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Engineering of a Novel Anti-Dioxin Bacillus Subtilis Probiotic for Poultry Feed

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Abstract

Introduction: Plastic waste incineration has increased dramatically in Asia, producing highly toxic by-products such as dioxins. The biomagnification and bioaccumulation of dioxins from the atmosphere to the soil, agriculture, feed, livestock, and finally to humans poses a serious concern for public and environmental health. This study aims to engineer a novel poultry feed additive for detoxification of poultry products.

Methods: The *Bacillus subtilis* feed probiotic will be created via insertion of a dioxin degradation system from *Sphingomonas wittichii* and thymine dependent biological containment system. This method is appropriate given that dioxin's primary form of contact with humans is through ingestion. All experimentations will be done in triplicates and with appropriate control groups.

Results: For the experimental group (recombinant *B. subtilis* growing on PCDD- and PCDF-contaminated media), an increase in catechol is expected in comparison to the control groups. This will be quantified via liquid chromatography. In addition, a decrease in PCDD and PCDF levels will be expected and measured via mass spectrometry. It is postulated that the chickens will not undergo significant changes after intake of the probiotic in the animal trials. The laboratory observations in measuring biodegradation efficiency are expected to persist into animal trials.

Discussion: Although good combustion practice is the top method for removing dioxins, it is impractical in developing countries due to its costs. Therefore, recombinant bacterial chicken feed probiotic is the most cost effective in terms of removing dioxins from contaminated animal products despite its few limitations.

Conclusion: This study implicates a gap in literature in developing preventative measures for toxic plastic waste disposal by-products which could be mediated through increased research of the application of dioxin-degrading enzymes. Possibilities for further research include examination of dioxin impacted farm animals and the effects of *B. subtilis* as a probiotic.

Keywords

Bacillus subtilis; *Sphingomonas wittichii*; probiotic; dioxin; dioxin dioxygenase; chicken feed

Introduction

Since China's ban on waste imports in 2018, numerous Southeast Asian countries have experienced a surge in plastic waste [1]. There are reports of nations, such as Indonesia, resorting to incineration to deal with their growing waste crisis [2]. Unregulated incineration has led to high levels of dioxins, a by-product of waste combustion, being incorporated into animal products

Dioxins include organic polychlorinated rings such as polychlorinated dibenzo p-dioxins (PCDDs) and polychlorinated dibenzo-furans (PCDFs) [3]. They are carcinogenic persistent organic pollutants (POPs) that may lead to deficiencies in the reproductive and endocrine systems with prolonged exposure [4]. Due to dioxin's lipophilicity, it accumulates in adipose tissue of fish and animals [5]. Humans can be exposed to dioxins via skin-contact and ventilation, but the primary form of contact is through the ingestion of dioxin-contaminated foods (Fig. 1) [6].

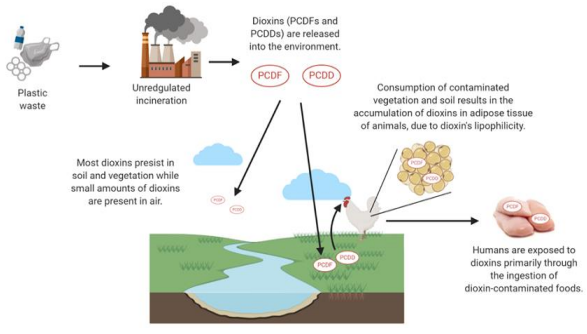

Figure 1. Sources, pathways, and effects of dioxin contamination. This figure illustrates how dioxins (PCDFs and PCDDs) are released into the environment and eventually enter our food chain. Other animal products, such as beef and dairy products, may be contaminated via similar pathways but are not shown in this figure.

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Approximately 200 pg TEQ/g of fat was detected in Indonesian eggs, which is 70 times more than the European Food Safety Authority (EFSA) tolerable daily intake (TDI) [7]. Schulz et al.'s 2005 study found that cows and sheep, who consumed feed grown on soils with high levels of dioxin, had higher concentrations of the toxin in their tissues and milk [8]. This exhibits the necessity of dioxin detoxification in animal feeds [8].

Current methods aimed to reduce dioxin emissions during waste incineration include: good combustion practice coupled with end-of-pipe treatment, addition of suitable inhibitors and selective catalytic reduction [3]. It was concluded that good combustion practice coupled with end of pipe treatment was the best of the practices in terms of cost and efficiency in removing dioxins [3]. However, it requires precise combustion temperatures and times which is impractical for large volumes of waste [3]. As a result of dioxin's stable aromatic ring, it persists in nature without degrading, however an enzyme that can readily degrade them is *S. wittichii* RW1's dioxin dioxygenase, coded by *dxnA1* and *dxnA2* genes [9]. This enzyme has the ability to perform oxygenolytic attacks and degrade mono-/di-chlorinated dibenzo-p-dioxins and dibenzofurans into their respective catechols and salicylates, which are further metabolized so they can enter pyruvate metabolism and TCA cycle respectively [9]. The oxygenolytic attacks are driven by RW1's electron supply system, in which flavoprotein reductase (*RedA2*) is protonated by NADH, which in turn transfers the electrons onto a ferredoxin (*Fdx1*) so it can reduce dioxin dioxygenase allowing it to catalyze the oxidation reactions [10]. This enzyme can be incorporated into animal feed in order to reduce the levels of dioxin consumption.

However, adding dioxin-degrading *S. wittichii* RW1 directly into animal feeds encounters a major problem as they are gram-negative, non-spore forming bacteria. They cannot survive under animal feed processing conditions and the acidic environment of the gastrointestinal (GI) tract [11]. However, this problem can be solved by using recombinant technology to insert the dioxin dioxygenase gene into a suitable host cell: *B. subtilis*. These spore-formers can withstand these harsh conditions and continue to metabolize dioxin in the GI tract [12]. In addition, as shown by Hayashi et al.'s 2018 study, *B. subtilis* are gastrointestinal probiotics which reduced *Salmonella enterica* (SE) colonization, increased microbiota diversity in the cecum allowing for increased ecosystem stability, and mediated expression/secretion of mucus by goblet cells to allow colonization of other favourable microbes [13]. We hypothesize that the engineered *B. subtilis* probiotic feed additive produced as a result of transforming *B. subtilis* so that it contains the enzyme dioxin dioxygenase from *S. wittichii* RW1 will properly degrade dioxin in fat deposits of poultry.

Methods

All experiments will be conducted in triplicates.

Chemicals and Culture Conditions

PCDD and PCDF will be purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *S. wittichii* RW1 (DSM 6014) will be purchased from the Leibniz Institute SMZ-German Collection of Microorganisms and Cell Cultures and will be used for the isolation and amplification of *dxnA1*, *dxnA2*, *RedA2*, and *Fdx1* fragments. *S. wittichii* RW1 will be cultured in a phosphate-buffered mineral medium with DBF as the sole carbon source (medium DSM 457) at 30 °C [14]. The *B. subtilis* host strain 168 will be purchased from the American Type Culture Collection (ATCC) and will be used as the expression host. *B. subtilis* 168 will be cultured at 37 °C in an aerobic batch culture in TY (tryptone/yeast extract) medium [15]. Starvation medium will be made using the OpenWetWare protocol.

Containment System

To prevent bioengineered *B. subtilis* from entering the natural environment as viable spores, an appropriate biological containment system must be incorporated. A passive system based on thymine (or thymidine) auxotrophy will be employed [16]. Thymine is required for the survival and growth of *B. subtilis* and produced with two thymidylate synthases (TSase), which are encoded by two unlinked genes: *thyA* (TSaseA) and *thyB* (TSaseB). Inactivation of *thyA* and *thyB* with chimeric genes can be done with a two-step process as described by Hosseini, Curilovs and Cutting [17]. The transformed colonies will lyse and die without thymine (or thymidine) supplements. Transformants will be selected in exposure to successively higher levels of trimethoprim. One important benefit of this method is that no additional antibiotic resistance genes need to be introduced. This containment system also ensures that viable bioengineered *B. subtilis* or their spores do not enter our food chain and cause potential health problems.

Generation of *Bacillus Subtilis* Competent Cells

To increase the competence of our *B. subtilis* 168, we will be growing the cells in a competence starvation medium under agitation and subsequently induce *comK* expression in a method described by Nijland et al [18]. The *comK* expression will be driven by a multicopy plasmid pLK, containing the *comK* gene and the IPTG-inducible *Pspac* promoter [19]. This plasmid will be inserted into *B. subtilis* 168 via an optimised protoplast transformation method described by Mirabdollah et al [20].

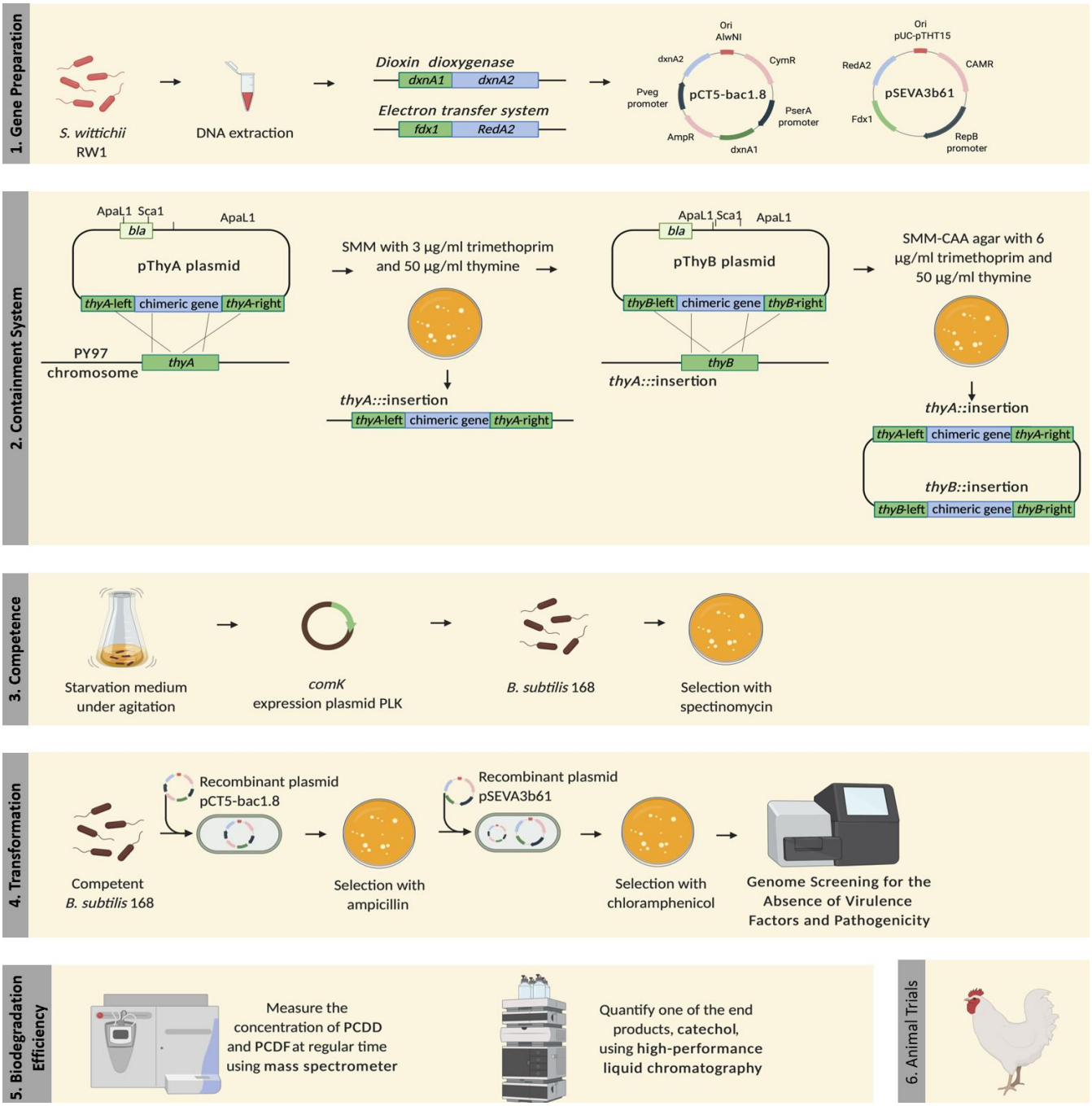

Figure 2. Diagrammatic flow of experimental methods. This figure represents the overall procedure involving creating and testing a recombinant dioxin-degrading *B. subtilis* 168. 1) Gene preparation: genomic DNA of *S. wittichii* RW1 will be extracted, and genes *dxnA1, dxnA2, fdx1, and RedA2* will be amplified via PCR. These genes will be inserted into two distinct plasmids. 2) Containment system: a passive system based on thymine (or thymidine) auxotrophy will be incorporated where *thyA* and *thyB* will be inactivated with chimeric genes. 3) Competence: competent *B. subtilis* 168 cells will be generated and selected. 4) Transformation: pCT5-bac1.8 and pSEVA3b61 will be transformed into competent *B. subtilis* 168 and successful transformants will be selected. 5) Biodegradation efficiency: BE will be assessed using mass spectrometer and high-performance liquid chromatography. 6)Animal trials: clinical trials will be conducted if in-vitro tests are completed and successful.

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Gene Amplification and Preparation

A study by Armengaud et al. elucidated that the expression of active dioxin dioxygenase required coexpression of the dioxin dioxygenase subunit genes *dxnA1*, *dxnA2* and the electron donor system encoded by *Fdx1* ferredoxin and the *RedA2* reductase derived from *S. wittichii* RW1 [19]. Genomic DNA of *S. wittichii* RW1 will be extracted with the Qiagen genomic DNA extraction kit using a method from Armengaud et al [19]. PCR amplification of dioxygenase cistrons (*dxnA1 and dxnA2)* will be carried out with primers AJ118 (ATGGCIAARMGIAAYGCIGT) and AJ124 (CATYTCDATRTARTGIGT) [19]. Primers for the electron transfer system cistrons (*RedA2* and *Fdx1*) will be obtained from Armengaud et al [19].

Construction of Expression Vectors and Transformation

A two-plasmid expression system will be designed with one plasmid pCT5-bac1.8 (Addgene plasmid #119871) from Dr. Tom Ellis's lab containing the genes of the α and β subunits and the other plasmid pSEVA3b61 (Addgene plasmid #58310) from Dr. Tom Ellis's lab containing the genes of the *Fdx1* ferredoxin and the *RedA2* reductase. The inserts will be under constitutive expression with the PserA promoter and the repB promoter for the dioxin dioxygenase and electron transfer system genes respectively. These high copy number plasmids will be inserted into *B. subtilis* 168 via the previous protoplast transformation method in a two step process [20]. The pCT5-bac1.8 transformed *B. subtilis* 168 will be selected with ampicillin plates. Successful transformants will subsequently be inserted with plasmid pSEVA3b61 and selected with chloramphenicol at 6 µg/mL.

Biodegradation Efficiency

To measure the biodegradation efficiency (BE), benchtop mass spectrophotometers will be used to measure the concentration of PCDD and PCDF at regular time intervals [21]. To verify BE, we will also quantify one of the end products, catechol, with high-performance liquid chromatography, shown by Singh et al. [22]. Since 10^7 - 10^8 CFU/kg feed daily is the approximate probiotic dose for broiler chickens, the consumption rate of the anti-dioxin probiotic will be adjusted from this standard depending on the BE of the recombinant bacteria [23]. Lastly, even though *B. subtilis* bacterium is considered as GRAS (generally recognized as safe), phenotypic characterization and genome screening will be done to ensure the absence of virulence factors and pathogenicity [24,25].

Animal Trials

Upon completion and success of our in-vitro testing, recombinant *B. subtilis* will be sent to Eurofins USA, where clinical trials will be performed to further ensure the safety and efficacy of our probiotic product. Depending where the product is released, specific regulations and guidelines will need to be followed; however, European Union (EU) and Scientific Committee on Animal Nutrition (SCAN) regulations should also be consulted as they are well-established [26].

Results

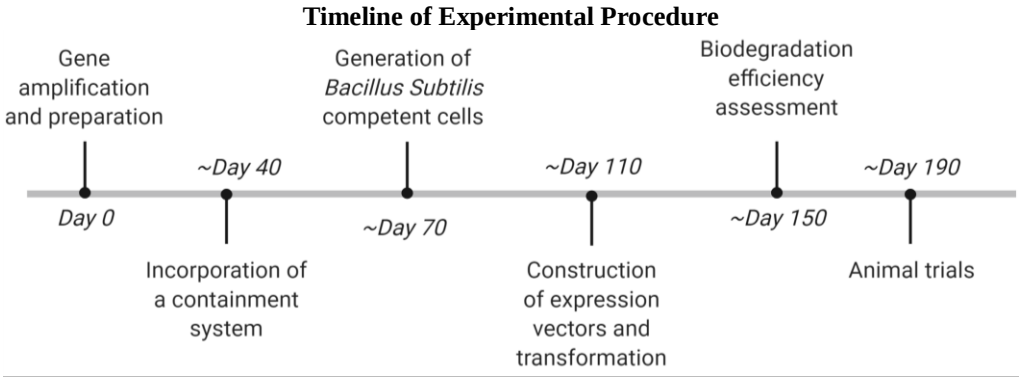

Figure 3. Timeline of the experiment. Time required to optimize and troubleshoot the procedure is included.

We expect no presence of virulence and pathogenic factors in the genome screening of the recombinant *B. subtilis* 168. Bacterial growth on pure PCDD and PCDF and dioxin saturated soil samples from Indonesia will be measured to assess the biodegradation efficiency of the recombinant *B. subtilis* 168. Various control groups will be implemented for this experiment (Table 1). Growth on both types of media will be examined with and without thymine supplements for control groups A-F and recombinant group G. Group A (unaltered *B. subtilis* 168) and B (*B. subtilis* 168 comprising of solely the containment system) will be expected to have unchanged levels of dioxin and catechol in their growth media since they do not carry the dioxin dioxygenase genes along with the electron transfer genes.

Group D (*B. subtilis* 168 comprising of the expression plasmid for the dioxin dioxygenase genes and the containment system) will be expected to have little to no changes in the levels of dioxin and catechol in their growth media since they do not the electron transfer genes to assess the validity of the necessity of electron transfer genes. Group C (*B. subtilis* 168 comprising of 2 expression plasmids without the containment system) and E (unaltered *S. wittichii* RW1) will be expected to have decreased levels of dioxin and catechol in their growth media since they carry both the dioxin dioxygenase genes along with the electron transfer genes. Control groups B and E that carry the containment system and are grown on media without thymine supplements will be expected to be unviable. Dioxin and catechol levels will be measured before experimental growth. We expect to detect an increase in catechol during recombinant *B. subtilis* 168 (Group G) growth on PCDD and PCDF as compared to the control groups A to E and with quantification via liquid chromatography (Table 1). We expect to measure a decrease in PCDD and PCDF levels as compared to the control groups A to E with measurement via mass spectrometry. In the animal trials, we expect no phenotypic or behavioural changes in the poultry. We also expect our laboratory observations in measuring BE to persist into animal trials.

Table 1. Expected Observations of Experimental and Control Groups.

Control Group	Expected Observations
Group A: Unaltered *B. subtilis* 168	Dioxin and catechol levels remain unchanged; no thymine supplement is required.
Group B: *B. subtilis* 168 comprising of solely the containment system	Dioxin and catechol levels remain unchanged; thymine supplement is required.
Group C: *B. subtilis* 168 comprising of the 2 expression plasmids without the containment system	Dioxin and catechol levels are decreased; no thymine supplement is required.
Group D: *B. subtilis* 168 comprising of the expression plasmid for the dioxin dioxygenase genes and the containment system	Dioxin and catechol levels remain unchanged or slightly decreased; thymine supplement is required.
Group E: Unaltered *S. wittichii* RW1	Dioxin and catechol levels are decreased; no thymine supplement is required.
Group F: Recombinant *B.subtilis* 168 comprising of the 2 expression plasmids with the containment system	Dioxin and catechol levels are decreased; thymine supplement is required.

Table 1 depicts the expected changes in dioxin and catechol levels in the control groups A-E grown on thymine and non-thymine supplemented media as well as in the experimental group F.

Discussions

The human tolerable daily intake (TDI) levels for dioxins at International Toxic Equivalent (I-TEQ) as set by EFSA = 0.25 pg TEQ/kg bw/day [27]. This research protocol aims to reduce the chronic dietary exposure to PCDDs and PCDFs via poultry consumption. Future directions could look at reducing the impact of other exposure routes and uncertainties to attain the goal of 0.25 pg TEQ/kg bw/day for countries with a TDI above this criterion.

This study has a few limitations worth noting. There is a lack of research on the BE of dioxin dioxygenase as well as on bacterial transformation utilizing dioxin dioxygenase genes. Therefore, it is difficult to assess the efficiency of our anti-dioxin probiotic as well as the transformation abilities of the dioxin dioxygenase and electron transfer genes.

Research has concluded that in terms of cost and efficiency, good combustion practices is the top method for removing dioxins [3]. However, the impracticality of such practice for large volumes of waste makes minimizing dioxin release costly [3]. Mass production of a recombinant bacterial chicken feed probiotic is expected to be relatively low cost due to the high reproduction rate of bacteria. In addition, the decreased level of dioxins makes export of poultry products possible as many countries have regulations that require dioxin concentrations to be below a specific threshold [6]. Therefore, a novel anti-dioxin feed additive probiotic carries a positive economic implication.

Implications for further research stems from the novelty of creating an anti-dioxin probiotic. While methods for removing dioxins from the environment exist, they are impractical for certain countries [3]. Additionally, there is a lack of methods in literature for removing dioxins from livestock. While this study utilizes the genes of *S. wittichii* RW1 for dioxin degradation as it is the most well studied, it is clear that there is a gap in literature in the study of these enzymes and their application. Further research could also look into advancing the potential of *B. subtilis* as a probiotic. This study focuses specifically on chickens due to the emphasis in current literature on the contamination of chickens and chicken eggs with dioxin as well as the main

form of human contamination being through the ingestion of dioxin-contaminated foods [6,7,8]. In further research, the extension of the creation of an anti-dioxin probiotic for other highly impacted farm animals could be investigated as well.

Conclusions

This proposed research is novel in that it will be utilizing engineered food additive probiotic in detoxification of dioxins for the first time. Current methods for remediating dioxin in the environment are unable to keep up with the rapid accumulation of plastic waste. While previous studies have examined and characterized possible dioxin degrading enzymes, we have identified a large gap in literature in the application of these enzymes. Therefore, the development of an anti-dioxin feed additive probiotic is necessary as a preventative measure, particularly for Asian and developing countries. This study highlights the consequences of waste mismanagement practices. It is hopeful that further research will prompt shifting of societal attitudes towards plastic consumption and legislator attitudes towards waste disposal methods in both developing and developed countries.

List of Abbreviations Used

EFSA: European Food Safety Authority
TDI: tolerable daily intake
PCDDs: polychlorinated dibenzo p-dioxins
PCDFs: polychlorinated dibenzo-furans
POPs: persistent organic pollutants
RedA2: flavoprotein reductase
Fdx1: ferredoxin
GI tract: gastrointestinal tract
BE: biodegradation efficiency
GRAS: generally recognized as safe
EU: European Union
SCAN: Scientific Committee on Animal Nutrition
ATCC: American Type Culture Collection
TY: tryptone/yeast extract

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Ethics Approval and/or Participant Consent

This study will be seeking animal ethics approval from the Animal Care Committee (ACC) of the University of Waterloo. In future steps, the successful recombinant B. subtilis will be sent to Eurofins USA where they will also be screened for ethics approval before conducting in vivo animal clinical trials. All work will conform with the Canadian Council on Animal Care (CCAC) and Animals for Research Act standards and guidelines.

Authors' Contributions

KG: Contributed to study design and planning, optimized the methodology, revised the manuscript critically and gave final approval of the version to be published.
AH: Contributed to study design and planning, optimized the methodology, revised the manuscript critically and gave final approval of the version to be published.
OL: Contributed to study design and planning, optimized the methodology, revised the manuscript critically and gave final approval of the version to be published.

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