Scientia Psychiatrica
Vol. 7, No. 3, 2026, pp. 149–159 · e-ISSN 2715-9736 journal homepage: scientiapsychiatrica.com

ORIGINAL RESEARCH ARTICLE

Limited Associations between Neuroactive Gut Microbial Pathways and Symptom Severity in Children with Autism

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ARTICLE INFO

Article history
Received: May 22, 2026
Revised: June 19, 2026
Accepted: July 10, 2026
Available online: July 26, 2026
Published: July 30, 2026

Keywords
Autism spectrum disorder
Gut microbiome
Metagenomics
Neuroactive pathways
Symptom severity

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DOI
10.37275/scipsy.v7i3.217

ABSTRACT

Background. Microbial genes that produce or transform neuroactive compounds may contribute to autism heterogeneity, but their relation to dimensional symptoms is unclear.

Objective. To test whether neuroactive microbial pathways selected a priori were associated with symptom severity in autistic children and improved out-of-sample prediction beyond clinical covariates.

Methods. This secondary cross-sectional analysis used linked metagenomic and clinical data from 818 children (464 autistic). Six of twelve a priori neuroactive MetaCyc pathways, detected in at least 10% of autistic participants, were analyzed. Social Responsiveness Scale, Second Edition (SRS-2), Anxiety Scale for Children–Autism Spectrum Disorder (ASC-ASD), and Child Behavior Checklist (CBCL) Internalizing scores were regressed robustly on centered log-ratio pathway abundance, age, sex, and batch, with Benjamini–Hochberg correction within outcome. Sensitivity analyses varied covariates and transformation, modeled pathways jointly, and examined influential observations, sparse detection, and cross-validated ridge prediction.

Results. Sample sizes were 464 (SRS-2), 459 (ASC-ASD), and 144 (CBCL Internalizing). PWY-5088 was inversely associated with SRS-2 ($\beta = -0.110$, 95% CI −0.200 to −0.020; $p = 0.017$); the predefined false-discovery threshold was narrowly missed ($q = 0.102$), and the estimate persisted in sensitivity analyses. No pathway was consistently associated with ASC-ASD or CBCL Internalizing. Pathways added minimal SRS-2 prediction gain (mean $\Delta R^2 = 0.004$) and worsened ASC-ASD prediction (mean $\Delta R^2 = -0.022$).

Conclusion. Neuroactive pathway profiles were not broad or clinically useful correlates of symptom severity. A small inverse association between PWY-5088 and social-communication severity merits replication with longitudinal metabolomics; it is neither a diagnostic biomarker nor evidence of causality.

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1. Introduction

Autism spectrum disorder is a neurodevelopmental condition defined by differences in social communication and by restricted or repetitive behavior. Language, cognition, sensory processing, adaptive function, anxiety, and physical health vary widely within it. Reported prevalence has risen across many settings. The clinical task has therefore moved on. It is no longer only to recognize autism but to understand what drives its heterogeneity, which shapes support needs and co-occurring symptoms. Gastrointestinal symptoms, selective eating, disturbed sleep, anxiety, and medication exposure are common in autistic children, and each can affect daily functioning. The gut microbiome is therefore studied as one part of a wider biological and environmental system. It is not a single explanation for autism.[1–4]

The microbiota–gut–brain axis offers several routes by which intestinal microbes might relate to neurobehavioral phenotypes. Microbes transform dietary substrates, modify bile acids and tryptophan metabolites, produce short-chain fatty acids, engage epithelial and immune signaling, and change how much of a neuroactive molecule is available.[5–8] Microbial genes annotated to gamma-aminobutyric acid, glutamate, glutamine, propanoate, and related pathways give a functional view that may sit closer to

mechanism than taxon abundance does. Yet a metagenomic pathway shows only what a community encodes. It does not show that a metabolite was made, absorbed, carried into the central nervous system, or able to alter neuronal signaling. This matters: different organizms can yield the same annotation, flux depends on substrate supply and ecological context, and peripheral and central neurotransmitter pools are physiologically separate.

Case–control studies have found differences in microbial taxa, functional profiles, metabolites, or gut–brain modules between autistic and non-autistic participants, but their direction and size differ considerably between cohorts.[9–20] Age, geography, diet, and stool consistency all contribute. So do gastrointestinal symptoms, sequencing protocol, medication use, and the handling of compositional, zero-inflated data. Large reanalyses indicate that diet and other autism-associated behaviors may explain part of the microbiome signature. Multi-kingdom and multi-omic studies suggest that coordinated functional signals can be detected when phenotyping and technical control are strong.[2–4,9–20] A cross-sectional case–control contrast cannot say whether microbial features come before autistic traits, follow autism-related diet and behavior, or reflect a two-way process.

Diagnostic-group contrasts leave open a further question: does microbial function track clinically meaningful variation inside the autistic population? Dimensional outcomes suit it better. Social responsiveness, anxiety, and internalizing symptoms vary widely among autistic children, and each can have its own biological, developmental, and measurement determinants. Recent clinical, intervention, multi-omic, and experimental studies have linked microbial composition or metabolites to gastrointestinal and behavioral measures. Many relied on modest samples, broad diversity metrics, many exploratory features, or in-sample model fit.[21–31] Fewer have defined neuroactive pathways a priori, controlled false discoveries within symptom domains, tested composition-aware alternatives, and checked whether associations add reproducible predictive information beyond basic clinical covariates.[32,33]

This study addresses that gap. It combines pathway-level inference with out-of-sample evaluation in a comparatively large linked metagenomic cohort; its novelty lies in an a priori panel of neuroactive microbial pathways tested across three complementary symptom domains. Robust regression, outcome-specific multiplicity control, alternative abundance transformations, joint-pathway and influence analyses, and repeated cross-validated incremental prediction are combined. The approach separates an association that is statistically detectable under some specifications from a pathway profile that is broadly informative or predictive; we aimed to determine whether neuroactive gut microbial pathway abundances were associated with social-communication severity, autism-specific anxiety, and internalizing symptoms in autistic children, and whether the pathways improved out-of-sample prediction beyond age, sex, and technical covariates.

2. Methods

2.1. Study design and setting

Study design and data source. We conducted a secondary cross-sectional analysis of de-identified participant-level data derived from a population-based, age- and sex-matched metagenomic study of the gut microbiome in autism; the source resource combined shotgun metagenomic profiles with demographic and clinical phenotypes from children aged 2–12 years. The public analysis record and source article describe recruitment, biospecimen processing, sequencing, quality control, and primary cohort construction.[3,4] The present analysis was defined around a single question: whether microbial pathways with neuroactive annotations explained variation in dimensional symptoms within autistic participants.

2.2. Participants

Participants and analytic sample. The open metadata listed 836 children, and 818 unique children had linked profiles. The linked analytic file contained these 818 children: 464 autistic participants and 354 non-autistic controls. Diagnostic group was used to describe the cohort and audit linkage, whereas all symptom-association and prediction models were restricted to autistic participants. Participant identifiers were checked for duplication, outcome ranges were screened, and pathway fields were reconciled against the source dictionary. No duplicate participant identifiers or impossible core values were identified. The analysis unit was one participant. Because exposure and outcome were observed contemporaneously, all coefficients were interpreted as cross-sectional associations.

2.3. Variables and instruments

Neuroactive pathway exposures. Twelve MetaCyc pathways were selected a priori because their annotations implicated gamma-aminobutyric acid, glutamate, glutamine, or related neuroactive metabolism. Pathway abundance had been generated from quality-controlled shotgun metagenomes using the source-study functional pipeline. Six pathways were detected in at least 10% of autistic participants and were eligible for primary inference: GLUDEG-I-PWY (GABA shunt), PWY-4041 (gamma-glutamyl cycle), PWY-5088 (L-glutamate degradation VIII to propanoate), PWY-5505 (L-glutamate and L-glutamine biosynthesis), PWY-6549 (L-glutamine biosynthesis III), and PWY-8190 (L-glutamate degradation XI); the prevalence threshold was applied without reference to outcome associations to avoid outcome-driven feature selection.

Outcomes and covariates. The primary outcome was the Social Responsiveness Scale, Second Edition total T score (SRS-2), a caregiver-reported standardized measure of social-communication characteristics and restricted or repetitive behavior. Higher T scores

indicate greater severity. Two secondary outcomes captured clinically important co-occurring domains: the Anxiety Scale for Children–Autism Spectrum Disorder total score (ASC-ASD), developed to assess anxiety presentations relevant to autistic children, and the Child Behavior Checklist Internalizing Problems score (CBCL Internalizing).[34–36] The source dataset contained SRS-2 for all 464 autistic participants, ASC-ASD for 459, and CBCL Internalizing for 144. Complete-case samples were defined separately for each outcome.

The minimally adjusted model included age, sex, and technical batch because these variables can influence symptom distributions or metagenomic measurement; the expanded sensitivity model additionally included available indicators of diet, medication exposure, and stool characteristics from the source dataset. Covariates were defined a priori and were not selected using statistical significance. Continuous outcomes and exposures were standardized to support comparison across pathways and domains. Sex was modeled as recorded in the source file. Batch was treated as a categorical technical factor.

2.4. Data collection procedure

Pathway abundance processing. Relative abundance data are constrained and contain many zeros. For the primary analysis, a small pseudocount was added consistently and pathway values were centered log-ratio (CLR) transformed against the eligible-pathway geometric mean, then standardized within autistic participants. The resulting coefficient represents the standard-deviation difference in outcome per standard-deviation increase in relative pathway abundance. Two alternative specifications were examined: $\log_{10}$-transformed abundance and an expanded composition-aware model using the same centered-log-ratio exposure with additional clinical covariates. PWY-5088 was detected in 19.4% of autistic participants, so detection-indicator and positive-abundance models were also used to distinguish prevalence from abundance among detected samples.

2.5. Statistical analysis

We summarized participant characteristics using means and standard deviations or counts and percentages. Pathway detection prevalence was calculated in autistic participants. Pearson and Spearman correlations were used as descriptive bivariate analyses, with multivariable models defining inference. For each eligible pathway and outcome, we fit a separate linear regression with standardized outcome as the dependent variable and standardized pathway exposure, age, sex, and batch as predictors. Heteroskedasticity-consistent HC3 standard errors were used because symptom variance and pathway distributions were not assumed to be homoscedastic. Coefficients are reported with 95% confidence intervals and two-sided p values.

Multiplicity was controlled separately within each outcome family across the six eligible pathways using the Benjamini–Hochberg procedure; a false-discovery-rate threshold of $q < 0.10$ was set as the inferential criterion because the pathway panel was focused and the analysis was intended to identify signals for replication. This threshold does not convert exploratory biological evidence into confirmation; effect magnitude, interval width, consistency, and predictive performance were considered together. No unsupported post hoc subgroup testing was used to obtain statistical significance.

Several robustness analyses were performed. First, pathway estimates were repeated with expanded clinical covariates and $\log_{10}$ abundance. Second, all six pathways were entered simultaneously with age, sex, and batch to assess whether the principal estimate was independent of correlated pathway information; variance-inflation factors quantified collinearity. Third, observations with Cook's distance greater than 4/n were excluded in an influence sensitivity analysis. Fourth, PWY-5088 was parameterized as detection versus non-detection and as abundance among detected samples. Fifth, autistic participants with and without CBCL Internalizing data were compared on observed characteristics to characterize outcome availability. Repeated analyses were interpreted by consistency of direction, effect size, and uncertainty rather than by isolated p values.

Incremental prediction was evaluated for SRS-2 and ASC-ASD because their sample sizes supported repeated resampling. Ridge-regression pipelines compared a clinical-only model containing age, sex, and batch with a clinical-plus-pathway model containing those variables and the six eligible pathway features. Preprocessing and penalization were performed within training folds. We used 20 repetitions of five-fold cross-validation with matched splits for the two models. Out-of-sample R^2, root-mean-square error, and prediction–outcome correlation were recorded. The distribution of paired ΔR^2 values, rather than a single favorable split, was used to judge added predictive information. CBCL prediction was not emphasized because only 144 autistic participants had outcome data.

Analyses used Python with pandas, NumPy, SciPy, scikit-learn, and statsmodels; the frozen scripts, intermediate tables, machine-readable results, validation report, and figures were retained with the case. Functional profiling and reporting followed contemporary reproducible microbiome-analysis and reporting principles.[37,38]

2.6. Ethical approval

Ethical approval and consent are reported in the Declarations. The source study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee (Ref. 2021.550), and written informed consent was obtained from parents or legal guardians.[3] The present de-identified secondary analysis was reviewed by the CMHC Research Center Ethics Committee Indonesia (No. 2026/0248). No participant was recontacted, no intervention was assigned, and trial registration was not applicable.

3. Results

3.1. Cohort and data coverage

The linked cohort included 818 children, of whom 464 were autistic and 354 were non-autistic. Mean age was 8.28 years (SD 1.97) overall, 7.87 years (SD 2.07) in autistic participants, and 8.81 years (SD 1.70) in non-autistic controls. Females comprised 27.3% of the total cohort and 14.4% of autistic participants. Mean SRS-2 T score was 67.69 (SD 9.95) in autistic participants compared with 52.38 (SD 6.40) in controls. Participant characteristics are detailed in Table 1, and cohort linkage with outcome-specific analytic samples is shown in Figure 1.

Table 1. Cohort characteristics and outcome availability

Characteristic	All (n = 818)	Autistic (n = 464)	Non-autistic (n = 354)
Age, mean (SD), years	8.28 (1.97)	7.87 (2.07)	8.81 (1.70)
Female, n (%)	223 (27.3)	67 (14.4)	156 (44.1)
SRS-2 T score, mean (SD)	61.06 (11.46)	67.69 (9.95)	52.38 (6.40)
ASC-ASD available, n (%)	813 (99.4)	459 (98.9)	354 (100.0)
CBCL Internalizing available, n (%)	474 (57.9)	144 (31.0)	330 (93.2)

Values are descriptive and use the linked participant-level file. SD, standard deviation; SRS-2, Social Responsiveness Scale, Second Edition; ASC-ASD, Anxiety Scale for Children–Autism Spectrum Disorder; CBCL, Child Behavior Checklist; CBCL Internalizing, Child Behavior Checklist Internalizing Problems score.

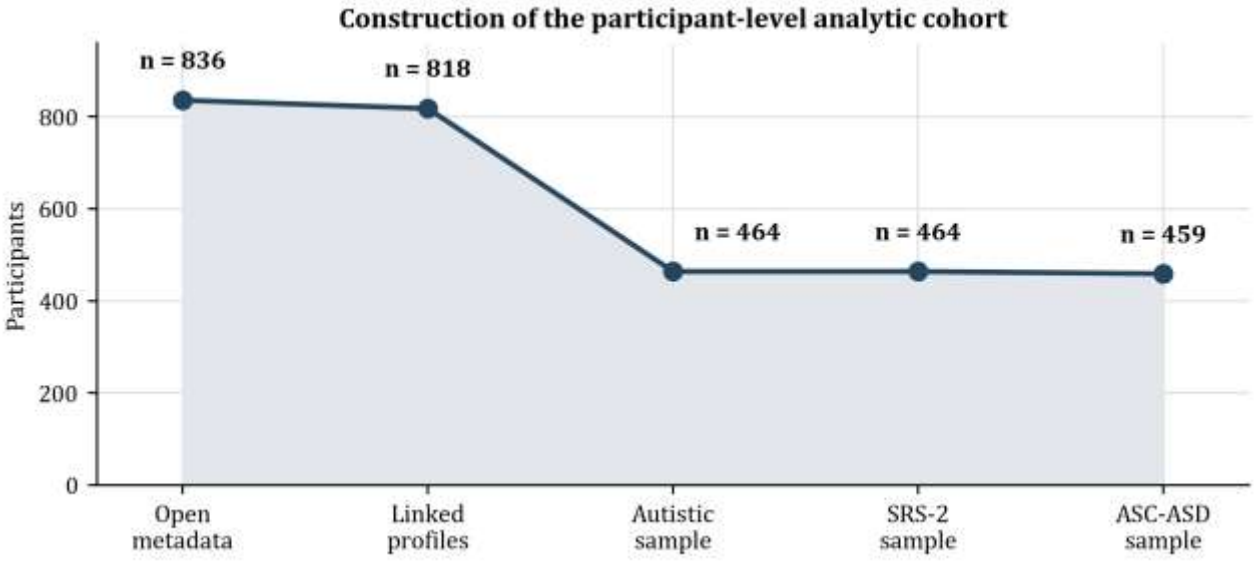


Figure 1. Cohort linkage and outcome-specific analytic samples. The open metadata listed 836 children; the linked dataset comprised 818 unique children, and association analyses were restricted to 464 autistic participants. ASC-ASD was available for 459 participants; CBCL Internalizing (144 participants) is not plotted.

SRS-2 was complete for all 464 autistic participants, and ASC-ASD was available for 459 (98.9%). CBCL Internalizing was available for 144 (31.0%), making this analysis less precise and more vulnerable to selection related to outcome availability. Autistic participants with CBCL data were slightly younger than those without CBCL data (mean 7.55 vs 8.02 years; standardized mean difference −0.228; p = 0.032); other observed comparisons did not show material imbalance. The CBCL findings were therefore treated as secondary, with emphasis on confidence intervals rather than absence of significance.

3.2. Pathway prevalence

Six of the twelve a priori neuroactive pathways met the 10% detection threshold in autistic participants. Pathway-specific detection prevalence is detailed in Figure 2. Detection varied substantially across functions, indicating that a simple abundance-only coefficient could combine ecological prevalence and within-positive abundance. PWY-5088, annotated as L-glutamate degradation VIII to propanoate, was detected in 19.4%. The eligibility rule excluded sparse pathways before outcome testing and reduced the number of primary comparisons. No pathway value was redefined using symptom data.

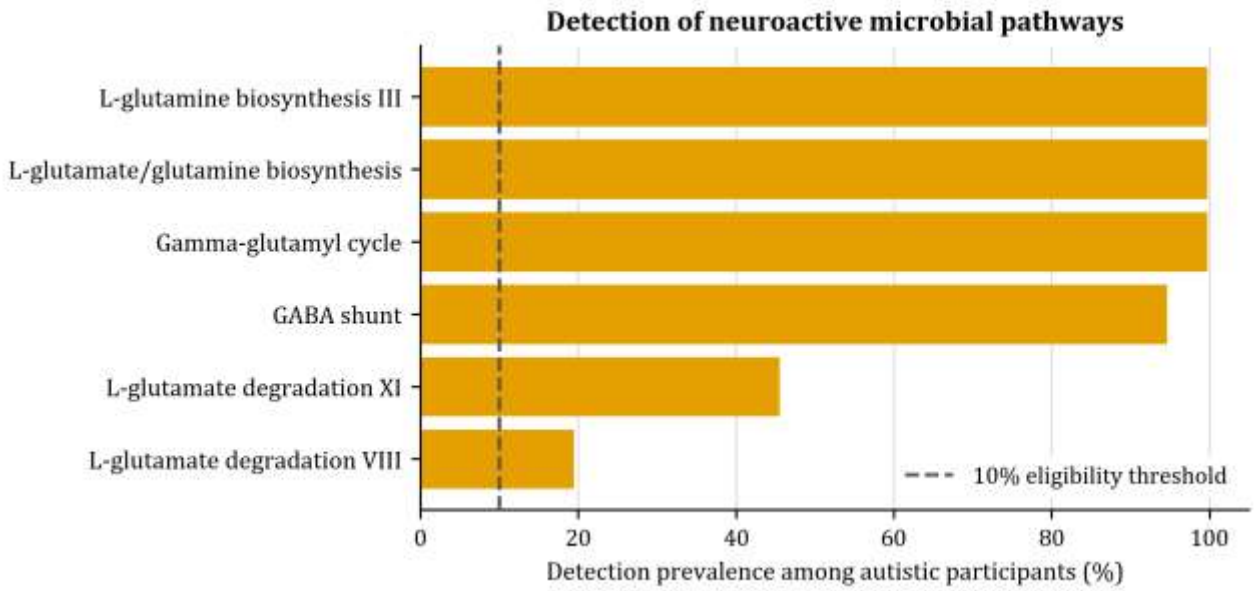


Figure 2. Detection prevalence of neuroactive microbial pathways among autistic participants. Six pathways meeting the outcome-independent threshold of detection in at least 10% of participants entered primary inference.

3.3. Primary adjusted associations

In the primary SRS-2 models, PWY-5088 had a negative standardized association (β = −0.110, 95% CI −0.200 to −0.020; p = 0.017). The corresponding q value was 0.102, narrowly above the predefined q < 0.10 threshold. Thus, the estimate was nominally detectable but did not meet the primary false-discovery criterion. The other five eligible pathways had smaller or less precise SRS-2 estimates: GLUDEG-I-PWY β = −0.046, PWY-4041 β = 0.014, PWY-5505 β = −0.030, PWY-6549 β = −0.061, and PWY-8190 β = −0.018; none met outcome-specific FDR control. The primary association pattern for SRS-2 and ASC-ASD is detailed in Figure 3, and the principal PWY-5088 estimates are summarized in Table 2.

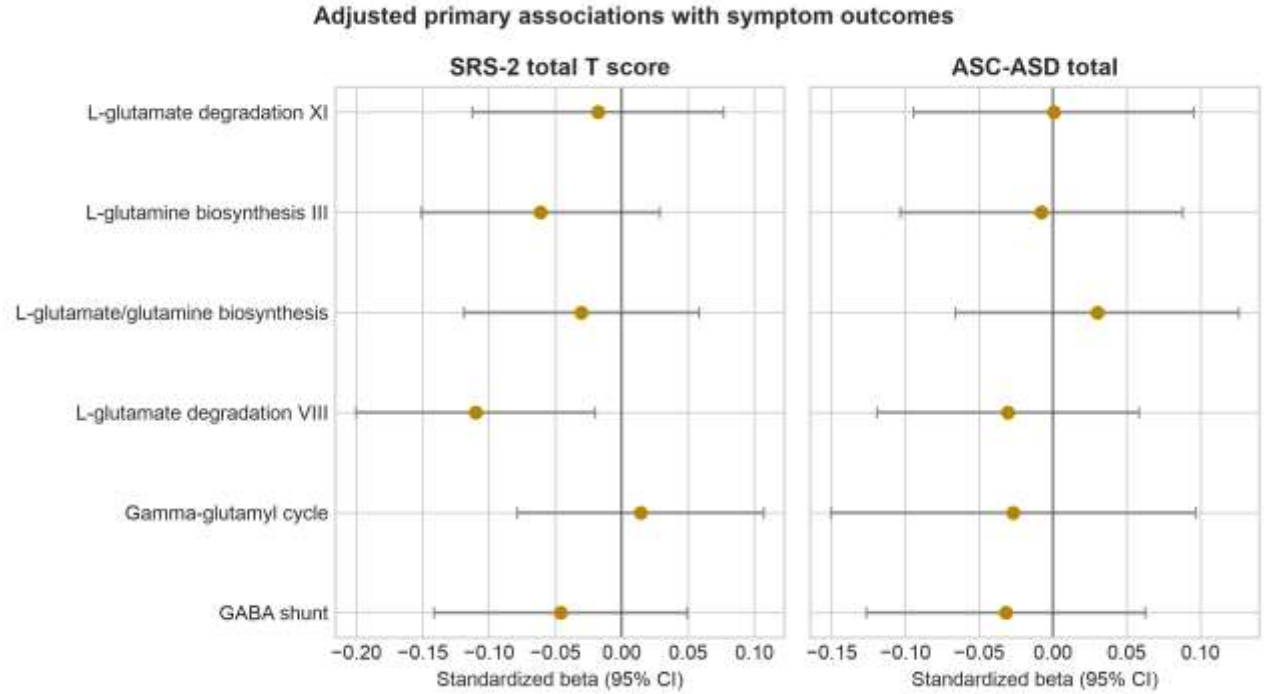


Figure 3. Adjusted primary associations between eligible neuroactive pathways and standardized SRS-2 and ASC-ASD scores. Points are standardized coefficients and bars are 95% confidence intervals from age-, sex-, and batch-adjusted linear models with HC3 robust standard errors. Multiplicity was controlled separately within outcome.

Table 2. Principal PWY-5088 estimates and incremental prediction

Outcome/analysis	n	Standardized β	95% CI	p	q or ΔR^2
SRS-2, primary CLR + HC3	464	−0.110	−0.200 to −0.020	0.017	q = 0.102
SRS-2, expanded covariates	464	−0.112	−0.202 to −0.023	0.014	q = 0.083
SRS-2, $\log_{10}$ abundance	464	−0.119	−0.207 to −0.031	0.008	q = 0.048
SRS-2, joint six pathways	464	−0.135	−0.230 to −0.039	0.006	—
SRS-2, influence sensitivity	440	−0.140	−0.230 to −0.050	0.002	—
ASC-ASD, primary	459	−0.030	−0.119 to 0.058	0.501	q = 0.992
CBCL Internalizing, primary	144	−0.120	−0.307 to 0.066	0.205	q = 0.888
SRS-2, repeated CV	464	—	—	—	mean ΔR^2 = 0.004
ASC-ASD, repeated CV	459	—	—	—	mean ΔR^2 = −0.022

CLR, centered log-ratio; HC3, heteroskedasticity-consistent standard errors; CV, cross-validation; CI, confidence interval; q, Benjamini–Hochberg false-discovery-rate q value; ΔR^2, mean out-of-sample R^2 of the clinical-plus-pathway ridge model minus that of the clinical-only model; PWY-5088, L-glutamate degradation VIII to propanoate; SRS-2, Social Responsiveness Scale, Second Edition; ASC-ASD, Anxiety Scale for Children–Autism Spectrum Disorder; CBCL Internalizing, Child Behavior Checklist Internalizing Problems score. Benjamini–Hochberg q values were calculated within each outcome family for the pathway-wise models. Joint, influence, and prediction analyses were interpreted as robustness or generalization analyses rather than additional primary tests.

No eligible pathway was consistently associated with ASC-ASD. For PWY-5088, the primary ASC-ASD estimate was β = −0.030 (95% CI −0.119 to 0.058; p = 0.501; q = 0.992). Across the six pathways, corrected results indicated no evidence of a broad pathway–anxiety pattern. Similarly, primary CBCL Internalizing estimates were imprecise. PWY-5088 had β = −0.120 (95% CI −0.307 to 0.066; p = 0.205; q = 0.888), and no pathway met the false-discovery criterion. Figure 3 displays the SRS-2 and ASC-ASD estimates and intervals, most of which were centered near zero; the CBCL intervals were wider because of the smaller sample (the PWY-5088 estimate is in Table 2).

3.4. Robustness and multivariable pathway analyses

The inverse PWY-5088–SRS-2 estimate was similar after adding diet, medication, and stool covariates (β = −0.112, 95% CI −0.202 to −0.023; p = 0.014; q = 0.083) and under $\log_{10}$ abundance (β = −0.119, 95% CI −0.207 to −0.031; p = 0.008; q = 0.048). These estimates are detailed in Table 2. The descriptive Pearson correlation was −0.113 (p = 0.015), whereas Spearman ρ was −0.075 (p = 0.105), suggesting that rank-monotonic evidence was weaker than the standardized linear relation. These specifications support a small negative association but also demonstrate sensitivity of multiplicity-adjusted classification to modeling choices.

When all six pathways were entered jointly, collinearity was modest (maximum variance-inflation factor 2.10). The adjusted PWY-5088 SRS-2 coefficient was −0.135 (95% CI −0.230 to −0.039; p = 0.006), while the joint model explained 3.5% of outcome variance and had an adjusted R^2 of 1.3%. After excluding 24 observations exceeding Cook distance 4/n, the estimate remained negative (n = 440; β = −0.140, 95% CI −0.230 to −0.050; p = 0.002). Joint and influence analyses therefore did not indicate that the association was solely attributable to pathway collinearity or a small set of influential observations.

Sparse-pathway sensitivity gave a more qualified picture. PWY-5088 detection versus non-detection was associated with a lower SRS-2 score in direction but not conventionally precise (β = −0.223, 95% CI −0.459 to 0.014; p = 0.065). Among the 90 participants with detected PWY-5088, the positive-abundance coefficient was −0.176 (95% CI −0.389 to 0.037; p = 0.104). Neither component independently established a robust effect, although both directions were consistent with the main estimate. For CBCL, a detection-indicator association was observed in a small outcome subset, but the positive-abundance estimate among only 28 detected participants was near zero and imprecise; this isolated result was not interpreted as confirmatory.

3.5. Incremental prediction

For SRS-2, the clinical-only ridge model had mean out-of-sample R^2 −0.008, whereas the clinical-plus-pathway model had mean R^2 −0.004. The paired mean ΔR^2 was 0.004, its percentile interval spanned approximately −0.002 to 0.014, and 16 of 20 repetitions favored adding pathways. Mean root-mean-square error changed from 9.975 to 9.954 points. Although the paired difference was usually positive, both absolute R^2 values were slightly below zero, showing that neither model predicted SRS-2 better than a held-out-sample mean benchmark to a clinically useful degree.

For ASC-ASD, the clinical-only model had mean out-of-sample R^2 0.007 and the clinical-plus-pathway model −0.015. Mean ΔR^2 was −0.022, the interval was approximately −0.037 to −0.013, and none of the 20 repetitions favored adding pathways. Prediction–outcome correlation also declined when pathways were added. The numerical prediction summary is detailed in Table 2, and Figure 4 shows the mean out-of-sample R^2 of the clinical-only and clinical-plus-pathway models. The regression signal for one SRS-2 pathway therefore did not translate into a strong multifeature predictor, and pathway profiles did not generalize across symptom domains.

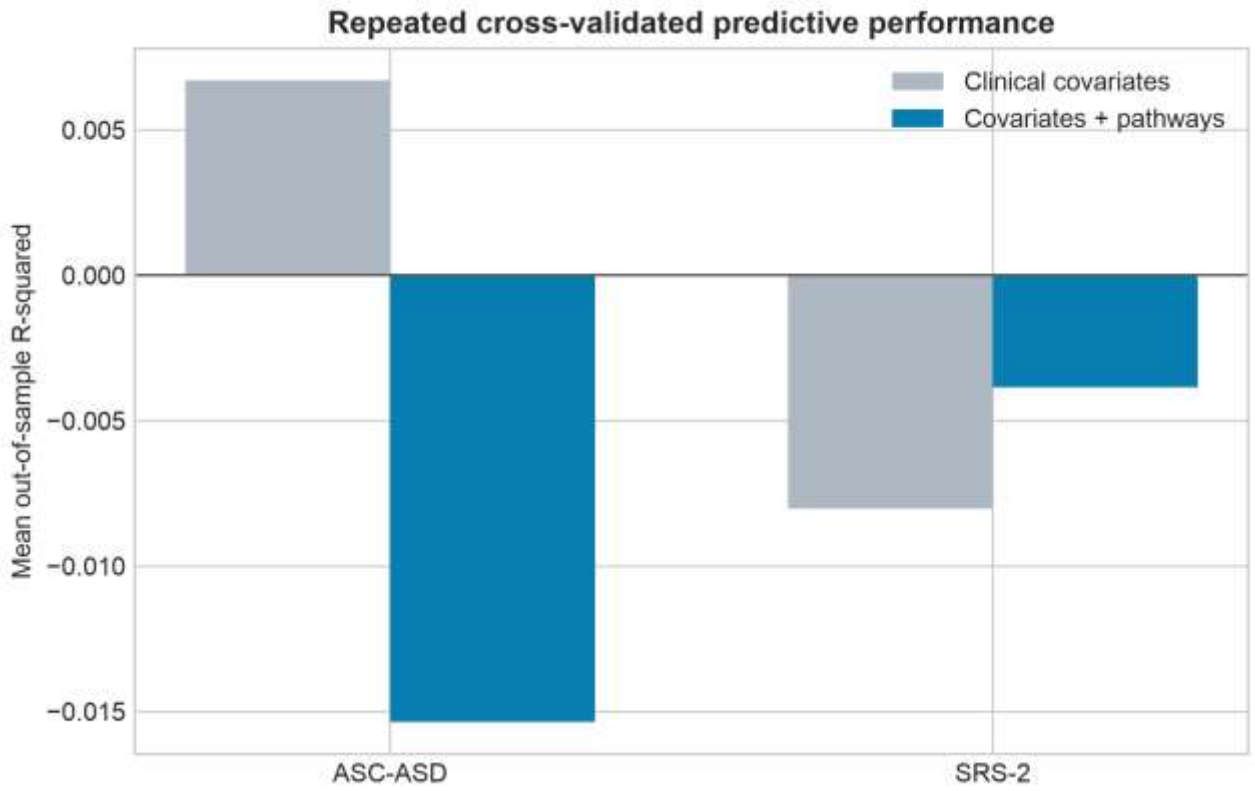


Figure 4. Repeated cross-validated incremental prediction. Bars show the mean out-of-sample R^2 of the clinical-only (covariates) and clinical-plus-pathway ridge models across 20 repetitions of five-fold cross-validation; the paired ΔR^2 values are given in Table 2. Absolute predictive performance remained limited.

4. Discussion

4.1. Principal findings

Summary of results. This study found limited evidence that neuroactive gut microbial pathways selected a priori broadly tracked dimensional symptom severity in autistic children; the main signal was a small inverse association between PWY-5088 (L-glutamate degradation VIII to propanoate) and SRS-2 severity. The primary q value narrowly missed the predefined false-discovery threshold. Even so, the size and direction of the estimate persisted with expanded covariate adjustment, an alternative transformation, simultaneous adjustment for the other pathways, and removal of influential observations. Eligible pathways were not consistently related to autism-specific anxiety or CBCL Internalizing, and together they added negligible SRS-2 prediction and worsened ASC-ASD prediction. The findings support a focused hypothesis about one functional pathway and argue against a broad, immediately useful neuroactive-pathway signature of symptom severity.

4.2. Comparison with the existing literature

Consistency with prior studies. This restrained reading fits a literature that holds both biologically suggestive findings and marked heterogeneity between cohorts. Recent experimental work shows that microbiome-directed perturbations and microbial metabolites can change autism-relevant behavior or neuroimmune signaling in animal models.[39–43] Human studies have linked autism to compositional, functional, or metabolomic features, at times pointing to glutamate, tryptophan, or short-chain-fatty-acid axes.[9–24,28–30,32,33] Large studies, however, also show that dietary preference, gastrointestinal context, geography, and technical design can account for much of the variation seen.[2–4,9–20] The present result adds a specific within-autism pathway estimate and not another case–control signature. Its small size and narrow domain specificity help explain why a plausible biological signal may fail to give portable clinical prediction.

4.3. Interpretation and mechanism

Biological interpretation of PWY-5088. The PWY-5088 annotation connects L-glutamate degradation to propanoate production, which permits several plausible but unproven biological readings. Glutamate takes part in microbial nitrogen and carbon metabolism. Propanoate is a short-chain fatty acid that can affect epithelial energy balance, immune signaling, and host metabolism.[44,45] Higher relative abundance of genes assigned to this pathway could reflect an ecological capacity to consume luminal glutamate, to make downstream organic acids, or to belong to a broader functional guild; it does not establish lower neural glutamate, greater propionate exposure in the brain, or a direct microbial effect on social behavior. Metagenomic abundance measures potential. Transcription, substrate availability, metabolite concentration, host absorption, hepatic processing, blood–brain transport, and receptor response were not measured; the inverse coefficient should therefore be read as a pathway-level association worth mechanistic

follow-up, and not as proof of a neurotransmitter mechanism.

Association and prediction. The contrast between association and prediction is especially instructive. A coefficient can be estimated with reasonable precision in the full sample and still add little to held-out prediction, particularly when the effect is small, the exposure is sparse, and baseline covariates explain little symptom variance. The mean SRS-2 ΔR^2 of 0.004 means that the pathways added, on average, less than half of one percentage point of out-of-sample variance. Absolute R^2 stayed slightly negative, so the apparent paired improvement was not useful prediction. For ASC-ASD, adding the pathways consistently worsened performance. These results argue against presenting the panel as a biomarker, and they show why in-sample p values or model fit cannot stand in for external or cross-validated performance.

Anxiety and internalizing outcomes. Consistent associations with ASC-ASD and CBCL Internalizing were absent. That should not be read as evidence that the microbiome is irrelevant to anxiety or emotional symptoms. It means that this a priori pathway panel, in this cross-sectional sample and measurement framework, gave no reproducible linear information about those outcomes. Anxiety in autistic children is heterogeneous and shaped by developmental level, intolerance of uncertainty, sensory experience, communication, environment, sleep, and co-occurring health problems. CBCL coverage was limited, and the available group was slightly younger, which reduced precision and may have changed the clinical spectrum represented; the wide CBCL intervals include small beneficial and small adverse associations. The proportionate conclusion is uncertainty and limited current evidence, not equivalence.

Magnitude of the association. The size of the association also needs quantitative context. A standardized coefficient of about −0.11 equals a little more than one tenth of a symptom-score standard deviation for each standard-deviation difference in relative pathway abundance, given the measured covariates. Even if replicated without bias, a difference of this scale is unlikely to discriminate individual children or settle support needs by itself; the joint six-pathway model had an adjusted R^2 of only 1.3%, so most between-child variation stayed unexplained. That is expected for a complex developmental phenotype shaped by biological, psychological, family, and environmental processes. It also shows why microbiome results belong in probabilistic systems and should not be presented as isolated determinants.

4.4. Implications for practice and policy

Clinical use. Clinically, the pathway panel should not be used to diagnose autism, stratify children, recommend microbiome products, or guide dietary restriction; the effect was small, domain specificity was limited, and predictive performance was clinically inadequate. The most defensible translational value is prioritization. PWY-5088 can enter independent, preregistered multi-omic studies that include explicit metabolite measurements and replication criteria; the largely null and weakly predictive findings also help calibrate expectations for functional microbiome markers and lower the risk of overstating associations that are only statistically detectable.

Analytic hierarchy. The analytic hierarchy may offer a useful template for future microbiome studies; the primary model asks whether a predefined association survives control of a defined multiplicity family. Robustness analyses ask whether its direction and size hold under defensible modeling decisions. Sparse decomposition asks which aspect of the exposure carries the estimate. Cross-validation asks whether the feature set improves prediction for unseen participants. The questions are related but not interchangeable. PWY-5088 moved partway through this hierarchy: it showed a stable small coefficient, uncertain sparse components, and negligible predictive contribution. Reporting where a signal stops matters as much as reporting where it starts.

Future research. Future studies should align diet, medication, gastrointestinal phenotype, stool consistency, symptom assessment, and biospecimen timing prospectively. Repeated sampling within children could separate stable between-person differences from short-term ecological fluctuation, and could show whether pathway changes precede symptom changes. Targeted and untargeted metabolomics should measure glutamate-related intermediates and short-chain fatty acids in stool and blood. Metatranscriptomics could assess active expression. Strain-resolved analysis may identify which organizms carry PWY-5088 and whether different community contexts yield similar pathway annotations. Causal questions will need triangulation across longitudinal cohorts, host–microbe mechanistic models, negative controls, and carefully justified interventions, not extrapolation from a single cross-sectional association.

4.5. Strengths and limitations

Strengths. Several design choices strengthen the analysis. Pathways were chosen by biological annotation and screened with an outcome-independent prevalence rule. Several symptom domains were assessed with instruments suited to autistic children; the primary models used a composition-aware transformation, robust standard errors, and outcome-specific false-discovery control. Sensitivity analyses covered measured diet, medication and stool factors, alternative abundance scaling, pathway collinearity, influential observations, and sparse detection. The linked sample was larger than in many earlier autism microbiome studies, and repeated matched cross-validation tested incremental predictive value directly. All major numerical outputs were independently reconciled with machine-readable source results, and the analytic scripts and source record support reproducibility.

Limitations. Limitations remain important. First, the cross-sectional design cannot establish temporal order. Autism-related eating patterns, gastrointestinal symptoms, medication use, family routines, or unmeasured health factors may shape the microbiome, so reverse causation and residual confounding are plausible. Second, functional profiles were inferred from stool metagenomes. They do not capture transcription, pathway flux, fecal or circulating metabolites, mucosal activity, vagal signaling, or central neurochemistry. Third, zeros may reflect biological absence or detection limits. The PWY-5088 association pointed the same way in the detection and positive-abundance analyses, but neither sparse component was precise on its own, which tempers claims of robustness. Fourth, the sample came from one population and sequencing pipeline, and geographic, dietary, developmental, and laboratory differences may limit transportability.

Limitations (continued). Fifth, symptom measures were caregiver reported and may share contextual variance, although the constructs and availability differed. The smaller CBCL subset limited power and prediction. Sixth, the primary q of 0.102 fell just outside the stated threshold. Favorable q values in sensitivity analyses add consistency but do not make the primary test positive after the fact. Seventh, repeated cross-validation is an internal test of transport and not validation in an independent cohort. Hyperparameter selection and preprocessing were nested within training folds, yet performance across entirely new sites, instruments, and batches remains unknown. Finally, the pathway panel was deliberately focused. Other microbial functions, species–pathway interactions, viruses, fungi, metabolites, and host genomic or immune features were outside its scope.

5. Conclusion

Overall, neuroactive microbial pathway profiles were neither broad correlates nor useful predictors of symptom severity in autistic children. PWY-5088 had a small inverse association with social-communication severity. That association was consistent across several analytic specifications, narrowly missed primary FDR control, and stayed uncertain under sparse-pathway decomposition. No comparable signal extended to autism-specific anxiety or internalizing symptoms. The results support targeted longitudinal and metabolomic replication. They also underline that pathway potential, clinical relevance, and causality are separate evidentiary questions.

Declarations

Use of Generative Artificial Intelligence. None.

Author Contributions. H.S.: conceptualization, methodology, software, formal analysis, data curation, visualization, writing – original draft, writing – review & editing. S.L.R.: validation, investigation, writing – review & editing. F.M.: supervision, project administration, writing – review & editing.

Funding. This research received no external funding.

Conflict of Interest. The authors declare that they have no competing interests.

Ethical Approval. The source study was approved by the Joint Chinese University of Hong Kong–New Territories East Cluster Clinical Research Ethics Committee (Ref. 2021.550). The de-identified secondary analysis was reviewed by the CMHC Research Center Ethics Committee Indonesia (No. 2026/0248)

Informed Consent. Written informed consent was obtained from parents or legal guardians in the source study. No participant was recontacted for this secondary analysis, which used de-identified data. Consent for publication is not applicable because the manuscript contains no identifiable individual information.

Data Availability. The source study data and associated materials are available through Zenodo (doi:10.5281/zenodo.17730818), subject to the terms of that record.

Acknowledgements. The authors acknowledge the investigators, participating families, and data curators of the source cohort.

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