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Identification of highly toxic metabolites produced by the bacterial biotransformation of the Indian bay leaf methanolic extracts *in vitro* using GC-MS: a new perspective for food-borne illnesses.

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Food-borne illnesses are on the rise due to unhygienic methods of preparation and serving especially by the street vendors. While the pathogens originating from the unhygienic food are directly causing various illnesses, we reasoned whether the microbes in the human gut microbiome (hGMb) may contribute to these illnesses leading to an even worse situation. In this study we evaluated such possibilities using the Indian bay leaf (IBL), a most commonly used spice in the preparation of biryani. The methanolic extracts of IBL were air-dried and were added to *Lysinibacillus spp.* containing bacterial cultures for biotransformation of any metabolites from IBL into possible toxins. *Lysinibacillus spp.* were previously identified on IBLs. GC-MS analysis of supernatants from the overnight bacterial cultures revealed multiple toxic compounds that are carcinogenic, neurotoxic, endocrine/hormone disruptors, cardiovascular inducing compounds, etc. GC-MS analysis of bacterial cultures without IBL extracts did not contain any of the above mentioned toxic substances suggesting that the microbes in hGMb can potentially biotransform the compounds in the IBL mixed with biryani into toxic substances that can cause various illnesses. However, the composition of hGMb differs from person to person, which may make this situation better or worse.

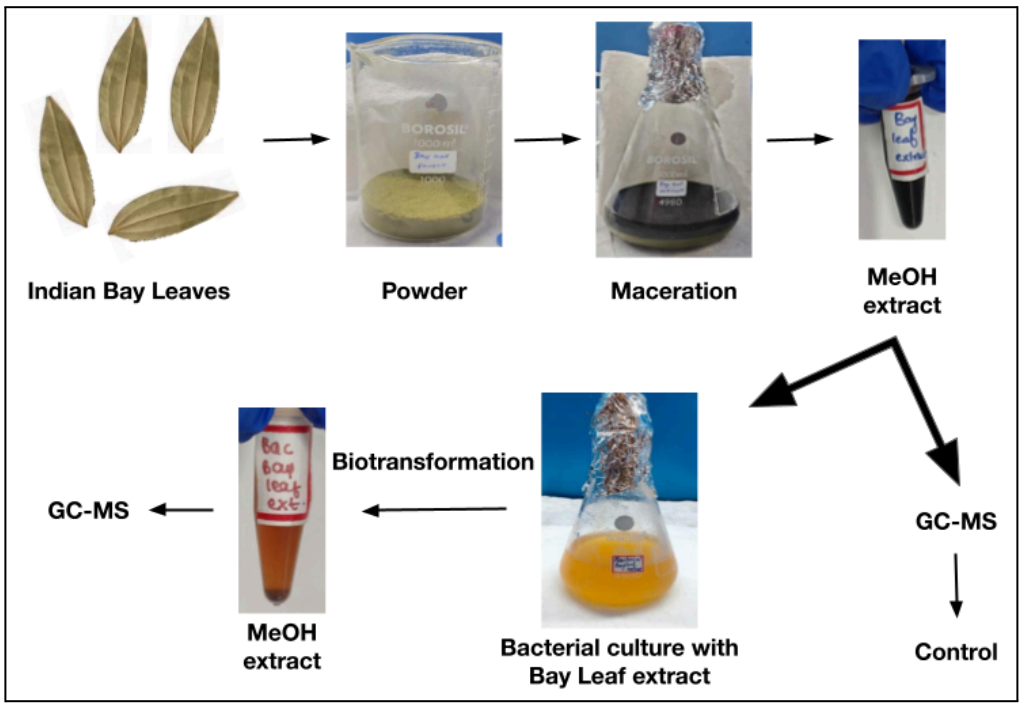

Figure 1. Overall process of GC-MS analysis using bacterial transformation of the Indian Bay Leaf extract.

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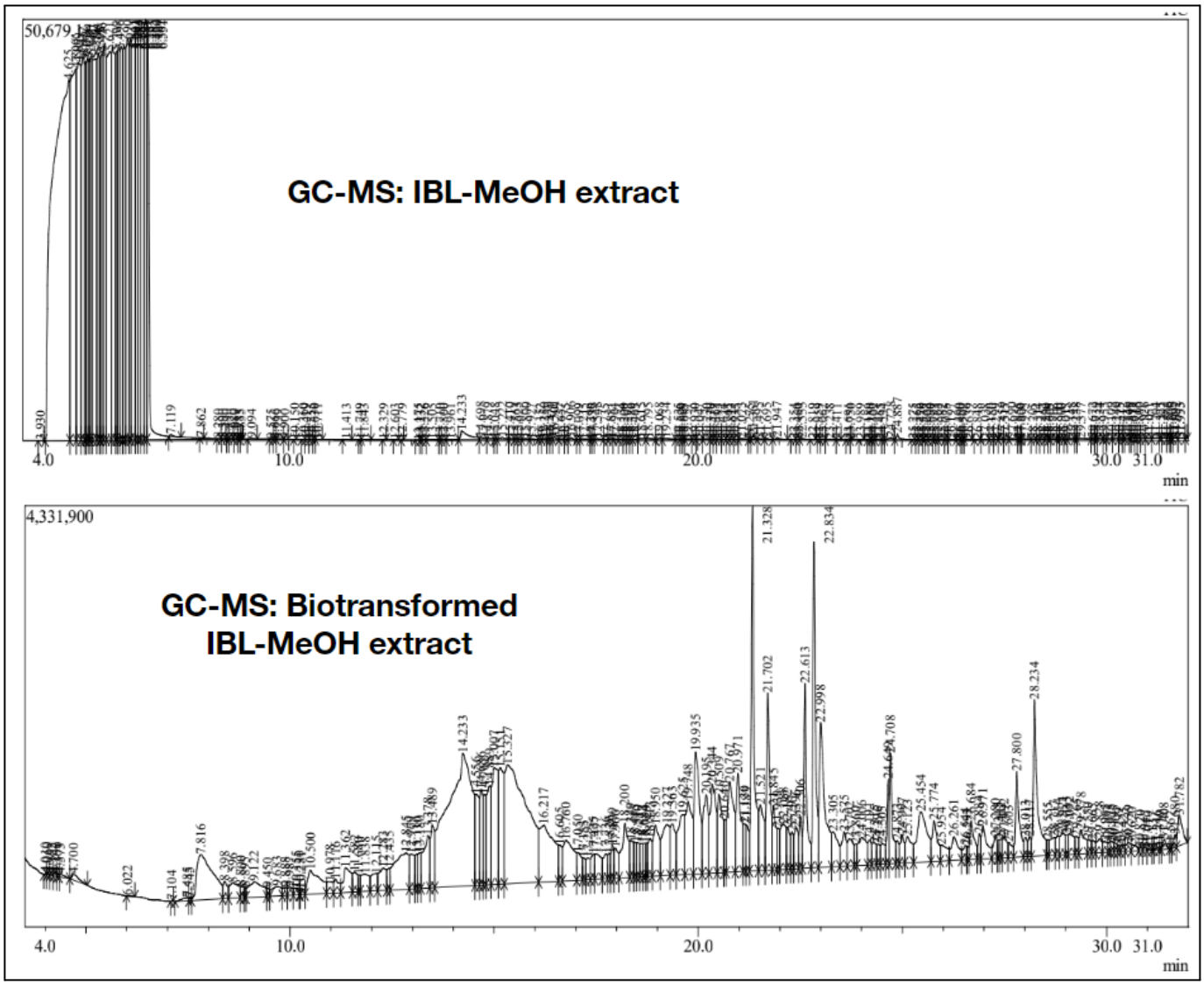

Figure 2. GC-MS spectra of IBL-MeOH (50,679,173 hits/peaks) and biotransformed IBL-MeOH (4,331,900 hits/peaks) extracts.

Food-borne illnesses have been common problems world wide in both under developed and fully developed countries [1]. India is famous for various street food vendors with a variety of street foods and snacks such as the panipuri, chat, etc. that are commonly consumed by many people on a daily basis [2]. Biryani, a rice-based food cooked with spices, has been added to the Indian street food list in the past few decades [3]. Previously we have shown that the roadside biryani sample contains *Enterobacter spp*. [4]. The major ingredients in biryani are spices that elevate the flavor of the dish overall. The Indian bay leaf (IBL) (*Cinnamomum tamala*) is a major herb in the spice mix that is critical for the flavor of Indian spicy foods such as biryani. While the California bay leaf *(Umbellularia californica*) is known to cause headaches due to the presence of umbellulone (a monoterpene ketone) [5] and methemoglobinemia [6], other mountain species of bay leaf are not recommended for culinary usage due to their poisonous nature [7]. Methemoglobinemia is a condition in which the iron atom in the hemoglobin exists in ferric (Fe^{3+}) state instead of ferrous (Fe^{2+}) state which impairs it from carrying oxygen in the blood and can be considered as a blood disorder which may lead to the formation of hemichromes, the degradation forms of hemoglobin. It has been previously shown that the bay leaves can be used as insect repellents [8].

The human gut microbiome (hGMb) contains a diversity of microbes and varies person to person depending on their food habits, living styles, daily stress, relationships, etc. [9].

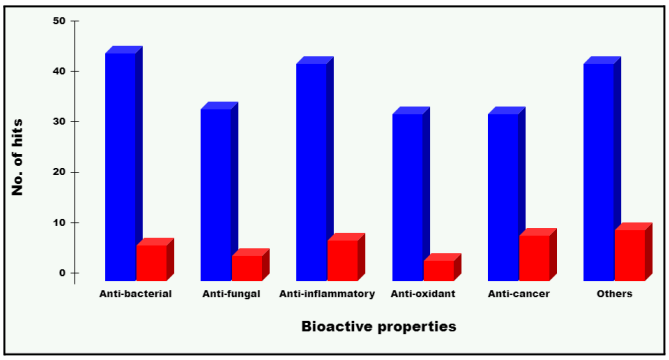

Figure 3. Histogram showing the number of hits (compounds) with various bioactive properties obtained in the GC-MS analysis before (blue bars) and after (red bars) bacterial biotransformation of the IBL-MeOH extracts.

A healthy balance in the hGMb is critical for one's health. However, the microbes in the hGMb may also play a pivotal role in the process of microbial biotransformation of food, drugs, etc. that are consumed on a daily basis. Such biotransformations may either lead to good health or sometimes may be lethal [10-14]. We have previously shown that the Indian Bay Leaf (IBL) from farmers' market contained *Lysinibacillus spp*. [15]. In this study, we hypothesized whether consumption of the IBL-containing food such as biryani is safe for humans. We evaluated the complete phyto- chemical profile of IBL methanolic extracts using GC-MS to identify any toxic compounds. Followed by this analysis, the dried methanolic extract of IBL was further incubated with *Lysinibacillus spp*. bacterial cultures *in vitro* (representing the hGMb) to investigate the possible biotransformation process and outcomes using GC-MS.

Materials & Methods:

Preparation of IBL methanolic extracts: The IBLs were purchased from the local farmers market and were tested for any moisture. After confirming that they are dry, they were blended into a dry powder. One hundred and fifty g of the dry powder was then thoroughly mixed with 250 ml. methanol (MeOH) in a clean conical flask. The flask was closed with a cotton plug and an aluminium foil on the top. Maceration was allowed to continue for one week at room temperature. The macerated mixture was then filtered through a clean muslin cloth folded into two layers. The cloth was squeezed to obtain all the liquid MeOH extract into a round bottomed flask for distillation. More than 95% solvent was removed from the extract and the remaining extract was air-dried for a few hours in a chemical fume hood. The final air-dried MeOH extract of IBL was split into two equal halves, one for the direct GC-MS analysis serving as a control and the other half for bacterial biotransformation.

Bacterial biotransformation: Five ml of pre-sterilized LB broth was taken in sterile culture tubes. One hundred µl of IBL-MeOH extract was added to the LB broth and thoroughly mixed. One tube was used as a control in which the IBL-MeOH was not added. A loopful of *Lysinibacillus spp*. bacterial cells were used to inoculate each tube. The inoculated cultures were incubated at 37 ℃ overnight. Cells were pelleted by centrifugation at 5,000 rpm for 20 min. Supernatants were used further for GC-MS analysis. Biotransformations were performed with different bacterial species which will be published separately.

Preparation of biotransformed samples for GC-MS: The supernatants from bacterial cultures were carefully collected into conical flasks to which 150 ml of MeOH per flask was added. Macerations were continued for 3 days at room temperature followed by distillation to remove the MeOH in the chemical fume hood. The final air-dried MeOH extracts were used for GC-MS analysis. All GC-MS spectral acquisitions were outsourced and the final spectra were analyzed to evaluate the full phytochemical profile of IBL-MeOH extracts vs. IBL-Biotransformed-MeOH extracts.

GC-MS data analysis: The GC-MS spectra of IBL-MeOH extract was compared to the biotransformed IBL-MeOH extract and total number of hits/compounds were analyzed. Compound identities were compared between the two spectra followed by a summary list of toxic compounds from both spectra with respect to human health and disease.

Results and Discussion:

Biotransformation significantly decreased the number of bioactive compounds in the MeOH extract: The GC-MS spectra of IBL-MeOH and biotransformed IBL-MeOH extracts showed significant differences in their overall number of hits/compounds (Figure 2). The total number of hits in IBL-MeOH extract and biotransformed IBL-MeOH extract were 50,679,173 and 4,331,900, respectively. A 11.7-fold decrease in the hits was seen after the biotransformation. One could reason that this decrease in the number of hits can be due to the decrease in the number of volatile compounds post-biotransformation. However this should be further confirmed by directly comparing the LC-MS and GC-MS profiles of the samples to capture any missing hits in the GC-MS. As shown in Figure 3, the number of hits possessing various bioactivities decreased significantly suggesting that the IBL-MeOH extract had almost 12-fold higher medicinal properties before the biotransformation. Further, the biotransformation can also be bacterial species-specific in the variable hGMb. We next investigated the possible routes of biotransformation for the compounds that have toxicity profiles classified from high to low toxicity to humans.

Compound	Toxicity Level	Effect	Associated Disease
7,12-Dimethylbenz[a]anthracene (DMBA)	High	Carcinogen, mutagen, immunosuppressant	Cancer (Ovarian, Skin and Breast cancer), immune suppression
Bis(2-ethylhexyl) phthalate (DEHP)	High	Endocrine disruptor, reproductive and liver toxicant	Hormonal disorders, infertility, liver disease, cancer
Morphinan-6-ol (Morphine derivative)	High	Neurotoxic, respiratory depressant, addictive	Opioid addiction, respiratory failure
Boldenone	High	Endocrine disruptor, hepatotoxic, cardiovascular risk	Hormonal imbalance, liver disease, heart disease
Dimethylamphetamine	High	Stimulant, neurotoxic	Addiction, neurodegeneration
1,2-15,16-Diepoxyhexadecane	High	Mutagenic, carcinogenic	Cancer
Acetamide, N-[4-(phenylamino)phenyl]-	High	Carcinogenic, mutagenic	Bladder cancer, DNA damage
Dihydroergotamine	Moderate-High	Vasoconstrictive, circulatory issues	Ergotism, gangrene, neurological damage
1,4,8,11-Tetraazacyclotetradecane-2,5,9,12-tetrone	Moderate-High	Cytotoxic, mutagenic	Cancer, cellular damage
Piperazine	Moderate	CNS depressant, hepatotoxicity	Neurological disorders, liver dysfunction
N-[2-(Cyclopropylcarbonyl)-1-benzofuran-3-yl]-2-(4-methyl-1-piperazinyl) acetamide	Moderate	Neurotoxic, hepatotoxic	Neurological disorders, liver disease
2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	Moderate	Possible neurotoxic effects	Neurological disorders
4-Fluoro-1-methyl-5-carboxylic acid, ethyl ester	Moderate	Hepatotoxic, neurotoxic	Liver damage, neurological disorders
Isoeugenol	Moderate	Allergenic, hepatotoxic	Allergies, liver damage
1,6-Dioxacyclododecane-7,12-dione	Low-Moderate	Possible endocrine disruptor	Hormonal imbalance
Perhydro-htx-2-one, 2-depentyl-, acetate ester	Low	Respiratory and skin irritant	Skin dermatitis, respiratory irritation

Table 1. Potential toxic compounds identified in biotransformed IBL-MeOH extract using GC-MS.

Identification of toxic compounds in the biotransformed IBL-MeOH extracts: A total of 16 compounds were identified with various levels of toxicity such as high, moderate-high, moderate, low-moderate and low in the biotransformed IBL-MeOH extract using GC-MS (Table 1). By comparing the list of compounds obtained in the GC-MS spectra of the IBL-MeOH extracts before and after biotransformation we identified a few potential routes. As shown in Figure 4, the hydroxylation of eucalyptol and transformation of eugenol to either isoeugenol of methoxy eugenol were predicted from the standard databases. The alarming finding in this study is that most of these toxic compounds are known to cause major health issues such as various types of cancers, multiple endocrine disorders, neuronal disorders, hepatotoxicity. Additionally, biryani, which contains IBL as the main spice for the flavor, has become the new norm for the diet of the majority of the Indian population.

Epidemiological coincidences provide a new perspective for food-borne illnesses: Out of the 16 identified toxic compounds, almost 50% were classified as highly toxic that are responsible for the major diseases such as cancer, endocrine disorders, neurotoxicity, hepatotoxicity, etc. Co-incidentally the recent Indian epidemiological studies suggest a rise in these diseases. Looking at the cancer epidemiology data from the past 3 decades in India a 12% rise has been estimated in the year 2025 in spite of the limitations in the methods of data collection [16-19]. Endocrine disorders such as thyroid disorders, diabetes, polycystic ovarian syndrome, congenital adrenal hyperplasia are also evidently on the rise [20-22]. Neurological disorders are gaining more attention in India in recent years due to the increase in the number of cases and improved treatment facilities in the country [23, 24]. IBL being one of the majorly used spices in most of the Indian food items (e.g. biryani), raises a major concern for unforeseen food-borne illnesses in the present and potentially in the future.

Lysinibacillus spp. colonization in the human gut microbiome: Lysinibacillus spp is uncommonly found in the hGMb. However, recent studies confirmed that these bacteria are capable of causing human diseases through contaminated foods for example [25-27]. Similar to any other bacterial species, the *Lysinibacillus spp.* are capable of lateral gene transfer [28] that prompted us to check their biotransformation capabilities on the dry spices such as the IBL that are commonly used in Indian cuisine. In the current study we have shown using GC-MS that *Lysinibacillus spp.* can perform biotransformation of compounds present in the IBL-MeOH extract *in vitro* when incubated in the laboratory.

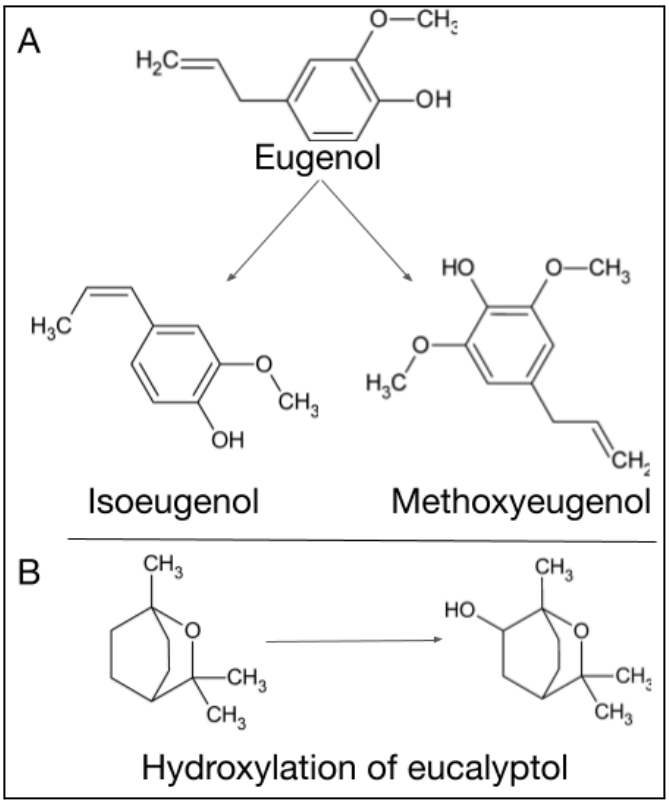

Figure 4. Possible biotransformations predicted by comparing the GC-MS spectra of IBL-MeOH and biotransformed IBL-MeOH extracts.

Conclusion and Future directions:

In this study we performed an *in vitro* demonstration that the *Lysinibacillus spp.* that are present on dry spices such as the IBL are capable of biotransformation of compounds to toxic compounds that could cause various potential food-borne illnesses. Taken together, these results suggest that the *Lysinibacillus spp.* that are capable of colonizing in the hGMb can potentially be capable of performing a similar biotransformation event that we showed here *in vitro*. However, further *in vivo* studies are needed to confirm this statement.

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Conflict of interest: The authors declare no conflict of interest in this study.

Author contributions: B.S. designed and performed all the wet lab experiments. M.V.S.A., L.A., V.M.A., M.K., H.T.M. and A.A. assisted B.S. in various aspects of the project. R.S.Y. is the principal investigator who designed the project, trained all students, secured required material for the project, provided the laboratory space and facilities needed. R.S.Y. wrote, edited and finalized the manuscript.

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