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Transcriptomic Reanalysis Reveals Complement, Circadian, and Toll-Like Receptor Signaling Remodeling in *Lactiplantibacillus plantarum*-Induced Innate Immune Memory in Human Monocytes

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Abstract: The commensal bacterium *Lactiplantibacillus plantarum* is recognized for its ability to cause a state of innate immune memory in monocytes, characterized by the tolerogenic phenotype and lowered pro-inflammatory responses. This study provides an extensive re-examination of the transcriptomic dataset originating from human CD14 + monocytes primed with live or heat-killed *L. plantarum* (GSE159496). Using a computational approach, 1,954 differentially expressed gene calls that corresponded to 1,397 unique genes were obtained through three pairwise comparisons. In addition, several novel biological pathways that were not investigated in the original study were discovered. Priming with live *L. plantarum* resulted in a marked downregulation of the complement system (12.5% decrease in its module score, $p < 0.001$), indicating an important mechanism of immune evasion. Additionally, a significant alteration of the circadian rhythm pathway was revealed, indicating its connection to metabolic time management in sustaining long-term immune tolerance. Toll-like receptor (TLR) signaling was coordinately repressed, with 17 pathway genes—including the receptors TLR2 and TLR8—significantly downregulated in live primed cells (p.adjust = 3.0×10^{-6}). Other significantly modulated novel pathways included a downregulation of glycosylation and post-translational modifications (−13.2%, $p < 0.01$) and an upregulation of amino acid sensing/mTOR signaling (+11%, $p < 0.001$) and xenobiotic detoxification (+12%, $p < 0.01$). These findings point to a multi-layered adaptive response where *L. plantarum* not only dampens canonical inflammatory signaling but also actively remodels cellular processes related to pathogen recognition, metabolic sensing, and stress responses. This reanalysis provides a more granular model of probiotic-induced immune memory.

Keywords: *Lactiplantibacillus plantarum*, innate immune memory, Toll-like receptor signaling, host–microbe interactions

1. Introduction

The innate immune system, once thought to lack memory, can form long-term functional adaptations in response to stimuli, a phenomenon known as trained immunity or immune tolerance [1, 2]. This process metabolically and epigenetically reprograms innate immune cells, including monocytes and macrophages [3]. Prior exposure enhances (training) or suppresses (tolerance) the response to a second challenge [2]. It has not yet been established what role this mechanism plays with respect to beneficial commensal bacteria, as most previous literature has focused on pathogen-derived stimuli. There is ongoing interaction between commensal bacteria and the host immune system, which trains immune cells and helps to maintain homeostatic balance [4].

The probiotic *Lactiplantibacillus plantarum* serves to modify the immune response of the host [5]. Previous studies indicated that the priming of human monocytes with *L. plantarum*, specifically in its live form, results in a long-term memory state that has tolerogenic properties [6]. This memory state is associated with increased intracellular survival of the bacterium, decreased pro-inflammatory cytokine production such as TNF, a reduction in both glycolytic and respiratory rates, and diminished NF-κB- and interferon-driven signaling [6].

In addition to those mechanisms contributing to tolerogenic memory, other processes are likely to participate in these tolerogenic events. Understanding how these mechanisms work at a biological and mechanistic level will, in turn, provide a meaningful basis for future therapeutic approaches [7]. Identifying

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the signaling pathways through which these commensal organisms act will help to rationally design next-generation probiotics and postbiotics [8]. There is great promise in the engineered manipulation of immune responses to provide new therapies for immune-mediated disease, such as inflammatory bowel disease (IBD), rheumatoid arthritis, and allergy [9]. Induction of a tolerogenic state by specific microbes may restore homeostasis and add to the effectiveness of anti-inflammatory therapy [9].

Recent work has made it increasingly clear that innate immune memory is not a single fixed program. Monocytes can move along a continuum that includes priming, enhanced training, and tolerance, with the final phenotype depending on the nature, strength, and duration of the initiating signal and on the interval before restimulation. One mechanism by which such alternative states emerge is through the mechanism of interferon-dependent chromatin alteration and regulation of genes that are inflammatory with the involvement of both STAT1 and IRF1 [10]. This proposition is important for the commensal organisms since continuous contact with microbes at the level of mucosal membranes does not always lead to the excessive inflammatory reaction in line with the classical notion of trained immunity. A properly working organism can maintain a more stable state in which important antimicrobial or homeostatic functions are conserved and damaging cytokine effects are controlled. New studies on innate memory indicate that it is accompanied by metabolic changes that actually play a role in chromatin regulation of innate immunity instead of just coming together with it. For instance, prolonged lactylation of histones has been shown to be a molecule that connects metabolism to long-lasting locus history through the epigenetic mechanism [11]. More generally, the current understanding of the phenomenon includes the notion of mTOR/HIF-1α activity with glycolysis, mitochondrial functioning, and metabolism-sensitive enzymes in the same regulatory network determining whether myeloid cells are hyperreactive or hyporeactive due to the initial event [12].

The recent discoveries help in understanding immune memory induced through probiotics. *L. plantarum* is considered to do more than just act as a simple bacterial strain in the human microbiota as various subspecies can modulate the activity of both the innate and adaptive immune system, as well as epithelial signaling and imbalance in mucosal immunity [13]. Moreover, homeostasis between the host and microbes is achieved by mechanisms other than cytokine action. For instance, the complement system has been shown to facilitate the communication between the microbiota and the host, where the complement activity can play a role in the antimicrobial defense and in the promotion of tolerance toward bacteria [14]. Finally, it is worth noting that circadian regulation exists within the body as well: macrophages can self-regulate their inflammatory response to stimuli based on the time of day with the help of their intrinsic circadian clocks [15]. Pattern-recognition signaling is similarly context dependent. Recent work with *L. plantarum* has shown that bacterial components can engage TLR2 together with other innate receptors to shape regulatory cytokine production, illustrating how the same broad receptor family can support either inflammatory or homeostatic outcomes depending on the microbial ligand and signaling context [16]. Taken together, these observations suggest that the tolerogenic memory induced by live *L. plantarum* may reflect coordinated remodeling across several regulatory systems rather than suppression of a single inflammatory pathway. This possibility motivated us to examine complement, circadian, glycosylation, Toll-like receptor (TLR), and metabolic modules together in the present transcriptomic reanalysis.

We hypothesized that biological mechanisms beyond those already described contribute to the induction and maintenance of *L. plantarum*-induced immune tolerance and that such mechanisms can be uncovered by systematic reanalysis of existing transcriptomic data [17]. We therefore performed a comprehensive reanalysis of publicly available RNA-seq data from human monocytes primed with live or heat-killed *L. plantarum*, using rigorous differential expression analysis, pathway enrichment analysis [10, 11], and module score quantification. We focused in particular on pathways governing complement activation, circadian timing, protein glycosylation, and TLR signaling, none of which were examined in the original report [6].

2. Methods

2.1. Original experimental design and data source

This study is a computational reanalysis of the publicly available transcriptomic dataset GSE159496 [12], generated and originally published by Pellon et al. [6]. No new experiments were performed, and no new human or animal samples were collected for the present work; the ethical approvals covering the original sample collection are described in the source publication [6]. In the original study, human CD14 + monocytes were isolated from healthy donors. These cells were then subjected to one of three conditions, each with three biological replicates (n = 3): (1) Unprimed, where cells were exposed to culture medium (control); (2) Live_primed, where cells were primed with live *L. plantarum* WCFS1; and (3) HeatKilled_primed, where cells were primed with heat-inactivated *L. plantarum* WCFS1. Primed cells were rested for six days to establish a memory state, and RNA was extracted for high-throughput sequencing [6].

2.2. Computational reanalysis methodology

Raw gene-level count data were downloaded from the Gene Expression Omnibus and processed through a bioinformatics pipeline in R (version 4.5.2) [12, 13]. We retained 16,246 genes with a minimum of 10 reads in at least three samples. The retained count data were normalized using the variance-stabilizing transformation (VST) method from the DESeq2 package to correct for library size differences and stabilize variance across the mean, making it suitable for visualization and module score analysis [18].

We analyzed differential expression with DESeq2, which models gene counts with a negative binomial distribution [18]. Three pairwise comparisons were conducted: (1) Live_primed vs. Unprimed, (2) HeatKilled_primed vs. Unprimed, and (3) Live_primed vs. HeatKilled_primed. A Benjamini–Hochberg adjusted p-value (padj) below 0.05 and an absolute log2 fold change greater than 1 defined a gene as differentially expressed [19].

We identified enriched pathways from the differentially expressed gene (DEG) lists using clusterProfiler [20]. Gene sets from Gene Ontology (GO: Molecular Function), KEGG, and Hallmark (MSigDB) databases were tested for overrepresentation using a hypergeometric test. A significance threshold of p.adjust < 0.05 and q-value < 0.2 was applied [17, 21–23].

2.3. Module score calculation and statistical validation

A module score for each sample quantified the activity of newly identified pathways. This score is defined as the mean

VST-normalized expression of all genes within a specific pathway gene set [18]. We used Welch's t-test to assess differences in pathway module scores, which average individual gene fluctuations. We then applied a Benjamini–Hochberg correction for multiple comparisons [19]. We correlated pathway module scores to measure co-regulation. All visualizations, including principal component analysis (PCA) plots, volcano plots, heatmaps, and boxplots, were generated using ggplot2 and associated packages [24]. The complete analysis workflow, including the software used and the manuscript item produced at each stage, is summarized schematically in Figure 1.

2.4. Data and code availability

The transcriptomic dataset analyzed in this study is publicly available from the Gene Expression Omnibus under accession GSE159496 [22]. All scripts used for data preprocessing, differential expression analysis, pathway enrichment analysis, module score calculation, statistical testing, and figure generation have been deposited in a public repository at https://github.com/ahmedalfahad/L-plantarum-innate-memory-reanalysis. The repository includes a README file documenting the R version and the exact version of every required package, the provenance of all input files, and step-by-step instructions for reproducing each figure, table, and reported statistic in this manuscript.

The analysis proceeded from the public data source (GSE159496) through low-count filtering and variance-stabilizing normalization; differential expression testing with DESeq2; overrepresentation analysis against the GO Molecular Function, KEGG, and Hallmark gene set collections; thematic prioritization of pathways not examined in the original study; module score quantification with Welch's t-test and Benjamini–Hochberg correction; and finally biological interpretation. The software used and the manuscript item generated at each stage are indicated in the right-hand column.

3. Results

3.1. Transcriptomic reprogramming in monocytes by *L. plantarum*

PCA of variance-stabilized expression data identified global transcriptomic changes from *L. plantarum* priming [18]. The first principal component (PC1) explains 54% of the variance and separates Live primed samples from Unprimed and Heat Killed primed samples (Figure 2(A)). Live bacterial priming establishes a long-term transcriptional state distinct from that of heat-killed bacteria or the control.

Live bacteria drove differential gene expression. Comparing Live primed with Unprimed monocytes showed the largest number of DEGs, with 624 downregulated and 605 upregulated genes. In contrast, the Heat Killed vs Unprimed comparison revealed 334 DEGs (228 downregulated, 106 upregulated), while the direct comparison of Live vs Heat Killed identified 391 DEGs, highlighting viability-specific effects (Figure 2(B)). Across all three comparisons, these calls correspond to 1,397 unique genes.

There is a core group of 40 DEGs shared across the three comparisons, with common regulation of SLAMF1, an immune gene that is downregulated, and HAMP, an antimicrobial peptide that is upregulated, as shown in Figure 2C. The Live vs Unprimed comparison has the largest set of uniquely affected genes (722 genes), indicating the extent of transcriptional reprogramming driven by live bacteria. Of the DEGs unique to the Live vs Unprimed comparison, there are members of the newly identified pathways, including ST3GAL2, a downregulated glycosylation gene, and ACSL4, an acyl-CoA synthetase involved in lipid metabolism (Figure 2(C)).

The magnitude and significance of gene expression differences are shown in the volcano plots for the three comparisons. In the Live vs Unprimed comparison, gene regulation displays a wide and symmetric distribution of both upregulated and downregulated genes, with important examples including the downregulated chemokine CCL15 and interleukin IL1A and the upregulated antimicrobial peptide HAMP and the receptor CD22 (Figure 2(D)). The Heat Killed vs Unprimed comparison indicates a lesser extent of transcriptional regulation, with fewer DEGs reaching significance on this plot (Figure 2(E)). The Live vs Heat Killed volcano plot isolates only those genes whose differential expression occurs as a consequence of the loss of bacterial viability, including SLC2A1 (upregulated) and CCL15 (downregulated), highlighting viability-specific effects (Figure 2(F)).

3.2. Identification of novel downregulated pathways

Our comprehensive enrichment analysis confirmed the downregulation of known inflammatory pathways such as TNF-α signaling via NF-κB, cytokine–cytokine receptor interaction, and IL-17 signaling, consistent with the original study, across the Hallmark, KEGG, and GO Molecular Function databases [25–27]. Results for all three comparisons—Live vs Unprimed, Heat Killed vs Unprimed, and Live vs Heat Killed—are presented together in Figure 3(A)–(C). However, we also identified several novel, significantly downregulated pathways that were not previously explored.

The most striking novel finding was the profound downregulation of the complement system. Live primed monocytes significantly repressed 31 complement cascade genes, including C1QB, C1R, and C1S. Live priming lowered the module score for this innate immunity pathway by 12.5% relative to the unprimed group ($p < 0.001$, Figure 4(A) and (B)).

We also found altered expression of the circadian rhythm pathway. A module of 22 core clock and circadian-regulated genes, including IL6, IL1B, and TNF, was significantly downregulated in Live primed monocytes, with a 14.2% decrease in module score ($p < 0.01$) (Figure 4(C) and (D)) [28, 29]. These data indicate that *L. plantarum* priming is associated with reduced expression of circadian-related genes, a pattern that may intersect with metabolic reprogramming and the tolerogenic state.

Furthermore, we identified significant downregulation of pathways related to glycosylation and post-translational modifications. This module, comprising 30 genes, showed a 13.2% decrease in expression in the Live primed group ($p < 0.01$), suggesting altered regulation of processes that influence monocyte surface receptors and secreted proteins and may therefore affect cell-cell communication and pathogen recognition (Figure 4(E) and (F)).

Consistent with this reduction in glycosylation-related transcription, TLR signaling was also coordinately repressed. Seventeen genes of the KEGG TLR signaling pathway were significantly downregulated in Live primed monocytes (p.adjust $= 3.0 \times 10^{-6}$, fold enrichment 4.73), including the pattern-recognition receptors TLR2 and TLR8 together with downstream signaling components and target genes (MAP2K3, STAT1, TNF, IL1B, IL6, IL12B, CD40, CD80, CCL3, CCL4, CCL5, CXCL8,

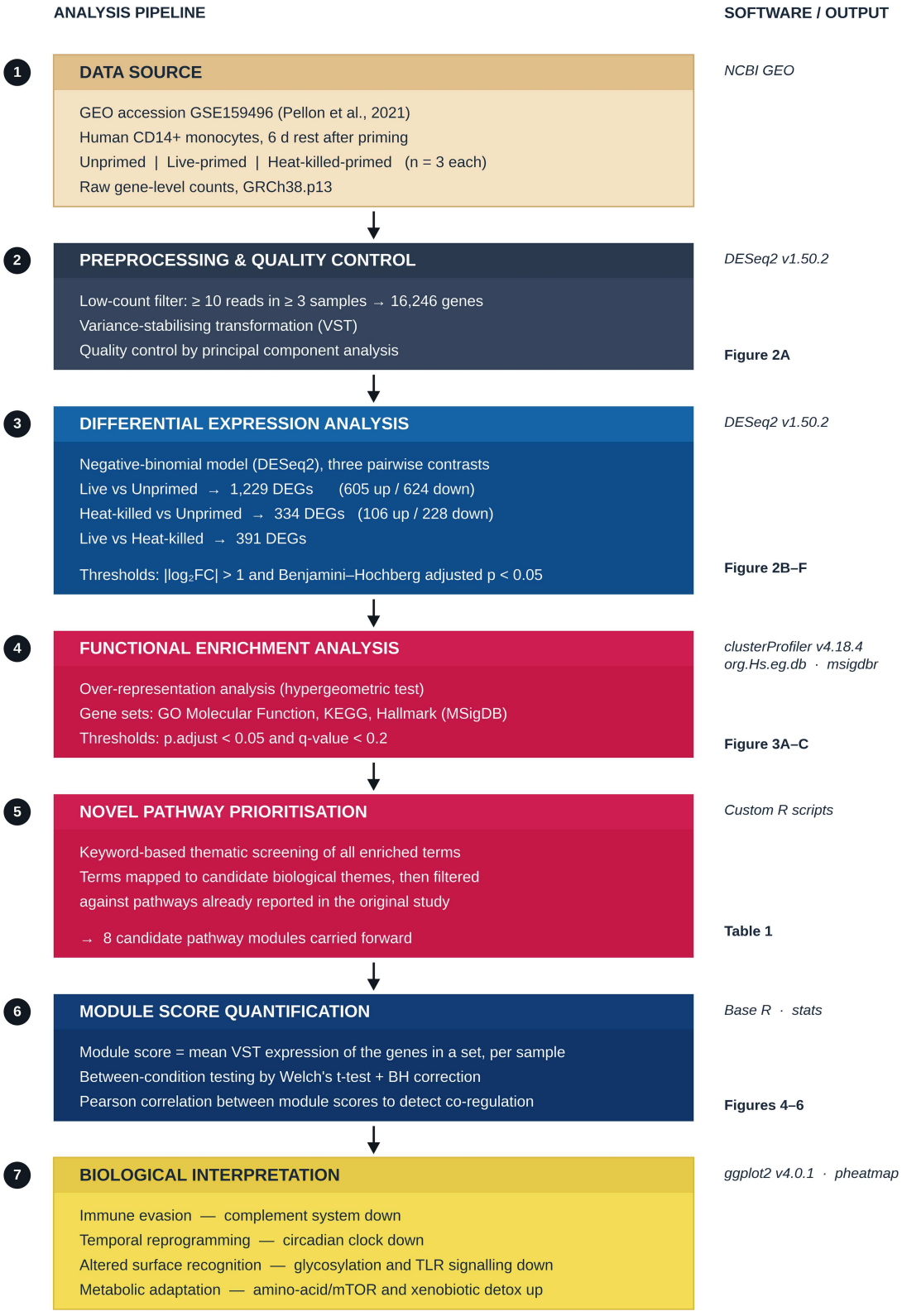

Figure 1. Schematic overview of the computational workflow

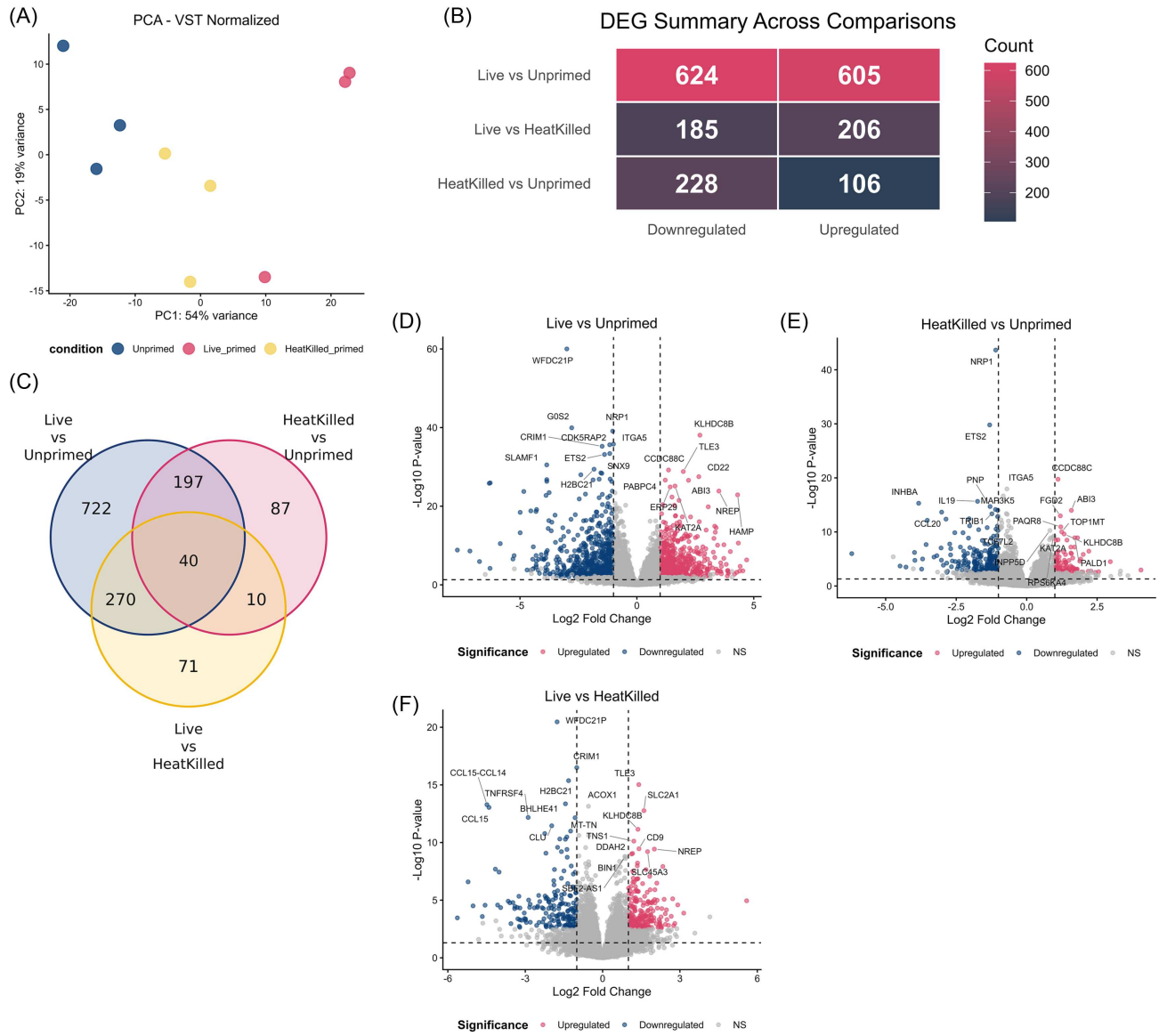

Figure 2. Transcriptomic changes in monocytes following *L. plantarum* priming. (A) PCA plot of VST [18] normalized data showing distinct clustering of Unprimed, Live primed, and Heat Killed primed samples. PC1 explains 54% of the variance, and PC2 explains 19%. (B) Heatmap summarizing the number of upregulated and downregulated differentially expressed genes (DEGs) for each of the three comparisons. (C) Venn diagram showing the overlap of DEGs between the Live vs Unprimed, Heat Killed vs Unprimed, and Live vs Heat Killed comparisons. (D) Volcano plot illustrating DEGs in Live primed versus Unprimed monocytes. Key downregulated genes include CCL15 and IL1A; key upregulated genes include HAMP and CD22. (E) Volcano plot illustrating DEGs in Heat Killed primed versus Unprimed monocytes, showing a less extensive transcriptional change compared to live priming. (F) Volcano plot illustrating DEGs that are dependent on *L. plantarum* viability (Live primed versus Heat Killed primed). Key viability-specific genes include SLC2A1 (upregulated) and CCL15 (downregulated). Thresholds throughout: |log2FC| > 1, padj < 0.05

CXCL9, and CXCL10). Seven pathway genes (CCL5, CD40, CD80, CXCL9, CXCL10, CXCL11, and STAT1) remained significantly reduced in the direct Live versus Heat Killed comparison (p.adjust = 4.1×10^{-3}), indicating that this transcriptional pattern is associated with bacterial viability (Figure 3(A) and (C)).

3.3. Identification of novel upregulated pathways

In addition to suppressive mechanisms, our analysis revealed several upregulated pathways indicative of an active cellular adaptation to the priming stimulus. A key finding was the significant upregulation of amino acid sensing and mTOR signaling. This 16-gene module was upregulated by 11% in Live primed monocytes ($p < 0.001$), suggesting an activation of nutrient-sensing pathways that are central to controlling metabolic reprogramming [30, 31] and cell fate decisions [32] (Figure 5(A) and (B)).

We also uncovered upregulation of xenobiotic detoxification pathways. This 7-gene module, which includes cytochrome P450s and other detoxification enzymes, was increased by 12% in the Live primed condition ($p < 0.01$). This pattern is consistent with a cellular stress-adaptation response and may reflect altered handling of bacterial metabolites in the tolerogenic state (Figure 5(C) and (D)).

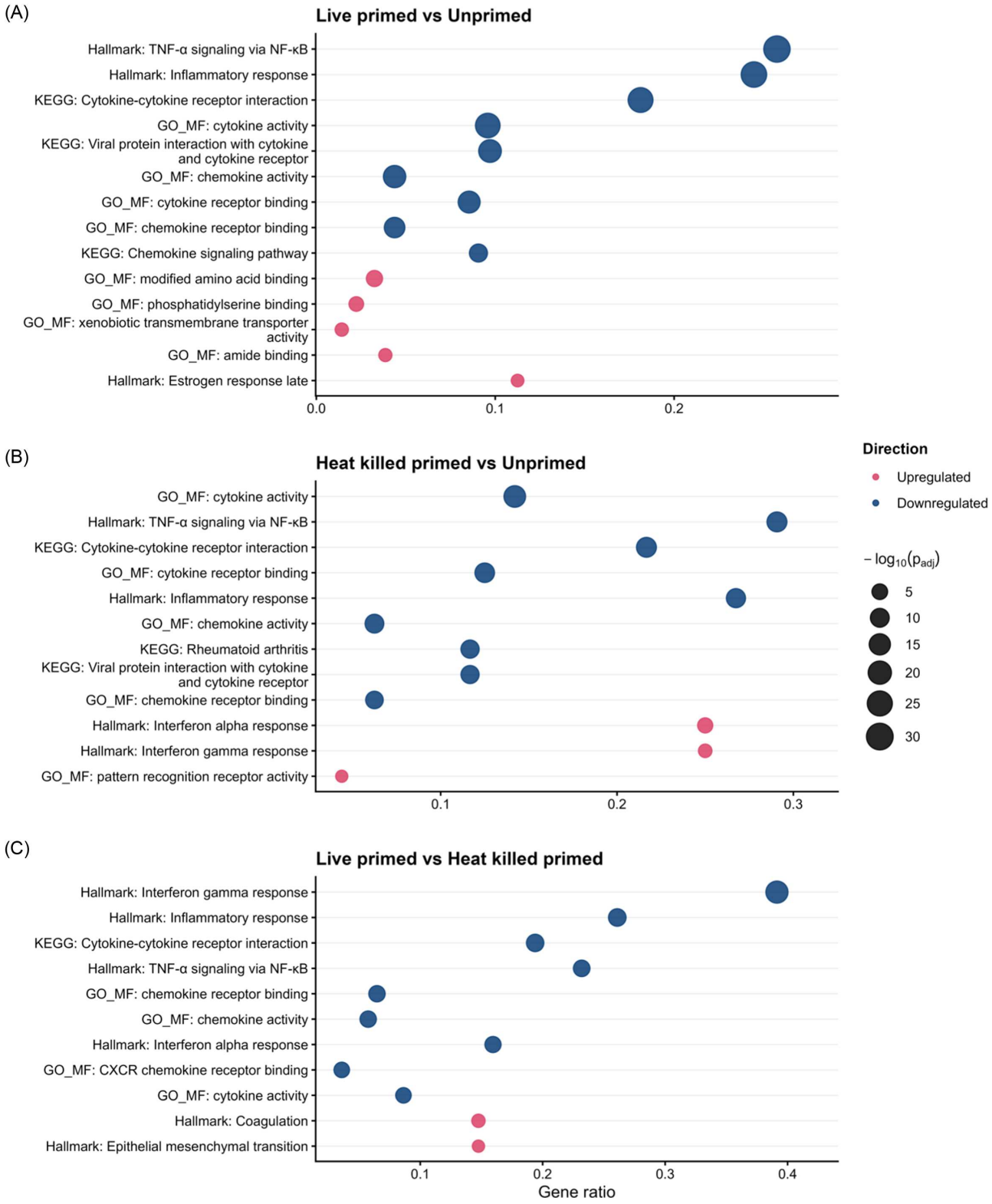

Figure 3. Pathway enrichment results for all three pairwise comparisons, combined into a single figure to allow direct comparison between conditions. (A) Live primed versus Unprimed. (B) Heat Killed primed versus Unprimed. (C) Live primed versus Heat Killed primed. For each comparison, the most significantly enriched terms across the GO Molecular Function, KEGG, and Hallmark databases are shown. Dot position indicates the gene ratio, dot size indicates −log10 (p.adjust) on a scale shared by all three panels, and color indicates the direction of regulation. Canonical inflammatory pathways are downregulated in all three comparisons, with the most extensive changes in live primed cells

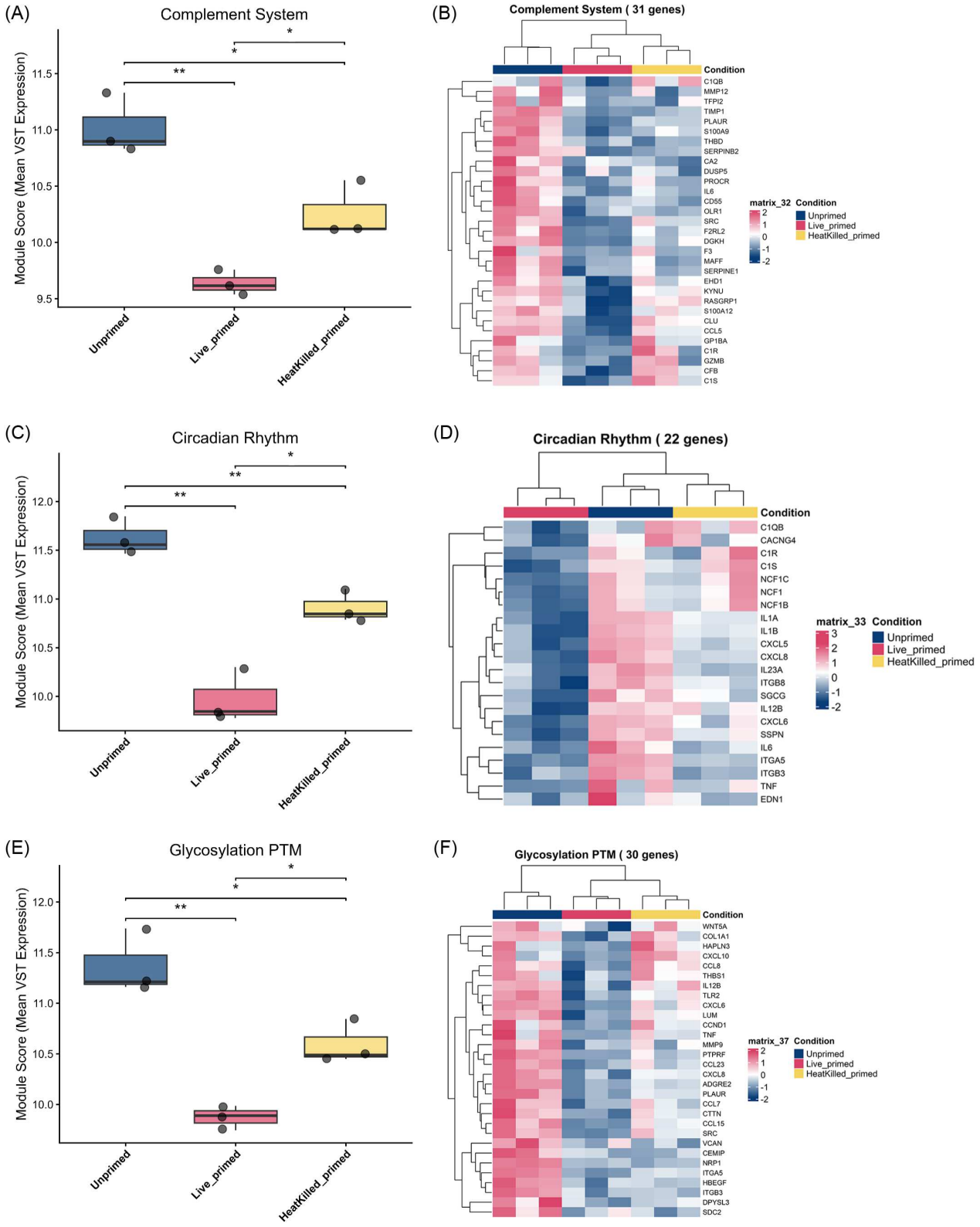

Figure 4. (A) Live primed monocytes had a lower complement system module score than Unprimed monocytes ($p < 0.001$). (B) Live primed monocytes express lower levels of complement system pathway genes, including C1QB, C1R, and C1S. (C) Live primed monocytes exhibit downregulation of the circadian rhythm module ($p < 0.01$). (D) Heatmap illustrating the co-downregulation of core clock and circadian-regulated genes (e.g., IL6, IL1B, TNF) in Live primed monocytes [20, 25]. (E) Boxplot of the glycosylation and post-translational modification (PTM) module score, demonstrating significant reduction in Live primed monocytes ($p < 0.01$). (F) Heatmap illustrating the downregulation of genes related to glycosylation and PTMs in Live primed monocytes

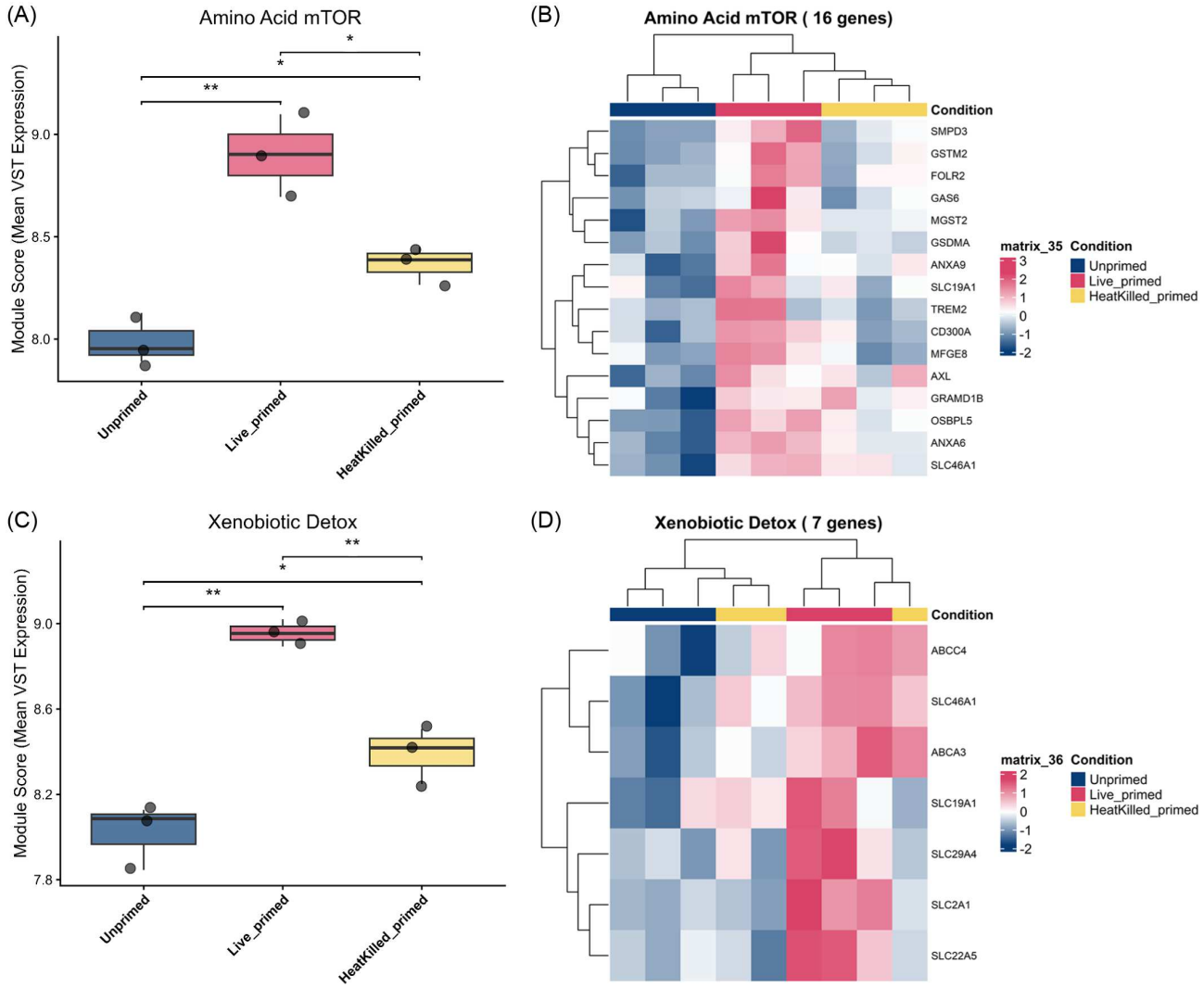

Figure 5. (A) Boxplot of the amino acid sensing and mTOR signaling module score, demonstrating significant upregulation in Live primed monocytes ($p < 0.001$). (B) Heatmap illustrating the upregulation of genes within the amino acid sensing and mTOR signaling pathway in Live primed monocytes. (C) Boxplot of the xenobiotic detoxification module score, demonstrating significant upregulation in Live primed monocytes ($p < 0.01$). (D) Live primed monocytes increased expression of xenobiotic detoxification genes, such as cytochrome P450s and GSTA1

3.4. Co-regulation and summary of novel pathways

We correlated the module scores to test the relationship between these pathways. The correlated downregulation ($r > 0.85$, $p < 0.001$) of complement, circadian rhythm, and glycosylation pathways suggests a unified program of immune suppression. An inverse correlation between suppressive and adaptive pathways, like amino acid/mTOR signaling, indicates reciprocal regulation between immune evasion and metabolic adaptation ($r < -0.75$, $p < 0.01$) (Figure 6(A) and (B)).

We identified new pathways and summarized their regulatory direction, key genes, and biological function in Table 1, which brings together the candidate mechanisms of *L. plantarum*-induced innate immune memory identified in this reanalysis.

4. Discussion

This transcriptomic reanalysis supports the original observation that live *L. plantarum* priming is followed by a persistent, low-inflammatory memory-like state in human monocytes [6], while extending that model to several pathways not emphasized in the source study. The present findings point to coordinated changes in complement-related transcription, circadian-associated genes, glycosylation, and TLR signaling, together with increased amino acid/mTOR and xenobiotic detoxification pathway activity. Recent work has increasingly framed innate immune memory as a spectrum that includes training, priming, and tolerance, with overlapping metabolic and epigenetic mechanisms but different functional outcomes [10, 12]. In that context, the pattern observed here is most appropriately described as tolerogenic innate immune memory rather than uniformly enhanced trained immunity. Importantly, the present analysis identifies associations within a transcriptional state; it does not by itself establish that any individual pathway is necessary or sufficient for the phenotype.

One of the clearest findings was the coordinated reduction of complement-related transcripts, including C1QB, C1R, and C1S. This builds upon the initial statement regarding lowered

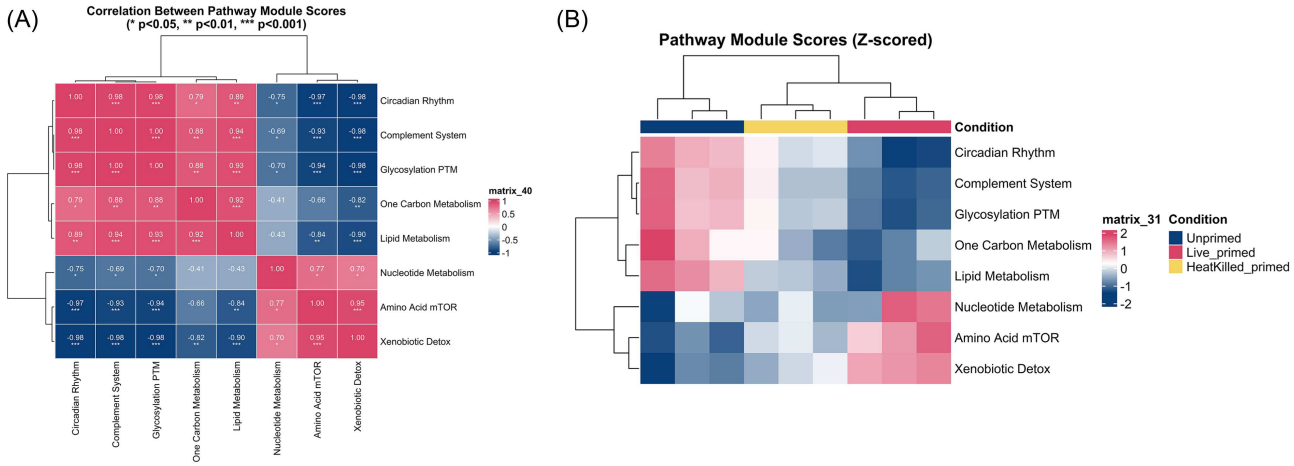

Figure 6. (A) Pearson correlations between pathway module scores indicated positive co-regulation among suppressive pathways. (B) The conditions clustered based on pathway activity. Live primed samples showed higher usage of nucleotide metabolism, amino acid mTOR, and xenobiotic detoxification. Heat Killed primed samples upregulated pathways for lipid metabolism, one-carbon metabolism, glycosylation, the complement system, and circadian rhythm

Table 1. Novel pathways identified in *L. plantarum*-induced innate memory

Pathway	Genes	Direction	Fold change	p-value	Key genes	Biological function
Complement system	31	Down	0.875	< 0.001	C1Q, C3, C5, CR1	Immune evasion, reduced opsonization
Circadian rhythm	22	Down	0.858	< 0.01	CLOCK, PER, CRY, BMAL1	Metabolic timing disruption
Glycosylation	30	Down	0.868	< 0.01	MGAT, FUT, ST3GAL	Altered receptor function
One-carbon metabolism	38	Mixed	0.964	< 0.05	MTHFD, SHMT, MTR	Epigenetic regulation
Amino acid/mTOR	16	Up	1.11	< 0.001	MTOR, SLC7A5, RPS6KB1	Nutrient sensing, adaptation
Xenobiotic detox	7	Up	1.12	< 0.01	CYP1A1, GSTA1, NQO1	Stress response, detoxification
Nucleotide metabolism	8	Mixed	1.02	ns	PPAT, IMPDH2, RRM2	Nucleotide pool regulation
Lipid metabolism	4	Down	0.912	< 0.05	FASN, ACSL, CPT1	Membrane remodeling

inflammatory signaling after *L. plantarum* priming [6]. The complement has a significant role in recognizing microbes, opsonization, and enhancing inflammatory responses; microbes can also affect this process by different means [33]. Furthermore, recent research in the field of microbiota shows that complement is not only an antimicrobial mediator—the components of complement produced on site facilitate local communication and thus can affect the microbial community composition and level of inflammation [14]. In this light, the fact that the complement module in live cell-primed monocytes is reduced may indicate that the process observed is actually a part of an overall transition toward a decrease in inflammatory response. It would, however, be premature to label this finding a direct complement-evasion mechanism. The dataset contains transcript abundance rather than measurements of complement protein, cleavage products, opsonization, or phagocytic function. Thus, the biologically plausible interpretation is that complement-associated transcription is attenuated in the tolerogenic state, whereas its functional consequences remain to be established. Independent priming experiments measuring C3-derived products, complement-dependent opsonophagocytosis, and intracellular complement activity would be needed to test this possibility.

A second feature was the 14.2% reduction in the circadian-associated module. BMAL1- and CLOCK-centered programs regulate immune-cell metabolism, trafficking, and inflammatory responsiveness [28, 29], and recent analyses of macrophage chronobiology further show that cell-autonomous clocks can gate cytokine production, migration, and metabolic activity [15]. The present result therefore provides a plausible link between the low-inflammatory phenotype and altered temporal regulation of monocyte function. Nevertheless, the data were collected at a single post-priming time point and were not generated as a circadian time series. Reduced expression of a circadian-associated gene set cannot therefore demonstrate loss of rhythmicity, phase shifting, or altered oscillation amplitude. The finding is better interpreted as circadian-related transcriptional remodeling that may intersect with immune-metabolic adaptation. Time-resolved experiments measuring BMAL1, CLOCK, PER, and CRY together with

cytokine responses would be required to determine whether a true change in cellular rhythmicity accompanies *L. plantarum*-induced tolerance.

Changes in glycosylation-related genes and TLR signaling provide a complementary view of altered microbial sensing. Glycosylation influences protein folding, receptor stability, ligand recognition, and cytokine signaling [34, 35]; consequently, reduced expression of glycosylation machinery could modify the way monocytes display or regulate immune receptors. In parallel, live primed cells showed coordinated repression of TLR2, TLR8, MAP2K3, STAT1, and multiple inflammatory cytokine and chemokine genes. Contemporary TLR literature supports a central role for these receptors in linking microbial recognition to NF-κB- and interferon-associated transcription [36, 37]. Recent work with *L. plantarum* also illustrates that probiotic signaling can involve combined pattern-recognition pathways, including TLR2 and Mincle, in a strain- and context-dependent manner [16]. Taken together, our findings are consistent with attenuation of a TLR-associated transcriptional program after the initial priming event, which could contribute to reduced responsiveness upon subsequent stimulation. They do not, however, establish receptor-level desensitization or prove that reduced TLR2 or TLR8 expression is the cause of tolerance. Transcript abundance may not parallel surface receptor protein, trafficking, phosphorylation, or signaling competence. Restimulation with defined TLR agonists, coupled with receptor protein, phosphoprotein, and cytokine measurements, would be necessary to demonstrate pathway-specific functional tolerance.

The increased amino acid/mTOR and xenobiotic detoxification modules indicate that the tolerogenic state is not simply one of global transcriptional suppression. mTOR integrates nutrient availability with metabolism, growth, survival, and immune-cell differentiation [30–32], and current models of innate immune memory emphasize that metabolic pathways can act as regulatory checkpoints whose effects depend on stimulus and cellular state [12]. This helps reconcile increased nutrient-sensing pathway activity with the metabolic quiescence reported in the original *L. plantarum* study [6]. Metabolic intermediates can also participate in longer-lived transcriptional regulation; for example, persistent histone H3K18 lactylation provides evidence that metabolic state can be coupled to epigenetic memory [11]. Thus, the mTOR-related signal seen might be indicative of cellular maintenance, longevity, or adaptation related to chromatin instead of representing general pro-inflammatory activation. At the same time, xenobiotic detoxification profile might indicate the adaptation in response to exposure to live bacteria. However, these explanations remain hypotheses because the dataset lacks data on metabolomic, phosphoproteomic, and chromatin analyses. By measuring mTOR activity, some important markers of epigenetic modification, and metabolite profiles after priming cells by live or heat-killed stimuli and perturbing metabolism, it would be possible to find out whether these processes influence the formation of memory or whether they are just side effects.

The remodeling documented in this study has broader implications with respect to how priming by commensals might interact with the pathogenesis of chronic immune-mediated diseases. Recent conceptual frameworks have changed the innate immunity memory spectrum from being training, priming, and tolerance to being a disease axis, whereby signals of microbial origin act on the gut–bone marrow axis to determine the level of inflammatory status of circulating monocytes [38]. This latter axis, when dysfunctional, promotes the chronic activation of myeloid cells and thus results in constant inflammation in diseases such as IBD that manifest even when remission is achieved, as epigenetic changes on inflammatory genes have been reported to occur [39, 40]. The scenario presented here, where TLR, complement, and glycosylation-associated genes are downregulated while genes implicated in amino acid/mTOR pathways are upregulated, signifies that the helper signaling in monocytes is reinstated in a tolerogenic state.

The pattern seen in this study—simultaneous downregulation of TLR, complement, and glycosylation-related transcription coupled with upregulation of the amino acid/mTOR pathway—fits the tolerogenic form of the same axis in which signals originating from commensal flora influence programming of monocytes away from hyperreactivity. Recently, it has been recognized that mTOR is an important regulatory point that controls the supply of nutrients and signals from microorganisms that determine myeloid cell fate [39]. Specifically, it has been suggested that a selective activation of the PI3K/Akt/mTOR signaling pathway through probiotics could potentially explain how particular bacteria can decrease intestinal immune dysregulation [41], and transcription-related data support this hypothesis in regard to monocytes. If the action of *L. plantarum* on the regulation of immune cells via mTOR-dependent mechanisms results in the stabilization of the tolerogenic chromatin state, this would explain the previously reported efficiency of the bacterium in various animal models of inflammation [9, 13]. It is possible to test this hypothesis by using inhibitors of the PI3K/mTOR signaling pathway during the priming stage and carrying out chromatin accessibility analysis. The present study further illustrates the scientific value of systematic computational reanalysis of publicly deposited transcriptomic datasets. Reanalysis with updated pathway gene set databases and module-level activity scoring can reveal biological programs that are statistically undetectable by per-gene approaches in small-replicate experiments and yet are biologically coherent and experimentally tractable. This is not merely a methodological observation: analogous transcriptomics-based computational strategies have proven productive across distinct biological contexts, identifying novel kinase complexes and virulence-associated gene networks in fungal pathogens that were not apparent from phenotypic screens alone [42], and uncovering regulatory immunity axes in the tumor microenvironment through bioinformatics integration of multi-omics signals [43]. The concept of tolerogenic trained immunity in disease is itself an emerging field in which computational reanalysis of archived clinical datasets is beginning to identify mechanistic signatures that would be prohibitively costly to capture through prospective experiments alone [44]. Together, these observations strengthen the argument for continued investment in open data repositories, meticulous metadata curation, and publicly deposited analysis code—all of which this study illustrates—as an infrastructure that enables each experiment to achieve scientific impact far beyond its intended meaning. The identification of the complement, circadian, glycosylation, mTOR, and xenobiotic modules in the three-condition dataset of nine samples demonstrates how much information about biology may remain dormant in data that has been well collected but poorly analyzed.

There are some limitations that should be considered while interpreting the obtained results. The analysis includes only one transcriptome dataset containing three biological replicates for each experimental condition, making the analysis somewhat not rigorous. The source dataset also constrains which microbiota-derived signals can be evaluated. For example, bile acid-mediated signaling is an important component of microbiota-immune communication [38], but it could not be meaningfully assessed because

the original design did not include bile acid exposure and no relevant gene set reached significance. Replication in independent donors, time-resolved profiling, functional pathway assays, and intestinal or other barrier-relevant models will therefore be important for determining which of the candidate pathways identified here are reproducible and mechanistically involved in *L. plantarum*-induced tolerogenic memory.

The TLR result further illustrates why probiotic signaling should not be reduced to a simple pro- versus anti-inflammatory classification. *L. plantarum* can engage TLR2 and other pattern-recognition receptors in a strain- and context-dependent manner, and recent work has shown that combined TLR2 and Mincle signaling can regulate IL-10 production in dendritic cells exposed to *L. plantarum* [16]. As a result, the two processes described above will work in concert. Thus, whereas the engagement of the receptor and the final state of transcription of the pathway seemed to be at odds with each other, intense initial receptor stimulation may in fact lead to an adaptive response at the level of the receptor or chromatin, thus curbing the downstream effects. This assumption fits the modern view of tolerance, training, and priming as being parts of the same signaling and epigenetic programs. The current state of the dataset with regard to reduced levels of STAT1 signaling is also in line with the idea of the role of transcription and interferon response in mediating the altered responsiveness of monocytes. Importantly, the current analysis does not allow to evaluate why TLR2 and TLR8 receptors demonstrated a reduced expression pattern—whether it is due to the change in the levels of TLR2 and TLR8 receptor proteins, modified trafficking, or malfunctioning of downstream effects.

The coexistence of low-inflammatory activity with high activity of the amino acid/mTOR pathway may seem like a contradictory notion at first glance; however, contemporary innate-memory theories validate this assumption. Metabolic pathways act as more than just markers of activation; they can serve as regulatory checkpoints depending on the stimulus, timing, and cellular state [12]. Furthermore, the recent evidence that histone H3K18 lactylation is able to last long after the initial stimulus suggests that the products of metabolism may be involved in regulation of the transcription process [11]. Therefore, in the case of monocytes preconditioned with *L. plantarum*, the increased activity of sensing nutrients may contribute to survival, biosynthetic maintenance, or chromatin remodeling in the absence of a globally pro-inflammatory phenotype, which is still a hypothesis because the dataset does not comprise any information on metabolomic, phosphoproteomic, and chromatin changes. Nevertheless, it generates testable predictions: viable-bacterium priming should produce coordinated changes in mTOR activity and selected epigenetic marks, and pharmacologic perturbation of these pathways should alter the magnitude or persistence of the tolerogenic response. These experiments would help distinguish whether mTOR signaling is mechanistically required for the observed memory state or is simply a correlated feature of cellular adaptation.

Ethical Statement

We confirm that the human-derived data analyzed in this study were obtained from the publicly available dataset GSE159496. The present study involved only computational reanalysis of existing data; no new human or animal samples were collected.

Conflicts of Interest

The authors declare that they have no conflicts of interest to this work.

Data Availability Statement

The data analyzed in this study are publicly available in the NCBI Gene Expression Omnibus under accession GSE159496: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE159496. This study reanalyzed existing data and generated no new experimental data.

The analysis scripts are deposited at https://github.com/ahmedalfahad/L-plantarum-innate-memory-reanalysis.

Author Contribution Statement

Hanan Yassin Muhsin: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Azhaar Raheem Hussein AL-Fartwsy**: Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Amenah Hussein Mousa**: Validation, Investigation, Data curation, Writing – review & editing, Visualization. **Ahmed AbdulJabbar Suleiman**: Conceptualization, Software, Validation, Resources, Writing – original draft, Supervision, Project administration.

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