typhi* was incubated in coculture with two putative LAB (CB + SAL and TOM + SAL), two commercial LAB (PRO1 + SAL and PRO2 + SAL). Sterile synthetic wastewater was used as control for the experiments (SAL alone). At time 0 (0h) and subsequently (24h, 48h and 72h), aliquots were removed, serially diluted and plated on XLD Agar to determine *S. typhi* colony counts. Each value is the mean of three experiments. The viability of *S. typhi* was monitored in a coculture with putative and commercial LAB (Table 3). A relatively constant antimicrobial activity against *S. typhi* appeared after 24h in coculture of both putative and commercial LAB. Notably, the viability of *S. typhi* decreased rapidly after 24h (Table 3) of contact with LAB (±2 log units), thus a coculture with LAB sevely inhibited Salmonella growth. In contrast, sterile synthetic wastewater (results not shown) and the one spiked with Salmonella only (SAL alone) did not show any antimicrobial effects as shown in Table 3.

Table 4. shows LAB viability in synthetic wastewater as a function of contact time. Putative LAB overnight culture was incubated in sterile synthetic wastewater (CB only and TOM only), *S.typhi* (CB + SAL and TOM + SAL). Commercial LAB isolates were also inoculated and incubated in sterile synthetic wastewater with *S.typhi* (PRO1 + SAL and PRO2 + SAL). At the start and at specific intervals, aliquots were removed, serially diluted and plated on MRS Agar to determine bacterial colony counts. Each value is the mean of three experiments.

Table 5 shows an evaluation of antimicrobial activity of putative LAB on *E. coli* O157 in synthetic wastewater. The antimicrobial activity of putative LAB was examined as a function of contact time. *Escherichia coli* O157 viability was monitored in a coculture with putative LAB (CB + *E. coli* O157 and TOM + *E. coli* O157), and commercial LAB (PRO1 + *E. coli* O157 and PRO2 + *E. coli* O157) were used as reference strains. A relatively constant antimicrobial activity against *E. coli* O157 appeared after 24h in coculture of LAB. There was a slight decrease in the viability of *E. coli* O157 after 24h (±1 log unit) of contact with both putative and commercial LAB. After 48h a sharp decrease in the concentration of *E. coli* O157 was observed (±2 log units). However, *E. coli* O157 on its own shows a progressive increase with time, thus no antimicrobial effects observed in synthetic wastewater inoculated with *E. coli* O157 only. A progressive increase in the concentrations of putative LAB was also observed on MRS agar (Table 4). *Escherichia coli* was inoculated in sterile synthetic wastewater (*E. coli* O157 only), putative LAB overnight culture (CB + *E. coli* O157 and TOM + *E. coli* O157) and commercial LAB stains (PRO1 + *E. coli* O157 and PRO2 + *E. coli* O157). At the start and at specific intervals, aliquots were removed, serially diluted and plated on XLD Agar to determine bacterial colony counts and each value is the mean of three experiments.

Table 3: Viability of *S.typhi*in coculture with LAB on XLD agar

		Log CFU/mL		
Time (h)	0	24	48	72
PRO1 + SAL	7.43	3.69	0	0
PRO2 + SAL	7.42	4.81	0	0
CB + SAL	7.39	4.94	0	0
TOM + SAL	7.47	4.88	0	0
SALonly	≥8.47	≥8.47	≥8.47	≥8.47
Key: SAL denotes *Salmonella typhi*				

Table 4: Viability of lactic acid bacteria on MRS agar over time

		Log CFU/ml		
Time (h)	0	24	48	72
PRO1 + SAL	6.68	≥7.47	≥7.47	≥7.47
PRO2 + SAL	6.65	≥5.48	≥7.47	≥7.47
CB + SAL	6.32	≥5.48	≥7.47	≥7.47
TOM + SAL	7.08	≥5.48	≥7.47	≥7.47
CBonly	6.51	≥5.48	≥7.47	≥7.47
TOMonly	7.4	≥5.48	≥7.47	≥7.47
PRO1only	7.47	≥7.47	≥7.47	≥7.47
PRO2only	7.47	≥7.47	≥7.47	≥7.47

Table 5: Antimicrobial activities of putative LAB on *E. coli* O157 on Hicrome Agar

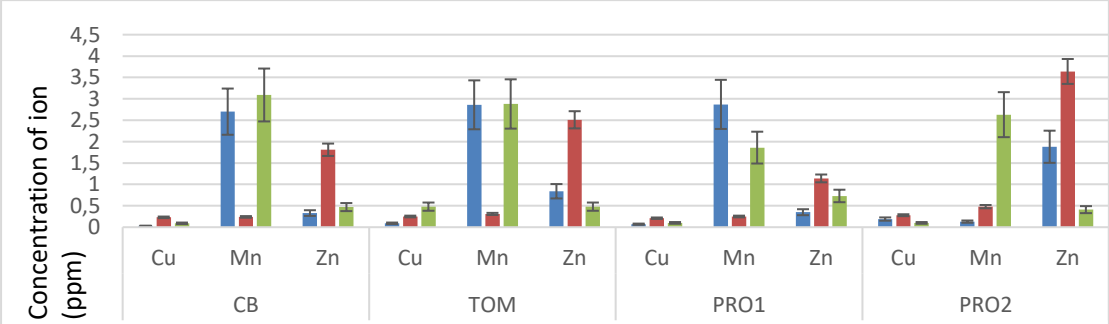
	Log CFU/mL			
Time (h)	0	24	48	72
PRO1 + O157	5.4	4.79	3.48	0
PRO2 + O157	5.43	4.48	3.6	0
CB + O157	5.48	4.6	3.48	0
TOM + O157	5.45	4.3	3.3	0
E. coli O157 only	≥5.48	≥5.48	≥5.48	≥5.48

Strain CB significantly reduced aqueous metal concentrations in the wastewater between the following periods: Cu (Days 7 and 10), Mn (Days 1 and 7) and Zn (Days 7 and 10) ($p < 0.05$) (Fig. 5). Strain CB also significantly reduced aqueous metal concentrations in the wastewater between the following periods: Mn (Days 1 and 7) and Zn (Days 7 and 10), but led to a steady increase in aqueous concentration of Cu between Days 1 and 10. Strain PRO1 significantly reduced aqueous metal concentrations in the wastewater between the following periods: Cu (Days 7 and 10), Mn (Days 1 and 7) and Zn (Days 7 and 10) ($p < 0.05$).

Strain PRO2 significantly reduced aqueous metal concentrations in the wastewater between the following periods: Cu (Days 7 and 10) and Zn (Days 7 and 10) ($p < 0.05$), however significantly increased the aqueous concentration of Mn between Days 1 and10 ($p < 0.05$) (Fig. 5).

Only strain CB significantly reduced aqueous concentration of ammonium sulphate [$(NH_4)_2SO_4$ +] between Days 1 and 7 ($p < 0.05$) (Fig. 6).

The results in Fig. 6 show that only CB was effective in reducing the concentration of $(NH_4)_2SO_4$ in the wastewater samples. Whereas, TOM did not significantly reduce the concentration of $(NH_4)_2SO_4$.



Abbreviation	Local / common name	Source of product
		English/Latin name
CB	Chambwa (Shona)	*P. curatellifolia* cake
TOM	Decomposing tomatoes	*Solanum lycopersicum*
PRO1	Lactibiane WYAGE (France)	*Lactobacillus acidophilus* LA 201, *Lactobacillus plantarum* LA 301 and *Lactobacillus casei* LA 205
PRO2	Probiotic FORT (France)	*Bifidobacterium lactis*, *Lactobacillus acidophilus*

Fig. 5: Dynamics in heavy metal (Cu, Mn and Zn) concentration in synthetic wastewater exposed to lactic acid bacteria

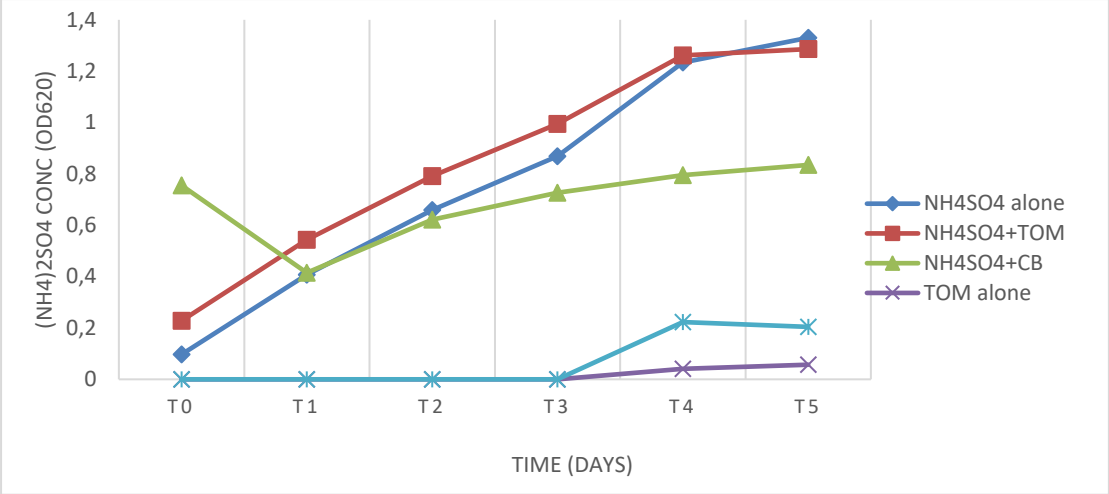


Fig.6: Dynamics in ammonium concentration in synthetic wastewater innoculated with lactic acid bacteria over time (T0, T1 = Day 1.....T5 = Day 5)

So far, attempts to utilise LAB in aquatic environments has been limited to aquaculture, particularly to control diseases of fish (Verschuere *et al.*, 2000; Jahangiri *et al.*, 2018; Zorriehzahra *et al.*, 2016). The experimental data analysed above provides evidence that the selected strains of LAB (isolated from rotten tomatoes (TOM) and *P. curatelifolia*, compared to commercial probiotic products (Lactibiane WYAGE - containing *Lb. acidophilus* LA 201, *Lb. plantarum* LA 301 and *Lb. casei* LA 205 (France), and Probiotic FORT - contaning *B. Lactis* and *Lb acidophilus*) (France): (i) have great probiotic properties, (ii) survive in wastewater, and (iii) inhibit *Escherichia coli* O157:H7 and *Salmonella enterica* subsp. *Enterica* (Serovar Typhi) and (iv) modulate concentrations of Cu, Mn, NH_4 and Zn in wastewater. This paper presents new strains of LAB with potential for use in wastewater treatment (removal of diarrhegenic microorganisms and undesired nutrients (including heavy metals) from wastewater. CB and TOM were shown to harbour great probiotic properties including: (i) being catalase negative, (ii) resistance to low pH, (iii) resistance to bile salts, (iv) tolerance to high concentrations of sodium chloride (NaCl) and (v) relatively high antibiotic resistance (as they may complement antibiotic therapies. The mechanisms that underlie the observed pathogen and nutrient removal properties of these LAB strains appears to be multifaceted. In the current study, results have shown that the LAB strains (CB and TOM) remove *E. coli* O157:H7 and *S. typhi* through their strong inhibition capacities (high *in vitro* MIC values that were comparable to those of streptomycin and kanamycin and the reduction of concentrations of *E. coli* O157:H7 and *S. typhi* as observed in the formulated synthetic wastewater. It would be desirable to show herein any changes in the concentrations of antimicrobial metabolites such as bacteriocins, nisins and others in wastewater (subject of subsequent research). This research has shown that the presence of LAB in the synthetic wastewater enhances the development of biofilms on an incoporated sand filter (results not shown). Experimental data have also provided evidence that strains of LAB (CB and TOM) as well as the commercial probiotic products [Lactibiane WYAGE - containing *Lb. acidophilus* LA 201, *Lb. plantarum* LA 301 and *Lb. casei* LA 205 (France),

and Probiotic FORT - contaning *B. Lactis* and *Lb acidophilus*) (France)] significantly reduced aqueous Cu, Mn, NH_4 and Zn concentrations in the wastewater particularly between days 7 and 10 of coincubation. Results herein are in sync with those of Monachese *et al.* (2012), Andrea *et al.* (2017), Halttunen *et al.* (2008), Mrvčić *et al.* (2009) and others. Novotnik *et al.* (2019) showed that LAB employ redox metabolism to modulate the concentration of aqueous manganese in wastewater. Furthermore, Zhai *et al.* (2015) have shown that antioxidative properties of LAB may also useful in the removal of metals such as cadnium. The sample wastewater was shown to have the following concentrations of the selected nutrient: (i) manganese > 3mg/L = 10 times higher than World Health Organisation recommended limits for drinking water (0.3mg/L) (WHO, 2011), (ii) zinc > 3.5mg/L, greater or equal to the WHO recommended limit of 3mg/L (WHO, 2011) and (iii) limited or no copper. High Mn concentrations in the municipal water, which ends up recycled into drinking water, is thought to cause neurotoxicity and is implicated in poorer neurobehavioural performances in children (Iyare, 2019). While Zn is used to control diarrhoea (Putri *et al.*, 2019), to have antioxidant and antiinflammatory effects (Prasad, 2014; Silva *et al.*, 2019) and to help bone development (Fathi and Farahzadi, 2017), high concentrations (above the WHO recommended limits) reportedly interferes with copper uptake in cells, hence, many of its toxic effects are in fact due to copper deficiency (Plum *et al.*, 2010). The proposed prototype is an extentions to the application of LAB in aquaculture. Lactic acid bacteria are shown herein to offer great potential as probiotic bacteria for use as therapy / prophylaxis against diarrhoea and as a tool for the removal of selected nutrients including heavy metals and diarrhoeagenic bacteria from municipal wastewaters. These strains can therefore be useful as therapy or prophylaxis against diarrhoea caused by *E. coli* , *S. typhi* and other pathogens. Further, the new strains of LAB (CB and TOM) can be available as tools for the management of urban and mining effluent. These products are seen as innovations with potential to impact the LIC where conventional water reticulation systems are inadequate and often release partially or un-treated wastewater into the environment – a major risk factor for diarrhoeal diseases in these countries. Urban centres such as Bindura (Zimbabwe) that are flanked by mining activities have an additional challenge to handling of municipal wastewater, that is the release of heavy metals from mining activities into the environment. Further preclinical studies towards the development of probiotic products for use against diarrhoea were underway. A prototype with these properties is also under development. It is important to identify the exact mechanisms that underlie the observed removal of pathogens and nutrients from wastewater. The proposed prototype offers an opportunity to develop a wastewater treatment system that complement the existing wastewater reticulation systems in LIC.

Conclusion

In the present study, two selected lactic acid bacteria isolated from environments within Zimbabwe, namely CB (strain of LAB from *P. curatellifolia* fruit cake) and TOM (strain of LAB from decomposed tomatoes) were shown to harbour great *in vitro* probiotic properties [resistance to low pH (pH2 and 3), tolerance to bile salts and NaCl, as well as significant antimicrobial activities

against strains of *E. coli* O157:H7 and *S. typhi* . The probiotic properties highlighted here were shown to be comparable to those of commercially available probiotic products (PRO1, probiotic Lactibiane WYAGE and PRO2, probiotic FORT). Further, compared to commercial probiotic products, strains CB and TOM also showed ability to modulate the concentration of selected nutrients (Cu, Mn, NH4 + and Zn) with a significant net reduction occuring between Day 7 or 10 (> 2 log CFU/ml). The selected indigenous LAB bacteria were also shown to deploy biofilms on surfaces of sand particles, which is thought to be one of the key mechanisms employed by the lactic acid bacteria to hold and kill *E. coli* and *S. typhi* in wastewater. Under the optimal conditions, CB and TOM can therefore significantly reduce concentrations of enteric pathogens in wastewater by at least 2-fold within a period of up to 2 days, and that of selected nutrients (Cu, Mn, NH4 + and Zn) within a period of between 7 and 10 days. With further studies to optimise the activities of CB and TOM, a prototype is being developed with potential to be employed in the bioremediation of the water contaminated with nutrients including heavy metals and enteric pathogens.

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Conflict of Interest
The author declares that there is no conflict of interests regarding the publication of this manuscript. In addition, the ethical issues, including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy have been completely observed by the authors.

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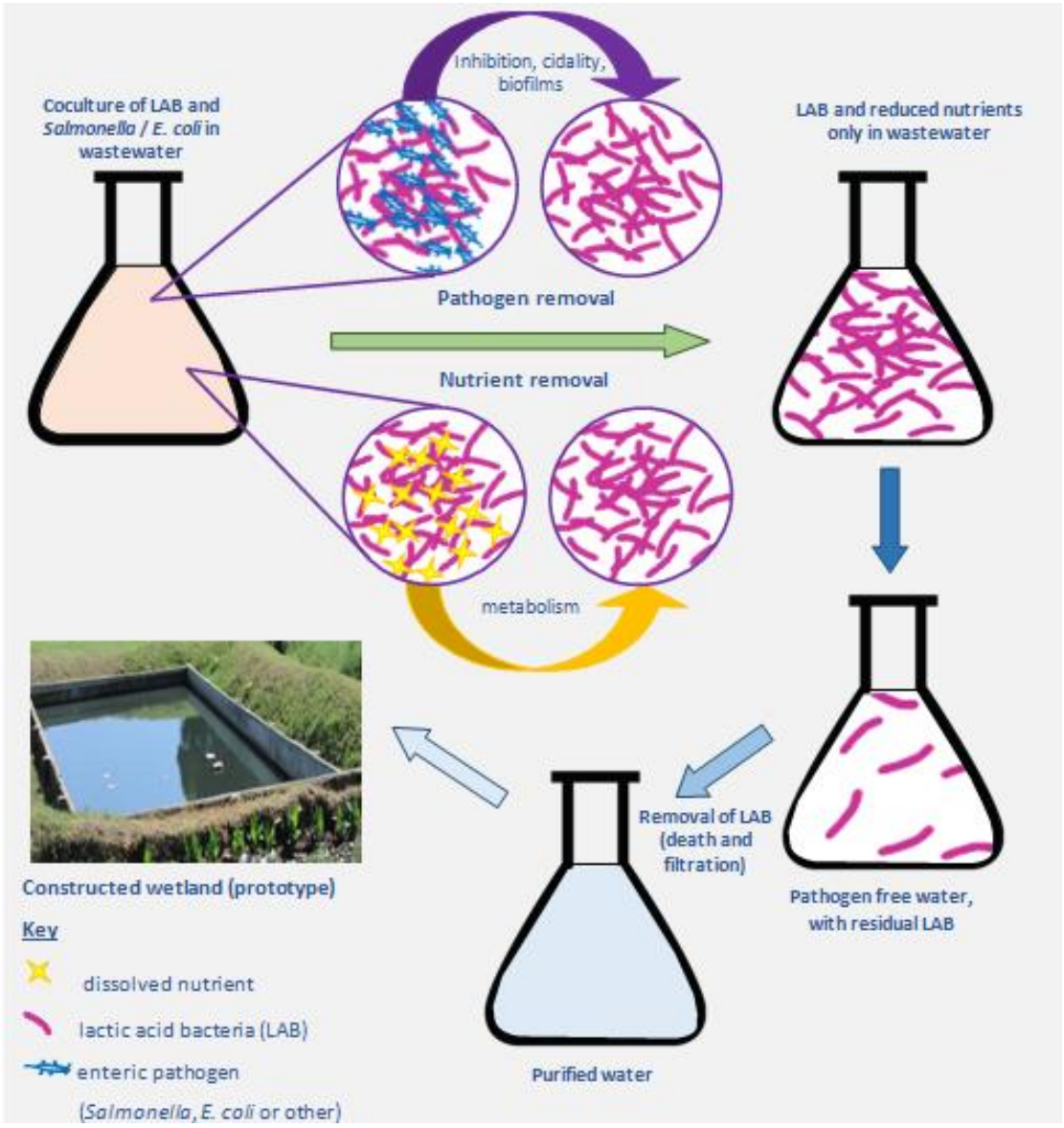
Graphical abstract



Graphical abstract: Model showing the removal of enteric pathogens and nutrients from wastewater by lactic acid

Highlights

- The provided LAB has great potential as a tool for the removal of selected heavy metals and diarrhoeagenic bacteria from municipal wastewaters;
- The selected lactic acid bacteria have strong antimicrobial activities *in vitro* and *in situ.*
- The selected LAB strains were shown to inhibit or kill the enteric bacteria. Biofilms are thought to be used by the LAB as a hold and kill mechanism;
- The selected lactic acid bacteria were shown to modulate concentrations of selected nutrients (Cu, Mn, NH4 + and Zn), resulting in a significant net reduction in the concentration of the nutrients 7 to 10 days *in situ.*