Gene Guard Detox Introduction. Over the past 50 years, industrial development has dramatically increased the number and types of toxins that humans are exposed to. Awareness of these threats to human health are also increasing and specific links have been established concerning the negative impacts of pervasive toxic chemicals. These clinical studies are rapidly informing the general population with the warning to avoid exposures to a number of toxins, although to date avoiding such ubiquitous chemical exposure is nearly impossible and no therapeutic options exist to mitigate the harmful effects of the toxins once consumed. Gene Guard Detox mission is to provide simple low-cost solutions to reduce or eliminate the health threats of toxins such as glyphosate, plasticizing chemicals (BPA), forever chemicals (PFOA), dioxins, heavy metals, most of which have been directly linked to elevated disease risk.

Results. Our scientific inquiries began with glyphosate, an herbicide and drying agent used in more than 750 products on the US market, and registered for use in more than 130 countries. Approximately 8.6 billion kg of glyphosate have been applied globally since 1974. It was known that some environmental bacteria are capable of metabolizing glyphosate, converting them to innocuous products. We reasoned that a search for bacteria under the FDA definition of Generally Recognized As Safe (GRAS) would provide a strategy for removing ingested glyphosate in humans as a probiotic. We initiated a screen of more than 100 GRAS isolates for such activity by growing these isolates in the laboratory in the presence of glyphosate. We were able to identify 3 unique isolates that removed 50% or more of input glyphosate. These results were confirmed by replicate experiments. We next turned our attention to the mechanism through which these bacteria removed glyphosate from culture media. We subjected bacterial cultures that grew to saturation in the absence of glyphosate and placed those cells in a salt solution containing glyphosate to determine whether glyphosate was still removed in the absence of growth or metabolism. The results of this experiment showed that after a 2hr incubation at room temperature, comparable amounts of glyphosate was removed compared to that achieved with living cultures. We concluded that glyphosate removal was not due to metabolism but rather through binding of the toxin to the cell. We extended this result by repeating the study using heat killed bacteria to ensure that no metabolism was occurring. This experiment resulted in slightly higher glyphosate removal compared to live cells showing unequivocally that glyphosate removal occurs through binding mechanisms or cellular binding. This result is of high commercial value since the probiotic retains/enhances binding activity after heat inactivation of cells, our strains may be added to a wider number of foods and beverages, thereby broadening the number of potential licensees.

To confirm the efficacy of our *in vitro* findings, we used mice that were fed a standard mouse chow for 1 week. Experimental mice (n=12) were given a probiotic mixture of the three glyphosate binding strains orally once daily for comparison to control mice that were not (n=12). After the 1-week intervention, stool, serum and liver were collected for glyphosate measurements. We noted that stool from probiotic fed mice contained 40% more glyphosate compared to control mice. This observation is important as it indicates a reduction in glyphosate absorption by the mice. This interpretation was consistent with results showing that serum from probiotic fed mice contained more than 50% less glyphosate as control mice. Finally, we noted that liver samples from probiotic fed mice contained 20% less glyphosate compared to control

mice. This result is potentially highly significant as it suggests that by reducing the absorption of dietary glyphosate, mice were able to detoxify peripheral tissues that had accumulated glyphosate over time. We anticipate that when repeated over a longer time frame, these results will display larger reductions in tissue glyphosate, thereby enhancing the potential health benefits of our product offerings.

We next turned our attention to the most common plasticizer, Bis phenol A (BPA). This toxin has been unequivocally shown to be an endocrine disruptor and promoter of various cancers. Using the same 3 strains that bound glyphosate, we observed that strain 1, removed 50% of input BPA, whereas the other strains 2 and 3 removed over 60% of input BPA. Importantly, heat killed bacteria displayed enhanced BPA binding, removing over 70% of input BPA. Average human exposures to BPA are estimated to be 0.68 micrograms/day. Based on our findings, we anticipate that BPA removal in humans may approach 100% as our *in vitro* results used significantly higher input quantities of BPA.

Future studies and expectations.

Gene Guard Detox has defined several goals to expand the breadth of toxin remediation products that can be commercialized over the next year.

1. **Toxin target screening.** The EPA has defined the top 10 environmental toxins. We used this list to guide our future experimentation **Table 1**.

Compound	Examples	Status
glyphosate	Glyphosate	Completed *in vitro* and *in vivo*
phthalates	Bisphenol A (BPA), di-n-butyl phthalate (DBP), butyl benzyl phthalate (BBP), Polyvinyl chloride (PVC)	Completed BPA *in vitro*
PFAS	Perfluorooctanoic acid (PFOA), Perfluorooctanoic sulfonic acid (PFOS)	PFOA in progress *in vitro*
PCBs	Polychlorinated biphenyl (PCB), 2,2',3,3'-tetrachlorobiphenyl	Future testing
dioxins	Polychlorinated dibenzo-p-dioxins (PCDD), polychlorinated dibenzofurans (PCDF)	Future testing
Volatile organic compounds	Benzene, ethylene, tetrachloroethylene	Future testing
DDT	Dichlorodiphenyltrichloroethane	Future testing

paraquat	N, N'-dimethyl-4,4'-bipyridium dichloride	Future testing
neonicotinoids	Imidacloprid, Thiamethoxam, Clothianidin	Future testing

These toxins will be used to challenge live and heat-killed cultures derived from a bank of GRAS isolates numbering >100 including predominately *Lactobacillus* spp. and *Bifidobacterium* spp.. In all cases, our goal will be to achieve binding activity that approximates or exceeds the daily dietary exposures that humans are exposed to. We are optimistic of success in these efforts since these toxins share chemical properties with one another, making it more likely that the positive results obtained to date will translate to yet to be tested toxins and additional successes. We will also expand our bank of GRAS isolates to ensure that we are able to identify the best binding activities available and seek proper patent protections for these applications.

2. **Prebiotic identification.** While we envision that most licensees of our products will use them in the form of post-biotics (heat inactivated probiotics), a significant segment of users will use them as traditional probiotics. As such, we intend to enhance our IP by defining proprietary formulations that further enhance binding capacity of future probiotic products. Based on our results to date, the number of probiotic cells is directly proportional to the total amount of toxin that may be bound. Prebiotics are compounds that promote the growth of select bacteria. In the gut, input probiotics must compete with the multitudes of resident bacteria. Prebiotics that enhance the relative abundance of our input probiotic strains are expected to increase total toxin binding activity.

   We will conduct *in vitro* anaerobic cultivation of human stool with a variety of prebiotics including inulin, GOS, FOS, lactose, lactitol and others to identify which prebiotics. The stool preparations will be spiked with our isolates at ~0.1% of total bacteria. Prebiotics are food for bacteria and those selected are expected to enhance the representation (number) of our strains in the digestive tract. We will confirm the impact of each prebiotic using DNA sequencing technology. The resulting sequence data will be used to compare control cultures containing our isolates to those treated with prebiotics to identify those that have the greatest impact on elevating our strains' abundance in the community. We expect lactose to perform the best as a prebiotic for our strains, however inclusion of additional prebiotics is important as it provides an opportunity to offer prebiotic alternatives in formulations to accommodate those subjects with lactose intolerance.

3. **Verification of prebiotic effect in rodent model.** Mice (n=6) will be used to test prebiotic effects *in vivo.* On day 1-7 mice will be provided $1X10^8$ probiotic strains by oral gavage daily. Prebiotic identified in the previous goal will be provided in the drinking water at 1% concentration. Fecal samples will be collected on day 0, day 2, 4, 6 and 7. These fecal pellets will be used to isolate genomic DNA from communities formed. The V3-V4 region

of 16S rDNA will be amplified by PCR for library construction and sequenced. The resulting sequence data will be used to compare control cultures containing 3 isolates to those treated with prebiotic to confirm activity *in vivo.*

4. **Additional Mouse studies.** Based on investor feedback, additional mouse testing for longer durations will be carried out. A similar design as used previously will be conducted supplementing drinking water with compounds that mice colonies are unlikely to have exposure to. These studies will be carried out for an extended period (4-6 weeks) compared to our 1-week trial to enhance overall effects, primarily the assessment of tissue detoxification resulting from probiotic reductions of daily absorption. The number and type of probiotic strains used in this study will be determined based on the *in vitro* binding results obtained. We expect that this intervention will result in similar levels of increased stool toxins being observed coupled to decreased serum levels of toxins analyzed. By extending the trial period, we expect to observe increased tissue detoxification since peripheral tissues will experience an extended period of time wherein toxins are not absorbed to appreciable extents, thereby allowing tissues to naturally engage in detoxification of toxins, resulting in reduced stores of toxic compounds.

5. **Human clinical trials.** It is our expectation that the results obtained *in vitro* and in mouse studies will translate to human usage. We see value in conducting human trials to fortify our claims to consumers and generate quantitative data pertaining to the efficacy of toxin remediation. This trial will be designed to enroll 50 human subjects that consume a standard western diet (non-organic foods). Since tissue analysis is not feasible, we will assess pre- and post-intervention toxin levels to assess the efficacy of post-biotic interventions by analyzing stool, urine and serum samples. Human subjects under written informed consent will take post-biotic doses twice daily with lunch and dinner meals for 8 weeks. We expect that the levels of toxin in stool to be low in pre-intervention analysis and higher once the intervention begins and remain steady throughout the trial. We expect urine levels to be high in pre-intervention analysis and lower in post-intervention measurements. We expect serum levels to be high in pre-intervention analysis and substantially lower in post-intervention measurements.

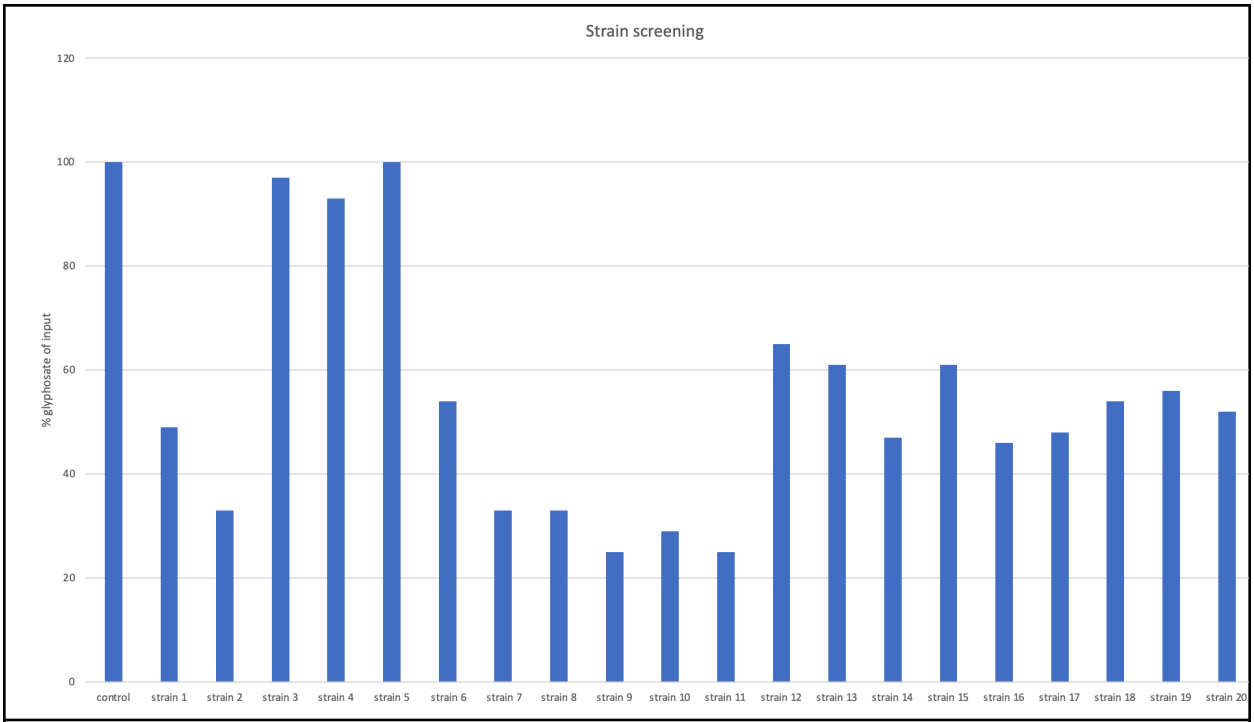

Figure 1. GRAS isolate screening. We screened a bank of GRAS isolates and rescreened those with apparent activity as shown. The strains displaying the greatest reduction in glyphosate were identified and sequenced. Redundancy was then eliminated resulting in 3 strains for further testing.

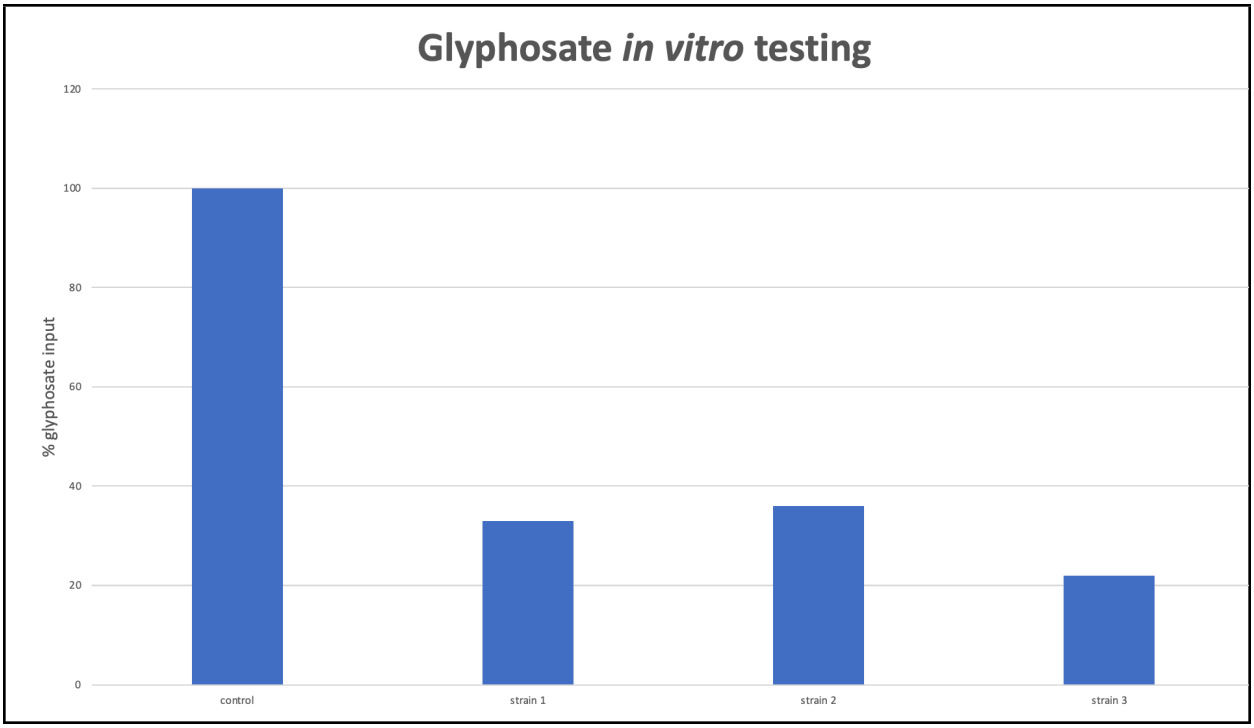

Figure 2. Best isolates for glyphosate removal. Repeat testing using 3 best isolates. Media containing 1 mM glyphosate were grown overnight with each of 3 isolates to confirm glyphosate removal.

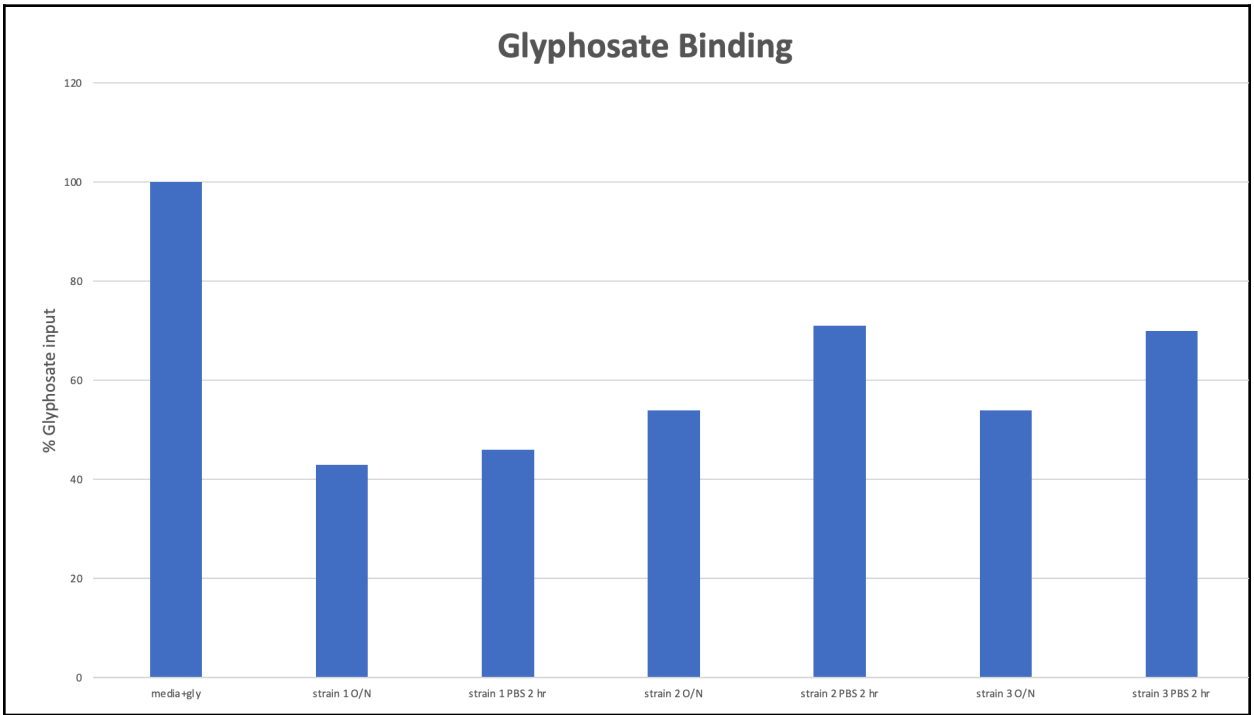

Figure 3. Mechanism of glyphosate reduction. To determine whether glyphosate removal was based on metabolism (degradation), we cultured isolates in 1 mM glyphosate O/N to be compared with the same number of cells in PBS incubated with 1 mM glyphosate for 2 hr at RT. The results showed comparable glyphosate removal.

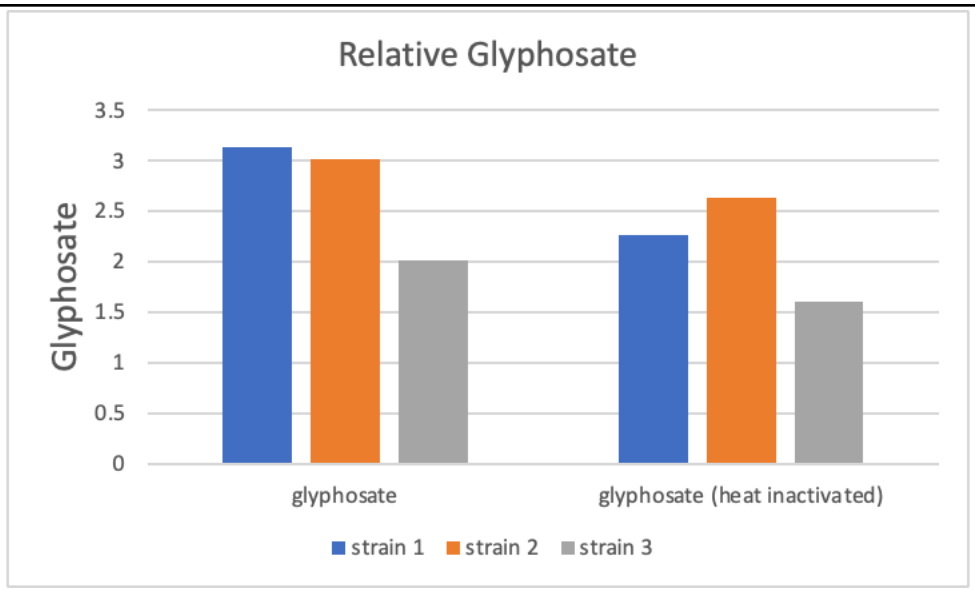

Figure 4. Heat inactivation of isolates. Mechanism of glyphosate removal is through cell surface binding. Activity is retained and elevated in heat inactivated cells

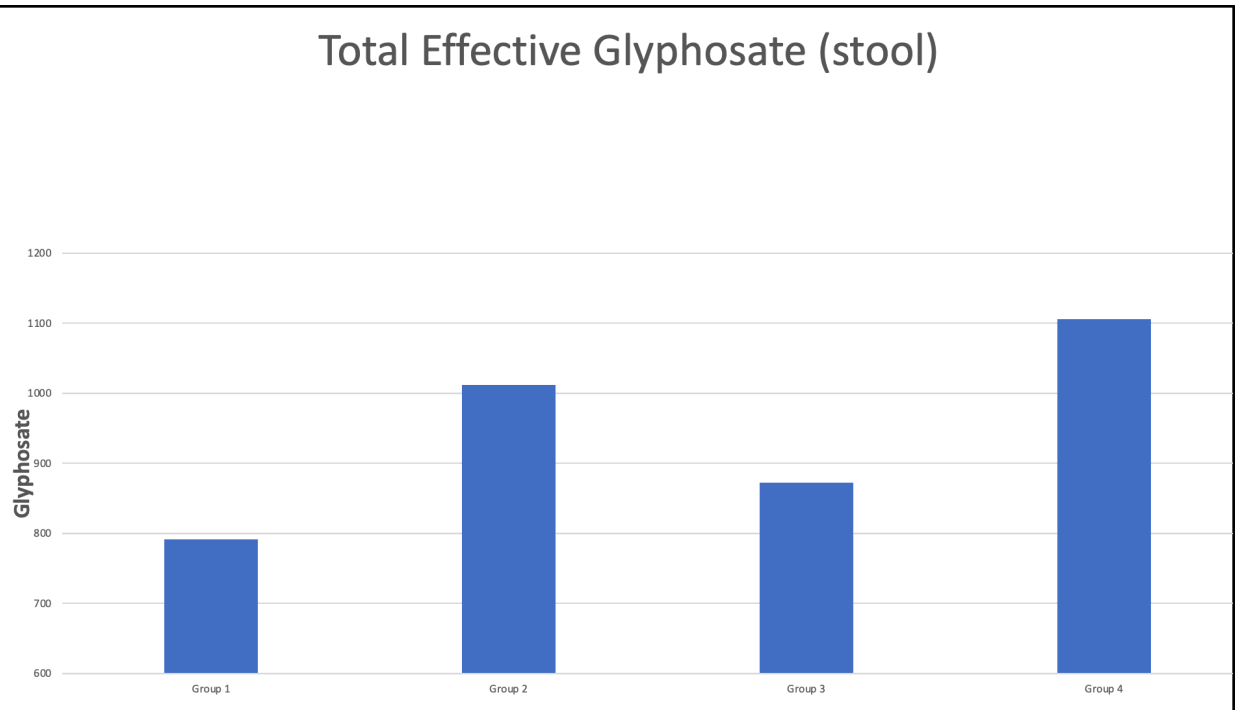

Figure 5. ***In vivo testing:*** **Mouse Study Design and stool glyphosate.** C57Bl/6 wild-type mice were randomly divided into 4 groups (n=12). All mice consumed standard chow containing glyphosate (not added exogenously). Experimental mice (groups 2-4) received probiotic once daily orally. **Group 1**: served as control, provided regular chow without probiotic. **Group 2**: provided regular chow with a mixture of 3 probiotic strains ($1X10^6$ cells). **Group 3**: provided regular chow with a mixture of 3 probiotic strains ($1X10^7$ cells). **Group 4**: provided regular

chow with a mixture of 3 probiotic strains ($1X10^8$ cells). After 7 days, stool, serum and liver tissue were collected for glyphosate measures. **Conclusion:** Mice receiving probiotic strains retain higher glyphosate (40%) in stool (group 4) due to binding, less absorbed in the intestine.

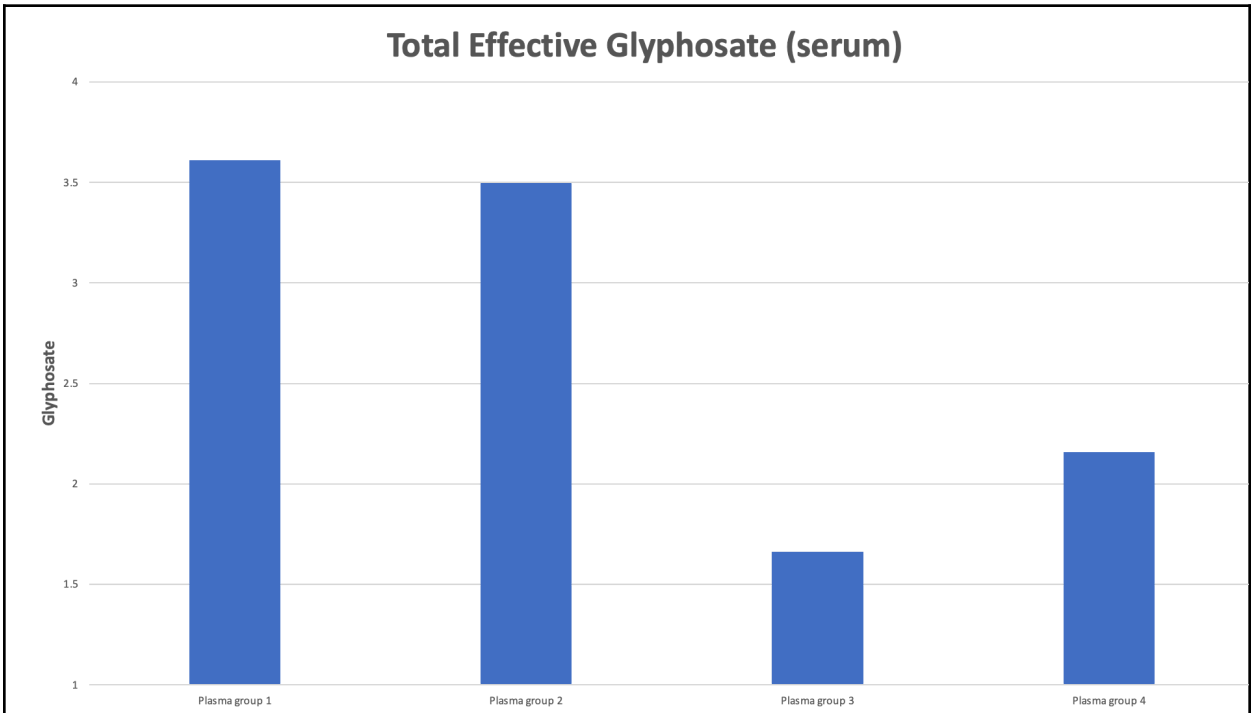

Figure 6. ***In vivo testing:*** **Serum glyphosate.** After a 1-week intervention, glyphosate levels in the serum were reduced by >50% (group 3), consistent with stool results and reduced absorption.

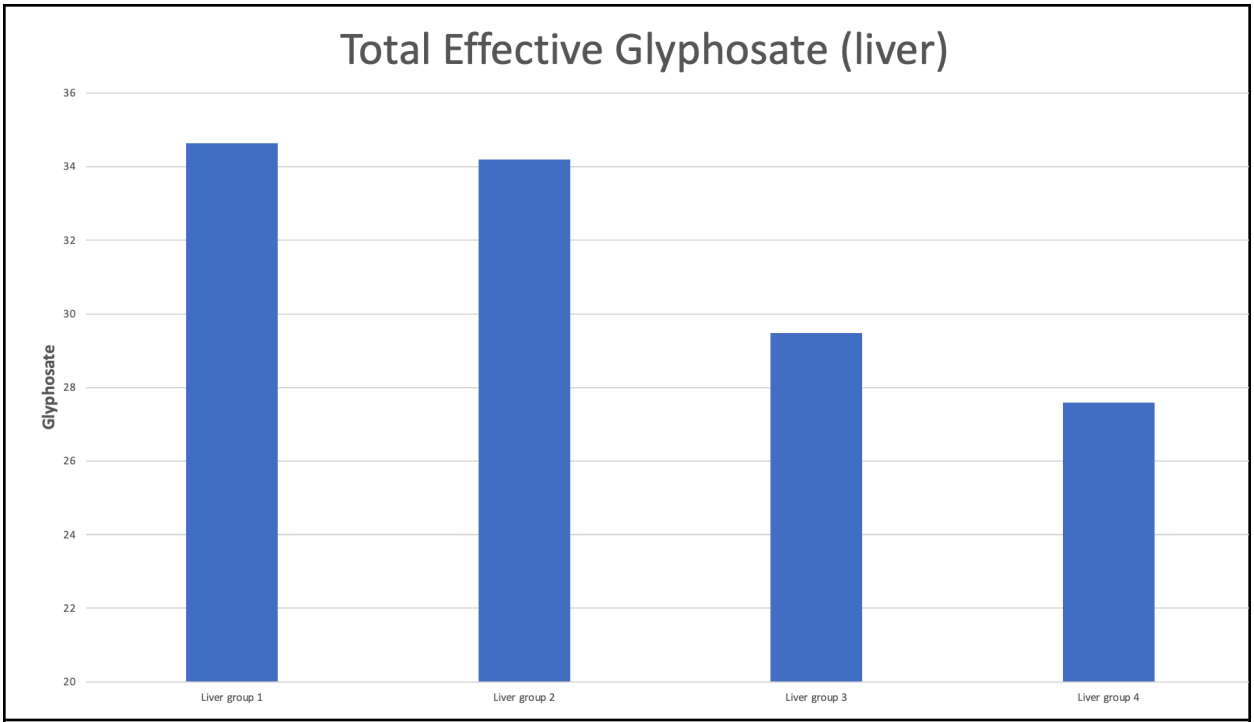

Figure 7. ***In vivo testing:*** **Liver glyphosate.** After a 1-week intervention, glyphosate levels in the liver were reduced by 20% (group 3), suggesting a tissue detoxification.

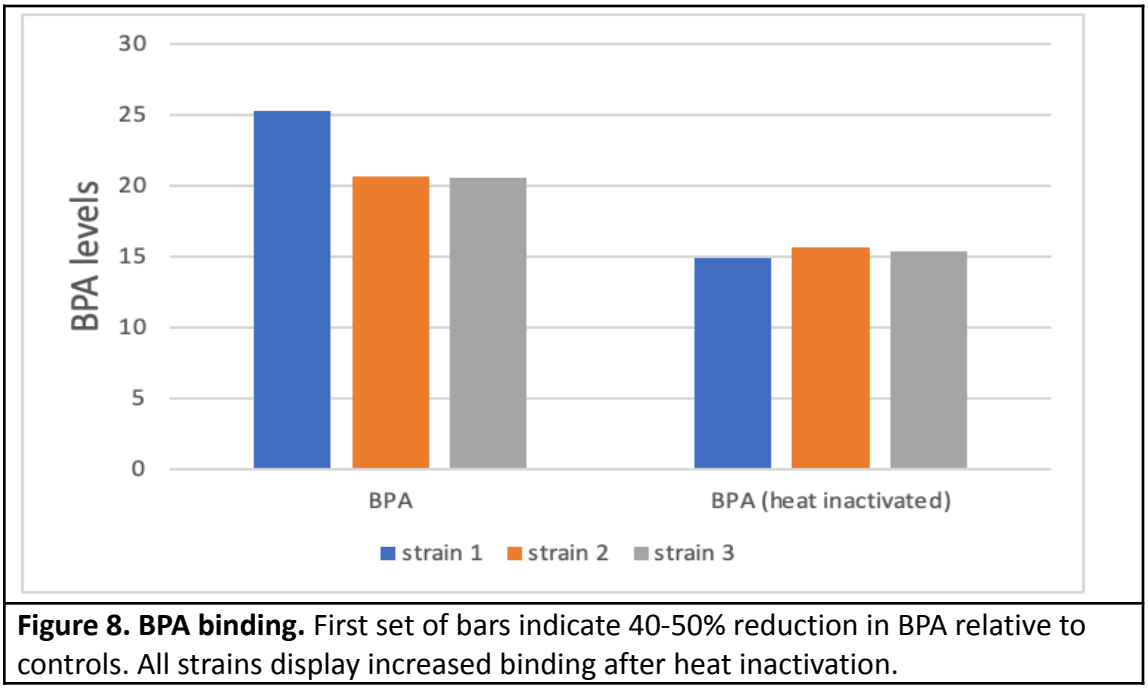

Figure 8. BPA binding. First set of bars indicate 40-50% reduction in BPA relative to controls. All strains display increased binding after heat inactivation.