

Article

Antimicrobial Efficacy and Transcriptomic Mode of Action of GS-2 Against Foodborne Pathogens

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Abstract

The transition toward reusable food packaging driven by European circular economy regulations introduces new food safety challenges related to microbial persistence and cross-contamination. GS-2 is a novel, food-safe antimicrobial formulation developed for use as a coating on reusable packaging materials. In the present study, the intrinsic antimicrobial activity of GS-2 was evaluated in liquid suspension against foodborne bacterial (*Escherichia coli* O157:H7, *Listeria monocytogenes*, and *Salmonella enterica* Agona) and fungal pathogens (*Aspergillus flavus* and *Aspergillus niger*), and the transcriptomic response of *L. monocytogenes* following sublethal GS-2 exposure was investigated. GS-2 exhibited concentration-dependent microbicidal activity, with strong reductions observed at ≥ 2.8 –3.0% against bacterial strains and *A. flavus*, while *A. niger* demonstrated resistance. Among bacteria, *L. monocytogenes* showed the greatest sensitivity, with populations reduced below detection limits at 3% GS-2. To elucidate mechanistic responses, RNA sequencing was performed on *L. monocytogenes* exposed to sublethal GS-2 concentrations (0.1–1.0%). Transcriptomic analyses using discrete and continuous models revealed a pronounced adaptive stress response at 0.5% GS-2, characterized by coordinated upregulation of pathways involved in carbon, nitrogen, and nucleotide metabolism. At higher sublethal concentrations (1%), transcriptional profiles shifted toward growth arrest and cellular damage management. These findings support GS-2 as a promising antimicrobial for enhancing the microbial safety of reusable food packaging systems.

Keywords: reusable packaging materials; antimicrobial coating; foodborne pathogens; RNA sequencing; bacterial stress response; differential gene expression



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1. Introduction

Growing environmental awareness and the global shift toward sustainable agricultural practices have prompted the European Union (EU) to enact measures targeting the reduc-

tion in single-use packaging, particularly plastics. The foundation of this effort resulted in the EU Packaging and Packaging Waste Directive in 1994, which outlined categories of packaging intended for reuse, such as primary, secondary, and tertiary materials [1]. Subsequent initiatives such as the European Commission's "A European Strategy for Plastics in a Circular Economy" further advanced these goals by establishing a target that at least 10% of all packaging be reusable, rather than solely recyclable, by the year 2030 [2]. More importantly, this framework integrates food safety considerations for reusable food-contact materials, introducing additional compliance challenges for U.S. exporters who must meet both environmental sustainability and food-safety requirements [3].

The evolving European Circular Economy regulations are expected to affect U.S. exporters of fresh produce, particularly those shipping tree nuts, citrus fruits, and sweet potatoes to EU markets [2,4]. When adopting reusable packaging systems for these commodities, microbial food safety becomes a central concern. Between 2020 and 2025, the United States documented 23 outbreaks of foodborne illness linked to fresh produce, primarily associated with *Escherichia coli* O157:H7 (*E. coli* O157:H7), *Listeria monocytogenes* (*L. monocytogenes*), and *Salmonella enterica* (*S. enterica*) [5]. The economic impact of outbreaks caused by these pathogens was estimated at approximately \$7.7 billion in 2018 [6]. Beyond bacterial hazards, spoilage microorganisms, particularly fungi, pose additional risks to the safety and quality of fresh produce. Fungal species such as *Aspergillus flavus* (*A. flavus*) and *Aspergillus niger* (*A. niger*) are common culprits of storage mold and fruit rot, with proliferation often occurring during ripening and long-term storage at fluctuating temperatures of 12.8–40 °C [7–10].

Microbial contamination of fresh produce can occur at multiple points throughout the production and handling continuum. During pre-harvest, both soil and irrigation water serve as major reservoirs of pathogenic and spoilage microorganisms, including bacteria, fungi, and viruses [11,12]. Post-harvest operations such as manual sorting, packing, processing, transport, and food preparation further increase the risk of introducing or spreading contaminants [13]. The recent transition toward reusable packaging systems introduces additional food safety concerns, as contaminated containers can facilitate cross-contamination between successive batches of produce [14]. In industrial practice, reusable packaging is typically sanitized using chemical disinfectants such as sodium hypochlorite (bleach), quaternary ammonium compounds, or peracetic acid [15,16]. While these agents are widely employed, concerns persist regarding the formation of chemical residues and the inconsistent effectiveness of decontamination when sanitizers are improperly diluted or applied. Compounding this issue, biofilm formation on produce or contact surfaces can protect microbial cells from disinfectant penetration, reducing the efficacy of sanitization protocols [16,17]. Consequently, there is an urgent need for innovative antimicrobial technologies capable of preventing pathogen transfer through reusable packaging. To address this challenge, Wintermute Biomedical has developed GS-2, a novel, water-soluble, and food-safe antimicrobial formulation designed as a surface coating [18]. The chemistry of GS-2 combines capric acid (a fatty acid), L-arginine (an amino acid), and thymol (a monoterpene phenol) [18].

Fatty acids such as capric acid possess well-established antimicrobial activity against a wide range of bacterial species [19]. However, their poor water solubility has historically constrained their application in food systems [20,21]. Wintermute Biomedical overcame this limitation through a patented process that converts fatty acids into water-soluble ammonium carboxylate salt with L-arginine, allowing them to be applied using conventional spray and coating systems without the need for organic solvents or surfactants [18]. This innovation was developed for use as an antimicrobial coating on reusable food-contact packaging, providing the rationale for evaluating its antimicrobial efficacy against food-

borne pathogens and investigating its concentration-dependent mode of action under controlled liquid-suspension conditions in the present study.

Prior research has shown the effectiveness of GS-2 against pathogens such as *E. coli* O157:H7, *L. monocytogenes*, and *S. enterica* [18,22]. GS-2 was shown to reduce pathogen transfer from reusable packaging materials such as acrylonitrile butadiene styrene (ABS), cardboard, high-density polyethylene (HDPE), and polypropylene (PP) onto grape tomatoes by $\sim 1.5\text{--}5 \log \text{ CFU/in}^2$ [22]. When the reusable packaging materials were stored for up to 42 days at two different storage conditions with GS-2 (4 °C + 90% relative humidity or 18 °C + 45% relative humidity), GS-2 was still able to retain its effectiveness for 14–42 days against pathogens [22]. However, from previous research, the mode of action of GS-2 against pathogens remained unclear. The objective of this study was to evaluate the antimicrobial efficacy of GS-2 against bacterial (*E. coli* O157:H7, *L. monocytogenes*, and *S. Agona*) and fungal pathogens (*A. flavus* and *A. niger*) in liquid suspension across increasing GS-2 concentrations and to elucidate its concentration-dependent mode of action using RNA-seq-based transcriptomic analysis of *L. monocytogenes* under sublethal exposure conditions.

2. Materials and Methods

2.1. Preparation of Microbial Inocula

Three bacterial strains (*Escherichia coli* O157:H7 TW14359 (*E. coli* O157:H7), *Listeria monocytogenes* LS810 (*L. monocytogenes*), *Salmonella Agona* 447967 (*S. Agona*)) and two fungal strains (*A. niger* van Tieghem 16888 (*A. niger*) and *Aspergillus flavus* MC21 (*A. flavus*)) were used in this study. All three bacterial strains were chosen due to their instigations in previous outbreaks: *E. coli* O157:H7 TW14359 for a 2006 spinach outbreak, *Listeria monocytogenes* LS810 from a 2011 cantaloupe outbreak, and *Salmonella Agona* 447967 for a 1998 toasted oats cereal outbreak [23–25]. For long-term storage, the bacterial strains were maintained at $-80\text{ }^{\circ}\text{C}$ in Tryptic Soy Broth (TSB, Difco, Becton Dickinson, Franklin Lakes, NJ, USA) supplemented with 20% glycerol (VWR International, Radnor, PA, USA). All three bacterial strains were rifampicin-resistant and maintained on Brain Heart Infusion Agar (BHIA, Difco, Becton Dickinson, NJ, USA) supplemented with 200 $\mu\text{g/mL}$ Rifampicin powder (Chem-Impex International, Dale, IL, USA) (BHIA^{rif}) as working stocks to eliminate background microorganisms. *A. niger* and *A. flavus* were not rifampicin-resistant and were maintained on potato dextrose agar (PDA, Difco, Becton Dickinson, Franklin Lakes, NJ, USA) as working stocks. Working stocks were stored at 4 °C for a maximum of 4 weeks.

For inoculation, the bacterial strains were prepared with overnight incubation at 37 °C in 10 mL TSB for 18 h. The overnight cultures were retrieved, 1 mL was taken and spun at $13,000 \times g$ for 1 min, and the supernatant was decanted. The resulting pellets were washed twice with 1 mL Butterfield's Phosphate Buffer (BPB) and resuspended in Tryptic Soy Broth (TSB, Difco, Becton Dickinson, NJ, USA). BPB stock was prepared by dissolving 68 g of potassium phosphate (Difco, Becton Dickinson, Franklin Lakes, NJ, USA) in 1 L of sterile distilled water (sdH_2O) and adjusted to pH 7.2. Subsequently, 1.25 mL of the dissolved potassium phosphate solution was added to 1 L of sterile distilled water to create BPB for use in this study. For *A. flavus* and *A. niger*, cultures were prepared 5 days prior to inoculation at 37 °C in 10 mL potato dextrose broth (PDB, Difco, Becton Dickinson, Franklin Lakes, NJ, USA).

2.2. Efficacy of GS-2 Against *A. flavus*, *A. niger*, *E. coli* O157:H7, *L. monocytogenes*, and *S. Agona* on Day 0 and After Day 3

The GS-2 formulation evaluated in this study was supplied by Wintermute Biomedical (Missoula, MT, US) as a ready-to-use formulation, comprising a water-soluble ammonium

carboxylate salt of capric acid and L-arginine compounded with thymol (i.e., the GS-2 with thymol formulation described in [18]); accordingly, all GS-2 concentrations reported herein refer to dilutions of this complete formulation rather than of capric acid alone. GS-2 stock was diluted with sdH₂O to prepare working solutions of 0.1–15.7%. Five mL of each GS-2 concentration was pipetted into 15 mL centrifuge tubes (VWR International, Radnor, PA, USA). In each centrifuge tube, 5 mL of bacterial or fungal inoculum was individually pipetted and vortexed for 30 s to ensure mixing. For the control, 5 mL of bacterial or fungal inoculum was vortexed with 5 mL of sdH₂O. The resulting inoculations were $\sim 10^7$ CFU/mL for *A. flavus* and *A. niger* and $\sim 10^9$ CFU/mL for the bacteria. The fungal inoculum ($\sim 10^7$ CFU/mL) represented the highest reproducible spore concentration that could be obtained under the culture conditions used, whereas bacterial cultures routinely reached approximately 10^9 CFU/mL. The samples were then stored at 22 ± 1 °C for 3 days.

On days 0 and 3, the samples were retrieved, and serial dilutions in BPB were performed and plated on BHIA^{rif} for bacterial strains and PDA for *A. flavus* and *A. niger*, respectively. *E. coli* O157:H7 and *S. Agona* plates were incubated at 37 °C for 24 h, and *A. flavus*, *A. niger*, and *L. monocytogenes* plates were incubated for 48 h at 37 °C prior to viable cell enumeration. A broth microdilution assay was performed to assess growth inhibition across a range of GS-2 concentrations. However, complete inhibition of visible growth was not observed within the concentrations tested; therefore, an MIC could not be established. Consequently, quantitative time-kill assays were used to characterize antimicrobial activity and identify sublethal concentrations for transcriptomic analysis. This format directly measures the microbicidal and fungicidal activity of GS-2 over a defined contact time and was specifically chosen to identify the sublethal concentrations required for transcriptomic analysis, rather than the growth-inhibitory thresholds captured by conventional broth- or agar-dilution MIC assays.

2.3. Bacterial RNA Extraction

From the results of 2.2, RNA extraction proceeded with only *L. monocytogenes*. Ten GS-2 concentrations (0.1–1.0%) were evaluated, as these GS-2 concentrations resulted in sublethal reductions permitting RNA recovery. After 30 s of treatment and before storage in 2.2, 0.5 mL of *L. monocytogenes* with GS-2 was immediately removed and added to 1 mL of RNeasy Protect Bacteria Reagent (Qiagen Inc., Germantown, MA, USA) and vortexed to mix and stabilize the bacterial RNA. Total RNA was extracted from *L. monocytogenes* samples using the RNeasy Mini Kit (Qiagen Inc., Germantown, MA, USA) according to the manufacturer's instructions. Total RNA was eluted in 50 µL of RNase-free water. The RNA concentration was determined using a fluorometer (Qubit 3.0, Life Technologies, Carlsbad, CA, USA) with the RNA HS Assay Kit (Life Technologies, Carlsbad, CA, USA). The quality of the RNA was examined using the High Sensitivity RNA ScreenTape on a TapeStation 4200 (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Samples with RIN scores > 7 and electrophoretic profiles that showed clear rRNA bands with no evidence of degradation were used for subsequent library construction. All samples were processed in a single batch to minimize potential batch effects.

2.4. Library Construction and RNA-Sequencing

For transcriptomic analysis, each GS-2 concentration (0.1–1.0%) and the untreated control were evaluated using at least four independent biological replicates. The library for sequencing was constructed using the Stranded Total RNA Prep with Ribo-Zero Plus (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. The RNA UD Indexes Set A and B (Illumina, San Diego, CA, USA) were used for indexing. To sequence the 48 samples, individual libraries were prepared for each sample and then pooled

into a single multiplexed library. The pooled library was quantified using a fluorometer (Qubit 3.0, Life Technologies, Carlsbad, CA, USA) with the High Sensitivity DNA Kit (Life Technologies, Carlsbad, CA, USA) and normalized to 4 nM according to the recommended workflow for the NextSeq 2000 system. The library pool was denatured and diluted following the NextSeq 2000 Denature and Dilute Libraries protocol (Illumina, San Diego, CA, USA) and supplemented with 5% PhiX Control v3 (Illumina, San Diego, CA, USA) as an internal sequencing control. Sequencing was performed on a NextSeq 2000 (Illumina, San Diego, CA, USA) using 600-cycle P1 chemistry to generate paired-end 150 bp reads.

2.5. Quality Control and Pre-Processing of RNA-Seq Data

FastQC v0.12.1 and MultiQC v1.16 [26,27] were used to assess the quality of the raw paired-end Illumina sequencing reads (using the ‘fastqc’ and ‘multiqc’ commands, respectively, and default settings). The raw paired-end Illumina sequencing reads underwent trimming/filtering using fastp v0.23.4 [28], using the ‘fastp’ command with the following options: (i) forward reads supplied to the ‘-i’ option; (ii) reverse reads supplied to the ‘-I’ option; (iii) forward and (iv) reverse reads, output using the ‘-o’ and ‘-O’ options, respectively; (v) ‘--detect_adapter_for_pe’ enabled (to detect adapters for paired-end data); (vi) ‘--length_required 36’ (to discard reads shorter than 36 bp); (vii) ‘--trim_poly_x’ enabled (to enable polyX trimming in 3’ ends); (viii) ‘--correction’ (to enable base correction in overlapped regions for PE data); (ix) ‘--cut_front’, ‘--cut_front_window_size 4’, and ‘--cut_front_mean_quality 20’ (to move a sliding window of size 4 from 5’ to tail, dropping the bases in the window if mean quality < 20, stopping otherwise); (x) ‘--cut_right’, ‘--cut_right_window_size 4’, and ‘--cut_right_mean_quality 20’ (to move a sliding window of size 4 from front to tail; if one window with mean quality < 20 is met, the bases in the window and the right part are dropped); (xi) ‘--thread 8’ (8 threads).

FastQC and MultiQC were used to assess the quality of the resulting fastp-trimmed reads as described above. Inspection of the resulting MultiQC report revealed that the fastp-trimmed reads would benefit from an additional round of trimming, and as such, the fastp-trimmed reads were supplied as input to Trim Galore v0.6.10 [29], which was used to perform a second round of trimming, using the ‘trim_galore’ command and the following options: ‘--cores 1 --fastqc --paired --gzip --stranded_illumina’. FastQC reports produced by Trim Galore were aggregated using MultiQC as described above. The resulting trimmed paired-end reads were deemed to be of adequate quality and were used in all subsequent steps.

2.6. Transcriptome Assembly and Genome Assembly Acquisition

Two methods were used to produce a reference to which trimmed Illumina paired-end RNA-seq reads could be mapped: (i) a reference transcriptome and (ii) a reference genome. For (i), a reference transcriptome was assembled by concatenating the (a) forward and (b) reverse trimmed reads for all samples into two separate files, and supplying the concatenated trimmed RNA-seq read files to rnaSPAdes v3.15.5 [30] (i.e., to the ‘-1’ and ‘-2’ options, respectively); rnaSPAdes was executed using the following command: ‘spades.py --rna -1 cat_trimmed_1.fastq.gz -2 cat_trimmed_2.fastq.gz -o out -t 8 --ss rf --tmp-dir/scratch/ -m 32’. The quality of the resulting assembled transcriptome was evaluated using rnaQUAST v2.3.0 [31] (with the ‘--ss’ and ‘--prokaryote’ options, to denote that the transcriptome was assembled using strand-specific RNA-seq data and that the genome was prokaryotic, respectively), and the assembled transcriptome was annotated using Prokka v1.14.6 (with options ‘--kingdom Bacteria --cpus 4 --addgenes --locustag transcripts --centre --compliant’) [32]. In addition to the assembled transcriptome, (ii) the genome of *Listeria monocytogenes* strain MOD1_LS810 (NCBI GenBank Assembly accession

GCA_016306305.1), and its associated annotation files (e.g., '.gff') were downloaded from NCBI's Assembly database [33]. All RNA-seq analysis steps were performed with (i) the assembled transcriptome and then repeated using (ii) the MOD1_LS810 reference genome, as detailed below.

2.7. Read Mapping, Quantification, and Exploratory Analyses

The 'bwa index' command from BWA v0.7.17-r1188 [34] was used to index the reference transcriptome/genome (default settings; see Section 2.6 above). Trimmed Illumina paired-end reads were mapped to the reference transcriptome/genome using 'bwa mem' and 28 threads, and the 'samtools sort' command from SAMtools v1.12 [35] was used to produce a sorted BAM file ('bwa mem -t 28 reference.fna sample_1.fq.gz sample_2.fq.gz | samtools sort -@ 2 -O bam -> sample.bam'). The resulting sorted BAM file was indexed using 'samtools index' (2 threads and default settings), and then 'samtools view' was used to count the number of mapped reads for each sample ('samtools view -F 0x4 sample.bam | cut -f 1 | sort | uniq | wc -l > sample.counts'). Reads were quantified using the 'featureCounts' function from the Rsubread package (v2.18.0) [36] of R v4.4.1 [37] with the following parameters:

```
Rsubread::featureCounts (files = bamfiles, # BAM files used as input
  annot.ext = "reference.gff", # GFF file for the reference transcriptome/genome
  isGTFAnnotationFile = T, # specify that reference is GTF file
  GTF.featureType = c ("CDS", "rRNA", "tmRNA", "tRNA"), # specify feature
  types used
  GTF.attrType = "locus_tag", # specify attribute type
  nthreads = 1, # use 1 thread
  countMultiMappingReads = T, # count multimappers
  fraction = T, # assign fractional counts to multimappers
  isPairedEnd = T, # specify that reads are paired-end
  strandSpecific = 2) # specify that reads are reverse-stranded
```

Exploratory analyses were conducted in R and plotted using ggplot2 v3.5.1 [38]. Briefly, reads mapping to protein-coding genes underwent a variance-stabilizing transformation (vst) using the 'vst' function from the DESeq2 v1.44.0 package [39]. The resulting vst-transformed counts were supplied as input to the following: (i) the 'pvclust' function from pvclust v2.2.0 [40], to perform average linkage hierarchical clustering with one thousand bootstrap replicates ('method.hclust = "average", method.dist = "euclidean", nboot = 1000'); (ii) the 'umap' function from umap v0.2.10.0, to construct a UMAP (default settings) [41]; (iii) the 'prcomp' function from R's stats package, to perform Principal Components Analysis (PCA; with parameters 'scale = F, center = T').

2.8. RNA-Seq Data Processing and Differential Gene Expression Analysis

Differential gene expression analyses were performed in R v4.4.1 (R Foundation for Statistical Computing, Vienna, Austria) using the DESeq2 package v1.44.0 [39]. Two complementary modeling approaches were used: (i) a continuous concentration–response model and (ii) a discrete pairwise comparison model.

For the continuous modeling approach, raw count matrices were transformed using the variance stabilizing transformation (VST) implemented in DESeq2. The VST-transformed matrix was subsequently standardized by transcript across all samples. The association between transcript abundance and GS-2 concentration was evaluated using Spearman rank correlation (cor.test function, base R), and significance was defined as $p < 0.01$.

These nominal p -values are unadjusted for multiple comparisons; after Benjamini–Hochberg false-discovery-rate correction, no transcript remained significant (minimum adjusted $p \approx 0.17$). The continuous model is therefore presented as a descriptive visualization of monotonic expression trends across the GS-2 concentration gradient and was not used for statistical inference.

For the discrete modeling approach, differential expression was evaluated using the design formula:

$$\text{Expression} \sim \text{GS-2 concentration}$$

Differentially expressed genes (DEGs) were identified by contrasting each GS-2 concentration against the control sample using the results function in DESeq2 with Benjamini–Hochberg false discovery rate (FDR) correction. Genes were considered significantly differentially expressed at adjusted p -value (p_{adj}) < 0.05 . Differentially expressed genes are additionally reported at a broad nominal (unadjusted) threshold of $p < 0.01$ to describe the full breadth of the concentration response; all inferential conclusions are based on the FDR-adjusted (p_{adj}) threshold.

Visualization of group-level transcript profiles was performed using the standardized VST matrix to generate heatmaps and correlation plots with ggplot2 v3.5.2 [38] and custom visualization functions from the ohchibi package v0.0.0.9000 [42]. Functional annotation of significant DEGs was conducted using the KEGGREST package v1.32.0 (keggLink and keggGet functions) to map genes to KEGG Orthology (KO) identifiers and pathways [43]. Pathway enrichment visualizations were produced using ggplot2 and ohchibi.

2.9. Statistical Analysis for GS-2 Microbicidal Activity

Each treatment was conducted with three biological replicates and three technical replicates each for every strain tested. A total of 2160 data points were collected for statistical analysis. *A. flavus*, *A. niger*, *E. coli* O157:H7, *L. monocytogenes*, and *S. Agona* populations from 2.2 were converted to log values and analyzed using unpaired t -tests comparing day 0 and 3 using GraphPad Prism, version 10.3.1 (GraphPad Software, LLC, Boston, MA, USA). p -values of < 0.05 were considered statistically significant.

3. Results

3.1. Concentration-Dependent Antimicrobial Activity of GS-2 Against Bacterial and Fungal Foodborne Pathogens

GS-2 was effective at reducing *E. coli* O157:H7, *L. monocytogenes*, *S. Agona*, and *A. flavus* populations at higher concentrations (2.8–3.0%) over a period of three days (Figure 1). For *E. coli* O157:H7 and *S. Agona*, populations were significantly ($p < 0.05$) higher on day 3 compared to day 0 at intermediate concentrations (1.8–2.1%). However, at 2.8% GS-2, populations were significantly ($p < 0.05$) reduced on day 3 by ~ 0.7 – 1.0 log CFU/mL compared to day 0 (Figure 1A,C), indicating that GS-2 produced modest but significant reductions at higher concentrations (2.8–3.0%) against these two Gram-negative bacterial strains. Among the bacterial pathogens tested, GS-2 demonstrated the greatest microbicidal activity against *L. monocytogenes*. Populations were significantly ($p < 0.05$) reduced on day 3 compared to day 0 beginning at 1.5% GS-2, and at 1.7% GS-2, reductions of ~ 1.8 log CFU/mL were observed (Figure 1B). By 2.1% GS-2, populations were reduced by ~ 2.5 log CFU/mL, and by 3.0%, counts fell by > 2 log CFU/mL to below the detection limit (1 log CFU/mL) by day 3 (Figure 1B).

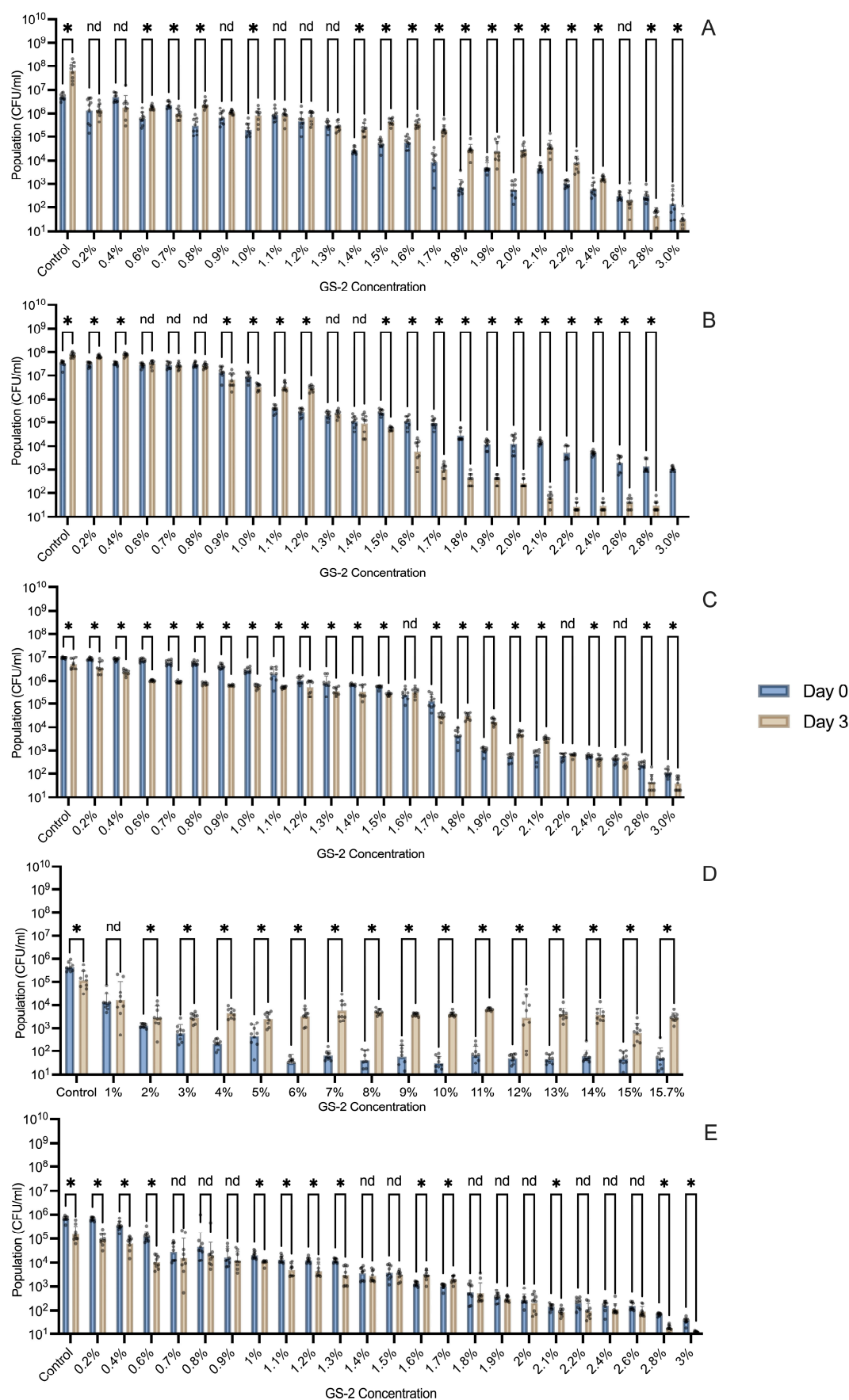


Figure 1. Populations (log CFU/mL) of *E. coli* O157:H7 (A), *L. monocytogenes* (B), *S. Agona* (C), *A. niger* (D) and *A. flavus* (E) after GS-2 treatments on day 0 and 3. GS-2 concentrations were 0–3% for

E. coli O157:H7, *L. monocytogenes*, *S. Agona* and *A. flavus*; 0–15.7% for *A. niger*. Data are presented as mean $\pm$ standard deviation of three biological replicates and three technical replicates each. Asterisks (*) represent significant differences between day 0 and day 3 populations within the same GS-2 concentration ($p < 0.05$). nd represents no significant differences between day 0 and day 3 populations within the same GS-2 concentration ($p > 0.05$).

For fungal strains, treatment with 3.0% GS-2 resulted in significantly ($p < 0.05$) decreased *A. flavus* populations of ~ 0.8 log CFU/mL by day 3, nearing the detection limit (Figure 1E). In contrast, GS-2 appeared to exhibit primarily fungistatic activity against *A. niger*, as populations did not significantly ($p > 0.05$) decrease across the concentrations evaluated from day 0–3 (Figure 1D). Higher GS-2 concentrations were also tested against *A. niger* ($\geq 15.7\%$) because preliminary studies using the same 0.1–3% GS-2 concentrations resulted in no reduction in *A. niger* populations. However, even with the higher GS-2 concentrations (1–15.7%) against *A. niger*, populations on day 3 were significantly ($p < 0.05$) increased by ~ 2 log CFU/mL after the highest GS-2 concentration of 15.7% (Figure 1D). Collectively, these results suggest that GS-2 may serve as an effective antimicrobial intervention against key foodborne bacterial pathogens, although its efficacy against certain fungal contaminants such as *A. niger* may be limited.

3.2. Transcriptomics Response of *L. monocytogenes* to Sublethal GS-2 Concentrations

Using the continuous concentration–response model for a subset of genes that were positively expressed, these genes exhibited significant ($p < 0.01$) positive correlation with standardized expression increasing with increasing GS-2 concentrations. There is higher variability within the subset of genes after GS-2 0.5%, 0.6%, 0.9%, and 1% treatment, as shown with the larger magnitudes of standardized expression: ~ -0.1 – 0.35 , ~ -0.15 – 0.19 , ~ 0.1 – 0.3 , ~ -0.17 – 0.32 , respectively, compared to the other GS-2 concentrations that had ranges of ± 0.1 (Figure 2A). The fitted trend line and 95% confidence intervals illustrate that although individual genes display variability in magnitude, the overall correlation with positively expressed genes and GS-2 concentration is positive. This pattern suggests that GS-2 may induce a coordinated activation of genes that respond as part of shared regulatory pathways rather than each gene reacting independently.

Conversely, another subset of *L. monocytogenes* genes that were negatively expressed demonstrated significant ($p < 0.01$) negative correlations, with standardized expression decreasing steadily with increasing GS-2 concentrations (Figure 2B). As with the positively correlated genes, down-regulated genes displayed a clear downward trend, with the strongest declines observed at concentrations of 0.5%, 0.9%, and 1% (Figure 2B). The smooth regression fit indicates that these responses reflect dose-dependent repression (Figure 2B), potentially corresponding to pathways that are inhibited under stress conditions triggered by GS-2. Together, these two opposing directional patterns indicate that GS-2 triggers a biphasic transcriptional response in *L. monocytogenes*, in which distinct groups of genes are either progressively activated or progressively repressed across the 0.1–1.0% concentration gradient. The positively correlated genes display concentration-dependent increases in expression, consistent with the induction of stress-responsive or protective pathways, whereas the negatively correlated genes show graded declines in expression, suggesting suppression of functions that may be energetically costly or incompatible with antimicrobial stress.

To complement the continuous dose–response patterns of Figure 2, analysis of differential expression using discrete pairwise contrasts and continuous dose–response modeling revealed distinct patterns in how *L. monocytogenes* responds to GS-2 exposure (Figure 3). In the discrete model, the number of significant genes (nominal $p < 0.01$) varied markedly across concentrations. The highest number of differentially expressed genes occurred

at 0.5% GS-2, where 93 upregulated genes and over 79 downregulated genes were detected at the nominal ($p < 0.01$) threshold relative to the control. Concentrations of 0.9% and 1.0% GS-2 also resulted in substantial transcriptional changes of 41 upregulated and 24 downregulated and 26 upregulated and 23 downregulated genes, respectively (Figure 3). In contrast, lower concentrations (0.2% and 0.4%) produced comparatively fewer significant genes, where both had zero overall upregulated genes (Figure 3A) and three and one downregulated genes, respectively (Figure 3B). This may indicate that early transcriptional shifts were minimal until GS-2 reached intermediate concentrations such as 0.5%. After Benjamini–Hochberg FDR correction, 27 genes remained significant at 0.5% GS-2 ($p_{adj} < 0.05$; 15 upregulated, 12 downregulated), and 0.5% was the only concentration with more than one FDR-significant gene, confirming it as the pivotal sublethal dose.

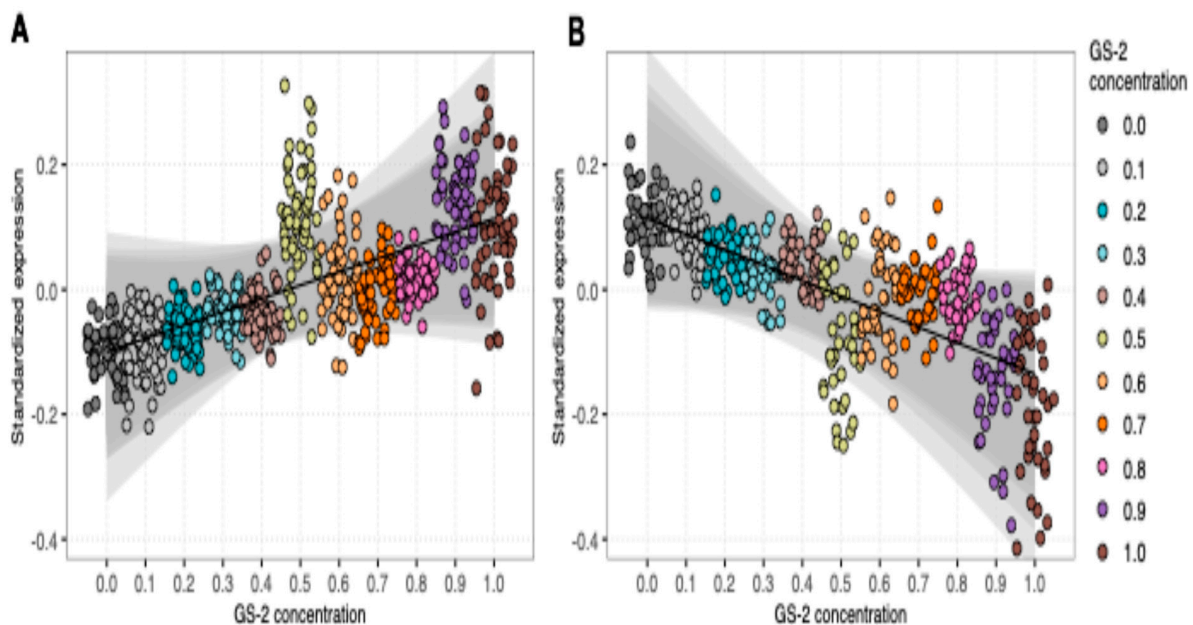


Figure 2. Scatterplots showing *L. monocytogenes* genes identified using the exploratory continuous model (nominal, unadjusted $p < 0.01$) after GS-2 treatment (0.1–1.0%). Genes were classified as positively correlated (A) or negatively correlated (B) based on their expression in response to increasing GS-2 concentrations. Each dot represents the average standardized gene expression (y-axis) at each GS-2 concentration (x-axis). Black lines represent the fitted linear model for each significant gene, and the black line represents the overall category-level trend.

The continuous model, which evaluates monotonic correlations across the full concentration gradient rather than discrete contrasts, identified 54 genes with increasing expression patterns and 41 genes with decreasing patterns across the increasing gradient of GS-2 concentrations 0.1–1.0% (Figure 3). The continuous model highlights coordinated transcriptomic trajectories that may not be fully apparent from the discrete model alone. Together, these results demonstrate that discrete and continuous modeling provide complementary insights. The discrete model identifies concentration-specific transcriptional responses with a pronounced peak at 0.5% GS-2, and the continuous model detects genes exhibiting expressional shifts across the entire gradient. This combined analysis reveals both the specific GS-2 concentrations where gene expression changes occur and the genes that shift gradually across the full 0.1–1.0% range.

To estimate the changes in *L. monocytogenes* gene expression after GS-2 treatment, log₂ fold-change heatmaps revealed that at concentrations $\geq 0.5\%$, but especially at 0.5%, 0.9% and 1%, there were a higher number of genes that were significantly ($p < 0.01$) highly expressed ($\geq \log_2$ fold-change 0.3) or highly negatively expressed ($\leq \log_2$ fold-change -0.3) (Figure 4A).

Out of the three GS-2 concentrations, 0.5% resulted in the highest number (~85% of genes) of highly expressed or negatively expressed genes compared to the control (Figure 4A). GS-2 0.9% had the second highest, with ~70% of genes, and GS-2 1% with 60% of genes (Figure 4A). In contrast, GS-2 0.1–0.4% had the highest number of genes that had low- to moderately expressed or negatively expressed genes compared to the control (Figure 4A).

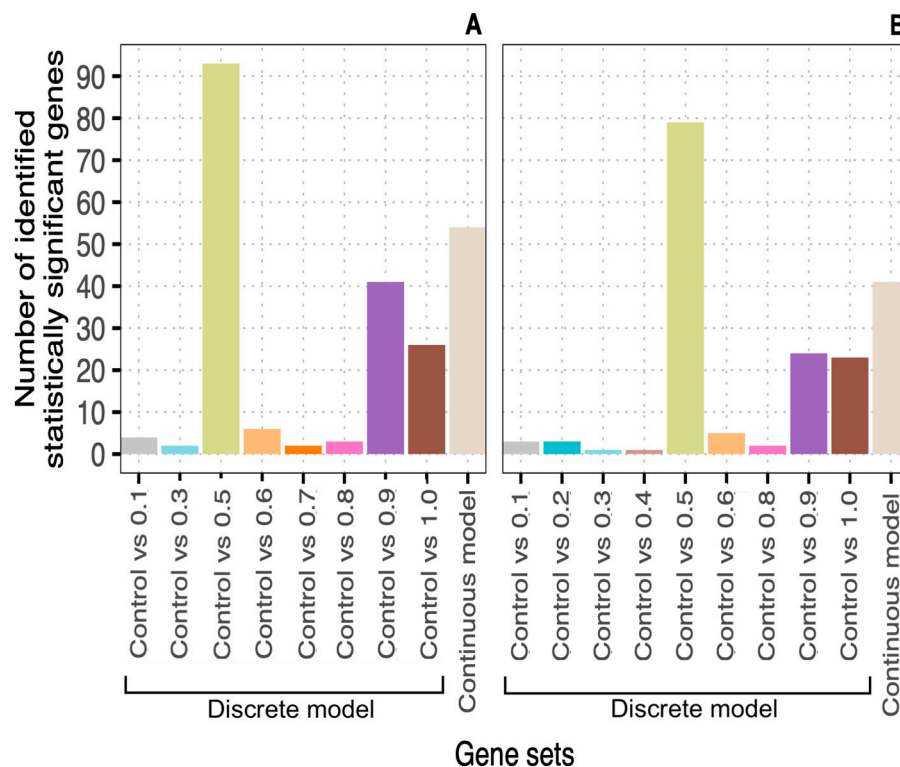


Figure 3. Differential gene counts of *L. monocytogenes* detected across GS-2 concentrations 0.1–1.0% relative to the control using discrete and continuous transcriptomic models (x-axis) to identify the number of statistically significant genes (y-axis). Bar represents genes significant at the nominal (unadjusted) significance threshold ($p < 0.01$). Benjamini-Hochberg FDR-adjusted ($padj < 0.05$) results are described in the Results. The plot is faceted into two panels with overall increasing (A) or decreasing (B) gene expression patterns as GS-2 concentrations increase.

With continuous modeling, the colored regions (black to yellow) were the genes that were significantly ($p < 0.01$) increasing (positive Spearman ρ coefficient) or decreasing (negative Spearman ρ coefficient) with increasing GS-2 concentrations (Figure 4B). The gray regions indicate that the genes do not significantly ($p > 0.01$) display a consistent pattern of change across the gradient (Figure 4B). Together, these integrated results indicate that GS-2 elicits both concentration-specific transcriptional responses detectable through discrete modeling and broader expression shifts through continuous modeling.

All the significantly increased and decreased *L. monocytogenes* genes from Figure 4 were mapped to KEGG pathways to assess whether specific biological functions were enriched across GS-2 treatments. For the discrete modeling of genes with overall increased expression as GS-2 concentrations (0.1–1%) increased, 0.5% treatment yielded the largest number of mapped genes, with ≥ 4 genes assigned to the pathway 00260 Glycine, serine and threonine metabolism, a pathway involved in amino acid biosynthesis and one-carbon metabolism [44,45] (Figure 5A). This was followed by three pathways with three mapped genes each: 00670 One carbon pool by folate (supplies one-carbon unit required for nucleotide biosynthesis and methylation reactions), 00240 Pyrimidine metabolism (responsible for the synthesis and recycling of pyrimidine nucleotides required for DNA and RNA synthesis), and 00250 Alanine, aspartate and glutamate metabolism (supports amino acid

biosynthesis and nitrogen metabolism) [44,45]. At 0.5% GS-2, an additional 16 pathways contained two genes each, and 34 pathways contained one mapped gene (Figure 5A), indicating the broad transcriptomic impact of this concentration. Across the remaining discrete contrasts, 1% GS-2 had the second-highest number of KEGG pathways represented (26 pathways), followed by 0.9% (24 pathways), 0.6% (9 pathways), 0.3% (5 pathways), and 0.1% and 0.7% (3 pathways each). For the continuous modeling, which identifies genes exhibiting monotonic expression shifts across the entire concentration gradient, three KEGG pathways contained three mapped genes: 00400 Phenylalanine, tyrosine and tryptophan biosynthesis, 00300 Lysine biosynthesis, and 00999 Biosynthesis of various plant secondary metabolites (Figure 5A). These pathways differed from those highlighted in the discrete contrasts, reflecting the distinct types of transcriptional information captured by the two analytical frameworks.

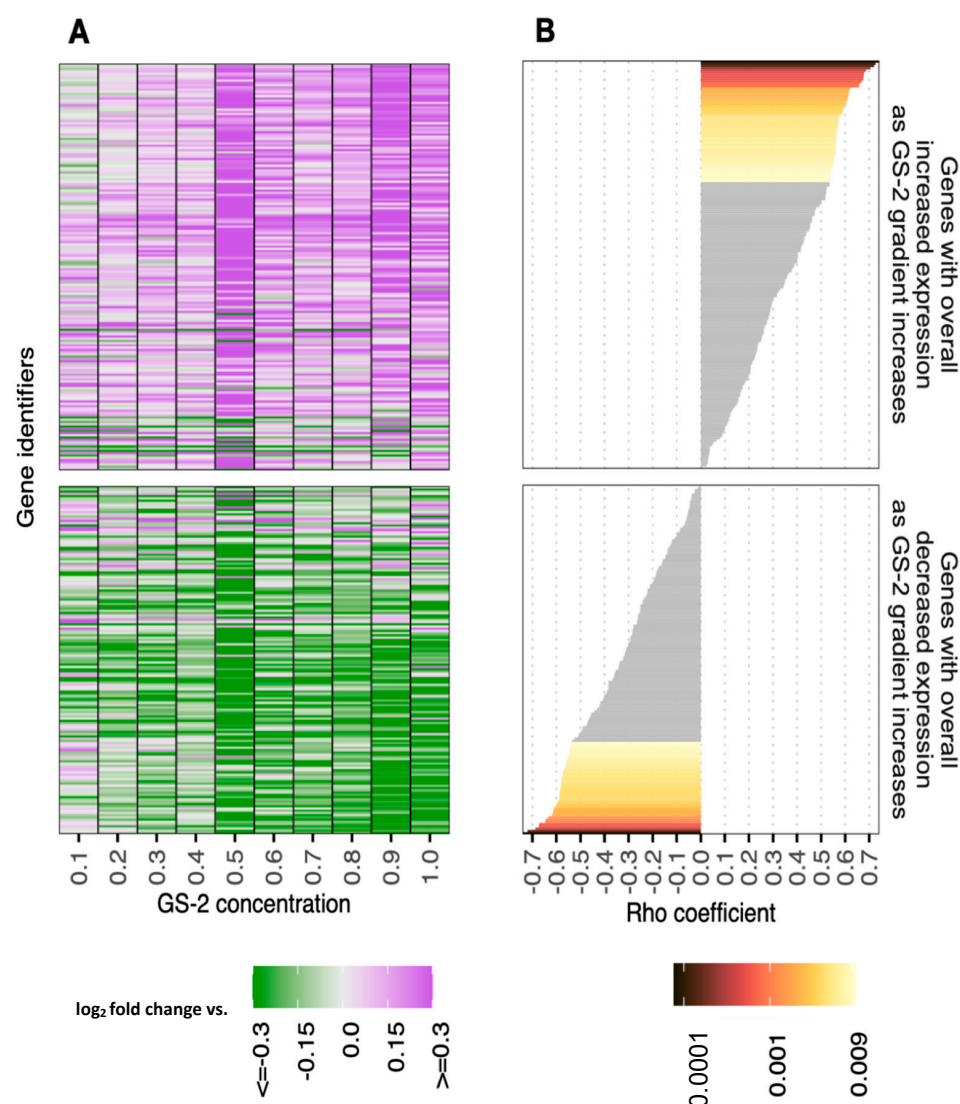


Figure 4. From the statistically significant *L. monocytogenes* genes after GS-2 treatment (0.1–1%) (y-axis), the $\log_2$ fold-change estimates for each significantly differentially expressed gene ($p < 0.01$) across individual GS-2 concentrations relative to the control using the discrete model (A) illustrate the direction and magnitude of concentration-specific transcriptional shifts. The continuous model for the same gene set using Spearman correlation coefficients (ρ), with positive and negative values indicating steadily increasing or decreasing expression across the GS-2 gradient; associated nominal (unadjusted) p -values (color scale) denote statistical significance ($p < 0.01$) and gray indicates non-significance ($p > 0.01$) (B).

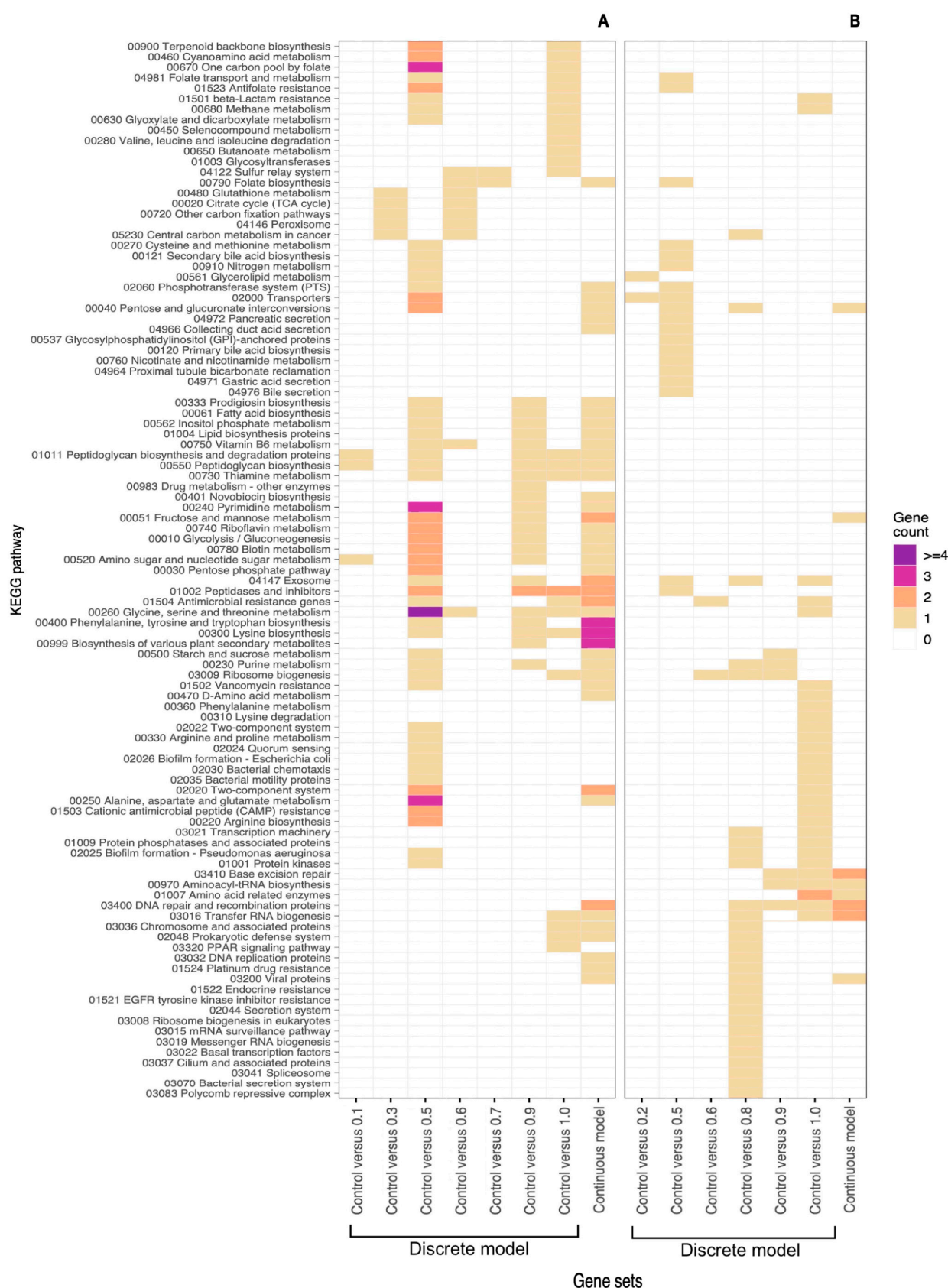


Figure 5. Heatmap showing the KEGG pathways (y -axis) enriched by *L. monocytogenes* genes after GS-2 treatment (0.1–1%). The plot is faceted across the x -axis into the two categories: Genes with overall increased expression as the gradient of GS-2 concentrations (0.1–1%) increases (A) and genes with overall decreased expression as the gradient of GS-2 concentrations (0.1–1%) increases (B), delineating the overall patterns of expression inferred by the discrete and the continuous models.

While the specific KEGG pathways with the highest number of mapped genes differed between discrete and continuous modeling, this divergence is expected because the two approaches capture fundamentally different aspects of the GS-2 response. The discrete model identifies genes that change significantly at individual concentrations relative to the control, often capturing threshold-like or nonlinear responses that occur only at specific GS-2 levels such as the strong transcriptomic shift at 0.5% GS-2. In contrast, the continuous model detects genes that display gradual, monotonic increases or decreases across the entire 0.1–1.0% gradient, regardless of whether any single concentration produces a large enough shift to reach significance in a pairwise comparison. As a result, the sets of genes entering the KEGG analysis differ between the two methods, leading to different pathways being ranked highest.

For the genes with overall decreased expression as the gradient of GS-2 concentrations (0.1–1%) increases, there were no pathways with more than two genes mapped (Figure 5B). GS-2 at 1% was the only concentration that had one pathway with 2 genes: 01007 Amino acid related enzymes (Figure 5B). Across the discrete contrasts, 1% and 0.8% GS-2 both had the highest number of KEGG pathways represented (29 pathways each), followed by 0.5% (19 pathways), 0.9% (7 pathways), and 0.2% and 0.6% (2 pathways each) (Figure 5B). Continuous modeling had three KEGG pathways with 2 genes mapped each: 03410 Base excision repair (repairs damaged DNA resulting from oxidative stress), 03400 DNA repair and recombination proteins, and 03016 Transfer RNA biogenesis (used for maturation of transfer RNAs essential for protein synthesis) [44,45] (Figure 5B). This pattern suggests that increasing GS-2 stress may gradually suppress functions related to DNA repair and translational machinery.

4. Discussion

4.1. Differential Antimicrobial Efficacy Across Microbial Taxa

When GS-2 at concentrations 0.1–3% was inoculated against microbes, population reductions in *A. flavus*, *E. coli* O157:H7, *L. monocytogenes*, and *S. Agona* were shown at concentrations 2.8–3% (Figure 1A–C,E). For *L. monocytogenes*, populations were decreased to below the detection limit with 3% GS-2 (Figure 1B). The enhanced sensitivity of *L. monocytogenes*, Gram-positive bacteria, compared to Gram-negative bacteria may reflect on how GS-2 attacks cells. Gram-positive cells possess a thick peptidoglycan cell wall, but lack an outer membrane, which leaves them exposed and vulnerable to antimicrobial compounds that can disrupt lipid bilayers [46]. In contrast, Gram-negative cells have a thinner peptidoglycan cell wall, but have additional barriers such as an outer membrane containing lipopolysaccharides that surround the thinner peptidoglycan cell wall [46,47]. The outer membrane of Gram-negative cells offers protection against antimicrobials [48], which can include GS-2. The GS-2 formulation evaluated here also contained thymol, a monoterpene phenol previously shown to enhance the activity of GS-2 against Gram-positive bacteria [18]. Thymol therefore likely contributes to the initial permeabilization of the *L. monocytogenes* cell envelope, which is consistent with the heightened sensitivity of this Gram-positive organism. In addition, at intermediate concentrations (1.8–2.1%), *E. coli* O157:H7 and *S. Agona* populations increased rather than decreased over the 3-day exposure (Figure 1A,C); this pattern is inconsistent with a purely osmotic or nonspecific growth-inhibitory effect and instead points to a concentration-dependent, formulation-specific antimicrobial action.

Against fungi, GS-2 displayed varied fungicidal activity with limited activity against *A. niger*, but strong fungicidal activity against *A. flavus* (Figure 1D,E). The initial fungal inoculum ($\sim 10^7$ CFU/mL) was lower than that used for the bacterial assays ($\sim 10^9$ CFU/mL) because this represented the highest reproducible spore concentration that could be ob-

tained under the culture conditions employed. Although the lower starting inoculum reduces the maximum measurable log reduction before reaching the detection limit, antimicrobial efficacy was evaluated as log reductions relative to each organism's initial population. Therefore, the observed concentration-dependent responses remain appropriate for assessing GS-2 activity within each organism. The differences between the cell wall of *A. flavus* and *A. niger* are not fully understood; however, the conidial cell wall of *A. flavus* contains a significantly higher number of proteins and percentage of β -1, 3-glucan branching compared to *Aspergillus fumigatus* (*A. fumigatus*), but *A. fumigatus*' conidial surface is covered by rodlet layers while *A. flavus* has exposed polysaccharides [49,50]. The cell wall of *A. niger* has been known to be similar to *A. fumigatus*, with cell wall amino acids in the same classes (I–VII) [51–54]. The rodlet layers in the conidial cell wall of *A. niger* can reduce the effectiveness of GS-2, which could potentially explain the effectiveness of GS-2 against *A. flavus* and not *A. niger*. From prior research, it has been shown that *A. niger* was also more resistant against chemical disinfectants such as chlorine ≤ 3 ppm and peracetic acid ≤ 100 ppm compared to *A. flavus* [55]. These findings may suggest that differences in conidial surface organization and accessibility, particularly the presence of protective rodlet layers, may potentially play a more influential role in GS-2 susceptibility than cell wall polysaccharide composition; however, further research can provide a clearer insight.

4.2. Mechanistic Insights from Sublethal Transcriptomic Responses

From the three bacterial strains, RNA sequencing was conducted on *L. monocytogenes* at sublethal GS-2 concentrations (0.1–1%). GS-2 at 0.5% was the concentration that had the highest variability and the highest number of statistically significant increasing and decreasing genes compared to the control (71–93 genes) ($p < 0.01$) (Figures 2 and 3). Comparatively, the higher sublethal GS-2 concentrations (0.9% and 1%) also had high variability, but both had a lower number of statistically significant increasing and decreasing genes compared to the control (23–41 genes) ($p < 0.01$) (Figures 2 and 3). Interestingly, GS-2 at 0.6–0.8% had a significantly lower number of increasing and decreasing genes compared to the control (2–6 genes) ($p < 0.01$) (Figure 3).

The pronounced transcriptional response at 0.5% GS-2 likely reflects a stress level at which *L. monocytogenes* was still able to actively regulate gene expression, resulting in coordinated increases and decreases across many genes. The relatively low number of differentially expressed genes at 0.6–0.8% GS-2 suggest a non-linear response in *L. monocytogenes*, where 0.5% represents an initial activation threshold for a broad stress program, but intermediate-high concentrations (0.6–0.8%) fall into a “plateau” region in which many stress pathways are already engaged and additional increases in GS-2 do not produce large, consistent expression shifts relative to the control. In addition, at intermediate GS-2 concentrations (0.6–0.8%), bacterial populations may consist of a mixture of actively responding, injured, and metabolically suppressed cells, resulting in inconsistent gene expression patterns across replicates and fewer statistically significant genes. At the highest sublethal concentrations (0.9–1%), although variability remained high, the reduced number of statistically significant genes suggests that the high GS-2 concentrations may cause increased cellular damage that limits the capacity for consistent, regulated transcriptional responses.

4.3. Sublethal GS-2 Exposure Elicits Coordinated Metabolic and Stress Responses

To gain insight into how *L. monocytogenes* adapts to sublethal GS-2 exposure and help contextualize the concentration-dependent transcriptional patterns observed in the discrete and continuous models from Figures 2–4, differentially expressed *L. monocytogenes* genes after GS-2 treatment were mapped to KEGG pathways. Across the GS-2 gradient (0.1–1.0%),

the strongest enrichment of upregulated pathways occurred at 0.5% GS-2, whereas down-regulated pathways were distributed across higher concentrations, particularly at 0.8% and 1% (Figure 5). This asymmetry may suggest that GS-2 elicits a robust, active stress response at intermediate concentrations (0.5%), followed by progressive suppression of cellular functions (0.8% and 1%) as GS-2 concentrations approach lethality.

At 0.5% GS-2, KEGG pathways that had ≥ 3 upregulated genes mapped (Figure 5A) were associated with nitrogen redistribution (00250 Alanine, aspartate and glutamate metabolism) and nucleotide metabolism (00240 Pyrimidine metabolism, 00260 Glycine, serine and threonine metabolism and 00670 One carbon pool by folate) [56–63]. Together, the upregulation of these pathways supports the idea that at the sublethal concentration of 0.5% GS-2, *L. monocytogenes* retains the capacity for carbon, nitrogen and nucleotide metabolism, which is consistent with an adaptive stress response rather than transcriptional shutdown.

At 1% GS-2, KEGG pathways associated with translational regulation (03016 Transfer RNA biogenesis), chromosomal protection and replication arrest (03036 Chromosome and associated proteins), emergency stress and defense responses (02048 Prokaryotic defense system) and membrane-associated stress responses (03320 PPAR signaling pathway) were uniquely enriched (Figure 5A) [44,64–66]. The important aspect to note is that while these KEGG pathways only had one upregulated gene mapped to each, these KEGG pathways were not enriched at the lower GS-2 concentrations and were enriched for the first time at GS-2 1% (Figure 5A). The unique enrichment of these KEGG pathways may indicate a shift from adaptive metabolic responses at 0.5% GS-2 to damage-control and survival-oriented mechanisms. Activation of tRNA biogenesis and chromosome-associated pathways suggests translational regulation, replication arrest, and genomic protection under severe stress, while enrichment of defense and lipid-associated signaling pathways is consistent with activation of emergency response systems. These patterns suggest that at 1% GS-2, *L. monocytogenes* may enter a stress state characterized by growth arrest and cellular-damage management, a pattern consistent with cumulative cellular stress that may include perturbation of the cell membrane.

KEGG mapping of downregulated *L. monocytogenes* genes was sparse, with most KEGG categories containing only a single mapped gene (Figure 5B). The only category containing more than one downregulated gene was after 1% GS-2 treatment: 01007 Amino acid related enzymes (Figure 5B), a broad KEGG functional grouping [67,68]. Sparse KEGG pathway mapping was observed across all GS-2 concentrations, reflecting the fact that KEGG pathway analyses capture only those differentially expressed genes with curated functional annotations [69]. Under the highest sublethal stress conditions of 1% GS-2, downregulation seemed to occur primarily at the level of individual genes rather than through coordinated suppression of entire pathways. As a result, most narrow KEGG pathways contained only a single downregulated gene, and only one broad functional category (01007 Amino acid related enzymes) accumulated two downregulated genes.

It is important to clarify that the antimicrobial efficacy experiments conducted in this study were performed in solution and were designed primarily to establish concentration-dependent microbial reductions and define sublethal exposure levels for transcriptomic analysis. These experiments were not intended to replicate GS-2 performance as a dried surface coating on reusable packaging materials. The physicochemical behavior of GS-2 in solution differs from that of a surface-applied formulation, where factors such as coating adherence, drying dynamics, surface interactions, and environmental conditions influence antimicrobial performance. Surface efficacy of GS-2 on packaging materials has been previously demonstrated [22], whereas the present study focuses on mechanistic insights under controlled liquid exposure conditions.

5. Conclusions

Overall, the transcriptomic profiling presented here indicates that sublethal GS-2 exposure elicits a coordinated, concentration-dependent stress response in *L. monocytogenes*, characterized by adaptive activation of carbon, nitrogen, and nucleotide metabolism at lower sublethal concentrations and a shift toward growth arrest and cellular-damage management at higher concentrations. These transcriptomic signatures, together with the differential susceptibility observed across taxa, including the relative resistance of *A. niger* compared with *A. flavus*, are consistent with, but do not by themselves directly demonstrate, a mode of action involving disruption of the microbial cell membrane; additional or other mechanisms may also contribute and warrant direct investigation. The antimicrobial effects of GS-2 appear to accumulate in a dose-dependent manner, ultimately overwhelming microbial stress-response mechanisms and resulting in microbicidal and fungicidal activity at higher concentrations. While transcriptomic analyses in this study focused on the Gram-positive model organism *L. monocytogenes*, the observed stress-response patterns provide a mechanistic framework for understanding GS-2 activity under the liquid-suspension conditions examined in this study. Future studies extending RNA sequencing to Gram-negative bacteria and fungal species would further elucidate whether comparable stress-response mechanisms contribute to GS-2 susceptibility across diverse microbial taxa.

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