Effect of Probiotic-Based Vaccination on Lactate Availability in Immune Inductive Tissues

Honors Thesis

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Abstract

Lactic acid bacteria (LAB) colonize the gastrointestinal tract and generally confer immune benefits to their host. Because of this, they are emerging as viable vaccine vectors, particularly against mucosal pathogens. LAB secrete high quantities of lactate as a byproduct of their metabolism, and lactate has been increasingly recognized as an immunomodulator, often in an anti-inflammatory capacity. We are studying how lactate metabolism of LAB-based vaccines affects lactate availability in immune inductive sites using the bacterial platform *Lactobacillus acidophilus*. Using Mirasol® Pathogen Reduction Technology system, we inactivated a recombinant *L. acidophilus* expressing the model antigen ovalbumin. We first verified that inactivation inhibited bacterial growth and reduced lactate metabolism while preserving antigen expression. We then administered the live and inactivated vaccines orally to BALB/c mice and measured lactate concentrations in harvested immune inductive sites (Peyer's patches and ileal lumen contents) using gas chromatography mass spectrometry. Lactate levels in the ileum and Peyer's patches were unchanged following administration with probiotic-based vaccines, suggesting their administration does not disrupt the metabolic equilibrium of these tissues that may trigger an anti-inflammatory state.

Introduction

Probiotic vaccines

Vaccines are critical for preventing disease, and are estimated to save 4 million lives each year (CDC, 2025). Vaccines provide protection from disease by introducing the host's immune system to an antigen, a unique piece of a pathogen that the host targets in an adaptive immune response. By vaccinating against these antigens, individuals generate an immune response that protects them against future infections by that specific pathogen. There are many types of FDA-approved vaccines, including live-attenuated, protein conjugated, and more recently mRNA vaccines (Research, 2025).

Recently, probiotic-based vaccines have been developed as promising novel vaccine candidates that confer unique benefits. Probiotic-based vaccines are bacteria that have been modified to present antigens. In this model, the bacteria retain the health benefits of being natural probiotics while becoming vectors for the antigen(s) of interest. These innate probiotic benefits notably includes stabilizing the gut microbiome, whose composition is correlated with improved immune responses and vaccine effectiveness (Zimmermann & Curtis, 2018). Practically, probiotic-based vaccines are easy to produce, store and transport at room temperature, and administer orally or intranasally (LeCureux & Dean, 2018; Vilander & Dean, 2019). They traffic via gut-associated lymphoid tissues (GALT) such as Peyer's patches to specifically elicit mucosal immune responses, demonstrated by higher secretory IgA (sIgA) titers than comparable intramuscular vaccines (Leontieva et al., 2024).

Commonly used probiotics in vaccines are *Lactobacillus* sp., or lactobacilli. They are classified by the FDA as generally-regarded as safe (GRAS) and are commensal organisms that are highly prevalent in the natural gut microbiome (Stoeker et al., 2011). They modify the host environment to protect against pathogens by producing inhibitory compounds, reducing toxin availability, competing for nutrients and adhesion sites, stimulating mucus production, and stabilizing the epithelial barrier (Vieco-Saiz et al., 2019; Tang et al., 2023). Lactobacilli are acid and bile resistant and are agonists of pathogen recognition receptors (PRR), including TLR-2, NLRs, and C-type lectin receptors (Vilander & Dean, 2019). As adjuvants, lactobacilli can stabilize or increase pro-inflammatory cytokines levels as well as T-cell stimulation (Sørensen et al., 2019; Alqazlan et al., 2021; Hart et al., 2004). When used in probiotic-based vaccines, lactobacilli can elicit immune responses with IgM, IgG, and IgA antibodies (Zhang et al., 2025; Chau et al., 2023; Abdo et al., 2019).

Lactobacilli are easy to genetically manipulate, making them ideal platforms for a probiotic-based vaccine with co-expression of antigen and adjuvant(s) (Kajikawa et al., 2012; Vilander & Dean, 2019). This study utilizes a recombinant *Lactobacillus acidophilus* (rLA) as a model probiotic-based vaccine. rLA has been engineered to express three components: (1) chicken ovalbumin (Ova) peptide 323-339, (2) *Salmonella enterica* serovar Typhimurium flagellin (FliC), and (3) *Escherichia coli* type I fimbriae (FimH). Ova 323-339 is a well-established antigenic peptide that binds major histocompatibility complex (MHC) class II and strongly induces T cell responses to Ova (McFarland et al., 1999; L.-Z. Sun et al., 2010). FliC and FimH are incorporated as adjuvants to boost the efficacy of the probiotic-based vaccine (Vilander & Dean, 2019). FliC is a TLR5 agonist and induces NF-kB activation (Kajikawa et al., 2012). FimH is a TLR4 agonist and enhances adhesion to microfold (M) cells, improving vaccine localization to Peyer's patches (GALT) such as Peyer's patches (Vilander et al., 2023).

Lactate and inflammation

Lactobacilli are classified as lactic acid bacteria (LAB), and a key feature of their metabolism is their high production of the metabolite lactate. Lactate stabilizes the gut microbiome and, notably, is associated with reduced acute inflammation (Louis et al., 2022; Caslin et al., 2021; Li et al., 2022). Immunomodulation by lactate can be mediated through receptors and transporters on a variety of immune cells, both innate and adaptive (Yang et al., 2020; Haas et al., 2015). Lactate can affect cytokine production, phenotype polarization, and even changes in epigenetic lactylation patterns (S. Sun et al., 2021).

LAB probiotic-based vaccines have been demonstrated to be effective (see above), but it remains unclear if their high lactate secretion may be counterproductive to a vaccine's intended inflammatory response. For example, high concentrations of lactobacilli in the gut microbiome have been associated with reduced vaccine effectiveness (Harris et al., 2017). In the context of lactobacilli, homofermentative LAB, which only product lactic acid, induce greater regulatory phenotypes than heterofermentative LAB (De Roock et al., 2010; Vissers et al., 2010). These phenotypes include T-regulatory (Treg) polarization, anti-inflammatory cytokine production, and downregulation of activation markers. *Lactobacillus acidophilus*, the species used in the construction of rLA, is a homofermentative LAB (Anjum et al., 2014).

On the tissue level, lactate concentrations can be modified during infection to support maintenance of the pathogen. For example, infection with *Salmonella enterica* serovar Typhimurium elevated lactate levels in the cecum by an approximate factor of 3 (Gillis et al., 2018). Therefore, it is important to establish the effect of administration with both a live and inactivated LAB probiotic-based vaccine may have on lactate concentrations, particularly in immune inductive sites such as Peyer' patches. Inactivation reduces lactate secretion, and it establishes whether shifts in lactate concentrations are attributable to lactate secreted by the administered LAB or due to metabolic changes in the host or other microbiota.

Vaccine inactivation

Our construct was inactivated using Mirasol™ Pathogen Reduction Technologies (PRT), a UV-directed inactivation system. UV inactivation of lactobacilli can enhances immune responses (Van Hoffen et al., 2010), and Mirasol™ PRT improves upon traditional UV inactivation with the addition of riboflavin. Riboflavin, better known as vitamin B2, is a safe addition when generating a probiotic-vaccine and can be degraded to FMN and FAD coenzymes (Goodrich et al., 2006). UV light and riboflavin produce greater DNA fragmentation, degradation, and base modifications than UV light alone (Goodrich et al., 2006).

Inactivation via DNA damage prevents cell replication while preserving protein structures. This maintains stably expressed proteins, such as epitopes on probiotic-based vaccines, while reducing activity of enzymatic proteins in active pathways such as that of lactate metabolism. We hypothesize that lactate secretion by rLA increases lactate concentration in immune inductive tissues while inactivated rLA (IrLA) does not change lactate concentration. To establish this, we evaluated lactate secretion of IrLA *in vitro* and its effect on lactate availability *in vivo.*

Methods

Inactivation

Table 1: Features of relevant constructs

Strain	**Description and origin**	**Reference**
Lactobacillus acidophilus NCFM	Human intestinal isolate	(Altermann et al., 2005)
NCK56	Wild type	(Kajikawa et al., 2012)
GAD390	*slpA-OVA323-339*-expressing	This study
	pTRK1034-fliC-expressing	(Kajikawa et al., 2011)
	N-term-fimH-expressing	(Vilander et al., 2023)

NCK56 and GAD390 (Table 1) were grown in 5 mL cultures overnight, passaged into 45 mL pre-warmed media overnight, and then passaged up to 350 mL final volume for 3.5 hours for the bacteria to reach exponential phase. NCK56 was grown in MRS media (BD; 288130) while GAD390 was grown in MRS media supplemented with 5 μg/mL erythromycin (Fisher Biotech; BP920-25). For the final passage, 7 mL NCK56 was added to 343 mL media while 42 mL GAD390 was added to 308 mL media. After 3.5 hours, concentration was evaluated using OD600 (SmartSpec™ 3000; Bio-Rad; 269BR) and both cultures were resuspended to a final concentration of 1 x 10^8 CFU/mL in 300 mL media. Cultures were kept on ice until inactivated.

The full volume of cultures plus 35 mL riboflavin (Terumo BCT) were transferred to platelet bags (Terumo BCT; 10090) compatible with Mirasol™ PRT system. The riboflavin was exposed to as little light as possible prior to inactivation, and the maximum number of bubbles were removed from the platelet bag. The bacteria were inactivated with 5000 J of energy using standard instrument settings. The inactivated samples were frozen at -20°C.

Evaluation of inactivation

Bacterial replication, epitope expression, and lactate secretion were used as metrics of the success of the inactivation. Together, these indicate the viability of the bacteria as well as its stability as a vaccine vector.

Replication

NCK56 and GAD390 were plated (N=2) to evaluate colony growth from live, riboflavin supplemented, and inactivated cultures. NCK56 was grown on MRS with 15 g/L agarose (Difco™; BD; 214530) and GAD390 was grown on MRS with 15 g/L agar and 5 µg/mL erythromycin. 100 µL of cultures were plated at concentrations of 1:1,000 and 1:10,000 in 1X PBS using EddyJet spiral plater (IUL) using Log Mode 100 µL. CFU/mL were calculated using SphereFlash (IUL; software version 1.0.1.15) using the Transmitted Fuzzy template (input values = 0, 85.0, and 0.3) and inoculation mode Log Mode 100 µL.

Epitope expression

Epitope expression following inactivation was evaluated using flow cytometry. NCK56 and GAD390 cultures were grown in 5 mL cultures at 42°C overnight and then passaged up to 50 mL final volume overnight. NCK56 was grown in MRS media while GAD390 was grown in MRS media supplemented with 5 µg/mL erythromycin. For the final passage, 1 mL NCK56 was added to 49 mL media while 6 mL GAD390 was added to 44 mL media. iGAD390 samples were thawed overnight at 4°C. Live and thawed inactivated cultures were centrifuged (Allegra X-15R; Beckman Coulter) at 3700 RPM (3186 x g) at 12°C for 10 minutes and washed twice with 25 mL 1X PBS. Concentration of cultures was evaluated using OD600. For each culture, 1×10^7 cells in 100 µL was added to a 96-well V-bottom staining plate (Caplugs; 333-8001-01V) and centrifuged at 3700 RPM for 10 minutes. Media supernatant was flicked into waste. Cells were resuspended and stained with the primary antibody followed by the secondary antibody in 1% BSA (Equitech-Bio; BAH65) with 1:1000 Kathon (SupelCo; 5-00135) for 20 minutes on ice while rocking (AROS160™ orbital shaker; Thermolyne; speed = 90). Between stains, cells were washed twice with 100 µL 1X PBS (Corning; 21-040-CV) at 3700 RPM for 5 minutes. After the final wash, cells were resuspended in flow buffer containing 1X PBS, 1% BSA, 5 mM EDTA (Corning; 46-0350CI), and 0.5% sodium azide (Sigma-Aldrich; S-2002). 5 µL propidium iodide (Invitrogen; P-21129) was added to evaluate membrane permeability of samples. Unstained and secondary-antibody-only negative controls were included.

Table 2: Summary of flow cytometry reagents used in evaluation of epitope expression

	Reagent	**Vendor**	**Catalog**	**Concentration**	**Laser**
Primary antibodies	Rabbit anti-chicken Ova 323-339	Alpha Diagnostic	OVA3231-A	1:100	NA
	Mouse anti-FliC	BioLegend	629702	1:200	NA
	Mouse anti-FimH mAb824	Gifted by Dr. Evgeni V. Sokurenko	N/A	1:1000	NA
Secondary antibodies	Donkey anti-rabbit FITC	BioLegend	406403	1:100	FL-1
	Goat anti-mouse PE	Invitrogen	P-21129	1:100	FL-2
Viability dye	Propidium iodide	Thermo Scientific	BMS500PI	N/A	FL-4

Samples were filtered using 40 µm filter caps (Greiner Bio-One; 542040) and then transferred to flow dilution tubes (Genesee Scientific; 14-360). Tubes were vortexed and then placed in the flow cytometer (Gallios™; Beckman Coulter). Flow cytometry analysis was completed using FlowJo™ (software version 10.8.1), filtering to exclude debris based on extremely low forward scatter/side scatter. Gates were set so that all negative controls (unstained, secondary-antibody-only, and NCK56 wild type bacteria) were less than 2% positive for any GAD390-specific epitope.

Lactate concentration

Lactate secretion in bacterial media

Each live and inactivated sample was resuspended to 2.5 x 10^{10} CFU/mL in STI buffer, composed of 20 mg/mL soy trypsin inhibitor (Sigma-Aldrich; T9128-10G) and 8.5 mg/mL bicarbonate buffer (EMD; SX0320-1). 200 µL was added to 4.8 mL media and cultured for 4 hours. NCK56 was grown in MRS media and GAD390 was grown in MRS media supplemented with 5 µg/mL erythromycin. Cultures were centrifuged at 3700 RPM for 5 minutes. The supernatant, containing secreted lactate, was filtered using a 40 µm filter cap and 0.22 um syringe filter (Celltreat; 229746). Samples were stored at -20°C.

Lactate concentrations of the samples were evaluated using gas chromatography mass spectrometry (GC-MS), conducted by Colorado State University's Analytical Resources Core (https://www.research.colostate.edu/bio/, RRID: SCR_021758). The following experimental methods were provided by the ARC staff:

Succinic acid-13C4 and lactic acid standard were purchased from Sigma-Aldrich. N-methyl-N-trimethylsilyltrifluoroacetamide with 1% trimethylchlorosilane (MSTFA) was purchased from Thermo Scientific. Solvents mentioned elsewhere were purchased from Fishersci.

Samples were first randomized and each sample was assigned with a new BIO-ID for processing. Thawed samples were briefly vortexed and mixed with methanol in a 1:4 ratio. The mixture was mixed, incubated at -20°C for 3 h, and then centrifuged at 15,000 g and 4°C for 10 min. After centrifugation, 50 µL of supernatant was diluted with 950 µL of 80% methanol. Then 10 µL of diluted sample was added with 50 µL of internal standard solution containing 50 ug/mL of succinic acid-13C4 in 50% methanol, mixed, and dried under nitrogen for 2 hours.

The dried sample extracts were resuspended in 50 µL of pyridine and 50 µL of MSTFA, and samples were vigorously vortexed for 30 s, incubated at 60 °C for 30 min, followed by centrifugation at 3,000 g x 10 min at 4°C. The supernatants of derived samples were transferred to glass inserts and stored at 4°C until analysis. A QC sample was generated by combining a small aliquot of each study sample, which was then split into aliquots of 50 µL to be injected after every 3 study samples. Metabolites were detected using a Trace 1310 GC coupled to a Thermo ISQ mass spectrometer. Samples (1 µL) were injected at a 10:1 split ratio to a TG-5MS column (30 m x 0.25 mm x 0.25 µm, Thermo) with a 1.2 mL/min helium gas flow rate. GC inlet was held at 285°C. The oven program started at 80°C for 30 s, followed by a ramp of 15°C/min to 330°C, and an 8 min hold. Masses between 50-650 m/z were scanned at 5 scans/sec under electron impact ionization. Transfer line and ion source were held at 300 and 260°C, respectively.

GC-MS data was processed using Chromeleon 7.2.10 software (Thermo Scientific). The quantification/confirming ions and GC retention time for each analyte can be found in Table 3. Peak areas were extracted for target compounds detected in biological samples and normalized to the peak area of the appropriate internal standard or surrogate in each sample. Absolute quantitation (mg/mL) was calculated using the quadratic equation generated from the calibration curve.

Table 3: GC-MS data processing method

Compound	Retention time (min)	MS quantification m/z	MS confirming m/z
Lactic acid	4.19	191	219
Succinic acid-13C4	6.49	251	176

Lactate in ileum and Peyer's patches

This phase of the experiment was completed with IACUC approval (Colorado State University, #4463, 5/23/23). 20 BALB/c mice from Jackson laboratories (10 male/10 female) were maintained by Colorado State University's Lab Animal Resources (https://www.research.colostate.edu/LARHome/, RRID: SCR_022157) with free access to food and water. They were housed in pathogen-free conditions in commercially available individually ventilated cages with autoclaved bedding and enrichment. Mice were monitored daily, and all were euthanized at the end of the study by carbon dioxide inhalation followed by cervical dislocation.

200 µL of each live and inactivated sample resuspended to 2.5 x 10^{10} CFU/mL in STI buffer was administered to BALB/c via oral gavage (Instech; FTP-20-38 and BD; 309659). After 4 hours, mice were sacrificed and their small intestine harvested and placed in 5 mL 1X PBS on ice. The last 4 cm of the small intestine, an approximate for the ileum, was collected. The ileum was flushed with 1X PBS using a 30-gauge needle (BD; 305106) and the ileal fluid collected. Peyer's patches were harvested and placed in 1 mL 80% methanol (Fisher Chemical; A412P-4) in DI water (MilliQ BioPak; CDUFBI0A1) in bead beating tubes for tissue processing (Fisher Scientific; 15-340-151). The ileum was then sliced open, and mucus was collected manually and placed in the tube with ileal fluid. The ileal fluid was centrifuged at 1500 rpm for 5 minutes and its supernatant was filtered through 40 µm filter cap and 0.22 µm filter syringe. The Peyer's patches samples and ileal fluid samples were stored at -20°C.

Lactate concentrations of the samples were evaluated using GC-MS conducted by Colorado State University's Analytical Resources Core as above, with the following modifications:

Prior to incubation, Peyer's patch samples were homogenized using a Fisherbrand Bead Mill 24, with 5 cycles of processing at a speed of 4 m/s for 15 seconds per cycle, with a 5 second dwell time between cycles. 100 µL of supernatant were added to 50 µL internal standard solution. For ileal fluid samples, 200 µL of supernatant were added to 50 µL internal standard solution.

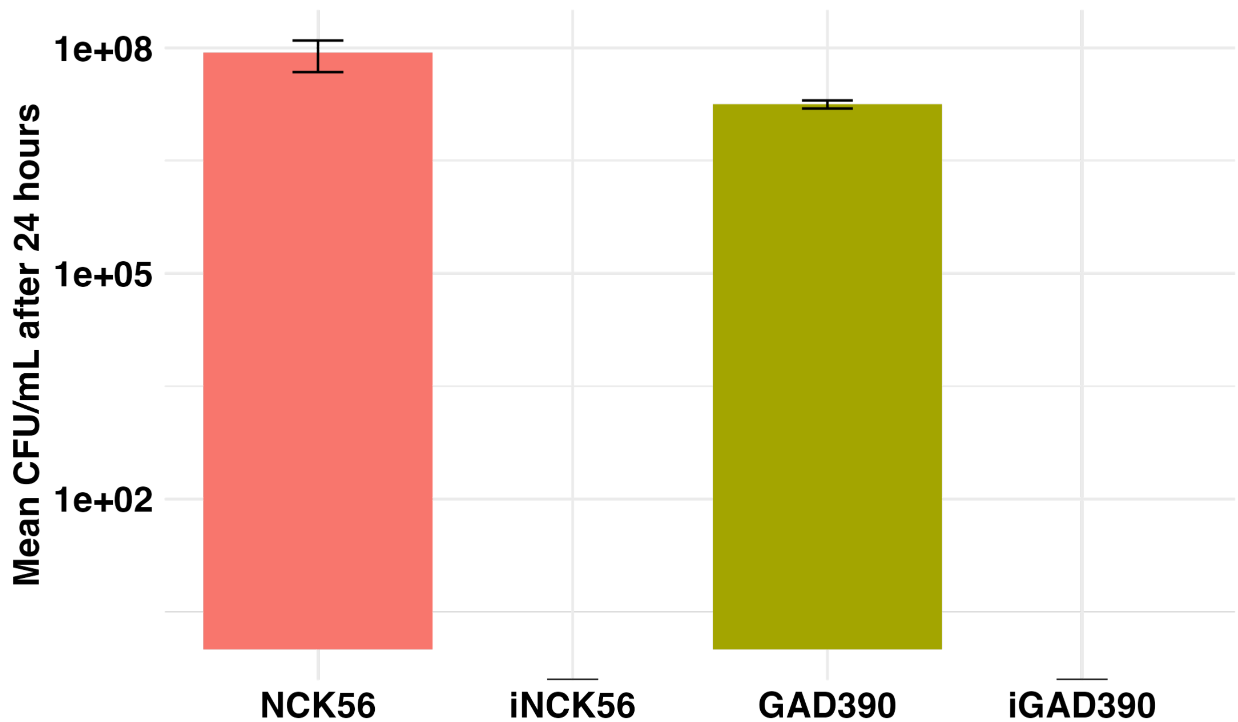
Results

Evaluation of inactivation

<u>Replication</u>

The ability of the bacteria to replicate and produce colony forming units was severely inhibited (Figure 1). At both 1,000-fold and 10,000-fold dilutions, NCK56 and GAD390 reached near 1 x 10^8 CFU/mL, the anticipated concentration based on optical density. All inactivated samples (iNCK56 and iGAD390) produced 0 colonies after both 24 and 48 hours.

Figure 1: Inactivation eliminated replication of NCK56 and GAD390. Replication was evaluated 24 hours following inactivation based on CFU/mL cultured on MRS plates (N=2). Data presented as Mean ± SD. Figure and statistical analysis generated using RStudio (software version 2023.12.1). NCK56 = live wild type L. acidophilus*; iNCK56 = inactivated NCK56; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390.*

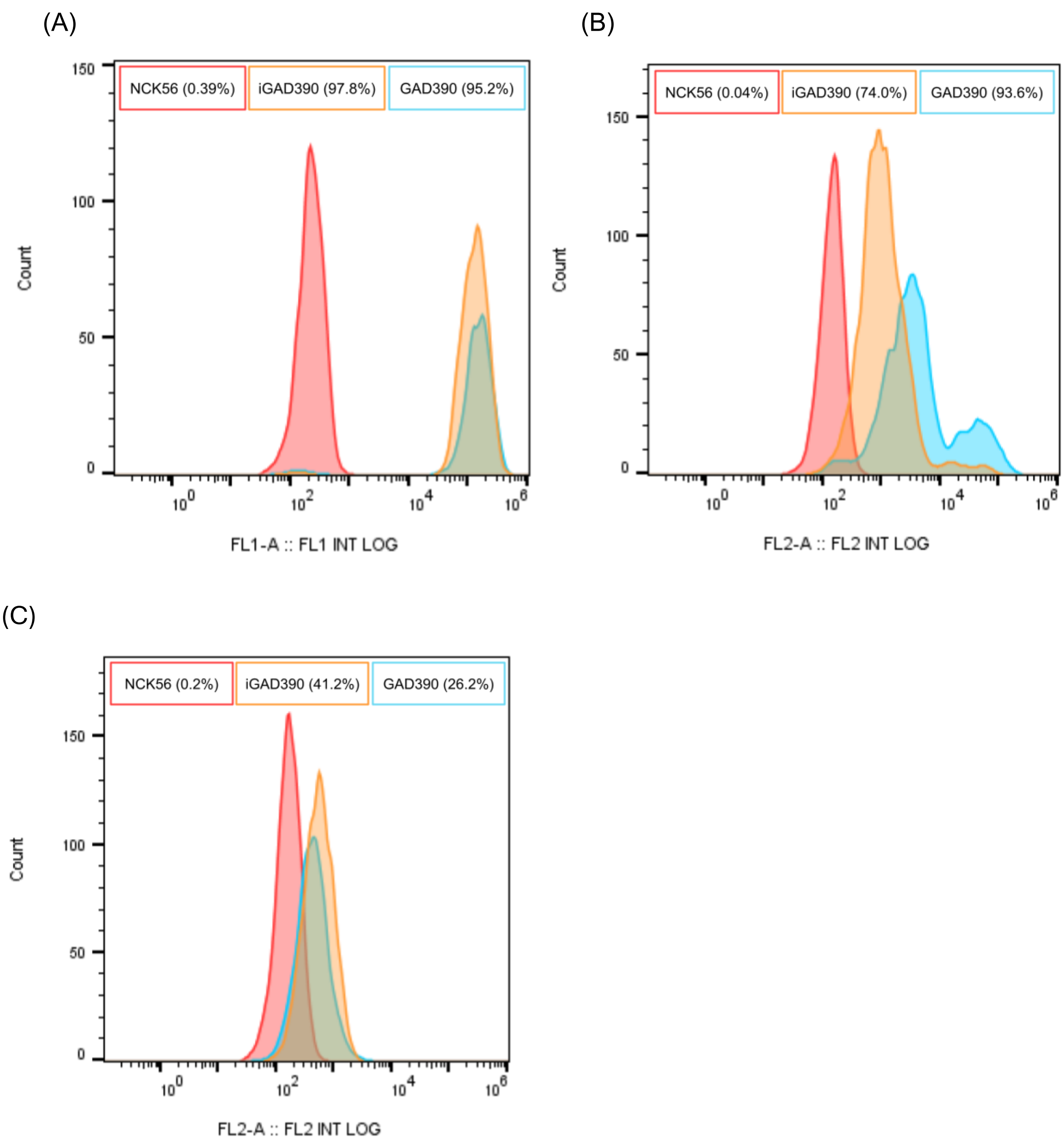


Epitope expression

Epitope expression was well maintained in GAD390 following inactivation. 95.2% of GAD390 events expressed Ova 323-339, which is very similar to the iGAD390's expression level of 97.8% (Figure 2A). Inactivation caused the greatest reduction in FliC expression, reducing event count expression from 93.6% to 74.0% on GAD390 and iGAD390 respectively (Figure 2B). FimH event count expression increased following inactivation from 26.2% to 46.2% (Figure 2C).

Membrane integrity was reduced following inactivation. Cells positive for propidium iodide, an exclusion dye, increased from 1.5% and 3.55% for live NCK56 and GAD390 respectively to 78.3% and 37.9% following inactivation.

Figure 2: GAD390 epitope expression was maintained after inactivation. (A) Expression of Ova 323-339 peptide (B) Expression of FliC adjuvant (C) Expression of FimH adjuvant. Figures generated using FlowJo™ (software version 10.8.1). NCK56 = live wild type L. acidophilus*; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390.*



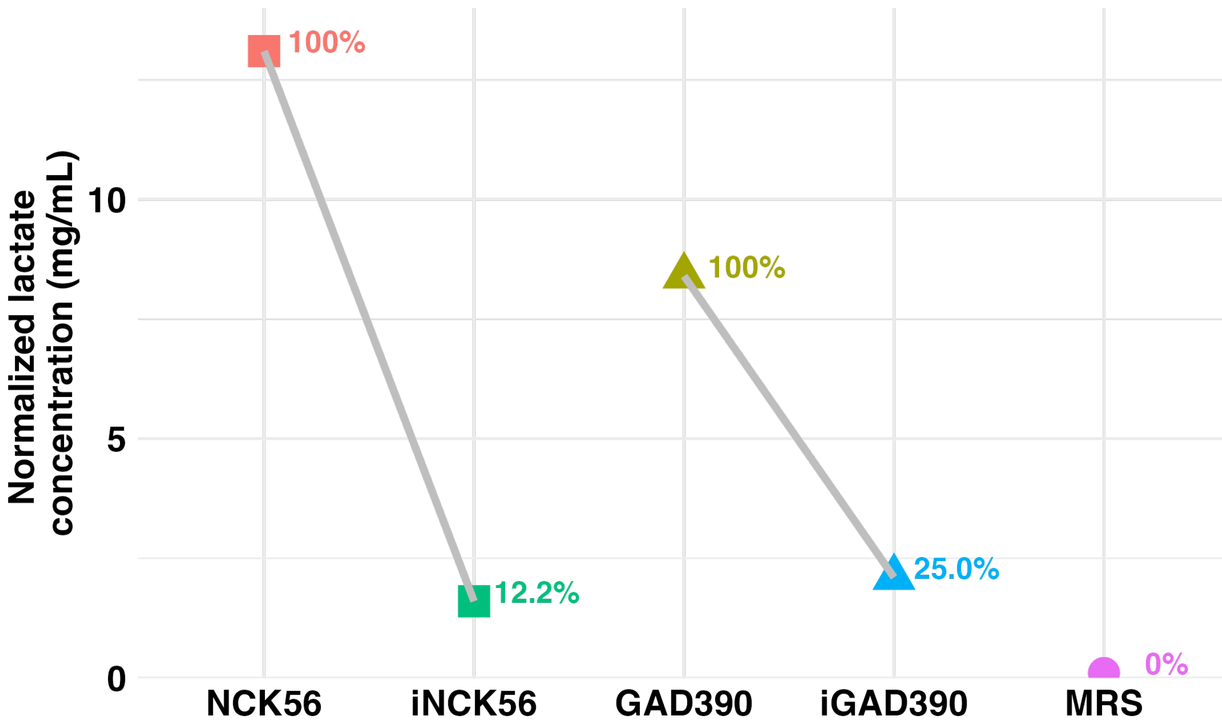
Lactate concentration

To measure lactate concentration, we evaluated several methods and ultimately used GC-MS. These other methods included alamarBlue (Thermo Scientific; DAL1025), Lactate-Glo^{TM} (Promega; J5021), and bromophenol blue (Sigma-Aldrich; B-8026). AlamarBlue contains resazurin, which is reduced to fluorescent resarufin in the presence of NAD(P)H and is an indicator of metabolic activity (Vieira-da-Silva & Castanho, 2023). Lactate-Glo^{TM} converts lactate to pyruvate, producing NADH as a byproduct which reduces pro-luciferin to luminescent luciferin. Bromophenol blue is a pH indicator with a gradient of yellow-green and blue between pH 3.0 and 4.6 respectively. These methods of measuring lactate secretion in bacterial culture media were unsuccessful as *Lactobacillus* species release antioxidants, even after cell death, which disrupt the reduction of these reagents (see Supplemental Results Figures 1-3) (Chooruk et al., 2017). GC-MS avoids this problem by measuring lactate directly and was therefore used for all presented experiments.

Lactate secretion in bacterial media

The concentration of lactate in bacterial media was considerably reduced 4 hours following Mirasol^{TM} PRT inactivation (Figure 3). Live cultures of both NCK56 and GAD390 produced high quantities of lactate (13.1 and 8.4 mg/mL respectively) as expected for lactobacilli. This was reduced to 1.6 and 2.1 mg/mL respectively following inactivation, which are reductions of 87.8% and 75.0% respectively.

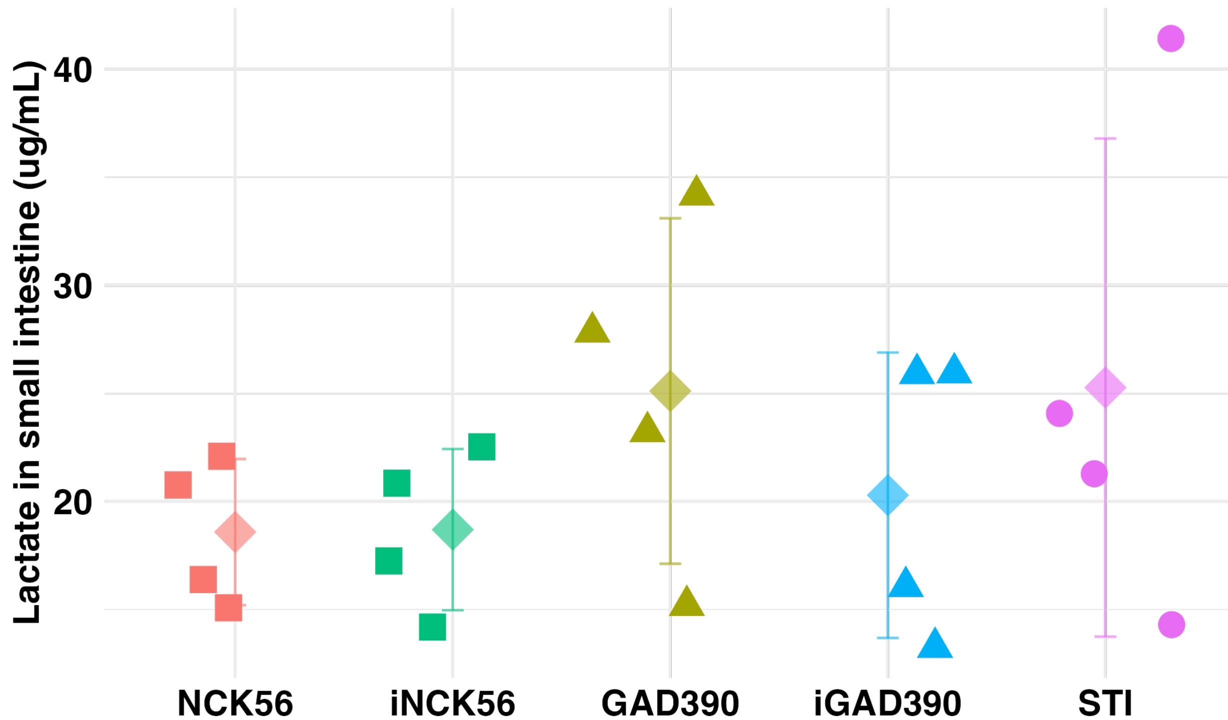
Figure 3: Inactivation reduced lactate concentration in bacterial media after 4 hours. Figure was generated in RStudio (software version 2023.12.1). NCK56 = live wild type L. acidophilus*; iNCK56 = inactivated NCK56; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390; MRS = MRS culture media.*



Lactate in ileum

Lactate quantities in the ileal fluid were not significantly different across any samples. Mice (N=4) dosed with live GAD390 had the highest of level of lactate, even compared to their live NCK56 counterparts (Figure 4). There was a similar but lesser increase between the means of mice dosed with iGAD390 and iNCK56. No samples exceeded basal lactate levels of mice dosed with STI buffer.

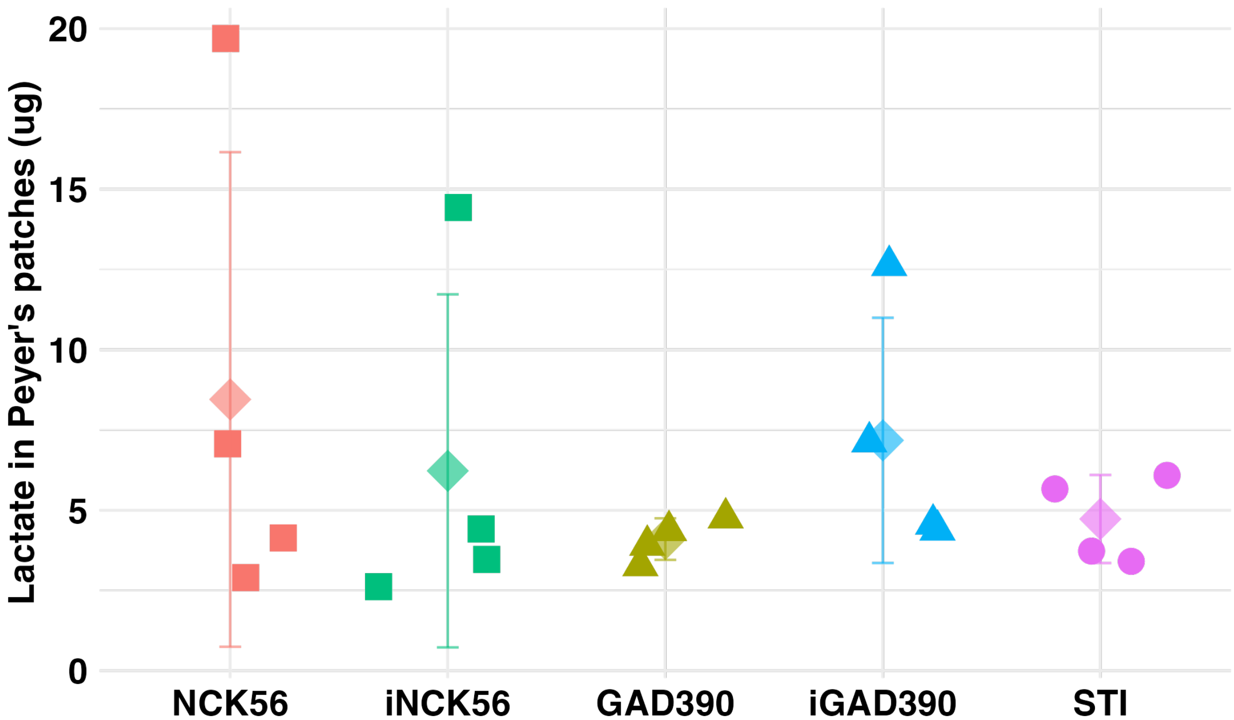
Figure 4: Raw quantities of lactate in the ileum remained statistically constant. Samples (N=4) collected 4 hours following administration and evaluated using GC-MS. Figure and statistical analysis generated using RStudio (software version 2023.12.1). NCK56 = live wild type L. acidophilus*; iNCK56 = inactivated NCK56; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390; STI = soy trypsin inhibitor dosing buffer.*



Lactate in Peyer's patches

Like lactate amounts in the ileum, there were no significant differences in the amount of lactate in Peyer's patches between samples groups (Figure 5). Mice (N=4) dosed with iGAD390 and NCK56 produced the highest raw amount of lactate, followed by mice dosed withGAD390 and then iNCK56.

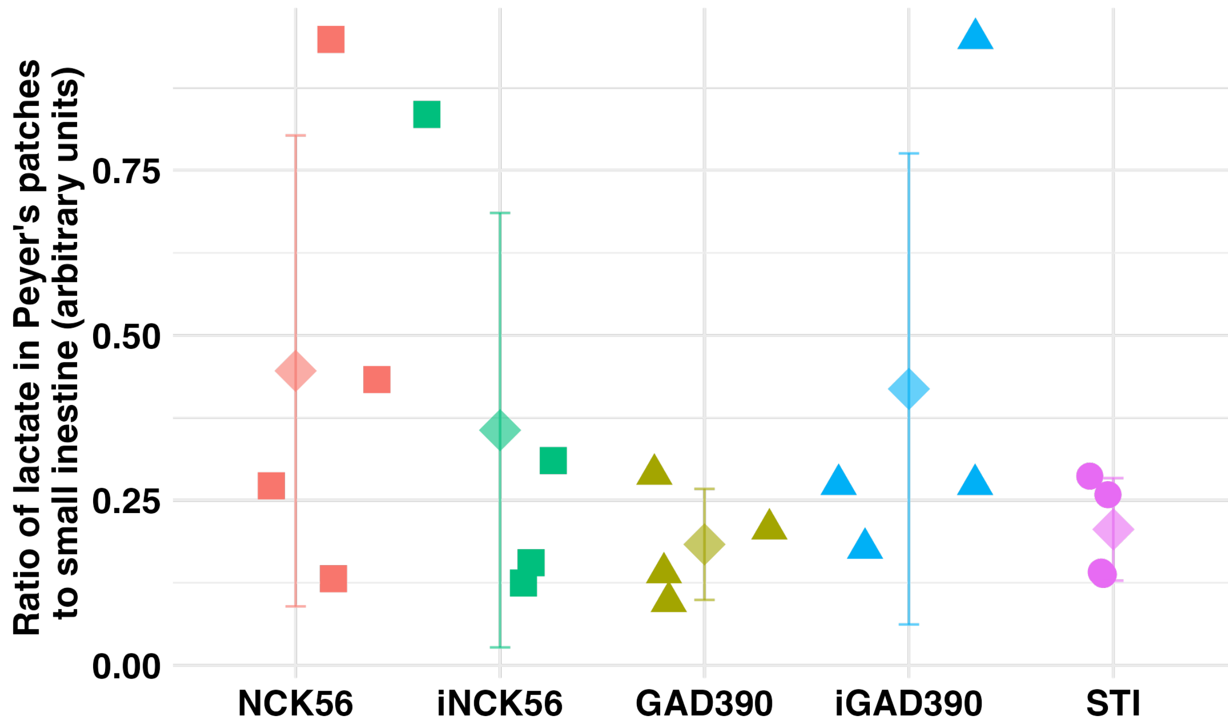
Figure 5: Raw quantities of lactate in the Peyer's patches remained statistically constant. Samples (N=4) collected 4 hours following administration and evaluated using GC-MS. Figure and statistical analysis generated using RStudio (software version 2023.12.1). NCK56 = live wild type L. acidophilus*; iNCK56 = inactivated NCK56; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390; STI = soy trypsin inhibitor dosing buffer.*



Relative levels of lactate between Peyer's patches and ileum

There were no significant differences between levels of lactate in the Peyer's patch and the ileum. Mice (N=4) dosed with NCK56 and iGAD390 produced the highest relative amounts of lactate, followed by mice dosed with iNCK55 and GAD390.

Figure 6: Relative quantities of lactate in the Peyer's patches compared to ileum remained statistically constant. Ratio of paired samples (N=4) were calculated as lactate in Peyer's patch (Figure 5) divided by lactate in ileum (Figure 4). Figure and statistical analysis, including outlier evaluation, generated using RStudio (software version 2023.12.1). NCK56 = live wild type L. acidophilus*; iNCK56 = inactivated NCK56; GAD390 = live recombinant* L. acidophilus; *iGAD390 = inactivated GAD390; STI = soy trypsin inhibitor dosing buffer.*



Discussion

This study examined the role of lactate secretion in LAB probiotic-based vaccine colonization using Mirasol^{TM} PRT inactivation. This mechanism of inactivation considerably reduced lactate secretion, and evaluating lactate quantities in the ileum and Peyer's patches shed light on colonization patterns of LAB.

Replication

Mirasol^{TM} PRT inactivation mechanistically targeted DNA, and it was therefore expected that inactivation would reduce replication of LAB. Elimination of replication suggested all LAB were sufficiently inactivated, producing, ideally, a uniform population of LAB for administration.

Epitope expression

Inactivation preserved an acceptable expression of epitopes, particularly for Ova 323-339. This showed no reduction in expression, which may be attributable to its expression *via* the stable SlpA protein. The greatest reduction in epitope expression was observed in the plasmid-expressed flagellin FliC adjuvant, with a decrease from 93.6% to 74.0%.

FimH expression increased following inactivation, although this was likely because FimH was expressed more highly in exponential phase (iGAD390 was inactivated in exponential phase while GAD390 was stained in stationary phase). FimH expression from other studies has generally been between 30 and 50% in exponential phase, suggesting inactivation does not considerably reduce expression.

There was a slight increase in membrane permeability following inactivation, but this did not appear to be correlated with epitope expression as Ova 323-339 was perfectly maintained.

Lactate concentration

<u>Lactate secretion in bacterial media</u>

Live NCK56 secreted the highest concentration of lactate. Live GAD390 secreted lactate at lower concentrations, likely due to the energy intensity required to maintain its epitopes. Lactate secretion was considerably reduced for both samples following inactivation. This suggested that even while Mirasol™ PRT inactivation did not directly target metabolism, the downstream effects of its DNA damage reduced metabolism output and lactate secretion.

<u>Lactate in ileum</u>

The basal concentration of lactate in the ileum was approximately 25 ug/mL, which corresponds with 0.28 mM (Figure 4). This is similar to levels observed in the cecum of germ-free mice of 0.38 mM (Gillis et al., 2018). There was no statistical difference between the baseline concentration and the concentration following administration with either live or inactivated samples. This indicates that probiotic-based vaccines do not shift the concentration of lactate in the ileum. This stable lactate level is likely beneficial, as it suggests the vaccine administration is not dramatically shifting the metabolic landscape of the gut the way an infection may.

<u>Lactate in Peyer's patches</u>

Like in the ileum, levels of lactate remained statistically even in the Peyer's patches (Figure 5). This suggests administration with *L. acidophilus* does not change the concentration of lactate in immune inductive sites. On the level of the tissue, this may indicate that immune cells are not shifting towards a regulatory phenotype as they tend to in a lactate-rich environment. As the Peyer's patches are the first immune inductive site reached by probiotic-based vaccines, it is important that their presence is not shifting the entire tissue towards a potentially anti-inflammatory state.

Future directions

While administration with live or inactivated probiotic-based vaccines does not change lactate levels in the ileum or Peyer's patches, their lactate secretion may still have localized effects that are not detectable on a tissue-wide level. One of these effects may be the ability of probiotic-based vaccines to localize to tissues such as Peyer's patches. This could be evaluated using a similar design to this study which replaces lactate measurements with bacterial cultures, flow cytometry, or imaging to observe bacteria localization directly.

Once localization patterns are established, it is important to also examine if inactivation status correspond with downstream immune stimulation. This could first be established *in vitro* looking at dendritic cell activation and T-cell activation. GAD390 is an ideal model for this system as T-cells with receptors specific to its MHC-II restricted ovalbumin antigen can be harvested in large quantities from transgenic mice.

Subsequently, it would be important to establish immune stimulation *in vivo*. Immune activation would best be evaluated with a mouse study examining specific antibody production in both serum and feces. This is an important metric for evaluating vaccine efficacy, particularly for probiotic-based vaccines where induction of IgA is a key feature of mucosal immunity.

Acknowledgements

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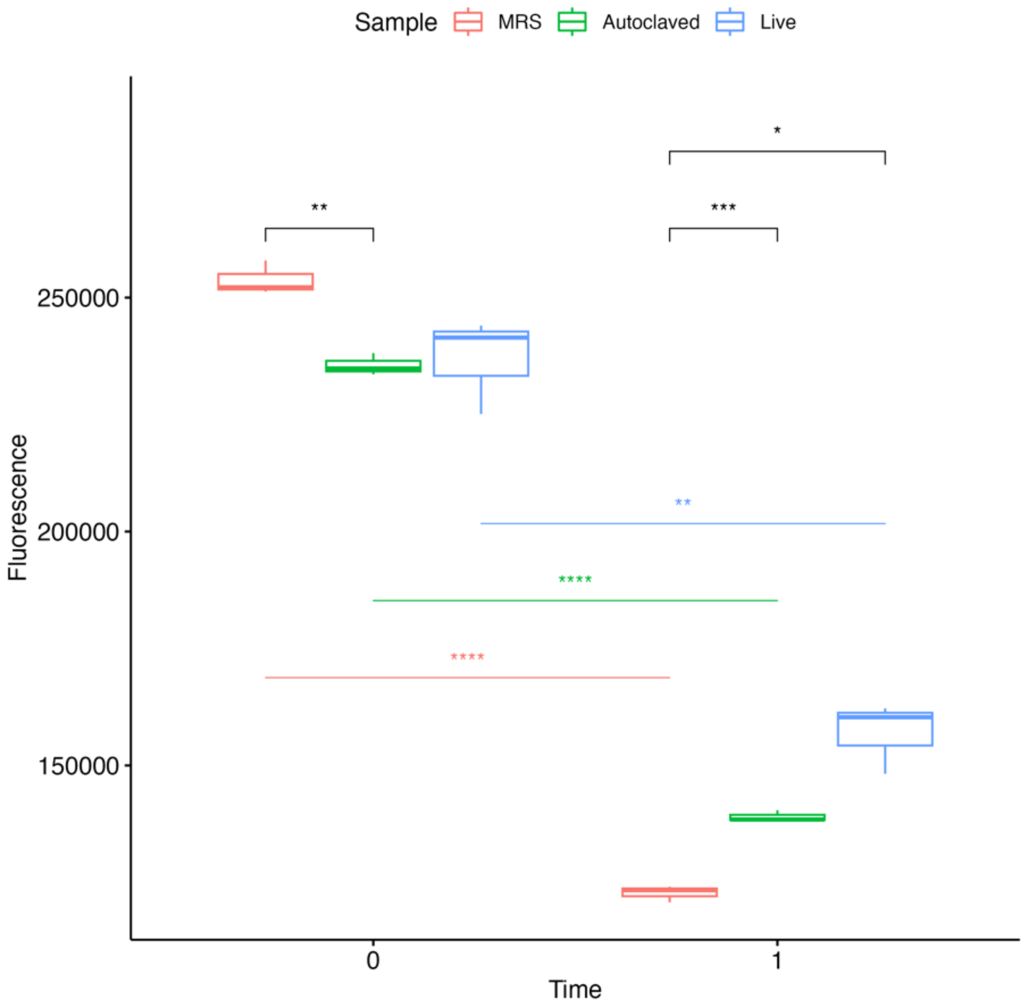
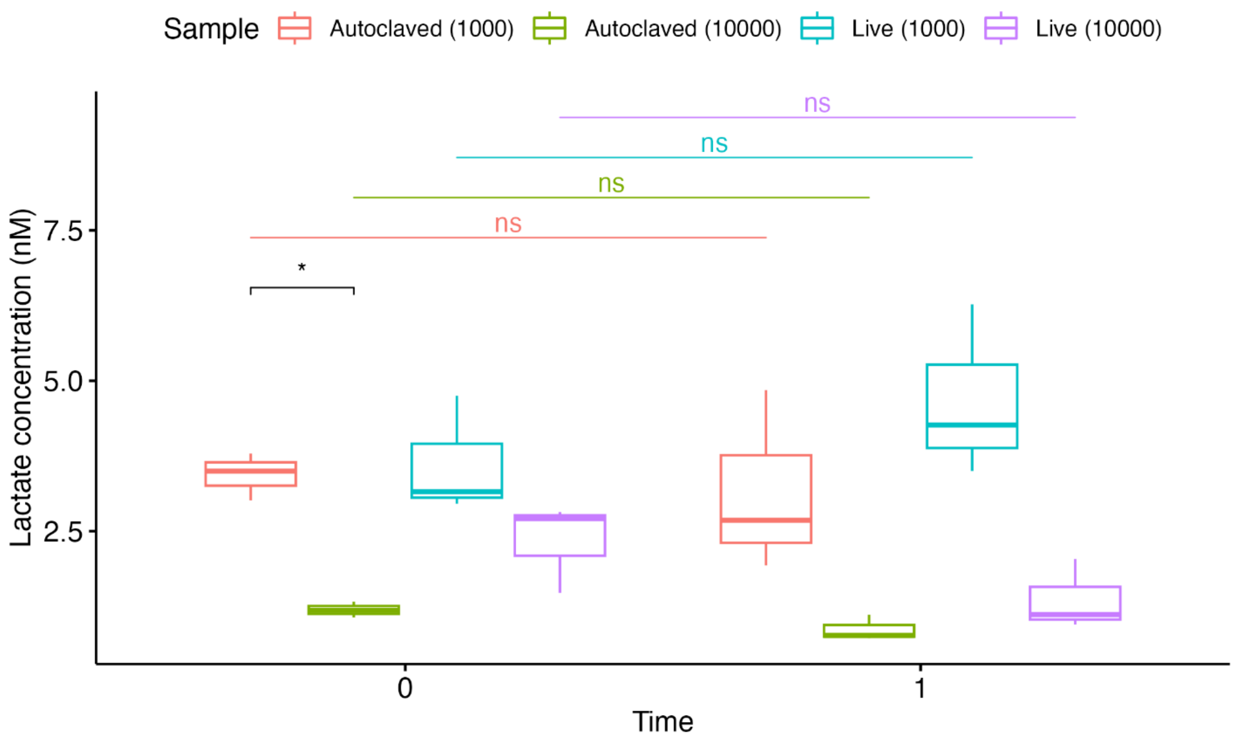
Supplemental Results

Supplemental Figure 1: alamarBlue fluorescence did not properly assess lactate concentrations in LAB. MRS media and autoclaved negative controls were expected to have constant levels of fluorescence but both decreased over time. Samples were incubated in 200 μL 10% alamarBlue in MRS media for 1 hour in 96-well fluorescent plates (Greiner Bio-One; 655209). Fluorescence was read on microplate reader (Synergy H1; BioTek; software version Gen 5 3.11.19) with excitation at 545 nm and emission at 590 nm. MRS = blank MRS culture media; Autoclaved = autoclaved GAD85 (probiotic-based vaccine); Live = live GAD85.

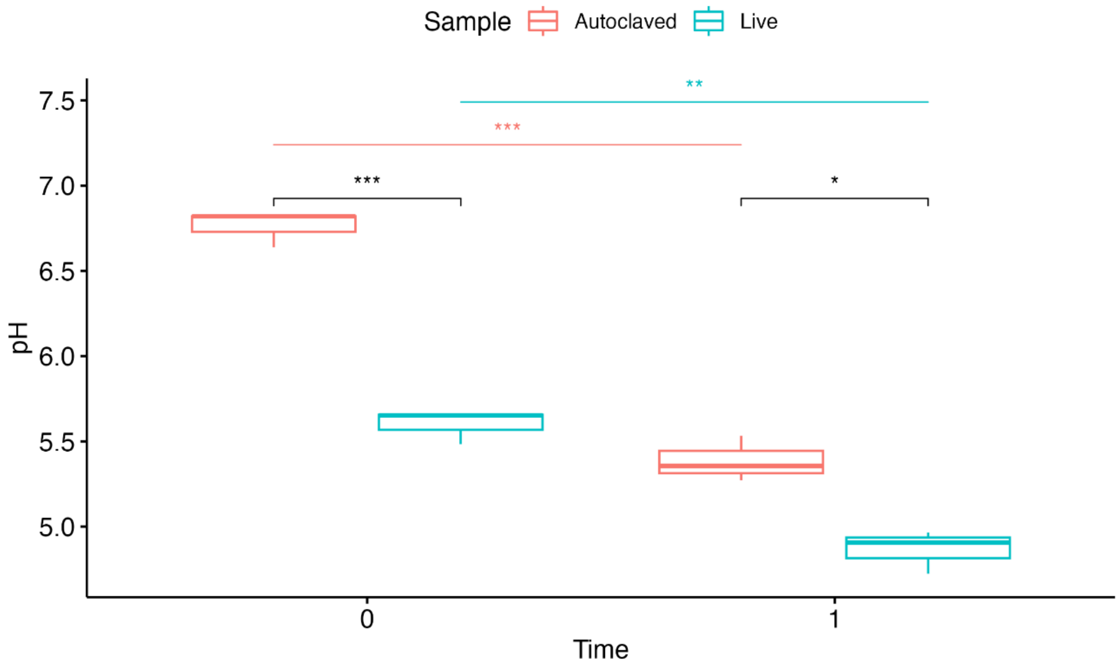


Supplemental Figure 2: Lactate-Glo did not properly assess lactate concentrations in LAB. Live samples, which were expected to increase over time, showed little change in luminescence and was inconsistent between 1,000- and 10,000-fold dilutions. Samples were run according to the manufacturer's protocol for cell culture medium (extracellular) in 96-well flat bottom plate (Greiner Bio-One; 608281), except samples were diluted either 1:1,000 or 1:10,000 based on preliminary data for expected lactate concentrations. Luminescence was read on microplate reader. Autoclaved (1000) = autoclaved GAD85 diluted 1:1000; Autoclaved (10000) =

autoclaved GAD85 diluted 1:10000; Live (1000) = live GAD85 diluted 1:1000; Live (10000) = live GAD85 diluted 1:10000.



Supplemental Figure 3: Bromophenol blue did not properly assess lactate concentrations in LAB. Autoclaved samples, which should have remained constant, showed reduced pH. 10 µL 0.1% w/v bromophenol blue in DI water was added to 100 µL of samples in MRS for 1 hour in 96-well plate. Absorbance was read on a microplate reader at 590 nm. A standard curve was developed using the following standards: pH = 2.67, 3.24, 3.67, 4.12, 4.76, 5.21, 5.68, and 6.28. Autoclaved = autoclaved GAD85; Live = live GAD85.



Sample
Autoclaved
Live
**
***
***
*
7.5
7.0
6.5
6.0
5.5
5.0
pH
0
1
Time

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