Summary

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Environmental pollution due to hazardous metals can be generated from both natural as well as anthropogenic sources. Most commonly found heavy metals at the contaminated sites are cadmium (Cd), lead (Pb), arsenic (As), mercury (Hg), chromium (Cr). Most commonly, they enter into human body through contaminated food or water. The most sensitive mammalian organs to the injurious effects of environmental heavy metal pollutants are liver and kidney. Oxidative stress is mainly responsible for heavy metal pathogenesis. Recently used bioremediation processes as well as physical and chemical methods for heavy metals detoxification are either too expensive or ineffective. Chelation therapy is one of the widely used strategy for metal toxicity where chelators can bind with metals to enhance their excretion from body by forming complexes. Combination therapy involving administration of antioxidants with chelators found to be more effective in restabilishing the disturbed antioxidants status due to heavy metal intoxication.

PQQ which is a potent antioxidant, also acts as growth factor, enhances reproductive capabilities as well as maintain neuronal and mitochondrial function, also reduces inflammation, dislipidemia and liver fibrosis. It also serves as a co-factor for glucose dehydrogenase, *E. coli* genome encodes for glucose dehydrogenase apoprotein but lacks the ability to synthesize PQQ co-factor. Thus synthesizing the PQQ within *Ec*N enables it to continuously secrete PQQ in gut and hence reduces the dependency of humans for PQQ on plant based diet. PQQ serves as a co-factor for glucose dehydrogenase allowing the *Ec*N to convert glucose to gluconic acid which is a prebiotic molecule. Gluconic acid as a prebiotic further utilized by gut microflora resident in lower part of gastrointestinal tract resulting in the production of short chain fatty acids (SCFAs).

The intestinal microbiota constitutes 1,000 different species with their collective genome found to contain 100 times more genes than the entire human genome. Gut microbiota in form of SCFAs provide energy to gut epithelial cells, produce essential vitamins, perform immunomodulation via their metabolites and also competes with potential pathogens for nutrients and niches, thereby provides “colonization resistance”, stimulation and development of the immune system, improvement of intestinal health by the regulation of microbiota, reducing symptoms of lactose

intolerance, synthesizing and enhancing the bioavailability of nutrients and decreasing the risk of several other diseases. Probiotic *Ec*N is non-pathogenic, non-invasive, does not produce cytotoxins or enterotoxins and found to be therapeutically effective against diarrhea, ulcerative colitis and chronic constipation. *Ec*N is also known to hinder the reactive oxygen species and tightens the tight junctions of intestinal wall.

Gut microbiota prevents absorption of ingested metal by binding and sequestering them on their cell membranes. In general, gut microbiota are not known to produce citric acid. Nevertheless, upto 9 mM of citric acid was obtained in various bacteria by overexpression of an artificial citrate operon (*csYF-citC*) consisting of NADH insensitive *E. coli cs* Y146F mutant gene along with *S. typhimurium* Na^+ dependent citrate transporter (*citC*) gene. The present study was done to evaluate the effect of combination therapy involving *Ec*N-20 producing PQQ with orally supplemented citric acid and *Ec*N-21 possessing artificial citrate operon (*csYF-citC*) and *pqq* gene cluster producing citric acid along with PQQ against Cd induced hepatotoxicity and nephrotoxicity.

vgb gene encoding for *Vitreoscilla* haemoglobin (VHb) enhanced the growth and survival of probiotic *E. coli* CFR16 in oxygen limiting conditions of gastrointestinal tract and protected against carbon tetrachloride induced oxidative damage due to its peroxidase activity. Hence, *vgb* gene was inserted in genome of *Ec*N to enhance its survival within gut and to locate its colonization *gfp* gene was also inserted. Nevertheless, *Ec*N-2 was not effective in reducing Cd toxicity due to low copy number of *vgb* gene within genome as compared to high copy number within plasmid in *E. coli* CFR16. However, *Ec*N-20 producing PQQ supplemented with citric acid orally is an effective strategy against Cd induced liver and kidney damage as compared to orally given PQQ and citric acid. This strategy found to be more effective for liver as compared to kidney. However, *Ec*N-21 was less effective which could be attributed to lower levels of secreted citric acid.

Therefore, increased levels of chelators required to be achieved. Organic acids such as 2-Ketogluconic acid can chelate Cd as well as Pb. Gluconic acid produced by genetically modified *Ec*N further utilized by gluconate dehydrogenase enzyme encoded by *gad* operon using FAD as cofactor converting gluconic acid into 2-ketogluconic acid within the periplasm. Therefore, *Ec*N-23 harbouring *pqq* gene

cluster and *gad* operon producing PQQ and sufficient levels of 2-ketogluconic acid was developed to chelate Cd which could prevents against its toxic effects. Interestingly, *Ec*N-23 producing PQQ and 2-ketogluconic acid was found to be effective against Cd induced liver and kidney damage as compared to *Ec*N-22 producing PQQ only and *Ec*N-2 supplemented with orally given PQQ and 2-ketogluconic acid.

To explore the effect of *Ec*N-23 on other heavy metals, Pb was considered as it is well implicated for heavy metal mediated immunotoxicity. Exposure to Pb increases susceptibility to infections by inducing shift from Th1 mediated immune response to Th2 which eventually causing altered cytokines release. If animals are pre-exposed to Pb then they are more sensitive to LPS mediated septic shock involving series of events such as elevation in iNOS activity, neutrophil infiltration, mast cell degranulation and lipid peroxidation. Hence, the present strategy was designed to evaluate the effect of synbiotic *Ec*N-23 producing PQQ, 2-ketogluconic acid and gluconic acid against Pb induced immunotoxicity in LPS (*E.coli* O55:B5)/GalN (Galactosamine) treated rats. *Ec*N-23 was also found to be effective in preventing the immunotoxicity of Pb in the colon and liver in LPS/GalN treated rats.

To determine the long term effects of *Ec*N-23 as well as its effects on coexposure studies, Cd and Pb coexposure experiments were conducted. Coexposure of Cd and Pb can generate reactive oxygen species leading to liver and Kidney damage, can also induce apoptosis henceforth impairing their function. Additive effect of Cd and Pb is neurotoxic too by disabling blood brain barrier and can also cause dislipidemia. Therefore, the present strategy was designed to evaluate the effect of symbiotic *Ec*N-23 producing PQQ, 2-ketogluconic acid, gluconic acid against long term (4 months) co-exposure of Cd and Pb. Remarkably, *Ec*N-23 was found to be effective for long term coexposure of Cd+Pb in liver and kidney damage as well as against dislipidemia without affecting essential metals homeostasis which illustrates its safety and usefulness for therapeutic purposes.

Citric and 2-ketogluconic acids do not chelate arsenic (As) due to the negative charge of arsenate and arsenite. Detoxification of As involves the biotransformation of As to methylated As species by microorganisms using *arsM* genes encodes for As(III) S-adenosylmethionine (SAM) methlyltransferases (ArsM) which eventually

forms volatile nontoxic trimethylarsine gas as end product. Hence, synbiotic *Ec*N-20 had been genetically modified to synthesize ArsM along with PQQ and gluconic acid and its potential had been analyzed for the detoxification of the hazardous arsenic metal in rat diet. EcN-24 producing ArsM, PQQ and gluconic acid showed protective effect against As induced oxidative stress and dislipidemia as compared to EcN-20 producing PQQ.

In conclusion, with prevailing techniques available to detoxify heavy metals are either ineffective or expensive. Combination therapy involving supplementation of antioxidants with chelators comes forth as a boon to resolve the associated health effects of metal toxicants. However, utilizing the gut microbiota as a continuous delivery agent to produce constitutive remedial factors like antioxidants, chelators, prebiotics and other detoxifying enzymes could arise new era of combination therapy involving synbiotics, probiotics and prebiotics as healthy and novel alternative to existing approaches to overcome heavy metal toxicity.