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In vitro Assessment of Chromium, Lead, Cadmium and Nickel Tolerance of *B. clausii*, a Prospective Probiotic Microorganism for *in vivo* Bioremediation

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Researches have demonstrated the ability of probiotic microorganisms to prevent and treat ailments especially those associated with the gastrointestinal tract. Probiotics like *Lactobacillus* spp. have also displayed the property of bioremediation of heavy metals under *in vitro* as well as *in vivo* conditions. The aim of this study was to assess the effects of chromium, lead, cadmium and nickel stress on the properties of *B. clausii*, a probiotic species of genus *Bacillus*. The minimum inhibitory concentration (MIC) of the organism under test was determined for Cr (VI), Pb (II), Cd (II) and Ni (II), followed by assessment of morphological and biochemical properties of the *B. clausii*, antibiotic sensitivity and probiotic efficacy by acid and bile tolerance assays. *B. clausii* exhibited exceptionally high MICs for the tested heavy metals. The organism did not exhibit any change in its morphological and biochemical characteristics after exposure to heavy metal stress. This stress also did not affect the test organism's probiotic behaviour as assessed by acid and bile tolerance assays where it showed maximum growth at 3 hrs. for pH 3 and 0.3% bile concentration, respectively. However, after exposure to the four heavy metals, *B. clausii* showed a tremendous increase in its antibiotic sensitivity. The above study has indicated the capacity for *B. clausii* to survive in and tolerate high levels of Cr (VI), Pb (II), Cd (II) and Ni (II) while showing no change in its characteristics. Therefore, *B. clausii* appears to be an ideal candidate for potential bioremediation of Cr (VI), Pb (II) Cd (II) with Pb (II), Cd (II) and Ni (II), *in vivo*.

Keywords: Bioremediation; *Bacillus clausii*; Heavy Metals; MIC; Probiotic.

In 2001, Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO) defined probiotics as "live microorganisms, which, when administered in adequate amounts, confer a health benefit on the host"[1]. However, probiotic food consumption has been a part of different cultures around the world for many centuries. Probiotic organisms generally belong to LAB (lactic acid producing bacteria) and include *Lactobacillus, Lactococcus, Bifidobacterium* and many other organisms[2]. Microorganisms of various other genera such as *Bacillus*, not belonging to LABs, have been identified and declared as probiotics as they exhibited probiotic characteristics[3]. In recent times, researchers have focussed on benefits of probiotics and many have established their health and nutritional benefits in humans. Beneficial

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effects of probiotics include improvement of gut health, reduction in symptoms of Irritable Bowel Syndrome (IBS)[4], antibiotic associated diarrhoea[5,6], infectious diarrhoea caused by rotavirus[7-9], traveller's diarrhoea[10] and necrotizing enterocolitis[11].

Many studies have been performed on bioremediation of heavy metals by microorganisms[12-14] and have reported an interesting observations that probiotics such as *Lactobacillus* spp. and *Bacillus* spp. also have the ability to bioremediate heavy metals[15-19]. This has been attributed to the structural components of their cell wall such as teichoic acid, lipoteichoic acid and peptidoglycan[20].

Heavy metals such as lead, chromium, nickel, cadmium, mercury and arsenic reach human gut through food and water laden with them. These heavy metals enter the food chain as a result of anthropogenic activities and cause varying levels of toxicity in humans[21]. Hence, these microorganisms are the best and most effective agents to reduce toxicity caused by heavy metals since they are safe in terms of human usage, are ingested via diet, show gut remediation and can eliminate the heavy metals by defaecation[22].

In this study, *B. clausii* was evaluated for tolerance and survival capacity in presence of Cr (VI), Pb (II), Cd (II) and Ni (II) and the effect of exposure of these heavy metals on it.

MATERIALS AND METHODS

Materials

Commercially available probiotic *Bacillus clausii* was procured from local chemist. It is marketed by Sanofi Synthelabo Pvt Ltd. Powai, Mumbai, India as a spore suspension under the name "Enterogermina®". Potassium dichromate, lead nitrate, cadmium sulphate and nickel sulphate are the salts of heavy metals which have been selected for this study and were used as sources of Cr (VI), Pb (II), Cd (II) and Ni (II), respectively. Antibiotic discs were procured from HiMedia, India for kanamycin (30 µg), norfloxacin (10 µg), chloramphenicol (30 µg), amoxycillin (10 µg), ciprofloxacin (5 µg), and ampicillin (10 µg).

Methods

Minimum Inhibitory Concentration (MIC)

MIC of Cr (VI), Pb (II), Cd (II) and Ni (II) for *B. clausii* was assessed by method of Mistry et al[23] with some modifications. Spores were germinated in nutrient broth (NB) and inoculated on nutrient agar (NA) plates supplemented with two-fold concentration of respective heavy metals ranging from 1-512 ppm. These plates were incubated at 37°C for 24 hrs. The lowest concentration of the respective heavy metal which inhibited the growth of probiotic was considered as the MIC of that heavy metal for *B. clausii*.

Characterization of Heavy Metal Resistant *B. clausii*

Various characterization parameters of *B. clausii* were evaluated before and after 24 hrs. exposure to Cr (VI), Pb (II), Cd (II) and Ni (II). This was done to assess whether the respective heavy metal exposure affects characteristics of the test organism.

Morphological Characterization

Observations on cell size, shape, colony characteristics and staining properties were performed on *B. clausii* before and after exposure to test heavy metals.

Biochemical Characterization

Catalase Test

Unexposed and exposed cultures of *B. clausii* for Cr (VI), Pb (II), Cd (II) and Ni (II) were cultured on NA slants. Few drops of H_2O_2 were added to the slant after 24 hrs. and observed for immediate bubbling[24].

Methyl Red and Voges Proskauer (MRVP) Test

MRVP test was performed to determine glucose fermentation products. MR test was done according to methodology of Clark and Lub[25] while for VP test method of Voges and Proskauer[26] modified by Barritt[27] was followed. This was done on unexposed and exposed *B. clausii* to Cr (VI), Pb (II), Cd (II) and Ni (II) respectively, in sets of glucose phosphate broth tubes. The tubes were inoculated and incubated for 72-96 hrs. Methyl red was added to one tube of the set to check development of acid. To the other tube, 12 drops of reagent VP-I was added followed by 2-3 drops of reagent VP-II. The tubes were exposed to air and were shaken intermittently. One tube remained uninoculated and acted as control.

Sugar Fermentation Test

Sugar fermentation test was performed according to procedure of Cappuccino and Sherman[28]. A set of sugar fermentation tubes were

prepared with different sugars (lactose, sucrose, glucose, sorbitol and mannitol). Phenol red was added to the broth as pH indicator to check for acid production and Durham's tube were placed in the broth to check for gas production. The broth was inoculated with unexposed and exposed *B. clausii* to each heavy metal respectively. An uninoculated tube was kept as control.

Antibiotic Sensitivity Test

To analyse whether Cr (VI), Pb (II), Cd (II) and Ni (II) exposure caused any change in antibiotic sensitivity of *B. clausii*, Kirby-Bauer disc diffusion method was performed using antibiotic discs[29]. Zone of inhibition was measured after 24 hrs. for the unexposed and exposed cultures of *B. clausii*.

Probiotic Characterization

Efficacy of *B. clausii* as a probiotic was studied by acid and bile tolerance assays after exposure to heavy metals under study. For both assays, methodology of Hassanzadazar et al[30] was followed with some modifications.

For acid tolerance assay, *B. clausii* exposed to Cr (VI), Pb (II), Cd (II) and Ni (II) respectively, was added to different broths of pH 2, 3 and 4. Growth of exposed *B. clausii* was monitored by viable cell count that was performed by pour plate technique for every hour for 3 hrs. Prior to plating, ten- fold serial dilution of the inoculum was prepared in 0.1% peptone water. All the experiments were done in triplicates. Cell counting was done after 24 hrs. of incubation at 37°C.

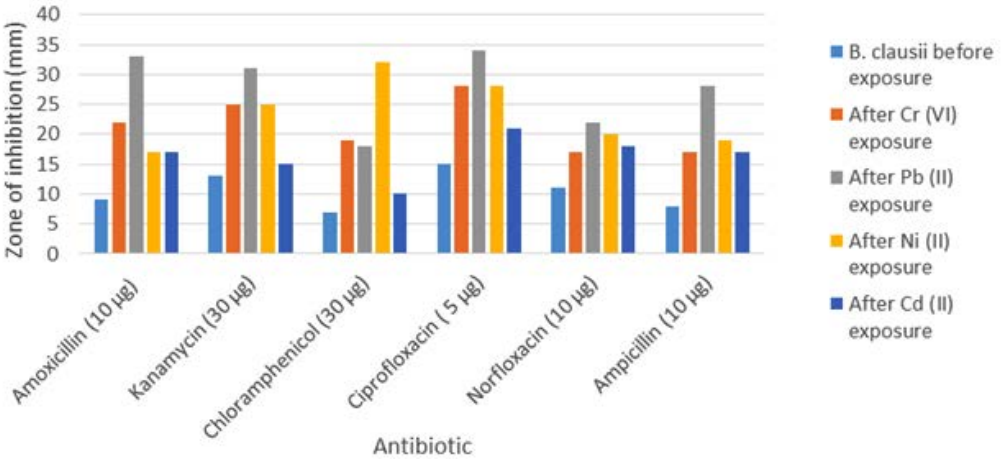

Fig. 1. Diameter of zone of inhibition (mm) for *B. clausii* before and after exposure to Cr (VI), Pb (II), Cd (II) and Ni (II)

Table 1. Comparison of morphological and biochemical characteristics of *B. clausii* before and after exposure to Cr (VI), Pb (II), Cd (II) and Ni (II)

S.no.	Characteristics	Unexposed *B. clausii*	*B. clausii* exposed to Cr (VI), Pb (II), Cd (II) and Ni (II)
1.	Colony characteristics	Creamish, opaque, small	Creamish, opaque, small
2.	Gram's staining	Gram positive	Gram positive
3.	Endospore staining	Endospore present	Endospore present
4.	Catalase test	Positive	Positive
5.	Glucose fermentation	Acid formation and gas production	Acid formation and gas production
6.	Sucrose fermentation	Acid formation and gas production	Acid formation and gas production
7.	Lactose fermentation	No acid and gas production	No acid and gas production
8.	Mannitol fermentation	Acid formation and gas production	Acid formation and gas production
9.	Sorbitol fermentation	Acid formation and gas production	Acid formation and gas production
10.	Methyl red test	Positive	Positive
11.	Voges-Proskauer test	Negative	Negative

For bile tolerance assay, NB with bile salt concentration of 0.2, 0.3 and 0.4% were prepared. *B. clausii* after 24 hrs. exposure to Cr (VI), Pb (II), Cd (II) and Ni (II) was added to the respective broths. Viable cell count was performed by pour plate technique. The inoculum was prepared hourly for 3 hrs. by ten- fold serial dilution in 0.1% peptone water. Colony count was done after 24 hrs. incubation at 37°C.

RESULTS AND DISCUSSION

Minimum Inhibitory Concentration (MIC)

In presence of nickel and chromium, *B. clausii* showed growth till 128 ppm and hence MIC was obtained as 256 ppm. In case of cadmium exposure, MIC was obtained at 64 ppm. However, after lead exposure, *B. clausii* showed good growth till 512 ppm and thus no MIC was recorded.

Earlier, environmental species of genus *Bacillus* have shown bioremediation of Cr (VI)[31], Pb (II)[32], Cd (II)[33] and Ni (II)[34]. The genetic determinants for heavy metal tolerance might be borne either on plasmids, transposons or genomic DNA[35,36]. In fact, these determinants are responsible for increased tolerance of microorganisms for heavy metals and are reported more in heavy metal polluted sites rather than in unpolluted sites[37].

The results of the assay for MIC indicate combined resistance of *B. clausii* and high MIC values for the heavy metals tested. The earlier studies conducted by Silver[38] and Alam and Imran[39]

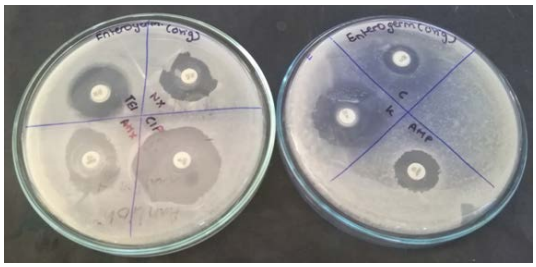

Fig. 2. Zone of inhibition of *B. clausii* before exposure to heavy metals

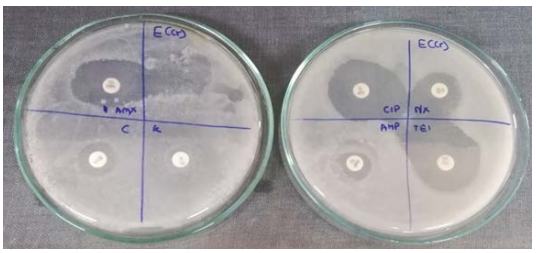

Fig. 3. Zone of inhibition of *B. clausii* after exposure to Cr (VI)

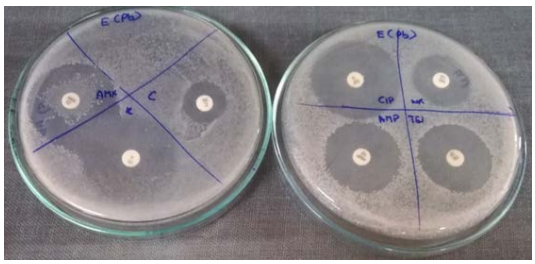

Fig. 4. Zone of inhibition of *B. clausii* after exposure to Pb (II)

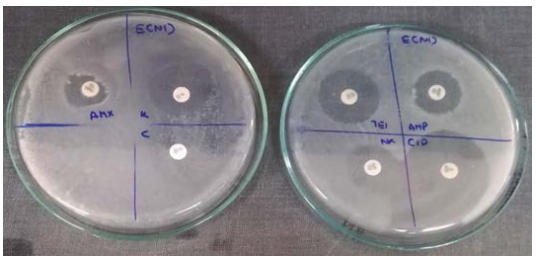

Fig. 5. Zone of inhibition of *B. clausii* after exposure to Ni (II)

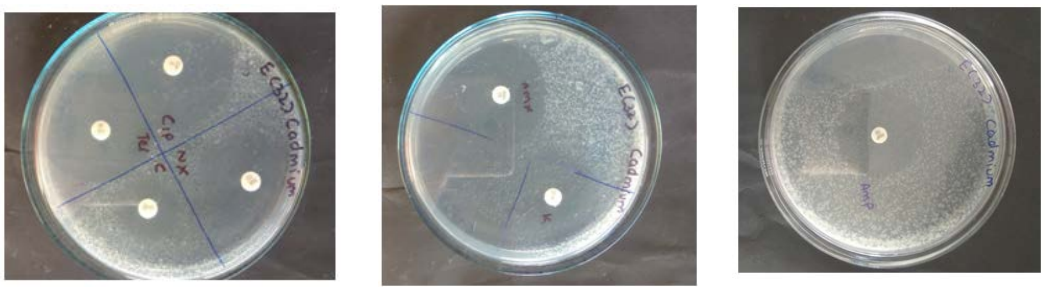

Fig. 6. (a, b, c): Zone of inhibition of *B. clausii* after exposure to Cd (II)

also have reported similar combined resistance of *B. clausii*. Further studies have reported that bacteria which are multi-heavy metal resistant have greater MIC values as compared to bacteria showing resistance to a single heavy metal[39-41].

According to WHO, the guideline values for Cr (VI), Pb (II), Cd (II) and Ni (II) in drinking water are 0.05 mg/L, 0.01 mg/L, 0.003mg/L and 0.07mg/L, respectively[42]. *B. clausii* has shown MIC values much above the permissible values of these heavy metals in drinking water, indicating that it has the potential for bioremediation of the above mentioned heavy metals.

Morphological and Biochemical Characterization

Vegetative cells of *B. clausii* exposed to heavy metal stress did not exhibit any change in their various morphological characteristics like cell shape, cell size, colony characters etc. Similar observations were made when heavy metal exposure did not affect the biochemical properties test organism. These results indicate that 24 hrs. exposure of *B. clausii* to Cr (VI), Pb (II), Cd (II) and Ni (II) could not modify or transform the organism's basic characteristics as depicted in Table 1.

Antibiotic Sensitivity Test

An organism can be either tolerant or resistant to antibiotics. For an organism to be resistant, it must grow in constant presence of low concentration of antibiotic. If an organism is categorized as tolerant, it means that it can survive for short duration of high concentration of antibiotic[43]. This helps to determine the

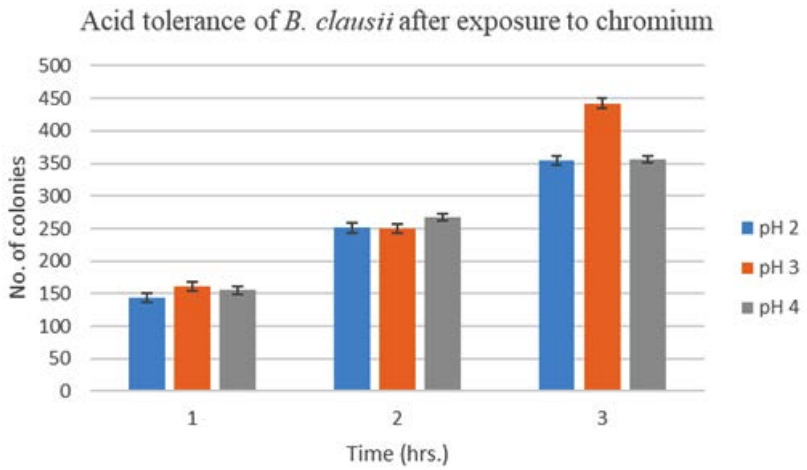

Fig. 7. Acid tolerance of *B. clausii* after exposure to chromium

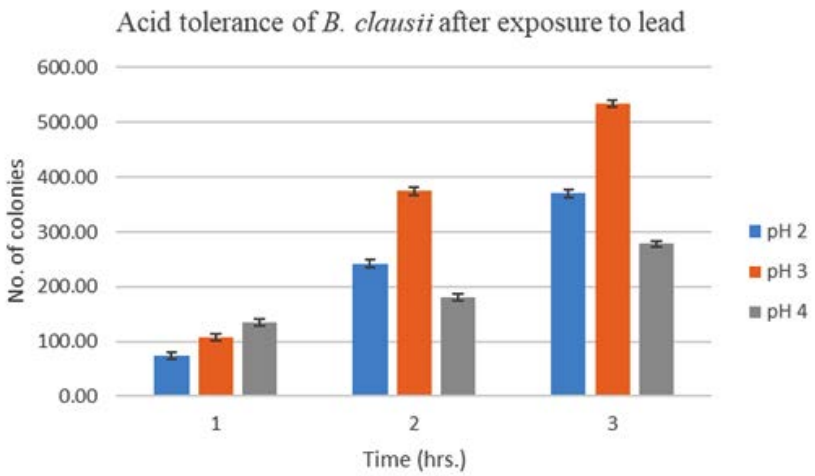

Fig. 8. Acid tolerance of *B. clausii* after exposure to lead

successful clinical use of antibiotics. In this study, upon exposure to heavy metal stress, a marked increase was observed in antibiotic sensitivity of *B. clausii* for the antibiotics used (Fig 1). Against amoxicillin, Pb (II) exposed *B. clausii* showed maximum change (from 9 mm to 33 mm) (Fig 2, Fig 4) while minimum change was recorded for Ni (II) (Fig 2, Fig 5) exposed and Cd (II) exposed culture (from 9 mm to 17 mm) (Fig 2, Fig 6b). Lead exposed culture recorded maximum (from 15 mm to 34 mm) (Fig 2, Fig 4) and cadmium exposed culture recorded minimum (from 15 mm to 21 mm) change against ciprofloxacin (Fig 2, Fig 6a). After exposure to lead, *B. clausii* showed doubling of antibiotic sensitivity (from 11 mm to 22 mm) against norfloxacin (Fig 2, Fig 4) while minimum was for chromium exposed culture (from 11 mm to 17 mm) (Fig 2, Fig 3). The zone of inhibition increased from 8 mm to 28 mm after lead exposure which was maximum for ampicillin (Fig 2, Fig 4) while minimum increase was for Cr (VI) (Fig 2, Fig 3) and Cd (II) (Fig 2, Fig 6c) exposed *B. clausii* (from 8 mm to 17 mm respectively). Pb (II) exposed test organism recorded maximum change against kanamycin (13 mm to 31 mm) (Fig 2, Fig 4) while Cd (II) exposed *B. clausii* showed negligible change (13 mm to 15 mm) (Fig 2, Fig 6b). For chloramphenicol, after exposure to nickel, *B.*

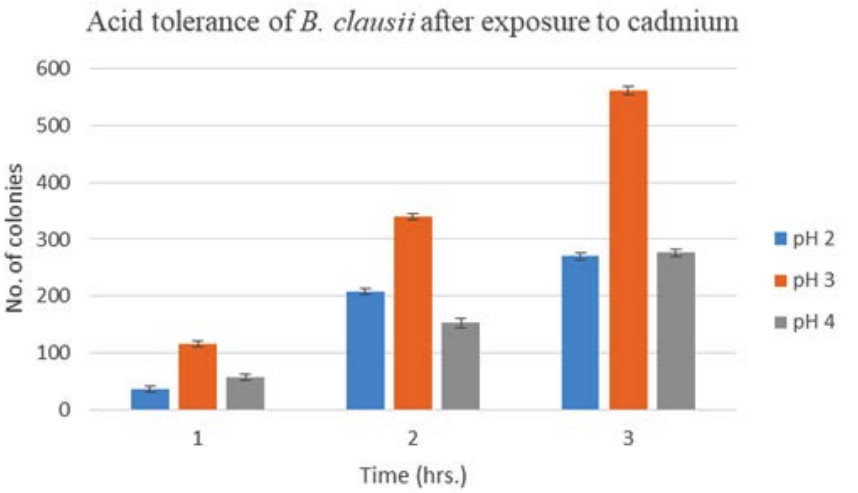

Fig. 9. Acid tolerance of *B. clausii* after exposure to cadmium

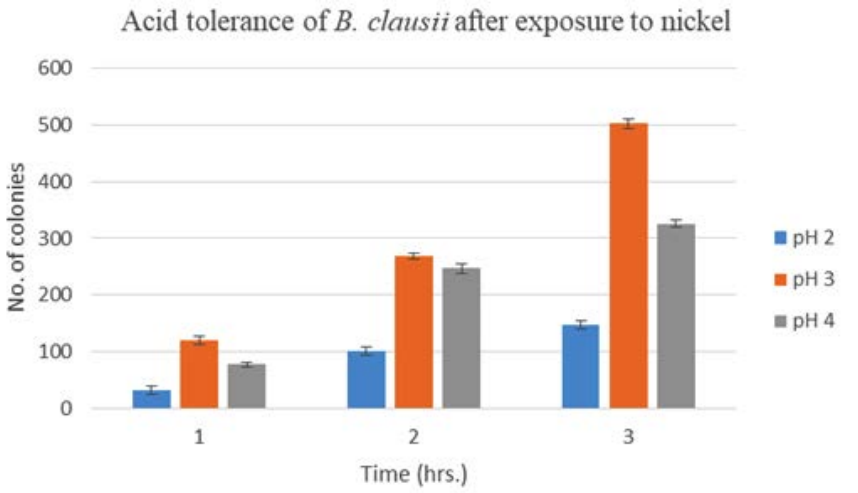

Fig. 10. Acid tolerance of *B. clausii* after exposure to nickel

clausii showed a tremendous increase in the zone of inhibition from 7 mm to 32 mm (Fig 2, Fig 5) while negligible change was found in Cd (II) exposed test probiotic (7 mm to 10 mm) (Fig 2, Fig 6a). This behaviour may be related to presence of proteins involved in chloramphenicol and kanamycin resistance have also been identified in proteome of *B. clausii*[44]. Thus, lead exposure caused maximum change in antibiotic sensitivity while cadmium exposure led to minimum change. According to above definition, *B. clausii* can be considered as resistant to the antibiotics tested against and can therefore be used to replenish the microflora of the gut after antibiotic treatment, even after heavy metal exposure. The results of above assay indicate that the antibiotic resistance of *B. clausii* decreases upon exposure to Cr (VI), Pb (II), Cd (II) and Ni (II). Similar results obtained by Tuckfield and McArthur[45], Habi and Daba[46] and Belapurkar *et al*[19], which also support our findings. Various researchers have postulated multiple reasons for this observation. The reasons can be inactivation of enzymes and proteins by heavy metals involved in antibiotic resistance[47], irreversible inhibition of

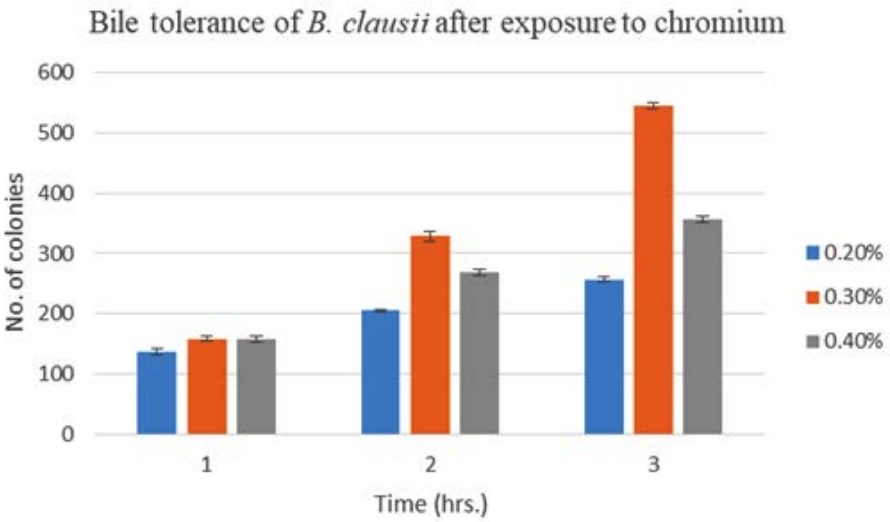

Fig. 11. Bile tolerance of *B. clausii* after exposure to chromium

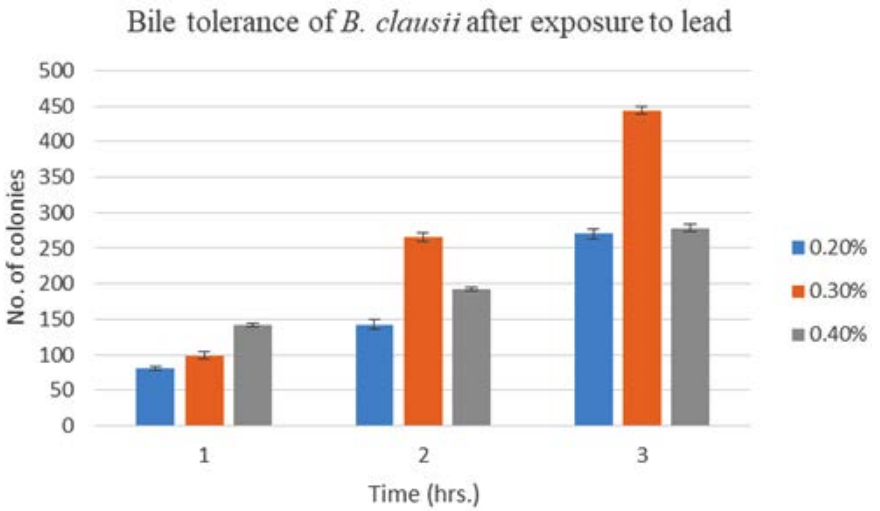

Fig. 12. Bile tolerance of *B. clausii* after exposure to lead

ribosome function by heavy metals[47] or chelation of antibiotics by heavy metals thus reducing their concentration[48].

From the above results, it can also been concluded that *B. clausii* has potential to resist and tolerate both heavy metals and antibiotics. In bacteria, antibiotic resistance and heavy metal tolerance are genetically linked through R- plasmid[49-51]. According to the study of Alam and Imran[39] selective pressure of the heavy metals causes co-selection of antibiotic resistance, albeit indirectly.

Probiotic Characterization

As *B. clausii* is a probiotic microorganism, it can survive low pH conditions of stomach as well as in presence of bile salts in the intestinal tract. To determine the effect of heavy metal stress on its probiotic efficacy of *B. clausii*, acid and bile tolerance assays were performed.

Acid Tolerance Assay

When Cr (VI) exposed *B. clausii* was inoculated in pH 2, 3 and 4 respectively and growth was monitored for 3 consecutive hours, it was observed that maximum CFU (colony forming

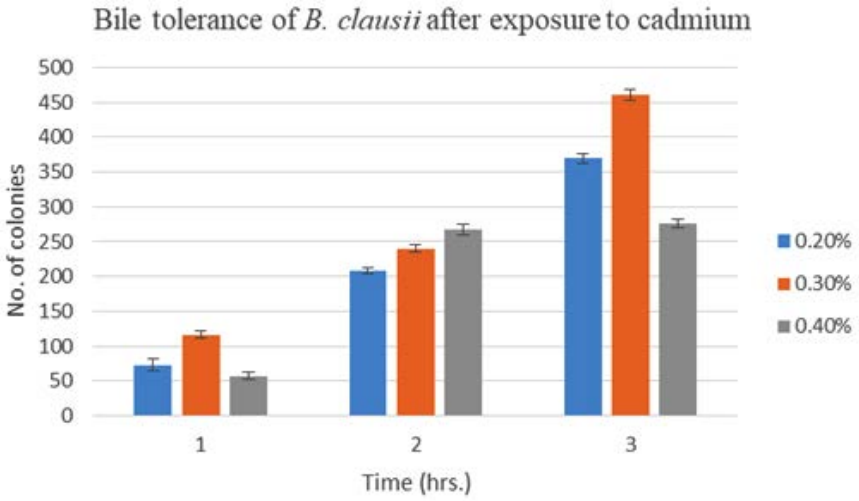

Fig. 13. Bile tolerance of *B. clausii* after exposure to cadmium

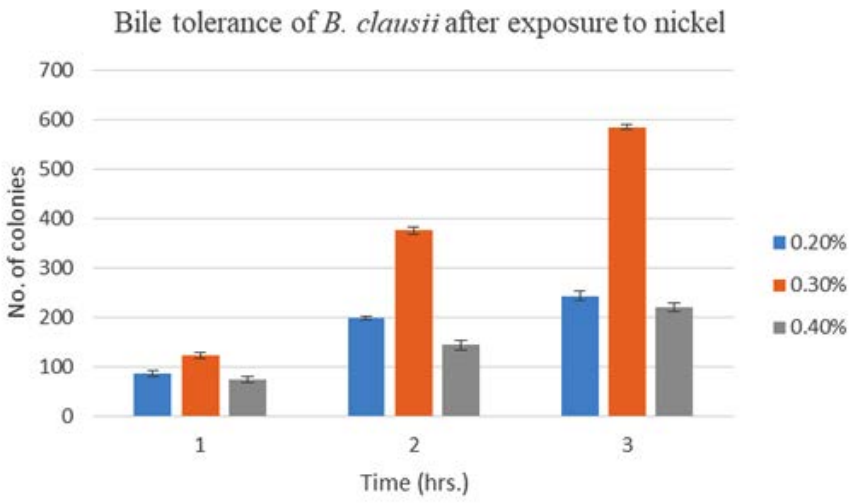

Fig. 14. Bile tolerance of *B. clausii* after exposure to nickel

units) were obtained at pH 3 after 3 hrs. (Fig. 7). Similar result was obtained for Pb (II), Cd (II) and Ni (II) exposed *B. clausii* (Fig. 8-10). This is in corroboration with a study conducted by Sahadeva et al[52] which says that for an organism to be acid tolerant it must show maximum growth at pH 3 after 3 hrs.

Mechanisms of acid tolerance are more prominent in Gram positive bacteria like *Bacillus* spp. as they attain higher cell densities which enhance biofilm formation and communication amongst cells[53]. These mechanisms are (i) change in types of fatty acids of cell membrane[54] (ii) exchange of proton for an amino acid in presence of amino acid carboxylase[55,56] (iii) conserving structures of macromolecules like proteins with the help of chaperones[54,57,58] and (iv) counteracting the low pH with production of alkaline molecules like ammonia[59]. Since the test organism belongs to genus *Bacillus*, it probably suggests use one or more of these mechanisms. Interestingly, despite high heavy metal stress, acid tolerance of *B. clausii* was not affected as concluded by the results of this assay.

Bile Tolerance Assay

For a probiotic to be categorized as bile tolerant, it must have maximal growth at 0.3% bile salt concentration after 3 hrs.[60,61] When *B. clausii* exposed to heavy metals under study was inoculated in bile salt concentrations of 0.2%, 0.3% and 0.4%, highest CFUs were observed at 0.3% bile after 3 hrs. Hence, subjection of *B. clausii* to heavy metal stress did not change its bile tolerance property (Fig. 11-14).

In contrast to defined mechanisms of acid tolerance, the methods by which bacterial cell counters bile stress is not well defined. One of the mechanism is with the use of enzyme bile salt hydrolase[62]. Apart from this, complex gene expression is also known to be responsible for bile tolerant property of bacteria[63-66].

It may be noted that even under heavy metal stress, *B. clausii* is able to retain its probiotic efficacy by probable use of different mechanisms and can be applicable for bioremediation of heavy metal, *in vivo*.

CONCLUSION

Anthropogenic and natural activities are the causes of Cr (VI), Pb (II), Cd (II) and Ni (II) contamination of soil and water and air. These are toxic heavy metals which reach humans through the food chain and cause detrimental effects on their health. Chromium and lead are systemic toxicants while nickel and cadmium are carcinogens. When the effects of these heavy metals were tested on a probiotic microorganism, *B. clausii*, no change was observed in its morphological and biochemical properties and its probiotic efficacy. Hence, it can be concluded that *B. clausii*, a commercial probiotic has the potential for *in vivo* bioremediation of Cr (VI), Pb (II), Cd (II) and Ni (II) and can therefore benefit the society at large by reducing the toxicity of these heavy metals in the human gut.

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REFERENCES

1. FAO/WHO. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Cordoba, Argentina: Report of a Joint FAO/WHO Expert Consultation. 2001; pp 5.
2. Todar K. The Good, the Bad and the Deadly, 1st edn. Wyoming, USA. 2004
3. Elshaghabee F.M.F., Rokana N., Gulhane R.D., Sharma C., Panwar H. *Bacillus* as potential probiotics: status, concerns, and future perspectives. *Front. Microbiol.* 2017; **8**: 1-15.
4. Marteau P., Seksik P., Jian R. Probiotics and intestinal health effects: a clinical perspective. *Br. J. Nutr.* 2002; 88(1): 51-7.
5. McFarland L.V. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *Am. J. Gastroenterol.* 2006; **101**(4): 812–22.
6. Sazawal S., Hiremath G., Dhingra U., Malik P., Deb S., Black R. E. Efficacy of probiotics in prevention of acute diarrhoea: a meta-analysis of masked, randomised, placebo-controlled trials. *Lancet Infect. Dis.* 2006; **6**(6): 374–82.
7. Szajewska H., Mrukowicz J. Z. Probiotics in the treatment and prevention of acute infectious diarrhea in infants and children: a systematic review of published randomized,

doubleblind, placebo-controlled trials. *J. Pediatr. Gastroenterol. Nutr.* 2001; **33**(4): 17–25.

8. Isolauri E., Kirjavainen P.V., Salminen S. Probiotics: a role in the treatment of intestinal infection and inflammation? *Gut.* 2002; **50**(3): 54–9.
9. Shah N.P. Functional cultures and health benefits. *Int. Dairy. J.* 2007; **17**(11): 1262–77.
10. McFarland L.V. Meta-analysis of probiotics for the prevention of traveler's diarrhea, *Travel Med. Infect. Dis.* 2007; **5**(2): 97–105.
11. Deshpande G., Rao S., Patole S. Probiotics for prevention of necrotising enterocolitis in preterm neonates with very low birth weight: a systemic review of randomized controlled trials. *Lancet.* 2007; **369**(9573): 1614–20.
12. Syed S., Chinthala P. Heavy metal detoxification by different *Bacillus* species isolated from solar salterns. *Scientifica* 2015; 1-8.
13. Imam S.A., Rajpoot I.K., Gajjar B., Sachdeva A. Comparative study of heavy metal bioremediation in soil by *Bacillus subtilis* and *Saccharomyces cerevisiae*. *Indian J. Sci. Technol.* 2016; **9**(47): 1-7.
14. Oaikhena E.E., Makaije D.B., Denwe S.D., Namadi M.M., Haroun A.A. Bioremediation potentials of heavy metal tolerant bacteria isolated from petroleum refinery effluent, *Am. J. Environ. Protect.* 2016; **5**(2): 29-34.
15. Ibrahim F., Halttunen T., Tahvonen R., Salminen S., Probiotic bacteria as potential detoxification tools: assessing their heavy metal binding isotherms. *Can. J. Microbiol*. 2006; **52**(9): 877–85.
16. Halttunen T., Finell M., Salminen S. Arsenic removal by native and chemically modified lactic acid bacteria. *Int. J. Food. Microbiol*. 2007; **120**(1–2): 173–8.
17. Halttunen T., Collado M.C., El-Nezami H., Meriluoto J., Salminen S. Combining strains of lactic acid bacteria may reduce their toxin and heavy metal removal efficiency from aqueous solution. *Lett. Appl. Microbiol.* 2008; **46**(2): 160–5.
18. Belapurkar P., Goyal P., Kar A. *In vitro* evaluation of bioremediation capacity of a commercial probiotic, *Bacillus coagulans*, for chromium (VI) and lead (II) toxicity. *J. Pharm. Bioallied Sci.* 2016; **8**(4): 272-6.
19. Belapurkar P., Goyal P., Kar A. Potential assessment of *Bacillus coagulans* for bioremediation of Zn (II) and Ni (II): an *in vitro* study. *Eur. J. Biomed. Pharm. Sci.* 2018; **5**(1): 627-32.
20. Delcour J., Ferain T., Deghorain M., Palumbo E., Hols P. The biosynthesis and functionality of the cell-wall of lactic acid bacteria. *Antonie Van Leeuwenhoek*. 1999; **76**(1-4): 159-84.
21. Ahmed S., Islam M. R., Ferdousi J., Iqbal T. S. Probiotic *Lactobacillus* spp. with bioremediation potential of toxic heavy metals. *Bangladesh J. Microbiol.* 2017; **34**(1): 43-6.
22. Robinson J.B., Tuovinen O.H. Mechanisms of microbial resistance and detoxification of mercury and organomercury compounds: physiological, biochemical, and genetic analyses. *Microbiol. Rev.* 1984; **48**(2): 95–124.
23. Mistry K., Desai C., Lal S., Patel K., Patel B. Hexavalent chromium reduction by *Staphylococcus* sp. isolated from chromium (VI) contaminated landfill. *Int. J. Biotechnol. Biochem.* 2010; **6**(1): 117 29.
24. MacFaddin J.F.: Biochemical Tests for Identification of Medical Bacteria, 3rd edn. Philadelphia, PA: Lippincott Williams & Wilkins. 2000; pp 113
25. Clark W.M., Lubs H.A. The differentiation of bacteria of the colon-aerogenes family by the use of indicators. *J. Infect. Dis.* 1915; **17**(1): 160–73.
26. Voges O., Proskauer B. Beitraege zur ernaehrungsphysiologie und zur differential diagnose der bakterien der hemmorrhagischen septicamie. *Z. Hyg.* 1898; **28**: 20–32.
27. Barritt M. M. The intensification of the Voges-Proskauer reaction by the addition of α-naphthol. *J. Pathol. Bacteriol.* 1936; **42**(2): 441–54.
28. Cappuccino J.G. Sherman N., Microbiology: A Laboratory Manual, 8th ed. Pearson Benjamin Cummings, San Francisco, CA, USA (2008)
29. Bauer A.W., Kirby W.M.M., Sherris J.C., Turck M. Antibiotic susceptibility testing by a standardized single disk method. *Am. J. Clin. Pathol.* 1966; **45**(4): 493-6.
30. Hassanzadazar H., Ehsani A., Mardani K., Hesari J. Investigation of antibacterial, acid and bile tolerance properties of lactobacilli isolated from Koozeh cheese. *Vet. Res. Forum.* 2012; **3**(3): 181-5.
31. Elahi A., Rehman A. Comparative behavior of two gram positive Cr^{6+} resistant bacterial strains *Bacillus aerius* S1 and *Brevibacterium iodinum* S2 under hexavalent chromium stress. *Biotechnol. Rep. (Amst.).* 2019; **21**: 1-8.
32. Qiao W., Zhang Y., Xia H., Luo Y., Liu S., Wang S., Wang W.. Bioimmobilization of lead by *Bacillus subtilis* X3 biomass isolated from lead mine soil under promotion of multiple adsorption mechanisms. *R. Soc. Open. Sci.* 2019; **6**(2): 1-10.
33. Shameer S. Biosorption of lead, copper and cadmium using the extracellular polysaccharides (EPS) of *Bacillus* sp., from solar salterns. *3 Biotech.* 2016; **6**: 1-10.

34. Uthra K., Kadirvelu K. Biosorption of nickel using mixed cultures of *Pseudomonas aeruginosa* and *Bacillus subtilis*. *Def. Life. Sci. J.* 2017; **2**(4): 442-7.
35. Cervantes C., Chavez K., Vaca S. Mechanisms of bacterial resistance to heavy metals. *Rev. Latinoam. Microbiol.* 1991; **33**(1): 61-70.
36. Carattoli A. Plasmid-mediated antimicrobial resistance in *Salmonella enterica*. *Curr. Issues Mol. Biol.* 2003; **5**(4): 113-22.
37. Coral M. U., Korkmaz H., Arikan B., Coral G. Plasmid mediated heavy metal resistances in *Enterobacter* spp. isolated from Sofulu landfill, in Adana, Turkey. *Ann. Microbiol.* 2005; **55**(3): 175-9.
38. Silver S. Bacterial resistances to toxic metal ions-a review. *Gene.* 1996; **179**(1): 9-19.
39. Alam M., Imran M., Metal tolerance analysis of Gram negative bacteria from hospital effluents of Northern India. *J. Appl. Pharm. Sci.* 2017; **7**(04): 174-80.
40. Basu M., Bhattacharya S., Paul A.K., Isolation and characterization of chromium-resistant bacteria from tannery effluents. *Bull. Environ. Contam. Toxicol.* 1997; **58**(4): 535-42.
41. Karbasizaed V., Badami N., Emtiazi G., Antimicrobial, heavy metal resistance and plasmid profile of coliforms isolated from nosocomial infections in a hospital in Isfahan. *Iran Afr. J. Biotechnol.* 2003; **2**(10): 379-83.
42. World Health Organization. Guidelines for drinking-water quality: first addendum to the 4th ed. Geneva. 2017.
43. Fridman O., Goldberg A., Ronin I., Shoresh N., Balaban N. Q. Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature.* 2014; **513**(7518): 418-21.
44. Khatri I., Sharma G., Subramanian S. Composite genome sequence of *Bacillus clausii*, a probiotic commercially available as Enterogermina®, and insights into its probiotic properties. *BMC Microbiol.* 2019; **19**(1): 1-15.
45. Tuckfield R.C., McArthur J.V. Spatial analysis of antibiotic resistance along metal contaminated streams. *Microb. Ecol.* 2008; **55**(4): 595-607.
46. Habi S., Daba H. Plasmid incidence, antibiotic and metal resistance among Entererobacteriaceae isolated from Algerian streams. *Pak. J. Biol. Sci.* 2009; **12**(22): 1474-82.
47. Hölzel C.S., Müller C., Harms, K.S., Mikolajewski S., Schäfer S., Schwaiger, K., Bauer J. Heavy metals in liquid pig manure in light of bacterial antimicrobial resistance. *Environ. Res.* 2012; **113**: 21–7.
48. Zhou Y., Xu Y.B., Xu J.X., Zhang X.H., Xu S.H., Du Q.P. Combined toxic effects of heavy metals and antibiotics on a *Pseudomonas fluorescens* strain ZY2 isolated from swine wastewater. *Int. J. Mol. Sci.* 2015; **16**(2): 2839–50.
49. Seget P.Z., Cycoñ J., Kozdrój J. Metal tolerant bacteria occurring in heavily polluted soil and mine spoil. *Appl. Soil. Ecol.* 2005; **28**(3): 237-46.
50. Jayaprakashvel M., Vijay S., Karthigeyan C.P., Hussain A.J. Isolation and characterization of mercury resistant marine bacteria from the coastal area of Chennai, India. *Int. J. Adv. Res. Eng. App. Sci.* 2015; **4**(8): 64-76.
51. Das S. N., Mandal M., Mandal S. Plasmid mediated antibiotic and heavy metal co-resistance in bacterial isolates from Mahananda river water (Malda, India). *Transl. Med. (Sunnyvale)*. 2016; **6**(4): 1-6.
52. Sahadeva R.P.K., Leong S.F., Chua K.H., Tan C.H., Chan H.Y., Tong E.V., Wong S.Y.W., Chan H.K. Survival of commercial probiotic strains to pH and bile. *Int. Food Res. J.* 2011; **18**(4): 1515-22.
53. Oh D.H., Marshall D. L..Monolaurin and acetic acid inactivation of *Listeria monocytogenes* attached to stainless steel. *J. Food. Prot.* 1996; **59**(3): 249–52.
54. Quivey R.G., Kuhnert W.L., Hahn K. Genetics of acid adaptation in oral *Streptococci. Crit. Rev. Oral Biol. Med.* 2001; **12**(4): 301–14.
55. Cotter P. D., Hill C. Surviving the acid test: responses of Gram-positive bacteria to low pH. *Microbiol. Mol. Biol. Rev.* 2003; **67**(3): 429–53.
56. Gale E.F.: The bacterial amino acid decarboxylases. In: *Advances in Enzymology and Related Areas of Molecular Biology* (Nord FF, ed). John Wiley & Sons, Inc. 2006; pp 1–32.
57. Huesca M., Goodwin A., Bhagwansingh A., Hoffman P., Lingwood C.A. Characterization of an acidic-pH-inducible stress protein (hsp70), a putative sulfatide binding adhesin from *Helicobacter pylori*. *Infect Immun.* 1998; **66**(9): 4061–67.
58. Lau S.K., Fan R.Y., Ho T.C., Wong G.K., Tsang A.K., Teng J.L., Chen W., Watt R.M., Curreem S.O., Tse H., Yuen K.Y., Woo P.C. Environmental adaptability and stress tolerance of *Laribacter hongkongensis*: a genome-wide analysis. *Cell Biosci.* 2011; **1**(1): 1- 27.
59. Goswami G., Panda D., Samanta R., Boro R.C., Modi M.K., Bujarbaruah K.M., Barooah M. *Bacillus megaterium* adapts to acid stress condition through a network of genes: Insight from a genome-wide transcriptome analysis. *Scientific Reports.* 2018; **8**(1): 1-12.
60. Hyronimus B., Le Marrec C., Hadj Sassi A., Deschamps A. Acid and bile tolerance of spore-forming lactic acid bacteria. *Int. J. Food*

Microbiol. 2000; **61**(2-3): 193-7.

61. Zhou X.X., Pan Y.J., Wang Y.B., Li W.F. *In vitro* assessment of gastrointestinal viability of two photosynthetic bacteria, *Rhodopseudomonas palustris* and *Rhodobacter* sphaeroides. *J. Zhejiang Univ. Sci. B.* 2007; **8**(9): 686-92.
62. Moser S.A., Savage D.C. Bile salt hydrolase activity and resistance to toxicity of conjugated bile salts are unrelated properties in lactobacilli. *Appl. Environ. Microbiol.* 2001; **67**(8): 3476-80.
63. Sánchez B., Champomier-Vergès M.C., Anglade P., Baraige F., De Los Reyes-Gavilán C.G., Margolles A., Zagorec M. Proteomic analysis of global changes in protein expression during bile salt exposure of *Bifidobacterium longum* NCIMB 8809. *J. Bacteriol.* 2005; **187**(16): 5799-808.
64. Sánchez B., Champomier-Vergès M.C., Stuer-Lauridsen B., Ruas-Madiedo P., Anglade P., Baraige F., De Los Reyes-Gavilán C.G., Johansen E., Zagorec M., Margolles A. Adaptation and response of *Bifidobacterium animalis* subsp. *lactis* to bile: A proteomic and physiological approach. *Appl. Environ. Microbiol.* 2007; **73**(21): 6757-67.
65. Andriantsoanirina V., Allano S., Butel M.J., Aires J. Tolerance of *Bifidobacterium* human isolates to bile, acid and oxygen. *Anaerobe*. 2013; **21**: 39-42.
66. Ruiz L., Margolles A., Sánchez B. Bile resistance mechanisms in *Lactobacillus* and *Bifidobacterium. Front. Microbiol.*2013; **4**: 1-8.