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Molecular dissection of putative mechanisms underlying the beneficial effects of fecal microbiota transplantation and traditional fermented ewe-dairy on anxiety-like symptoms: A bioinformatics and systems biology study

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Abstract

Background: Evidence suggests that fecal microbiota transplantation (FMT) alleviates anxiety-like behaviors in animal models; however, the underlying molecular mechanisms remain unclear. Additionally, fermented dairy consumption has been associated with improved anxiety-related phenotypes. This *in silico* study investigated the molecular and systems-level effects of FMT and traditional Iranian fermented ewe dairy on anxiety-like symptoms in mice.

Methods: RNA-seq datasets from anxiety-model mouse cortex tissues and healthy controls, together with 16S rRNA microbiome data from healthy mouse feces and fermented ewe dairy products, were retrieved from the NCBI SRA database. Differential expression and microbiome analyses were performed. Predicted microbial proteins were screened for key enzymes involved in anxiolytic metabolite biosynthesis. Extracellular, surface-associated, and signaling proteins were identified and mapped to

human orthologs. Microbiome-host interactions were explored using MicrobioLink2, and overlaps between fecal and dairy microbiomes were assessed.

Results: A total of 239 differentially expressed genes (DEGs) were identified in anxiety-model mouse cortex tissue. The fecal and dairy microbiomes were predicted to produce 4,156 and 7,287 proteins, respectively, including 1,572 and 2,724 extracellular, surface-associated, or signaling proteins. Both microbiomes contained key enzymes involved in anxiolytic metabolite biosynthesis, including GABA-related pathways. Interaction networks linked microbiome-derived proteins to host DEGs and downstream pathways, revealing shared proteins between the two microbiomes.

Conclusion: The identified enzymes and microbiome-host interactions, including involvement of the serotonin neurotransmitter release cycle, provide putative molecular explanations for the beneficial effects of FMT. Shared protein components suggest that traditional Iranian fermented ewe dairy may possess intrinsic anxiolytic potential, warranting further experimental validation.

Keywords: Gut-brain axis, fecal microbiota transplantation, microbial metabolites, multi-omics, anxiety disorders, fermented ewe dairy products

INTRODUCTION

Anxiety represents the body's natural response to stressful and threatening circumstances; however, excessive anxiety can give rise to pathological conditions and anxiety disorders[1,2]. Anxiety disorders rank among the most prevalent psychiatric illnesses worldwide, with recent estimates indicating that they affect approximately 4.4% of the global population[3]. These disorders—which include panic disorder, social anxiety disorder, post-traumatic stress disorder, generalized anxiety disorder, specific phobias, and obsessive-compulsive disorder—can impair quality of life, diminish self-esteem, and disrupt daily functioning across domains such as work, learning, creativity, and interpersonal relationships[2,4,5]. Moreover, chronic anxiety disorders may contribute to the emergence of more severe mental health conditions, including depression[6].

A multitude of factors influence the development of such disorders, among them

genetic predisposition, lifestyle, nutrition, smoking and alcohol consumption, environmental exposures, and sex hormones[7,8]. From a neurophysiological and neuroanatomical perspective, numerous brain regions and circuits are implicated. Several areas of the cerebral cortex play key roles in regulating emotions—particularly fear—and stress responses; these include the orbitofrontal cortex, the ventromedial cortex, and the limbic cortex, which interconnect with the amygdala, a central hub for emotional processing. Dysfunction within these cortical territories, or in the cerebral cortex more broadly, can precipitate anxiety disorders[8-11].

Another factor that has recently captured researchers' attention is the gut microbiome and its overarching influence on the brain and mood states[12]. The past few years have witnessed a surge in investigations centered on the gut-brain axis (GBA). This axis constitutes a bidirectional biochemical and neural communication pathway between the brain and the gastrointestinal tract—specifically the gut—whose regulation can be shaped, directly or indirectly, by the gut microbiome through endocrine, neural, or immune routes. Available evidence suggests that this axis becomes disrupted during illness or under stressful conditions, leading to intestinal dysbiosis, alterations in mood and behavior, and inflammation[13]. The gastrointestinal system harbors well over one hundred trillion bacteria that coexist as commensals and mutualists. This bacterial community is largely unique to each individual and is influenced by genetics, diet, age, host metabolism, geography, psychotropic or antibiotic use, and ultimately stress[14]. Examination of the gut microbiome in individuals suffering from mental disorders such as anxiety and depression reveals overt differences when compared with healthy individuals: the gut microbiome of affected patients displays diminished diversity and is depleted of certain beneficial species that normally reside in a healthy gut[15]. Reports further document neurochemical alterations arising from aberrant gut microbiome activity, including reduced expression of genes associated with neuroplasticity and serotonin receptors[16]. Collectively, these findings point to a bidirectional relationship between disturbances in the gut microbiome and psychiatric disorders, including anxiety disorders.

Despite the availability of pharmacotherapies for these conditions, many patients avoid medication because of adverse side effects or unsatisfactory treatment responses. Yet, as systemic disorders, mental illnesses necessitate systemic therapeutic approaches.

Microbiome transplantation—commonly referred to as fecal microbiome transplantation (FMT)—entails transferring healthy donor gut bacteria into a recipient's intestine with the aim of re-establishing a healthy microbial flora in individuals suffering from dysbiosis or related disruptions[17]. This therapeutic strategy, which has lately drawn considerable research interest, is currently being used for a broad spectrum of conditions, including multiple sclerosis, metabolic disorders, neurological diseases such as Parkinson's disease, and psychiatric disorders such as autism[18-21].

To date, numerous clinical-phase studies have examined the effects of FMT on depression, autism, anorexia nervosa, and alcoholism, and both the positive therapeutic outcomes and the underlying mechanisms have been delineated to a reasonable extent. For anxiety disorders, however, the vast majority of such investigations remain in preclinical stages, still far from being accepted as a validated therapeutic modality. Although reports document beneficial effects of FMT on anxiety-like symptoms in animal models (e.g., mice), the molecular underpinnings and the precise mechanisms driving these effects have remained elusive and poorly characterized. The prevailing focus on irritable bowel syndrome (IBS), coupled with the insufficient examination of other psychiatric disorders per se (rather than solely their psychosomatic complications), has compounded this gap in knowledge[22]. A pressing need thus exists for deeper, more granular investigations in this arena, with the hope that identifying and elucidating the molecular mechanisms behind this treatment may eventually facilitate its translation into clinical and routine practice.

Reports on the influence of dairy consumption on mental health are inconsistent. Some studies indicate a higher prevalence of psychiatric disorders among individuals with high intakes of milk and dairy products, whereas others suggest a protective effect of these foods against such conditions. However, a 2024 Mendelian randomization analysis revealed that consumption of semi-skimmed milk may lower the risk of developing psychiatric disorders, including depression and anxiety[23]. Furthermore, a study of 7,387 Iranian adults reported an association between the consumption of full-fat and semi-fat dairy products and a reduced risk of depression and anxiety symptoms[24].

Metabolites produced by bacteria isolated from the natural microbiome of traditional

fermented dairy products exhibit beneficial health properties, such as antioxidant activity and immunomodulatory effects[25,26]. It is therefore plausible that consumption of traditional fermented dairy products may exert a positive influence on anxiety symptoms and, through the activity of its natural flora, modulate the gut-brain axis.

Given the high global prevalence of anxiety disorders, along with the side effects and resistance associated with single-target therapies, there is a clear need for further research aimed at identifying novel therapeutic strategies—particularly systemic treatments. The potential of FMT as an emerging systemic therapy for these disorders is noteworthy. The obscurity surrounding the molecular mechanisms underlying its therapeutic effect is one factor that has hindered its acceptance as a routine treatment for anxiety disorders; elucidating these mechanisms could therefore facilitate its broader adoption. On the other hand, since the anxiolytic effects of Iranian dairy products have been documented in prior studies, investigating the microbiome of traditional Iranian dairy products could yield valuable insights for developing anti-anxiety therapeutic approaches. Accordingly, the aim of the present study is to examine the molecular factors underlying the beneficial effects of fecal transplantation and consumption of traditional Iranian ewe dairy products in mice exhibiting anxiety-like symptoms using bioinformatic and systems-biology methodologies.

METHODS

Collection of raw RNA-seq data of brain tissue and 16S rRNA microbiome data from healthy mouse feces and traditional Iranian ewe dairy products

Raw RNA-seq data from the cortical tissue of mice exhibiting anxiety-like symptoms and healthy control animals were obtained from the SRA database (www.ncbi.nlm.nih.gov/sra) (Bioproject: PRJNA843035)[27]. Additionally, raw 16S rRNA metabarcoding data of the fecal microbiome of healthy mice (Study: SRP020353; SRR834785, SRR834787, SRR834791) and the microbiome of traditional Iranian ewe milk (Bioproject: PRJNA624311; SRR11575337), yogurt (SRR11524343), and doogh (SRR11612429), produced by nomads of Qasr-e Shirin, Iran, were retrieved from the same repository[28,29].

RNA-seq analysis and identification of differentially expressed genes (DEGs) in the cortical tissue of anxiety-like mice

Gene expression analysis of the RNA-seq data was performed on the Galaxy Europe server (usegalaxy.eu)[30] using the available tools, with the aim of identifying differentially expressed genes (DEGs) in the brain tissue of mice exhibiting anxiety-like symptoms. First, read quality was evaluated with FastQC (Galaxy Version 0.74+galaxy1). When data quality was insufficient, reads were trimmed using Trim Galore (Galaxy Version 0.6.10+galaxy0) to attain the desired quality. The reads were then aligned to the mouse genome (mm10) with HISAT2 (Galaxy Version 2.2.2+galaxy0). Subsequently, the aligned reads were processed with FeatureCounts (Galaxy Version 2.1.1+galaxy0) to quantify expression levels based on the number of reads mapping to each gene. Gene annotation was conducted via the annotateMyIDs tool (Galaxy Version 3.18.0+galaxy0). Finally, DESeq2 (Galaxy Version 2.11.40.8+galaxy2) was employed for differential expression analysis, comparing gene expression between the cortical tissue of control mice and those with anxiety-like symptoms, and significantly differentially expressed genes (DEGs) were identified.

Microbiome analysis and prediction of proteins produced by the natural flora of healthy mouse feces and traditional ewe dairy products

Microbiome analysis of the 16S rRNA metabarcoding data from fecal and dairy samples was again carried out on Galaxy Europe to generate the data required for subsequent protein prediction—specifically, an OTU table. For each raw dataset derived from fecal or dairy samples, quality was first assessed with FastQC, and trimming (when needed) was performed using Trimmomatic (Galaxy Version 0.39+galaxy2). Because the downstream tools required inputs in FASTA format, the data were converted from FASTQ to FASTA using the FASTQ-to-FASTA converter (Galaxy Version 1.0.2+galaxy2). To reduce data volume prior to clustering, duplicate sequences were removed with VSEARCH-dereplicate (Galaxy Version 2.8.3.0). Dereplicated sequences were clustered using the VSEARCH cluster_fast algorithm (using the VSearch clustering tool Galaxy Version 2.8.3.0). Taxonomic classification and assignment of representative sequences were achieved with the Assign Taxonomy tool (Galaxy Version 1.9.1.0) against the taxonomy reference, greengenes (13_8) - 64_otu_taxonomy. The outputs from clustering and taxonomic assignment were supplied to the Make OTU table tool (Galaxy Version 1.9.1.0), generating an OTU table. The resulting OTU table and the dereplicated sequence output were used by PICRUSt2 (Galaxy Version 2.5.3+galaxy0) to predict proteins potentially produced by

the microbiome. The list of predicted KEGG Orthology (KO) identifiers was saved for downstream analyses.

Retrieval of amino acid sequences and names of microbiome-produced proteins and enzymes corresponding to KO identifiers

Prokaryotic proteins corresponding to KO identifiers were retrieved using the KEGG (www.genome.jp/kegg) and UniProt (www.uniprot.org) databases. Owing to the large number of KO identifiers, a custom Python script was developed and executed to expedite and simplify the process. The script automatically queried the KEGG and UniProt APIs and mapped the input KOs to their corresponding UniProt IDs, protein names, and amino acid sequences of prokaryotic proteins. A subset of the extracted data was manually verified to ensure accuracy. The script is provided in a supplementary file [Supplementary File 1].

Screening the protein list for key enzymes in the biosynthetic pathways of anxiolytic metabolites

A literature review was performed to identify known anxiolytic metabolites, their biosynthetic pathways, and the key enzymes catalyzing each step. The names of these enzymes were then searched within the predicted protein lists derived from both microbiome sources—i.e., healthy mouse feces and ewe dairy products. Production of a given metabolite was considered plausible when the majority of the enzymes belonging to its biosynthetic pathway were identified; such metabolites and their associated pathways were selected and categorized.

Preparation of microbiome and RNA-seq data outputs for the MicrobioLink2 pipeline

Because the MicrobioLink2 pipeline focuses on interactions between surface or extracellular proteins/enzymes and the host tissue, and since the pipeline is designed for human tissues, preparatory steps were necessary prior to its execution. To filter for surface, extracellular, or signal peptide-containing proteins, the amino acid sequences obtained earlier were submitted to subcellular location prediction tools—namely PSORTb (version 3.0.3; psort.org/psortb) and DeepLocPro(version 1.0; services.healthtech.dtu.dk/services/DeepLocPro-1.0)—as well as SignalP (version 6.1; services.healthtech.dtu.dk/services/SignalP-5.0), which predicts the presence of signal

peptides based on amino acid sequence. Proteins possessing at least one of these two features—an appropriate subcellular location or a signal peptide—were deemed suitable for the subsequent stage, and a list of their corresponding UniProt IDs was compiled.

Separately, human orthologs of all expressed genes—particularly the DEGs derived from the RNA-seq analysis of the mouse cortex—were obtained using g:Profiler. Any gene lacking a human counterpart was excluded at this stage. Ultimately, two files were compiled: a Transcriptome file containing the full set of genes expressed in the cortex of mice exhibiting anxiety-like symptoms, and an Endpoint file containing the DEGs.

Implementation of the MicrobioLink2 pipeline and identification of interactions and downstream pathways

To detect potential microbe-host protein-protein interactions and evaluate their downstream effects on host signaling pathways, the MicrobioLink2 pipeline was applied according to its official protocol[31]. Initially, raw transcriptomic data (the Transcriptome file) were filtered by Z-score to reduce noise and exclude lowly expressed genes. This was accomplished with the script *z-score_filter_terminal.py*, which accepts a CSV file containing gene expression values and returns a filtered dataset; the default threshold of Z-score = -3.11 was used to retain genes with significant expression under the conditions examined.

In the second step, amino acid sequences of the human orthologs of the remaining genes were retrieved from UniProtKB using the script *get_human_fasta.py* to support subsequent analyses. In parallel, the third step employed *download_bacterial_proteins.py* to obtain bacterial protein sequences and their domain annotations. The input for this script was a file of UniProt IDs corresponding to the microbial surface/extracellular or signaling proteins predicted earlier. The outputs of these two steps provided the molecular entities and the basis for predicting domain-motif interactions.

The fourth step involved predicting interactions between the human orthologs and the bacterial proteins via the script *DMI.py* and the ELM database. Potential molecular contacts between bacterial domains and motifs in the human orthologs were identified,

and preliminary host-microbe interaction networks were constructed. Subsequently, in the next step, to ensure the biological structural plausibility of the predicted interactions, those whose motifs were located outside disordered regions or within globular domains of the human orthologs were removed. This filtering was performed with *idr_prediction.py*, which relies on the external script, *iupred2a.py*. Only interactions that possessed features compatible with realistic biological conditions were carried forward.

In the sixth step, the host-microbe interaction results and transcriptomic data were reformatted for modeling using *tiedie_input_processing.py*. This generated the standard input files upstream.input, downstream.input, and pathway.sif, which define source nodes, target nodes, and network links for the TieDIE analysis. The seventh step conducted network propagation using the TieDIE algorithm executed by *tiedie.py*, thereby linking the upstream effects of microbial proteins to the downstream responses of host proteins. Next, in the eighth step of the pipeline, the TieDIE outputs were combined with the initial data and prepared for visualization with *tiedie_output_processing.py*. This produced the final network file (usecase_final_network.txt) and a node- attribute table.

In the ninth step, the resulting networks were visualized using Cytoscape (version 3.10.3). The files from step eight were imported into Cytoscape, and cross- kingdom interactions together with key network nodes were displayed graphically by leveraging node and edge attributes.

Finally, to biologically interpret the interaction networks, enrichment and functional analysis were carried out with the script enrichr_id_database_ranking.py and the gget library, which interfaces with the Enrichr service. Analyses were performed at two levels: direct targets of bacterial proteins (within the HMI network) and signaling pathways derived from the TieDIE model. Enriched biological pathways were ranked by combined score and adjusted *p* value, and the top pathways were visualized as bar charts. The list of pathways attaining statistical significance (adj. *p*-value < 0.05) was subsequently screened for the presence of any pathway with the potential for anxiety modulation.

All steps were executed within the Anaconda environment (version 2024.10) to ensure compatibility among the Python libraries and external tools such as mygene, IUPred2A, and gget.

Assessment of the predicted metaproteome of traditional ewe dairy products for shared features

To explore any potential effects analogous to those obtained for the fecal microbiome, the overlap between the predicted protein complements of the ewe milk, yogurt, and doogh microbiome and the fecal microbiome proteins was examined at two levels: (i) before the MicrobioLink2 pipeline—i.e., the entire set of surface/extracellular or signaling proteins—and (ii) after the pipeline—i.e., the subset of proteins that participated in and influenced the predicted interactions.

RESULTS

Differentially expressed genes in the cortex of mice exhibiting anxiety-like symptoms and their human orthologs

Differential expression analysis with the DESeq2 package identified 239 significantly differentially expressed genes (DEGs) using the thresholds FDR < 0.05 and $|\log_2 \text{Fold Change}| \geq 0.3$. Of these, 230 had identifiable human orthologs [Figure 1, Table 1].

Table 1. Genes displaying the largest expression changes ($|\log_2 FC| \geq 0.9$) and their corresponding human orthologs.

Gene ID	Human ortholog	Base mean	Log_2(FC)	StdErr	*P*-value	*P*-adj
Serinc2	SERINC2	181.823	1.35603	0.181964	9.18e-14	7.41e-11
Tnfrsf25	TNFRSF25	200.504	1.34373	0.211625	2.16e-10	8.21e-08
Cdkn1a	CDKN1A	343.467	1.28709	0.132523	2.68e-22	8.65e-19
Egr1	EGR1	6901.71	1.06126	0.289471	0.000246	0.015824
Dok3	DOK3	141.605	1.06082	0.202015	1.51e-07	3.49e-05
Fosl2	FOSL2	1037.35	1.02453	0.313281	0.001074	0.046141
Egr3	EGR3	1576.82	0.991719	0.076916	4.9e-38	6.34e-34
Itga10	ITGA10	125.143	0.950298	0.193565	9.13e-07	0.000159

Gpr3	GPR3	159.264	0.924511	0.181245	3.38e-07	6.93e-05
Trib1	TRIB1	603.921	0.913012	0.262638	0.000508	0.026920
Klf4	KLF4	139.659	0.900836	0.184991	1.12e-06	0.000190
Bace2	BACE2	55.1133	-0.941245	0.274219	-3.432455	0.000598
Dbp	DBP	1155.12	-0.958028	0.085257	-11.23692	2.69E-29
Pcsk9	PCSK9	52.7580	-1.027139	0.284889	-3.605401	0.000311
Angpt1	ANGPT1	75.2495	-1.038087	0.304820	-3.405564	0.000660

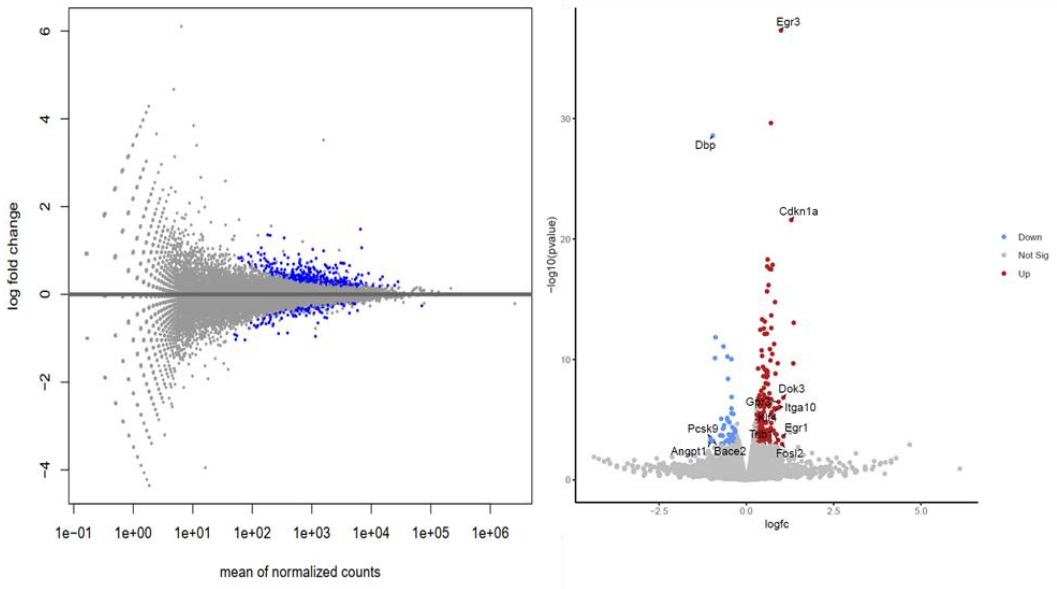

Figure 1. Differential gene expression analysis of mouse brain tissues exhibiting anxiety-like behaviors following RNA-seq profiling. Left: MA plot displaying the relationship between mean normalized expression counts and $\log_2$ fold change ($\log_2$FC). Blue points represent significantly differentially expressed genes (adjusted $p < 0.05$), while gray points indicate non-significant genes. Right: Volcano plot illustrating the distribution of differentially expressed genes according to log fold change and statistical significance (-log10 p-value). Red and blue points indicate significantly upregulated and downregulated genes, respectively, using thresholds of adjusted $p < 0.05$ and $|\text{logFC}| \geq 0.3$. Labeled genes correspond to the most strongly dysregulated transcripts ($|\text{logFC}| \geq 0.9$).

Predicted proteins from the fecal microbiome of healthy mice and from traditional ewe dairy products, and their subcellular localizations

Microbiome analysis and subsequent protein prediction yielded 4,156 proteins for the

fecal microbiome and 7,287 proteins for the microbiome of the dairy products. From these, 1,572 and 2,724 proteins, respectively, were selected for the MicrobioLink2 pipeline. The retained proteins were those predicted to be extracellular or surface-exposed (according to PSORTb and DeepLocPro-1.0) or to carry a signal peptide, TAT signal peptide, or lipoprotein signal peptide (as determined by SignalP).

Key enzymes in the biosynthetic pathways of anxiolytic metabolites

A curated list of established anxiolytic metabolites, their biosynthetic routes, and the corresponding key enzymes and transcription factors was compiled. Screening these key proteins against the predicted proteomes of the healthy mouse fecal microbiome and the ewe dairy microbiome confirmed the presence of components involved in the production of anxiolytic metabolites, like gamma-aminobutyric acid (GABA). A detailed account of these findings is presented in Table 2.

Table 2. Key proteins associated with the biosynthesis of anxiolytic metabolites detected among the predicted proteins of the examined microbial floras.

Microbiome Source	Target Metabolite	Associated Biosynthetic Pathway	Protein Name	Detected UniProt ID(s)	Protein Functional Category	Citation
Feces	GABA	GABA Shunt	Glutamate dehydrogenase	A0A031WDM8, A0A059ZD70, A0A023WNY3	Enzyme	(32-34)
			Glutamate decarboxylase	A0A059XY27	Enzyme	
		Polyamine Degradation	Ornithine decarboxylase	A0A011UC74	Enzyme	
			Proline dehydrogenase	A0A023WZX2	Enzyme	

	Valeric Acid	Valeric Acid Biosynthesis Pathway	Acetyl-CoA Synthase/Carbon Monoxide Dehydrogenase	A0A031WFJ0	Enzyme	(35)
			Acyl-CoA synthetase	A0A023WU66, A0A075WMS2, A0A060NFW8,	Enzyme	
			Acetyl-CoA acetyltransferase	A0A023WTC4	Enzyme	
			3-Hydroxyacyl-CoA dehydrogenase	A0A024EHL2, A0A023WXH4, A0A075WA35	Enzyme	
			Enoyl-CoA hydratase	A0A011UN55, A0A023WZX6, A0A024EHL2, A0A080M452, A0A060DNU9, A0A023X1G3	Enzyme	
			Enoyl-CoA reductase	A0A023WTL7	Enzyme	
	L-lysine	Diaminopi	Aspartokinase	A0A011U8	Enzyme	(36-3

	melate (DAP) pathway (for lysine biosynthesis from aspartate; succinylase variant in E. coli, acetylase variant in B. subtilis)		U1, A0A060HAI9		8)
		Aspartate-Semialdehyde Dehydrogenase	A0A023WT27	Enzyme	
		Tetrahydrodipicolinate Synthase	A0A011UM46	Enzyme	
		N-Acetyldiaminopimelate Deacetylase	A0A031WJZ1	Enzyme	
	Lysine Biosynthesis regulator	lysR family regulator	A0A011VIU9, A0A024E725, A0A023WY40, A0A023WUF7	Transcription Factor	
Butyrate	Butyrate Biosynthesis Pathway (Pyruvate Pathway)	3-Ketoacyl-CoA Thiolase	A0A023WUD4	Enzyme	(39)
		Hydroxybutyryl Dehydrogenase	A0A031WIT5	Enzyme	
		Enoyl-CoA Hydratase	A0A011UN55, A0A023WZX6, A0A024EHL2, A0A080M452,	Enzyme	

				A0A060DNU9, A0A023X1G3		
		Butyrate Biosynthesis Pathway (4-Aminobutyrate Pathway)	4-Hydroxybutyrate Dehydrogenase	A0A0A2FSX8	Enzyme	
			4-Hydroxybutyrate CoA-Transferase	A0A069AAR6	Enzyme	
			4-Hydroxybutyryl-CoA Dehydratase	A0A031WIT5	Enzyme	
		Butyrate Biosynthesis Pathway (Lysine Pathway)	Lysine2,3-Aminomutase	A0A060I2K3	Enzyme	
			Lysine5,6-Aminomutase	A0A0C7NXI6	Enzyme	
			3,5-Diaminohexanoate Dehydrogenase	A0A0A2FUS1	Enzyme	
			3-Keto-5-Aminohexanoate Cleavage Enzyme	A0A068R0D5	Enzyme	
Ewe Dairy	GABA	GABA Shunt	Glutamate dehydrogenase	A0A031WDM8, A0A059ZD70, A0A023WNY3	Enzyme	(32-34)
			Glutamate decarboxylase	A0A059XY27	Enzyme	
		Polyamine Degradation	Ornithine decarboxylase	A0A011UC74	Enzyme	
			Gamma-aminobutyraldeh-yde dehydrogenase	A0A024E9F3	Enzyme	

		Proline dehydrogenase	A0A023WZX2, A0A1N6M0D5	Enzyme	
	GABA Transmembrane Transport	GABA permease	A0A024ED42	Permease	
Valeric Acid	Valeric Acid Biosynthesis Pathway	Acetyl-CoA Synthase/Carbon Monoxide Dehydrogenase	A0A031WFJ0	Enzyme	(35)
		Acyl-CoA synthetase	A0A0A0NJC7, A0A023WU66, A0A075WMS2, A0A060NFW8, A0A024EIJ8	Enzyme	
		Acetyl-CoA acetyltransferase	A0A023WTC4	Enzyme	
		3-Hydroxyacyl-CoA dehydrogenase	A0A075WA35, A0A023WWZ5, A0A024EHL2, A0A023WXH4	Enzyme	

	Enoyl-CoA hydratase	A0A248W0N8, A0A024EJI9, A0A024EJI9, A0A0B6SC27, A0A011UN55, A0A023WZX6, A0A024EHL2, A0A0A1F6W8, A0A060LYH2, A0A024E8A6, A0A059XXX1, A0A080M452, A0A060DNU9, A0A0C5VLB0, A0A023X1G3, A0A024E4U5, A0A076Z4	Enzyme

			X0, A0A024E484		
		Enoyl-CoA reductase	A0A023WTL7, A0A024E7L8	Enzyme	
L-lysine	Diaminopimelate (DAP) pathway (for lysine biosynthesis from aspartate)	Aspartokinase	A0A011U8U1, A0A060HAI9, A0A068R7H4	Enzyme	(36-38)
		Aspartate-Semialdehyde Dehydrogenase	A0A023WT27	Enzyme	
		Tetrahydrodipicolinate Synthase	A0A011UM46	Enzyme	
		N-Acetyldiaminopimelate Deacetylase	A0A031WJZ1	Enzyme	
	Lysine Biosynthesis regulator	LysR Family Regulator	A0A011VIU9, A0A024E725, A0A023WY40, A0A075LJI7, A0A099MX	Transcription Factor	

				U8, A0A023WMV6, A0A024HIH2, A0A024E6U9, A0A060DSD2, A0A024EK72 A0A023WRZ3, A0A023WUF7, A0A231MZN0		
			LysR Family Protein	A0A069B7T2	Transcription Factor	
Butyrate	Butyrate Biosynthesis Pathway (Pyruvate Pathway)	3-Ketoacyl-CoA Thiolase		A0A023WUD4	Enzyme	(39)
		Hydroxybutyryl Dehydrogenase Enoyl-CoA Hydratase		A0A031WIT5 A0A248W0N8, A0A024EJI9, A0A024EJI9, A0A0B6SC27,	Enzyme	

		A0A011UN55, A0A023WZX6, A0A024EHL2, A0A0A1F6W8, A0A060LYH2, A0A024E8A6, A0A059XXX1, A0A080M452, A0A060DNU9, A0A0C5VLB0, A0A023X1G3, A0A024E4U5, A0A076Z4X0, A0A024E484	
Butyrate Biosynthesis Pathway (4-Amino	4-Hydroxybutyrate Dehydrogenase	A0A0A2FSX8	Enzyme
	4-Hydroxybutyrate CoA-Transferase	A0A069AAR6	Enzyme
	4-Hydroxybutyryl-	A0A031WI	Enzyme

	butyrate Pathway)	CoA Dehydratase	T5	
	Butyrate Biosynthesis Pathway (Lysine Pathway)	Lysine2,3-Aminomutase	A0A060I2K3	Enzyme
		Lysine5,6-Aminomutase	A0A0C7NXI6	Enzyme
		3,5-Diaminohexanoate Dehydrogenase	A0A0A2FUS1	Enzyme
		3-Keto-5-Aminohexanoate Cleavage Enzyme	A0A068R0D5	Enzyme

GABA = Gamma-aminobutyric acid.

Protein-protein interactions between bacterial and cortical proteins and their downstream pathways

Domain-motif interactions were predicted between proteins from the fecal microbiome of healthy mice and human orthologs of proteins expressed in the cortical tissue of mice exhibiting anxiety-like symptoms, using the scripts and databases described earlier. The resulting interaction network was visualized in Cytoscape [Figure 2]. Functional and enrichment analysis of these interactions revealed the top 20 downstream pathways affected by the predicted associations [Figure 3]). Moreover, inspection of the statistically significant pathways (adj. p-value < 0.05) indicated that the observed interactions may also trigger specific pathways implicated in the modulation of anxiety [Table 3].

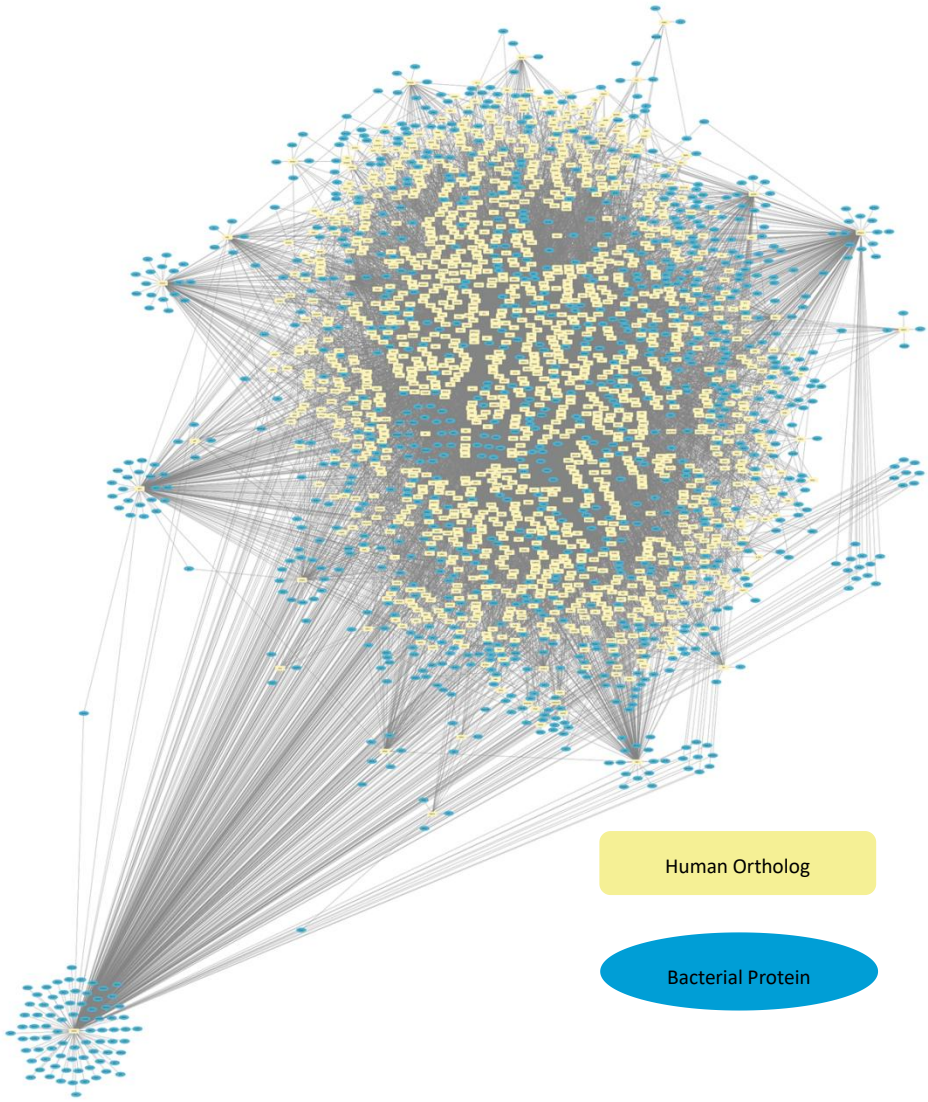

Figure 2. Predicted domain-motif interactions between proteins from the fecal microbiome of healthy mice and human orthologs of proteins expressed in the cortex of mice with anxiety- like symptoms.

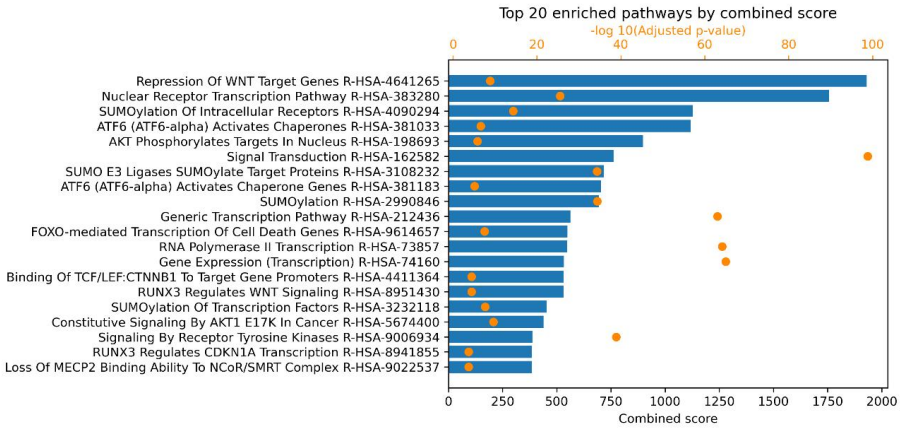

Figure 3. Top 20 biological pathways influenced by the predicted gut-brain interactions.

Pathways were selected based on the highest combined scores and adjusted p values derived from functional enrichment analysis.

Table 3. Biological pathways associated with gut-brain interactions and anxiety modulation.

Path_name	*P*_val	Z_score	Combined_score	Overlapping_genes	Adj_p_val	Database
Serotonin Neurotransmitter Release Cycle R-HSA-181429	0.001467	5.065575	33.047216	'UNC13B', 'SNAP25', 'SYN3', 'SYN2', 'TSPOAP1', 'SYN1', 'PPFIA3', 'VAMP2'	0.004807	Reactome_2022
Dopamine Neurotransmitter Release Cycle R-HSA-212676	0.000466	4.873790	37.385775	'UNC13B', 'SNAP25', 'CASK', 'APBA1', 'SYN3', 'SYN2', 'TSPOAP1', 'SYN1', 'PPFIA3', 'VAMP2'	0.001720	Reactome_2022
Circadian Clock R-HSA-400253	3.508483e-17	8.614256	326.383511	'CRTC2', 'CRTC3', 'HDAC3', 'SMARCD3', 'CRTC1', 'BHLHE41', 'CREM', 'RORA', 'NR3C1', 'HIF1A', 'NPAS2', 'RXRA', 'NFIL3', 'NRIP1', 'EP300', 'TBL1X', 'MEF2D', 'PPARGC1A',	1.66068e-15	Reactome_2022

				'NCOA1', 'NCOA2', 'SREBF1', 'MEF2C', 'CREBBP', 'CSNK1D', 'CSNK1E', 'NR1D1', 'KLF15', 'SIRT1', 'PER1', 'CREB1', 'NCOR1', 'CRY2', 'BHLHE40', 'CRY1', 'FBXL3', 'PPARA', 'CLOCK', 'BMAL1'		
BMAL1:CLOCK,NPAS2 Activates Circadian Gene Expression R-HSA-1368108	2.664421e-06	6.834506	87.724468	'NCOA1', 'NCOA2', 'CREBBP', 'SMARCD3', 'BHLHE41', 'KLF15', 'NPAS2', 'RXRA', 'TBL1XR1', 'BHLHE40', 'TBL1X', 'CLOCK'	1.953909e-05	Reactome_2022

Overlap between the predicted proteins of the healthy mouse fecal microbiome and those of ewe dairy products

The results of the comparative analysis that was performed to identify shared proteins between the healthy mouse fecal microbiome and the microbiome of ewe dairy products at two levels (i) and (ii) confirmed the existence of overlapping proteins at

both levels. This finding indicates that common protein molecules are present not only within the global proteomes but also among those engaged in and influenced the predicted gut-brain interaction. Such overlap supports the notion that the ewe dairy microbiome may exert biological effects analogous to those of the gut microbiome, owing to the presence of these shared molecular components.

DISCUSSION

Anxiety disorders, owing to their rising prevalence and the considerable burden they impose on sufferers, have drawn sustained attention from the scientific community. According to the latest 2021 estimate by the World Health Organization (WHO), approximately 4.4% of the global population—equating to roughly 359 million individuals—are affected by these conditions, rendering anxiety disorders the most prevalent class of mental disorders worldwide[3]. While a normal level of anxiety can enhance performance, the excessive anxiety characteristic of anxiety disorders can lead to functional impairment, disruptions to daily life, and even the development of depression[2,4,5,40].

Conventional treatments for these conditions encompass psychotherapeutic approaches such as cognitive-behavioral therapy (CBT), short-term pharmacotherapy with anxiolytics, long-term pharmacotherapy with agents such as selective serotonin reuptake inhibitors (SSRIs), or a combination thereof[41]. Nevertheless, a number of challenges confront both clinicians and patients during the treatment course: limited access to and the high cost of long-term psychotherapy, the inability of CBT to address the needs of every patient, the emergence of drug resistance, and the adverse effects of these medications, which, being one-dimensional therapeutics, may not sufficiently address such systemic disorders. These obstacles can, in turn, discourage patients from continuing treatment if the expected outcomes remain elusive[42]. Side effects of anxiolytic drugs include excessive drowsiness, markedly diminished alertness and concentration, respiratory depression, memory impairment, weight gain or loss, sexual dysfunction, high addictive potential and dependence, withdrawal symptoms upon discontinuation, and, in some cases, an increase in suicidal ideation and risk[43,44].

Given the well-established bidirectional communication between the gut microbiome and the brain via the gut-brain axis, and the contribution of axis dysfunction to the

pathogenesis of mental disorders[45-48], fecal microbiome transplantation (FMT) has emerged as a novel therapeutic avenue for certain conditions, including depression and autism[22,49]. However, FMT has not yet gained approval or acceptance for the treatment of anxiety disorders and remains at the preclinical stage. Nonetheless, the beneficial effects of FMT on anxiety-like symptoms in translationally relevant animal models, such as mice, have been repeatedly documented[22.50,51]. To our knowledge, however, no study to date has thoroughly elucidated the molecular underpinnings of these positive effects.

Elucidating the molecular mechanisms underlying the positive effects of microbiome transplantation on anxiety and anxiety-like symptoms could advance this novel therapeutic approach toward clinical application and regulatory approval. Consequently, the objective of the present study was to investigate these underlying mechanisms using bioinformatics and systems biology tools. Collectively, the analyses and findings of this study indicate a potential role for the healthy gut microbiome in ameliorating anxiety symptoms, likely through the production of anxiolytic metabolites and the activation of pathways that may ultimately attenuate such symptoms. These points will be discussed in detail below.

In a 2025 experimental study, Xu and colleagues demonstrated that oral supplementation with gamma-aminobutyric acid (GABA) alleviated anxiety-like behaviors in a chronic restraint stress mouse model by increasing GABA levels in the prefrontal cortex, upregulating anti-inflammatory cytokines (IL-10 and TGF-β1), and reversing complement system dysregulation (C3, C4b, Cfh, Cfi), highlighting its immunomodulatory role in anxiety reduction[52]. GABA, a primary inhibitory neurotransmitter under physiological conditions, may cross the blood-brain barrier in appreciable quantities via several transport systems (transcytosis, carrier-mediated transport, or simple diffusion of hydrophobic molecules). Upon crossing, it can bind to its cognate receptors, triggering downstream signaling pathways. Alternatively, GABA may exert positive effects on sleep quality and anxiety through other routes, such as enhancing parasympathetic tone via the gut-brain axis through its influence on the peripheral nervous system (PNS)[45,53-55].

Smriga and Torii reported that oral L-lysine administration positively influences

anxiety symptoms. This amino acid appears to act as a specific antagonist for the 5-HT4 receptor—but not for other serotonin receptors such as 5-HT1A, 2A, 2B, 2C, or 3—thereby mitigating serotonin-induced anxiety as well as stress- and gut-related anxiety symptoms linked to serotonin signaling[56].

A 2022 study by Cristiano C. *et al.* showed that oral or injectable administration of short-chain fatty acids (SCFAs), such as butyrate, improved depression- and anxiety-like behaviors in rodent models[57]. These fatty acids are capable of crossing the blood-brain barrier, where they exert neuro-anti-inflammatory and neuro-protective effects[58-60].

Valeric acid, likewise, has documented anxiolytic properties. Loeb *et al.* and Vishwakarma *et al.* reported that its effects are mediated through agonism of the GABA-A receptor, elevation of GABA levels via inhibition of alpha-ketoglutarate dehydrogenase (up-regulation of GABA shunt activity) and GABA-transaminase (reducing GABA degradation), and enhancement of synaptic availability and postsynaptic effects of this neurotransmitter[61,62].

Our results revealed the presence of several key proteins and enzymes involved in the biosynthesis and transport of these four metabolites—GABA, L-lysine, butyrate, and valeric acid—as well as certain proteins implicated in regulating the expression of related pathway genes, among the predicted proteome of the healthy mouse fecal microbiome. This finding suggests that this microbial community may possess the potential capacity to produce these metabolites [Figure 4]. It should be noted, however, that our predictions are based on metabarcoding data. More direct experimental approaches—such as *in vitro* assays of metabolite production by members of this microbiome, or genomics and proteomics studies (as opposed to metagenomics and metaproteomics) on specific species and strains within this community—are required to confirm the present findings.

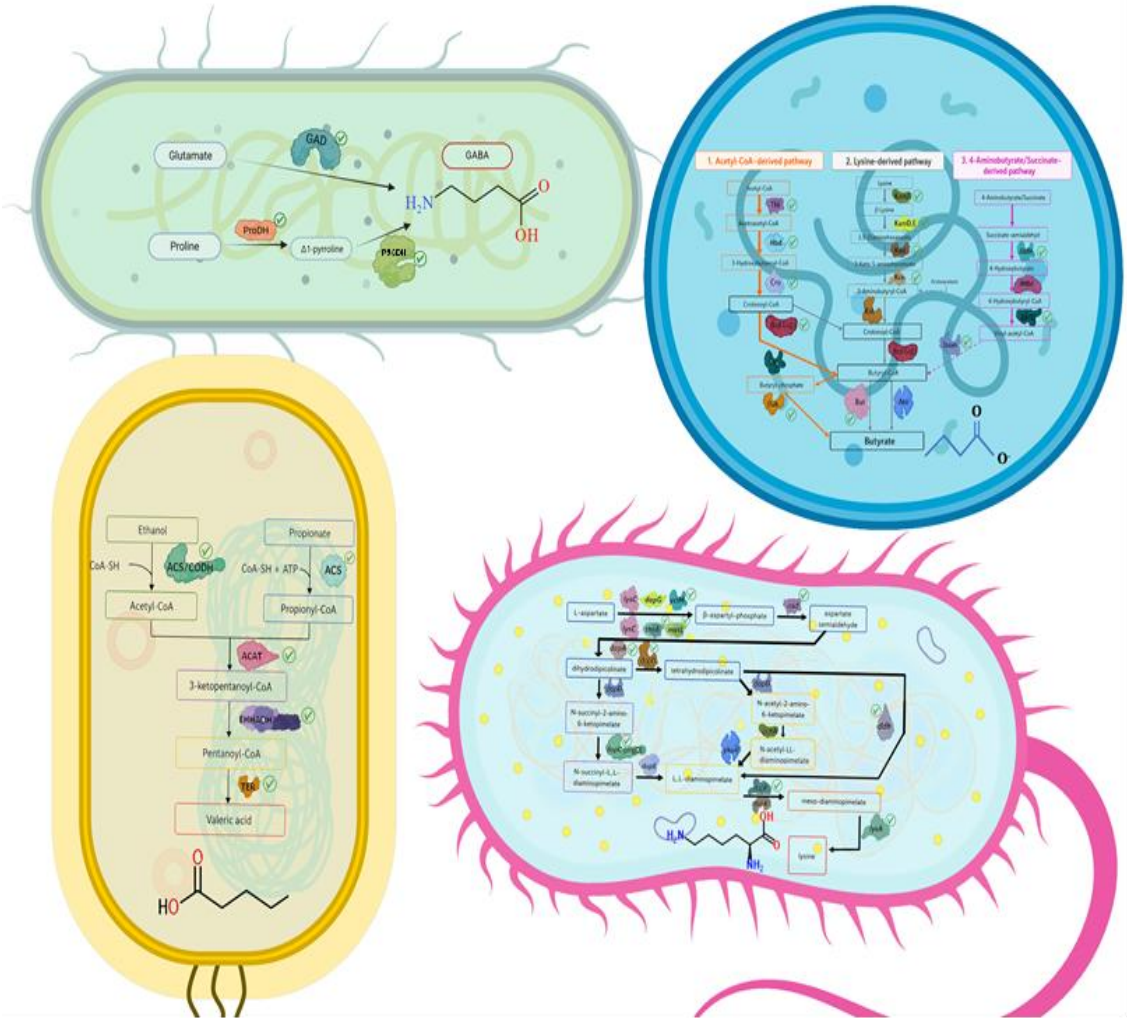

Figure 4. Bacterial biosynthetic pathways of metabolites with demonstrated anxiolytic activity, alongside key enzymes for each pathway. The pathways depicted are: (top left) GABA biosynthesis, (top right) butyrate biosynthesis, (bottom right) L-lysine biosynthesis, and (bottom left) valeric acid biosynthesis. Enzymes identified among the predicted proteins of the studied microbiome are marked with a check symbol. GAD: Glutamate decarboxylase, proDH: Proline dehydrogenase, P5CDH: Δ1-pyrroline dehydrogenase, Thl: Thiolase, Hbd: Beta-hydroxybutyryl-CoA dehydrogenase, Cro: Crotonase, Bcd: Butyryl-CoA dehydrogenase, Ptb: Phosphate butyryltransferase, Buk: Butyrate kinase, KamA: Lysine-2,3-aminomutase, KamD,E: Lysine-5,6-aminomutase, Kdd: 3,5-diaminohexanoate dehydrogenase, Kce: 3-keto-5-aminohexanoate cleavage enzyme, Kal: 3-aminobutyryl-CoA ammonia lyase, But: Butyryl-CoA:acetate CoA transferase, Ato: Butyryl-CoA:acetoacetate CoA transferase, AbfH: 4-hydroxybutyrate dehydrogenase, 4Hbt: Butyryl-CoA:4-hydroxybutyrate CoA transferase, AbfD: 4-hydroxybutyryl-CoA dehydratase, Isom: Vinylacetyl-CoA 3,2-isomerase,

ACS/CODH: Acetyl-CoA Synthase/Carbon Monoxide Dehydrogenase, ACS: Acyl-CoA synthetase, ACAT: Acetyl-CoA C-acetyltransferase, EHHADH: 3-hydroxyacyl-CoA dehydrogenase and enoyl-CoA hydratase, Ter: Enoyl-CoA reductase, LysC, DapG: Aspartokinase, YclM: Aspartokinase 3, ThrA: Bifunctional aspartate kinase and homoserine dehydrogenase, MetL: Bifunctional aspartate kinase and homoserine dehydrogenase 2, Asd: Aspartate-semialdehyde dehydrogenase, DapA: 4-hydroxy-tetrahydrodipicolinate synthase, DapB: 4-hydroxy-tetrahydrodipicolinate reductase, Ddh: 2-hydroxyacid dehydrogenase, DapD: Tetrahydrodipicolinate succinylase, PatA: Putrescine aminotransferase, ykuR: N-acetyldiaminopimelate deacetylase, dapC: Succinyldiaminopimelate transaminases, dapE: Succinyl-diaminopimelate desuccinylase, DapF: Diaminopimelate epimerase, DapX: N-acetyl-LL-diaminopimelate aminotransferase, LysA: Diaminopimelate decarboxylase.

Among the neurotransmitters recognized for their role in alleviating anxiety and depression and promoting positive mood states, serotonin and dopamine stand out. Both are monoamine neurotransmitters that are not confined to the central nervous system but are widely distributed throughout the body, where they fulfill diverse physiological roles. These include the maintenance of homeostasis, regulation of emotions, mood, and sleep, contributions to learning, and modulation of immune and gastrointestinal function. Dysregulation of the synthesis or release of these two transmitters can precipitate a spectrum of physical and psychiatric disorders, and has even been implicated in carcinogenesis[63,64].

Evidence indicates that dopamine agonists can exert anxiolytic-like effects through activation of the dopamine receptors. For instance, findings from recent studies underscore the therapeutic potential of dopaminergic modulation in the medial prefrontal cortex (mPFC). The administration of the D1 receptor agonist SKF38393 into the mPFC has been shown to exert robust anxiolytic effects by reversing the neuronal hyperactivity induced by glutamatergic disruption in the ventral hippocampus, suggesting that D1 receptor activation can serve as a regulatory mechanism to mitigate anxiety-like states[65]. Similarly, several studies demonstrated that serotonin attenuates anxiety symptoms via binding to receptors such as 5-HT2A and subsequent engagement of cortico-limbic circuits. Activation of these receptors dampens amygdala

reactivity, strengthens connectivity within the ACC-DMN-CEN networks, enhances cognitive flexibility, promotes neuroplasticity in the hippocampus and prefrontal cortex, increases the excitability of pyramidal neurons, reinforces top-down regulation of the amygdala, modulates stress-related hormones in the hypothalamus, reduces impulsivity, and improves behavioral control[66]. Direct and indirect consequences of impaired synthesis and synaptic levels of dopamine and serotonin have been documented in the onset or exacerbation of anxiety, post-traumatic stress disorder, obsessive-compulsive disorder, depression, and suicidal ideation. For instance, studies of COVID-19 patients have revealed that the disease can trigger anxiety symptoms in approximately 30% of cases, an effect attributed to reduced biosynthesis of these neurotransmitters through diminished absorption of their precursor amino acids, notably tryptophan[67-72].

Functional and enrichment analysis performed in the present study revealed that pathways governing the release of dopamine and serotonin may be activated as a consequence of protein-protein interactions between the fecal microbiome of healthy mice and the human orthologs of proteins expressed in the cortex of mice exhibiting anxiety-like symptoms. Thus, one possible mechanism underlying the beneficial effect of fecal microbiome transplantation on anxiety-like behaviors could involve extracellular, surface-associated, or signaling proteins from the fecal microbiome engaging cortical tissue to elevate serotonin and dopamine levels within the relevant neurological circuits[Figure 5].

Disruption of circadian rhythms is associated with an increased risk of anxiety and its comorbid conditions. Toki *et al.* demonstrated that perturbing circadian rhythms in mice heightened anxiety-like behaviors, diminished social interaction, and impaired object recognition memory[73]. Conversely, Ashkenazy *et al.* showed that reinforcing circadian entrainment via light therapy ameliorated certain anxiety- and depression-like symptoms in rats[74]. Among mouse models of anxiety disorders, circadian disruption is more pronounced in paradigms involving social stress or prenatal stress than in other chronic stress models[75]. Notably, among the downstream pathways identified through the predicted protein-protein interactions in this study, pathways related to the circadian clock (Circadian Clock R-HSA-400253) and its regulatory components (BMAL1:CLOCK,NPAS2 Activates Circadian Gene Expression R-HSA-1368108) were observed. This finding suggests that fecal

microbiome transplantation may contribute to the alleviation of anxiety symptoms—or anxiety-like symptoms in mice—by influencing circadian rhythm regulation, potentially through the modulation of genes such as BMAL1 and CLOCK [Figure 5].

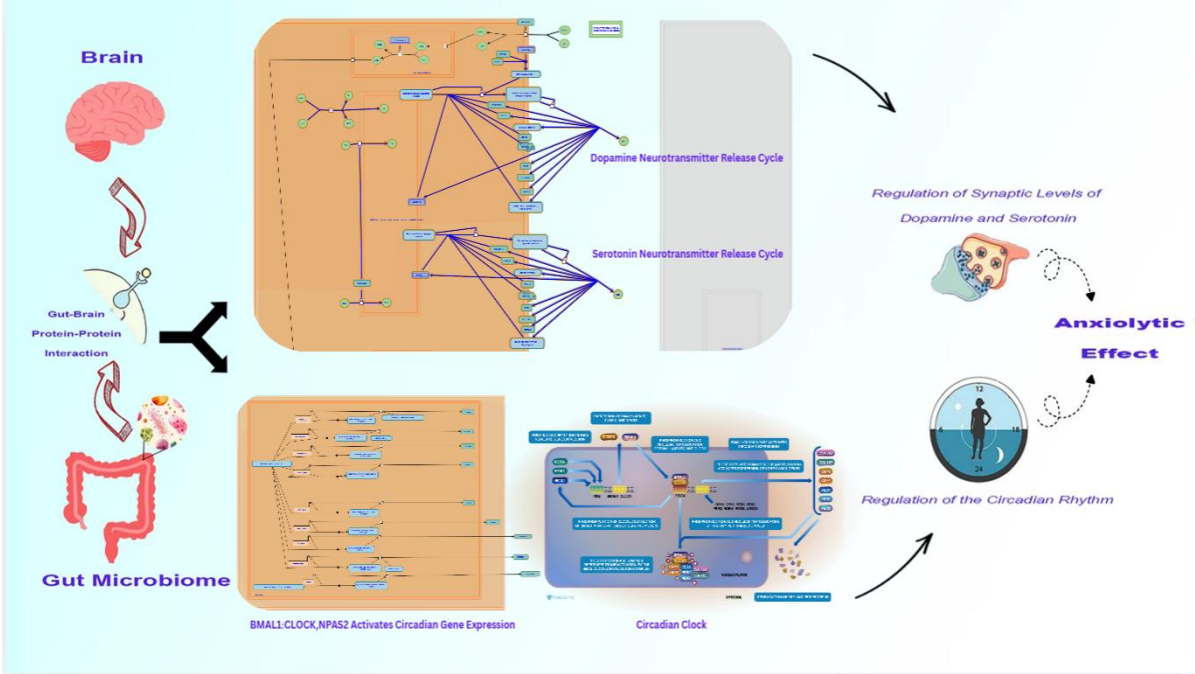

Figure 5. Downstream gut-brain protein-protein interaction pathways that may regulate synaptic levels of serotonin and dopamine, as well as circadian rhythms, thereby potentially mediating anxiolytic effects. Pathways were extracted from the Reactome database (reactome.org), which was also employed during the MicrobioLink2 enrichment and functional analysis stages.

Another facet of this study concerned the potential impact of traditional dairy products obtained from Iranian nomadic ewe on anxiety and anxiety-like symptoms. A body of previous research has linked dairy consumption with anxiety relief. For instance, a 2022 study conducted on young Azorean university students found that fermented dairy products exerted a positive modulatory effect on anxiety[76]. Iran, with its mosaic of ethnic groups, traditions, and dietary customs, is home to a remarkable diversity of local dairy products and thus possesses substantial potential for such functional foods; the primary producers of these indigenous items are rural and nomadic pastoralists[77]. In addition, as mentioned earlier, an investigation of Iranian adults (20-69 years) associated high-fat milk intake with a reduced risk of depression, and semi-skimmed milk with a lower likelihood of anxiety symptoms[24]. Dairy matrices serve as effective

carriers for delivering probiotics, prebiotics, and postbiotics to the human body[78], and a growing body of evidence supports the beneficial effects of probiotic consumption on anxiety and depression[79]. Characterizing the microbial composition of a given dairy product can therefore shed light on the mechanisms by which it influences anxiety. To explore this possibility, the microbiome of traditional dairy products made from ewe milk by nomads in the Qasr-e Shirin region of Iran was analyzed. The results suggest that these local products, by virtue of their rich microbial communities, may alleviate anxiety symptoms. In particular, the predicted proteome of the microbial microbiome from ewe milk, yogurt, and doogh (a traditional fermented yogurt drink) contained proteins potentially involved in the biosynthesis and transport of anxiolytic metabolites—namely, valeric acid, GABA, butyrate, and L-lysine—and exhibited considerable proteomic overlap with the healthy mouse fecal microbiome, both globally and among the subset of proteins that participate in host-microbe interactions. This overlap hints at similar downstream effects; hence, the consumption of traditional ewe-milk dairy products may help modulate anxiety symptoms.

CONCLUSION

The bioinformatic analyses performed in this study indicate that fecal microbiome transplantation (FMT) may contribute to the amelioration of anxiety symptoms through several avenues: the production of anxiolytic metabolites such as GABA, valeric acid, butyrate, and L-lysine; activation of signaling pathways that regulate the synaptic levels of serotonin and dopamine; and modulation of circadian rhythms. These findings underscore the promise of FMT as a systemic, multi-pathway therapeutic intervention for anxiety disorders. Also, consumption of traditional Iranian ewe dairy products may have a similar positive effect through the production of the aforementioned anxiolytic metabolites and activation of similar pathways. Importantly, the present work is entirely *in silico*; complementary *in vitro* and *in vivo* (animal and clinical) investigations are imperative to validate these results and delineate the precise mechanisms of action. Such studies could provide new insights into the understanding and treatment of anxiety disorders, the most common psychiatric conditions worldwide.

DECLARATIONS

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Authors' contributions

Study conception, design, and execution, and preparation of the initial draft: Negar Mojiri;

Supervision and manuscript revision: Zahra Rezvani; both authors approved the final version.

Availability of data and materials

The original data used in this study are mentioned and included in the paper. In case of any additional queries, please contact the corresponding authors. Further data will be made available on request.

AI and AI-assisted tools Statement

The authors acknowledge that artificial intelligence (AI) technologies were employed to facilitate specific aspects of the research and writing process. AI language models, including ChatGPT, Grok, and Gemini, were used to assist with the refinement and troubleshooting of Python code. In addition, these AI tools—along with DeepSeek V4 Pro—played a significant role in the academic translation of this article from Persian into English and in editing the final text. The prompts provided to these tools were carefully engineered to ensure content accuracy and preserve scientific integrity. Following the use of such services, the author reviewed and edited the output where needed. The authors take full responsibility for the content and accuracy of this work.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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