

Short-term *Bacillus clausii* administration remodels the peripheral T-cell receptor repertoire through convergent CDR3 sequence architectures

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Abstract

Background

The intestinal microbiota is a major regulator of systemic immunity, yet its influence on the structural organization of the T-cell receptor (TCR) repertoire remains poorly understood. While probiotic administration has been shown to modulate immune function through changes in cytokine production and immune-cell activity, whether these interventions reshape the architecture of the circulating T-cell repertoire at single-clonotype resolution has not been established. We therefore investigated the longitudinal impact of short-term *Bacillus clausii* administration on the peripheral TCR β repertoire using ultra-deep immune repertoire sequencing.

Methods

Peripheral blood mononuclear cells were collected from a healthy individual immediately before and after completion of a 10-day oral *Bacillus clausii* regimen. TCR β repertoires were analyzed using complementary nucleotide- and amino acid-based clonotype definitions. Repertoire richness, diversity, clonal evenness, V(D)J gene usage, repertoire overlap, differential clonotype abundance, CDR3 sequence composition, motif enrichment, and hierarchical sequence clustering were evaluated to characterize quantitative and structural changes induced by probiotic administration.

Results

Short-term probiotic administration was associated with marked quantitative remodeling of the peripheral TCR β repertoire. Repertoire richness and diversity decreased substantially, accompanied by increased clonal focusing and expansion of dominant clonotypes. Despite this contraction of the low-frequency clonotype compartment, the overall structural organization of the repertoire remained remarkably stable. CDR3 length distribution, TRBV/TRBJ gene usage, and dominant V-J rearrangements were largely preserved, while abundance-weighted repertoire similarity remained exceptionally high, indicating conservation of the principal antigen-experienced T-cell populations. Differential abundance analysis demonstrated that repertoire remodeling was driven predominantly by expansion of pre-existing clonotypes rather than widespread emergence of new clones. Moreover, expanded clonotypes segregated into multiple clusters sharing conserved CDR3 sequence motifs despite heterogeneous TRBV and TRBJ usage, revealing convergent receptor architectures that were independent of germline gene composition.

Conclusions

These findings demonstrate that short-term *Bacillus clausii* administration does not induce wholesale restructuring of the peripheral T-cell repertoire but instead promotes selective quantitative remodeling within a preserved structural framework. Expansion of pre-existing clonotypes together with the emergence of convergent CDR3 sequence architectures suggests that microbiota-directed interventions fine-tune the organization of the adaptive immune repertoire rather than replacing it. This study

establishes ultra-deep immune repertoire sequencing as a powerful approach for investigating the systemic immunological effects of probiotics and provides a conceptual framework for understanding how microbiota-targeted interventions shape adaptive immunity.

Introduction

The intestinal microbiota has emerged as one of the principal regulators of systemic immune homeostasis, influencing host physiology far beyond the gastrointestinal tract. Continuous bidirectional communication between commensal microorganisms and the immune system shapes lymphocyte development, immune tolerance, inflammatory responses, and resistance to infection. Perturbations of this finely balanced ecosystem have been implicated in a broad spectrum of pathological conditions, including inflammatory bowel disease, autoimmune disorders, cancer, metabolic diseases, and impaired responses to immunotherapy (1). Consequently, therapeutic modulation of the gut microbiota has become an increasingly attractive strategy for manipulating systemic immunity.

Among microbiota-directed interventions, probiotics have attracted considerable attention because of their ability to restore microbial homeostasis while exerting measurable immunomodulatory effects. In particular, *Bacillus clausii*, a spore-forming Gram-positive bacterium widely used in clinical practice, survives gastric acidity and transiently colonizes the intestine, where it interacts with epithelial cells, resident immune populations, and microbial communities. Experimental and clinical studies have shown that *B. clausii* influences cytokine production, enhances epithelial barrier integrity, modulates dendritic-cell maturation, and promotes both pro-inflammatory and regulatory immune pathways depending on the immunological context (2–5). Despite these observations, our understanding of how probiotic administration affects the adaptive immune system remains remarkably limited.

Most previous studies have evaluated probiotic-induced immune modulation using conventional immunological readouts, including circulating cytokines, antibody responses, leukocyte phenotyping, or bulk transcriptional profiling (6). While these approaches have established that probiotics can influence systemic immune function, they provide only indirect information about the adaptive immune repertoire. The adaptive immune response is fundamentally encoded within the extraordinarily diverse population of T-cell receptors (TCRs), whose clonotypic composition determines antigen recognition, immunological memory, and the capacity to respond to future immune challenges (9–11). Consequently, understanding whether probiotics influence adaptive immunity ultimately requires characterization of T-cell receptor dynamics at single-clonotype resolution.

The complementarity-determining region 3 (CDR3) of the TCR β chain represents the principal determinant of peptide recognition and therefore provides a molecular record of adaptive immune history (12, 13). High-throughput immune repertoire sequencing (Rep-Seq) has transformed the study of adaptive immunity by enabling comprehensive characterization of millions of individual clonotypes with nucleotide-level resolution. Beyond measuring repertoire diversity, Rep-Seq allows quantitative investigation of clonal expansion, repertoire overlap, V(D)J gene usage, sequence convergence, and

structural organization of antigen receptor repertoires. These approaches have revealed characteristic repertoire remodeling in viral infections, cancer, autoimmune diseases, vaccination, and immunotherapy (14–16). In contrast, remarkably little is known about whether microbiota-directed interventions induce comparable changes in the organization of the peripheral adaptive immune repertoire.

Importantly, modulation of the T-cell receptor repertoire does not necessarily imply generation of new antigen specificities. Adaptive immune responses may instead arise through quantitative redistribution of pre-existing clonotypes, selective expansion of structurally related receptors, or subtle remodeling of repertoire architecture while preserving the dominant immunological memory compartment (17). Distinguishing between these fundamentally different modes of repertoire adaptation requires simultaneous analysis of clonal diversity, repertoire topology, sequence composition, and structural convergence. Whether short-term probiotic exposure induces broad replacement of circulating T-cell populations or selectively reorganizes an already established repertoire has not previously been investigated.

To address this question, we performed a longitudinal high-resolution analysis of the peripheral TCR β repertoire before and after a complete course of *Bacillus clausii* administration. Using complementary analyses of clonotype abundance, repertoire diversity, V(D)J gene usage, CDR3 sequence composition, motif enrichment, and hierarchical sequence clustering, we sought to determine whether probiotic administration alters the quantitative composition, structural organization, and sequence architecture of the circulating adaptive immune repertoire.

Our results demonstrate that short-term probiotic exposure does not globally restructure the peripheral T-cell repertoire but instead selectively remodels its quantitative organization through expansion of pre-existing clonotypes exhibiting convergent CDR3 sequence architectures, revealing a previously unrecognized level of adaptive immune modulation associated with microbiota-directed intervention.

Materials and Methods

Study design

A longitudinal exploratory study was conducted to investigate the effects of short-term probiotic administration on the peripheral T-cell receptor (TCR) repertoire. Peripheral blood was collected from a healthy adult volunteer (male, 60 y/o) immediately before initiation of probiotic treatment (PRE) and after completion of a 10-day oral regimen of *Bacillus clausii* (Enterogermina®, Sanofi), administered according to the manufacturer's recommendations (4×10^9 spores/day). Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation, processed and cryopreserved for immune repertoire sequencing. Because the study included a single individual sampled at two longitudinal time points, all statistical analyses were considered exploratory and intended to generate biological hypotheses rather than establish population-level inference.

TCR β repertoire sequencing

Cryopreserved PBMCs were thawed by immediate immersion of the vials in a 37°C water bath, and then washed in complete media. After washing PBMC in phosphate buffered saline (PBS), RNA isolation was performed using the RNeasy Plus Micro Kit (QIAGEN) with the QIAcube connect instrument (QIAGEN). The isolated RNA was used to generate sequencing libraries via Omniscope's single-molecule TCR β assay (osTCR) (Omniscience, Inc., Delaware, USA). Raw sequencing data were processed to assemble and align TCR contigs, extracting CDR3 nucleotide sequences alongside V and J gene annotations to quantify clonotype expansion. Functional clonotypes were defined by productive CDR3 rearrangements. A productive rearrangement is defined as an in-frame V(D)J recombination lacking premature stop codons. Depending on the biological question, subsequent analyses utilized either exact CDR3 nucleotide sequences or amino acid sequences combined with TRBV and TRBJ gene assignments. Only productive rearrangements that passed all quality-control filters were retained for downstream analysis.

Repertoire diversity analysis

Global repertoire organization was characterized using complementary measures describing richness, diversity, clonality, and abundance distribution. Diversity was evaluated using Shannon entropy, Hill numbers ($q = 1$ and $q = 2$), and the Inverse Simpson index. Clonal inequality was quantified using the Gini coefficient and Pielou-derived clonality. Rarefaction analysis was performed to normalize repertoire richness for sequencing depth. To evaluate abundance organization, cumulative frequencies of the dominant clonotypes, Lorenz curves, and rank-abundance distributions were generated.

Longitudinal repertoire comparison

Longitudinal similarity between pre- and post-treatment repertoires was evaluated using both qualitative and quantitative similarity indices. Presence-absence similarity was assessed using the Jaccard index, Sørensen-Dice coefficient, and overlap coefficient. Abundance-weighted similarity was quantified using the Morisita-Horn index, allowing discrimination between turnover of low-frequency clonotypes and preservation of dominant T-cell populations. Differential clonotype abundance was calculated as log₂ fold change between longitudinal samples after frequency normalization.

Analysis of V(D)J gene usage

TRBV and TRBJ gene usage frequencies were calculated from productive clonotypes. Global V- and J-segment distributions were compared between time points, and differential gene usage was calculated as the difference in normalized frequencies between PRE and POST samples. V-J pairing frequencies were visualized as heatmaps representing the abundance of individual germline recombination events.

CDR3 sequence analysis

To investigate structural remodeling of the antigen-recognition repertoire, complementary analyses of CDR3 amino acid composition were performed. Differential motif enrichment was assessed using overlapping amino acid k-mers of length three, four, and five residues. Position-specific amino acid frequencies were visualized using sequence logos generated from expanded clonotypes. Pairwise sequence similarity among expanded CDR3 β clonotypes was calculated using Levenshtein distance, followed by hierarchical clustering to identify groups of structurally related receptors. Sequence clusters were annotated according to their dominant conserved C-terminal amino acid motifs.

Statistical analysis

Because this study represents an exploratory longitudinal analysis of a single individual, formal hypothesis testing was intentionally minimized. Continuous repertoire metrics were compared descriptively between longitudinal samples. To determine the significance of differential clonal expansion across time points, we utilized a statistical noise model provided by Omniscope, generating a p-value under the null hypothesis of no difference in expansion between timepoints. Visualizations of these differential expansions were generated via the Omniscope Immune Portal (osIP). All other graphical analyses were generated in R (version 4.5.1) using the packages immunarch, ggplot2, ComplexHeatmap, circlize, igraph, and custom scripts developed for repertoire analysis. Unless otherwise indicated, all analyses were performed using normalized clonotype frequencies.

Results

Short-term *Bacillus clausii* administration induces quantitative focusing of the peripheral TCR β repertoire

To determine whether short-term probiotic administration alters the quantitative organization of the circulating adaptive immune repertoire, we performed a longitudinal analysis of peripheral TCR β clonotypes before and after completion of a 10-day *Bacillus clausii* treatment regimen. Following quality control and error correction, the pre-treatment repertoire comprised 565,007 sequencing records corresponding to 564,788 productive clonotypes, whereas the post-treatment sample contained 157,165 sequencing records and 156,866 productive clonotypes. Frequency normalization confirmed comparable quantitative scaling between datasets, enabling direct longitudinal comparison of repertoire architecture.

Across both time points, a total of 620,253 unique amino acid CDR3 clonotypes were identified. Of these, 44,911 clonotypes were shared between the pre- and post-treatment repertoires, while 469,465 and 105,877 clonotypes were uniquely detected before and after treatment, respectively. This distribution immediately suggested extensive remodeling within the low-frequency clonotype compartment while indicating persistence of a substantial fraction of the pre-existing repertoire.

To determine whether these compositional changes affected the global organization of the repertoire, we quantified multiple complementary measures of diversity and clonal evenness. All diversity metrics consistently demonstrated a marked reduction in repertoire complexity following probiotic

administration. Shannon entropy decreased from 9.49 to 8.00, corresponding to a reduction of nearly 80% in the effective number of clonotypes (Hill number $q = 1$), which declined from 13,289 to 2,986. Likewise, dominant-clone diversity (Hill $q = 2$; Inverse Simpson index) decreased from 55.87 to 28.63, indicating that repertoire contraction extended beyond the rarest clonotypes and also affected the distribution of intermediate-frequency clones.

This reduction in diversity was accompanied by increased clonal inequality. The Gini coefficient increased from 0.687 to 0.746, while clonality ($1 - \text{Pielou's evenness}$) rose from 0.278 to 0.329, indicating that a progressively smaller fraction of clonotypes accounted for an increasingly larger proportion of the circulating repertoire. Consistent with this observation, the most abundant clonotype expanded from 11.3% to 17.1% of the total repertoire, whereas the cumulative contribution of the ten most abundant clonotypes increased from 29.5% to 36.4%. These changes indicate that probiotic administration promoted a redistribution of clonotype frequencies toward a more polarized repertoire dominated by a limited number of expanded T-cell populations (Table 1).

The ecological consequences of this redistribution were further illustrated by Lorenz curve and rank-abundance analyses (Fig. 1). Although both repertoires retained the characteristic long-tailed distribution of adaptive immune receptor populations, the post-treatment repertoire exhibited a more pronounced deviation from the line of equality together with a substantially truncated low-frequency tail. Conversely, the hierarchical organization of the most abundant clonotypes remained largely unchanged, indicating that repertoire remodeling primarily reflected contraction of the rare clonotype compartment rather than disruption of the dominant clonal hierarchy. Differential abundance analysis further supported this interpretation, showing that most highly abundant clonotypes remained quantitatively stable, whereas the majority of low-frequency clonotypes underwent selective expansion or contraction following treatment.

Collectively, these findings demonstrate that short-term *Bacillus clausii* administration induces quantitative focusing of the peripheral TCR β repertoire. Rather than producing indiscriminate changes across the entire clonotype landscape, probiotic exposure preferentially contracts the extensive reservoir of low-frequency clonotypes while increasing the relative contribution of expanded T-cell populations, thereby generating a more focused yet highly organized adaptive immune repertoire.

The dominant peripheral T-cell repertoire remains structurally preserved despite quantitative remodeling

Although probiotic administration induced a marked contraction of repertoire diversity, the overall structural organization of the circulating TCR β repertoire remained remarkably stable. To distinguish changes in clonotype composition from alterations in repertoire architecture, we evaluated complementary measures of longitudinal repertoire similarity that differentially weight clonotype identity and abundance.

Presence–absence metrics revealed limited overlap between the two time points. The Jaccard index was 0.072, while the Sørensen–Dice coefficient reached only 0.135, indicating that a substantial proportion of unique amino acid CDR3 clonotypes was detected exclusively at one sampling point. Likewise, the overlap coefficient showed that only 29.8% of the smaller post-treatment repertoire was contained within the baseline repertoire. Together, these unweighted metrics demonstrate extensive turnover within the low-frequency clonotype compartment, consistent with the large number of private clonotypes identified before and after treatment.

A fundamentally different picture emerged when clonotype abundance was incorporated into the analysis. The abundance-weighted Morisita–Horn similarity index reached 0.933, indicating that the quantitative organization of the dominant repertoire remained highly conserved despite the apparent loss of numerous low-frequency clonotypes. Thus, although thousands of rare clonotypes were no longer detected following treatment, the major expanded T-cell populations that account for most of the circulating repertoire exhibited remarkable longitudinal stability.

This conclusion was further supported by analysis of the shared clonotype compartment. The 44,911 clonotypes detected at both time points represented only a minority of the total clonotype diversity but accounted for the majority of repertoire abundance. Importantly, their cumulative contribution increased from 58.0% of the pre-treatment repertoire to 75.3% following probiotic administration, indicating that repertoire remodeling was accompanied by preferential enrichment of clonotypes already present before treatment rather than replacement by newly emerging T-cell populations (Table 2).

The structural resilience of the repertoire was further reflected in the preservation of fundamental molecular characteristics. The distribution of CDR3 amino acid lengths remained virtually superimposable between the two sampling points (Fig. 2), with both repertoires displaying the characteristic unimodal distribution centered at 14–15 amino acids. No appreciable differences were observed in peak position, distribution width, or terminal tails, indicating that probiotic administration did not alter the overall structural constraints governing TCR β CDR3 organization.

Taken together, these findings demonstrate that short-term *Bacillus clausii* administration induces quantitative remodeling without structural disruption of the peripheral TCR β repertoire. While the low-frequency clonotype compartment undergoes extensive reorganization, the dominant antigen-experienced repertoire retains its overall abundance hierarchy, molecular composition, and structural framework. These observations identify structural resilience as a defining feature of probiotic-associated repertoire remodeling, suggesting that microbiota-directed immune modulation primarily reorganizes the quantitative distribution of pre-existing clonotypes rather than altering the fundamental architecture of adaptive immunity.

Repertoire remodeling is driven by selective expansion of pre-existing clonotypes

To determine whether the observed repertoire remodeling resulted from the emergence of new clonotypes or from quantitative redistribution of pre-existing T-cell populations, we compared the abundance of individual CDR3 β clonotypes between the pre- and post-treatment repertoires (Fig. 3).

Global abundance analysis revealed that the majority of clonotypes clustered closely around the identity line, indicating highly comparable frequencies before and after probiotic administration. Only a relatively small subset of clonotypes deviated substantially from this distribution, demonstrating that repertoire remodeling was not characterized by widespread changes across the entire clonotype landscape but instead reflected selective quantitative alterations affecting a restricted number of T-cell populations.

Classification of clonotype dynamics demonstrated that the predominant mechanism underlying repertoire remodeling was expansion of clonotypes already present in the baseline repertoire. Most highly abundant clonotypes detected after treatment were readily identifiable before probiotic administration and exhibited increased frequencies rather than de novo appearance. Conversely, clonotypes detected exclusively after treatment represented only a minor fraction of the repertoire and remained confined almost entirely to the lowest abundance classes (Suppl. Table 1).

Consistent with this observation, the dominant clonotype (**CASSSGFGYTF**) underwent the largest quantitative expansion, increasing from 11.35% of the circulating repertoire before treatment to 17.15% after probiotic administration. Additional high-frequency clonotypes, including **CASNQDRETYEQYF** and **CASSGRIVGGFNEQYF**, also displayed substantial expansion while remaining detectable at both longitudinal time points. Together, these observations demonstrate that the major quantitative changes induced by probiotic administration occurred within clonotypes already established in the circulating T-cell compartment rather than through recruitment of previously undetected receptor specificities (Fig. 3).

Importantly, clonotypes exhibiting the strongest positive fold changes were not restricted to a single abundance class. While numerous low-frequency clonotypes showed considerable dispersion in fold-change values, reflecting the expected stochastic variability associated with rare populations, the most abundant clonotypes generally remained remarkably stable or displayed moderate coordinated expansion. Likewise, clonotypes showing pronounced contraction were relatively uncommon and were predominantly observed within lower-frequency populations. These findings indicate that repertoire remodeling does not represent indiscriminate expansion or contraction but rather selective amplification of specific clonotype subsets within an otherwise stable abundance hierarchy.

Taken together, these results demonstrate that short-term *Bacillus clausii* administration reshapes the peripheral TCR β repertoire primarily through quantitative amplification of pre-existing clonotypes rather than widespread de novo clonal emergence. This pattern is consistent with modulation of an established adaptive immune compartment, in which previously circulating T-cell populations undergo selective redistribution while the overall clonal framework remains preserved.

Germline V(D)J architecture remains largely preserved following probiotic administration

To determine whether quantitative repertoire remodeling was accompanied by changes in germline gene utilization, we compared TRBV and TRBJ segment usage before and after *Bacillus clausii* administration (Fig. 4). Despite the pronounced redistribution of clonotype frequencies described above, the overall organization of germline gene usage remained remarkably stable across the two longitudinal samples.

The global TRBV and TRBJ usage profiles were highly comparable before and after treatment, with the same dominant gene segments accounting for the largest proportion of the repertoire in both conditions. TRBV12-4 remained the most frequently represented V segment, while TRBJ2-7 was consistently the predominant J segment, indicating preservation of the principal germline architecture underlying the circulating TCR β repertoire. Although several individual gene segments exhibited measurable quantitative differences between time points, these changes were modest relative to the overall stability of the repertoire-wide V(D)J distribution.

Differential usage analysis identified a limited number of gene segments showing preferential enrichment following treatment, including TRBV12-4 and TRBJ1-2. However, these quantitative shifts occurred within an otherwise conserved germline landscape and did not alter the global hierarchy of V- or J-segment utilization. Likewise, gene segments displaying relative contraction after treatment remained detectable at both time points, suggesting quantitative redistribution rather than selective disappearance of specific germline rearrangements.

Analysis of V-J pairing further supported the preservation of repertoire architecture. The global pattern of V-J combinations remained highly consistent between the two sampling points, with the dominant TRBV12-4/TRBJ1-2 rearrangement maintaining its position as the most abundant pairing throughout the longitudinal analysis. No novel highly represented V-J combinations emerged following treatment, nor was there evidence for replacement of the dominant recombination framework by alternative germline architectures.

Interestingly, expansion mapping demonstrated that multiple clonotypes carrying the same TRBV12-4/TRBJ1-2 rearrangement possessed distinct CDR3 amino acid sequences, indicating that expansion occurred across several independent clonotypes sharing a common germline framework rather than through amplification of a single rearranged receptor. Conversely, identical or closely related CDR3 sequences were also observed across different V-J combinations, suggesting that clonotype expansion cannot be explained solely by preferential usage of individual germline segments.

Collectively, these findings indicate that probiotic-associated repertoire remodeling occurs within a largely preserved V(D)J framework. The maintenance of global germline gene usage and dominant V-J pairing demonstrates that short-term *Bacillus clausii* administration does not substantially alter the mechanisms governing TCR repertoire generation. Instead, quantitative remodeling is superimposed

upon a stable recombinatorial architecture, implying that changes arise primarily through redistribution of clonotype abundance rather than reorganization of germline gene utilization.

Expanded clonotypes converge toward shared CDR3 sequence architectures

To determine whether the clonotypes selectively expanded following probiotic administration shared common structural characteristics, we performed complementary analyses of CDR3 β amino acid composition, including differential k-mer enrichment and position-specific residue conservation (Figs. 5 and 6). Despite the apparent diversity of expanded clonotypes, all three analytical approaches converged toward a common observation: repertoire remodeling was associated with the preferential expansion of clonotypes sharing conserved CDR3 sequence architectures rather than randomly distributed receptor sequences.

Differential k-mer analysis revealed reproducible enrichment of a restricted set of amino acid motifs within the post-treatment repertoire. Glycine-, serine-, and aromatic residue-containing motifs, including SSG, SSS, GFG, and FGY, were consistently enriched across overlapping 3-mer, 4-mer, and 5-mer analyses. Importantly, identical sequence cores remained significantly enriched as k-mer length increased, indicating that these signatures reflected expansion of clonotypes sharing extended structural sequence features rather than isolated amino acid substitutions. Conversely, motifs enriched in glutamine-, alanine-, and threonine-containing residues showed consistent depletion after treatment, suggesting selective redistribution of specific structural elements within the CDR3 repertoire rather than generalized changes in amino acid composition (Fig 5).

Position-specific amino acid profiling further demonstrated that expanded clonotypes were characterized by highly conserved residue organization. Although the central portion of the CDR3 region retained substantial sequence diversity, multiple amino acid positions displayed strong enrichment for glycine, serine, tyrosine, glutamine, glutamate, and phenylalanine residues. Notably, several of these conserved residues directly corresponded to the motifs identified by the k-mer analysis, providing independent confirmation that the observed enrichment reflected shared molecular organization rather than analytical bias. The recurrent conservation of aromatic residues within the C-terminal region, together with preservation of characteristic sequence patterns across multiple expanded clonotypes, indicates that repertoire remodeling preferentially involved receptors sharing common physicochemical properties (Fig. 6).

Taken together, these complementary analyses demonstrate that short-term *Bacillus clausii* administration is associated with structural convergence of the peripheral TCR β repertoire. Rather than promoting indiscriminate clonal expansion, probiotic-associated repertoire remodeling preferentially amplifies clonotypes sharing conserved CDR3 sequence motifs and higher-order structural architectures. The convergence of independent sequence analyses toward the same molecular

signatures suggests that quantitative repertoire remodeling occurs within constrained regions of CDR3 sequence space, revealing an additional level of adaptive immune organization beyond conventional measures of clonotype diversity or V(D)J gene usage.

Hierarchical clustering

To investigate higher-order relationships among expanded clonotypes, hierarchical clustering based on pairwise CDR3 sequence similarity was performed (Fig. 7). This analysis identified nine major sequence families, each defined by a characteristic conserved C-terminal motif, including **NEQFF**, **QETQYF**, **YEQYF**, **GELFF**, **TEAFF**, **SPLHF**, **NQPQHF**, **YGYTF**, and **NVLTF**. Within each cluster, clonotypes exhibited high sequence similarity despite heterogeneous TRBV and TRBJ gene usage, indicating that sequence organization was primarily determined by CDR3 amino acid composition rather than by shared germline rearrangements. Interestingly, multiple TRBV and TRBJ gene segments contributed to individual sequence clusters, while identical germline segments were distributed across several independent CDR3 families. This many-to-many relationship demonstrates that structurally related receptors can arise from distinct V(D)J recombination events and that similar germline rearrangements may generate multiple structurally divergent CDR3 configurations. Consequently, the sequence clusters identified here represent shared structural solutions that are largely independent of individual TRBV or TRBJ usage. Sequence logo analysis highlighted highly conserved amino acid signatures within each cluster. Although the central portion of the CDR3 displayed variable degrees of diversity, the C-terminal region was remarkably conserved, with characteristic motifs defining each cluster (e.g., NEQFF, QETQYF, GELFF, and TEAFF). In contrast, clusters such as SPLHF, NQPQHF, YGYTF, and NVLTF exhibited greater variability in the central residues while preserving conserved flanking amino acids, consistent with the maintenance of shared structural constraints despite sequence diversification. Across all clusters, aromatic and polar residues—including tyrosine (Y), phenylalanine (F), glutamine (Q), glutamate (E), and glycine (G)—were recurrently enriched, reflecting common physicochemical features of the CDR3 repertoire. Mapping clonotype abundance onto the hierarchical clustering further demonstrated that expanded clonotypes were distributed across several independent sequence families rather than concentrated within a single dominant cluster. Thus, probiotic-associated repertoire remodeling involved coordinated expansion of multiple structurally related clonotype groups instead of selective amplification of one immunodominant receptor family. Collectively, these observations indicate that the adaptive response preserved after probiotic administration is organized around several recurrent CDR3 architectures that remain detectable despite substantial heterogeneity in germline gene composition.

Discussion

The present study provides evidence that short-term *Bacillus clausii* administration remodels the peripheral T-cell receptor repertoire through selective reorganization of its quantitative architecture rather than wholesale replacement of its structural framework. Although probiotic administration induced a marked contraction of repertoire richness and increased clonal focusing, the dominant antigen-

experienced compartment remained remarkably stable. Expanded clonotypes largely originated from T-cell populations already present before treatment and were organized into multiple convergent CDR3 sequence families despite heterogeneous germline gene usage. Collectively, these observations suggest that short-term microbiota-directed intervention can reshape the internal organization of an established adaptive immune repertoire without evidence of wholesale repertoire replacement, consistent with the concept that the intestinal microbiota continuously regulates systemic adaptive immunity through dynamic modulation of existing immune networks rather than by inducing entirely new immune responses (1-2).

This distinction has important conceptual implications. Changes in immune diversity are frequently interpreted as evidence of immune activation or suppression. However, adaptive immune repertoires are dynamic ecosystems in which clonotypes continuously fluctuate because of homeostatic turnover, antigen exposure, stochastic recombination, and memory-cell maintenance (9-11). Consequently, reductions in repertoire richness alone provide only limited insight into the biological mechanisms governing repertoire adaptation. Our findings instead suggest that probiotic administration induces quantitative repertoire focusing, characterized by redistribution of clonotype abundance toward an increasingly polarized yet structurally preserved immune landscape. Rather than reflecting a generalized loss of immune competence, this reduction in diversity may represent selective reallocation of clonal resources within an otherwise resilient adaptive immune system, an interpretation that aligns with recent advances in systems immunology and adaptive immune repertoire analysis (18).

The remarkable preservation of repertoire architecture observed in this study further supports this interpretation. Despite the disappearance of a large proportion of low-frequency clonotypes, abundance-weighted similarity between the two longitudinal repertoires remained exceptionally high, while CDR3 length distribution and overall V(D)J gene usage was largely maintained. These findings indicate that probiotic-associated immune modulation occurs without disruption of the principal antigen-experienced T-cell populations that dominate the circulating repertoire. Instead, the peripheral adaptive immune system appears to behave as a highly resilient network capable of accommodating substantial quantitative fluctuations while preserving its global structural organization. Similar patterns of repertoire resilience have been described following vaccination, acute viral infections, and immune reconstitution, where extensive changes within rare clonotypes coexist with remarkable stability of dominant memory populations.

One of the most informative observations emerging from this study is that repertoire remodeling was driven predominantly by expansion of clonotypes already present before probiotic administration. *De novo* clonotypes represented only a minor component of the post-treatment repertoire and remained confined to very low frequencies, whereas the most abundant expanded clonotypes were consistently detectable at baseline. Although immune repertoire sequencing alone cannot distinguish naïve from memory T-cell populations, this pattern is consistent with preferential quantitative modulation of established circulating clones rather than broad recruitment of previously absent receptor specificities. Such a mechanism is biologically plausible considering that probiotics are generally believed to exert

their immunomodulatory effects indirectly through regulation of epithelial integrity, microbial metabolites, dendritic-cell activation, and cytokine production within the gut-associated lymphoid tissue rather than through direct antigen-specific stimulation of peripheral T cells.

Beyond quantitative redistribution, our analyses uncovered a second level of repertoire organization that is not captured by conventional diversity metrics. Independent analyses of k-mer enrichment, position-specific amino acid composition, and hierarchical clustering consistently demonstrated that expanded clonotypes shared conserved CDR3 sequence architectures despite heterogeneous TRBV and TRBJ usage. These observations indicate that repertoire remodeling preferentially involved structurally related receptors rather than random collections of expanded clonotypes. From an immunological perspective, this finding is consistent with the concept of structural convergence, whereby independent V(D)J recombination events generate receptors exhibiting similar antigen-binding solutions. Comparable patterns of convergent CDR3 organization have previously been described in antiviral immunity, cancer, and vaccine responses, where unrelated clonotypes converge toward common structural motifs capable of recognizing similar peptide–MHC complexes. Although the antigenic drivers underlying the present observations remain unknown, the identification of multiple independent CDR3 families sharing conserved sequence motifs strongly suggests that probiotic-associated repertoire remodeling is constrained within discrete regions of receptor sequence space.

Importantly, the present findings also challenge the common assumption that adaptive immune remodeling should primarily be interpreted through changes in germline V(D)J usage. While modest quantitative differences in individual TRBV and TRBJ segments were observed, the overall recombinatorial architecture remained remarkably stable throughout the intervention. Instead, the most informative biological signal emerged from analysis of CDR3 organization itself. This observation highlights an increasingly recognized principle in immune repertoire biology: germline gene usage provides only a partial description of adaptive immune organization, whereas the structural composition of CDR3 sequences more directly reflects functional receptor architecture. Consequently, characterization of sequence convergence may provide substantially greater biological resolution than traditional analyses based exclusively on V- and J-segment frequencies.

Taken together, these observations suggest that microbiota-directed interventions may influence adaptive immunity through reorganization of repertoire topology rather than generation of new adaptive immune specificities. In this model, probiotic administration subtly redistributes the relative abundance of pre-existing T-cell populations while preserving the global structural backbone of the circulating repertoire. Such a mechanism would allow continuous fine-tuning of immune surveillance without compromising established immunological memory, providing a biologically efficient strategy through which transient alterations of the intestinal ecosystem exert systemic immunological effects.

Several limitations should be considered when interpreting these findings. First, this study represents an exploratory longitudinal analysis performed in a single individual and therefore cannot establish the generalizability of the observed repertoire dynamics. Second, while the single-molecule TCR β

sequencing approach utilized here provides a remarkably deep and quantitative view of the repertoire, it inherently does not provide information regarding antigen specificity, paired $\alpha\beta$ receptor composition, or functional T-cell phenotype. Consequently, although the observed structural convergence strongly suggests non-random repertoire organization, definitive identification of the cognate antigens driving clonotype expansion will require complementary approaches integrating paired $\alpha\beta$ immune repertoire sequencing, single-cell transcriptomics, peptide–MHC multimer technologies, structural modeling, and computational specificity prediction frameworks.

Despite these limitations, the present work demonstrates that ultra-deep immune repertoire sequencing provides a sensitive framework for investigating the systemic immunological consequences of microbiota-directed interventions. Rather than viewing probiotic administration simply as a stimulus that increases or decreases immune activity, our findings support a more nuanced model in which the adaptive immune system undergoes architectural remodeling through selective redistribution of structurally related clonotypes. We propose that this concept of adaptive immune architecture represents a useful framework for interpreting how microbial interventions influence systemic immunity and may ultimately prove more informative than conventional measurements of repertoire diversity alone.

Declarations

All methods were carried out in accordance with relevant guidelines and regulations.

The study has been approved by the Institutional Ethical Committee of the Istituto Nazionale Tumori - IRCCS - "Fond G. Pascale" – NAPOLI IT (Protocol n. 57/22 OSS "HepAnt – Tumor antigen discovery for innovative cancer immunotherapies in HCC: from benchside to bedside" and subsequent amendments).

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Ethics approval and

The study was conducted on samples obtained by the last author L.B. No need for approval by the Institutional Ethical Committee of the Istituto Nazionale Tumori - IRCCS - "Fond G. Pascale" – NAPOLI IT

Consent for publication

N/A.

Consent to participate

N/A

Availability of data and material

The processed AIRR-seq format TCRB contig datasets generated and analysed during the current study are included in this published article and its Supplementary Information files. The raw sequencing files are not publicly available due to intellectual property and confidentiality restrictions.

Competing interests

The authors declare no potential conflicts of interest.

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Authors' contributions

AM performed all the wet lab analyses and bioinformatics analyses; AMS, JLM, MS, MG, MC, HH performed all the NGS analyses and additional confirmatory statistical analyses; MT and LB designed and coordinated the study; AM, MT and LB drafted the manuscript. All Authors approved the manuscript.

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Tables

Table 1. Quantitative Metrics, Diversity Indices of the AA CDR3 Repertoire.

Category	Metric	PRE	POST
Dataset & Alignment Yield	Input rows	565,007	157,165
	Repaired frequency cells	564,788	156,866
	Raw frequency sum after repair	1.0002	1.0003
Alpha-Diversity & Evenness	Shannon entropy	9.4947	8.0018
	Inverse Simpson	55.8682	28.6340
	Gini coefficient	0.6867	0.7455
	Clonality (1-Pielou)	0.2780	0.3289
Effective Repertoire Size	Hill q=1 [exp(Shannon)]	13,289.54	2,986.41
	Hill q=2 [Inverse Simpson]	55.86	28.63
Clonal Dominance	Top 1 AA clonotype cumulative frequency	0.11	0.17
	Top 10 AA clonotypes cumulative frequency	0.29	0.36

Table 2. Overlap Analysis of the AA CDR3 Repertoire.

Category	Metric	PRE	POST
Repertoire Overlap & Intersection	Shared AA CDR3 clonotypes	44,911	
	PRE-only AA CDR3 clonotypes	469,465	
	POST-only AA CDR3 clonotypes	105,877	
	Union AA CDR3 clonotypes	620,253	
Beta-Diversity Similarity Indices	Jaccard index AA	0.072	
	Sørensen-Dice index AA	0.13	
	Overlap coefficient AA	0.29	
	Morisita-Horn AA	0.93	
Shared Repertoire Weight	Shared AA abundance	0.58	0.753

Figures

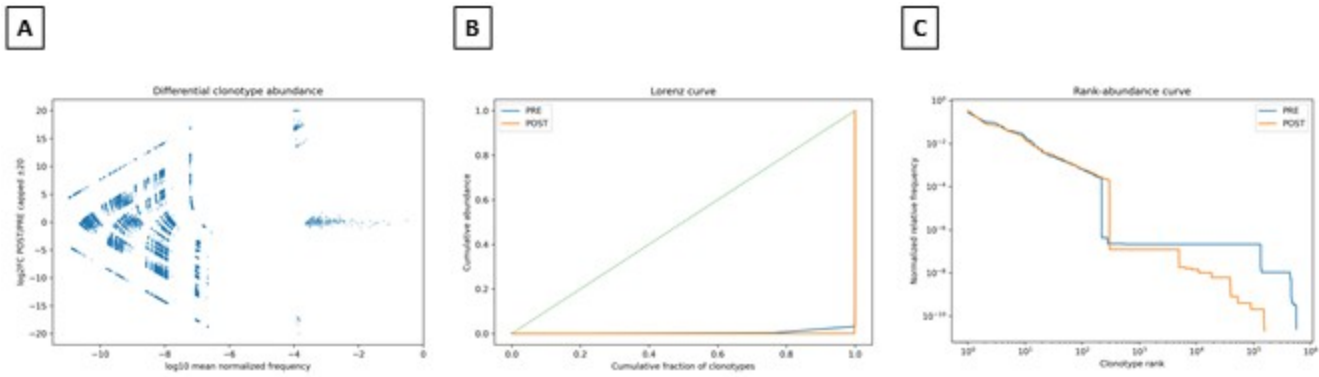


Figure 1

Short-term *Bacillus clausii* administration induces quantitative focusing of the peripheral TCR β repertoire. (A) Differential abundance analysis of amino acid CDR3 β clonotypes comparing pre-treatment (PRE) and post-treatment (POST) repertoires. Each point represents one clonotype plotted according to its mean normalized abundance and log2 fold change. Most highly abundant clonotypes remained stable across the two longitudinal samples, whereas quantitative remodeling predominantly affected low-frequency clonotypes. (B) Lorenz curves illustrating clonotype frequency inequality before and after treatment. The increased deviation of the POST repertoire from the line of equality demonstrates enhanced clonal dominance and reduced repertoire evenness. (C) Rank-abundance distributions of normalized clonotype frequencies. Both repertoires retain the characteristic long-tailed organization of adaptive immune receptor populations; however, the POST repertoire exhibits a shorter low-frequency tail and increased contribution of dominant clonotypes, indicating quantitative focusing rather than global restructuring of repertoire organization.

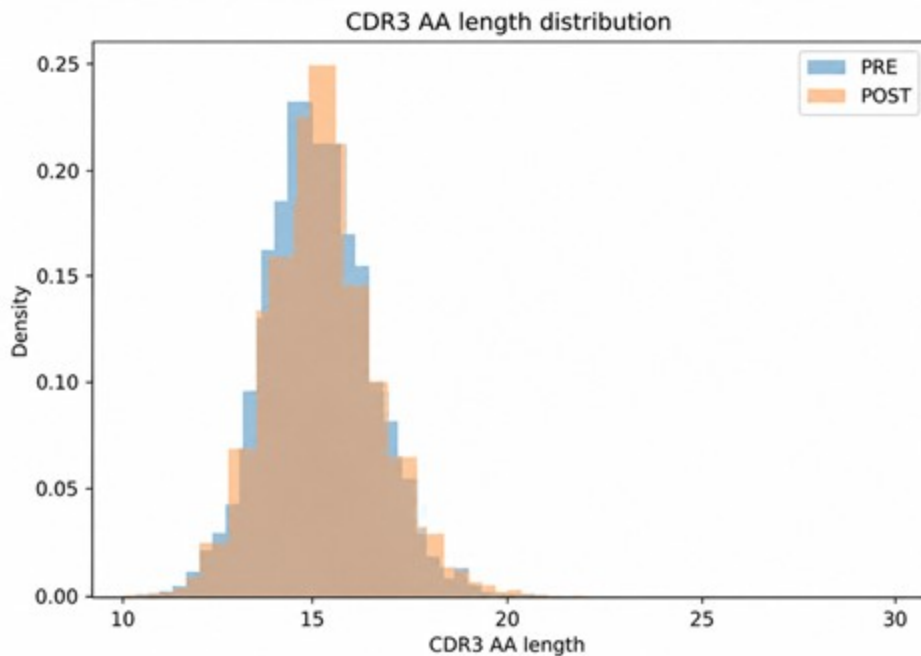


Figure 2

Preservation of CDR3 length distribution following probiotic administration. Density distribution of productive TCR β CDR3 amino acid lengths in the PRE and POST repertoires. Both samples display highly overlapping unimodal distributions centered at approximately 14–15 amino acids, with no appreciable changes in peak position or overall distribution, indicating preservation of the structural constraints governing CDR3 length despite quantitative repertoire remodeling.

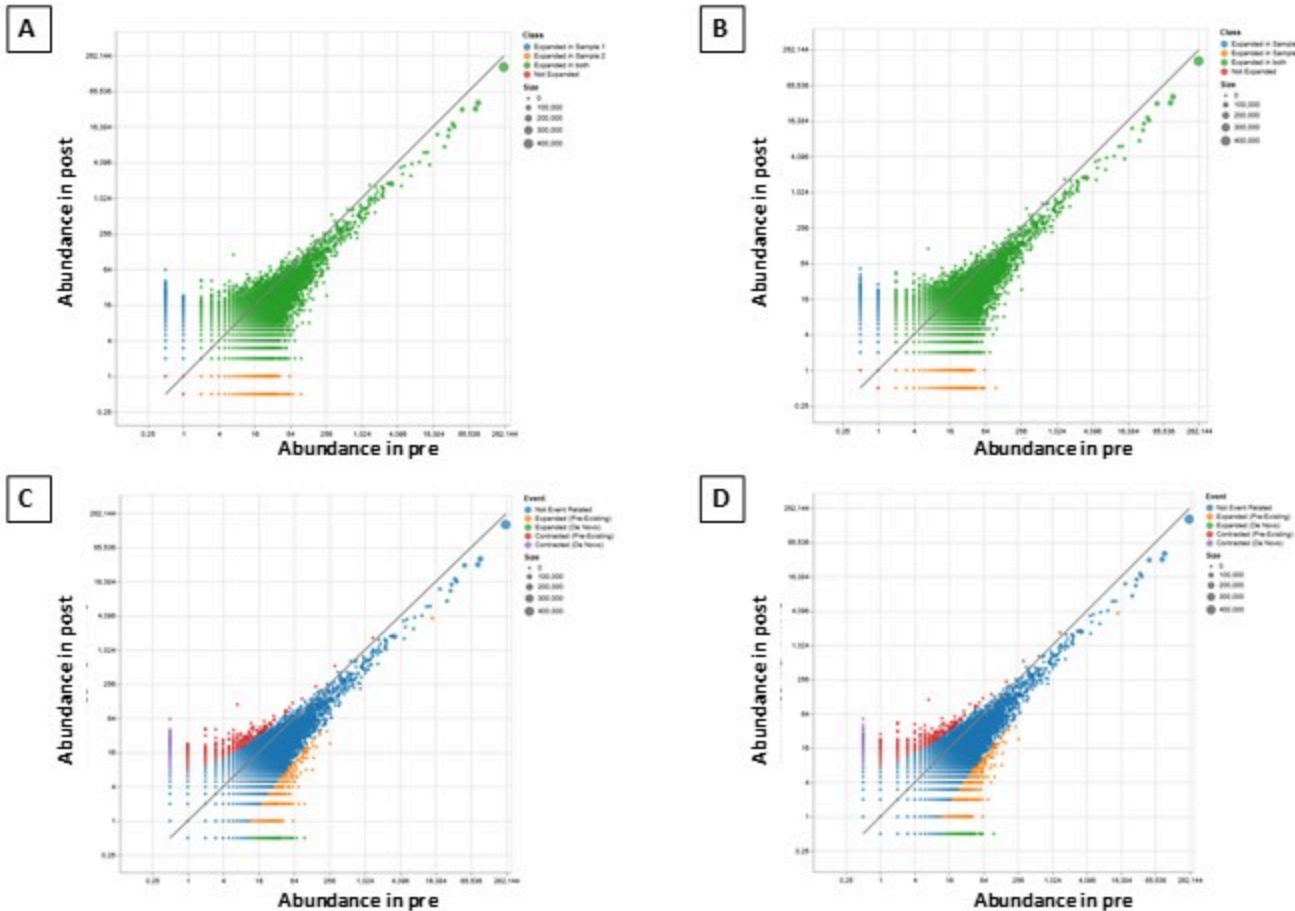


Figure 3

Quantitative remodeling is driven by expansion of pre-existing clonotypes. Scatter plot comparing normalized clonotype frequencies between PRE and POST repertoires. Each point represents one productive amino acid CDR3 β clonotype. Plots **A** and **B** display a deterministic clonotype classification, with colors corresponding to clonotypes expanded in PRE (blue), POST (orange), both (green), and not expanded (red). In plots **C** and **D** a statistical noise model is applied to determine the significance of differential expansion, generating a p -value under the null hypothesis of no difference. In these plots, colors indicate distinct longitudinal behaviors: no significant difference between PRE and POST and not event related (blue), significantly expanded and pre-existing (orange), significantly expanded and *de novo* (green), significantly contracted but still present in post timepoint (red), and significantly contracted to the point of disappearance (purple). As shown, the majority of expanded high-frequency clonotypes were already detectable before treatment, whereas *de novo* clonotypes remained confined to the low-frequency compartment. These observations demonstrate that probiotic-associated repertoire

remodeling results primarily from quantitative redistribution of established T-cell populations rather than widespread emergence of novel clonotypes.

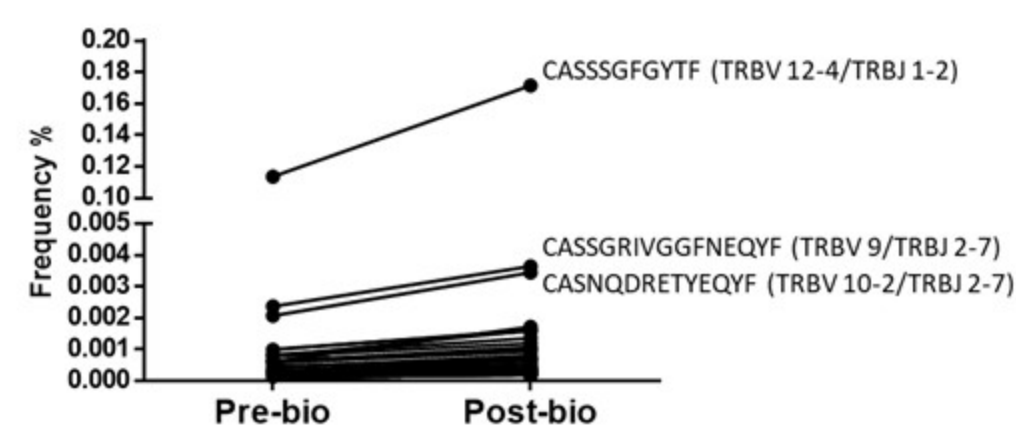


Figure 4

Figure 3. Top pre-existing clonotypes expanded after treatment. The expansion pattern of the top 20 clonotypes identified in the post-treatment sample is represented. The sequence of clonotypes with the highest frequency is reported.

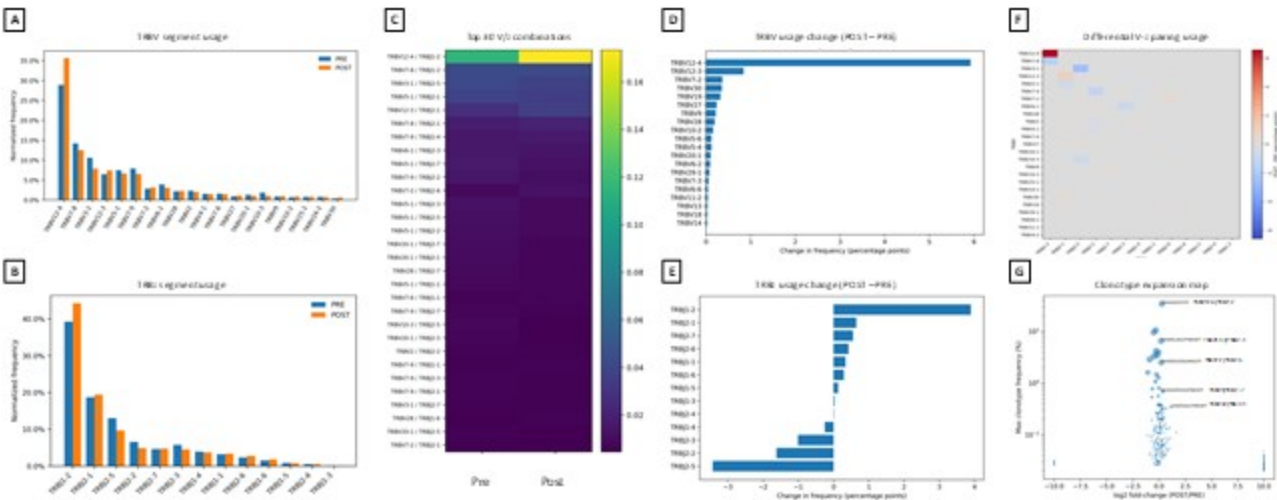


Figure 5

Figure 4. Germline V(D)J architecture remains largely preserved following probiotic administration. (A) Relative TRBV gene usage in PRE and POST repertoires. (B) Relative TRBJ gene usage before and after treatment. (C) Heatmap of TRBV–TRBJ pairing frequencies demonstrating preservation of the dominant recombination architecture across both longitudinal samples. (D) Differential TRBV usage (POST–PRE). (E) Differential TRBJ usage (POST–PRE). (F) Clonotype expansion map highlighting expanded clonotypes sharing the dominant TRBV12-4/TRBJ1-2 rearrangement. (G) Representative expanded clonotypes carrying identical germline rearrangements but distinct CDR3 sequences. Overall, probiotic-

associated repertoire remodeling occurs within a largely conserved germline framework, indicating that quantitative changes primarily affect clonotype abundance rather than V(D)J recombination architecture.

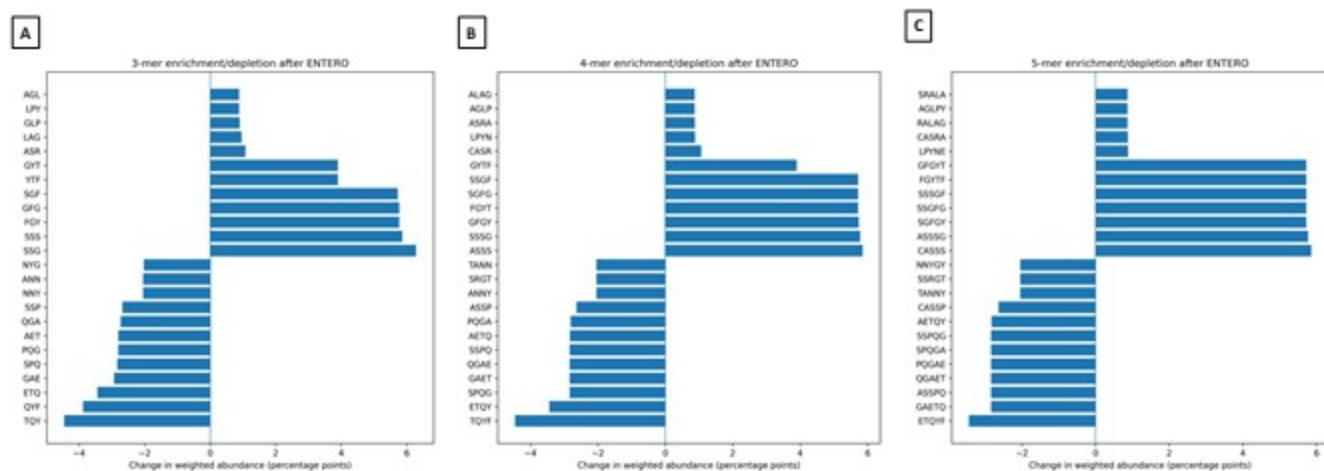


Figure 6

Figure 5. Expanded clonotypes exhibit conserved CDR3 sequence motifs. Differential amino acid motif enrichment analysis performed using overlapping **3-mer (A)**, **4-mer (B)** and **5-mer (C)** sequence windows extracted from productive CDR3 β sequences. Positive values indicate motifs enriched following probiotic administration, whereas negative values identify motifs depleted after treatment. Glycine-, serine-, and aromatic residue-rich motifs remain consistently enriched across increasing k-mer lengths, demonstrating expansion of clonotypes sharing common structural sequence elements rather than isolated amino acid substitutions.

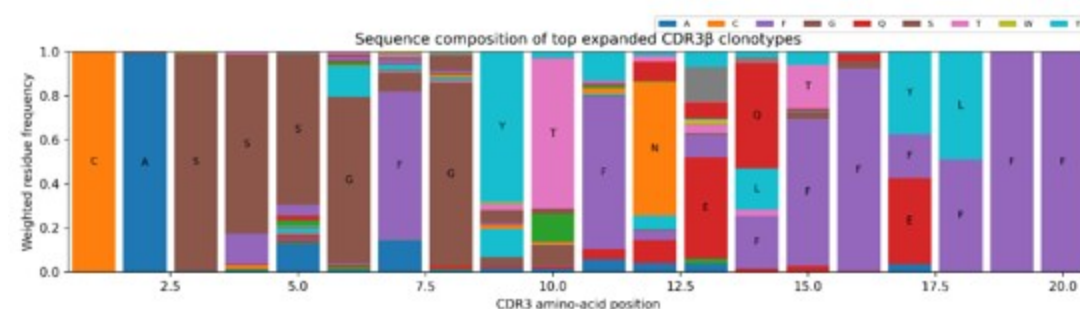


Figure 7

Figure 6. Position-specific amino acid conservation within expanded CDR3 β clonotypes. Sequence logo generated from the 50 most expanded CDR3 β clonotypes following probiotic administration. Letter height represents residue conservation at each CDR3 position. Conserved glycine, serine, glutamine, tyrosine and phenylalanine residues define characteristic sequence signatures shared among expanded clonotypes, whereas greater variability is maintained within the central CDR3 region. These findings indicate that expanded clonotypes share conserved structural organization despite sequence diversity.

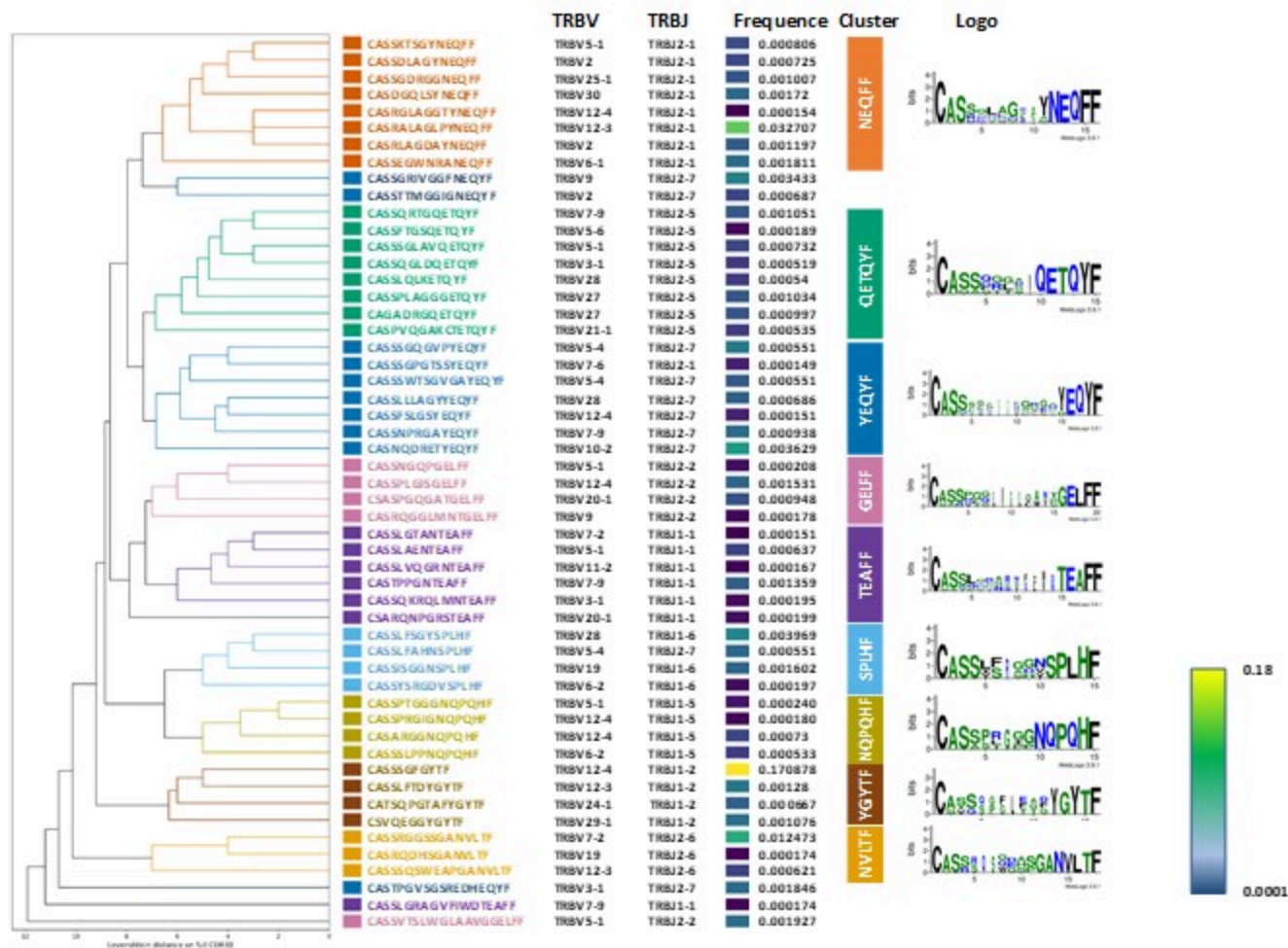


Figure 8

Figure 7. Expanded clonotypes converge toward shared CDR3 sequence architectures independently of germline gene usage. Hierarchical clustering of expanded CDR3 β amino acid sequences based on pairwise Levenshtein distance. Rows correspond to individual clonotypes annotated according to TRBV usage, TRBJ usage, normalized clonotype frequency, and representative CDR3 sequence. Sequence logos summarize the conserved amino acid composition of each cluster. Nine major sequence families were identified, each characterized by conserved C-terminal motifs (NEQFF, QETQYF, YEQYF, GELFF, TEAFF, SPLHF, NQPQHF, YGTYF and NVLTF). Individual clusters contain clonotypes derived from multiple TRBV and TRBJ gene segments, demonstrating that structurally related CDR3 architectures arise independently of germline V(D)J gene usage. Expanded clonotypes are distributed across several convergent sequence families rather than concentrated within a single dominant lineage, indicating that probiotic-associated repertoire remodeling preferentially amplifies structurally related receptor architectures.

Supplementary Files

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